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Review

Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45

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ABSTRACT

CD45 is a leukocyte specific transmembrane glycoprotein and a receptor-like protein tyrosine phosphatase (PTP). CD45 can be expressed as several alternatively spliced isoforms that differ in the extracellular domain. The isoforms are regulated in a cell type and activation state-dependent manner, yet their function has remained elusive. The Src family kinase members Lck and Lyn are key substrates for CD45 in T and B lymphocytes, respectively. CD45 lowers the threshold of antigen receptor signalling, which impacts T and B cell activation and development. CD45 also regulates antigen triggered Fc receptor signalling in mast cells and Toll-like receptor (TLR) signalling in dendritic cells, thus broadening the role of CD45 to other recognition receptors involved in adaptive and innate immunity. In addition, CD45 can affect immune cell adhesion and migration and can modulate cytokine production and signalling. Here we review what is known about the substrate specificity and regulation of CD45 and summarise its effect on immune cell signalling pathways, from its established role in T and B antigen receptor signalling to its emerging role regulating innate immune cell recognition and cytokine production.

Contents

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1. Introduction—a historical perspective

CD45 is a leukocyte specific glycoprotein that was first identified and characterized using antisera and monoclonal antibodies in the late sixties and seventies. Multiple isoforms of CD45 were identified that were differentially expressed in a cell type and activation specific manner [reviewed in 1,2]. In the eighties, CD45 was cloned [3] and its large cytoplasmic domain was found to have sequence similarities with the, then, newly sequenced protein tyrosine phosphatase, PTP1B [4]. CD45 was quickly established as a receptor-like protein tyrosine phosphatase [5] and around the same time, CD45 was shown to be required for antigen-induced T cell proliferation [6]. Soon after, the Src family kinase, Lck, was identified as a substrate for CD45 in T cells [7,8] and Lyn as a CD45 substrate in B cells [9,10]. Many subsequent publications in the late eighties and nineties defined the impact of CD45 on T cell receptor (TCR) and B cell receptor (BCR) signalling [reviewed in 11–14]. In the nineties, CD45$^{-/-}$ mice [15–17] established the importance of CD45 in TCR and BCR signalling in vivo and identified a role for CD45 in T and B cell development [reviewed in 18,19]. Since then, these mice, together with CD45 E613R knock-in mice [20] have provided much of the new information on CD45 [reviewed in 21]. The Janus kinases (JAKs) were identified as potential CD45 substrates [22] and the function of CD45 broadened to include effects on cytokine signalling as well as NK receptor and TLR signalling. CD45 functions have been explored in mast cells, macrophages and dendritic cells and roles have been identified in leukocyte adhesion and migration. Here, we discuss the substrates, specificity and regulation of CD45 and then focus on the effect of CD45 on Src family kinases and how CD45 affects various signalling pathways in leukocytes.

2. CD45 substrates

2.1. Substrate specificity

In vitro, CD45 exhibits a broad specificity for tyrosine phosphorylated peptides at physiological pH [23,24]. Crystallization of the majority of the cytoplasmic domain of CD45 with a tyrosine phosphorylated peptide suggests a preference for a hydrophilic residue at pY-1 and a negatively charged amino acid at pY-2 or -3 [25]. No differences in the rate of dephosphorylation were observed when peptides from the C-terminal negative regulatory site of Lck, Fyn and Src were compared, or when these were compared to a peptide corresponding to the phosphorylation site in the activation loop [24,26]. This indicates no selectivity between these kinases at the peptide level and implies that factors other than recognition at the catalytic site define the substrate specificity of CD45.

2.2. Src family kinases

In leukocytes, certain Src family kinase members have been identified as CD45 substrates: Lck, and to a lesser extent Fyn in T cells [7,8,27,28]; Lyn in B cells [9,10,29,30]; Hck and Lyn in macrophages [31], and Lyn, Hck and Fyn in dendritic cells [32], see Table 1. Interestingly, not all Src family kinases appear to be substrates for CD45. Fgr is not hyperphosphorylated in macrophages or dendritic cells lacking CD45 [31,32] and Src is not affected in CD45$^{-/-}$ T cells [28] or dendritic cells [32]. In B cells, Fyn and Blk may be CD45 substrates as they can be hyperphosphorylated in the absence of CD45 [30,33]. These kinases require CD45 for their BCR inducible tyrosine phosphorylation [33] but unlike Lyn, do not associate with CD45 [34].

Lck and Lyn are the best characterized CD45 substrates in T and B cells respectively, where CD45 can dephosphorylate both the C-terminal, negative regulatory site (Y505 in Lck and Y508 in Lyn) and the positive regulatory site (Y394 in Lck and Y397 in Lyn) in the activation loop [reviewed in 12,13,35,36]. Phosphorylation of Src family kinases at the negative regulatory site by Csk results in an intramolecular interaction with the SH2 domain, creating a folded inactive conformation [37,38]. Dephosphorylation at this site by CD45 opens up the molecule, creating a "primed" molecule. Clustering of these primed Src family kinases results in the transphosphorylation of the activation loop, which displaces it from the catalytic site and creates an active kinase by allowing substrate access. Dephosphorylation at this site by CD45 or other PTPs such as SHP1, 2 and PEP down regulate Src family kinase activity and return the Src family kinase to the primed state. Thus by acting at both sites, CD45 promotes the turnover of Src family kinase activity and works to keep these Src family kinases in the primed, dephosphorylated state. It is likely that the phosphorylation of Src family kinases is highly dynamic, being constantly phosphorylated and dephosphorylated. Any disruption to this equilibrium will lead to the activation or inactivation of the Src family kinase.

The observation that CD45 dephosphorylates Lck to a greater extent than Fyn in T cells suggests that Lck is a preferred substrate for CD45. To determine if this preference occurs at the level of the enzyme–substrate interaction, we sought to characterize this interaction in vitro using purified recombinant proteins. Lck, Fyn and Src all bound to the same extent to the cytoplasmic domain of CD45 indicating no discrimination at the protein level (Felberg and Johnson, unpublished). Further analysis of the CD45-Lck complex revealed that the non-catalytic domains of both CD45 and Lck contributed to the interaction [39–41]. The unique amino-terminal region (N), the SH2 domain and the kinase domain of Lck all bound to the first domain of CD45 (D1) containing the membrane proximal wedge region and the catalytic phosphatase domain of CD45. Only the kinase domain of Lck bound to the non-catalytic domain (D2) of CD45 [41]. Neither the

Table 1
Phosphorylation status of proteins directly or indirectly affected by the loss of CD45 in leukocytes.

Hyperphosphorylated proteins	Cell type	Phosphorylated by	References
Lck Y505[a]	T cell lines, thymocytes	Csk	[7,145]
Lck Y394	T cell lines, thymocytes	Lck	[46,96,97]
Fyn Y528	T cell line	Csk	[27,28]
Lyn Y508	B cell lines	Csk	[10,29]
Lyn Y397	B cell line	Lyn	[29]
Hck Y501	BMDM[b]	Csk	[31]
PTPα Y789	T cells	Src family kinases	[51]
Cbp/PAG	T cells and T cell lines	Fyn	[51,52]
SHIP	BMDC[b]		[32]
DAP12	NK cells	Src family kinases	[47]
CD22	B cells	Lyn	[114]
Hyperphosphorylated proteins after stimulation	**Cells**	**Stimulation**	**References**
JAK1, 2, 3	B cells, thymocytes,	IFNα	[22]
JAK2	BMMC[b]	IL-3	[22]
JAK3	B cells	IL-4	[22]
CD22	B cells	BCR crosslinking	[115]
Hypophosphorylated proteins after stimulation	**Cells**	**Stimulation**	**References**
CD3ζ, CD3ε	Thymocytes	None & CD3/TCR	[46]
Zap70	Thymocytes, T cells	CD3/TCR	[46,146]
HS-1, cbl	Thymocytes	CD3/TCR	[46]
CD19	B cells	BCR	[112]
Src family kinases not affected by the lack of CD45	**Cells**		**Refs**
Fgr	BMDM[b] and BMDC[b]		[31,32]
Src	T cells and BMDC[b]		[28,32]

[a] Numbering as in mouse proteins.
[b] Bone marrow derived macrophages, dendritic cells and mast cells respectively.

acidic region in D2 nor the C-terminal 78 residues of CD45 were required for the interaction. Furthermore, the interaction of D2 with Lck was localized to sub-domain X containing helix G in the kinase domain, a region conserved within the Src family kinases [41]. These interactions, which likely contribute to substrate recruitment and specificity, all occur independently of the tyrosine phosphorylation state of Lck [39,41]. The non-catalytic interactions are relevant in the cell, as a CD45 construct lacking the active phosphatase domain associated with Lck in T cells [42]. This construct sequestered Lck away from the active CD45 phosphatase, resulting in reduced TCR induced calcium signals and IL-2 production, but interestingly, still allowed MAPK signalling to proceed, leading to CD69 expression.

To investigate how CD45 can dephosphorylate two spatially distinct and functionally opposing sites in Lck, the CD45–Lck interaction was analyzed using various phosphorylation site mutants of Lck [40]. In one case, CD45 binds to the "closed" form of Lck and dephosphorylates the C-terminal phosphorylation site, which is normally bound internally to the Lck SH2 domain. In the other case, CD45 binds the "open" form of Lck and dephosphorylates the exposed tyrosine on the activation loop. Fig. 1 provides two hypothetical models of how this might be achieved by CD45, taking into account the known in vitro interactions. The phospho-tyrosine independent binding of CD45 to the SH2 domain of Lck [39], perhaps mediated by the membrane proximal wedge region, may help disrupt the intramolecular phosphotyrosine–SH2 domain interaction thereby exposing the phosphotyrosine residue and facilitating its interaction with the catalytic site of CD45. A comparison of the dephosphorylation rates of Lck revealed that dephosphorylation of Y394 in the activation loop of the open form of Lck preceded at a faster rate than dephosphorylation of Y505 in the closed form of Lck [40]. This difference was attributable to the protection of phosphoY505 by the SH2 domain of Lck as a mutant defective in this interaction was dephosphorylated at a similar rate to the open Y394 phosphorylated form, as were the kinase domains alone [40]. This indicates that at least in vitro, CD45 has a slight preference for the positive regulatory tyrosine when full length, closed Lck is compared, but no preference when both phosphorylated Lck molecules are in the open conformation. Thus CD45 shows no major site preference for Lck in vitro, suggesting that any observed differences in vivo must be due to differences in substrate or enzyme localisation or to the intervention of regulatory proteins.

2.3. Other potential substrates

2.3.1. Src family kinase substrates

Evidence that CD45 can promote the inactivation of Src family kinases has complicated the identification of CD45 substrates, as many putative CD45 substrates are also Src family kinase substrates. It is thus difficult to distinguish whether CD45 directly dephosphorylates the potential substrate or indirectly prevents phosphorylation by inactivation of the kinase.

While Src family kinases may have a basal level of activity in leukocytes, they are typically activated on receptor recognition of an extracellular ligand. Major substrates for Lck and Lyn include key immunomodulatory proteins such as the CD3 chains, Zap70, Cbl, Cbp/PAG, Igα/β chains, FcRγ, DAP12, Dectin-1, CD22, PIR-B, SIRPα, CD19 and CD21 [43–45]. The majority of these proteins have immunoreceptor tyrosine based activation or inhibitory motifs (ITAM or ITIM) and their phosphorylation leads to the recruitment of SH2 domain-containing signalling proteins and either an activatory or inhibitory signal. The phosphorylation state of many of these proteins are affected by the loss of CD45 and some have been implicated as potential CD45 substrates [46] and see Table 1. DAP12 is phosphorylated by Src family kinases and is hyperphosphorylated in CD45 null NK cells [47], suggesting it is either a direct substrate for CD45 or indirectly affected by CD45. Cbp is normally constitutively phosphorylated by Fyn in lipid rafts, leading to the recruitment of Csk and the phosphorylation of Src family kinases at the negative regulatory site [48–50]. Cbp is hyperphosphorylated in CD45$^{-/-}$ T cells. It is rapidly dephosphorylated upon TCR stimulation, possibly by the recruitment of CD45 to lipid rafts. Cbp is therefore either directly or indirectly affected by CD45 [51,52].

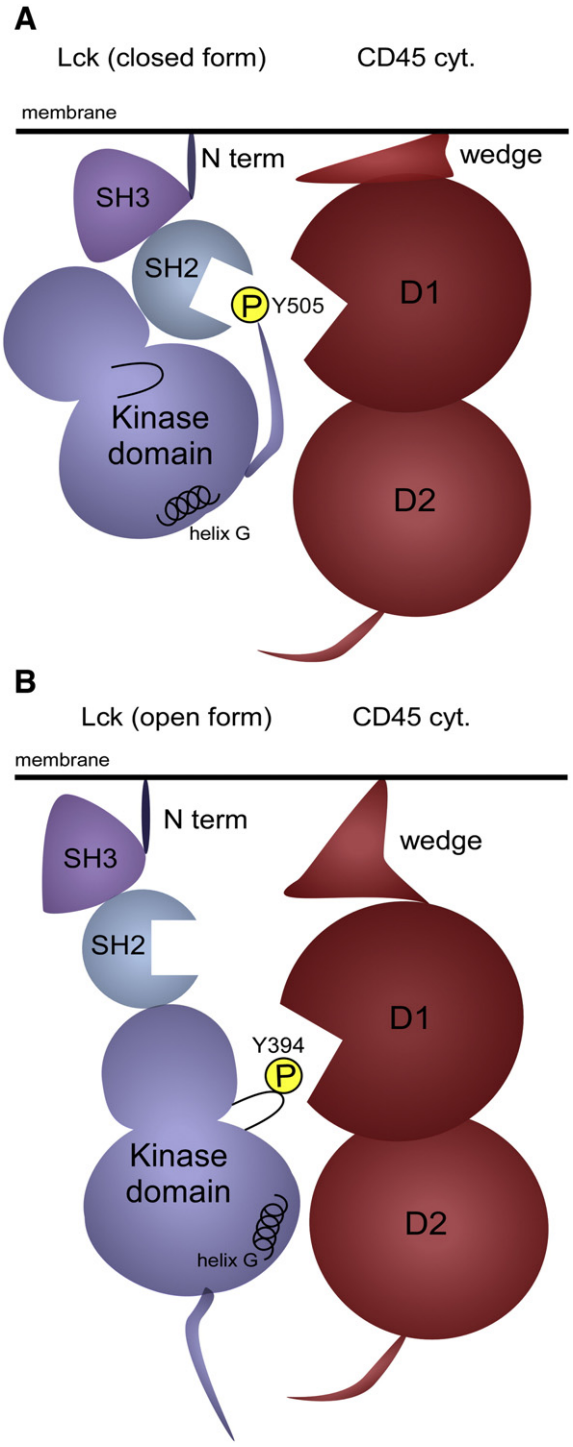

Fig. 1. Schematic model of how CD45 might interact with Lck to dephosphorylate both the negative regulatory site (Y505) and the positive regulatory site (Y394). A, shows the possible association of the cytoplasmic domain (CD45 cyt.) with the closed form of Lck and B, the possible association of CD45 with the open form of Lck. The cytoplasmic domain of CD45 includes the membrane proximal wedge region, the catalytically active domain 1 (D1), the inactive domain 2 (D2) and the C-terminal 78 amino acids. The unique amino terminal region (N term), the SH3 and SH2 domains, as well as the activation loop and the small and large lobe of the kinase domain of Lck are shown. The helix G region in subdomain X of Lck that may interact with CD45-D2 is indicated. It is notable that in this schematic, the wedge region in B had to be extended, in order for CD45 access Y394.

Another potential CD45 and Src family kinase substrate in T cells is the receptor-like protein tyrosine phosphatase, PTPα. Recombinant CD45 can directly dephosphorylate PTPα in vitro suggesting that it may be a direct substrate for CD45 [51]. However, both Src family kinases and CD45 can regulate the phosphorylation of PTPα at Y789, which is required for its efficient dephosphorylation of Fyn [51]. Interestingly, PTPα dephosphorylates Fyn but not Lck in T cells [51,53].

2.3.2. Janus kinases

The only other tyrosine kinases that have been implicated as substrates for CD45 are those belonging to the Janus kinase (JAK) family. JAK kinases are hyperphosphorylated in CD45 deficient T cells, B cells and mast cells in response to IFNα, IL-4, and IL-3 cytokines, respectively [22]. Recombinant CD45 D2 bound to JAK2 and recombinant CD45 could dephosphorylate JAK2 in vitro, supporting the idea that JAKs are direct substrates for CD45. Although inhibition of Src family kinases did not affect JAK phosphorylation in this study, Src family kinases can impact the JAK/STAT pathway and regulate cytokine signalling [54,55], suggesting that CD45 regulation of Src family kinase activity could also impact this signalling pathway.

3. Regulation

Although CD45 has been known to be a tyrosine phosphatase for almost 20 years, surprisingly little is understood about how its phosphatase activity is regulated. The lack of evidence for ligand-induced activation of CD45 (a common mechanism for receptor tyrosine kinases) led to the view that the phosphatase activity of CD45 is not switched on by a specific extracellular ligand. Instead it is thought to be constitutively active, providing tonic dephosphorylation and helping to maintain Src family kinases in their primed, unphosphorylated state. In the absence of evidence for the regulation of the catalytic activity of CD45, scientists turned towards investigating the regulation of CD45 activity by its expression level and location in the cell.

3.1. Expression levels

Mice heterozygous for the targeted disruption of CD45 express lower levels of CD45, yet display enhanced positive and negative T cell selection compared to wild-type mice [56], implying that normal levels of CD45 have an inhibitory component. However, this was not supported by wild-type mice overexpressing the CD45RO transgene which showed enhanced TCR signalling and increased negative selection [57]. Recently, a more systematic study was performed to evaluate the effect of expressing different levels of the CD45RO isoform in $CD45^{-/-}$ mice [58]. Interestingly, about 3% of the wild type level of CD45 is required to restore T cell numbers and 10–20% is required to restore peripheral T cell function. Expression of low levels of CD45 reduced TCR signalling, but expression of intermediate levels of CD45, like the heterozygous mice, showed a hyperactive TCR response [58]. This is consistent with higher levels of CD45 being inhibitory, possibly by the dimerization of CD45. However, examination of the phosphatase activity showed a linear increase with increasing expression levels [58]. Further analysis showed that low levels of CD45 reduced Y505 phosphorylation in the cell more efficiently than Y394 phosphorylation, which required higher levels of CD45 to see an effect [58]. This implies that lower levels of CD45 are predominantly activatory for Lck whereas higher levels are more inhibitory. Why would higher levels of CD45 be required to dephosphorylate Y394, as in vitro studies suggest it is not because CD45 has a lower affinity for this site [40]? The low signal for phospho Y394 [58] suggests that it is present at low levels in the cell and thus would require higher enzyme concentrations for its efficient dephosphorylation. Also, active Lck may be sequestered in lipid rafts or signalling complexes where CD45 has limited access. Increasing the levels of CD45 may increase the level that partitions into this fraction. Although different expression levels of CD45 can affect Lck and T cell function, it is not clear whether changes in CD45 expression levels occur physiologically to regulate Lck activity. In T cells, it is the CD45 isoforms that change upon activation, not the overall expression level [59]. Interestingly, B cell maturation and function was not fully restored when $CD45^{-/-}$ mice were reconstituted with single CD45RO, CD45RB or CD45RABC isoforms that were approaching levels present in heterozygous mice [60,61]. Thus it would appear that B cells require high levels of the specific CD45RABC isoform to restore B cell function, possibly to dephosphorylate the activating tyrosine in Lyn.

3.2. Localisation

The majority of CD45 resides in the fluid part of the plasma membrane leaving only a small percentage that partitions into lipid rafts [62]. However, the location of CD45 in the membrane is not static and its association with lipid rafts is dynamic and cholesterol dependent [62]. Lck is distributed both inside and outside of lipid rafts and has been reported to be hyperphosphorylated and less active in a glycolipid enriched Triton-X-100 insoluble fraction where CD45 was excluded [63]. This is consistent with the presence of Cbp and Csk in lipid rafts [49]. Thus, in lipid rafts the equilibrium between Csk and CD45 may be shifted towards Csk, providing a reservoir of phosphorylated CD45 substrates, including inactive Src family kinases. Upon TCR activation, Cbp becomes rapidly dephosphorylated possibly due to the transient recruitment of CD45 to the lipid rafts [52], which would quickly shift the equilibrium in favour of an active Src family kinase. Although CD45 is required for the dephosphorylation and priming of CD4-associated Lck outside lipid rafts [64], TCR activation recruits CD4-associated Lck to lipid rafts where clustering would activate Lck and promote the phosphorylation and activation of lipid raft dependent Fyn [65]. The absence of Csk and CD45 from these domains would be expected to lead to sustained Src family kinase activation and downstream signalling. Consistent with this, the engineered inclusion of CD45 to these lipid domains decreases TCR signalling [66]. Upon antigen recognition, a small percentage of CD45 is transiently associated with the TCR, then excluded as the immune synapse forms [67] and later recruited to the central supramolecular activation cluster (c-SMAC), consistent with its ability to down regulate signalling at later time points [68]. If one equates immune synapse formation with the coalescence of lipid rafts, then the distribution of CD45 agrees with its dynamic association with lipid rafts. While the presence of lipid rafts makes sense to compartmentalize signalling, their existence has been questioned, largely due to the lack of definitive techniques to identify and characterize them in the cell [69]. As well, more than one type of lipid raft may exist and may dynamically form and vary with cholesterol concentration.

CD44-associated Lck is present in lipid rafts in T cells and upon CD44 ligation, Lck is clustered and activated [70,71]. In the absence of CD45, Lck is phosphorylated at Y394 and signalling is sustained whereas in CD45 positive T cells, CD45 is recruited to the CD44-Lck clusters, Y394 is not detectably phosphorylated and signalling is transient [72]. This is an example where CD45 is recruited to dephosphorylate Y394 and down regulate Lck activity. How CD45 is rapidly translocated to these clusters is not known, but one would expect it to be triggered by the activation signal and may be dependent on the actin cytoskeleton. Indeed, Src family kinase activity is required for CD44-induced actin rearrangement [72] and CD45 has been reported to associate with the actin-associated proteins, fodrin and spectrin [73,74].

These data show that the localisation of both CD45 and the Src family kinases in and out of lipid domains and the c-SMAC can affect the phosphorylation state of Src family kinases. Generally, the majority of CD45 is located outside of lipid rafts where its major role is to dephosphorylate the negative regulatory site and “prime” Src

family kinases. Upon receptor mediated clustering, the Src family kinases are activated and often relocalize to lipid rafts, where the concentration of CD45 is much lower and the kinases can enjoy sustained signalling. The later recruitment of CD45 to these domains will then primarily dephosphorylate the activation site and down regulate activity.

Changes in CD45 isoforms alter the extracellular region of CD45, affecting both the length and glycosylation of the molecule. These changes may be predicted to lead to different ligand interactions, but despite much effort, no CD45-specific or isoform specific ligands have been found. Since 25% of CD45's weight is carbohydrate [75], it is perhaps not surprising that carbohydrate moieties on CD45 interact with various lectin molecules. These include galectin-1 [76,77], which binds galactose residues; CD22, which binds sialic acid residues [78]; and the C-type lectin MGL on antigen presenting cells, which recognizes terminal N-acetyl galactosamine residues [79]. Although these interactions are not exclusive to CD45, they can alter the distribution of CD45 in the membrane, which in turn can affect substrate accessibility. Indeed, the interaction between MGL and CD45 reduces TCR induced signalling [79] and an interaction with the galectin lattice in resting T cells can direct CD45 to lipid raft domains [80]. Thus both extracellular and intracellular factors that alter the distribution of CD45 have the potential to alter its accessibility to certain substrate pools.

3.3. Intrinsic factors

3.3.1. Protein interactions

Although ionomycin treatment decreases the phosphorylation of serine residues in CD45 and reduces its phosphatase activity [81], surprisingly little else is known about how the phosphatase activity of CD45 is regulated in the cell, particularly in response to physiological stimuli. Although D2 of CD45 interacts with the catalytic D1 domain and is required for optimal phosphatase activity [24] it is not known whether this interaction is regulated or whether D2 has other functions besides binding substrate [40,41]. Interactions with other proteins in the cell could influence the proximity of CD45 to substrates. In this respect, the transmembrane region of CD45 associates with CD45-associated protein (CD45AP) in T cells, a lymphocyte specific linker protein that also binds to Lck [82,83]. A recent report suggests that CD45AP promotes the interaction of CD45 with its substrates [84]. However, the impact of CD45AP regulating substrate dephosphorylation in vivo is unclear, as studies from CD45AP$^{-/-}$ mice are inconsistent. One study shows a reduced CD45-Lck association and altered T cell activation [85] whereas another study sees no effect [86].

Like other PTPs, the presence of a catalytic cysteine in CD45 makes it susceptible to regulation by reversible oxidation. Increased levels of reactive oxygen species, typically generated by NADPH oxidase, have been shown to negatively regulate various PTPs [reviewed in 87] and one report suggests that this may be the case for CD45 in neutrophils [88].

3.3.2. The wedge region

The crystal structure of the majority of the cytoplasmic domain of human CD45 [25], as with all other receptor PTPs crystallized to date, indicate that the membrane proximal region prior to domain 1 forms a helix-turn-helix and is referred to as the wedge region [89]. Mutation of a glutamic acid (E) to an arginine (R) at the apex of this wedge region in CD45 by Weiss' group has produced interesting functional effects, both as a chimeric protein with the epidermal growth factor receptor (EGFR) [90] and as a knock-in mutation in mice [20,91–93]. A chimeric EGFR-CD45 molecule that was no longer able to augment TCR signalling when EGF was added, led to the proposal that dimerization of CD45 may down regulate its activity [90]. A point mutation at the apex of the wedge region of CD45 prevented this inhibitory effect induced by EGF. This, together with the structure of PTPα D1 which crystallized as a dimer [94], led to the inhibitory dimerization model where the wedge region of one molecule of CD45 is predicted to bind to the active site of another and block activity [90]. While it is clear that the wedge mutation has an activating effect, it has been difficult to establish that CD45 dimerises in the cell to down regulate phosphatase activity. Indeed, the cytoplasmic domain of CD45 crystallized as a monomer and the structure determined that the dimeric conformation observed for PTPα D1, was not possible for the two-domain phosphatase CD45 [25]. In addition, no evidence was found for dimerization of the two-domain CD45 phosphatase in vitro, whereas the single CD45 D1 protein existed primarily as a dimer [24].

Generally, the wedge mutant mice exhibit a hyperactive phenotype, typically opposite to that observed for the CD45$^{-/-}$ mice [21]. However, the phosphorylation states of Lck and Lyn are not opposite, in fact they more closely resemble that of the CD45$^{-/-}$ mice. This suggests that Src family kinases are poor substrates for the wedge mutant, which could be attributed to an inefficient phosphatase, an inability to bind substrate or to mis-localisation. In B cells and mast cells, the wedge mutant phenotype most closely mirrors that of Lyn$^{-/-}$ cells, which also exhibit a hyperactive phenotype [91,92]. The wedge mutant appears to prevent Lyn activation more effectively than the absence of CD45, perhaps by preferentially dephosphorylating the activation site. However, in thymocytes expressing the wedge mutant, Lck is hyperphosphorylated at Y394, suggesting the opposite [93]. Clearly more work is needed to understand this interesting dichotomy.

4. The function of CD45 in leukocytes

4.1. The effect of CD45 on pre-TCR and TCR signalling

Analysis of CD45 deficient T cell lines revealed a positive role for CD45 in proximal TCR signalling events [11]. This was attributed to the ability of CD45 to dephosphorylate the negative regulatory tyrosine of Lck (Y505), a priming effect that is required for the participation of Lck in CD3 ITAM phosphorylation and the activation of Zap70 [reviewed in 12–14]. Consistent with this, the T cell defects in the CD45$^{-/-}$ mice were rescued with a constitutively active Lck, mutated at Y505 to phenylalanine [95]. However, analysis of some CD45 deficient cell lines revealed that Lck is also hyperphosphorylated at the positive regulatory site, Y394, and more active in an in vitro kinase assay, showing that CD45 can also downregulate Lck activity [96]. This was also observed in CD45$^{-/-}$ thymocytes [97] and led to the suggestion that Lck is more active in CD45 negative cells, not less active [reviewed in 35,36]. Although a doubly phosphorylated Lck is an active kinase, indicating the dominance of Y394 phosphorylation [98], it is not clear whether individual Lck molecules in CD45 negative T cells are phosphorylated on both sites or whether there are two pools of Lck that are affected differently by the absence of CD45. In support of the latter, CD45 preferentially primes CD4-associated Lck [99,100] whereas forced localization of CD45 to lipid rafts reduces CD3 induced responses [66], indicating opposing effects of CD45 on different pools of Lck.

Three independent CD45 knockout mouse lines were generated by targeting exon 6 [15], 9 [16] or 12 [17] and revealed a significant role for CD45 in T cell development. The accumulation of immature CD4$^-$CD8$^-$ double negative, DN3 cells (CD25$^+$ CD44$^-$) and a reduced number of DN4 (CD25$^-$ CD44$^-$) cells suggested a partial block in this pre-TCR dependent transition. This was attributed to reduced Lck participation in pre-TCR signalling, as the defect was overcome by crossing CD45-RAG deficient mice with mice expressing a constitutively active Lck [101]. Also, Lck$^{-/-}$ mice had a severe defect at this transition [102]. However, the major defect in thymocyte development in the CD45$^{-/-}$ mice is the severely reduced transition of CD4$^+$CD8$^+$ double positive (DP) thymocytes to single positive thymocytes, which results in only 5–10% of the normal numbers of mature T cells exiting into the periphery [15,16]. Both positive and negative selection was affected by CD45. Crossing

CD45$^{-/-}$ mice with TCR transgenic mice determined that there was an increased threshold for both positive and negative selection events, resulting in a lack of positive selected cells and the retention, instead of deletion, of cells normally destined for negative selection [17].

The few mature T cells that were present in the CD45$^{-/-}$ mice were incapable of CD3/TCR-induced proliferation [15], consistent with a positive role for CD45 in TCR signalling and/or an anergic phenotype. Both Lck and Fyn are hyperphosphorylated in CD45$^{-/-}$ thymocytes and both basal and TCR-stimulated CD3ζ and CD3ε phosphorylation are reduced, as is TCR-induced Zap70 recruitment and the tyrosine phosphorylation of proteins such as Cbl and HS1 [46]. However, TCR signalling was not ablated, as increases in inositol triphosphate (IP3) and calcium were detected [46]. This may be attributed to Syk activation, which is down regulated in mature T cells and is not as dependent on CD45 and CD3 ITAM phosphorylation as Zap70 [103]. Alternatively, it may be due to some CD45 independent Lck activation as Lck is hyperphosphorylated at its positive regulatory site, in addition to its negative regulatory site, in CD45$^{-/-}$ thymocytes [97].

Thymocytes from mice expressing the CD45 wedge mutant showed a slight increase in phosphorylation at the negative regulatory site of Lck (Y505) and a substantial increase at Y394, the positive regulatory site, compared to CD45$^{+/+}$ mice, suggesting a hyperactive kinase. This led to enhanced positive selection that was consistent with enhanced calcium and pERK signals induced after TCR stimulation [93]. However, these signals are down-regulated in mature T cells. This suggests, at least in thymocytes, that the wedge mutant is defective in its ability to dephosphorylate the positive regulatory tyrosine and down regulate Lck activity.

4.2. The effect of CD45 on BCR signalling

Initial studies with CD45-deficient B cell lines indicated that there was reduced B cell receptor signalling [reviewed in 104]. In the CD45 null J558Lμm3 plasmacytoma cell line, Lyn was hyperphosphorylated at the negative regulatory site Y508 and was not further phosphorylated upon antigen stimulation [10]. Fyn and Blk were not inducibly phosphorylated upon antigen stimulation, yet Igα and Igβ were phosphorylated and Syk and Btk were activated, albeit to a much lesser degree than in the CD45^{+} cells [10,33]. PLCγ was phosphorylated but IP_3 levels were reduced and calcium signals were abolished [105,106]. This indicates that the lack of CD45 affects Src family kinase-mediated signalling, but does not completely block Syk or Btk activated signals. In keeping with this partial block of BCR signalling events, analysis of CD45$^{-/-}$ mice revealed more subtle B cell developmental defects than was observed for T cell development. In the absence of CD45, B cell development occurs but the amount and proportion of the different B cell subsets are significantly altered. There is a slight accumulation of pro-B cells in the bone marrow [107] and an overall increase in CD19^{+} B cells in the spleen. There are increased numbers of immature IgM^{hi} IgD^{lo} B cells (T1), IgM^{hi} IgD^{hi} B cells (T2) and marginal zone B cells and a reduced percentage of recirculating mature IgD^{hi} B cells (FO)[16,91,108]. In the peritoneum, CD45$^{-/-}$ mice had a decreased percentage of B1 cells whereas the wedge mutant had an increased percentage [91]. These changes in B cell development in the CD45$^{-/-}$ mice are all consistent with decreased BCR signalling strength, resulting in an increased signalling threshold. This was elegantly demonstrated using the hen-egg lysozyme (HEL) transgenic model. In B cells expressing CD45, circulating HEL autoantigen stimulates negative selection, but in CD45$^{-/-}$ B cells, the HEL antigen stimulates positive selection and the accumulation of a pool of mature long-lived IgD^{hi} B cells [109]. Decreased BCR signalling in CD45$^{-/-}$ B cells is consistent with the failure of crosslinked surface IgM to induce proliferation [16,108]. ERK phosphorylation and the extracellular entry of calcium were affected by the lack of CD45, but other tyrosine phosphorylation signals such as Igα/β and PLCγ phosphorylation were not. Indeed, other B cell functions such as T-dependent and T-independent antigen-specific antibody production and class switching were intact in CD45 exon 6-deficient B cells when functional CD4^{+} T cells were provided [110].

The reduced signalling implicated CD45 in the positive regulation of Lyn, which, together with Fyn and Blk, have a positive effect on BCR signalling [reviewed in 18,19]. These Src family kinases can also phosphorylate CD19 [111], an important co-stimulatory molecule in B cells. CD19 is hypophosphorylated in CD45$^{-/-}$ B cells [112], consistent with CD45 promoting Src family kinase activity. However, both in CD45 deficient B cell lines and splenic B cells, Lyn was more active in an in vitro kinase assay, despite being hyperphosphorylated at the negative regulatory tyrosine, suggesting that the loss of CD45 may activate Lyn [9,29,30]. Related to this, Lyn was also shown to have a non-redundant, negative role in BCR signalling [113]. Only Lyn can phosphorylate the ITIM motifs present in negative regulatory molecules such as CD22, PIR-B and FcγRIIb1 [reviewed in 44,45]. This unique effect of Lyn became apparent in Lyn$^{-/-}$ mice, which are hyper-responsive to BCR ligation and have high levels of circulating IgM and autoreactive antibodies [113]. Lyn$^{-/-}$ mice have reduced numbers of mature B cells in the periphery as well as reduced marginal zone B cells. HEL reactive Lyn$^{-/-}$ B cells were deleted whereas wild-type B cells survived, became anergic and entered the periphery [114]. This is opposite to many of the characteristics of the CD45$^{-/-}$ B cells, suggesting that the loss of CD45 may indeed result in a hyperactive Lyn that enhances its negative effects. Consistent with this, CD22 had increased basal and induced phosphorylation in a CD45 deficient B cell line, which led to the enhanced recruitment of SHP1, a tyrosine phosphatase capable of down-regulating Src family kinase activity and BCR signalling [115]. Interestingly, mice deficient in both CD45 and functional SHP1 (CD45$^{-/-}$ *me*^{v}/*me*^{v} mice) showed normal B cell maturation and surface IgM induced proliferation, indicating that the increased BCR signalling threshold induced by loss of CD45 could be reversed by the loss of SHP1 [112]. Although the loss of CD45 reduces the effectiveness of BCR signalling, it is not easy to determine whether this is due to reduced Lyn-mediated positive BCR signalling, or enhanced Lyn-mediated negative signalling, or a combination of the two. It is also possible that CD45 may directly dephosphorylate the negative regulators. Perhaps the simplest explanation is that there are two pools of Lyn, a Y508 phosphorylated, inactive pool, that when dephosphorylated is primed and ready to participate in BCR signalling, and an active, Y397 phosphorylated pool that perhaps resides in lipid rafts and phosphorylates negative regulators. CD45 would activate the first pool and inactivate the second, both of which would promote BCR signalling. However the picture is more complex as Fyn and Blk, which have a positive, but non-essential, role in BCR signalling [116,117], may also be CD45 substrates [30,33].

Interestingly, the B cell defects observed in the CD45 E613R wedge mutant mice are almost opposite to that found in the CD45$^{-/-}$ mice and eerily similar to the Lyn$^{-/-}$ mouse, showing hyper-responsive BCR signalling, lymphoproliferation and expansion of B1 cells, which on a mixed genetic background leads to the development of autoantibodies and glomerulonephritis [91]. However, in the wedge mutant B cells, Lyn is constitutively hyperphosphorylated at the negative regulatory site, as in CD45$^{-/-}$ B cells [30] and has low constitutive and BCR-inducible Y397 phosphorylation compared to CD45$^{+/+}$ B cells [91]. This is consistent with it being a less active kinase and mirroring the phenotype of Lyn$^{-/-}$ B cells.

4.3. The effect of CD45 on Fc and NK receptor signalling

Fc receptor (FcR) signalling shares some similarities with antigen receptor signalling in that subunits of the Fcγ and Fcε receptors have ITAM motifs that are phosphorylated by Src family kinases and recruit Syk [reviewed in 118]. CD45 is important for IgE triggered degranulation in primary mast cell cultures and CD45$^{-/-}$ mice are resistant

to IgE-dependent systemic anaphylaxis [119]. IgE mediated degranulation and pro-inflammatory cytokine production is reduced in bone marrow derived mast cells (BMMC) [92]. Lyn was hyperphosphorylated on the negative regulatory site, Y508 and no calcium flux was observed upon antigen induced IgE crosslinking in the CD45$^{-/-}$ BMMC [92]. Data from Lyn and Fyn deficient mice indicated a distinct contribution from both in downstream signalling pathways: Lyn was important for calcium mobilisation and Fyn for PI3K activation and degranulation [120,121 and reviewed in 122]. However, Lyn$^{-/-}$ and Lyn$^{-/-}$Fyn$^{-/-}$ BMMC have enhanced IgE mediated degranulation, indicating a predominant negative effect of Lyn, despite the upregulation of Fyn in the Lyn$^{-/-}$ mice [120,123]. This suggests that CD45 affects both Lyn and Fyn activity in BMMC. CD45 wedge mutant BMMC showed an opposite phenotype to CD45$^{-/-}$ BMMC, despite Lyn being hyperphosphorylated at Y508. BMMC expressing the wedge mutant more closely mimicked Lyn$^{-/-}$ BMMC, with enhanced degranulation and enhanced Fyn dependent PI3K activation [92], suggesting that, like B cells, Lyn may be more active in CD45$^{-/-}$ BMMC and less active in wedge mutant BMMC.

FcγRs mediate the phagocytosis of IgG coated particles and antibody-dependent cell cytotoxicity (ADCC) in NK cells [reviewed in 118]. The loss of CD45 did not affect ADCC in NK cells [124], suggesting that FcγR signalling did not require CD45. However, subsequent experiments with FcγRIII (CD16) and NK receptors Ly49D and NKG2D showed that although CD45 was not required for ADCC it was for IFNγ production [125,126]. In addition, CD45 was required for CD16-stimulated granulocyte-macrophage colony stimulating factor (GM-CSF) and Ly49D induced RANTES, MIP-1α and 1β production. Interestingly, these receptors are linked to the ITAM containing signalling proteins, DAP12, FcRγ chain or CD3ζ. As with ITAM signalling in lymphocytes, both Src and Syk family kinases are involved and CD45 was required for Syk phosphorylation and calcium increase. However, Syk/Zap70 deficient NK cells are also deficient in ADCC, raising the question as to how CD45 can differentially affect the Ly49D triggered signal leading to cytokine production but not the signal leading to cytotoxicity, particularly as both appear to utilize Syk. Perhaps a clue to this is Ly49D induced PI3K activation, which occurred in the absence of CD45 [126].

4.4. The effect of CD45 on TLR signalling

Src family kinases and other signalling molecules such as PI3K can affect TLR signalling but their place in the pathway is not well defined and their effect can differ depending on the cell type or TLR stimulated [reviewed in 127]. Down regulation of Hck in a macrophage cell line reduced LPS-induced TNFα production [128] and Lyn$^{-/-}$ bone marrow derived dendritic cells (BMDC) produced less IL-12 [129,130]. However, normal or enhanced levels of IL-1, IL-6 and TNFα were observed after LPS/IFNγ stimulation of BMDC from Hck, Lyn and Fgr triple knockout mice [131], leaving the role of Src family kinases in TLR-induced cytokine production uncertain and potentially complex. In CD45$^{-/-}$ BMDC, TLR2 and TLR9 stimulation increased the production of inflammatory cytokines such as IL-12, TNFα and IL-6 [32,132] yet only a slight effect was observed with LPS [32]. However, reduced levels of IFNβ were observed after stimulation with LPS or poly(I:C), a TLR3 ligand [32]. CD45$^{-/-}$ mice were much less efficient at secreting IFNα in response to lymphocytic choriomeningitis virus infection and were unable to clear the infection [133]. Our current hypothesis is that CD45 has a negative effect on MyD88-dependent TLR signalling leading to pro-inflammatory cytokine production and a positive effect on MyD88-independent, TRIF-dependent signalling leading to IFNβ production. Lyn, Hck and Fyn were all hyperphosphorylated in CD45$^{-/-}$ BMDC and Lyn was not activated upon LPS stimulation suggesting that Src family kinase dysregulation contributes to the effect of CD45 on TLR induced cytokine secretion. However, other signalling pathways (for example from ITAM receptors, cytokine receptors and adhesion molecules) and TLR-induced feedback loops can all impact TLR signalling, making the interpretation of CD45's effect on TLR signalling more difficult.

4.5. The effect of CD45 on cytokine signalling

In T cells, CD45, Lck and Zap70 were shown to associate with the interferon receptor, IFNαR1 upon addition of IFNα [134]. These molecules were important for the anti-proliferative effects of IFNα in Jurkat T cells, but not for the inhibition of viral replication or induction of interferon responsive genes mediated via JAK/STAT1/2 pathways [134]. However, another group found that JAK1 was hyperphosphorylated on pY1022/1023 in CD45$^{-/-}$ thymocytes, B cells and Jurkat T cells in response to IFNα [22]. This resulted in more effective suppression of IFNα induced viral replication of the Coxsackievirus B3 (CVB3) in CD45 deficient Jurkat T cells and protection from lethal CVB3 infection in vivo [22]. In addition, IFNα or TNF stimulation of CD45$^{-/-}$ BMDC resulted in enhanced IL-6 secretion [132] and the treatment of CD45$^{-/-}$ BMMC with IL-3 led to JAK2 hyperphosphorylation and hyperproliferation [22]. Erythropoietin induced more erythroid colony forming units (CFU) and IL-3 more neutrophil/macrophage CFU from CD45$^{-/-}$ bone marrow progenitors, consistent with a negative regulatory effect of CD45 on cytokine signalling [22]. However, in another study there was no difference in IL-3 induced CFU between CD45$^{-/-}$ and wild-type bone marrow [15]. Interestingly, Lyn$^{-/-}$ mice have increased myeloid and erythroid progenitors and increased erythroid CFU from the spleen in response to IL-3, GM-CSF or CSF-1 [55], illustrating that Lyn can also negatively regulate cytokine signalling [54]. IL-3 induced a hyperproliferative response in BMMC from both CD45 deficient and CD45 wedge mutant mice [92]. Lyn was hyperphosphorylated at its negative regulatory site in both cases, suggesting that Lyn activity is down regulated. Thus CD45 may negatively regulate cytokine signalling by activating Lyn and directly or indirectly down-regulating JAK activity.

4.6. The effect of CD45 on leukocyte adhesion and migration

4.6.1. T cell and macrophage adhesion

CD45 can affect integrin-mediated adhesion in T cells and macrophages [31,135] and CD44-mediated adhesion and spreading in T cells [71,72]. CD45$^{-/-}$ bone marrow derived macrophages (BMDM) adhered more rapidly to tissue culture plastic than wild type macrophages, but $\alpha_M\beta_2$ mediated adhesion to plastic dishes was not sustained unless macrophage-colony stimulating factor (M-CSF) was present [31]. The $\alpha_M\beta_2$ integrin CR3, CD45 and Lyn all colocalised with F-actin at focal adhesion sites, which formed in both CD45$^{+/+}$ and CD45$^{-/-}$ cells. The Src family kinases Hck and Lyn, but not Fgr, were hyperphosphorylated at their negative regulatory site in the absence of CD45, yet were more active in an in vitro kinase assay, implying that CD45 could also down regulate Hck and Lyn activity in macrophages [31]. In CD45 deficient T cell lines, enhanced adhesion to fibronectin fragments was observed for $\alpha_5\beta_1$ (VLA5), but not $\alpha_4\beta_1$ (VLA4) mediated adhesion [135]. The effect of CD45 on $\alpha_5\beta_1$-mediated adhesion requires CD45 phosphatase activity as well as the transmembrane domain of CD45, which mediates the association with CD45AP.

In CD44-mediated cell adhesion, CD45 is important for down regulating Src family kinase activity [71,72]. CD44 ligation clusters Lck and in CD45 deficient T cells this led to sustained tyrosine phosphorylation, Fyn recruitment, Pyk2 and PLCγ phosphorylation, PI3K activation and elongated cell spreading. This was attributed to sustained Lck activation, as Lck Y394 phosphorylation was evident in the CD44 clusters [72]. In contrast, CD44 ligation in CD45 positive T cells led to the recruitment of CD45 to the CD44-Lck clusters, minimal Y394 phosphorylation, transient tyrosine phosphorylation and limited, round cell spreading. This indicates that CD44-associated Lck can be activated in the absence of CD45 and that recruitment of CD45 down regulates Lck

activity. CD45 recruitment may result in the transient activation of Lck due to the competing activities of CD45 and activated Lck. Lymphocytes are highly mobile cells experiencing many transient interactions, perhaps one role of CD45 is to facilitate transient interactions and limit unwanted stable adhesion.

4.6.2. Chemotaxis in neutrophils and T cells

The first paper to suggest that CD45 may affect leukocyte migration was the inhibition of leukotriene B4 and C5a stimulated migration of neutrophils by some CD45 mAbs [136]. Further work noted that during chemokine induced neutrophil migration, CD45 segregates to the front of the cell [137]. Subsequently, $CD45^{-/-}$ neutrophils were found to be hyper-responsive to MIP-1α (CCL3) and MIP-2 (CXCL1) [138]. The $CD45^{-/-}$ neutrophils showed increased peak Ca^{2+} flux responses, ERK1/2 activation and actin polymerization in response to MIP-1α and MIP2. Enhanced in vivo migration was also observed, as there was an increased accumulation of $CD45^{-/-}$ neutrophils in the peritoneum in response to thioglycollate stimulation. Interestingly, this phenotype was similar to that observed in $Hck^{-/-}$ $Fgr^{-/-}$ double-deficient neutrophils and to PIR-$B^{-/-}$ neutrophils [138]. The lack of PIR-B phosphorylation in $Hck^{-/-}$ $Fgr^{-/-}$ cells led the authors to conclude that Hck and Fgr negatively regulate chemokine signalling in neutrophils by tonic phosphorylation of the negative regulator, PIR-B. Thus CD45 may regulate neutrophil migration by regulating the ability of Src family kinases to phosphorylate the negative regulator, PIR-B.

In contrast to neutrophils, CXCL12 (SDF-1) induced migration was reduced in CD45 deficient and Lck deficient Jurkat T cells [139–141]. Although MAPK activity was enhanced in CD45 deficient Jurkat T cells in response to CXCL12, downstream induction of tyrosine phosphorylated proteins such as Pyk2, FAK, p130Cas and paxillin was reduced [140]. There was an inducible association of CD45 with CXCR4 upon CXCL12 addition but this was lost upon treatment with methyl-β-cyclodextrin, a cholesterol-depleting drug that disrupts lipid rafts [140]. Interestingly, CD3 stimulation inhibited CXCL12 induced chemotaxis and CXCL12 stimulation reduced subsequent TCR signalling [139], suggesting that the two pathways are interconnected. Indeed, CXCL12 signalling reduces subsequent TCR induced tyrosine phosphorylation of Zap70, LAT and SLP-76 [139]. CXCR4 was reported to physically associate with the TCR utilizing the Zap70 binding ITAMs to prolong ERK activation and increase intracellular calcium and AP1 activation. This also contributes to the costimulatory effect of CXCL12 on TCR induced cytokine secretion [142]. Thus, CXCL12-induced signalling shares common pathways with TCR signalling and is positively regulated by CD45. CD45 can also modulate CXCL12-induced migration in vivo as $CD45^{-/-}$ bone marrow mononuclear cells show reduced migration to CXCL12 and reduced homing of c-kit^{+} bone marrow progenitors [143]. We have also observed reduced CXCL12-induced migration of $CD4^{-}CD8^{-}$ DN thymocytes (Lai et al, manuscript submitted).

5. Perspective

CD45 can both positively and negatively regulate immune cell signalling by positively or negatively regulating Src family kinase activity. CD45 has a positive effect on TCR signalling and T cell migration by activating Lck. Lck has a positive effect in these signalling pathways by phosphorylating ITAMs in the TCR associated CD3 chains. In BCR and FcR signalling in B cells and mast cells, CD45 has a positive effect that can be achieved by both activating and inactivating Lyn. CD45 can dephosphorylate the negative regulatory tyrosine thus priming the Src family kinases, Lyn, Fyn and possibly Blk, for participation in receptor signalling. Secondly, CD45 can dephosphorylate the activatory tyrosine, thereby limiting the ability of Lyn to phosphorylate negative regulators such as CD22. This dual function of CD45 in the same cell can be achieved by compartmentalising signalling and having separate pools of Lyn, one of which requires CD45 for activation and one that does not. The location of Src family kinases in or out of lipid rafts and their accessibility to CD45 are key factors that determine the extent of Src family kinase activation.

In some cases such as CD44-mediated adhesion, neutrophil migration, cytokine signalling and TLR2 and 9 stimulated cytokine production, CD45 has a negative effect. In these cases, CD45 could either down regulate a positive effect of Src family kinases or upregulate a negative effect. In CD44-mediated adhesion, CD45 is not required for Lck activation, but is recruited to down regulate Lck activity. In cytokine signalling, CD45 down regulates the JAK/STAT pathway, either by down regulating JAK activation or by activating the negative effect of Lyn. Again, the ability of CD45 to down regulate a positive signal may relate to its ability to access that particular pool of Src family kinases.

Src family kinase activity is determined by the net balance of positive and negative regulators that control its phosphorylation state. Through Src family kinase regulation, CD45 is emerging as a regulator of ITAM and ITIM mediated immunoreceptor signalling in leukocytes. These proteins themselves are emerging as invasive regulators of various immune signalling pathways. Besides directly regulating major immune signals such as antigen receptor, Fc receptor and NK receptor signalling, evidence is accumulating for their ability to integrate signals and modulate other receptor signalling pathways such TLR, cytokine and adhesion signalling, see [144] for a recent review.

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