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Lymphocyte Phosphatase-Associated Phosphoprotein (LPAP) as a CD45 Protein Stability Regulator

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Abstract—Lymphocyte phosphatase-associated phosphoprotein (LPAP) is a binding partner of the phosphatase CD45, but its function remains poorly understood. Its close interaction with CD45 suggests that LPAP may potentially regulate CD45, but direct biochemical evidence for this has not yet been obtained. We found that in the Jurkat lymphoid cells the levels of LPAP and CD45 proteins are interrelated and well correlated with each other. Knockout of LPAP leads to the decrease in the surface expression of CD45, while its overexpression, on the contrary, caused its increase. No such correlation was found in the non-lymphoid K562 cells. We hypothesize that LPAP regulates expression level of CD45 and thus can affect lymphocyte activation.

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Keywords: LPAP, CD45, T cell receptor, lymphocyte activation

INTRODUCTION

Lymphocyte phosphatase-associated phosphoprotein (LPAP) was first described as a molecule bound to the phosphatase CD45 [1]. CD45 protein plays an important role in lymphocyte activation, and it has been studied quite well [2]. LPAP, unlike its partner, has not been thoroughly studied. LPAP has no homologues in the human proteome and its function is still unknown. There is only indirect evidence of its role in T cell activation and B cell development [3]. Close association of LPAP with the CD45 phosphatase and its multiple phosphorylation, including the ERK-dependent Ser-163 phosphorylation, suggest that this protein is a participant of the activation cascade [4, 5]. The CD45 molecule is able to regulate the Lck kinase required to trigger activation cascade of lymphocytes after stimulation of the T cell antigen-specific receptor (TCR). By interacting with CD45, LPAP can affect signal transduction from the TCR in lymphocytes.

Formation of a tight complex between LPAP and CD45 suggests that these proteins are functionally related. Since LPAP does not have phosphorylated tyrosines, it is not a direct substrate of CD45 phosphatase, which dephosphorylates only modified tyrosines. There is some evidence that LPAP prevents formation of CD45 dimers, which are characterized by lower phosphatase activity than the monomeric form, and, thus, indirectly regulates activity of CD45 [6].

Several observations have shown that the CD45 molecule is important for maintaining stability of LPAP and its mouse homolog CD45-AP. First, in the lymphoid cell lines with knockdown of the phosphatase CD45, the LPAP protein is synthesized, but is rapidly

Abbreviations: KO, knockout; LPAP, lymphocyte phosphatase-associated phosphoprotein; mKO, monoclonal culture with knockout of the gene of interest; pKO, polyclonal culture with knockout of the gene of interest; PMA, phorbol-12-myristate-13-acetate; TCR, T cell antigen-specific receptor.

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degraded [1]. Second, in the T cell line experiments with induced CD45 expression, it was established that accumulation of the LPAP protein begins only after activation of the CD45 synthesis [7]. Knockdown of CD45 by shRNA reduces the level of LPAP protein [8]. Data on the reverse effect of LPAP on CD45 are limited and contradictory. In the lymphocytes of two LPAP-knockout mouse models, the level of CD45 was reduced [9, 10]. In the third LPAP knockout mouse line, this effect was not observed [11], as well as in the Jurkat cells with LPAP knockdown [12].

Some data indicate that the effects of CD45 on the level of LPAP protein are carried out at the post-transcriptional level [1, 13]. The Jurkat WT ($CD45^+$) and J45.01 ($CD45^-$) cells have high levels of LPAP mRNA, but the protein is detected only in the wild type Jurkat cells. When the construct encoding CD45 was transfected into the J45.01 cells, the level of LPAP protein was restored [1]. Transcriptional analysis of the $CD45^+$ and $CD45^-$ variants of T cells has shown that production of LPAP and CD45 mRNAs are regulated independently [13].

Thus, the body of published data indicates existence of a relationship between the LPAP and CD45 protein levels. We have suggested that LPAP acts as a chaperone for CD45 that controls stability and level of the CD45 protein. Jurkat cells with the CD45 or LPAP knockouts and a series of cell lines with different levels of the LPAP protein were produced to test this assumption. These cell lines showed correlation between the LPAP and CD45 protein levels. Existence of correlation was confirmed in the clonal and polyclonal populations. Our results suggest that the LPAP function could involve regulation of the CD45 expression in the cell.

MATERIALS AND METHODS

Cell culture, antibodies, and flow cytometry. Jurkat and K562 cells were cultivated in a RPMI-1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, and gentamicin (80 mg/liter) (Paneco, Russia) at 37°C in a humidified atmosphere containing 5% CO_2. Mouse monoclonal antibodies CL7 (IgG2a, anti-LPAP), LT45 (IgG2a, anti-CD45), EC101 (IgG1, anti-CD59), MC7E7 (IgG1, anti-CD98) were produced previously in our laboratory [4]. Antibodies CD69-PE (BioLegend, USA), OKT3 (eBioscience, USA), anti-Flag M2 (Sigma, USA) were also used.

For intracellular staining, 1 ml of PBS containing 1% paraformaldehyde was added to the cell pellet and cells were incubated for 10 min at room temperature. The cells were next washed twice in PBS, the cell pellet was resuspended in a permeabilization buffer (PBS containing 0.1% saponin, 0.1% BSA, 0.05% NaN_3). The cells in the permeabilization buffer were mixed with the antibody CL7-Alexa 594 or LT45-Alexa 594 and incubated for 30 min, then the cells were washed twice in the permeabilization buffer. Surface staining and washing were carried out in PBS. The stained cells were analyzed with a CytoFLEX S flow cytometer (Beckman Coulter, USA). The cells were sorted using FACSAria II (Becton Dickinson Biosciences, USA).

Generation of Jurkat cell lines with a double-nicking knockout of LPAP or CD45 using CRISPR/Cas9 method. Two target sequences for *PTPRCAP* and *PTPRC* genes encoding LPAP and CD45, respectively, were selected using the online http://www.genome-engineering.org/resource [14]. Oligonucleotides were synthesized in the company Evrogen (Russia) (table).

Oligonucleotides were annealed and cloned into a pKS-gRNA-BB vector at the BbsI restriction site [15, 16]. To perform knockout, Jurkat cells (1.5×10^6) were transfected using a Neon electroporation system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Electroporation mixture in a buffer R contained 0.5 μg of plasmids encoding guide RNAs gR-LPAP-1 and gR-LPAP-3 for LPAP knockout or gRNA-CD45-1 and gRNA-CD45-2 for CD45 knockout, and 3 μg plasmids pcDNA3.3-Cas9 D10A (#41816, Addgene, USA). In the case of LPAP knockout, cells were seeded into a 96-well plate 24 h after transfection, tested for LPAP expression three weeks later, and knockout clones were selected. In the case of CD45 knockout, 5 days after transfection, CD45 expression in the cells was assessed, and the CD45-negative population was expanded and sorted.

Generation of LPAP knockout Jurkat cell lines using the SORTS method. Knockout was performed using the SORTS (Surface Oligopeptide knock-in for Rapid Target Selection) method [17]. Briefly, principle of the method was as follows. Using CRISPR/Cas9 technology, a short DNA construct was inserted into the gene of the target protein (a process also called knock-in), which blocked expression of the endogenous protein. At the same time, in the knockout cells, a marker peptide was expressed on the plasma membrane. Presence of a label made it possible to select cells with a knockout, and the use of two labels made it possible to select cells with a knockout of two alleles. For this purpose, two oligonucleotides with homology arms were synthesized in the Evrogen company: 5′-2A-LPAP and 3′-2A-LPAP (table).

The resulting oligonucleotides were used as primers to obtain donor DNA. Plasmids pUCHR-mClover-AID-P2A-CD5HA2-bglpA and pUCHR-mClover-AID-P2A-CD5Flag2-bglpA, intended for introducing into the cell genome constructs encoding the HA or Flag peptide tag, respectively, were used as templates for PCR [17]. PCR products were run on a 1% agarose gel, a fragment of approximately 450 bp was excised, and then

Oligonucleotides for *PTPRCAP* and *PTPRC* gRNAs

Name	Sequence
5′-gR-LPAP-1	CACCGCATCCCGAGCCCTAAGGTGC
3′-gR-LPAP-1	AAACGCACCTTAGGGCTCGGGATGC
5′-gR-LPAP-3	CACCGCGCTGCCACCCGAGCCCAAG
3′-gR-LPAP-3	AAACCTTGGGCTCGGGTGGCAGCGC
5′-gRNA-CD45-1	CACCGAAATGACAGCGCTTCCAGAA
3′-gRNA-CD45-1	AAACTTCTGGAAGCGCTGTCATTTC
5′-gRNA-CD45-2	CACCGAGGTGATATTACCCTCAGTC
3′-gRNA-CD45-2	AAACGACTGAGGGTAATATCACCTC
5′-2A-LPAP	CGAAAGTGGTCTTGGTCACCCAGCCTGCCCCACACCAGGCCCCACCCCAGGTGCTGAGCCCTCTG AGCCCCTGCCTGTCTCCCACAGGCTCTGCCCTGC**GGATCCGGCGCAACAAAC**
3′-2A-LPAP	GGCGGCGCCAGGCCAGTGCTAGGCCAGTGGCCAGCAGTAGGAGCAGCAGCAGCAGCAGGACAA CGGTGACAGAGCTGGAGCCCACGCTGTCCTCCG**CACACAAAAAACCAACACAC**

Note. Sequences of homology arms are underlined, and regions of complementarity to the template plasmid are highlighted in bold.

isolated from the gel using a GeneJET Gel Extraction Kit (#K0692, Thermo Fisher Scientific) according to the manufacturer's instructions. Product concentrations were measured using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific).

To perform knockout, Jurkat cells (1.5×10^6) were transfected using a Neon electroporation system as described above. Electroporation mixture in a buffer R contained 0.5 μg of plasmids encoding guide RNAs gR-LPAP-1 and gR-LPAP-3, 3 μg of plasmid pcDNA3.3-Cas9 D10A (#41816, Addgene), 0.4 μg of a donor DNA (purified PCR product, see above) encoding the HA tag, and 0.7 μg of a donor DNA encoding the Flag-tag. Five days after transfection, cells were tested for effectiveness of mono- and biallelic knockin based on expression of HA and Flag epitope tags using flow cytometry. Cells were expanded and several rounds of positive population sorting were performed. For simplicity, the resulting cells were designated as LPAP^{pKO} (polyclonal knock out).

Generation of cell lines with stable expression of LPAP. HEK293T cells were seeded at 0.1 million per well of a 24-well plate, and after a day of cultivation, transfection was performed using a Lipofectamine 2000 reagent (Invitrogen, USA). For this, three plasmids were used: 0.87 μg of a HIV-1 packaging vector pCMVΔ8.2R (#12263, Addgene), 1.3 μg of a pUCHR-LPAP-wt transfer vector, 0.27 μg of a pCMV VSVG plasmid (# 8454, Addgene), encoding protein G from vesicular stomatitis virus. After 6 h, the medium was changed; after 48 hours, the supernatant was collected and filtered through a 0.45-μm filter. For transduction, target cells were seeded at 0.1 million per well of a 24-well plate and 250 μl of supernatant containing lentivirus was added. After 2 days, transduction efficiency was assessed using flow cytometry. The pool of transduced cells was cloned, and the clones were tested using flow cytometry.

Cell activation. Cells were activated by adding 10 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma, USA) followed by 4 h cultivation. Alternatively, cells were activated by culturing for 24 h in plates with wells pre-coated with anti-CD3 antibody OKT3 (eBioscience, USA) at a concentration of 0.1 to 10 μg/ml. Activation was stopped by adding cold PBS to the cells.

Immunoprecipitation. Cells were suspended in a cold lysis buffer containing 1% (w/v) Triton X-100, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 mM sodium orthovanadate (all reagents from Merck, USA). After 30 min of incubation on ice, nuclei and insoluble cell membranes were removed by centrifugation for 30 min at 4°C (20,000*g*). Immunoprecipitation was performed using CL7 or LT45 antibody covalently immobilized on an AffiGel-Hz carrier (Bio-Rad, USA). Immunoprecipitates were washed three times in a lysis buffer, the protein was eluted in a sample buffer for SDS-PAGE (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, and 0.05% bromophenol blue) by heating for 5 min at 80°C.

Electrophoresis and Western blotting (WB). Eluted proteins were separated using electrophoresis

in 10 or 18% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions in a Laemmli buffer system. After SDS-PAGE, proteins were transferred to a PVDF membrane using a semi-dry method. The membrane was blocked with 5% dry skim milk in PBS supplemented with 0.1% Tween 20. Antigens were detected using primary and then secondary antibodies against mouse IgG labeled with horseradish peroxidase (GE Healthcare, USA). The signal was detected with a ChemiDoc XRS System (Bio-Rad) using chemiluminescence reagents from Millipore (USA).

Statistical data processing. For statistical analysis and data visualization, the GraphPad Prism 8 program (GraphPad Software, USA) was used. Data were compared using a one-sample *t*-test. Results are shown as a mean ± standard deviation (SD). Correlation was assessed using the nonparametric Spearman test.

RESULTS

Levels of LPAP and CD45 proteins decrease in the absence of the partner protein. It was previously found that in the absence of CD45 phosphatase, the level of LPAP in the cell decreases [1]. At the same time, data on the reverse effect of LPAP on CD45 expression are contradictory. In order to evaluate mutual influence of these two proteins, we generated the CD45 or LPAP knockout Jurkat cell lines using the CRISPR/Cas9 technology.

It was found that in the CD45 knockout Jurkat T cell line, the level of LPAP was only 10% of the amount of the protein in the parental line, which corresponded to the level of autofluorescence of the wild-type cells (Fig. 1, a, b). To examine relationship between the LPAP and CD45 expression in more detail, we generated a panel of the Jurkat LPAP^{KO} sublines using two

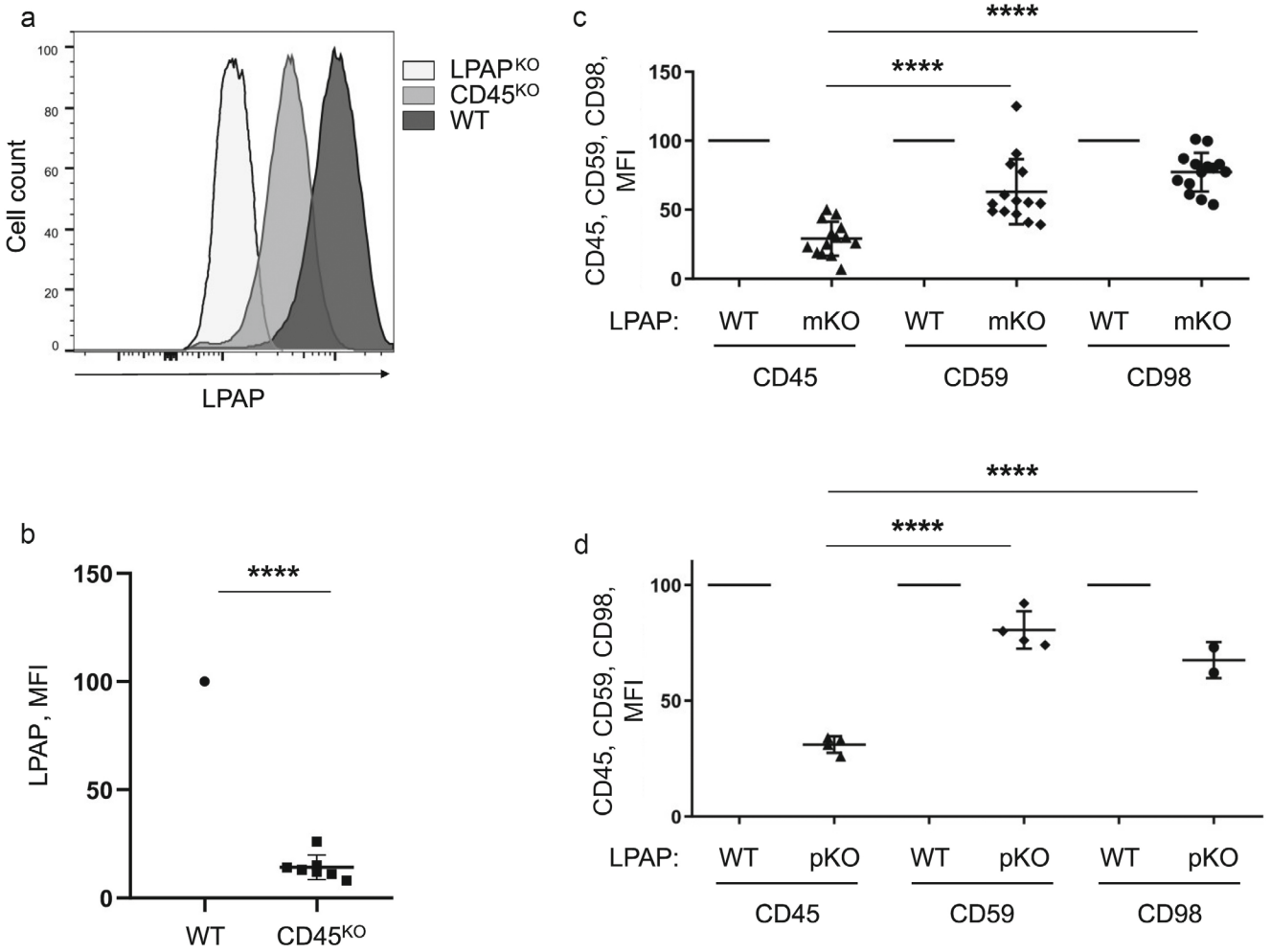

Fig. 1. Levels of LPAP and CD45 proteins decrease in the absence of a partner protein. a) Representative cytograms of LPAP expression on the wild-type (WT) Jurkat cells, as well as on the CD45^{KO} or LPAP^{KO} cells. b) LPAP expression based on the results of testing 7 CD45^{KO} clones; c, d) Expression of CD45 and "irrelevant" proteins CD59 and CD98 in the Jurkat LPAP^{mKO} (c) or LPAP^{pKO} (d) cultures. Protein expression levels of LPAP, CD45, CD59 and CD98 were determined using flow cytometry. Normalized CD45 expression level was compared with a reference value of 100 using a one-sample Student's *t*-test (b). To compare protein levels in the wild-type and knockout cells, ANOVA was used with *post hoc* analysis using Tukey's test (c, d). **** $p < 0.0001$.

different approaches. First, we used the "traditional" method of knocking out the *LPAP* gene using the CRISPR/Cas9 technology, and after subsequent cloning we obtained a series of monoclonal cultures of Jurkat LPAP^{KO} (hereinafter referred to as LPAP^{mKO}). And next, using the recently developed SORTS method [18], we obtained a polyclonal Jurkat population with the knockout of LPAP (hereinafter referred to as LPAP^{pKO}). The second approach has some advantages. Firstly, the Jurkat cell line is heterogeneous, and when working with individual clones of this line, there is a high risk that the observed phenotype is due to characteristics of a particular clone, and not to the experimental impact. Secondly, it has been shown that the cell clones can be very different from both the parental population and polyclonal population of the sorted knockout cells due to consolidation of the off-target genomic changes [17-19].

In all Jurkat LPAP^{mKO} cultures, the CD45 expression was reduced to 30% of the wild-type level (Fig. 1c). The CD45 level in the Jurkat LPAP^{pKO} cells was 37% of the CD45 level in the wild-type cells (Fig. 1d). Agreement between the data obtained for the individual clones and for the polyclonal population allows us to exclude contribution of interclonal variability to the observed phenotype. As a control, we used the CD59 and CD98 molecules as "irrelevant" proteins, for which there is no data on their interaction with LPAP. The level of "irrelevant" proteins CD59 and CD98 also decreased, but to a significantly lesser extent than CD45 (Fig. 1c; $p < 0.0001$). This indicates specific nature of the decrease in the CD45 protein level in the LPAP knockout cells.

CD45 expression correlates with the level of LPAP. After we discovered that the LPAP knockout resulted in the decrease in the CD45 protein level, we decided to investigate whether restoration of the level of LPAP in the cells could increase the phosphatase expression. For this purpose, we selected one of the Jurkat LPAP^{mKO} clones and introduced the gene encoding LPAP^{wt} into it using stable lentiviral transduction. The resulting population was then cloned and the effect of restoration of the expression of LPAP and CD45 proteins was assessed using flow cytometry. When testing 21 Jurkat subclones, we found high level of correlation between the CD45 and LPAP expression (Spearman correlation coefficient $r = 0.77$, $p < 0.0001$) (Fig. 2a). In contrast, for the clones tested, there was no significant correlation between the LPAP expression and expression of the irrelevant protein CD98 ($r = 0.02$) (Fig. 2b). As an additional control, we used the Jurkat WT clones, in which there was also no correlation between the levels of LPAP and CD45 (Fig. 2c). This control shows that the correlation shown in Fig. 2a is not the result of a cloning procedure.

Since the described result was shown in the cells derived from a single Jurkat LPAP^{KO} clone and could be due to characteristics of the randomly selected clone, we decided to verify the results with the data obtained from a polyclonal knockout. For this purpose, the gene encoding LPAP^{wt}, or the *GFP* gene in the control sample, was introduced into the Jurkat LPAP^{pKO} cells by lentiviral transduction. During transduction, three increasing doses of the virus were used (#1, #2, #3), after which expression of LPAP and CD45 in the resulting cultures was analyzed using flow cytometry. With increase of the LPAP level (Fig. 2d), the amount of CD45 in the cells also increased (Fig. 2, e, f). Difference between the levels of surface and total CD45 observed in the pKO cells did not change upon re-expression of LPAP (Fig. 2e). This indicates that in the absence of LPAP, CD45 is degraded and is not retained and accumulated in the vesicular system of the cell.

In the cells with enhanced LPAP expression the CD45 level increases. In the previous step, we found correlation between the LPAP and CD45 levels in the Jurkat cells with the LPAP levels ranging from 0% to 100% and CD45 levels ranging from 30% to 100%, relative to their endogenous expression levels. However, the question arises whether this correlation would persist with further increase in the level of LPAP in the cells. Using lentiviral transduction, a construct containing the LPAP-Flag-IRES-GFP cassette was introduced into the Jurkat cells. After two rounds of GFP^{hi} cell sorting, the level of LPAP increased markedly (Fig. 3a). In order to distinguish between the endogenous LPAP and exogenous LPAP-Flag, electrophoresis was performed in the 18% PAAG. Increase in the intensity of the band with the increased molecular weight was observed, which corresponded to the LPAP protein with the Flag peptide tag, compared with the intensity of the endogenous LPAP bands (Fig. 3b).

We hypothesized that in the Jurkat lymphoid cells there is a special mechanism for homeostatic regulation of LPAP levels, which cannot be bypassed by lentiviral transduction. Then we took the K562 cells of the erythromyeloid lineage, which carry CD45 on the surface, but are practically devoid of the endogenous LPAP (MFI = 3000). After lentiviral transduction, the K562 cells expressed high levels of LPAP-Flag (MFI = 116,000) (Fig. 3c). At the same time, the CD45 protein level also increased 3.1-fold (Fig. 3d). Thus, both the increased LPAP expression and its ectopic expression led to the increased CD45 expression.

LPAP knockout reduces CD3-induced expression of the activation molecule CD69. One of the early manifestations of T cell activation is upregulation of the CD69 protein exposure on the plasma membrane. In order to evaluate possible contribution of LPAP to intracellular signal transduction, we compared expression of the CD69 molecule on the Jurkat WT and Jurkat LPAP^{pKO} cells upon activation with PMA or antibodies

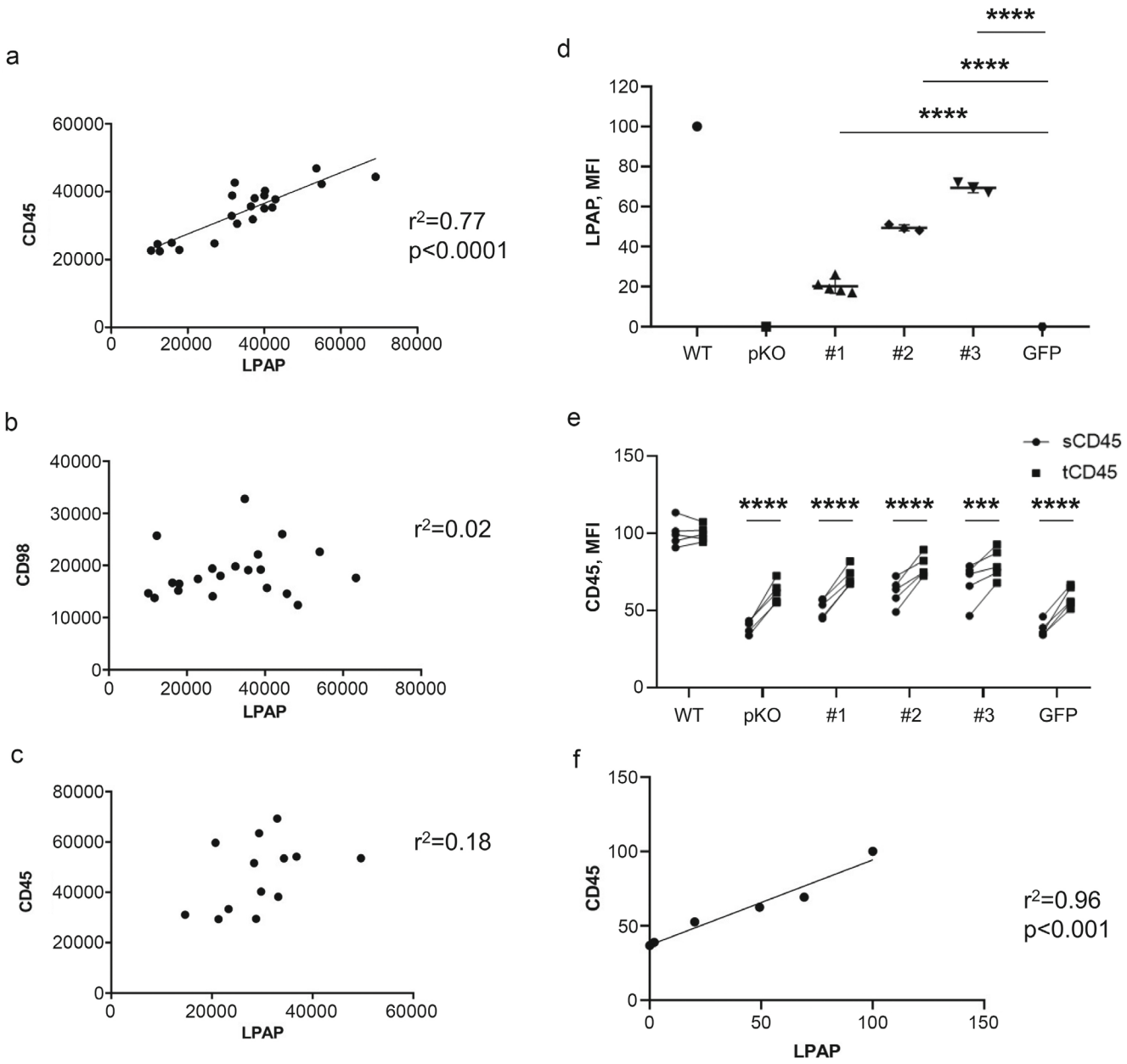

Fig. 2. CD45 levels correlate with LPAP protein levels. a, b) Correlation between the levels of LPAP and CD45 (a) or LPAP and CD98 (b) in the Jurkat LPAP^{KO} clones with LPAP^{WT} re-expression. c) Correlation between LPAP and CD45 levels in the Jurkat LPAP^{WT} clones; d, e) LPAP (d) and CD45 (e) levels in the Jurkat LPAP^{pKO} cells stably transduced with the increasing doses of virus to re-express LPAP^{wt} (#1, #2, #3) or express GFP. LPAP expression is normalized to the expression in the Jurkat LPAP^{wt} cells. For CD45, values are shown for the surface (sCD45) and total (tCD45) levels. f) Correlation between LPAP and CD45 levels in the Jurkat LPAP^{KO} clones stably transfected with LPAP^{wt}. Expression levels were determined from MFI values, which were normalized to the Jurkat WT mean expression after subtracting the background level in the Jurkat CD45^{KO} or Jurkat LPAP^{KO} cells. Individual values as well as mean values ± SD are shown. Normalized expression level of LPAP in the transduced cells was compared with the value of zero in the GFP-expressing cells using a one-sample Student's *t*-test (d). ANOVA with *post hoc* analysis using Tukey's test was used to compare surface (s) and total (t) CD45 levels in the Jurkat LPAP^{pKO} cells and transducers. *** $p < 0.001$, **** $p < 0.0001$. Mean values for LPAP and sCD45 levels from (e) were used to calculate the correlation (f).

against the CD3 receptor. When stimulated with PMA, the pKO cells expressed 40% less CD69 molecules than the wild-type cells (Fig. 4a). We then used a more physiological stimulus, CD3 cross-linking using the OKT3 antibody. Since the CD69 expression on the OKT3-activated Jurkat cells has a bimodal distribution (Fig. 4b, left panel), we compared the LPAP knockout cells and the wild-type cells by two parameters: percentage of the activated cells and MFI of the CD69$^+$ cells from the population with high level of CD69 (gating to the corresponding populations is shown in Fig. 4b, middle and right panels).

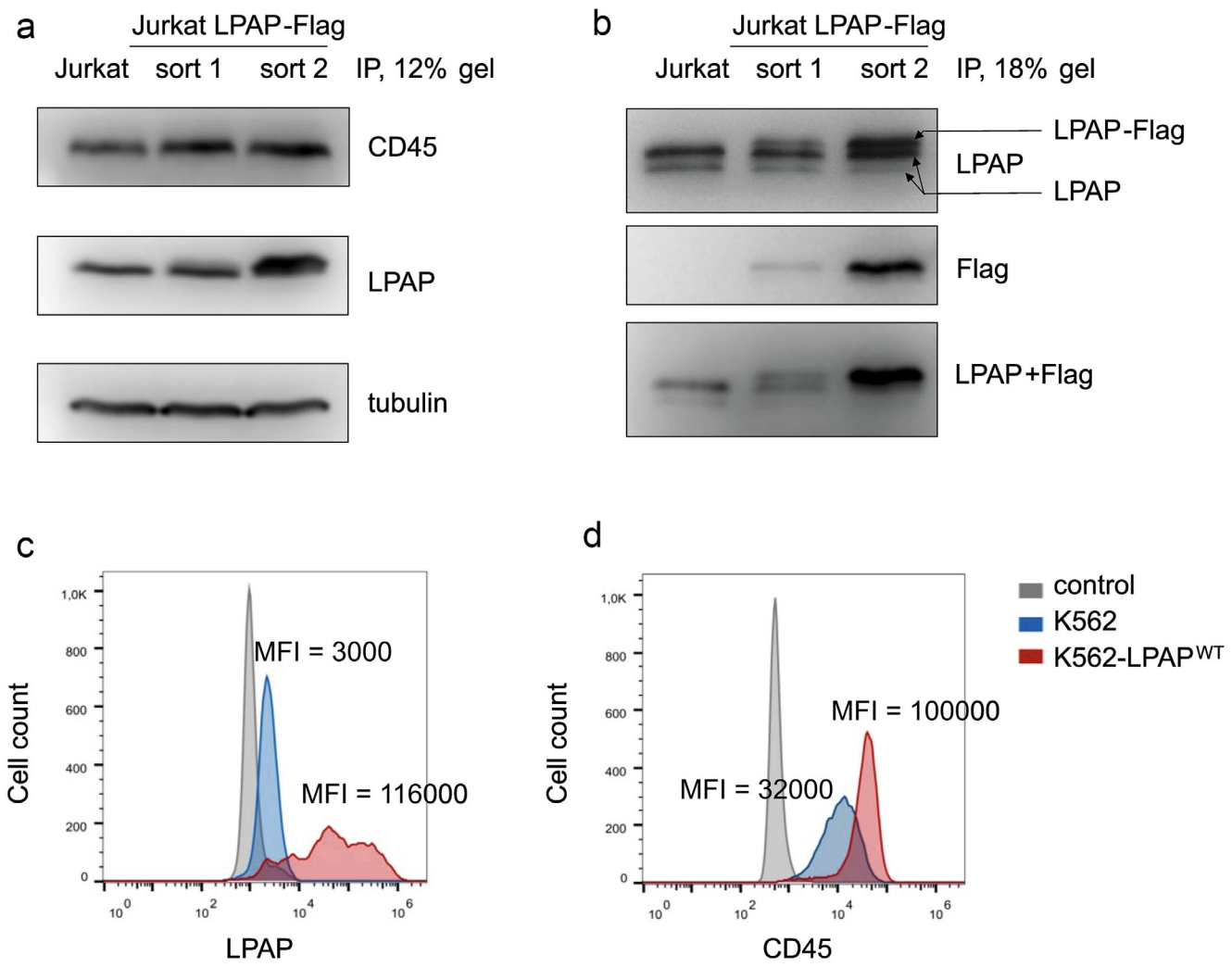

Fig. 3. In the cells with enhanced expression of LPAP, the level of CD45 increases. a, b) Level of LPAP protein was analyzed in the Jurkat and Jurkat LPAP-Flag cell lines after the first and second sorting (sort 1 and sort 2). Cells were lysed, LPAP or CD45 proteins were isolated by immunoprecipitation (IP), samples were separated by electrophoresis on a 12% (a) or 18% (b) gel, and Western blotting was performed with the indicated antibodies. The lower blot (b) was stained with antibodies against Flag and LPAP. The LPAP and LPAP-Flag bands are indicated by arrows. c, d) Expression of LPAP and CD45 on the surface of wild-type K562 cells, as well as of the cells stably transduced with LPAP-Flag.

Jurkat cells are characterized by low level of CD3 expression, which is detected only on some cells. We hypothesize that these $CD3^+$ cells respond to stimulation with the OKT3 antibody, resulting in the bimodal distribution of CD69. When activated via CD3, a significant difference between the Jurkat WT and $LPAP^{pKO}$ cells was observed in the samples with high concentration of OKT3 (1 and 10 μg/ml). Moreover, in the case of the pKO population, almost all cells were activated, but the level of CD69 on them was reduced compared to the wild-type cells (Fig. 4b, left panel). This difference could be due to the different levels of CD3 on the two cell types (Fig. 4c, left panel). In the pKO population, percentage of the $CD3^+$-positive cells was higher (Fig. 4c, middle panel), while MFI of the $CD3^+$-positive population was lower compared to the wild-type cells (Fig. 4c, right panel), which is consistent with the result for CD69 expression.

DISCUSSION

Role of the LPAP protein in the cell remains unknown, but formation of a tight complex with CD45 indicates that the LPAP function may be associated with regulation of this phosphatase. Changes in the phosphorylation status of LPAP upon T cell activation suggest that LPAP is a participant of the T cell lymphocyte receptor signaling cascade [5].

Some studies suggest that the CD45 phosphatase is required to maintain LPAP stability [7, 13]. This is in good agreement with our data that in the CD45 knockout Jurkat cells, the level of LPAP decreases by 90%. We previously showed that the knockdown of CD45 using shRNA reduced LPAP expression by more than twofold [8]. Finally, the Jurkat-derived cell line J45.01, which has only 5-8% of CD45 [20], expresses three

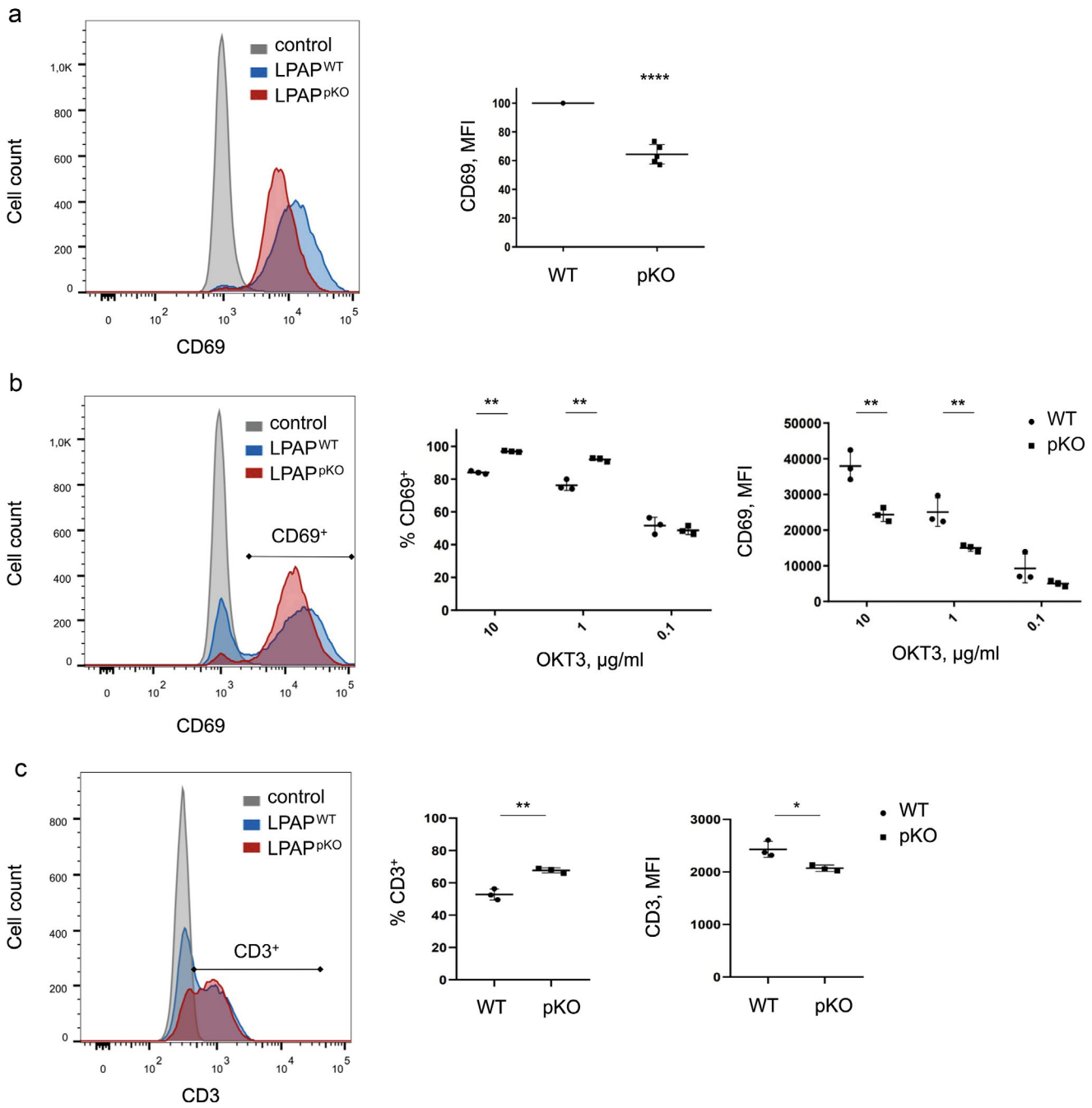

Fig. 4. Comparison of CD69 and CD3 expression on the wild-type and LPAP knockout Jurkat cells. a) CD69 expression on the cells activated with PMA. b) Expression of CD69 on the cells activated with the OKT3 antibody. c) CD3 expression on the cells activated with the OKT3 antibody. Levels of CD69 (b) and CD3 (c) were compared using a two-sample *t*-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

times less LPAP than the wild type [8]. Thus, there is consistent evidence that when the CD45 level decreases, the amount of LPAP in the cell decreases significantly.

To answer the question about the possible effect of LPAP on the CD45 protein levels, we generated a panel of LPAP knockout Jurkat sublines. It was found that in the knockout population, the level of CD45 was reduced to 30% of the wild-type levels. This is the most pronounced effect of all those described in the literature [9-11]. It could be suggested that LPAP affect not stability of the CD45 phosphatase, but rather its localization. However, our data on the effect of LPAP on total and surface CD45 levels (Fig. 2, e, f) do not support this assumption. We further showed that when LPAP expression was restored, the CD45 level increased, and there was a correlation between the levels of these proteins. Using lentiviral transduction of Jurkat cells,

we were unable to achieve a significant increase in the LPAP levels compared to the wild-type cells. On the other hand, cells of the erythromyeloid line K562, which do not carry endogenous LPAP, were capable of stable overexpression of this protein. We hypothesize that there is a tight mechanism in the lymphoid cells that controls the level of LPAP and this mechanism is absent in the nonlymphoid cells. It should be noted that the CD45RB isoform is predominantly present on the Jurkat cells, and the CD45R0 isoform is predominantly present on the K562 cells. We have previously shown that LPAP is associated with all isoforms approximately equally [8], therefore, different behavior of LPAP on the Jurkat and K562 cells cannot be explained by association with different CD45 isoforms.

The data we obtained previously, as well as the results presented in this work, indicate that the CD45 phosphatase maintains stability of LPAP and influences its phosphorylation status, and the LPAP protein, in turn, is able to regulate the level of CD45. Stability of the CD45 protein is probably not entirely determined by the LPAP molecule. This leads to the fact that even with the complete knockout of LPAP, at least 30% of the CD45 protein level is detected in the wild-type cells. Indirect evidence suggests that LPAP may influence the strength of T cell responses to low-affinity ligands [21] and may also be involved in the regulation of B cell differentiation [3]. It could be assumed that all this occurs indirectly, through the regulation of CD45 levels.

The group of Schraven et al. (1991) showed that without CD45, LPAP is synthesized, but is quickly degraded [7]. It could be assumed that the opposite is true regarding the CD45 stability. It is possible that in the absence of a partner protein, the regions responsible for recognition by the degradation system are exposed in the CD45 and LPAP proteins. The balance of activity of ubiquitin ligases and deubiquitinating proteins is important for regulation of the signaling pathways, including the T cell receptor cascade [22]. For example, ubiquitination of the TCR chains by CBL-b is required for receptor degradation and attenuation of the signaling cascade during the late stages of T cell activation. Another example of a protein whose main function is to bind and maintain partner stability is the transmembrane polypeptide type 6 (CMTM6), which interacts with the programmable cell death receptor ligand [23].

Bioinformatics analysis predicts that most of the cytoplasmic region of LPAP does not have a pronounced structure [24], and, hence, LPAP can be classified as an intrinsically disordered protein (IDP). Such proteins can acquire a certain conformation when interacting with a partner molecule [25]. The intrinsically disordered proteins, due to their ability to interact with various partners, often act as adapter proteins or scaffold proteins for the assembly of multicomponent complexes. They can interact with high specificity and moderate affinity, which is necessary for the strictly time-regulated processes and makes them important participants in signaling cascades [26]. It is possible that LPAP mediates interactions of CD45 with other proteins.

The question of LPAP contribution to activation of the signaling cascade of T lymphocytes still remains open. In our work, the LPAP knockout Jurkat populations expressed fewer CD69 molecules, when activated with PMA or OKT3. Based on these data, direct effect of LPAP on the signaling cascade could be suggested, however, such interpretation is complicated by the possible indirect effect of LPAP through regulation of the CD45 expression. An indication of the possible involvement of LPAP in T lymphocyte activation is observation that the reduced LPAP expression on the tumor-infiltrating lymphocytes is a potential marker of triple-negative breast cancer [27].

CONCLUSION

Our data suggest that the main function of LPAP is to modulate the level of CD45 protein. This raises a number of questions for further research. How does the interaction of these two proteins maintain their stability and what is the mechanism of degradation of CD45 and LPAP in the absence of a partner protein? Why is CD45 protein stable in the myeloid cells that do not constitutively express LPAP? Is there a specific mechanism of CD45 regulation in the lymphoid cells associated with LPAP? Answers to these questions will help reveal new details of lymphocyte activation.

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