CONSEQUENCES OF REGULATED PRE-mRNA SPLICING IN THE IMMUNE SYSTEM

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Abstract | Alternative splicing is widely recognized to be a ubiquitous and crucial mechanism for generating protein diversity and regulating protein expression. Numerous immunologically relevant genes have been found to undergo alternative splicing; however, there has been little effort to develop a coherent picture of how alternative splicing might be used as a general mechanism to regulate the function of the immune system. In this review, I summarize the mechanisms by which splicing is controlled in T cells, and discuss the role of alternative splicing and alternative isoform expression in the regulation of T-cell activation and function.

SPLICING
The processing of pre-mRNA such that introns are removed and exons are joined directly to one another.

CASSETTE EXONS
Exons that are included in only a percentage of the final mRNA transcripts that are derived from a given pre-mRNA.

MUTUALLY EXCLUSIVE EXONS
Two or more exons in a single pre-mRNA that are never both included in the final mRNA transcript.

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A functional immune system requires both a high degree of diversity and the ability of individual cells to rapidly adapt and respond to changing environmental conditions. It is therefore to be expected that such a system would rely heavily on several mechanisms of gene regulation to achieve the required diversity and flexibility of function. In recent years, much attention has focused on understanding the crucial role of transcriptional regulation in the immune system; however, much less attention has been given to another widespread mechanism of gene regulation, namely alternative pre-mRNA SPLICING.

Current studies indicate that primary transcripts from more than 75% of all human genes undergo alternative splicing, with a bias towards genes that are expressed in the nervous and immune systems[1,2]. Indeed, a literature search with the keywords 'alternative splicing' and 'immune system' yields hundreds of papers. Nevertheless, we are only now beginning to appreciate the importance of alternative splicing in regulating the function of the immune system. Many of the genes that have been shown to be alternatively spliced in the immune system belong to families or functional groups, indicating that several key events in an immune response are likely to be influenced by changes in splicing. In addition, recent studies have begun to clarify some of the mechanisms by which the splicing of genes can be altered in response to antigen stimulation. Such studies have indicated that similar or overlapping mechnisms might influence the splicing of multiple genes to allow for coordinate regulation of functionally related proteins. These recent advances are reviewed here with particular emphasis on the role of alternative splicing in T-cell activation and effector function.

Basic patterns of alternative splicing

The term alternative splicing is used to describe any situation in which a primary transcript can be spliced in more than one pattern to generate multiple, distinct mRNAs. This includes the selective inclusion or exclusion of CASSETTE EXONS, the differential use of MUTUALLY EXCLUSIVE EXONS or the differential use of particular SPLICE SITES (FIG. 1). Although there are many patterns of alternative splicing, from a mechanistic standpoint they can generally be viewed simply as splice-site recognition by the splicing machinery — a ribonuclearprotein complex known as the SPLICEOSOME. Several recent reviews have detailed the general mechanisms by which splicing is regulated[3,4]. Essentially, the decision as to whether a particular exon, or exon region, is included in the final mRNA transcript mainly depends on whether the surrounding splice sites are recognized and bound appropriately by the spliceosome (BOX 1). In other words, if the spliceosome binds the splice sites that flank an exon, it is

SPLICE SITES
Conserved sequences at the exon–intron boundaries that direct the splicing machinery and determine the precise location of pre-mRNA cleavage and exon joining.

SPLICEOSOME
A ribonucleoprotein complex that is involved in splicing of nuclear pre-mRNA. It is composed of five small nuclear ribonucleoproteins (snRNPs) and more than 50 non-snRNPs that recognize and assemble on exon–intron boundaries to catalyse intron processing of the pre-mRNA.

SR PROTEIN
A group of highly conserved, serine (S)- and arginine (R)-rich splicing-regulatory proteins. In most experimental systems, binding of an SR protein to an exon leads to exon inclusion.

HETEROGENEOUS NUCLEAR RIBONUCLEOPARTICLE PROTEIN
(HnRNP protein). A class of diverse RNA-binding proteins that associate with nascent pre-mRNA and can influence splicing. Several experimental systems have shown that binding of an hnRNP protein to an exon leads to exon skipping.

EXONIC SPLICING SILENCERS
(ESSs). Sequences in an exon that promote exon skipping.

EXONIC SPLICING ENHANCERS
(ESEs). Sequences in an exon that promote exon recognition and inclusion.

INTRONIC SPLICING SILENCERS
(ISSs). Sequences in an intron that promote skipping of a flanking exon.

INTRONIC SPLICING ENHANCERS
(ISEs). Sequences in an intron that promote recognition and inclusion of a flanking exon.

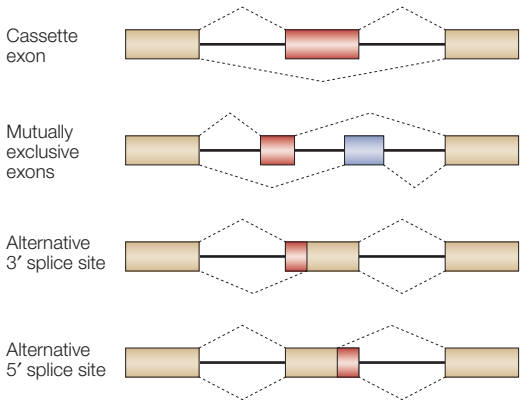


Figure 1 | **Basic patterns of alternative splicing.** Exons and introns are represented by boxes and solid lines respectively. Dashed lines indicate patterns of splice-site joining. Constitutive exons, that is, those that are included in all mRNA isoforms, are in yellow. Red and blue areas indicate the exons, or exon portion, that are differently included in the various alternative-splicing patterns. Cassette exons are complete exons that are differentially included in the final mRNA transcript, whereas mutually exclusive exons indicate a situation in which multiple cassette exons are used in a mutually exclusive manner. Alternative 3′ or 5′ splice sites are patterns in which splice sites within an exon are used instead of those that result in inclusion of the full exon.

included in the final mRNA, but if the spliceosome cannot bind these splice sites, the exon is omitted from the final transcript. For most regulated exons, the spliceosome-bound and spliceosome-unbound states exist in a dynamic equilibrium in any given cell. The balance of this equilibrium determines the ratio of the RNA isoforms that are expressed. Spliceosome binding is determined by the competing activities of various auxiliary regulatory proteins, such as members of the SR PROTEIN or HETEROGENEOUS NUCLEAR RIBONUCLEOPARTICLE (HNRNP) PROTEIN families, which bind to non-splice-site regulatory sequences and alter the binding of the spliceosome to a particular splice site (BOX 1). Such regulatory sequences are known as EXONIC SPLICING SILENCERS (ESSs), EXONIC SPLICING ENHANCERS (ESEs), INTRONIC SPLICING SILENCERS (ISSs) and INTRONIC SPLICING ENHANCERS (ISEs). Although there are numerous systems in which the mechanisms and consequences of alternative splicing have been studied[5,6], here I focus on the regulation of splicing during T-cell activation, and the role of regulated splicing in modulating the function of the immune system.

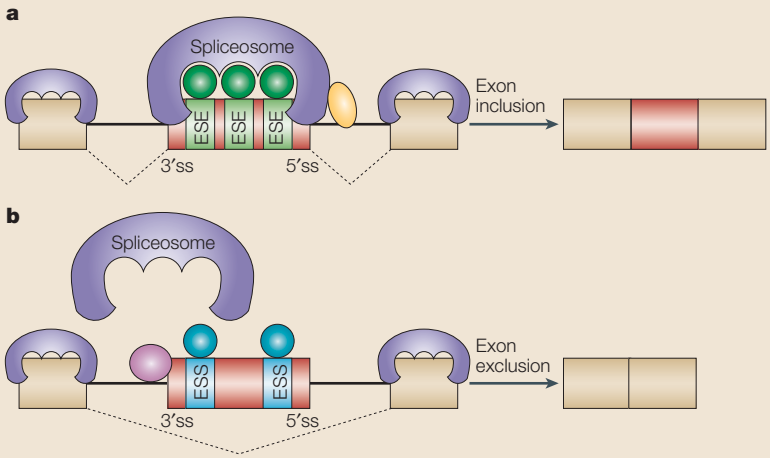
Functional consequence of alternative splicing

The important feature of alternative splicing, in terms of influencing cellular function, is that it allows for the creation of multiple distinct mRNA transcripts from a given gene[7,8]. In many cases, each of these mRNA transcripts has the potential to encode a unique protein, often with distinct or opposing functions. In other cases, some of the mRNA transcripts might not encode a functional polypeptide, but rather function as a discard pathway for excess pre-mRNA to prevent overexpression of the alternative mRNA-encoded protein[9]. Finally, the expression of alternative mRNA transcripts and proteins encoded by a single gene can be regulated in a tissue, temporal or stimuli-dependent manner to control cellular function.

The literature is now full of examples of genes in the immune system for which there is some evidence of a functional role of alternative splicing. This includes the generation of immunological diversity through alternative splicing of transcripts encoding MHC molecules[10] and transporter associated with antigen processing 2 (REF. 11); the expression of soluble forms of Fcγ receptors that alter Fc-dependent function[12]; alterations of T-cell receptor (TCR) signalling through the addition of residues in the cytoplasmic domain of the TCR ζ-chain[13]; and modulation of intracellular signalling and intercellular communication through the expression of various isoforms of cytokines, cytokine receptors, kinases, phosphatases and adaptor proteins (TABLES 1,2). For some of these genes, the evidence for a change in function due to the generation of alternatively spliced transcripts is based solely on analysis of the mRNA transcripts, with no confirmation that distinct protein isoforms are expressed *in vivo*[13–15]. Therefore, the role of alternative splicing in regulating the function of these proteins, although intriguing, is still theoretical. However, for other genes, there has been confirmation that multiple protein isoforms are encoded from a single gene, and we have gained insight into how the function and expression of the protein isoforms differ (TABLES 1,2). Particularly with regards to T-cell activation and function, analysis of examples of alternative splicing that have been confirmed at the protein level to generate distinct protein isoforms indicate a compelling model in which alternative splicing might be used to modulate many of the responses of a T cell to antigen challenge. Moreover, the fact that the splicing patterns of an increasing number of genes have been shown to be influenced by antigen stimulation implies a feedback mechanism in which alternative splicing has a crucial role in modulating the threshold of T-cell activation and maintaining T-cell homeostasis[16–19].

T-cell activation and migration. One of the first events after antigen recognition by the TCR is activation of intracellular protein tyrosine kinases (PTKs). Many of these PTKs have been shown to be alternatively spliced in T cells (TABLE 1). The SRC-family PTK FYN has two isoforms that arise from the splicing of two mutually exclusive exon 7s, which encode parts of the SRC homology 2 (SH2) and kinase domains of the protein[20]. One isoform of FYN (FYNT) is mainly expressed by haematopoietic cells, whereas the other isoform (FYNB) was originally identified in brain tissues. Although both isoforms have been detected in T cells, functional studies have shown that FYNT is more efficient than FYNB at mediating TCR-induced calcium mobilization[21,22]. This difference in function is conferred by sequence differences in the kinase domains of the two FYN isoforms[21]. Interestingly, the normally low expression of the FYNB isoform by T cells increases in virally infected T cells[23], and it is possible that this might decrease T-cell activation, which could be of benefit to the virus. By contrast,

Box 1 | **General mechanisms for splicing regulation**



In mammalian splicing, the splice sites (ss) flanking an exon are recognized by the spliceosome (or spliceosome components along with regulatory proteins, as described below) in a cooperative manner referred to as exon definition[3,4,88]. This exon-definition complex must form in order for an exon to be included in an mRNA transcript. Spliceosome binding is mainly directed by interactions between the intron sequences at the 5′ and 3′ splice sites, and the RNA and protein components of the spliceosome. However, as mammalian splice sites are poorly conserved, they are often not sufficient to bind the spliceosome with high affinity. It has been shown experimentally that proteins bound to non-splice-site sequences in an exon or intron can influence the efficiency of spliceosomal binding by mechanisms that include — but are probably not limited to — spliceosomal recruitment (see figure, part a) or steric hindrance (see figure, part b). Sequences that bind to proteins that promote spliceosome recognition of an exon are known as exonic or intronic splicing enhancers (ESEs or ISEs), whereas sequences that are required to inhibit recognition of an exon are known as exonic or intronic splicing silencers (ESSs or ISSs). Two important conclusions from such a model, which have been verified experimentally, are first, that mutation of sequences located far from a splice site can strongly influence splicing (for an example see the discussion of CD45 regulation) and second, that as the two states depicted above are often in a dynamic equilibrium, subtle changes in the balance of ESS- and ESE-binding proteins can alter the ratio of mRNA isoform expression.

activation of T cells with phorbol esters results in increased expression of FYNT[19], and this could provide a mechanism for amplifying T-cell activation.

A kinase-defective form of the SRC-family member LCK has also been implied by the expression of variant mRNA transcripts, although it has not been shown whether this variant LCK protein is translated by normal T cells[24]. The *SYK* (spleen tyrosine kinase) gene is also alternatively spliced to produce two isoforms, one of which (SYKB) lacks a unique portion of the linker domain that differentiates SYK from other related kinases[25,26]. Although the kinase domain is identical in both isoforms, only SYK, and not SYKB, can promote TCR-mediated signalling[27]. This difference is due to the fact that SYKB is not recruited to the TCR complex because it does not interact with the phosphorylated immunoreceptor tyrosine-based activation motif of the TCR ζ-chain[27]. Although it is unclear whether the relative expression of SYK and SYKB is regulated in T cells, there is evidence that SYKB might be more resistant to proteolysis than SYK[27] and therefore might have prolonged expression.

IMMUNOLOGICAL SYNAPSE
A stable region of contact between a T cell and an antigen-presenting cell that forms through cell–cell interaction of adhesion molecules. The mature immunological synapse contains two distinct, membrane domains: a central cluster of T-cell receptors, known as the central supramolecular activation cluster (cSMAC) and a surrounding ring of adhesion molecules known as the peripheral SMAC.

Other molecules that are crucial to early events in T-cell activation are the cell-surface adhesion molecules. These proteins control IMMUNOLOGICAL SYNAPSE formation, co-stimulation and/or T-cell migration. Two members of the immunoglobulin superfamily of adhesion molecules that are alternatively spliced in T cells are intercellular adhesion molecule 1 (ICAM1)[28] and platelet/endothelial cell-adhesion molecule 1 (PECAM1; also known as CD31)[29]. The genes encoding these proteins both contain cassette exons, the inclusion or exclusion of which alters the extracellular or cytoplasmic domains of these transmembrane proteins, respectively. Such alternative splicing might be predicted to change the adhesion and/or signalling properties of the proteins. Indeed, it has been shown that the ICAM1 isoform that lacks the third immunoglobulin domain cannot bind to lymphocyte function-associated antigen 1 (LFA1), and is deficient in functioning as an accessory molecule in the presentation of antigen to T cells[28,30]. In at least some cell types, expression of this smaller form of ICAM1 is upregulated by stimulation with lipopolysaccharide (LPS)[28]. Although the functional consequence of PECAM1 alternative splicing has not yet been determined, it is interesting to note that prolonged stimulation of Jurkat T cells with the phorbol ester phorbol myristate acetate (PMA) results in an increased production of the smaller PECAM1 isoforms[31]. Given the potential role of PECAM1 as an inhibitor of T-cell activation, a stimulation-induced change in PECAM1 splicing might be indicative of a feedback mechanism, as has been proposed for other genes such as *CD45* (see later).

One of the most striking examples of alternative splicing of a cell-adhesion molecule is that of CD44, a protein involved in T-cell homing. The *CD44* gene has 10 'variable' cassette exons, which are either all omitted from the final mRNA transcript or are included in various combinations (FIG. 2a). At least 20 isoforms of CD44 have been described at the mRNA level[32], with at least six of these being confirmed to produce protein[33] (FIG. 2a). The variable exons of *CD44* all encode portions of the membrane-proximal extracellular domain of the protein, and the presence of some of the variable exons, such as v3, v6 and v7, has been shown to increase the association of CD44 with various proteins or with the extracellular matrix polysaccharide hyaluronan[33]. Although a clear functional distinction between the CD44 isoforms has not been determined, a strong correlation has been observed between malignant severity of tumours and expression of CD44 isoforms that include the variable exons v4–v7, v3 or v9 (REFS 34–36). In addition, the expression profile of CD44 isoforms in T cells is altered after activation, such that naive T cells mainly express the smallest CD44 isoform that lacks all variable exons, whereas activated T cells express multiple CD44 isoforms, including those that contain variable exons v1, v3, v4, v5, v7 or v10 (REFS 18,37). Interestingly, antibodies specific for these larger molecular-weight variants of CD44 block *in vivo* activation of T cells[18], thereby indicating that the alternative splicing of CD44 is crucial for T-cell function.

FOUR-HELICAL BUNDLE
A structural motif in proteins in which four α-helices are packed together.

DOMINANT-NEGATIVE INHIBITOR
A protein variant that inhibits the proper function of the co-expressed wild-type protein.

Cytokine response. The secretion of, and response to, various cytokines has a crucial role in T-cell development, proliferation and effector function. Notably, many studies indicate that almost all aspects of cytokine signalling (cytokine production, cytokine-receptor expression, and the expression and activity of signal-transduction molecules) might be influenced by alternative splicing[38,39]. Interleukin-6 (IL-6) is a cytokine that has been implicated in the differentiation of T cells[40]. Similar to many cytokines, including IL-2 and IL-4, IL-6 has an overall structure of a FOUR-HELICAL BUNDLE[41–43]. Skipping of exon 4 from IL-6 pre-mRNA results in the deletion of two of the four helices, although the overall fold and stability of the molecule is maintained[44]. The IL-6 receptor (IL-6R) is composed of subunits that only associate in the presence of IL-6 (REF. 45). Importantly, although the smaller isoform of IL-6 can still bind to one subunit of the IL-6R (IL-6Rα), it lacks the binding sites that are required to recruit the signalling subunit of the receptor (IL-6Rβ)[44]. Therefore, the exon-exclusion isoform of IL-6 is predicted to function as a DOMINANT-NEGATIVE INHIBITOR of IL-6 signalling. Such an IL-6 inhibitor could potentially block the differentiation of $CD4^+$ T cells to T helper 2 cells[40]. Alternative splicing similar to that of IL-6 has also been observed for IL-4 and IL-2, two cytokines that are essential for T-cell maturation and proliferation[14,15]. As for IL-6, the alternative IL-4 and IL-2 mRNA transcripts lack an exon that encodes residues that are crucial for the formation of an active receptor complex, such that the alternative isoforms would be predicted to function as dominant-negative signalling inhibitors[46,47]. Unfortunately, a lack of appropriate antibodies has prevented proof that such inhibitory IL-4 and IL-2 proteins are naturally expressed. However, given the potential regulatory importance of such molecules in T-cell differentiation and proliferation, further investigation is clearly warranted to determine whether the expression and function of IL-2 and IL-4 are influenced by alternative splicing.

In addition to the production of variant cytokines, alternative splicing also gives rise to variant cytokine receptors. Soluble versions of many cytokine receptors have been detected in the serum, and for at least several cytokines, including IL-4, IL-5 and IL-7, the expression of a soluble receptor isoform has been shown to be a result of alternative splicing[39]. For some receptor subunits, such as IL-4Rα, the soluble isoform is encoded by an mRNA transcript that includes a mutually exclusive exon containing a stop codon, which is inserted before the exons encoding the transmembrane and extracellular

Table 1 | **Splice variants shown to be translated in T cells**

Gene family	Gene	Splicing event	Consequence on protein	Functional consequence	Expression pattern in T cells	Refs
PTK	*FYN*	Mutually exclusive exons	Change in SH2 and kinase domains	FYNT is better than FYNB at Ca^{+2} flux and IL-2 production. Both are the same in terms of phosphorylating tyrosines	FYNB increased on infection of T cells with HTLV-1. FYNT increased on PMA stimulation of Jurkat T cells	19,21, 23
PTK	*SYK*	Cassette exon	Deletion of portion of linker region	Altered binding to ITAMs. Less support of TCR-induced signalling	ND	25,27
Adhesion molecule (Ig superfamily)	*ICAM1*	Cassette exons	Altered number of immunoglobulin domains	Different adhesion properties to LFA1	Some isoforms can differ on stimulation with LPS	28
Adhesion molecule (Ig superfamily)	*PECAM1* (*CD31*)	Cassette exons	Altered cytoplasmic domain	ND	Increased skipping of cassette exons on PMA stimulation of Jurkat T cells	29,31
Adhesion molecule	*CD44*	Cassette exons	Altered membrane-proximal extracellular domain	ND	Increased inclusion of cassette exons on antigen stimulation of naive T cells or PMA stimulation of Jurkat T cells	18,33
PTP	*CD45*	Cassette exons	Altered extracellular domain	Change in dimerization capacity Change in phosphatase activity	Increased skipping of cassette exons on antigen stimulation of naive T cells or PMA stimulation of Jurkat T cells	16,59
Cell-surface receptor	*CTLA4*	Cassette exon	Change in inclusion of transmembrane domain	Transmembrane versus soluble form of molecule	Increased inclusion of cassette exon (transmembrane form) on stimulation of naive T cells	17,68
Apoptosis	*TID1*	Mutually exclusive exons	Altered carboxyl terminus	Pro-apoptotic versus anti-apoptotic form of molecule	Anti-apoptotic form increases on antigen stimulation of T_H2 cells. Putative causative factor in T_H2-cell resistance to AICD	85
Apoptosis	*CD95*	Cassette exon	Change in inclusion of transmembrane domain	Transmembrane versus soluble receptor	Transmembrane form is selectively increased on stimulation of PBMCs	86

AICD, activation-induced cell death; CTLA4, cytotoxic T-lymphocyte antigen 4; HTLV-1, human T-cell leukaemia virus type 1; ICAM1, intercellular adhesion molecule 1; Ig, immunoglobulin; IL-2, interleukin-2; ITAM, immunoreceptor tyrosine-based activation motif; LFA1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; ND, not determined; PBMC, peripheral-blood mononuclear cell; PECAM1, platelet/endothelial cell-adhesion molecule 1; PMA, phorbol myristate acetate; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SH2, SRC homology 2; SYK, spleen tyrosine kinase; TCR, T-cell receptor; T_H2, T helper 2; TID1, tumorous imaginal discs (*Drosophila*) homologue.

domains[48]. In other cases, such as for IL-7Rα, the soluble form is generated as a result of exclusion of a cassette exon that encodes the transmembrane domain[49]. Regardless of the pattern of alternative splicing, the production of a soluble form of a cytokine receptor is thought to be tightly regulated and can have a wide variety of effects on cytokine-mediated signalling, including the inhibition or activation of cytokine signalling depending on whether the soluble receptor competes or synergizes with the membrane-bound receptor for binding to the cognate cytokine[39].

Finally, alternative splicing has been observed for many of the intracellular molecules that are involved in the transduction of a signal from a cytokine receptor to downstream targets. One of these is the PTK PYK2, which although not specific for cytokine-induced signal-transduction pathways, is activated downstream of receptors for IL-2, IL-7 and tumour-necrosis factor[50–52]. Inclusion of a unique cassette exon in the PYK2 mRNA alters one of the proline-rich domains of the protein. As described earlier for SYK, this alternative splicing of PYK2 does not change the catalytic activity of the protein, but does alter the ability of PYK2 to interact with an unidentified protein of approximately 115 kDa and this might have consequences on the signalling capability of PYK2 (REF. 53). Another kinase involved in cytokine signalling is IL-1R-associated kinase 1 (IRAK1), a serine/theronine kinase that is associated with IL-1R[54]. Two variants of IRAK1 are expressed by thymocytes. Use of an alternative 3′ splice site in exon 12 leads to expression of an IRAK1 isoform that lacks 30 amino acids at the end of its kinase domain[55]. This variant isoform therefore lacks kinase activity, although it retains the ability to interact with many IRAK1-binding partners. This smaller isoform is also more stable on IL-1 induction than its full-length counterpart, indicating that it might function as part of a negative-feedback loop in which IRAK1 function is decreased in response to IL-1 stimulation[55]. IL-1 signalling is also influenced by alternative splicing of the adaptor protein MyD88 (myeloid differentiation primary-response gene 88). Full-length MyD88 protein interacts with the IL-1R and recruits IRAK4 to phosphorylate and activate IRAK1 in response to IL-1 binding[56,57]. The gene encoding MyD88 contains a cassette exon that encodes a small domain that is required for association with IRAK4. When this cassette exon is skipped, the resulting isoform of MyD88 (MyD88s) is not capable of recruiting IRAK4, and functions as a dominant-negative inhibitor of IL-1 signalling[57,58]. Although it is not yet clear what function this inhibitory isoform has *in vivo*, it is interesting to note that expression of the small isoform has been shown to specifically increase after LPS activation of monocytes through Toll-like receptor 4 (TLR4), a member of the TLR/IL-1R superfamily which, similar to IL-1R, initiates a MyD88-dependent signalling cascade[58].

T-cell homeostasis. Once a T cell has been activated and has carried out its required effector functions, its activity must be attenuated to prevent hyperproliferation or hyperactivity of the immune response. T cells have various mechanisms to achieve this crucial dampening of the immune response, including the recently recognized effects of alternative splicing of CD45 and cytotoxic T-lymphocyte antigen 4 (CTLA4). CD45 is a transmembrane protein tyrosine phosphatase that is essential for activation of resting T cells[16]. The gene

Table 2 | **Cytokine-related alternative splicing in T cells**

Gene family	Gene	Splicing event	Consequence on protein	Functional consequence	Expression pattern in T cells	Refs
Cytokine	*IL-6*	Cassette exon	Deletion of α-helices B and C	Loss of interaction with IL-6Rβ (the signalling component of the receptor). Inhibition of signalling by full-length IL-6	ND	44
Cytokine	*IL-15*	Alternative 3′ splice site	Alters translational start and leader sequence of peptide	Differential translation efficiency and targeting of peptide	ND	87
Cytokine receptor	*IL-4Rα*	Mutually exclusive exons	Altered carboxyl terminus	Transmembrane versus soluble receptor	ND	48
Cytokine receptor	*IL-7Rα*	Cassette exon	Change in inclusion of transmembrane domain	Transmembrane versus soluble receptor	Present in many T-cell lineages. Expression might be increased in leukaemic cells	49
PTK	*PYK2*	Cassette exon	Deletion of a portion of one of the two proline-rich regions	Altered binding to various protein partners	PYK2-H, with a shorter proline domain is expressed by T and B cells. The longer version, PYK2, is expressed in the brain	53
Serine/threonine kinase	*IRAK1*	Alternative 3′ splice site	Loss of 30 amino acids at terminus of the kinase domain	Change in kinase activity and stability	Both forms are expressed by thymocytes	55
Intracellular adaptor protein	*MyD88*	Cassette exon	Deletion of intermediary domain	Change in ability to interact with IRAK4, resulting in loss of signalling	Increased expression of the smaller isoform on LPS stimulation of monocytes	57,58

IL, interleukin; IRAK, IL-1R-associated kinase; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary-response gene 88; ND, not determined; PTK, protein tyrosine kinase; PYK2, protein tyrosine kinase 2; R, receptor.

MIXED LEUKOCYTE REACTION
A tissue-culture technique for testing T-cell reactivity. The proliferation of one population of T cells, induced by exposure to inactivated MHC-mismatched stimulator cells, is determined by measuring the incorporation of ^{3}H-thymidine into the DNA of dividing cells.

encoding CD45 contains three cassette exons that are independently regulated, such that the gene encodes five isoforms of CD45 protein that differ in their extracellular domains[16] (FIG. 2b). Importantly, splicing of the transcript encoding CD45 is significantly altered in response to prolonged activation of T cells[16,59]. Naive T cells express high levels of the larger isoforms of CD45 (CD45RA), whereas activated T cells express predominantly the smaller CD45 isoforms (CD45R0). The CD45 protein has an inherent ability to dimerize, with the smaller isoforms showing the greatest tendency of dimerization[60]. In its dimeric form, the intracellular phosphatase activity of CD45 is inhibited, presumably due to steric occlusion of the catalytic domain[61]. As CD45 phosphatase activity promotes T-cell signalling, such dimerization would be predicted to increase the threshold for T-cell activation. Indeed, cell lines that only express the smaller isoform of CD45 have been shown to respond more weakly to TCR ligation than cells expressing similar levels of the larger isoform[60]. The reduced ability of the small CD45 isoforms to support T-cell activation is consistent with a model in which the change in CD45 splicing, from CD45RA to CD45R0, after T-cell activation, functions as a feedback mechanism to attenuate prolonged TCR signalling[60,62]. Such a model is supported by a study that showed a correlation between a single-nucleotide polymorphism (SNP) that inhibits the activation-induced repression of CD45RA expression and susceptibility to the autoimmune disease multiple sclerosis[63]. The idea that the expression of CD45R0 by effector T cells has a dampening effect on T-cell signalling is not inherently contradictory to the observation that memory T cells (which are by definition CD45R0^{+}) respond strongly to TCR ligation. It has been shown that the requirement for CD45 in TCR signalling can be abrogated by increased expression of SYK[64]. Therefore, memory and effector T cells are likely to have different threshold requirements for CD45 function due to differences in the expression levels of intracellular signalling molecules.

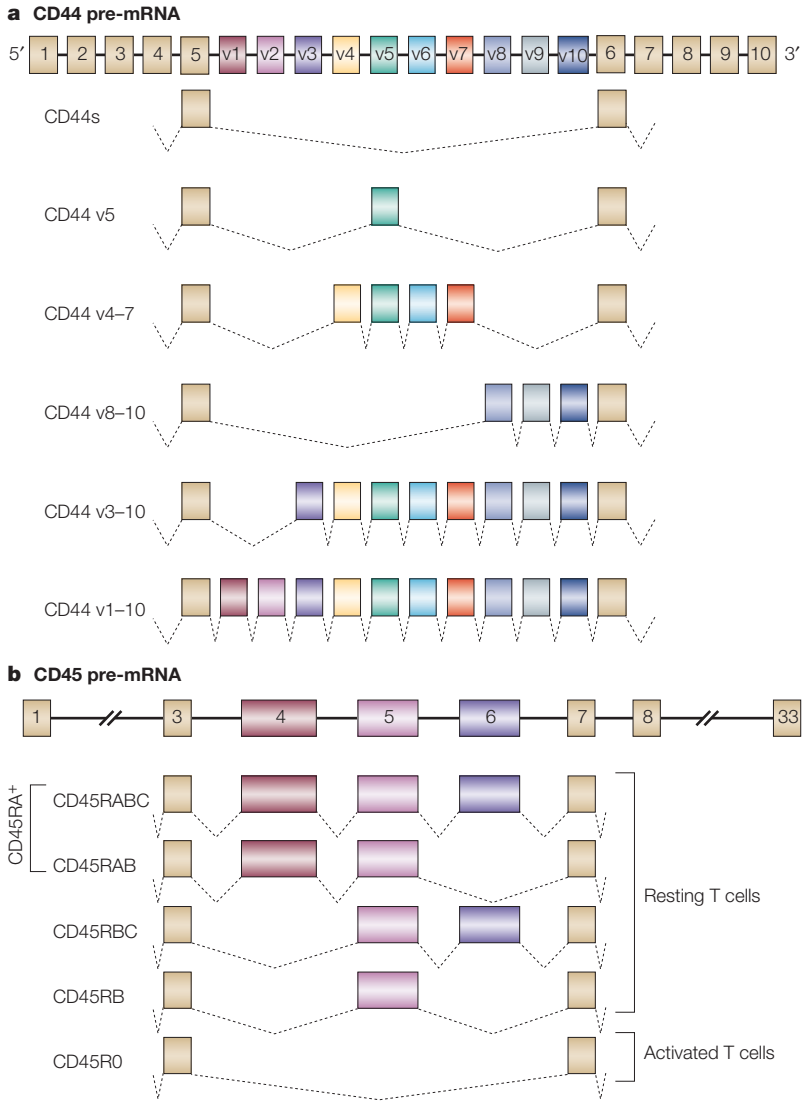


Figure 2 | **Alternative splicing of CD44 and CD45 pre-mRNA. a** | Schematic of the CD44 pre-mRNA, which has a variable cassette exon cluster (v1–v10), and the six distinct mRNA transcripts (generated by alternative splicing of the CD44 pre-mRNA) that are known to be translated. **b** | Schematic of the CD45 pre-mRNA, which contains 33 exons in total. Exons 4, 5 and 6 are variable cassette exons, which are alternatively spliced to give rise to five mRNA transcripts (and resulting protein isoforms). The expression of the various exons is repressed after T-cell stimulation, resulting in predominant expression of the CD45R0 isoform.

Another mechanism by which T cells block prolonged T-cell activation is through the expression of CTLA4. CTLA4 competes with CD28 for binding to CD80 and CD86, thereby inhibiting the CD28-dependent co-stimulatory signal required for naive T-cell activation and transmitting an inhibitory signal[65,66]. In naive T cells little, if any, CTLA4 is expressed on the cell surface, whereas cell-surface expression of CTLA4 is strongly induced on T-cell activation[67]. This increase in cell-surface expression is due not only to increased transcription and protein transport, but is also due to a change in alternative splicing. As described earlier for IL-7Rα, the exon in the *CTLA4* gene that encodes the transmembrane domain functions as a cassette exon[17,68]. This exon is excluded from most of the mRNA transcripts that are translated in resting T cells, therefore what little protein is expressed is mainly a soluble form of CTLA4. This soluble form of CTLA4 can be detected in at least a subpopulation of human serum samples and can possibly function as a general suppressor of the immune system, as it has been shown to inhibit cell proliferation in a MIXED LEUKOCYTE REACTION[17,68]. By contrast, on stimulation of T cells, the splicing of the CTLA4 pre-mRNA is altered so that almost all the final mRNA transcripts include the cassette exon[17,68]. This induced inclusion of the transmembrane-encoding exon ensures that high levels of CTLA4 are expressed on the surface of the activated T cells so as to prevent specific hyperstimulation. Taken together, the studies regarding *CD45* and *CTLA4* indicate that the alternative splicing of these genes might act in concert to modulate the threshold for T-cell activation.

Diseases associated with aberrant splicing in the immune system. An increasing number of human diseases have been shown to be caused by mistakes in

alternative splicing[69–71]. In particular, aberrations in many of the alternative-splicing events described earlier have been linked to human diseases and this observation in itself provides further evidence of the importance of alternative splicing in modulating the function of the immune system. The connection between the altered expression of CD44 and CD45 isoforms and susceptibility to cancer or multiple sclerosis, respectively, has already been described here[34–36,63]. Altered isoform ratios of CTLA4 have also been linked to impaired immune function[72,73]. In particular, one study found that a CTLA4 genotype linked to Graves' disease corresponds to less exclusion of the transmembrane exon, leading to decreased expression of soluble CTLA4 (REF. 73). Variation in the ratio of expression of membrane and soluble forms of cytokine receptors has similarly been shown to correlate with disease. Selectively increased expression of the soluble form of IL-6R correlates with increased disease severity in conditions such as rheumatoid arthritis, asthma, systemic sclerosis and multiple myeloma[74]. Moreover, the expression of truncated, soluble isoforms of IL-7Rα is higher in patients with acute lymphoblastic leukaemia than in normal individuals[75], whereas the expression of soluble forms of IL-5R is high in patients with asthma[76]. Finally, mRNA transcripts encoding the putative truncated, inhibitory IL-4 variant that was discussed earlier have been shown to be markedly higher in patients with systemic sclerosis than in normal controls[77]. So, understanding the regulation of splicing in the immune system is not only crucial to understanding normal immune function, but it might also help us to determine the causal mechanisms behind numerous human diseases.

Regulation of alternative splicing

The abundance of genes in the immune system that are alternatively spliced, and the connections between splicing and disease, strongly imply that alternative splicing is a crucial mechanism for regulating and fine-tuning the function of the immune system. Clearly, if we are to fully understand the regulation of immune function, we need to have a more complete understanding of how the alternative splicing of implicated genes is controlled at different stages during development and activation of the immune system. Surprisingly, however, little work has been done to identify the sequences, proteins and mechanisms by which splicing is regulated in the immune system. Indeed, of all the genes discussed earlier, only the splicing of *CD44* and *CD45* has been studied in any mechanistic detail. Interestingly, a comparison between even these two regulatory events gives an initial indication of the commonalities and complexities of alternative splicing in T cells.

As described earlier, the *CD44* gene contains 10 variable cassette exons, which are thought to be regulated independently of one another (FIG. 2a). Inclusion of CD44 variable exons v1, v3, v4, v5, v7 and v10 has been shown to be increased after stimulation of T cells[37]. The mechanisms controlling inclusion of exon v5 in activated T cells is the best understood of any signalling-induced splicing event. Deletion analysis of CD44 exon v5 reveals the presence of an ESS and an ESE in this exon[37] (FIG. 3a). Although the function of the ESE is not clear, the ESS is thought to repress inclusion of exon v5 in resting T cells by functioning as a high-affinity binding site for the known splicing repressor protein hnRNP A1, as mutation of the ESS most strongly inhibits hnRNP A1-dependent skipping of exon v5 in co-transfection experiments[78]. Activation-induced inclusion of exon v5 in T cells depends on the RAS–ERK (extracellular signal-regulated kinase) signalling pathway[37,79], and is enhanced by overexpression of the STAR (signal transduction and activation of RNA) family protein SRC-associated in mitosis, 68 kDa (SAM68)[80]. SAM68 binds to the ESS of exon v5 in *in vitro* assays, therefore suggesting a model in which SAM68 could compete with hnRNP A1 for binding to exon v5, thereby relieving hnRNP A1-dependent repression of splicing[80]. The fact that SAM68 is phosphorylated by ERK indicates that this phosphorylation might function as a switch to increase usage of exon v5 after TCR ligation, although mutation of the putative phosphorylation sites on SAM68 does not block the ability of SAM68 to promote ERK-induced inclusion of exon v5 (REF. 80).

Such studies showing a role for hnRNP A1 and SAM68 in controlling the splicing of CD44 exon v5 do not rule out the potential involvement of other regulatory proteins. Indeed, overexpression of both the single-stranded nucleic-acid-binding protein YB1 (Y box-binding protein 1) and the RNA helicase p72 have been shown to influence the inclusion of exon v5. Both YB1 and p72 were identified as proteins that bind to CD44 variable exon v4 (REFS 81,82) (FIG. 3b). In transfection studies with a construct containing both *CD44* exons v4 and v5, overexpression of YB1 and p72 was shown to increase inclusion of exon v4 in a manner that was dependent on the presence of an ESE that is A/C-rich (ACE) in exon v4 (REFS 81,82). Surprisingly, the inclusion of exon v5 was also increased by YB1 and p72, despite the fact that exon v5 does not seem to have an ACE-like sequence. The precise mechanism by which YB1 and p72 regulate CD44 exon v4 or v5 inclusion has yet to be determined.

Similar to *CD44*, *CD45* contains multiple variable cassette exons (4, 5 and 6) that are independently regulated. At least some combination of the CD45 variable exons are included in about 40–90% of mRNA transcripts in resting T cells, but these exons are predominantly excluded from the mRNA in activated T cells[16] (FIG. 2b). Each of these exons contains unique ESE and ESS sequences that determine their level of inclusion in resting T cells[83] (A. Tong, J. Nyugen and K.W.L., unpublished observations). The functional role of these sequences has been emphasized by the finding of a naturally occurring, synonymous SNP (C77G) that disrupts the ESS of CD45 exon 4 (REF. 83). This SNP results in abnormally high levels of exon 4 inclusion in T cells, and as described earlier, correlates with susceptibility to multiple sclerosis[63,84]. In addition to the ESS and ESE activities, each of the variable exons contains

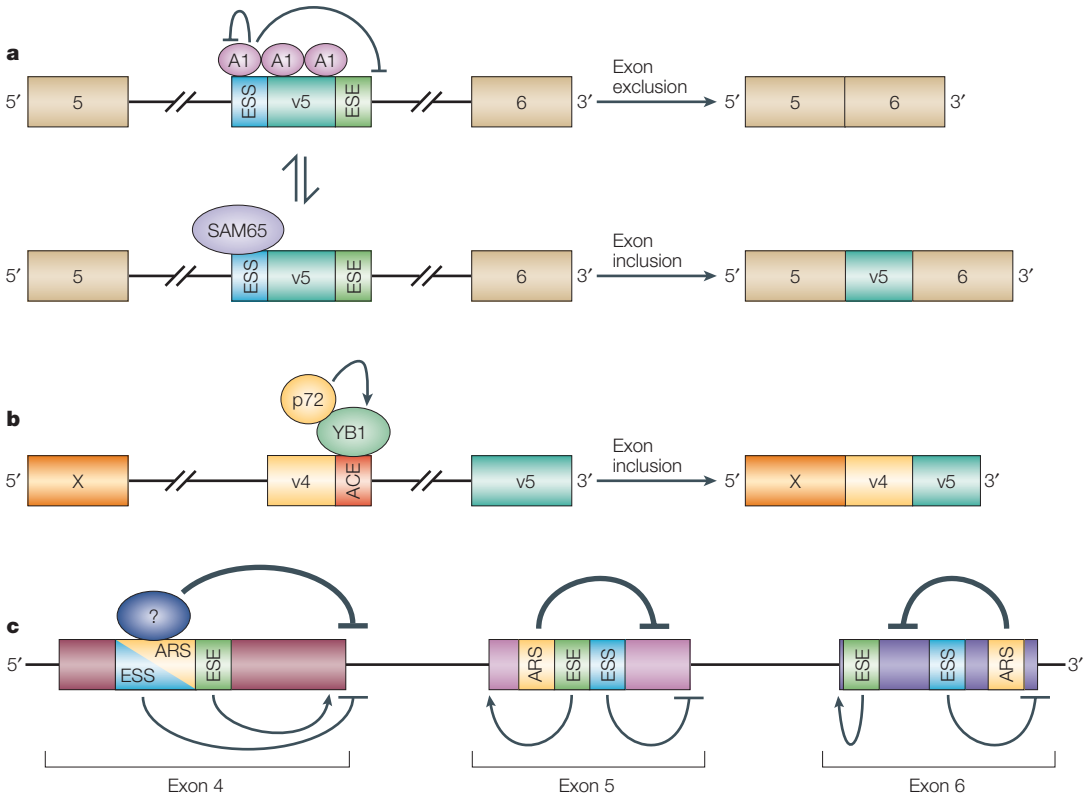


Figure 3 | **Proposed models for the regulation of CD44 and CD45 alternative splicing. a** | Proposed model for the regulation of CD44 exon v5. In this model, the proteins hnRNP A1 (heterogenous nuclear ribonucleoparticle protein A1) and SAM68 (SRC-associated in mitosis, 68 kDa) compete for binding to the exonic splicing silencer (ESS) region of the exon. Binding of hnRNP A1 leads to exon skipping, whereas binding of SAM68 relieves this repression. **b** | Proposed model for the regulation of CD44 exon v4. Y box-binding protein 1 (YB1) has been shown to bind to the A/C-rich exonic splicing enhancer (ACE ESE) of CD44 exon v4 and to promote inclusion of exon v4 in an ACE-dependent manner. The RNA helicase p72 also increases exon v4 inclusion in an ACE-dependent manner. **c** | Schematic of the regulation of CD45 variable exons. In exon 4, the activation-responsive sequence (ARS) overlaps with the ESS (indicated by the dual colours). A complex of as-yet-unknown proteins binds to this dual ESS/ARS sequence. Arrows and bars indicate the activation or repression of exon usage, respectively, with the thickest bars indicating activation-induced exon repression. Except for exon 4, in which regulation is known to occur at the 5′ splice site, the location of the arrows is not meant to suggest mechanism.

a consensus sequence motif that is known as the activation-responsive sequence (ARS)[19] (FIG. 3c). The ARS is responsible for mediating CD45 EXON SKIPPING after T-cell activation, as mutation of key residues in the ARS in any of the three exons abolishes activation-induced exon repression[19]. Moreover, insertion of the ARS-containing region from CD45 exon 4 into an heterologous, unregulated exon is sufficient to confer exon repression on T-cell activation[19]. At least for CD45 variable exon 4, the ARS binds a protein complex that inhibits exon recognition by the spliceosome and leads to exon skipping (C. Rothrock, A. House and K.W.L., unpublished observations). However, the components of this protein complex, the mechanism by which this complex represses exon recognition and the means by which the activity or abundance of this complex increases after T-cell activation remain to be determined.

Although T-cell signalling leads to inclusion of the CD44 variable exons and skipping of the CD45 variable exons, there are still similarities between the two pathways. As shown for CD44, the signalling pathway that leads to CD45 alternative splicing includes activation of RAS, although the role of ERK in the regulation of CD45 splicing is less clear[59]. Despite this similarity, the T-cell signalling pathways that induce changes in CD44 and CD45 splicing do diverge at some point. For example, the regulation of CD45 splicing, but not that of CD44, requires *de novo* protein synthesis[37,59]. This perhaps reflects the requirement for CD44 alternative splicing during early T-cell effector functions, such as homing to sites of infection, whereas CD45 alternative splicing only needs to occur later in the time-course of an antigen response, to maintain homeostasis.

EXON SKIPPING
Exclusion of an exon from a pre-mRNA into the final mature mRNA transcript.

Conclusions and future perspectives

Although alternative splicing of CD44 and CD45 are regulated by overlapping, but distinct mechanisms, there is reason to believe that other genes may be regulated by mechanisms that have more in common with CD45 splicing. The ARS that controls the activation-induced repression of CD45 variable exons is also found in the regulated exons of other genes[19], including *FYN*, *CTLA4* and *PECAM1*, which influence the threshold of

T-cell activation. This raises the possibility that the regulated splicing of these genes, and/or other genes with overlapping functional roles, might be coordinately regulated at the level of alternative splicing to achieve even tighter or more robust control over the activity of the immune system.

Obviously, much work remains to be done in understanding the mechanisms and ramifications of regulated alternative splicing in the immune system. Delineation of the sequences that are crucial to the splicing regulation of various genes would help us to interpret or anticipate potentially deleterious nucleotide mutations in these genes, as has been done for *CD45* (REF. 83). Identification of more of the proteins, pathways and mechanisms by which splicing regulation occurs would allow for the further identification of concerted changes in splicing, and clarify the ways in which changes in splicing are induced to yield functionally significant outcomes. The immune system, with its complex developmental pathways and finely tuned responses, has always been an important and fascinating model of the intricacy and complexity of biological regulation. The role of alternative splicing in the control of immune responses is just one more layer of regulation that we must understand to fully appreciate this highly evolved and crucial system.

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Competing interests statement

The author declares no competing financial interests.

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