CD5 expression promotes IL-10 production through activating MAPK/Erk pathway and upregulating TRPC1 channels in B lymphocytes

Soizic Garaud[1]*, Taher E Taher[2]*, Marjolaine Debant[1], Miguel Burgos[3], Sarra Melayah[1], Christian Berthou[1] , Kaushal Parikh[4], Jacques-Olivier Pers[1], Damien Luque-Paz[1,5], Gilles Chiocchia[6], Maikel Peppelenbosch[7], David A Isenberg[8], Pierre Youinou[1], Olivier Mignen[3], Yves Renaudineau[1,5$], Rizgar A Mageed[2$¶]

[1]INSERM ERI29/EA2216, réseau epigenetique and réseau canaux ioniques du Cancéropôle Grand Ouest, Brest University Medical School, France; [2]Centre for Experimental Medicine and Rheumatology, Queen Mary University of London, UK; [3]Canalopathy and Calcium signaling, INSERM UMR1078, Brest, France; [4]Centre for Experimental and Molecular Medicine, Academic Medical Center, Meibergdreef 9, The Netherlands; [5]Laboratory of Immunology and Immunotherapy, CHU Morvan, Brest, France; [6]Institut Cochin, INSERM U567, Paris, France; [7]Department of Gastroenterology and Hepatology, Erasmus MC, Gravendijkwal 230, Rotterdam, The Netherlands; [8]Centre for Rheumatology, University College London, UK

Running Title: CD5 promotes MAPK/Erk activation and IL-10 production in B cells

Key words: CD5, B cells, MAPK/Erk, calcium, TRPC1.

Footenotes: *S Garaud and TE Taher contributed equally to this study. [$]Y Renaudineau and RA Mageed contributed equally as senior authors to this study.

Correspondence:

Professor R. A. Mageed,
Centre for Experimental Medicine and Rheumatology,
William Harvey Research Institute,
Barts and the London School of medicine and Dentistry,
Queen Mary University of London,
Charterhouse Square, London EC1M 6BQ, UK
E-mail: r.a.mageed@qmul.ac.uk
Tel. No. +44-20-78823989
Fax No.: +44-20-78826121

Conflict of interest: The authors declare no conflict of interest.

Abbreviations: Ab: antibody; BCR: B cell receptor; BTK: Bruton's tyrosine kinase; Erk: extracellular signal-regulated kinases; E1A; exon 1A of CD5; E1B: exon 1B of CD5; PLCγ: phospholipase C gamma; SH2: Src homology 2; SHIP: SH2-containing inositol phosphatase; SHP1: SH2 domain-containing protein tyrosine phosphatase-1 Syk: spleen tyrosine kinase; Y: tyrosine.

ABSTRACT

CD5 is constitutively expressed on T cells and a subset of mature normal and leukemic B cells in patients with chronic lymphocytic leukaemia (CLL). Important functional properties are associated with CD5 expression in B cells, including STAT3 activation, IL-10 production and the promotion of B lymphocyte survival and transformation. However, the pathway(s) through which CD5 influences the biology of B cells and its dependence on B cell receptor (BCR) co-signaling remain unknown. In this study we show that CD5 expression activates a number of important signaling pathways including Erk1/2 leading to IL-10 production through a novel pathway independent of BCR engagement. This pathway is dependent on extracellular calcium (Ca^{2+}) entry facilitated by upregulation of the transient receptor potential channel 1 (TRPC1) protein. We also show that Erk1/2 activation in a sub-group of CLL patients is associated with TRPC1 overexpression. In this subgroup of CLL patients, small inhibitory RNA (siRNA) for CD5 reduces TRPC1 expression. Furthermore, siRNAs for CD5 or for TRPC1 inhibit IL-10 production. The findings provide new insights into the role of CD5 in B cell biology in health and disease and could pave the way for new treatment strategies for treating patients with B-CLL.

INTRODUCTION

CD5 is expressed on T cells and a sub-population of B cells, B1 cells.[1] B1 cells make up a large subset of B lineage cells during early life but their frequency among all B cells declines with age.[2] The B1 subset plays important roles in the immune system; produces natural antibodies and contributes to innate immunity. However, these cells can also give rise to leukemic B cells in patients with chronic lymphocytic leukaemia (CLL).[3] The involvement of CD5 in the pathophysiology of B-CLL remains to be conclusively established but there is evidence that CD5 is involved in B-CLL development, at least through IL-10 production.[4] Thus, $CD5^+$ B cells produce IL-10 and are the main B cell source of the cytokine.[5] This ability is relevant to B-CLL pathophysiology since IL-10 acts as a growth factor for B cells owing to its stimulatory[6,7] and anti-apoptotic properties.[8] Furthermore, IL-10 production is associated with the outcome of CLL[9] and with a malignant genotype.[10]

Recently, we revealed that CD5 induces IL-10 production by activating the signal transducer and activator of transcription 3 (STAT3) and nuclear factor of activated T cells 2 (NFAT2) in a subset of B-CLL cells.[11,12] Interestingly, the activation of these transcription factors influences disease progression in patients with B-CLL.[13,14]

CD5 is a member of the conserved scavenger receptor cysteine-rich (SRCR) superfamily.[15] It has a cytoplasmic tail with no enzymatic activity but has a conserved motif with a threonine and 4 tyrosine residues. Two tyrosines (Y429 and Y441) serve as docking sites for phosphorylated Src homology 2 (SH2) domain containing proteins.[16] In T and B lymphocytes, CD5 associates with Src kinases, e.g. Lyn, which phosphorylates the SH2 domain of CD5 creating docking sites for Lck, Zap70, PI3K, c-Cbl and the SH2/SH3 RasGap.[17,18] In contrast, the phosphatase SHP1 binds CD5 on Y378.[19] In the yeast two-hybrid system, CD5 associates with CAM kinase IIδ and casein kinase II (CK2) that phosphorylate CD5 serine 459 (Ser^{459}) and serine 461 (Ser^{461}).[20,21] $CD5^+$ B lymphocytes exhibit delayed JNK activation and lack the

ability to induce p38 MAPK and NF-κB activation upon BCR cross-linking although Erk1/2 and NFAT2 are constitutively active.[22] Furthermore, CD5 reduces intracellular Ca^{2+} mobilization upon BCR engagement.[23] Based on these findings, CD5 has been implicated in B lymphocyte tolerance and leukemic transformation.[24]

In this study, we report changes in multiple intracellular signaling pathways resulting from CD5 expression. Thus, CD5 promotes constitutive MAPK activation through a Ca^{2+}-dependent pathway leading to Erk1/2 phosphorylation (pErk1/2) and IL-10 production. This IL-10 production is independent of BCR engagement but is associated with the expression of a non-selective channel permeable to Ca^{2+}, transient receptor potential channel 1 (TRPC1). In addition, CD5 promotes the activation of the PI3K/Akt/mTOR pathway which has important roles in B cell survival and proliferation. These effects occur through the ability of CD5 to activate a range of key kinases.[25] Furthermore, we show that in pErk1/2 positive CLL B cells, siRNA to CD5 suppresses TRPC1 expression while siRNAs for CD5 or TRPC1 inhibit IL-10 production.

MATERIALS AND METHODS

Patients

Twenty six patients who fulfilled the criteria for CLL[26] were recruited at the Centre of Ressources Biologiques (CRB)-santé in Brest (Table 1). Disease assessment included Binet stage determination, progression free survival (PFS), CD38 expression, cytogenetic abnormalities and lymphocyte counts. Informed consent was obtained from all patients and the Ethical committee at Brest University Medical School Hospital approved the study. B cells were enriched to >96% using an enrichment kit (StemCell Technologies).

Cell culture

The CD5-negative hairy B cell leukemia cell line Jok-1[27], which has phenotypic characteristics of B CLL cells[28], was transfected with cDNA for either the membrane isoform of CD5, E1A (Jok-E1A) or the cytoplasmic E1B isoform (Jok-E1B).[11] Cells were maintained in RPMI-1640 containing 10% fetal calf serum (FCS), antibiotics and 0.5mg/ml G418 (Sigma-Aldrich). For activation, 10^6 cells/mL were stimulated with 10µg/mL goat F(ab')2 anti-human IgM coated onto Sepharose beads (Bio-Rad). For inhibition experiments, 10^6 cells/ml were incubated for 48h with 50-100µM PD98059 (inhibits Mek1; Calbiochem), 100µM lanthanum (La^{3+}; blocks extracellular Ca^{2+} entry; Sigma-Aldrich) or 50µM LY294002 (inhibits PI3K; Sigma-Aldrich), and 10ng/mL rapamycin (inhibits mTOR; Pfizer, NY). The level of IL-10 in culture supernatants was quantified by ELISA (BD OptiEIA™).

Antibodies

Antibodies (Abs) to Erk1/2, phosphorylated-Erk1/2 (pErk1/2), Syk/pSyk, Btk/pBtk, PLCγ2/pPLCγ2, SHP1/pSHP1, SHIP/pSHIP were from Insight Biotechnology. Abs to Lyn, c-Cbl, Vav1, CD79a, S6K/pS6K T389, STAT3/pSTAT3 S727, STAT1/pSTAT1 S727, Akt/pAkt S473 were from Abcam. The anti-CD5 clone UCHT2, the rabbit anti-extracellular TRPC1, and the mouse anti-β-actin Abs were from BD Biosciences, and Sigma-Aldrich, respectively.

Western blotting and immunoprecipitation

Cell lysates in 1% NP-40 buffer (1% NP-40, 150mM NaCl, 2mM EDTA, 10mM Tris-HCl pH 7.4, 5mM NAF) containing protease/phosphatase inhibitors were separated on 10% SDS-PAGE, blotted onto PVDF membranes, probed with Abs and revealed with HRP-conjugated secondary Abs and enhanced chemiluminescence (ECL) (Amersham-Pharmacia). For immunoprecipitation, lysates were cleared by centrifugation and carried out with Abs coupled to protein G-Sepharose, washed and analysed by Western blotting.

Flow cytometry

Expression of TRPC1 was detected with specific rabbit Ab followed by FITC-conjugated goat $(Fab')_2$ anti-rabbit IgG (Immunoresearch). Data were acquired and analysed using the FC500 flow cytometer (Beckman-Coulter) relative to staining with the isotype control. Results were expressed as mean fluorescence intensity (MFI).

Intracellular calcium (iCa^{2+}) level measurement

Imaging was used to monitor iCa^{2+} mobilization in B cells loaded for 30min at 37°C with 2µM Fura-2/AM. B cells in 6 independent experiments were washed and attached onto cell-Taq pre-coated coverslips. Fura-2-fluorescence was excited sequentially at 340 and 380nm, emission recorded at 520nm and excitation/emission ratios calculated. Extracellular Ca^{2+} depletion was used to measure iCa^{2+} release. Repletion at 1.8mM Ca^{2+} was used to determine Ca^{2+} entry and subsequent addition of 100µM La^{3+} used to block entry. In selected experiments, ratios were normalized to basal values (F0) at the beginning of each experiment and provided as ($\Delta F/F0$).

Transfection with small interfering RNA (siRNA)

10^6 cells were transfected with siRNA at 3pM using a B cell nucleofector transfection kit (Lonza). Small interfering RNAs (siRNAs) to CD5 RNA plus control siRNA were purchased from Ambion (Life Technologies). The siRNAs to TRPC1 (3'-GCAUCGUAUUUCACAUU CU-3'; 5'-UGAGCCUCUUGACAAACGA-3') were obtained from Eurogentec.

Kinome array analysis

The kinome array (Pepscan Systems) was performed as described.[25] Briefly, 10^6 Jok-1, Jok-E1A or Jok-E1B cells were lysed in 50µL lysis buffer and analysed. The array has 1024 peptides representing phosphorylation sites in protein substrates of all known kinases spotted onto glass slides. Ten µL of the peptide array in incubation mix (50% glycerol, 50µM ATP, 0.05% v/v Brij-35, 0.25mg/ml BSA, [^{33}P] ATP (1MBq)) was added to the lysates, loaded onto the chips and allowed to phosphorylate for 90min at 37°C. Washed and dried slides were exposed to a phosphor-imager for 72h and data acquired (Storm™, Amersham-Biosciences). The level of incorporated radioactivity, which corresponds to the level of phosphorylation was quantified by array software Scanalyze (Eisen Software). Differential kinase activation in Jok-E1A and Jok-E1B cells were quantified as significant fold changes in the ratio of phosphorylated peptides compared with un-transfected Jok-1 cells. All analyses were carried out in triplicates and repeated on two separate occasions.

Construction of CD5 mutants

Two deletion mutants, S398M^{start} and S415M^{start} and 3 proteins with amino acid replacements were generated. Both deletion mutants had the extracellular domains and transmembrane (Tm) regions deleted. The SHP-1 and the CaM-binding motifs were removed from the S398M^{start} mutant plus the first CK2 motif from the S415M^{start} mutant. These mutants were generated by PCR using ATG-containing sense/anti-sense primers, cloned into the pDNR-dual and subcloned into the pLPcmv vector using the Cre-recombinase system (BD-Biosciences). The E1A-CD5 cDNA was mutated at 3 amino acid positions using the Quick-Change Site-Directed Mutagenesis kit (Agilent Technologies). Point mutations were induced into the serine phosphorylation sites (^{422}AS^{423} → ^{422}VD^{423}; ^{428}EYS^{430} → ^{428}AAA^{430}; ^{459}SDS^{461} → ^{459}VDG^{461}). All constructs were validated by sequencing.

cDNA microarray

This was performed according to Agilent Technologies' instructions as described.[29] Thirteen µg mRNA were reverse-transcribed and fluorescence-labelled using cyanine 3-CTP-RNA Quick Amplification kit. Labelled cDNAs were hybridized to the Agilent Whole Human

Genome Oligo Microarray (4x44k). Each sample was hybridized with 3 arrays in biological replicates and slides washed, dried and the fluorescence quantified using a scanner (Agilent-G2565AA). The signals were analysed after subtracting background outliers using the Feature Extraction Software. Signal values were calculated as the ratio between the intensity of signals from the Jok-E1A or Jok-E1B cells to Jok-1 cells. The data can be viewed in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database (accession number GSE50714). Data normalization, quality control and probe list processing were all carried out with GeneSpring GX using the feature extractor plug-in as.[29]

RT-PCR and quantitative RT-PCR

RNA was extracted using the RNeasy kit (Qiagen) and reverse-transcribed using oligo-dT. RT-PCR was used for *CD5* (sense: 5'-TCGGACGGCTCAGCTGGTATGAC-3'; antisense: 5'-TGC CATCCGTCCTTGAGGTAGAC-3'); *TRPV2* (sense: 5'-TCACCGCTGTTGCCTACCATCA 3'; antisense: 5'-AGGGCTACAGCGAAGCCGAAAA-3'); *TRPC1* (sense: 5'-ACCTTCCATT CGTTCATTGG-3'; antisense: 5'-TGGTGAGGGAATGATGTTGA-3', and GAPDH (sense: 5'-TGCACCACCAACTGCTTAGC-3', antisense: 5'-GGCATGGACTGTGGTCATG AG-3'). Amplification used 150ng cDNA, 20ng genomic DNA, 200nM primers and 2.5unit Taq polymerase (Thermo-Fisher Scientific). The protocol consisted of denaturation at 94°C for 5min, 40 cycles of 94°C for 40s, 60°C for 40s and extension at 72°C for 1min and one last cycle at 72°C for 10min. For quantitative RT-PCR, taqman gene expression assays FAM/MGB probes (Hs 00901640_m1-human TRPV2, Hs 00608195_m1 human TRPC1, and Hs 99999905_m1 human GAPDH) were from Applied Biosystems (Foster City, USA). For CD5, 500nM specific primers were used (sense: 5'-TCGGACGGCTCAGCTGGTATGAC-3'; antisense: 5'-TGCCAT CCGTCCTTGAGGTAGAC-3') plus 1XSYBR green PCR master mix (Applied Biosystems). The level of mRNA was normalized to GAPDH and cycle thresholds compared using the $2^{-\Delta\Delta ct}$ method.

Gene ontology and the analysis of biological pathways

The FatiGO web-interface was used to carry out data mining using the Gene Ontology database (www.geneontology.org). The signaling pathways were grouped by functional classes and pathways.

Statistical analyses

Differences between the lines were analysed using student's t test and/or the Mann-Whitney U test when appropriate. *P* values were determined using the GraphPad Prism Version 6.0 statistical software package and values less than 0.05 were considered significant.

RESULTS

CD5 promotes constitutive activation of pErk1/2

A key signaling molecule in normal and B-CLL cell survival and IL-10 production is Erk1/2.[13] An association between CD5 expression, constitutive Erk1/2 phosphorylation (pErk1/2) and IL-10 production has been suggested.[22] To assess this directly, we compared pErk1/2 levels in untransfected and CD5-transfected Jok-1 cells. The results revealed that Jok-1 transfection with either the membrane isoform of CD5 (Jok-E1A) or the cytoplasmic isoform (Jok-E1B) enhanced constitutive pErk1/2 markedly (Figure 1a). To explore the molecular mechanism(s) underpinning increased pErk1/2 by CD5, we transfected Jok-1 cells with CD5 mutants (Figure 1b). Transfection with CD5 lacking the extracellular-transmembrane domains, $S398M^{start}$ or $S415M^{start}$, or with mutations in the intracellular domain, $^{422}AS^{423} \rightarrow {}^{422}VD^{423}$ or $^{459}SDS^{461} \rightarrow {}^{459}VDG^{461}$ did not affect constitutive pErk1/2 and IL-10 production (Figures 1c/d). However, transfection with CD5 mutated in the intracellular domain $^{428}EYS^{430} \rightarrow {}^{428}AAA^{430}$ reduced pErk1/2 and IL-10 production with levels of both similar to untransfected Jok-1 cells (IL-10: 99.7±5.5pg/mL with native CD5 *versus* 19.3±5.5pg/mL in $^{428}AAA^{430}$ CD5, $P<0.05$). This indicates that the $^{428}EYS^{430}$ motif, which encompasses the Src kinase docking site Y^{429}, irrespective of the subcellular location of CD5, is critical for constitutive pErk1/2 and IL-10 production.

Constitutive Erk1/2 phosphorylation is independent of BCR engagement

The canonical BCR-dependent Erk1/2 phosphorylation involves activating the Syk/Btk/PLCγ2 pathway, which is regulated by two phosphatases, SHP1 and SHIP.[30] To determine if constitutive Erk1/2 phosphorylation in Jok-E1A/E1B cells occurs as a result of an association between CD5 and the BCR, we determined the phosphorylation status of Syk, Btk, PLCγ2, SHP1 and SHIP in non-activated Jok-1 and Jok-E1A/E1B cells. The results showed that pSyk, pBtk, pPLCγ2 and pSHIP were not different in Jok-1 compared with Jok-E1A/E1B cells (Figure 2a). However, the level of pSHP1 was higher in Jok-E1A/E1B cells compared with Jok-1 cells as previously reported in $CD5^+$ CLL B cells.[18]

To further test the hypothesis that constitutive pErk1/2 is BCR-independent in $CD5^+$ B cells, we carried out immunoprecipitation with anti-CD5 mAb (clone UCHT2) and Western blotting (Figure 2b). These experiments confirmed that CD5 was not associated with the BCR complex (CD79a) when the BCR is not engaged, while engagement with the $F(ab')_2$ anti-IgM resulted in co-precipitation of CD79a with CD5. In contrast, SHP1 co-precipitated with CD5 in Jok-E1A cells only when the BCR was not engaged. These data are consistent with our previous findings showing that CD79a associates with CD5 in B-CLL only after BCR engagement.[31]

To rule out that the findings are due to defective BCR-mediated signaling in Jok-1 cells, the kinetics of PLCγ2 and Erk1/2 phosphorylation were studied before and after BCR engagement with the $F(ab')_2$ anti-IgM. As shown Figure 2c, the level of pPLCγ2 was similar in all 3 lines before BCR engagement. PLCγ2 phosphorylation increased after 5min and continued until 30min post-BCR engagement confirming that BCR-mediated signaling is functional in Jok-1 cells (Figure 2c). Phosphorylation of Erk1/2 in the three lines with BCR engagement was highest at 2.5min and declined thereafter (Figure 2d). Importantly, levels of pErk1/2 increases were merely additive and proportional to the baseline in the 3 lines. The data, therefore, indicates that enhanced Erk1/2 phosphorylation by CD5 occurs independently of the BCR and through different pathways.

CD5 expression induces multiple signaling pathways

To identify the signaling pathway(s) through which CD5 enhances constitutive pErk1/2, the effect of the plasma membrane and intracellular CD5 isoform expression on intracellular signaling was assessed by kinome array analyses. Significant changes ($P<0.05$) in the phosphorylation of 1024 substrates of all known kinases are reported when the increase was ≥2 folds higher, while decreases are reported when levels were half or less. The phosphorylation of 154 substrates was increased and of 29 decreased in Jok-E1A/E1B cells

compared with Jok-1 cells (key ones shown in Table 2). Analyses of kinases and biological pathways reflected in these changes using FatiGO revealed that at least 2 pathways, one that drives Ras/Erk, Calmodulin and PKC through Ca^{2+} activation and the other, the one that drives PI3K/Akt/mTOR pathway, were either activated, or activation enhanced in the presence of CD5 (summarized in Figure 3a). Activation of PI3K/Akt/mTOR was further studied by Western blotting (Figure 3b) which confirmed an increase in the constitutive phosphorylation of Akt and S6K in Jok-E1A/E1B cells compared with Jok-1 cells. Activation of the PI3K/Akt/mTOR pathway could be related to the association between CD5 and the p85 unit of PI3K as established by immunoprecipitation with a mAb to CD5 (Figure 3c). Moreover, we established a link between CD5 and Lyn as well as with the U3 ubiquitin ligase c-Cbl, and the kinase Vav1 as previously described in thymocytes.[32]

Comparing the effect of membrane *versus* cytoplasmic CD5 revealed overlaps between the effect of the two isoforms on kinase activation but also differences on how they impact signaling (Table 3). Both isoforms activated the Ca^{2+}-dependent Ras/Erk, PKC and the PI3K/Akt/mTOR pathways.

CD5 expression impacts the Ca^{2+} pathway

The kinome analysis indicated that constitutive Erk1/2 phosphorylation in $CD5^+$ B cells is dependent on the Ca^{2+} pathway. To verify this proposition, we carried out single-cell video microscopy and observed an elevated resting initial fluorescence ratio suggesting an increase in basal level of iCa^{2+} in Jok-E1A compared with Jok-1 cells (340/360: 1.155±0.009, n=1,723, in Jok-E1A *versus* 1.067±0.007, n=1,623, in Jok-1; $P<0.001$, Figure 4a and b). Jok-E1B cells were excluded from this analysis as these cells constitutively express the fluorescent marker GFP. Based on the observation that such effects could be reversed when depleting Ca^{2+} from media in the absence of stimulation (Figure 4b), we next assessed whether this increase could

be the consequence of an elevated constitutive extracellular Ca^{2+} entry. To test this, we carried out Ca^{2+} repletion experiments followed by the addition of the non-selective plasma membrane Ca^{2+} channel blocker La^{3+}. The results revealed that with Ca^{2+} repletion, iCa^{2+} increased in resting Jok-E1A ($\Delta F/F0$: 0.16±0.01 Jok-E1A *versus* 0.08±0.01 Jok-1, $P<0.001$) (Figure 4c-d). In addition, the experiments confirmed that this effect can be reversed with La^{3+}. These findings confirm that the effect of CD5 is dependent on membrane Ca^{2+} channels.

We next carried out inhibition experiments to confirm the dependence of Erk1/2 phosphorylation by CD5 on Ca^{2+} using PD98059 and La^{3+}. PD98059 inhibits MEK/Erk activation while La^{3+} inhibits extracellular Ca^{2+} entry. In addition, since increases in iCa^{2+} and Ca^{2+} influx in lymphocytes could involve the PI3K/Akt/mTOR pathway, which is activated in resting $CD5^+$ B cells as shown in this study, pErk1/2 activation was evaluated in the presence of LY294002, which inhibits PI3K, and rapamycin, which inhibits mTor.[33,34] Interestingly, LY294002 and rapamycin had no effects on pErk1/2 in contrast to PD98059 and La^{3+} (Figure 5a). To confirm this observation, we assessed whether phosphorylation of STAT1/3 S727 also occur independently of the PI3K/mTor pathway.[35] Again, PD98059 and La^{3+}, but not LY294002 or rapamycin, inhibited pSTAT1/3 S727 in Jok-E1A/E1B. Furthermore, PD98059 and La^{3+} also inhibited IL-10 production (Figure 5b). These data, therefore, indicate that iCa^{2+} increase and constitutive Erk1/2-STAT1/3 phosphorylation when CD5 is expressed in B cells and bypasses the PI3K/Akt/mTOR pathway and may result from transient upregulation of Ca^{2+} membrane channel(s).

CD5 expression alters the transcriptome of B lymphocytes

To provide further insight into the impact of CD5 on B cell biology we analysed the transcriptome of the Jok-E1A and Jok-E1B lines compared with the Jok-1 line using the whole human genome oligonucleotide microarray. The analyses revealed that the expression level of 621 unique genes changed in Jok-E1A cells compared with Jok-1 cells. The analyses revealed that the expression level of 621 genes changed in Jok-E1A cells compared with Jok-

1 cells. The expression of 502 (80.8%) genes increased by >1.5 fold in CD5-E1A compared with Jok-1 cells while the expression of 119 (19.1%) genes decreased by >1.5 in CD5-E1A compared with Jok-1 cells. These results show similarities of some of the altered genes with a previous study in Daudi cell B cells transfected with CD5 (Supplementary data). Thus, among genes whose expression is altered by >2.0 folds, the expression of 45 genes was found up-regulated and 7 down regulated in both cell lines. Some of the genes whose expression was altered were genes encoding cytokines and chemokines (IL-10, IL2RG, and CCL3), signaling molecules (MKNK2, RGS1), apoptosis inhibitors (Bcl-2), transcription factors (NF-KB2, Spi-C), and cell surface receptors (CD83, CD74, CD54/ICAM1, CD69).[10] With the exception of 4 genes (*TRIM68*, *FRDM6*, *DYNLRB1* and *FLJ11710*), no differences were observed between Jok-E1A and Jok-E1B cells.

Analysis of changes in Ca^{2+}-permeable channel expression revealed upregulation of genes encoding the cationic channel TRPV2 and the transient receptor potential channel 1 (TRPC1) in both Jok-E1A and Jok-E1B cells compared to Jok-1 cells. Upregulation of both *TRPV2* and *TRPC1* genes was confirmed by RT-PCR in Jok-E1A/E1B (Figure 6a).

CD5 drives TRPC1 expression and IL-10 production in pErk1/2 positive B-CLL cells

To verify that CD5 drives IL-10 production through up-regulating TRPC1 and/or TRPV2, B cells from 26 patients with CLL segregated into two groups according to the phosphorylation status of pErk1/2 by Western blotting (Figure 6b, and data not shown). As expected, pErk1/2 activation was associated with IL-10 production (P<0.01) in B CLL cells (Figure 6c). TRPV2 was detectable at low levels in B cells from some CLL patients but no differences were observed between pErk1/2^{+} and pErk1/2^{-} B-CLL with regards to *TRPV2* transcripts by quantitative RT-PCR (Fig 6c). In contrast, *TRPC1* transcripts were detectable at significantly higher levels in pErk1/2^{+} B-CLL patients compared with pErk1/2^{-} B-CLL patients (P<0.001). Of note was that *TRPV2* and *TRPC1* were not detectable in B or T cells from healthy controls

(data not shown). Flow cytometry confirmed that the TRPC1 protein was expressed on B cells from pErk1/2^{+} CLL patients (MFI TRPC1: 1.9±1.3 in pErk1/2^{+} CLL *versus* 0.4±0.1 in pErk1/2^{-} CLL, $P< 0.05$) (Figure 6d). This data is consistent with the expression of TRPC1 in the Jok-E1A line (MFI: 5.9±2.4 *versus* 0.4±0.3 in Jok-1, $P<0.01$). The level of Erk1/2 phosphorylation and TRPC1 expression were independent of age, sex, CLL stage, disease progression, CD38 expression or the cytogenetic status of patients.

Finally, to confirm that CD5 induces TRPC1 expression and, promotes IL-10 production, we used siRNA for CD5 and TRPC1 to transfect B-CLL cells from 3 pErk1/2^{+} and 3 pErk1/2^{-} patients. After two days of culture, CD5 reduction was determined at mRNA level in CD5 and TRPC1 siRNA transfected B-CLL cells from both groups. The expression of TRPC1 was reduced with cd5-siRNA and TRPC1-siRNA in pErk1/2^{+} B-CLL cells (Figure 6e). In both cases the siRNAs resulted in inhibiting IL-10 production in pErk1/2^{+} B-CLL cells. Collectively, these results indicate that in pErk1/2^{+} B-CLL cells, CD5 promotes IL-10 production through a BCR-independent Ca^{2+}-dependent pathway that involves the non-selective Ca^{2+} channel protein TRPC1.

DISCUSSION

This study reveals that CD5 directly alters the biology of B cells including inducing IL-10 production. The molecular pathways through which CD5 modulates B cell biology appear to be mediated through Erk1/2 activation in a Ca^{2+}-dependent pathway and involving the non-selective Ca^{2+} channel TRPC1. Interestingly, the changes induced by CD5 are distinct from its negative modulating effects on BCR-signaling. Furthermore, the data reveal that pathways induced by CD5 in B cells are similar to those activated in B-CLL since induced CD5 expression replicates several characteristics of neoplastic B cells including constitutive basal Erk1/2 phosphorylation. This observation is consistent with previous studies[13] as is the ability of CD5 to activate STAT1/3[35] and IL-10 production;[8] all features of neoplastic B-CLL cells. In addition, CD5 expression led to perturbation in Ca^{2+} homeostasis leading to increased basal iCa^{2+}.[36]

Consistent with our observation that expression of CD5 induces biological changes distinct from its role in modulating effects on BCR-mediated signaling is the distinct effect on Ca^{2+} mobilization in the two settings.[31] The characteristics noted in $CD5^+$ B cells are similar to anergic[37,38] B cells and $CD5^+$ transitional B cells.[39-41] Such "anergic signature" was previously shown to be a characteristic feature of B-CLL cells.[42] Interestingly, the anergic phenotype of B cells was shown to be reversed in hen egg lysozyme (HEL) transgenic mice when the mice were made CD5 deficient.[24]

The current study also provides an in-depth insight into pathways leading to the constitutive activation of Erk1/2 and IL-10 production in B-CLL cells. Thus, the study shows that a Ca^{2+} influx-dependent pathway is involved in constitutive Erk1/2 phosphorylation and IL-10 production. Unlike conventional $CD5^-$ B2 cells in which Erk1/2 phosphorylation is mediated through Syk/BTK/PLCγ2 and PI3K activation following BCR engagement,[43] constitutive

Erk1/2 phosphorylation by CD5 occurs independently of this pathway. This is revealed based on the finding that Syk, BTK and PLCγ2 were not activated in unstimulated $CD5^+$ B cells and that inhibition of PI3K with LY294002 was ineffective in suppressing constitutive Erk1/2 phosphorylation in contrast to the effectiveness of the non-selective Ca^{2+} channel blocker La^{3+}. The newly-identified pathway is compatible with the observations that inhibition of Erk1/2 phosphorylation in B-CLL cells does not occur immediately after BTK inhibition[44] and that Erk1/2 phosphorylation in leukemic B cells in patients with CLL failed to mobilize Ca^{2+} upon BCR cross-linking.[13,42]

Since $CD5^+$ B cells in healthy individuals and patients with autoimmune diseases, such as systemic lupus erythematosus and also patients with CLL cells express both isoforms of CD5[45] but at different levels, we studied whether the two isoforms impact intracellular signaling differently. The results revealed that there were no major differences in the effect the two isoforms have on intracellular signaling in B cells except that the E1B-CD5 down-regulated the level of CD5 expression on the membrane.[12,45] These results indicate that the ^{428}EYS^{430} motif is functional in both isoforms. This is in agreement with previous studies showing that the CD5 Y^{429} is constitutively phosphorylated in B-CLL cells,[10] most probably by Lyn,[18] and that this has a positive effect on transcription but a negative one on BCR-mediated signaling.

Mechanisms through which CD5 plays a dual role in modulating B cell signaling and biology, however, remains unclear. This is in part due to the capacity of CD5 to activates a large array of kinases and phosphatases as shown in our current study. Consistent with the inhibitory effect of CD5 on BCR-mediated signaling, we observed that CD5 associates with SHP1 and c-Cbl in resting cells but with CD79a following BCR engagement. The positive effect of CD5 on gene transcription, however, appears to be due to the recruitment of key kinases including Lyn, the p85 unit of PI3K and Vav1. However, the molecular mechanism(s) through which CD5 modulates Ca^{2+} homeostasis and the role of TRPC1 in the process remains to be defined.

The expression of TRP channels has been associated with cancer, and in particular TRPC1 overexpression was described in transformed $CD5^+$ chicken DT-40 cell line and in human B lymphoblast cell lines (BLCL).[46,47] In DT-40 cells, TRPC1 was linked with intracellular Ca^{2+} increase and NFAT2 activation,[46] a signaling cascade that leads to cytokine/chemokine production in B-CLL cells. Interestingly, mice deficient in *TRPC1* have defective B cell functions similar to what is seen in *NFAT2* deficient mice.[48] Consequently, TRPC1 up-regulation in $CD5^+$ B cells may be an important mechanism that promotes B-CLL cell survival.

CONCLUSIONS

This study provides molecular evidence that CD5 expression *per se* alters B cell biology including constitutive activation of key signaling pathways leading to IL-10 production. Pathways and transcription factors activated by CD5 include those involved in regulating B cell survival, proliferation, cytokine/chemokine production and transformation. The findings reported in this study could help in better understanding of the biology and regulatory properties of $CD5^+$ B cells in health and in diseases including in patients with B-CLL and how it could, potentially, contribute to B cell abnormalities. These findings could have beneficial effects in designing new treatment strategies, particularly in CLL patients identified as refractory to currently available treatments. Such treatment strategies could involve the use of monoclonal antibodies to membrane proteins relevant to B-CLL cell transformation such as TRPC1 or CD5 as mono or combination therapies.[49] Alternatively, signaling pathways mediated by CD5 and involved in B-CLL cell transformation could be targeted. For example, high basal Ca^{2+} levels,[50] or upstream kinases could be targeted as has successfully been used to treat patients with autoimmune diseases.[51]

Acknowledgments: This study was supported by a grant from Arthritis Research-UK to Rizgar Mageed and by grants from the "Cancéropole Grand Ouest", the “Région Bretagne”, and the “Ligue contre le cancer” to Yves Renaudineau. We thank Catherine Riou and Professor Valérie Ugo (Brest) for the clinical material, Ms Simone Forest and Geneviève Michel for helping with typing the manuscript.

Supplementary information accompanies the manuscript on *Cellular & Molecular Immunology*'s website (http://www.nature.com/cmi/).

RFERENCES

1 Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* 2002; **20**: 253-300.

2 Hardy RR, K. Hayakawa K. CD5 B cells, a fetal B cell lineage. *Adv Immunol* 1994; **55**: 297-339.

3 Hayakawa K, Formica AM, Colombo MJ, Ichikawa D, Shinton SA, Brill-Dashoff J *et al*. B cells generated by B-1 development can progress to chronic lymphocytic leukemia. *Ann N Y Acad Sci* 2015; e-pub ahead of print 23 April; doi: 10.1111/nyas.12768.

4 Gary-Gouy H, Harriague J, Bismuth G, Platzer C, Schmitt C, Dalloul AH. Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. *Blood* 2002; **100**: 4537-4543.

5 Burdin N, Rousset F, Banchereau J. B-cell-derived IL-10: production and function. *Methods* 1997; **11**: 98-111.

6 Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**: 683-765.

7 Defrance T, Vanbervliet B, Brière F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* 1992; **175**: 671-682.

8 Kitabayashi A, Hirokawa M, Miura AB. The role of interleukin-10 (IL-10) in chronic B-lymphocytic leukemia: IL-10 prevents leukemic cells from apoptotic cell death. *Int J Hematol* 1995; **62**: 99-106.

9 Fayad L, Keating MJ, Reuben JM, O'Brien S, Lee BN, Lerner S *et al*. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. *Blood* 2001; **97**: 256-263.

10 Gary-Gouy H, Sainz-Perez A, Marteau JB, Marfaing-Koka A, Delic J, Merle-Beral H *et al*. Natural phosphorylation of CD5 in chronic lymphocytic leukemia B cells and analysis of CD5-regulated genes in a B cell line suggest a role for CD5 in malignant phenotype. *J Immunol* 2007; **179**: 4335-4344.

11 Garaud S, Le Dantec C, de Mendoza AR, Mageed RA, Youinou P, Renaudineau Y. IL-10 production by B cells expressing CD5 with the alternative exon 1B. *Ann N Y Acad Sci* 2009; **1173**: 280-285.

12 Garaud S, Morva A, Lemoine S, Hillion S, Bordron A, Pers JO *et al*. CD5 promotes IL-10 production in chronic lymphocytic leukemia B cells through STAT3 and NFAT2 activation. *J Immunol* 2011; **186**: 4835-4844.

13 Apollonio B, Scielzo C, Bertilaccio MT, Ten Hacken E, Scarfò L, Ranghetti P *et al*. Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood* 2013; **121**: 3879-3888.

14 Li P, Grgurevic S, Liu Z, Harris D, Rozovski U, Calin GA *et al*. Signal transducer and activator of transcription-3 induces MicroRNA-155 expression in chronic lymphocytic leukemia. *PLoS One* 2013; **8**: e64678.

15 Sarrias MR, Grønlund J, Padilla O, Madsen J, Holmskov U, Lozano F. The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. *Crit Rev Immunol* 2004; **24**: 1-37.

16 Gary-Gouy H, Bruhns P, Schmitt C, Dalloul A, Daëron M, Bismuth G. The pseudo-immunoreceptor tyrosine-based activation motif of CD5 mediates its inhibitory action on B-cell receptor signaling. *J Biol Chem* 2000; **275**: 548-556.

17 Gary-Gouy H, Lang V, Sarun S, Boumsell L, Bismuth G. In vivo association of CD5 with tyrosine-phosphorylated ZAP-70 and p21 phospho-zeta molecules in human CD3+ thymocytes. *J Immunol* 1997; **159**: 3739-3747.

18 Tibaldi E, Brunati AM, Zonta F, Frezzato F, Gattazzo C, Zambello R *et al*. Lyn-mediated SHP-1 recruitment to CD5 contributes to resistance to apoptosis of B-cell chronic lymphocytic leukemia cells. *Leukemia* 2011; **25**: 1768-1781.

19 Perez-Villar JJ, Whitney GS, Bowen MA, Hewgill DH, Aruffo AA, Kanner SB. CD5 negatively regulates the T-cell antigen receptor signal transduction pathway: involvement of SH2-containing phosphotyrosine phosphatase SHP-1. *Mol Cell Biol* 1999; **19**: 2903-2912.

20 Bauch A, Campbell KS, Reth M. Interaction of the CD5 cytoplasmic domain with the Ca2+/calmodulin-dependent kinase IIdelta. *Eur J Immunol* 1998; **28**: 2167-2177.

21 Raman C, Kuo A, Deshane J, Litchfield DW, Kimberly RP. Regulation of casein kinase 2 by direct interaction with cell surface receptor CD5. *J Biol Chem* 1998; **273**: 19183-19189.

22 Wong SC, Chew WK, Tan JE, Melendez AJ, Francis F, Lam KP. Peritoneal CD5+ B-1 cells have signaling properties similar to tolerant B cells. *J Biol Chem* 2002; **277**: 30707-30715.

23 Gary-Gouy H, Harriague J, Dalloul A, Donnadieu E, Bismuth G. CD5-negative regulation of B cell receptor signaling pathways originates from tyrosine residue Y429 outside an immunoreceptor tyrosine-based inhibitory motif. *J Immunol* 2002; **168**: 232-239.

24 Hippen KL, Tze LE, Behrens TW. CD5 maintains tolerance in anergic B cells. *J Exp Med* 2000; **191**: 883-890.

25 Parikh K, Poppema S, Peppelenbosch MP, Visser L. Extracellular ligation-dependent CD45RB enzymatic activity negatively regulates lipid raft signal transduction. *Blood* 2009; **113**: 594-603.

26 Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood* 2011; **117**: 5019-5032.

27 Gross HJ, Merling A, Moldenhauer G, Schwartz-Albiez R. Ecto-sialyltransferase of human B lymphocytes reconstitutes differentiation markers in the presence of exogenous CMP-N-acetyl neuraminic acid. *Blood* 1996; **87**: 5113-5126.

28 Loisel S, André PA, Golay J, Buchegger F, Kadouche J, Cérutti M *et al*. Antitumour effects of single or combined monoclonal antibodies directed against membrane antigens expressed by human B cells leukaemia *Mol Cancer* 2011; 10:42.

29 Devauchelle-Pensec V, Cagnard N, Pers JO, Youinou P, Saraux A, Chiocchia G. Gene expression profile in the salivary glands of primary Sjogren's syndrome patients before and after treatment with rituximab. *Arthritis Rheum* 2010; **62**: 2262-2271.

30 Richards JD, Davé SH, Chou CH, Mamchak AA, DeFranco AL. Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J Immunol* 2001; **166**: 3855-3864.

31 Nédellec S, Renaudineau Y, Bordron A, Berthou C, Porakishvili N, Lydyard PM *et al*. B cell response to surface IgM cross-linking identifies different prognostic groups of B-chronic lymphocytic leukemia patients. *J Immunol* 2005; **174**: 3749-3756.

32 Roa NS, Ordoñez-Rueda D, Chávez-Rios JR, Raman C, García-Zepeda EA, Lozano F et al. The carboxy-terminal region of CD5 is required for c-CBL mediated TCR signaling downmodulation in thymocytes. *Biochem Biophys Res Commun* 2013; **432**: 52-59.

33 Buhl AM, Pleiman CM, Rickert RC, Cambier JC. Qualitative regulation of B cell antigen receptor signaling by CD19: selective requirement for PI3-kinase activation, inositol-1,4,5-trisphosphate production and Ca2+ mobilization. *J Exp Med* 1997; **186**: 1897-1910.

34 Chen J, Fang Y. A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem Pharmacol* 2002; **64**: 1071-1077.

35 Frank DA, Mahajan S, Ritz J. B lymphocytes from patients with chronic lymphocytic leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues. *J Clin Invest* 1997; **100**: 3140-3148.

36 Muggen AF, Pillai SY, Kil LP, van Zelm MC, van Dongen JJ, Hendriks RW *et al*. Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* 2015; **29**: 321-328.

37 Healy JI, Dolmetsch RE, Timmerman LA, Cyster JG, Thomas ML, Crabtree GR *et al*. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity* 1997; **6**: 419-428.

38 Chumley MJ, Dal Porto JM, Cambier JC. The unique antigen receptor signaling phenotype of B-1 cells is influenced by locale but induced by antigen. *J Immunol* 2002; **169**: 1735-1743.

39 Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood* 2005; **105**: 4390-4398.

40 Limnander A, Depeille P, Freedman TS, Liou J, Leitges M, Kurosaki T *et al*. STIM1, PKC-delta and RasGRP set a threshold for proapoptotic Erk signaling during B cell development. *Nat Immunol* 2011; **12**: 425-433.

41 Limnander A, Zikherman J, Lau T, Leitges M, Weiss A, Roose JP. Protein kinase Cdelta promotes transitional B cell-negative selection and limits proximal B cell receptor signaling to enforce tolerance. *Mol Cell Biol* 2014; **34**: 1474-1485.

42 Muzio M, Apollonio B, Scielzo C, Frenquelli M, Vandoni I, Boussiotis V *et al*. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood* 2008; **112**: 188-195.

43 Kawauchi K, Ogasawara T, Yasuyama M. Activation of extracellular signal-regulated kinase through B-cell antigen receptor in B-cell chronic lymphocytic leukemia. *Int J Hematol* 2002; **75**: 508-513.

44 Cheng S, Ma J, Guo A, Lu P, Leonard JP, Coleman M et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia* 2014; **28**: 649-657.

45 Garaud S, Le Dantec C, Berthou C, Lydyard PM, Youinou P, Renaudineau Y. Selection of the alternative exon 1 from the cd5 gene down-regulates membrane level of the protein in B lymphocytes. *J Immunol* 2008; **181**: 2010-2018.

46 Mori Y, Wakamori M, Miyakawa T, Hermosura M, Hara Y, Nishida M et al. Transient receptor potential 1 regulates capacitative Ca(2+) entry and Ca(2+) release from endoplasmic reticulum in B lymphocytes. *J Exp Med* 2002; **195**: 673-681.

47 Roedding AS, Li PP, Warsh JJ. Characterization of the transient receptor potential channels mediating lysophosphatidic acid-stimulated calcium mobilization in B lymphoblasts. *Life Sci* 2006; **80**: 89-97.

48 Yildirim E, Carey MA, Card JW, Dietrich A, Flake GP, Zhang Y *et al*. Severely blunted allergen-induced pulmonary Th2 cell response and lung hyperresponsiveness in type 1 transient receptor potential channel-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 2012; **303**: L539-549.

49 Loisel S, André PA, Golay J, Buchegger F, Kadouche J, Cérutti M *et al*. Antitumour effects of single or combined monoclonal antibodies directed against membrane antigens expressed by human B cells leukaemia. *Mol Cancer* 2011; **10**: 42.

50 Muggen AF, Pillai SY, Kil LP, van Zelm MC, van Dongen JJ, Hendriks RW *et al*. Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* 2015; **29**: 321-328.

51 O'Shea JJ, Laurence A, McInnes IB. Back to the future: oral targeted therapy for RA and other autoimmune diseases. *Nat Rev Rheumatol* 2013; 9: 173-182.

FIGURE LEGENDS

Figure 1: Constitutive Erk1/2 activation and IL-10 production in CD5-expressing B cells is dependent on the phosphorylation of Y429 in the CD5 molecule. (**a**) The upper panel depicts Western blotting analysis of phosphorylated Erk1/2 (pErk1/2) in untransfected Jok-1 cells and Jok-1 cells transfected with membrane (E1A-CD5) or cytoplasmic (E1B-CD5) CD5. The lower panel shows total level of Erk1/2. **(b)** Cartoons representing full length CD5 and mutants generated in this study to identify sites in CD5 involved in Erk1/2 activation. CD5 has 3 extracellular domains (1-3), a transmembrane (Tm) region and a cytoplasmic domain. Truncated CD5 molecules ($S398M^{start}$ and $S415M^{start}$) are named according to their start codons. **(c)** Western blotting of constitutive Erk1/2 phosphorylation in untransfected Jok-1 cells (labelled c), cells transfected with native CD5 (1) or with the mutants generated as shown in the cartoons; and **(d)** ELISA results for the level of IL-10 produced by the corresponding cells in (**c**). The cells were cultured for 48hrs. The data in (**c**) and (**d**) represent three independent experiments. Statistical analyses were by the Mann-Whitney U test for IL-10 production. * indicates $P<0.05$ for statistical difference in IL-10 production between Jok-1 cells transfected with the $^{428}AAA^{430}$ compared with the full length CD5 molecule.

Figure 2: Constitutive Erk1/2 phosphorylation in CD5-expressing B cells is BCR-independent. (a) Western blotting (WB) analysis of Syk, BTK, PLCγ2, SHP1 and SHIP phosphorylation in Jok-1, Jok-E1A and Jok-E1B cells. **(b)** Anti-CD5 immunoprecipitation (IP: αCD5) followed by Western blotting of Jok-E1A cell for the association between CD5 and the BCR complex in resting and $F(ab')_2$ anti-human IgM (α-IgM) stimulated Jok-E1A cells. The left panel shows CD5, CD79a, and SHP1 in the Jok-E1A cell lysate (WB) tested as controls. The panel on the right depicts the association between CD5 and CD79a after α-IgM stimulation following immunoprecipitation (IP) with anti-CD5 mAb **(c)** Western blotting for the kinetics of PLCγ2 phosphorylation in unstimulated Jok-1, Jok-E1A and Jok-E1B cells or cells stimulated

with anti-IgM. The upper 3 panels depict the kinetics of PLCγ2 activation from times 0-10min after BCR engagement with the anti-IgM. The blots are for phosphorylated PLCγ2 (pPLCγ2), middle for total PLCγ2 protein (PLCγ2) and lower for β-actin protein. The bottom 3 panels depict the level of pPLCγ2 at time points 0, 20 and 30 minutes with anti-IgM. **(d)** Analysis of the kinetics of pErk1/2 following BCR engagement arranged as in (**c**). The two graph panels to the right of the Western blots represent semi-quantification data for the level of pPLCγ2 as in (**c**) and pErk1/2 in (**d**) of the signaling molecules represented as the ratio of band intensity for the phosphorylated proteins to that of the total protein.

Figure 3: Key signaling pathways affected by CD5 expression. (a) A cartoon representing the main kinases and signaling pathways whose activities are affected by CD5 expression in Jok-1 B cells. Only the major kinases and signaling pathways are shown based on the kinome array analysis and Western blotting in the current study and data from the literature. **(b)** Western blotting showing phosphorylation (top) and total protein levels (bottom) of Akt, and S6K in Jok-1, Jok-E1A and Jok-E1B cells. **(c)** Immunoprecipitation with anti-CD5 mAb in Jok-E1A cells reveals that CD5 associates with Lyn, the p85 regulatory unit of PI3K, c-Cbl, and Vav1. Representative of 3 independent experiments. TK: tyrosine kinase; ITAM: immune receptor tyrosine-based activation motifs.

Figure 4: CD5 expression modulates the Ca^{2+} pathway in B cells. (a) CD5 expression in Jok-E1A cells increases the basal level of intracellular Ca^{2+} (iCa^{2+}) compared with Jok-1 cells. **(b)** Histograms representing basal levels of iCa^{2+} in Jok-1 and Jok-E1A cells. The increase in basal iCa^{2+} in Jok-E1A cells is sensitive to extracellular Ca^{2+} depletion (no Ca^{2+}) as can be noted in (**a**) and confirmed in (**c**). Re-addition of extracellular Ca^{2+} to resting Jok-1 and Jok-E1A cells as shown in (**c**) reveals a high extracellular and constitutive Ca^{2+} influx in Jok-E1A. This influx can be reversed in the presence of lanthanum (La^{3+}) and ratios normalized to basal values (F0) indicated as (ΔF/F0). The mean and standard error of the mean (SEM) of the ΔF/F0 values in (**c**)

are from 6 independent experiments are presented in histograms in (**d**). *** indicate $P<0.001$ values for the difference between the two cell lines as determined by Student's t test.

Figure 5: CD5 promotes the phosphorylation of Erk1/2 and STAT1/STAT3 S727 and IL-10 production that are dependent on extracellular Ca^{2+} entry. (**a**) Analysis of constitutive Erk1/2 phosphorylation; STAT1 S727 phosphorylation; STAT3 S727 phosphorylation and (**b**) IL-10 production in Jok-1, Jok-E1A and Jok-E1B cells after 48h culture in the presence of PD98059 (at 50µM for Western blotting and at 100µM for IL-10 production), lanthanum (La^{3+}), rapamycin (Rapa) or Ly294002 (Ly29). PD98059 inhibits MEK1 and 2; La^{3+} blocks extracellular Ca^{2+} entry; rapamycin inhibits PI3-K/mTOR and Ly294002 inhibits PI3K/Akt. Cells cultured without inhibitors are used as controls and marked "**c**". Levels of IL-10 produced by cells cultured either alone or with the above indicated inhibitors were determined by ELISA and IL-10 levels are expressed as percentage of basal values and % reduction is presented as mean and SEM for 3 independent experiments. Basal value of IL-10 in Jok-1 cells was 32±6.9pg/mL and 105±8.7pg/mL for the CD5 transfected cells. * indicates $P<0.05$ for the statistical difference in the level of IL-10 production in the presence of a given inhibitor compared with the cultured cells without inhibitors using the Mann-Whitney U test.

Figure 6: TRPC1 regulates extracellular Ca^{2+} entry by CD5 in Jok-1 B cells and B cells from Erk1/2^{+} B-CLL patients. (a) transcripts of *CD5, TRPV2, TRPC1* and *GAPDH* in Jok-1, Jok-E1A and Jok-E1B B cells determined by RT- PCR. (**b**) B-CLL patients were divided into two groups based on the phosphorylation status of Erk1/2 protein by Western blotting. # indicates B cells from CLL patients positive for constitutively-phosphorylated Erk1/2. (**c**) Levels of *IL-10* (n=26 patients), *TRPC1* (n=26) and *TRPV2* (n=12) transcripts relative to *GAPDH* mRNA as determined by real time-PCR in B cells from pErk1/2^{+} and pErk1/2^{-} B-CLL patients. ** indicates $P<0.01$ and *** indicates $P<0.001$ for the statistical differences using Student's t test in the relative level of *IL-10* and *TRPC1* transcripts between pErk1/2^{+} and pErk1/2^{-} B-CLL

patients, respectively. (**d**) Representative FACS of extracellular TRPC1 protein expression (black histograms) in CLL#2 (pErk1/2^{-}), CLL#15 (pErk1/2^{+}), Jok-1, and Jok-E1A cells. MFI= Mean fluorescence intensity for each cell is indicated and isotype controls are presented as grey histograms. (**e**) Histograms depicting levels of *TRPC1, CD5* and *IL-10* transcripts in B cells from pErk1/2^{+} (black histograms) and pErk1/2^{-} (white histograms) B-CLL patients following transfection with control siRNAs (c-siRNA), CD5-siRNA and TRPC1-siRNA. The top two histograms depict relative levels of *CD5* (left) and *TRPC1* (right) transcript to *GAPDH* mRNA. The lower two histograms represent relative levels of *IL-10* transcripts to those of *GAPDH* in pErk1/2^{+} (left panel) and pErk1/2^{-} (right panel). B cells from three pErk1/2^{+} and three pErk1/2^{-} B-CLL patients were studied in these experiments. * indicates $P<0.05$ for the statistical difference in the level of transcripts for *CD5*, *TRPC1* and *IL-10* when using siRNA for CD5 or TRPC1 compared with c-siRNA. The statistical analyses were carried out using Student's t test.

Table 1. Demographic, clinical and immunological information on patients with chronic lymphocytic leukaemia (CLL) included in the study.

CLL	Age years	Sex	Binet	Follow-up (years)	PFS (months)	Ly ($x10^9$/L)	$CD19^+$ $CD5^+$ $CD38^+$ (%)	Cytogenetic	pErk1/2 status*
1	53	F	B	4	36	38,2	1%	del13q14	neg
2	55	M	A	10	>120	41,7	0%	del13q14	neg
3	65	M	C	2	26	42,1	88%	del13q14	neg
4	86	F	A	7	>84	23,9	2%	del13q14	neg
5	73	M	A	8	102	38,2	7%	del13q14	neg
6	74	M	B	6	57	25,8	2%	del13q14	neg
7	67	F	A	5	>60	14,3	4%	normal	neg
8	63	M	B	1	10	56	30%	del11q/ATM, del 13q14	neg
9	65	F	B	3	18	20.6	0%	del13q14	neg
10	85	M	A	9	>108	35,6	8%	normal	neg
11	53	F	A	8	>96	26,8	0%	del13q14	neg
12	67	F	A	4	>54	9,5	64%	trisomy 12	neg
13	77	F	A	6	>77	20,4	0%	del13q14	neg
14	88	F	A	20	>240	19,8	7%	ND	pos
15	77	M	B	16	126	96	50%	del13q14	pos
16	71	M	B	7	72	69,2	20%	trisomy 12, del13q14	pos
17	83	M	A	5	65	57,8	11%	del13q14	pos
18	76	M	A	10	>120	31,3	7%	del13q14	pos
19	79	M	A	2	>24	14,9	4%	ND	pos
20	74	F	B	10	123	45,7	7%	del13q14	pos
21	74	F	A	5	>57	41.8	48%	del13q14	neg
22	77	M	A	3	>36	16.7	26%	normal	pos
23	66	M	A	12	>130	62.5	0%	del13q14	neg
24	63	F	B	9	90	60.4	8%	del13q14	neg
25	84	F	B	5	46	61.9	23%	del17p/TP53, del13q14	pos
26	56	F	B	7	36	35.3	1%	del13q14	pos

PFS: progression free survival; Ly: lymphocytes numbers**. *** Indicates CLL patient divided on the basis of the phosphorylation status of MAPK Erk1/2 in their B cells into neg= negative ($pErk1/2^-$) ; pos= positive ($pErk1/2^+$). ND= not determined.

Table 2. Kinase activation profiles and change in their activity in Jok-E1A and Jok-E1B relative to untransfected Jok-1 cells.

Peptide with phosphorylation site	Protein substrate	Kinase	Function	E1A /Ctrl	E1B/ Ctrl	Change
FIGEHYVHVNA	HGFR/MET (Y1349)	Abl, autoP	TK receptor	4.7	3.5	Up
PESIHSFIGDG	MTOR (S2481)	Akt, autoP	Akt/mTOR	2.7	2.2	Up
EVPRRSGLSAG	MBD3 (S24 S26)	Aurora A		4.4	3.4	Up
PGMKIYIDPFT	EPHB1 (Y594)	autoP	TK receptor	4	3.9	Up
DIKSDSILLTS	CDC42/PAK5 (S573)	autoP, Ca^{2+}	Prot Kinase	6.1	5	Up
CEEEFSDSEEE	HDAC1 (S421 S423)	autoP, CK2	Cell cycle, DNA repair	2	2.4	Up
ETPAISPSKRA	dUTPase/DUT (S99)	CDC2	Enzyme	5.6	5.2	Up
GDAAETPPRPR	MEK1/MAP2K1 (T286)	CDK1	MAPK/Erk	5.3	4.3	Up
DPWGGSPAKPS	EPN1 (S357)	CDK1	Vesicle formation	7.5	9	Up
SASPYTPEHAA	TP73 (T86)	CDK1/2, CDC2, autoP	Cell cycle	5.6	6.1	Up
LSRMGSLRAPV	E2F1 (S364)	CHK2	TF	3.3	3.2	Up
PELARYLNRNY	HRS/HGS (Y329)	EGFR/MET	Vesicle formation	4	4.9	Up
DYDDMSPRRGP	HNRNPK (S284)	Erk	RNA binding	3.5	2.7	Up
AEVLPSPRGQR	TOP2A (S1213)	Erk1, CDK1	Cell cycle	3.7	3.5	Up
GPHRSTPESRA	PSEN1 (S353 S357)	GSK3B	MAPK/Erk	4.1	4.2	Up
RSGLCSPSYVA	MYC (S71)	MAPK/JNK	TF	2.3	2.3	Up
EKPRLSFADRA	PKC theta (S676)	nd	PKC	4.9	4.1	Up
ALRRESQGSLN	RGS14 (S260)	PKA	GTPase	9.8	11	Up
SAWPGTLRSGM	HSPB8 (T63)	PKC	Small HSP	3.5	3.2	Up
TTCVDTRWRYM	HIR/KCNJ4 (T53)	PKC	K receptor	3.1	2.8	Up
KSFTRSTVDTM	CD88/C5AR1 (S334 S338)	PKC	G protein	3.8	3.6	Up
AGIQTSFRTGN	DDX5 (S557)	PKC, autoP	RNA binding	2.7	2.9	Up
LLREASARDRQ	TRPV1 (S801)	PKCalpha, Ca^{2+}	Ca receptor	3	2.8	Up
EHRKSSKPIME	HES1 (S37-38)	PKCalpha, Ca^{2+}	TR	6.3	5.2	Up
ESLESTRRILG	SNAP23 (S23 T24)	PKCalpha, Ca^{2+}	Vesicle	2.5	2.8	Up
EGKHLYTLDGG	Rack1/GNB2L1 (Y228)	Src kinase	G protein	3.4	2.2	Up
SRLSAYPALEG	CD5 (Y465)	Src kinase	PI3K	2.8	2.9	Up
EVERTYLKTKS	GRIN2A (Y1105)	Src kinase (Fyn)	Ca receptor	4.1	3.1	Up
PCTTIYVAATE	CD150/SLAM (Y307)	Src kinase (Fyn)	ITAM receptor	2.7	2	Up
EEGEGYEEPDS	CD19 (Y409)	Src kinase (lyn)	ITAM receptor	2.3	2.5	Up
GTDLEYLKKVR	OGT (Y989)	Src kinase, INSR	Enzyme	2.5	2.3	Up
GSPSVRCSSMS	SMAD2 (S464 S465 S467)	TGFBR, BMPR1	TF	3.8	4.7	Up
FMRRTSLGTEQ	PTGER4 (S222)	unknown	G protein	5	5	Up
LDRFLSLEPVK	CCND1 (S90)	unknown	Cell cycle	2.2	4	Up
HSLPFSLPSQM	CBL (S623)	unknown	TK regulation	2.9	2.8	Up
TDGNRSSHSRL	BID (S64 S65)	unknown	TF	2.4	2.3	Up
ASKMDTCSSNL	F2R/PAR1 (S406)	unknown	G protein	0.3	0.5	Down

The table lists peptide substrates whose phosphorylation status is different in Jok-1 cells transfected with the E1A or E1B isoforms of CD5, corresponding protein substrates, kinases whose activity is altered and the ratio of activity of the kinase in the transfected cells compared with untransfected Jok-1 cells (according to phosphositeplus database at http://www.phosphosite.org). Ratio: refers to the ratio of activity of the kinase in Jok-E1A/E1B cells compared with Jok-1 cells. The analyses were carried out in triplicates for each cell line and the analysis repeated on two separate occasions. Differences were analyzed using student's t test. $P<0.05$ are considered significant and shown in the table. Change: indicates whether activity of the kinase in question was upregulated (Up), or down regulated (Down). Abbreviations: CK2: casein kinase 2; Ctrl: control, refers to activity of the kinase in the untransfected Jok-1 cells; RTK: receptor tyrosine kinase; TF: transcription factor; TK: tyrosine kinase; TF: transcription factor; TR: transcription repressor; autoP: autophosphorylated.

Table 3. Altered phosphorylation of kinase substrates by CD5 expression in Jok-1 B cells.

	CD5-E1A		CD5-E1B	
Kinase	**up**	**down**	**up**	**down**
PI-3K/Akt/mTOR	6	0	3	0
CaMkII	4	0	1	0
Cell cycle (CDK, CDC)	12	0	15	0
CK1/CK2	2	4	4	1
GSK3B	2	0	5	0
NF-κB	1	0	1	0
Jak/STAT	1	0	0	0
Ras-Erk	6	0	9	1
PKA	9	0	11	0
PKC	11	0	11	3
Src kinases	9	0	12	0

Table 3 lists the number of target peptide substrates whose phosphorylation status is upregulated (up) or down regulated (down) in Jok-E1A and Jok-E1B cells when compared with untransfected Jok-1 cells. The analysis was carried out as described in Table 2 legend and is drawn on data summarized in the same Table.

Figure 1

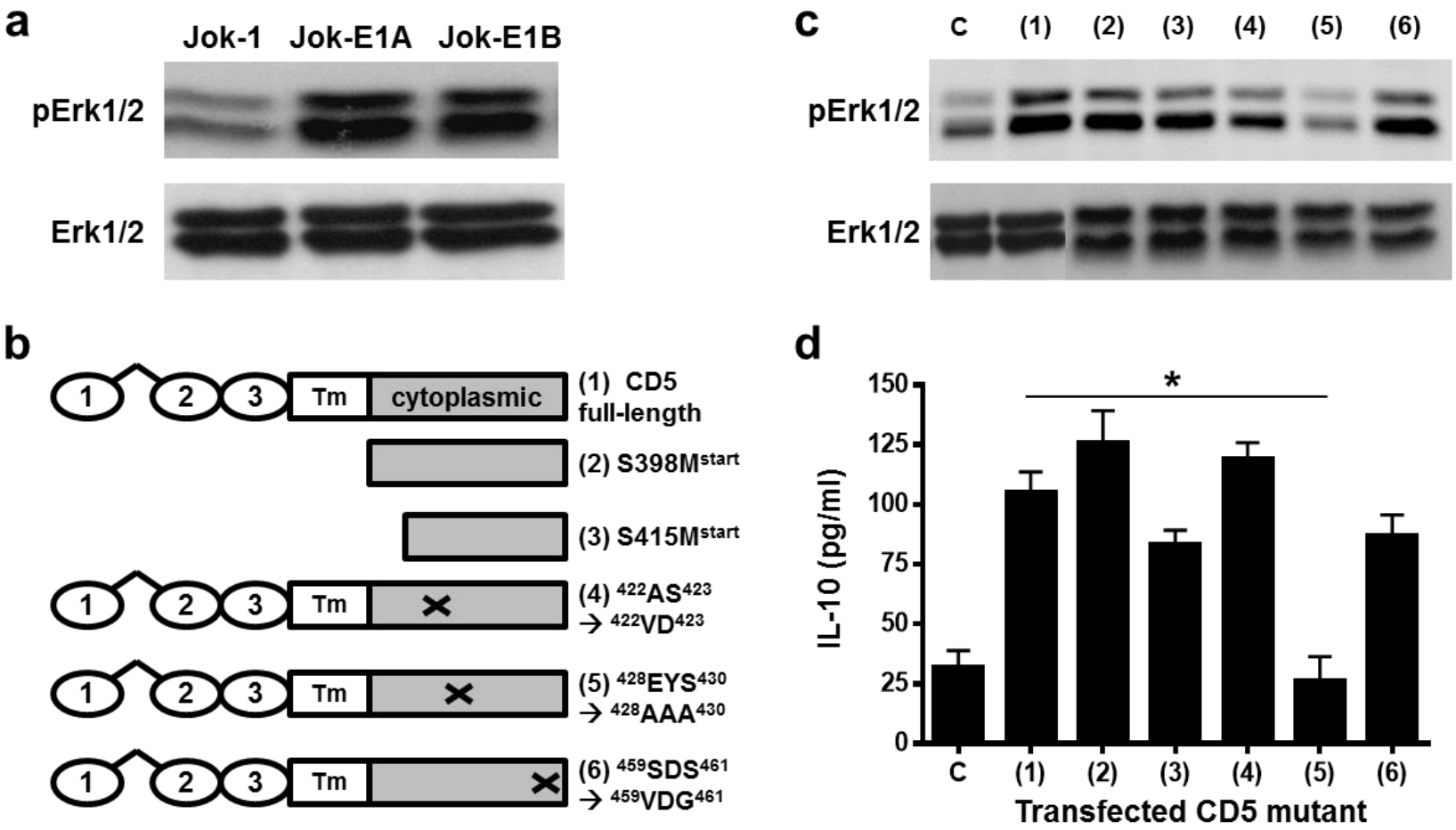

a
Jok-1
Jok-E1A
Jok-E1B
pErk1/2
Erk1/2
b
1
2
3
Tm
cytoplasmic
(1) CD5 full-length
(2) S398M start
(3) S415M start
(4) 422AS423 → 422VD423
(5) 428EYS430 → 428AAA430
(6) 459SDS461 → 459VDG461
c
C
(1)
(2)
(3)
(4)
(5)
(6)
pErk1/2
Erk1/2
d
IL-10 (pg/ml)
150
125
100
75
50
25
0
*
C
(1)
(2)
(3)
(4)
(5)
(6)
Transfected CD5 mutant

Figure 2

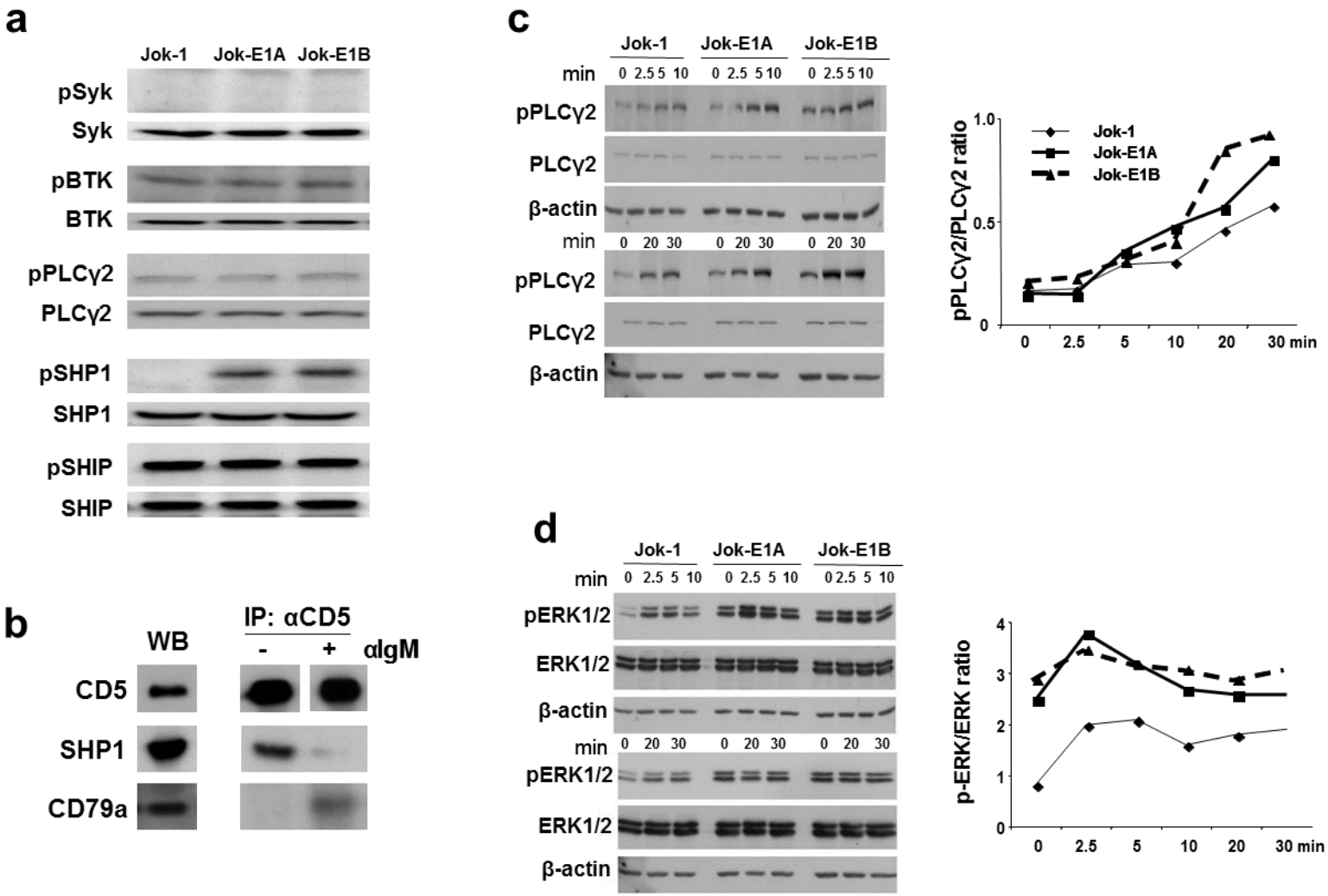

a
Jok-1
Jok-E1A
Jok-E1B
pSyk
Syk
pBTK
BTK
pPLCγ2
PLCγ2
pSHP1
SHP1
pSHIP
SHIP
b
WB
IP: αCD5
-
+
αIgM
CD5
SHP1
CD79a
c
Jok-1
Jok-E1A
Jok-E1B
min
0 2.5 5 10
0 2.5 5 10
0 2.5 5 10
pPLCγ2
PLCγ2
β-actin
min
0 20 30
0 20 30
0 20 30
pPLCγ2
PLCγ2
β-actin
pPLCγ2/PLCγ2 ratio
1.0
0.5
0
Jok-1
Jok-E1A
Jok-E1B
0
2.5
5
10
20
30 min
d
Jok-1
Jok-E1A
Jok-E1B
min
0 2.5 5 10
0 2.5 5 10
0 2.5 5 10
pERK1/2
ERK1/2
β-actin
min
0 20 30
0 20 30
0 20 30
pERK1/2
ERK1/2
β-actin
p-ERK/ERK ratio
4
3
2
1
0
0
2.5
5
10
20
30 min

Figure 3

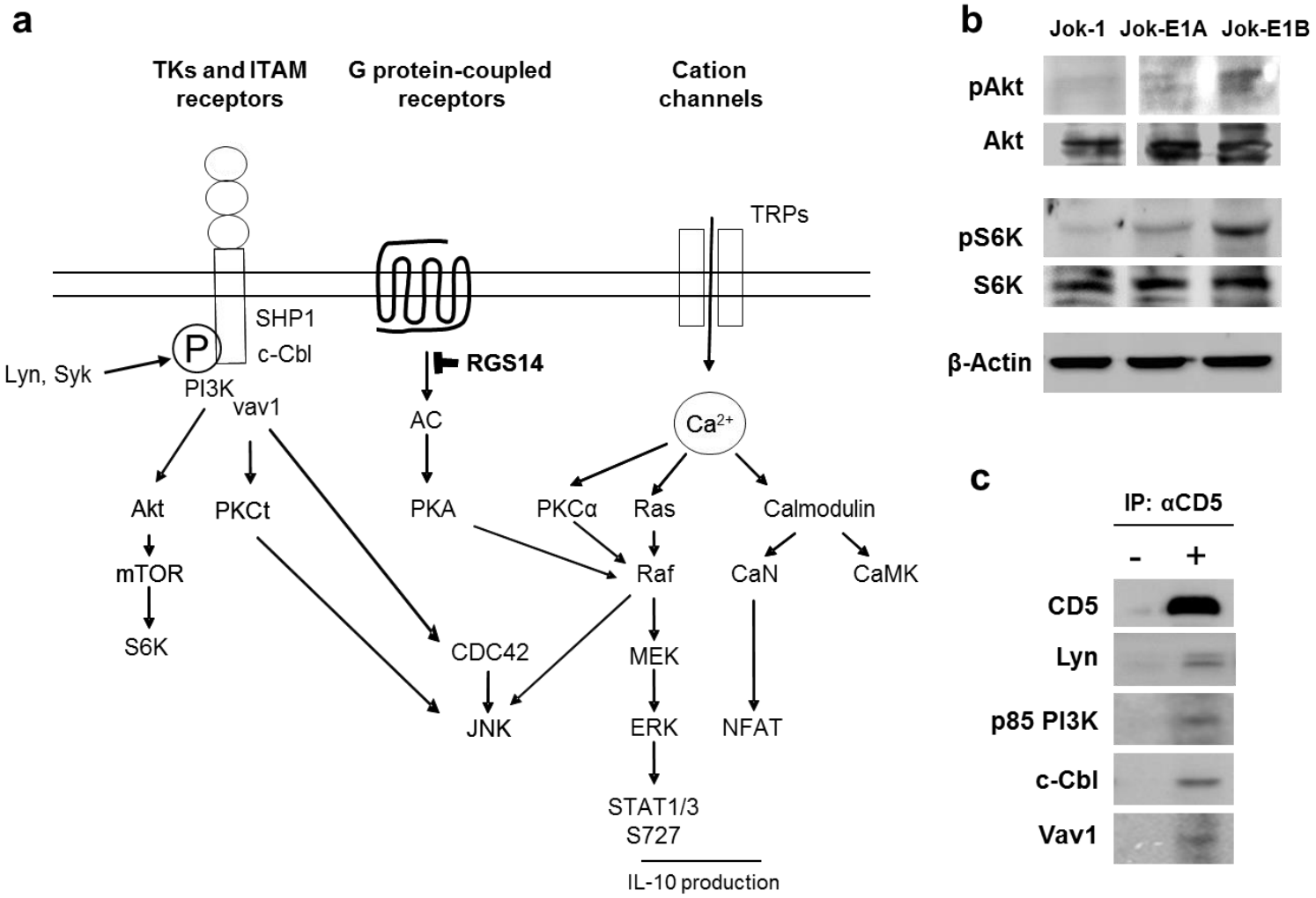

a
TKs and ITAM receptors
G protein-coupled receptors
Cation channels
TRPs
SHP1
c-Cbl
P
Lyn, Syk
PI3K
vav1
RGS14
AC
Ca2+
Akt
PKCt
PKA
PKCα
Ras
Calmodulin
mTOR
Raf
CaN
CaMK
S6K
CDC42
MEK
JNK
ERK
NFAT
STAT1/3
S727
IL-10 production
b
Jok-1
Jok-E1A
Jok-E1B
pAkt
Akt
pS6K
S6K
β-Actin
c
IP: αCD5
-
+
CD5
Lyn
p85 PI3K
c-Cbl
Vav1

Figure 4

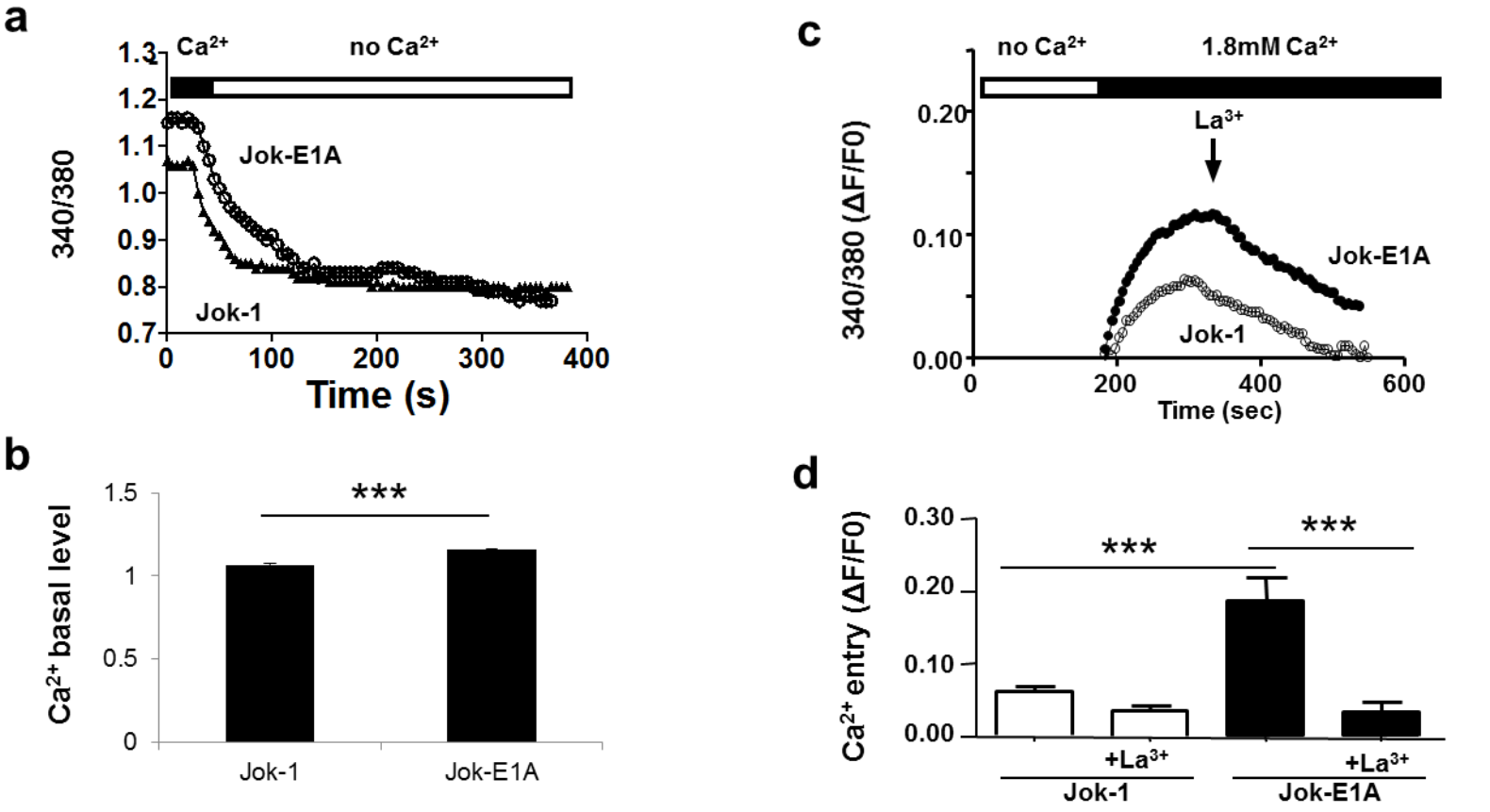

a
Ca2+
no Ca2+
340/380
Jok-E1A
Jok-1
Time (s)
b
Ca2+ basal level
***
Jok-1
Jok-E1A
c
no Ca2+
1.8mM Ca2+
La3+
340/380 (ΔF/F0)
Jok-E1A
Jok-1
Time (sec)
d
Ca2+ entry (ΔF/F0)
***
***
+La3+
+La3+
Jok-1
Jok-E1A

Figure 5

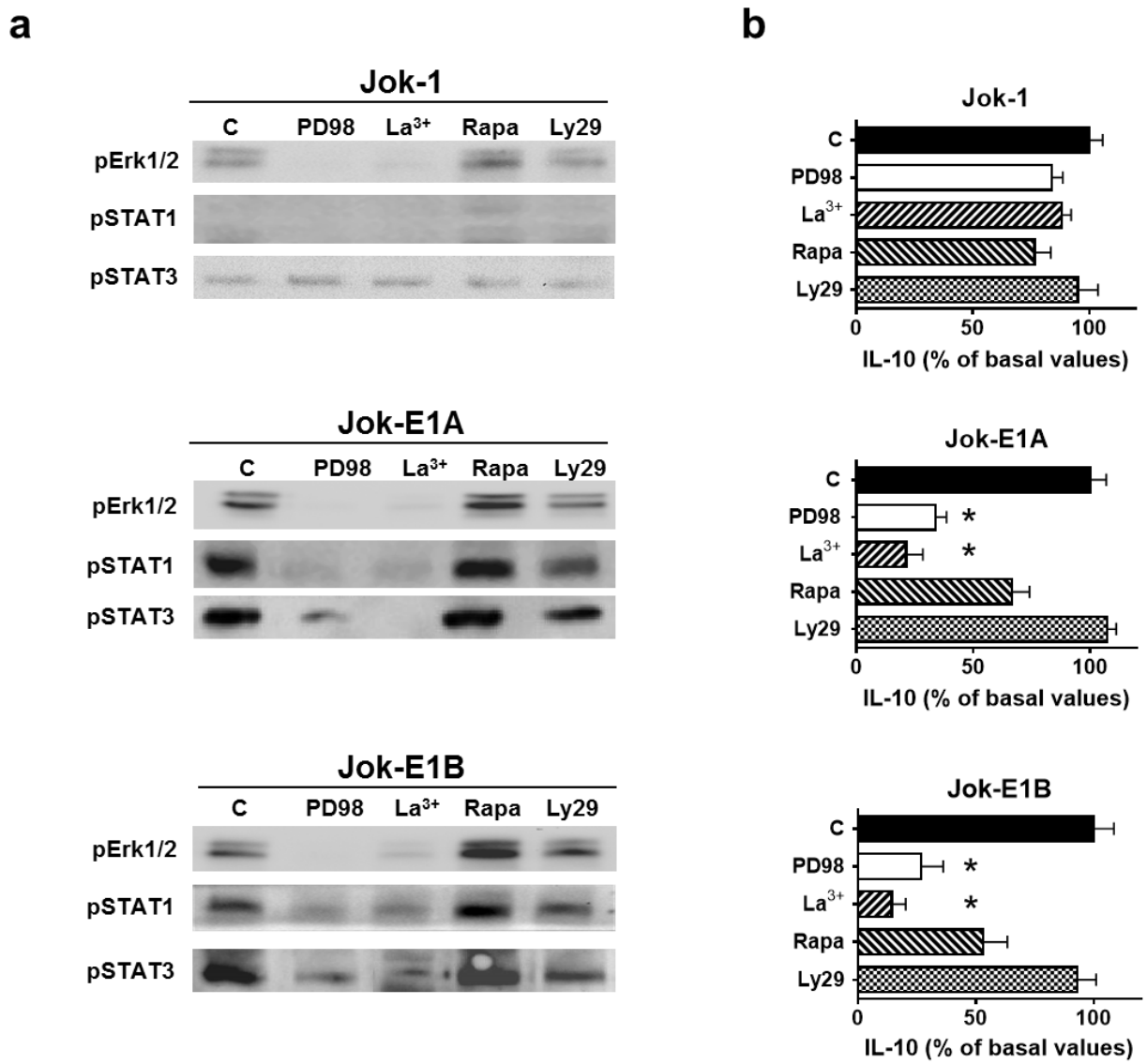
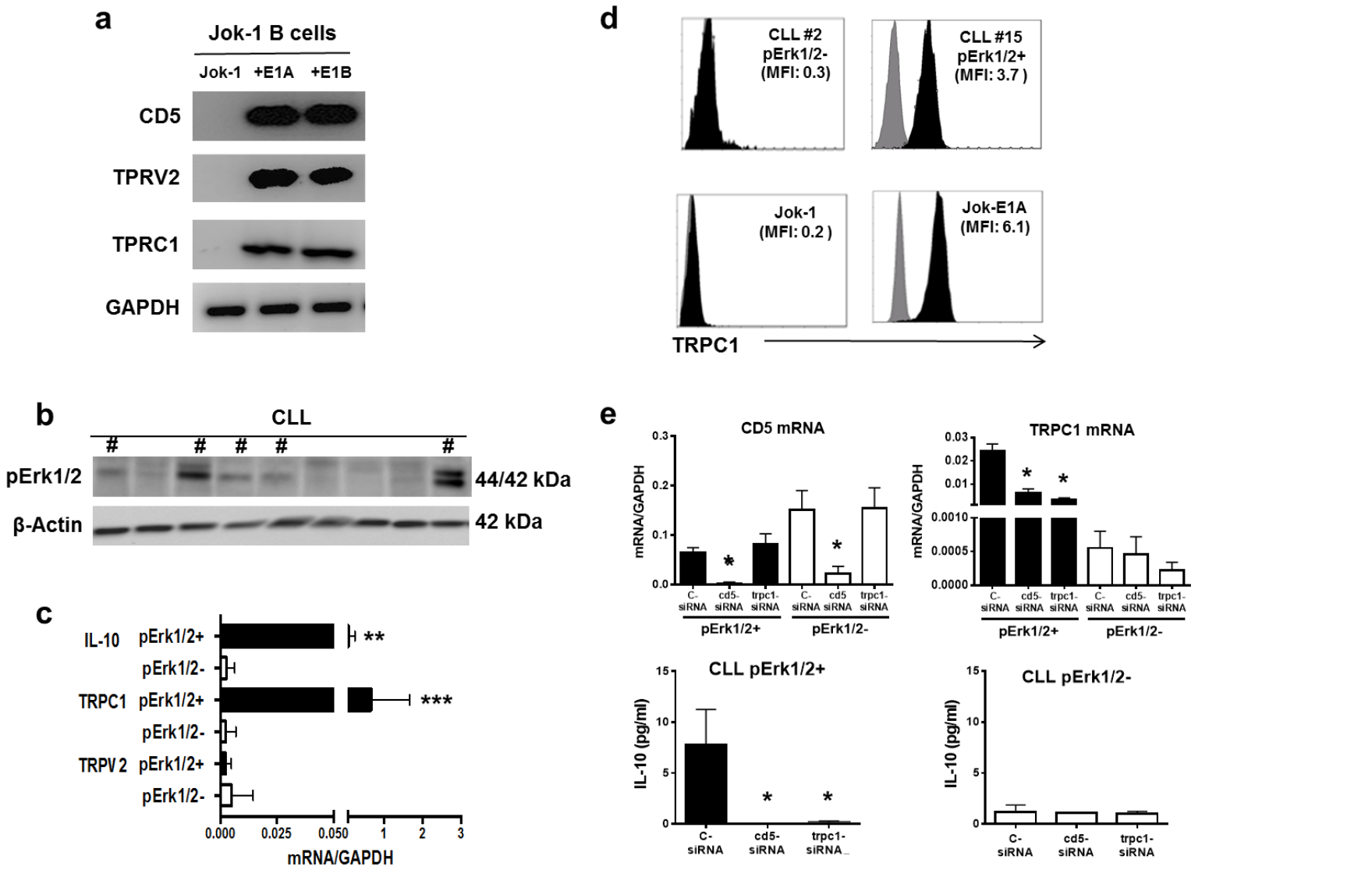

a
Jok-1
C
PD98
La3+
Rapa
Ly29
pErk1/2
pSTAT1
pSTAT3
Jok-E1A
C
PD98
La3+
Rapa
Ly29
pErk1/2
pSTAT1
pSTAT3
Jok-E1B
C
PD98
La3+
Rapa
Ly29
pErk1/2
pSTAT1
pSTAT3
b
Jok-1
C
PD98
La3+
Rapa
Ly29
0
50
100
IL-10 (% of basal values)
Jok-E1A
C
PD98
La3+
Rapa
Ly29
*
*
0
50
100
IL-10 (% of basal values)
Jok-E1B
C
PD98
La3+
Rapa
Ly29
*
*
0
50
100
IL-10 (% of basal values)

Figure 6


a
Jok-1 B cells
Jok-1
+E1A
+E1B
CD5
TPRV2
TPRC1
GAPDH
b
CLL
pErk1/2
β-Actin
44/42 kDa
42 kDa
c
IL-10
TRPC1
TRPV2
pErk1/2+
pErk1/2-
**
***
0.000
0.025
0.050
1
2
3
mRNA/GAPDH
d
CLL #2
pErk1/2-
(MFI: 0.3)
CLL #15
pErk1/2+
(MFI: 3.7)
Jok-1
(MFI: 0.2)
Jok-E1A
(MFI: 6.1)
TRPC1
e
CD5 mRNA
TRPC1 mRNA
mRNA/GAPDH
C-siRNA
cd5-siRNA
trpc1-siRNA
pErk1/2+
pErk1/2-
CLL pErk1/2+
CLL pErk1/2-
IL-10 (pg/ml)