


# Selected signalling proteins recruited to the T-cell receptor–CD3 complex

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## Introduction

Immune responses to infectious pathogens serve to maintain body homeostasis. Among various immune cells, T cells play an important role to fulfil this critical function. A T-cell response to foreign antigen is initiated by the binding of the T-cell receptor (TCR)–CD3 complex to a

## Summary

The T-cell receptor (TCR)–CD3 complex, expressed on T cells, determines the outcome of a T-cell response. It consists of the TCR- $\alpha\beta$  heterodimer and the non-covalently associated signalling dimers of CD3 $\epsilon\gamma$ , CD3 $\epsilon\delta$  and CD3 $\zeta\zeta$ . TCR- $\alpha\beta$  binds specifically to a cognate peptide antigen bound to an MHC molecule, whereas the CD3 subunits transmit the signal into the cytosol to activate signalling events. Recruitment of proteins to specialized localizations is one mechanism to regulate activation and termination of signalling. In the last 25 years a large number of signalling molecules recruited to the TCR–CD3 complex upon antigen binding to TCR- $\alpha\beta$  have been described. Here, we review knowledge about five of those interaction partners: Lck, ZAP-70, Nck, WASP and Numb. Some of these proteins have been targeted in the development of immunomodulatory drugs aiming to treat patients with autoimmune diseases and organ transplants.

**Keywords:** protein–protein interaction; signal transduction; T-cell activation; T-cell receptor–CD3 complex.

foreign peptide bound to an MHC molecule presented on an antigen-presenting cell. Information of this binding is transmitted into the cytosol to activate many signalling proteins.<sup>1,2</sup> The final targets are transcription factors, to alter the gene expression profile, metabolic enzymes, to change metabolic activity,<sup>3</sup> and cytoskeletal rearrangement. Together this leads to cell proliferation and effector

Abbreviations: CAR, chimeric antigen receptor; ERK, extracellular signal-regulated kinase; ITAMs, immunoreceptor tyrosine-based activation motifs; LAT, linker for the activation of T cells; Lck, lymphocyte-specific protein tyrosine kinase; Nck, non-catalytic region of tyrosine kinase; PRS, proline-rich sequence; SH, Src-homology; SLP-76, SH2-domain-containing leucocyte protein of 76 000 MW; TCR, T-cell receptor; TSAd, T-cell specific adaptor protein; VCA, verprolin homology domain-cofilin homology domain-acidic region; WASP, Wiskott–Aldrich syndrome protein; WAS, Wiskott–Aldrich syndrome; ZAP-70,  $\zeta$  chain-associated protein kinase of 70 000 MW

molecule production and secretion, which are crucial for T-cell-mediated immune responses.<sup>4</sup>

The TCR-CD3 complex is a multisubunit protein complex. It is composed of an antigen-binding TCR $\alpha\beta$  heterodimer non-covalently associated with the non-variable signal transduction subunits; the CD3 heterodimers CD3 $\epsilon\gamma$  and CD3 $\epsilon\delta$  as well as the CD3 $\zeta\zeta$  homodimers.<sup>5-7</sup> The cytoplasmic tails of CD3 $\epsilon$ , CD3 $\delta$ , and CD3 $\gamma$  each contain one immunoreceptor tyrosine-based activation motif (ITAM) and that of CD3 $\zeta$  contains three ITAMs, hence one TCR-CD3 complex comprises 10 ITAMs. The conserved amino acid sequence of the ITAMs is D/ExYxxLx (6-8)YxxL. Antigen binding to TCR- $\alpha\beta$  results in phosphorylation of the ITAM residues, leading to recruitment and activation of multiple downstream signalling molecules including enzymes and adaptor proteins.<sup>1,4</sup> As there is a myriad of signalling molecules, regulated protein-protein interactions are one of the critical mechanisms for regulating specificity in signal transduction. Over the past decades a large number of proteins have been reported to be recruited to the TCR-CD3 complex. Here, we review recent data on five (direct or indirect) interaction partners of the TCR-CD3 complex, including the lymphocyte-specific protein tyrosine kinase (Lck), CD3 $\zeta$ -associated protein kinase of 70 000 MW (ZAP-70), non-catalytic region of tyrosine kinase (Nck), Wiskott-Aldrich syndrome protein (WASP), and the inhibitor of Notch-1 signalling Numb (Table 1). Other proteins associated with the TCR-CD3 complex have been discussed elsewhere and they are not covered in this review.<sup>8-13</sup> The effects of some mutations of these proteins on TCR signalling is shown in Table 2.

T cells develop in the thymus where self-reactive T cells are deleted by a process called negative selection, which is based on a strong signal elicited by high-affinity binding to the self-peptide MHC.<sup>14,15</sup> A lack of, or mutation in, the critical proteins involved in TCR-CD3 signalling, such as ZAP-70 and WASP, causes a reduction of the TCR-CD3 signalling strength that allows autoreactive T cells to escape from negative selection and reach peripheral tissues.<sup>16-20</sup> These autoreactive T cells can be activated in response to self-peptide, which consequently

leads to tissue injury known as autoimmune disease.<sup>15,21</sup> Hence, chemical agents blocking specifically the T-cell activation process are promising therapeutic interventions for the treatment of T-cell-driven diseases. Here, we cover the information on some inhibitors that target the signalling proteins at the TCR-CD3 as they may have a potential to be used for the treatment of autoimmune disorders and in organ transplantations.

## Lck

Members of the Src family of protein tyrosine kinases modulate signal transduction downstream of transmembrane receptors in most, if not all, cell types. In T cells, Lck is a member of the Src family of 56 000 MW. TCR-CD3 engagement with an antigenic peptide MHC triggers the phosphorylation of the ITAM tyrosines by Lck.<sup>22</sup> Phosphorylated ITAMs then become a docking site for ZAP-70, which is also activated by Lck upon binding to the ITAMs.<sup>23</sup> Subsequently, ZAP-70, together with Lck, phosphorylates downstream signalling molecules, to activate TCR-CD3-controlled signalling cascades.

Lck contains an N-terminal membrane anchor region (SH4 domain), a unique domain, an Src-homology 3 (SH3) domain, an SH2 domain, a catalytic kinase domain and a short C-terminal tail (Fig. 1a). The SH4 domain is post-translationally modified by the addition of lipids, including myristoylation and palmitoylation, which allows the attachment of Lck to the plasma membrane. A serine 59 residue in a unique domain of Lck can be phosphorylated by the extracellular signal-regulated kinase (ERK)<sup>24</sup> and phosphorylation of this residue inhibits Lck activity.<sup>25</sup> In addition, Lck activity is tightly regulated by a conformational state mainly depending on the phosphorylation and dephosphorylation of two tyrosine residues (Y394 and Y505) on the catalytic kinase domain and the C-terminal tail, respectively.<sup>26</sup> Phosphorylation of Y505 by the C-terminal Src kinase mediates an intramolecular interaction with the SH2 domain, resulting in an inactive or closed conformation of Lck. When Y505 is dephosphorylated by the phosphatase CD45 or SHP-1, the SH2 domain detaches from Y505, so promoting an opened

**Table 1.** Selected proteins interacting with the T-cell receptor (TCR) -CD3 complex

Proteins associated with TCR-CD3	Binding domain of the associated protein	Binding motif of the TCR-CD3	Effect on TCR signalling	References
Lck	SH2	Phospho-ITAM	Enhancement	38
ZAP-70	SH2	Phospho-ITAM	Enhancement	86
Nck	SH3.1 and SH2	Proline-rich sequence (PRS) and Phospho-ITAM within CD3 $\epsilon$	Enhancement	40, 65
WASP	SH3 domain bind to Nck	Indirect via Nck	Unknown	79
Numb	Phosphotyrosine binding (PTB) domain	NPDY motif within CD3 $\epsilon$	Decrease	84

**Table 2.** Mutations of T-cell receptor (TCR) -CD3 binding proteins with their effects on TCR signalling

TCR-CD3 binding proteins	Mutations	Effects on TCR signalling	References
Lck	R154K (SH2 mutant)	Inhibits Lck association with ZAP-70 and CD3 $\zeta$	38
	Y192F	Inhibits Lck association with TSA $\Delta$ , Itk, Pyk2 and SHP-1 and enhances tyrosine-phosphorylated proteins	87
	Y394F	Closed conformation with decreased kinase activity	35,88
	Y505F	Open conformation with increased enzymatic activity	35,88
	Y505F, K273R	Open conformation but lacking kinase activity	35
	Y315F	Inhibition of Vav-ZAP-70 interaction and reduction of tyrosine phosphorylation	89
	Y319F	Impairment of Ca <sup>2+</sup> mobilization, Ras activation and activation of phospholipase C $\gamma$ 1	54
	W131A	Increases kinase activity of ZAP-70	90
	Y315, 319A	Open conformation with increased kinase activity of ZAP-70	51
	Y315, 319F	Closed conformation with ZAP-70 kinase inactive	51
ZAP70	D461N	Inactivates the kinase domain known as 'kinase dead'	50
	Y493F	Inactivates ZAP-70 catalytic activity	91
Nck	Nck1(W38K) (SH3.1 mutant)	Impairs the binding of Nck1 to CD3 $\epsilon$ and decreases ERK activation	65
	Nck1(W143K) (SH3.2 mutant)	Impairs the binding of Nck1 to Cbl	92
	Nck1(W229K) (SH3.3 mutant)	Impairs the expression of CD69 expression and ERK phosphorylation	63
	Nck1(R308K) (SH2 mutant)	Impairs the binding of Nck1 to CD3 $\epsilon$ Abrogates the binding of Nck1 to ADAP	65,93
WASP	WASPAC (deletion of amino acids 444–502 at C terminus)	Inhibits actin polymerization but enhances the activation of NFAT transcription factor and ERK phosphorylation in human T cells	94
	L46P (WH1 mutant)	Impairs the chemotactic migration of human T cells and actin cytoskeleton reorganization	95
	A47D (WH1 mutant)	Impairs the chemotactic migration of human T cells and actin cytoskeleton reorganization	95
Numb	$\Delta$ Numb (condition deletion of <i>Numb</i> )	Normal CD3 $\zeta$ phosphorylation in murine T cells	96

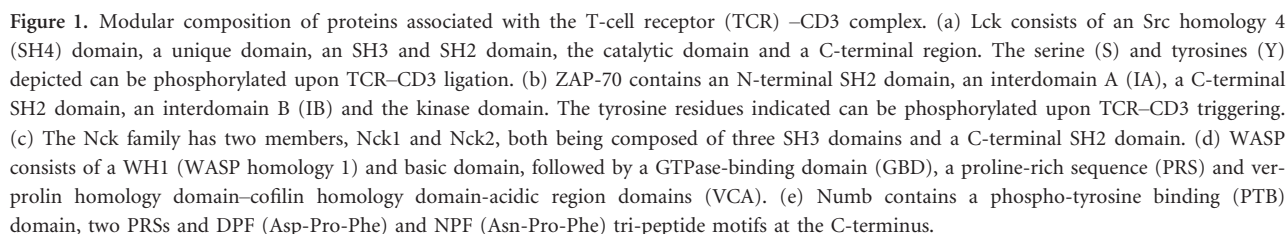
conformation. The opened conformation allows phosphorylation of Y394 by Lck trans-autophosphorylation.<sup>26,27</sup> However, doubly phosphorylated tyrosines Y394 and Y505 might also exist and confer a dominant effect of kinase activity over the inhibitory Y505.<sup>28</sup>

Different pools of Lck have been identified, including Lck in the cytoplasm, Lck anchored to the plasma membrane and Lck associated with the co-receptors CD4 and CD8.<sup>29</sup> Approximately 40% of Lck is already active (phosphorylated at Y394) in resting T cells and upon TCR engagement the amount of active Lck increases, as seen by Forster resonance energy transfer.<sup>30–32</sup> In addition, the distribution of Lck to its correct destination may regulate the function of Lck in phosphorylating its substrates.<sup>26</sup> Several lines of evidence have also suggested that initial phosphorylation of the CD3's ITAMs is mediated by free Lck, whereas the co-receptor-associated Lck acts as an adaptor molecule to bring the CD4 or CD8 molecule to the phosphorylated TCR-CD3 complex in a later step.<sup>33,34</sup> Furthermore, it has been suggested that the conformation of Lck determines its distribution. Lck in the opened conformation might allow clusters of Lck, whereas the closed conformation inhibits the clustering. TCR triggering induces the clustering of Lck with the

phosphorylated TCR-CD3, suggesting that conformation-driven Lck clustering may determine its localization to perform its activity.<sup>35</sup> These findings suggest that Lck recruitment to the TCR-CD3 complex induces the phosphorylation of the ITAMs.

Lck can be co-immunoprecipitated with the TCR-CD3 complex upon TCR-CD3 ligation, suggesting that these two proteins can interact directly or indirectly with each other.<sup>36</sup> An indirect interaction might be mediated by RhoH, a haematopoietic-specific Rho GTPase.<sup>37</sup> The direct interaction might be mediated by the SH2 domain of Lck and phosphorylated ITAMs.<sup>38,39</sup> In addition, the SH2 domain of Lck can also interact with lipid within the plasma membrane upon TCR activation. This binding might be crucial for a lateral diffusion of Lck to interact with the triggered TCR-CD3 complex.<sup>39</sup> These data indicate that localization of Lck to TCR-CD3s that are phosphorylated on few tyrosines facilitates the phosphorylation of the other ITAM tyrosines within CD3.

Our own data have suggested that the resting TCR-CD3 is in a closed conformation, in which the ITAM tyrosines are not exposed, but hidden within the quaternary structure of the TCR-CD3 complex.<sup>40,41</sup> Upon peptide-MHC binding to TCR- $\alpha\beta$  an open CD3



As Lck expression is found only in T cells and natural killer cells, selective inhibitors that target Lck would potentially provide a safe treatment of diseases mediated by over-activation of T cells such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and organ graft rejection.<sup>43</sup> A large number of compounds have been reported that selectively inhibit Lck activity by binding to the ATP pocket of Lck's kinase domain.<sup>44</sup> Some of those inhibitors prevent the allograft rejection in mouse models,<sup>45,46</sup> and one inhibits the hind paw swelling in an adjuvant-induced rat arthritis model.<sup>47</sup>

ZAP-70 causes a profound combined immunodeficiency, which is characterized by an absence of CD8 T cells and a defective function of CD4 T cells.<sup>17,18</sup> Combined mutations of R192W and R360P in ZAP-70 cause an autoimmune syndrome. The former mutation results in decreased binding to phospho-CD3, whereas the latter mutation reduces an autoinhibitory mechanism.<sup>48</sup> These mutations that alter TCR signalling thresholds cause autoimmune diseases as phenotypically demonstrated by uncontrollable bullous pemphigoid, colitis and proteinuria.<sup>48</sup>

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70 is in an autoinhibited conformation mediated by the intramolecular interaction of Y315 and Y319 with the kinase domain.<sup>50</sup> Upon TCR engagement, the tandem SH2 domains of ZAP-70 are recruited to doubly phosphorylated ITAMs of the CD3 subunits. Binding to the CD3 subunits changes ZAP-70 conformation to an opened conformation with the release of Y315 and Y319 from the kinase domain. This facilitates the phosphorylation of Y315 and Y319 by either Lck<sup>51,52</sup> or by trans-autophosphorylation.<sup>53</sup> Likewise, the conformational change also gives rise to a more flexible kinase domain, resulting in phosphorylation of Y493, which is located within the activation loop of the kinase domain, by either Lck or by trans-autophosphorylation.<sup>51</sup> Phosphorylation of Y493 allows ZAP-70 to be catalytically active. Lck can bind with its SH2 domain to phospho-Y319 of ZAP-70 and is required to mediate the phosphorylation of various tyrosine residues on ZAP-70.<sup>54</sup> Mutation of ZAP-70's Y319<sup>53</sup> or Lck's SH2 domain<sup>54</sup> abrogates the Lck–ZAP-70 interaction and consequently impairs downstream signalling. Taken together, the activation of ZAP-70 relies on two steps: first binding of the tandem SH2 domains of ZAP-70 to doubly phosphorylated tyrosines within the ITAMs of CD3, causing a conformational change, and second the Lck- and ZAP-70-mediated phosphorylation of Y315, Y319 and Y493 resulting in full ZAP-70 activation.<sup>49–51</sup>

By comparing the different ITAMs among the CD3 subunits (CD3 $\zeta$ , CD3 $\delta$ , CD3 $\epsilon$  and CD3 $\gamma$ ), it is likely that ZAP-70 preferentially binds to fully phosphorylated CD3 $\zeta$ .<sup>9</sup> Recently, a 'catch-and-release' model for ZAP-70 activation has been proposed by Katz *et al.*<sup>55</sup> After recruitment of ZAP-70 to the phosphorylated TCR–CD3 complexes and ZAP-70 phosphorylation by Lck, activated ZAP-70 is released from the TCR–CD3 complexes into the plane of the plasma membrane. The association of ZAP-70 with the membrane might be mediated by the binding of the SH2 domains to lipids or of phosphotyrosines to other membrane-associated proteins. Consequently, empty phospho-TCR–CD3 complexes allow the recruitment of additional ZAP-70 molecules to the TCR–CD3 for activation of additional ZAP-70. The released ZAP-70 translocates within the membrane into adjacent protein islands to mediate phosphorylation of its substrates including the linker for the activation of T cells (LAT) and the SH2-domain-containing leucocyte protein of 76 000 MW (SLP-76).<sup>55</sup> Phosphorylated LAT and SLP-76 adaptor proteins have various interacting partners such as the phospholipase C- $\gamma$ 1, which is recruited to these two proteins to form the LAT/SLP-76 signalosome.<sup>56</sup> Forming of this signalosome results in T-cell activation, proliferation and differentiation.

As ZAP-70 is required to initiate T-cell activation, inhibition of ZAP-70 from interacting with the TCR–CD3 by small molecules may be used to treat patients with autoimmune diseases and organ transplants. High-throughput

screening of a library of 132 842 compounds has been conducted to find inhibitors that would disrupt the interaction of ZAP-70 with CD3 $\zeta$ .<sup>57</sup> A series of pyrimidine derivatives that can inhibit ZAP-70 activity have been identified and patented by researchers and Novartis companies.<sup>58</sup>

In recent years, chimeric antigen receptor (CAR)-expressing T cells have been used for tumour immunotherapy. CARs consist of an extracellular anti-tumour antigen single Fv fragment, a transmembrane region and the cytoplasmic tail of CD3 $\zeta$ . CAR signalling relies on tumour antigen-binding-induced CD3 $\zeta$  tail phosphorylation. An *in silico* model has suggested that the sensitivity of TCR signalling is modulated by the differential affinities of ZAP-70 to the ITAMs of CD3 $\zeta$ , and sequential phosphorylation of these ITAMs leading to a 'switch-like' response of TCR signalling.<sup>59</sup> Cytokine production by T cells could occur without phosphorylation of the CD3 $\zeta$ , CD3 $\delta$ , CD3 $\gamma$  chains when there are intact CD3 $\epsilon$  chains.<sup>60</sup> It has been suggested that no matter which ITAMs are phosphorylated, the number of ITAMs to be phosphorylated would determine the outcome of the T-cell response.<sup>61</sup> Hence, to obtain effective CAR-T cells with a strong anti-tumoral cytotoxic function but without producing too much cytokine, preventing the so-called cytokine storm, one may optimize CD3 $\zeta$  signalling by titrating the number of ITAMs to be phosphorylated and by using other CD3 chains than CD3 $\zeta$ .

## Nck

Nck is a 47 000 MW cytosolic adapter protein that is composed of three SH3 domains (SH3.1, SH3.2 and SH3.3) and one SH2 domain (Fig. 1c). In humans, two Nck isoforms exist; Nck1/Nck $\alpha$  and Nck2/Nck $\beta$ , which share 68% amino acid sequence similarity.<sup>62</sup> Although redundant roles of Nck1 and Nck2 have been reported, our previous work has shown that Nck1 and Nck2 molecules are functionally non-redundant in T-cell activation.<sup>63</sup> In response to TCR triggering, Nck is recruited to SLP-76 to mediate actin rearrangement, which is essential for immunological synapse formation, T-cell activation and cell movement.<sup>64</sup> Nck is doing so by binding to WASP.

In addition, inducible direct association of Nck to the TCR–CD3 complex occurs when the latter is triggered. For this association, Nck simultaneously uses its SH3.1 and SH2 domains.<sup>65</sup> The SH3.1 domain directly interacts with the PxxPxxDY sequence located within the proline-rich sequence (PRS) of the CD3 $\epsilon$ .<sup>40</sup> For this association to occur the TCR needs to be in its *Active* CD3 conformation and the tyrosine needs to be in the non-phosphorylated state.<sup>40,66</sup> The SH2 domain interacts with the second tyrosine of the CD3 $\epsilon$  ITAM, when this tyrosine is phosphorylated.<sup>65</sup> The functions of Nck–CD3 interaction is not well understood. A knock-in mouse



strain was generated in which the CD3 $\epsilon$  PRS was replaced with another sequence, abolishing the binding to the SH3.1 domain of Nck, but most likely also to Numb (see below).<sup>67</sup> The fact that Nck is a positive and Numb a negative regulator of signalling, might explain why the phenotype of the mutant T cells was mild. To only block the Nck-CD3 interaction, another knock-in mouse line with point mutations of the two central prolines of the PxxP motif of CD3 $\epsilon$  PRS to alanine has been generated.<sup>68</sup> Indeed, T cells from these knock-in mice do not recruit Nck to the TCR upon stimulation. In addition, this mutation is accompanied with impaired CD3 $\zeta$  phosphorylation and decreased ZAP-70 recruitment to the TCR-CD3 complex, as well as impaired ZAP-70 phosphorylation.<sup>68</sup> Moreover, the SH3.2 domain of Nck can bind to a proline motif in the unique domain of Lck.<sup>69</sup> Recently, another adaptor protein called the T-cell specific adaptor protein (TSAd) was identified that interacts with the Src family of proteins including Lck and promotes actin polymerization via interaction with Nck.<sup>70</sup> Nck and Lck contain multiple binding sites on TSAd. The Nck SH2 interacts with phospho-TSAd whereas the Nck SH3.1 and SH3.3 interact with TSAd PRS. The SH2 Lck binds to phospho-TSAd and the Lck SH3 binds to the TSAd PRS. Taken together, Nck recruitment to the TCR-CD3 complex may also bring Lck to TCR.

Interestingly, the importance of the Nck-CD3 interaction might depend on the antigen quality, as this interaction was critical for stimulation of T cells with weak (low-affinity) antigens, but not with strong (high-affinity) antigens.<sup>71</sup> Foreign antigens are often of high affinity and self antigen of low affinity.<sup>72</sup> Hence, the requirement of Nck recruitment for T-cell activation only by low (and not by high) affinity antigens has raised the possibility for inhibition of the Nck-CD3 interaction as a target for treatment of autoimmune diseases caused by self-reactive T cells. Borroto *et al.*<sup>73</sup> have chemically generated a low-molecular-weight inhibitor targeting a non-canonical pocket within the Nck SH3.1 domain. As expected, this inhibitor prevented the binding of Nck to the TCR-CD3 complex. T-cell activation in response to low-affinity antigens was strongly inhibited by this inhibitor, as seen in mouse models for psoriasis, asthma and multiple sclerosis. Interestingly, the T-cell response to a mouse pathogen acting as a strong high-affinity peptide was normal after treatment with this inhibitor. Altogether, these results indicate that this synthetic inhibitor could be a candidate to be evaluated in clinical trials to treat various T-cell-mediated autoimmune diseases.<sup>73</sup>

## WASP

WASP belongs to the WASP family of proteins consisting of WASP, N-WASP and WAVE/SCAR molecules.<sup>74</sup>

Mutation of WASP or lack of WASP expression causes the Wiskott-Aldrich syndrome (WAS), which is characterized by thrombocytopenia, eczema, increased susceptibility to infection and increased risk to develop autoimmune disease.<sup>20,75</sup> WASP contains a WASP homology 1 domain, a basic domain, a PRS, a GTPase-binding domain and a verprolin homology domain-cofilin homology domain-acidic region (VCA) domain (Fig. 1d). These domains are required for binding to different cytoskeleton-regulating proteins. For instance, the GTPase-binding domain binds CDC42,<sup>76</sup> whereas the PRS acts as a binding site for various SH3-containing proteins such as Nck.<sup>77</sup> The function of WASP at the SLP-76 signalosome in regulating actin skeleton dynamics is well described.<sup>78</sup>

As WASP is the binding partner of Nck,<sup>77</sup> we tested whether recruitment of Nck to the TCR-CD3 complex may also bring WASP to the TCR-CD3. We found that WASP is co-immunoprecipitated with the TCR-CD3 complex after T-cell activation.<sup>79</sup> However, whether this was mediated by Nck is not known. Although the function of WASP recruitment to the TCR-CD3 complex has not been investigated, these results suggest that there would be an alternative pathway of WASP (besides the SLP-76 signalosome) to regulate actin reorganization in the vicinity of the TCR-CD3 complex.

## Numb

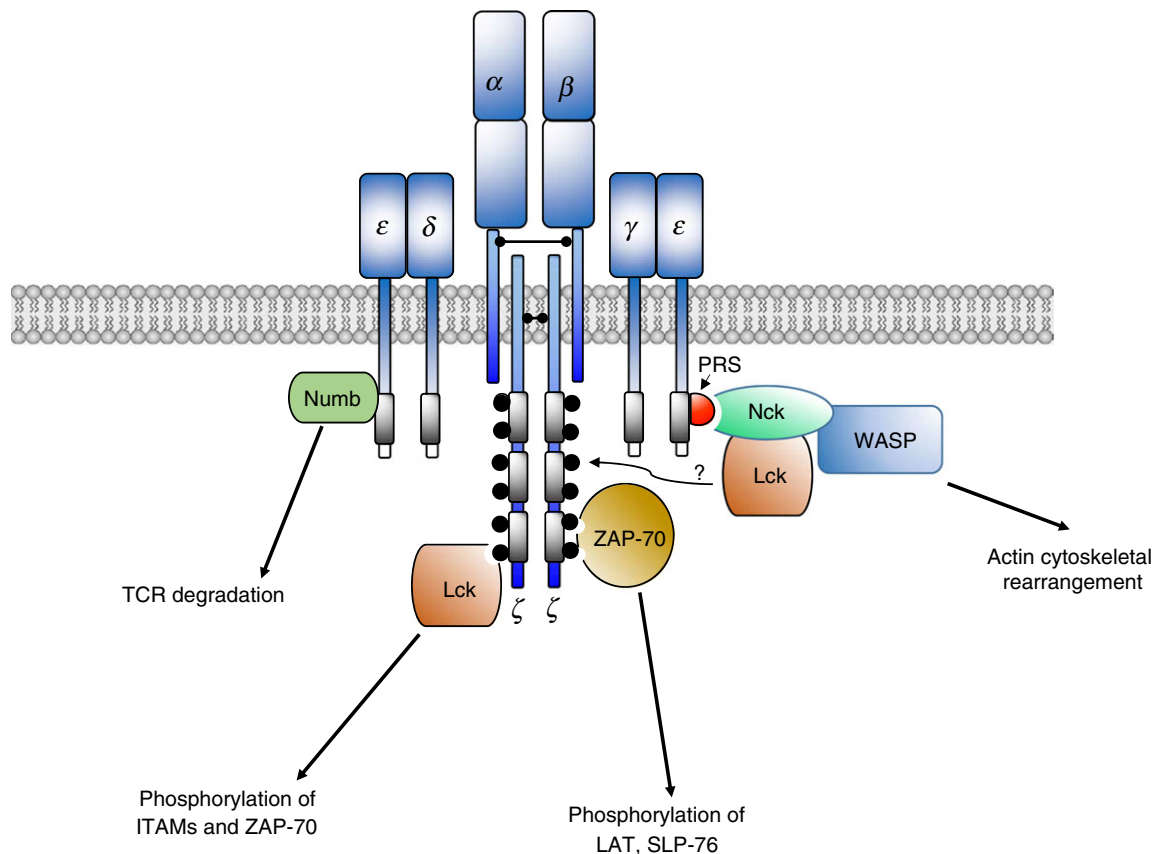
Numb is an adaptor protein that regulates receptor internalization. Numb is up-regulated in the active phase of multiple sclerosis<sup>80</sup> and type 1 diabetes.<sup>81</sup> Two homologues of Numb including Numb and Numb-like have been identified in mammals.<sup>82</sup> Numb is composed of a phosphotyrosine binding domain, several proline-rich regions at the centre of the molecule and two tri-peptide motifs (Fig 1e).<sup>82</sup> Numb is involved in the development of murine thymocytes by regulating pre-TCR signalling.<sup>83</sup> In addition, Numb may control TCR signalling in mature T cells. Constitutive expression of CD69 and interferon- $\gamma$ , as well as constitutively phosphorylated ERK, are found in the CD4<sup>+</sup> T cells from dominant negative Numb transgenic mice. Upon stimulation, CD4<sup>+</sup> T cells from these mice exhibit higher ERK, ZAP-70 and Akt phosphorylation than those of the wild-type mice, indicating that Numb may be required for a negative control of TCR-mediated signal transduction.<sup>84</sup> It was suggested that Numb plays a role in TCR degradation by simultaneously binding to both Cbl and a site within CD3 $\epsilon$  that overlaps with the Nck binding site, thus mediating TCR degradation.<sup>84</sup>

Numb can bind with its phosphotyrosine binding domain to the cytoplasmic tail of CD3 $\epsilon$  within the PRS to the sequence NP DY.<sup>84</sup> Indeed, an endocytosis motif in CD3 $\epsilon$  in this region has been identified,<sup>85</sup> suggesting that

Numb might be involved in TCR–CD3 endocytosis. Interestingly, Numb was suggested to constitutively associate with CD3 $\epsilon$ . So far, not much is known about the order of binding of the TCR–CD3 binding partners. Here, we propose that in resting T cells, CD3 $\epsilon$  is occupied with Numb that impedes TCR signalling. Upon TCR ligation, a conformational change of the CD3 $\epsilon$  may result in the release of Numb and exposure of the CD3 $\epsilon$  PRS, which is the site that interacts with Nck. Recruitment of Nck to the TCR also brings Lck to the TCR to facilitate ITAM phosphorylation. Full ITAM phosphorylation then releases Nck so that ZAP-70 can bind. Once the TCR signal is transmitted, ZAP-70 is replaced by Numb to mediate TCR degradation and these cause a deviation of T-cell activation. However, further studies are required to elucidate the mechanism underlying Numb-regulated TCR signalling and the related TCR degradation pathways.

## Conclusion

TCR–CD3 complex is the key molecule to initiate biochemical events in T-cell activation and differentiation that can lead to different outcomes, depending on the quantity and quality of the stimulus. Nevertheless, how stimulation of the TCR–CD3 complex can give rise to distinct outcomes still remains unclear. Based on the recent findings, we propose that distinct outcomes may be due to the different interaction partners to be recruited to the TCR–CD3 complex upon TCR–CD3 engagement (Fig. 2). These protein partners are involved in both enhance and decrease of TCR signalling and in different downstream signalling pathways. Lck can interact directly or indirectly with the TCR–CD3 complex and phosphorylate the ITAMs to initiate signal transduction. ZAP-70 directly interacts with the TCR–CD3 complex upon CD3 phosphorylation and activates downstream



**Figure 2.** Selected signalling proteins at TCR–CD3 complex. TCR–CD3 ligation induces a conformational change of CD3 $\epsilon$ , leading to the exposure of its proline-rich sequence (PRS). Nck is then recruited to the PRS within the cytoplasmic tail of the CD3 $\epsilon$ . Subsequently, Lck is associated with Nck upon TCR activation. Hence, Nck recruitment to TCR may also bring Lck to TCR–CD3 complex to mediate phosphorylation of the ITAM motif. In addition, Lck can directly interact with phospho-ITAM. When the second tyrosine of the CD3 $\epsilon$  ITAM is phosphorylated, Nck can bind with CD3 $\epsilon$  using its SH3.1 and SH2 domains in a co-operative manner. In proximity to the TCR–CD3 complex, Lck phosphorylates tyrosines in each ITAM of the CD3 chains. ZAP-70 is then recruited to bind to the phospho-ITAMs, where ZAP-70 itself is phosphorylated by Lck. WASP can be associated with Nck upon TCR activation to regulate actin polymerization. Numb can be associated with the CD3 $\epsilon$  to regulate in TCR degradation leading to a decrease in TCR signalling.

signalling cascades. Nck is recruited to CD3 $\epsilon$  and might co-recruit Lck and WASP to the TCR-CD3 complex. TCR-CD3-recruited WASP might control actin reorganization at TCR-CD3. Numb is a new binding partner of the TCR-CD3 complex and participates in TCR degradation to lessen TCR signalling after T-cell stimulation. However, the exact molecular mechanisms underlying the dynamic distributions of these proteins into and out of the TCR-CD3 complex still need further clarification.

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## Disclosures

The authors declare no conflict of interest.

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