THE PHYSIOLOGICAL ROLE OF DEUBIQUITINASE A20 IN INFLAMMATION AND RELATED DISEASES

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INTRODUCTION

Background

A20, also known as TNFAIP3 (tumour necrosis factor α-induced protein 3), is a protein involved in negative regulation of the inflammation and apoptosis[1]. It is a ubiquitin-editing enzyme that is capable of ubiquitination and deubiquitination[1]. However, it is its function as a deubiquitinase that makes it a potent regulator of several innate immune signals, specifically TNFα, via the nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) pathway[2]. Ubiquitination, as its name suggests, is ubiquitous in the body, so its implications in treating clinical diseases are extremely important.

A20 Gene Mutations and Phenotypes

Deubiquitinase A20 is encoded by the *TNFAIP3* gene[2]. Any mutations, including single nucleotide polymorphisms (SNPs), in this gene lead to changes in A20 expression[2]. Specifically, SNPs in the *Tnfaip3* gene is linked to susceptibility to inflammatory and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and many more diseases[1] as seen in **FIGURE 1**[3]. In the more extreme cases of A20 deficiency, spontaneous inflammation and perinatal lethality[4] occurs, as clearly demonstrated by the resulting phenotype of *Tnfaip3*$^{-/-}$ mice, especially when treated with sub-lethal doses of TNF, DSS, and LPS (pro-inflammatory ligands)[1,2]. Furthermore, bi-allelic mutations of the *Tnfaip3* gene contribute to a wide spectrum of lymphomas[1].

A20 Protein Structure[5]

Deubiquitinase A20, like any other protein, consists of a polypeptide with a C-terminus and an N-terminus. Each terminus of the polypeptide contains one specific, distinct, significant motif[5]. On the N-terminus half, there lies the ovarian tumour (OTU) domain which contains the C103 (cysteine-103) deubiquitin motif. This C103 ubiquitin motif, as suggested by its name, has deubiquitinase (DUB)

activity[6]. Similarly, on the C-terminus half, there are seven zinc finger motifs, with the fourth zinc finger (ZF4)[7] being the most significant in helping regulate the NFκB pathway. This fourth zinc finger functions to help recruit A20 and sequentially, bind to ubiquitin chains. Both of these motifs play an important role in the function of deubiquitinase A20[8].

Protein Ubiquitination

Proteins are ubiquitinated when they need to be localized to the proteasome to be degraded. In the process of ubiquitination[9], the small protein ubiquitin is attached to the target protein (the protein to be degraded). This attachment occurs through an isopeptide bond between the C-terminal of the carboxyl group on ubiquitin and the side chain of a lysine residue on the target protein. Since the protein ubiquitin itself also contains seven lysine residues, poly-ubiquitin chains can form[9]. The presence of seven different ubiquitination sites, K6, K11, K27, K29, K33, K48 and K63, allows for a multitude of linking combinations between ubiquitin proteins, allowing the formation of a variety of unique poly-ubiquitin chains, where different chains may signal different actions[10]. Specifically, K48 ubiquitin chains mark the target protein for degradation by the proteasome, while K63 ubiquitin chains are mainly used in signal transduction pathways[9]. These distinct lysine-specific actions are observed when comparing the K63 ubiquitination of TRAF6 and the ubiquitination of K48 IκB in the canonical NFκB pathway[9].

Poly-ubiquitination Mechanisms[2]

There are three main enzymes involved in the poly-ubiquitination mechanism[2] as shown in **FIGURE 2**[9]. First, the E1 ubiquitin activating enzyme, is responsible for creating a ubiquitin-E1conjugate, by attaching a ubiquitin protein from its C-terminus onto a cysteine residue on E1 using ATP hydrolysis[2]. Second, the E2 ubiquitin-conjugating enzyme, which transfers the ubiquitin previously attached to E1 to E2[2]. Last, the E3 Dubiquitin ligase, which transfers the ubiquitin previously attached to

E2 to the lysine residue on the target protein or on another ubiquitin protein part of a poly-ubiquitin chain[2].

The NFκB Pathways

NFκB is a transcription factor family that is stimulated by multiple ligands on varying receptors. In the cell, it is held in an inactive complex, where NFκB is bound to IκB in the cytoplasm[2]. Thus, NFκB activation occurs when this inactive complex dissociates, allowing NFκB to localize to the nucleus and act as a transcription factor[2]. This necessary dissociation is signalled to occur when the appropriate ligand binds to its corresponding receptor, instigating a signalling cascade[2].

The canonical pathway occurs when a toll-like receptor (TLR4)[11] is activated by its corresponding ligands like bacterial lipopolysaccharides (LPS), it recruits the adaptor protein MyD88. MyD88 recruits the IL-1 receptor associated kinases (IRAK), IRAK4 and IRAK1[2]. Sequentially, IRAK4 phosphorylates IRAK1[2]. This phosphorylation on IRAK1 recruits E3 ubiquitin ligase TRAF6[2]. Then, TRAF6 creates a poly-ubiquitin chain on its K63 residue[2].

As a non-canonical parallel pathway, when TNF receptor 1 (TNFR) is activated by TNFα, it recruits an adaptor protein TRADD[2]. TRADD recruits the receptor activating protein 1 (RIP1) and E3 ubiquitin ligases TRAF2[12]. TRAF2 creates a poly-ubiquitin chain on the RIP1 K63 residue[2].

This poly-ubiquitin chain ultimately recruits kinase TAK1 and the IKK complex[2]. Then, TAK1 phosphorylates the IKKβ subunit, thereby activating the IKK complex[2]. Once the IKK complex is activated, it phosphorylates IκB[2]. This phosphorylated IκB is then targeted for ubiquitination by TRAF on K48[2]. Finally, this poly-ubiquitin chain on IκB marks it for degradation, consequently, as IκB is degraded by the proteasome, NFκB is free to localize to the nucleus and activate transcription of various pro-inflammatory cytokine genes[2]. This pathway can be visualized in **FIGURE 3.**

THE ROLE OF A20 IN INFLAMMATION AND IMMUNITY

Mechanism of Inflammation

The ubiquitinase to deubiquitinase relationship is analogous to that of kinase to phosphatase relationship[2]. Since A20 is a deubiquitinase, it functions to remove the poly-ubiquitin chains attached by ubiquitinases. Both motifs of A20 assist it in such a task. These motifs bind ubiquitin, as well as supports its E3 ubiquitin ligase activity[2]. The C103 deubiquitin motif, specifically, restricts ubiquitination of RIP1[2]. The ubiquitination of RIP1, part of the pathway to ubiquitinate IKK in order to activate NFκB, also recruits A20, via its ZF4 motif[7], as a negative regulation mechanism[2]. A20, using its C103 deubiquitin motif, then functions to remove the K63 poly-ubiquitin chains on RIP1 and TRAF2/TRAF6 to restrict the activation of NFκB[2]. In addition, the C103 motif also inhibits E2-E3 enzyme interaction[2], thereby limiting the formation of ubiquitin chains to begin with. These two motifs of deubiquitinase A20 pose it as a key regulator in inflammation homeostasis via the NFκB pathway. Excessive inflammation is often linked to autoimmune disorders[2].

A20 in Myeloid Cells[13]

As known, A20 is a key regulator in the non-canonical NFκB pathway[2]. When the A20 gene *Tnfaip3* is knocked out in myeloid mouse cells, the mice develop enthesitis and paw inflammation[13]. This paw inflammation is dependent on IL-1β expression, suggesting idiopathic arthritis[13]. In *Tnfaip3*-deficient mice, increased levels of IL-1β, IL-6, IL-18 and TNFα are observed, along with elevated levels of Th17 cells and anti-collagen type II antibodies (classic signs of rheumatoid arthritis)[13]. In short, A20 regulates IL-1β and IL-18 release from macrophages, contributing to controlling the arthritis phenotype[13].

A20 in Dendritic Cells

During inflammation, monocyte derived dendritic cells (moDCs) are recruited to inflammatory sites[14]. When *Tnfaip3* is knocked out in all dendritic cells (DCs), most of their counts decreased while moDCs increased,[14] a sign of systemic inflammation leading to autoimmunity[14]. This suggests that A20 is involved in the survival of the decreased DCs and in the restriction of moDC numbers[14]. The apoptotic DCs release T-cell differentiating cytokines IL-12 and IL-13, which in turn increases Th1-cell and Th17-cell differentiation[14]. This results in three spontaneous phenotypes: inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) and in general, multi-organ inflammation[13]. In IBD and SLE, there are elevated levels of IL-6, whereas in multi-organ inflammation, there are increased levels of TNFα and IFNγ[13]. When the knockout mice receptors are agonized with TNFα, there is a spontaneous surge of pro-inflammatory cytokines, more so than the wild-type receptors[14]. In the wild-type receptors, 30 minutes after stimulation phosphorylation of IκB is downregulated since A20 is present[14]. In addition, DCs stimulate B-cell proliferation. When *Tnfaip3*-deficient DCs are used to stimulate B-cells, they proliferated much more so than the wild-type DCs. In short, pro-inflammatory cytokines and anti-apoptotic proteins in DCs and B-cell proliferation are heavily controlled by A20[13].

A20 in B-Cells

B-Cells are also regulated by A20 indirectly via DCs but also directly via B-cell receptor (BCR) signalling. When *Tnfaip3* is knocked out in B-cells, there is increased IL-6 production[13]. Additionally, there are more B-cells in this knockout due to increased anti-apoptotic protein expression, leading to enlarged spleens and secondary lymphoid organs[14]. The anti-apoptotic protein indicate that A20 is key regulator in B-cell selection, proliferation and survival[15]. These *Tnfaip3* B-cell specific knockout mice are also genetically predisposed to develop autoimmunity[15]. This suggests that A20 has a role in preventing the formation of autoantibodies, supported by the fact that *Tnfaip3* B-cell knock out mice

produce more autoantibodies than the wild type[15]. In short, A20 controls IL-6 production and anti-apoptotic protein expression in B-cells, resulting in the SLE phenotype[13].

A20 in T-Cells

Like BCRs, A20 also regulates T-cell receptors (TCR)-mediated NFκB activation[16]. When *Tnfaip3* is knocked out mature T cells, 80% of CD8+ T cells and 30% of CD4+ T-cells are affected, whereas with a *Tnfaip3* knockout in CD4+ T cells, 100% of both CD8+ and CD4+ T-cells are affected[13]. When the mature T cells are knocked out, the phenotype of inflammatory lung disease develops, along with elevated CD8+ T-cells[13]. In these T cells, when their TCR is bound, signals for NFκB activation, leading to more IL-17 and IFNγ release[13]. These cells end up dying from necroptosis and autophagy due to excessive inflammation[13]. In short, A20 regulates autophagy and necroptosis in T-cells, as well as IFNγ release, which is responsible for the inflammatory lung disease[13].

THE ROLE OF A20 IN VARIOUS DISEASES

A20 and Autoimmune Diseases

As stated earlier, SNPs in the *Tnfaip3* gene are linked with genetic predispositions to various autoimmune diseases. These autoimmune diseases result from excessive inflammation, as well as the production of autoantibodies. However, these phenotypes can also occur when the gene enhancers that bind to *Tnfaip3* are mutated[17]. When such an enhancer, named TT>A, is deleted *in vivo,* the humanized mouse experiences enhanced inflammatory responses, autoantibody product and commonly, inflammatory arthritis[17]. This specific enhancer is proposed to be in association with the autoimmune phenotype of SLE[17]. These observations establish that these autoimmune and inflammatory phenotypes can not only be linked to genetic mutations in the coding and non-coding regions of *Tnfaip3* but in its associated enhancers as well[17].

A20 and Inflammatory Bowel Disease

IBD occurs in the intestinal cells of the bowel. When the lamina propria cells are extracted from the gut of A20-deficient mice, they contained significantly increased numbers of macrophages and granulocytes, with enhanced expression of IL-12 and TNFα[18]. Additionally, T lymphocytes isolated from the same lamina propria cells contained elevated IFNγ levels[19]. Furthermore, A20 ablation in intestinal epithelial cells causes receptors to be hypersensitive to their ligands, like TNFR to TNFα and LPS to TLR, causing excessive ligand-induced inflammation[18].

A20 and Inflammatory Lung Disease[20]

In many inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF), are caused by prolonged NFκB activation in the cell[20]. Since the NFκB pathway starts with receptors like TNFα, TLR and TCR, such inflammatory lung diseases can be exacerbated by infections[20]. Normally, A20 is already regularly expressed upon exposure to infection, in order to terminate NFκB signalling. During infections, antigens or LPS bind to their corresponding receptors, activating the NFκB pathway, resulting in inflammation. This inflammation in a diseased lung is inflammation on top of the existing inflammatory lung disease. Specifically, in the airways, A20 negatively regulates TLR2 and TLR4-induced inflammation by reducing the production of IL-8[20].

A20 and Cancer

A20 is known to be a tumour suppressor in cancers, such as lymphomas[21], where A20 inhibits TNFα-induced apoptosis[19]. Lymphomas are associated with deletions in chromosome 6q[22], where *Tnfaip3* is located, which implicates A20 mutations for this phenotype[23]. In most lymphomas, nonsense or frameshift mutations prevented the translation of a full length A20 protein[23]. This partial A20 contains only the OTU domain of the N-terminus, making this truncated protein only capable of its DUB

activity[23]. With the loss of the C-terminus ZF4's E3 ubiquitin ligase capabilities, there is a loss of protein function[23]. This suggests that, in lymphomas, it is the C-terminus that contains the key region responsible for tumour suppression[23]. However, this tumour suppressor effect is not universal is across all cancers. Rather, it is dependent on what the dominant role of A20 in that specific cancer and tissue, whether it's inhibiting NFκB activation or inhibiting apoptosis[2]. For example, in pancreatic cancer cells, there is decreased A20 expression, whereas in glioma cells, the opposite is observed[24].

DISCUSSION

The Significance of A20

A20's key role in the ubiquitous NFκB pathway enables it to be significantly involved in many cells, as summarized in **FIGURE 4**. Its involvement in macrophages, dendritic cells, B-cells and T-cells means mutations in A20 impacts both innate and adaptive immunity, as well as inflammation. Not only does A20 affect each of these cell types individually and directly, but also indirectly, with plenty of crosstalk and chain reactions, like a complex, intertwined web. For instance, B cells are directly affected, but also affected via dendritic cells which in itself is directly affected by A20. Additionally, A20 is also involved in other signalling pathways, canonical and non-canonical, such as the Wnt[25], JAK/STAT and TGFβ signalling pathway[2]. Its heavy involvement in this vast number of cell types and pathways makes it a finicky target for therapeutics, requiring very precise and specific therapeutic methods, as opposed to the go-to broad spectrum treatments.

With such heavy involvement in various pathways, it is unsurprising that A20 mutations are the root of many diseases. This makes A20 a high area of interest since advances in this research would allow for the progression of clinical applications and treatments for the multitude of patients suffering from these widespread diseases, particularly the variety of autoimmune diseases and cancers.

Ultimately, A20 is truly significant as it is able to break the vicious cycle of signalling that begins with a pro-inflammatory cytokine or ligand binding to a receptor which signals to produce more pro-inflammatory cytokines. Without A20 keeping this system in check, there is constitutive signalling and inflammation that could potentially transform into an autoimmune disease or cancer in the long run.

Therapeutic Implications

As A20 is mutated or altered in its phenotypic diseases, it makes A20 a suitable target for therapies and treatments. In most conditions, A20 is dysregulated, or is suffering a complete loss of function. In these cases, constitutive expression of A20 rescues this mutant phenotype and alleviates the condition[8]. This signature mutation in A20 in many autoimmune diseases makes it an obvious biomarker for CD, SLE, and psoriasis, facilitating diagnosis of these diseases.

Currently, the most common treatment for A20-dysregulation/deficient diseases is anti-TNFα therapy, reducing the ligand instigating the signal cascade instead[8]. However, A20-specific therapeutic approaches are in development. A few miRNAs[6] that silence A20 have been discovered and are targets of therapy to increase A20 expression[8]. Gene therapy is on the rise to restore A20 function, the only caveat being the delivery system to execute this form of treatment[8].

Limitations

A20 mutations are associated with loss of function of the protein. This poses a problem in treatment, as most modern-day treatments are based on decreasing an overly expressed protein, as opposed to restoring the function of a protein[19]. Restoring the function of a protein is notably more difficult than knocking down the expression of a constitutively expressed protein. Currently, there is no pharmaceutical method to induce or simulate A20 expression[20]. The best method to restore protein function would be to transfer a functional gene of A20 via gene therapy and editing[20]. Unfortunately, there is currently no standard method of executing this delivery[20]. To make matters more complicated,

due to the ubiquitous nature of the NFκB pathway, it is too dangerous to directly inhibit the pathway entirely as it would hinder both the innate and adaptive immunity of a patient.

CONCLUSION

Despite the many advancements in the area of research regarding A20 throughout the years, there is still much more work to be done. A lot more research must be conducted before any findings can be applied in a clinical aspect, especially in the pharmaceutical and gene therapy fields in order to restore A20. With that being said, the outlooks in this area of study are promising, with positive outcomes on the horizon.

FIGURES

Figure 1: Tissue-specific A20 deficiency[3]

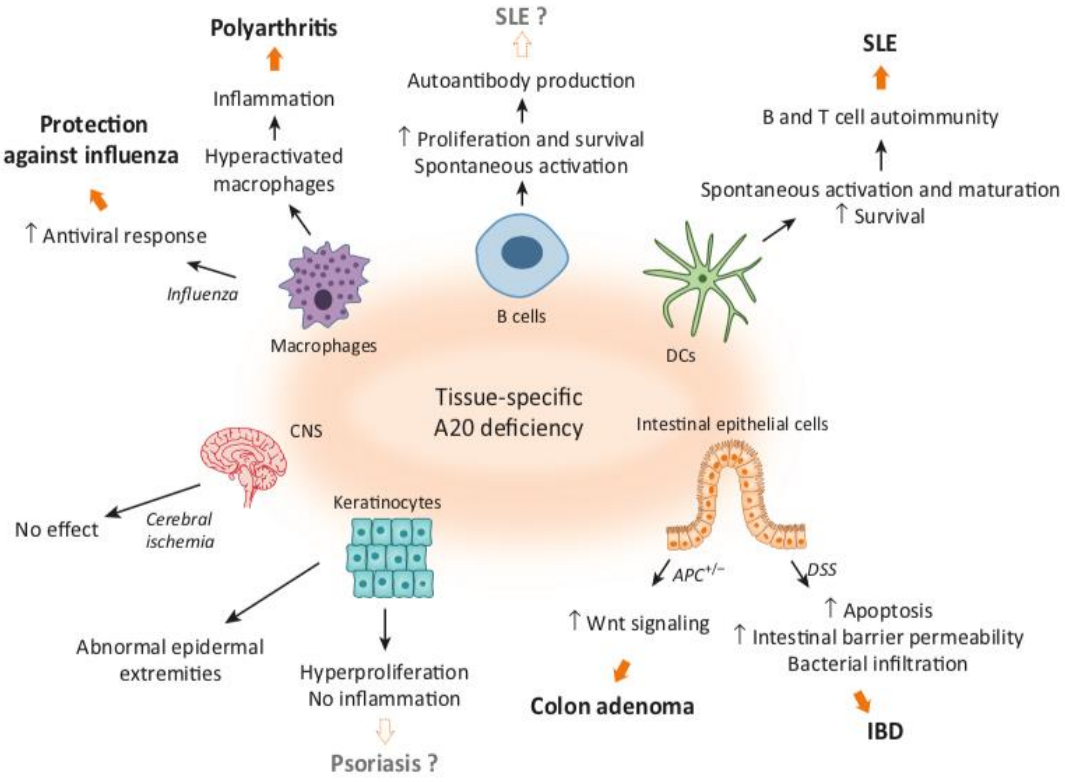


Figure 2: Mechanism of Ubiquitination[9]

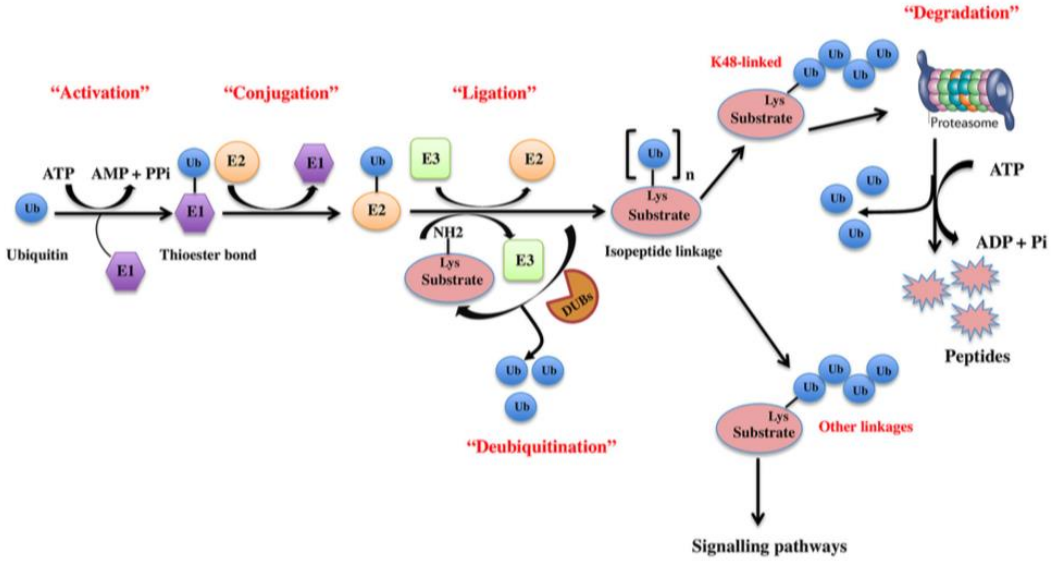


*Figure 3: The two pathways of NFκB activation**

*The number of ubiquitins and lysines is not representative of their actual numbers.

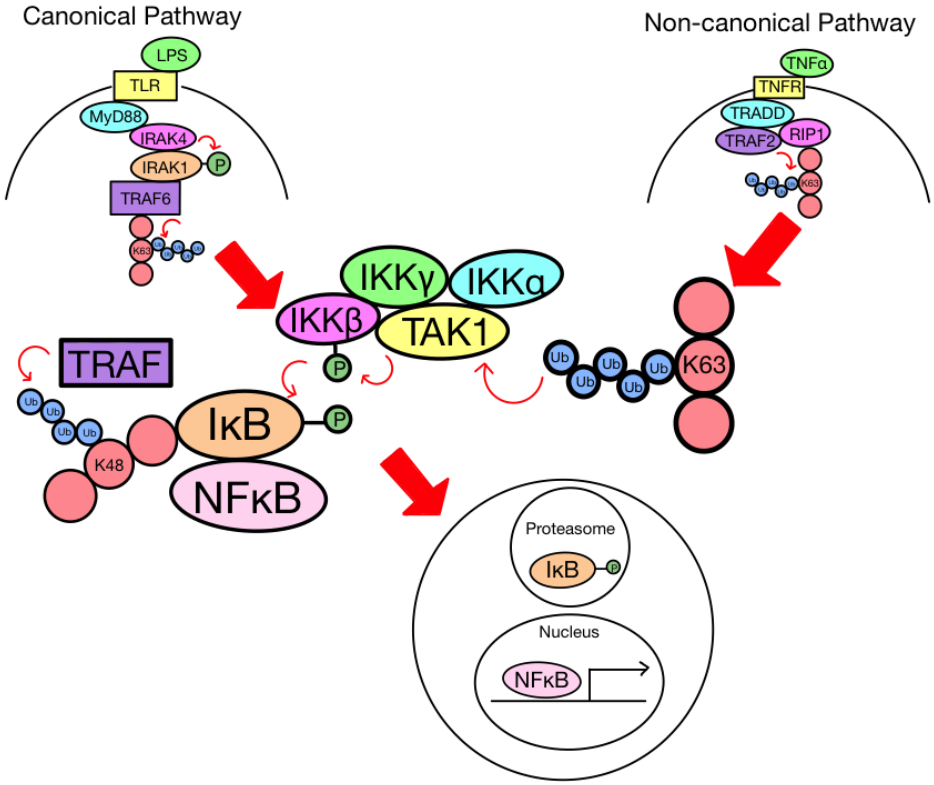
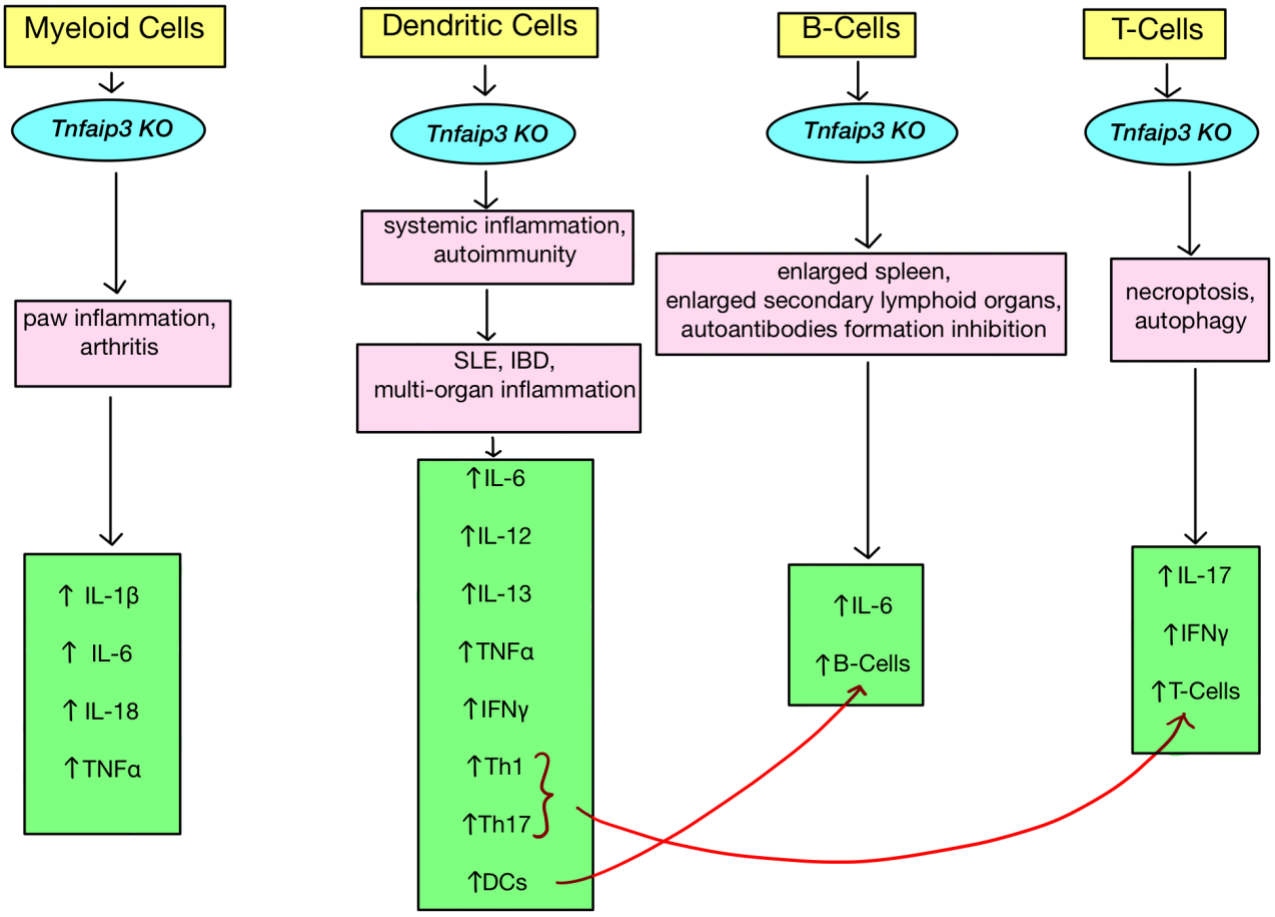


Figure 4: A20 Cell Type Summary Chart



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