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Buněčné a molekulární mechanizmy aktivace naivních T-lymfocytů

Cellular and molecular mechanisms of naive T-cell priming

Bakalářská práce

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Prohlášení

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Poděkování

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Abstrakt

Priming T-lymfocytů je komplikovaný signalizační proces zahrnující několik úrovní molekulární a časoprostorové regulace. To, zda je zahájena TCR signalizace, závisí na signalizačním prahu TCR, který je pravděpodobně nastaven pomocí molekul CD5 a CD6 během vývoje T-lymfocytů v thymu. Vlastní TCR signalizace („Signal 1“) zahrnuje několik drah, které vedou k produkci hlavních prozánětlivých transkripčních faktorů – NF-κB, NFAT a AP-1. Tyto transkripční faktory se účastní transkripce prozánětlivých cytokinů, z nichž nejdůležitější roli hraje IL-2. Molekulární úroveň primingu T-lymfocytů zahrnuje signalizaci z kostimulačních receptorů CD28, CD27 a HVEM, které jsou lokalizovány v imunologické synapsi. Signální dráhy kostimulačních molekul a TCR sdílejí významnou část signálních molekul, což zajišťuje, že aktivita kostimulačních molekul TCR signalizaci zesiluje. Krátce po aktivaci T-lymfocytů je zvýšena exprese koinhibičních molekul CTLA-4 a PD-1, které naopak TCR signalizaci a signalizaci z kostimulačních molekul tlumí. Souhra mezi kostimulačními a koinhibičními molekulami představuje „Signal 2“, který je zodpovědný za další šíření signálu během T-lymfocytové signalizace.

Klíčová slova

priming T-lymfocytů, TCR signalizace, kostimulace T-lymfocytů, koinhibice T-lymfocytů, TCR signalizační práh, imunologická synapse

Abstract

T cell priming is a complicated signalling process involving several levels of molecular and spatiotemporal regulation. Whether TCR signalling is initiated depends on the TCR signalling threshold which is thought to be set during the T cell development in thymus by CD5 and CD6. TCR intrinsic downstream signalling ("Signal 1") involves several pathways which result in the production of the main proinflammatory transcription factors, namely NF-κB, NFAT and AP-1. Those transcription factors participate in the transcription of proinflammatory cytokines such as IL-2. The molecular interface of T cell priming involves signalling from several types of costimulatory receptors, namely CD28, CD27 and HVEM, which are allocated to the immunological synapse. A significant overlap is present between the downstream signalling networks of TCR and costimulatory molecules which amplifies the transcription of proinflammatory genes. Shortly after T cell priming, coinhibitory molecules, namely CTLA-4 and PD-1, are upregulated to deliver negative signals to tune the stimulatory signalling. The interplay between costimulatory and coinhibitory molecules represents "Signal 2" that is responsible for further progression of T cell signalling.

Key words

T cell priming, TCR signalling, T cell costimulation, T cell coinhibition, TCR signalling threshold, immunological synapse

List of abbreviations

AP-1	activator protein 1
APC	antigen presenting cell
BCL10	B cell lymphoma 10
BTLA	B- and T-lymphocyte attenuator
CARMA1	CARD-containing MAGUK protein 1
CD	cluster of differentitation
Cdc42	cell division control protein 42 homolog
cIAP	cellular inhibitor of apoptosis
CRD	cysteine rich domain
Csk	C-terminal Src kinase
cSMAC	central supramolecular activation complex
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAG	diacylglycerol
DC	dendritic cell
dSMAC	distal supramolecular activation complex
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase
FOXO	forkhead box O
Gads	GRB2-related adaptor downstream of Shc
Grb2	growth factor receptorbound-2
HVEM	Herpesvirus entry mediator
IκB	inhibitor of NF-κB
IKK	IκB kinase
IP3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
Itk	interleukin-2-inducible T-cell kinase
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LFA	lymphocyte function-associated antigen 1
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAP	mitogen-activated protein kinase
MHC	major histocompatibility complex
mTORC	mammalian target of rapamycin complex
NEMO	NF-kappa-B essential modulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T-cells
NIK	NF-κB-inducing kinase
Nur77	nerve growth factor IB
PD-1	programmed cell death protein 1
PI3K	phosphoinositide 3-kinase

PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase
PLCγ1	phospholipase gamma 1
pSMAC	peripheral supramolecular activation complex
Rac	Ras-related C3 botulinum toxin substrate
Raf	rapidly accelerated fibrosarcoma
RasGRP1	RAS guanyl-releasing protein
Rheb	Ras homolog enriched in brain
RhoH	Ras homolog gene family, member H
SH	Src homology
SHP	Src homology region 2 domain-containing phosphatase
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SOS	son of sevenless
Src	sarcoma
TCR	T cell receptor
TRAF	TNF receptor associated factor
TSA d	T cell-specific adaptor protein
VLA-4	very late antigen-4
Zap70	Zeta-chain-associated protein kinase 70

Table of Contents

1 Introduction

T cells represent the centrepiece of adaptive immune responses. Once naïve T cells leave the thymus, they are fully competent to recognize antigen peptides presented by MHC molecules (pMHC), clonally expand, differentiate into effector cells and potentially differentiate into long lived memory cells. The process of antigen recognition and induction of differentiation is termed T cell priming[1].

T cell priming is tightly regulated to prevent spontaneous T cell activation that could result in harmful autoimmune diseases. Several levels of regulation are present at the molecular and cellular interface of T cell priming[2,3].

Regulation of T cell priming at the molecular level involves signalling from several types of receptors. The first step in naïve T cell activation is the pMHC recognition by the T cell receptor (TCR) followed by subsequent downstream signalling events, also referred to as "Signal 1". However, TCR signalling alone is not sufficient for full T cell activation and results in the induction of T cell anergy[4]. For full activation, T cells depend on additional signals from costimulatory and coinhibitory molecules termed as "Signal 2". Finally, the effector functions of the T cell are finely tuned by cytokine signalling in the local microenvironment termed as "Signal 3"[5,6].

All signalling events are also spatiotemporally regulated at the cellular level. Naïve T cells can be primed only by the professional antigen presenting cells such as dendritic cells (DC). At the beginning of the immune response, mature DCs transport captured antigen to the lymph node where they interact with naïve T cells and form a contact termed immunological synapse (IS)[7].

The IS represents a multiprotein signalling platform which comprises TCR, costimulatory and coinhibitory molecules and adhesion molecules that are spatially arranged for optimal signal transduction that leads to full T cell activation[8].

Although intensively studied, the process of naïve T cell priming is still not completely understood. Identification of the molecular and cellular pathways involved in T cell priming is important for the development of T cell transfer based cancer immune therapies as it would allow for *in vitro* generation of so-called third-party cancer specific T cells from healthy donors, that could establish long lasting immune control of malignant disease.

2 Receptors and pathways involved in T cell priming

2.1 TCR signalling

The TCR is a multiprotein complex capable of recognizing antigen peptides presented by MHC class I or class II molecules, delivering the first signals required for T cell activation.
In majority of T cells, TCR is structurally composed of αβ heterodimers associated with CD3 multimeric complex composed of CD3γ, CD3δ, CD3ε, and CD3ζ subunits which form δε, γε, and ζζ dimers (see Fig.1). Neither TCR nor associated CD3 possess kinase activity, instead, signalling occurs through the cytoplasmic ITAMs of the CD3 subunits[9].

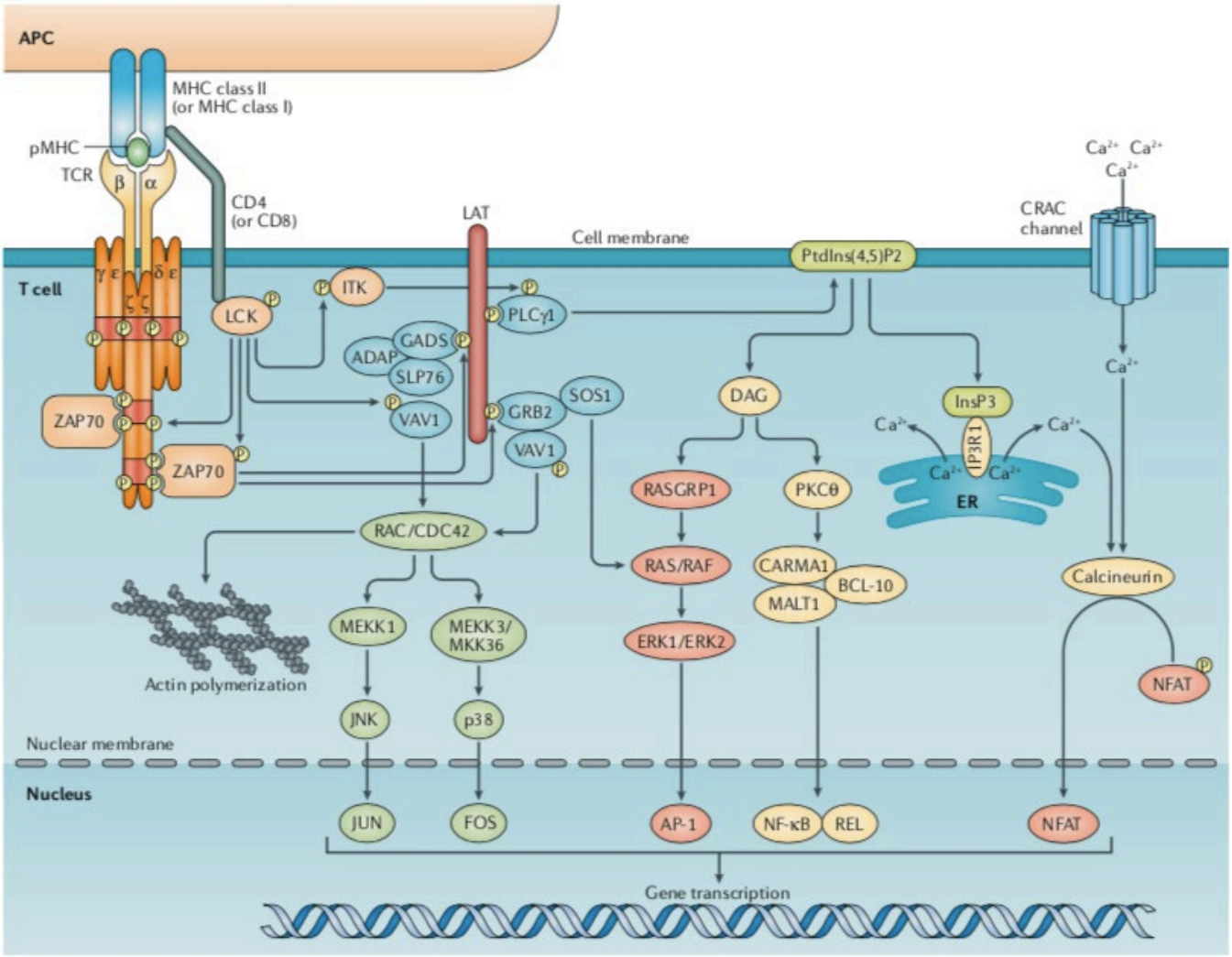

Fig. 1 TCR structure and signalling. TCR is αβ heterodimer associated with CD3, a multimeric complex consisting of δε, γε, and ζζ dimers. Upon pMHC II or pMHC I engagement with TCR, CD3 is phosphorylated by Lck brought into the complex by CD4 or CD8. Zap70 binds to phosphotyrosines on CD3. Lck further phosphorylates Zap70 and brings it into proximity with LAT. Tyrosine residues of LAT are then phosphorylated by Zap70. Phosphotyrosines of LAT subsequently bind PLCγ1 and adaptor proteins Grb2 and Gads. PLCγ1 cleaves PIP_2 into IP_3 and DAG. IP_3 diffuses into cytoplasm where it binds to IP3R1 on ER resulting in Ca^{2+} cytoplasmic influx. Ca^{2+} then binds to calcineurin allowing it to dephosphorylate NFAT which can then enter the nucleus. DAG recruits RASGRP1 and PKCθ. Both RASGRP1 and SOS1 are capable of activating Ras GTPase resulting in MAP kinase signalling: Ras/Raf/MEK/Erk/AP-1. PKCθ phosphorylaes CARMA1 which leads to CARMA1-BCL10-MALT1 signalosome responsible for NF-κB activation. Gads binds SLP-76 which can then recruit Vav1. Similarly, Grb2 can recruit Vav1 too. Vav1 signalling results in Jun and Fos production via two MAP kinase pathways of JNK and p38. Source: Gaud, G., Lesourne, R. & Love, P.E. Regulatory mechanisms in T cell receptor signalling. Nat Rev Immunol 18, 485–497 (2018).

2.1.1 Initiation of TCR signalling

One of the most important molecules for the initiation of TCR signalling is the Src family kinase Lck which is placed to the TCR-CD3 complex by the cytoplasmic domains of TCR co-receptors CD4 or CD8[10]. Lck fulfils 3 major functions – it creates docking sites for other signalling proteins by phosphorylating ITAMs of CD3, it activates ZAP70 and finally, Lck functions as a molecular bridge between ZAP70 and LAT. Phosphorylated ITAMs represent docking sites for SH2 domain-containing molecules such as Zap70, that is crucial for T cell function as Zap-70-deficient patients lack functioning T cells and therefore suffer from severe combined immunodeficiency syndrome (SCID)[11]. Prior to T cell activation, Zap70 remains within cytoplasm in its autoinhibited form. Upon stimulation, Zap70 recruitment to phosphorylated ITAMs uncovers its SH2 domain. However, for Zap70 to be fully activated, its phosphorylation of Y319 by Lck is needed. Moreover, Lck binds to phosphorylated Zap70 on Y319 through its SH2 domain while at the same time binding a proline-rich motif on LAT utilizing its SH3 domain. The engagement of both SH2 and SH3 domains prevents Lck to adopt inactive conformation which amplifies its signalling properties and it creates a molecular bridge between Zap70 and LAT which is important for the formation of LAT signalosome[12].

Zap70-dependent LAT phosphorylation further propagates the initial TCR signal via phosphorylation of LAT on four major sites: Y132, Y171, Y191 and Y226. Phosphorylated Y132 on LAT binds the SH2 domain of PLCγ1[13]. Phosphorylated Y171, Y191 and Y226 represent docking sites for the adaptor protein Grb2 which then associates with guanosine exchange factor SOS. Phosphorylated Y171 and Y191 of LAT bind the adaptor protein Gads which then associates with SLP-76[13].

SLP-76 is an adaptor protein that has been shown to utilize its proline-rich domain to bind SH3 domain of PLCγ1. SLP-76 further recruits a tyrosine kinase Itk which phosphorylates PLCγ1 on Y783 resulting in PLCγ1 activation[14].

LAT signalosome is not the only source of Itk. Itk can be alternatively activated by PIP_3 which is generated by the PI3K pathway (described in detail in chapter 2.1.5). When Itk is activated by PIP_3, it then interacts with SLP-76 via its SH2 domain connecting the LAT signalosome to the PI3K signalling network[15]. Similarly to Itk recruited by the LAT signalosome, Itk recruited by PI3K signalling has also been shown to phosphorylate PLCγ1[16].

2.1.2 NFAT pathway

Upon its phosphorylation by Itk, PLCγ1 generates a secondary messengers IP3 and DAG. IP3 can freely diffuse in cytoplasm where it binds to its receptor IP3R present within the membrane of ER. Upon IP3 binding Ca2+ is released from the ER to activate more signalling molecules such as calcineurin which further dephosphorylates NFAT resulting in NFAT nuclear translocation[17].

2.1.3 MAP kinase pathway

DAG generated by PLCγ1 remains anchored in the plasma membrane owning to its two fatty acid chains. DAG further recruits guanosine exchange factor RasGRP1 and PKCθ. Both guanosine exchange factors RasGRP1 and SOS (recruited through LAT signalosome, see chapter 2.1.1) can activate Ras GTPase which further propagates signalling via Ras/Raf/MEK/Erk/AP-1 pathway resulting in inflammatory cytokine expression[18].

AP-1 is a transcription factor consisting of two subunits, namely Fos and Jun. Another pathway that is involved in AP-1 production, specifically in the production of its Fos subunit, is mediated by Vav1. Vav1 is a cytoplasmic guanosine exchange factor for Rho/Rac GTPases that is recruited to phosphorylated Y112 and Y128 of an adaptor protein SLP-76[19]. Vav1 has a complicated protein structure which allows it to mediate both upstream and downstream signalling[20]. Vav1 activity propagates MAP kinase pathway of Rac/Cdc42/MEKK/JNK/Jun[9,21]. Fos MAP kinase pathway is also mediated by Vav1 resulting in signalling of Rac/Cdc42/MEKK/p38/Fos[9].

A study using phosphoproteomics visualized that Vav1 is able to negatively regulate Lck as Vav1 deficient Jurkat cells presented with increased phosphorylation of Lck substrates such as ITAMs of CD3 or Zap70. The study also revealed capability of Vav1 to mediate crosstalk between the TCR and CD28. This crosstalk was characterized by reduced phosphorylation on Y191 of CD28 in Vav1 deficient Jurkat cells as well as decreased colocalization of TCR with CD28[20].

2.1.4 Canonical NF-κB pathway

Canonical NF-κB pathway is promoted by the downstream signalling events of CARMA1-BCL10-MALT1 (CBM) signalosome. CBM signalosome is assembled upon PKCθ-mediated phosphorylation of CARMA1 which induces CARMA1 conformational changes that reveal its caspase recruitment domain (CARD)[22]. CARMA1 utilizes its CARD domain to bind to BCL10 which promotes BCL10 nucleation. BCL10 forms a filamentous network owning to CARD-CARD interactions between individual BCL10 molecules. C-terminal domain of BCL10 then interacts with MALT1. MALT1 is a paracaspase that can promote NF-κB signalling in two ways. Firstly, it binds TRAF6 which then promotes NF-κB activation. TRAF6 contributes to IKK activation, it is an E3 ubiquitin ligase mediating K63-linked polyubiquitination which both directly and indirectly activates IKK that is further responsible for NF-κB activation. TRAF6 directly polyubiquitinates IKK-γ/NEMO subunit of IKK which results in IKK activation and subsequent IκB phosphorylation. Phosphorylation of IκB results in its ubiquitination and subsequent degradation which releases NF-κB allowing for its rapid translocation into the nucleus. The polyubiquitination of IKK-γ/NEMO by TRAF6 is facilitated by a signalling adaptor protein p62. Depletion of p62 by shRNA has been shown to impair NF-κB production, however, interaction between TRAF6 and NEMO appeared to be undisturbed[23].

The indirect effect of TRAF6 on IKK is dependent on TRAF6 ability to undergo K63 auto-ubiquitination. TRAF6 then recruits TAK1 to its K63 polyubiquitinated chain. TAK1 further phosphorylates IKK-β, an IKK subunit. IKK-β then phosphorylates IκB. Phosphorylation of IκB results in NF-κB activation and its nuclear translocation[24].

Secondly, MALT1 is activated by K63-linked ubiquitination mediated by TRAF6 and it subsequently cleaves A20, a deubiquitinating enzyme functioning as one of the negative regulators of IKK/NF-κB signalling. To balance out MALT1 signalling, A20 is massively upregulated in the response of post-inductive TCR signalling and it is capable of removing K63-linked polyubiquitin chain from TRAF6 and MALT1 chains resulting in the deactivation of IKK[25].

Another negative feedback loop to regulate CBM signalling is driven by TAK1. Silencing of TAK1 increases CBM signalosome assembly and ubiquitination of MALT1[26].

2.1.5 PI3K/Akt pathway

PI3K/Akt pathway represents an important source of signals that promote T cell survival, cell growth and proliferation. The signalling starts upon TCR engagement, when PI3K phosphorylates PIP_2 to produce membrane-bound PIP_3 which serves as a docking protein for PH domain-containing molecules such as Itk, Akt or SIN1[27,28].

Akt is a serine-threonine kinase that activates mTOR, a kinase consisting of two subunits, namely mTORC1 and mTORC2. Akt activates mTORC1 by an inhibitory phosphorylation of a small GTPase TSC 1/2[29]. TSC 1/2 is a GTPase-accelerating protein which keeps Rheb GTPase in its inactive GDP form. Upon its phosphorylation, TSC 1/2 releases Rheb which then binds GTP and activates mTORC1[30]. mTORC1 enhances the expression of Myc which has been shown to promote metabolic shift leading to cell growth and cell cycle progression[31].

mTORC2 is kept inactive via SIN1 that supresses its intrinsic kinase domain. Binding of PIP_3 to the PH domain of SIN1 releases SIN1 from the interaction with mTORC2 leading to mTORC2 activation[32]. mTORC2 is then required for FOXO signalling which is a transcription factor for pro-apoptotic genes. Upon mTORC2 activation FOXO translocates from the nucleus to the cytoplasm resulting in the promotion of T cell survival[33].

2.1.6 Prevention of spontaneous TCR activation

To prevent spontaneous TCR autoactivation negative regulation of Lck is needed. RhoH, a small GTPase serves as a docking protein for Csk kinase which phosphorylates inhibitory tyrosine (Y505) on Lck preventing its autophosphorylation and subsequent downstream signallig. Inhibitory activity of Csk is counteracted by CD45 which dephosphorylates Y505 resolving in Lck activation. During early immunological synapse formation and T cell signaling massive tyrosine phosphorylation occurs, therefore it is not desired for a phosphatase not to be present in the center of an immunological synapse anymore. CD45 is pasively excluded to the periphery owning to its large extracellular domain[2,34]. Lck is also regulated by Zap70-dependent phosphorylation of Y192 within SH2 domain of Lck. When not phosphorylated, SH2 domain of Lck is responsible for its association with CD45. Activated Zap70 therefore serves as a negative regulator of Lck activity[3,35].

2.2 Fine-tuning of TCR signalling threshold

The magnitude of TCR downstream signalling is determined in the earliest moments after pMHC recognition. All T cell are selected for low affinity recognition of cognate pMHC and therefore are constantly receiving sub-threshold signals. For priming to occur, TCR signals have to exceed a given threshold that is specific for every cell and is determined during thymic development long before naïve T cell encounters foreign antigen. Two molecules have been implied in the determination and fine tuning of this threshold – namely CD5 and CD6 [36].

2.2.1 CD5 and CD6 tune TCR signalling

CD5 and CD6 molecules are constitutively expressed on the developing and mature T cells[37,38]. They impact T cell development in thymus and the activation of naïve T cells via altering TCR signalling in both coinhibitory and costimulatory manner. Both CD5, CD6 share the ability to recruit SH2 domain-containing molecules which allows them to form multiprotein signalosomes. CD5 can be phosphorylated through four major tyrosine residues which can then recruit Lck, PI3K, SHP1 and Csk[39–43]. Similarly, CD6 can be phosphorylated on nine tyrosine residues which can result in recruitment of Lck, Zap70, Itk, Vav1 and adapor proteins – SLP76, Grb2 and Gads[44–46]. The CD6 signalosome shares many similarities with the LAT signalosome, a recent study has found that LAT and CD6 signalosomes can interact via T cell-specific adaptor protein (TSA d). This interaction is facilitated by the SH2 domain of TSA d protein which binds phosphotyrosines of both CD6 and LAT[47].

Nowadays CD5 and CD6 are considered to be mainly inhibitory despite recruiting many downstream signalling molecules responsible for T cell activation. However, the outcome of their signalling seems to be context dependent – the majority of studies showing that CD5 is inhibitory were performed on developing T cell whereas the stimulatory effect of CD5 has been mainly demonstrated on mature T cells[48].

2.2.2 Molecular basis of CD5 cosignalling

CD5 belongs to to the superfamily of scavenger receptor cysteine-rich (SRCR) glycoproteins. Currently no physiological ligand has been described[49], however, it has been shown that CD5 doesn't require to bind ligands to exert its inhibitory function[50]. The inhibitory properties of CD5 could be partially mediated by recruitment of Csk kinase and

SHP1 phosphatase[43]. Recent study, however, pointed out that the inhibitory activity of CD5 was independent of SHP1[39].

The magnitude of CD5 mediated signalling has been shown to depend on the affinity of the pMHC-TCR complex established during thymic selection with the affinity of pMHC-TCR being positively corelated with CD5 expression on T cells[51]. The inhibitory activity of CD5 which tunes TCR signalling during T cell development was found to be dependent on its carboxy-terminal region which is necessary for c-Cbl ubiquitin ligase phosphorylation and subsequent degradative ubiquitination of Vav1[52].

Naïve T cells circulating in the periphery are constantly interacting with endogenous self-peptides on MHCs. Recent findings indicate that the interaction with self-peptides provides T cells with subthreshold (tonic) TCR signals that have been of importance in the maintenance of naïve T cell viability[49]. Several studies have demonstrated that the contact between naïve T cell and self-peptide is important for an adequate response to foreign antigens. T cells that have been deprived of encountering endogenous self-peptides presented with an insufficient response to foreign antigens. The insufficient response was characterized by reduced phosphorylation on ITAMs of TCR ζ chain[53] and significantly reduced IL-2 response in $CD4^{+}$ [54]. The increased reactivity found in T cells with higher affinity to self-peptides is correlated with high expression of CD5. This was demonstrated in a recent study where $CD5^{hi}$ $CD8^{+}$ and $CD4^{+}$ naïve T cell subsets showed increased expression of the Nur77-GFP reporter gene in response to self-peptide compared to $CD5^{lo}$. However, it remains unclear how CD5 specifically influences the capacity of T cells to respond towards a foreign antigen[55].

A study has shown that in naïve $CD4^{+}$ and $CD8^{+}$ T cells, increased expression of CD5 influences TCR subthreshold signalling during self-antigen encounters which translates into magnified IL-2 production upon foreign antigen encounter and subsequent T cell maturation indicating that in this specific context CD5 has a stimulatory function[54]. In another study, the increase in CD5 expression was found to be positively correlated with cytokine receptors clustering which resulted in an increased IL-2 sensitivity[56]. Naïve T cells expressing high levels of CD5 have been found to be more immunocompetent compared to the ones expressing low CD5 levels[49]. To promote these relatively more self-reactive and therefore more immunocompetent T cells, high expression of CD5 ensures that TCR signalling during thymic selection is finely tuned in favour of the slightly self-reactive T cells. This is possible owning to CD5 having mainly coinhibitory contribution to TCR signalling during T cell

development in thymus. Tuning the signalling of relatively self-reactive TCR by high levels of CD5 allows these T cells to survive the thymic selection[49].

The molecular mechanism underlying the duality of CD5 signalling is not clear. Recent study shows that CD5 actively adjusts NF-κB signalling in both developing and peripheral T cells. CD5 doesn't seem to influence Zap70, PLCγ or LAT expression levels, instead it was found to alter the levels of IκBα expression. During T cell development, CD5 expression level typically reflects thymocyte development with double negative thymocytes expressing very little CD5 which then increases as the thymocytes proceed to exhibit single positive phenotype. The increase in CD5 expression was found to be positively correlated with IκBα expression due to $CD5^{-/-}$ T cells being unable to maintain high expression of IκBα simultaneously with their inability to express p65, the NF-κB subunit. In contrast, $CD5^{hi}$ T cells expressed high levels of IκBα as well as high levels of p65. The high expression of p65 could be a possible explanation for why high expression of CD5 ensures that T cells are more immunocompetent upon foreign antigen encounter. At the same time the simultaneous high expression of IκBα could be a part of the inhibitory mechanism of CD5 that is independent of SHP1 ensuring that although $CD5^{hi}$ T cells have a significant depot of p65 they remain self-tolerant[57].

2.2.3 Molecular basis of CD6 cosignalling

CD6 utilizes its ectodomain through which it binds to CD166 which is abundantly expressed on reticular epithelia of thymus providing developing T cells with strong adhesion. CD6 and CD166 together form one of the strongest bond amongst surface adhesion molecules[58], however, CD6 doesn't require to bind ligands to exert its inhibitory function[59]. Nonetheless, CD6-CD166 bond could possibly enhance naïve T cell activation by elongating the contact with APCs when scanning for foreign antigen. The inhibitory effect of CD6 has been shown to be dependent on its cytoplasmic domain[59]. Recent data shows that CD6-CD166 interaction results in an increased association of CD6 with SLP76 and Zap70. Interestingly, the same interaction also results in an increased phosphorylation of CD6 linked SHP1 phosphatase[60]. SHP1 has been shown to negatively regulate $CD4^+$ and $CD8^+$ proliferation in response to TCR stimulation. When T cells lack SHP1, they become resistant to Treg suppression due to enhanced Akt phosphorylation which indicates that the PI3K/Akt pathway is more active in SHP1 deficient T cells leading to uncontrolled T cell activation[61]. Nonetheless, whether specifically SHP1 stands behind the inhibitory mechanism of CD6

remains unclear[60].

Similar to CD5, CD6 was also found to set TCR signalling threshold during T cell development in thymus. CD6 level of expression has also been shown to positively correlate with the transition from DN to SP $CD4^+$ and $CD8^+$ T cells. As CD6 expression increases, developing T cells receive more anti-apoptotic signals indicating that CD6 drives T cells through the process of positive selection[62]. In a recent study, $CD6^{-/-}$ mice presented with decreased positive selection of T cells represented by reduced single positive $CD4^+$ and $CD8^+$ T cell populations while the overall T cell count stayed the same within the population of $CD6^{-/-}$ T cells. The upregulation of CD6 was also found to correlate with the upregulation of the early activation marker CD69 which only supports the fact that CD6 plays key role in regulating TCR signalling in favour of T cells surviving negative selection. In the same study, peripheral T cells in $CD6^{-/-}$ mice presented with exacerbated autoimmune conditions which could be due to an increased $CD4^+$ response indicating for a coinhibitory function of CD6.[63]

3 Costimulation and coinhibition in early T cell signalling

The transition from naïve T cells to the activated T cells referred to as T cell priming is facilitated through the costimulatory receptors that can colocalize with TCR-CD3 complex to deliver the earliest costimulatory signals.[8] TCR signalling results in the production of three main transcription factors: AP1, NFAT and NF-κB[64]. In the process of T cell priming, the signalling pathways of costimulatory molecules intertwine with the signalling pathways of TCR which ultimately leads to the amplification of the signal. The costimulatory molecules, namely CD28, CD27 and HVEM, are constitutively expressed on T cell surface and participate in T cell priming in case TCR signalling occurs. During T cell priming, most of the coinhibitory receptors are not detectable[5].

To model the situation of early T cell signalling, the *Tidal model of T cell cosignalling* has been proposed. This model takes into consideration the overlapping expression of cosignalling molecules during T cell activation. After antigen encounter, early costimulatory molecules are upregulated to participate in T cell priming. This is followed by the recruitment of more costimulatory molecules to the immunological synapse. Shortly after however, T cell starts expressing coinhibitory signalling molecules which serve as a negative feedback. *The Tidal model* suggests that at the peak of *the tide* both costimulatory and coinhibitory molecules are expressed. The accumulation of signalling from both of these molecule groups at a given time is what influences the outcome of the naïve T cell signalling and is termed as Signal 2[5].

As signalling pathways downstream of TCR often overlap with signalling pathways downstream of CD28, CD27 and HVEM, this chapter will discuss how these early costimulatory molecules intertwine with TCR signalling. This chapter will also discuss the involvement of coinhibitory molecules in TCR signalling, namely CTLA-4 and PD-1 that are upregulated shortly after the costimulatory molecules.

3.1 Costimulatory molecules

3.1.1 CD28 cosignalling in T cell priming

CD28 is constitutively expressed on naïve T cell surface and in association with CD80/CD86 on APCs it is the predominant source of an early costimulatory signalling resulting in activation of anti-apoptotic pathways and production of cytokines. Similar to CD3, the cytoplasmic domain of CD28 also lacks intrinsic signalling properties but it contains conserved tyrosine-based motifs which bind SH2-conatining molecules upon phosphorylation as well as proline-rich domains which bind SH3-containing molecules[65].

Phosphorylation of the tyrosine residues by Lck and Fyn kinases occurs immediately after binding of CD28 to its CD80/CD86 ligands, which leads to CD28 cross-linking[66].

One of the prominent binding partners od CD28 is PI3K that binds to Y191 of CD28 via the p85α regulatory subunit[67]. This binding induces PI3K activation which activates Akt that delivers signals for cell cycle progression as well as anti-apoptotic signals[33].

Another important binding partner of CD28 is the adaptor protein Grb2 which links CD28 signalling to MAP kinase and NFAT pathways. Grb2 binds to YMNM motif of CD28 through its SH2 domain or it binds the PYAP motif through the SH3 domain[68,69].

The enhancement of the NF-κB pathway by CD28 is driven through its interaction with Gads and subsequent formation of the CBM signalosome[70].

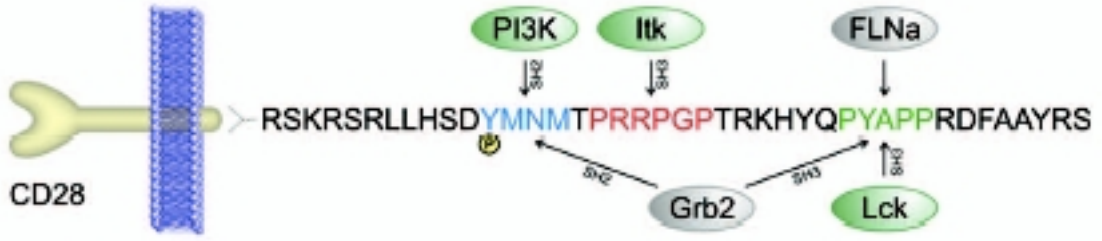

Fig. 2 CD28 cytoplasmic docking sites for signalling molecules. PI3K binds through its SH2 domain to the YMNM motif. Itk utilizes its SH3 domain to bind to PRRPGP motif. Grb binds either to the YMNM motif through its SH2 domain or it utilizes its SH3 domain to bind the PYAP motif which can be also bound by the SH3 domain of Lck. Source: Riha P, Rudd CE. CD28 co-signaling in the adaptive immune response. Self Nonself. 2010;1(3):231-240. doi:10.4161/self.1.3.12968

In a recent study, CD28 signalling *in vivo* was systematically characterized using phosphoproteomic analysis (see Fig. 3). In this study phosphorylation of CD28 downstream signalling molecules was revised upon selective CD28 blockade using CTLA-4 while TCR signalling remained intact which provided an insight into how CD28 operates under physiological conditions[71].

The inhibition of CD28 by CTLA-4 resulted in a decreased phosphorylation of Y191 which is responsible for recruitment of the p85α subunit of PI3K. The phosphorylation of PI3K and mTOR was also altered upon CD28 blockade. CD28-proximal signalling molecules such as Vav1, PLCγ and SHC1 presented with decreased tyrosine phosphorylation. Downstream signalling molecules of PKCθ such as CARMA1 and NF-κB have also exhibited decreased phosphorylation. Vav1, PLCγ, SHC1, PKCθ, CARMA1 and NF-κB need to be phosphorylated in order to exert their stimulatory function. Therefore, the decrease in their phosphorylation upon CD28 blockade indicates that CD28 is in charge of activating these specific molecules[71].

On the contrary, NFAT requires to be dephosphorylated to exhibit its stimulatory function. It has been shown that CD28 blockade results in an increased NFAT serine/threonine phosphorylation showing that CD28 is responsible for NFAT activation .

Together these phosphoproteomics data correspond with the signalling events taking place during TCR signalling. CD28 therefore enhances TCR signalling through quantitatively increasing the amount of stimulatory downstream signalling molecules which ultimately leads to T cell priming[71].

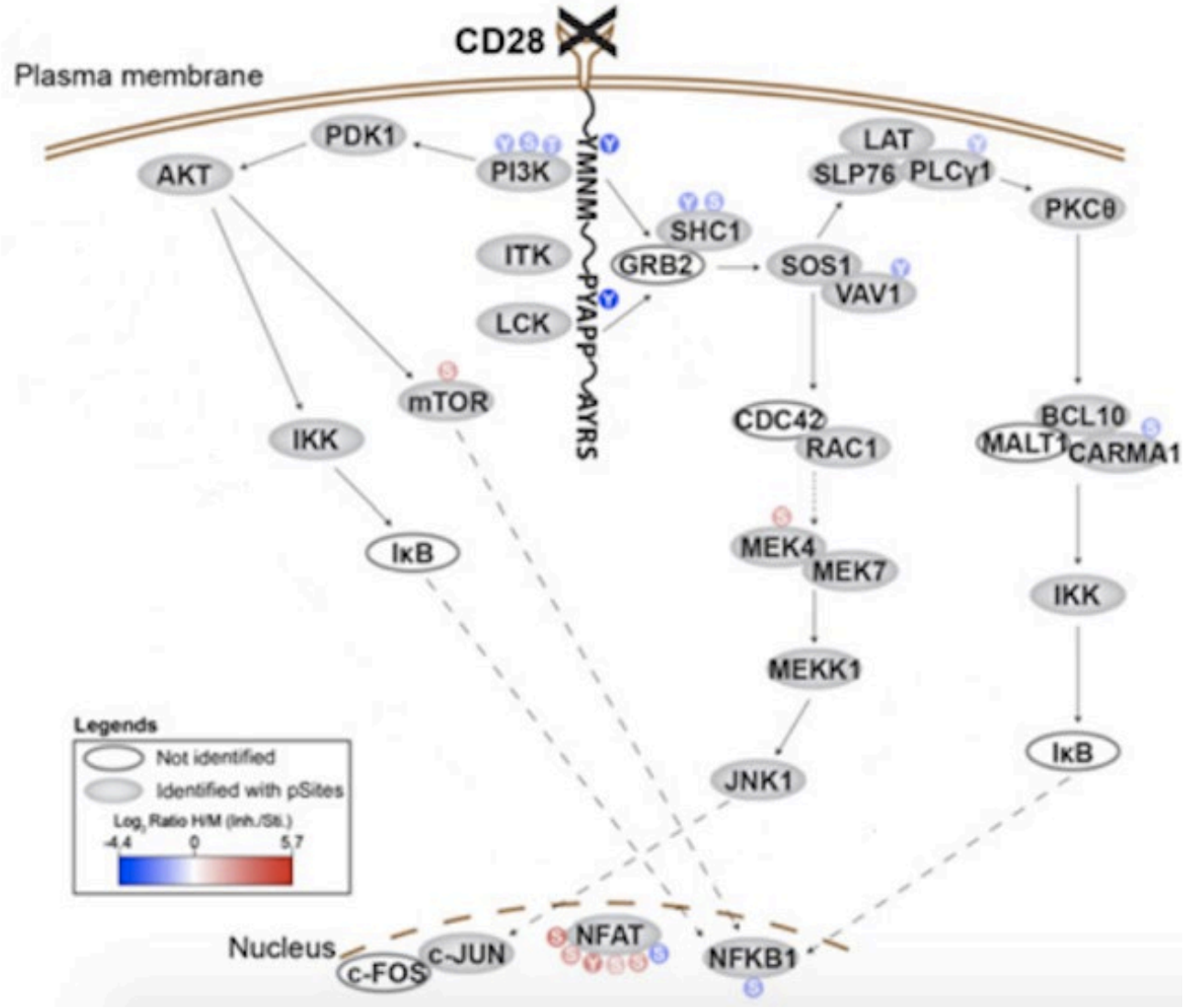

Fig. 3 CD28 pathway based on phosphoproteomics. Dowstream signalling molecules with phosphorylation sites regulated by inhibiton of CD28 are marked in gray. Phosphorylation of downstream signalling molecules marked in white hasn't been shown to be influenced by CD28 inhibition. Source: Tian, R. et al. Combinatorial proteomic analysis of intercellular signaling applied to the CD28 T-cell costimulatory receptor. Proc. Natl. Acad. Sci. 112, E1594–E1603 (2015).

3.1.2 CD27 cosignalling in T cell priming

CD27 synergizes with TCR-CD3 signalling during T cell priming to deliver one of the first costimulatory signals[8]. CD70 is the only known ligand for CD27 and its expression is tightly regulated. The expression of CD70 is transient and it's mainly expressed on mature DCs[72]. Upon CD27 upregulation, three CD27 monomers bind CD70 trimer on APCs forming a ligand-receptor trimeric complex[73]. Assembly of the trimeric ligand-receptor complex enables the cytoplasmic tail of CD27 to bind TRAF adaptor proteins. TRAFs further activate downstream signalling molecules. CD27 has been shown to bind TRAF2, TRAF3 and TRAF5[8].

TRAF2 and TRAF5 are responsible for both canonical and non-canonical NF-κB signalling (see Fig. 4) as well as for Jun activation[74–76]. On the contrary, TRAF3 has been shown to inactivate the non-canonical NF-κB pathway[75].

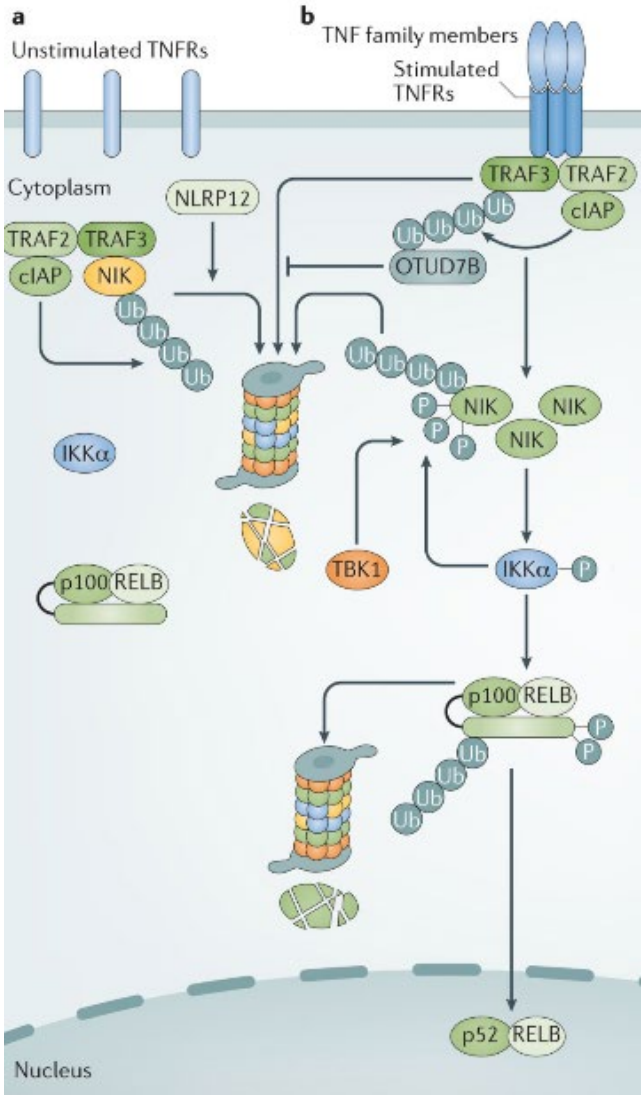

Fig. 4 The general model of non-canonical NF-κB pathway induced by TNFRSF signalling. The non-canonical NF-κB pathway is mediated by signalling from TNFRSF and results in gradual activation of NF-κB-inducing kinase (NIK). When TNFRs are left unstimulated, constitutively synthetized NIK undergoes a degradative polyubiquitination mediated by E3 ubiquitin ligase complex cIAP-TRAF2-TRAF3 complex. When TNFRs are stimulated, TRAF2, TRAF3 and cIAP are recruited to the cytoplasmic domain of TNFR. cIAP then mediates degradative K48 polyubiquitination of TRAF3 which results in TRAF3 degradation and subsequent cumulation of NIK in the cytoplasm. NIK then phosphorylates IKKα which further phosphorylates p100. Phosphorylated p100 then undergoes proteasomal processing resulting in the formation of NF-κB heterodimer RelB-p52 and its translocation into the nucleus. The non-canonical NF-κB pathway is negatively regulated by TRAF3 deubiquitination which prevents NIK enrichment in the cytoplasm. Direct negative regulation of NIK is facilitated by IKKα-mediated phosphorylation which also doubles as a negative feedback loop. Another phorphorylation leading to inactivation of NIK is facilitated by TBK1. Source: Sun, S. The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol 17, 545–558 (2017).

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The basis of the molecular interactions between downstream signalling molecules of CD27 signalling is still incomplete[77]. However, several studies have been made on the detailed mechanisms of the individual downstream molecules of CD27. For instance, a recent study on NIK has found that NIK deficiency in mice T cells results in an impaired T cell priming upon experimental autoimmune encephalomyelitis (EAE) induction. The impaired priming led to a decrease of antigen-experienced T cells which resulted in a complete resistance to EAE[78].

This could be due to several reasons. Firstly, the same study found that NIK deficient T cell displayed impairments an immunological synapse formation due to the dysregulation of F-actin expression[78]. F-actin couples with CD28, possibly the most important cosignalling molecule in T cell priming, hence the dysregulation of F-actin expression could have also negatively impacted the role of CD28 during T cell priming and IS formation[79]. The dysregulation in F-actin expression was characterized by an increased F-actin expression upon naïve T cell activation[78].

Secondly, additional analysis of NIK deficiency in T cell has found that NIK deficient T cells have shown decreased phosphorylation in Zap70, LAT, AKT, ERK1/2 and PLCγ in response of TCR engagement which implies that NIK could also regulate TCR signalling[78].

Another study has shown that NIK mutant T cell present with an impaired production of IL-2 upon anti-CD3 stimulation[80]. Moreover, a different study has shown that there could be a link between TCR and NIK as an increased activation of NIK and IKKα was observed upon TCR stimulation. However, the processing of p100/p52 was not observed simultaneously with the TCR downstream activation of NIK and IKKα[81]. Nevertheless, the information on the molecular mechanisms underlying direct engagement of NIK in TCR signalling still remains unknown[78].

Taken together, CD27 signalling has a close relationship to TCR signalling – both canonical NF-κB and Jun pathways have been meticulously studied and are both an established part of TCR signalling. However, the role and molecular mechanism of the non-canonical NF-κB signalling and its relationship between CD27 and TCR signalling is yet to be discovered.

3.1.3 HVEM cosignalling in T cell priming

Herpesvirus entry mediator (HVEM) consists of CRD1, CRD2, CRD3 and CRD4 ectodomains which are responsible for ligand-binding. HVEM has been shown to interact with LIGHT, BTLA and CD160. The interaction between HVEM and LIGHT is facilitated by CRD2 and CRD3 interaction. The interaction of HVEM with BTLA and CD160 is facilitated by CRD1 and CRD2 domains[82,83]. The outcomes of HVEM-mediated signalling are somewhat complicated as HVEM has both costimulatory and coinhibitory properties as well as the signalling of HVEM and its ligands is bidirectional[83].

All of the ligands of HVEM mentioned above – LIGHT, BTLA and CD160 – are orchestrated in a self-regulatory network[83]. HVEM and BTLA are constitutively expressed on naïve T cells, however, HVEM is kept inactive due to its *cis* interaction with BTLA. It has been estimated that up to 80% of HVEM on the T cell surface is bound in the cis HVEM-BTLA complex. It has been shown that the HVEM-BTLA complex is capable of bidirectional signalling hence the inhibitory activity is mediated by the cytoplasmic domain of BTLA which has the ability to recruit SHP-1 and SHP-2 phosphatase through its phosphorylated ITIM domain. The remaining amount of unbound HVEM has the potential to deliver costimulatory signalling through *trans* interactions during T cell priming. The *trans* interaction between LIGHT and HVEM has been shown to deliver costimulatory NF-κB through TRAF2 molecule[84].

Upon TCR triggering, LIGHT is transiently expressed on a T cell surface. Subsequently, BTLA in the *cis* HVEM-BTLA complex is displaced by LIGHT which results in *cis* HVEM-LIGHT internalization which terminates the coinhibitory signalling delivered by the *cis* HVEM-BTLA complex. The membrane-bound BTLA is then free to engage in a *trans* interaction with HVEM expressed on surrounding T cells which results in PI3/AKT signalling[83].

3.2 Coinhibitory molecules

3.2.1 CTLA-4

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a coinhibitory molecule that is recruited to the immunological synapse after CD28 is upregulated. CTLA-4 and CD28 are homologues and both share the same MYPPPY motif for binding CD80 or CD86 with the fact that CTLA-4 binds CD80/CD86 with significantly higher affinity[85]. Shared homology allows for competitive binding of CTLA-4 to CD80/86 which physically sequesters CD28 outside of cSMAC[86]. The search for differences between CD28-CD80 and CTLA-4-CD80 complexes are under way. Recent study shows that TRP50 on CD80 contributes to CD28 affinity as mutation of TRP50 abolishes CD28-CD80 complex formation. Based on mathematical modelling, CD28 might also possess bivalent nature similar to CTLA-4 meaning that it could also bind two molecules of CD80. However, it still remains to be determined what aspects partake in CTLA-4-CD80 high binding affinity[85].

The inhibitory activity of CTLA-4 is mediated through the recruitment of SHP2 and PP2A phosphatases which results in Zap70, LAT, ERK and JNK dephosphorylation.[87–89]

3.2.2 PD-1

Programmed cell death 1 (PD-1) is also a checkpoint inhibitor that is recruited to the immunological synapse during the early T cell signalling[90]. PD-1 binds two ligands to exert its inhibitory function, namely PD-L1 and PD-L2. After PD-1 engages with its ligands it is phosphorylated on ITIM and ITSM which results in the recruitment of SHP2. PD-1 mediated SHP2 signalling has been shown to dephosphorylate Lck, Zap70 and SLP-76 which counteracts TCR signalling and CD28 and CD27 cosignalling. The decreased phosphorylation of Lck, Zap70 and SLP-76 then resolves in the decline of Ras/Raf/MEK/Erk signalling as well as the decline of PI3K/AKT signalling[91–93].

It has been shown that PD-1 upon its activation by PD-1L preferentially dephosphorylates CD28, not TCR. The dephosphorylation of CD28 is mediated by the SHP2 recruited to PD-1[93].

4 Spatiotemporal regulation of T cell priming at the immunological synapse

The immunological synapse (IS) represents a specialized cellular structure that forms between T-cells and APCs. It incorporates four types of receptors – TCR, adhesion molecules, costimulatory and coinhibitory receptors. The signalling processes involved in IS formation represent the first step of T cell priming and ultimately decide if an immune response is initiated[8].

Structurally, the mature IS consists of central, peripheral and distal supramolecular radially symmetric activation complexes (cSMAC, pSMAC and dSMAC). cSMAC is enriched with TCR and CD28 microclusters that are responsible for the recruitment of the earliest signalling molecules. cSMAC also comprises coinhibitory molecules CTLA4 and PD-1 that deliver negative feedback during the early T cell signalling. Clustering of adhesion molecules is typical for pSMAC which contains LFA-1 and VLA-4. Both adhesion molecules have been shown cooperate with the early cosignalling molecules and even some cosignalling properties have been ascribed to LFA-1 alone. For dSMAC, the presence of CD45 is typical as CD45 is a phosphatase which could possibly interfere with the phosphorylation events that are typical for the early T cell cosignalling[8,34,94].

The formation and stability of IS is dependent on integrins, namely LFA-1 and VLA-4. In resting T cell, integrins remain in inactive states. TCR signalling triggers *inside-out* signalling responsible for immediate conformational changes and clustering of adhesion molecules. LFA-1-ICAM-1 interaction resolves in *outside-in* signalling responsible for actin reorganisation and recruitment of cosiganlling molecules as many of them have been proven to couple with actin[95]. LFA-1 activity is directly influenced by colocalization with tetraspanin CD9 which exerts inhibitory outcome on LFA-1 adhesive capacity by disrupting LFA-1 microcluster formation[95]. LFA-1 has the potential activate CD8+ cytotoxic T lymphocytes even in the absence of CD28-mediated costimulation , LFA-1 has also been shown to decrease T cell activation threshold[94]. VLA-4 centralizes in pSMAC, it has been shown that its ligation inhibits TCR microcluster movement allowing for prolonged transmission of downstream signalling[97].

The relevance of IS composition and formation lies in the clustering of cosignalling molecules and early activation markers. A close contact between cells within cSMAC is formed with the help of CD2 molecule, molecules with large extracellular domains such as CD45 are excluded to the periphery[2]. In the beginning of IS formation CD2-CD58 complex

colocalizes with TCR in microclusters within cSMAC. Shortly after, however, CD2-CD58 moves to LFA1-ICAM-1 rich pSMAC and continues to move further to dSMAC where it forms a corolla (petal-like circular structure). The corolla has the ability to capture CD28-CD80 and ICOS-ICOS-L. Corolla-mediated TCR signalling has been shown to augment T-cell activation by recruiting LAT. In contrast to that, high PD-1 levels significantly decrease CD2 signalling[98].

CD28 is allocated to cSMAC via size-based segregation. Both CD28 and LFA1 couple with F-actin. LFA-1-ICAM-1 forms a radially symmetric pSMAC, its F-actin coupling is what prevents molecules from either entering or escaping cSMAC. Simultaneously, CD28-CD80 is driven to form microclusters with TCR owning to its F-actin coupling and its relatively small extracellular domain[79]. How CD27 and HVEM are allocated to the IS remains unknown.

CTLA-4 is one of the checkpoint inhibitors within the IS, its recruitment to cSMAC steadily increases upon CD28 upregulation whereas CD28 is constitutively expressed on the naïve T cell surface in case TCR signalling occurs[8,99] .

PD-1 represents another checkpoint inhibitor within the IS. Live imaging of inhibitory synapse formation provided visualization of PD-1 dynamics showing that PD-1-PD-L1 complex centralizes in cSMAC in the early inhibitory IS formation and then it migrates to the periphery[90].

5 Conclusion

T cell priming is a complex process involving several levels of molecular and spatiotemporal levels of regulation. Firstly, the most important step leading to T cell priming is the initiation of the TCR signalling. Whether TCR signalling is initiated is manipulated by the TCR threshold which represents a minimum cumulative TCR signalling. TCR threshold is thought to be set during the T cell development in thymus by CD5 and CD6 molecules. It has also been found that TCR threshold is dependent on the earliest downstream signalling molecules.

Secondly, TCR intrinsic downstream signalling involves several pathways all of which are interconnected through the formation of the LAT signalosome. The main results of the TCR signalling is the production of the proinflammatory transcription factors, namely NF-κB, NFAT and AP-1. Those transcription factor participate in a proinflammatory gene transcription cytokines such as IL-2. However, TCR signalling alone can't initiate a successful immune response and results in T cell anergy.

Thirdly, T cell priming requires cosignalling molecules orchestration within the immunological synapse. Immunological synapse is a place of contact between T cells and DCs and it allows costimulatory molecules, namely CD28, CD27 and HVEM, to participate in naïve T cell priming by intertwining with the TCR signalling. The interaction of costimulatory molecules with TCR signalling lies either in the recruitment, phosphorylation or dephosphorylation of shared downstream signalling molecules which results in amplification of TCR signalling. However, TCR signalling alone can't initiate a successful immune response and results in T cell anergy. The effects of costimulatory molecules participating in T cell priming are shortly after their upregulation tuned by the upregulation of coinhibitory molecules, namely CTLA-4 and PD-1, which represent an immune checkpoint to prevent autoimmune reactions. The signalling events downstream of costimulatory and coinhibitory molecules represent the "Signal 2" during which it is being decided whether T cell can further progress in the process of differentiation to the "Signal 3" that represents signalling events mediated by the cytokines present in T cell microenvironment.

6 List of citations

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