The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology

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Nuclear factor (NF)-κB has an important role in immunity and inappropriate NF-κB activity has been linked with many autoimmune and inflammatory diseases. Multiple mechanisms normally ensure the proper termination of NF-κB activation. In this context, the intracellular ubiquitin-editing protein A20 (also known as Tumor Necrosis Factor Alpha-Induced Protein 3 or TNFAIP3) is a key player in the negative feedback regulation of NF-κB signaling in response to multiple stimuli. Moreover, A20 also regulates tumor necrosis factor (TNF)-induced apoptosis. Recent genetic studies demonstrate a clear association between several mutations in the human A20 locus and immunopathologies such as Crohn’s disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes. These findings further illustrate the importance of A20 in the resolution of inflammation and the prevention of human disease.

Properties of NF-κB signaling

Nuclear factor (NF)-κB is a transcription factor that regulates the expression of an exceptionally large number of genes in response to infection, inflammation and other stressful situations requiring rapid reprogramming of gene expression [1]. NF-κB represents a family of structurally related and evolutionarily conserved proteins (p100 or NF-κB2, p105 or NF-κB1, p65 or RelA, RelB, c-Rel), which exist as homo- or heterodimers. In resting cells, NF-κB is kept inactive by binding to inhibitor of κB (IκB) proteins, of which IκBα is best known. Upon stimulation with a wide variety of agonists including tumor necrosis factor (TNF), interleukin (IL)-1 and Toll-like receptor (TLR) ligands, IκBα is phosphorylated and subsequently polyubiquitinated and degraded by the proteasome, releasing NF-κB, which then accumulates in the nucleus and activates transcription of its target genes. NF-κB activating receptors share many signaling intermediates and they all employ intracellular adaptor proteins that provide modularity to NF-κB activation [2]. Many of the protein–protein interactions in NF-κB signaling are regulated by non-degradative polyubiquitination and deubiquitination (Box 1). A central event in NF-κB signaling is the activation of the IκB kinase (IKK) complex, which is comprised of the two catalytic subunits IKK1 and IKK2 (also known as IKKα and IKKβ) and the regulatory subunit, NEMO (NF-κB essential modulator, also known as IKKγ) (Figure 1). The activated IKK complex mediates phosphorylation of the inhibitory IκB protein, enabling its proteasome-dependent elimination. Gene targeting experiments showed that IKK2 and NEMO, but not IKK1, are required for NF-κB activation by TNF and TLRs [3]. Some receptors such as the lymphotoxin β receptor and CD40 receptor have the ability to activate an alternative NF-κB pathway, involving IKK1 and leading to p100 NF-κB processing to p52, which forms a dimer with RelB. The p52–RelB complex regulates specific genes involved in B-cell development and lymphoid organogenesis [1].

NF-κB can be activated in virtually every cell type. Defects in the regulation of NF-κB-dependent gene expression and apoptosis contribute to a variety of diseases including inflammatory and autoimmune diseases, neurological disorders and cancer. As such, controllable modulation of NF-κB signaling might be valuable in treating pathological conditions, and many companies are directing efforts into the development of NF-κB inhibitors. Because NF-κB activation is so crucial to many cellular processes, it is not surprising that a tight regulation of this pathway and the genes it induces is an absolute requirement. To achieve this, cells employ a multilayered control system to keep NF-κB signaling in check, and the combined action of different positive and negative regulators helps to fine-tune the immune response. Recently, substantial progress has been made in our understanding of the mechanisms that control the dynamics of NF-κB activation and several autoregulatory feedback loops terminating the NF-κB response have been described [4]. In this context, the ubiquitin-editing protein A20 (also referred to as TNF α-induced protein 3 or TNFAIP3) has been described as a key player in the termination of NF-κB signaling. The molecular and biochemical mechanisms by which this versatile protein modifier regulates NF-κB signaling have been reviewed in detail elsewhere [5]. Here we mainly focus on new findings that reveal a key role for A20 in controlling NF-κB-dependent inflammation. In particular, we discuss the recent discovery of A20 as a susceptibility gene for a plethora of different immune pathologies.
Figure 1. Inhibition of TNF- and TLR4-induced NF-κB activation by A20. Left-hand side: stimulation of TNF-R1 leads to the recruitment of the adaptor proteins TRADD, RIP1 and TRAF2 along with the E3 ubiquitin ligases cIAP1 and cIAP2, which then catalyze the attachment of K63-linked polyubiquitin chains to RIP1. These ubiquitin chains bind the TAB2 subunit of the TAK1 kinase complex, resulting in TAK1 activation. TAK1 phosphorylates and activates IKK2, which is also recruited through the binding of NEMO to K63-linked ubiquitinated RIP1. Recently, NEMO was shown to selectively bind linear head-to-tail ubiquitin chains [79], and to become itself modified by linear ubiquitin chains upon TNF and IL-1 treatment [80], the function of which is still unclear. IKK phosphorylates IκBα, resulting in its K48-linked ubiquitination and subsequent proteasomal degradation. This liberates NF-κB (here shown as a heterodimer of p65 and p50) to translocate to the nucleus and to promote target gene expression. A20 is a DUB that dampens TNF-induced NF-κB activation by disassembling K63-linked polyubiquitin chains from RIP1, and replacing them by K48-linked polyubiquitin chains.
Box 1. Regulation of signaling by ubiquitination

Ubiquitination is a post-translational modification of proteins that is recognized as a fundamental regulatory mechanism of signal transduction [20,83]. Ubiquitin is a 76 amino acid conserved protein that is covalently attached to other proteins in a highly regulated process involving the stepwise activation of the E1 ubiquitin activating enzyme, E2 conjugating enzymes and E3 ubiquitin ligases. The latter confer substrate specificity and enable the attachment of ubiquitin to a specific lysine in the target substrate. Each of the seven lysines in ubiquitin can themselves also be coupled to another ubiquitin, thus leading to a polyubiquitin chain on the original target protein. Recently, also head-to-tail (= linear) polyubiquitination has been demonstrated to be involved in NF-κB signaling [79,80]. The type of polyubiquitin chain determines the further fate of the target substrate. Polyubiquitin chain formation through K48 of ubiquitin directs proteasomal degradation of the modified protein. By contrast, K63 polyubiquitin chain linkages or linear polyubiquitination do not lead to degradation of the substrate but mediate a low-affinity binding of other proteins that contain specific ubiquitin-binding domains. This can stabilize transient protein–protein interactions and is widely used in NF-κB signaling. Ubiquitination is reversed by DUBs which are proteases that cleave ubiquitin chains from their substrate, making ubiquitination a very dynamic process. Several DUBs such as CYLD, A20 and Cezanne, have been demonstrated to remove K63-linked ubiquitin chains from specific target substrates in the NF-κB signaling pathway, thus negatively regulating NF-κB activation [81].

Immunoregulatory function of A20

A20 is a cytoplasmic zinc finger protein that was originally identified as a TNF-inducible protein and which has been characterized as a dual inhibitor of NF-κB activation and cell death [5]. In most cell types, basal A20 expression is very low but its transcription is rapidly induced upon NF-κB activation. Once expressed, A20 functions as a negative feedback regulator of NF-κB activation. The essential role of A20 in the regulation of NF-κB and apoptotic signaling was clearly demonstrated with the generation of a complete A20 knockout mouse [6]. Mice deficient for A20 are hypersensitive to TNF and die prematurely due to severe multi-organ inflammation and cachexia [6]. A20-deficient cells fail to terminate TNF-induced NF-κB responses and are more susceptible to TNF-mediated apoptosis. Besides its crucial role for the regulation of TNF receptor-dependent pro-inflammatory signals, A20 is also required for terminating NF-κB signaling in response to microbial products such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP) [7], which trigger TLR4 and Nucleotide-binding Oligomerization Domain containing 2 (NOD2) receptors, respectively. Deregulated TLR signaling in response to commensal bacteria was shown to be responsible for the multi-organ inflammation and premature death of A20 knockout mice [8]. Several reports suggest that A20 also restricts innate immune signaling in response to virus infection [9–11], but in vivo evidence for this is still lacking. Finally, A20 expression can also affect adaptive immunity. Two independent studies showed that RNA interference-mediated downregulation of A20 in dendritic cells (DCs) results in enhanced T-cell stimulatory capacity [12,13]. More specifically, silencing of A20 in human DCs results in an increase in their cytokine production following stimulation with the synthetic TLR3 ligand poly(I:C) and an increased T-cell stimulatory capacity [12]. An increased immunostimulatory potency of A20 silenced murine DCs was also demonstrated even in the absence of an activation stimulus [13]. Moreover, this latter study also reported that A20 silenced DCs inhibit regulatory T cells and hyperactivate tumor-infiltrating cytotoxic T lymphocytes and T-helper-cells that then become refractory to regulatory T-cell-mediated suppression. A20 silencing might thus provide a strategy to overcome regulatory T-cell-mediated suppression in an antigen-specific manner and increase the efficiency of vaccines against cancer and perhaps infectious diseases.

Mechanisms of NF-κB inhibition by A20

The molecular mechanism responsible for the NF-κB inhibitory function of A20 has been clarified by the elucidation of A20 as a ubiquitin-editing protein with dual functions: a deubiquitinating enzyme (DUB) activity mediated by its N-terminal ovarian tumor (OTU) domain and E3 ubiquitin ligase activity mediated by the C-terminal zinc finger containing domain (Figure 2) [14]. In the case of TNF signaling, Receptor Interacting Protein-1 (RIP1), an essential NF-κB signaling molecule, is polyubiquitinated by the E3 ubiquitin ligases cIAP-1 and -2 (Cellular Inhibitor of Apoptosis Protein-1 and -2) [15]. A20 DUB activity removes K63-linked polyubiquitin chains from RIP1, preventing its interaction with NEMO. Furthermore, A20 E3 ubiquitin ligase activity then promotes RIP1 K48-linked polyubiquitination, which triggers the proteasome-mediated degradation of RIP1 [14,16] (Figure 1). In addition, A20 is capable of targeting the associated signaling molecule TRAF2 to the lysosomes for degradation, an activity that does not require A20’s ubiquitin modifying property [17]. A20 can also dismantle K63-linked polyubiquitin chains from TRAF6 and RIP2, similarly turning off NF-κB activation by TRL4 and NOD2, respectively [7,18] (Figure 1). However, removal of K63-linked ubiquitin chains from TRAF6 and RIP2 is not followed by their A20-mediated K48-linked polyubiquitination and subsequent degradation. Because A20 is also able to inhibit NF-κB activation induced by TAK1 overexpression, which signals downstream of RIP1, RIP2 and TRAF6 [19], it is likely that A20 acts at multiple steps in the NF-κB signaling pathway. In this context, several other molecules that contribute to the proper activation of the IKK complex have been shown to undergo K63-linked polyubiquitination [20], making them potential targets for A20. The role of A20-mediated ubiquitin editing has so far only been studied in the context of its NF-κB
inhibitory function. Whether similar ubiquitin-editing mechanisms of A20 mediate the anti-apoptotic activity of A20 is still unclear, but it is supported by some very recent data. Death receptor stimulation was shown to induce cullin3 E3 ligase-mediated polyubiquitination of caspase-8 at the death-induced signaling complex, resulting in the interaction of caspase-8 with the ubiquitin-binding protein p62 (also known as sequestome-1). The latter promotes caspase-8 aggregation, leading to its full activation and driving commitment to cell death. Interestingly, A20 is recruited to caspase-8 in the death-induced signaling complex, where it reverses its ubiquitination and blocks the associated increase in caspase-8 activity, suggesting the existence of a dynamic balance in the ubiquitin-mediated activity of caspase-8 [21].

Little is known about the molecular mechanisms that regulate the ubiquitin-editing and NF-κB inhibitory function of A20. ABINs and TAX1BP1 are A20 binding proteins that have been proposed to recruit A20 to its ubiquitinated substrate by directly binding ubiquitin [22–24]. The E3 ubiquitin ligases Itch and RNF11 are two other components of the A20 ubiquitin-editing complex that are essential for the termination of TNF-mediated NF-κB and JNK signaling [24,25], but their precise role remains to be discovered. Finally, in the case of T cell receptor (TCR) and B cell receptor induced NF-κB activation, A20 is itself proteolytically processed by the paracaspase MALT1, compromising its NF-κB-inhibitory potential [26].

A20 in autoimmune and inflammatory diseases
Given its key function in the fine-tuning of NF-κB signaling and apoptosis, it can be expected that defects in A20 expression or function could lead to chronic inflammation and tissue damage. Here, we describe some recent findings that reveal a crucial link between polymorphisms in the human A20 locus and a wide spectrum of human autoimmune and inflammatory diseases (Table 1). In addition, we discuss the potential role of A20-mediated regulation of NF-κB signaling and/or apoptosis in the immunopathology of these diseases.

A20 and inflammatory bowel disease
Inflammatory bowel disease (IBD), and in particular Crohn’s disease (CD), is thought to result from a dysregulated interaction between the host immune system and its commensal microflora [27,28]. Recognition of bacterial products by TLRs represents a crucial component of the symbiosis between the commensal microflora and the host.

Table 1. Genetic variants in or near A20 at 6q23 (138.230274–138.246135) that are associated with inflammatory autoimmune pathology in humans

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mutation in A20 or SNPs identified</th>
<th>remark</th>
<th>Position (Mb)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerosis in mice in diabetic patients</td>
<td>E627A (mouse) rs 5029930 rs 610604</td>
<td>within intron</td>
<td>138.232377</td>
<td>[69]</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>rs 7753394 rs 2327832</td>
<td>outside gene</td>
<td>138.126940</td>
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<tr>
<td>Coeliac disease</td>
<td>rs 10499194/rs 13207033 rs 6922020</td>
<td>outside gene</td>
<td>138.044330</td>
<td>[39]</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>rs 5029937 rs 10499197 rs 7749323</td>
<td>within intron</td>
<td>138.236844</td>
<td>[41]</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>rs 5029939 rs 10499197 rs 7749323</td>
<td>within intron</td>
<td>138.237416</td>
<td>[43]</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>rs 13192841 rs 10499197 rs 7749323</td>
<td>outside gene</td>
<td>138.174209</td>
<td>[43]</td>
</tr>
<tr>
<td>Psoriasis</td>
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<td>outside gene</td>
<td>138.044330</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>rs 610604</td>
<td>within intron</td>
<td>138.241100</td>
<td>[55]</td>
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immune system and is essential for resistance to epithelial injury [29]. NF-κB signaling in intestinal epithelial cells was recently shown to be crucial for controlling intestinal immune homeostasis and limiting chronic inflammation, because intestinal epithelial cell-specific inhibition of NF-κB spontaneously caused severe chronic intestinal inflammation in mice [30,31]. An important role for the p65 (RelA) subunit of NF-κB in intestinal homeostasis was highlighted by a recent report that mice with specific deletion of RelA in intestinal epithelial cells exhibit increased susceptibility to chemically induced colitis [32]. An important step in the inflammatory process in these NF-κB-deficient mice involves the sensitization of NF-κB-deficient epithelial cells to TNF-induced apoptosis, compromising epithelial integrity and allowing bacterial translocation into the mucosa, thus leading to recruitment of inflammatory immune cells and chronic inflammation [30,32].

Mice genetically deficient in A20 also develop severe intestinal inflammation [6], leading to the hypothesis that expression of A20 is essential for intestinal homeostasis and suppression of NF-κB-dependent inflammation. In line with this, a recent genome-wide association study (GWAS) for seven most prevalent common inflammatory diseases, undertaken in the British population by the Wellcome Trust Case Control Consortium, identified A20 as a CD susceptibility gene [33]. An earlier independent study on 260 IBD-affected pairs from 139 families also associated a region of human chromosome 6q with the IBD phenotype [34], with one of the candidate genes in this locus being A20. Finally, mucosal biopsies from 69 CD patients were analyzed and confirmed a consistent down-regulation of mucosal A20 expression, which might hamper their ability to regulate pathological NF-κB activation induced by acute inflammatory responses [35]. Interestingly, single nucleotide polymorphisms (SNPs) in the A20 region on 6q23.3 were recently also identified as a disease risk factor in coeliac disease, an autoimmune disorder of the small intestine that occurs in genetically predisposed people and which is caused by intolerance to dietary gluten protein from wheat and related proteins from barley and rye [36].

The observation that A20 and MyD88 double-deficient mice are dramatically less inflamed and survive far longer than A20 single knockout mice suggests that steady state TLR signals can be profoundly proinflammatory and lethal [8]. Moreover, A20-deficient mice display splenomegaly due to infiltration of activated myeloid cells, which can be rescued in a MyD88-deficient background. Therefore, A20 is crucial for restricting intestinal inflammation as it prevents constitutive homeostatic MyD88 signals from becoming inflammatory, and commensal enteric bacteria seem to be the driving force inducing cachexia and myeloid cell proliferation via MyD88 signals in the absence of A20.

**A20 and rheumatoid arthritis**

Rheumatoid arthritis (RA) is the most common inflammatory arthritis affecting up to 1% of the adult population. Although the initiating cause of RA is still not fully understood, inflammatory cytokines such as TNF, IL-1, IL-6 and RANKL are of central importance for the chronic inflammatory state leading to disease. From these, TNF seems to have a primary role in RA because it regulates the expression of all other pro-inflammatory cytokines found in joints, making it an attractive therapeutic target. One effective treatment of severe RA consists of the inhibition of TNF through administration of TNF antagonists [37,38]. To identify susceptibility alleles associated with RA, a GWAS was carried out to identify genetic regions with potential DNA variants determining susceptibility to RA [39,40]. Two SNPs were unequivocally identified at 6q23. Although these variants are not located in a gene, they are thought to influence A20 as its nearest gene (~150 kb downstream of A20), probably by the presence of potential regulatory DNA elements in this region. Further fine-mapping studies also indicated an association of these and other polymorphisms in A20 and the 6q23 intergenic region with RA [41,42]. As A20 is required for termination of TNF-induced signals, these findings reveal A20 as a likely susceptibility locus for RA.

**A20 and systemic lupus erythematosus**

Another autoimmune disease for which the A20 gene was recently identified as being associated with, is systemic lupus erythematosus (SLE) [43,44]. This is a multigenic disease characterized by dysregulated interferon responses and loss of self-tolerance to cellular antigens. Autoantibody production results in local and systemic inflammation and organ failure. To identify genetic variants contributing to SLE, a GWAS was carried out, and an association between SLE and an A20 variant was identified. Evidence for two other SNPs that are associated with SLE were also found near A20, including the one previously described for RA [43]. When evaluating whether the A20 rs5029939 risk allele was associated with SLE clinical manifestations, heterozygous carriers were shown to have a twofold increased risk of developing renal or hematologic manifestations compared to homozygous non-risk subsets [45]. Based on the available evidence for a role of A20 variants in RA, a second study used data from the recently published GWAS [46] to specifically examine the potential association of A20 with SLE [44]. This study revealed that three independent SNPs in the A20 region are associated with SLE, including one coding and two non-coding polymorphisms. The coding SNP results in a F127C amino acid change in the OTU domain of the A20 protein. To test the biological impact of this SNP, the corresponding mutant A20 cDNA was transfected in HEK293T cells and shown to be modestly, but consistently, less effective at inhibiting TNF-induced NF-κB activity [44]. The epidemiological and genetic similarities between SLE and RA suggest that not only the same genes but also identical variants, might affect risk of each disease.

**A20 and autoimmune diabetes**

Type 1 diabetes (T1D) is a chronic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by the infiltration of inflammatory cells into the pancreatic islets of Langerhans, followed by the selective destruction of insulin-producing β-cells. As a result, insufficient insulin secretion results in hyperglycemia causing complications,
such as retinopathy, nephropathy and cardiovascular disease. One of the main mechanisms causing β-cell death is the intra-islet release of inflammatory mediators by activated immune cells [47], a process in which NF-κB is involved [48]. A transgenic mouse line in which NF-κB activation is attenuated specifically in β-cells conferred nearly complete protection against streptozotocin-induced T1D, with reduced intra-islet lymphocytic infiltration [48]. Analysis of 17 SNPs that have been reported in several autoimmune diseases (RA, SLE, CD, multiple sclerosis) also identified 6q25 containing A20 as a susceptibility locus for T1D [49], suggesting that the uncontrolled activation of NF-κB, due to reduced A20 expression, participates in T1D development. A20 is also a cardinal anti-apoptotic gene in β-cells [50,51]. Islet expression of A20 is rapidly upregulated in response to cytokine stimulation, and direct plasmid-mediated transfer of the A20 gene into the pancreas has been shown to protect mice in streptozotocin-induced T1D [52]. Adenovirus-mediated overexpression of A20 in rodent and human islets reduces cytokine-induced apoptosis and subsequent reactive oxygen species production by islets in vitro, but A20 overexpression has also been associated with improved islet survival in a syngeneic marginal islet mass transplantation model [50,51]. Moreover, loss of A20 expression renders β-cells susceptible to apoptotic death [53]. These data demonstrate that A20 is an important cytoprotective protein which could be a candidate for gene therapy in islet transplantation and therapeutic intervention in T1D.

A20 and psoriasis
Psoriasis is a common immune-mediated disorder with a multifactorial genetic basis that affects the skin, nails and joints. The most obvious cellular features of psoriasis are epidermal hyperplasia, altered keratinocyte differentiation and inflammation [54]. Individuals with psoriasis show increased risk for other autoimmune disorders, such as arthritis, atherosclerosis and CD [54]. So far, between 10 and 20 chromosome regions have been proposed to harbour psoriasis genes but only a few genes have been identified [54]. To identify new psoriasis susceptibility loci, a GWAS of 1409 psoriasis patients and 1436 controls was carried out [55]. Next to SNPs for genes involved in IL-23 signaling, loci including A20 and ABIN-1 (also known as TNFAIP3-interacting protein 1 or TNIP1) showed strong association with psoriasis [55]. Interestingly, in the mouse PL/J strain, carrying a CD18 hypomorphic mutation which gives rise to a skin disease resembling human psoriasis, a region of mouse chromosome 10 encompassing A20 was identified as contributing to the psoriasiform skin disease [56]. Notably, the same region of the mouse genome has been associated with atherosclerosis [57], a most prevalent co-morbidity of psoriasis [58].

A20 and atherosclerosis
Atherosclerosis has emerged as a cause of mortality in systemic autoimmune diseases such as RA and SLE and compelling evidence demonstrates that these diseases are risk factors for the development of atherosclerosis [59]. In addition, psoriasis and diabetes have been associated with atherosclerosis and cardiovascular mortality [58,60]. Atherosclerosis was once thought to be solely a disease of lipid accumulation in the vessel wall, but inflammation also seems to be an important driving factor, leading to the now largely accepted concept of atherosclerosis as a chronic inflammatory disease [61]. Several immune cells and mediators that take part in an inflammatory reaction can be identified in the atherosclerotic lesions in which vascular cells interact with immune cells. Cholesterol-containing low-density lipoproteins that accumulate in the intima activate the endothelium and promote recruitment of T cells and monocytes, the latter of which differentiate into macrophages and end up as pro-inflammatory foam cells. Local antigens trigger Th1 responses with secretion of proinflammatory cytokines that contribute to local inflammation and plaque growth. At the endothelial level, TLRs are a potential link between inflammation and atherosclerosis, as they are used not only to sense pathogens but also host-derived ligands such as oxidized lipoproteins or their component oxidized lipids and thereby initiate so-called sterile inflammation’. NF-κB has been implicated in the pathogenesis of atherosclerosis as it regulates the expression of many proinflammatory genes involved in the initiation and progression of atherosclerotic lesions [62]. However, NF-κB-dependent expression of anti-apoptotic genes could be seen as anti-atherosclerotic, as cell death becomes an important aspect at later stages of atherosclerosis in which it determines plaque stability [62].

An intercross between atherosclerosis susceptible (C57BL/6J ApoE<sup>−/−</sup>) and resistant (FVB/N ApoE<sup>−/−</sup>) mice identified an atherosclerosis-susceptibility locus on chromosome 10 of the mouse genome [57,63–65]. One of the candidate genes mapping to this locus is A20. Sequencing of the mouse A20 gene coding region from both parental strains revealed a SNP resulting in a single E627A amino acid change (C57BL/6J versus FVB/N). This mutation introduces a putative casein kinase 2 phosphorylation site in A20 from C57BL/6J that is not present in FVB/N, leading to less effective termination of TNF-stimulated NF-κB activation in the atherosclerosis susceptible C57BL/6J mice [57]. Vascular smooth muscle cells from these mice showed prolonged TNF-induced expression of the NF-κB target genes A20 and In<sub>Ba</sub> [57]. In a recent study, the role of A20 in atherosclerotic lesion development was further examined using A20 haplo-insufficient mice [65]. This study showed an increased aortic root atherosclerotic lesion area in A20 insufficient mice whereas A20 overexpressing transgenic mice had a decreased lesion area. The protective effect of A20 on atherosclerosis was associated with reduced expression of pro-atherosclerotic NF-κB target genes, such as the adhesion molecules VCAM-1, ICAM-1 and monocyte colony-stimulation factor (M-CSF), and reduced plasma levels of NF-κB-driven cytokines [65]. The atheroprotective effect of A20 is most likely caused by its ability to shut down NF-κB activation that is driven via a MyD88-dependent pathway, as MyD88–, TLR2– and TLR4-deficient mice, on an ApoE<sup>−/−</sup> background, also show decreased atherosclerosis [66–68]. A20 SNPs were also shown to be associated with increased atherosclerosis in diabetic patients [69]. Whereas the susceptibility effect in mice is due to the E627A substitution [57], the effect in humans is mediated by variants.
affecting the expression of the A20 gene, with lower mRNA levels being associated with increased cardiovascular risk and higher levels with protection [69]. These variants in the human A20 gene are part of a larger group of polymorphisms, including mutations in lymphotoxin-a and in an IkB homologue [70], influencing susceptibility to atherosclerotic through modulation of NF-κB activity [69].

A20 as a tumor suppressor in B-cell lymphomas
Persistent NF-κB activation has a crucial role in cancer development and progression [71], implicating a potential role for A20 as a tumor suppressor. Recently, different genetic studies have revealed that A20 is frequently inactivated by somatic mutations and/or deletions in multiple B-cell lymphomas, including mucosa-associated tissue (MALT) lymphoma, Hodgkin’s lymphoma, activated B-cell like diffuse large B-cell lymphoma and follicular lymphoma [72–78], which are all frequently associated with constitutive activation of the NF-κB pathway. Most of the A20 mutations were nonsense or frameshift mutations that prevent production of full-length protein. When re-expressed in lymphoma-derived cell lines carrying biallelic A20, non-somatic and/or deletions in multiple genetic studies have revealed that A20 is frequently inactivated by somatic mutations and/or deletions in multiple B-cell lymphomas, including mucosa-associated tissue (MALT) lymphoma, Hodgkin’s lymphoma, activated B-cell like diffuse large B-cell lymphoma and follicular lymphoma [72–78]. In addition, A20-deficient cells stably generated tumors in immunodeficient mice whereas the tumorigenicity was effectively suppressed by re-expression of A20 [75]. Altogether, these results reveal the intriguing role of A20 as a novel tumor suppressor protein whose inactivation might thus represent a common mechanism leading to constitutive or aberrant NF-κB activation that mediates cell proliferation and survival of subsets of B-lineage lymphomas. These studies, along with the recent identification of A20 as a susceptibility gene for multiple autoimmune diseases, also suggest that A20 might be a crucial molecular link between chronic inflammation and cancer.

Conclusions and perspectives
The list of proteins participating in the negative regulation of NF-κB activation and apoptosis continuous to accumulate, with new molecular mechanisms emerging frequently. A20 is a unique protein in the sense that it exerts both NF-κB inhibitory and anti-apoptotic activities, and it is now recognized as a key regulator in inflammation and immunity. The large number of GWAS studies over the past two years have uncovered A20 as a susceptibility locus for multiple autoimmune and inflammatory diseases. Changes in A20 expression or activity can alter the intensity or duration of immune responses that take part in the development and progression of these diseases. It remains to be resolved if and how the reported polymorphisms in or near the A20 locus affect the expression and/or function of A20. In this context, mice lacking A20 in specific cell types or expressing mutant versions of A20, combined with mouse models for human autoimmune and inflammatory diseases will be valuable tools. Another unresolved issue relates to the molecular mechanisms employed by A20 to regulate NF-κB activation and apoptosis. Whereas specific ubiquitinated proteins have been identified as A20 targets in the NF-κB signaling pathway, it is still completely unknown how A20 regulates apoptosis signaling. We predict that the vast expansion of studies on ubiquitination will reveal novel targets for A20 in addition to the identification of regulatory mechanisms. A detailed knowledge of these mechanisms should eventually lead to the development of novel therapeuic approaches that support the activity of A20.

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