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Genetic relationships between *A20/TNFAIP3*, chronic inflammation and autoimmune disease

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Abstract

A20 [also known as TNFAIP3 (tumour necrosis factor α-induced protein 3)] restricts and terminates inflammatory responses through modulation of the ubiquitination status of central components in NF-κB (nuclear factor κB), IRF3 (interferon regulatory factor 3) and apoptosis signalling cascades. The phenotype of mice with full or conditional *A20* deletion illustrates that A20 expression is essential to prevent chronic inflammation and autoimmune pathology. In addition, polymorphisms within the *A20* genomic locus have been associated with multiple inflammatory and autoimmune disorders, including SLE (systemic lupus erythaematosis), RA (rheumatoid arthritis), Crohn's disease and psoriasis. A20 has also been implicated as a tumour suppressor in several subsets of B-cell lymphomas. The present review outlines recent findings that illustrate the effect of A20 defects in disease pathogenesis and summarizes the identified *A20* polymorphisms associated with different immunopathologies.

Introduction

The NF-κB (nuclear factor κB) family of transcription factors plays a key role in controlling inflammatory and immune responses [1]. NF-κB activation can proceed by two distinct signalling cascades. Canonical NF-κB signalling is induced in response to pro-inflammatory cytokines [e.g. TNF (tumour necrosis factor)] and microbial infection and induces the expression of mainly pro-inflammatory and survival genes, whereas non-canonical NF-κB signalling is initiated by a subset of receptors (e.g. lymphotoxin β) and mainly regulates the development of lymphoid organs and adaptive immune responses [2]. Because A20 has been described as a regulator of canonical NF-κB signalling, the focus of the present review is on this pathway. In the canonical pathway, NF-κB dimers are sequestered in the cytoplasm by binding to IκB (inhibitor of NF-κB) proteins, of which IκBα is the best known. Upon encountering an inflammatory stimulus such as TNF or LPS (lipopolysaccharide), IκBα is phosphorylated followed by its ubiquitination and proteasomal degradation, releasing NF-κB for migration to the nucleus where it can drive gene expression [1]. Different receptors activate distinct NF-κB signalling pathways, which all converge at a central IKK (IκB kinase) complex composed of two related kinases, IKK1 and IKK2 (also known as IKKα and IKKβ), and a regulatory subunit NEMO (NF-κB essential modulator, also known as IKKγ).

Defects in the regulation of NF-κB-dependent gene expression contribute to a variety of diseases, including inflammatory and autoimmune diseases, neurological disorders and cancer. A tight regulation of NF-κB signalling is thus absolutely required. To achieve this, cells employ different control mechanisms to keep NF-κB signalling in check [3]. In this context, the ubiquitin-editing protein A20 [also known as TNFAIP3 (TNFα-induced protein 3)] has been described as a key player in the termination of NF-κB signalling and pro-inflammatory gene expression [4].

A20/TNFAIP3 is a cytoplasmic zinc-finger protein that is induced under inflammatory conditions and acts as a negative-feedback regulator of NF-κB activation in response to multiple stimuli, including TNF, IL (interleukin)-1, TLR (Toll-like receptor) and NLR [Nod (nucleotide-binding oligomerization domain)-like receptor] ligands. A20 was also shown to control antiviral signalling by acting as a negative regulator of IRF3 (interferon regulatory factor 3) signalling [5]. In addition to its NF-κB and IRF3 inhibitory properties, A20 is also a strong inhibitor of TNF-induced apoptosis [6]. The physiological importance of A20 as an anti-inflammatory protein is clearly demonstrated by the phenotype of A20-deficient mice, which are cachexic and develop severe multi-organ inflammation causing premature lethality [7]. Although little is known on the molecular mechanisms by which A20 controls apoptotic signalling, A20's NF-κB-inhibitory activities were shown to depend on its ubiquitin-editing function. The N-terminus has DUB (deubiquitinating) activity and can inhibit NF-κB signalling by removing Lys^{63}-linked polyubiquitin chains from specific NF-κB signalling molecules [8]. The C-terminal zinc-finger-containing domain, however, possesses E3

Key words: A20, autoimmunity, B-cell, inflammatory bowel disease (IBD), nuclear factor κB (NF-κB), tumour necrosis factor α-induced protein 3 (TNFAIP3).

Abbreviations used: DSS, dextran sodium sulfate; DUB, deubiquitinating; GWAS, genome-wide association study; IBD, inflammatory bowel disease; IκB, inhibitor of nuclear factor κB; IKK, IκB kinase; IRF3, interferon regulatory factor 3; NEMO, nuclear factor κB essential modulator; NF-κB, nuclear factor κB; NLR, Nod (nucleotide-binding oligomerization domain)-like receptor; PRR, pattern-recognition receptor; RA, rheumatoid arthritis; SLE, systemic lupus erythaematosis; SNP, single nucleotide polymorphism; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNFAIP3, TNFα-induced protein 3; TNIP1, TNFAIP3-interacting protein 1.

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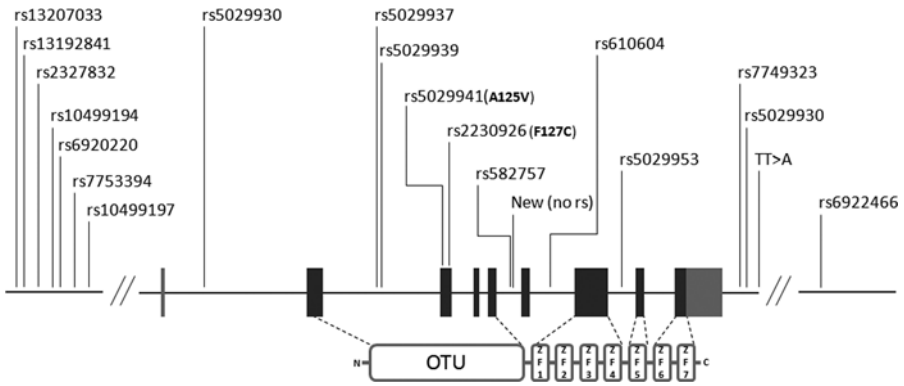
Figure 1 | Localization of polymorphisms associated with human immunopathologies in and around *A20/TNFAIP3*

The nine exons of human *A20* are represented as black/grey boxes, with the intronic regions in between. Untranslated exons are represented in grey. A schematic overview of the protein domains corresponding to specific exons of *A20* is displayed below the genomic structure. The N-terminal OTU (ovarian tumour) domain is essential for DUB activity and the C-terminal zinc finger (ZF 1–7) domains mediate E3 ubiquitin-ligase activity.



ubiquitin-ligase activity, promoting Lys[48]-linked polyubiquitination followed by proteasome-mediated degradation of its target [8]. Recently, A20 was also shown to affect the ubiquitination status of signalling proteins by preventing the interaction between E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases via competitive binding [9]. More information on the molecular mechanisms of NF-κB signalling and its regulation can be found elsewhere (e.g. [6]).

A20 and intestinal mucosal biology

Our intestinal microbiome poses a serious challenge to our immune system. The intestinal epithelium acts as a permeable barrier for efficient absorption of nutrients, but, at the same time, remains impermeable for luminal antigens, bacteria and bacterial products. Luminal bacteria are sensed by specialized innate immune receptors, called PRRs (pattern-recognition receptors), which are expressed by the epithelium and include TLRs, NLRs and C-type lectin receptors. Basal PRR stimulation leads to homoeostatic NF-κB signalling which does not cause spontaneous inflammation, but regulates intestinal barrier stability, epithelial proliferation, anti-microbial peptide production and anti-apoptotic responses [10]. This protective function of NF-κB in the intestinal epithelium is clearly demonstrated in mice that specifically lack NEMO or both IKK1 and IKK2 in the intestinal epithelium, and which develop spontaneous intestinal inflammation due to increased epithelial apoptosis, leading to bacterial mucosal infiltration [11]. We showed recently that specific deletion of A20 in the intestinal epithelium also increased the sensitivity of the epithelium to apoptosis [12]. Although these mice develop normally without any sign of spontaneous intestinal inflammation, they are hypersensitive to DSS (dextran sodium sulfate)-induced colitis and are unable to recover from DSS-induced intestinal damage. This DSS-hypersensitivity is associated with increased epithelial apoptosis [12]. Additionally, low-dose TNF injection causes massive epithelial apoptosis, leading to bacterial infiltration, bacteraemia and lethal sepsis within hours [12]. Interestingly, A20 expression is low at birth, and is strongly induced when the intestine becomes colonized by commensal bacteria [13]. In agreement with our observations in enterocyte-specific A20-deficient mice, it was shown that lethal inflammation in full A20-knockout mice is also triggered by the bacterial commensal flora initiating pro-inflammatory cytokine production and systemic inflammation [14]. Together, these data show that A20 in enterocytes mainly acts as a cytoprotective protein and suggest that defects in A20 expression or function could contribute to intestinal pathology.

Evidence for a role of A20 in human intestinal pathology also came from recent genetic studies identifying *A20* as a susceptibility locus for IBD (inflammatory bowel disease) (Table 1 and Figure 1). A linkage analysis study on 260 IBD patients from 139 Caucasian families associated a region of human chromosome 6q, containing the *A20* gene, to IBD [15]. In addition, a GWAS (genome-wide association study) for seven major common inflammatory diseases, on British people by the Wellcome Trust Case Control Consortium, identified A20 as a susceptibility gene for Crohn's disease [16]. Expression analysis on mucosal biopsies from 69 Crohn's disease patients confirmed a consistent down-regulation of *A20* [17], further indicating reduced or defective A20 function in IBD. Recently, a novel non-synonymous mutation in African-American patients in exon 3 (A125V) was found to be associated with increased risk of IBD, whereas the same mutation was protective for SLE (systemic lupus erythaematosis) [18]. Computer modelling predicted that this amino acid change could alter the DUB activity of A20, affecting its proper function [18]. Interestingly, an SNP (single nucleotide polymorphism) in the *A20* locus was also

Table 1 | **Genetic variants in or near *A20/TNFAIP3* (138188581–138204449) associated with different immunopathologies in humans (based on NCBI SNP database)**

SNP	Location	Nucleotide	Position	Disease association	Population
rs13207033	223 kb upstream	A/G	137965418	RA	American [26]
rs13192841	221 kb upstream	A/G	137967214	SLE	European [24]
rs2327832	215 kb upstream	A/G	137973068	Coeliac disease	European [19–21]
				RA	European [21,25,43]
rs10499194	186 kb upstream	C/T	138002637	RA	European/African-American [44], American [26], European [43,45,46]
				Juvenile idiopathic arthritis	[47]
				SLE/RA*	Japanese [48]
				Type 1 diabetes	European [28]
rs6920220	182 kb upstream	A/G	138006503	RA	American [26], European [25,45,46,49], European/African-American [44]
				Juvenile idiopathic arthritis	[47]
				Type 1 diabetes	European [28]
				SLE	European [23]
rs7753394	103 kb upstream	C/T	138085248	Crohn's disease	European [16]
rs10499197	56 kb upstream	G/T	138132516	SLE	European [23]
rs5029930	Intron 1	A/C	138190684	Coronary artery disease in Type 2 diabetes	American [50]
rs5029937	Intron 2	G/T	138195151	RA	European [46,51]
rs5029939	Intron 2	C/G	138195723	Systemic sclerosis	European [52]
				SLE	European [23], African-American [18]
rs5029941	Exon 3	C/T (A125V)	138196060	SLE/IBD†	African-American [18]
rs2230926	Exon 3	T/G (F127C)	138196066	SLE	European [24,53], African-American [18], Japanese [54], Chinese [30]
				SLE/RA	Japanese [48]
				Sjögren's syndrome/Crohn's disease/psoriasis/RA	European [22]‡
rs582757	Intron 5	A/G	138197824	Rheumatic heart disease	Chinese [55]
New (no rs)	Intron 5	C/–	138197889	RA	European [22]‡
rs610604	Intron 6	A/C	138199417	Psoriasis	Caucasian [34]
				Coronary artery disease in Type 2 diabetes	American [50]
rs5029953	Intron 7	A/G	138200760	SLE	African-American [18]
rs7749323	26 kb downstream	A/G	138230389	SLE	European [23]
rs5029930	28 kb downstream	A/C	138232377	Coronary artery disease in Type 2 diabetes	American [50]
Polymorphic dinucleotide	68 kb downstream	TT>A	138272732–138271733	SLE	European/Korean [29]
rs6922466	256 kb downstream	A/G	138444930	SLE	European [24]

*Risk factor in Caucasian population, protective in Japanese population [48].

†Protective in SLE, risk factor for IBD in African-American population [18].

‡Additional SNPs were identified in multiple but unspecified autoimmune disease patients according to Musone et al. [22].

identified as a risk factor in coeliac disease [19–21]. Finally, the non-synonymous SNP rs2230926/F127C was found to be associated with Crohn's disease in a Caucasian population with multiple autoimmune diseases [22] (Table 1).

In conclusion, the combined data from mouse disease models and human IBD samples identify A20 as a protein important for intestinal immune homoeostasis, and suggest that A20 deficiency or dysfunction could sensitize for IBD development. A20 restricts aberrant TLR- and NLR-induced NF-κB signalling in mucosal immune cells in response to the commensal microbiota, thereby preventing the production of harmful pro-inflammatory cytokines, and preserves intestinal barrier integrity in inflammatory conditions by preventing enterocyte apoptosis. On the basis of these findings, local enhancement of A20 function in the intestinal mucosa might therefore be a promising therapeutic strategy for the treatment of IBD.

A20 and autoimmune diseases

Next to the above described association of A20 with IBD and coeliac pathology, several more polymorphisms in or near the *A20* locus were described as being associated with inflammatory autoimmune pathology, including SLE [23,24], RA (rheumatoid arthritis) [25,26], psoriasis, multiple sclerosis [27] and Type 1 diabetes [28]. We recently published an overview of, at that time, all known A20 polymorphisms associated with disease [4]. Many of these have since been confirmed by multiple independent studies, often with patient and control cohorts from different populations. In addition, a number of new polymorphisms and mutations have been identified through genetic studies. An updated overview of the currently known disease-associated polymorphisms in the *A20* genomic locus is provided in Table 1 and Figure 1.

Most of the disease-associated *A20* polymorphisms reside outside the *A20* gene or in intronic sequences (Figure 1). Only two non-synonymous SNPs were found, in very close proximity in exon 3. These two SNPs (rs2230926/F127C and rs5029941/A125V) both affect the N-terminal DUB domain of A20, and functional studies on the SLE-associated F127C and A125V mutations were found to result in decreased inhibitory activity of A20 [18,24]. Using computer models, the structural implications of both mutations were predicted, showing that the A125V mutation could lead to conformational changes affecting the nearby catalytic core of the DUB domain, whereas the F127C mutation could influence the binding of target proteins [18]. In a recent study, all exons of *TNFAIP3* were sequenced in a collection of 123 individuals with multiple autoimmune diseases and 397 unrelated healthy controls, identifying 11 new coding variants, of which eight are non-synonymous mutations spread over the entire coding sequence [22]. This study also identified the F127C coding SNP as a mutation associated with the risk of Sjögren's syndrome, Crohn's disease, psoriasis and RA [22]. Additionally, a novel SLE-associated haplotype (TT>A) was recently identified in a conserved regulatory region downstream of *A20*, which results in reduced A20 expression. This polymorphism results in reduced DNA binding of NF-κB protein complexes [29].

The SLE-associated SNP (rs2230926) was first identified in Caucasians and was recently confirmed in a Chinese Han population [30]. The same GWAS identified some new SLE-susceptibility loci. One of the newly identified SNPs (rs10036748) is located in the *TNIP1* (TNFAIP3-interacting protein 1), also known as *ABIN1* (A20-binding inhibitor of NF-κB) locus, an A20-binding inhibitor of NF-κB and apoptosis signalling [31]. Another *TNIP1* SNP (rs7708392) associated with SLE risk was identified in a Caucasian population [32] and confirmed in a Japanese population [33]. Together with *A20*, *TNIP1* was also identified as a susceptibility gene for psoriasis [34]. It is worth mentioning that several genetic loci have been associated with more than one immunopathology, and many autoimmune patients are affected by multiple autoimmune diseases [22] (Table 1).

A20 and B-cell biology

Persistent NF-κB activation has a critical role in cancer development and progression [35]. Different genetic studies have suggested a role for A20 as a tumour suppressor, since A20 inactivation by somatic mutations and/or deletions, leading to constitutive NF-κB activation, is a frequent event in several subsets of B-cell lymphomas [36–39].

To study the role of A20 in lymphomagenesis, we and others generated B-cell-lineage-specific *A20*-knockout mice [40–42]. All three studies show that B-cell-specific A20 deficiency enhances B-cell proliferation and survival and leads to an autoimmune pathology, but does not lead to the spontaneous development of B-cell lymphomas. A20-deficient B-cells show increased CD40-, BCR- (B-cell receptor) and TLR-induced NF-κB responses *in vitro* [40–42]. Remarkably, according to Tavares et al. [40], A20-deficient B-cell survival results from the resistance of B-cells to Fas-induced apoptosis due to increased NF-κB-dependent expression of the anti-apoptotic protein Bcl-x. Moreover, their mice developed a lupus-like autoimmune pathology characterized by elevated numbers of germinal centre B-cells, autoantibodies and glomerular immunoglobulin deposits [40]. In contrast with these findings, studies with our A20-deficient mice show development of a progressive inflammatory phenotype, leading to an autoimmune syndrome only in old mice [41]. These mice do not display significant levels of antibodies against nuclear self-antigens (ANAs), which are the most common autoantibodies observed in SLE, but a general IgG autoreactivity to cardiolipin (diphosphatidylglycerol), a common autoantigen in autoimmune disease [41].

The fact that B-cell-specific *A20*-knockout mice do not develop B-cell lymphomas in naive conditions suggests that A20 deficiency may sensitize to lymphomagenesis only in co-operation with other B-cell oncogenes. Future studies should provide more insight into this aspect to clarify the role of A20 as a tumour suppressor in B-cells.

Concluding remarks

A20 exerts both NF-κB-inhibitory, IRF3-inhibitory and anti-apoptotic activities. GWASs have identified *A20* as a susceptibility gene for IBD, multiple autoimmune pathologies and subsets of B-cell lymphomas, and suggest that defects in A20 expression or function may contribute to disease pathogenesis. Although these genetic studies clearly define *A20* as a disease-susceptibility gene, more functional studies are needed to clarify the importance of A20 in disease pathogenesis. Mice lacking A20 in specific cell types or expressing mutant versions of A20 are important tools in these studies and will be very helpful to clarify the mechanisms by which A20 exerts its protective actions. Data from full *A20*-knockout mice suggest that the lethal phenotype is the consequence of uncontrolled innate immune responses triggered by intestinal bacteria, underscoring an essential anti-inflammatory function of A20 in these cells [7,14]. In contrast, A20 is dispensable for intestinal tissue development and enterocyte homoeostasis, but essential as a protective protein in conditions of inflammatory pressure [12]. A20 deficiency in B-cells does not lead to lymphomagenesis, but increases B-cell responses and survival, leading to the development of autoimmune pathology [40,41]. Future studies using tissue- and cell-specific *A20*-knockout mice will help to clarify further the role of A20 in autoimmune and inflammatory pathology.

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