Alternative Splicing in Immunomodulatory Genes

Subjects: Immunology

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The interaction between the tumor and immune cells within the tumor microenvironment is facilitated by signaling pathways driven by immunomodulatory proteins. Alternative splicing regulates the production of multiple immunomodulatory proteins with diverse functionality from a single mRNA transcript. Splicing factors are pivotal in modulating alternative splicing processes but are also subject to regulation. The dysregulation of alternative splicing may result from splicing factor (SF) abnormal expression levels and mutations in the cis and trans-acting elements and small nuclear RNA (snRNA) molecules. Aberrant splicing may generate abnormal mRNA transcripts encoding isoforms with altered functions that contribute to tumorigenesis or cancer progression.

alternative splicing isoforms immunomodulatory genes CTLA-4 PD-1 PD-L1 HLA-G STING TLR-4 MYD88

1. Cytotoxic T Lymphocyte-Associated Protein 4 (CTLA-4)

CTLA-4 also referred to as CD152 [1] is a co-inhibitory receptor expressed on the plasma membrane of activated cytotoxic T cells, helper T cells, T regs, memory T cells, natural killer cells [2], monocytes, granulocytes, B cells, skeletal muscle cells, placental fibroblasts [1] and in lung, bladder, ovarian, colon, renal, breast, uterine, rhabdomyosarcoma, melanoma and neuroblastoma cancer cell lines [3]. CTLA-4 is immunosuppressive, unlike its homolog CD28, as it can prevent antigen presentation and naive helper CD4+ T cell activation. It mediates immunosuppression by scavenging CD80 or CD86 through high affinity binding than CD28 and via trans-endocytosis in antigen-presenting cells (APCs) [4]. CTLA-4 is primarily localized intracellularly, and its translocation to the immunological synapse is dependent on the stimulatory signals caused by major histocompatibility complex (MHC) and T cell receptor (TCR) and CD28-CD80/86 binding. The exocytosis of vesicles containing CTLA-4 at the plasma membrane surface is dependent on the TCR signaling strength [5].

CTLA-4 gene comprises four exons with exon 1 encoding a signaling peptide (SP), exon 2 encoding the ligand binding domain (LBD), exon 3 encoding the transmembrane domain (TD) and exon 4 encoding the cytoplasmic tail (CT) [6]. CTLA-4 has two major isoforms generated by alternative splicing, namely sCTLA-4 and mCTLA-4, which are expressed in tumor cells and implicated in cancer immunosurveillance escape. They both bind to similar ligands namely CD80 and CD86 with greater affinity than CD28. The sCTLA-4 lacks exon 3 that codes for the transmembrane domain [7], binds to CD80 and blocks its interaction with CD28 in APCs consequently inhibiting T

cells activation [8]. mCTLA-4 depletes CD80 and CD86 from the APCs cell surface through trans-endocytosis [7]. Serum sCTLA-4 is associated with drug response and better patient survival in melanoma patients treated with ipilimumab[9]. sCTLA-4 levels can function as a predictor of disease recurrence in hepatocellular carcinoma (HCC) patients treated with radiofrequency ablation [10]. Selective sCTLA-4 blockade inhibits metastatic melanoma in mice suggesting its involvement in metastasis [11]. sCTLA-4 was shown to be associated with shorter survival in glioma patients[12]. Furthermore, sCTLA-4 was found to be a potential marker of disease progression in acute lymphoblastic leukemia patients as the overexpression of sCTLA-4 positively correlated with the percentage of leukemic B cells [13].

2. Programmed Death 1 (PD-1)

PD-1 belongs to the CD28/B7 subgroup of the immunoglobulin (Ig) superfamily, which is localized on the cell surface of the activated B cells, monocytes and T cells. PD-1 inhibits T-cell proliferation and survival when bound to programmed death ligand 1 (PD-L1) or programmed death ligand 2 (PD-L2) by blocking the IL-2, IFN-γ and TNF-α production. It is important for preventing autoimmunity and maintaining self-tolerance by inhibiting T-cell activation during the effector phase [5][14]. Various studies have shown that PD-1 deficiency in mice models generates autoimmunity exhibited by the occurrence of conditions in mice such as lupus-like arthritis, glomerulonephritis, fetal dilated cardiomyopathy [15] and fatal myocarditis [16]. The high expression of PD-1 promotes tumor immune surveillance escape and cancer progression, for example, in colorectal cancer. Furthermore, high PD-1 levels correlate with a poor prognosis in esophageal cancer, primary central nervous system lymphoma (PCNSL) [17] and cervical adenocarcinoma [18]. The blockade of PD-1 enhances NK cell activity and antibody secretion by activating the PD-1-expressing B-cells in addition to upregulating effector T cell functions in tissues and TME [15].

Five mRNA splice variants, namely *PD-1Δex2, PD-1Δex3, PD-1Δex2,3, PD-1Δex2,3,4* and *flPD-1*, were detected from human PBMCs. The *flPD-1* is also referred to as a full variant as it contains exons 1 (encoding leader peptide (LP)), 2 (extracellular IgV-like domain (EIgVD)), 3 (transmembrane domain (TD)) and 4 and 5 (intracellular domain (ID)). They are named according to the exons they lack; for example, the number after ex on the name denotes excised exon(s). *PD-1Δex2* lacks exon 2, which coded for the extracellular IgV-like domain meaning that the resulting isoform cannot bind the PD-L1 and PD-L2 ligands. *PD-1Δex3* variant encodes a soluble form of PD-1 (sPD-1), which can easily bind to PD-L1/L2 compared to the membrane-bound PD-1 and interferes with the interaction between PD-1 and PD-L1/2 [14]. The sPD-1 was shown to attenuate the inhibition of T cell activity in a TME and consequently restore anti-tumor immunity [19]. The *PD-1Δex2,3* variant is suggested to not encode an apparent functional putative protein as it lacks exons for both the intramembrane and the ligand binding domains. The translation of *PD-1Δex2,3,4* can create a premature STOP codon in exon 5 and generate a protein lacking the extracellular IgV-like, cytosolic and transmembrane domains [14].

3. Programmed Death Ligand 1 (PD-L1)

PD-L1 is a co-inhibitory molecule expressed on the cell surface of B cells, dendritic cells (DCs), natural killer cells, macrophages, T cells, MDSCs, endothelial, epithelial and tumor cells. The overexpression of PD-L1 is strongly linked to advanced disease and unfavorable prognosis in the bladder, breast, pancreatic, ovarian, melanoma, kidney, gastric and liver cancers [20]. PD-L1 facilitates epithelial–mesenchymal transition (EMT) in breast tumor stem cells and is linked to metastatic disease and unfavorable clinical outcomes in colorectal cancer. The expression of PD-L1 in ovarian cancer cells was shown to be upregulated by the presence of IFN-γ [21]. *PD-L1/CD274* gene consists of seven exons with exon 1 coding for the 5′ untranslated region (5′ UTR), exon 2 encoding signaling peptide (SP), exon 3 encoding the IgV-like domain (IgVD), exon 4 encoding IgC-like domain (IgCD), exon 5 encoding the transmembrane domain (TD), exon 6 encoding intracellular domain (ID) and exon 7 encoding a portion of the ID and a 3′ untranslated region (3′UTR) [22].

The alternative splicing of the *PD-L1* gene in colorectal cancer (CRC) generated isoforms a, b and c. Isoform a is considered a full-length isoform containing all exons from 1 to 7. Isoform b lacks exon 3 and was shown to potently inhibit T cell function more than isoforms a and c and promoted tumor cell immune escape. Furthermore, isoform b was shown to be correlated with an unfavorable prognosis and survival in colorectal cancer patients. Isoform c is a secreted form of PD-L1 lacking the membrane-binding and intracellular domains, capable of binding PD-1 and downregulating T-cell activity. It is implicated in the growth of tumors and metastasis; thus, it is considered a prognostic marker in CRC [23]. Hassounah et al. [24] detected another sPD-L1 protein capable of binding PD-1 and downregulating IL-2 and IFN-γ production in primary T cells. Four soluble PD-L1 isoforms, namely PD-L1-1, PD-L1-3, PD-L1-9 and PD-L1-12, have been detected in melanoma cancer cells and are formed by the presence of a stop codon before the transmembrane domain. They are associated with disease progression in melanoma patients receiving immune checkpoint blockade treatment[25]. Gong and colleagues [26] have detected five PD-L1 splice variants from NSCLC patients who relapsed from anti-PD-L1 therapy, consisting of a full-length variant encoding a membrane-bound isoform and four variants encoding the soluble form of PD-L1, among which only two were proven to be stable. These stable isoforms, namely PD-L1v229 and PD-L1v242, were shown to act as decoys and bind to PD-L1 blockade in vitro as a result promoting the PD-L1 and PD-1 interaction, which further suppressed the anti-tumor immunity.

4. Human Leukocyte Antigen G (HLA-G)

HLA-G has potent co-inhibitory effects on an anti-tumor immune response compared to other immune checkpoint molecules. The interaction between HLA-G with its receptors such as ILT2/CD85j/LILRB1 (ILT2), ILT4/CD85d/LILRB2 (ILT4) and KIR2DL4/CD158d (KIR2DL4) targets B cells, monocytes, NK and T cells; DCs and monocytes; and NK decidual cells, respectively [27]. HLA-G plays a significant role in maintaining fetal–maternal immune tolerance and is used in transplantation [28]. HLA-G inhibits the activity of immune cells via receptor binding, trogocytosis and chemotaxis impairment. The expression of HLA-G promotes tumor immune escape by modulating both the phenotype and function of immune cells leading to immune evasion and metastasis [29]. The conditions in the TME such as the presence of certain cytokines, glucocorticoids, heat shock and hypoxia contribute to the modulation of HLA-G expression [30].

The alternative splicing of *HLA-G* generates four membrane-bound isoforms, namely HLA-G1 to HLA-G4, and three soluble isoforms, HLA-G5 to HLA-G7. The extracellular protein section of HLA-G1 and HLA-G5 is complete with all three alpha domains, namely α-1, α-2 and α-3, linked to β2 microglobulin (β2M). The other isoforms have different extracellular protein structures and are not linked to β2M. For example, HLA-G2, HLA-G4 and HLA-G7 [31] and HLA-G3 lacks α-2, α-3 or both α-2 and α-3 domains, respectively. The isoforms without α-3 cannot interact with ILT4 or ILT2 receptors [27]. *HLA-G2*, *HLA-G4* and *HLA-G3* are generated by skipping exons 3, 4 and both exons 3 and 4, respectively. HLA-G1 to HLA-G4 isoforms are produced due to the presence of a stop codon in exon 6. The soluble isoforms resulted from the retention of intron 4 by HLA-G5 and HLA-G6 or intron 2 by HLA-G7 that generated a premature STOP codon before the transmembrane domain which led to their secretion. Exon 3 skipping also occurs in *HLA-G6* alternative splicing [32].

These isoforms are hypothesized to have distinct immunosuppressive functions. The HLA-G5 or -G6 has been shown to create an immunosuppressive environment around the tumor tissue. HLA-G6 has a negative correlation with pathological complete response (pCR) in the HER2+ breast cancer subtype, as the low expression levels of HLA-G6 were consistent with a high pCR rate [31]. M8 melanoma cells secreted the HLA-G5 isoform, which prevented NK cell-mediated cytotoxicity towards the target cell via the impairment of lytic granules polarization [30]. Soluble HLA-G (sHLA-G) detected in blood serum mainly consists of sHLA-G5 and sHLA-G1 (generated from proteolytic cleavage of HLA-G1) [33]. It has been demonstrated to be associated with tumor aggressiveness, tumor-node-metastasis stage, histological type, or a reduced survival period of breast, lung cancer and papillary thyroid carcinoma (PTC) patients [34]. Furthermore, sHLA-G is associated with advanced melanoma stage and tumor load[35]. The soluble HLA-G can serve as a diagnostic marker for distinguishing benign from malignant tumors [30]. HLA-G has great potential for use in overcoming drug resistance by blocking its expression or function [36]. Furthermore, HLA-G might serve as a possible marker for tumor susceptibility to chemotherapy and as a prognostic marker for advanced tumor stage and clinical outcome [30].

5. Simulator of Interferon Genes (STING)

STING is a regulatory protein consisting of four transmembrane domains (TM) located in the endoplasmic reticulum (ER), cytoplasmic ligand binding domain (LBD) and the C-terminal tail or domain (CTD). LBD can homodimerize and undergo conformational changes upon binding by cGAMP. The CTD is responsible for binding and phosphorylation by kinases such as TANK-binding kinase 1 (TBK1) [37]. STING is mostly expressed in innate, adaptive and non-immune cells. The modulation of inflammation by STING begins with the sensing of nucleic acid molecules in the cytosol that might have resulted from viruses, bacteria and dying cells (through phagocytosis) by cGAS, which then uses GTP and ATP to synthesize cGAMP. STING is activated by interacting with cGAMP and then is carried by iRhom and transported from the ER to the Golgi body, where it recruits and activates the TBK1 and the IkB kinase (IKK). TBK1 undergoes autophosphorylation and also phosphorylates STING. Interferon regulatory factor 3 (IRF3) is recruited by STING, phosphorylated by TBK1, and homodimerizes to enter the nucleus, where it activates the transcription of type I Interferons, chemokines and inflammatory cytokines. IKK kinase consisting of the IKKα and IKKβ activates NF-kB, which then enters the nucleus and induces transcription

by phosphorylating NF-kB inhibitor (IkB-α) [38][39][40]. In mouse prostate cancer cells, cytosolic DNA triggers the STING pathway and induces anti-cancer immunity [41]. Another piece of evidence proposes that the presence of STING in B16 melanoma cells is associated with the activation of anti-tumor immunity, which inhibits tumor progression [42]. Thus, several studies have proposed that STING activation is a promising strategy in cancer immunotherapy [40]. The downregulation of STING signaling impedes the DNA responses required for generating vital cytokines, including IFN-I, that mediate tissue repair and anti-tumor T cell priming [43].

A plasma membrane-bound isoform (pmSTING) with its C-terminus in the extracellular space has been detected in mouse and human cells. This isoform can be bound by an activated cGAMP to initiate signal transduction after the detection of extracellular DNA [44]. Other STING isoforms have been detected, including MITA-related protein (MRP), truncated isoform 2, truncated isoform 3, STING-β and tSTING-Mini. Isoform 2 lacks exon 4 and 7, isoform 3 lacks exon 7 and contains intron retention after exon 3 [45], and tSTING-Mini is generated from exon skipping of exons from 2 to 5 [46]. They all do not interact with TBK1 due to the lack of CTD, which is a binding domain for TBK1 [45]. Moreover, tSTING-Mini can produce a strong and fast antiviral response by inducing the phosphorylation of tIRF3 without interacting with tTBK1 [46]. MRP is another isoform detected in both mice and humans that inhibits IRF3 activation since it lacks TBK1 binding domain but can activate NF-κB [45][47]. This protein has a dimerization domain and, thus, can form homodimers with itself or heterodimers with STING and both inhibit STING interaction with other proteins such as TBK1 and block IRF3 activation while activating the NF-kB [48]. The other alternatively spliced STING isoform is STING-β. The transcription promoter region is at intron 5 of the STING allele. Therefore, its final transcript lacks exons 1 to 5, and its first exon has an extra ribonucleotide sequence at the 5′ end, causing the product isoform to have more than 25 amino acids at the N-terminus. Despite these extra amino acids, STING-β lacks the transmembrane domain, and thus, it cannot activate the TBK1/IRF3 as the normal membrane-bound STING [49]. This is because the transmembrane domain is reported to be critical for STING functioning as it is required for STING to relocate from the ER to the post-Golgi compartments during STING signaling. Any STING isoform without the TM domain is deemed non-functional [37]. Even though STING-β is non-functional on its own, it possesses a CTD domain that enables its binding to the STING, cGAMP and TBK1 as a result blocking them from interacting with their effectors. Therefore, STING-β expression inversely correlates with IFN-I production [49].

6. Toll-like Receptor 4 (TLR-4)

TLR-4 is a member of the pathogen recognition receptor (PRR) family expressed in natural killer cells, macrophages, T cells, neutrophils, DCs and in cancer cells [50]. In lung cancer cells, TLR-4 activation may enhance immunosuppressive cytokine production that promotes apoptotic resistance [51]. TLR-4 is mainly activated by pathogen-associated molecular patterns (PAMPs) in a tumor microenvironment. It is essential for DC activation, maturation, differentiation and migration, and it is suggested to be responsible for the transformation from conventional to immunosuppressive regulatory DC in TME. TLR-4 promotes angiogenesis in the tumor microenvironment. Moreover, TLR-4 expressed on tumor-associated macrophages (TAM) is responsible for the migration of the TAMs into the TME [52].

The human *TLR-4* gene consists of three exons with exon 1 encoding an SP and the section of the extracellular domain (ED). Then, exon 2 encodes another section of the ED, and exon 3 codes for the last section of the ED, TD and the cytoplasmic domain (CD) [53]. The extracellular domain is for the interaction with extracellular ligands, and the cytoplasmic domain is for interacting with TRIF and MyD88 [50]. Alternative splicing modulates a negative feedback mechanism that reduces inflammation. The stimulation of the TLR-4 signaling pathway by lipopolysaccharide (LPS) induces inflammation. On the other hand, LPS stimulation inhibits inflammation by altering the pre-mRNA splicing of genes that give rise to the proteins involved in the TLR-4 signaling pathway [54]. There is a pre-mRNA encoding soluble isoform of TLR-4 detected in mouse macrophages, which are induced during LPS stimulation that has an extra exon between exons 2 and 3 containing an in-frame STOP codon. This soluble isoform mediates a negative feedback mechanism of the TLR-4 pathway, as it reduces LPS-induced TNF-α production and NF-kB activation [54][55]. Another soluble TLR-4 isoform was detected in oral lichen planus (OLP) patients, which was shown to produce similar effects of negative regulation since cytokine production was inhibited in activated macrophages [56]. It is not known whether the dysregulated splicing of TLR-4 splicing contributes to the sTLR-4 secretion in humans [54]. sTLR-4 was detected in early-stage NSCLC patients and was suggested to be correlated with tumor metastasis and poor survival[57]. This isoform can combine with myeloid differentiation factor 2 (MyD-2) to form an sTLR4/MD-2 complex which can inhibit TLR-4 signaling by preventing the binding of the membrane-bound TLR-4 to its ligands [58].

7. Myeloid Differentiation Factor 88 (MYD88)

MyD88 is an inflammatory signaling adapter downstream of TLRs and IL-1R receptor families. It contains three domains, namely a C-terminal Toll or Interleukin-1 receptor (TIR) domain, an intermediate domain (ID) and an N-terminal death domain (DD). It activates IL-1R-associated kinase (IRAK) family kinases by linking it to IL-1R or TLR family members via its ID domain [59]. The activated IRAK family kinases result in several functional outputs, including the stimulation of NF-kB, MAPK and AP-1, causing MyD88 to be a central player in these inflammatory pathways. MyD88 signaling produces pro-inflammatory and IFN I cytokines [60][61]. MyD88 is highly expressed in colorectal cancer and plays a predominant role in promoting colorectal cancer cell proliferation, invasion and metastasis. The knocking down of MyD88 reduced the activity of NF-kB and AP-1 pathways that resulted in the inhibition of colorectal cancer progression [62].

MyD88 has eight isoforms with different functions, including the full-length (MyD88L) and short MyD88 (MyD88s) isoforms [59]. The *MyD88L* splice variant has five exons, and the *MyD88s* splice variant has four exons due to the skipping of exon 2. This exon 2 encodes an ID essential for linking activated TLRs to the IRAK-containing Myddosome during signal transduction. Thus, MyD88s does not have this function and is regarded as a TLRs signaling inhibitor. This isoform is produced as a result of a negative feedback loop in mice macrophages to inhibit the TLR signaling and pro-inflammatory cytokine production. *MyD88s* is generated via the inhibition of SF3A and SF3B during alternative splicing in mice's macrophages. MyD88L is responsible for the inhibition of the SF3A complex, which interacts with U2 snRNP during splicing. U2 snRNP is crucial for 3′ splice site recognition during the spliceosome assembly to the pre-mRNA. MyD88s splice variant is generated when the 3′ splice site intron 2 is

used rather than the 3′ splice site in intron 1, resulting in the skipping of exon 2 [63]. It could be assumed that the binding of MyD88L to the SF3A interferes with the recognition of the 3′ splice site in intron 1 by U2 snRNP. The mechanisms of how the binding of MyD88L to SF3A influences the attachment of U2 snRNP to the branch point near the 3′ splice site at the end of intron 2 are not known. However, this negative feedback inhibition does not apply in B cell lymphomas because the prolonged TLR activation produces isoforms that enhance TLR and NF-kB signaling, such as the MyD88L rather than MyD88s, which has an inhibitory effect [59]. Further studies are needed to understand the mechanisms of negative feedback inhibition by MyD88L and how the negative feedback loop does not apply in B cell lymphomas.

Methyltransferase-like 3 (METTL3) is another splicing factor involved in the modulation of alternative splicing of *MyD88s* in human dental pulp cells. An experimental study has proven that the depletion of the METTL3 leads to the production of MyD88s [64]. METTL3 is constantly overexpressed in CRC patients and is coupled with poor prognosis. It promotes CRC through the m6A-GLUT1-mTORC1 axis. However, METTL3 may have potential in the development of CRC-anti-cancer therapies since its combinatorial targeting with mTORC1 inhibited CRC growth [65]. Other MyD88 isoforms include MyD88N1 detected in liver, brain, heart and kidney tissues that lack the DD and the ID domains, as well as MyD88N2 detected only in brain tissue that does not code for any known functional domain [66].

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