

Alternative polyadenylation in CD2 expression

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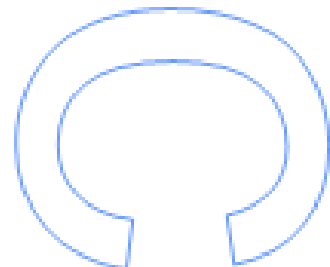
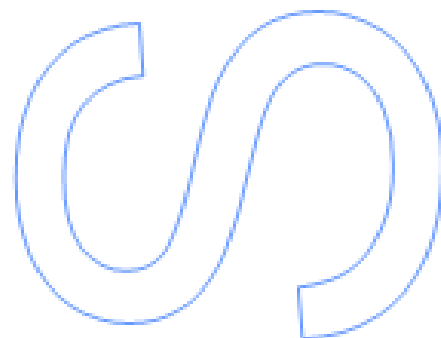
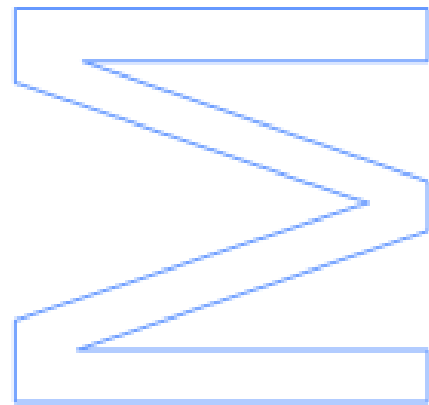
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abbreviations

A	Adenine
APA	Alternative Polyadenylation
APS	Ammonium Persulfate
BDNF	Brain-derived Neurotrophic Factor
Bp	Basepair
BSA	Bovine Serum Albumine
C	Cytosine
CD	Cluster of Differentiation
Clcn1	Chloride Channel 1
CPSF	Cleavage and Polyadenylation Specificity Factor
CstF	Cleavage Stimulation Factor
CF	Cleavage Factor
C2	Complement 2
dNTP	Deoxyribonucleotide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
ELAV	Embryonic Lethal Abnormal Vision
F	Forward
G	Guanine
GABA	γ -Aminobutyric Acid
HRP	Horseradish Peroxidase
hnRNP F	Heterogeneous Nuclear Ribonucleoprotein F
Ig	Immunoglobulin
MHC	Major Histocompatibility Complex
MRC	Medical Research Council
mRNA	Messenger RNA
miRNA / miR	MicroRNA
mAbs	Monoclonal Antibodies
NOVA	Neuro-Oncological Ventral Antigen
NK	Natural Killer
Nt	Nucleotide
ON	Overnight
PA	Polyadenylation
PAS	Polyadenylation Signal

PAP	Polyadenylation polymerase
PABP	Polyadenylation Binding Protein
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
PTB	Polypyrimidine Tract Binding-protein
PBMCs	Peripheral Blood Mononuclear Cells
PHA	Phytohemagglutinin
qPCR	Quantitative Polymerase Chain Reaction
R	Reverse
RACE	Rapid Amplification of cDNA Ends
RT	Reverse Transcriptase
RNA	Ribonucleic Acid
RNA Pol II	RNA Polymerase-II
RBP	RNA-binding protein
SDS	Sodium Dodecyl Sulfate
S.E.M.	Standard Error of the Mean
snRNP	Small Nuclear Ribonucleoprotein Particles
SMA	Spinal Muscular Atrophy
SMN2	Survival of Motor Neuron 2
T	Thymine
TEMED	Tetramethylethylenediamine
TF	Transcription Factor
TSS	Transcription Start Site
TCR	T Cell Receptor
U	Uracil
UTR	Untranslated Region
USE	U-rich Sequence Elements
UCSC	University of California Santa Cruz

Abstract

Gene expression in eukaryotes is regulated at different levels, including transcription and RNA processing. Alternative polyadenylation (APA) in the 3'-UTR, during 3' end pre-mRNA processing, has a fundamental role in gene expression in a variety of cellular programs: cellular differentiation and proliferation, leukocyte and neuron activation, and cancer. This important step in 3' end processing is mediated by several *cis*-elements and *trans*-acting factors. *Trans*-acting factors comprise RNA-binding proteins (RBP) that act as modulators. It has been reported that the shortening of the 3'-UTR through APA causes the loss of microRNAs (miRs) target sites in the mRNAs, decreasing the chance for silencing/degradation of the mRNA molecules, therefore resulting in the production of higher amounts of protein. Human T lymphocytes stimulated through T cell receptors express preferentially mRNA isoforms with shorter 3'-UTRs. CD2 encodes a transmembrane glycoprotein present in T cells that acts as an adhesion molecule and as a signal transducer during the immunological synapse formation. There are two mRNA isoforms for human CD2 generated by APA, differing in its 3'-UTR length. However, the physiological functionality of the formation of multiple CD2 mRNA species is still undisclosed. We show that CD2 3'-UTR sequence presents a high degree of conservation among several mammals. Analysis of the 3'-UTR led to the finding of a putative binding site for miR-320 in the longer 3'-UTR and several binding sites for RBPs, including NOVA1, a protein characteristically expressed in the brain. We observed that CD2 APA in the 3'-UTR is dependent on T cell activation, as proximal polyadenylation signal (PAS) is more efficiently used in activated T cells. Additionally, it has been described the expression of immune molecules in neurons, but their role in this system is still unknown. As the nervous system is similar to the immune system concerning many phenomenological, functional and molecular properties, the presence of CD2 in the brain and the involvement of APA in its expression were investigated. We found that CD2 is expressed in cerebellum, cortex, hippocampus and thalamus in human brains. We additionally found that the two CD2 mRNAs are present in the thymus and in a differentiated brain cell line however only the shorter CD2 isoform is produced in undifferentiated brain cells. Two working models for CD2 mRNA APA concerning the nervous and immunological systems were constructed based on the evidences found.

Keywords: *CD2, alternative polyadenylation, gene expression*

Sumário

A expressão génica em eucariotas é regulada a diferentes níveis, incluindo transcrição e processamento de RNA. Poliadenilação alternativa (APA) na 3'-UTR, durante o processamento a 3' do pré-mRNA, tem um papel fundamental para a expressão dos genes numa variedade de programas celulares: diferenciação celular e proliferação, activação de leucócitos e neurónios, e desenvolvimento de cancro. Este passo fundamental no processamento da extremidade 3' é mediado por vários elementos *cis* e factores *trans*. Factores *trans* compreendem proteínas de ligação ao RNA (RBP), que agem como moduladores. Tem sido relatado que o encurtamento da 3'-UTR através de APA provoca a perda de locais de ligação de microRNAs (miR) em moléculas de mRNA, diminuindo a possibilidade de silenciamento / degradação das mesmas, resultando assim na produção de quantidades mais elevadas de proteína. Linfócitos T humanos estimulados através de receptores de células T expressam preferencialmente isoformas de mRNA isoformas com 3'-UTRs menores. O CD2 codifica uma glicoproteína transmembranar, presente nas células T que actua como uma molécula de adesão e como um transdutor de sinal durante a formação de sinapses imunológica. Existem duas isoformas de mRNA para CD2 humano gerados por APA, diferindo no comprimento da sua 3'-UTR. No entanto, a funcionalidade fisiológica da formação de múltiplas espécies de mRNA de CD2 é ainda desconhecida. Nós mostramos que a sequência 3'-UTR de CD2 apresenta um elevado grau de conservação entre vários mamíferos. A análise da 3'-UTR levou à descoberta de um local de ligação putativo para miR-320 e para várias RBPs a nível da 3'-UTR, incluindo NOVA1, uma proteína caracteristicamente expressa no cérebro. Observou-se que a APA do CD2 na 3'-UTR é dependente de activação de células T, sendo o sinal de poliadenilação proximal é preferencialmente utilizado em células T activadas. Para além disto, tem sido descrita a expressão de moléculas do sistema imunológico em neurónios, mas o seu papel neste sistema é ainda desconhecido. Tendo em conta que o sistema nervoso é semelhante ao sistema imunitário relativamente a muitas propriedades fenomenológicas, funcionais e moleculares, a presença de CD2 no cérebro e o envolvimento de APA na sua expressão, foram investigados. Descobrimos que o CD2 é expresso em cerebelo, córtex, hipocampo e tálamo no cérebro humano. Adicionalmente, verificou-se que os dois mRNAs de CD2 estão presentes no timo e numa linha celular de cérebro diferenciada, no entanto, apenas a isoforma curta do CD2 é produzida na mesma linha celular mas num estado indiferenciado.

Dois modelos de trabalho de APA das isoformas de mRNA do CD2 para os sistemas nervoso e imunológico foram construídos com base nas evidências encontradas.

Palavras-chave: *CD2, poliadenilação alternativa, expressão génica*

Introduction

Gene Expression

Gene expression on eukaryotes requires the precise regulation of a series of events during development and differentiation. There are two main levels at which gene expression regulation can occur: transcriptional and post-transcriptional. The transcriptional control involves chromatin remodeling, DNA binding of regulatory proteins and interaction with the transcription machinery, whereas post-transcriptional regulation requires a fine-tuning of pre-mRNA processing, mRNA stability and export, and translation mechanisms. RNA-binding proteins and miRNAs play an essential role on post-transcriptional regulation [1-4].

Transcription control

Eukaryotic genomic DNA is assembled as chromatin in the nucleus and wrapped around an octamer of histones. Histone modifications modulation directly influences the chromatin structure and is critical for gene expression regulation by determining the accessibility of the DNA to be transcribed [5, 6]. DNA hypermethylation is associated with inactive chromatin and transcriptional repression by preventing the binding of RNA polymerase II (Pol II) and transcription factors (TFs) to the promoter. At this stage, if chromatin remodeling occurs and DNA becomes hypomethylated, this enables the binding of Pol II and TFs to the exposed promoter, allowing transcription initiation [7]. This type of epigenetic regulation has been shown to be an important feature for tissue-specific regulation, although in most cases the DNA methylation state and consequently the availability of the chromatin is constant across different cell types [1]. The most basic type of transcriptional control consists in the sequence-specific binding to a promoter of regulatory proteins that may act both as activators, enhancers or silencers [8]. The gene promoter consists of a region that overlaps the transcription start site (TSS) and that has a regulatory proximal region where TFs typically bind. This binding may activate, promote or prevent the transcription of the correspondent gene [1, 8].

Post-transcriptional regulation

The production of mature mRNAs demands several nuclear processes that endow with opportunities for regulation [9]. The production of a functional mRNA molecule requires correct transcription termination coupled with polyadenylation (PA) at the 3'end, and the splicing of introns and ligation of exons [10]. Splicing is accomplished by the spliceosome complex, which is formed by small nuclear ribonucleoprotein particles (snRNPs). Production of different mRNAs and consequently distinct protein isoforms occurs by alternative splicing, which is a highly regulated process that allows the production of several mRNA isoforms and contributes for protein diversity. Alternative splicing has thus a key role on several cellular processes such as cell death, cell growth, development, differentiation and disease, among others [11].

PA is of vital importance in pre-mRNA processing, allowing transcripts export to the cytoplasm and contributing for mRNAs stability and efficient mRNA translation [12]. The molecular mechanisms behind this intricate process are now clear. PA starts with the endonucleolytic cleavage at the pre-mRNA 3'end directed by *cis*-elements localized upstream and/or downstream of the PAS, U-rich regions and U/GU-rich regions respectively. The AAUAAA sequence is the canonical PAS that is recognized by the 3'-end processing machinery that cleaves the pre-mRNA and extends it by addition of a PA untemplated tail 15-30 nucleotides downstream of the PAS [10, 13, 14]. The core machinery responsible for the correct 3'-end processing consists of four main protein complexes: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor (CF) CF I_m and CF II_m, plus other associated proteins. The CPSF is composed by several subunits and is responsible for recognizing the PAS and catalyzing the cleavage reaction. The CstF complex recognizes U/GU-rich regions located downstream of the PAS [15, 16]. This elaborated 3'-end processing machinery comprises about 25 protein factors and around 60 individual proteins, such as symplekin, PA polymerase (PAP) and PA binding protein (PABP) (Figure 1) [10, 17]. The correct assembly and function of the 3'-end machinery is essential to produce a mature mRNA [10, 18, 19].

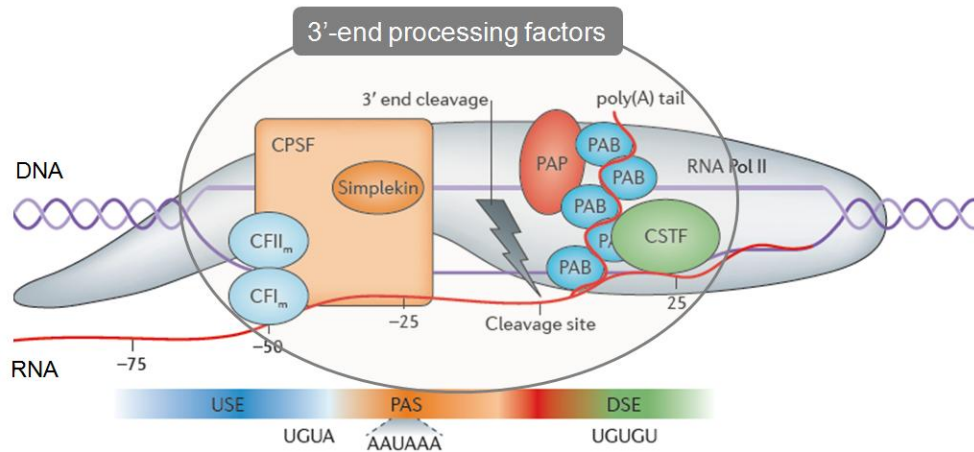


Figure 1 | **Core pre-mRNA 3' end processing machinery and *cis* regulatory elements.** The two cleavage factors (CF) and CPSF bind to the upstream and to the PAS regions respectively. CstF binds downstream of the PAS, and PAB proteins bind to the PA tail in the pre-mRNA. Adapted from Elkon *et al*, 2013.

Alternative polyadenylation (APA)

It has been known for some time that one gene may possess multiple PAS, that are used to produce different mRNA isoforms, by a mechanism named alternative polyadenylation [20, 21]. APA thus consists of a major gene expression regulatory mechanism for determining the downstream post-transcriptional fate of a mRNA molecule [20]. APA is recognized as a critical cellular mechanism, playing a direct role in gene regulation and it is now established that APA is not an exception but the rule, as it occurs in over half the human genes [22, 23]. Most genes possess the canonical PAS located at the 3'-UTR, but additional PAS may be present across the mRNA sequence [24]. There are four types of APA regarding the location of the PAS in the mRNA sequence [20]. Cleavage and PA in the 3'-UTR can occur in two different ways: with the PAS being located in tandem in the 3'-UTR or in an alternative terminating exon. A less frequent type of APA is the intronic APA, and even less frequent is the internal exon APA (Figure 2) [14, 15]. Whilst APA has an impact in the production of different proteins [25], when it occurs in the 3'-UTR it also plays a crucial role, tightly regulating gene expression [21, 26].

Genome-wide methodologies have shown that shorter 3'-UTR mRNA transcripts resulting of APA are often associated with cell proliferation and transformation, while longer isoforms are more common in differentiated cells [27-29]. Human primary cells use frequently distal PAS, whereas upon cell activation and in cancer the proximal PAS is more often used, resulting in an overall 3'-UTR shortening [27, 28]. It has been shown that in proliferating cells, such as embryonic and cancer cells, the proximal PAS is more frequently used, because eliminating large parts of the 3'-UTR enables the transcripts to escape miRNA targeting/degradation [28, 29]. In fact, it has been observed that shorter transcripts generally produce higher levels of protein [27, 28].

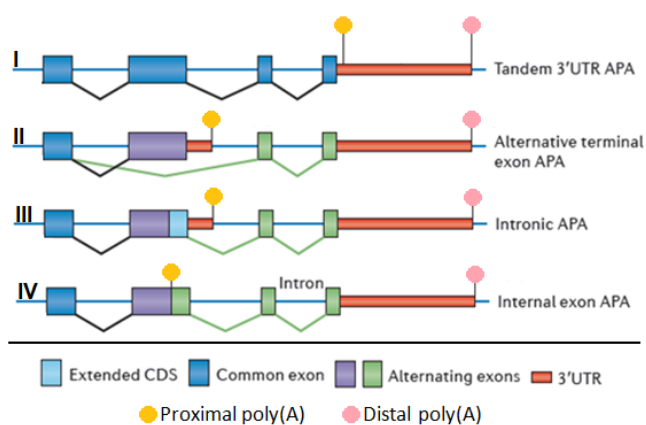


Figure 2 | **Four different mechanisms of APA depending on the PA sites location.** The APA marked with I corresponds to the simplest form of APA, in which PA sites are located in the same terminal exon. The transcripts produced differ only in the 3'-UTR length, and the protein produced is the same. APA as in shown in II, III and IV may affect the coding region. The situation defined as II in the figure uses PA sites in alternative terminating exons. III uses an intronic PA site and IV involves cleavage in an internal exon. APA II-IV is dependent on splicing.

Adapted from Elkon *et al.*, 2013.

RNA-binding proteins (RBPs) and microRNAs (miRNAs)

Two major regulators affecting pre-mRNA processing and metabolism, operating at the 3'-UTR level, are miRNAs and RBPs [20]. These *trans*-acting factors have a major impact in mRNA expression by interacting with *cis*-elements located within the 3'-UTR [20, 22].

RBPs - Recent experiments show that RBPs binding sites are enriched in the 3'-UTR regions of the mRNA, between the first and last PA sites, suggesting a clear link between APA and RBPs for gene expression regulation [29, 30]. RBPs associate with mRNAs and are the main players in post-transcriptional control. RBPs are involved in every step linking transcription to translation, determining the fate of the nascent mRNAs: splicing, PA, localization, stability and translation efficiency [31, 32]. Curiously enough, the genome regions encoding for these proteins are among the most conserved in the human genome [33]. In fact, a deregulated production of these proteins or factors required for RBPs assembly in the RNA, or the emergence of mutations, may have severe consequences and cause disease [34]. This arises either by the occurrence of mutations causing pre-mRNA processing, eg, splicing, defects or by RNA gain of function caused by disruption of alternative splicing [34]. An example of *cis*-acting mutations disrupting splicing is in the spinal muscular atrophy (SMA), a fatal autosomal recessive disorder. In this case, a single nucleotide substitution in the survival of motor neuron 2 (SMN2) gene, located in a splicing signal, is crucial for this disease [35]. Another critical splice change happens when a premature termination codon is inserted in the Clcn1 (a chloride channel) gene, leading to loss of function, and thus causing myotonia (delayed relaxation of the skeletal muscles) [36].

Many RBPs have been characterized for a long time and some of them were shown to have a critical function in PA [37, 38] and APA [39, 40]. More recently, with genome-wide methodologies, the importance of RBPs in APA has been further clarified [41]. The U1 snRNP (U1) protein, better known for its role in splicing, also plays an essential role in APA, specifically in the correct formation of mRNAs 3'ends, by preventing premature cleavage in PAS located in introns [42]. Another study links PTB (polypyrimidine tract binding protein) and PA efficiency in 3'end processing. It was shown in this study that PTB and CstF directly compete for the binding to the U-rich sequence element (USE) upstream of the C2 complement PAS, decreasing gene expression by deficient PA. Hence, when PTB is overexpressed there is a marked reduction in the efficient mRNA 3'end processing, with PTB acting as a negative

regulator [43]. PTB was more recently shown to also regulate pro-thrombin PA, by binding to an USE [44].

One other RBP that has a function in splicing but it is also important in the APA mechanism is SRp20. This protein belongs to the conserved SR protein family and has been shown to affect recognition of an alternative 3'-terminal exon by interacting with a PA factor binding to an APA site. This study was conducted in the gene coding for calcitonin and calcitonin gene-related peptide, whose pre-mRNA is alternatively processed in a tissue-specific manner. In thyroid C cells exon 4 is included, generating an mRNA with four exons and usage of the exon 4 PA site. In neuronal cells the exon 4 is excluded producing an mRNA with 5 exons (1-3 and 5-6) using exon 6 PA site. In this case, if SRp20 is overexpressed, the cells will include exon 4. In thyroid C cells, the addition of a mutant SRp20 form inhibits exon 4 inclusion, because this SRp20 mutant form will inhibit the binding of CstF to the exon 4 PA site preventing cleavage and addition of the PA tail [45].

Some RBPs thus have multiple functions, both in splicing and PA, and the relative cellular concentrations of specific RBPs modulate these pre-mRNA processing mechanisms.

miRNAs - miRNAs have been pointed out as extremely significant elements in post-transcriptional regulation. miRNAs consist of a class of highly conserved small non-coding RNAs of about 23 nt long [46]. miRNAs genes are mostly intronic, although some have been reported to locate in intergenic regions. The miRNAs biogenesis mechanics have been elucidated (Figure 3). miRNAs genes are transcribed in the nucleus by RNA Pol II or RNA Pol III and processed by Drosha. miRNAs are then exported into the cytoplasm, where they undergo further processing by Dicer resulting in an unstable miRNA duplex. Finally the duplex unwinds and one of the strands is incorporated into the RISC complex, becoming functional and targeting the mRNAs molecules [47].

The negative regulation (degradation or silencing) of the mRNA occurs by miRNAs targeting mRNAs. This occurs by 6-8 nt complementarity to recognition sequences that are mainly present in the 3'-UTR. It is estimated that around 30% of the human transcriptome is under miRNA regulation [48].

It is now well established that these small RNA molecules control several physiological and pathological processes, such as development, differentiation, proliferation and cancer cell progression [49, 50]. miRNAs are correlated with human cancers both by aberrant expression and by directly regulating oncogenes or tumor-suppressor genes. For example, a recent study found that miR-320 is significantly downregulated in

prostate cancer and when overexpressed in these cells causes a decrease in tumorigenesis both *in vitro* and *in vivo*. This miRNA targets genes of the Wnt/ β -catenin pathway decreasing the levels of cancer stem cell markers [50]. Recent evidence shows that miRNA regulation plays a crucial role in overall APA patterns shifts, by degrading longer isoforms, from a normal cellular state to cancer cell transformation state [49].

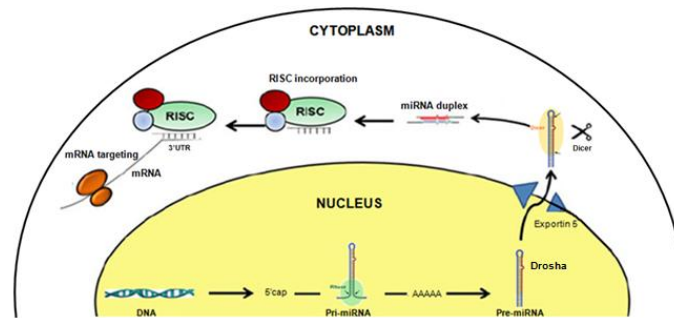


Figure 3 | **miRNAs biogenesis.** The primary-miRNA is transcribed in the nucleus, digested by RNase and processed by Drosha forming the pre-miRNA hairpin precursor. This pre-miRNA is exported by the exportin 5/Ran GTP complex into the cytoplasm, and is processed by Dicer into a miRNA duplex. The unwinding of this duplex and incorporation of one of the miRNA strands into RISC, makes it become functional and able to target mRNA molecules. Adapted from Arora *et al.* 2013.

APA modulation in different tissues and cell states

Pre-mRNAs can undergo alternative processing according to the development pattern, overall cell state or in a tissue-specific manner resulting in differential gene expression [51, 52]. APA can shape gene activity by altering the resultant protein, stability and localization or translation efficiency of a transcript. This often occurs in a tissue- or developmental stage-specific manner [53, 54].

Most alternative cleavage and PA events vary amid tissues and the majority of tissue-specific APA appears to be species specific, suggesting that the change in PAS choice is conserved in mammals [21, 52]. In fact, recent studies found that tissue-specific APA events are widespread and that human tissues have an overall PA site selection specificity [24, 55]. These different APA patterns have also been characterized in diverse developmental stages and, recently, global APA changes revealed to be associated with certain human diseases [26, 56]. Namely, in cases of dedifferentiation and increased proliferation, overall APA favors a general shortening in the 3'-UTR length [28]. When a gene generates shorter 3'-UTR isoforms through APA this facilitates the deletion of *cis* regulatory elements that may be targeted by RBP or miRNAs [20].

Recent genome-wide studies revealed that in the brain, nervous system, ovulated oocytes and zygotes, transcripts usually show mRNA 3'-UTR lengthening through APA, unlike what was observed in blood, ovaries, placenta, developing spermatocytes and proliferating T cells, where the tendency is to preferentially use the proximal PA site [20, 24]. A recent study on gene expression analysis determined that the embryonic development is accompanied by the production of transcripts exhibiting longer 3'-UTRs while generation of induced pluripotent stem cells from differentiated cells is marked by an overall shortening of the 3'-UTR [24].

Furthermore, coordinated changes in APA have been detected in specific situations such as T-cell activation and neuronal activity [27, 57].

APA in the nervous system

Long 3'-UTR transcripts are characteristic from the nervous system and brain, reflecting a preferential usage of distal PA sites [54, 58].

One of the first examples of APA modulation was found in the nervous system, more specifically in the brain-derived neurotrophic factor (BDNF) gene, essential for neurons survival. This neurotrophic factor has two PA sites, and generates two transcripts differing in their 3'-UTR length. These transcripts have different translation efficiencies, thus APA provides a precise temporal and spatial control of protein expression within neurons [24, 59].

Another study evaluating the transcriptome produced in hippocampal neurons after membrane depolarization showed a striking shift in the general APA pattern, preferentially producing shorter 3'-UTR transcripts. These transcripts were truncated mRNAs with functions differing from the original full-length isoforms [57].

NOVA2, a RBP characteristically expressed in the brain with a known role in alternative splicing events, has been shown to also participate in APA, favoring longer 3'-UTR isoforms production [60], which indicates that RBPs play roles in both splicing and PA. ELAV, a brain specific RBP, produces the same lengthening effect by repressing cleavage at proximal APA sites [61].

Another interesting aspect of APA in the brain resides in the fact that, according to the latest data, brain-specific APA sites seem to lack in most cases the canonical PAS [56] and thus may be more prone to regulation. Other recent studies show that, in the human brain, gene expression variation mainly occurs in an age-dependent manner, correlating to robust developmental changes [61].

APA in the immune system

APA consists of a common regulatory mechanism in several physiological processes, namely in immune responses [27].

Using genome-wide RNA-Seq methodologies it was shown that upon T cell activation there is a general increased usage of the proximal PA site. Accordingly, mRNAs with seed matches to proliferation-associated T cell miRNAs, show a shortening of their 3'-UTRs suggesting that this could be a mechanism of escaping gene silencing by miRNAs [27]. One of the most well established examples of how APA has a functional role at the immunity level regards B cells, T cells and monocytes. After stimulation of these cells, the increased levels of CstF-64 promoted the usage of weaker, proximal immunoglobulin heavy chain (IgM) PA sites [25]. Regarding CstF-64, it was also shown that plasma B cells exhibited lower levels of this *trans*-acting protein than those found in their precursors (pre-B cells), causing a different usage of APA signals in the Ig genes [25]. In this case, the production of different transcripts constitutes a cellular- and developmental stage-specific APA mechanism [25].

Regarding the IgM heavy chain, there are two mRNA transcripts being produced through alternative processing. One encodes for the secreted form and another for the membrane-bound form and they differ in their 3'ends (Figure 4). APA coupled to alternative splicing regulates the production of one or the other. The secreted form is produced when there is usage of the intronic PA site, favored by increased levels of CstF-64, while the membrane-bound IgM emerges when the cleavage happens at the distal site in plasma cells [24].

Linking cancer and immunity, and more specifically B cells, there is the example of certain mantle cell lymphomas. In this pathological situation a shift in the APA pattern occurs in B cells leading to the shortening of cyclin D1 3'-UTR, possibly enabling its escape from miRNA targeting which in turn results in a cyclin D1 overexpression [28].

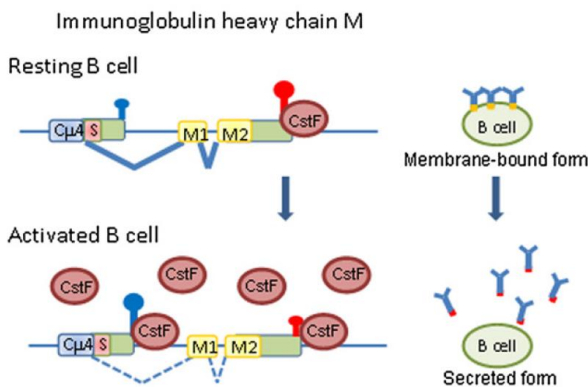


Figure 4 | **APA in IgM heavy chain.** In a resting state, the cell presents regular levels of CstF, favoring the distal PA site. After receiving a stimulus, the cells become activated and have an increased level of CstF which accumulates near the proximal weaker PA site, forcing its usage. The cleavage at this region generates only secreted IgM isoforms. Adapted from Giammartino *et al.*, 2011.

CD2

The main step to develop an adaptive immune response is the binding of the T-cell receptor (TCR)/CD3 exposed on the surface of T lymphocytes to a specific peptide-major histocompatibility (pMHC) complex [62]. This occurs whenever an antigen is presented to T cells by antigen-presenting cells (APCs). Auxiliary signals are needed at this point, being provided by adhesion molecules, namely CD2 and its ligand CD58 [62]. This binding helps to define the distance between membranes and supports T cell activation by facilitating the TCR-pMHC complex interaction. Thus, CD2 plays a critical role in the immunological synapse formation, needed for the emergence of immune responses [63], acting both as an adhesion and as a signaling molecule, initiating an intracellular signaling cascade.

Signaling can be induced by the clusters formed in membrane microdomains, through CD2-CD58 interaction [64]. Thus, CD2 binding increases T cell proliferation and cytokine secretion, in a ZAP70-dependent process [65]. Inhibition of this interaction results in an abortive T cell response. This inhibition is relevant in autoimmune diseases, such as rheumatoid arthritis in which T cells show higher expression levels for CD2 and CD58. Molecules that provide inhibition of this interaction may act as immunosuppressants and monoclonal antibodies (mAbs) against CD2 inhibit T cell activation or adhesion [63]. However, in mice, T cells deficient for CD2 appear to be only mildly affected, suggesting that CD2 may play a somewhat redundant role in T cell activation [64]. Recent studies state that the combined deficiency in co-receptors CD2 and CD28 leads to profound defects in activation and proliferation of T cells [64], while lack of CD28 alone is not able to fully impair T cell functioning. These two molecules seem to set off overlapping, though distinct, initial steps of T cell activation. Both CD2 and CD28 help to span the distance of 15 nm which corresponds to the distance required for the TCR-peptide/MHC complex to be formed [62].

CD2 is a glycoprotein expressed in T cells, thymocytes, NK cells and a subset of thymic B cells. CD2 and its ligand belong to the immune-globulin superfamily (IgSF) surface receptors. These co-receptors' extracellular domains are comprised of a N-terminal non disulfide bonded Ig variable (V) domain and a membrane-proximal truncated Ig constant-2 (C2) domain containing two intrachain disulfide bonds. Besides the extracellular domain, CD2 contains a transmembrane domain and a cytoplasmic domain [65].

The most important feature of CD2 for this work is the fact that the gene coding for CD2 is able to produce two different mRNA isoforms through APA in the 3'-UTR.

Hence, since CD2 is expressed and has a functional role at the immune synapse, we addressed the question of whether APA is relevant for CD2 expression and if this protein is expressed at the nervous synapse. We investigated if regulation of CD2 expression is tissue-specific, showing different APA patterns in T lymphocytes and in the brain.

Aims

There are three main goals in this project:

- Understanding how APA regulates CD2 expression in human primary T cells in a resting vs. activated state.
 - *In silico* analysis for CD2 mRNA and genomic sequences conservation will be evaluated and APA sites and predicted binding sites for microRNAs and RNA-binding proteins (RBPs) in the 3'-UTR of CD2 will be identified
 - Mapping the 3'ends in both activated and resting states of T cells by 3'RACE, and perform RT-qPCR to quantitatively evaluate the proximal or distal PA site usage in the two cellular states.
- Ascertain if this cluster of differentiation, typically an adaptive immunity molecule, is present in the brain.
 - Detection methods will be used, such as RT-PCR and immunohistochemistry to investigate the presence of CD2 in different rat and human brain tissue, and in which neuronal cells CD2 is expressed.
- Evaluate a possible tissue- and developmental-specific APA in rat thymus (immune system) and brain cells (nervous system).
 - 3'RACE will be performed on samples from thymus and a brain cell line in different differentiation states.

Material & Methods

***In silico* analysis**

The UCSC genome browser was accessed to check for APA sites and CD2 mRNA isoforms coding and 3'-UTR sequences. Conservation values were assessed by gathering the sequences retrieved in NCBI nucleotide database using the *Geneious v4.8* software [66].

The prediction of putative binding sites for RBPs and miRNAs was determined using the SFmap and micro.org database, respectively. The miRNAmap database was used to confirm expression tissues. Primers were designed in NCBI's tool Primer-BLAST, and analyzed in the bioinformatic appliance OligoAnalyzer (Integrated DNA Technologies, Inc).

Human PBMCs isolation

Human blood buffy coats were obtained from volunteer blood-donors (Hospital S.João). Peripheral blood mononuclear cells (PBMCs) were extracted using Lympholyte®-H (Cedarlane labs), according to the manufacturer's instructions. This protocol provides a density gradient separation medium that isolates lymphocytes and monocytes from human peripheral blood. Cells were counted on an automated cell counter (Countess®, Life Technologies).

Human T lymphocytes extraction

The isolated PBMCs were resuspended at approximately 1×10^8 cells/mL. Primary T lymphocytes were extracted from the PBMCs cell suspension using EasySep negative selection kit (STEMCELL technologies). With this kit unwanted cells are recognized by specific antibody complexes which recognize CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123 positive cells. Unwanted immunolabelled cells are retained in a specific fraction using magnetic beads while T cells are enriched by separating the desired fraction into a new tube. T cells were counted as previously described and cultured at 1×10^6 cells/mL.

Cell Culture

Cells were grown and maintained in culture at 37 °C with 5% CO₂ atmosphere, at a concentration between 1 to 2 x 10⁶ cells/mL, in RPMI 1640 medium modified with glutaMAX and phenol red, and supplemented with 10% fetal bovine serum (FBS) and 5% of pen/strep antibiotic (penicillium/streptomycin). All the reagents mentioned are from Gibco®, Life Technologies cell culture.

Primary T lymphocytes were kept in culture for 48 h.

Jurkat T cell line cells were split in a 1:3 ratio every 2-3 days.

Cell Activation

Both primary T cells and Jurkat T cells (1 x 10⁶ cells/mL) were activated with PHA (phytohemagglutinin) in a final concentration of 1 µg/mL, for 48 h. Activated cells were harvested for further RNA extraction.

Wistar rat tissue handling

Fresh rat tissue was obtained from Teresa Summavielle's laboratory and used immediately for total RNA extraction.

Cryopreserved rat brain sections were obtained from Teresa Summavielle's laboratory, and processed for immunohistochemistry analysis.

Human tissue handling

Prepared paraffin slides of different human brain sections and fresh-frozen Human brain tissue were obtained from the Medical Research Council (MRC), UK brain bank network. Tissue samples were maintained at - 80 °C until processing for total RNA extraction.

Total RNA extraction

The total RNA enriched with small RNAs from cell suspensions or tissue samples was extracted using mirVana™ kit protocol (Ambion, Life Technologies), briefly described below.

For Cell suspensions, primary T lymphocytes and/or Jurkat T cells were counted and 10^2 to 10^7 cells were pelleted and further washed in cold PBS 1 x, before proceeding to RNA extraction. Cells were lysed in the lysis/binding buffer, provided in the kit.

For tissue samples approximately 250 mg of **tissue** was obtained from each sample and the remaining frozen at $-80\text{ }^{\circ}\text{C}$. Tissue samples were homogenized in the kit's lysis/binding buffer using a plastic pestle (brain tissue is very soft, and a plastic pestle is enough to disrupt the tissue).

For organic extraction, lysates (either **tissue** or cell **suspensions**), were incubated with a miRNA Homogenate Additive and extracted with one volume of Acid-Phenol:Chloroform to one volume of lysate. The aqueous phase was recovered to continue with the total RNA isolation procedure. 1.25 volumes of 100% ethanol were then added to the samples and mixed thoroughly. This mixture was passed through a filter cartridge and the samples/columns were washed using miRNA wash solution 1. At this point the DNA is removed by performing a DNase (DNase I recombinant, RNase-free, 10 U/ μL , Roche) treatment on column for 15 min. Samples were then washed again with miRNA wash solution 1. Another wash step was performed using miRNA solution 2, and flow-through was discarded. RNA was eluted with 100 μL of 95°C pre-heated elution solution.

RNA quantification was performed in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and the RNA stored at $-80\text{ }^{\circ}\text{C}$.

RT-PCR

Reverse transcription reaction

cDNA was prepared using SuperScript III™ Reverse Transcriptase enzyme (Invitrogen, Life Technologies) and 1 µg of total RNA following manufacturer's guidelines. Shortly, the RNA was denatured for 5 minutes at 65 °C with 1 µL of dNTPs (10 mM), 1 µL of 50 µM Random Hexamers and Nuclease-free water (Ambion) to a final volume of 14 µL. Samples were placed on ice for another 5 minutes. A reverse transcription mix was prepared with 4 µL of the cDNA synthesis buffer (5x), 1 µL of DTT (0.1 M), 0.5 µL of RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl) and 0.5 µL of SuperScript III RT (200 units/µl, Invitrogen), added to the samples to a final volume of 20 µL and gently mixed. The samples were incubated for 10 minutes at 25 °C, 60 minutes at 50 °C and 10 minutes at 70 °C to inactivate the RT enzyme. Negative reactions, without SuperScript III, were performed to discard genomic DNA contamination. cDNAs were stored at -20 °C.

PCR

For PCR reactions 2 μL of cDNA was used, with 2 μL of GoTaq buffer (5x), 2 μL of MgCl_2 (25 mM), 1 μL dNTPs, 1 μL of each primer (forward and reverse) and 0.2 μL of GoTaq DNA Polymerase (5 U/ μL , Promega). Initial denaturation was performed at 95 $^\circ\text{C}$ for 2 minutes followed by 30 cycles of: 95 $^\circ\text{C}$ for 1 minute, annealing with each primer pair optimized temperature for 30 seconds (see Table 1) and extension at 72 $^\circ\text{C}$ for 1 minute. Final extension was performed at 72 $^\circ\text{C}$ for 5 minutes. PCR samples were analysed by gel electrophoresis on a 1.5% agarose gel stained with SYBR Safe® (Life Technologies).

Table 1 | Sequences of primers used for PCR reactions.

Target	Primer pair	Sequence 5' – 3'
Human CD2	hCD2 F	TCGGAGAAATGATGAGGAGCTG
	hCD R	TGCTGGATTCTGAGGGGTTG
Human 18S (reference gene)	h18S F	GCAGAATCCACGCCAGTACAAGA
	h18S R	CCCTCTATGGGCCCGAATCTT
Rat CD2	ratCD2 F	CTCCATCAGGCACACAGGTT
	ratCD2 R	ATTAGGGGGTGGCAACGAAA
Rat YWHAZ (reference gene)	ratYWHAZ F	GATGAAGCCATTGCTGAACTTG
	ratYWHAZ R	GTCTCCTTGGGTATCCGATGTC

3'RACE**Reverse transcription reaction**

cDNA for 3'RACE reaction was prepared as described above, using SuperScript III enzyme (Invitrogen) and 1 μg of total RNA following manufacturer's guidelines. However, during the denaturation step at 65 $^\circ\text{C}$, 1 μL of 10 μM oligonucleotide Adaptor Primer (AP) was added to the reaction. Everything else was done as mentioned before for the reverse transcription reaction in RT-PCR reaction.

3'RACE

This reaction was performed using 2 µL of cDNA, 1 µL forward primer (10 µM), 1 µL of Anchor primer (10 µM), 1 µL of dNTPs (10 mM), 10 µL of Phusion HF Buffer (5x), 0.5 µL of Phusion™ Hot Start polymerase (2 U/µL, New England Biolabs) and Nuclease-free water to a final volume of 50 µL. Initial denaturation was performed at 98 °C for 30 seconds, followed by 35 cycles performed with the following temperatures: 15 seconds at 98 °C (denaturation), 20 sec for annealing (temperature specific for each primer pair, see Table 2) and 30 seconds at 72 °C for extension. Final extension step was performed at 72 °C for 7 minutes. 3'RACE reactions were analyzed by gel electrophoresis on a 1.5% agarose gel like mentioned above (see RT-PCR method). The interest bands were cut from the agarose gel and the DNA purified with the QIAquick Gel Extraction kit (Qiagen), according to manufacturer's instructions. DNA quantification was performed in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

Table 2 | Sequences of primers used for 3'RACE reactions.

Target	Primer	Sequence 5' – 3'
Human CD2	hCD2 F	AGACAAGAGCCCACAGA
Rat CD2	ratCD2 F 1	GTCAACTGTCCAGAGAAAGGTC
	ratCD2 F 2	CTCCATCAGGCACACAGGTT
Oligo (dT) Adaptor primer	AP	GACCACGCGTATCGATGTGCGACTTTTTTTTTTTTTTTTTVN
Anchor primer	AP R	GACCACGCGTATCGATGTGCGAC

Cloning into TOPO® vector

DNA obtained from the gel extraction was prepared for TOPO® TA Cloning Reaction (Invitrogen, Life Technologies). First, dATPs were added to the purified DNA in a mixture of 15 µL DNA, 2 µL dATPs (10 mM), 8 µL GoTaq buffer (5x), 8 µL MgCl₂ (25 mM), 0.4 µL GoTaq enzyme and Nuclease-free water to a final volume of 40 µL. Reaction of dATPs addition was performed at 72 °C for 10 minutes. PCR product was then purified using QIAquick PCR Purification kit (Qiagen) and DNA was eluted in nuclease-free water. Cloning was performed using TOPO® TA Cloning kit (Invitrogen, Life Technologies), with a mixture of 5 µL DNA, 0.5 µL vector and 0.5 µL of salt solution, incubated for 20 minutes at room temperature (RT).

Transformation of competent bacteria

For transformation, 5 µL of the TOPO-cloning reaction were mixed with 50 µL of TOP10 chemically competent *E.coli* (Invitrogen, Life Technologies). Cells were incubated on ice for 10 minutes and then incubated at 42 °C for 1 minute (heat shock) and put back on ice for 5-10 minutes. 200 µL of LB medium were added and cells incubated for at least 30 minutes at 37 °C in an orbital shaker. The cells were plated and grown in LB plates with kanamycin (100 µg/mL) at 37 °C, overnight (ON). The colonies were then screened by colony PCR analysis.

Colony PCR

For colony PCR, at least 16 colonies were chosen for analysis. Each colony was immediately incubated in the PCR mixture (1 µL of M13 F and M13 R primers, 0.5 µL dNTPs (10 mM), 2 µL MgCl₂ (25 mM), 2 µL GoTaq Buffer (5x), and 0.06 µL of GoTaq DNA Polymerase (5 U/µL, Promega), and nuclease-free water to a final volume of 10 µL. Initial denaturation was carried for 5 minutes at 95 °C, followed by 30 cycles with 30 seconds at 95 °C (denaturation), 30 seconds at 55 °C (annealing) and 2 minutes at 72 °C (extension). Final extension was performed at 72 °C for 5 minutes. After incubation in the PCR mixture, the selected colonies were streaked to a LB agar plate with kanamycin and incubated at 37 °C ON. Colony PCR products were analyzed in an agarose gel, and the confirmed positive were selected. The positive plated colonies were scrapped and incubated in 3 mL of LB medium supplemented with kanamycin (100 µg/mL) and grown in an orbital shaker incubator at 37°C ON.

Mini-preps (QIAprep® Miniprep, Qiagen) of the selected positive clones were prepared according to manufacturer's guidelines and plasmid DNA sent for sequencing.

Table 3 | Sequences of primers used for colony PCR.

Target	Primer	Sequence 5' – 3'
M13	M13 F	AGCATCAAATGGCGTGGAGA
	M13 R	AGCATCAAATGGCGTGGAGA

Real-time qPCR

For quantification of CD2 mRNA isoforms the standard curve method was performed. First, a standard curve with 10-fold serial dilutions of cDNA was created to evaluate and optimize primer pair efficiency. For this reaction 2 μ l of cDNA diluted at least three times was analyzed by real-time PCR (1 μ l of each primer (10 pmol), 2 μ l of cDNA, 10 μ l of iQ SYBR green supermix (BioRad), and nuclease-free water to a final volume of 20 μ l. Experiments were quantitated by the comparative CT method ($2^{\Delta Ct}$) to evaluate overall expression levels. To access the ratio between both mRNA CD2 isoforms a variant of this expression was applied consisting of $2^{\Delta Ct \text{ CD2 total}} / 2^{\Delta Ct \text{ CD2 pA2}}$.

Northern blot analysis

Premade Northern blot (BD Biosciences, Clontech), containing approximately 2 μ l of polyA+ RNA per lane from different rat tissues was initially prehybridized for 2 hours and then hybridized in Prehybridization/Hybridization buffer (Ambion) for 18 hours at 42 °C with a CD2 full length specific probe. The blot was washed with decreasing concentrations of saline-sodium citrate (SSC). First wash solution with 0.01 % SDS and the other solutions with 0.1% SDS. Finally the membrane was exposed to an autoradiography X-ray film (Kodak) and intensifying screen at -80 °C for labelled RNA visualization. The levels of CD2 mRNA were normalised to the levels of β -actin mRNA retrieved with a β -actin specific probe.

Antibodies

Monoclonal antibodies used were OX34 (IgG2a) (Jefferies *et al* 1985, JEM 162) or OX54 (IgG1) (Clark 1988, JEM 167), both recognizing rat CD2, for human CD2 recognition the monoclonal antibody used was RFT11 (Royal Free Hospital, London, U.K). As negative control antibodies OKT3 (IgG2a) (Hoffman 1980, PNAS 77) against human CD3, and OX21 (IgG1) reacting to Human C3b inactivator (Hsiung 1982, Biochem J 203).

Protein extraction for western blot analysis

All procedures were performed at 4 °C. Cortex, cerebellum, striatum and hippocampus were homogenized in dissection buffer (50 mM Tris-acetate, pH 7.4, 10% sucrose, 5 mM EDTA and 1 mM PMSF, a serine protease inhibitor), the homogenate was centrifuged at 800 x g for 20 minutes, and the supernatant recovered and centrifuged at 16,000 x g for 30 minutes. The pellet obtained was resuspended in Triton X-100 lysis buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mg/ mL BSA, 1 mM PMSF and 1% Triton X-100) and kept on ice for 30 minute to ensure full lysis. Following a centrifugation at 11,000 x g for 10 minutes, the supernatant was recovered. Protein lysates were quantified using Bradford's Bio-Rad Colorimetric Protein Assay (Bio-Rad) according to the manufacturer's instructions. Briefly the protein levels in our samples were determined by measuring the OD_{595 nm} of a colorimetric reaction between an acidic dye and our protein solution. The comparison a calibration curved with pre-made BSA standards allows for determination of the protein concentration. Protein lysates were stored at -80°C until Western Blot analysis.

SDS-PAGE (PolyAcrilamid Gel Eletrophoresis)

Protein lysates were separated by SDS-PAGE using discontinuous 3% (stacking) and 10% (separating) polyacrilamid gels. The separating-gel solution was prepared with 10% acrylamid/bisacrylamid (30:0.8), 1.5 M Tris-Cl pH 8.8, 10% SDS, 10% APS and 0.1% TEMED, and the stacking-gel solution with 3% acrylamid/bisacrylamid, 1 M Tris-Cl pH 6.8, 10% SDS, 10% APS and 0.2% TEMED. APS and TEMED were only added just before gel casting. For gel casting, first the separating gel was added and covered with a ddH₂O layer until complete polymerization (approximately 45 minutes). After polymerization of separating gel the ddH₂O layer was removed, the stacking solution gel was added, the comb placed and the gel allowed to polymerize for about 15 minutes. After removing the comb, running buffer (25 mM Tris-Cl, 192 mM glycine and 0,1% SDS) was added to the chamber and the gel loaded with 24 µL of each sample, with the same amount of protein, and non-reducing buffer (0.5 M Tris-Cl pH 6.8, 5% SDS, 20% glycerol and 0.05% bromophenol blue). Samples were previously boiled for 5 minutes. The gel ran for 1 hour and 10 minutes at 150 Volts (V).

Western blot

Proteins were transferred (transfer buffer composed by 25 mM Tris-Cl, 192 mM glycine and 20% methanol, pH 8.3) to the nitrocellulose membranes Hybond C-extra (Amersham, GE Life Sciences) for 1 hour at 100 V, 4° C in a Mini Trans-Blot (Bio-Rad) apparatus. The membrane was removed and molecular weight markers were indicated. The membrane was incubated in a TBS-T solution (20 mM Tris-Cl, 137 mM NaCl, 0.1% Tween 20, pH 7.6) with 5 % powder milk for 1 hour, washed for 15 minutes, and washed again twice for 5 minutes, agitating in a TBS-T solution. The primary antibody diluted in TBS-T was added and was left incubated at room temperature for 1 hour, agitating. Membrane was washed again and the secondary diluted antibody (1:50000 goat anti-mouse-HRP in TBS-T) was added and incubated for 1 hour. Membrane was washed as before. Excess washing buffer was discarded and the membrane put in a cling-film mixed with a solution in a ratio of 40:1 of the detection solutions A and B from ECL Plus kit (Amersham, GE Life Sciences) for 5 minutes. Membrane was transferred to a new cling-film sheet and put on an exposing cassette. The film was exposed under red light for a few seconds.

Brain sections immunohistochemistry

Cryopreserved wistar rat brain sections

Brain sections were washed four times with PBS to remove the cryoprotection solution. The UltraVision Detection System Anti-Polyvalent, HRP/DAB (ThermoScientific) was used. Briefly, after washing, the samples were incubated in a hydrogen peroxide 3% solution for 15 minutes to reduce non-specific background and washed three times with PBS. Ultra V Block reagent was applied and sections were left incubating for 5 minutes at room temperature and a 2% BSA solution was applied for 1 hour to block nonspecific background staining. The BSA solution was then removed and sections were incubated with the primary antibody for 2 hours and then washed four times in PBS. Next, the Biotinylated Goat Anti-Polyvalent component was applied and incubated for 10 minutes, at which point another four washes with PBS were done. Then Streptavidin-Peroxidase was applied and incubated for 10 minutes, washing again after this incubation period. 1-2 drops of DAB Chromogen was added to 1 mL of DAB Substrate and mixed agitating, and finally applied to the brain sections left incubating for 5 minutes. After another PBS wash, the sections were transferred to gelatinized slides where they were left drying out for 6 hours at 4 °C. Finally the slides were assembled with permanent mounting medium (Histomount, National Diagnostics).

Human brain paraffin slides

Paraffin slides were incubated at 60 °C for 30 minutes, after this time the slides were subjected to deparaffinization/rehydration by being submersed in xylene for 5 minutes twice, then in absolute ethanol for 5 minutes as well, decreasing the ethanol concentration; in 96% ethanol for 3 minutes twice, and 5 minutes in 70% ethanol. The slides were then rehydrated in ddH₂O and the antigen retrieval was performed incubating the slides in citrate buffer (10 mM, pH 6) for 1 hour in a water bath at 100 °C. Slides were left cooling at RT for about 20 minutes and then washed with TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.4) for 1 minute. The slides were cleaned, not allowing the tissues to dry, and the samples were delimited with a PAP pen (abcam ab2601). Staining was performed using UltraVision Detection System Anti-Polyvalent, HRP/DAB (ThermoScientific) like mentioned for cryopreserved wistar rat brain sections. For dehydration, slides were washed in increasing ethanol concentrations (70%, 96%, 100%) for 3 minutes and then washed in xylene twice for 1 minute. To finish, coverslips were mounted with DPX Mounting Medium (Leica, Biosystems).

Results

Human CD2 mRNA has two different APA sites in the 3'-UTR and putative binding sites for RBPs and miRNAs

CD2 encodes a transmembrane glycoprotein present mostly in T cells, acting both as an adhesion molecule and as an activating signal transducer during the immunological synapse formation [64, 67]. To identify putative CD2 PA sites in the 3'-UTR a bioinformatic analyses for the CD2 3'-UTR was performed, using the UCSC Genome database (genome.ucsc.edu/), detecting the presence of two PA sites (Figure 1).

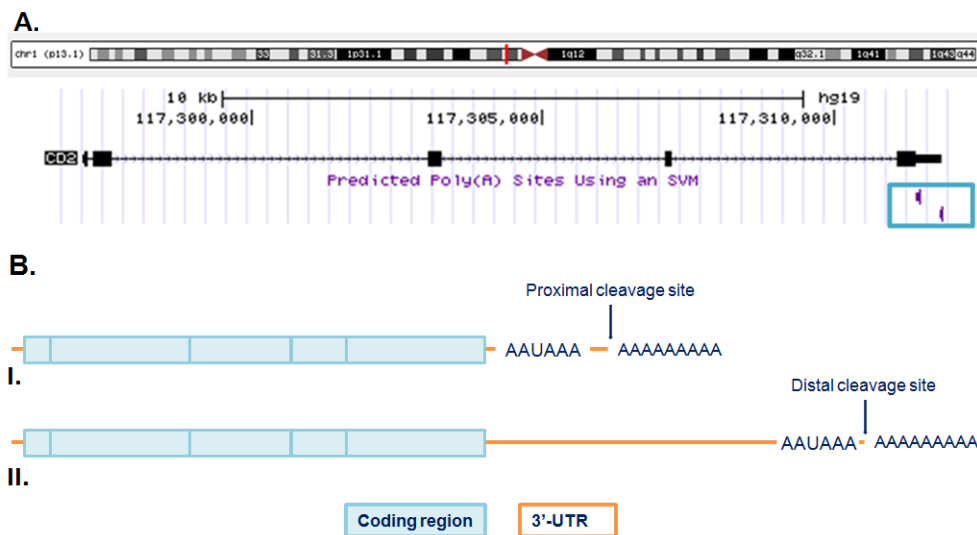


Figure 1 | **Predicted PA sites for human CD2.** **A.** The scheme represents the location at the chromosome level and genomic sequence of human CD2. The red bar represents the region where CD2 gene is located, in chromosome one. The genomic sequence encoding for this protein is 14,766 bp long. Under the schematic representation of the chromosome, black boxes represent exons (5 exons) and the space in between exons corresponds to introns. The narrow black box at the end represents the 3'-UTR. Two predicted PA sites are depicted in purple surrounded by a blue rectangle. Adapted from UCSC Genome Bioinformatics. **B.** Schematic representation of CD2 mRNA isoforms, with different 3'-UTR Lengths. **I.** Short CD2 mRNA isoform **II.** Long CD2 mRNA isoform.

To evaluate the presence of *trans*-acting factors potentially binding to CD2 3'-UTR, two other bioinformatic tools were utilized. Putative binding sites for RBPs were predicted using SFmap (sfmap.technion.ac.il/), which consists of a computational tool able to map RBPs predicted to bind to the sequence inserted. In this case, only the 3'-UTR sequence was inserted because the goal is to understand if there are any RBPs regulating the PAS usage in the 3'-UTR. Three RBPs possessing putative binding sites were identified with a high score in CD2 3'-UTR: hnRNP F, SRp20 and NOVA1 (Figure 4).

Finally, putative miRNAs targeting CD2 3'-UTR were unveiled using **microRNA.org**, a site that provides knowledge on predicted miRNAs targets, with experimentally observed expression patterns. We found miR-128, miR-544, miR-137, miR-216b and miR320 as potential miRNAs targeting CD2 3'-UTR (Figure 2). After retrieving the putative miRNAs targeting CD2 3'-UTR, it was important to evaluate the pattern of expression of these miRNAs, thus miRNAs expressed in brain and T cells were selected, as these were our models of study, using miRNAMap database (Figure 3). miR-320 was selected as a miRNA of interest to study in CD2 regulation, because although it is widely expressed in several tissues and cells, it is the only miRNA from those previously identified that is expressed in both T and nervous cells. Moreover, miR-320 potentially targets only the longer 3'-UTR, leading to the question of whether CD2 APA has a role in CD2 miR-320 silencing.

After gathering this data a schematic representation of CD2 3'-UTR was made, highlighting the two PA sites and putative binding sites for RBPs - Srp20, NOVA1 and hnRNP F and a miRNA - miR-320 (Figure 4).



Figure 2 | **Predicted miRNAs targeting CD2 3'-UTR**. Representation of CD2 3'-UTR mRNA sequence 445 nt long. The two PA sites are underlined with a red outline (AAUAAA) and possibly targeting miRNAs are shown on top of the sequence. Adapted from **microRNA.org**.

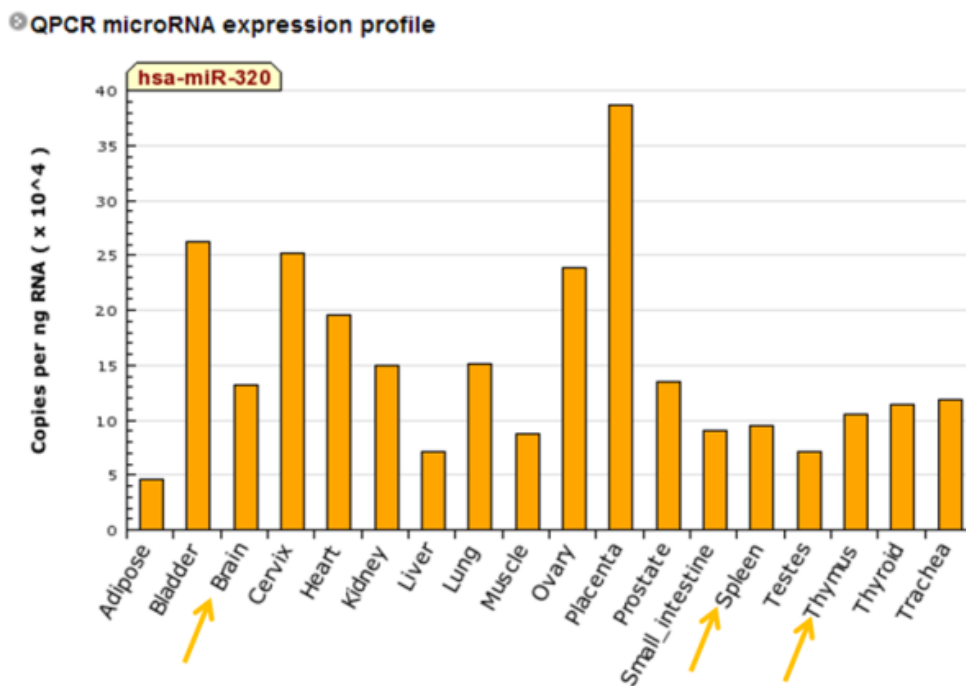


Figure 3 | **miRNA-320 expression profile.** Expression pattern for miR-320 in human tissues, through real-time PCR. From the miRNAs depicted in Figure 2, only miR-320 is expressed in both in T cells (thymus and spleen) and in the brain. Adapted from miRNAmap.mbc.nctu.edu.tw/.



Figure 4 | **CD2 3'-UTR sequence.** 3'-UTR sequence of CD2 cDNA, showing two canonical PAS (AATAAA) in red, and the respective cleavage sites marked by a darker red arrow. In purple, yellow and green boxes are identified putative binding sites for hnRNP F, NOVA1 and SRp20, respectively. In blue the potential miR-320 targeting region.

CD2 mRNA isoforms are conserved in several mammal species

To further underline the importance of these APA sites and putative binding sites for *trans*-acting factors, CD2 mRNA sequence conservation was evaluated. CD2 mRNA sequences from several different mammalian species were gathered from NCBI nucleotide database (ncbi.nlm.nih.gov/nuccore). Sequence alignments were performed using the default settings of MUSCLE 3.6 running in Geneious v4.8 [66].

CD2 mRNA coding and 3'-UTR sequence present a high level of conservation among several mammals, revealing a 70,9% pairwise percent identity value for the CD2 sequence including its 3'-UTR (Figure 5). The proximal pA1 signal (AAUAAA) is the canonical signal, conserved in human, mouse and rat, however the distal pA2 signal was not annotated in rodents.

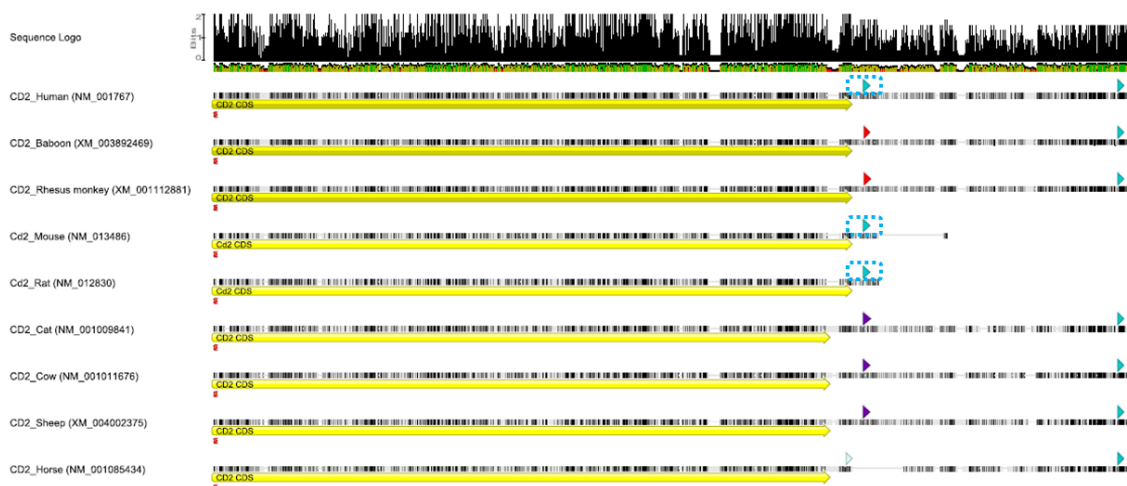


Figure 5 | **CD2 mRNA sequence alignment in several mammals.** CD2 mRNA coding (yellow long arrow) and 3'-UTR sequence conservation among several mammals, revealing a 70,9% pairwise percent identity value. Blue arrows surrounded with a blue dashed line identify the proximal pA1 signal (AAUAAA), which is conserved in human, mouse and rat. The downstream blue arrows present in every sequence except for the CD2 mRNA sequence in rodents, highlight the distal pA2 signal, conserved in all other mammal species depicted.

The genomic 3'-UTR sequences from rodents and human, including the proximal and distal PAS, were aligned and the overall 3'-UTR sequence of CD2 was shown to be conserved. The high level of conservation is reflected in a 66,2% of pairwise percent identity and 53,2% of identity. The three aligned sequences showed a conserved proximal pA1 signal (AATAAA) while the pA2 signal was only conserved in human and mouse (AATAAA) but not in rat (AACAAA) (Figure 6). Furthermore, two possible CD2 transcripts with different 3'-UTR lengths were found for the different species in NCBI nucleotide database. Taken together, these results suggest that CD2 might be subjected to APA in the 3'-UTR, originating two mRNA of different 3'-UTR lengths.

Interestingly, this analysis also revealed that hnRNP F putative binding sites are also conserved (Figure 6 I) and that some level of conservation is present in the potential NOVA1 binding sites (Figure 6 II, II, IV).

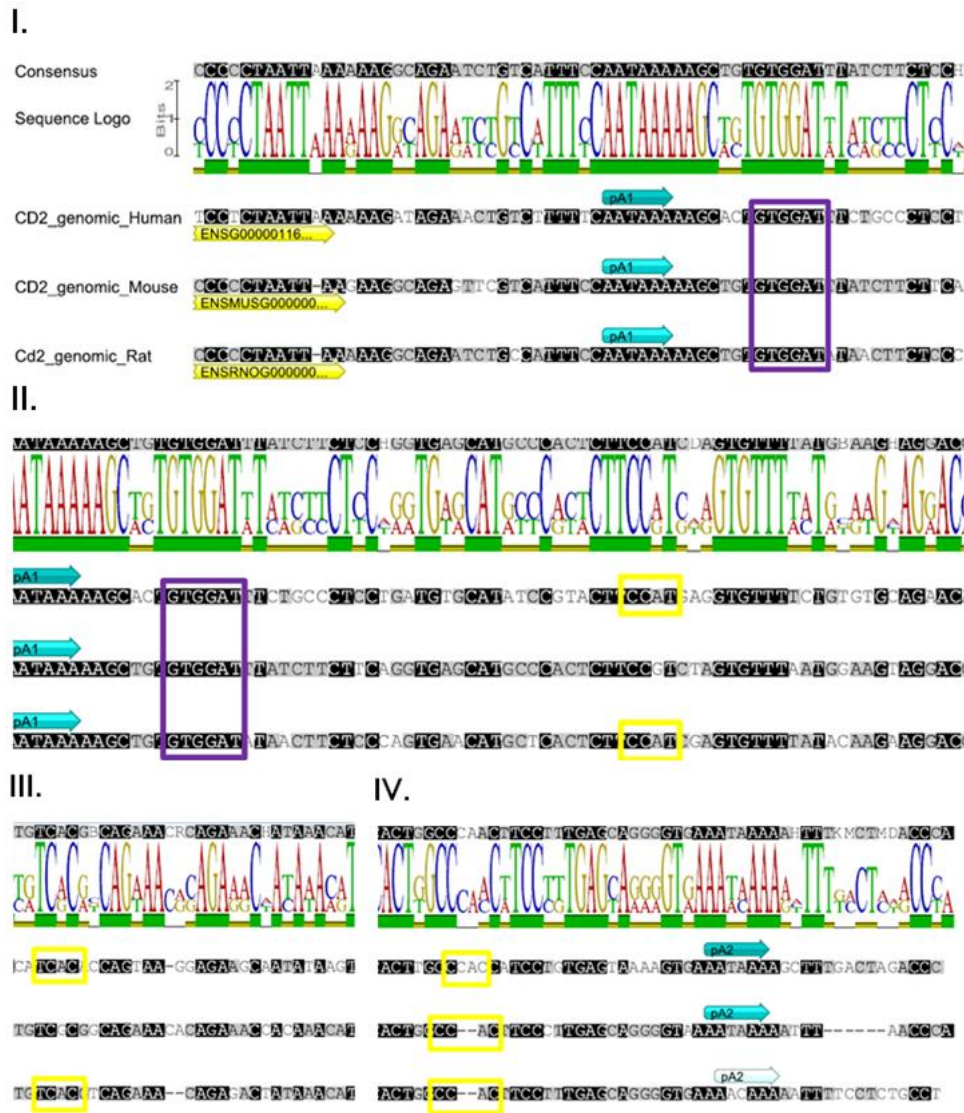


Figure 6 | Close-up on genomic alignment for CD2 3'-UTR sequences (human, mouse and rat). I. 3'-UTR sequence showing the proximal conserved pA1 signal (blue arrow, AATAAA) and a putative binding site for hnRNP F (purple box, GTGGAT). II. and III. 3'-UTR sequence presenting two conserved putative binding sites in human and rat for NOVA1 (yellow boxes, CCAT [II.] and TCAC [III.]). IV. 3'-UTR sequence depicting a putative binding site for NOVA1 (yellow box, CCAC) and the predicted pA2 signal (blue arrow, AATAAA). The rat genomic sequence shows a weaker PAS (light blue arrow, AACAAA). Adapted from **Geneious** software.

Human primary T lymphocytes express two CD2 mRNAs differing in their 3'-UTR lengths

CD2 expression in human T lymphocytes may be regulated by several so far unexplored pre-mRNA processing mechanisms. CD2 may undergo APA, since in humans two possible CD2 transcripts with 3'-UTR lengths of about 70 and 440 nt were found in the UCSC genome database (see Figure 1), with the shorter transcript containing a canonical PAS upstream of the predicted cleavage site and the longer transcript possessing another canonical PA site. To determine which of these isoforms are expressed in human primary T cells subjected to different conditions and map their 3'ends we performed 3'RACE analyses (position of RACE forward primer is depicted in Figure 7).

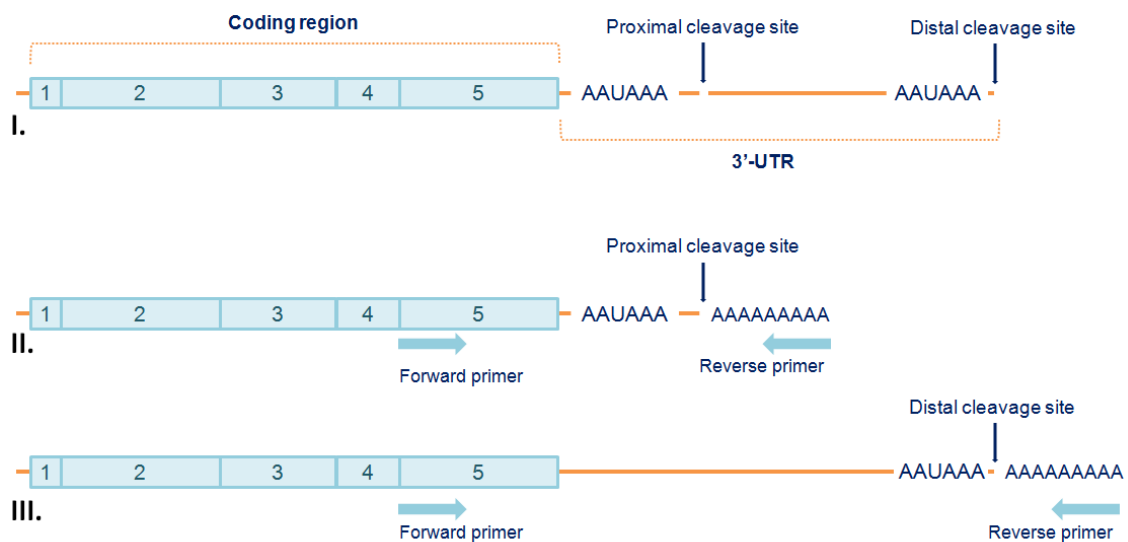


Figure 7 | **Schematic representation of CD2 mRNAs.** I. CD2 longer mRNA depicting five exons in the coding region, two PAS and respective cleavage sites at the 3'-UTR. II. CD2 shorter mRNA isoform, showing the location of the primer pair for 3'RACE analysis. Forward primer located at the beginning of the 5th exon and reverse primer binding to the PA tail. III. CD2 longer mRNA isoform, with the respective primer pair used for 3'RACE. The primer pair used is the same for mapping both isoforms.

We kept human primary T cells isolated from PBMCs from healthy donors in culture for 48 h in two different conditions: resting and activated with PHA (phytohaemagglutinin). Figure 8 shows a representative experiment: in resting and activated primary T lymphocytes both CD2 transcripts were clearly identified, with 370 and 750 nt, as expected for the proximal and distal PAS, respectively.

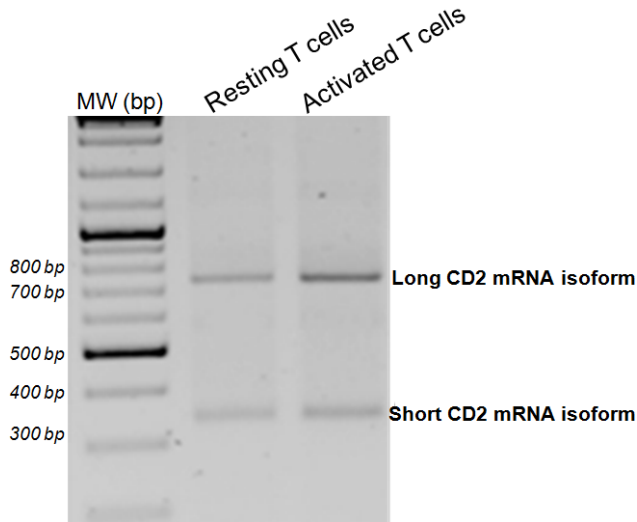


Figure 8 | **CD2 products of a 3'RACE reaction for primary human T lymphocytes.** The first lane corresponds to the DNA ladder, with molecular weights in base pairs (bp). The other two lanes correspond to resting and activated human primary T cells, showing both two CD2 mRNA isoforms. The short isoform is about 370 bp and the long isoform is about 740 bp. Primer pair used in this reaction is depicted in Figure 7.

The amplicons obtained were confirmed by sequencing, and the mRNA 3'ends mapped (Figure 9). We found that, in both resting and activated states, human T cells express two CD2 mRNAs with 3'-UTRs of different lengths, due to APA.

Short CD2 mRNA isoform sequence

CAGACCTCGAGTTCAGCCAAAACCTCCCCATGGGGCAGCAGAAAACCTCATT
GTCCCCCTTCCTCTAATTAAAAAAGATAGAAACTGTCTTTTTC**AATAAAA**AAG
 CACTGTGGATTTCTGCCCTCCTGATGTGCATATCCGTA **AAAAAAAAAAAAA**

Long CD2 mRNA isoform sequence

CAGACCTCGAGTTCAGCCAAAACCTCCCCATGGGGCAGCAGAAAACCTCATT
GTCCCCCTTCCTCTAATTAAAAAAGATAGAAACTGTCTTTTTC**AAATAAAA**AAG
 CACTGTGGATTTCTGCCCTCCTGATGTGCATATCCGTA**CTTCCATGAGGTG**
 TTTTCTGTGTGCAGAACATTGTACCTCCTGAGGCTGTGGGCCACAGCCAC
 CTCTGCATCTTCGAACTCAGCCATGTGGTCAACATCTGGAGTTTGTGGTCT
 CCTCAGAGAGCTCCATCACACCAGTAAGGAGAAGCAATATAAGTGTGATTG
 CAAGAATGGTAGAGGACCGAGCACAGAAATCTTAGAGATTTCTTGTCCTCCT
 CTCAGGTCATGTGTAGATGCGATAAATCAAGTGATTGGTGTGCCTGGGTCT
 CACTACAAGCAGCCTATCTGCTTAAGAGACTCTGGAGTTTCTTATGTGCC
 TGGTGGACACTTGCCACCATCCTGTGAGTAAAAGTGA**AATAAAA**AGCTTTG
 ACTAG **AAAAAAAAAAAAA**

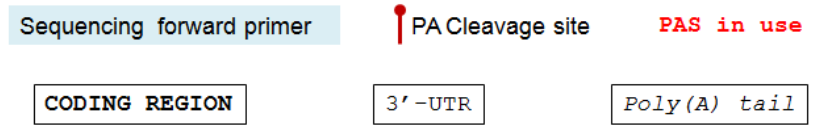


Figure 9 | **Mapping of CD2 PAS in the 3'-UTR. Sequencing results for CD2 3'RACE products.** After cloning the 3'RACE products illustrated in Figure 8, the plasmids were sequenced using a forward primer located near the 3'end of exon 5 (light blue box). The short CD2 mRNA isoform uses the proximal PAS (in bold, colored red) located 23 nt downstream of the coding region and is cleaved for PA tail addition 40 nt downstream. The long CD2 mRNA isoform uses the pA2 signal and is cleaved for PA tail addition 12 nt downstream.

CD2 proximal PAS is preferentially used upon T cell activation

Previous studies have unveiled a global mRNA shortening upon activation of T lymphocytes in mice [27].

To determine if in humans the choice of the PAS of CD2 was affected by the cell activation state, and since the 3'RACE technique is not quantitative, we analyzed the expression levels of the two CD2 mRNA isoforms in human primary T lymphocytes and in a Jurkat T cell line, both in a resting and activated (with PHA) states by RT-qPCR. Two specific primer pairs were designed as depicted in Figure 10.

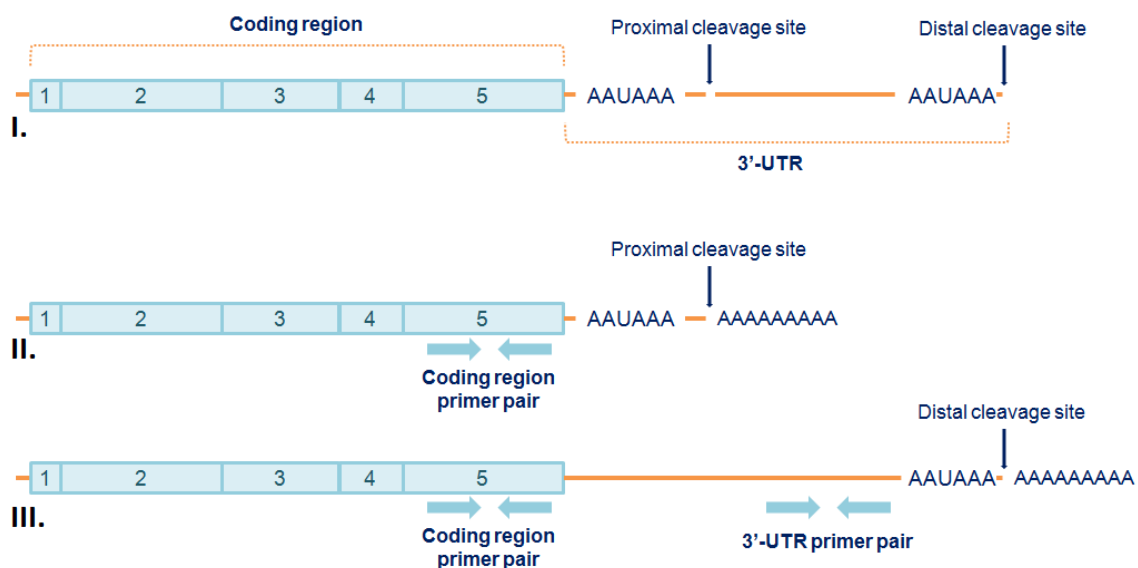


Figure 10 | **Scheme of primer pairs used in RT-qPCR analyses.** I. CD2 mRNA sequence depicting five exons in the coding region, two PAS and respective cleavage sites at the 3'-UTR. II. Short CD2 mRNA isoform is amplified by the primer pair located at the terminal exon (exon 5). III. Long CD2 mRNA isoform will be amplified by both the primer pair designed for the coding region and by the primer pair designed at the 3'-UTR. The primer pair in the coding region will hence amplify both mRNA isoforms, while the primer pair located at the 3'-UTR will amplify only the long isoform.

We started by comparing the overall CD2 expression levels in human primary T cells and the Jurkat cell line by RT-qPCR, using the primer pair located at the terminal exon (indicated as Total in Figure 11), in resting (R) and activated (A) states. This primer pair quantifies both CD2 mRNAs, ie, total CD2. It is clear that CD2 mRNA is present in much higher levels in primary T cells when compared to values obtained from the T cell line (Figure 11, compare levels of Total CD2 mRNA in A and B). We then quantified the levels of expression for the longer CD2 mRNA. In Jurkat cells, there is a very low expression level for the longer isoform in a resting state. We can also observe that upon activation with PHA, there is a slight increase in CD2 mRNA levels, while the longer isoform is less produced (Figure 11 B). This effect is not observed for human

primary cells (Figure 11 A). The elongated CD2 3'-UTR is generally poorly expressed in both primary T lymphocytes and Jurkat cell line.

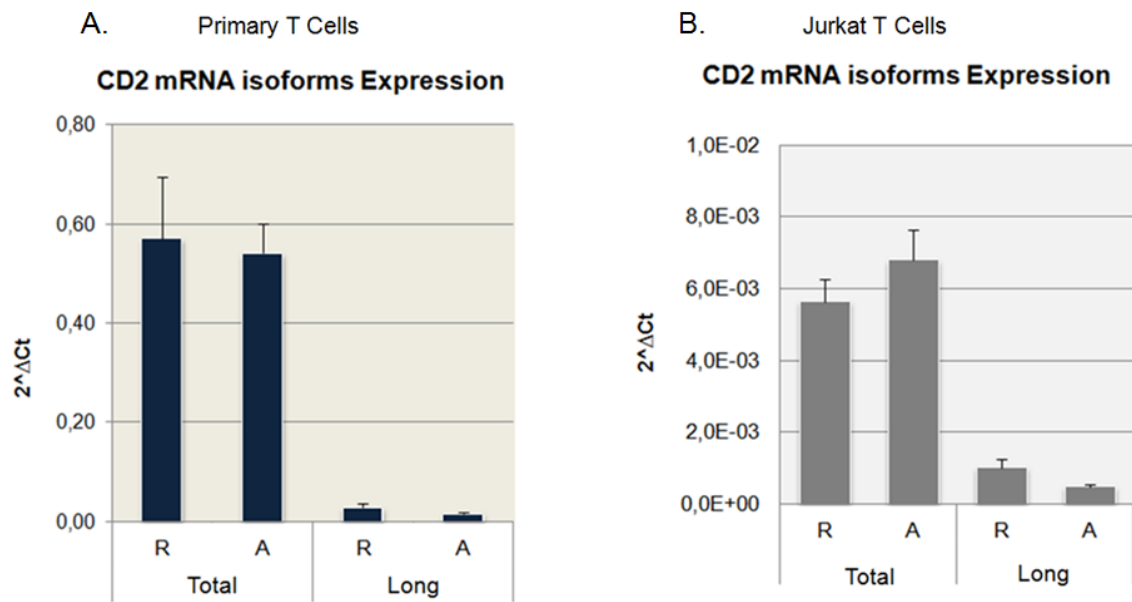


Figure 11 | **CD2 mRNA Expression in Jurkat and Primary T cells.** (A) Primary T lymphocytes. Levels of CD2 mRNA isoforms in resting and activated human primary T lymphocytes were quantified by RT-qPCR. (B) Jurkat T cells. Levels of CD2 mRNA isoforms in resting and activated Jurkat cells were quantified by RT-qPCR. The primer pairs used for this quantification are depicted in Figure 10. The results obtained were normalized for the reference gene. Error bars show s.e.m. from at least three independent experiments.

To quantify a possible effect of APA in the levels of the two CD2 mRNA isoforms, we analyzed the total levels of CD2 transcripts and CD2 pA2 mRNA levels by RT-qPCR. In primary T cells there is a decrease of 40% in CD2 pA2 mRNA levels upon activation, while total CD2 transcripts show no variance in the same conditions (Figure 12). This indicates that there is a preferential usage of the proximal PAS upon activation. In Jurkat T cells this effect is even more pronounced: while the total CD2 mRNA has a slight increase upon activation, the longer isoform shows a decrease of approximately 50%. These results are in agreement with previous studies where a general switch towards the proximal PAS was observed in activated mouse T cells [27]. Our results thus suggest that this APA switch is conserved in other species.

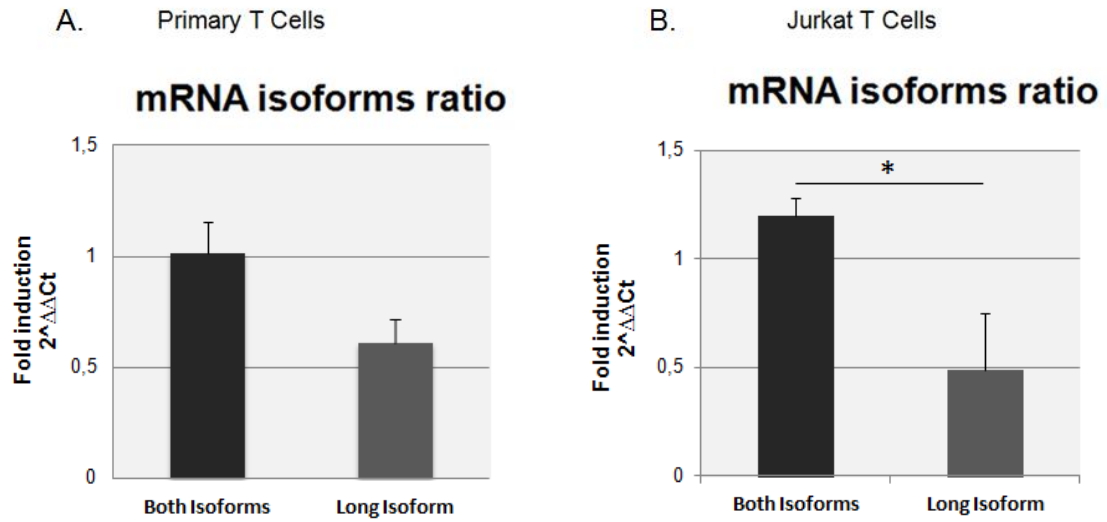


Figure 12 | **T cell activation causes preferential usage of the proximal PAS in CD2 3'-UTR.** (A) In primary T lymphocytes there are no overall changes in CD2 mRNA expression upon activation, showing however a decrease of almost one half in CD2 long mRNA isoform expression. Hence, a preferential usage of the proximal PA site in CD2 is taking place, upon activation (B) Upon T cell activation, Jurkat cells show a slight increase in CD2 overall expression, while CD2 long mRNA isoform depicts a decrease of about 50%. Hence, a preferential usage of CD2 proximal PAS is also seen for Jurkat cells upon activation. Levels of total and pA2 CD2 mRNAs were quantified by RT-qPCR. A - activated T cells; R - resting T cells. Primer pairs used are depicted in Figure 10. For the two models, error bars show s.e.m. from at least three independent experiments. * p < 0.05

CD2 mRNA and protein are expressed in human and rat brain

Previous results obtained in the laboratory showed, by Northern blot analysis, the presence of CD2 mRNA in different rat tissues – heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis (Figure 13 **A.**). Very irrigated tissues, such as heart and spleen show a very strong band for CD2. Other tissues such as lung and liver (mildly irrigated) also show a significant band while tissues such as brain, kidney and testis show a light band for CD2. Skeletal muscle has no presence of CD2 mRNA. As CD2 has a known function in T cells, it was surprising to detect CD2 expression in the brain, because this is a blood-free tissue due to the blood-brain barrier. This result led to further analyze the presence of this immune molecule in the nervous system by Western blot. CD2 expression was detected from cell lysates of different parts of the brain by immunoblotting using the anti-CD2 antibody OX-34. Dilutions of cell lysates of the high-expressing T cell line C58 were used as positive controls. These previous results showed CD2 protein expression in different rat brain areas, more significant in the cortex and striatum (Figure 13 **B.**).

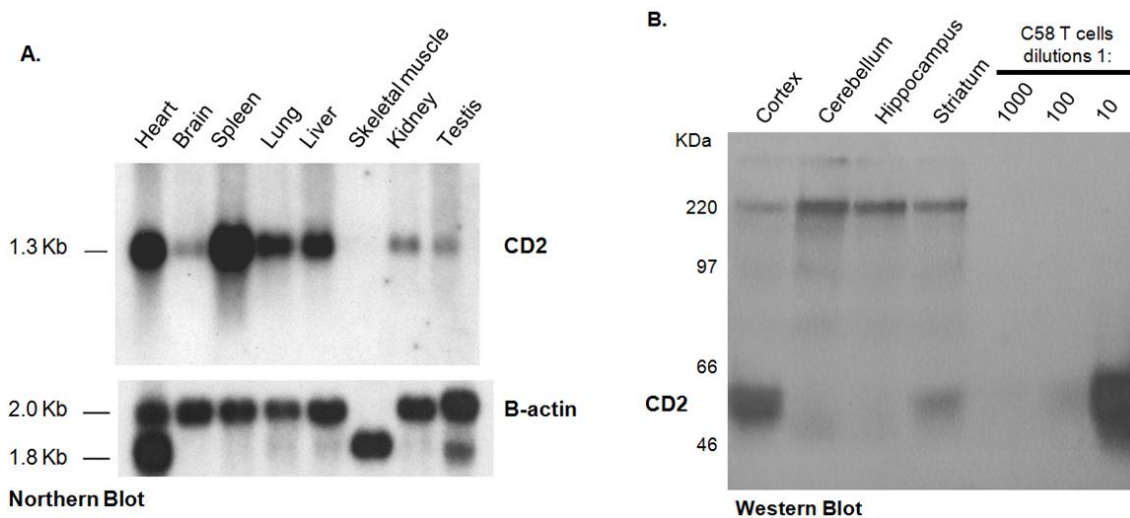


Figure 13 | **CD2 mRNA and protein expression in rat.** (A) Northern blot on several different rat tissues with a CD2 specific probe. (B) CD2 protein expression by immunoblotting. CD2 was detected from cell lysates of different brain areas (cortex, cerebellum, hippocampus and striatum). The antibody used for this detection was anti-CD2 OX-34. Dilutions of cell lysates of the T cell line C58 were used as positive controls.

To further explore and analyze these results, we performed a RT-PCR with specific primers for CD2 coding region in four rat brain areas: cerebellum, cortex, hippocampus and thalamus using YWHAZ as a reference gene and thymus tissue as a positive control (Figure 14 **I.**). As it can be clearly observed, CD2 mRNA is detected in all tissues mentioned. Previous immunohistochemistry results from the laboratory showed

that CD2 is clearly expressed in pyramidal cells in different cortical regions and in Purkinje cells in the cerebellum, in rat brain sections (Figure 14 II.).

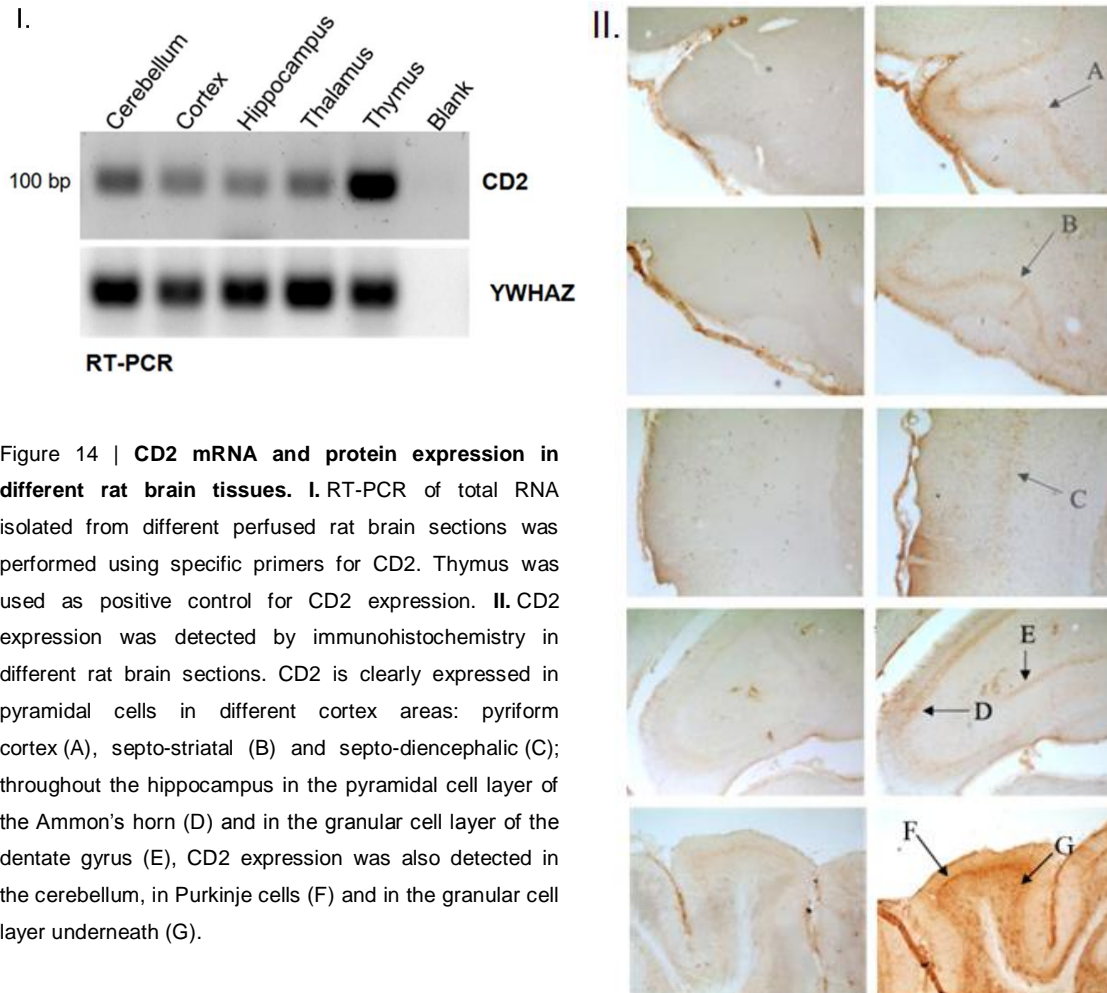


Figure 14 | **CD2 mRNA and protein expression in different rat brain tissues.** I. RT-PCR of total RNA isolated from different perfused rat brain sections was performed using specific primers for CD2. Thymus was used as positive control for CD2 expression. II. CD2 expression was detected by immunohistochemistry in different rat brain sections. CD2 is clearly expressed in pyramidal cells in different cortex areas: pyriform cortex (A), septo-striatal (B) and septo-diencephalic (C); throughout the hippocampus in the pyramidal cell layer of the Ammon's horn (D) and in the granular cell layer of the dentate gyrus (E), CD2 expression was also detected in the cerebellum, in Purkinje cells (F) and in the granular cell layer underneath (G).

We then wanted to address the question: is CD2 also present in the human brain? To evaluate CD2 expression in the human brain, we obtained human brain tissue and brain sections from the UK MRC brain bank (mrc.ac.uk/brainbanksnetwork). Using these samples, we performed RT-PCR and evaluated CD2 localization in the brain by immunohistochemistry. Strikingly, we were able to detect CD2 mRNA by RT-PCR in the human brain, which is more evident in the cerebellum, cortex and thalamus, but also present in hippocampus (Figure 15 I.). Importantly, we also detected CD2 protein in the human brain sections by immunohistochemistry (Figure 15 II.). These results showed that CD2 is present in Purkinje cells (cerebellum) and hippocampal pyramidal neurons, as we had previously seen in the rat model, and suggest that its function in the brain is conserved in human and rat.

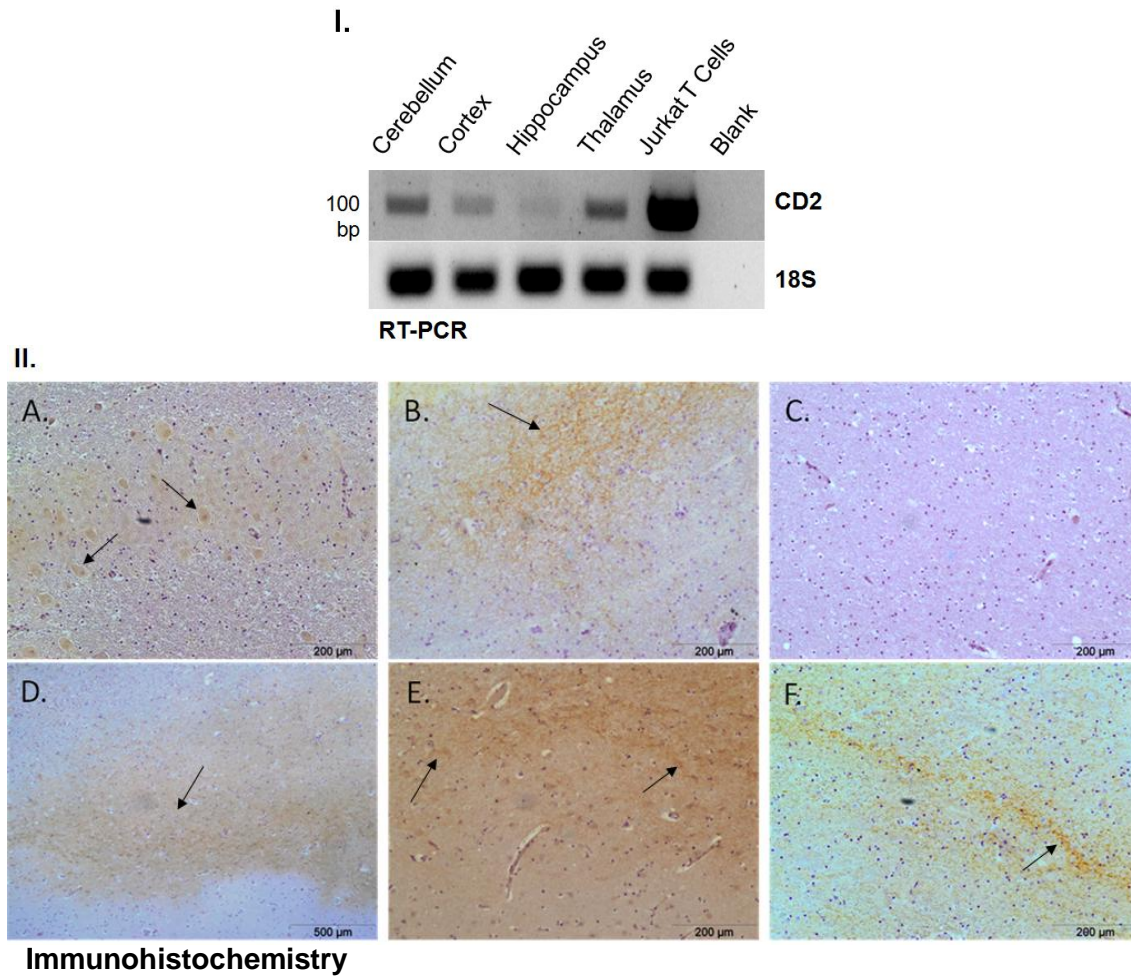


Figure 15 | **CD2 mRNA and protein expression in different human brain tissues.** I. RT-PCR of total RNA isolated from different human brain sections was performed using specific primers for CD2. Jurkat T cells were used as positive control for CD2 expression. II. CD2 expression by immunohistochemistry in human brain sections. CD2 is detected in Purkinje cells in the cerebellum (A), in frontal cortical regions (B), in hippocampal pyramidal neurons (D, E) and in the thalamus (F). C corresponds to the negative control.

As shown in Figure 14 and 15, CD2 is expressed in several rat and human brain areas and this seems to be specific for GABAergic brain cells. We wanted to evaluate the overall expression of CD2 in the different areas, in comparison with a tissue that has high levels of CD2 – thymus. Expression levels of CD2 mRNA in rat cerebellum, cortex, hippocampus, thalamus and thymus were quantified by RT-qPCR (Figure 16). After RNA isolation from the different rat tissues, a qPCR was conducted with primers specific for CD2 and YWHAZ, used as a reference gene. As expected, mRNA expression for CD2 was significantly lower in the four selected brain regions in comparison with the mRNA levels obtained in the thymus. This result is consistent with the findings in immunohistochemistry, where only a few cells seem to be marked with the anti-CD2 antibody. Even though all brain areas showed very low expression levels

of CD2 mRNA when compared to the positive control, thalamus shows the higher expression level for CD2 (Figure 16).

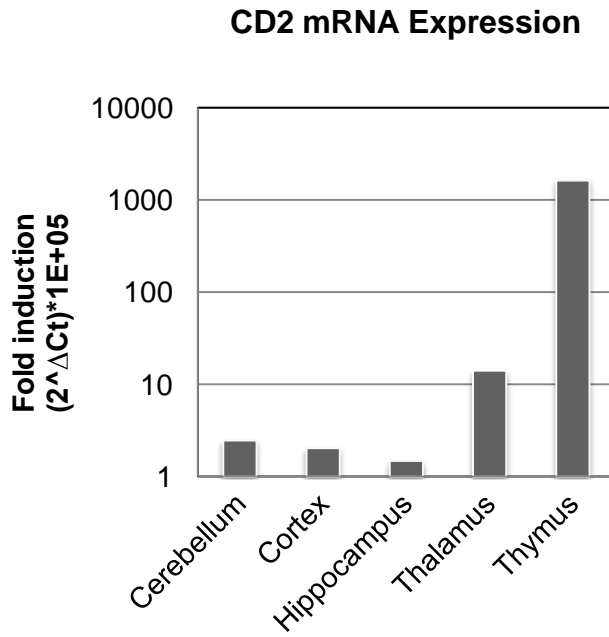


Figure 16 | RT-qPCR for CD2 mRNA in different rat brain areas. Levels of CD2 mRNA in rat brain and thymus were quantified by RT-qPCR.

Rat CD2 APA is tissue- and developmental-specific

Since the brain expresses the larger proportion of the genome by alternative processing than any other tissue, resulting in a high level of mRNA diversity [53], we next investigated CD2 APA in rat brain. A 3'RACE was performed for the selected brain areas, and as it can be seen the short CD2 mRNA isoform is detected in all samples except in the cerebellum (Figure 17 A).

As APA has been shown to depend on the cellular state [21] we wanted to gain insight on a possible role of CD2 APA in brain cell differentiation. Thus, we performed a 3'RACE analysis, in a rat brain cell line (OLN). This cell line consists of an oligodendroglia cell line derived from primary rat brain glial cultures [68]. Cells were kept in DMEM 10% FBS, maintaining a non-differentiated state (OLN n-dif.). To get differentiated cells (OLN dif.) we changed the medium composition to DMEM 1% FBS, after one day in culture to induce differentiation of the oligodendroglia cells (OLN dif.). As it can be seen in Figure 17 B, the pattern of CD2 APA mRNA is altered upon OLN differentiation. For the different brain areas and the non-differentiated OLN only the shorter isoform was detected. However in the thymus and the differentiated OLN the longer isoform was also detected. Hence, these results indicate that CD2 APA is tissue- and developmental-specific in the rat.

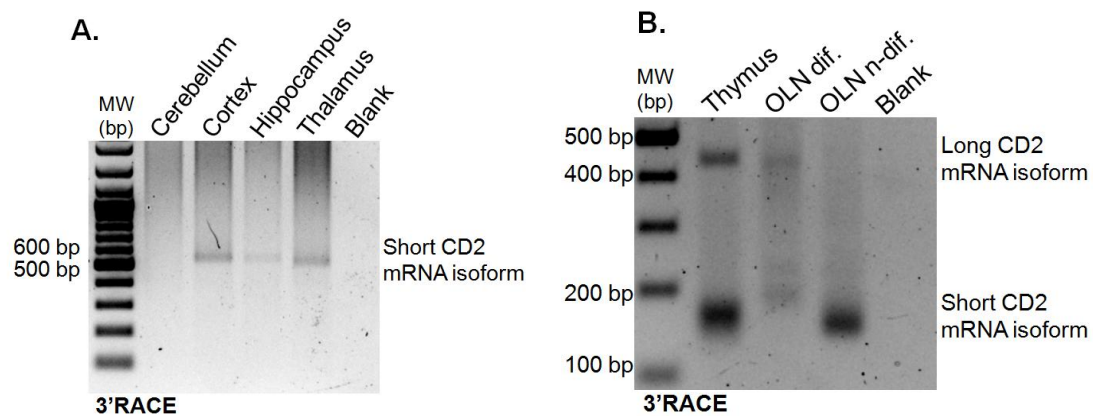


Figure 17 | **3'RACE for rat CD2 mRNA.** (A) 3'RACE reaction for several rat brain areas. A specific CD2 forward primer was used. The band depicted at about 500 bps illustrates the shorter and annotated CD2 mRNA isoform. (B) 3'RACE reaction in thymus (positive control) and in a rat brain cell line (OLN) in two different differentiation states – differentiated (OLN dif.) and non-differentiated (OLN n-dif.). Another specific CD2 forward primer was used. In this reaction the expected size for the shorter isoform was about 170 bp, while for the long isoform (according to the genomic alignment depicted in Figure 3) the size would be approximately 450 bps.

Discussion

Regulation at the RNA level is composed by several layers and is an essential event for cell identity, development and to answer to surrounding stimulus. In this intense cellular activity, PA represents an important player, being coordinated at the spatial and temporal level with transcription and splicing. APA allows transcriptome and proteome diversity, along with expression level fluctuations, being directed by a complex of several protein factors and *cis* elements [24].

The emergence of different mRNA transcripts by APA depends on the overall cellular state and cell type and recent studies state that an elevated number of APA events occur in the coding genes, and more than half of those are tissue-specific [54], clearly stating the importance of this regulatory mechanism [22]. Moreover, the localization of some mRNAs molecules depends on sequences contained in the 3'-UTR determined by APA. This mechanism has an elevated impact on polarized cells, such as neurons. mRNA molecules seem to acquire their localization signals through APA modulation during development [26].

APA extensive modulation is specific for several biological processes indicating an active control towards PA site selection. PA sites highly regulated during cell events such as proliferation/differentiation were enriched with downstream U-rich elements [29]. PA factors influencing PA site usage are also differentially expressed in different human tissues, and through bioinformatic analyses it has been observed a correlation between the expression of PA factors and the 3'-UTR length. Usually, when expression of PA factors is low, the distal PA site is selected. Conversely, high expression of genes coding for 3'-end-processing factors leads to an overall choice of the proximal site [29].

Another important feature related to the PA choice is RNA Pol II transcription dynamics. When a slower transcription rate is imposed by RNA Pol II, there is a tendency to choose the proximal PA site. Splicing also seems to play a key role in regulating APA, through U1 snRNP. This small nuclear ribonucleoprotein induces splicing but also favors the distal PA site selection [29].

In this work we intended to unveil the possibility of a tissue-specific APA event and further understand the intervenients and their influence in determining APA modulation and consequent PA signal choice.

CD2 undergoes APA

The first step towards investigating a possible tissue-specific APA modulation in CD2 was to evaluate the possibility for generating different mRNA isoforms through APA. To answer this question, I started by doing an extensive bioinformatic analyses on the CD2 molecule.

Using UCSG genome database for humans, we could confirm that the CD2 gene codes for two mRNA transcripts differing in their 3'-UTR length. These two isoforms are confirmed by EST (expressed sequence tag) data. Then we further investigated possible *trans*-acting factors, RBPs, that could bind to CD2 3'-UTR as well as potential target sites for miRNA.

During these analyses SFmap provided a somewhat extended list of predicted proteins binding to CD2 mRNA sequence, and we chose the proteins with the highest putative binding scores: hnRNP F, NOVA1 and SRp20.

hnRNP F is a non-tissue-specific heterogenous ribonuclear protein that binds to pre-mRNA molecules and participates in mRNA metabolism, namely in splicing and APA [69]. In fact, there is a study revealing the involvement of this RNA-binding protein in a pre-mRNA splicing neural specific event, even though this protein is widely expressed [70]. This protein seems to have higher affinity for poly(G) sequences in mRNA [71], possibly targeting CD2 3'-UTR in a GUGGAU sequence, between the proximal PA site and the respective cleavage site [69].

NOVA1 is a neuron-specific alternative splicing factor having an impact in determining splice sites in pre-mRNA molecules. More recently it has been discovered a large number of Nova-RNA interactions at the 3'-UTR level, revealing that Nova proteins not only influence splice site choice at the pre-mRNA level but also regulate APA in the brain [60]. In CD2 3'-UTR, NOVA1 has several putative binding sites. More specifically, this protein has partially overlapping putative binding sites near pA2. The finding that these proteins are important in post-transcription mRNA regulation in the brain and that they have putative binding sites for CD2 3'-UTR, strengthen the possibility of CD2 (typically present in the immune synapse) also being present in the brain, possibly located at the neuronal synapse.

It has been demonstrated that SRp20 (an SR protein) has an effect on the recognition of an alternative 3'zterminal exon, by affecting the efficiency of binding of a PA factor (CstF) to an APA site in a tissue-specific manner, and that the levels of this protein alter during differentiation [45, 72]. SRp20 has one putative binding site in CD2 3'-UTR, between the proximal cleavage site and pA2.

To investigate potential miRNA target sites in the CD2 3'-UTR, we used microRNA.org database, and found five miRs. miR-128 has a target site immediately after the proximal PAS, which means that this miR may target both CD2 isoforms for degradation/silencing. Thus, we excluded this miR from our analyses. The other four potential miRs target sites are localized between the two PAS. However, we wanted to identify only miRs that were expressed in both nervous and immune systems. Hence, resorting to miRMap we came to the conclusion that only miR-320, targeting CD2 mRNA immediately downstream of pA2, was expressed in both the immune and nervous system.

CD2 mRNA sequence conservation in mammal species

To further validate CD2 mRNA 3'-UTR isoforms, and the *trans*-acting elements putatively binding to it, the next step was to evaluate the conservation level of CD2 sequence. To accomplish this we used Geneious v4.8 software. After aligning CD2 coding and 3'-UTR sequences of several mammals, it was clear that CD2 mRNA sequence is very conserved among the selected species, showing more than 70% pairwise percent identity. The proximal PAS is conserved (the canonical signal) only in human and rodents. However, in rodents the 3'-UTR is only annotated until the proximal PA and cleavage site. At this point we decided to retrieve the genomic sequences for CD2 mRNA (human and rodents) and perform an alignment. From this alignment it was clear that the 3'-UTR between these three species has a high conservation level, over 66% pairwise percent identity. The proximal pA1 signal is conserved among these species, but pA2 is conserved only in human and mouse. For rat, this potential signal corresponds to a weaker PAS, AACAAA.

To analyze the strength of the putative binding site of the *trans*-acting elements we also analyzed the conservation levels of the putative binding sequences. The sequence for hnRNP F binding is conserved in the three species analyzed. However the same does not occur for SRp20 as the putative sequence for the binding of this protein is not present in rodents. Regarding the binding sequences for NOVA1, from the five sites present in human, two are conserved in human and rat, and a third one is conserved throughout the three species. The conserved sequences for putatively binding proteins among the three species would be the most interesting to further study, not only because conserved sequences located in a non-coding region are more likely to contain regulatory elements but also because the possibility of studying different mammals (human and rodents) confers stronger proof for their regulatory roles.

CD2 mRNA isoforms are differentially expressed upon T cell activation

Based on recent work done in mouse primary T lymphocytes [27], we decided to evaluate the presence of two CD2 mRNA transcripts in primary human T lymphocytes in both an activated and resting states. Through a 3'RACE and sequencing analysis, we concluded that both mRNA isoforms are expressed in both cellular states, and determined the cleavage sites of the correspondent pre-mRNAs.

Then we wanted to address the question of whether a shift in APA pattern occurs upon T cell activation, and thus we activated primary human T lymphocytes and Jurkat T cells. First we analyzed the expression levels on both cell types, and observed that in primary T cells the expression levels are considerably higher, compared to the levels present in the Jurkat cell line. Then we investigated the 3'-UTR length upon activation with PHA. In both cellular models we observed a shortening of the 3'-UTR length of CD2 mRNA upon activation. These are important results, as they agree with previous published data for mouse T cells, but had not been described before in human cells.

Therefore, we answered the question of whether CD2 produces different mRNA isoforms according to the cellular state in human T cells.

CD2 expression and APA

After preliminary results showed that CD2 mRNA and protein are present in different rat brain areas, we went to uncover the exact localization of this transmembrane protein in the human brain. The presence of this molecule in the brain is surprising, since CD2 is a well-known immune molecule expressed in T lymphocytes, and its presence in the brain has never been mentioned before. Through immunohistochemistry technique, we were able to identify CD2 expression in four different human and rat brain regions (cerebellum, cortex, hippocampus and thalamus). These results are consistent with those previously obtained in the rat model. Cells expressing CD2 at the surface seem to be GABAergic neurons (cells that produce γ -aminobutyric acid, a neurotransmitter), namely Purkinje cells in the cerebellum and hippocampal pyramidal cells. The neurotransmitter (GABA) conferring the name to the cells in which we found CD2 is especially important during brain development [73]. A compelling aspect of our findings, which would be interesting to investigate in the future, is the physiological relevance of CD2 expression in neurons. Namely, if CD2 acts as a cell adhesion molecule or as a signaling transducer molecule at the neuronal synapse, similarly to its function at the immunological synapse. Another important question to address is what CD2's ligand in neurons. It would be interesting to investigate if CD58, a CD2 ligand typically expressed in APCs, is present and can also bind CD2 in the brain, or if there are other possible CD2 ligands in the nervous system.

When we started to search for CD2 mRNA isoforms in the rat brain, we could only see the short isoform by 3'RACE in tissues. However, when we used thymus as a positive control for the reaction and a brain cell line (OLN93) in different differentiation status, we could see an extra band with the expected size for pA2 (according to the genomic alignments done previously). We then designed new RT-PCR primers, based on the genomic alignment initially done, for the region between the proximal pA1 site and pA2, and we could confirm that the distal pA2 signal, although a weaker PAS, is also recognized in the rat.

Upstream of pA2 there is a predicted NOVA1 binding site, conserved in human, mouse and rat, suggesting that CD2 mRNA may be a target for regulation through NOVA1. Since NOVA1 is a RNA-binding protein characteristically expressed in the brain it is possible that expression of CD2 in the brain could be dependent on NOVA1. It will be relevant in the future to investigate the role of NOVA1 in CD2 expression in the brain, using siRNA knockdown and overexpression studies using reporter genes and knockout mice [74].

However it could be interesting to address the possibility of hnRNP F, which is widely expressed and has been shown to affect APA, regulate CD2 in the brain or in the immune system, or possibly in both.

Previous results from the laboratory showed that miR-320 is expressed in resting T lymphocytes. Therefore, the decrease in the CD2 longer isoform levels upon T cell activation is not likely to be due to miR-320 silencing, since this miRNA is also present when the T cells are in a resting state. Nevertheless, it is necessary to quantify miR-320 in activated T cells, because if it is overexpressed in an activated state, it may account for the decrease in the long mRNA CD2 isoform observed.

Working models

Our working model in T cells is represented in Figure 1. In accordance with what has been seen in mice T cells [27], the significant decrease on the long CD2 mRNA isoform upon activation observed may be due to miR-320 overexpression when cells are activated. However, other players may be inducing this shift, such as hnRNP F and SRp20, which are widely expressed, as well as NOVA1. Although NOVA1 has been described as brain-specific [54] we could detect it in T cells through RT-PCR. It is also possible that other factors such as transcription factors and chromatin remodeling may act on CD2 expression explaining the preferential usage of pA1 upon T cell activation. Future studies will be essential to further clarify this model.

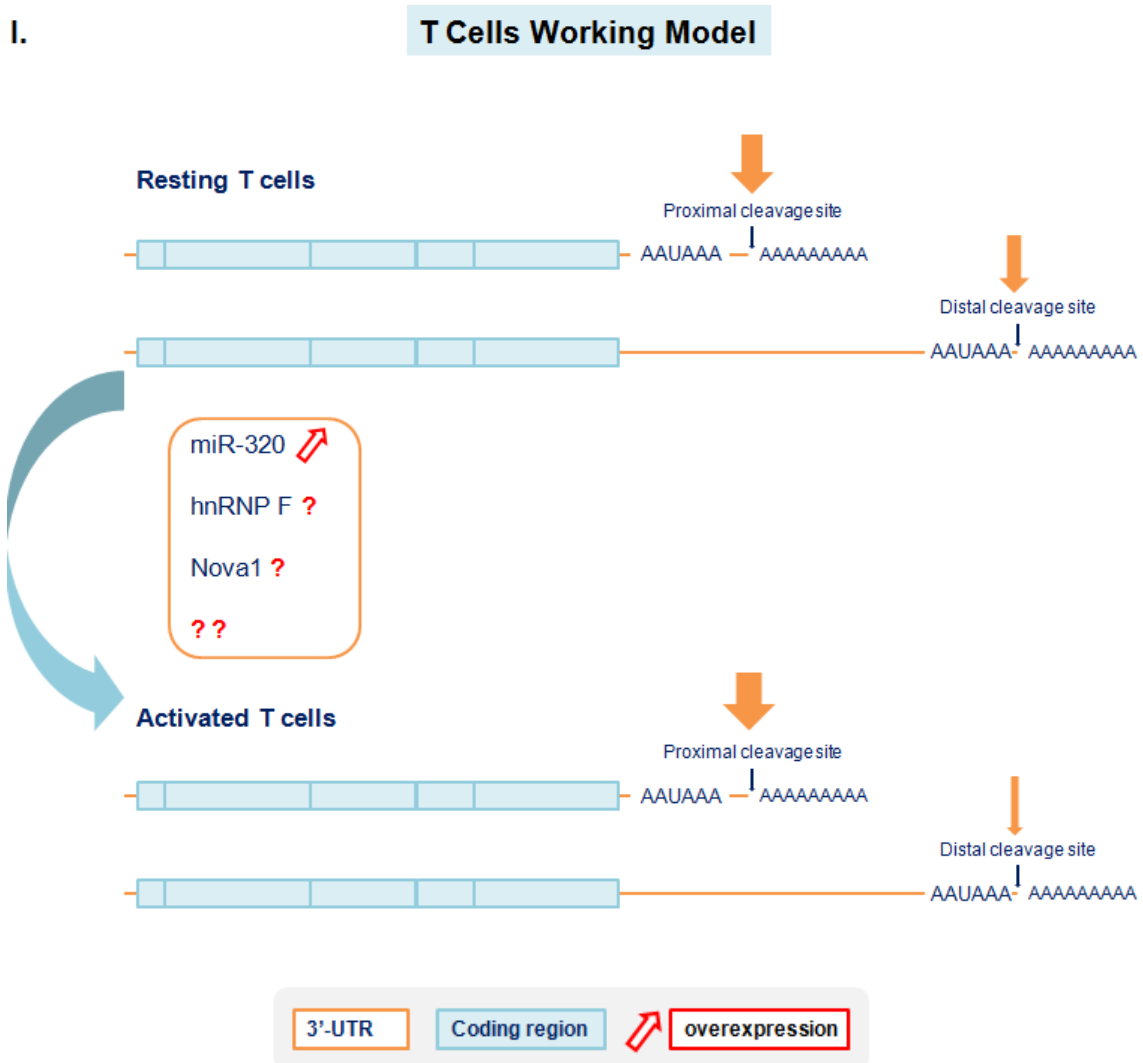


Figure 1 | **T cells working model.** Schematic representation of CD2 APA pattern in a resting and activated T cells.

Differentiation of a brain cell line alters CD2 APA expression. According to our 3'RACE analysis the shorter CD2 isoform is only expressed in differentiating cells, in agreement with what was observed for other genome-wide studies [29]. However, differentiated cells exhibited both isoforms expression. The protein factors we took into account for the T cell working model, as well as SRp20, may also have a role in CD2 APA in the brain. But again several other factors may have a role regulating this event, and further studies are needed to confirm and explore our results.

II.

Brain Working Model

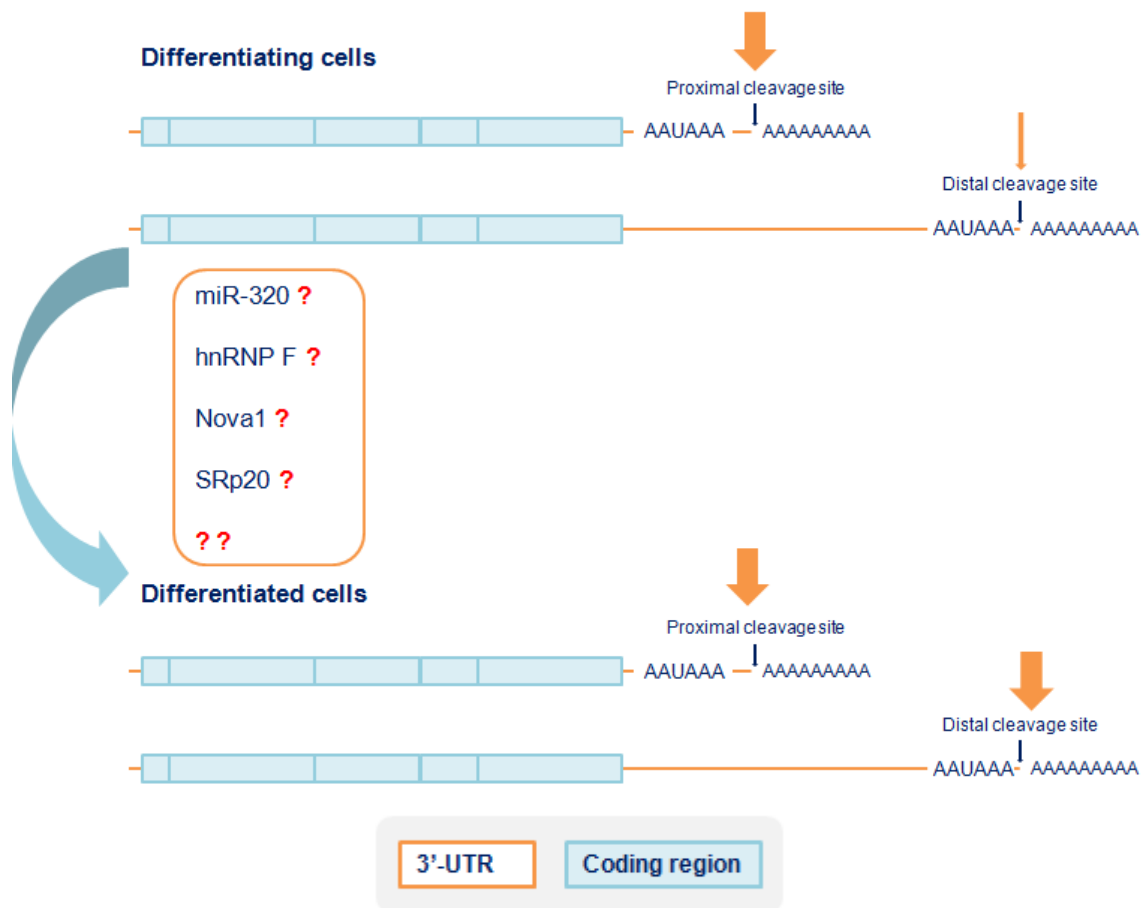


Figure 2 | **Brain working model.** Schematic representation of CD2 APA pattern in brain cells in a differentiating and differentiated state.

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