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The Three As: Alternative Splicing, Alternative Polyadenylation and their impact on Apoptosis in Immune Function

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Summary

The latest advances in next-generation sequencing studies and transcriptomic profiling over the past decade have highlighted a surprising frequency of genes regulated by RNA processing mechanisms in the immune system. In particular, two control steps in mRNA maturation, namely alternative splicing and alternative polyadenylation, are now recognized to occur in the vast majority of human genes. Both have the potential to alter the identity of the encoded protein, as well as control protein abundance or even protein localization or association with other factors. In this review we will provide a summary of the general mechanisms by which alternative splicing (AS) and alternative polyadenylation (APA) occur, their regulation within cells of the immune system, and their impact on immunobiology. In particular, we will focus on how control of apoptosis by AS and APA is used to tune cell fate during an immune response.

Keywords

Alternative Splicing; Alternative Polyadenylation; Apoptosis; T cell activation

The central dogma of biology states that DNA is transcribed into RNA, and RNA is translated into protein. At the RNA level, there are a multitude of processing events involved in the maturation of a nascent transcript (pre-mRNA) to a final protein-encoding messenger mRNA, each of which has the potential to expand the protein repertoire to ultimately impact cell activity[1–5]. Alternative splicing (AS) occurs during RNA processing and has the potential of altering protein identity and expression and therefore cellular function[6–9] (Fig. 1,2). Similarly, alternative polyadenylation (APA) impacts the length of the final mRNA, with the potential to truncate the encoded protein or alter the presence of regulatory elements in the 3' untranslated region (3' UTR) to control protein expression[10,11] (Fig. 2). In recent years there has been a growing body of literature revealing the breadth to which both AS and APA are controlled in the immune system, an increased understanding of the mechanisms driving these processes, and a recognition of the extent to which AS and APA impact the response of lymphocytes to immune challenge (see[12,13] and references below). In this review, we summarize the general mechanisms driving AS and APA and

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Conflict of Interest

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provide both classic and recent examples of how AS and APA are regulated in immune cells. Moreover, as a model for the functional consequence of AS and APA in immunology, we focus on AS and APA events in genes encoding apoptotic signaling proteins. Immune cells are especially interesting in the context of apoptosis as dysregulated apoptosis in immune cells plays a large role in autoimmunity, where prolonged and unregulated stimulation has damaging effects to the host[14,15]. Thus, the dynamic AS and APA changes that occur in apoptotic related genes in activated immune cells, combined with the reliance on apoptosis to downregulate the activity of immune cells following immune perturbance, provides an important framework for understanding the critical nature of mRNA processing events such as AS and APA in tuning the reactivity and homeostasis of the immune system.

Alternative splicing

Alternative splicing (AS) is a co-transcriptional process that occurs during RNA processing and gives rise to unique RNA transcripts in the cell[6,16]. The most common types of AS patterns include whole exons that are selectively included or removed from the mature RNA transcript, otherwise known as cassette exons[17] (Fig. 1). Other known patterns of AS events are described in Figure 1, all of which alter the identity of the resulting mRNA. Remarkably, genome-wide studies suggests that approximately 95% of human genes undergo AS to yield at least one, and often several, alternative isoforms[17,18]. Because AS increases the number of unique mRNA transcripts in a cell, AS increases proteome diversity[2,5,6]. Protein isoforms that arise from alternative splicing have been shown to exhibit changes in enzymatic activity, localization, or stability, and thus to impact cell activity[6,7]. While the impact of every alternative splicing event in each gene has yet to be characterized, there are numerous studies that exemplify the necessity of regulated AS events for the maintenance of homeostatic cell activity and demonstrate how the dysregulation of AS can lead to disease[19–21]. In addition, beyond single examples of AS events impacting cell activity, concerted changes in AS have been observed in genome-wide studies and shown to function cooperatively to govern global cell states and tissue maintenance[22–25]. Therefore, understanding how AS is regulated and coordinated in cells is critical to understanding many physiologic responses, including the response of immune cells to pathogen challenges.

Mechanisms of Alternative Splicing

The spliceosome is a large macromolecular complex that is essential for constitutive splicing of RNA and can be regulated to give rise to AS products[16,26]. The spliceosome is made up of several small nuclear ribonucleoparticles (U1, U2, U4–U6 snRNPs), each of which is comprised of a small RNA and multiple proteins (Fig. 2a). These snRNPs sequentially bind to the 5’ and 3’ splice sites and branch point sequences on pre-mRNA to direct and catalyze the cleavage of introns and the ligation of exons during constitutive splicing[6,16,26]. The location and efficiency of splicing carried out by the spliceosome can be regulated by a variety of factors including splice site strength, transcription elongation rates, single nucleotide polymorphisms (SNPs), and RNA binding protein (RBP) activity[6,16,26]. In this review, we focus mostly on the impact of RBPs on alternative splicing, as RBPs most often regulate condition-specific splicing changes in cells by binding to intronic or exon sequences near splice sites on an immature RNA transcript to block or enhance spliceosomal

association[16] (Fig. 2b). Sequences that recruit RBPs to enhance splicing are called exonic splicing enhancers (ESE) and intronic splicing enhancers (ISE), while sequences that inhibit splicing are called exonic splicing silencers (ESS) and intronic splicing silencers (ISS)[16]. Notably, the same sequence motif can function as any of these activities, as the definition is based on the location of the sequence and the ultimate impact on splicing, which results from both the RBP that is recruited and the context of the surrounding sequences (see below). Changes in expression, post-translational modifications, and/or localization of RBPs between cellular states alter the balance of activities on these enhancer and silencer sequences to ultimately alter AS patterns[16,27] (Fig. 2b).

RBP Regulation of Alternative Splicing

Splicing regulatory RBPs in humans can largely be divided into three main classes – SR proteins, hnRNP proteins, and hnRNP-like proteins (Fig. 3). Each class of proteins typically contain RNA binding motifs and one or more functional domains that mediate protein-protein interactions. SR proteins are defined by containing at least one RRM (RNA Recognition Motif)-type RNA binding domain as well as a domain comprised of serine-arginine (SR) dipeptides that facilitates homotypic interactions[6,28], while hnRNP and hnRNP-like proteins are more heterogeneous in their domain composition and can contain both hnRNPK-homology (KH) (not shown) or arginine-glycine (RGG) RNA binding motifs, as well as RRM motifs. Traditional hnRNP proteins are defined based on their initial co-purification and discovery and are typically more abundant and/or ubiquitously expressed than hnRNP-like proteins, which are RBPs that have hnRNP-like domains but have been isolated independently[29,30]. All of these three classes of RBPs typically bind to short sequence motifs with a range of promiscuity and redundancy[6,31,32]. This allows transcripts to simultaneous ensure the ability to bind a necessary subset of RBPs while still adhering to coding or other regulatory constraints.

Importantly, the RBPs discussed above function in a highly combinatorial manner such that even modest changes in the abundance or activity of individual RBPs or core spliceosomal proteins can result in large splicing changes in particular transcripts[6,16]. Moreover, each gene is regulated by a discrete subset of RBPs, with each RBP typically regulating several hundred splicing events[6,16]. Overall, this combinatorial pattern of RBP association results in a complex network of coregulation, in which any change in RBP expression or activity can potentially impact the splicing of some or many transcripts (e.g. see Fig. 4 below). Consistently, signal- or developmental-responsive changes in AS are typically mediated by changes in RBP abundance due to either regulated transcription or mRNA stability of the messages encoding RBPs, or changes in activity or localization mediated by post-translational modification of the protein itself (e.g. see reviews[27,33]). Examples of such regulation of AS via RBPs in activated immune cells are detailed later in this review.

Alternative Polyadenylation

Beyond AS regulation, protein expression in cells is also controlled at the RNA-level through the presence or absence of regulatory sequences located in the 5’ and 3’ untranslated regions (UTRs) of mRNA[10,34]. In particular, 3’ UTRs are the primary site of miRNA

association and function[10,11] as well as containing binding sites for proteins that regulate translation, mRNA stability, and subcellular localization of mRNA[10,11,35]. Indeed, even protein phosphorylation state, protein complex assembly, and overall protein activity has been shown to be influenced by the presence of regulatory elements in 3'UTRs[36,37].

Importantly, the length of the 3'UTR of a given gene is not static, but rather can vary in a tissue and condition-specific manner through the process of alternative polyadenylation (Fig. 1, 2c,d). Polyadenylation itself, which occurs on virtually all mRNAs, as well as some non-coding RNAs, involves the cleavage of the nascent RNA during transcription, followed by the subsequent addition of ~200 adenosine bases to the exposed 3' hydroxyl end[38]. Alternative polyadenylation (APA) refers to instances in which cleavage, and subsequent polyadenylation, occurs at different positions on a transcript in a condition or cell-type specific manner[11] (Fig. 1). APA results in the truncation of the open reading frame if cleavage occurs prior to the final coding exons (e.g. either version of the alternate terminal exon, Fig, 1). On the other hand, when both or all of the cleavage events are downstream of the stop codon, APA results in 3'UTRs of distinct lengths and sequence (Fig. 1, Tandem 3'UTR). Since 3'UTR identity determines the ultimate expression and regulation of the upstream open reading frame, as described above, APA after the stop codon can have a profound effect on protein expression or function[10,11,35,39]. Notably, recent transcriptome-wide studies have observed APA in over half of human protein-coding genes[11,40]. Therefore, regulation of APA provides an additional prevalent level of RNA-based control of cellular activity.

Mechanisms and Regulation of Alternative Polyadenylation

Cleavage and polyadenylation is mediated by a large multi-component complex consisting of a core CPSF (Cleavage and Polyadenylation Stimulation Factor) subcomplex as well as additional enhancer subcomplexes CFI (cleavage factor I), CFII (cleavage factor II) and CstF (cleavage stimulatory factor), each of which are comprised of 2–6 proteins[38] (Fig. 2c). These four subcomplexes (aka the Cleavage and Polyadenylation Factors or CPAFs) associate in a cooperative manner with genomic-encoded sequence motifs comprising a hexameric AAUAAA and upstream and downstream U/G-rich sequences (collectively known as the Polyadenylation Sequence or PAS). The AAUAAA hexamer is the primary binding site for the CPSF complex and directs the location of cleavage, whereas CFI binds to an upstream UGUA and CstF associates with a U/G-rich element downstream of the core hexamer[38] (Fig. 2c). Importantly, since each of these interactions is relatively weak, the cooperative assembly of CPSF with CFI and CstF helps ensure efficiency of binding and accurate positioning of the overall complex; the strengthening of one interaction can compensate for suboptimal (aka "weak") interactions at other regions[11]. Indeed, much regulation of APA is thought to be controlled by the expression level of various CPAFs, in which increases in abundance of proteins such as the RNA-binding component of CstF (CstF64) increases the use of weak APA site through promoting occupancy of low affinity sites through mass action ([41,42] and see also below).

Additionally, the efficiency of CPAF complex recruitment to specific locations on the pre-mRNA substrate can also be controlled by RBPs in a manner analogous that the regulation

of AS (Fig. 2d). Several SR, hnRNP and hnRNP-like RBPs have been shown to promote recruitment of CPAFs to weak PAS sites or to compete with complexes such as CFI or CstF for their cognate binding sites[43–45]. As described above for the regulation of splicing, changes in the expression or activity of RBPs in response to environmental or developmental cues can redirect the activity of the CPAFs on individual substrate pre-mRNAs to alter 3'UTR identity of specific genes. Other recent reviews provide an excellent comprehensive discussion of the mechanisms and regulation of APA[11,38], while below we focus on specific examples of APA regulation in the immune system.

AS and APA in the Immune System

Much early work on gene expression in the immune system made use of microarrays and the use of exon junction or isoform-specific probes to infer splicing or polyadenylation changes allowed several early studies demonstrating widespread AS changes across the transcriptome during activation of human CD4+ and CD8+ T cells[46] and APA changes upon activation of T cells, B cells and monocytes[39]. However, the microarray platform was never optimized to detect APA or AS[47,48]. Therefore, most early predictions of AS or APA in the immune system were incomplete and not well validated experimentally[12]. In the past few years, however, there has been a tremendous increase in our appreciation for AS and APA throughout biology due to advances in next-generation sequencing such as increasing the number and length of sequencing reads obtained by short-read RNA-Seq, the emergence of long-read RNA-Seq, and the development of robust methods to quantify AS and APA from both such RNA-Seq data[42,49,50].

One of the first studies to apply RNA-Seq data for AS prediction in the immune system was from our own lab, studying activation of primary and cultured human T cells[51]. This work resulted in the identification of ~200 genes that exhibited robust, validated AS changed upon activation of Jurkat cells with PMA and primary CD4+ T cells with PHA[51]. Since then, many groups have used RNA-Seq data to gain a genome-wide view of AS and/or APA in various primary immune cell types (e.g. see[52–61] and Table 1 for several examples). Together these studies have confirmed the broad impact AS and APA have on gene expression during the innate and adaptive immune responses. In addition, the deposition in public repositories of RNA-Seq data from these and other studies is a tremendous benefit for future discovery, as this enables investigators to directly compare transcriptomes from a wide range of conditions to seek further insight. For example, RNA-Seq data collected for gene expression studies by one group, may be used to investigate AS or APA by others (e.g.[52]). It is anticipated, therefore, that our appreciation for the depth of regulation of immune cell function by AS and APA will grow tremendously in the near future. Already there are many clear examples of how AS and APA impact immune responses. Below we discuss some of the best studied instances of AS and APA in immune cells and what is known about the regulators and mechanisms driving AS and APA in T cell, B cells and macrophages.

Prototypical regulators and regulation of AS in Immune Cells

Two classic examples of AS regulated by immune activation, discovered more than 20 years ago, includes the regulated splicing of CD45 (PTPRC) upon T cell activation[62–64] and

the alternative splicing of TLR4 signaling proteins in LPS-treated macrophages[65,66] (Fig. 4). Both examples display induced changes in isoform expression, limiting the extent of cellular signaling in response to subsequent stimuli, thereby preventing prolonged activation and, in turn, promoting recovery of the immune system to a basal state. Consistently, defects in CD45 and MyD88 AS have been linked to hyperactivity of immune cells and the development of autoimmune diseases or cancer[67–70]. More recently, the functional impact of many additional activation-induced AS events in lymphocytes and macrophages have been studied in detail. Since several reviews have previously described many of these cases in depth[12,13], we focus below on only a few of the most extensively studied examples of AS to provide an overview of the functional impact of AS in the immune system and highlight what is known about the mechanisms, signaling pathways and RBPs connecting splicing to immune challenge.

CD45 and the global impact of hnRNP L and LL in T cell biology—CD45 is a transmembrane protein tyrosine phosphatase that is expressed on the cell surface of nucleated hematopoetic cells[71]. The intracellular phosphatase activity of CD45 plays a critical role in antigen signaling in lymphocytes by promoting signaling cascades triggered upon engagement of the T cell receptor (TCR) or B cell receptor (BCR). By contrast, the extracellular domain regulates CD45 phosphatase activity. As early as the late 1980s it was recognized that three exons (exons 4–6, also termed A-C in the older literature) within the gene encoding CD45 (now called *PTPRC*) undergo alternative splicing[72]. Exons 4–6 encode a portion of the extracellular domain of CD45, thus their differential inclusion impacts the ability of antibodies to interact with CD45 on the cell surface[71]. In particular, exclusion of exon 4–6 results in a smaller isoform that specifically interacts with CD45RO antibodies, while isoforms encoded by mRNAs that include exons 4, 5 or 6 react with antibodies CD45RA, RB and RC respectively[71,73]. The ability to detect different isoforms of CD45 by flow cytometry led to the recognition that isoform expression is a useful marker of cell identity and state. Namely B cell express an isoform (B220, also called RABC) that is recognized by the RA, RB and RC antibodies (includes all of exons 4–6), while naïve T cells predominantly express an isoform reactive to RA and RB (primarily includes exons 4&5)[71,73]. Perhaps most importantly for both immunology and understanding splicing regulation, T cells switch from the RA to the RO isoform upon antigen stimulation due to repression of exon 4 (and 5 and 6 to a lesser extent) inclusion upon activation[71,74]. The smaller (RO) isoform lacks bulky glycosylation and sialyation groups on the extracellular side and thus is more prone to homodimerization than the larger RA isoforms[75]. As dimerization causes steric hindrance of the intracellular phosphatase domain, the switch from RA to RO upon activation decreases phosphatase activity, effectively downregulating subsequent antigen signaling[75–77]. Consistently, several studies have suggested that the switch in CD45 expression from RA to RO is critical to maintain T cell homeostasis, and removing this “break” leads to immune hyperactivity[77] (and see discussion of C77G SNP below).

The CD45 RA to RO switch has been used extensively by immunologists to track T cell activation, but also provides an excellent model for mechanistic studies of activation-induced splicing regulation. Indeed, several groups, including our own, have examined

CD45 AS as a model for identifying RBPs that regulate splicing in immune cells and in response to antigen stimulation (Fig. 4). Each of the three variable exons of CD45 contain a conserved Activation Responsive Sequence (ARS) that represses inclusion (i.e. functions as an inducible Exonic Splicing Silencer (ESS))[78,79]. In naïve T cells, the ARS is primarily bound by hnRNP L, which exerts a weak level of repression[79,80]. However, upon T cell activation, two additional proteins – the hnRNP L homologue hnRNP L-like (hnRNP LL) and PSF (aka SFPQ) – also are recruited to the ARS elements and provide much greater repressive activity, thus promoting skipping of exons 4–6 and production of the RO isoform[81–84]. The signal-responsive recruitment of hnRNP LL and PSF are due to distinct regulatory mechanisms. HnRNP LL is poorly expressed in naïve T cells and undergoes significant transcriptional upregulation in response to antigen stimulation[82–84]. By contrast, PSF is hyperphosphorylated in naïve T cells by the kinase GSK3 and bound to a partner protein, TRAP150, that blocks access of RNA to the RNA-binding domain (RRM) of PSF[85,86]. Upon activation, GSK3 activity is downregulated and PSF thus loses phosphorylation on key residues that are critical for interact with TRAP150 – thereby allowing association between PSF and RNA[85]. The functional significance of the association of hnRNP L/LL/PSF complex with RNA is highlighted by the presence of a SNP within the ARS sequence of exon 4 (C77G) that results in aberrantly high levels of CD45RA, and corresponding low expression of CD45 RO[87–89]. This SNP weakens the overall association of proteins with the ARS and, consistent with the above functional consequence of the RA to RO switch, correlates with susceptibility to autoimmune diseases[67,90–92].

Finally, it is worth noting that in addition to hnRNP L, LL and PSF, CD45 exon 5 is also regulated by the presence of CTCF bound to DNA[93] (Fig. 4). CTCF is a DNA-binding protein that regulates genome architecture, functioning as both an insulator between genes and a regulator of transcriptional pausing within genes[93–95]. In the CD45 gene, binding of CTCF to the DNA encoding exon 5 results in pausing of RNA Polymerase II (Pol II) during transcription, which in turn promotes assembly of the splicing machinery on the weak splice sites of exon 5, thereby increasing inclusion of exon 5 in the final mRNA[93]. Such activity of CTCF in regulating splicing is consistent with a general mechanistic interplay between transcription and splicing[96–98]. In particular, numerous studies have demonstrated that the speed of Pol II elongation can directly impact AS patterns by influencing competition between splice sites or splicing regulatory elements[97]. In the case of CD45, the impact of CTCF in controlling Pol II elongation, and thus exon 5 inclusion, is regulated by both the expression level of CTCF in B cells and by methylation of its DNA target in T cells[93]. In particular, activated and memory T cells have increased DNA methylation as compared to naïve T cells and this increased methylation reduces binding of CTCF resulting in the observed loss of exon 5 inclusion upon T cell activation and in the transition from naïve to memory T cell state[93,99]. In thinking about this mechanism, it is worth noting that the transcription elongation factor ELL2 has also been implicated as a general regulator of splicing in B cells, although the specificity of this effect is not fully understood[100].

In sum, the regulated splicing of CD45 provides both an excellent example of the functional relevance of AS in lymphocytes and also has led to the identification and characterization of many factors involved in tuning splicing in a condition-specific manner in T cells. In particular, the induced expression of hnRNP LL and regulated activity of PSF that

is observed upon T cell activation provide prototypical examples of how RBPs can be regulated in a signal-dependent manner[27], while regulation of CTCF binding via DNA methylation represents a general model for how epigenetic marks may influence splicing[101].

PSF has a complex and poorly identified RNA recognition sequence, and depletion of PSF has not yet been linked to widespread splicing changes in lymphocytes[86,102]. Similarly, although genome-wide studies have identified a number of regulated splicing events in T cells that are directly bound and regulated by CTCF, the functional consequence of these remain unexplored[99]. By contrast to PSF and CTCF, hnRNP L and hnRNP LL both clearly play a major role in shaping immune function. These homologues are both highly expressed in activated T cells, bind efficiently to CA-rich elements in RNA, and have been shown to widely regulate splicing in T cells[82–84,103–106]. Analysis of in vivo binding of hnRNP L in primary human CD4+ cells shows enriched binding to genes involved in Wnt and TCR signaling[104], and knock-out of hnRNP L in developing thymocytes results in increased Lck activity, a defect in transition to CD4+CD8+ double positive thymocytes and lack of migration of single positive cells to the periphery[103]. Similarly, several lines of evidence suggest that hnRNP LL has widespread impact on AS and function in both B and T cells. hnRNP LL increases upon differentiation to memory T cells and plasma cells from naïve T and B cells respectively and regulates the splicing of many genes involved in immune function and the differentiated state[82–84,105,106]. Moreover, mice with a hypermorphic allele of hnRNP LL exhibit a decrease in circulating naïve and memory CD4+ T cells due to lack of persistence of these cells in the periphery[84]. Given that hnRNP LL is highly enriched in lymphoid cells over many other tissues, this protein may be uniquely evolved to regulate splicing during the adaptive immune response.

MKK7, LEF1 and regulation by CELF2—Another RBP that is highly regulated in lymphocytes is the hnRNP-like CUG-binding protein and ELAV-Like Factor (CELF2, Fig. 3). During thymocyte development and in response to T cell activation, CELF2 is regulated at the level of transcription, by mRNA stability and the level of splicing, to alter the expression of active protein[107,108]. Transcription of CELF2 is induced by NFkB, which binds near the transcription start site of CELF2 in TNF or PMA-activated cells[107]. By contrast, the stability of the CELF2 mRNA is promoted in a JNK-dependent manner[107,109]. Together these signaling events result in an increase in CELF2 mRNA and protein.

As its name implies, CELF2 binds to CUG and UG-rich repeats in RNA[110]. When this binding occurs in or around exons in a nascent transcript, the result is often CELF2-dependent changes in splicing[110,111]. Indeed, both targeted and whole-transcriptome RNA-Seq studies have identified hundreds of genes that are regulated in a CELF2-dependent manner in cultured Jurkat T cells, many of which overlap with JNK-regulated AS events in primary CD4+ cells and/or AS events that are regulated upon the DN to DP transition in thymic development[107,109,111,112]. Two of the best characterized of these CELF2-dependent splicing events, in terms of both mechanism and functional impact, are observed in the genes encoding the LEF1 transcription factor and the MAP kinase, MKK7 (Fig. 4).

In the case of LEF1, CELF2 binds to a regulatory element downstream of variable exon 6 to promote its inclusion[108,111]. Inclusion of exon 6, in turn, adds 28 amino acids to a regulatory

domain of LEF1 which promotes its ability to activate transcription from the TCRalpha promoter[108]. Thus, increased expression of CELF2 during thymocyte development is a likely driver of TCRalpha expression as cells progress from the DN to DP state[107], although this has not been directly proven in vivo.

In contrast to its activity in promoting exon inclusion in LEF1, CELF2 inhibits inclusion of exon 2 in the gene encoding the JNK kinase MKK7[109]. Specifically, binding of CELF2 immediately upstream of MKK7 exon 2 blocks spliceosome assembly at this exon and promotes exon skipping[111]. Exon 2 encodes 16 amino acids that disrupt the docking domain through which MKK7 interacts with its substrate JNK, therefore CELF2-induced skipping of exon 2 enhances MKK7 phosphorylation of JNK and subsequent JNK signaling[109]. As mentioned above, expression of CELF2 itself is enhanced by JNK signaling, thus the regulation of MKK7 by CELF2 provides a feedforward regulatory loop to augment JNK signaling, and subsequent cytokine release, in response to T cell activation[109]. Together, LEF1 and MKK7 provide two examples in which CELF2-dependent splicing clearly tunes the activity of T cells to promote optimal development and responsiveness of the immune response. Moreover, the differential activity of CELF2 on LEF1 and MKK7 underscore a common rule in splicing regulation, namely, RBPs can have both positive and negative impacts on exon inclusion depending on the location and context of their binding site[16,111]. Although the mechanistic basis of such differential activity has not been fully characterized, one explanation is that when a RBP binding site directly overlaps a splice site, competitive binding leads to exon skipping; while binding of an RBP adjacent to a site required for spliceosome assembly can promote splicing through cooperative protein-protein interactions[16,111].

Regulation of AS in innate immunity – TLR4 signaling and hnRNP M—As alluded to above, the innate immune response is also tuned by alternative splicing. This is best demonstrated in the TLR4 signaling pathway (Fig. 4). Toll-like receptor 4 (TLR4) is a transmembrane protein, one of several pattern recognition receptors (PRRs) that recognize features of microbial infection, and is found to be critical for tailoring the immune activation response of APCs including dendritic cells and macrophages[113]. When bound by an agonist such as lipopolysaccharide (LPS), TLR4 nucleates an intracellular complex via MyD88 that recruits several IL-1 receptor-associated kinases (IRAK) including IRAK4 and IRAK1[13,114]. When co-associated with MyD88, IRAK4 phosphorylates IRAK1, which in turn propagates signaling leading to activation of NF-kB[114]. Notably, TLR4, MyD88 and IRAK1 all undergo AS in response to LPS stimulation in a manner that attenuates further signaling[13]. In the case of TLR4, LPS stimulation of monocytes or macrophages leads to inclusion of a so-called “poison” exon (i.e. one that includes a stop codon), prior to the transmembrane domain, such that the resultant encoded protein is secreted and competitively binds to soluble LPS, thus limiting engagement of cell-bound receptors[65,115].

Similarly, in response to LPS stimulation exon 2 of the gene encoding MyD88 is skipped, resulting in an in-frame deletion, removing the portion of MyD88 that is necessary for IRAK4 association. The resulting smaller MyD88 isoform (MyD88s), functions as a dominant negative of TLR4 signaling as incomplete complex assembly on TLR4 no longer leads to IRKA1 phosphorylation[66,116]. Finally, IRAK1 also exists as multiple isoforms

through AS, which differ in their ability to interact with and activate downstream effectors in inflammatory signaling[117–119]. Surprisingly, despite the clear functional significance of the LPS-mediated AS of TRL4, MyD88 and IRAK-1, little is known about the mechanisms through which this splicing regulation is conferred. Although changes in the expression of core splicing components have been shown to alter the balance of some of these splicing choices[69,120,121], much work remains to uncover the signaling pathways and RBPs that tune LPS sensitivity of macrophages via AS of these key genes.

Although gene-specific regulators of TLR4, MyD88 and IRAK1 splicing have not yet been identified, one RBP that has been conclusively linked with AS and TLR4 signaling is hnRNP M[53]. HnRNP M is a repressor of splicing of weak introns in IL-6 and other genes involved in the innate immune response, reducing the expression of fully processed transcripts. Consistently, hnRNP M attenuates innate immune signaling as demonstrated by the fact that macrophages depleted of hnRNP M exhibit higher expression of pro-inflammatory cytokines, such as IL-6, upon LPS activation and reduced infection with virus or Salmonella[53]. In other words, knockdown of hnRNP M leads to hyperactivation of macrophages in response to viral and bacterial pathogens. This repressive activity of hnRNP M is partially countered by TLR4 signaling, which phosphorylates hnRNP M, thereby reducing its association with IL-6 and other target genes and resulting in an increase in IL-6 expression[53]. In sum, while much more remains to be discovered regarding the molecular mechanisms of AS during the innate immune response, it is clear that regulation of splicing plays an essential role in shaping the response of the innate immune system to viral and bacterial pathogens.

Prototypical regulators and regulation of APA Regulation in Immune Cells

Compared to AS, our understanding of the mechanisms and impact of APA regulation in immune cells, and across all of biology, is still relatively limited. AS has been studied in more depth and detail than APA perhaps due to the notion that changes in AS more frequently have a predictable consequence on protein expression than changes in APA. Nevertheless, the transcriptome-wide regulation of APA in human cells, particularly the global trends displayed in anti-CD3/CD28 activated CD4+ T cells described above ([39] and Table 1), highlights the need to investigate the regulation of APA in the immune system. Indeed, already there are a few well documented examples of APA playing a robust role in the behavior of the immune system, and control of APA in immune cells has been observed to be driven both by changes in the expression of core CPAFs as well as via the activity of specific RBPs. Below, we review several of the best characterized examples of the mechanism and impact of APA in immune cells to both provide an overview of what is known and to motivate further studies.

Ig heavy chain and regulation by altering abundance of a core CPAF: CstF64

—One of the first examples of regulated APA in human cells was identified through the study of the switch from membrane-bound to secreted forms of immunoglobin heavy chain (IgH) that occurs for at least the mu isotype of IgH during B cell activation. In 1980, the groups of Baltimore, Hood and Wall simultaneously reported that the membrane-bound form of IgH expressed in pre-B and primary B cells, and the secreted form of IgH that

predominates in plasma cells, are generated by differential processing of the 3' end of the gene encoding the Ig heavy chain[122–124]. The gene encoding IgH has two alternative PAS sites[125]. The first is after the exons encoding the constant region of IgH (PASs), while the second is after two additional exons that encode the transmembrane domain (PASm) (Fig. 5). In primary B cells the constant-region exons are spliced to the transmembrane exons. By contrast, upon differentiation to plasma cells the IgH message is cleaved at the first PAS site immediately after the constant-region exons. This prevents the transmembrane exons from being transcribed or spliced to the rest of the message, such that the encoded protein lacks a membrane-anchoring domain and thus is secreted into the extracellular environment[125].

As described above, PAS sequences and their surrounding contexts can differ in the affinity with which they recruit core components of the cleavage and polyadenylation machinery (see Fig. 2c, d). In particular, the sequence that recruits the protein CstF64 is highly degenerate, thus CstF64 exhibits a wide range of binding affinities to these target sites. The CstF64 binding site adjacent to the PASm in IgH is a high affinity "strong" site[126]; thus even at low cellular concentrations of CstF64 this protein binds to this distal site in IgH to promote recruitment of the CPSF complex and cleavage of the transcript. By contrast, the CstF64 binding site at the proximal PASs has lower affinity for CstF64[126]. Therefore, CstF64 does not nucleate the CFAP complex at this more proximal PAS site unless the abundance of CstF64 is high. Primary B cells have a relatively low concentration of CstF64, consistent with the skipping of the proximal PAS site, and use of the distal site, in IgH under these conditions[126]. However, upon B cell differentiation, such as triggered by LPS stimulation, the expression of CstF64 is dramatically upregulated[126], resulting in "filling" of the proximal site by CstF64 and cleavage at this site before the distal site is even transcribed. Thus, regulation of IgH is a clear example of the functional implications of changing the expression level of core CFAP factors, as discussed in the previous section. Given the central role of CstF64 in APA, it is almost certain that the change in CstF64 expression during B cell differentiation drives APA of other physiologically-relevant targets. Notably, CstF64 expression is also higher in effector versus naïve T cells, where this difference has been implicated in at least one study to drive altered isoform expression of the NF-AT transcription factor[127]. Additionally, LPS-stimulated macrophages exhibit increased CstF64, which has been attributed to changes in both abundance and APA of downstream genes[128]. Therefore, CstF64 regulation is foundational in shaping 3' UTR identity, gene expression and cellular function not just in B cells, but throughout both the adaptive and innate immune system.

CELF2 – a RBP regulator of APA as well as AS in T cells—Just as CELF2 binding to target RNAs can alter binding of splicing factors to their cognate sequences, binding of CELF2 near PAS sites also can sterically hinder binding of CPAFs. Notably, the optimal binding site for CELF2 (UG-repeats[110]) is similar to that of CFIm25 (UGUA[129]) or CstF64 (U/UG-rich sequences[130]). Therefore, one can readily appreciate that subtle difference in RNA sequence, or in the relative concentration of CELF2 versus CFIm25 or CstF64, can result in the preferential binding of CELF2 over the CPAFs. Indeed, competitive binding between CELF2 and CFIm25 and CstF64 has recently been demonstrated in biochemical assays and has been shown in cells to alter APA[43]. Given that CELF2 is regulated by

JNK signaling in activated T cells, it is likely that CELF2 may drive much of the APA that has previously been observed upon antigen stimulation of CD4+ primary T cells. Indeed, CELF2 regulates a wide program of activation-induced APA in the Jurkat model T cell line[43]. However, the functional consequence of CELF2-mediated APA regulation on immune cell function remains relatively poorly studied and merits much further attention. Interestingly, one study recently suggested that hnRNP LL might also be a specific regulator of APA, as depletion of hnRNP LL increases the ratio of the above-described soluble versus membrane-bound IgH[131]; however, this effect appears to be at the level of splicing and is inconsistent with the increase in both hnRNP LL expression and soluble IgH observed in the transition from B cells to plasma cells[106].

Regulation of Apoptosis in T cells via AS and APA

Apoptosis

A newly emerging example of the functional impact of AS and APA in the immune system is in regard to apoptosis. Apoptosis is a tightly regulated form of controlled cell death that occurs in response to both extracellular stimuli and intracellular stress[132,133]. Hallmarks of apoptotic cells include cellular shrinkage, phosphatidylserine exposure on the extracellular membrane, DNA fragmentation, and cellular blebbing[132]. In the immune system, apoptosis plays a critical role in maintaining homeostasis and limiting responsiveness of immune cells. For example, one of the outcomes of T cell engagement with antigen is Activation-Induced Cell Death (AICD), where cells are eliminated after multiple rounds of stimulation through the triggering of apoptosis[134,135]. Such AICD is critical to control the degree and duration of the T cell response to enable a return to homeostasis and prevent hyper-reactivity[134]. In addition, T cells undergo apoptosis in the presence of a weak stimulation, or in the absence of co-stimulation, to prevent spurious immune responses[136,137]. Consistently, defects in apoptosis regulation in immune cells has been linked to autoimmune disease[138–140].

Both the cell-extrinsic or intrinsic triggering of apoptosis activates signaling cascades that involve extensive cross-talk between two major protein families: Caspases and Bcl-2 proteins[141–144] (Fig. 6). Caspases are cysteine proteases that are categorized into two different groups. Initiator caspases (Casp-8, −9, −10) function at the apex of apoptotic signaling to link apoptotic triggers to downstream signaling events[144]. The initiator caspases exist initially as pro-form monomers and are activated through their dimerization to cleave target proteins. Executioner caspases (Casp-3, −6, −7), on the other hand, exist as inactive caspase dimers and are activated through cleavage by initiator caspases to promote cellular responses[144]. By contrast to the proteolytic activity of caspases, BCL2 family members control mitochondrial membrane permeabilization and can either promote or inhibit apoptotic signaling[133,143]. There are approximately 20 Bcl-2 family members, characterized by their relatively conserved Bcl-2 homology (BH) domains that promote protein interaction and dimerization. Pro-apoptotic BCL2 family proteins include Bax, Bak, Bid and Bim; while Bcl-xL, Bcl-w, MCL1 and Bcl2 are examples of family members that inhibit apoptosis[133,143].

The extrinsic pathway of apoptosis is initiated by the activation of a death receptor on the cell surface, such as Fas (CD95), TRAIL receptors, or TNF receptors[141,145]. Upon

oligomerization of death receptors, pro-caspase 8 and FADD are recruited to the intracellular domain of the death receptor to promote dimerization and activation of pro-caspase 8[145]. In addition to activating the executioner caspases, activated Casp-8 also cleaves the BCL2 family member, Bid, to produce the cleavage product tBid[133]. tBid links the extrinsic and intrinsic pathways together[146]. The intrinsic apoptotic pathway consists of signaling events amongst numerous Bcl-2 family members that culminate in the breakdown of the outer mitochondrial membrane, known as mitochondrial outer membrane permeabilization or MOMP[133,147]. Not only can the intrinsic pathway be activated by the events downstream of the extrinsic pathway, but the intrinsic pathway can also be directly activated by the presence of reactive oxygen species (ROS), nutrient deprivation, cellular stress, and by many other means[141,142]. MOMP leads to the subsequent release of cytochrome c from the mitochondria[147]. Once released, cytosolic cytochrome c nucleates the oligomerization of APAF-1 and Casp-9 into a complex named the apoptosome[144]. The apoptosome is essential for the activation of the enzymatic activity of the initiator Casp-9. Once activated, Casp-9 cleaves the above-mentioned effector caspases to promote apoptosis, in a manner analogous to the extrinsic pathway[133,144].

Alternative splicing has been well documented to generate pro- or anti-apoptotic forms of Caspases, Bcl-2 family members or other apoptotic signaling proteins, to impact cellular viability[148–150] (Fig. 6). For example, the alternative splicing of Bcl-x, was one of the earliest examples of physiologic relevance of AS in humans, as altering the ratio between the long anti-apoptotic (Bcl-xL) and short pro-apoptotic (Bcl-xS) directly impacts cell survival[150,151]. More recently, APA of several apoptosis related genes has also been described (see below). Given the critical role of apoptosis in shaping the reactivity of immune system, understanding the role of AS and APA in setting the threshold of sensitivity for apoptosis is essential for appreciating the impact of AS and APA in immune responses. Below we discuss recent evidence for how apoptotic-related AS and APA is controlled in T cells to shape immune response and homeostasis. We also highlight critical questions that remain to be answered in hopes of motivating further study.

Regulation of Apoptosis in the Immune System by Alternative Splicing

FAS—Perhaps the most fully characterized example of how AS is used to tune apoptosis in the immune system is with regards to the extrinsic apoptosis receptor Fas (also known as CD95 or APO-1) (Fig. 6). Fas belongs to the tumor necrosis factor receptor family (TNF-R)[145] and is the key trigger for the above-described AICD in activated T cells[152]. Upon activation, both Fas and its ligand, FasL, are upregulated in T cells[152]. Engagement of Fas by FasL results in trimerization of Fas on the cell surface and recruitment and activation of Casp-8/10[142]. Interestingly, at the lower concentration of Fas and FasL found in naïve T cells, the Fas/FasL interaction may provide a co-stimulatory effect to boost early T cell activity and proliferation[153,154].

Fas is regulated not only by changes in gene expression but also at the level of AS (Fig. 7). Exclusion of exon 6 from the final mRNA transcript produces a soluble Fas protein that lacks the transmembrane region to anchor the receptor to the extracellular membrane[155–158]. As for TLR4 discussed above, the soluble Fas receptor competes with

membrane-bound Fas for FasL ligation, thereby limiting Fas signaling to the cell[155,158]. Activation of T cells promotes exon 6 inclusion, favoring the pro-apoptotic, membrane bound form of Fas required for AICD[157,159]. However, defects in this switch have been commonly observed and linked to a variety of diseases, including autoimmune phenotypes such as lupus (SLE), autoimmune lymphoproliferative syndrome (ALPS) and leukemias, all of which exhibit increased skipping of Fas exon 6 consistent with this form decreasing apoptotic signaling[155,159–161].

The regulation of Fas exon 6 inclusion has been attributed to hundreds of genes and a variety of signaling pathways[162]. Most directly, TIA-1 and PTB bind immediately around exon 6 and act in competition with each other, with TIA-1 binding downstream of Fas exon 6 to promote its inclusion, while PTB binds upstream and represses use of exon 6[163,164]. T cell activation increases both the expression and phosphorylation of TIA-1 to promote exon 6 inclusion, while IL-7 has been shown to decrease PTB via the activity of the miRNA, mir-124, also leading to increased exon 6 inclusion[165–167]. Interestingly, caspase cleavage of core splicing factors such as U2AF65 further influences the balance of Fas exon 6 inclusion to create a feedback loop that tunes Fas expression during apoptosis[168]. Moreover, some correlations been made between dysregulated RBPs and dysregulated Fas splicing, such as the finding of increased expression of the splicing protein SPF45 in glioblastoma, which drives increased exon 6 skipping and is a potential therapeutic target for glioma[169,170]. However, further insight into the regulation of Fas AS in disease is certain to yield valuable information regarding the treatment of immunologic pathologies.

Bcl-x—As mentioned above, Bcl-x was among the first genes shown to encode pro- and anti-apoptotic isoforms through alternative splicing[150,151]. Bcl-x is a transmembrane protein that lies in the outer mitochondria membrane to regulate MOMP events within the intrinsic apoptotic signaling cascade[171] (Fig. 6). Two well characterized AS isoforms of Bcl-x exist: the anti-apoptotic longer isoform, Bcl-xL, and the pro-apoptotic shorter isoform Bcl-xS due to an alternative 5’ splice site within exon 2 (Fig. 7). Both isoforms are in-frame and produce stable proteins: Bcl-xL is 233 amino acids long while Bcl-xS lacks 63 amino acids, resulting in the removal of BH1 and BH2 domains from the protein. Bcl-xL prevents apoptosis by binding to Bax and tBid via its BH1 and BH2 domains, thereby preventing Bax and tBid from inducing MOMP[150,171–173]. Bcl-xS, on the other hand, not only lacks the ability to interact with and suppress Bax and tBid, but also can dimerize with Bcl-xL thereby functioning as a dominant-negative to the anti-apoptotic activity of the long isoform[150,174]. Consistently, overexpression of Bcl-xL, or knocking down Bcl-xS, increases cellular survival; while overexpressing Bcl-xS, or repressing Bcl-xL, increases the cellular apoptosis[171,175].

As described for Fas, the mechanisms of AS of Bcl-x have also been extensively studied. A host of factors, including SR proteins SRSF 2,3,7,9 and 10 and hnRNPs F/H, A1, K and PTB, all regulate the relative use of the competing alternative 5’ splice sites in Bcl-x exon 2 to tune the balance of expression of the short and long isoforms (recently reviewed in[150]). However, most of the studies regarding Bcl-x AS have been done in the context of Hela and 293 cells, or in response to DNA damage, rather than in cells of the immune system.

In T cells, Bcl-xL expression plays a critical role in survival during T cell development in the thymus and also effector responses in the periphery[176,177]. Naïve peripheral T cells express both Bcl-x isoforms at low levels. T cell activation results in an increased expression of both isoforms, but favors expression of Bcl-xL as a mechanism to promote the survival and function of effector T cells, particularly with CD28 co-stimulation[177]. For the most part, the induced expression of Bcl-xL upon T cell activation is due to transcription, with apparent little impact on AS[177]. Notably however, two identified regulators of Bcl-x AS, SF3B1(SAP155) and Sam68, are regulated respectively by PI3K and Fyn, two kinases activated by antigen engagement of T cells[178–180]. Therefore, it seems plausible that splicing of Bcl-x, in addition to transcription, maybe regulated in at least some stages of T cell activation. Moreover, as clearly shown in B cells, dysregulation of Bcl-x splicing often is a driver of cellular transformation and cancer[181]. An interesting question warranting further study, therefore, is whether dysregulation of Bcl-x is observed in other diseases of the immune system such as immunodeficiencies or autoimmunity.

Bim—Another Bcl-2 family member that is regulated at the level of alternative splicing is Bim (Fig. 7). Bim is a pro-apoptotic BH3-only protein that interacts with other Bcl2 family proteins such as MCL1, Bcl-xL, and BCL2 to promote apoptotic signaling[133] (Fig. 6). Numerous lines of evidence demonstrate a critical role for Bim activity in regulating T cell development, survival, and function[135,182]. For example, knock-out of Bim in mice results in a severe block of T cell development in the thymus[183], while in peripheral T cells, the absence of Bim reduces rates of AICD induced in both *in vivo* infection and *in vitro* stimulation[184,185]. Similarly, in human T cells knock-down of Bim by siRNA also protects cells from AICD[185].

Bim is expressed as 3 major AS isoforms that arise due to alternative splicing. The three major isoforms exist due to the selective inclusion of exons 2a and 2c, and the intronic region between these two exons, which is also referred to as exon 2b (Fig. 7). BimEL is the longest isoform with the inclusion of exons 2a, 2b, 2c. BimL includes exons 2a and 2c, while BimS includes only exon 2[186]. While the functional differences between these isoforms has not been clearly documented, at least one study suggests that BimS has the greater pro-apoptotic activity than the longer isoforms[187]. Consistent with this notion, and the role of Bim in AICD, stimulation of CD4+ T cells results in a reduction in BimEL and an increase in BimS[188]. However, despite the clear AS changes in Bim between naïve and activated T cells, the mechanisms and RBPs involved in this regulation are entirely unknown. Moreover, given that most studies with Bim have focused on only one isoform, it would be of interest to directly compare the roles of the alternate isoforms in more cell types and conditions through manipulation of isoform expression by CRISPR or treatment with antisense oligonucleotides. Additionally, biochemical studies are needed to better understand if BimS, BimL or BimEL exhibit differential binding partners with other BCL2 family interaction members. Clearly the study of Bim is ripe for uncovering additional mechanisms and functional implications of AS in the immune system.

cFLIP—A final example of regulation of apoptosis by AS with relevance to the immune system is cFLIP (cellular FLICE-like inhibitory protein). cFLIP, encoded by *CFLAR*, is

a negative regulator of the extrinsic pathway signaling events activated by death-receptor signaling (Fig. 6). cFLIP is structurally related to caspase-8, but lacks proteolytic activity; therefore, the competitive binding and heterodimerization of cFLIP to caspase-8 inhibits caspase-8 cleavage and subsequent activation[189,190]. Three major AS isoforms of cFLIP are expressed at the protein level in T cells including: cFLIPL, cFLIPS, cFLIPR (Fig. 7). All major cFLIP isoforms contain two DED domains, which allows for the heterodimerization with caspase-8[189]. The longest isoform, cFLIPL, also contains the catalytically inactive caspase-like domain. By contrast, cFLIPR and cFLIPS are truncated prior to the caspase-like domain due to inclusion of intron 6 or poison exon 7, respectively, both of which introduce a premature stop codon[189]. All isoforms maintain the capability to be a negative regulator of caspase-8 and −10 activation due to the DED domains; however, cFLIPL function is more complex, as under certain experimental conditions and concentrations it also can enhance Casp-8 activity via its caspase-like domain[191,192]. The cFLIP isoforms also appear to differ in their effects on T cell differentiation, in that siRNA knockdown of cFLIPL increased IFNgamma secretion in Th1 cells and IL4 production in Th2 cells, while siRNA knockdown of cFLIPS decreased IL4 production and GATA3 expression of Th2 cells[193].

cFLIP is yet another example where isoform levels are differentially regulated upon T cell activation. Specfically, cFLIPS, but not cFLIPL, is upregulated in primary T cells activated with either PHA or anti-CD3 antibody and upon restimulation of effector T cells with anti-CD3 and anti-CD28 antibodies[194–196]. cFLIPS is also selectively upregulated by activated T cells polarized to the Th2 subset compared to Th1 and Th0 (unpolarized) cells, in a manner which is dependent on STAT6 signaling[193]. NFAT, p38alpha, and DHX32 have all been implicated to be specific inducers of cFLIPS expression and activity in T cells, although it is not clear if this is due to splicing or to selective stabilization of the cFLIPS protein[191,194,197,198]. Although the mechanisms by which RBP activity may regulate AS changes in cFLIP are not well characterized in T cells, a few studies have begun to characterize the role of RBP activity mediating cFLIP AS in other cell types. The knockdown of RNA binding proteins hnRNPA2 and hnRNPB1, and the combined deletion of RBM5,6, and 10, have both been demonstrated to increase the inclusion of exon 7 in glioblastoma cancer cells and HeLa cells, respectively, to promote expression of cFLIPS[199,200].

One strong piece of evidence in favor of functional relevance of cFLIP AS is the discovery of a SNP that has been implicated to regulate inclusion levels of cFLIP isoforms. The Shwerk group discovered a SNP in the 3’ splice site of intron 6 (rs10190751A) that is characterized to enhance levels of cFLIPR compared to cFLIPS in humans[201]. Comparison of the frequency of rs10190751A between a population of healthy cells and primary follicular lymphoma samples revealed a significant association with the SNP in follicular lymphoma samples compared to control[201], consistent with cFLIPS having a stronger anti-apoptotic impact than other isoforms[195,196]. The molecular mechanism by which rs10190751A increases cFLIPS is yet to be determined, although this likely involves changes in the binding of splicesomal components or trans-acting factors to regulate the abundance of cFLIPS. Additionally, the phenotypic consequences of having the SNP allele in a cancer context remains an important unanswered question.

Regulation of Apoptosis in the Immune System by Alternative Polyadenylation

CD5—Although, to date, there is little evidence for APA regulation of caspases, Bcl-2 proteins or other core apoptotic machinery, the expression of several regulators of apoptosis is controlled through APA. One example of this in T cells is the gene encoding the CD5 type-1 transmembrane glycoprotein (Fig. 8). CD5 is a regulator of TCR signaling whose expression is tightly controlled and regulated during T cell development and activation[202,203]. Upon T cell activation, tyrosine residues in intracellular domain of CD5 become phosphorylated, forming a docking site for multiple signal transduction proteins including PI3K, rasGAP and CK2[204]. One major consequence of CD5 activity is the regulation of activation induced cell death (AICD)[202,203,205–208]. CK2 is a pro-survival kinase and the CD5/CK2 signaling axis promotes cell survival in thymocytes[208]. Consistently, depletion of CD5/CK2 in mice reduces their susceptibility to experimental autoimmune encephalomyelitis (EAE) through increased apoptosis and accelerated AICD[205]. Separate from CDK2 signaling, CD5 also protects tumor-specific T cells from AICD by regulating the expression of FasL and thus activation of Caspase-8[209].

Although there have been limited studies detailing the mechanisms controlling CD5 expression, recently a group demonstrated that in T cells CD5 expression is regulated at the RNA level by APA[210]. Three polyadenylation sites are utilized in the 3’UTR region of CD5 (pa1, pa2, pa3) in resting Jurkat and primary T cells (Fig. 8). Upon T cell activation, there is a preference for the proximal PAS sites[210]; consequently, the length of the CD5 3’UTR becomes shorter. Use of luciferase reporter assays demonstrated that use of the most proximal PAS site of CD5 results in increased protein production as compared to the distal PAS sites[210]. The 3’ UTR sequences between the CD5 promixal and distal PAS sites contains a miR-204 binding site. Mutation of the miR-204 binding site in the longer 3’UTR phenocopies use of the proximal PAS in the luciferase assay[210], demonstrating that the switch from distal to proximal PAS promotes CD5 expression by evading miRNA-mediated repression. Although the mechanism driving CD5 APA is not conclusively proven, biochemical studies suggest that binding of the hnRNP protein PTB near the CD5 proximal PAS promotes its use[210]. Obvious remaining questions of interest are whether there is a correlation between PTB and miR-204 levels and CD5 expression in the thymocytes and peripheral T cells, and if the perturbance of PTB and miR-204 have effects on T cell effector functions through CD5 regulation. Moreover, given that both the shortening of the 3’UTR of CD5 and the result of removal of a miRNA binding site, are consistent with the global studies on APA during T cell activation[39], it will be interesting to determine if PTB has a more widespread impact of APA in T cells beyond CD5.

GIMAP5—GIMAP, GTPase immune-associated protein, is a family of small putative GTPases also referred to as immune-associated nucleotide-binding proteins (IANs)[211,212]. There are 7 GIMAP proteins encoded in the human genome, several of which play a role in T cell activity[211,212]. Notably, GIMAP5 not only plays an important anti-apoptotic role in T cells, but dysregulation of APA of this gene leads to autoimmune disease[211,213] (Fig. 8).

The BB (Biobreeding) rat was the first model system to suggest GIMAP5 activity on apoptosis in T cells. The BB rat is prone to spontaneously acquire type-1 diabetes and also

develops life-long lymphopenia. At least the lymphopenia in the BB rat is due to a frame-shift mutation of GIMAP5 that produces a truncated protein product[214–216]. The expression of this truncated GIMAP5 protein results in increased apoptosis rates and a shortened lifespan of T cells in the BB rat model, with a corresponding 10-fold reduction in CD4+ and CD8+ T cells[216–218]. Recent studies have associated this phenotype with impaired calcium signaling and loss of mitochondrial integrity in T cells, therefore insinuating a role of the intrinsic apoptosis pathway in mediating the increased apoptosis rates[219–221]. Parsing out the role of GIMAP5 in human T cells have led to contradicting results as one study found overexpressing this protein leads to apoptosis in Jurkat T cells and in PHA/IL2 activated primary human T cells grown without apoptotic inducers[222], while another study showed that overexpression of GIMAP5 leads to increased resistance to apoptosis in Jurkat T cells induced with gamma-radiation and okadaic acid[223]. Overall, these studies suggest that GIMAP5 is regulating T cell apoptosis; however, more work is necessary to detail the exact role and context of GIMAP5 function.

Interestingly, GIMAP5 is regulated through APA in at least some autoimmune diseases. A SNP in the 3'UTR region of GIMAP5 is enriched in a subset of SLE patients compared to healthy controls and is also correlated with the increased presence of IA-2 autoantibodies in patients with type-1 diabetes[224,225]. The SNP (rs6598) converts the canonical hexamer sequence utilized by polyadenylation machinery (AATAAA) to the sequence, AATAGA (Fig. 8). The presence of the AATAGA hexamer sequence is correlated with a longer 3'UTR when comparing individuals that is homozygous for the allele against heterozygous and WT controls[225]. This SNP confirms an association between dysregulated GIMAP5 APA and disease correlation; however, it remains unknown how the change in 3'UTR alters GIMAP5 expression or function. Importantly, regardless of mechanism, this GIMAP5 SNP highlights the need to investigate sequence variations outside of coding sequences as potential drivers of disease and suggests the potential for additional unanticipated regulation of APA impacting apoptosis, as well as other activities of immune cells.

Conclusions and Future Directions

The regulation of gene expression in immune cells is, by necessity, highly complex to enable accurate and robust response to the many different challenges faced by innate and adaptive immune systems. Here we lay out existing evidence for a broad impact by both regulated splicing (AS) and 3' end processing (APA) in shaping the function of immune cells and the immune response. We further describe some of the regulatory proteins and mechanisms that have been most widely implicated in the control of AS and APA in lymphocytes and macrophages. Most importantly, we highlight the many questions that remain unanswered with regards to AS and APA in immunity. In particular, we focus on the regulation of apoptosis during the immune response as a field ripe for further discovery. Indeed, beyond the gene regulation examples described above, RNA-Seq studies have already suggested AS changes in Bax and Casp-8 in T cells; and APA of cFLIP[109,226]; although none of these have been validated nor has the functional impact been explored. In addition, there are AS isoforms of other apoptosis signaling proteins that have been well studied in other cell types, but have yet to be investigated in immune cells, such as Casp-9[227]. In sum, the continued focus on transcriptomics in immune cells is predicted to yield many additional examples

of how apoptosis and other critical features of immune regulation are controlled by AS and APA. Finally, given the proven ability to regulate isoform expression for therapeutic value[228], further discovery of AS and APA in the immune system is also critical as it is predicted to suggest novel therapeutic avenues for the treatment of immunologic disease.

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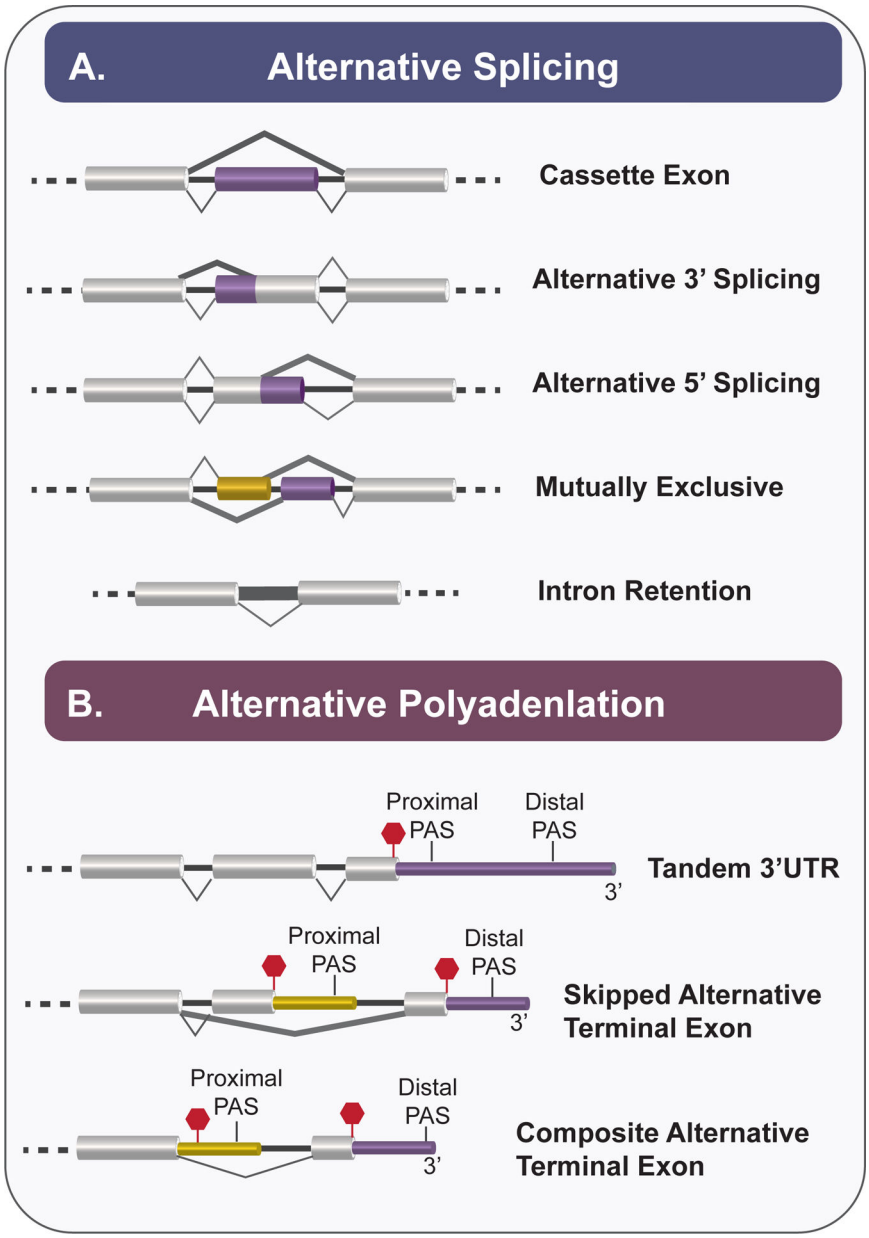

Figure 1. An illustration of alternative splicing and polyadenylation patterns.

A. Schematic of alternative splicing patterns including: cassette exon, alternative 5' or 3' splice site selection, mutually exclusive exons, and intron retention. Constitutive exons are depicted in grey and alternatively splicing exons are depicted in purple or yellow. Thick lines represent alternatives to the canonical (thin line) splicing patterns. **B.** Examples of alternative polyadenylation patterns include tandem 3'UTR, skipped alternative terminal exon, and composite alternative terminal exon. While the proximal and distal PAS are in proximity to each other within tandem 3'UTRs, proximal PAS may be located within upstream sequences and its inclusion may be subjected to regulation like alternative splicing. Thick lines represent alternatives to the canonical (thin line) splicing patterns. Red hexagon represents translation stop codon. Purple and yellow regions indicate 3'UTRs.

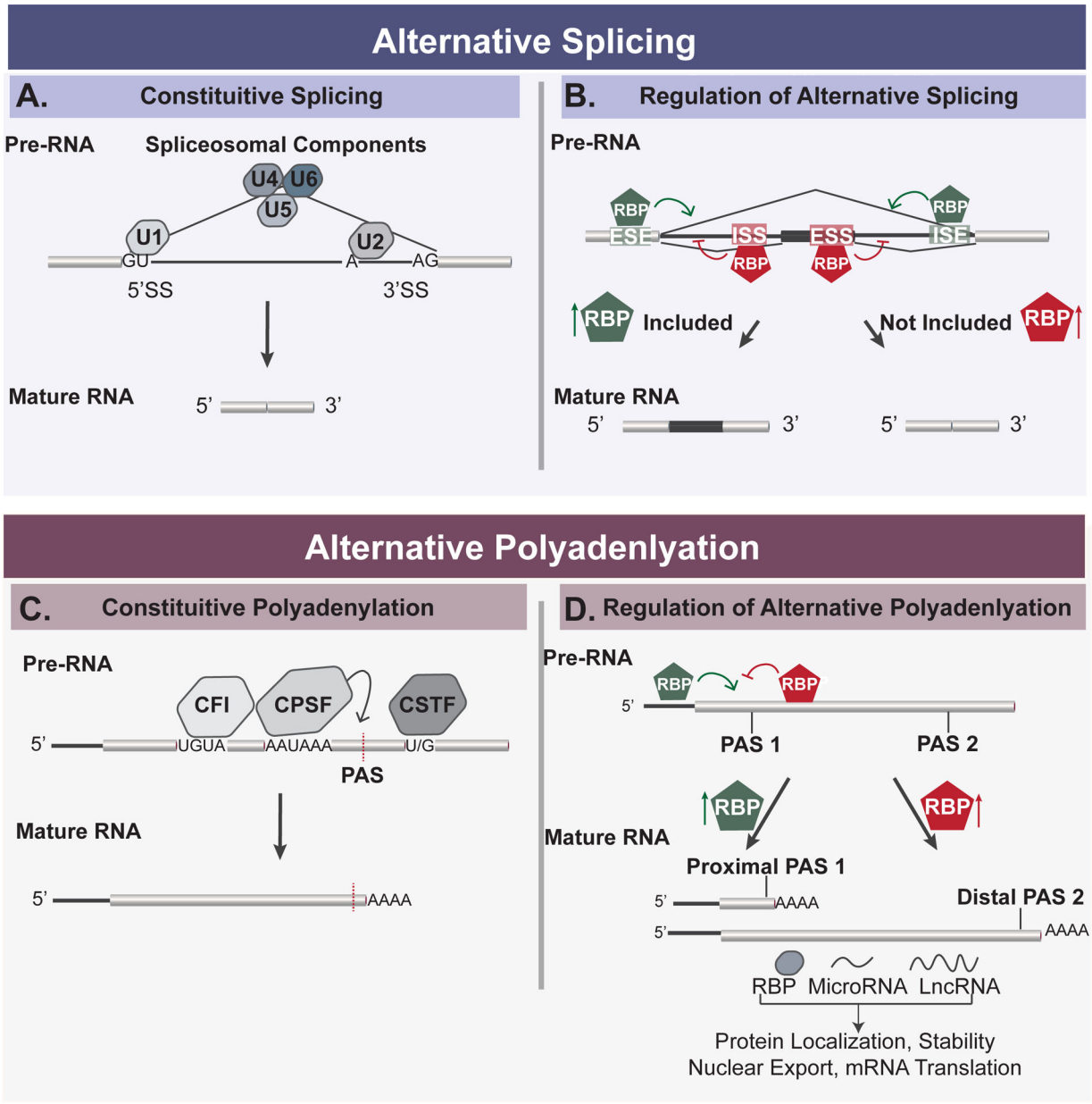

Figure 2. Constitutive splicing and polyadenylation are regulated by RBPs that give rise to alternative splicing and alternative polyadenylation products.

A. Diagram of constitutive splicing. Spliceosomal components, or snRNPs are depicted as U1–U6 and bind to 5' and 3' splice sites. snRNPs are essential for constitutive splicing of introns of pre-RNA to produce mature RNA. **B.** The regulation of alternative splicing is influenced by RNA binding proteins (RBPs) that may either enhance (green hexagon) or repress (red hexagon) exon inclusion. RBPs enhance splicing by binding to exon splicing enhancer (ESE) sequences or intron splicing enhancer (ISE) sequences, or repress splicing by binding to exon splicing silencer (ESS) sequences or intron splicing silencer (ISS) sequences. Due to the combinatorial regulation of RBPs, exon inclusion is mediated by the increased or decreased presence of RBPs, as depicted by arrows. **C.** Cleavage at the polyadenylation site (PAS) of pre-RNA is mediated by a large multi-component

complex containing a core CPSF, CFI, CstF subcomplex. **D.** The regulation of alternative polyadenylation is regulated by the activity of RBPs that may either enhance (green hexagon) or repress (red hexagon) the usage of PAS sites.

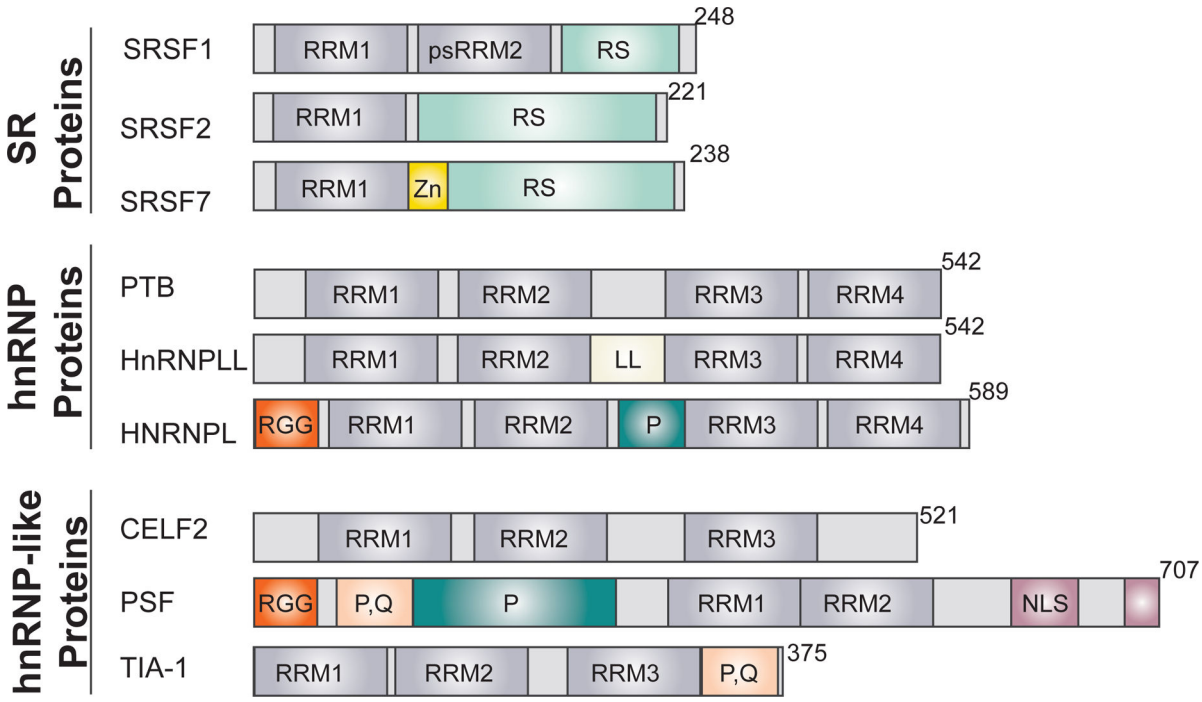

Figure 3. RNA binding proteins characterized to regulate alternative splicing and alternative polyadenylation.

RRM, RNA recognition motif (a classic 2 alpha helix, 4 beta-strand fold); psRRM2, pseudo-RRM (similar fold to an RRM but lacking key residues); P, proline-rich region; RGG, arginine/glycine/glycine repeat region; P, Q, proline/glutamine rich sequence; RS, arginine/serine dipeptide repeat sequence; Zn, zinc finger; NLS, nuclear localization sequence; LL, unique L-like linker sequence.

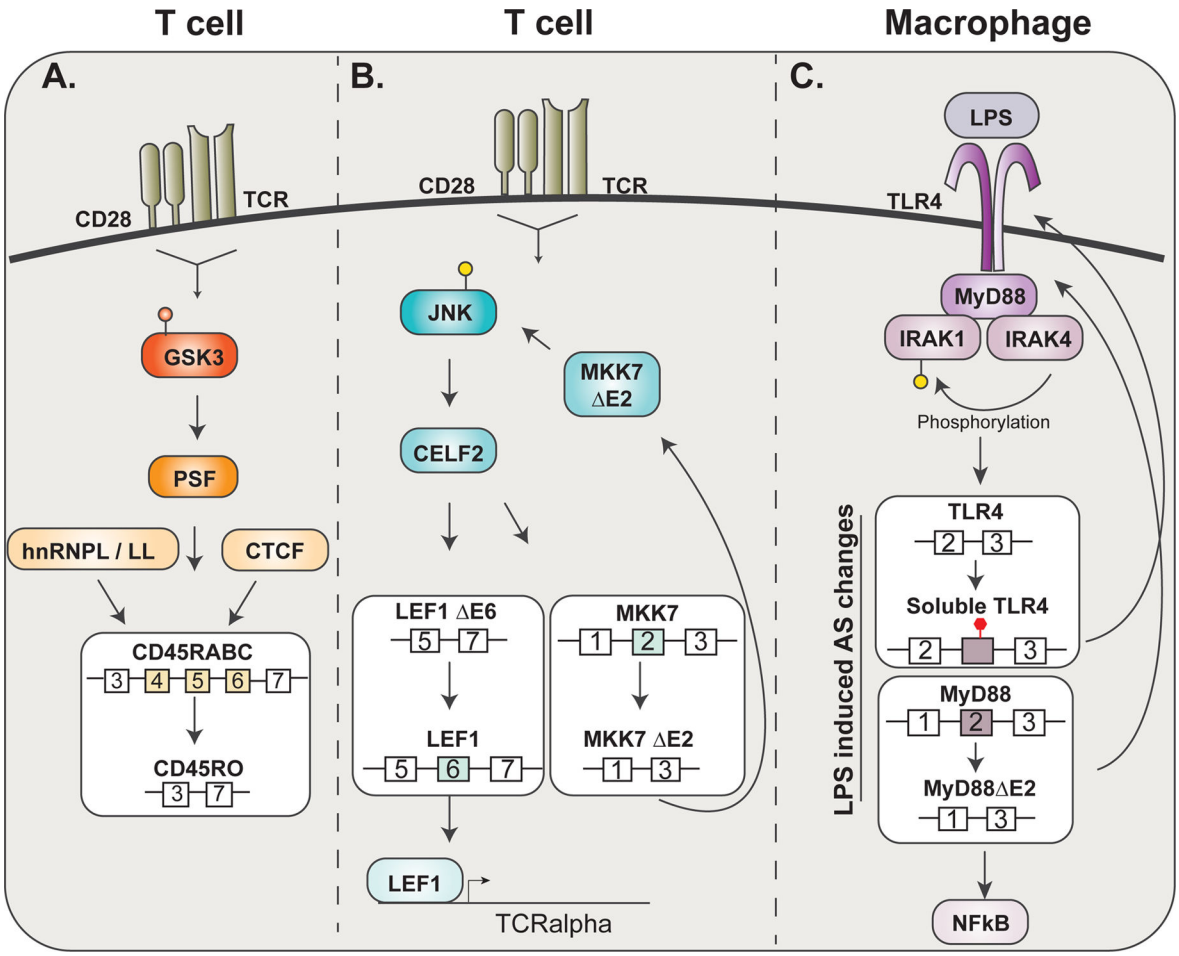

Figure 4. Classic examples of AS in the immune response.

A. Upon T cell activation, GSK3 inactivation leads to subsequent PSF activity to then influence CD45RABC splicing to CD45RO along with hnRNPL/LL and CTCF. **B.** Upon T cell activation, the activation of JNK kinase signaling regulates CELF2 activity to influence the splicing of LEF1 and MKK7. A feed-forward loop is established as MKK7 ΔE2 phosphorylates JNK to then further regulate CELF2 activity. **C.** LPS incudes macrophage activation through TLR4 signaling. TLR4 recruits MyD88 which further recruits IRAK4 (only the long form of MyD88) and IRAK1. IRAK1 is phosphorylated by IRAK4, when co-recruited, to promote NFkB signaling.

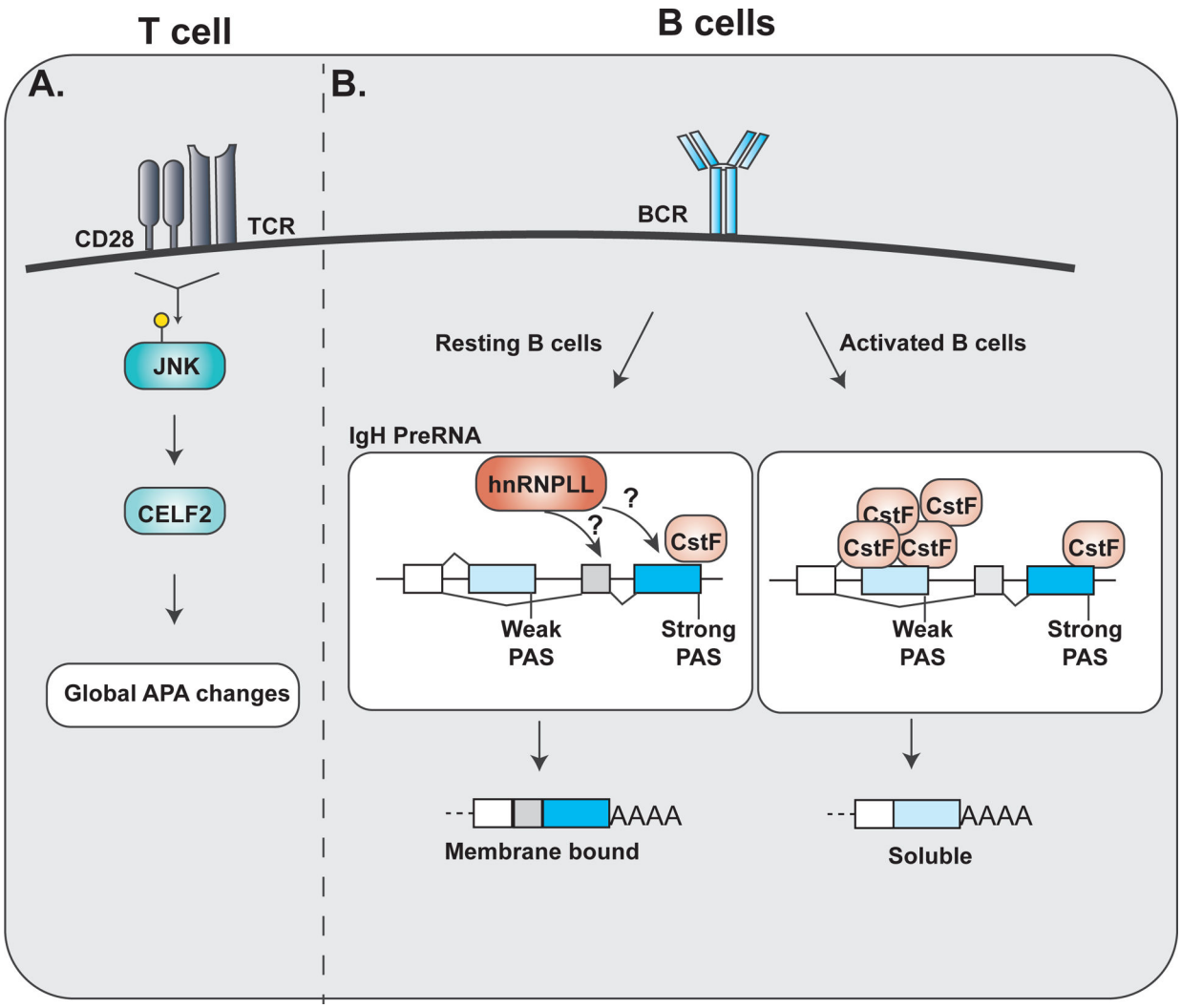

Figure 5. Classic examples of APA in the immune response.

A. Upon T cell stimulation phosphorylated JNK activates CELF2 activity to mediate global APA changes within cells. **B.** Within resting B cells, low levels of CstF binding to the strong distal Pas site and leads to increased production of membrane bound immunoglobulin heavy chain (IgH). hnRNPLL activity is also attributed to the selection of the strong PAS site selection in resting B cells. Upon B cell activation, increased expression of CstF promotes usage of the weaker proximal PAS site and increased production of soluble IgH.

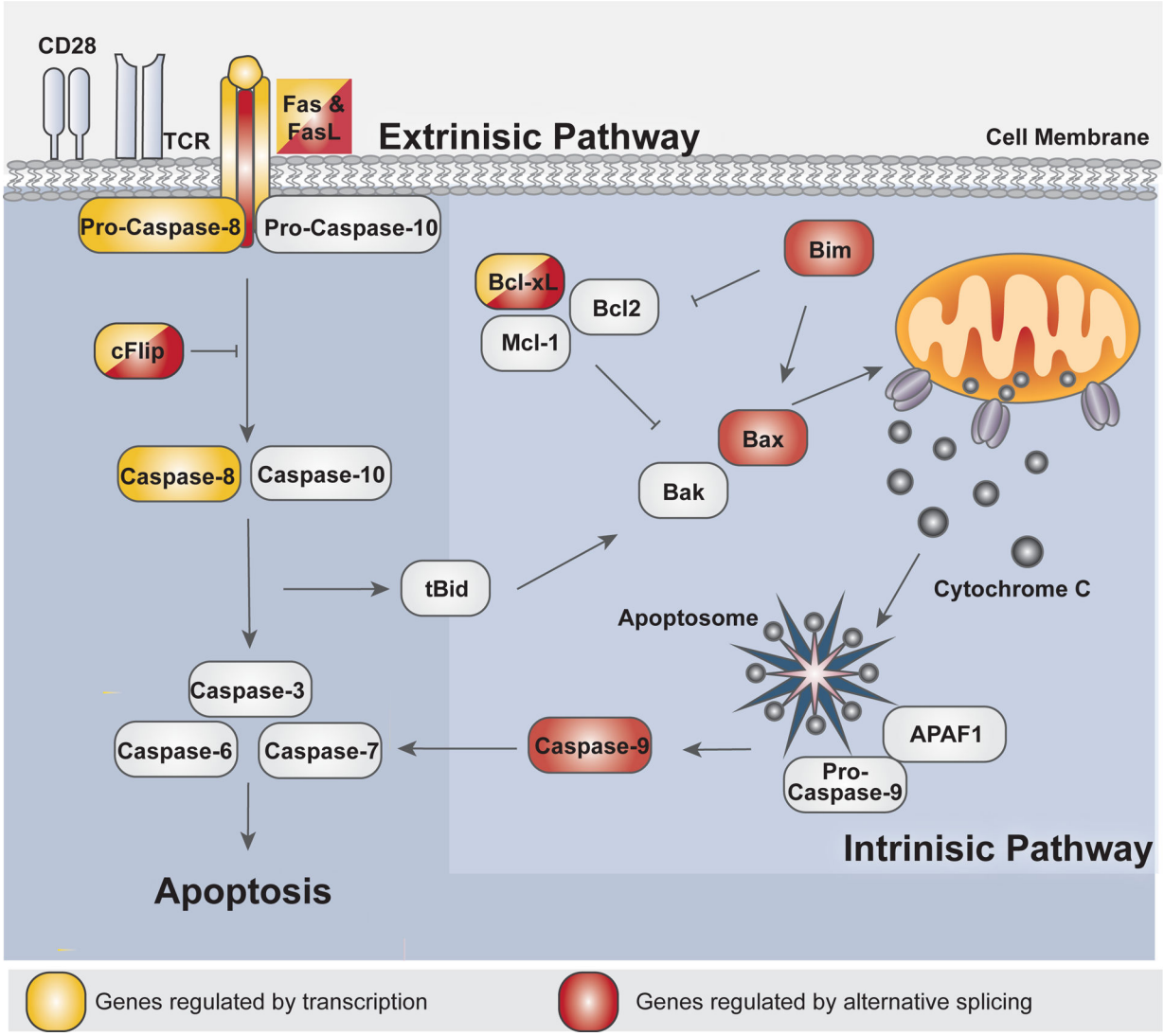

Figure 6. Signaling regulators of the apoptosis signaling pathway are regulated at the level of alternative spicing.

The extrinsic pathway and intrinsic signaling pathway both converge to activate effector caspase activity (Caspase-3,−6–7) to promote apoptosis within the cell. The extrinsic pathway events are activated upon Fas/Fas ligand signaling. Caspase-8/10 are subsequently activated and then cleaves and activates downstream effector caspases. Caspase-8/10 activation may be blocked by cFLIP activity. The cleavage of tBid by Caspase-8/10 then promotes intrinsic pathway signaling. The intrinsic pathway includes pro-apoptotic and anti-apoptotic BCL2 family protein members to regulate mitochondrial outer-membrane depolarization (MOMP) and the release cytochrome C into the cytoplasm. Upon MOMP, activated caspase-9 later cleaves and activates effector caspases. Fas[152], FasL[152], and Caspase-8[132], labeled in yellow, are characterized to be regulated at the level of transcription in T cell. Casp9[227], Bax[109], and Bim[188], labeled in red, are characterized to be regulated at the level of AS. cFLIP[194,229] and Bcl-x[151] are labeled in red and yellow are characterized to be regulated at both the level of transcription and AS.

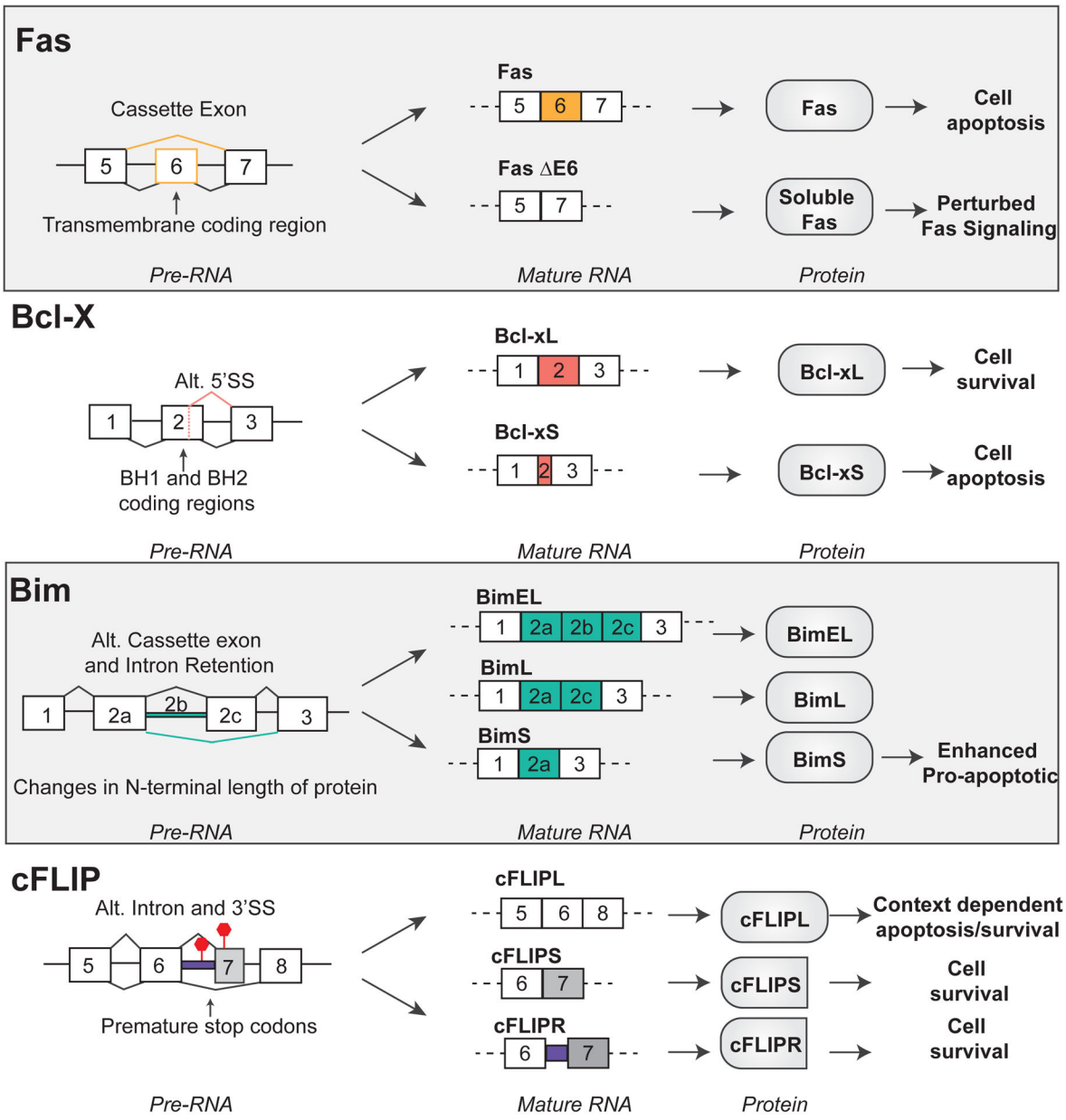

Figure 7. Canonical apoptosis signaling mediators are regulated at the level of AS in T cells.

Fas, Bcl-x, Bim, and cFLIP are regulated at the level of alternative splicing. Diagrams depict the pattern of alternative splicing at the pre-RNA and mature RNA level. The impact on protein and cell physiology are illustrated for each alternative splicing event.

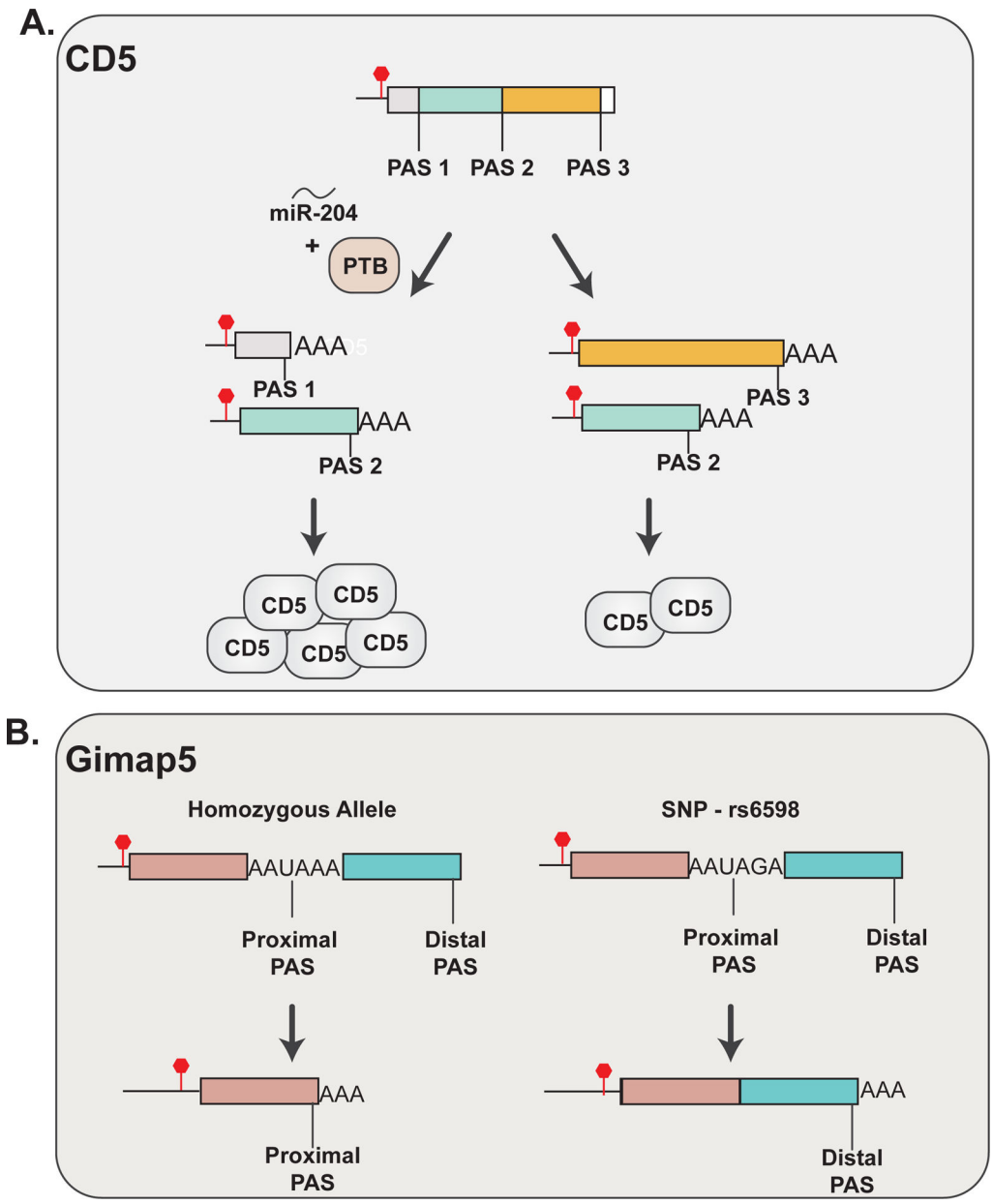

Figure 8. Genes characterized to regulate apoptosis in T cells are regulated at the level of APA.

A. Three polyadenylation sites are utilized in the 3'UTR region of CD5. Proximal PAS usage (PAS 1, PAS2) are regulated by miR-204 and PTB activity. Proximal PAS usage correlates with increased protein output, as opposed to distal PAS usage. **B.** The presence of a SNP (rs6598) within the hexamer sequence of Gimap5 increases the usage of the distal PAS, compared to WT allele.

Table 1:

Selected RNA-Seq based studies of splicing and APA in Immune Cells.

	Study	Organism/Cell Type	Stimulus	Analysis method	Findings	Reference
Alternative Splicing	Radens et al. RNA 2020	Human Naïve CD4+ T cells	Anti-CD3/CD28	MAJIQ	Identified hundreds of splicing changes and compared these to polarized Th cells	1
	Liu et al., NAR 2018	Human M1 and M2 Macrophages	GM-CSF and M-CSF	rMATs, MISO, SpliceTrap	1933 and 1262 events in 1327 and 903 genes are specific to GM- and M-CSF conditions	2
	Lin et al., 2016	Human Macrophages	Monocyte Differentiation	PennDiff, DEXseq, and IUTA	Detected 233 M1-induced AS events	3
	Kalam et al., PLoS Pathogens 2017	Human Macrophages	*Mycobacterium tuberculosis*	rMats	1057 and 583 splicing events were found unique to H37Ra and H37Rv Mtb strains by 48hrs respectively	4
	Pai et al., PLoS Genetics 2016	Human Macrophages	Bacterial infection	MISO	Identified hundreds of retained introns and alternative exons	5
	West et al., Cell Reports 2019	Mouse RAW 264.7 Macrophages	Salmonella Infection with hnRNP M KD	MAJIQ	94 LSVs in uninfected versus hnRNP M KD macrophages and 67 LSVs in *Salmonella*-infected versus hnRNP M KD macrophages	6
	Janssen et al., G3 2020	Mouse Avelolar Macrophages	LPS	DEXseq	1,000 genes with altered isoform usage on Day 3 *vs.* baseline (Day 0) upon LPS challenge	7
Alternative Polyadenylation	Kalam et al., PLoS Pathogens 2017	Human Macrophages	*Mycobacterium tuberculosis*	DaPars	increase in the uses of distal poly-A sites and increased expression of transcripts with longer 3'-UTR	4
	Gruber et al., Nat Comm 2014	Mouse T cell	Anti-CD3/ CD28/IL2	3'end seq	3,116 genes undergo alternative polyadenylation (APA) at tandem poly(A) sites	8
	Beisang et al., Gene 2014	Human T cells	Anti- CD3/CD28	3'end seq	2352 3′UTRs length changed over time following T cell activation and 203 of these transcripts were CELF1 targets	9
	Singh et al., Nat Comm 2018	Human T cells, B cells	NA	3'end seq	Intronic polyadenylation isoforms are widely expressed in immune cells, differentially used during B-cell development or in different cellular environments, and can generate truncated proteins lacking C-terminal functional domains	10
	Pai et al., PLoS Genetics 2016	Human Macrophages	Bacterial infection	MISO	Identified hundreds of genes with shortened 3' UTRs upon infection	5