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Phosphoinositide 3-kinase (PI3K) and nutrient sensing mTOR (mammalian target of rapamycin) pathways control T lymphocyte trafficking

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Summary

PI3K and mTOR are evolutionarily conserved regulators of cell metabolism. Here we show PI3K and mTOR determine the repertoire of adhesion and chemokine receptors expressed by T lymphocytes. Key lymph node homing receptors, CD62L (L-selectin) and CCR7, are highly expressed on naive T lymphocytes but downregulated following immune activation. CD62L downregulation occurs via ectodomain proteolysis and suppression of gene transcription. PI3K p110δ controls CD62L proteolysis via mitogen-activated protein (MAP) kinases whereas PI3K p110δ control of CD62L transcription is mediated by the nutrient sensor mTOR via regulation of the transcription factor KLF2. PI3K-mTOR nutrient sensing pathways also determined expression of the chemokine receptor CCR7 and regulate lymphocyte trafficking *in vivo*. Hence, lymphocytes utilize PI3K and mTOR to match metabolism and trafficking.

Keywords

CD62L; L-selectin; CCR7; PI3K; p110δ; rapamycin; MAP kinases; KLF2

Introduction

The lipid product of phosphoinositide 3-kinases (PI3Ks), phosphoinositide (3,4,5) tri-phosphate ($PI(3,4,5)P_3$), binds to the pleckstrin homology (PH) domains of proteins and controls an array of signaling molecules including serine or threonine kinases such as PKB (also known as AKT), Tec family tyrosine kinases and guanine nucleotide exchange proteins

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Author Contributions

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Competing Interests Statement

The authors declare no competing financial interests.

for Rac and Rho-family GTPases[1]. T cell activation induces rapid and sustained production of PI(3,4,5)P_3 in a response that is essential for T cell immune responses[2-4]. One important and evolutionarily conserved role for PI3Ks in T cells is to regulate cell metabolism and protein synthesis[5]. However, PI3Ks also regulate actin dynamics[6] and accordingly there has been a past focus on the role of PI3Ks in regulating leukocyte motility and chemotaxis[7-10]. The impetus for these studies came from the realization that the correct localization of lymphocytes is essential for effective immune responses. Thus, understanding the molecular mechanisms and signal transduction pathways that control lymphocyte trafficking is important.

Naive T lymphocytes constantly circulate around the body via the blood, lymphatics and secondary lymphoid organs. Immune responses are initiated within secondary lymphoid organs, such as peripheral lymph nodes, when T cells encounter primed antigen presenting cells expressing cognate antigen-major histocompatibility complexes together with appropriate costimulatory molecules. Lymph node entry requires the coordinated migration of cells and is dependent on chemokine receptors such as CCR7 and molecules that mediate lymphocyte adhesion such as CD62L (L-selectin) and integrins11›12. T cell entry into lymph nodes from the blood occurs in specialized high endothelial venules (HEVs). The first step of transmigration is CD62L mediated tethering and rolling of naive lymphocytes on the endothelium of HEVs12›13. CD62L, which is highly expressed constitutively on naive and memory T lymphocytes12 is thus essential for the entry of these cells into peripheral lymph nodes14›[15].

Immune activation of T cells induces striking changes in their migratory patterns. Effector T lymphocytes migrate to a greater extent to non-lymphoid tissues and sites of inflammation and have a reduced capacity to home to peripheral lymph nodes compared to naive and memory T cells[12,16]. Changes in the trafficking behavior of T cells are important for immune responses and are mediated by changes in the expression of chemokine receptors and adhesion molecules. For example, activated T cells downregulate CCR7 and CD62L but upregulate expression of tissue homing receptors such as the integrins VLA-4 and cutaneous lymphocyte associated antigen[17]. CD62L expression at the membrane is controlled by a balance of two activities – the rate of *CD62L* gene transcription and the rate of CD62L proteolytic cleavage[18-20]. The proteolytic cleavage and shedding of CD62L from the cell surface of T lymphocytes is an immediate response to triggering of antigen receptors[21] whereas *CD62L* gene transcription is lost following cytokine controlled differentiation of effector T cells[18,22]. This transcriptional loss of CD62L is frequently used as a marker to distinguish naive and antigen experienced T cells in the blood and lymphoid organs during immune responses. Effector and effector memory $CD8^+$ cytotoxic T lymphocytes (CTLs) are $CD62L^{lo}$ and preferentially home to peripheral tissues whereas central memory CTLs express high amounts of CD62L similar to naive T cells and home to lymph nodes[12,16].

The signal transduction pathways that dynamically modulate CD62L and CCR7 expression during immune activation of T cells are not characterized. However, the differing abilities of the cytokines interleukin 2 (IL-2) and IL-15 to downregulate CD62L expression in $CD8^+$ T cells[23,24] parallels the relative strength of these cytokines to stimulate PI3K-mediated cell growth responses[25]. Antigen-primed $CD8^+$ T cells cultured in the presence of IL-2 thus sustain PI3K signaling at high levels, synthesize high amounts of protein and downregulate CD62L. In contrast, T cells maintained with IL-15 can only sustain low-level PI3K signaling, synthesize less protein and fail to downregulate CD62L expression24›[25]. These data are purely correlative, however the idea that PI(3,4,5)P_3 abundance might control expression of T cell homing receptors is intriguing because there is a large body of work that link PI3Ks to the control of lymphocyte metabolism, cell growth, chemotaxis and chemokinesis[7,8,10]. However, it has never been considered that PI3Ks might regulate

lymphocyte recirculation by regulating the plasma membrane expression of lymph node homing receptors CD62L and CCR7. Historically, loss of CD62L and CCR7 expression is used to discriminate naive and antigen-experienced T cells. The present study shows that CD62L and CCR7 expression rather report the PI3K and mTOR {A000094} http://www.signaling-gateway.org/molecule/query?afcsid=A000094 signaling status of a T cell and are not simply an epigenetic consequence of immune activation. PI(3,4,5)P_3 and mTOR signaling are thus shown to be essential determinants for CD62L and CCR7 expression and to be key regulators of T cell migration *in vivo*.

Results

PI3K regulates T cell receptor-induced CD62L shedding

Triggering of the T cell receptor (TCR) complex with cognate antigen-major histocompatibility complexes induced rapid downregulation of CD62L from the cell membrane of naive T cells (Fig. 1a). This initial acute loss of CD62L surface expression following immune activation of T cells is predominantly mediated by proteolytic cleavage[21,26]. Resting T cells exhibited basal constitutive cleavage of CD62L but during immune activation accelerated proteolysis occurred, resulting in increased amounts of the soluble cleavage product of CD62L in the culture supernatants of TCR-activated T cells (Fig. 1b). The acute cleavage of CD62L takes place proximal to the cell membrane and is mediated by a disintegrin and metalloprotease (ADAM) 17, also known as tumor necrosis factor (TNF)— converting enzyme (TACE)[27]. Mutation of the ADAM17 target sites in CD62L thus prevents CD62L shedding21›26. To confirm the contribution of proteolytic cleavage of CD62L in TCR-mediated downregulation we examined the ability of TCR triggering to downregulate the expression of a mutant of CD62L that has the membrane proximal region of CD62L replaced with that of P-selectin, thus removing the proteolytic target sequences for ADAM17. Crosslinking of TCR complexes with CD3 antibodies downregulated cell surface expression of CD62L on wild-type T cells and also downregulated expression of wild-type CD62L expressed under the control of the *hCD2* promoter in CD62L-deficient T cells (CD62L(WT))(Fig. 1c). However, TCR triggering did not downregulate surface expression of CD62L molecules with mutated proteolytic target sequences (CD62L(MUT))(Fig. 1c).

To test the involvement of PI3K in TCR-induced shedding of CD62L, we performed experiments with LY294002, a pharmacological inhibitor of PI3K. LY294002 suppressed the shedding of CD62L induced by T cells activated with cognate peptide-MHC complexes or crosslinking CD3 antibodies (Fig. 1d). Class 1 PI3Ks comprise a p110 catalytic subunit and an adapter regulatory subunit. Four p110 isoforms exist (p110α, p110β, p110γ and p110δ) and three adapter subunits, p85α, p85β and p55γ[28].

In T cells, the p110δ PI3K

PI3K p110 delta {A001770}

http://www.signaling-gateway.org/molecule/query?afcsid=A001770 catalytic subunit produces the PI(3,4,5)P_3 that is generated in response to TCR triggering3›29›30. We therefore examined CD62L regulation in T cells where homologous recombination has been used to substitute wild-type p110δ for a catalytically inactive mutant p110δ(D910A)[29]. T cells from these mice fail to produce PI(3,4,5)P_3 in response to TCR triggering[3]. T cells expressing p110δ(D910A) failed to downregulate CD62L in response to TCR triggering (Fig. 1e) although these cells shed CD62L in response to the pharmacological stimulus phorbol 12,13, dibutyrate (PDBu), which bypasses the TCR.

CD62L transcription is suppressed by PI3K p110δ

Low CD62L expression in effector cytotoxic T cells reflects low gene transcription[22]. To look at the involvement of PI3K in the regulation of CD62L mRNA expression we examined the impact of PI3K inhibition on CD62L expression in $CD8^+$ cytotoxic T cells. P14 TCR transgenic T cells that express a $V_\alpha 2V_\beta 8.1$ TCR were primed for two days with the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) peptide, gp33-41 (KAVYNFATM), presented by the MHC class I molecule, $H\text{-}2D^b$. Thereafter cells were cultured in IL-2 to generate cytotoxic effector T cells (CTLs). As controls, antigen-primed T cells cultured with IL-15 were used. IL-15 is a potent mitogen and sustains exponential T cell clonal expansion of antigen-primed $CD8^+$ T cells but does not support differentiation of effector cytotoxic T cells, rather it generates cells that phenocopy memory T cells[23,24]. $CD8^+$ T cells cultured with IL-2 expressed low amounts of CD62L whereas cells cultured with IL-15 were $CD62L^{hi}$ (Fig. 2a). These differences in CD62L expression reflected differences in cellular abundance of its mRNA, which was low in CTLs (Fig. 2b). Yet CTLs did not globally downregulate all surface receptors. CD25, the α subunit of the IL-2 receptor is highly expressed in CTLs, reflecting abundant expression of CD25 mRNA in CTLs (data not shown). We found no evidence for increased shedding of CD62L in IL-2 stimulated cells, rather there was less CD62L proteolytic cleavage in T cells cultured with IL-2 versus IL-15 reflecting the much lower *de novo* synthesis of CD62L in IL-2-stimulated T cells (Fig. 2c).

IL-2 and IL-15 differ in the strength of the PI3K signal they can induce[25]. Antigen-primed T cells maintained in IL-15 have a low cellular content of $PI(3,4,5)P_3$ (120,000 molecules per cell) compared to 540,000 molecules per cell in cells stimulated with IL-2 and show reduced activation of PI3K mediated signal transduction pathways (Supplementary Fig. 1 online). The ability of IL-2 to downregulate CD62L thus correlates with its ability to strongly activate PI3K signaling pathways. To probe the role of PI3K in IL-2 downregulation of CD62L, antigen-primed T cells were cultured with IL-2 in the presence or absence of the PI3K inhibitor LY294002. Effector $CD8^+$ T cells cultured with IL-2 expressed low amounts of CD62L protein and mRNA whereas effector T cells cultured in IL-2 plus LY294002 were $CD62L^{hi}$ and expressed abundant CD62L mRNA (Fig. 2d,e). PI3K p110δ is required for antigen receptor function but not for IL-2 induced proliferation of T cells29›30. Hence, p110δ(D910A) T cells show attenuated proliferation in response to TCR stimulation, this can be overcome when activated through the TCR in the presence of strong co-stimulation provided by CD28 and in the presence of IL-2 (ref. 29). p110δ(D910A) $CD8^+$ T cells thus responded to IL-2 stimulation and proliferated exponentially over a period of 4 days when cultured in IL-2 to produce large granular cells indistinguishable from wild-type T lymphoblasts (Fig. 2f). PI3K-PKB pathways regulate the expression of two key nutrient receptors in T cells, namely, CD71, the transferrin receptor and CD98, a critical component of L-type amino acid transporter complexes25›31.The expression of CD71 and CD98 was comparable on wild-type and p110δ(D910A) T cells cultured in IL-2 (Fig. 2g). Yet IL-2-stimulated p110δ(D910A) T cells maintained high surface expression of CD62L as compared to the low amounts of CD62L seen in control CTL populations (Fig. 2g). These data revealed a selective role for p110δ in IL-2 signal transduction in that it is required for IL-2-mediated downregulation of CD62L but not for IL-2-induced expression of nutrient receptors, mitogenesis or cell growth. Further evidence for the selective effect of p110δ loss on CD62L expression in T cells comes from analyzing the expression of P-selectin and E-selectin ligands in IL-2-stimulated p110δ(D910A) $CD8^+$ T cells. The upregulation of P-selectin and E-selectin ligands normally accompanies effector T cell differentiation[12]. IL-2 stimulated p110δ(D910A) $CD8^+$ T cells expressed high amounts of P-selectin and E-selectin ligands, equivalent to those seen in control CTLs (Fig. 2h). Thus, p110δ selectively controls expression of L but not E or P selectin ligands in T cells.

Different PI3K pathways control CD62L expression

TCR stimulated p110δ(D910A) T cells have multiple signaling defects including reduced activation of the MAP kinases Erk1 and Erk2 (ref. 29). This latter defect could be relevant to the PI3K dependence of TCR-induced CD62L shedding because chemokine-induced shedding of CD62L in neutrophils has been shown to be mediated by Erks (ref. 32) which phosphorylate ADAM17 and control its trafficking to the cell surface[33-35]. We accordingly explored the role of Erk1 and Erk2 in TCR-induced shedding of CD62L. Down regulation of CD62L in antigen stimulated P14 $CD8^+$ T cells was blocked by the inhibitor PD184352 (Fig. 3a), which prevents activation of the upstream kinase MEK1 and hence Erk activation. The inhibition of PI3K and Erks also blocked downregulation of CD62L in $CD4^+$ and $CD8^+$ T cells polyclonally activated with CD3 antibodies (Supplementary Fig. 2 online). We also examined the role of Erks in the transcriptional downregulation of CD62L that occurs in effector CTLs. Inhibition of Erk activation with PD184352 did not prevent CD62L downregulation in IL-2 maintained CTLs (Fig. 3b).

PI3K initiates signaling mediated by the mammalian target of rapamycin, mTOR kinases which are evolutionarily conserved serine-threonine kinases that play a central role in integrating signals from nutrients (amino acids and energy) to regulate cell growth and cell cycle progression[36]. We therefore assessed the impact of rapamycin, which inhibits the mTOR-raptor complex, on CD62L expression. TCR-induced downregulation of CD62L on primary $CD8^+$ T cells was not blocked by rapamycin (Fig. 3c). In contrast, downregulation of CD62L surface expression in CTLs was rapamycin sensitive. Treatment of CTLs cultured with rapamycin prevented IL-2-induced downregulation of CD62L mRNA and treated cells showed increased surface expression of CD62L (Fig. 3d,e). Note rapamycin did not globally change the expression of activation markers as the expression of CD44 was unaffected by rapamycin treatment (Fig. 3f). PI3K thus appears necessary for acute proteolytic cleavage of CD62L because of its role in regulating MAP kinases whereas PI3K regulates CD62L transcription via mTOR.

The transcription factor KLF2 has been shown to be a key modulator of the expression of homing receptors involved in the regulation of lymphocyte migration[37-39]. Previous studies have identified that KLF2 directly regulates CD62L transcription by binding to the *CD62L* promoter and is both necessary and sufficient for expression of CD62L[38,39]. We therefore examined whether the PI3K-mTOR sensitivity of *CD62L* transcription reflects a role for these signals in controlling expression of KLF2. We performed real-time PCR analysis of KLF2 mRNA in antigen-primed $CD8^+$ T cells cultured in IL-15 or maintained in IL-2 (CTLs) and cultured in the presence or absence of LY294002 or rapamycin (Fig. 4a-c). KLF2 mRNA abundance was low in effector CTLs but greatly increased in CTLs treated with the PI3K inhibitor LY294002 (Fig. 4b). KLF2 mRNA expression was also strikingly increased in CTLs treated with rapamycin (Fig. 4c). The expression of KLF2 in CTLs was thus negatively regulated by PI3K and mTOR. Activation of PI3K and mTOR induced loss of KLF2 and subsequently prevented expression of KLF2 target genes such as *CD62L*. Another KLF2 gene target that has been identified encodes the sphingosine 1 phosphate receptor 1($S1P_1$) which controls lymphocyte egress from secondary lymphoid organs[37-39]. IL-15-treated $CD8^+$ T cells that have lower PI3K signaling, showed increased $S1P_1$ mRNA expression as compared to IL-2-treated $CD8^+$ T cells (Fig. 4d). Moreover, treatment of IL-2-treated $CD8^+$ T cells with the PI3K inhibitor LY294002 (Fig. 4e) or the mTOR inhibitor rapamycin (Fig. 4f) resulted in increased expression of the KLF2 gene target *S1P1* (refs. 38, 39).

Loss of PTEN downregulates CD62L

The above results showed that PI3K signals were required for downregulation of CD62L but did not establish whether they were sufficient. $PI(3,4,5)P_3$ is normally dephosphorylated by the $PI(3,4,5)P_3$ 3′ phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) to produce $PI(4,5)P_2$. The loss of PTEN in T cells results in accumulation of $PI(3,4,5)P_3$ and activation of downstream targets[40]. Accordingly, to assess whether $PI(3,4,5)P_3$ production was sufficient to downregulate CD62L we looked at the surface phenotype of T cells following deletion of PTEN. In these experiments, T cell-specific *Pten*-deficient mice (PTEN KO(T)) were created by backcrossing mice with PTEN alleles floxed by *lox*P cre excision sequences to *Lck*-Cre transgenic mice that express the Cre recombinase selectively in T cells[40]. CD62L expression is upregulated during thymus development with the highest CD62L expression found on CD4 and CD8 single-positive (SP) thymocytes (Fig. 5a). Strikingly, CD62L expression in PTEN KO(T) thymocytes was reduced compared to wild-type controls. PTEN KO(T) double-positive (DP) thymocytes thus expressed lower amounts of CD62L than wild-type DP cells (Fig. 5a). PTEN KO(T) SPs were also $CD62L^{lo}$ (Fig. 5a). The loss of CD62L was seen in both $CD4^+$ and $CD8^+$ SPs and in peripheral $CD4^+$ and $CD8^+$ PTEN KO(T) cells (Fig. 5a and Supplementary Fig. 3 online). The impact of PTEN loss on CD62L expression was mediated by changes in CD62L mRNA expression, which was lower in PTEN KO(T) versus wild-type thymocytes (Fig. 5b). We observed no increase in CD62L shedding in PTEN KO(T) thymocytes that might have accounted for the deceased CD62L expression (Fig. 5c). Rather PTEN loss appeared to result in decreased shedding of CD62L in thymocytes. However the failure of PTEN KO(T) thymocytes to effectively produce CD62L mRNA and protein makes it impossible to make any firm conclusions as to whether the increased production of $PI(3,4,5)P_3$ as a result of PTEN deletion would be sufficient to directly regulate proteolytic cleavage of CD62L. The impact of PTEN loss on CD62L expression was also seen in activated peripheral T cells. Antigen receptor activated T cells cultured in IL-15 normally express high amounts of CD62L whereas antigen primed PTEN KO(T) cells cultured in IL-15 have low levels of CD62L(Fig. 5d), reminiscent of the low expression of CD62L on IL-2 cultured T cells. Low amounts of CD62L expressed on the surface of $CD8^+$ PTEN KO(T) cells cultured in IL-15 was not due to increased shedding of surface CD62L (Fig. 5e), rather these changes in CD62L expression were reflected by the expression of CD62L mRNA, which was downregulated in PTEN KO(T) IL-15 cultured T cells (Fig. 5f).

Consistent with the notion that KLF2 directly regulates *CD62L* expression, the loss of CD62L in PTEN KO(T) cells correlated with downregulation of KLF2 mRNA (Fig. 5g). KLF2-deficient thymocytes show several defects in expression of T cell trafficking molecules and one consequence of these defects is that mature T cells accumulate in KLF2 null thymi because they fail to exit to peripheral tissues[39]. PTEN KO(T) thymocytes had a markedly increased frequency of mature SP cells (Fig. 5h), as defined by low expression of CD24 (HSA), indicating that PTEN deletion resulted in retention of mature T cells in the thymus. Hence, activation of PI3K signaling immediately derails lymphocyte trafficking *in vivo*.

PI3K-mTOR signaling controls expression of CCR7

The chemokine receptor CCR7 is key for the coordinated migration of T cells into secondary lymphoid organs and also controls their motility and positioning within lymphoid tissues[12]. Immune activation of T cells results in downregulation of CCR7 expression, which is important for ensuing T cell immune responses. For example, downregulation of CCR7 during an antiviral immune response is important for virus clearance because it facilitates the release of effector CTLs from the splenic white pulp and promotes their migration to peripheral tissues[41]. The transcription factor KLF2 can regulate *Ccr7*

expression and KLF2-deficient thymocytes simultaneously lose expression of CD62L and CCR7 (ref. 39). Accordingly, the ability of PI3K and mTOR signaling pathways to downregulate expression of KLF2 might also have consequences for CCR7 expression. To examine this possibility we compared CCR7 expression on antigen-primed $CD8^+$ T cells cultured in IL-15 that express abundant KLF2 versus $CD8^+$ T cells cultured in IL-2 that expressed much less KLF2. Antigen-primed $CD8^+$ T cells cultured in IL-2 downregulate CCR7 expression whereas cells cultured in IL-15 remained CCR7 high (Fig. 6a). Strikingly, antigen-primed $CD8^+$ T cells cultured with IL-2 in the presence of the PI3K inhibitor LY294002 failed to downregulate CCR7 (Fig. 6b) indicating that the loss of CCR7 in these cells was dependent on PI3K. Additional experiments revealed the production of $PI(3,4,5)P_3$ was sufficient to downregulate CCR7 expression. Hence, CCR7 expression was lost in $CD8^+$ PTEN KO(T) cells even when cultured with IL-15 (Fig. 6c). We next examined the role of mTOR in regulating CCR7 expression. Antigen-primed $CD8^+$ T cells cultured with IL-2 in the presence of the mTOR inhibitor rapamycin failed to downregulate CCR7 (Fig. 6d). Importantly, the CCR7 receptors expressed on the T cells cultured in either rapamycin or LY294002 were functional (Fig. 6e). Antigen-primed $CD8^+$ T cells cultured in IL-2 (effector CTLs) lost the capacity to chemotax in response to the CCR7 ligand CCL19 whereas rapamycin or LY294002 treated cells retained their ability to chemotax in response to CCL19 (Fig. 6e).

The loss of CD62L and CCR7 by activated T cells is an important mechanism that prevents effector T cells re-entering secondary lymphoid organs and facilitates their redirection to peripheral tissues. The ability of mTOR inhibitors to prevent loss of CD62L and CCR7 would allow immune-activated T cells to continue to traffic to secondary lymphoid tissues and hence dilute their recruitment to peripheral tissues as required for these cells to exert their effector function. To test this hypothesis *in vivo*, adoptive transfer experiments were performed comparing the ability of control effector cytotoxic T cells (CTLs) or rapamycin-treated CTLs to home to secondary lymphoid tissues. In these experiments, $CD8^+$ P14 T cells were activated using specific peptide gp33 for 2 days, followed by 2 days culture in IL-2 in the presence or absence of rapamycin. IL-2-stimulated (as controls) or IL-2 plus rapamycin-stimulated $CD8^+$ T cells were labeled with either CFSE (carboxyfluoroscein succinimidyl ester) or CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine), mixed at a ratio of 1:1 and transferred into C57BL/6 hosts. After 24 hours the mice were sacrificed and tissue was analyzed for the presence of the transferred cells. Striking differences were seen in the *in vivo* trafficking behavior of rapamycin-treated and control CTLs (Fig. 6f). The rapamycin-treated cells retained the ability to home to secondary lymphoid organs compared to control CTLs and hence accumulated in lymph nodes and spleen. Thus, mTOR controls the trafficking patterns of effector CTL.

Discussion

The present study identifies that $PI(3,4,5)P_3$ and mTOR mediate antigen receptor- and cytokine-induced downregulation of CD62L and CCR7, two crucial molecules that regulate lymphocyte recirculation. Inhibition of PI3K prevented both the proteolytic cleavage pathways and transcriptional mechanisms that down-modulate CD62L expression in activated T cells. Elevation of cellular $PI(3,4,5)P_3$, via deletion of PTEN, induced loss of CD62L and CCR7 expression and immediately derailed lymphocyte trafficking *in vivo*. PI3K and mTOR were also essential for CCR7 downregulation by effector T cells. $PI(3,4,5)P_3$ and mTOR are signaling molecules more usually associated with the control of T cell metabolism. In particular, mTOR has an evolutionary conserved role as a nutrient sensor and functions to match cell growth to a cell's metabolic capacity and nutrient availability. The ability of mTOR to control CD62L and CCR7 expression and *in vivo*

trafficking of CTLs indicates that mechanisms have evolved to synchronize lymphocyte trafficking to nutrient availability and hence cellular energy status.

How important is modulation of CD62L and CCR7 expression during T cell immune responses? It is well known that changes in cell surface CD62L and CCR7 expression can substantially modify lymphocyte recirculation patterns and have a major impact on immune responses[15,41,42]. The transcriptional downregulation of CD62L during immune activation serves to prevent activated T cells re-entering peripheral lymph nodes thereby favoring their migration to peripheral tissues. The acute downregulation of CD62L in T cells via endoproteolytic cleavage has biological significance as blocking CD62L cleavage on T cells perturbs their recirculation and inhibits antiviral T cell responses[21,26,43]. The loss of expression of CCR7 by T cells after immune activation is equally important as continued expression of CCR7 favors retention of effector T cells to secondary lymphoid tissues rather than allowing their relocation to peripheral tissues where the cells exert effector function[41].

There are multiple isoforms of PI3K but the p110δ catalytic subunit of PI3K is shown herein to be the important isoform for CD62L regulation in T cells. Previous studies have shown that the p110δ isoform is predominant in TCR-PI3K signaling. Accordingly, the fact that p110δ is the TCR-coupled PI3K that controls CD62L expression is unsurprising as loss of p110δ prevents $PI(3,4,5)P_3$ production in antigen stimulated T cells[3]. It was however unexpected that IL-2 regulation of CD62L expression would be dependent on p110δ. Hence, although PI3K activity is essential for IL-2 signal transduction, p110δ(D910A) T cells grow and proliferate normally in response to IL-2. It was therefore concluded that other PI3K isoforms such as p110α or p110γ mediate IL-2 signal transduction. The fact that IL-2 downregulation of CD62L expression is dependent on p110δ affords the insight that the IL-2 receptor is coupled to multiple isoforms of PI3K that mediate distinct downstream functions.

$PI(3,4,5)P_3$ modulates the activity of several different signal transduction pathways including those mediated by MAP kinases, Erk1 and Erk2, and mTOR. A salient finding was that both these PI3K regulated signaling pathways control CD62L expression. TCR-induced proteolytic cleavage of CD62L was shown to be dependent on TCR-PI3K induced activation of Erk1 and Erk2 whereas a PI3K-mTOR pathway controlled CD62L and CCR7 expression by regulating the cellular abundance of KLF2, a key transcription factor for CD62L and CCR7. The PI3K-mTOR-KLF2 pathway also controlled expression of $S1P_1$ which controls egress of lymphocytes from secondary lymphoid organs. The importance of MAP kinases for the regulation of cytokine gene transcription during T cell activation is known. A role for MAP kinases in regulating the proteolytic cleavage of CD62L gives new insight that MAP kinases also control T cell trafficking. Similarly, the mTOR inhibitor rapamycin is used clinically as an immunosuppressant but it has not been appreciated that regulation of lymphocyte trafficking might contribute to its clinical efficacy. It was originally thought that rapamycin suppressed immune responses because of the evolutionarily conserved role for mTOR kinases as nutrient sensors that regulate protein synthesis and cell cycle progression of T cells[5,44]. More recently, it has been suggested that mTOR inhibition promotes the generation of regulatory T cells that suppress immune responses[45,46]. The present demonstration that rapamycin prevents downregulation of CD62L, CCR7 and $S1P_1$ receptor expression and controls lymphocyte trafficking *in vivo* gives new insight about how rapamycin modulates immune responses. The ability of rapamycin to redirect activated effector T cells to secondary lymphoid organs could result in destruction of antigen-primed dendritic cells and hence prematurely terminate immune responses[47]. The containment of activated cytotoxic T cells within secondary lymphoid organs would also prevent immune destruction of target cells in peripheral tissues. Moreover, secondary lymphoid organs provide the cytokine and stromal stimuli that promote T cell metabolism and survival. The use of common signaling pathways to control

T cell metabolism and expression of lymph node homing receptors would ensure that during immune responses lymphocytes match metabolic competence to migration patterns.

Methods

Mice and cells

P14 LCMV TCR transgenic mice[48] and C57BL/6 (wild-type) mice were bred and maintained in the WTB/RUTG, University of Dundee in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines. CD62L null mice expressing transgenes encoding either a non-cleavable mutant CD62L (CD62L(MUT)), or wild-type cleavable CD62L (CD62L(WT)) under the control of the *hCD2* promoter have been described[26]. These mice have T lymphocyte restricted expression of either wild-type CD62L or a non-cleavable CD62L mutant that was generated by replacing the membrane proximal region (MPR) of wild-type CD62L with the MPR of P-selectin, thus removing the proteolysis site of CD62L. Mice containing a knockin mutation of PI3K wherein wild-type alleles of the p110δ catalytic subunit of PI3K were substituted with a point mutation (D910A), which is a catalytically inactive form of p110δ, p110δ(D910A), have been described[29]. *PTEN*$^{fl/fl}$ *Lck*-Cre$^{+/-}$ (PTEN KO(T)) mice were generated as described[40] by crossing mice with floxed PTEN alleles with mice expressing Cre recombinase under the control of the proximal p56^{lck} promoter (*Lck*-Cre$^{+/-}$).

To activate primary T cells, spleens and/or lymph nodes were removed, disaggregated and red blood cells lysed. Cells were cultured in RPMI 1640 containing L-glutamine (Invitrogen), heat-inactivated 10% FBS (Gibco), 50 μM β-mercaptoethanol (β-ME, Sigma) and penicillin/streptomycin (Gibco). Single cell suspensions from lymph node preparations or splenocytes were adjusted to 2×10^6 or 5×10^6 cells/ml respectively. Cells from non-TCR transgenic mice were stimulated with 5 μg/ml of the CD3 monoclonal antibody (2C11) to trigger the TCR. Cells isolated from P14 LCMV mice were stimulated with soluble LCMV specific peptide gp33-41 (1 nM) (gp33-41 KAVYNFATM was synthesized by the CRUK protein production laboratory, London). Where indicated, phorbol 12,13-dibutyrate (PDBu, Calbiochem) was used at a final concentration of 20 ng/ml. Where indicated, cells were incubated with specified kinase inhibitors. The PI3K inhibitor LY294002 (Promega) was used at a final concentration of 10 μM, the mTOR inhibitor, rapamycin (Calbiochem), was used at a final concentration of 20 nM, the MEK inhibitor PD184352 (synthesized in-house by the Division of Signal Transduction Therapy, Dundee) was used at a final concentration of 2 μM. Cells were incubated at 37 °C with 5% CO_2 throughout for indicated times. To generate lymphoblasts, murine CD8$^+$ T cells were grown from spleen or lymph node preparations cultured for 48 h in the presence of the stimulus (either 2C11 or gp33-41 peptide), washed and resuspended at 2×10^5 cells/ml with cytokines at a final concentration of 20 ng/ml IL-2 (Chiron) or 20 ng/ml IL-15 (Peprotech). To generate p110δ(D910A) lymphoblasts, p110δ(D910A) splenocytes were stimulated using 5 μg/ml of the CD3 monoclonal antibody (2C11) in the presence of 20 ng/ml IL-2 for 48 h, washed and resuspended at 4×10^5 cells/ml with 20 ng/ml IL-2. Where indicated, SP CD4$^+$ T cells and CD4$^+$CD8$^+$ DP T cells were sorted from the thymi of PTEN KO(T) and wild-type littermate controls using a FACS Vantage cell sorter with Diva upgrade (Becton Dickinson).

Flow cytometry

Fc receptors were blocked using Fc Block (BD Pharmingen) in RPMI, 0.5% FBS for 15 min at 4 °C. 100 μl of cells at a concentration of 1-2 × 10^7 cells/ml were then stained with saturating concentrations of antibody at 4 °C for 30 min in RPMI, 0.5% FBS. Antibodies used were: CD62L-PE (phycoerythrin)(Clone MEL-14), CD4-FITC (fluorescein isothiocyanate) (L3T4)(RM4-5), TCRβ-biotin (Clone H57-597), HSA (CD24)-PE (Clone

M1/69), CD25-FITC (Clone 3C7) and CD71-PE (Clone C2F2), CD44-PeCy5 (phycoerythrin cyanine 5) (Clone IM7) from BD Pharmingen; CD8-TriColour (Clone 510) and Streptavidin-APC (allophycocyanin) from Caltag, CD98-PE (Clone RC388) from eBiosciences. For CCR7 staining, cells were labeled with mouse CCL19-Fcγ and detected using PE-conjugated anti-human Fcγ (both from eBiosciences). For P- and E-selectin ligand staining, cells were labeled with recombinant P-selectin Fc and E-selectin Fc (R&D Systems) and detected using APC-conjugated anti-human Fcγ (Jackson ImmunoResearch). Cells were washed and resuspended in RPMI, 0.5% FBS prior to acquisition on a FACS Calibur or LSR (Becton Dickinson). A minimum of 10^4 relevant events were collected, stored ungated and the data analyzed with FlowJo software (TreeStar Inc). Live cells were gated according to their forward scatter (FSC) and side scatter (SSC).

CD62L shedding assay

Cells were prepared at 1 to 2 × 10^6 cells/ml in RPMI 1640 + L-glutamine, heat-inactivated 0.5% FBS, 50 μM β-ME and penicillin/streptomycin. 100 μl cells were added to wells of a 96-well plate in triplicate. Where indicated, cells were treated with 1 nM specific peptide gp33-41 to stimulate the TCR or incubated in the presence or absence of the PI3K inhibitor LY294002 (10 μM). The cells were then incubated for 1 h at 37 °C in 5% CO_2. Soluble CD62L in the supernatant was measured using the DuoSet mouse sL-Selectin/CD62L ELISA kit (R&D Systems). The amount of soluble CD62L present in the supernatant is expressed as pg CD62L released per 1 × 10^5 cells.

Quantitative real-time PCR

RNA was purified using the RNeasy RNA purification Mini Kit (Qiagen) (Genomic DNA was digested with RNase-free DNase (Qiagen) following manufacturer instructions) and reverse-transcribed using the iScript cDNA synthesis kit (BioRad). Quantitative PCR was performed in 96-well plate format using iQ SYBR Green based detection (BioRad) on a BioRad iCycler. 18*S* mRNA was used for normalization.

Primers

18S forward: 5′-ATCAGATACCGTCGTAGTTCCG-3′

18S reverse: 5′-TCCGTCAATTCCTTTAAGTTTCAGC-3′

CD62L forward: 5′-ACGGGCCCCCGTGTCAGTATGTG-3′

CD62L reverse: 5′-TGAGAAATGCCAGCCCCGAGAA-3′

KLF2 forward: 5′-TGTGAGAAATGCCTTTGAGTTTACTG-3′

KLF2 reverse: 5′-CCCTTATAGAAATACAATCGGTCATAGTC-3′

S1P1 forward: GTG TAG ACC CAG AGT CCT GCG-3′

S1P1 reverse: AGC TTT TCC TTG GCT GGA GAG-3′

PI(3,4,5)P_3 quantification

Estimation of the intracellular concentrations of PI(3,4,5)P_3 in CD8(IL-2) and CD8(IL-15) cells were made using a time-resolved fluorescence resonance energy transfer (TR-FRET) displacement assay as described previously[49].

CCL19 Transwell chemotaxis assay

Membrane inserts of Transwell chemotaxis plates (CoStar) were coated with 2 μg/ml fibronectin (Sigma) overnight at 4 °C. The membranes were blocked using 2% heat-

inactivated FBS in PBS for 1 h at 37 °C. 5×10^5 cells in 100 μl RPMI containing 0.5% heat-inactivated FBS were placed in the upper chamber of the Transwell chemotaxis plate in triplicate. 600 μl diluted CCL19 (200 ng/ml, R&D Systems) was placed in the lower chambers and the percentage of cells migrating across the 5 μm pore size membrane was determined by flow cytometry after a 3 h incubation at 37 °C in 5% CO2.

Adoptive Transfer

Murine P14 $CD8^+$ T cells were grown from spleen preparations cultured for 48 h in the presence of gp 33-41 peptide, washed and resuspended at 2×10^5 cells/ml with a final concentration of 20 ng/ml IL-2 in the presence or absence of the mTOR inhibitor rapamycin (20 nM) for 48 h. 50×10^6 CD8(IL-2) cells were loaded with CellTracker Orange (CMTMR; Invitrogen) and 50×10^6 CD8(IL-2+rapamycin) cells were loaded with CFSE (Invitrogen). The cells were mixed equally and 10×10^6 cells were injected into the tail vein of C57BL/6 mice. 24 h later the mice were sacrificed and blood, spleen and lymph nodes were removed for analysis. The number of CMTMR-labeled CD8(IL-2) cells recovered and the number of CFSE-labeled CD8(IL-2+rapamycin) cells recovered was expressed as a percentage of the total number of recovered cells. (n = 9 mice)

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4.00 for Macintosh, GraphPad Software. A non-parametric Mann Whitney test was used where the number of experiments performed was not sufficient to prove normal distribution. When comparing KLF2 mRNA expression in CD8(IL-2) and CD8(IL-15) cells, a one-sample t test was used to calculate P values with the theoretical mean set to 1.00. Figures show the mean of at least 3 experiments, performed in triplicate, with error bars showing the s.e.m.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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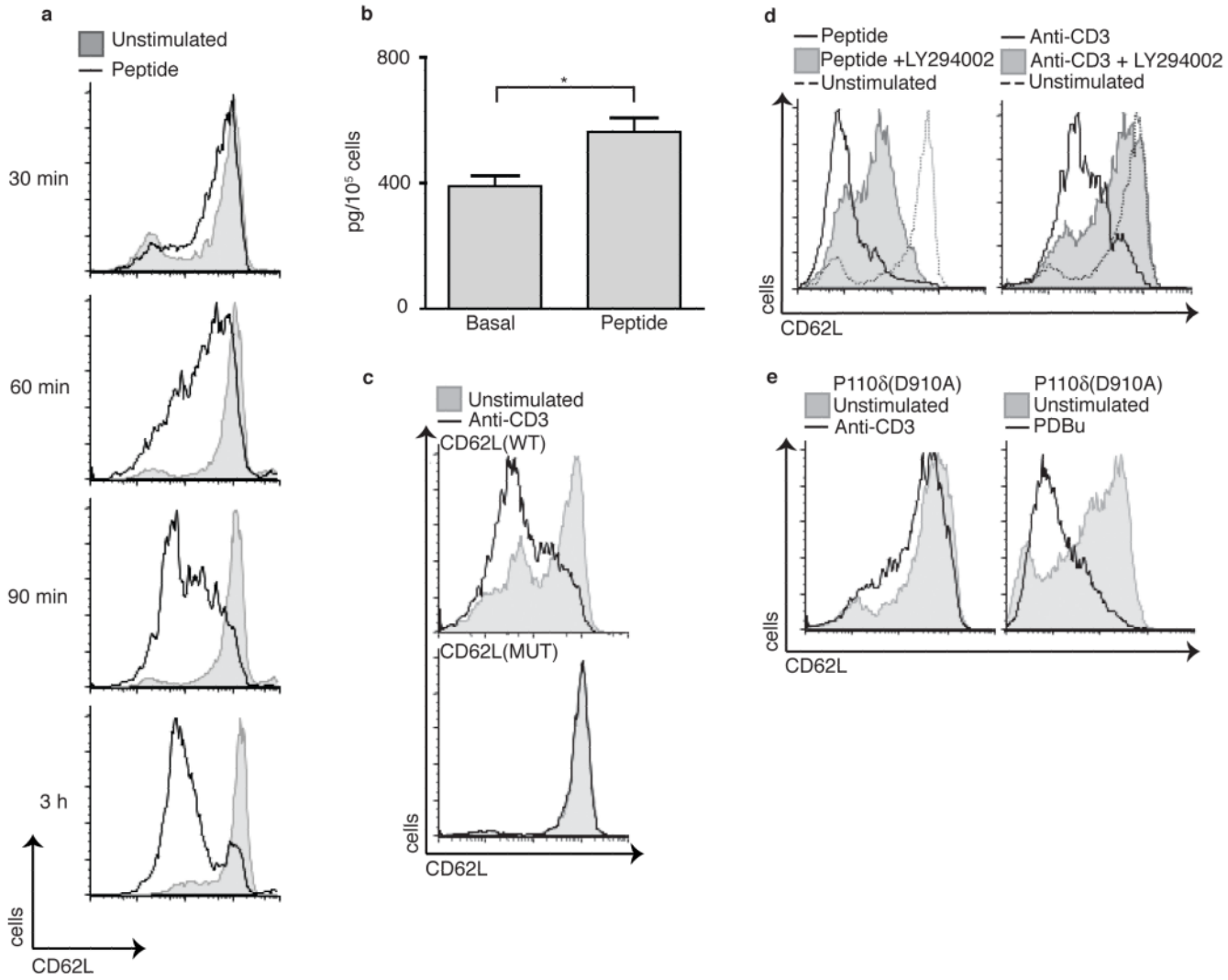

Figure 1. CD62L shedding from TCR activated CD8$^+$ T cells is PI3K dependent

a) Flow cytometric analysis of CD62L (L-selectin) cell surface expression on P14 TCR transgenic CD8$^+$ T cells either unstimulated or stimulated with specific peptide gp33-41 for 3 hours. **b)** Amount of CD62L (pg/10^{5}cells) shed from P14 T cells either unstimulated or stimulated with specific peptide gp33-41 for 1 hour. ($p < 0.05$, error bars mean ± s.e.m.) **c)** CD62L cell surface expression on unstimulated CD8$^+$ T cells or CD8$^+$ T cells stimulated with CD3 antibodies for 4 hours from mice expressing shed-capable CD62L, CD62L(WT) (upper panel), or shed-resistant CD62L, CD62L(MUT) (lower panel). **d)** CD62L surface expression on P14 TCR transgenic (left panel) or wild-type (WT) CD8$^+$ T cells (right panel) unstimulated or stimulated with peptide gp33-41 or CD3 antibodies for 4 hours in the presence or absence of LY294002 (10 μM) **e)** CD62L cell surface expression on splenic T cells from p110†(D910A) mice stimulated with CD3 antibody (left panel) or PDBu (right panel) for 4 hours. Data are representative of 5 (**a**), 2 (**b**) and 3 (**c,d,e**) experiments.

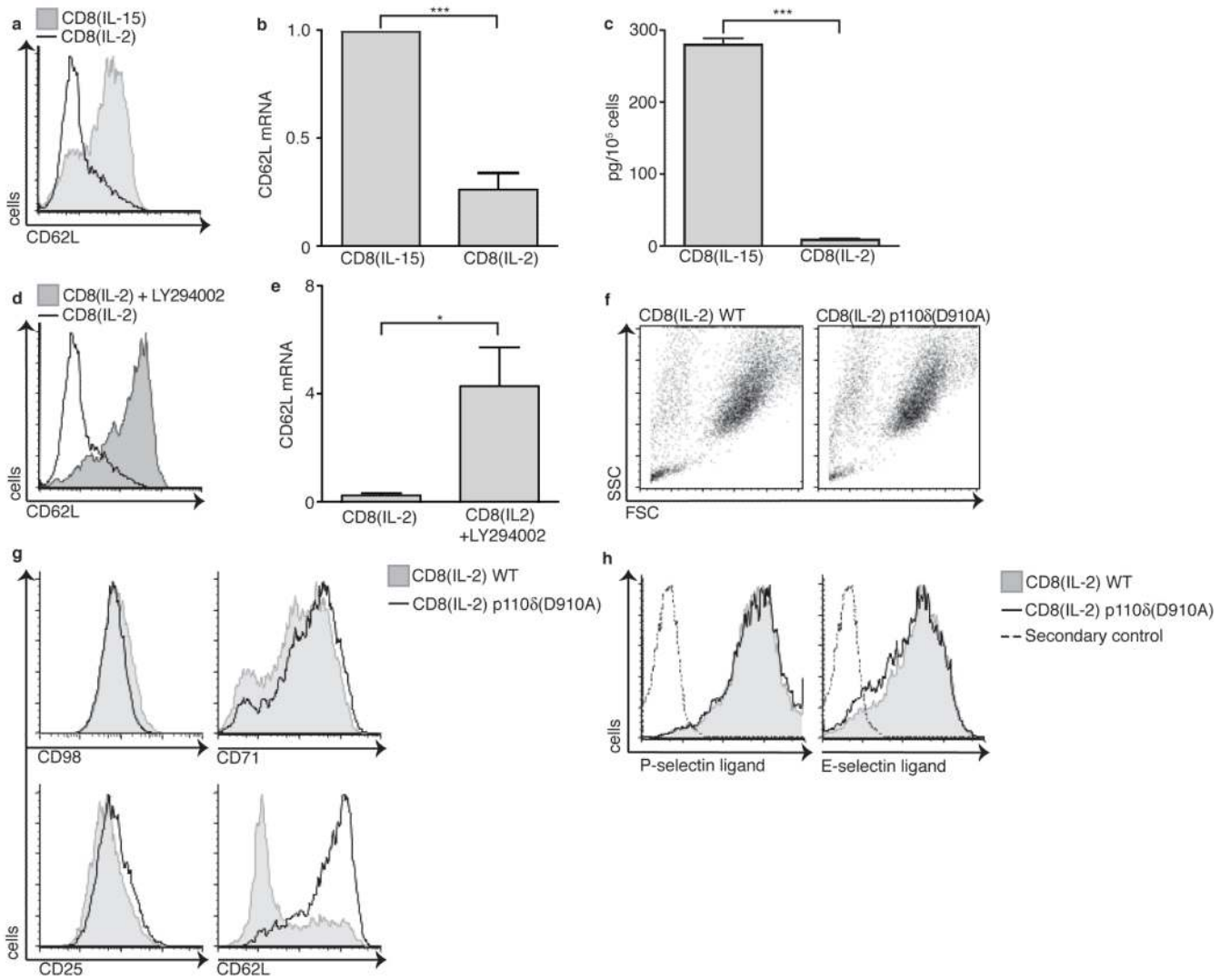

Figure 2. CD62L downregulation in effector CD8$^+$ T cells is PI3K dependent

a) Histograms show cell surface expression of CD62L on P14 CD8$^+$ T cells activated for 2 days with peptide gp33-41 and thereafter maintained in either IL-15 (CD8(IL-15)) or IL-2 (CD8(IL-2)) for 48 hours. **b)** Relative expression of CD62L mRNA in P14 CD8(IL-2) versus CD8(IL-15) cells ($p < 0.05$, error bars mean ± s.e.m.). **c)** Amount of CD62L (pg/10^5cells) shed from P14 CD8(IL-15) versus CD8(IL-2) cells ($p < 0.0005$, error bars mean ± s.e.m.). **d**) CD62L cell surface expression on P14 CD8(IL-2) cells in the presence or absence of the PI3K inhibitor LY294002 (10 μM) for 48 hours. **e)** Relative expression of CD62L mRNA (compared to P14 CD8(IL-15) cells) in P14 CD8(IL-2) cells cultured with or without LY294002 (10 μM) ($p < 0.05$, error bars mean ± s.e.m.). **f)** Forward and side light scatter of WT (left) or p110†(D910A) (right) CD8(IL-2) cells. **g)** Flow cytometric analysis of CD98, CD71, CD25 and CD62L expression on wild-type or p110δ(D910A) CD8(IL-2) cells. **h)** Histograms show P-selectin or E-selectin ligand expression on wild-type or p110δ(D910A) CD8(IL-2) cells. Data are representative of 6 (**a,b,d,e**), 2 (**c**), 4 (**f,g**) and 3(**h**) experiments.

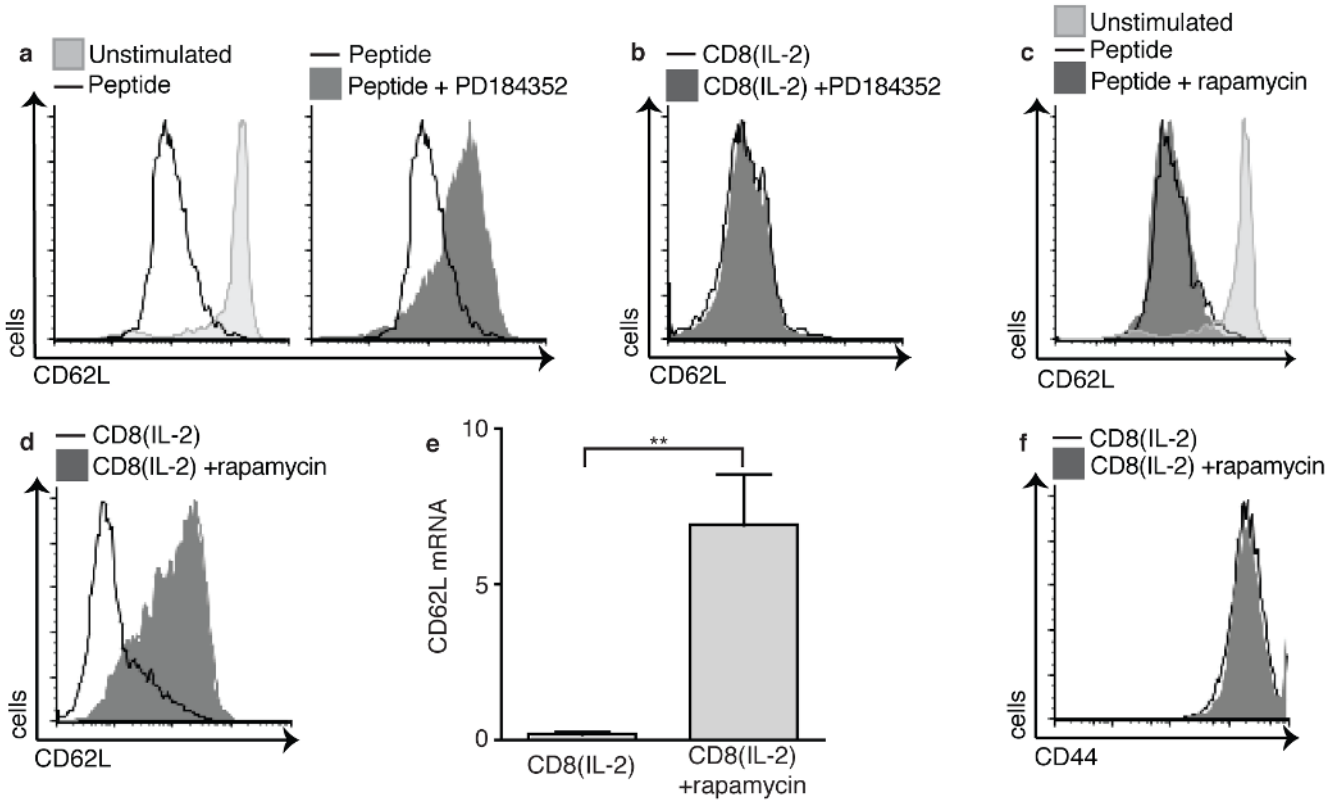

Figure 3. TCR induced CD62L shedding is ERK dependent whereas IL-2 induced downregulation of CD62L transcription is mTOR dependent

a) CD62L expression on P14 T cells either unstimulated or peptide gp33-41 stimulated for 4 hours (left panel) in the presence or absence of PD184352 (2 μM) (right panel). **b)** CD62L expression on P14 T cells stimulated with peptide gp33-41 for 48 hours and thereafter maintained in IL-2 for 48 hours (CD8(IL2)) in the presence or absence of PD184352 (2 μM) **c)** CD62L expression on primary P14 $CD8^+$ T cells either unstimulated or peptide gp33-41 stimulated in the presence or absence of rapamycin (20nM). **d)** CD62L surface expression on P14 CD8(IL2) cells maintained for 48 hours in the presence or absence of rapamycin (20nM). **e)** CD62L mRNA in P14 CD8(IL-2) cells maintained for 48 hours in the presence or absence of rapamycin (20nM). mRNA expression is relative CD62L mRNA present in P14 $CD8^+$ cells maintained in IL-15 (CD8(IL15)) for 48 hours. (n=6, $p < 0.005$, error bars mean ± s.e.m.), **f)** CD44 expression on P14 CD8(IL-2) cells maintained for 48 hours in the presence or absence of rapamycin (20nM). All data are representative of 3-6 experiments.

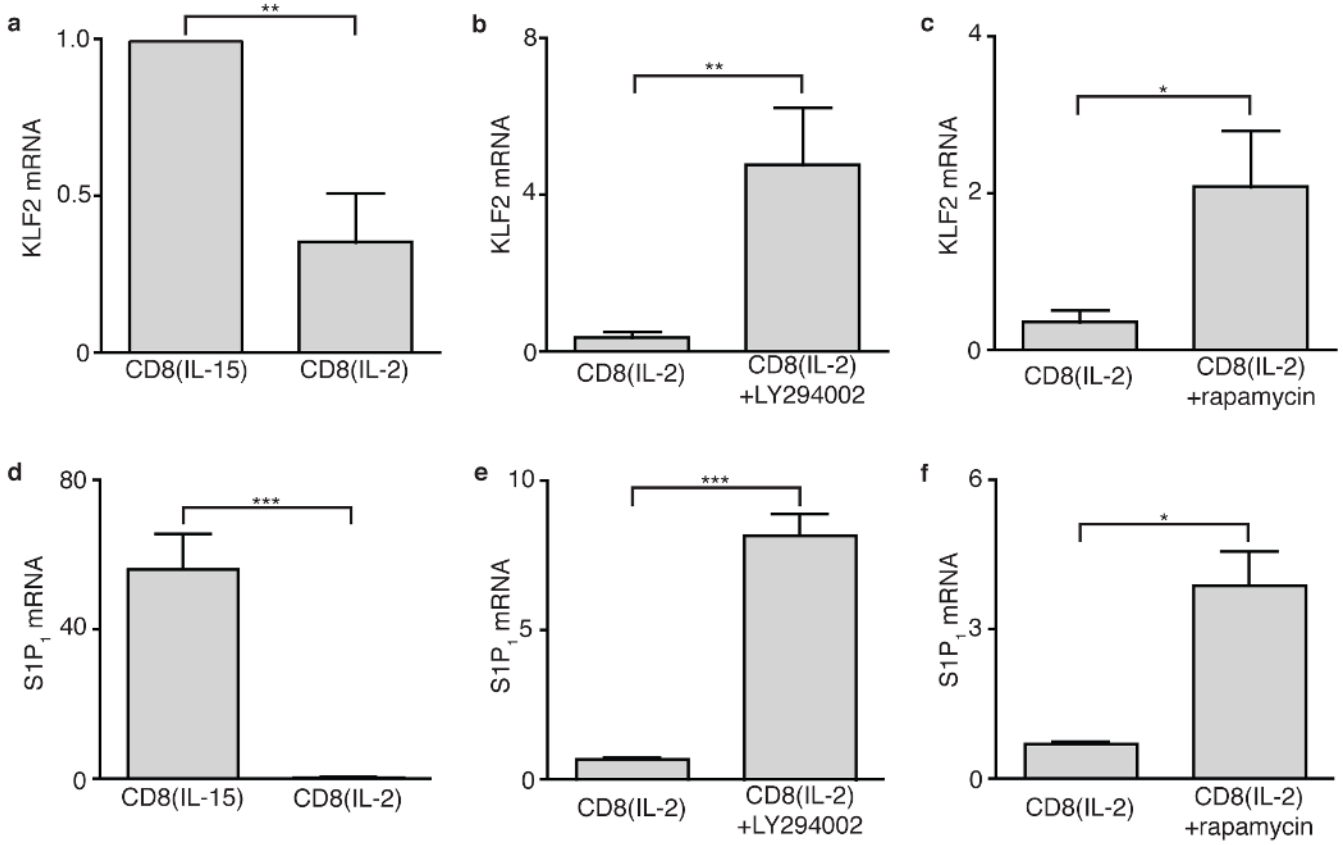

Figure 4. KLF2 and S1P$_1$ expression is regulated by PI3K and mTOR signaling

a) Relative expression of KLF2 mRNA in P14 CD8$^+$ cells activated for 2 days with gp33-41 and thereafter maintained in either IL-15 (CD8(IL-15)) or IL-2 (CD8(IL-2)) for 48 hours (*n*=6). **b)** Relative expression (compared to CD8(IL-15) cells) of KLF2 mRNA in P14 CD8(IL-2) cells maintained with or without LY294002 (10 μM) for 48 hours (*n*=6). **c)** Relative expression (compared to CD8(IL-15) cells) of KLF2 mRNA in P14 CD8(IL-2) cells maintained with or without rapamycin (20nM) for 48 hours (*n*=6). **d)** Relative expression of S1P$_1$ mRNA in P14 CD8(IL-2) or P14 CD8(IL-15) cells (*n*=4). **e)** Relative expression of S1P$_1$ mRNA in P14 CD8(IL-2) cells maintained with or without LY294002 (10 μM) for 48 hours (*n*=4). **f)** Relative expression of S1P$_1$ mRNA in P14 CD8(IL-2) cells maintained with or without rapamycin (20nM) for 48 hours. (*n*=4). (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, error bars mean ± s.e.m)

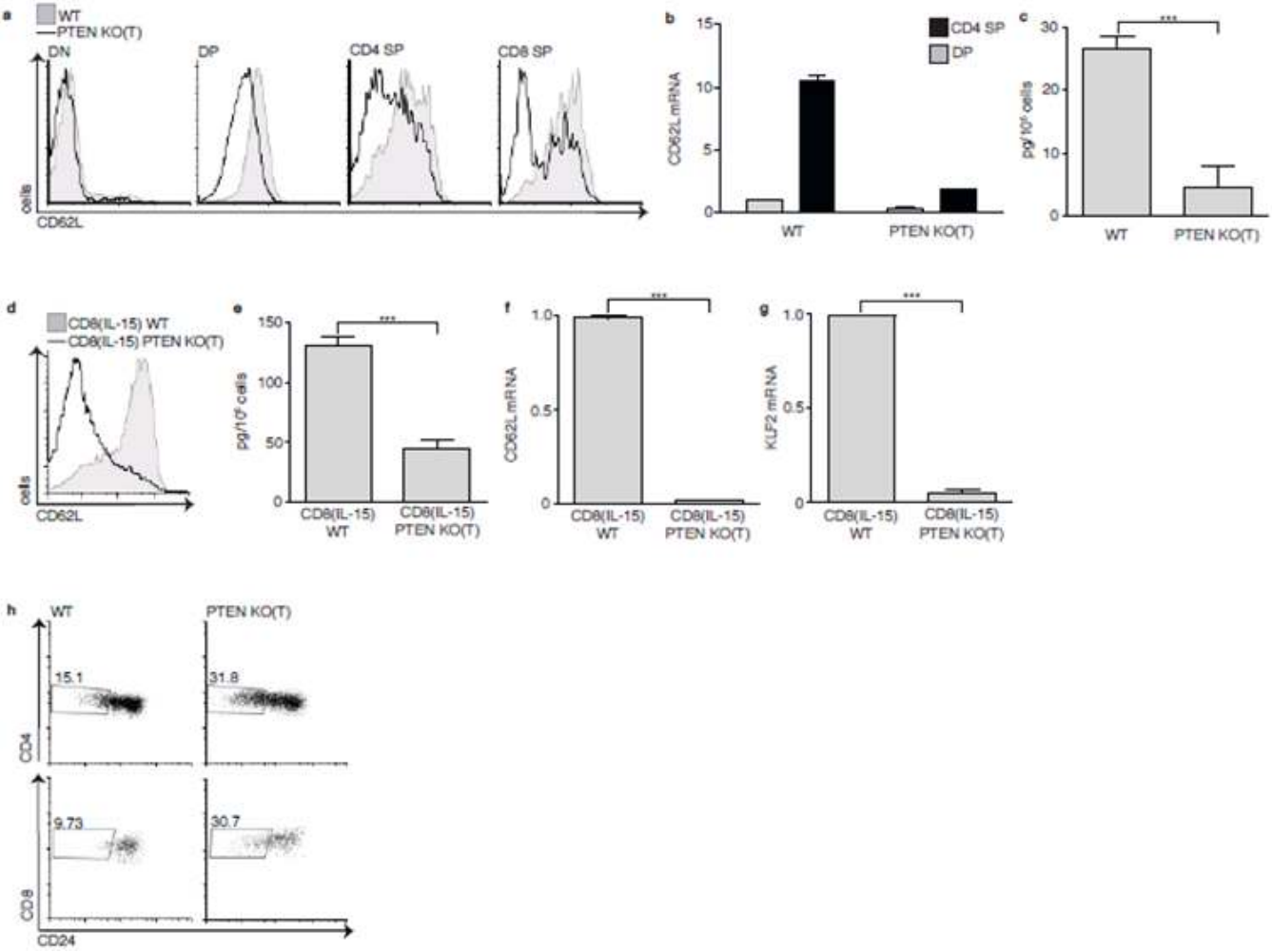

Figure 5. Loss of PTEN is sufficient to downregulate CD62L expression

a) CD62L surface expression on PTEN KO(T) versus wild-type (WT) thymocytes (*n*=3). **b)** CD62L mRNA expression in sorted DP and CD4 SPs from PTEN KO(T) and WT controls. Expression of CD62L mRNA is shown relative to WT DP cells (*n*=3). **c)** Amount of CD62L shed (pg/10^5 cells) over 1 hour from PTEN KO(T) or WT thymocytes. The data show an average of 2 experiments performed in triplicate ($p < 0.0005$, error bars mean ± s.e.m.). **d)** CD62L surface expression on PTEN KO(T) or WT CD8(IL-15) cells. **e)** Amount of CD62L shed (pg/10^5 cells) during 1 hour from PTEN KO(T) or WT CD8(IL-15) cells The data show an average of 2 experiments performed in triplicate. ($p < 0.0005$, error bars mean ± s.e.m.) **f)** Relative expression of CD62L mRNA from PTEN KO(T) or WT CD8(IL-15) cells. (*n*=3, $p < 0.0005$, error bars mean ± s.e.m.). **g)** Relative expression of KLF2 mRNA from PTEN KO(T) or WT CD8(IL-15) cells (*n*=3, $p < 0.0005$, error bars mean ± s.e.m.). **h)** CD24 expression on CD4 or CD8 single positive PTEN KO(T) or WT thymocytes (% of CD24 low expressing cells is indicated). (*n*=6)

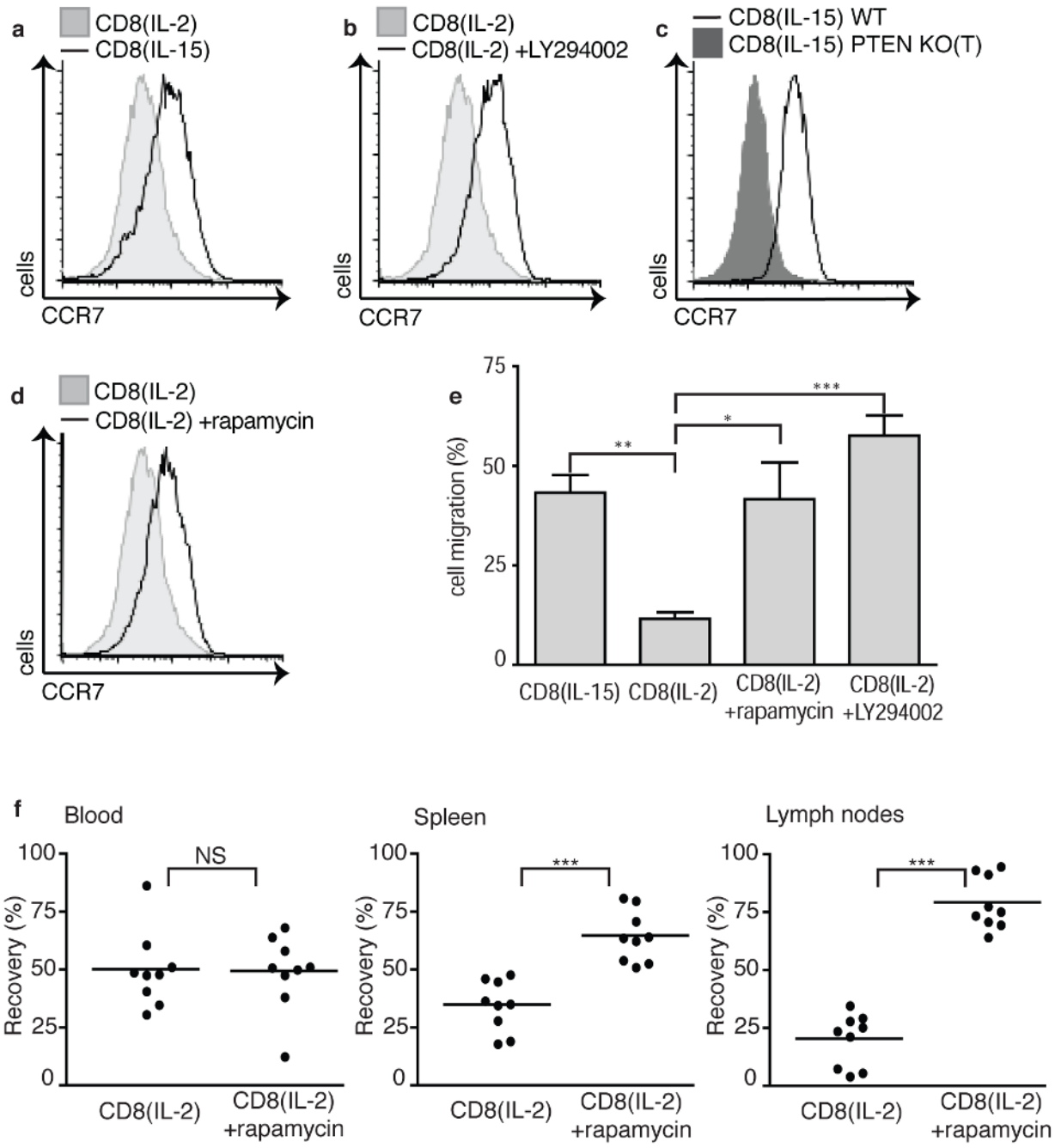

Figure 6. CCR7 downregulation on activated T cells is dependent on PI3K and mTOR

a) CCR7 surface expression on P14 CD8$^+$ cells activated for 2 days with gp33-41 and thereafter maintained for 48 hours in either IL-15 (CD8(IL-15)) or IL-2 (CD8(IL-2)). **b)** CCR7 surface expression on CD8(IL-2) cells cultured for 48 hours with or without LY294002 (10 μM). **c)** CCR7 surface expression on PTEN KO(T) or wild-type (WT) CD8(IL-15) cells. **d)** CCR7 surface expression on CD8(IL-2) cells cultured with or without rapamycin (20nM) for 48 hours. **e)** Transwell migration to CCL19 of CD8(IL-15) or CD8(IL-2) cells maintained for 48 hours in the presence or absence of either LY294002 (10 μM) (CD8(IL-2+LY294002)) or rapamycin (20nM) (CD8(IL-2+rapamycin)) (n=3, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, error bars mean ± s.e.m). **f)** P14 CD8(IL-2) cells were

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either untreated or treated with rapamycin for 48 hours, labeled with CFSE or CMTMR respectively, and mixed at a ratio of 1:1 prior to injection into C57Bl/6 hosts. The data show the percentage of CD8(IL-2) versus CD8(IL-2+rapamycin) cells as a percentage of the total number of transferred cells recovered in the blood, lymph nodes or spleen 24 hours after transfer (n= 9, *** $p < 0.0005$, bar shows mean).