T Cell Activation Regulates CD6 Alternative Splicing by Transcription Dynamics and SRSF1

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T cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1

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T he CD6 cell-surface Ag is a type I transmembrane glycoprotein mostly expressed on thymocytes and mature T and B1a lymphocytes (T and B1 cells) (1). CD6 belongs to the scavenger receptor cysteine–rich (SRCR) superfamily of protein receptors, containing three cysteine-rich domains in its extracellular region (2). Human CD6 is encoded by 13 exons, of which the first 7 encode the amino terminal sequence and the extracellular and transmembrane regions, whereas the remaining 6 exons code for the cytoplasmic tail. Each of the three extracellular SRCR domains (d1–d3) is coded by a separate exon (e3–e5).

The functional role of CD6 has not been definitively established. Whereas it is undeniable that increased expression of CD6 results in repression of TCR-mediated signaling (3, 4), the direct binding of CD6 with specific Abs or recombinant ligand may enhance cellular responses, presumably through the induced aggregation of protein kinases associated with the cytoplasmatic tail of CD6 (3, 5, 6). Notwithstanding the uncertain nature of CD6, possibly even having a dual role, the fact is that CD6 impacts on cell growth and differentiation, and misregulation of the function of CD6 may result in physiological imbalances and autoimmunity. CD6 has been associated with several autoimmune diseases such as multiple sclerosis (7, 8), rheumatoid arthritis (9), psoriasis (10), and Sjögren’s syndrome (11) and has been considered as a possible therapeutic target for some of these pathologies (12).

The known ligand for CD6 is the Ig super family receptor CD166, expressed on conventional APC and also in thymic epithelia, with the CD6–CD166 interaction being determinant on thymocyte selection (13). CD166 is additionally expressed in the epithelial layer of the blood–brain barrier and enables the transmigration of CD4+ T cells into the brain (14). The interaction between CD6 and CD166 involves the binding of the membrane distal Ig super family domain of APC surface-expressed CD166 to the membrane-proximal SRCR domain 3 of CD6 (15). Importantly, this interaction is fairly strong and can help with strengthening and stabilizing T cell–APC contacts (16). Localization and function of both CD6 and CD166 at the immunological synapse (IS) are determined by and reliant on this binding, and conversely, a naturally occurring alternative splicing–dependent CD6 isoform that lacks domain 3, CD6Δd3, fails to target and recruit CD166 to the IS (17).

CD6Δd3 mRNA is expressed in 40% of T lymphocytes, being the dominant isoform in one-quarter of this subpopulation in rats...
Alternative splicing of exon 5 in the CD6 gene is physiologically and functionally relevant, as the differential expression of the membrane-proximal SRCR domain coded by that exon determines CD6 and CD166 localization or exclusion from the IS. In this study, we show that activation-induced CD6 exon 5 skipping in T cells involves the downregulation and recruitment of the SRSF1 splicing factor to an ISE in CD6 intron 4 that together with an increase in the RNA pol II transcription rate of the CD6 gene contribute to the production of a CD6 isoform devoid of the ligand-binding domain.

Materials and Methods

Cell isolation and drug treatment

Buffy coats from healthy donors were provided by Hospital São João, Serviço de Imunohematoterapia (Porto, Portugal), and PBMCs were isolated by density-gradient separation using Lympholyte-H (Cedarlane Laboratories). T cells were isolated from PBMCs using the Easysep Human T Cell Enrichment Kit (StemCell Technologies). Cells were maintained in RPMI 1640 Glutamax with 10% FBS, 1% sodium pyruvate, and 1% penicillin-streptomycin (Life Technologies). T cells were stimulated overnight with PHA-P at 10 μg/ml or with a combination of anti-CD3 (OKT3) at 2 μg/ml and anti-CD6 (MEM-98) at 10 μg/ml. In chromatin-modulation experiments, 6 × 10⁶ cells were treated with 0.5 μM TSA or with 10 μM camptothecin for 1 h at 37°C.

Plasmids

To construct the CD6 minigene, the genomic DNA region from exon 4–6 was amplified by PCR and cloned into the pCMVdi vector (kindly given by Juan Valcárcel, Centre de Regulación Genómica, Barcelona, Spain). Intron 4 mutants were made by PCR site-directed mutagenesis using Phusion DNA polymerase (Finnzymes). T7 epitope–tagged vectors pCT7SSF2, pCT7S5RF20, and pCGTA1A used for overexpression of the splicing factors SRSF1, SRSF3, and hnRNP A1, respectively, were a kind gift from Javier Cáceres (Medical Research Council, Edinburgh, U.K.).

Transfections

Cells were transfected by nucleofection using the Amaxa Human T Cell Nucleofector kit (Lonza). A total of 2 μg each CD6 minigene mutant or T7-tagged expression vectors were used for cell transfections (Supplemental Fig. 4A). For the knocking down experiments, PBMCs were transfected with small interfering RNAs (siRNAs; Sigma-Aldrich) (SRSF1, 5′-AGCAUGUG-CGGCAGUCAUGU-3′; SRSF3, 5′-UCAGUAGUAGUAACUGUGUC-3′; SRSF3#2, 5′-GAGAGGUGUGUAACGAA-3′; hnRNP A1#1, 5′-CCAC-UUAACUGUGAAGAAUUU-3′; and hnRNP A1#2, 5′-CUUUUUGUG-GUGUGUGUGUGGA-3′) at a final concentration of 50 nM for SRSF1 and SRSF3 and 300 nM for hnRNP A1 (Supplemental Fig. 4B, 4C). Total RNA and protein extracts were isolated after 48 h of incubation at 37°C, 5% CO₂.

Cell fractionation and RNA extraction

Cells were washed twice with ice-cold PBS and centrifuged for 5 min at 290 × g at 4°C. The cell pellet was resuspended in 1 ml RSB buffer (10 mM Tris-Cl [pH 7.4], 10 mM NaCl, and 3 mM MgCl₂) and incubated 3 min on ice. Cells were pelleted at 1500 × g for 3 min at 4°C, the supernatant was discarded, and the cells were lysed by gentle resuspension in 150 μl RSBG40 buffer (10 mM Tris-Cl [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, 10% glycerol, and 0.5% Nonidet P-40). Samples were centrifuged at 4500 × g for 3 min at 4°C. The supernatant (cytoplasmic fraction) was collected in a new Eppendorf tube (Eppendorf), and 1 ml TRizol (Invitrogen) was added to extract cytoplasmic RNA according to the manufacturer’s protocol. The nuclei pellet was also resuspended in 1 ml TRizol (Invitrogen) to extract the nuclear RNA.

RT-PCR

Total RNA from human primary T cells was isolated using TRizol (Invitrogen), and 500 ng RNA for each condition was treated with DNase I (Roche). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. A total of 25% of the RT reaction volume was used to analyze endogenous CD6 exon 5 alternative splicing pattern by PCR amplification with Go Taq DNA polymerase (Promega). In the case of CD6 minigene mutants, cDNA was synthesized with a plasmid RT primer, and radiolabeled primers were used in a low-cycle (20 cycles) PCR reaction. Quantitative PCR (qPCR) reactions were performed with a 1:10 cDNA dilution using IQ SYBR Green
Supermix (Bio-Rad) and following the manufacturer’s instructions. Primer sequences are available upon request. Due to donor variability, the resting CD6 exon 5 splicing pattern was confirmed prior to analysis.

Western blotting
Whole-cell lysates were prepared, resolved, and transferred with the iBlot gel transfer device (Life Technologies). Incubations with primary Abs diluted in TBST containing 3% nonfat dried milk were followed by washes with TBST, incubation with the appropriate secondary Abs in TBST/dried milk, and detection using enhanced chemiluminescence (ECL Prime; Amersham/GE Healthcare). Abs used were: anti-SRSF1 (AK96, a kind gift from Adrian Krainer, Cold Spring Harbor Laboratory), anti-hnRNP A1 (9H10; kind gift from Gideon Dreyfuss, Howard Hughes Medical Institute), and anti-SR20 (7B4-sc13510; Santa Cruz Biotechnology).

Chromatin and RNA immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed using human T cells as previously described (37). The relative occupancy of the immunoprecipitated protein at each DNA site was estimated as follows: 2^{Ct (input)} - 2^{Ct (IP)} (where Ct (input) and Ct (IP) are mean threshold cycles (Ct) of quantitative RT-PCR done in duplicate on DNA samples from input and specific immunoprecipitations, respectively). Abs used: rabbit polyclonal anti–Pol II (N20; Santa Cruz Biotechnology), anti–histone H3 (ab7971; Abcam); anti-H3K36me3 (ab9050; Abcam); anti-H3K9me3 (ab8898; Abcam), and anti-H3K9Ac (ab10812; Abcam). Primer sequences are available upon request.

RNA immunoprecipitation (RIP) was performed using the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore). A rabbit mAb anti–SRSF1 (ab133689; Abcam) was used. The relative occupancy of the immunoprecipitated protein at each RNA site was estimated as described above for the ChIP assay.

UV-crosslinking immunoprecipitation assays
A total of 5000 cpm of intron 4 premRNA probes were incubated in 50-μl reaction mixtures containing 100 μg PBL nuclear extracts, 32 mM HEPES (pH 7.9), 1.56 mM MgCl2, 0.5 mM ATP, 20 mM creatine phosphate, and 2.6% polyvinyl alcohol. Samples were incubated at 30˚C for 20 min, UV-crosslinked in a Hoefer UVC 500 Ultraviolet Crosslinker (254 nm for 9 min, 4 cm from light source; Hoefer), and treated with 5 μg RNase A (1 μg/μl) at 37˚C for 30 min. A 10% aliquot of the cross-linked samples was loaded on a 12% SDS-PAGE. For immunoprecipitation, 100 μg tissue culture supernatant of monoclonal anti–SRSF1 Ab (AK96) and 35 μg Pol II antibody (Abcam) were added to 80% cross-linked material, and 0.1 M KCl Buffer D was added to a final volume of 200 μl. The mixture was incubated overnight at 4˚C in a rotative wheel. Beads were washed twice with ice-cold Binding Buffer I (20 mM HEPES [pH 7.9], 150 mM NaCl, and 0.05% Triton X-100) and two times with ice-cold Binding Buffer II (20 mM HEPES [pH 7.9], 150 mM NaCl, and 1% Triton X-100). After the last wash, the supernatant was completely removed, and beads were resuspended in 35 μl 2X SDS-loading dye and heated for 5 min at 95˚C. After centrifugation, the supernatant was loaded on a 12% SDS-PAGE. Gels were fixed for 35 min at room temperature in a fixing solution before drying. Gels were exposed to a radiographic film.

Results
Modulation of chromatin structure affects CD6 exon 5 skipping in human primary T lymphocytes
We have previously shown that skipping of exon 5 of CD6 is induced by T cell activation, resulting in an increase of the CD6Δd3 mRNA isoform, which is translated into a CD6 poly-peptide that lacks the domain of interaction with its ligand, CD166 (schematically represented in Fig. 1A) (17). To investigate the molecular mechanisms regulating T cell activation–dependent alternative splicing of CD6, we first confirmed the pattern of exon 5 skipping in human primary T lymphocytes activated with the mitogenic lectin PHA-P. As expected, PHA induced a switch in the exon 5 alternative splicing pattern, resulting in a >2-fold decrease of the ratio between the CD6 full-length mRNA isoform (CD6FL) and the isoform omitting exon 5 (CD6Δd3) (Fig. 1B, 1C). This switch is also observed in purified T cells activated with anti-CD3 and anti-CD6 mAbs (Supplemental Fig. 1).

It was also apparent that activation with PHA induced an increment in the levels of the full-length mRNA isoform, suggesting a general transcriptional induction (Fig. 1b). To understand how transcription of CD6 was regulated upon T cell activation, we purified T lymphocytes from blood and analyzed changes in RNA pol II occupancy in the CD6 gene and CD6 expression levels occurring in resting and activated T cells using ChIP (Fig. 2). Upon T cell activation, an overall increase of RNA pol II occupancy throughout the CD6 gene was observed (Fig. 2b), concurring with an increase in CD6 transcription (Fig. 2c).

Accumulating evidence indicates that alternative splicing is influenced by chromatin histone modifications (43), in particular H3K36me3 and H3K9me3 (32, 33). To investigate whether these regulatory mechanisms could also govern CD6 exon 5 alternative splicing, we analyzed the pattern of H3K36me3 and H3K9me3 in the CD6 gene, in resting and activated T cells. H3K36me3 levels were increased in the body of the gene compared with the first exon and the intergenic region (Supplemental Fig. 2A), as it occurs in actively transcribed genes (44). H3K9me3 levels that were described to be enriched in a subset of alternative exons (33) were decreased in the body of the CD6 gene in comparison with the intergenic region (Supplemental Fig. 2B). However, there were no differences in H3K36me3 and H3K9me3 levels either between resting and activated T cells or comparing exon 5 with the neighboring exons. This indicates that these epigenetic marks are not modulated in the CD6 gene by T cell activation, and they do not have a function in CD6 alternative splicing.

It has been shown that active genes show altered H3K9 acetylation levels upon T cell activation (45). We thus characterized histone H3K9 acetylation in CD6 in resting and activated T cells (Fig. 3A). By contrast with the methylation marks analyzed, we detected an overall increase in H3K9Ac in activated T cells. These differences are statistically significant at the 5’ end of the gene as expected and also in exon 5. Treatment of HeLa cells with inhibitors of histone deacetylases (HDACs) have been shown to
cause alterations in the splicing pattern of alternatively spliced exons (46). We thus hypothesized that upon T cell activation, the CD6 gene could undergo alterations in the chromatin structure to facilitate the accessibility of the transcription machinery, resulting in the increased CD6 mRNA levels and exon 5 skipping observed. When we treated T cells with an inhibitor of HDACs (TSA), an increase in the levels of transcripts lacking exon 5 was observed (Fig. 3B), suggesting that the open chromatin state promotes skipping of exon 5. Conversely, when T cells were incubated with an inhibitor of topoisomerase I, camptothecin, which hampers RNA pol II elongation, the opposite effect in the alternative splicing pattern was observed: inclusion of exon 5 was promoted (Fig. 3B). We quantified both CD6 mRNA isoforms and confirmed that TSA as well as camptothecin have significant but opposite effects in the splicing pattern of exon 5 (Fig. 3C), suggesting that the RNA pol II transcription rate plays a determinant role in the skipping or inclusion of exon 5 in CD6 pre-mRNA.

CD6 intron 4 contains a complex set of splicing regulatory elements

Using the UCSC Genome Browser, we found several peaks of conservation in intron 4, distant from the splice sites.

FIGURE 2. Characterization of RNA pol II occupancy levels and CD6 expression in resting and activated T cells. (A) Schematic representation of human CD6 locus. Exons are represented by boxes, and introns are shown as a thin line. The region for specific qPCR primers pairs is underlined. (B) Graphic representation of RNA pol II occupancy levels in CD6 gene in resting and activated T cells evaluated by ChIP assays showing an increase of RNA pol II occupancy upon T cell activation (n = 5). (C) CD6 expression levels in resting and activated PBMCs were analyzed by qPCR, revealing an increase of expression upon cell activation with PHA (n = 3). Error bars represent SEM. The asterisk indicates statistical significance (*p < 0.05 Student t test).

FIGURE 3. CD6 H3K9Ac levels in resting and activated T cells and CD6 chromatin structure modulation. (A) Graphic representation of H3K9Ac levels in CD6 gene in resting and activated T cells evaluated by ChIP assay shows an increase of acetylation upon T cell activation. (B) Semiquantitative RT-PCR showing the TSA and camptothecin effects in CD6 exon 5 alternative splicing pattern. (C) CD6 isoform expression levels were analyzed by qPCR. Primers were designed to span the 4 to 5 and 4–6 exon junction regions. Error bars represent SEM. n = 3. The asterisk indicates statistical significance (*p < 0.05, Student t test).
conserved in 46 species of vertebrates, suggesting the presence of important regulatory elements (Supplemental Fig. 3A).

We therefore generated a functional minigene containing the genomic fragment spanning exons 4–6 to characterize the cis elements involved in CD6 alternative splicing (Supplemental Fig. 3B). Importantly, when transfected into PBMCs, this minigene recapitulates the alternative splicing pattern of the endogenous CD6 gene, with the majority of transcripts containing exon 5 (Supplemental Fig. 3C, left panel). Moreover, upon PHA-induced T cell activation, there is the same enrichment of CD6Dd3 over CD6FL as observed in endogenous CD6 (Supplemental Fig. 3C, right panel, compared with Fig. 1B, Supplemental Fig. 1). This indicates that the minigene contains all of the necessary sequences to promote CD6 exon 5 alternative splicing and can be used to characterize the cis-regulatory elements involved.

To characterize the conserved region in intron 4, we engineered the minigene to introduce specific deletions originating the constructs depicted in Fig. 4A. Minigene mutant i4Δ1, containing a 436-nt deletion in the central region of intron 4 (nt 193–629) (Fig. 4A), was transfected into resting T cells, and the splicing pattern was analyzed by RT-PCR. A complete switch in exon 5 alternative splicing was observed, with a 3-fold reduction in the mRNA isoforms ratio (relative increase of CD6Dd3 over CD6FL) (Fig. 4B). A mutant having a smaller deletion (i4Δ6) induced a statistically significant similar reduction, indicating that an ISE for exon 5 inclusion is contained in the region between nt 297 and 629 of intron 4 (Fig. 4B). This is a sequence-specific effect, as confirmed by replacement of the i4 297–629 sequence by an unrelated sequence (i4Δ6 ctrl) (Fig. 4B).

To fine-map the ISE identified, three mutants containing deletions of ∼100 nt were generated, each one lacking one-third of the i4Δ6 deleted region (A, Δ297–383; B, Δ384–481; and C, Δ482–629), and transfected into T cells (Fig. 4C). Intriguingly, deletion of any of the three regions (A, B, and C) induced only a partial 2-fold reduction in the CD6FL/CD6Δd3 ratio when compared with unmodified i4 (Fig. 4C), suggesting that several regulatory elements may be present. To further dissect this region, mutant minigenes having combined or overlapping sequences excluded in the nt 297–629 region were transfected and their splicing pattern analyzed (Fig. 4D). Deletion of nt 297–481 (AB) induced a reduction of ∼3-fold in the CD6FL/CD6Δd3 isoforms ratio compared with wild-type (WT) i4. By contrast, mutant BC (Δ384–629) did not induce any alterations in the ratio (Fig. 4D). In addition, deleting regions A and C together again introduced a significant reduction in the CD6FL/CD6Δd3 ratio (Fig. 4D). Taken together, these results suggest that both silencer and enhancer elements coexist in region BC, as contrasted with the effect (increased exon 5 skipping) of deleting regions B or C independently; deletion of B together with C (BC) does not result in significant differences in comparison with the WT. We therefore constructed an additional mutant (D, Δ451–544) for which the deleted sequences comprise part of region B and part of region C. Interestingly, this deletion causes a significant increase in exon 5 inclusion (2-fold increase in the CD6FL/CD6Δd3 ratio), suggesting that it contained an inhibitory sequence for exon 5 inclusion (Fig. 4E).

CD6 exon 5 alternative splicing is regulated by SRSF1, SRSF3, and hnRNPA1

As alternative splicing of exon 5 of the CD6 gene is regulated by T cell activation, we investigated variations in the expression of relevant splicing factors in resting and activated T cells by RT-PCR. Different mutants, each one having different deletions in intron 4, were created by PCR-directed mutagenesis and used for transient transfection in human T cells. Graphic representation of the CD6FL/CD6Δd3 ratios in intron 4 mutants and CD6 WT minigene determined by semiquantitative RT-PCR. Error bars represent SEM. n = 3; (E) n = 2. The asterisks indicate statistical significance (*p < 0.05, **p < 0.01, Student t test). E, enhancer; S, silencer.
qPCR and immunoblotting. Within the nt 297–629 regulatory sequence of intron 4, there are several putative binding sites for splicing factors such as SRSF1, SRSF2, SRSF3, SRSF5, SRSF6, hnRNPA1, hnRNPA2B, hnRNPH/F, and PTB as determined by bioinformatics analyses (SFmap Web site: http://sfmap.technion.ac.il/index.html). We focused on those presenting the highest SFmap algorithm scores (i.e., SRSF1, SRSF3, and hnRNPA1).

Upon PHA-induced T cell activation, there were no significant alterations in mRNA or protein expression of either hnRNPA1 or SRSF3 (Fig. 5A, 5B); however, SRSF1 mRNA and protein levels were decreased by ~50% in activated T cells. To investigate the role of differential SRSF1 levels in CD6 alternative splicing, we used siRNA depletion and overexpression assays. Remarkably, overexpression of SRSF1 resulted in 75% reduction in the expression of the CD6Δd3 isoform (Fig. 5C). Overexpression of SRSF3 and hnRNPA1 also induced significant changes in CD6Δd3 expression (2-fold decrease and 1.5-fold increase, respectively), suggesting that changes in their protein expression, if produced, may also affect CD6 alternative splicing (Fig. 5C). The converse effect on CD6Δd3 isoform expression was observed when all of these splicing factors were siRNA knocked down individually (Fig. 5D).

Given the observed decrease in SRSF1 expression upon T cell activation and the effect of this factor on CD6 alternative splicing, we hypothesized that the lower recruitment of SRSF1 to the regulatory element of intron 4 was the limiting factor for exon 5 inclusion. To investigate this, we performed RIP with a specific Ab for SRSF1 and CD6 primers. Indeed, upon cell stimulation with PHA, a marked decrease of the recruitment of SRSF1 to the intron 4 was evident when compared with untreated cells (Fig. 5E). Importantly, recruitment of SRSF1 to CD6 was also prevented by TSA. As SRSF1 protein levels are not altered by TSA treatment (Fig. 5F), these results suggest that recruitment of SRSF1 to CD6 pre-mRNA is acetylation dependent.

To demonstrate that SRSF1 binds to i4Δ6, we performed UV crosslinking and immunoprecipitation assays using the sequence deleted from i4Δ6 as a pre-mRNA template. As can be seen, SRSF1 binds to this element in intron 4 (Fig. 5G). Overall, our results suggest that not only is SRSF1 less expressed upon T cell activation, but it is also strikingly less recruited to an ISE present in intron 4 of the CD6 primary transcript, all resulting in significant increase of exon 5 skipping (Fig. 6).

Discussion

Upon Ag recognition, T cell activation is determined by the balance between activatory and modulatory signals. The first type of signals include the affinity of a given TCR to a specific antigenic peptide presented in the context of MHC complexes displayed at the surface of APCs and also all of the signaling machinery that relays the information received at the cell surface down to the nucleus. This consists of a well-structured pathway that sequentially activates effector enzymes and adapters, such as the Src family kinases Lck and Fyn that phosphorylate the ITAMs of the CD3 complex and the kinase ZAP70 that, upon binding to phosphorylated ITAMs, becomes activated and phosphorylates the membrane adapter LAT, which in turn serves as a docking platform for many downstream effectors such as PLC-γ, PI3K, GADS, Grb2/Sos1 connecting to the Ras–MAPK pathway, Itk, and SLP-76, among others (47).
In contrast, there are a few receptors such as CD5 and CD6 that limit TCR-mediated responses at the onset of activation. In the case of CD5, this is achieved through the phosphorylation-dependent recruitment of signaling inhibitory enzymes such as the phosphatase Src homology region 2 domain-containing phosphatase-1, the ubiquitin ligase Cbl, and the Ras GTPase-activating protein (48, 49). The kinases that promote these associations by phosphorylating tyrosine residues of the cytoplasmic tail of CD5 are the same Lck and Fyn that induce positive signaling via the TCR/CD3 complex, although subsequently, CD5 activation can nonetheless modulate the kinase’s activity (50, 51). Regarding CD6, it is still not clear how signaling attenuation is achieved, as none of the CD5-associated inhibitors have been reported to associate with CD6. On the contrary, upon activation and phosphorylation, CD6 becomes packed with effector enzymes and adapters such as Lck, Fyn, Zap70, Itk, and SLP-76, all connoted with productive signaling and full T cell activation (3, 6).

One hypothesis to explain the inhibitory properties of CD6 is that the receptor may function as a decoy adapter, having high affinity for the signaling mediators and thus removing much of the activation potential away from the sites of TCR-mediated signaling initiation. On this line, it is attractive to ponder that upon T cell activation, to promote the skipping of the exon that encodes the ligand-binding domain is a very enticing way of removing this affinity for the signaling mediators and thus removing much of the potential away from the sites of TCR-mediated signaling.

Splicing and polyadenylation are predominantly cotranscriptional events, allowing for crosstalk between these processes. In particular, the RNA pol II transcription elongation rate was demonstrated to affect alternative splicing (52), and we have previously shown the same effect in alternative polyadenylation (53). According to the RNA pol II kinetic coupling model that integrates alternative splicing with transcription kinetics, an increase of the RNA pol II elongation rate affects exon inclusion, depending on the strength of the splice sites (54).

Kadener et al. (38) showed that a compact chromatin structure of a replicated reporter plasmid acted as a barrier to RNA pol II elongation, leading to a higher exon inclusion. This effect was reverted by inhibition of HDAC, using TSA, promoting histone acetylation and a subsequent chromatin opening. Schor and collaborators (39) characterized a change in chromatin structure upon neuronal cell depolarization that induces the skipping of the alternative exon 18 of NCAM mRNA. These results led us to hypothesize that the chromatin structure could affect exon 5 alternative splicing and prompted us to investigate the H3K9 acetylation profile of the CD6 gene. Our results showed that T cell treatment with the HDAC inhibitor TSA promotes skipping of exon 5, mimicking the effect of T cell activation on CD6 alternative splicing. Conversely, when we decreased the RNA pol II elongation rate, we promoted inclusion of exon 5 (Fig. 3B).

Taken together, our results indicate that the chromatin structure alterations that occur upon T cell activation and an increase in the transcription rate of CD6 may favor exon 5 skipping from the mature mRNA. The fact that the most mature rat single-positive thymocytes, which have higher CD6 expression than double-negative thymocytes, present increased levels of exon 5 skipping seems to corroborate our interpretation that the levels and rate of transcription of the CD6 gene are coresponsible for the generation of an alternative transcript.

The selection of 5’ and 3’ splice sites in pre-mRNA is governed in part by RNA sequences, the “splicing code,” and RNA binding proteins (55–57). To investigate the importance of RNA motifs and the trans-acting factors in the regulation of CD6 alternative splicing, we used a minigene that mimics the endogenous CD6
CD6 ALTERNATIVE SPlicing REGULATION IN T CELL ACTIVATION

Using RIP, we detected a decrease of SRSF1 recruitment to the CD6 primary transcript in activated T cells that could be explained not only by a decrease of SRSF1 expression, but also by the effect of an increased chromatin acetylation level has in the recruitment of this splicing factor, as shown by others (39). Our ChIP experiments for H3K9ac revealed an increase of CD6 acetylation level upon T cell activation. Accordingly, when we treated cells with TSA, we observed less SRSF1 recruitment to the CD6 primary transcript. A similar effect was observed in neuroblastoma cells by Schor and collaborators (66), showing that chromatin acetylation upon cell membrane potential depolarization led to an accumulation of SRSF1 in the nuclear speckles, being less recruited to the primary transcripts compromising the splicing of nascent RNA. Although it was previously shown that TSA treatment did not affect the acetylation levels of T7-tagged SRSF1 transfected into HEK-293T cells (66), it is still possible that TSA causes SRSF1 acetylation, leading to a differential recruitment to CD6 pre-mRNA. In fact, acetylation of nonhistone proteins such as SRSF2 was already described and promotes its proteosomal degradation (67).

The working model we propose for the regulation of CD6 exon 5 alternative splicing upon T cell activation is depicted in Fig. 6. In resting conditions, T cells have a basal CD6 transcription level, with sufficient SRSF1 levels to bind to the intron 4 regulatory element. In these conditions, SRSF1 promotes the inclusion of exon 5 in the majority of mature mRNAs. Upon T cell activation, an increase of CD6 acetylation levels and a consequent chromatin structure modification facilitates CD6 transcription. Under these activation conditions, there is a deficient recruitment of SRSF1 to intron 4, which is necessary for exon 5 inclusion, leading to exon skipping and the production of CD6Δ exon 3. Taken together, we showed that the human T cell activation–induced alternative splicing of the CD6 gene is regulated at multifactorial levels, with chromatin structure, transcription rate, and splicing factor SRSF1 having fundamental roles. As CD6 has been associated with several autoimmune diseases, the molecular mechanisms regulating CD6 alternative splicing upon activation of human T cells provide new insight into a physiologically relevant molecule.

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Disclosures
The authors have no financial interests of conflict.

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Supplementary Figure 1 – T cell CD6 exon 5 alternative splicing pattern (a) Semi-quantitative RT-PCR analysis of CD6 exon 5 alternative splicing pattern in resting, PHA stimulated purified T cells activated with CD3+CD6 mAbs.
Supplementary Figure 2 – H3K36me3 and H3K9me3 CD6 occupancy (a), (b) Chromatin Immunoprecipitation (ChIP) for H3K36me3 and H3K9me3 marks along the CD6 gene, using resting and activated T cells.
Supplementary Figure 3 - Analysis of CD6 exon 5 alternative splicing using a minigene system. (a) Conservation analysis (comparative genomics tool, UCSC genome browser), of CD6 exon 5 genomic region revealed several peaks of conservation in intron 4. (b) The genomic region from exon 4 to exon 6 was cloned into a mammalian expressing vector carrying a CMV promoter and a SV40 poly A signal. (c) Peripheral blood mononuclear cells of healthy donors were transiently transfected with the CD6 minigene and the exon 5 alternative splicing pattern was analysed by RT-PCR and compared with the endogenous CD6 (left panel) and minigene transfected cells were PHA stimulated reproducing CD6 exon 5 activation induced alternative splicing. To distinguish the minigene transcripts from CD6 endogenous transcripts a plasmid-specific primer was used in the RT reaction.
**Supplementary Figure 4 – SRSF1, SRSF3 and hnRNPA1 overexpression and Knockdown efficiency** (a) Western blot gel showing T7 epitope tagged vectors pCGT7SF2, pCGT7SRp20 and pCGT7A1 expression in PBMCs 48h upon cells transfection in overexpression experiments (NT- non transfected cells) (b) SRSF1, hnRNP A1 and SRSF3 protein levels after siRNA transfection showing a knock down efficiency of 94%, 91% and 97% respectively 48h upon siRNA transfection (c) SRSF1, hnRNP A1 and SRSF3 mRNA levels, analysed by qPCR, 48h upon siRNA transfection.