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# Identification of a novel transcript variant of the human CD6 gene that lacks exon 9

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ABSTRACT

CD6 is a transmembrane glycoprotein, mainly expressed by all T cells and a subset of B cells. It has been shown that the human CD6 gene has six reported transcript isoforms, including full length and Δ3 isoforms. These are CD6a, CD6b, CD6c, CD6d, CD6e and CD6Δ3. In every isoform, particular exon(s) are known to be alternatively spliced out. In our findings, we observed a novel transcript isoform of CD6, where exon 9 is spliced out. Semi-qPCR and nucleotide sequencing confirmed the presence of a novel isoform of CD6 i.e. CD6f in human mononuclear cells. Quantitative expression showed significant high expression of CD6f over the full length transcript variant i.e. CD6a. Interestingly, their expressions get further increased upon polyclonal activation.

## 1. Introduction

CD6 is a cell surface glycoprotein that is involved in the development and activation of T cells (Gimferrer et al., 2004; Orta-Mascaró et al., 2016). CD6 is found to be involved in the formation of immunological synapse (IS) between T cells and macrophages (mφ), thus regulating T cell activation. CD6 gene contains 13 exons and first five exons encode the extracellular domains (d1, d2 and d3 domains). These extracellular domains of CD6 belong to Scavenger Receptor Cysteine Rich (SRCR) family and its d3 domain interacts with CD166, also known as activated leukocyte adhesion molecule (ALCAM) (Chappell et al., 2015). Exon 7 encodes the transmembrane domain and exons 8–13 encode cytoplasmic domain of CD6 protein. Different isoforms of CD6 have been observed in T cells derived from healthy individuals (Bowen et al., 1997; Kobarg et al., 1997). Importantly, these isoforms are also shown in mitogen-activated B cells from patients with chronic lymphocytic leukemia (CLL) (Bowen et al., 1997). Pre-mRNA splicing regulates the expression of these isoforms at the transcriptional level (da Glória et al., 2014). Although the possible role of different isoforms of CD6 in the formation of immunological synapse (IS) and T cell signaling is shown (Castro et al., 2007; Oliveira et al., 2012), but the functional significance of its different isoforms is poorly understood particularly in activation and proliferation of T cells.

Several isoforms of CD6 are reported, including an isoform (Δ3), where ligand binding domain (d3) is omitted. Five other isoforms are also reported in human i.e. CD6a, CD6b, CD6c, CD6d and CD6e. However, an isoform of CD6, where exon 9 is spliced out, has been reported in mouse (Bonet et al., 2013; Whitney et al., 1995). There is no such report available, which shows the presence of CD6f in T cells of human. In the present study, we identified a similar transcript isoform of human CD6 gene, where exon 9 is omitted and is a possible analog of mouse CD6f isoform. We measured the relative mRNA expression of the CD6f isoform in comparison to CD6a isoform in mononuclear cell from peripheral blood of healthy human samples. We also quantitatively measured its expression in mononuclear cells upon polyclonal activation.

## 2. Materials and methods

### 2.1. Recruitment of healthy subjects

Human peripheral blood mononuclear cells (PBMCs) were isolated from form healthy lab volunteers with no history of chronic illness in the past, any treatment with antibiotics / steroids in last three months. In total, 24 healthy subjects were included in the study (n = 24; Age, Mean ± S.D., 29.25 ± 2.21; M/F, 14/10). These were first informed about the study and then their consents were taken. Samples were collected in EDTA tube (BD Vacutainer™ K2 EDTA, Cat. No. 367856, Becton Dickinson, Franklin Lakes, NJ) from Institute Health Center, Motilal Nehru National Institute of Technology (MNNIT), Allahabad (U.P.), India. Approval from the Institute Ethics Committee was obtained (Ref. No.: MNNIT/IEC/2017-18/20).

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### 2.2. Reagents

RPMI medium (Cat. No. 31800-022, Gibco life technologies, Grand Island, NY 14072 USA with L-glutamine, without HEPES and $NaHCO_3$) were used as a nutrient medium. L-Glutamine (cat-TCL012, Himedia, Mumbai, India), HEPES (Cat No. MB016-25 G, Himedia, Mumbai, India) and NaHCO3 (Cat No. 14 4-5 5-8, Fisher scientific, Mumbai, India) were added in the media along with antibiotics (Pen-Strep-Ampho Sol™; Cat No. P4333-20ML Sigma-aldrich, St. Louis, USA). The medium was supplemented with 10% fetal calf serum (FCS) (Cat. No. 10270106, Gibco life technologies, Grand Island, NY 14072 USA) for proper maintenance of cells. Ficoll gradient solution was purchased for isolation of mononuclear cells (Cat. No. LS00 1-5 00MLHiSep™ LSM 1077Himedia, Nashik India). To perform polyclonal activation experiments, Phorbol 12-myristate 13-acetate (PMA) (Cat. No. P8139, Sigma-Aldrich, St. Louis, USA) and Ionomycin (Cat. No. I0634-1MG, Sigma-Aldrich, St. Louis, USA) were used in culture along with PBMCs. Trizol™ reagent was procured for isolation of total RNA (Cat. No. 9109, RNAiso Plus TakaRa Clontech, Kusatsu, Shiga Japan). Anti-human CD6 domain 1 (SRCR1; Clone: UMCD6/3F7B5, Cat. No. sc-65249, Santa cruz, biotechnology Inc, Texas, United States) and anti-human CD62 L (Clone: DREG-56, Cat. No. A16359, Invitrogen Waltham, MA, USA) antibodies were used to measure CD6 expression and activation of cells, respectively.

### 2.3. Isolation of mononuclear cells from peripheral blood

Mononuclear cells (MNCs) were isolated from peripheral blood (with anticoagulant i.e EDTA) using Ficoll hypaque density gradient centrifugation method. Briefly, the anticoagulated blood was first mixed with incomplete RPMI / PBS, then layered on the gradient solution and centrifuged at 2000 rpm for 20 min at RT. The interphase (buffy coat) was isolated and washed thrice with incomplete RPMI / PBS. Finally, the cells were suspended in complete RPMI (10% FCS). The viability of cells was checked by trypan blue dye exclusion test and was always found more than 95–98%.

### 2.4. Culture of mononuclear cells

For culture experiments, $2 \times 10^6$cells/ml mononuclear cells (MNCs) were cultured in 24 well culture plate (0.5 ml/ well, flat bottom plates, Cat. No. 702001, Nest biotechnology, Jiangsu, China) in presence of PMA (20 ng/ml, Sigma-Aldrich Co, St. Louis, USA) and Ionomycin (2 μm, Sigma-Aldrich Co, St. Louis, USA) for 12 h at 37 °C in $CO_2$ incubator (5%) at Central Facility, Department of Biotechnology, M.N.N.I.T. Allahabad. After incubation, cells were harvested and washed thrice.

### 2.5. Isolation of total RNA using Trizol Method and cDNA synthesis

Briefly, freshly isolated and/or cultured MNCs were subjected to a Trizol method for isolation of total RNA. Quality was assessed by running total RNA in 1% agrose gel electrophoresis as well as purity (260/280 ratio) was measured on a PicoGene™ spectrophotometer (Cat. No. GX-SX2-AG, Thermo Scientific, Massachusetts, USA). Contamination of genomic DNA was eliminated with DNase-I enzyme digestion following instruction according to manufacturer's with DNase-I (Cat. No. EN0521, Thermo Scientific, Massachusetts, USA). cDNA were prepared with Reverse Transcriptase reaction mixture, which includes 1 μg DNase-I treated RNA, both Random Hexamer (Cat. No. S0142, Thermo Scientific, Massachusetts USA) and OligodT primer (Cat. No. S0131, Thermo Scientific, Massachusetts, USA), Ribolock™ RNase Inhibitor (1 μl, Cat. No. -EO0381, Thermo Scientific, Massachusetts USA), 4 μl of MMLV Protoscript™ reaction buffer (5X), MMLV Protoscript™ Reverse Transcriptase (1 μl, Cat. No. M0368 L, New England BioLab, Ipswich, Massachusetts, US) and 1 μl of dNTP (10 mM, Cat. No. R0192, Thermo Scientific, Massachusetts USA). Nuclease free water was added to make final volume 20 μl. PCR program set for cDNA conversion included $25^0$ C for 5 min, $42^0$ C for 60 min and 72 for 10 min.

### 2.6. Real Time PCR (qPCR) to amplify human cd6a and cd6f mRNA

Quantitative PCR was performed in StepOneplus™ real time PCR machine (stepone Plus Applied biosystems, Marsiling, Singapore). The qPCR reaction mixture included 5 μl SYBR premix Ex Taq (Cat. No. RR420, TaKaRa, Mountain View, CA 94043USA), both forward and reverse primers (each 0.5μM), cDNA (diluted if required). Finally, nuclease free water was added to make up the volume of reaction (10 μl). In order to distinguish CD6a and CD6f isoform, separate overlapping/junctinal primers were designed (Eurofins Genomics India Pvt Ltd), In the case of CD6a isoform, forward and reverse primers were designed for the junctions of exon 4–5 and exon 8–9 respectively. Similarly, in case of CD6f isoform, forward and reverse primers were designed for the junctions of exon 8–10 and exon 11–12, respectively. U6 mRNA was taken as a house keeping control for calculation of fold change in the expression. The sequences of the primers are given here; for CD6a isoform: FP 5'-AACCTCTGCAGCCAGTC-3, RP5'-CAGCATGAAAACTTCT TTGG-3'; for CD6f isoform: FP 5'-ATCACCATCCCCAAAGAAGATTC-3', RP 5'-GTGCTGGAGCTGTCATCAG-3'; U6: FP 5'-GTGCTCGCTTCGGCAG CACATATAC-3', RP 5'-AAAAATATGGAACGCTTCACGAATTTG -3'. PCR conditions, including the annealing temperature (AT), were optimized by semi-Q PCR (AT: CD6a, 53°C; CD6f, 53.7°C; U6, 59°C). qPCR program PCR was conducted under the standard conditions [7 min at 95°C, (20 s at 95°C and 30 s at AT) x 40 cycles]. Differences in gene expression were calculated by the comparative Ct method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

### 2.7. Amplification and sequencing of CD6f isoform

As discussed above, a set of overlapping primer was used for the amplification of the CD6f isoform in semi-Q PCR. Reaction mixture included 2 μl of reaction buffer (10x), forward and reverse primers (0.5μM each), 5 μl dNTP (1.25 mM; Cat. No. R0192, Thermo Scientific, Massachusetts USA), 0.2 μl of Dream Taq™ DNA polymerase (Cat. No. EP0702, Thermo Scientific, Massachusetts USA) and 1 μl of cDNA. Nuclease free water was added to make up the total volume up to 20 μl. Semi-Q PCR was performed with a Prima-96 PCR machine (Himedia, India). Following PCR program was used for amplification of CD6f isoform: 5 min at 95 °C, (10 s at 95 °C, 30 s at 53.7 °C and 72 °C for 45 s) x 35 cycles, 10 min at 72 °C. Finally, PCR product was run in 2% agrose gel electrophoresis with 100bp DNA ladder (Cat. No. SM0241, ThermoScientific, Massachusetts, USA) and observed in the gel documentation system (UVITEC, Cambridge). The resulting PCR product was subjected to purification and sequencing (Chromous Biotech Pvt. Ltd, India).

### 2.8. Surface staining of cells

Resting and activated cells were harvested and washed with PBS (2x). Cells were first incubated with monoclonal CD6 primary antibody (CD6d1/SRCR1; Clone: UMCD6/3F7B5) in staining buffer (PBS + BSA + Azide) for 20 min on ice. Then after, cells were washed twice with PBS and were incubated with FITC conjugated secondary antibody and PECy7 anti- human CD62 L antibody for 20 min on ice. After washing twice with PBS, cells were fixed in 2% formalin, then resuspended in staining buffer.

### 2.9. Flow cytometer and data analysis

Stained samples were acquired in four color flow cytometer (Beckton Dickinson, C6 Accuri). Samples were acquired immediately after staining. However, it can be kept at 4 °C in the dark till the

acquisition is performed within 24 h of sample preparation (17). Acquired data were analyzed on Flowjo 10.1.1. software. During analysis of data, all the gates were applied using fluorescence minus one (FMO) control (Supplementary information, Fig. S2). Briefly, scatter gated lymphocytes were analyzed for the expression of CD6d1 (FITC) and CD62L (PECy7) in bivariate dot plot. Overlaid histograms are preferred to demonstrate the level of expressions of CD6d1 in resting and activated samples.

### 2.10. Statistical analysis

All the data are presented in mean with standard deviation, as these are normal continuous variables. The comparison of all continuous variables between the studied group *t*-test is used. P-value less than 5% level of significance have been regarded as significant results. The analysis is done using SPSS 15.0. Graphs and figures were made in GraphPad Prism 5 version.

## 3. Results

### 3.1. A new isoform of CD6 in healthy human subjects, where exon 9 is spliced out

Besides the full length isoform of CD6 i.e. CD6a, other isoforms of CD6 (CD6b, CD6c, CD6d and CD6e) is characterized by variable length of cytoplasmic domain, which is generated by alternative splicing of different exons (from exon 7–12, Fig. 1A). In order to check the possibility of CD6f (Δexon9) in human T cells, mononuclear cells (MNCs) were isolated and checked for the expression of CD6f transcript variant (Δexon9) using semi-qPCR (CD6a and CD6f, Fig. 1B and C respectively) as well as qPCR (Fig. 1D). Finding shows presence of CD6f transcriptional variants in human T cells from healthy subjects (n = 15, Fig. 1C and D). More importantly, CD6f transcript isoform was expressed more abundantly than the full length CD6a variant (n = 15, p < 0.014, unpaired t test).

### 3.2. Nucleotide sequencing of PCR product confirms the splicing of exon 9 and thus the presence of CD6f in human mononuclear cells

The semi-qPCR with primer pair specific to possible combination of exons in CD6f isoform was performed (Fig. 2A) and checked for the expression (Fig. 1C). After the nucleotide sequencing of the PCR product (Chromous Biotech Pvt. Ltd, India), four color chromatograms shows the result of sequencing run (Fig. 2A). The four color chromatograms show the sequences of junctions of exon 8 and 10 (forward end) and exon 11–12 (reverse end), respectively. The constructed complete sequence of the PCR product (from forward and reverse ends) is shown here with (Fig. 2B). Semi-qPCR and nucleotide sequencing confirmed the presence of the novel isoform of CD6 in human MNCs. The sequences of the PCR product from both the ends are given in the supplementary information (Fig. S1).

### 3.3. Expression of CD6f increases after polyclonal activation

We analyzed the expression of the human CD6f isoform (Δexon9) upon polyclonal activation of mononuclear cells. The activation of T cells was confirmed by the expression of IL-2 in resting and activated samples by qPCR (Fig. 3B, n = 9, $p < 0.002$, paired t test). qPCR analysis of CD6f isoform showed significantly higher expression in activated samples (Mean ± S.D., 52.42 ± 41.77) as compared to resting samples (Fig. 3A; n = 9; Mean ± S.D., 4.37 ± 4.85, $p < 0.029$, paired t test). Though, the relative analysis of CD6a also showed increased expression upon activation, but the levels of expression of CD6f in resting as well as activated samples were significantly higher than that of CD6a (Fig. 3A; n = 9).

### 3.4. Polyclonal activation increases the expression of CD6 on T cells

Firstly, the activation of MNCs were confirmed by the down regulation of CD62L using flowcytometry experiments (Fig. 4A, B, E and F). Finding showed high expression of CD6 in lymphocytes upon activation (n = 5; Mean ± S.D., 2252.20 ± 1501.53) as compared to resting lymphocytes (Mean ± SD., 1452.40 ± 713.60; $p = 0.062$) after 6 h polyclonal stimulation. Upon 12 h of polyclonal activation, it

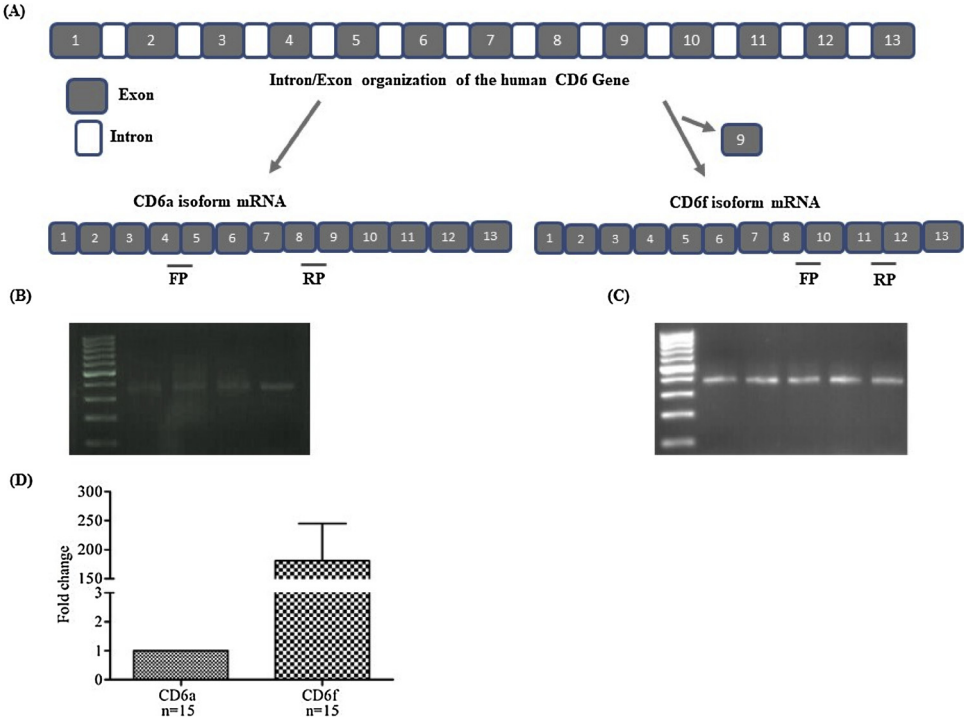


**Fig. 1.** New isoform of CD6 in healthy human subjects. (A) Schematic layout of the intron/exon organization of human CD6 gene is shown. Splicing out of exon 9 ends up with a new combination of exons in the CD6 mRNA i.e. CD6f. Agarose gel image of semi-qPCR with two primer pairs to check the expression of (B) CD6a and (C) CD6f transcripts in human peripheral blood mononuclear cells (MNCs). In first set of primers for CD6a, the forward and reverse primers are directed at the junction of exon 4–5 and exon 8–9 respectively. The junctions of exon 8–10 and exon 11–12, as shown in the schematic layout, are the sites, where forward and reverse primers bind to specifically amplify the CD6f transcript isoform. (D) Bar diagram shows the fold change in expression ($2^{-\Delta\Delta Ct}$) of the CD6f transcript variant in MNCs compared to the full length transcript variant of CD6 i.e. CD6a (p < 0.014, unpaired t test). Data is represented as mean ± S.D and significance is shown as the p value (< 0.05).

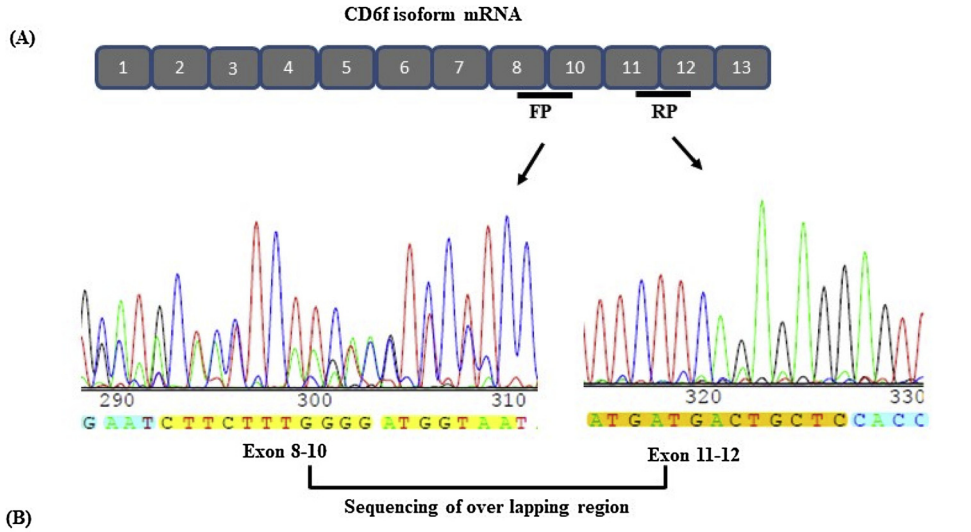


**Fig. 2.** Nucleotide sequencing of PCR product confirms the splicing of exon 9. (A) The forward and reverse primers are directed at the junction of exon 8–10 and exon 11–12 respectively. Using this primer set, the amplified semi-qPCR product was sequenced commercially. The four color chromatogram of the forward end and reverse end are shown. The junctions are highlighted in two colors. (B) The constructed nucleotide sequence of forward and reverse ends of amplified PCR product of CD6f is shown.

was interesting to note the down regulation of CD6d1 domain in activated MNCs (Fig. 4H, Mean ± S.D., 1190.00 ± 300.50) as compared to resting cells (Mean ± S.D., 2618.00 ± 801.05; $p = 0.044$). This must be noted that anti-CD6 antibody is directed against the d1 domain of CD6 protein and detects all isoforms, as d1 domain is found in all of them.

### 3.5. Discussion

Several genes in human generate multiple mRNA transcripts arising from alternative splicing, diverse promotor selection and different polyadenylation (Johnson et al., 2003). mRNA transcript generated by these processes encode different protein isoforms, which are assigned for distinct biological function(s). Human proteome is large and diverse due to protein isoforms, but the function(s) of these isoforms have not been yet deciphered (Uhlén et al., 2015). Importantly, different patterns of splicing and thus their resultant transcript variants are critical to regulation as well as the function of the respective cells. Similarly, human CD6 gene generates different mRNA transcripts by alternative splicing, which splice out different exons in combinations and form several isoforms of the same protein. CD6 gene has been reported to encode six isoforms at mRNA and protein levels, but only few of them are established in terms of their functional significance in the immune system (Bowen et al., 1997). It is important to identify these isoforms and understand their functions in development of the immune system.

CD6 gene is primarily transcribed in T cells and also in a subset of B cells. Several mRNAs transcripts have already been identified in CD6 gene. The extracellular and intracellular domains of CD6 are encoded by exon 1–5 and exon 7–13, respectively. Exon 7 encodes for transmembrane region. Besides full length variant (i.e. CD6a), CD6Δ3 transcript variant (i.e. Δexon5), where extracellular domain d3 is omitted, is well documented. Cytoplasmic domain of full length CD6a is encoded by all seven exons (7–13). At least four isoforms of CD6 are identified on the basis of missing exons from 7 to 13 in different combinations. Among these four transcript variants, CD6b is missing exon 8 (Δexon8), CD6c is missing exon 8 and 9 (Δexon8–9), CD6d is missing exon 8 and exon 12 (Δexon 8 & 12) and CD6e isoform are missing exon 9 and exon 12 (Δexon9&12) (Bowen et al., 1997). Our finding is the first report,

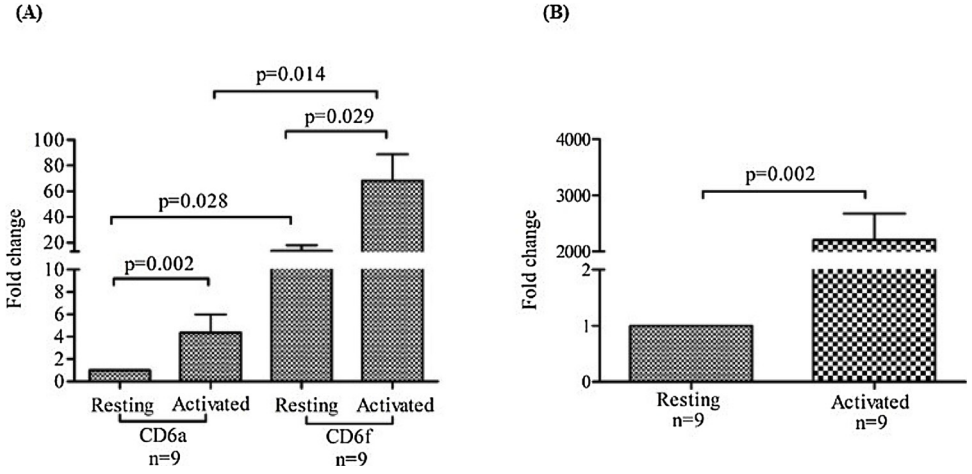


**Fig. 3.** Increased expression of CD6f upon polyclonal activation (A) Bar diagram shows the fold change in expression ($2^{-\Delta\Delta Ct}$) of CD6a (full length) and CD6f transcript variants in resting as well as polyclonal activated cells derived from human peripheral blood mononuclear cells (MNCs, n = 9, p = 0.002 and p = 0.029 all paired t test; p = 0.028 and p = 0.014 all unpaired t test). (B) Relative increase in expression of IL-2 confirms the activation of cells upon polyclonal activation (n = 9, p = 0.002 paired t test). Data is represented as mean ± S.D and significance is shown as the p value.

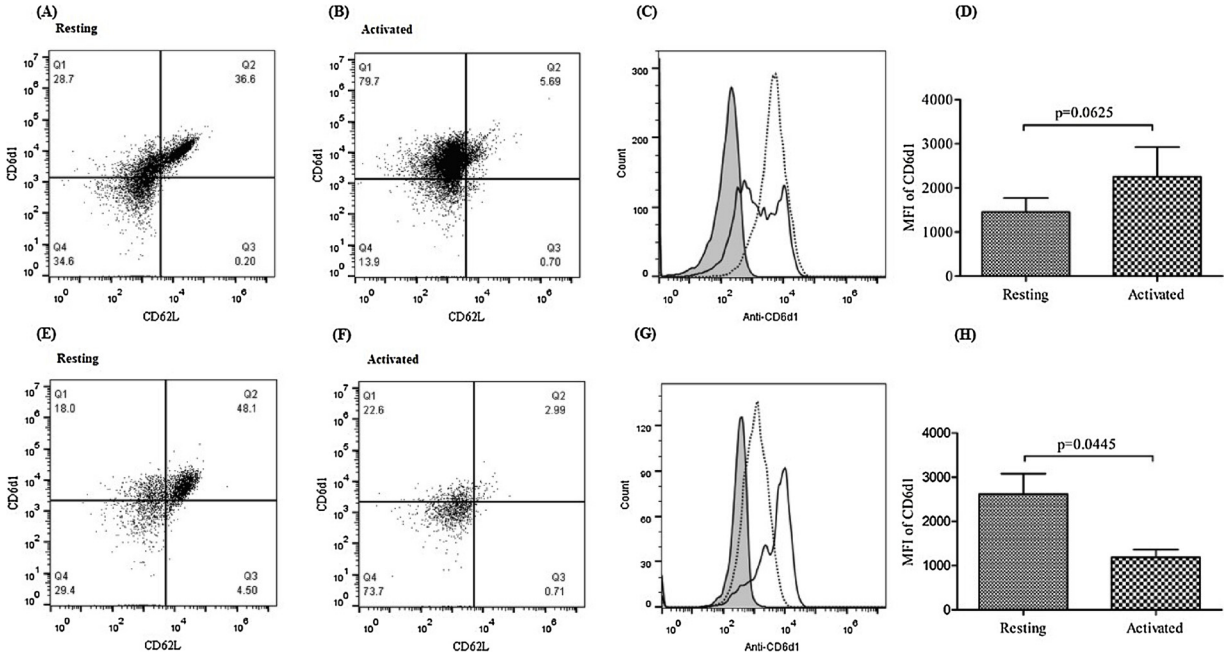


**Fig. 4.** Expression of CD6 protein upon polyclonal activation: Flowcytometry plot shows expression of CD6d1 (*y axis*) and CD62L (*x axis*) in (A) resting as well as (B) activated mononuclear cells (MNCs) after 6 h of polyclonal stimulation with PMA and ionomycin. (C) Overlaid histogram shows the expression of CD6d1 in the unstained (*filled*), resting (*solid line*) and activated (*dotted line*) MNCs after 6 h of polyclonal stimulation. (D) Bar diagram shows the MFI of CD6d1 expressing cells in resting and activated MNCs after 6 h of polyclonal activation ($p = 0.0625$, *Wilcoxon Signed Ranked test*). Similar to panel A–B, (E) resting and (F) activated MNCs are shown for their expression of CD6d1 and CD62L after 12 h of polyclonal stimulation.(G) Overlaid histogram shows the expression of CD6d1 in the unstained (*filled*), resting (*solid line*) and activated (*dotted line*) MNCs after 12 h of polyclonal stimulation. (H) Bar diagram shows the MFI of CD6d1 expressing cells in resting and activated MNCs after 12 h of polyclonal activation ($p = 0.0445$, *Wilcoxon Signed Rank test*). Data is shown as mean ± S.D. and significance is considered if $p$ value is less than 0.05. MFI, median fluorescence intensity.

which shows the omission of exon 9. This has been confirmed by the nucleotide sequencing as well. This newly identified mRNA transcript of human CD6, as earlier reported in mice, is hereby designated as a CD6f transcript (and or Δexon9) variant (Robinson et al., 1995a,b).

We also showed that the relative mRNA expression of CD6f isoform is significantly higher in both resting as well as polyclonal activated MNCs (Fig. 3). Interestingly, we failed to observe an increase in the expression of CD6 protein using flowcytometry at 12 h, contrary to what we observed in the case of mRNA expression. To corroborate our observation, we estimated its protein level expression at 6 h of polyclonal activation and interestingly observed increased expression. Increased expression of CD6 protein followed by its disappearance from the surface at 12 h, particularly when mRNA expressions of CD6f and CD6a were heightened, indicates the possible shedding of CD6 protein in the culture milieu. Though we have not checked in our study; but it has already been documented in literature (Carrasco et al., 2017). However, the mechanism of shedding and its importance is not yet worked out. Furthermore, we have observed increased mRNA expression of other isoforms (CD6b, CD6c, CD6d, and CD6e) in MNCs upon polyclonal activation at 12 h (*unpublished observation*). This should also be noted that we have used monoclonal antibody to d1 domain of CD6 for flowcytometry purpose and all isoform of CD6 retains d1 domain (clone: UMCD6/3F7B5). Thus, these isoforms cannot be discriminated using this clone of antibody, which binds to d1 domain. The other clone of antibody which binds to extracellular d3 domain can discriminate only Δ3 variant from the rest of the isoform (*d1 minus d3*; clone: OX126). Since it stains all other isoforms except Δ3, thus it cannot be used for flowcytometry experiment to selectively distinguish other isoforms. Moreover, unlike Δ3 isoform, splicing of various exons in other isoforms results in omission of different peptide segments in their cytoplasmic tail and these isoforms cannot be stained by the available two clones of antibodies.

The downstream signaling pathway of CD6 is not very clear. CD6 has a long cytoplasmic tail and it is devoid of intrinsic catalytic activity, but contains several signal-transducing consensus motifs (Robinson et al., 1995a,b). According to available literature, it possesses two proline-rich motifs containing SH3 domain-binding consensus sequence, three serine/Thr-rich motifs, three protein kinase C (PKC) phosphorylation-site motifs, 10 casein kinase 2 (CK2) phosphorylation site motif and nine Tyr residues (Robinson et al., 1995a,b). This cytoplasmic tail is known to be constitutively phosphorylated (Cardenas et al., 1990), and become hyper phosphorylated on ser and Tyr residues upon T cell activation (Cardenas et al., 1990; Wee et al., 1993). Except Δ3 isoform of CD6, other isoforms's including CD6f show variation in their cytoplasmic regions, as the splicing occurs between exon 8 to exon 13 (all encode for cytoplasmic domain). The cytoplasmic domain of CD6f which lack region encoded by exon 9, shows following missing residues i.e. tyrosine 486/489/503, serine 478/480/482/484/494 and threonine 500/501. Serine residue cluster (480/482/484), which is missing in CD6f isoform, is embedded into a consensus site for CK2. This CK2 site contributes to the constitutive phosphorylation of CD6 and critically important for ligand induced CD6 mediated MAPK activation (Bonet et al., 2013). Therefore, the proportional dominance of CD6f over CD6a in resting as well as activated cells highlights an important regulatory mechanism of T cell activation, which needs to be understood.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.06.004.

## References

Bonet, L., Farnós, M., Martínez-Florensa, M., Martínez, V.G., Lozano, F., 2013. Identification of functionally relevant phoshorylatable serine clusters in the cytoplasmic region of the human CD6 lymphocyte surface receptor. FEBS Lett. 587, 2205.

Bowen, M.A., Whitney, G.S., Neubauer, M., Starling, G.C., Palmer, D., Zhang, J., Nowak, N.J., Shows, T.B., Aruffo, A., 1997. Structure and chromosomal location of the human CD6 gene: detection of five human CD6 isoforms. J. Immunol. 158, 1149.

Cardenas, L., Carrera, A., Yague, E., Pulido, R., Sanchez-Madrid, F., De Landazuri, M., 1990. Phosphorylation-dephosphorylation of the CD6 glycoprotein renders two isoforms of 130 and 105 kilodaltons. Effect of serum and protein kinase C activators. J. Immunol. 145, 1450.

Carrasco, E., Escoda-Ferran, C., Climent, N., Miró-Julià, C., Simões, I.T., Martínez-Florensa, M., Sarukhan, A., Carreras, E., Lozano, F., 2017. Human cD6 Down-Modulation following T-cell activation compromises lymphocyte survival and Proliferative responses. Front. Immunol. 8, 769.

Castro, M.A., Oliveira, M.I., Nunes, R.J., Fabre, S., Barbosa, R., Peixoto, A., Brown, M.H., Parnes, J.R., Bismuth, G., Moreira, A., 2007. Extracellular isoforms of CD6 generated by alternative splicing regulate targeting of CD6 to the immunological synapse. J. Immunol. 178, 4351.

Chappell, P.E., Garner, L.I., Yan, J., Metcalfe, C., Hatherley, D., Johnson, S., Robinson, C.V., Lea, S.M., Brown, M.H., 2015. Structures of CD6 and its ligand CD166 give insight into their interaction. Structure 23, 1426.

da Glória, V.G., de Araújo, M.M., Santos, A.M., Leal, R., de Almeida, S.F., Carmo, A.M., Moreira, A., 2014. T cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1. J. Immunol. 193, 391.

Gimferrer, I., Calvo, M., Mittelbrunn, M., Farnós, M., Sarrias, M.R., Enrich, C., Vives, J., Sánchez-Madrid, F., Lozano, F., 2004. Relevance of CD6-mediated interactions in T cell activation and proliferation. J. Immunol. 173, 2262.

Johnson, J.M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P.M., Armour, C.D., Santos, R., Schadt, E.E., Stoughton, R., Shoemaker, D.D., 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science 302, 2141.

Kobarg, J., Whitney, G.S., Palmer, D., Aruffo, A., Bowen, M.A., 1997. Analysis of the tyrosine phosphorylation and calcium fluxing of human CD6 isoforms with different cytoplasmatic domains. Eur. J. Immunol. 27, 2971.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402.

Oliveira, M.I., Gonçalves, C.M., Pinto, M., Fabre, S., Santos, A.M., Lee, S.F., Castro, M.A., Nunes, R.J., Barbosa, R.R., Parnes, J.R., 2012. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. Eur. J. Immunol. 42, 195.

Orta-Mascaró, M., Consuegra-Fernández, M., Carreras, E., Roncagalli, R., Carreras-Sureda, A., Alvarez, P., Girard, L., Simões, I., Martínez-Florensa, M., Aranda, F., 2016. CD6 modulates thymocyte selection and peripheral T cell homeostasis. J. Exp. Med. 213.

Robinson, W.H., Prohaska, S.S., Santoro, J.C., Robinson, H.L., Parnes, J.R., 1995a. Identification of a mouse protein homologous to the human CD6 T cell surface protein and sequence of the corresponding cDNA. J. Immunol. 155, 4739.

Robinson, W.H., de Vegvar, H.E.N., Prohaska, S.S., Rhee, J.W., Parnes, J.R., 1995b. Human CD6 possesses a large, alternatively spliced cytoplasmic domain. Eur. J. Immunol. 25, 2765.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C T method. Nat. Protoc. 3, 1101.

Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., 2015. Tissue-based map of the human proteome. Science 347, 394.

Wee, S., Schieven, G.L., Kirihara, J.M., Tsu, T.T., Ledbetter, J.A., Aruffo, A., 1993. Tyrosine phosphorylation of CD6 by stimulation of CD3: augmentation by the CD4 and CD2 coreceptors. J. Exp. Med. 177, 219.

Whitney, G., Bowen, M., Neubauer, M., Aruffo, A., 1995. Cloning and characterization of murine CD6. Mol. Immunol. 32, 89.