The soluble cytoplasmic tail of CD45 regulates T-cell activation via TLR4 signaling

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**Introduction**

CD45 is a type I transmembrane glycoprotein that is expressed on all nucleated hematopoietic cells [1]. It is regarded as the prototypic receptor-like protein tyrosine phosphatase (PTP) and has important functions in the regulation of both, T- and B-cell antigen receptor signaling [1]. Its extracellular domain consists of three fibronectin type III-like domains, a cysteine-rich domain and a variable N-terminal domain [1]. The latter is encoded by up to three alternatively spliced exons, which can give rise to the different CD45 isoforms [2]. A single transmembrane domain links the extracellular domain to the intracellular part of CD45, which contains two tandemly duplicated PTP homology domains (D1 and D2). However, of these motifs, only D1 is an active phosphatase, while D2 may only contribute to enzyme stability [1].

Apart from its transmembrane signaling function, our group has described an alternative function for CD45 [3].

The soluble cytoplasmic tail of CD45 (ct-CD45) is a cleavage fragment of CD45, that is generated during the activation of human phagocytes. Upon release to the extracellular space, ct-CD45 binds to human T cells and inhibits their activation in vitro. Here, we studied the potential role of TLR4 as a receptor for ct-CD45. Treatment of Jurkat TLR4/CD14 reporter cells with ct-CD45 induced the upregulation of the reporter gene NF\(\kappa\)B-eGFP and could be blocked by inhibitors of TLR4 signaling. Conversely, ct-CD45 did not promote the NF\(\kappa\)B-controlled eGFP induction in reporter cells expressing TLR1, TLR2, and TLR6 transgenes and did not lead to the activation of the transcription factors NF\(\kappa\)B, AP-1, and NFAT in a Jurkat reporter cell line expressing endogenous TLR5. Moreover, ct-CD45 binds to recombinant TLR4 in an in vitro assay and this association was reduced in the presence of oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine. Blockade of TLR4 with mAb HTA125 partially reversed the ct-CD45-mediated inhibition of T-cell proliferation. Interestingly, targeting of TLR4 with mAb W7C11 also suppressed T-cell proliferation. In summary, the results of this study demonstrate that ct-CD45 acts via a noncanonical TLR4 activation pathway on T cells, which modulates TCR signaling.
Stimulation of human phagocytes with cell wall components of yeast (zymosan) or the phorbol ester PMA induces sequential cleavage of CD45, which is initiated by shedding of the CD45 ectodomain upon cleavage by serine proteases and metalloproteinases. This initial proteolytic event is succeeded by γ-secretase-mediated cleavage and release of the CD45 intracellular domain. This soluble fragment termed “cytoplasmic tail of CD45” (ct-CD45) is 95 kDa in size and contains both phosphatase homology domains. After its processing, ct-CD45 is released from dying phagocytes and was found to act as a cytokine-like factor on human T lymphocytes that potently inhibits their proliferation independent of the protein’s phosphatase activity [3].

Further studies showed that ct-CD45 may be detected in human plasma or serum under steady-state conditions [4]. Interestingly, adult human plasma showed higher ct-CD45 levels than plasma derived from umbilical cord blood. Furthermore, plasma ct-CD45 was reduced in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) compared to healthy controls. Cultivation and activation of primary human T cells in the presence of adult plasma depleted from ct-CD45 revealed higher proliferation rates compared to undepleted plasma. In line with these findings, exogenous ct-CD45 inhibited the proliferation and cytokine production of T cells activated via CD3 and via CD3/CD63 stimulation. However, T cells that received costimulation via CD28 were not susceptible to the inhibitory effects of ct-CD45 [4].

T cells activated in the presence of ct-CD45 were characterized by increased expression of two putative regulators of T-cell quiescence, Schlafen family member 12 (SLFN12) and Krueppel-like factor 2 (KLF2). Cell-cycle analysis revealed arrest of ct-CD45-treated cells at the G0/G1 phase and was associated with increased expression of the cyclin-dependent kinase (CDK) inhibitor p27kip1, whereas cyclin D2 and D3 as well as CDK2 and CDK4 were downregulated [4, 5]. Taken together, these data suggested a potential role of ct-CD45 in the maintenance of T-cell quiescence during the steady state.

The receptor responsible for the initiation of ct-CD45-mediated effects was unknown. A screen of a human T cell-derived cDNA library revealed protein associated with TLR4 (PRAT4A) as a potential receptor for ct-CD45 [6]. Nevertheless, experiments using the Biacore system could not demonstrate direct interaction of the two proteins [4, 5]. PRAT4A was found to regulate cell-surface expression of TLR4 [7], thus, suggesting that ct-CD45 could be an endogenous ligand for TLR4. TLR4 has decisive roles in the sensing of both pathogen- and damage-associated molecular patterns on innate immune cells, which are delivered by microbe-derived and endogenous ligands, respectively [8]. Yet, TLR4 is also an important receptor on human T cells as well [9–13]. Here, we demonstrate that ct-CD45 specifically binds to TLR4 and signals via this pathway leading to noncanonical TLR4 activation on T cells. These findings indicate that TLR4 signaling can control TCR signaling.

### Results

#### ct-CD45 signals in TLR4-expressing reporter cells

A plethora of endogenous molecules has been described to ligate TLR4 [8]. Based on our own previous findings, we hypothesized that TLR4 could be a potential receptor for ct-CD45 [4].

To verify this hypothesis, we used a TLR4 reporter cell system to analyze TLR4-dependent downstream signaling initiated by ct-CD45. TLR4/CD14 double-transfected Jurkat T cells, that express an eGFP reporter gene under the control of NFκB promoter elements, were stimulated with titrated amounts of soluble ct-CD45. Reporter gene activation was observed in a dose-dependent manner (Fig. 1A), indicating ct-CD45-mediated initiation of downstream signaling. Jurkat cells do not express TLR4 as demonstrated by flow cytometry and qPCR (Supporting Information Fig. S2A and B). Furthermore, this cell line does not activate NFκB nuclear translocation upon treatment with the canonical TLR4 ligand LPS in the functional reporter cell assay in the absence of TLR4 transgene (Supporting Information Fig. S2B).

To exclude, that ct-CD45 activate other TLR besides TLR4, we treated Jurkat NFκB GFP TLR6, TLR2/6, TLR2/1, and TLR2/1/6 reporter cells with different concentrations of recombinant ct-CD45. However, ct-CD45 did not significantly activate NFκB in this setting (Fig. 1B, Supporting Information Fig. S1A).

To investigate whether ct-CD45 would influence other signaling transduction pathways independent of TLR4, a Jurkat triple parameter reporter (TPR) cell line was used that expresses the fluorescent proteins CFP, Egfp, and mCherry under control of NFκB, NFAT, and AP-1 promoter elements. These TPR cells do not have functional TLR4 but display significant cell-surface expression of TLR5 [14]. Jurkat cells (WT) or TLR6, TLR2/6, TLR2/1, and TLR2/1/6 reporter cells do not express TLR4 as demonstrated by flow cytometry and in the functional reporter cell assay (Supporting Information Figs. S1A and S2A). Treatment of TPR cells with ct-CD45 or LPS did not lead to enhanced basal activation of NFκB, AP-1, or NFκB (Fig. 1C and Supporting Information Fig. S4), demonstrating that signaling induced by ct-CD45 requires TLR4.

#### Targeting of TLR4 inhibits ct-CD45-induced signaling

We then analyzed whether inhibitors that act via blocking the access of ligands to the TLR4 extracellular domain would influence ct-CD45-mediated reporter gene activation. For this purpose, a neutralizing TLR4 monoclonal antibody (W7C11) and oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC), an endogenous TLR4 ligand, were used. The oxidized phospholipid oxPAPC has been described to interfere with LPS signaling by competing with TLR4 accessory proteins, such as MD2, for receptor binding, thus, inhibiting LPS-mediated downstream signaling [15]. Both, W7C11 mAb and oxPAPC suppressed ct-CD45-induced NFκB activation (Fig. 2A), suggesting either...
Figure 1. ct-CD45 induces TLR4-dependent NFκB activation. (A) Jurkat TLR4 NFκB GFP reporter cells were treated with the indicated concentrations of ct-CD45. LPS (1 ng/mL) and PMA/Ionomycin (P/I, 100 nM) were used as positive controls. Reporter gene activation was determined 18 h after treatment via flow cytometry. Representative flow cytometry histograms and bar charts of pooled experiments are shown. Open histograms illustrate the fluorescence intensity of unstimulated reporter cells. Filled histograms demonstrate reactivity of reporter cells upon treatment. The gating strategy applied for these analyses is demonstrated in Supporting Information Fig. S5. (B) Jurkat TLR6, TLR2/6, TLR2/1, and TLR2/1/6 NFκB GFP reporter cells were treated with ct-CD45 at the indicated concentrations or with LPS or P/I as controls. Measurements were performed via flow cytometry as above, displaying bar charts from pooled experiments. (C) NFκB/AP-1/NFAT triple parameter reporter (TPR) cells, that do not express any TLR transgene, were mock stimulated with PBS or treated with ct-CD45 (20 μg/mL). Activation of NFκB (CFP), AP-1 (mCherry), and NFAT (eGFP) promoters was assessed 18 h after treatment via flow cytometry. (A-C) Error bars indicate mean ± SEM. *p < 0.05 and **p < 0.01 (two-tailed Student’s paired t-test). Only statistically significant differences are indicated. Data are pooled from (A) 5 (n = 5, except for condition ‘P/I’, where n = 3), (B) 3 (n = 3), and (C) 2 (n = 2) independent experiments with one flow cytometric measurement per experimental condition in each experiment.
Figure 2. ct-CD45-mediated NFκB activation is blocked by various inhibitors of TLR4 signaling. (A) Jurkat TLR4 NFκB GFP reporter cells were left unstimulated (PBS) or treated with ct-CD45 (20 μg/mL) and received in parallel medium (mock) or the TLR4 signaling inhibitors mAb W7C11 (5 μg/mL) or oxPAPC (30 μg/mL). (B) Reporter cells were preincubated with medium or CLI-095 at a final concentration of 1 μg/mL for 6 h then ct-CD45 was added at the indicated concentrations. (A,B) The cells were incubated for another 18 h. Reporter gene activation was analyzed by measuring mean fluorescence intensity (MFI) of the NFκB-induced eGFP reporter gene via flow cytometry. Data displays mean ± SEM. *p < 0.05, **p < 0.01, ns, not significant ([A] two-tailed Student’s paired t-test and [B] two-way ANOVA with Bonferroni’s post-test). (A, B) Data are pooled from 4 (n = 4, except condition ’W7C11’, where n = 3) independent experiments with one flow cytometric measurement per experimental condition in each experiment.

inhibition of ct-CD45 binding or interference with subsequent TLR4 activation.

To further verify that these effects were indeed TLR4-dependent, the reporter cells were pretreated with the small-molecule inhibitor CLI-095 (also known as TAK-242), which selectively inhibits signaling mediated via the TLR4 intracellular domain [16]. CLI-095 potently inhibited ct-CD45-mediated eGFP activation (Fig. 2B and Supporting Information Fig. S3A), indicating that TLR4 was required for ct-CD45 signaling.

ct-CD45 binds to TLR4

To analyze whether ct-CD45 can physically associate with TLR4, a modified in vitro TLR4 binding assay, which had been previously utilized for the characterization of other TLR4 ligands [17, 18] was implemented. In this assay system, ct-CD45 and the canonical TLR4 ligand LPS were coated to microtiter plates and were incubated with recombinant TLR4 protein. Bound TLR4 was then detected via the anti-TLR4 mAb W7C11 and alkaline-phosphatase-labeled anti-mouse IgG. OD, optical density (absorbance). (A,B) Error bars indicate mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 (one-way ANOVA with Bonferroni’s post-test). Only statistically significant differences are indicated. Data are pooled from (A) 4 (n = 4) and (B) 3 (n = 3) independent experiments.
The ct-CD45-TLR4 interaction can be blocked by the TLR4 antagonist oxPAPC. (A) Ct-CD45 (A) and (B) LPS were coated to 96-well microtiter plates at 5 μg/mL (52 nM) and 100 μg/mL (1.1 μM), respectively. For blocking, recombinant TLR4/LPS were coincubated either with oxPAPC (100 μg/mL), PTP inhibitor (100 μM), recombinant CD14 (5 μg/mL), or recombinant MD2 (5 μg/mL). (A,B) Bound TLR4 protein was detected using W7C11 and alkaline-phosphatase-labelled anti-murine IgG. PBS/HSA 1% was used as a negative control (100% binding) against which the effects of the inhibitors were compared. (A,B) Error bars indicate mean ± SEM. *p < 0.05 (two-tailed Student’s paired t-test). Only statistically significant differences are indicated. Data are pooled from five independent experiments (n = 5).

LPS does not interfere with ct-CD45-mediated inhibition of T-cell proliferation

While the stimulatory effects of TLR4 on innate immune cells are widely accepted, the analyses of direct TLR4 effects on T lymphocytes have yielded variable results and might be context dependent. For example, TLR4-deficiency in T cells was described to aggravate experimental colitis [10], while it was reported to be protective in an EAE model [19].

Since our prior results have demonstrated an inhibitory role for ct-CD45 on T cells [3, 4], we first sought to investigate if the canonical TLR4 ligand LPS negatively regulates T-cell activation in our system. Thus, primary human T cells were stimulated with plate-bound anti-CD3 and ct-CD45 immobilized via anti-His mAb in the presence of LPS or control (anti-His). T-cell proliferation was measured via methyl-3H thymidine incorporation and is displayed as relative proliferation rate (100% corresponding to control). Error bars indicate mean ± SEM. *p < 0.05, ns, not significant (two-tailed Student’s paired t-test). Data are pooled from four independent experiments using different T-cell donors per experiment (n = 4).

LPS-stimulated cells and controls (Fig. 5). Despite a lack of effect by LPS on T-lymphocyte growth, we tested if this TLR4 ligand affected ct-CD45-mediated suppression of T-cell activation. While ct-CD45 treatment leads to distinct inhibition of T-cell growth, no further enhancement of this effect was observed in the presence of LPS (Fig. 5), suggesting that ct-CD45 and LPS trigger different signaling routes via TLR4.

**TLR4 blockade antagonizes ct-CD45-mediated inhibition of T-cell activation**

Since experiments performed with reporter cells suggested TLR4 as the cellular receptor mediating ct-CD45 signal transduction, it was hypothesized that TLR4 blockade on primary T cells might reverse ct-CD45-induced suppression of T-cell growth. To test this, human T cells were activated with plate-bound CD3 in the presence of immobilized ct-CD45 and subsequently treated in parallel with either TLR4 neutralizing mAb or CLI-095. While ct-CD45 alone potently inhibited T-cell growth, addition of the TLR4 neutralizing mAb HTA125 significantly antagonized ct-CD45 effects, since T cells had enhanced proliferation rates compared to cells treated with control antibody (Fig. 6A). Inhibition of the TLR4 intracellular domain via CLI-095 also showed a tendency for improved T-cell growth (Fig. 6A), albeit the results were not statistically significant.

The capacity of other TLR4 inhibitors to reverse ct-CD45-mediated inhibition was also tested. Interestingly, it was found that the TLR4 neutralizing mAb W7C11 strongly abolished T-cell activation (Fig. 6B). In summary, these results further suggest that TLR4 can mediate suppressive effects on T cells independent of the canonical pathway induced by LPS.
of T-cell activation thresholds, since it preferentially suppresses immunoregulatory characteristics [4]. However, ct-CD45 is not a component of human plasma and appears to contribute to its acted as an inhibitor of T-cell activation. We found that ct-CD45 then indicated that the resulting cleavage fragment—ct-CD45—during the activation of human phagocytes [3]. Further studies CD45, which is the prototypic receptor-like PTP, as a target for RIP with potential biological activity [20]. Our group has identified served cellular process that generates soluble protein fragments regulated intramembrane proteolysis (RIP), an evolutionarily con-

Figure 6. ct-CD45-mediated inhibition of T-cell proliferation is antagonized by TLR4 blockade. Primary human T cells were preincubated with soluble anti-TLR4 mAb HTA125 (5 μg/mL), isotype control antibody (5 μg/mL), or the small-molecule inhibitor CLI-095 (100 ng/mL) for 1 h and were then stimulated with plate-bound CD3 or CD3/ct-CD45. (B) T cells were preincubated with soluble W7C11 mAb (5 μg/mL) or isotype control (5 μg/mL) and were subsequently stimulated with plate-bound anti-CD3. (A,B) T-cell proliferation was assessed by addition of methyl-3H-thymidine on day 3 and further cultivation of the cells for another 18 h. Results are displayed as relative proliferation rates (100% corresponding to [A] CD3 stimulation only and [B] to IgG1 isotype ctrl). Error bars indicate mean ± SEM. *p < 0.05, ns, not significant (two-tailed Student’s paired t-test). (A,B) Data are pooled from four independent experiments using different T-cell donors per experiment (n = 4).

Discussion

A substantial number of immune receptors are subject to regulated intramembrane proteolysis (RIP), an evolutionarily conserved cellular process that generates soluble protein fragments with potential biological activity [20]. Our group has identified CD45, which is the prototypic receptor-like PTP as a target for RIP during the activation of human phagocytes [3]. Further studies then indicated that the resulting cleavage fragment—ct-CD45—acted as an inhibitor of T-cell activation. We found that ct-CD45 is a component of human plasma and appears to contribute to its immunoregulatory characteristics [4]. However, ct-CD45 is not a mere inducer of T-cell anergy, but rather an extrinsic regulator of T-cell activation thresholds, since it preferentially suppresses T-cell activation induced via CD3/TCR (signal 1) but not in the presence of costimulation via CD28 (signal 2) [4]. This hypothesis is supported by the finding, that ct-CD45-treated T cells have gene expression profiles that are also found in quiescent lymphocytes, such as low expression of cyclins, but high levels of the CDK inhibitor p27kip1 or of the putative quiescence factors KLF2 and SLFN12 [4, 5]. However, the cell-surface receptor that initiates the biological functions of ct-CD45 in T cells has not yet been identified.

In this study, the idea that ct-CD45 effects could be mediated via this TLR was tested, particularly, as it appears to be a promiscuous receptor for several endogenous TLR ligands [8]. Using a reporter system based on the Jurkat T-cell line, it was possible demonstrate that TLR4 expression is required for ct-CD45-induced NFκB activation. In addition, reporter gene activation mediated via ct-CD45 could be inhibited using various TLR4 signaling inhibitors. Furthermore, ct-CD45 also did not activate NFκB and two other major transcription factors, AP1 and NFAT, in a TPR system expressing endogenous TLR5, but not TLR4. Interestingly, basal NFκB signaling has been shown to be necessary in naïve T lymphocytes for maintaining transcription of the IL-7 receptor α-subunit, which promotes the survival of these quiescent cells in vivo [21]. Thus, it is tempting to speculate that low-level TLR4 activation via plasma-derived ct-CD45 might also contribute to basal NFκB activation in human T cells and aid them in preserving their quiescent phenotype and responsiveness to antigenic stimulation.

TLRs are well known for their recognition of conserved PAMPs, which is a prerequisite for the activation of innate immune cells and the ensuing induction of adaptive immunity toward a pathogen [22]. However, an increasing number of endogenous TLR ligands have been identified, that can provide a mechanistic explanation for the induction and maintenance of sterile inflammatory responses such as those found in systemic inflammatory response syndrome or autoimmune disorders like RA [23]. Not all endogenous TLR ligands promote inflammation. The oxidized phospholipid oxPAPC is an endogenous antagonist of both TLR2 and TLR4 that appears to compete with TLR accessory proteins, which interact with the microbial ligands of these receptors [15]. Nevertheless, even agonistic signaling via TLR4 can be diverted leading to different functional outcomes. Prolonged stimulation with LPS is known to result in a state of tolerance, which is characterized by reduced expression of inflammatory cytokines both in vitro and in vivo [24]. This effect is dose-dependent and can be enhanced upon repeated administration of LPS. Furthermore, Piccinini and colleagues demonstrated that different TLR4 ligands may induce divergent signaling pathways leading to the expression of both overlapping and distinct gene products [25]. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products. In this study, it was found that the infectious stimulus LPS promotes the expression of both overlapping and distinct gene products.
may explain the differential functional outcomes observed for ct-CD45 and LPS treatment on human primary T cells.

While LPS had no effect on the growth of primary human T lymphocytes, ct-CD45 inhibited T-cell proliferation induced via CD3 stimulation, which could be partially inhibited by TLR4 blockade via the TLR4 mAb HTA125 or the small-molecule inhibitor CLI-095. Intriguingly, application of another TLR4 blocking antibody (W7C11) prevented the proliferative responses of human T cells independent of concomitant ct-CD45 treatment. These results are in line with a report demonstrating inhibition of T-cell activation by oxPAPC [26], which would suggest divergence of signaling pathways upon TLR4 engagement. TLR4 signaling has mainly been associated with the induction of inflammation, however, there have been reports describing an inhibitory role for TLR4 signaling in T cells. González-Navajas et al. [10] demonstrated that TLR4-deficiency aggravates a mouse model of experimental colitis in a T cell-dependent manner. Mechanistically, this observation could be explained by inhibitory tonic signaling of TLR4 mediated by commensal-derived LPS, which leads to inhibition of ERK 1/2 and the T-cell receptor signaling pathway [10]. Another report demonstrated that TLR4 signaling inhibited T-cell chemotaxis toward CXCL12 via the upregulation of suppressor of cytokine signaling 3 (SOCS3) [9, 12]. Nevertheless, it remains difficult to understand why LPS and ct-CD45 have different effects on T cells although both are activating NFκB. TLR4 signals through TRIF and MyD88. Our data obtained with the NFκB reporter suggest that ct-CD45 activates the MyD88 route. Recently, Adelaja et al. demonstrated that six distinct signaling codons convey discrete information to distinguish stimuli and enable appropriate responses [27]. It is intriguing that LPS and ct-CD45 trigger such distinct signaling codons and it will be interesting to explore this possibility in future studies.

LPS-mediated signaling via TLR4 involves several accessory proteins to form the TLR4 receptor complex [28]. LPS is initially bound in plasma by the LPS-binding protein and then transferred to CD14, which exists in soluble form as well as membrane-bound via GPI. Both LPB and CD14 are required to enhance the sensitivity of TLR4 toward LPS [29]. CD14 is also necessary for the endocytosis of TLR4 upon LPS-binding and initiation of TRIF-dependent signaling [30]. CD14 transfers LPS to the TLR4-associated protein MD2 and signaling is initiated by homodimerization of LPS/TLR4/MD2 complexes [31]. Thus, it could be possible that ct-CD45 is the ligand of one of these accessory molecules or interacts with the complex to signal via TLR4. The results of this study do not fully answer the question if ct-CD45/TLR4 interaction is direct or indirect which needs to be studied in further detail including surface plasmon resonance assays and binding studies to TLR4 on the surface of cells.

Moreover, TLR4 signaling might be even more complex than outlined above and involve additional interaction partners to enable signaling via different endogenous ligands. Recent studies suggest that TLR4 signaling involves recruitment of TLR4 and other accessory proteins, including, CD36 or CD44, to lipid rafts [32]. CD36 is a scavenger receptor that binds multiple microbial PAMPs, including LPS, but also endogenous ligands such as β-amyloid. It has been reported to associate with TLR4-TLR6 heterodimers and induce sterile inflammation upon recognition of β-amyloid or oxidized low-density lipoproteins [33]. CD44 has been described as a receptor for hyaluronan. Intriguingly, while CD44 seems to promote TLR4-dependent inflammation [34], it negatively regulates cytokine production induced via TLR4 or other TLR in the presence of bacterial PAMPs [35]. In the light of these findings, it appears plausible that ct-CD45 interacts with receptors engaged in lipid raft-mediated TLR4 signaling. Ct-CD45 might have parallels to soluble CD83, which was described to alter TLR4 signaling on monocytes by binding to the TLR4 coreceptor MD2 [36]. In this study, the association of soluble CD83 with the TLR4/MD2 complex depended on the coexpression of CD44v6 [36]. Thus, ct-CD45 binding to cells might correlate with the expression of an accessory protein that could enable high avidity binding to the TLR4 complex.

Ct-CD45 is present at high levels in human plasma during the steady state, but is significantly reduced in plasma derived from patients suffering from SLE or RA, which suggests that ct-CD45 contributes to the pathogenesis of these autoimmune diseases [4]. However, these results had to be interpreted with caution, since nearly the entire patient population studied was under treatment with potent immunosuppressive drugs [4]. Indeed, it was found that the reduction of ct-CD45 levels was correlated with certain immunosuppressive treatment regimens, since patients receiving methotrexate in the RA group or azathioprine in the SLE group had the lowest ct-CD45 levels [4]. TLR ligands both of microbial and endogenous origin have been implicated in the pathogenesis of SLE and RA [8]. Our present results indicate that ct-CD45 is a TLR4 agonist that potentially diverts TLR4 signaling pathways towards expression of immunoregulatory mediators. Thus, it is tempting to suggest that these patients might benefit from the exogenous administration of ct-CD45 as a therapeutic drug. In summary, we could demonstrate that ct-CD45 acts as an immunoregulatory factor on human T lymphocytes via noncanonical TLR4 activation. Thus, TLR4 signaling upon engagement with the endogenous ligand ct-CD45 controls TCR signaling.

### Material and methods

#### Media, reagents, and chemicals

Primary cells and Jurkat reporter cell lines were maintained in RPMI 1640 medium (Sigma, St. Louis, MO), supplemented with 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (Gibco, Grand Island, NY).

Lipopolysaccharide (LPS, ultrapure) from *Escherichia coli* as well as the TLR4 inhibitors, oxPAPC and CLI-095, were obtained from InvivoGen (Toulouse, France). The molecular weight of LPS was calculated according to Morrison and Leive [37]. The CD45 PTP inhibitor was from Calbiochem (Darmstadt, Germany). PBS was supplied from Lonza (Verviers, BE). 4-Nitrophenyl phosphate disodium salt hexahydrate was bought from Sigma Aldrich.
Human serum albumin contained within the pharmaceutical formulation Albomin was from Biotest (Dreieich, Germany).

The anti-6x-His tag mAb (4E3D10H2/E3) in unlabeled form as well as with an Alexa Fluor 647 conjugate was supplied by Thermo Fisher Scientific (Waltham, MA). The TLR4 (CD284) mAbs HTA125 and W7C11 were obtained from Thermo Fisher and InvivoGen, respectively. For cell surface stainings, goat anti-mouse IgG labeled with Alexa Fluor 647 (Jackson Immunoresearch, Newmarket, UK) and goat anti-mouse IgG conjugated to Oregon Green 488 (Thermo Fisher) were used as secondary reagents. Goat anti-mouse IgG with an alkaline phosphatase conjugate was acquired from Sigma Aldrich. The CD3 (OKT3) mAb for T-cell activation was obtained from Jansen–Cilag (Vienna, AT). All reagents used in this study were endotoxin free according to the information of the manufacturer.

The CD45 catalytic domain comprising the amino acids 632–1304 with an N-terminal His-tag was purchased from BPS Bioscience (San Diego, CA). The endotoxin level of the recombinant CD45 is less than 1 EU/µg. Recombinant human TLR4 protein (His-tagged) was supplied by Sino Biological (Beijing, PR. China). Recombinant CD14 and MD2 were bought from R&D Systems (Minneapolis, MN).

Primary cell isolation

Primary human T lymphocytes (CD3+) were isolated from buffy coats that were purchased from the Austrian Red Cross. Buffy coats were diluted at a 1:2 ratio in RPMI medium containing 10 U/mL heparin and total PBMC were isolated by standard density gradient centrifugation for 30 min (900 g, RT, no brake) via Ficoll–Paque Plus (GE Healthcare, Chalfont St. Giles, UK). Subsequently, T lymphocytes were purified using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were subjected to incubation with a cocktail of biotin-labeled antibodies to deplete remaining CD11b+, CD14+, CD16+, CD19+, CD33+, and MHC class II-positive cells by magnetic column-based separation. Bulk CD3+ T cells were collected in the flow through of isolation [38, 39].

Cell lines

Jurkat E6.1 TPR cells transduced with NFkB-CFP, NFAT-eGFP, and AP-1-mCherry constructs as well as the single reporter cell lines Jurkat NFkB-eGFP-TLR4/CD14, Jurkat NFkB-eGFP-TLR2/6, Jurkat NFkB-eGFP-TLR6, Jurkat NFkB-eGFP-TLR2/1, and Jurkat NFkB-eGFP-TLR2/1/6 were generated at our institute as previously described [14].

Reporter gene assays

Jurkat NFkB-eGFP-TLR4/CD14, Jurkat NFkB-eGFP-TLR2/6, TLR6, TLR2/1, and TLR2/1/6, and Jurkat TPR reporter cells were cultured in 24-well plates at densities between 5 and 8 × 10^4 cells/well. The cells were incubated with soluble ct-CD45 at concentrations ranging from 7.5 to 60 µg/mL (as indicated in the figure legends) for 18–20 h at 37°C, 5% CO2. In some experiments, Jurkat NFkB-eGFP-TLR4/CD14 cells were cotreated with CLI-095 (1 µg/mL or 100 ng/mL), W7C11 (5 µg/mL), or oxPAPC (30 µg/mL). Expression levels of fluorescent reporter genes under control of the respective transcription factors were analyzed by flow cytometry. Viable cells were gated according to their FSC/SSC characteristics (Supporting Information Fig. S5).

RNA isolation and qPCR

Total cellular RNA was isolated usingpeqGOLD TriFast reagent (peqLab, Erlangen, DEU). For isolation, 5 × 10^5 cells/mL were lysed in 500 µL of TriFast reagent and isolated according to the manufacturer’s protocol. A total of 1 µg of total RNA per sample was reverse transcribed using H-Minus-Reverse Transcriptase (Thermo Fisher Scientific Inc.) and oligo-dT18 primers. Quantitative real-time PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green qPCR master mix (Quanta Biosciences, Gaithersburg, MD) for detection. Detection was performed according to the manufacturer’s protocol. cDNA was amplified using a standard program (10 min at 95°C, 40 cycles of 15 s at 95°C/15 s at 60°C/45 s at 72°C).

GAPDH was used as an endogenous reference gene. Primers specific for TLR4, CD14, TLR2, and TLR1 were designed using Primer 3 Plus software and were custom synthesized by Sigma–Aldrich. Sequences were as follows: TLR4 fwd 5´-CGTGGAGGTGTTGCGGGATT TT-3´; CD14 fwd 5´-ACGCCGAACCTTGTGAGC-3´ and CD14 rev 5´-GGGAGGTTGTCGGGGATT TT-3´; TLR1 fwd 5´-TTGGCTGTGA CACTGACCTC-3´ and TLR1 rev 5´-ACTGGACACA CACTCTGAG-3´; TLR2 fwd 5´-GCTGTCCTGACGGTGACTGCTGTC-3´ and TLR2 rev 5´-CTTGTGACCTGCCCTGGAG-3´.

T-cell proliferation assays

For T-cell activation, 96-well MAXISORP Nunc-Immuno plates (Thermo Fisher Scientific) were coated either with CD3 (OKT3) mAb at 3 µg/mL or a combination of CD3 and anti-6x His tag mAb at 3 µg/mL each at 4°C overnight. Plates were washed twice with PBS. In assays using ct-CD45, recombinant protein (His-tagged) was added at the indicated concentration to CD3/anti-His precoated plates and was incubated for 4 h at 37°C in a humidified atmosphere (5% CO2). After a final washing step (2×), primary human T cells were added at 1 × 10^5 cells per well. For TLR4 blockade, the cells were preincubated with the anti-TLR4 mAbs HTA125 or W7C11 (5 µg/mL each), IgG2a isotype control antibody (5 µg/mL), the small-molecule inhibitor CLI-095 (100 ng/mL), or oxPAPC (30 µg/mL) for 1 h at 37°C before plating. In some experiments, LPS (5 µg/mL) was added in
soluble form to the wells as indicated. T cells were cultivated for 3 days and proliferation was determined by addition of 0.05 mCi methyl-3H-thymidine/well. After further culture for 18 h, the cells were lysed with deionized water and harvested onto filter plates (Millipore Corp., Bedford, MA). The plates were dried for 3 h at 37°C before addition of 25 μL/well MicroScint™-O cocktail (PerkinElmer, Waltham, MA) and were then counted on a Microbeta 2 scintillation counter (PerkinElmer). All assays were performed in triplicate.

ct-CD45-TLR4 binding assay

Ct-CD45 binding to TLR4 was analyzed using an assay system described previously [17, 18]. Ninety-six-well microtiter plates (Corning Life Sciences, Tewksbury, MA) were coated with 50 μL of LPS or ct-CD45 solution at the indicated concentrations overnight at 4°C. Afterwards, plates were washed five times and blocked with 50 μL/well of Albiomin solution (2% in PBS) overnight at 4°C. After washing, recombinant TLR4 His-tagged protein was added at 10 μg/mL and incubated for 2 h at room temperature. For blocking experiments, either of the following reagents (oxPAPC, CD45 PTP inhibitor, LPS, ct-CD45, rec. CD14, rec. MD2) were coinubated with TLR4 at the concentrations indicated in the figure legend. Unbound TLR4 was removed by washing. TLR4 binding was detected using anti-TLR4 mAb (W7C11, 2 μg/mL) and (after washing) subsequent incubation with anti-mouse IgG (1:500) conjugated to alkaline phosphatase. Both antibodies were incubated for 1 h at room temperature (RT). Bound antibody was visualized by adding 4-nitrophenyl phosphate disodium at 1 mg/mL for 40 min (RT) in the dark and measured on an ELISA microplate reader (Bio-Rad Laboratories).

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Statistical analysis: Data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) applying Student’s paired t-test or ANOVA with Bonferroni’s post-test as indicated in the respective figure legend. p values: *p < 0.05; **p < 0.01; ***p < 0.001.

Conflict of interest: Brian Crowe and Birgit Reipert were employees of Baxalta Innovations GmbH when the studies were performed and are stockholders of Takeda Pharmaceutical Company Limited. Alexander Puck, Sarojinidevi Künig, Madhura Modak, Lara May, Pia Fritz, Claire Battin, Katharina Radakovic, Peter Steinberger, and Johannes Stöckl are or have been employees of the Medical University of Vienna, Vienna, Austria and have no financial or commercial conflict of interest. All authors have read and approved the final version of the manuscript.

Author contributions: A.P., S.K., M.M., L.M., C.B., K.R., P.F. performed experiments and analyzed the data. P.S., B.M.R., B.A.C. provided essential reagents and gave experimental advice. J.S. supervised the research and analyzed data. A.P. and J.S. wrote the manuscript.

Data availability statement: All data that support the findings of this study are available from the corresponding author upon request.

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Abbreviations: CDK: cyclin-dependent kinase · ct-CD45: soluble cytoplasmic tail of CD45 · KLF2: Kruppel-like factor 2 · oxPAPC: oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine · PRAT4A: protein associated with TLR4 · PTP: protein tyrosine phosphatase · RA: Rheumatoid arthritis · RIP: regulated intramembrane proteolysis · RT: room temperature · SLFN12: Schlafen family member 12 · TPR: triple parameter reporter