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Regulation of immune cell development through soluble inositol(1,3,4,5)tetrakisphosphate

Karsten Sauer^{1,*} and Michael P. Cooke²

¹ Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

² Genomics Institute of the Novartis Research Foundation, 10675 John J. Hopkins Drive, San Diego, CA 92121, USA

Preface

The membrane lipid phosphatidylinositol(3,4,5)trisphosphate (PtdInsP₃) regulates membrane receptor signalling in many cells, including immunoreceptor signalling. Here, we review recent data that have indicated essential functions for the soluble PtdInsP₃ analogue inositol(1,3,4,5)tetrakisphosphate (InsP₄) in T cell, B cell and neutrophil development and function. Decreased InsP₄ production in immunocytes causes immunodeficiency in mice and might contribute to inflammatory vasculitis in Kawasaki disease in humans. InsP₄-producing kinases could therefore provide attractive drug targets for inflammatory and infectious diseases.

Because it can be combinatorially phosphorylated on six hydroxyl groups, the cyclic poly-alcohol D-*myo*-inositol is an ideal cellular information carrier. Its many derivatives form an “inositol code”, whose most prominent members are soluble **inositol phosphates** (InsPs) [G] and their membrane-lipid counterparts, the **phosphoinositides** [G]^{1, 2} (Fig. 1, Box 1). In 1983, the identification of inositol(1,4,5)trisphosphate [Ins(1,4,5)P₃, InsP₃ here] as a **second-messenger** [G] that mediates the receptor-induced Ca²⁺ mobilization in most mammalian cells first demonstrated physiological importance for soluble InsPs³. More recently, intriguing functions for several higher-order InsPs have been identified (Box 1). Moreover, the phosphoinositide-lipids phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂) and PtdIns(3,4,5)P₃ (PtdInsP₃) regulate signalling by many receptors in many cell types^{4–9}. PtdInsP₃ is generated by phosphoinositide 3-kinase (PI3K) and metabolized by lipid-phosphatases, including phosphatase-and-tensin-homologue (PTEN), SH2-domain-containing inositol polyphosphate 5-phosphatase 1 (SHIP1) and SHIP2¹⁰. The key to phosphoinositide function is the phosphorylation status of their cytosol-exposed InsP headgroups. These bind to specific domains in signalling or cytoskeletal proteins to control their membrane recruitment. Defects in phosphoinositide metabolism or function contribute

*Correspondence to: K.S. (ksauer@scripps.edu).

Further information

Databases

Entrez Gene: <http://www.ncbi.nlm.nih.gov/gene>

Itpkb | Itpkb | ITPKB | InsP3KB

NCBI Structure: <http://www.ncbi.nlm.nih.gov/Structure>

Itpkb | Itpkb | ITPKB

OMIM: <http://www.ncbi.nlm.nih.gov/omim>

Kawasaki syndrome | Kawasaki disease | Mucocutaneous Lymph Node Syndrome | Infantile Polyarteritis

Web site links

Karsten Sauer's homepage: <http://www.scripps.edu/ims/sauer>

Michael P. Cooke's homepage: <http://www.gnf.org/technology/immunology>

to many diseases, including cancer, leukaemias, immunodeficiencies, autoimmune, neurodegenerative, allergic and inflammatory disorders^{4–9}.

Box 1

Higher-order InsP metabolism and functions

The figure below summarizes important aspects of the mammalian higher-order InsP metabolism with known enzymes. For additional details, see REF.^{44, 74, 76, 114} Orange, enzymes with genetically documented functions in immunocytes. Purple, InsPs found in immunocytes. Bold purple, antigen-receptor modulated InsPs^{17, 18, 23, 25, 45, 58–66, 115}.

Higher-order InsPs have recently been implicated in regulating major cellular processes in eukaryotes:

- Protein membrane-recruitment (InsP₄, reviewed here)
- Apoptosis (*PP*-InsPs/Inositol-pyrophosphates)^{116, 117}
- Cytoskeletal & vesicle dynamics, exo-/endocytosis, cytokinesis (InsP₄, InsP₆; *PP*-InsPs in *S. pombe*)^{44, 117–119}
- Cyclin-CDK inhibition in the *S. cerevisiae* phosphate-starvation response (InsP₇)¹²⁰
- Protein-protein interactions (InsP₄)¹²¹
- Ca²⁺-mobilization (InsP₃, InsP₄, reviewed here; InsP₆)^{122, 123}
- ClC-3 Cl[−]-channels (Ins(3,4,5,6)P₄)¹²⁴.
- O₂/haemoglobin binding in nucleated erythrocytes (Ins(1,3,4,5,6)P₅)⁴⁴
- Antioxidant-function
- mRNA-export from the nucleus (InsP₆, yeast)^{114, 125, 126}
- Translation termination (InsP₆, yeast)¹²⁷
- Gene transcription (InsP₇, yeast)¹²⁸
- mRNA-editing (InsP₆)¹²⁹
- Chromatin-remodeling (InsP₄, InsP₅, InsP₆, yeast)^{130, 131}
- DNA repair (InsP₆, non-homologous end-joining; *PP*-InsPs, yeast hyper-recombination)^{132, 133}
- Telomere length (*PP*-InsPs, yeast)^{134, 135}

InsPs may modulate protein function by acting as

- Essential structural cofactors
- Triggers for induced fit or allosteric structural changes
- Direct antagonistic competitors for phosphoinositide ligands
- Nonenzymatic protein phosphorylating agents (*PP*-InsPs)¹³⁶

Most of these findings were obtained in yeast or non-immunocytes, reviewed in REFs.^{1, 14, 33, 44–47, 74, 76, 117, 137}. InsP-pyrophosphorylation further expands the inositol code and enables pyrophosphate-transfer onto protein substrates. It will be interesting to determine whether InsP₃ 3-kinase deficiency affects specific higher-order InsP-isomer levels in immunocytes, and to analyze whether altered isomers mediate aspects of InsP₄

Membrane

DAG

PLC γ

PtdIns(4,5)P₂

PI3K

PTEN

PtdIns(3,4,5)P₃ (PtdInsP₃)

SHIP

PtdIns(3,4)P₂

IPMK

Ins(1,4,5)P₃ (InsP₃)

IPK1

Ins(1,3,4,5)P₃

Ins(1,3,4,5,6)P₅

Ins(1,3,4,5)P₄

Ins(1,2,3,4,5,6)P₆ (InsP₆)

Ins(3,4,5,6)P₄

PP-InsP₃ (InsP₇)

PP-InsP₄

Soluble

Inositols in immunoreceptor signalling

TCR/BCR-engagement also triggers membrane recruitment and activation of PI3Ks, which phosphorylate PtdIns(4,5) P_2 at the 3-position of its inositol-ring into PtdIns P_3 . PtdIns P_3 then recruits several important signalling proteins to cellular membranes by binding to their **pleckstrin-homology (PH)** [G] or other domains³¹. Among these, the TEC-family protein-tyrosine-kinases (TFKs) **ITK** [G], TEC and BTK are essential for antigen-receptor signalling. In particular, TFKs phosphorylate and activate PLC γ 1 and PLC γ 2^{27, 28}. Furthermore, PtdIns P_3 -binding to the AKT PH domain controls recruitment and activation of this key regulator of lymphocyte survival, proliferation, differentiation and activation^{5, 6, 32}.

The crucial roles of PI3K, PTEN, SHIP1 and SHIP2 in immunocyte development, survival, proliferation and function, and in preventing leukaemia, indicate the importance of carefully controlling PtdInsP₃ turnover in immunocytes (Fig. 1, Box 1)^{5, 6, 32}.

InsP₃ 3-Kinases convert InsP₃ into InsP₄

Besides mediating Ca²⁺ release, InsP₃ can also be converted to InsP₄ by one of four mammalian InsP₃ 3-kinases: the IP₃-3K/IP3K/ITPK gene-family members A, B and C, and the distantly related, predominantly nuclear InsP multikinase (IPMK/IPK2) (Fig. 1,2, Box 1, Table 1)^{11–15, 25, 33–39}. ITPKA is abundant in the nervous system. It was also found in myeloid precursors with little expression in lymphoid tissues^{18, 20}. *ItpkA*^{−/−} mice showed altered **long-term potentiation [G]** in the hippocampus and memory deficits without reported immunological phenotypes^{40, 41}. ITPKB is abundant in brain and haematopoietic cells^{18, 33}. *ItpkB*^{−/−} mice showed defects in lymphocyte development and myelopoiesis^{16–23}. Published data do not indicate an important role for the ubiquitously expressed ITPKC in mouse development, viability, fertility or immune function²³. *ItpkC*^{−/−} thymocytes showed unaltered InsP₃ 3-kinase activity²³. In humans, however, genetic data may indicate an intriguing ITPKC role in inhibiting peripheral T cell function⁴². No roles for IPMK in the immune system have been reported (Table 1), although IPMK might regulate JAK–STAT signalling in flies⁴³.

Biochemical studies and genetic studies in yeast suggest many potential molecular functions for InsP₄, reviewed in REFs.^{1, 11–14, 33–35, 44–47}. InsP₄ binding to their PH domains may regulate membrane recruitment, activation or protein-interactions of Ras GTPase-activating proteins (RASA2/GAP1^m, RASA3/GAP1^{IP4BP})^{48, 49}, BTK, AKT, or certain regulators of vesicular trafficking (synaptotagmins, centaurin-α1/p42^{IP4}). A long-standing controversy concerns InsP₃ 3-kinase roles in regulating Ca²⁺ mobilization in mammalian cells^{14, 44, 45}. Early studies suggested that they limit InsP₃ accumulation by converting it into InsP₄. Others suggested that InsP₄ or a metabolite inhibit Ca²⁺ mobilization, possibly by antagonizing with InsP₃-binding to InsP₃-receptors^{50, 51}. In rat hepatocytes, InsP₄ can induce nuclear Ca²⁺-uptake⁵². In other studies, InsP₄ augmented InsP₃-mediated Ca²⁺ mobilization through an enigmatic mechanism or might even mimic InsP₃^{44, 45}. Genetic data may argue against RASA3-involvement⁵³. In RBL-2H3 or Jurkat cells, exogenous InsP₄ facilitated InsP₃-induced SOCE by inhibiting an InsP₃-metabolizing 5-phosphatase (Fig. 3)⁵¹. Interestingly, InsP₄ alone could not induce SOCE in these cells⁵¹. Conversely, InsP₄ directly activated Ca²⁺-channels in endothelial or neuronal cells^{54–57}. Thus, InsP₃ 3-kinases and InsP₄ can have diverse effects on Ca²⁺-mobilization depending on experimental conditions, cell type, upstream-receptor, their subcellular localization, composition of the InsP₃-receptors or Ca²⁺-channels involved, or other factors⁵¹. This demonstrates the need for genetic and biochemical *in vivo* studies with purified, primary cells.

Similar to PtdInsP₃, InsP₄ can be metabolized through dephosphorylation. The best characterized route is its dephosphorylation by a 5-phosphatase into Ins(1,3,4)P₃, a precursor for many other InsPs (Box 1)^{17, 18, 23, 25, 45, 58–66}. Several phosphatases can dephosphorylate the 5-positions of InsP₃ and InsP₄⁶⁷. Which phosphatases do this in immunocytes is unknown. *In vitro*, the immunocyte-expressed SHIP1, SHIP2 or PTEN can dephosphorylate InsP₄ to Ins(1,3,4)P₃ or Ins(1,4,5)P₃/InsP₃, respectively (Fig. 1)^{68–71}. However, the PTEN/SHIP-deficiency of Jurkat cells⁷² which contain an InsP₄-inhibitable 5-phosphatase⁵¹ indicates relevance for other enzymes.

Besides acting as an InsP₃ 3-kinase, or a non-conventional PI3K of uncertain physiological relevance⁷³, IPMK can also phosphorylate InsP₃, InsP₄, Ins(1,4,5,6)P₄ or Ins(1,3,4,6)P₄ at the 3, 5 or 6-positions to generate Ins(1,3,4,5,6)P₅^{11–14, 25, 33–36, 39, 74}. This likely is the major eukaryotic pathway for producing higher-order InsPs (Box 1)^{14, 75–77}. Because of its

early position in this pathway, some InsP_4 functions might really be mediated by other InsPs .

Studies of $\text{ItpkB}^{-/-}$ mice recently unveiled important *in vivo* functions of ITPKB and InsP_4 and provided novel insight into how InsP_4 acts molecularly.

InsP_4 controls T cell development

$\text{ItpkB}^{-/-}$ mice independently generated by two groups show severe peripheral T cell lymphopaenia and immunodeficiency. The cause is a profound block of T cell development at the $\text{CD4}^+\text{CD8}^+$ double-positive (DP) thymocyte-stage due to impaired positive selection^{17, 18, 23}. Retrovirally expressed wildtype but not catalytically impaired ITPKB restored the development of $\text{ItpkB}^{-/-}$ thymocytes. Hence, the catalytic activity of ITPKB is required in thymocytes. During positive selection, low avidity/affinity TCR engagement induces DP thymocyte maturation. High avidity/affinity TCR stimulation induces apoptosis during negative selection. This prevents the maturation of detrimental self-reactive T cells. Most TCR signalling-mediators are involved in both processes. One study reported defective negative selection in $\text{ItpkB}^{-/-}$ mice expressing the transgenic HY TCR²³. However, the higher deletion in the controls compared to $\text{ItpkB}^{-/-}$ thymocytes likely reflects the presence of mature thymocytes in the control but not $\text{ItpkB}^{-/-}$ sample and not increased negative selection, because mature T cells can induce DP cell death through a cytokine storm³⁰. Moreover, the HY-TCR system may have limited physiological relevance, because expression of this transgenic mature TCR starts before the DP stage, alters the phenotype of DP cell precursors and causes deletion at the DP stage⁷⁸. In contrast, endogenous mature TCRs are expressed mainly on DP and later stages, and significant negative selection occurs after the DP stage. Therefore, detailed studies in more appropriate models are required to conclusively determine whether ITPKB has a role in negative selection. An essential role for ITPKB in positive but not negative selection would be intriguing. Of all the TCR signalling-mediators studied, only RASGRP1, calcineurin B and ERK1/2 are exclusively required for positive but not negative selection^{30, 79, 80}.

InsP_4 controls TCR signalling through ITK

$\text{ItpkB}^{-/-}$ thymocytes show strongly decreased TCR-induced InsP_4 production²³. Total InsP_3 3-kinase activity was reduced by ~50%, indicating that ITPKB might be redundant with other InsP_3 3-kinases in thymocytes. InsP_3 production and Ca^{2+} -mobilization were normal^{18, 23}. This was unexpected, because InsP_3 mobilizes Ca^{2+} and ITPKB converts InsP_3 into InsP_4 , which can itself control Ca^{2+} mobilization (discussed above)^{14, 44, 45}. Although more detailed analyses are needed, the InsP_4 -role in a positive feedback-loop of PLC γ 1 activation discussed below (Fig. 1·3) may explain this conundrum. Impaired InsP_3 -turnover through ItpkB could allow the residual PLC γ 1-activity in $\text{ItpkB}^{-/-}$ thymocytes to produce normal InsP_3 levels¹⁷. Because of the unchanged InsP_3 levels, the failure of catalytically inactive ITPKB to restore $\text{ItpkB}^{-/-}$ thymocyte development indicated a specific requirement for InsP_4 for positive selection. Indeed, addition of a cell-permeable InsP_4 -ester can allow $\text{ItpkB}^{-/-}$ thymocyte maturation in NTOC [G] (Y. H. Huang and K. Sauer, unpublished observations). Mechanistic analyses showed that in $\text{ItpkB}^{-/-}$ DP cells, TCR-induced ERK activation was defective due to impaired ITK membrane recruitment and activation, causing decreased PLC γ 1 activation and DAG production^{17, 18}. TCR stimulation primarily activates ERK through RASGRP1-mediated RAS-activation^{26, 81, 82}. RASGRP1 is recruited to cellular membranes through DAG. The ability of exogenously provided phorbol-12-myristate-13-acetate (PMA), a DAG-analogue, to restore ERK activation in $\text{ItpkB}^{-/-}$ thymocytes and allow their maturation showed, for the first time, that DAG is essential for thymocyte positive selection. It moreover confirmed InsP_4 as an essential

mediator of ITK- and PLC γ 1-dependent DAG production, RAS and ERK activation. But how does InsP $_4$ act?

A first clue came from the finding that InsP $_4$ binds to the ITK PH domain *in vitro*¹⁷. At high, probably super-physiological, concentrations, InsP $_4$ competed with PtdInsP $_3$ for ITK PH domain binding. Surprisingly, physiological, low μ M concentrations of soluble InsP $_4$ promoted ITK PH domain binding to PtdInsP $_3$ -coated beads¹⁷. DP thymocytes contain an ITK pool whose ability to bind PtdInsP $_3$ is transiently increased by TCR stimulation. In *ItpkB*^{-/-} thymocytes, which produce decreased InsP $_4$ after TCR stimulation, basal ITK binding to PtdInsP $_3$ is reduced and TCR stimulation does not increase it. Moreover, TCR-induced ITK membrane recruitment and activation were perturbed. This suggests that TCR-induced InsP $_4$ promotes Itk–PtdInsP $_3$ interactions, and therefore ITK membrane recruitment and activation in DP thymocytes. The precise underlying mechanism remains to be determined. However, the ability of full-length ITK or its PH domain alone to oligomerize opens the possibility that InsP $_4$ binding to one ITK subunit induces allosteric changes in the other ITK subunits that cooperatively increase the affinity of their PH domains for PtdInsP $_3$ ^{17, 27, 28, 83}. These findings identified the first physiological “InsP $_4$ receptor” and the first *in vivo* function for InsP $_4$. Moreover, they indicate that in some cases, oligomerization might be important for the functions of PH domains, which mediate protein membrane recruitment downstream of many different receptors in many cell types^{17, 31, 84}.

Positive selection needs the TCR signal amplifier InsP $_4$

Based on these data, InsP $_4$ establishes a **positive feedback-loop [G]** of PLC γ 1 activation by ITK downstream of the TCR that is required for production of sufficient DAG to activate RAS and ERK and trigger positive selection (Fig. 3)¹⁷. Rescued *ItpkB*^{-/-} thymocyte maturation upon retroviral expression of wildtype but not CaM-binding-deficient ITPKB indicates that Ca²⁺–CaM-binding is required for ITPKB activation *in vivo*^{17, 85}. *In vitro* data support this view^{11–15, 85}. TCR-induced InsP $_4$ production requires Ca²⁺ and ITPKB^{18, 23, 25, 62, 63} and can be inhibited by CaM inhibitors (J. Huang and K. Sauer, unpublished observations). These findings add a **feedforward [G]** loop of ITPKB activation by Ca²⁺–CaM downstream of ITK and PLC γ 1 to our model. In DP thymocytes, the combination of feedback- and feedforward-activation loops establishes an InsP $_4$ - and Ca²⁺-dependent signal amplifier that is required for mild TCR stimuli to trigger positive selection.

Depending on ligand affinity/avidity, an enigmatic signal-splitter downstream of Ca²⁺ has been postulated to direct the outcome of TCR engagement towards positive or negative selection⁸⁰. The Ca²⁺ dependence of ITPKB and its requirement for positive selection make the InsP $_4$ -mediated signal amplifier an attractive candidate signal-splitter component. A prediction would be that InsP $_4$ is only required for ERK activation in response to mild TCR stimuli, but dispensable for negative selection in response to stronger TCR stimuli. Interestingly, “strong” CD3/CD4-costimulation induced DAG production and ERK activation in *ItpkB*^{-/-} thymocytes^{17, 18} (and Y.H. Huang and K. Sauer, unpublished observations). Thus, it will be interesting to analyse in more physiological models whether negative selection is intact in *ItpkB*^{-/-} mice.

ItpkB^{-/-} thymocytes show 30–40% residual InsP $_3$ 3-kinase activity and InsP $_4$ production^{18, 23}. Thus, complete InsP $_4$ -deficiency could have more profound effects on TCR signalling and might impact processes that are intact in *ItpkB*^{-/-} mice. Reminiscent of the effects of PtdInsP $_3$ modulation on multiple aspects of thymocyte development^{5–9, 32}, *Itk*^{-/-} mice have defects in preTCR signalling, positive and negative selection. In particular, they accumulate **innate-like CD8⁺ T cells [G]** and atypical T helper 2 (T_H2)-phenotype $\gamma\delta$ **T cells [G]**^{27, 28, 86, 87}. It will be interesting to investigate whether co-disruption of multiple InsP $_3$ 3-kinases completely inhibits TCR-induced InsP $_4$ accumulation and ITK activation in

thymocytes and elicits similar phenotypes. However, the positive selection defect in *Itk*^{-/-} and even *Itk*^{-/-}*Rlk*^{-/-} mice is milder than in *ItpkB*^{-/-} mice. Thus, other *InsP*₄ targets likely contribute to *InsP*₄-function in positive selection. Indeed, *InsP*₄ modulates binding of PtdInsP₃ to other thymocyte-expressed *InsP*₄-binding proteins, including TEC and RASA3⁴⁸, with similar dose-responses as ITK¹⁷. RASA3 inactivates RAS. Its PH domain can bind *InsP*₄, PtdIns(4,5)P₂ or PtdInsP₃^{44, 45, 49, 88, 89}. Impaired RASA3-sequestration from RAS by *InsP*₄ has been proposed to explain the decreased RAS/ERK activation in *ItpkB*^{-/-} DP cells¹⁸. However, no *in vivo* data are available and further studies are needed. RAS is primarily activated by DAG-recruited RASGRP1 in T cells (Fig. 3). Impaired DAG production in *ItpkB*^{-/-} DP cells¹⁷ blocks RASGRP1 recruitment and RAS activation upstream of RASA3 and should thus be dominant over any potential effects of *InsP*₄-deficiency on RASA3. Hence, other *InsP*₄ effectors are more likely to mediate ITK-unrelated *InsP*₄ functions in positive selection.

Altogether, ITPKB and *InsP*₄ are essential for thymocyte development. A profound block of positive selection in *ItpkB*^{-/-} mice results primarily from defective TCR-induced DAG production. This in turn reflects the disruption of an essential signal-amplifier composed of a feedforward loop of ITPKB activation by Ca²⁺-CaM, and of a positive feedback loop of ITK and PLCγ1 activation by *InsP*₄. The amplifier is probably needed to allow mild TCR stimuli to elicit positive selection. Phenotypic differences between *Itk*^{-/-} and *ItpkB*^{-/-} mice indicate involvement of other *InsP*₄-effectors. Whether *InsP*₄ has any role in thymocyte negative selection is still unclear.

Possible roles of *InsP*₄ in mature T cells

TCR stimulation induces *InsP*₃ 3-kinase activity and *InsP*₄ production in human Jurkat T cells^{25, 62}. Peripheral T cells express all three ITPKs. Owing to the developmental defects and severe T cell lymphopenia in *ItpkB*^{-/-} mice, analyses of specific ITPKB roles in peripheral T cells will require conditional *ItpkB*^{-/-} mice, RNAi [G] or selective small-molecule inhibitor studies.

A recent study found an intriguing association of a human *ITPKC* allele that decreased *ITPKC* mRNA splicing efficiency and abundance with susceptibility to Kawasaki syndrome, the leading cause of childhood-acquired-heart-disease in developed countries⁴². Acute-phase patients have T cell infiltration into the coronary artery wall and increased IL-2 production, suggesting that T cell hyperactivation could contribute to the disease. Suggesting a potential ITPKC-function in limiting peripheral T cell activation that could be perturbed in Kawasaki-disease patients, ITPKC mRNA levels increased after PMA/Ionomycin stimulation^{33, 42}. *ITPKC* overexpression decreased, and *ITPKC* knockdown increased, phytohaemagglutinin- and PMA-induced NFAT activation and IL-2 mRNA expression in Jurkat cells⁴². The mechanism is unknown, but could involve some of the aforementioned, controversial *InsP*₃ 3-kinase/*InsP*₄-roles in Ca²⁺-mobilization, or other functions of ITPKC, *InsP*₄ or its metabolites (Fig. 3, Box 1)^{14, 44, 45}. Mechanistic studies of ITPKC in primary T cells are needed to elucidate it.

A negative *InsP*₄ role in peripheral T cells would contrast with its positive role in thymocytes. Several potential explanations could be envisioned: First, the functions of *InsP*₄ could differ depending on TCR signal intensity or the developmental stage of T cells. Positive selection is triggered by mild and/or transient TCR signals in DP thymocytes. Negative selection is mediated by strong and/or sustained TCR signals and might be less impaired in *ItpkB*^{-/-} mice^{17, 23, 30}. Peripheral T cells also generate strong TCR signals that might be impacted differently by *InsP*₄-deficiency. Alternatively, the effects of *InsP*₄ might depend on its cellular concentration, subcellular localization or on the specific *InsP*₄-

effectors or metabolites present in a cell. It is unknown how ITPKC-modulation affects the levels of InsP_4 and its metabolites in peripheral T cells, and whether their InsP_4 -effector complement differs from that in thymocytes. Finally, different ITPKs might have distinct roles that could include noncatalytic functions and might depend on their subcellular localization^{41, 90, 91}. Testing all of these possibilities will require simultaneous ITPK-inhibition through small molecules, or their conditional co-disruption.

InsP_4 controls B cell development

ItpkB^{-/-} mice contain substantial B cell numbers. B cell development in the bone marrow of chemically-induced *ItpkB*^{-/-} mice is unchanged. However, the numbers of all splenic B cell subsets are markedly decreased, including transitional B cells undergoing selection, mature follicular and marginal zone B cells. Follicular B cells in *ItpkB*^{-/-} mice have downregulated IgM levels. Half of the splenic IgD⁺ cells express the plasma cell marker CD138^{16, 19}. They represent a pre-plasma cell checkpoint-stage enriched in models of B cell tolerance⁹². Indeed, mature B cells from *ItpkB*^{-/-} mice share many features with B cells from soluble hen-egg lysozyme (sHEL)/HEL-specific immunoglobulin (IgHEL) and Ars/IgArs transgenic models of B cell anergy⁹³. These include IgM-downregulation, impaired BCR-driven proliferation, reduced BCR-induced upregulation of surface-CD69, CD86 and MHCII, and decreased antibody-responses to T cell-independent antigens^{16, 19, 21}. These defects are specific to BCR stimulation. *ItpkB*^{-/-} B cell responses to LPS or CD40-specific antibodies were normal. These findings indicate that ITPKB might control B cell selection and tolerance induction.

In the sHEL/IgHEL and Ars/IgArs anergy models, constitutive self-antigen expression causes BCR desensitization, impaired BCR-activation of proximal tyrosine-kinases (LYN, SYK) and of downstream effectors including PLC γ 2, reduced InsP_3 production and impaired Ca^{2+} influx^{94, 95}. In contrast, anergic B cells from *ItpkB*^{-/-} mice showed normal BCR-activation of LYN, BTK, PLC γ 2, ERK1, ERK2, IKK α and IKK β with unaltered InsP_3 -production, but increased BCR-induced SOCE (Fig. 4)¹⁶. Normal Ca^{2+} responses to thapsigargin, which opens SOCE-channels by depleting ER Ca^{2+} -stores, indicated that SOCE-channels were unaltered. Addition of cell-permeable InsP_4 -esters decreased thapsigargin-induced SOCE and normalized BCR-induced SOCE in *ItpkB*^{-/-} B cells¹⁶. How InsP_4 inhibits SOCE in B cells is unknown. The mechanism might include perturbations of some of the aforementioned, controversial InsP_3 3-kinase/ InsP_4 -roles in Ca^{2+} -mobilization or other functions of InsP_4 or its derivatives (Fig. 4, Box 1)^{1, 11–14, 33–35, 44–47}. Altogether, B cells from *ItpkB*^{-/-} mice are anergic but have a dramatically different biochemical signature than other models of B cell anergy. How enhanced BCR-induced Ca^{2+} signaling causes *ItpkB*^{-/-} B cell anergy remains an important unresolved question.

An independent study described similar alterations in B cell development and T cell-independent antibody-responses in *ItpkB*^{-/-} mice²¹. The authors also reported decreased survival of *ItpkB*^{-/-} B cells *in vitro* and attributed this to accumulation of the pro-apoptotic protein BIM. Indeed, transgenic expression of the anti-apoptotic protein BCL-2 or BIM haplo-insufficiency increased B cell numbers in *ItpkB*^{-/-} mice. The authors further reported impaired BCR- or Ionomycin-induced Ca^{2+} influx in *ItpkB*^{-/-} B cells, and decreased BCR-induced ERK activation in BCL-2-expressing *ItpkB*^{-/-} B cells. They also showed that ATP-stimulation, or cell-permeable InsP_4 -ester-treatment of non-lymphoid COS cell-transfectants expressing ITPKB sequestered RASA3⁴⁸ from the plasma membrane and proposed that InsP_4 antagonizes RASA3 PH domain-binding to membrane-PtdIns(4,5) P_2 (Fig. 1)^{21, 49}. They proposed that InsP_4 increases B cell survival by sequestering RASA3 in the cytosol, resulting in sustained RAS/ERK activation, BIM-phosphorylation and -degradation. However, RASA3-localization and -function were not studied in B cells. The relevance of

the COS cell results for lymphocytes is unclear. Detailed analyses of RASA3-localization and -function in wildtype, *ItpkB*^{-/-} or *InsP*₄-ester-treated B cells are required to conclusively determine potential RASA3-roles in *InsP*₄ signalling in B cells.

Because ITPKB appears to control B cell selection, it was unclear if the signalling alterations in B cells from *ItpkB*^{-/-} animals reflect the induction and selective survival of anergic B cells, or an additional ITPKB function in BCR-signalling in mature B cells. To address this issue, *ItpkB*^{-/-} mice were crossed with IgHEL-transgenic mice. B cells from *ItpkB*^{-/-} IgHEL mice are also anergic¹⁹. They showed surface IgM downmodulation, IgD⁺CD138⁺ B cell accumulation and decreased proliferative antigen-responses. Early BCR signalling was unaltered. BCR-induced Ca²⁺ responses were greatly increased. This could be reversed by cell-permeable *InsP*₄-ester treatment. Transgenic soluble sHEL-antigen expression in IgHEL mice leads to anergy, characterized by surface IgM downregulation and impaired HEL-induced proliferation. In sHEL/IgHEL double-transgenic mice, *ItpkB* deficiency resulted in a tenfold decrease in the number of splenic IgHEL-specific B cells, and two-fold fewer immature IgHEL-specific B cells in the bone marrow¹⁹. Thus, ITPKB-deficiency converted anergy to deletion in the sHEL/IgHEL model, reminiscent of the impact of losing other negative regulators of BCR signalling, including CD22, SHP1 or LYN.

These data indicate that the main ITPKB-function in B cells is to inhibit BCR-induced Ca²⁺ signalling. They help to reconcile the discrepancies between the previous reports by suggesting that the increased BIM-expression in polyclonal *ItpkB*^{-/-} B cells in REF.²¹ may result from augmented negative selection of a B cell subset. Consistent with this model, *ItpkB*^{-/-} IgHEL mice show neither increased negative selection nor BIM accumulation. Similar considerations may explain the decreased ERK activation and Ca²⁺ influx in polyclonal *ItpkB*^{-/-} B cells in REF.²¹. In particular, the altered ionomycin-response in REF.²¹ but not REF.¹⁶ indicates a more extreme phenotype, perhaps owing to the selection of aberrant B cells over time or to differences in housing, health status, genetic background or age of the mice used in both studies.

***InsP*₄ broadens the B cell repertoire by limiting SOCE**

Based on these data, we propose a model in which ITPKB, via production of *InsP*₄, functions as a feedback-inhibitor of BCR-induced SOCE (Fig. 4). Increased SOCE in *ItpkB*^{-/-} mice alters B cell selection and tolerance induction. The outcome depends on the BCR-affinity for self-antigens. *ItpkB*-deficiency enhances Ca²⁺ signaling and promotes positive selection for low-affinity self-specific BCRs such as IgHEL. It induces anergy of B cells expressing low-to-moderately self-reactive BCRs in non-transgenic or *IgHEL* backgrounds, but deletion of normally anergic B cells with more strongly self-reactive BCRs, as seen in the sHEL/IgHEL system¹⁹. By feedback-inhibiting SOCE, ITPKB therefore extends the repertoire of immature B cells that survive negative selection. Interestingly, all B cells that mature in non-transgenic *ItpkB*^{-/-} mice become anergic regardless of their affinity for self. This might reflect distinct ITPKB or *InsP*₄-roles in mature B cells whose elucidation will require inducible ITPKB-deletion or the use of specific small-molecule ITPKB inhibitors.

B cells from *ItpkB*^{-/-} mice have features of anergic B cells that arise in models of self-tolerance, suggesting that ITPKB-deficiency can allow the positive selection of autoimmune-prone B cells. If some of these could escape anergy, they might cause autoimmune disease. However, in contrast to mice lacking other negative regulators of BCR signalling, including CD22, LYN or PTP1, *ItpkB*^{-/-} mice show no overt signs of autoimmunity. This could simply reflect a requirement for other immunocytes such as mature T cells, which are absent in *ItpkB*^{-/-} mice. However, mixed radiation-chimeras of

ItpkB^{-/-} bone marrow with wild-type T, B and myeloid cells also lacked autoimmune pathology (A. Miller and M. Cooke, unpublished observations). This might indicate a lack of required additional autoimmune insults. Alternatively, increased BCR-induced SOCE in *ItpkB*^{-/-} B cells might prevent autoimmunity by enabling more efficient deletion of autoimmune-prone B cells as seen in the sHEL/IgHEL system. Crossing *ItpkB*^{-/-} mice with appropriate autoimmune-prone strains or BCR-transgenic mice will address conclusively whether *ItpkB*-deficiency promotes or prevents autoimmune disease.

Roles of ITPKB in the innate immune system

Recent studies have shown important functions of ITPKB in the innate immune system. Contrasting with its positive roles in lymphocyte development, ITPKB may limit myelopoiesis. It moreover controls neutrophil function.

ItpkB limits myelopoiesis

ItpkB mRNA is expressed by all murine haematopoietic stem/progenitor cell populations (Fig. 5)²⁰. *ItpkC* mRNA is expressed by all myeloid lineages, and minimally by haematopoietic stem cells and common lymphoid progenitors. Common myeloid progenitors and megakaryocyte–erythrocyte progenitors express low *ItpkA* mRNA levels. *ItpkB*^{-/-} mice show increased proliferation and expansion of granulocyte–monocyte progenitors, increased neutrophil production and elevated peripheral blood neutrophil numbers (Fig. 5)²⁰. Thus, ITPKB limits myeloid differentiation *in vivo*. The precise mechanism is unclear, but might involve inhibition of the PI3K target AKT, whose PH domain can bind PtdInsP₃, PtdIns(3,4)P₂ or InsP₄, which can antagonize PtdInsP₃-binding^{22, 31, 96–98}. AKT is essential for myelopoiesis and can promote neutrophil and monocyte development⁶. Haematopoietic progenitor cell-enriched bone marrow cells from *ItpkB*^{-/-} mice show increased phosphorylation (activation) of AKT, and of the AKT target-site Y₁₄₅ on the cell-cycle inhibitor p21^{Cip1}. Y₁₄₅-phosphorylation decreases cell cycle-inhibition by p21^{Cip1}. It will be exciting to analyze whether AKT-inhibition normalizes myelopoiesis in *ItpkB*^{-/-} mice, and to explore potential *ItpkA/C*-roles in myelopoiesis.

InsP₄ controls neutrophil function

Stimulation with chemoattractants including *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induces InsP₃ 3-kinase activity and InsP₄ accumulation in neutrophils, which express mainly ITPKB^{22, 47, 99}. Bone-marrow-derived neutrophils (BMNs) from *ItpkB*^{-/-} mice show increased chemotaxis and superoxide production after treatment with fMLP or the complement-factor C5a. This correlates with increased AKT phosphorylation and actin polymerization²². Treatment with cell-permeable InsP₄-ester had opposite effects and inhibited fMLP-induced membrane-recruitment of the AKT PH domain in neutrophilic HL60 promyelocytic leukemia cells. AKT-PH domain–GFP fusion proteins co-precipitated InsP₄, InsP₅ and InsP₆. This suggests that ITPKB limits chemoattractant-induced neutrophil activation, possibly by inhibiting AKT membrane recruitment and activation through InsP₄-antagonism with AKT PH domain binding to PtdInsP₃ or PtdIns(3,4)P₂. Whether InsP₄ limits Akt-recruitment in other cell types *in vivo* is unknown. Overall, fMLP-induced Ca²⁺ responses were normal in *ItpkB*^{-/-} neutrophils²². However, the authors later mentioned decreased Ca²⁺ store-release but enhanced SOCE as unpublished data^{20, 47}. Defective Ca²⁺ homeostasis, Ca²⁺ mobilization or other ITPKB/InsP₄ functions could possibly explain the paradoxical finding of decreased viability of *ItpkB*^{-/-} BMNs *in vitro*²⁰ although PI3K–AKT signalling (which is increased in *ItpkB*^{-/-} BMNs) inhibits neutrophil spontaneous death⁴⁷.

Interestingly, *ItpkB* deficiency promoted neutrophil recruitment into inflamed peritoneal cavities in an acute peritonitis model. However, clearance of the injected bacteria was not

substantially improved²². Subsequent studies showed slightly decreased bacterial clearance *in vivo*, but increased killing of serum-opsonized bacteria *in vitro* by *ItpkB*^{-/-} neutrophils. Serum from *ItpkB*^{-/-} mice, which contains less opsonizing IgG than wildtype serum due to the aforementioned B cell defects, facilitated killing of bacteria less efficiently than wildtype serum⁴⁷. This indicates that ITPKB limits neutrophil recruitment and *in vitro* function. However, *in vivo*, ITPKB probably acts in several different cell types to control peritonitis responses. Although not investigated in detail, the decreased IgG titres in *ItpkB*^{-/-} mice^{16, 19, 21} might mask or counteract the neutrophil hyperactivity by preventing efficient opsonization of bacteria. Finally, altered Ca²⁺ homeostasis and mobilization in *ItpkB*^{-/-} neutrophils⁴⁷ and impaired additional functions of ITPKB/InsP₄ probably contribute to the complex effects of *ItpkB* deficiency on neutrophil function *in vivo*.

Conclusion

Its similarity to the cytoplasm-exposed PtdInsP₃ tetraphospho-inositol-headgroup allows InsP₄ to bind to the PH domains of the lymphoid-expressed PI3K-effectors ITK, TEC, BTK and AKT (Figs. 1, 3)^{17, 31, 96–98}. In thymocytes, InsP₄ binding to the ITK PH domain promotes PtdInsP₃ binding to ITK, probably through a cooperative-allosteric mechanism involving PH domain oligomerization¹⁷. In neutrophils, InsP₄ inhibits AKT PH domain – membrane interactions, probably by competing with PtdInsP₃- and PtdIns(3,4)P₂-binding. Overall, the phenotypes of *ItpkB*^{-/-} mice are consistent with one role for InsP₄ as a soluble regulator of PI3K, PTEN, SHIP1 and SHIP2 function. InsP₄ does this by controlling PH domain interactions of their lipid-substrates or products (Fig. 1). The phenotypic differences between mice lacking individual InsP₃ 3-kinases versus PI3K subunits probably reflect incomplete abrogation of InsP₄ or PtdInsP₃ production, respectively, the fact that InsP₄ can either promote or inhibit PtdInsP₃ function and might only control a subset of PtdInsP₃ targets, PtdInsP₃-independent functions of InsP₄ or its metabolites (Box 1), or non-catalytic ITPKB functions^{3, 4791}.

How InsP₄ inhibits SOCE in B cells and possibly neutrophils is unknown. The mechanism might include perturbations of some of the aforementioned, controversial InsP₃ 3-kinase/InsP₄-roles in Ca²⁺-mobilization, or other InsP₄ functions (Fig. 4)^{1, 11–14, 33–35, 44–47}.

InsP₄ acts as a precursor for multiple other InsPs, many of which have been found in immunocytes (Box 1)^{17, 18, 23, 25, 58–66}. Some InsP₄ functions could thus be mediated by InsP₄-derived InsPs rather than by InsP₄ itself. Based on the recently identified, intriguing higher-order InsP functions in non-lymphoid cells (Box 1), higher-order InsP studies in immunocytes should be very exciting.

Quantitative kinetic analyses of InsP₄, PtdInsP₃ and higher-order InsP-levels in *ItpkB*^{-/-} mice, the identification of all relevant InsP-targets through bioinformatics or affinity-chromatography, ITPKB structure-function analyses and conditional InsP₃ 3-kinase co-disruption in relevant cell types will be required to fully understand how InsP₃ 3-kinases govern immunocyte development and function, and to identify all underlying molecular mechanisms.

Although ITPKB is required for efficient selection of both T and B cells, its positive role in thymocyte positive selection strikingly contrasts with its negative role in B cell selection^{17, 18, 23}. In T cells, ITPKB likely extends the repertoire by amplifying weak TCR signals that would otherwise fail to elicit positive selection. In B cells, ITPKB expands the repertoire by inhibiting BCR signalling and preventing the deletion of B cells that would otherwise fail to develop. That a single gene achieves repertoire extension in opposite manners in T and B cells is fascinating. It probably reflects important differences in the rate-limiting steps for

selection of T cells (positive selection) versus B cells (negative selection). Interestingly, these differences mirror the differential impact of ITPKB on SOCE in both cell types. Feedback-inhibition of SOCE by InsP_4 does not control thymocyte positive selection but is crucial for extending the B cell repertoire. It will be interesting to determine whether this difference results from differences in InsP_4 or InsP_4 -metabolite concentrations, InsP_4 -effector or Ca^{2+} -channel repertoires^{33, 100} between both cell types.

Another difference between *ItpkB*^{-/-} thymocytes and splenic B cells is the defective TCR-induced ITK membrane recruitment and activation in *ItpkB*^{-/-} thymocytes¹⁷, which contrasts with the normal BCR-induced phosphorylation of BTK and PLC γ in *ItpkB*^{-/-} splenic B cells^{16, 19}. The highly related ITK, TEC and BTK PH domains can all bind PtdInsP_3 or InsP_4 ^{5, 17, 33, 46, 47}. The differences between *ItpkB*^{-/-} thymocytes and B cells could possibly be explained by differing affinities of the various PH domains for InsP_4 versus PtdInsP_3 ^{101–103}, or by PH domain-independent BTK activation in B cells⁵. Moreover, the possibility that sorted splenic B cell subpopulations from *ItpkB*^{-/-} mice might show stronger differences in BTK and PLC γ phosphorylation than the subtle effects found in total splenic B cells¹⁶ remains to be investigated.

T cells, B cells and neutrophils are key mediators of inflammatory diseases, including rheumatoid arthritis, diabetes or asthma. Defects in their development or function can cause immunodeficiencies such as AIDS or X-linked agammaglobulinemia, or promote cancer. T cells moreover cause organ-transplant rejection. All three cell types require ITPKB for their development or function. We therefore next discuss the therapeutic opportunities and liabilities of targeting ITPKs pharmacologically.

Therapeutic relevance

Because of its key roles in immunocyte development and function, cancer cell survival, proliferation and migration, and in other pathologic processes such as obesity, PI3K is a popular therapeutic target. The first PI3K inhibitors recently entered clinical trials for inflammatory disease and cancer indications. However, the ubiquitous functions of PI3K provide significant potential for toxicity, which might limit therapeutic applications^{5, 7, 8, 104}. Autoimmune-phenotypes in mice with altered PI3K function raise additional concerns for long-term therapies⁵. Moreover, the ability of IPMK to function as a Wortmannin-insensitive PI3K⁷³ might limit the efficacy of classic PI3K inhibitors that do not inhibit IPMK. Because their product, InsP_4 , controls PI3K function at the level of PtdInsP_3 , ITPKs are potential alternative targets. However, InsP_4 can affect PtdInsP_3 function positively, negatively or not at all depending on the specific PtdInsP_3 target involved¹⁷, and ITPKs have PtdInsP_3 -independent functions. Hence, the effects of ITPK inhibition are difficult to predict.

Based on the phenotypes of *ItpkB*^{-/-} mice (Table 1)^{16–18, 21, 23}, selective ITPKB-inhibition should primarily affect B cell, T cell and neutrophil development and function without significant effects on other tissues. However, broad ITPK-family inhibitors might have effects on tissues expressing other ITPKs, including the brain. Although it might be possible to prevent ITPK inhibitors from passing the blood–brain barrier, analyses of *ItpkA/B/C* double- or triple-deficient mice and studies with proof-of-concept ITPK inhibitors are required to address potential liabilities. Although InsP_4 can act as a precursor for higher-order InsPs with incompletely understood but important functions in mammalian cells (Box 1)⁷⁴, mouse embryonic fibroblasts lacking detectable *ItpkA*, *ItpkB* and *ItpkC*-expression are viable and still produce InsP_5 and InsP_6 ^{23, 75}. This suggests that preservation of higher-order InsP production through the broadly expressed, structurally diverse IPMK⁷⁷ might reduce the risk of potential side-effects of selective ITPK-inhibition.

Their biochemical properties and available high-throughput assays make ITPKs highly “druggable” [G]^{11–13, 25, 33, 105}. Compared with protein and lipid kinases, distinct structural features of the ITPKs could allow the rational design of highly selective, ATP- or substrate-competitive or allosteric ITPK inhibitors^{11, 33, 85, 106–108}. Indeed, several groups have identified small-molecule ITPK inhibitors. Some show high ITPK-selectivity over IPMK^{33, 109–112}.

The strong inhibitory effects of *ItpkB*-deficiency on thymocytes and B cells make broad immunosuppression for the treatment of transplant-rejection or severe autoimmune diseases the most likely indication for ITPKB inhibitors. A true evaluation of their therapeutic potential awaits validation with human cells. ITPKB is highly expressed by human peripheral T cells³³. The recent implication of genetic *ITPKC* polymorphisms in Kawasaki disease⁴² indicates the pathophysiological importance of ITPKs. Although the precise roles of ITPKs in disease aetiology are unknown, a negative role for ITPKC in human T cell activation⁴² could pose a potential liability for broad *Itpk*-inhibitors. Alternatively, this negative function of ITPKC might allow the development of ITPK inhibitors as adjuvants to promote T cell function in immunodeficiencies and infectious diseases.

Neutrophils have important roles in infections, allergies, inflammatory diseases and autoimmunity¹¹³. Hence, the pathophysiological relevance of the increased neutrophil chemotaxis and superoxide production, but decreased neutrophil viability in *ItpkB*^{−/−} mice²² requires exploration. Increased neutrophil recruitment in peritonitis models in *ItpkB*^{−/−} mice²² could possibly indicate opportunities for ITPKB-inhibition for anti-infective therapies, but might pose liabilities for immunosuppression. The feasibility of neutrophil modulation depends on the involvement of T and B cells in the targeted diseases, as despite the increased neutrophil number, clearance of intraperitoneal-injected bacteria was unaltered or even decreased in *ItpkB*^{−/−} mice through mechanisms that might involve decreased serum IgG titres^{22, 47}. Altogether, the validation of ITPKB as a therapeutic target will require more detailed analyses of its role in innate immunocytes and in peripheral T cells, analyses of *ItpkB*^{−/−} mice in relevant disease models and proof-of-concept studies with potent and selective small-molecule ITPKB inhibitors.

Two decades after its discovery, *ItpkB*^{−/−} mice have demonstrated important *in vivo* signalling functions for *InsP*₄ and unveiled the first physiological *InsP*₄ effectors⁸⁴. Like many other important discoveries, these findings pose more questions than they answer (Box 2). In non-immune cells and yeast, higher-order *InsPs* have recently been implicated in several important cellular processes (Box 1)^{1, 25, 74, 84}. Thus, it will be particularly exciting to investigate the functions of the higher-order *InsPs* in immunocytes (Box 1) and their potential contributions to *InsP*₄-signaling²⁵. Without any doubt, deciphering the lymphocyte “inositol code” will remain a rich area of research for years to come.

Box 2

Unanswered questions about *InsP*₄

- What is the precise relationship between membrane-PtdInsP₃ and its soluble partner *InsP*₄. How many events hitherto believed to be exclusively under PtdInsP₃ and hence PI3K control are exclusively or partly controlled by *InsP*₄⁸⁴?
- What determines whether a PH domain is regulated by *InsP*₄ and whether *InsP*₄ promotes or inhibits PtdInsP₃ binding? Does the mode of *InsP*₄ regulation depend on PtdInsP₃ levels, which can be high in unstimulated cells⁸⁴? Does

InsP₄ regulate other PH domain functions such as protein–protein interactions^{31, 138}?

- Which kinases account for the residual ITPK activity in *ItpkB*^{-/-} thymocytes^{18, 23}? Will their conditional co-disruption unveil immune phenotypes that more closely resemble those of mice with perturbed PI3K function? Is InsP₄ involved in thymocyte negative selection?
- How does InsP₄ regulate SOCE in B cells and in neutrophils? Does it act through similar mechanisms in both cell types?
- What functions does InsP₄ have in peripheral T cells? The positive role of ITPKB in thymocytes^{17, 18, 23} and the potential negative role of ITPKC in human T cells⁴² raise the testable possibility that different ITPKs might have diverse functions.
- TEC-family protein tyrosine kinases play key roles in peripheral T cell differentiation and function^{27, 28}. Are PtdInsP₃-binding TEC-family kinases in peripheral T cells controlled by InsP₄?
- InsP₄ can act as a precursor for many other InsPs (Box 1)^{17, 18, 23, 25, 58–66}. Which InsP₄ functions are really mediated by the other InsPs found in immunocytes^{17, 18, 23, 25, 45, 58–66, 115}?
- Which phosphatases turn over InsP₃ or InsP₄ in immunocytes? Are SHIP1, SHIP2 or PTEN^{68–71} involved?
- Do any of the recent findings about InsP₄ functions in immunocytes apply to non-lymphoid cells?

At a glance/Online summary

- By acting as a soluble analogue of the membrane lipid phosphatidylinositol(3,4,5)trisphosphate (here termed PtdInsP₃), inositol(1,3,4,5)tetrakisphosphate (here termed InsP₄) can control the functions of phosphoinositide 3-kinases (PI3Ks), PTEN, SHIP1 and SHIP2 — the kinases that produce, and the phosphatases that metabolize, PtdInsP₃ in lymphocytes. InsP₄ likely has additional functions that may include those of many InsP₄-metabolites. In the best understood mechanism so far, InsP₄ regulates the membrane recruitment of proteins by promoting or inhibiting the interactions of their pleckstrin homology domains (PH domains) with PtdInsP₃.
- In mouse thymocytes, positive regulation of ITK membrane recruitment by InsP₄ establishes a feedback loop of phospholipase Cγ1 activation downstream of the T cell receptor that is essential for the production of sufficient amounts of the second messenger lipid diacylglycerol to trigger positive selection and the generation of a mature T cell repertoire. These findings unveiled the first physiological function of InsP₄ as an essential soluble messenger molecule, two decades after its original discovery.
- Unexpectedly, defective function of the InsP₄-producing kinase ITPKC in humans might result in peripheral T cell hyperactivation and contribute to Kawasaki syndrome, the leading cause of acquired heart disease among children in developed countries.
- The InsP₄-producing kinase ITPKB is required for B cell development and functional competence, as shown by the B cell developmental defects and

energy in *ItpkB*^{-/-} mice. The mechanisms through which ITPKB controls B cell development are unclear, but might include inhibition of store-operated Ca²⁺ entry (SOCE) by InsP₄ through an as yet unknown mechanism.

- ITPKB limits SOCE and PtdInsP₃-mediated membrane recruitment of the PI3K effector kinase AKT in neutrophils. Defects in these processes and in the production of opsonizing IgG by B cells probably underlie a complex phenotype of increased neutrophil chemotaxis and superoxide production, but decreased neutrophil viability and reduced bacterial clearance in an acute peritonitis model in *ItpkB*^{-/-} mice.
- As key regulators of PI3K function with essential roles in the adaptive and innate immune system and unique structural features that facilitate the development of selective inhibitors, the InsP₄-producing ITPKBs are attractive targets for the development of novel anti-inflammatory therapies, improved vaccination strategies or avenues to overcome immunodeficiencies. However, more detailed studies in relevant disease models and with proof-of-concept small molecule inhibitors are required to establish indications, evaluate potential side effects and address toxicity concerns.

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Glossary terms

Positive or negative feedback loop	A cause-and-effect circuit where several processes occur in sequence, and where the output of a downstream process promotes or inhibits, respectively, an upstream process, forming a circular signalling loop. Negative feedback can increase the stability and accuracy of a signalling system by correcting unwanted changes. Positive feedback can amplify a signal by increasing the gain of the signal-transducing machinery
Feedforward amplification or inhibition	A cause-and-effect circuit where several processes occur in sequence, and where the output of an upstream process promotes or inhibits, respectively, a downstream process, usually bypassing one or more intermediate processes. This can set the gain for a signalling circuit, or specifically amplify or limit, respectively, one of several different downstream signalling branches
Phosphoinositides	A family of phosphorylated derivatives of the membrane lipid phosphatidylinositol that contain a hydrophobic diacylglycerol backbone esterified to a polar inositol headgroup
Inositol phosphate (InsP)	The consensus term for all phosphorylated D- <i>myo</i> -inositol derivatives. Mammalian cells produce many higher-order InsPs, including multiple phosphatidylinositol (PtdIns) or inositol (Ins) tris-, tetrakis- and pentakisphosphate isomers. To improve readability, we use the non-IUPAC acronyms ² PtdInsP ₃ for phosphatidylinositol(3,4,5)trisphosphate [PtdIns(3,4,5)P ₃], InsP ₃ for inositol(1,4,5)trisphosphate [Ins(1,4,5)P ₃] and InsP ₄ for

inositol(1,3,4,5)tetrakisphosphate [Ins(1,3,4,5) P_4] in this review. This does not imply that PtdIns P_3 , Ins P_3 and Ins P_4 are the only, or the most important InsPs

Second messenger	A signalling molecule such as Ins P_3 or DAG that is produced by a primary effector, usually an enzyme (PLC γ 1 in the case of Ins P_3 or DAG in T cells) that is in turn activated by a signal transducer (such as the TCR signalosome complex) downstream of a transmembrane receptor (such as the TCR) after its engagement by a first messenger ligand (peptide–MHC complexes in the case of the TCR). The second messenger may then activate one or more secondary effectors that further transmit signals into the cell
Kawasaki Disease	Mucocutaneous lymph node syndrome, infantile polyarteritis. An acute, self-limited vasculitis of infants. The leading cause of acquired heart disease among children in developed countries
Store-operated Ca^{2+} entry	(SOCE). A mechanism used by many cells to increase intracellular Ca^{2+} concentrations and transduce signals in response to cell surface receptor ligation. This first results in the release of Ca^{2+} from intracellular stores. STIM proteins sense this depletion of Ca^{2+} stores and trigger the opening of plasma membrane Ca^{2+} channels containing ORAI proteins that mediate capacitative Ca^{2+} influx. In some cell types, other Ca^{2+} channels may contribute to SOCE ^{29, 100}
Pleckstrin homology domain	(PH domain). A structurally conserved protein domain of ~120 amino acids that is found at least 305 times in the human genome. About 60 PH domains mediate protein membrane recruitment or protein–protein interactions by binding to phosphoinositides or to other proteins, respectively. The functions of most PH domains are unknown
ITK	(IL-2-inducible T cell kinase). A T cell and NK cell expressed TEC-family non-receptor protein-tyrosine-kinase (TFK) that is involved in T cell receptor signalling
Long-term potentiation	(LTP). A long-lasting enhancement of synaptic transmission between two neurons. LTP is thought to underlie learning and memory
Innate-like CD8 ⁺ T cells	An $\alpha\beta$ T cell subset characterized by high-level expression of CD44, CD122 and NK cell markers, indicative of an activated or memory phenotype. In contrast to conventional naive $\alpha\beta$ T cells, innate T cells are IL-15 dependent and can exert effector functions immediately upon stimulation. While conventional T cells are selected by peptide–MHC complexes on non-hematopoietic cells in the thymus, innate T cells are selected by MHC proteins on haematopoietic cells
$\gamma\delta$ T cells	A distinct lineage of T cells whose T cell receptor contains γ and δ chains instead of α and β chains. $\gamma\delta$ T cells are not classical MHC-restricted. Their antigenic ligands are largely unknown and may include non-peptides. $\gamma\delta$ T cells play particularly important roles in epidermal tissues. They contribute to pathogen defence, tumour surveillance, mucosal and skin barrier functions and, in mice, to skin wound healing

RNAi	RNA interference. An RNA-dependent gene silencing process initiated by short double-stranded RNA (dsRNA) molecules that can either be produced endogenously by the cell (micro RNA pathway), or exogenously provided by viruses or through laboratory techniques. The cellular ribonuclease Dicer cleaves dsRNAs into 21–25 base pair small interfering RNAs (siRNAs) whose “guide strand” binds to complementary regions in cellular mRNAs within the RNA-induced silencing complex (RISC). This results in degradation of the mRNA and/or translational blockade
Druggable Protein	A term frequently used in the pharmaceutical industry to denote a protein that can bind drug-like molecules with high affinity and selectivity ¹⁰⁵ . It usually has a property such as a catalytic activity, ion conductance or ligand-binding that is modulated by binding of the drug-like molecules, usually either small-molecule chemical compounds or biologicals. A protein is considered increasingly druggable, the more easily selective and potent modulators of its function with desired drug-like properties such as oral bioavailability, high absorption and tissue permeation can be developed
NTOC	Neonatal thymic organ culture. A method where neonatal thymic lobes are incubated in media <i>in vitro</i> . It allows one to monitor the effects of adding pharmacologic agents on thymocyte development

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Biographies

Karsten Sauer

Karsten Sauer received his Ph.D. in Biochemistry from the University of Tuebingen, Germany. After postdoctoral training at the Merck Research Laboratories, Rahway, New Jersey, USA, he joined the Genomics Institute of the Novartis Research Foundation, San Diego, California, as a group leader. He now is an Associate Professor at The Scripps Research Institute, La Jolla, California, USA, which he joined in 2006. His research interests are the signal transduction mechanisms controlling lymphocyte development and function, and the mechanisms through which signalling defects cause immune disorders.

Michael P. Cooke

Michael P. Cooke received his Ph.D in Biochemistry from the University of Washington, Seattle. After postdoctoral work at Stanford University, he was Director of Functional Genomics at SyStemix Inc., Palo Alto, California. Since 1999, he is Director of Immunology at the Genomics Institute of the Novartis Research Foundation, San Diego, California. His research includes the application of functional genomics to study the biology of haematopoietic stem cells and the adaptive immune system and the translation of these findings into novel therapeutics.

Fig. 1. $\text{Ins}(1,3,4,5)\text{P}_4$ regulates PtdInsP_3 function in immunocytes

After antigen-receptor engagement, the membrane-lipid $\text{PtdIns}(4,5)\text{P}_2$ — which has a membrane-embedded lipid, and a cytosol-exposed charged inositol phosphate (InsP) moiety — can either be converted into $\text{PtdIns}(3,4,5)\text{P}_3/\text{PtdInsP}_3$ by phosphoinositide 3-kinase (PI3K), or into the second messengers diacylglycerol (DAG) and soluble $\text{Ins}(1,4,5)\text{P}_3/\text{InsP}_3$ by phospholipase- $\text{C}\gamma$ 1 (PLC γ 1). The phosphatase-and-tensin-homologue phosphatase PTEN counteracts PI3K. SH2 domain-containing-inositol-polyphosphate 5-phosphatases 1/2 (SHIP) convert PtdInsP_3 into $\text{PtdIns}(3,4)\text{P}_2$. Primarily via their InsP -headgroups, $\text{PtdIns}(4,5)\text{P}_2$, PtdInsP_3 and $\text{PtdIns}(3,4)\text{P}_2$ can bind to pleckstrin homology (PH) domains in certain proteins and recruit them to membranes. InsP_3 mobilizes Ca^{2+} but can also be phosphorylated at its 3-position into $\text{Ins}(1,3,4,5)\text{P}_4/\text{InsP}_4$ by InsP_3 3-kinases (ITPKA, ITPKB, ITPKC, IPMK/IPK2)^{11, 17, 18, 23, 34, 35}. Owing to its similarity to the PtdInsP_3 -headgroup, InsP_4 can bind to certain PtdInsP_3 -binding PH domains and promote (green) or inhibit (red) PtdInsP_3 -binding. In $\text{CD4}^+\text{CD8}^+$ thymocytes, InsP_4 -promotion of PtdInsP_3 -binding to the ITK/TEC PH domains establishes a feedback-loop of PLC γ 1-activation. In neutrophils, InsP_4 -inhibition of PtdInsP_3 - or $\text{PtdIns}(3,4)\text{P}_2$ -binding to its PH domain may limit AKT membrane-recruitment. InsP_4 can also inhibit RASA3/GAP1^{IP4BP}-binding to $\text{PtdIns}(4,5)\text{P}_2$ or PtdInsP_3 ^{18, 21}. Whether this occurs in immunocytes is unknown. R_1 , R_2 , fatty-acid side-chains; Circled P, phosphate moiety. Orange, enzymes with demonstrated physiological relevance in immunocytes.

Fig. 2. Schematic comparison of the structures of ITPKA, ITPKB and ITPKC

Shown are the major known features of the three ITPK-gene family inositol(1,4,5)trisphosphate (InsP_3) 3-kinases, but not to scale. ITPKs contain a carboxy-terminal InsP_3 -kinase domain preceded by a Ca^{2+} -Calmodulin (CaM)-binding domain. Ca^{2+} -CaM binding can activate ITPKA and ITPKB and is likely required for ITPKB function in thymocytes^{17, 85}. The physiological roles of the highly divergent amino-terminal regions of the ITPKs are unknown, but they might include the regulation of ITPK activity, subcellular targeting or adaptor functions⁹⁰. ITPKs have several motifs that have been implicated in actin binding, nucleo-cytoplasmic shuttling (nuclear export sequence, NES), proteolytic processing (potential PEST motifs) or phosphorylation by protein kinase A or C (PKA, PKC), or by CaMKII. Biochemical studies indicate that PKA and PKC may control ITPK activity, possibly by interfering with Ca^{2+} -CaM binding. However, the *in vivo* relevance of all of these interactions is unknown. Several excellent reviews discuss them in detail^{11–15, 33, 35, 85}. Modified/Reproduced with permission from REF.³³.

Fig. 3. Ins(1,3,4,5)P₄ functions in TCR signalling

TCR-engagement activates protein-tyrosine-kinases (SFK, ZAP), which tyrosine-phosphorylate transmembrane-adaptors (TMAPs, LAT). This causes phosphoinositide 3-kinase/PI3K-activation. PI3K phosphorylates membrane-phosphatidylinositol(4,5)bisphosphate/PtdIns(4,5)P₂/PIP₂ to phosphatidylinositol(3,4,5)trisphosphate/PtdInsP₃/PIP₃. By binding to their PH domains, PtdInsP₃ recruits phospholipase-Cγ1 (PLCγ1) and its upstream-activator-kinase ITPK. ITPK is oligomeric with low PH domain-affinity for PtdInsP₃ (dark gray). Therefore, initial ITPK-recruitment is limited and only triggers low-level PLCγ1-activation. PLCγ1 hydrolyzes PtdInsP₂ into low diacylglycerol/DAG- and Ins(1,4,5)P₃/InsP₃-amounts. InsP₃ mobilizes Ca²⁺. Ca²⁺ binds calmodulin/CaM which binds to and activates ITPKs and calcineurin/CaN¹³⁹. CaN dephosphorylates and activates the transcription-factor NFAT²⁹. ITPKs phosphorylate InsP₃ into Ins(1,3,4,5)P₄/InsP₄ which may have several functions based on data from *ItpkB*^{-/-} mouse thymocytes^{17, 18, 23}, *ITPKC*-mutant human T cells⁴² and biochemical studies^{11–13, 25, 51, 83, 85}. InsP₄-binding to one ITPK-subunit allosterically increases the PH domain affinity of all ITPK-subunits for PtdInsP₃ (light gray). This promotes ITPK membrane-recruitment, causing full PLCγ1-activation and sufficient DAG-production to activate RAS–ERK and trigger thymocyte positive selection. ITPKB-deficiency perturbs this feedback-activation through decreased InsP₄-production and ITPK-recruitment. ITPKC-deficiency might cause peripheral T cell-hyperactivity, possibly through increased NFAT signalling. The cause could be impaired InsP₃-turnover by ITPKC

or InsP_4 -inhibited 5-phosphatases, or perturbed other functions of InsP_4 or its derivatives (Box 1)^{45, 51}.

Fig. 4. Ins(1,3,4,5) P_4 functions in BCR signalling

Schematic depiction of B cell receptor (BCR) signalling through ITPKB. Following BCR engagement and assembly of a “signalosome” multiprotein-complex, phospholipases $C\gamma 1$ and -2 ($PLC\gamma$) hydrolyse phosphatidylinositol(4,5)bisphosphate ($PtdInsP_2$) into the second messengers diacylglycerol (DAG) and Ins(1,4,5) P_3 ($InsP_3$). As in T cells, $InsP_3$ mediates the release of Ca^{2+} from the ER by binding to $InsP_3$ -receptors (IP_3R). The resulting depletion of Ca^{2+} stores activates plasma-membrane channels that mediate store-operated Ca^{2+} entry (SOCE). The resulting increase in intracellular Ca^{2+} levels is required for B cell proliferation and differentiation. Ca^{2+} also activates ITPKB (Fig. 3), which converts $InsP_3$ to Ins(1,3,4,5) P_4 ($InsP_4$). $InsP_4$ inhibits SOCE through an unknown mechanism^{16, 19}, but can also affect $InsP_3$ -receptors, $InsP_3$ -dephosphorylation by 5-phosphatases, other $InsP_4$ -effectors, or be converted to other $InsPs$ which might mediate aspects of $InsP_4$ -function in B cells. In any case, ITPKB acts as a feedback inhibitor of BCR-induced SOCE. Physiologically, this is required to prevent B cell anergy and extend the functional B cell repertoire by decreasing the number of B cells that are negatively selected.

Fig. 5. *Ins(1,3,4,5) P_4* controls haematopoiesis at multiple levels

Hematopoiesis originates from hematopoietic stem cells (HSC). Multipotent progenitors (MPP) give rise to common lymphoid (CLP) or myeloid (CMP) progenitors. CLPs initiate the B/T cell lineages through early-thymic-progenitors (ETP) and pro-B cells, respectively. ETPs develop through CD4⁻CD8⁻ (DN) and CD4⁺CD8⁺ (DP) stages into mature T cells. In the bone-marrow, pro-B cells develop via pre-B cells into immature B cells. These translocate into the spleen to mature through transitional stages into mature B cells. CMPs give rise to granulocyte-monocyte (GMP) and megakaryocyte-erythrocyte (MEP) progenitors and the indicated mature cell types. In mice, *ItpkB*-deficiency impairs DP thymocyte positive selection, blocking maturation into CD4⁺ and CD8⁺ T cells (red bar)^{17, 18, 23}. Human ITPKC loss-of-function may cause peripheral T cell-hyperactivation⁴². In *ItpkB*^{-/-} mice, perturbed splenic B cell development yields reduced numbers of B cells that are anergic^{16, 19, 21}. Conversely, *ItpkB*-deficiency causes GMP-accumulation (green arrows), the generation of hyperactive neutrophilic granulocytes with decreased viability, and mildly reduced erythrocyte numbers^{20, 22, 47}. Mast cells express *ItpkB* and produce IP₄ after stimulation¹¹⁵. Small-molecule ITPK-inhibition might augment their activation^{24, 109}, but the target-selectivity of the low-affinity ITPK-inhibitors used is unknown and genetic studies are needed.

Table 1Phenotypes of Ins(1,4,5) P_3 3-Kinase mutant mice and humans

Species	Gene	Mutation	Phenotype	References
Mouse	<i>ItpkA</i>	Knockout	Perturbed long-term potentiation in the hippocampus. Memory deficits. In the brain, reduced Ins(1,3,4,5) P_4 (Ins P_4) but normal Ins(1,4,5) P_3 (Ins P_3) levels. Normal glutamate-induced Ca^{2+} -mobilization in hippocampal neurons. Defective PAK1 accumulation and actin reorganization.	40, 41
	<i>ItpkB</i>	Knockout	Blocked thymocyte positive selection. Unclear if negative selection is affected (see text). Peripheral T lymphopenia. Thymocytes: 30–40% residual Ins P_3 3-kinase activity. Reduced TCR-induced Ins P_4 , but normal Ins P_3 levels. Normal TCR-induced Ca^{2+} -mobilization. Defective B cell development. Reduced B cell viability. Impaired T-cell-independent antibody responses. Impaired BCR- or Ionomycin-induced Ca^{2+} -influx. Rescued survival but decreased BCR-induced Erk- activation in Bcl-2-expressing <i>ItpkB</i> ^{-/-} B cells. Haematopoietic progenitor cell-enriched bone marrow cells: Increased Akt-and P21 ^{Cip1} -phosphorylation. GMP expansion. Overproduction of hyperactive neutrophils of reduced viability. Enhanced chemoattractant-induced neutrophil chemotaxis, superoxide production, Akt activation, actin-polymerization and SOCE, but reduced Ca^{2+} store release. Augmented neutrophil recruitment but defective clearance of <i>i.p.</i> injected bacteria in peritonitis models. Mildly decreased erythrocyte numbers.	20–23, 47
	<i>ItpkB</i>	ENU mutant	Blocked thymocyte positive selection; peripheral T lymphopenia. Retroviral expression of wildtype but not catalytically inactive ItpkB in thymocytes rescues positive selection. DP thymocytes: No residual ItpkB-protein detected. ~40% residual Ins P_3 3-kinase activity. Normal TCR-induced Ca^{2+} -mobilization. Impaired TCR-induced Itk membrane-recruitment and activation with resulting defects in PLC γ 1-activation, diacylglycerol-production, Raf, MEK and Erk-phosphorylation. Treatment with the diacylglycerol-analog PMA rescued <i>ItpkB</i> ^{-/-} thymocyte maturation in NTOC [G]. Reduction of all splenic B cell subsets. Peripheral B cell anergy. Shift of the BCR selection window towards anergy or deletion in BCR transgenic mouse models. Splenic B cells: No significant proximal BCR signaling defects. Elevated BCR- induced SOCE. This is reversed by cell- permeable Ins P_4 -ester treatment.	16–19, 33, 46, 85, 140
	<i>ItpkC</i>	Knockout	No defect reported. Unaltered Ins P_3 3- kinase activity in thymocytes.	23
	<i>ItpkB+ItpkC</i>	Double- knockout	Same thymic phenotype as <i>ItpkB</i> -knockout.	23
	<i>IPMK (Ipk2)</i>	Knockout	Embryonic lethal (day 9.5) due to morphological defects. Impaired production of higher-order InsPs in ES cells.	77
Humans	<i>ITPKC</i>	Loss-of-function polymorphism	Associated with susceptibility to Kawasaki-syndrome. Acute-phase patients have T cell infiltration into the coronary artery wall and increased IL-2 production.	42
	<i>ITPKC</i>	Knockdown in Jurkat cells	Increased phytohaemagglutinin- and PMA-induced NFAT-activation and IL-2 mRNA expression. Mechanism unclear.	42
	<i>ITPKC</i>	Over-expression in Jurkat cells	Reduced phytohaemagglutinin- and PMA-induced NFAT-activation and IL-2 mRNA expression. Mechanism unclear.	42