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 erythematosis), 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), 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-dependent 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 Lys63-linked polyubiquitin chains from specific NF-κB signalling molecules [8]. The C-terminal zinc-finger-containing domain, however, possesses E3
ubiquitin-ligase activity, promoting Lys48-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)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Nucleotide</th>
<th>Position</th>
<th>Disease association</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13207033</td>
<td>223 kb upstream</td>
<td>A/G</td>
<td>137965418</td>
<td>RA</td>
<td>American [26]</td>
</tr>
<tr>
<td>rs13192841</td>
<td>221 kb upstream</td>
<td>A/G</td>
<td>137967214</td>
<td>SLE</td>
<td>European [24]</td>
</tr>
<tr>
<td>rs2327832</td>
<td>215 kb upstream</td>
<td>A/G</td>
<td>137973068</td>
<td>Coeliac disease</td>
<td>European [19–21]</td>
</tr>
<tr>
<td>rs10499194</td>
<td>186 kb upstream</td>
<td>C/T</td>
<td>138002637</td>
<td>RA</td>
<td>European [21,25,43]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Juvenile idiopathic arthritis</td>
<td>European/African-American [44], American [26], European [43,45,46]</td>
</tr>
<tr>
<td>rs6920220</td>
<td>182 kb upstream</td>
<td>A/G</td>
<td>138006503</td>
<td>RA</td>
<td>American [21,25,43]</td>
</tr>
<tr>
<td>rs7753394</td>
<td>103 kb upstream</td>
<td>C/T</td>
<td>138085248</td>
<td>Crohn’s disease</td>
<td>European [16]</td>
</tr>
<tr>
<td>rs10499197</td>
<td>56 kb upstream</td>
<td>G/T</td>
<td>138132516</td>
<td>SLE</td>
<td>European [23]</td>
</tr>
<tr>
<td>rs5029390</td>
<td>Intron 1</td>
<td>A/C</td>
<td>138196848</td>
<td>Coronary artery disease in Type 2 diabetes</td>
<td>American [50]</td>
</tr>
<tr>
<td>rs5029937</td>
<td>Intron 2</td>
<td>G/T</td>
<td>138195151</td>
<td>RA</td>
<td>European [21,25,46,49]</td>
</tr>
<tr>
<td>rs5029939</td>
<td>Intron 2</td>
<td>C/G</td>
<td>138195723</td>
<td>Systemic sclerosis</td>
<td>European [23], African-American [18]</td>
</tr>
<tr>
<td>rs5029941</td>
<td>Exon 3</td>
<td>C/T (A125V)</td>
<td>138196060</td>
<td>SLE/IBD†</td>
<td>African-American [18], Japanese [54], Chinese [30]</td>
</tr>
<tr>
<td>rs2230926</td>
<td>Exon 3</td>
<td>T/G (F127C)</td>
<td>138196066</td>
<td>SLE</td>
<td>European [24,53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SLE/RA</td>
<td>European [22]‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sjögren’s syndrome/Crohn’s disease/pсориasis/RA</td>
<td>European [22]‡</td>
</tr>
<tr>
<td>rs582757</td>
<td>Intron 5</td>
<td>A/G</td>
<td>138197824</td>
<td>Rheumatic heart disease</td>
<td>Chinese [55]</td>
</tr>
<tr>
<td>rs610604</td>
<td>Intron 6</td>
<td>C/−</td>
<td>138197889</td>
<td>RA</td>
<td>Caucasian [34]</td>
</tr>
<tr>
<td>rs5029953</td>
<td>Intron 7</td>
<td>A/G</td>
<td>138200760</td>
<td>Psoriasis</td>
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<tr>
<td>rs7749323</td>
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<td>A/G</td>
<td>138230389</td>
<td>SLE</td>
<td>African-American [18]</td>
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<tr>
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<td>A/C</td>
<td>138232377</td>
<td>SLE</td>
<td>European [23]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coronary artery disease in Type 2 diabetes</td>
<td>American [50]</td>
</tr>
<tr>
<td></td>
<td>Polymorphic dinucleotide</td>
<td>TT&gt; A</td>
<td>138272732-138271733</td>
<td>SLE</td>
<td>European/Korean [29]</td>
</tr>
<tr>
<td>rs6922466</td>
<td>68 kb downstream</td>
<td>A/G</td>
<td>138444930</td>
<td>SLE</td>
<td>European [24]</td>
</tr>
<tr>
<td></td>
<td>256 kb downstream</td>
<td>A/G</td>
<td></td>
<td>SLE</td>
<td>European [24]</td>
</tr>
</tbody>
</table>

1Risk factor in Caucasian population, protective in Japanese population [48].
2Protective in SLE, risk factor for IBD in African-American population [18].
3Additional 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 homeostasis, 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 and autoimmune diseases [23,24], RA (rheumatoid arthritis) [25,26], psoriasis, multiple sclerosis, 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-, 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 (ANA), 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 cooperation 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 homeostasis, 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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