RNA Biology 7:4, 462-473; July/August 2010; © 2010 Landes Bioscience

Alternative splicing in multiple sclerosis and other autoimmune diseases

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Key words: alternative splicing, autoimmune disease, SNP, multiple sclerosis, interleukin 7 receptor alpha chain

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; HLA, human leukocyte antigen; hnRNP, heterogeneous nuclear ribonucleoprotein; *IL7R*, interleukin 7 receptor α chain; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cell; pre-mRNA, pre-messenger RNA; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single-nucleotide polymorphism; SR, serine/arginine-rich protein; TCRζ, T-cell receptor ζ chain

Alternative splicing is a general mechanism for regulating gene expression that affects the RNA products of more than 90% of human genes. Not surprisingly, alternative splicing is observed among gene products of metazoan immune systems, which have evolved to efficiently recognize pathogens and discriminate between "self" and "non-self", and thus need to be both diverse and flexible. In this review we focus on the specific interface between alternative splicing and autoimmune diseases, which result from a malfunctioning of the immune system and are characterized by the inappropriate reaction to self-antigens. Despite the widespread recognition of alternative splicing as one of the major regulators of gene expression, the connections between alternative splicing and autoimmunity have not been apparent. We summarize recent findings connecting splicing and autoimmune disease, and attempt to find common patterns of splicing regulation that may advance our understanding of autoimmune diseases and open new avenues for therapy.

Introduction

Alternative splicing is a highly elaborate process of gene regulation and a potent generator of protein diversity. Most metazoan protein-coding genes consist of short exons and much longer introns, which are retained and lost in mature mRNAs, respectively. For mRNAs to be transported to the cytoplasm where they can direct protein synthesis, introns need to be efficiently and accurately removed. This is achieved via the process of RNA splicing.[1] Considering that multiple mRNAs can be generated from a single pre-mRNA via different combinations of splicing patterns, and that splicing can be regulated in a variety of ways (tissue-specific, developmental stage-specific, stimulus-specific),[2] the resulting mRNA and protein diversity is truly enormous. According to some recent estimates,[3] up to 94% of all human genes undergo alternative splicing, which means that this mechanism of post-transcriptional regulation is critical for virtually all cellular processes.

Several layers of information, referred to as the "splicing code", are included in the pre-mRNA sequence (reviewed in refs. 2–5). These include the consensus 5' and 3' splice sites, a branch point sequence upstream of the 3' splice site, and a wide variety of intronic and exonic splicing elements (see below). The basic splicing machinery, known as the spliceosome, recognizes and binds to these elements and executes a highly complicated task of correctly defining and joining appropriate exons together. Two cellular spliceosomes termed "major" and "minor", work in parallel; here we will focus on the former. The major spliceosome, a multi-megadalton ribonucleoprotein particle, consists of both RNAs (small nuclear RNAs, snRNAs: U1, U2, U4/U6 and U5) and a variety of protein factors (which together with the snRNAs form five small nuclear ribonucleoprotein complexes, or snRNPs).

The mechanisms of spliceosome assembly and action have been described in detail elsewhere (reviewed in refs. 6–9). Briefly, the 5' splice site is recognized first by the U1 snRNP, followed by the U2 snRNP binding to the branch point site, which results in the formation of the pre-splicing complex. Addition of the U4/U5/U6 tri-snRNP to the pre-splicing complex produces a fully assembled spliceosome, which undergoes conformational changes resulting in the first catalytic step of the splicing reaction, branch formation. After subsequent conformational changes and proper alignment of the two exons to be spliced, the second catalytic reaction, ligation of the exons, occurs. Importantly, spliceosome function is executed through dynamic RNA-RNA, RNA-protein and protein-protein interactions.[1,10]

Mammalian splice and branch point sites are short sequences, which do not contain all the information necessary to direct correct exon definition. This information is provided by additional splicing regulatory elements that exist within both exons and introns. Based on their mode of action, these additional elements

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Submitted: 02/20/10; Accepted: 05/07/10
Previously published online:
www.landesbioscience.com/journals/rnabiology/article/12301

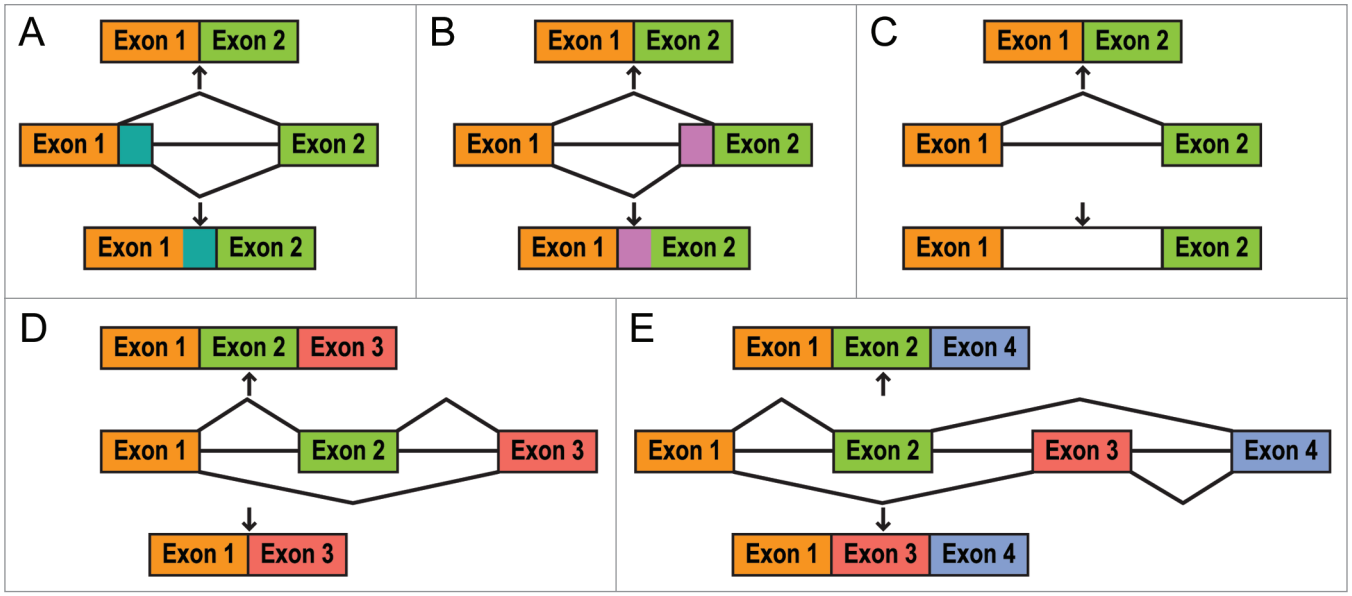

Figure 1. Alternative pre-mRNA splicing. (A) alternative use of 5′ splice sites; (B) alternative use of 3′ splice sites; (C) intron retention; (D) alternative use of a cassette exon; (E) alternative use of two mutually exclusive exons. For explanations, see text.

can be divided into at least four groups: exonic or intronic splicing enhancers (ESEs or ISEs) activate splicing from within exons or introns, respectively, while exonic or intronic splicing silencers (ESSs or ISSs) suppress splicing.[2,4,11] Occasionally, the same element is able to activate and suppress splicing of two mutually exclusive exons (reviewed in ref. 12). Most of the aforementioned cis-acting regulatory elements function through the binding of different trans-acting protein factors such as heterogeneous nuclear ribonucleoproteins, hnRNPs and SR (serine/arginine-rich) proteins. The mechanism by which many of these cis-elements and trans-acting factors regulate splicing is, however, still not completely understood.

The layers of regulation described above control both constitutive and alternative splicing of exons. Alternative splicing, which leads to the generation of multiple different transcripts from the same primary template, proceeds through several different pathways (**Fig. 1**): (a) use of alternative 5' splice sites; (b) use of alternative 3' splice sites; (c) intron retention; (d) a choice to include or skip an exon, and (e) a choice between two mutually exclusive exons.[5,13] Together with other events that lead to transcript diversity (e.g., alternative promoter usage and/or alternative polyadenylation)[3] the five processes described in **Figure 1** result in protein isoforms that can possess diverse or even opposite functions.

The extraordinarily high degree of proteome diversity generated by alternative splicing, as well as the number of genes that undergo this processing, implies that alternative splicing regulates virtually every aspect of cellular metabolism, and, as a consequence, every system in the human body. The immune system is no exception. Evolved to protect the body from remarkably diverse pathogens and to discriminate between "self" and "non-self", the immune system requires diversity of gene expression, and the contribution of alternative splicing to this diversity has only recently begun to be appreciated.

Many genes in the immune system have been shown to undergo alternative splicing (e.g., HLA, TCRζ-chain, a variety of cytokine and cytokine receptor transcripts; for an excellent review on the importance of alternative splicing in the immune system, see ref. 13). Nevertheless, our knowledge of the global involvement of alternative splicing in different aspects of immunity is still limited. Microarrays and other high-throughput technologies provide an invaluable tool to study global gene expression on a genome-wide scale. When applied to studying genes involved in T- and B-cell activation, microarray analysis using Affymetrix Human Exon 1.0 ST arrays reveals three distinct classes of genes that change as a function of immune activation in both T and B cells: (1) differentially expressed and alternatively spliced; (2) constitutively expressed and alternatively spliced, and (3) differentially expressed without alternative splicing.[14] Interestingly, of all the differentially expressed genes, about 44% are alternatively spliced at each time point of T-cell activation, while of all the constitutively expressed genes, 50–70% are alternatively spliced. This finding shows that there is potential for a significant increase in transcript diversity due to alternative splicing of constitutively expressed genes upon T-cell activation. These genome-wide approaches should reveal the global impact of alternative splicing on the immune system.

Alternative splicing and autoimmune diseases. As noted above, alternative splicing is critical for normal functioning of virtually every system in the human body. At the same time, it is also implicated in the development of various diseases, and many disease-associated missense mutations mediate their deleterious effects by altering the splicing pattern of the gene.[2,15] Autoimmunity, or the inappropriate reaction to self-antigens, underlies a number of diseases, some organ-specific and some systemic, and represents an exciting, yet complex, field of study. Many (if not all) autoimmune diseases are thought to be complex,

with both environmental and multiple genetic factors determining pathology. An understanding of the importance of alternative splicing in the development of autoimmune diseases is now just starting to emerge. As is the case for any other disease in which aberrant splicing plays a role, autoimmune diseases can result from mutations in both cis-acting regulatory elements and trans-acting factors. As reviewed in ref. 4, mutations in any of these elements can: (1) cause disease directly; (2) modify its severity; (3) confer susceptibility. Moreover, processing and presentation of antigens, both of "self" and "foreign" origin, can be influenced by alternative splicing (see below). Overall, deciphering the connections between alternative splicing and autoimmunity holds promise for a better understanding of both processes.

While a number of genes have been found to be associated with autoimmune diseases, we will focus this review on multiple sclerosis, a major immune-mediated neurological disorder, and discuss the data that point to a role of alternative splicing in this disease. We will then briefly outline how other autoimmune diseases may be affected by alternative splicing.

Multiple Sclerosis

Multiple sclerosis (MS) is a major neurodegenerative disorder involving the central nervous system (CNS). Disease features include penetration of lymphocytes into the CNS, induction of local inflammation and immune responses, demyelination and axon damage.[16] MS preferentially affects young adults and shows a strong gender preference with females being affected 2–3 times more frequently than males.[17] The course of the disease varies greatly among individual patients, ranging from a mild, or even asymptomatic, disease to severe disability. MS has been divided into four clinical subtypes as a means of predicting prognosis and response to therapy: relapsing remitting (85% of diagnosed MS cases), primary progressive (10%), progressive relapsing (5%) and secondary progressive (develops in 50–80% of relapsing remitting patients).[16] The prevalence is highest among individuals of Caucasian origin with more than 400,000 individuals affected in the US and more than 800,000 in Europe. MS is a disease with a strong genetic component; however, environmental factors (e.g., viral infection) have also been proposed to play a role.[16]

Multiple family-based and case-control studies have been performed on MS populations to identify disease susceptibility genes. However, for almost forty years the only genetic locus consistently linked to the disease has been the human leukocyte antigen locus, or HLA. This changed in 2007 when a SNP within the gene encoding the interleukin 7 receptor alpha chain (*IL7R*) was shown to be significantly associated with susceptibility to MS (see below). Importantly, alternative splicing of *IL7R* transcripts was affected by this MS-associated allele. We will review the data supporting this finding and then focus our attention on other mRNA transcripts that are potentially regulated by alternative splicing in MS.

IL7R and MS

The receptor for IL7 consists of two chains: the *IL7R* chain (CD127), which provides ligand specificity, and the common cytokine receptor γ_c-chain (CD132). *IL7R* is expressed almost exclusively on cells of the lymphoid lineage, and it is crucial for their survival and proliferation. *IL7R* expression is tightly regulated, strictly based on whether or not these cells need to receive survival, proliferation, or, in some cases, differentiation signals. *IL7R* is expressed at the double negative ($CD4^-CD8^-$) T-cell progenitor stage, absent at the double positive ($CD4^+CD8^+$) stage, and then re-expressed at the single positive ($CD4^+$ or $CD8^+$) stage. In addition to T-cell development in the thymus, naïve and memory T cells in the periphery require signaling through *IL7R* for their survival. The significance of this tightly regulated expression of *IL7R* has been noted and is reviewed elsewhere.[18]

Association of *IL7R* with susceptibility to MS has been suggested by a number of studies, however, this association was unambiguously established and replicated by three partially independent research groups.[19-21] A functional, non-synonymous single-nucleotide polymorphism (SNP) rs6897932 (T→C, T244I) within exon 6 of *IL7R* shows the strongest association with MS among all three studies. The result for rs6897932 is due to overtransmission of the "C" risk allele to offspring affected with MS and is independent of the known HLA effect.[19]

IL7R pre-mRNA consists of eight exons, with exon 6 coding for the transmembrane domain. Two isoforms of *IL7R* have been identified based on alternative splicing of exon 6: a membrane-bound isoform (*rIL7R*), in which exon 6 is included, and a soluble isoform (*sIL7R*), in which exon 6 is skipped (**Fig. 2**).[22,23] Importantly, both isoforms are capable of ligand (IL7) binding.

To test whether alternative splicing of exon 6 was differentially affected in transcripts from the "T" or the MS-associated "C" alleles, Gregory et al.[19] analyzed exon 6 inclusion both in vitro and in vivo. For the in vitro analysis, minigenes containing either "T" or "C" alleles of rs6897932, as well as parts of flanking introns, were created. When transfected into a variety of cell lines, transcripts from the minigenes containing the MS-associated "C" allele show an approximately two-fold increase in exon 6 skipping when compared with transcripts containing the "T" allele. Based on additional mutagenesis analysis (transversions to either "G" or "A" at the SNP position, as well as substitutions around the SNP), the authors concluded that the disease-associated "C" allele affects exon 6 alternative splicing by augmenting the action of an exonic splicing silencer (ESS).

The in vitro results were supported by at least two lines of evidence in vivo: first, when peripheral blood mononuclear cells (PBMC) from healthy controls are analyzed by quantitative real-time PCR for allele-specific *IL7R* expression, a significantly lower expression of the exon 6–7 amplicon is observed for carriers of the "C" allele.[19] Second, semi-quantitative RT-PCR analysis of PBMC from MS patients who are homozygous for the "C" allele showed a 4–5-fold increase in exon 6 skipping compared to patients who are homozygous for the "T" allele (Gregory et al., unpublished results). Additionally, Lundmark et al.[20] reported that expression of *IL7R* mRNA was elevated in the cerebrospinal fluid of individuals with MS in comparison with individuals with other neurological diseases. This suggests that changes in *IL7R* expression have pathophysiological significance, although no distinction between the two *IL7R* isoforms was made. Lastly,

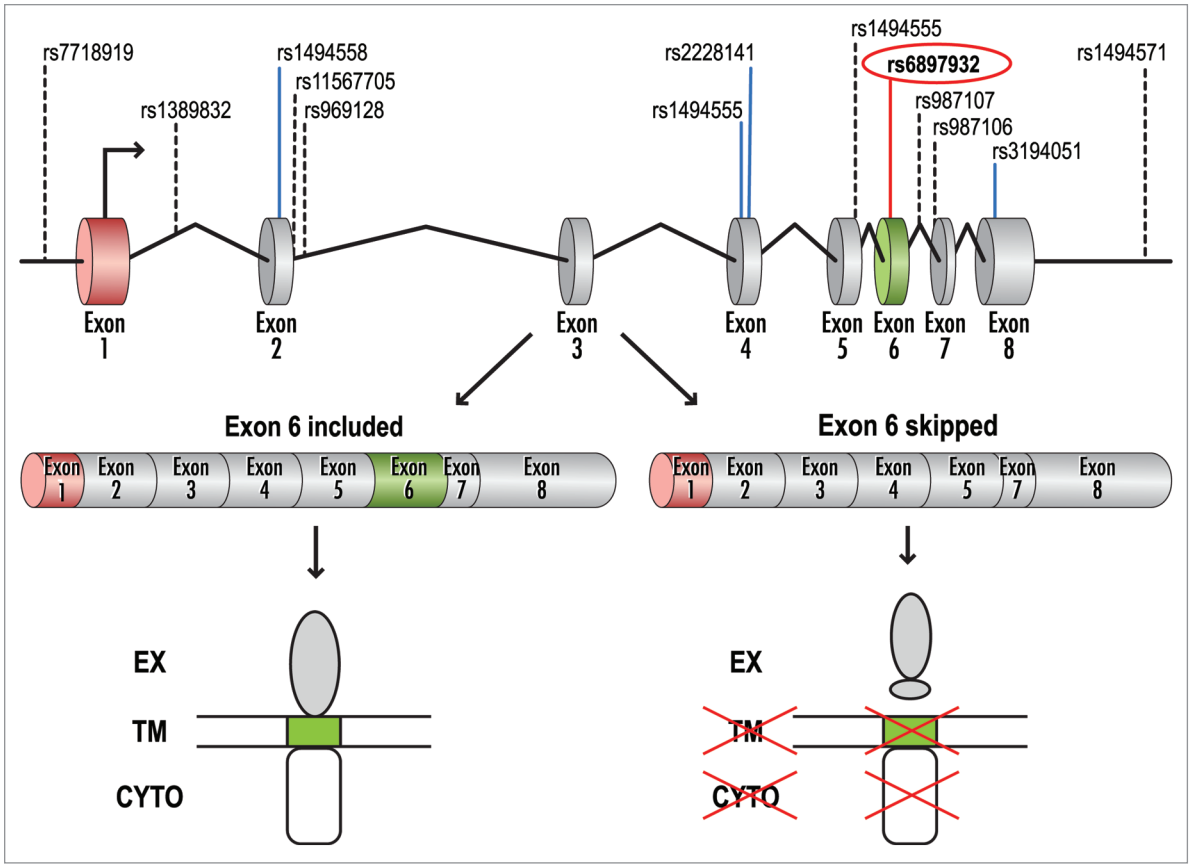

Figure 2. Schematic representation of *IL7R* pre-mRNA splicing. Genomic and pre-mRNA structure of the *IL7R* gene is shown (top), with SNPs selected for genotyping in reference 19, listed above. The MS-associated SNP, rs6897932, is highlighted. Alternative splicing of the *IL7R* pre-mRNA leads to the production of two isoforms: a membrane-bound (*rIL7R*, exon 6 included) or soluble (*sIL7R*, exon 6 skipped). The transmembrane isoform of the receptor consists of the extracellular (EX), transmembrane (TM) and cytoplasmic (CYTO) domains. Skipping of exon 6 leads to a translation reading frame shift and translation termination due to a premature stop codon. Adapted in part from reference 23.

McKay et al.[24] established that expression of *sIL7R* mRNA was significantly elevated in whole blood samples from patients with two types of MS (primary progressive and relapsing remitting), and that increased expression of *sIL7R* correlated with a particular *IL7R* haplotype that was more common in the primary progressive MS patients. Taken together, these data suggest that: (1) there is a decrease in the ratio of transmembrane to soluble isoforms of *IL7R* in MS, and (2) aberrant alternative splicing of *IL7R* exon 6 causes this change in isoform ratio.

Clearly, the causality of *IL7R* in MS pathogenesis has not been formally proven, and the exact role of the increased production of *sIL7R* in MS has not been unraveled. One can envision, however, a number of plausible explanations that could connect aberrant splicing of exon 6, increased *sIL7R* production and MS. As discussed earlier, developing T cells in the thymus as well as naïve and memory T cells in the periphery require signaling through *IL7R* for survival and proliferation. As reviewed in ref. 18, *IL7R* ligand, IL7, is expressed constitutively; however, its amount is thought to be very low and just sufficient to maintain a finite number of T cells. Therefore, lower expression of the transmembrane *IL7R*, due to increased skipping of exon 6, could lower IL7-mediated signaling below a critical threshold and compromise survival and proliferation of T cells (in this delicately balanced system, even a small decrease in *rIL7R* would be sufficient to generate an effect). Additionally, sequestration of the ligand by the increased amounts of the *sIL7R* isoform could deprive T cells of their survival and proliferative signals. It must be noted, however, that the connection between this antagonistic mode of action for *sIL7R* and the development of MS, as proposed here, remains unproven.

Developing a hypothesis in which aberrant signaling through *rIL7R*, and, thus, compromised T-cell survival and proliferation, contribute to the etiology of MS is challenging for two reasons: (1) other unknown environmental and genetic factors may contribute to the development of the disease, and (2) the possible commensurate decrease in T-cell populations is contrary to a disease model in which there is acute T cell proliferation and inflammatory response that results in axon demyelination. Two studies that identified an increase in $CD8^+$ T cells from PBMC in MS patients[25,26] also identified a relative decrease in $CD8^+$ T cells in the CNS, in line with the assumption that a decrease in T-cell populations is associated with the disease.[26] It is also possible that lower expression of *rIL7R* by $CD8^+$ T cells in MS patients renders these cells more susceptible to viral attack. Indeed, various

viruses have been implicated in the development of MS,[27,28] and in chronic viral infections a phenomenon known as CD8+ T cell exhaustion, which is characterized by poor function and viability of memory T cells, as well as low levels of *rIL7R* expression by these cells, has been described.[18,29,30] Thus, it is possible that in individuals who genetically express lower levels of *rIL7R*, CD8+ T-cell exhaustion occurs in response to chronic viral infection. Viral infection can subsequently induce autoimmunity, resulting in demyelination and axon damage.

CTLA-4: A General Marker of Autoimmunity?

Cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) is another candidate for association with MS. Several genetically associated polymorphisms have been identified within the *CTLA-4* gene, and multiple attempts have been made to establish their role in susceptibility to MS and other autoimmune diseases (reviewed in ref. 31). Yet, reports on the association of *CTLA-4* with MS have been inconsistent.[32-41] However confusing the association of *CTLA-4* with MS, it is worth considering this gene as a potential contributor to the disease.

CTLA-4 is critical for negative regulation of T-cell proliferation. By binding to its ligands (CD80 and CD86) on antigen-presenting cells, *CTLA-4* counteracts the activity of the T-cell costimulatory molecule CD28, which binds to the same ligands to activate T-cell proliferation. Negative regulation of T-cell proliferation by *CTLA-4* results in the induction of T-cell anergy, and, eventually, peripheral tolerance.[42] Together with the data from experiments in *CTLA-4*-knockout mice, which develop lymphoproliferative disorders characterized by infiltration of lymphocytes into multiple organs and tissue destruction,[43] and *CTLA-4*-knockdown animals, which rapidly progress to autoimmune diabetes,[44] these results suggest a role of *CTLA-4* in the development of autoimmunity.

Two isoforms of *CTLA-4* are produced by alternative splicing in human cells: a "full-length" transmembrane isoform (*flCTLA-4*), in which exons 1 through 4 are included, and a soluble isoform (*sCTLA-4*), in which exon 3 is skipped.[45,46] Both *CTLA-4* isoforms are capable of binding CD80 and CD86. Interestingly, production of these isoforms seems to be regulated by T cell activation: resting T cells express *sCTLA-4*, but downregulate its expression upon activation, whereas expression of f*lCTLA-4* rapidly increases upon activation and remains constant until the end of the immune response.[13,47,48] Considering that regulation of *CTLA-4* expression is critical for providing proper downregulation signals for activated T cells, and that availability of the transmembrane isoform could affect signaling through *CTLA-4*, it is attractive to propose that aberrant differential expression of *CTLA-4* isoforms, either due to alternative splicing, or other mechanisms, could contribute to autoimmune diseases.

Is alternative splicing of *CTLA-4* important for the development of other autoimmune diseases? Abundant data implicate *CTLA-4* in a number of autoimmune diseases (autoimmune hypothyroidism, Graves' disease, type I diabetes,[31] and Myasthenia gravis,[49] to name a few), and therefore, *CTLA-4* has been proposed as a "general susceptibility gene to autoimmunity."[33] Interestingly, an abnormal splicing spectrum of *CTLA-4* isoforms has been detected in patients with Myasthenia gravis,[49] and recent data suggest that when the levels of *sCTLA-4* are reduced by RNAi, the potency of regulatory T cells is significantly reduced.[50] These two lines of evidence underscore the importance of *CTLA-4* alternative splicing in the development of autoimmune diseases.

CD45, An Intriguing Example with an Uncertain Association

CD45, a prototypic receptor tyrosine phosphatase, is essential for activation and development of T cells.[51] Alternative splicing of exons 4–6 within the *CD45* extracellular domain results in the production of isoforms *CD45RABC* (exons 4–6 included), *CD45RAB* (exons 4 and 5 included), *CD45RBC* (exons 5 and 6 included), *CD45RB* (exon 5 included), and *CD45RO* (exons 4–6 skipped). Naïve T cells express longer isoforms at higher levels, while activated and memory T cells express shorter isoforms (*CD45RO*).[13,52] In resting T cells, *CD45* phosphatase activity is needed to maintain a signal-competent pool of protein kinases of the Src family, which initiates a signal transduction cascade and induces T-cell activation upon antigen encounter. Importantly, this phosphatase activity can be regulated by isoform-differential homodimerization: the alternatively spliced isoforms differentially homodimerize in primary T cells, with the smallest isoform (*CD45RO*) being able to dimerize more efficiently and rapidly than the larger isoforms.[52] *CD45* dimers have significantly reduced phosphatase activity, which implies that this splicing switch is critical for preventing prolonged TCR signaling and undesirable tissue injury.[52]

Several SNPs that affect alternative splicing of exon 4 in *CD45* have been identified.[53,54] Some of these SNPs have been proposed to be associated with MS.[53] For example, the SNP 77C→G in exon 4 results in increased production of the longer isoforms. Considering that the longer isoforms are associated with more efficient TCR signaling, failure to attenuate prolonged TCR activation could contribute to the inflammatory response resulting in axon demyelination in MS. In contrast, the nonsynonymous SNP 138G→A in exon 6 results in increased production of the shorter isoform *CD45RO*,[55,56] with the "G" allele having a significant protective effect in Graves' disease, another autoimmune disorder.[55] Other studies, however, have argued against a significant association between *CD45* and MS.[57-62] Alternative splicing of *CD45* is one of the best-characterized splicing events in the immune system, both at the level of cis-acting regulatory elements,[63] and trans-acting protein factors.[64-67] Pending resolution of the conflicting reports on association with MS, these studies could prove valuable for the understanding of alternative splicing in this disorder.

Other MS Susceptibility Genes with Alternative Splicing Connections

OAS1. Haplotype "GA" of the 2',5'-oligoadenylate synthetase 1 (*OAS1*) gene, defined by the "G" allele of a SNP at the 3' splice

site of exon 7 and the "A" allele of a SNP in exon 3, has been linked to increased *OAS1* enzyme activity as well as MS and type 1 diabetes susceptibility.[68-71] Importantly, different alternatively spliced mRNA isoforms are produced depending on which allele is present at the 3' splice site of exon 7.[69] However, functional data establishing the role of these alternative splicing products and their corresponding protein counterparts in susceptibility to MS are lacking. It is tempting to speculate that involvement of *OAS1* in the pathogenesis of this disease reflects possible viral etiology. *OAS1* is one of the critical components of the interferon antiviral response; it is activated in the presence of double-stranded viral RNAs and catalyzes polymerization of ATP into 2',5'-linked oligoadenylates, which bind to and activate the latent ribonuclease, RNase L, resulting in degradation of viral RNAs.[69] It is necessary to establish the role(s) that different alternatively spliced *OAS1* isoforms play in both defense against viruses and MS pathogenesis. Caution, however, needs to be taken when assessing the contribution of individual *OAS1* SNPs to the development of MS and other autoimmune diseases, due to the long-range linkage disequilibrium between *OAS1* polymorphisms,[68,71] as well as recently reported inconsistencies in association of *OAS1* with type 1 diabetes.[72,73]

KLC1. One report suggests a possible involvement of a particular variant of the kinesin light-chain 1 (*KLC1*) gene in protection against MS.[74] Kinesin is involved in trafficking of the myelin synthesis apparatus[74,75] (hence a direct connection to MS), and a protective SNP within intron 13 of the *KLC1* gene has been hypothesized to affect splicing. To our knowledge, no data supporting this hypothesis exist; however, it is of interest that multiple splicing isoforms of *KLC1* have been identified.[76] If the SNP in intron 13 is, in fact, involved in protection against MS, it might shed light on the role of the alternatively spliced kinesin isoforms in MS.

Alternative Splicing of Candidate Autoantigens, Splicing Factors and More

Potential autoantigens. Autoantigen isoforms generated by alternative splicing could potentially exhibit different immunogenic properties and are extremely attractive candidates for detailed splicing analysis. Interestingly, Ng[77,78] and colleagues applied bioinformatics analyses to determine the extent of alternative splicing within 45 randomly selected human autoantigens and randomly selected, non-autoantigenic genes. Based on these analyses, alternative splicing occurs in transcripts of 100% of autoantigens, and only in about 42% of randomly selected genes. Although these results need to be re-interpreted in light of recent reports suggesting that almost every gene in the human genome can be alternatively spliced,[3] they suggest that autoantigen transcripts are more prone to splicing regulation. Moreover, splicing by the minor spliceosome (involving U12-type introns) is predicted to occur more frequently in autoantigen transcripts than in transcripts from non-autoantigens.[77] The authors[77,78] suggest that alternative splicing can modulate imunogenicity of different spliced forms by: (1) creating novel antigenic epitopes; (2) altering surface accessibility; (3) enabling expression by tissues not normally expressing autoantigens, and (4) altering patterns of posttranslational modifications. Thus, alternative splicing may be responsible, at least in part, for the generation of untolerized epitopes, and, as a result, autoimmunity.

Several candidate autoantigens have been identified in MS, namely, myelin basic protein (*MBP*), myelin oligodendrocyte glycoprotein (*MOG*), and myelin proteolipid protein (*PLP*), as well as others. Transcription from a large *Golli* (for "gene expressed in the oligodendrocyte lineage")-*MBP* transcription unit gives rise to *Golli-MBP* and classic *MBP* pre-mRNAs, both of which are alternatively spliced, producing different protein isoforms. Processing of these protein isoforms by the enzymes of the matrix metalloproteinase (*MMP*) family can generate similar sets of immunogenic peptides.[79-81] Moreover, while expression of classic *MBP* isoforms is restricted to myelin-forming cells in the CNS, the *Golli-MBP* alternatively spliced transcripts are expressed in the thymus, spleen and lymph nodes, as well as on the cells of the myeloid lineage, providing possible MS autoantigens outside of the CNS. Therefore, it is plausible that these *MBP*-containing *Golli-MBP* isoforms, processed by *MMPs* into immunogenic peptides, are recognized by autoreactive T cells which, if they cross the blood-brain barrier, would recognize neuronal *MBP* as an antigen.

Myelin oligodendrocyte glycoprotein (*MOG*) is a minor integral membrane protein found on the outermost surface of the myelin sheath. Complex alternative splicing of the *MOG* pre-mRNA gives rise to up to seventeen different isoforms (both transmembrane and secreted).[82] Investigation of the six major mRNA isoforms shows that they: (1) localize differentially (cell surface vs. endoplasmic reticulum vs. secreted) and (2) are translated.[83,84] Although analysis of the MS brain tissue samples did not reveal any disease-specific differences in the *MOG* isoform patterns,[83] it is still possible that *MOG* isoforms generated by alternative splicing could be involved in MS pathogenesis. Secreted forms of MOG can have a protective effect if they interact with anti-*MOG* antibodies and thus prevent demyelination, while CNS-specific forms can induce an autoimmune response when recognized by specific anti-*MOG* antibodies upon the death of oligodendrocytes and subsequent exposure of novel *MOG* antigens.[83]

Finally, use of alternative 5' splice sites within exon 3 of the gene coding for the main component of CNS myelin, myelin proteolipid protein (*PLP*), gives rise to two alternatively spliced isoforms: the longer *PLP* isoform and the shorter *DM20* isoform (reviewed in refs. 81, 85–89, and also see **Fig. 1**). *DM20* lacks exon 3B and codes for a protein product that is 35 amino acids shorter than the *PLP* isoform. Interestingly, despite its major role in the CNS, *PLP* is also expressed outside of the CNS, most importantly, in the thymus, with intrathymic expression largely restricted to the short *DM20* splice variant. In a study using a model of MS known as experimental autoimmune encephalomyelitis (EAE), Klein et al.[90] show that the thymic expression of *DM20* is sufficient to confer T-cell tolerance to all epitopes of the *PLP* protein in EAE-resistant mice. In contrast, one of the *PLP* epitopes in EAE-susceptible mice is encoded by the CNS-specific exon 3B of *PLP*, which is skipped in the *DM20* splice variant. Thus, EAE-susceptible mice are prone to developing the

disease due to the lack of tolerance to this particular epitope of the *PLP* protein. As the expression of *PLP* in the human thymus is also restricted to the *DM20* isoform,[90] an "uncensored" repertoire of T cells that escape central tolerance and are autoreactive to the exon 3B-encoded epitope, exists. Once damage to myelin by as-yet-undefined triggers has occurred, and full-length *PLP* has been presented, these specific autoreactive T cells may be activated and gain access to the CNS, where they can initiate an inflammatory response and thus contribute to MS.[90]

PLP/DM20 alternative splicing has been characterized in detail.[86-89] Several cis-acting regulatory elements, including G-rich sequences, have been found in exon 3B. Two of these G-rich elements, named G_1 and M2, bind hnRNPs H and F, which recruit U1 snRNP to the *DM20* 5' splice site and regulate the *PLP/DM20* ratio.[89] These mechanistic data, although not yet connected to the development of MS or changes in T-cell tolerance, are invaluable for understanding *PLP/DM20* splicing regulation and its potential involvement in MS pathogenesis.

Viral splicing? The involvement of viral infection in MS pathogenesis has been debated for quite a while and has been reviewed elsewhere.[28,91] The human endogenous retrovirus HERV-H has been proposed to be associated with MS, and interestingly, one study reported that a combination of two HERV-H splice variants, the so called "envelope" (env) and "protease-envelope" (protease-env) splice variants, can be found in 40% of MS patients but only in 10% of controls, which, the authors speculated, might be a sign of the activated state of the virus and may even lead to increased production of the HERV-H *env* protein.[92] In another study, a possibility that alternative splicing plays a role in generating different isoforms for yet another MS-associated human endogenous retrovirus, HERV-W, has been suggested.[93] Involvement of alternatively spliced viral transcripts in MS pathogenesis is an untested, albeit intriguing, hypothesis.

Alternative splicing and demyelination. As described earlier, regulated expression of the myelin protein components is important for establishing proper myelination patterns and can potentially be affected in MS. A classic demyelination model in the mouse is the quakingviable (qk^v) mutation. The mutation results from the deletion of an upstream regulatory sequence in the *qkI* gene, leading to reduced transcription of the *QKI* protein, a single hnRNP K homology (KH) RNA-binding domain protein.[94-96] Qk^v mice have a twitching phenotype and show severe demyelination of the CNS, with reduced levels of *QKI* correlating with the severity of demyelination. Interestingly, different *QKI* protein isoforms are produced by alternative splicing in a developmentally-regulated manner.[94-96] Several lines of evidence have established that some of these isoforms serve as splicing factors to regulate expression of myelin structural genes (*MBP*, *PLP* and myelin-associated glycoprotein, *MAG*). For example, isoform *QKI-5* binds to an intronic splicing silencer (ISS) within intron 12 of the *MAG* pre-mRNA and represses exon 12 inclusion in wild-type mice.[96] In the qk^v mice, however, where less *QKI* protein is present, more isoform with exon 12 included is produced.[96] *QKI-5* also regulates splicing of *PLP* (by switching the splicing towards the *DM20* isoform) and *MBP* (by preferentially including exon 6 and excluding exon 5) pre-mRNAs, implying that aberrant splicing regulation is caused by reduced levels of *QKI* protein in the mutant mice and lies at the heart of the demyelination phenotype.

Although no data regarding the involvement of the human homolog of *QKI* in demyelination in MS are available, the quaking model presents an interesting example of an important event in MS pathology that is modulated by alternative splicing. It would be interesting to establish whether similar misregulation of *QKI* homologs is observed in MS.

Other Autoimmune Diseases

Alternative splicing affects more than 90% of the genes in the human genome, so it would not be surprising if most of the genes involved in autoimmunity would have at least one alternatively spliced isoform. Both mechanistic and functional data on alternatively spliced genes in autoimmunity are still lacking (as most of the reports simply propose alternative splicing involvement without follow-up confirmation). Regardless of this, a trend is apparent: most of the genes involved in autoimmunity are alternatively spliced, and a vast area of research aimed at the mechanistic determination of splicing pathways and the role of alternative splicing in developing autoimmunity is now open. Some of the genes proposed to be alternatively spliced in autoimmune diseases are listed in **Table 1**. A thorough description of all of these genes and the alternative processing of their transcripts is beyond the scope of this review; thus, we chose to focus on some selected examples.

Myasthenia gravis. Myasthenia gravis (MG) is an autoimmune disease characterized by impaired transmission across the neuromuscular junctions and consequent muscle weakness.[97] MG is mediated by autoantibodies against the nicotinic acetylcholine receptor (*nAchR*). Inhibitors of acetylcholinesterase (*AChE*), an enzyme that degrades acetylcholine, have been used widely for the treatment of MG, but an unexpected side effect caused by alternative splicing of *AChE* transcripts calls their use into question. Two isoforms of *AChE*, the major (synaptic) isoform, *AChE-S*, and a normally rare readthrough isoform, *AChE-R*, are non-selectively blocked by these inhibitors.[98] These isoforms are differentially induced under stress, and destruction of *nAChR* by MG antibodies, followed by cholinergic imbalance, is a stress condition that results in a splicing switch towards production of the readthrough isoform. Unexpectedly, treatment with non-selective *AChE* blockers contributes to elevated *AChE-R* production. Because *AChE-R* is soluble and secreted, and capable of non-synaptic hydrolysis of acetylcholine, its elevated expression exacerbates muscle weakness. Interestingly, an antisense oligonucleotide that selectively binds to this particular spliced isoform has been tested in the rat model of MG, and its administration results in improved survival, neuromuscular strength and clinical status in the animals tested.[98] This antisense oligonucleotide is being evaluated as a potential anti-MG drug in clinical trials.[99]

An aforementioned alternative splicing event among *CTLA-4* transcripts has also been proposed to be associated with MG

Table 1. Alternatively spliced genes that are implicated in autoimmune diseases

Autoimmune disease	Alternatively spliced pre-mRNA	Consequence	Ref.
Multiple sclerosis	*IL7R* (skipping of exon 6)	more *sIL7R* produced	19, 22–24
	OAS1 (new 3' splice site)	new isoforms	68
	KLC1 (SNP in intron 13)	?	74
	MBP	multiple isoforms	79–81
	MOG	multiple isoforms	82–84
	PLP (alternative 5' splice sites)	two isoforms	85, 90
Myasthenia gravis	*AChE*	two isoforms	98, 99
	CTLA-4	different isoforms	100
Systemic lupus erythematosus	*BANK1* (SNP at branch point)	isoform lacking exon 2	106
	LILRA2 (activation of cryptic 3' splice site)	novel isoform lacking 3 amino acids	107
	TCRζ (skipping of exon 7; alternative splicing of 3'-UTR)	different isoforms	108–110
	IRF5 (alternative 5' splice sites)	specific exon 1 used	114
	RasGRP1	aberrant splice variants	115
	CD72 (13-nucleotide repeats and 4 bp deletion in intron 8)	isoform lacking exon 8	116
	IL20R (alternatively spliced in the mouse model of SLE)	soluble receptor	117
	CR2 (SNPs)	decreased splicing efficiency of exon 11	118
Ulcerative colitis	*PTPσ* (skipping of exons 8, 9)	isoform lacking Ig-like domain	101
Scleroderma	*IL4*	truncated isoform	119, 120
Rheumatoid arthritis	*IL6R*	soluble receptor	111, 112
	TNFR2 (skipping of exons 7, 8)	novel soluble receptor	113
	Tenascin-C	increase in large isoform	121
	CD1d	soluble isoforms	122
	CD44	multiple splice variants	123–125
	Fibronectin	isoform containing EDA region	126
	CD137	soluble receptor isoforms	127
Autoimmune inner ear disease	*IL1R2*	two isoforms	128
Type 1 diabetes	*TAP2*	two isoforms	129
	IA2	differential isoform expression	130
Kawasaki disease	*ITPKC* (SNP in intron 1 disrupts a poly-G run)	decreased splicing efficiency	102

Abbreviations: *AChE*, acetylcholinesterase; *BANK1*, B-cell scaffold protein with ankyrin repeats; *CR2*, complement receptor 2; EDA, extra domain A; *IA2*, insulinoma-associated tyrosine phosphatase-like protein; Ig-like, immunoglobulin-like; *IL4*, interleukin 4; *IL1R2*, interleukin-1 receptor type II; *IL6R*, interleukin 6 receptor; *IL7Rα*, interleukin 7 receptor α chain; *IL20R*, interleukin 20 receptor; *IRF5*, interferon regulatory factor 5; *ITPKC*, inositol 1,4,5-triphosphate 3-kinase C; *KLC1*, kinesin light-chain 1; *LILRA2*, leukocyte immunoglobulin-like receptor A2; *MBP*, myelin basic protein; *MOG*, myelin oligodendrocyte glycoprotein; *OAS1*, 2',5'-oligoadenylate synthetase 1; *PLP*, proteolipid protein; *PTPσ*, protein-tyrosine phosphatase sigma; *RasGRP1*, Ras guanyl-nucleotide releasing protein 1; *TCRζ*, T-cell receptor ζ chain; *TAP2*, transporter 2, ATP-binding cassette, subfamily B; *TNFR2*, receptor for tumor necrosis factor α2; 3'-UTR, 3'-untranslated region. A question mark ("?") designates isoforms that have been predicted, but not shown, to arise from alternative splicing.

(ref. 100, and also see the above discussion of the role of *CTLA-4* in multiple sclerosis and general autoimmunity).

Ulcerative colitis. Ulcerative colitis (UC), a subgroup of inflammatory bowel disease, is a chronic debilitating intestinal illness. A three-marker SNP haplotype within the receptor protein-tyrosine phosphatase sigma (*PTPσ*) gene has been found to be significantly associated with UC.[101] The presence of the haplotype results in a novel splicing event, skipping of exons 8 and 9, which leads to the deletion of the entire third immunoglobulin domain in the *PTPσ* ectodomain. Although the functions of the deleted domain, as well as the consequences of its deletion, are still unclear, it is possible that the new isoform possesses either different dimerization or ligand-binding abilities. *PTPσ* is thought to maintain its substrates, E-cadherin and β-catenin, in a dephosphorylated state, which is necessary for preventing the disassembly of the adherens junction, and aberrant alternative splicing associated with the disease-susceptible haplotype is likely to interfere with this or other *PTPσ* functions. Importantly, *PTPσ* knockout mice develop mild colitis, and are susceptible to developing severe colitis when challenged with specific colitis inducers,[101] supporting the role of *PTPσ* in the development of UC.

Kawasaki disease. A pediatric systemic vasculitis of unknown etiology, Kawasaki disease has been proposed to involve T-cell activation and infiltration of coronary artery walls.[102] A functional

SNP *itpkc_3* (G→C) in intron 1 of the inositol 1,4,5-triphosphate 3-kinase C (*ITPKC*) gene is significantly associated with disease susceptibility and formation of coronary artery aneurysms. The presence of the "C" risk allele results in reduced *ITPKC* splicing when minigene constructs are employed in in vitro splicing assays.[102] Since it is thought that *ITPKC* serves as a negative regulator of T-cell activation, it seems likely that a reduction in splicing will lead to lower levels of functional *ITPKC*, an increase in T-cell activation, and subsequent inflammation.

At the nucleotide level, the disease-associated "C" allele disrupts a "GGG" run, and is located right next to the 5' splice site of intron 1. Poly-G runs constitute splicing enhancer elements and are important regulators of splicing, which are known to serve as binding sites for hnRNP H; recent work has shown that the activity of the poly-G runs depends on the strength of the adjacent 5' splice site (it is about 4-fold higher when adjacent to an intermediate-strength 5' splice site as compared to a weak 5' splice site, and about 1.3-fold higher when compared to a strong 5' splice site).[103] The 5' splice site of *ITPKC* intron 1 conforms to the consensus sequence. Therefore, the activity of hnRNP H would depend on both of these elements. It is likely that the disease-associated "C" allele disrupts the poly-G enhancer element, interferes with hnRNP H binding, and leads to reduced *ITPKC* splicing. Although direct regulation of this splicing event by binding of hnRNP H has yet to be proven experimentally, this is an excellent example of how recent advances in bioinformatics analyses of alternative splicing, combined with genetic data, reveal the mechanistic aspects of splicing regulation.

Systemic lupus erythematosus. Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease.[104] SLE is predominant among young women and is characterized by the presence of autoantibodies against a variety of antigens. Unknown etiology, coupled with the ability of SLE to involve any or all organ systems in its pathogenesis, makes this disease particularly interesting and important to study.

Multiple SLE susceptibility genes have been identified, and despite some inconsistency in the results, it is very likely that many genes that are crucial for SLE pathogenesis have already been discovered.[105] Interestingly, some of them are proposed to affect splicing (**Table 1**). For example, three SNPs in the *BANK1* (B-cell scaffold protein with ankyrin repeats) gene together confer susceptibility to SLE. One of these SNPs, located at the branch-point site within intron 1, affects the relative splicing efficiency and upregulates production of a novel *BANK1* isoform that lacks exon 2.[106] As a result, this *BANK1* isoform lacks a putative inositol 1,4,5-triphosphate receptor (IP3R)-binding domain and is proposed to act in a dominant-negative or dose-dependent manner to attenuate *BANK1*-mediated signaling.[106] The precise role of the attenuated signaling of *BANK1* in SLE is still unclear, as is the role of *BANK1* in B cell receptor-mediated signaling, but considering the crucial role of B cells in the pathogenesis of SLE, it is likely that aberrant *BANK1* functioning is involved in pathogenesis.

Leukocyte immunoglobulin-like receptor A2 (*LILRA2*) is a stimulatory receptor that is highly expressed on monocytes, macrophages, myeloid denritic cells and a subset of natural killer cells. A SNP G→A that disrupts a 3' splice site of intron 6 in the *LILRA2* pre-mRNA, has been associated with SLE in a Japanese population.[107] The presence of the SLE-associated allele activates a cryptic 3' splice site 9 nucleotides downstream of the original 3' splice site. This leads to production of a novel protein isoform that lacks three amino acids in the linker region and is expressed on the surface of monocytes. Since the ligands for *LILRA2* are still undetermined, it is hard to predict how the three amino acid deletion affects ligand binding; however, the authors speculate that either signaling through Toll-like receptors or regulation of a specific subpopulation of T helper cells could be affected by this deletion. Both hypotheses still remain to be tested, and an independent confirmation of the association of *LILRA2* with SLE in a different population is needed.

Regulation of the T-cell receptor ζ chain (TCRζ) pre-mRNA by alternative splicing is yet another example of the potential importance of alternative splicing in SLE.[108-110] Several alternatively spliced isoforms of TCRζ have been reported, including a form lacking exon 7, as well as an isoform in which the 3'-untranslated region is alternatively spliced. Production of these two isoforms leads to reduced expression of both the TCR/CD3 complex and the ζ protein itself on the cell surface, as well as reduced production of interleukin 2 (IL2) upon stimulation. Moreover, both isoforms are less stable than the full-length transcripts, and their more rapid degradation contributes to diminished expression of the TCRζ protein. The multi-subunit T-cell receptor (TCR)/CD3 complex uses TCRζ chains for signal transduction, so abnormalities in the expression of the TCRζ chain can lead to T-cell dysfunction, loss of tolerance and the development of autoimmunity.

Other genes regulated at the level of alternative splicing have been associated with SLE and other autoimmune diseases (**Table 1**). Discussing them in detail goes beyond the scope of this review, but from the number of studies that have revealed a possible connection between alternative splicing and SLE pathogenesis, one can conclude that alternative splicing regulation is important for conferring the risk to SLE and contributing to its pathogenesis.

Concluding Remarks

Cellular gene expression is regulated at many levels, all of which contribute to the final amount and diversity of gene products. Given that nearly all gene products undergo alternative splicing, the extent to which expression of genes is regulated by this processing is truly extraordinary. Moreover, many disease-associated missense mutations exert their deleterious effect by altering the splicing pattern of the gene.

The immune system, which recognizes and fights a seemingly endless variety of "foreigners", but should not react towards the equally diverse "self" components, thrives on diversity and flexibility, with alternative splicing being one of the major contributors. At the same time, when the immune system malfunctions, for example, when it inappropriately reacts towards "self" determinants, alternative splicing is likely to be involved. Until recently this connection was not immediately apparent. This was due to a number of reasons: first, in many cases, splicing

of a particular disease-associated gene has been proposed, but not confirmed, at the RNA or protein level; second, even if the presence of alternatively spliced isoforms at the RNA level was confirmed, data connecting the corresponding protein isoforms with pathogenesis were lacking. Moreover, in the majority of cases, mechanistic studies to decipher how splicing of each particular susceptibility gene works, e.g., by characterizing its cis-acting regulatory elements and trans-acting factors, have not been performed. Lastly, inconsistency between studies trying to ascertain genetic effects that, although real, are small, adds to the confusion and sometimes makes even thoroughly performed mechanistic studies inapplicable to the analysis of splicing in autoimmunity.

Regardless of these difficulties, we have summarized evidence that indicates an important role of alternative splicing in autoimmunity and highlighted the following common trends: first, as the case of *IL7R* exemplifies, alternative splicing can produce soluble counterparts to transmembrane cytokine receptors, and these soluble isoforms can be associated with disease. Other examples include the involvement of the soluble receptor for interleukin 6 (*sIL6R*) in the pathophysiology of inflammatory bowel disease and rheumatoid arthritis and possible involvement of a novel isoform of the soluble receptor for tumor necrosis factor α [*DS-TNFR2(Δ7,8)*] in rheumatoid arthritis.[111-113] Second, since all autoimmune diseases share one unique feature—production of autoantigens—generation of different autoantigen isoforms by alternative splicing can potentially lead to creation of new immunogenic epitopes. Although attractive, this hypothesis needs thorough experimental testing, which may prove difficult due to the mere fact that in many cases the causative autoantigens have not been identified.

Overall, revealing the connections between alternative splicing and the pathogenesis of autoimmune diseases is a complex, yet exciting, task. Thorough studies on both the mechanism of splicing regulation as well as the role that alternatively spliced isoforms play in etiology or pathogenesis, are needed in each case. This will not only reveal how alternative splicing of particular genes operates, but will also shed light onto some general mechanisms of alternative splicing involvement in the pathogenesis of autoimmunity.

Acknowledgements

The authors thank Dr. Shelton Bradrick for critical reading of this document and many useful comments. The authors acknowledge support from grants NIH R01 CA127727 (M.G.B.) and NIH R01 NS060925-01 (S.G.).

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