LIPID SIGNALING IN T CELL DEVELOPMENT AND FUNCTION

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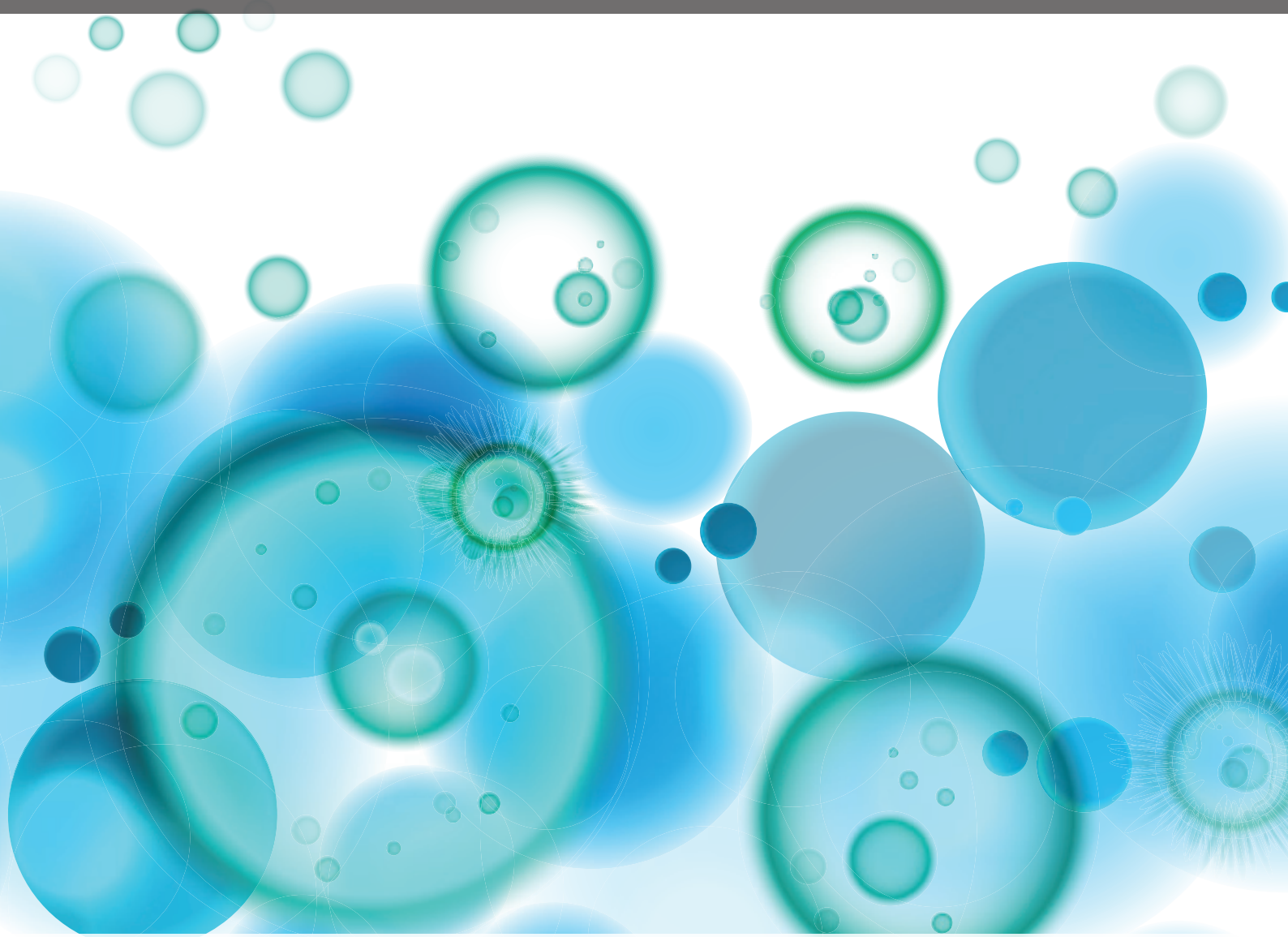

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LIPID SIGNALING IN T CELL DEVELOPMENT AND FUNCTION

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Lipids are best known as energy storing molecules and core-components of cellular membranes, but can also act as mediators of cellular signaling. This is most prominently illustrated by the paramount importance of the phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) signaling pathways in many cells, including T cells and cancer cells. Both of these enzymes use the lipid phosphatidylinositol(4,5)bisphosphate (PIP2) as their substrate. PLCs produce the lipid product diacylglycerol (DAG) and soluble inositol(1,4,5)trisphosphate (IP3). DAG acts as a membrane tether for protein kinase C and RasGRP proteins. IP3 is released into the cytosol and controls calcium release from internal stores. The PI3K lipid product phosphatidylinositol(3,4,5)trisphosphate (PIP3) controls signaling by binding and recruiting effector proteins such as Akt and Itk to cellular membranes. Recent research has unveiled important signaling roles for many additional phosphoinositides and other lipids. The articles in this volume highlight how multiple different lipids govern T cell development and function through diverse mechanisms and effectors. In T cells, lipids can orchestrate signaling by organizing membrane topology in rafts or microdomains, direct protein function through covalent lipid-modification or non-covalent lipid binding, act as intracellular or extracellular messenger molecules, or govern T cell function at the level of metabolic regulation. The cellular activity of certain lipid messengers is moreover controlled by soluble counterparts, exemplified by symmetric PIP3/inositol(1,3,4,5)tetrakisphosphate (IP4) signaling in developing T cells. Not surprisingly, lipid producing and metabolizing enzymes have gained attention as potential therapeutic targets for immune disorders, leukemias and lymphomas.

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EDITORIAL
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Editorial: Lipid signaling in T cell development and function

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Keywords: lipid, PI3K, T cell, inositol, eicosanoid, vitamin D_3, adipokine, TNF

Best known as energy storing molecules and core-components of cellular membranes, lipids are also important regulators of cell signaling. They can compartmentalize signaling by organizing membrane-topology into specialized sub-domains in the plasma membrane or into intracellular microvesicles (1, 2). Covalent lipid-modification of proteins can direct their localization to particular membrane compartments. Non-covalent membrane lipid interactions control the mechanics of T cell receptor (TCR) signal transduction (3, 4). Membrane lipids can also act as second-messengers, as exemplified by the phosphoinositide-3-kinase (PI3K) lipid-product phosphatidylinositol(3,4,5)trisphosphate (PIP_3) in lymphocytes, the topic of several reviews here, and of a recent dedicated Research Topic in *Frontiers in Immunology* (5). Other lipids can also act as intracellular or extracellular messengers, or govern cell function at the level of metabolism. Not surprisingly, lipid producing and metabolizing enzymes and lipid downstream-effectors have gained considerable attention as potential therapeutic targets for immune disorders, blood cancer, and even aging (5, 6) (and this Research Topic), and certain lipids are being used as therapeutics (7).

The 11 reviews in this Research Topic highlight some of the most important, or most recently discovered lipid functions in T cells. So and Croft review evidence that besides the TCR and costimulatory CD28, members of the Tumor-Necrosis-Factor (TNFR) superfamily contribute to sustained PI3K-pathway activation in T cells and beyond (8). Based on their studies of the TNFR OX40, the authors suggest a model where ligand-induced TNFR oligomerization concentrates PI3K and Akt close to TCR/CD28 signalosomes. This may contribute to the well-established TNFR-requirements for T cell clonal expansion, survival, and memory. The authors also review evidence for TNFR-mediated PI3K control in other cells and discuss important open questions, such as which precise molecular interactions link TNFRs to PI3K/Akt. Wang and colleagues review the components and mechanisms of PI3K-signaling in lymphocytes (9). They discuss how PI3Ks are activated to produce PIP_3, the mechanisms limiting PIP_3 production, and the role of PIP_3 removal by lipid-phosphatases. Next, the authors discuss how PIP_3 specifically binds to effector proteins such as Akt and Tec-kinases via their PH domains to control lymphocyte biology. They also discuss a less well-appreciated mechanism of how soluble inositol-phosphates can control PI3K-signaling by acting as PIP_3-analogs. An increasing number of studies suggest that this non-canonical way of controlling PI3K-function has broad importance in hematopoiesis (9–11). The authors conclude by reviewing how protein-ligands of PIP_3-binding domains provide yet another level of control as exemplified by their recent work on calmodulin–PH domain interactions. A complementing review by Srivastava and colleagues focuses on PIP_3 metabolizing lipid-phosphatases in T cells (12). The paramount tumor suppressor function of PTEN, critical functions in effector and regulatory T cells, and recent efforts to target them pharmacologically underscore the importance of PIP_3 removal by lipid-phosphatases. Among them, PTEN reverses the PI3K-reaction, whereas SHIP1/2 also control signaling by producing $PI(3,4)P_2$, which recruits and controls effector proteins additional to Akt and Tec-family kinases.

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But PIP_3 and its immediate derivatives are not the only important phosphoinositides in immune cells. Nunès and Guittard discuss recent evidence for functions of PI5P in TCR signaling (13). They introduce the enzymes governing PI5P metabolism, review PI5P-binding domains in effector proteins such as Dok, and discuss potential PI5P functions in T cells. While inositol-phosphates are produced through hydrolysis of the phosphodiester-bond between the glycerol and inositol-phosphate moieties of phosphatidylinositols, phosphatidylinositol-deacylation by phospholipase A generates glycerophosphoinositols. Containing both glycerol- and phosphoinositol-moieties, glycerophosphoinositols are soluble and may have intracellular signaling roles. They can also be excreted to potentially exert paracrine functions. Patrussi et al. discuss metabolism and potential immunomodulatory roles for these little-studied messengers (14). In particular, glycerophosphoinositol(4)phosphate can augment TCR and CXCR4 chemokine receptor signaling in T cells. Unknown cellular receptors for, and physiological relevance of glycerophosphoinositols indicate exciting research opportunities in this young field.

Other phosphoinositide derivatives with important functions in T cells are the PI3K-substrate phosphatidylinositol(4,5)bisphosphate (PIP_2), diacylglycerol (DAG) and phosphatidic acid (PA). Jun and colleagues review how these lipids orchestrate intricate interactions of Ras guanine-nucleotide-exchange-factors, Ca^{2+}, adaptor- and effector proteins to control both kinetics and topology of Ras-activation in T cells (15). The authors discuss the roles of allosteric and feedback mechanisms, Ras-acylation, and complex interactions between the Ras- and PI3K-pathways. A complementing review by Krishna and Zhong discusses how diacylglycerol-kinases (DGK) phosphorylate DAG into PA to control T cell development and function, in particular to maintain self-tolerance (16). The authors review roles for known DAG-effectors and little-understood PA-effectors in T cells, potential contributions of DAG or PA metabolism, and DGK functions in other immune cells. Improved antiviral and antitumor activities of DGK-deficient T cells might indicate potential translational relevance, but as the authors point out, more studies are needed.

Moving beyond phosphoinositides, Nicolaou and colleagues (7) and Lone and colleagues (17) review the diverse functions of polyunsaturated fatty acid (PUFA)-derivatives in T cells. PUFA-lipids can modulate membrane-associated signalosomes by altering membrane composition, or act as precursors of secreted signaling-lipids. Both reviews first discuss biosynthesis and metabolism of eicosanoids, including prostanoids, leukotrienes, fatty acid epoxides, and endocannabinoids, and then review their diverse and often complex functions in T cell biology and disease. They further discuss the therapeutic potential of PUFA, a particularly interesting topic given the exploration of dietary PUFA as antiinflammatory agents, and the interest in targeting the prostanoid PGE_2 to improve immunotherapies for cancer or infections (7, 18).

Another lipid-derived messenger with important immune-regulatory functions is the cholesterol-derivative vitamin D_3. Kongsbak and colleagues review how its binding to the transcription-factor vitamin-D-receptor (VDR) controls T cell development, differentiation, and function (19). They describe the mechanisms controlling VDR expression and activity, and discuss roles for VDR downregulation in promoting autoimmune diseases or in dampening innate immunity during certain infections. Autoimmune disease-reversal by VDR-agonist/antibiotic combinations suggests translational relevance and possible contributions to the long-known but ill-understood links between microbial infections and the etiology of autoimmmunity. Available as nutrients, vitamin D_3, the lipid-related vitamin A, and short-chain fatty acids (20) all are potential lead-agents for therapeutic immunomodulation.

Finally, Procaccini and colleagues discusses how adipokine-hormones – produced by fat tissue and best known to influence energy-homeostasis and neuroendocrine function – may link metabolism with immunity (21). This area has recently gained increasing attention because of reported links between obesity, chronic inflammation, and various diseases, including cancer. The authors introduce the cellular and molecular components linking fat tissue and immune system and then review the often controversial, potential immunoregulatory roles of leptins, adiponectins, and other adipokines.

Altogether, the reviews in this Research Topic highlight how recent progress has profoundly altered and expanded our understanding of lipid functions in T cell biology but also raised many interesting questions. Rather than merely acting as membrane components and energy stores, lipids have emerged as important and multifaceted signaling molecules both inside and outside of T cells. We believe that this *Frontiers in Immunology* Research Topic provides its readers with a broad and stimulating basis to follow these important developments. In this sense, we thank all the authors for their outstanding contributions.

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Regulation of PI-3-kinase and Akt signaling in T lymphocytes and other cells by TNFR family molecules

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Activation of phosphoinositide 3-kinase (PI3K) and Akt (protein kinase B) is a common response triggered by a range of membrane-bound receptors on many cell types. In T lymphocytes, the PI3K-Akt pathway promotes clonal expansion, differentiation, and survival of effector cells and suppresses the generation of regulatory T cells. PI3K activation is tightly controlled by signals through the T cell receptor (TCR) and the co-stimulatory receptor CD28, however sustained and periodic signals from additional co-receptors are now being recognized as critical contributors to the activation of this pathway. Accumulating evidence suggests that many members of the Tumor Necrosis Factor receptor (TNFR) superfamily, TNFR2 (TNFRSF1B), OX40 (TNFRSF4), 4-1BB (TNFRSF9), HVEM (TNFRSF14), and DR3 (TNFRSF25), that are constitutive or inducible on T cells, can directly or indirectly promote activity in the PI3K-Akt pathway. We discuss recent data which suggests that ligation of one TNFR family molecule organizes a signalosome, via TNFR-associated factor (TRAF) adapter proteins in T cell membrane lipid microdomains, that results in the subsequent accumulation of highly concentrated depots of PI3K and Akt in close proximity to TCR signaling units. We propose this may be a generalizable mechanism applicable to other TNFR family molecules that will result in a quantitative contribution of these signalosomes to enhancing and sustaining PI3K and Akt activation triggered by the TCR. We also review data that other TNFR molecules, such as CD40 (TNFRSF5), RANK (TNFRSF11A), FN14 (TNFRSF12A), TACI (TNFRSF13B), BAFFR (TNFRSF13C), and NGFR (TNFRSF16), contribute to the activation of this pathway in diverse cell types through a similar ability to recruit PI3K or Akt into their signaling complexes.

Keywords: PI3K, AKT, TNFSF, TNFRSF, TRAF, signalosome

INTRODUCTION

The response of T lymphocytes to extrinsic stimuli has been known for many years to involve activation of phosphoinositide 3-kinase (PI3K) that results in a sustained rise in the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3 or $PI(3,4,5)P_3$), produced from phosphatidylinositol (4,5)-bisphosphate (PIP_2 or $PI(4,5)P_2$), and translocation of a subset of proteins containing pleckstrin homology (PH) domains to the plasma membrane, such as Akt (protein kinase B) and phosphoinositide-dependent kinase 1 (PDK1). Akt activity is regulated by the binding of PIP_3 or phosphatidylinositol (3,4)-bisphosphate ($PI(3,4)P_2$) to its PH domain, and by phosphorylation on threonine 308 by PDK1 and on serine 473 by the mammalian target of rapamycin complex 2 (mTORC2). Although PDK1 and mTORC2 may be central to Akt function, they likely have activities unrelated to Akt. For example, PDK1 also phosphorylates and activates other AGC protein kinases without binding to PIP_3, such as 70 kDa ribosomal protein S6 kinases (S6Ks), 90 kDa ribosomal protein S6 kinases (RSKs), serum/glucocorticoid-regulated kinases (SGKs), and protein kinase Cs (PKCs) (Finlay and Cantrell, 2011). Importantly, the signaling network regulated by PI3K and Akt plays an integral role in promoting T cell activation, differentiation, and survival, and also participates in suppressing the induction of Foxp3-expressing regulatory T cells (T_{reg}) that otherwise would limit the T cell response (Fruman and Bismuth, 2009; Huang and Sauer, 2010; Josefowicz et al., 2012; Okkenhaug, 2013). Activated Akt potentially regulates many downstream molecules, directly or indirectly through phosphorylation, that contribute to maximizing the T cell response. These include blocking the activity of forkhead box O (Foxo) transcription factors such as Foxo1 that promote differentiation of inducible T_{reg}, suppressing the activity or expression of pro-apoptotic molecules such as Bad and Bim, antagonizing the expression of cell cycle inhibitor proteins, and promoting T cell functionality and survival by increasing glucose uptake and glycolysis, and through augmenting IκB Kinase and NF-κB activity.

The range of membrane receptors that participate in triggering this PI3K/Akt axis in T cells may have been underappreciated. Recognition of antigen by the T cell receptor (TCR) in the context of signaling from the co-stimulatory receptor CD28 has long been known to promote PI3K activity. CD28 is constitutively expressed on T cells, and engagement by B7 molecules (CD80 and CD86) directly recruits the p85 regulatory subunit of PI3K (p85 PI3K) through a pYMNM (phospho-Tyr-Met-Asn-Met) motif located

in CD28's cytoplasmic tail (Pages et al., 1994). The overall signaling activity of CD28, including through the PI3K and Akt pathway, participates in the initial activation and division of T cells in many situations, although extensive studies have also suggested that the interaction with p85 is dispensable for many functions of CD28 in naïve T cells (Fruman and Bismuth, 2009). As well as CD28 and related molecules like ICOS, many additional receptors on T cells may contribute to PI3K and Akt activity. Sustained and periodic signaling from these receptors over time is increasingly being recognized as vital for continued T cell differentiation and survival, further suppression of T_{reg} development, the generation of memory, and the reactivation of memory T cells. Members of the tumor necrosis factor receptor (TNFR) superfamily constitute many of these receptors (Croft, 2003, 2009; Watts, 2005; So et al., 2006). Although all of the TNFR family members discussed in this review are strong activators of NF-κB, and NF-κB certainly plays a role in many of the functional consequences of triggering these receptors, increasing evidence suggests that their ability to also target PI3K and Akt may be integral to their function. In T cells, these receptors include TNFR2 (TNFRSF1B), OX40 (TNFRSF4), 4-1BB (TNFRSF9), HVEM (TNFRSF14), and DR3 (TNFRSF25), which are either constitutively expressed or induced after activation (**Figure 1**). There are several other TNFR molecules that control T cell function, such as CD27 (TNFRSF7), CD30 (TNFRSF8), and GITR (TNFRSF18), that have yet to be described to promote activation of PI3K or Akt, however it is highly likely that they also have the ability to target this pathway (**Figure 1**). This review summarizes recent findings on the potential importance of TNFR family signaling in stimulating the PI3K-Akt pathway in T cells as well as in other cell types, and

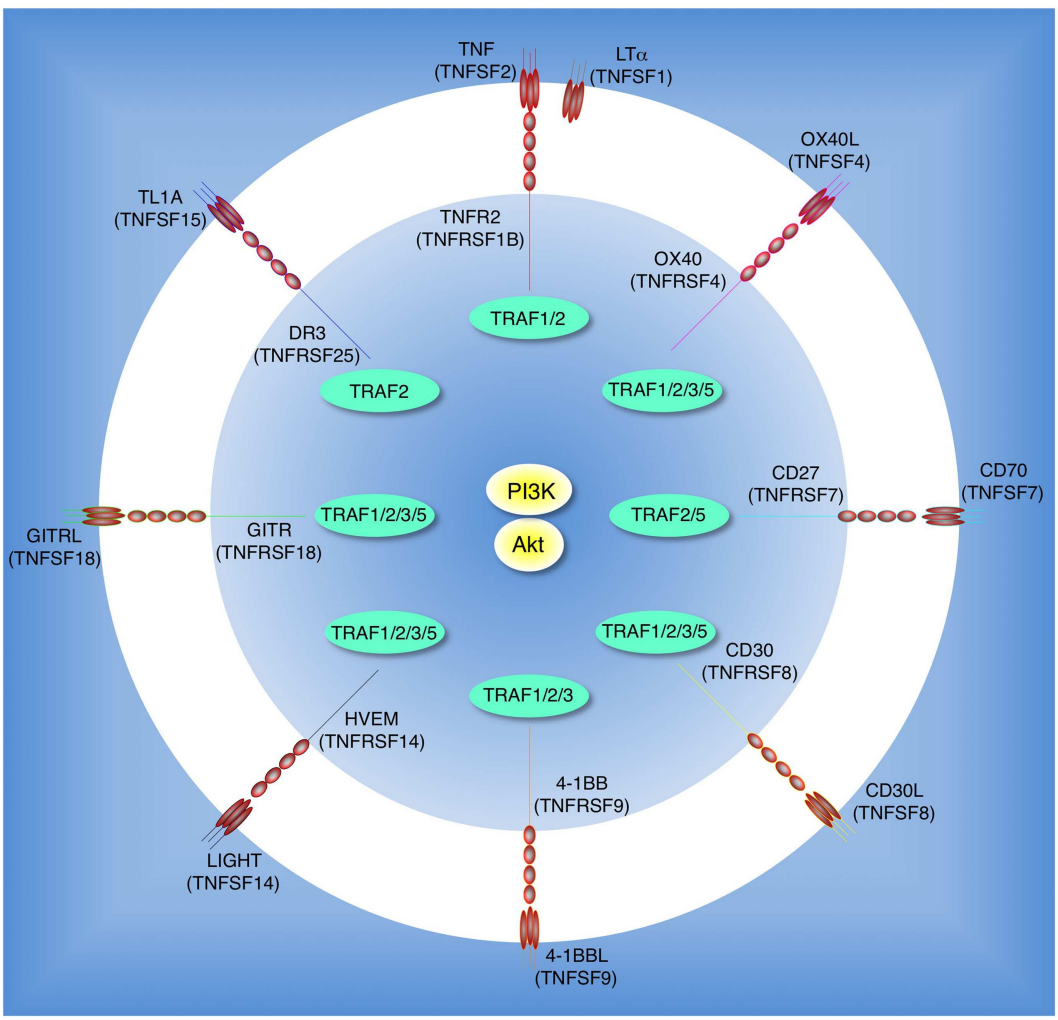

FIGURE 1 | Tumor necrosis factor receptor family molecules that possibly activate the PI3K-Akt pathway in T cells. Molecular interactions between TNF receptor superfamily (TNFRSF) members and TNF ligand superfamily (TNFSF) members: TNFR2 (TNFRSF1B) and TNF (TNFSF2) or LTα (TNFSF1); OX40 (TNFRSF4) and OX40L (TNFSF4); CD27 (TNFRSF7) and CD70 (TNFSF7); CD30 (TNFRSF8) and CD30L (TNFSF8); 4-1BB (TNFRSF9) and 4-1BBL (TNFSF9); HVEM (TNFRSF14) and LIGHT (TNFSF14); GITR (TNFRSF18) and GITRL (TNFSF18); DR3 (TNFRSF25) and TL1A (TNFSF15). Interactions between TNFR-associated factors (TRAFs) and TNFRSF molecules are indicated in the inner circle. CD27, CD30, and GITR have yet to be described to promote activation of PI3K or Akt, but this is likely given their overlapping TRAF-binding capacity.

discusses the likely mechanism of how TNFR family molecules organize signalosomes on the membrane to sustain lipid signaling. The initial sections will present a brief overview of reported activities of TNFR family members on T cells that have been described to augment PI3K or Akt activity. We will then discuss the potential molecular connections that allow these molecules to link to PI3K or Akt, and lastly review PI3K and Akt related activation by other TNFR family members in non-T cells.

REGULATION OF T CELL CO-SIGNALING BY TNFRSF MEMBERS: TNFR2, OX40, 4-1BB, HVEM, AND DR3

TNFR2 (TNFRSF1B)

TNFR2 is mainly expressed in cells of the immune system including T cells. The ligand TNF (TNFSF2) is produced by activated macrophages, T cells, and many other cell types, and exists as a transmembrane trimer whose proteolysis also leads to a soluble form. TNFR2 is more efficiently triggered by transmembrane TNF than by soluble TNF (Grell et al., 1995; Faustman and Davis, 2010). TNFR2 is constitutively expressed on T cells and increases its expression after T cell activation. Interaction of TNF with TNFR2 is co-stimulatory to TCR-mediated T cell activation and effector T cell differentiation (Kim and Teh, 2001, 2004; Aspalter et al., 2003; Kim et al., 2006) and TNFR2-deficient T cells possess a defect in survival during the early phase of clonal expansion that correlates with a defect in survivin, Bcl-2, and Bcl-$_{XL}$ expression (Kim and Teh, 2004; Kim et al., 2006). Importantly, TNFR2 was found to sustain Akt activity in T cells stimulated through the TCR and CD28. Given the described activities of PI3K-Akt in promoting expression of the aforementioned anti-apoptotic and cell cycle related molecules in various cell types including T cells, this data suggested that TNFR2 triggered Akt signaling may have participated in regulating expansion and survival of these effector-type T cells (Kim and Teh, 2004). The differentiation of effector T cells from a naïve population is counter to the differentiation of regulatory T cells (iT$_{reg}$), and activation of PI3K and Akt has been shown to block induction of Foxp3 and iT$_{reg}$ development (Haxhinasto et al., 2008; Sauer et al., 2008). In line with this, neutralization of TNF has also recently been found to enhance development of iT$_{reg}$ cells (Zhang et al., 2013). TGF-β-induced Smad3 phosphorylation directs transcription of Foxp3 and formation of iT$_{reg}$, and phosphorylation of Akt through TNF-TNFR2 interaction was described to facilitate Akt-Smad3 interaction and suppress Foxp3 expression, potentially explaining in part why TNF would block iT$_{reg}$ differentiation (Zhang et al., 2013).

Substantiating that Akt is a general target of TNF signaling, Akt phosphorylation has also been shown to be enhanced through TNFR1 and/or TNFR2 in various cell types, such as HEK293 cells (Ozes et al., 1999), HeLa cells (Ozes et al., 1999; Pastorino et al., 1999), HepG2 cells (Reddy et al., 2000), U937 cells (Reddy et al., 2000), endothelial cells (Zhang et al., 2003a), fibroblasts (Hanna et al., 1999; Zhang et al., 2001), myocytes (Hiraoka et al., 2001), cortical neurons (Marchetti et al., 2004), and hepatocytes (Osawa et al., 2001).

OX40 (TNFRSF4)

OX40 is induced on activated T cells while its ligand, OX40L (TNFSF4), is inducible on professional antigen-presenting cells (APCs). OX40-OX40L interactions positively regulate conventional T cell responses and can negatively affect T$_{reg}$ differentiation (So et al., 2008; Croft et al., 2009; Croft, 2010; Ishii et al., 2010). OX40 functions later than CD28, and potentially later than TNFR2, providing signals to promote continued division and survival, and hence clonal expansion of effector and memory T cells (Gramaglia et al., 2000). OX40 signaling was shown to augment and sustain PI3K-Akt activity when antigen was presented to T cells, again correlating with its ability to promote continued expression of molecules that control cell cycle progression as well as anti-apoptotic Bcl-2 family members (Rogers et al., 2001; Song et al., 2004, 2005). Importantly, a dominant-negative version of Akt reproduced many of the defects associated with a lack of OX40 expression, and introduction of a constitutively active version of Akt into T cells that lacked OX40 almost fully reversed the defect in clonal expansion and survival exhibited by these T cells (Rogers et al., 2001; Song et al., 2004, 2005). OX40 signaling also antagonizes the differentiation of Foxp3+ or IL-10+ iT$_{reg}$ (Ito et al., 2006; So and Croft, 2007; Vu et al., 2007). No formal proof has been provided that OX40 inhibition of Foxp3 and iT$_{reg}$ development is mediated in part by Akt activation, but again this is likely.

4-1BB (TNFRSF9)

4-1BB is another inducible molecule on activated T cells that can be triggered by 4-1BBL (TNFSF9) expressed on activated APCs (So et al., 2008; Snell et al., 2011; Vinay and Kwon, 2012). 4-1BB ligation can again promote T cell clonal expansion, differentiation, and expression of cytokines, and can enhance the survival of effector and memory T cells through upregulation of Bcl-$_{XL}$. 4-1BB signaling also has the ability to inhibit TGF-β-driven conversion of naïve CD4$^+$ T cells into iT$_{reg}$ either through direct activity or indirectly via upregulation of IFNγ production (Madireddi et al., 2012). 4-1BB was found to promote phosphorylation of Akt in T cells, and proliferative responses mediated by 4-1BB were blocked by a PI3K inhibitor, coincident with suppressing cyclin expression and promoting the cell cycle regulatory molecules p27^{kip1} (Lee et al., 2002, 2003). Some studies suggested that 4-1BB induced the anti-apoptotic Bcl-2 family molecules, Bcl-2 and Bcl-xL, in murine T cells in a PI3K-Akt independent manner (Lee et al., 2002, 2003). However, suppression of apoptosis and induction of c-FLIP$_{short}$ and Bcl-$_{XL}$ by 4-1BB in human peripheral blood T cells was blocked by targeting PI3K or Akt (Starck et al., 2005).

HVEM (TNFRSF14)

HVEM can interact with a number of different ligands, however its primary activating ligand in the TNF family is LIGHT (TNFSF14). HVEM is widely expressed on many cell types, including being constitutive on T cells. LIGHT in contrast is inducible on T cells as well as certain APCs such as DC and B cells upon activation (Steinberg et al., 2011; Ware and Sedy, 2011). Ligation of HVEM by LIGHT provides stimulatory signals that additionally can impact activation, differentiation, or survival of T cells. In line with HVEM controlling Akt activation at later stages of T cell responses, HVEM-deficient T cells were shown to display reduced Akt activity at the peak of the effector response that correlated with defective expression of Bcl-2 and reduced T cell survival.

Furthermore, the defect in T cell survival was rescued by ectopic expression of an active form of Akt (Soroosh et al., 2011).

Substantiating Akt as a downstream target of HVEM, the ability of LIGHT to induce macrophage migration and vascular smooth muscle cell proliferation also correlated with activation of PI3K and Akt (Wei et al., 2006), and LIGHT was found to promote PI3K-Akt phosphorylation in osteoclast precursor cells, supporting osteoclast differentiation (Hemingway et al., 2013).

DR3 (TNFRSF25)

DR3 is constitutively expressed by T cells and is upregulated following T cell activation, while TL1A (TNFSF15), the ligand for DR3, is induced in APCs (Meylan et al., 2011). Interaction of TL1A with DR3 also provides costimulatory signals to T cells in concert with antigen/TCR signaling and this can contribute to enhanced production of pro-inflammatory cytokines, and increased clonal expansion and differentiation of T cells. Although no studies have been conducted as yet on conventional T cells, ligation of DR3 was shown to promote T_{reg} proliferation that was blocked by an Akt inhibitor (Schreiber et al., 2010). Stimulation of DR3-expressing human acute monocytic leukemia THP-1 cells with TL1A, or anti-DR3 antibodies, also induced phosphorylation of Akt concomitant with upregulation of expression of βig-h3, an extracellular matrix protein. This was blocked by an inhibitor of PI3K and inhibitors of PKC, suggesting that PKC activation by DR3 may be involved in PI3K-Akt activation via this receptor in this cell type (Lee et al., 2010). Lastly, E-selectin (CD62E) has been suggested to be an alternate ligand for DR3, and E-selectin was found to activate the PI3K-Akt pathway via DR3 in HT29 colon carcinoma cells (Porquet et al., 2011).

TNF RECEPTOR OLIGOMERIZATION, MEMBRANE LIPID MICRODOMAINS, AND THE T CELL TNFR SIGNALOSOME

Although the studies described above show that TNFR2, OX40, 4-1BB, HVEM, and DR3 can enhance PI3K and Akt activation in T cells or other cell types, a primary question is whether this is a direct activity, or indirect through modulating or enhancing signaling through other non-TNFR molecules including the TCR or CD28. Moreover, as these TNFR molecules do not have obvious PI3K-binding motifs, similar to the pYMNM motif of CD28, it is not clear how they would directly link to PI3K or Akt or how the connection with the lipid mediators is facilitated.

TNF family ligands share the TNF homology domain (THD), which binds to cysteine-rich domains (CRDs) of the TNF family receptors. TNF ligands are synthesized as either membrane-bound or soluble trimeric proteins. Many biochemical and functional studies show that the transmembrane ligands can robustly activate receptors whereas the soluble trimeric ligands differ in their ability to be activating molecules. Some TNFR molecules, such as TNFR1 and CD40, are thought to be pre-clustered at the cell surface in the absence of their cognate TNF ligand (Chan, 2007), which likely aids their ability to respond to the soluble ligand. This is exemplified by TNF, which is highly active in soluble form when recognizing TNFR1. In contrast, studies of trimers of molecules such as OX40L and 4-1BBL have suggested they do not have functional effects when soluble, implying their receptors are not pre-assembled into clusters. However, artificially generated oligomerized versions of soluble trimeric ligands, including OX40L, 4-1BBL, and GITRL work as highly efficient agonists in T cells and other cell types (Haswell et al., 2001; Zhang, 2004; Stone et al., 2006; Muller et al., 2008; Zhou et al., 2008; Wyzgol et al., 2009). Other variants on this theme are molecules like APRIL whose soluble form can be oligomerized naturally through interaction with polysaccharide side chains of heparin sulfate proteoglycans, allowing effective signals through its receptors TACI or BCMA (Ingold et al., 2005; Kimberley et al., 2009); or BAFF that also binds to TACI, and is unable to activate this receptor as a single trimer, but can assemble as an ordered structure comprising 20 trimers (60-mer) and then gains the ability to be a strong TACI agonist (Liu et al., 2002; Bossen et al., 2008). In sum, these results suggest that oligomerization of most TNFR molecules, beyond the basic trimer complex that would be formed after ligation of a single trimeric ligand, is a prerequisite for efficient recruitment and activation of signaling moieties.

All TNFR family molecules also promote intracellular kinase activation at least in part through adaptor proteins called TNFR-associated factors (TRAFs). For example, TNFR2 has the potential ability to recruit and/or directly bind TRAFs 1 and 2 (Rothe et al., 1994); OX40: TRAFs 1, 2, 3, and 5 (Kawamata et al., 1998); 4-1BB: TRAFs 1, 2, and 3 (Jang et al., 1998); HVEM: TRAFs 1, 2, 3, and 5 (Marsters et al., 1997); and DR3: TRAF2 (Chinnaiyan et al., 1996). TRAF proteins already can exist as trimers in the cytosol before binding to the cytoplasmic tails of TNF receptors (Park et al., 1999), suggesting that oligomerization of the receptors will then additionally result in oligomerized scaffolds of at least one, but more likely multiple, TRAF molecules.

Another facet that might be important to the ability of TNFR family molecules to link to PI3K and Akt directly is the regulated movement of TNFR oligomers into detergent-insoluble cholesterol- and sphingolipid-rich plasma membrane microdomains (DIM or lipid rafts). Here the spatiotemporal regulation of protein–protein interactions and dynamic protein networks may orchestrate to allow any biological outcome (Dykstra et al., 2003; Viola and Gupta, 2007). DIM are estimated as $<$20 nm diameter in a living cell (Eggeling et al., 2009), indicating that molecules that translocate into DIM are likely condensed into a small area. Although not investigated for many TNFR molecules to date, particularly in T cells, several members of the family have been visualized to concentrate in DIM after stimulation by their ligands including OX40 (So et al., 2011a,b) and 4-1BB (Nam et al., 2005). Therefore, translocation into lipid-rich microdomains might be a common and important feature of the TNFR family. Moreover, TRAF2 can interact with Filamin-A, which functions as a scaffold for DIM formation (Leonardi et al., 2000; Arron et al., 2002), and with Caveolin-1, which is a component of DIM (Feng et al., 2001), suggesting that recruitment of this molecule might promote or maintain localization of TNFR molecules in these lipid-rich areas. TRAF2 binding is shared by all TNFR family molecules that have been described to co-stimulate T cells (**Figure 1**), including those shown to date to promote PI3K and Akt activation (see above), implying TRAF2 may be a critical link to PI3K and/or Akt. Perhaps of equal significance, PIP_2 is enriched and constitutively associated with DIM, and at least

in T cells, a proportion of total cellular PI3K and PDK1 are constitutively associated with detergent-insoluble fractions (Pike and Casey, 1996; Dykstra et al., 2003; So et al., 2011a). Therefore, it is reasonable to suggest that these lipid-rich microdomains are likely to play a critical role in triggering PI3K-Akt signaling by facilitating the localization of oligomerized TNFR and TRAF molecules with PI3K, PIP_2, and PDK1 (Lasserre et al., 2008). This would enhance the likelihood of PIP_3 production, and membrane recruitment and phosphorylation of Akt, assuming PI3K can be activated.

Only studies of one molecule to date have shown a direct link of a TNFR family molecule to PI3K and Akt in T cells. However, there is strong rationale that the findings will be generalizable. We clearly demonstrated in several studies that OX40 signaling strongly synergizes with antigen signals to augment Akt activity in recently activated or effector T cells (Song et al., 2004; So et al., 2011a,b). After interaction of OX40 with transmembrane OX40L, OX40 moved into DIM and immunoprecipitation experiments revealed that it organized a signalosome containing many molecules including TRAF2, the IKK complex, and PKCθ and the CARMA1-BCL10-MALT1 complex, that regulate NF-κB1 activation, and also including p85 PI3K and Akt (So et al., 2011a,b). The formation of this complete OX40 signalosome in T cells was dependent on TRAF2 and on translocation of OX40 into DIM, but independent of antigen/TCR stimulation (So et al., 2011a,b). Moreover, in the absence of TRAF2 or by disrupting DIM, OX40 could not complex with either PI3K or Akt. Interestingly, OX40 was unable to induce significant cellular phosphorylation of PI3K, PIP_3 accumulation, or Akt activation, unless antigen was presented to the T cells, even though antigen/TCR signaling had no obvious impact on recruitment of PI3K or Akt to the OX40 signalosome. The explanation for this was not clear, but we only found a moderate amount of PDK1 associated with the OX40 complex suggesting that this might in part contribute to the inability of OX40 ligation in isolation to lead to phosphorylation of Akt, and why antigen recognition was essential. However, more recent data have shown that OX40 associates with an E3 ligase that appears to limit its ability to activate Akt in T cells (Croft, unpublished). This then suggests that OX40 does possess the capacity to activate Akt independently of other receptors, but regulatory elements may keep this ability in check in T cells providing control over this aspect of OX40 biology to the TCR. Importantly, these data then imply that OX40 functions in T cells by quantitatively enhancing the amount of PI3K and Akt that is available to be activated in the lipid-rich microdomain environment. We hypothesize that the higher ordered oligomerized TNFR-ligand modules that are organized in DIM of T cells essentially offer functional hot spots of concentrated PI3K and Akt in the vicinity of the TCR/CD28 signalosome (**Figure 2**). These data also highlight that TRAF adaptors are likely to play critical roles in linking the TNFR family to PI3K and Akt in T cells. Our studies show that TRAF2 is important for OX40 to recruit both PI3K and Akt into its signaling complex, but whether TRAF2 directly binds one or both molecules is not yet clear. Other TRAFs, particularly TRAF6, may be also critical as described below in non-T cells, although how much this might vary from a T cell to another cell type is also not clear.

REGULATION OF PI3K AND AKT BY TNFRSF MEMBERS IN NON-T CELLS: CD40, RANK, FN14, TACI/BAFFR, AND NGFR

CD40 (TNFRSF5)

Signaling through CD40 after ligation by CD40L (TNFSF5) is important for promoting the activation, division, and maturation of APCs, and isotype switching of B cells (Graham et al., 2010; Gommerman and Summers deLuca, 2011). CD40 can directly bind to TRAFs 1, 2, 3, 5, and 6 (Pullen et al., 1998) and CD40 engagement leads minimally to translocation of CD40, TRAF2, TRAF3, and TRAF6 into DIM where CD40 activates downstream signaling cascades (Hostager et al., 2000; Vidalain et al., 2000; Arron et al., 2001). Cross-linking CD40 has been found to activate PI3K in the Daudi human B lymphoblastoid line (Ren et al., 1994), and promote Akt phosphorylation, downregulation of $p27^{kip1}$, and upregulation of Bcl-$_{XL}$, in primary murine B cells that was blocked by a PI3K inhibitor (Andjelic et al., 2000). CD40-induced proliferation and survival of B cells deficient in a negative regulatory adaptor molecule was also suppressed by introduction of dominant-negative Akt (Aiba et al., 2006). After triggering CD40 on murine bone marrow-derived DCs, p85 PI3K was furthermore found to be recruited to CD40 correlating with enhanced Akt activation (Arron et al., 2001). Similarly, CD40L induced Akt phosphorylation and survival in human monocyte-derived DCs that was blocked with a PI3K inhibitor (Yu et al., 2004). In other cells, stimulation of CD40 on human microvascular endothelial cells also induced PI3K and Akt phosphorylation, concomitant with an increase in cell survival and proliferation, and these functional activities were suppressed by PI3K inhibitors and a dominant-negative version of Akt (Deregibus et al., 2003).

Although the adaptors required for CD40 to connect to PI3K and Akt have not been investigated in every situation, several pieces of evidence suggest TRAF6 and/or TRAF2 are crucial. For example, CD40 was shown to block apoptosis induced by the Fas death receptor in a PI3K and Akt dependent manner, and this was abrogated in B cells that were deficient in TRAF6 (Benson et al., 2006). A CD40 signalosome containing TRAF2, TRAF6, and p85 PI3K was also visualized in endothelial cells (Deregibus et al., 2003) and a signalosome of TRAF6 and PI3K in DCs (Arron et al., 2001). Lastly, fibroblasts lacking TRAF2 or TRAF6 displayed impaired Akt phosphorylation that was triggered by CD40 engagement (Davies et al., 2005).

RANK (TNFRSF11A)

RANK interactions with RANKL (TNFSF11) regulate bone remodeling, lymph node formation, establishment of the thymic microenvironment, and mammary gland development during pregnancy (Leibbrandt and Penninger, 2008). RANK has the potential to recruit TRAFs 1, 2, 3, 5, and 6 (Wong et al., 1998; Darnay et al., 1999) and triggering of RANK with RANKL has been shown to promote activation of the PI3K-Akt pathway in osteoclasts and DCs. Similar to CD40, RANK was visualized to induce a signalosome containing TRAF6 and p85 PI3K in these cell types. PP1, an inhibitor for Src family kinases, inhibited Akt phosphorylation mediated by RANK, indicting that c-Src is an upstream regulator of PI3K. In accordance, the kinase activity of c-Src was upregulated in the RANK signalosome and RANK was

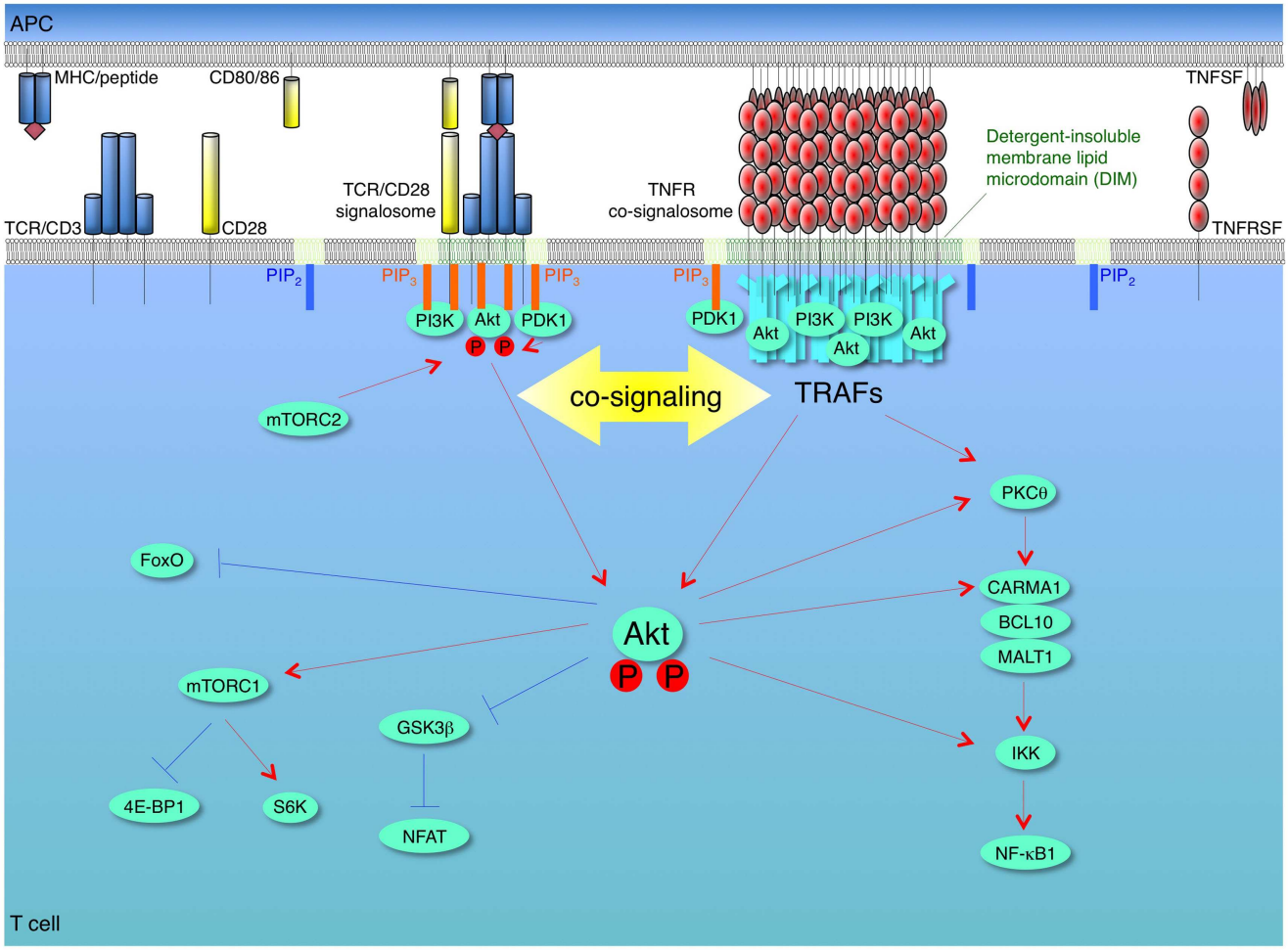

FIGURE 2 | Model of synergy between TCR/CD28 and TNFR signalosomes for activation of the PI3K-Akt pathway in T cells. T cells are activated firstly by recognition of antigen by the T cell receptor (TCR)/CD3 complex when it is displayed by the major histocompatibility complex (MHC) on antigen-presenting cells (APCs). The second co-stimulatory signal is delivered through CD28 by interaction with its ligands CD80 and/or CD86. These combined signals can activate phosphoinositide 3-kinase (PI3K), which leads to conversion of PIP_2 into PIP_3 at the plasma membrane. The pleckstrin homology (PH) domain containing proteins, Akt (protein kinase B) and phosphoinositide-dependent kinase 1 (PDK1), are recruited to the membrane PIP_3, and then Akt is phosphorylated by PDK1 and by the mammalian target of rapamycin complex 2 (mTORC2). This promotes translocation of Akt from membrane to cytosol, thereby allowing regulation of downstream pathways through phosphorylation of target molecules, such as glycogen synthase kinase 3β (GSK3β), forkhead box O (Foxo), and IκB kinase (IKK). Akt phosphorylates and inactivates two negative regulators of mTORC1, tuberous sclerosis complex 2 (TSC2) and proline-rich Akt substrate of 40 kDa (PRAS40), which results in activation of mTORC1. Akt contributes to NF-κB activation through phosphorylation of IKK and interaction with protein kinase C θ (PKCθ) or caspase-recruitment domain (CARD)-membrane-associated guanylate kinase (MAGUK) protein 1 (CARMA1). After recognition of trimeric TNF ligand superfamily (TNFSF) molecules on APCs, TNF receptor superfamily (TNFRSF) molecules on T cells are trimerized and oligomerized and recruit trimeric TNFR-associated factors (TRAFs) to their cytoplasmic TRAF-binding motifs. The TNFSF-TNFRSF complex then translocates into detergent-insoluble membrane lipid microdomains (DIM). The TNFRSF-TRAF superclusters recruit and allow the efficient accumulation of PI3K and Akt in concentrated depots in close proximity to the TCR/CD28 signalosome, which results in a quantitative contribution of TNFR signalosomes to enhancing and sustaining PI3K and Akt activation triggered by the TCR/CD28 signalosome. The TNFR signalosomes also can promote activation of NF-κB irrespective of TCR/CD28 signaling. Red lines show activating signals, blue lines show inhibitory signals.

found unable to activate Akt without the enzymatic activity of c-Src (Wong et al., 1999; Arron et al., 2001; Xing et al., 2001).

TRAF6 again may be critical for the ability of RANK to target the PI3K-Akt pathway, and certain elements might also limit Akt activation. Upon stimulation of RANK in the RAW264.7 monocyte/macrophage cell line, phosphorylation of PI3K and Akt was upregulated and this was further amplified by introduction of dominant-negative SHP-1. TRAF6 was found to interact with RANK and SHP-1, and SHP-1 antagonized the association between RANK and TRAF6 (Zhang et al., 2003b). Similar to other TNFR molecules, RANK may also function in the context of DIM. After engagement by RANKL in osteoclasts, TRAF6 was shown to translocate into DIM where c-Src is constitutively resident, and disruption of DIM reduced Akt activation and concomitantly blocked osteoclast differentiation, survival, and bone resorption activity (Ha et al., 2003).

FN14 (TNFRSF12A)

FN14 is another TNFR molecule. It is expressed on epithelial cells, endothelial cells, and other non-hematopoietic cells and engages TWEAK (TNFSF12) and promotes a number of differentiation activities depending on the cell type. Stimulation of FN14 on mouse osteoblastic MC3T3-E1 cells induced Akt phosphorylation and RANTES production in a PI3K-dependent manner (Ando et al., 2006). TWEAK also promoted expression of ICAM-1 and VCAM-1 on human gingival fibroblasts that was blocked with an inhibitor of PI3K (Hosokawa et al., 2006), and Akt phosphorylation and matrix metalloprotease-9 (MMP-9) expression in mouse C2C12 myotubes was suppressed by targeting PI3K or introducing a dominant-negative version of Akt (Kumar et al., 2009). Similar results were also reported with TWEAK activation of FN14 on: renal tubular epithelium cells where PI3K inhibitors prevented upregulation of cyclin D1 and cell proliferation (Sanz et al., 2009); cardiomyocytes where FN14-mediated proliferation was also blocked by interfering with PI3K activity (Novoyatleva et al., 2010); and human gingival fibroblasts where the PI3K-Akt pathway contributed to induction of CCL20 (Hosokawa et al., 2012). FN14 has the potential to directly recruit and bind TRAFs 1, 2, 3, and 5 (Brown et al., 2003) but no studies to date have attempted to link a specific TRAF to the ability of FN14 to phosphorylate and activate PI3K or Akt.

TACI (TNFRSF13B)/BAFFR (TNFRSF13C)/BCMA (TNFRSF17)

TACI, BAFFR, and BCMA are mainly expressed on B cells and play critical roles in survival of B cells at distinct stages of development by engaging APRIL (TNFSF13) and/or BAFF (TNFSF13B) (Rickert et al., 2011). BAFFR can activate the PI3K-Akt pathway in mature B cells (Patke et al., 2006; Otipoby et al., 2008; Woodland et al., 2008) and BAFF-mediated proliferative and survival responses were defective in B cells lacking p110δ PI3K (Henley et al., 2008). PKCβ was found to interact and directly phosphorylate Akt on serine 473 after ligation of BAFFR, with Akt phosphorylation being greatly reduced in PKCβ-deficient B cells (Patke et al., 2006). Follicular lymphoma B cells were additionally found to respond to APRIL-TACI stimulation by phosphorylating p85 PI3K, Akt, mTOR, 4E-BP1, and p70S6K, and PI3K inhibitors blocked these APRIL-induced activities and cellular proliferation (Gupta et al., 2009).

Stimulation of human myeloma cells expressing TACI, BAFFR, and BCMA with BAFF or APRIL has also been shown to activate the PI3K-Akt pathway concomitant with protection against apoptosis (Moreaux et al., 2004); and lastly human adipose-derived stem cells additionally phosphorylated Akt after exposure to APRIL or BAFF (Zonca et al., 2012). Similar to other TNFR family members, TACI, BAFFR, and BCMA may directly recruit and bind TRAFs 2, 5, and 6 (Xia et al., 2000), TRAFs 2 and 3 (Xu and Shu, 2002), and TRAFs 1, 2, 3, 5, and 6 (Hatzoglou et al., 2000; Shu and Johnson, 2000), respectively, suggesting that TRAF2 and/or 6 may again mediate PI3K-Akt signaling, although no studies have addressed this as yet.

NGFR (TNFRSF16, $p75^{NTR}$)

Lastly, NGFR that is mainly expressed on neurons and glia during development of the central nervous system, and is induced after many types of nervous system injury, also appears to utilize PI3K and Akt for certain activities. NGFR only binds neurotrophins [nerve growth factor (NGF); brain-derived neurotrophic factor (BNDF); and neurotrophin-3 and -4 (NT-3 and -4)]. NGFR may primarily work as a co-receptor and cooperates with the Trk receptor tyrosine kinase family (Trk-A, -B, and -C), Sortilin-family receptors, and Nogo receptor/Lingo-1 (Ibanez and Simi, 2012). Many studies (e.g., refs Soltoff et al., 1992; Yao and Cooper, 1995; Jackson et al., 1996; Vaillant et al., 1999; Takano et al., 2000) have shown that NGF activates PI3K and induces PIP_3 production, and that the Akt pathway works as a key regulator of neurotrophin-induced neuronal survival. NGFR signaling is initiated in caveolae, which are a special type of DIM and serve as signaling platforms (Bilderback et al., 1999; Huang et al., 1999), but the contribution of individual TRAF molecules to activation of PI3K and Akt has not yet been reported. NGFR can directly bind TRAFs 2 and 6 (Khursigara et al., 1999; Ye et al., 1999), and TRAF6 recruitment has been suggested to be essential for signal transduction activity (Vilar et al., 2009), again implying this receptor may connect to the PI3K-Akt pathway via similar TRAF adaptors as other members of the TNFR family.

CONCLUSION

In conclusion, many stimulatory TNFR family members have been reported to augment PI3K and Akt activation in diverse cell types, suggesting that this pathway can be a major contributor to the functional effects mediated by these molecules. After interaction with their transmembrane, and in some cases soluble, TNF family ligands, TNFR molecules oligomerize and organize signalosomes in membrane lipid microdomains. The cytoplasmic domain of TNFR family members does not have the consensus motif that can directly bind PI3K. Rather, TNFR molecules bind to overlapping but distinct subsets of TRAF adaptor proteins, and these adaptors initiate many of the signals delivered by the receptors. Increasing evidence suggests that TRAF2 and/or TRAF6 are required for recruitment of PI3K or Akt into TNFR signalosomes, but whether these TRAF molecules directly bind to PI3K or Akt is not clear. In overexpression studies, and in MEFs stimulated with IGF-1 or IL-1, TRAF6 was precipitated with Akt, and furthermore TRAF6 induced K63-linked ubiquitination and membrane localization of Akt (Yang et al., 2009). Thus it is possible that this is also a primary activity when TRAF6 is recruited to a TNFR molecule. However, not all TNFR family members that have been reported to promote PI3K and Akt activation appear to bind or recruit TRAF6, but they may all interact with TRAF2. This implies that TRAF2 could be the crucial adaptor in some cases, but whether TRAF2 possesses the same activity as TRAF6 in being able to complex with, and ubiquitinate, Akt is presently unknown.

In T cells, TNFR family molecules are crucial for clonal expansion and survival, and for the generation of T cell memory, and increasing evidence suggests these functional effects are in part mediated by enhancing antigen-initiated PI3K and Akt activity. Some TNFR family molecules in non-T cells appear to have the capacity to activate PI3K and Akt without signaling from other receptors, suggesting they may directly phosphorylate PI3K and/or recruit other kinases that can perform this function, and they

may also recruit kinases such as PDK1 that phosphorylate Akt. In contrast, most evidence suggests that in T cells the ability of TNFR molecules to promote phosphorylation of PI3K and Akt is restricted unless the TCR recognizes antigen. This makes sense as T cells are governed by many checkpoints that limit their response in an attempt to control autoreactivity. Thus, it is likely that the higher-order TNFR-TRAF superclusters induced within lipid-rich microdomains of T cells then allow the efficient accumulation of PI3K and Akt in concentrated depots in close proximity to the TCR signalosome, and the function of this would be to either relieve a molecular checkpoint that limits Akt phosphorylation, or would be to simply provide more molecules of Akt available to the TCR. Further work in this area is required to fully understand the nature of TNFR signalosomes and how they may differ from molecule to molecule and in T cells versus other cell types.

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Lipid and protein co-regulation of PI3K effectors Akt and Itk in lymphocytes

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The phosphoinositide 3-kinase (PI 3-kinase, PI3K) pathway transduces signals critical for lymphocyte function. PI3K generates the phospholipid PIP_3 at the plasma membrane to recruit proteins that contain pleckstrin homology (PH) domains – a conserved domain found in hundreds of mammalian proteins. PH domain–PIP_3 interactions allow for rapid signal propagation and confer a spatial component to these signals. The kinases Akt and Itk are key PI3K effectors that bind PIP_3 via their PH domains and mediate vital processes – such as survival, activation, and differentiation – in lymphocytes. Here, we review the roles and regulation of PI3K signaling in lymphocytes with a specific emphasis on Akt and Itk. We also discuss these and other PH domain-containing proteins as they relate more broadly to immune cell function. Finally, we highlight the emerging view of PH domains as multi-functional protein domains that often bind both lipid and protein substrates to exert their effects.

Keywords: PI3K, lymphocyte activation, pleckstrin homology domain, Akt signaling, Itk signaling

LYMPHOCYTE ACTIVATION RECEPTORS SIGNAL THROUGH CLASS I PI3Ks

Phosphoinositide 3-kinase (PI3K) activation is important for lymphocyte survival, activation, differentiation, and migration. Many lymphocyte surface receptors activate class 1 PI3Ks, which phosphorylate phosphatidyl inositol 4,5-bisphosphate [$PI(4,5)P_2$, PIP_2] at the D-3 hydroxyl group of the myo-inositol ring to generate phosphatidyl inositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$, PIP_3]. Two subclasses, 1A and 1B, are activated by distinct receptor types (**Figure 1**). Receptors or signaling adapters that are phosphorylated at YxxM sequence motifs signal though class IA PI3K, which includes p85α and p85β regulatory subunits and p110α, p110β, and p110δ catalytic subunits. These receptors include CD19, CD28, and ICOS co-receptors; IL-2, IL-7, IL-3, IL-15, and GM-CSF cytokine receptors (1–6); and receptors coupled to TRIM, DAP10, and MyD88 adapter proteins (7–11). Receptor ligation leads to tyrosine phosphorylation at the YxxM motif and subsequent recruitment of PI3K regulatory subunits through one or both Src homology 2 (SH2) domains. Regulatory subunits are then phosphorylated by Syk or Jak family tyrosine kinases to trigger activation of their constitutively associated catalytic subunits (3).

G-protein-coupled receptors (GPCRs) signal through Class 1B PI3K, which includes p101 regulatory and p110γ catalytic subunits (12). These classic, seven transmembrane domain receptors include chemokine receptors and signal through heterotrimeric G proteins, Gα and Gβγ to promote cell migration. GPCR ligation dissociates the Gβγ dimer, allowing its binding to p101 regulatory subunits and subsequent activation of associated p110γ catalytic subunits. Activation of p110γ catalytic activity can also be induced by Ras activation (Ras-GTP) to promote migration of neutrophils (13).

Although many receptors activate class 1 PI3K, the magnitude and kinetics of PI3K activation differs greatly among receptors, depending on ligand binding kinetics and feedback circuitry that can either amplify or dampen PI3K signaling (14). Additionally, co-ligation of receptors, such as the T cell receptor (TCR) and the CD28 co-receptor, can cooperate to potentiate and sustain PI3K activation and PIP_3 generation.

PIP_3 ASSOCIATION WITH PLECKSTRIN HOMOLOGY DOMAINS

PI3K activation induces PIP_3 accumulation, which comprises less than 5% of PIP_2 levels and less than 1% of total membrane lipids (15). Despite its low overall abundance, super-resolution microscopy has revealed ~100 nm membrane clusters of PIP_3 that create high local PIP_3 concentrations (16). High affinity and specificity binding between PIP_3 and pleckstrin homology (PH) domains of PI3K effectors helps to recruit and activate these effectors at the plasma membrane (**Figure 2**). Like protein–protein interactions that are induced by phosphorylation, PIP_3 interactions with PH domains allow rapid transduction of downstream signals without new protein synthesis.

The PH domain is an evolutionarily conserved structural fold found in proteins expressed in organisms ranging from yeast to mammals (17). The core of the PH domain is a seven-strand β-barrel that is encoded by approximately 120 amino acids and is composed of two anti-parallel β sheets and a C-terminal α helix (**Figure 3**). The mammalian genome contains roughly

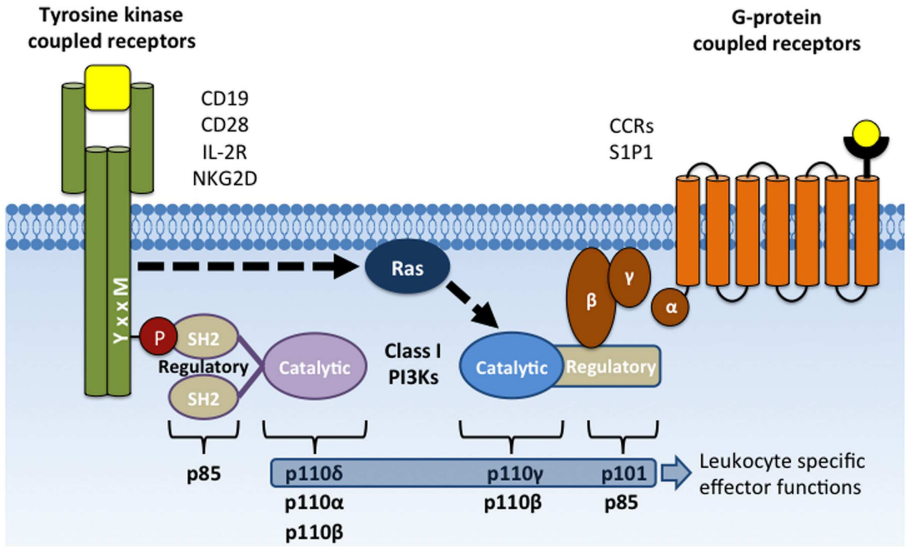

FIGURE 1 | Activation of class I PI3Ks by YxxM signaling subunits and GPCRs. Membrane receptors that activate PI3K include CD19, CD28, and NKG2D co-receptors, cytokine receptors (e.g., IL-2R), G-protein-coupled receptors (chemokine receptors), and Fcγ receptor I and III. Class IA PI3Ks are recruited to the plasma membrane through SH2 domain interactions with phosphorylated YxxM motifs. Class IB PI3Ks are recruited and activated by direct interaction with the Gβγ subunit following GPCR activation. Activated PI3K phosphorylates the membrane lipid $PI(4,5)P_2$ to form $PI(3,4,5)P_3$.

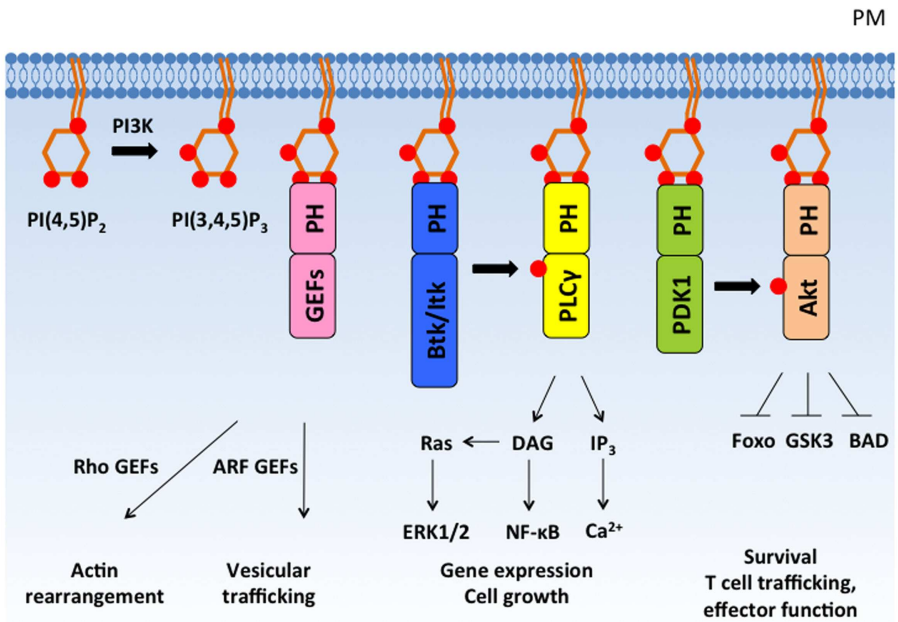

FIGURE 2 | $PI(3,4,5)P_3$ recruits PH domain-containing proteins to the plasma membrane and regulates diverse cellular responses. PI3K phosphorylates $PI(4,5)P_2$ to form $PI(3,4,5)P_3$, which recruits PH domain-containing signaling proteins to the plasma membrane. PH domain-containing proteins are activated at the plasma membrane and mediate important cellular responses such as cytoskeleton rearrangement, cell growth, proliferation, and survival. PM, plasma membrane; GEF, guanine nucleotide exchange factor.

300 PH domains found in proteins that perform diverse functions including cellular activation, cytoskeletal reorganization, vesicular trafficking, and cell cycle control. Approximately, 15% of PH domains, including Akt and Itk, bind to phosphoinositides with high specificity and affinity (K_d: nanomolar – low micromolar range). PH domains generally interact with phosphoinositides through positively charged lysine and arginine residues within the basic motif KXn(K/R)XR (18). However, not all PH domains bind to PIP_3. Several PH domains interact with phosphoinositides that are selectively enriched in other membrane compartments,

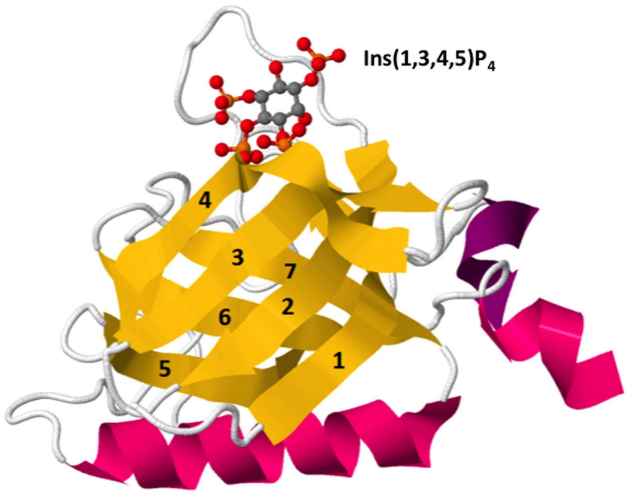

FIGURE 3 | Crystal structure of Btk PH domain in complex with Ins(1,3,4,5)P_4 (PDB ID: 2Z0P). The PH domain is comprised of a β-barrel formed by seven β-strands (yellow, 1–7) capped by an α-helix (pink). The hyper-variable loops of the β-barrel form the binding surface for lipid ligands such as Ins(1,3,4,5)P_4 [top, shown by ball-and-stick model: red (oxygen), orange (phosphorus), and gray (carbon)].

such as PI4P within the Golgi membrane (19) or PIP_2 at the resting plasma membrane (17). Thus, conveying lipid specificity to PH domains constitutes a key mechanism for spatially sequestering distinct effector proteins within cells. Regulating the abundance of lipids either in resting or activated cells controls basal and induced effector activity. Additionally, regulated production of lipid ligands such as PIP_3 within specific membrane nano-domains can induce polarized activation of downstream effectors in a robust but transient manner. This is because PIP_3 abundance is not only spatial restricted but also finely controlled by receptor-induced PI3K-dependent PIP_3 generation and by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and SH2 domain-containing inositol 5′-phosphatase (SHIP) phosphatase-dependent PIP_3 metabolism.

PROTEIN PHOSPHATASES INHIBIT PI3K ACTIVATION WHILE INOSITOL PHOSPHATASES REDUCE PIP_3 LEVELS

PI3K signaling is negatively regulated at distinct steps in its signaling cascade by both protein and lipid phosphatases. Protein tyrosine phosphatases SHP-1 and SHP-2 inhibit PI3K activation by preventing early receptor signaling and by directly down-regulating PI3K activity, the latter of which is accomplished by de-phosphorylation of phospho-tyrosine residues within signal adapter proteins and PI3K regulatory subunits (71). Inhibitory receptors that restrict lymphocyte activation through SHP-1 or SHP-2 include inhibitory killer-cell immunoglobulin-like receptors (KIR) on NK cells (72), CD22 on B cells (73), and CTLA-4 and PD-1 on T cells (74, 75). Phosphorylated immunoreceptor tyrosine-based inhibition motifs (ITIM) within the cytoplasmic domains of KIRs, CD22, and CTLA-4 recruit SHP-1 and SHP-2 to prevent activating signals at the plasma membrane (72, 74, 75). Persistent T cell activation signals can also be inhibited by SHP-1 and SHP-2 recruitment to the immunoreceptor tyrosine-based switch motif (ITSM) in PD-1, an inhibitory receptor expressed on chronically stimulated T cells (76, 77). For a detailed discussion regarding the requirements of SHP-1 and SHP-2 in T cell development, differentiation, and effector function, refer to Ref. (78).

In T cells, CTLA-4 can also directly repress Akt signaling by recruiting the Ser/Thr phosphatase PP2A (77), which dephosphorylates the T308 (79, 80) and possibly S473 (79), residues required for Akt activity. Thus, CTLA-4 utilizes a dual approach to antagonize CD28 and PI3K signaling: SHP-2-dependent inhibition of TCR signaling by CD3ε de-phosphorylation and PP2A-dependent de-phosphorylation of Akt (74, 77, 81).

Lipid and inositol phosphatases also prevent PI3K effector activation. PTEN and SHIP both dephosphorylate membrane PIP_3. However, while PTEN converts PIP_3 back to its lipid precursor $PI(4,5)P_2$ to prevent further activation of PI3K effectors, SHIP converts PIP_3 into $PI(3,4)P_2$, a lipid that retains the ability to bind the Akt PH domain (82). In the latter case, subsequent de-phosphorylation of $PI(3,4)P_2$ into PI(3)P by the inositol phosphatase, INPP4B is required to "turn off" Akt membrane recruitment (83). Inhibitory receptors including FcγIIB on B cells and mast cells and Ly49A and Ly49C on NK cells contain ITIM motifs that recruit SHIP through its SH2 domain (84, 85). Membrane receptors with cytosolic PDZ domains recruit PTEN to control PIP_3 levels. Although the functional significance of PDZ domain-containing receptors on lymphocyte activation requires additional investigation, maintaining appropriate PTEN levels is crucial for the control of immune cell homeostasis and function (86).

GENERAL AND CELL TYPE-SPECIFIC Akt FUNCTIONS

Akt belongs to the AGC family of Serine/Threonine kinases. The three Akt isoforms are differentially expressed in various cell types but are 77–83% sequence identical. Akt activity prevents apoptosis, promotes protein expression, and regulates cellular metabolism (20–23). Akt mediates these general cellular functions through direct phosphorylation of RxRxxS*/T* motifs (24) found in a plethora of cellular targets including forkhead box transcription factors, TSC2, GSK3, and BAD, which are discussed in detail elsewhere (20). A somatic mutation in Akt that replaces glutamate with lysine at residue 17 (hereafter referred to as E17K) leads to cellular transformation and has been identified in human breast, colorectal, and ovarian cancer (25, 26). The E17K mutation is located in the lipid binding pocket of Akt's PH domain and dramatically increases its affinity for membrane lipids, causing constitutive Akt signaling (27). Ectopic expression of E17K in hematopoietic stem cells is sufficient to induce development of lymphoblastic T cell lymphoma within 6–8 weeks following transfer into recipient mice (28). Similarly, conditional deletion of the Akt targets Foxo1/3/4 in mice leads to development of the same type of lymphomas 15–25 weeks after induction of Foxo deletion (29).

In lymphocytes, Foxo proteins regulate the gene expression of Rag recombinases, Ikaros, CCR7, IL-7R, TCF7, Eomes, and Foxp3, which are critical for controlling lymphocyte development, trafficking, and differentiation (30–37). Akt phosphorylation of Foxo1 and Foxo3 leads to their degradation and down-regulates Foxo-dependent gene expression (31, 38). Genetic ablation of

both Foxo1 and Foxo3 causes a multi-focal autoimmune disease due to defective Foxp3 expression and T regulatory (Treg) cell specification and function (34). Similarly, retroviral expression of constitutively active myristoylated Akt (myrAkt) in $CD4^{-}CD8^{-}$ thymocytes impairs Treg development *in vivo* following intrathymic transfer. Importantly, the inhibitory effect of myrAkt is on *de novo* but not established Foxp3 expression (39). In contrast, broad expression of myrAkt as a transgene under the control of the CD2 promoter leads to increased regulatory T cell numbers *in vivo* and enhanced suppressive activity (40). Interestingly, conventional $CD4^{+}$ T cells expressing transgenic myrAkt are less responsive to TGFβ suppression and fail to differentiate into the Th17 lineage in response to TGFβ and IL-6 *in vitro* (40).

A proper balance of Akt activity is also required for appropriate $CD8^{+}$ T cell maturation, effector function, and memory development (41). Uzel and colleagues recently published a study on patients with somatic dominant active p110δ (a catalytic subunit of PI3K) expression (42). T cell blasts from these patients have increased phosphorylation of AKT at T308 and S473, a decline in Foxo1, increased S6 activation, and glucose uptake. This hyperactive Akt/mTORC1 axis causes CD8 T cells to proliferate more vigorously, differentiate more readily into effector cells, and undergo cellular senescence. Sustained Akt activity in these patients also impairs development of CD8 memory T cells, which require a metabolic "switch" from glycolysis to fatty acid oxidation (41, 43). Furthermore, defective CD8 responses result in recurrent sinopulmonary infections and chronic viremia due to Epstein-Barr virus (EBV) and/or cytomegalovirus (CMV) infection (42). Cantrell and coworkers published a surprising finding demonstrating distinct roles for PDK1 and Akt in promoting cellular metabolism and effector responses of CD8 T cells, respectively (44). T cells expressing a catalytically inactive p110δ or treated with an Akt inhibitor are defective for Akt T308 phosphorylation. Akt-defective CD8 T cells proliferate normally in response to IL-2 but are unable to express proper lymphoid homing receptors and cytotoxic effector proteins (44). In contrast, conditional deletion of PDK1, the upstream activator of Akt, leads to defective glucose uptake and metabolism, resulting in reduced CD8 T cell proliferation. This indicates that PDK1 promotes proliferation in an Akt-independent manner (44). It remains to be determined whether PDK1 and Akt have distinct roles in cell types in which multiple functions have been attributed to Akt activity.

TEC FAMILY KINASES REGULATE IMMUNE CELL DEVELOPMENT AND FUNCTION

The Tec family of non-receptor tyrosine kinases, including Tec, Btk, Itk/Emt/Tsk, Rlk/Txk, and Bmx/Etk, are differentially expressed in immune cells. Each Tec family member contains an N-terminal PIP_3-binding PH domain except Rlk, which contains a cysteine-string motif that results in Rlk palmitoylation. In general, Tec kinases activate PLCγ to trigger Ca^{2+} and diacylglycerol (DAG) signaling. Mimicking Ca^{2+} and DAG activation with the addition of calcium ionophores and phorbol myristate acetate (PMA) is sufficient to induce many aspects of lymphocyte activation, differentiation, and effector responses *in vitro*. The requirement for Tec kinases in immune functions is apparent from the profound defects observed in human patients carrying mutations in Tec kinases and in mouse models of single and combined Tec kinase deficiencies.

In 1993, Btk was first identified in patients with X-linked agammaglobulinemia (XLA), an inherited immunodeficiency disease characterized by profound hypogammaglobulinemia due to severely decreased B cell numbers (45). XLA patients carry Btk mutations that prevent the maturation of pro-B cells into pre-B cells. Pre-B-cell receptor signaling at the pro-B to pre-B transition requires Btk activation by the Src kinase Lyn (46–48). A Btk mutation database generated from approximately 400 XLA patients indicates that the majority of missense mutations in the Btk PH domain are in the putative PIP_3-binding pocket (49–51). The XLA missense mutants F25S, R28H, T33P, V64F, and V113D dramatically reduce Btk binding to PIP_3 *in vitro* and disrupt Btk activation in B cells (52, 53). A similar mutation in mice, R28C also abolishes Btk binding to PIP_3 and results in murine X-linked immunodeficiency (Xid) disease (53). These findings demonstrate the importance of PI3K-dependent PIP_3 generation for the membrane recruitment and activation of Btk in promoting B cell receptor signaling during maturation and humoral immune responses.

While disruption of PIP_3 association causes hypo-B-cell responses, enhanced PIP_3 association also leads to B cell dysfunction. The Btk E41K mutant significantly increases Btk PH domain affinity for phosphoinositides and results in constitutive membrane localization when expressed ectopically in COS-7 cells (52, 53). Btk E41K expression allows cytokine-independent growth of the pro-B-cell line Y16 (54), demonstrating its gain-of-function activity. However, mice expressing a Btk E41K transgene controlled by the MHC class II locus are more severely B cell-deficient than even Xid mice (55). Lack of IgM^{high} cells in the bone marrow suggest that constitutive Btk E41K activation leads to inappropriate deletion of immature B cells by mimicking strong BCR signals that promote apoptosis of auto-reactive B cells (55). Thus, appropriate levels of Btk activation are critical for developmental progression of B cells, productive B cell activation and differentiation, as well as deletion of auto-reactive cells.

The first patients identified with Itk mutations were initially diagnosed with Hodgkin's lymphoma but subsequently characterized to have an underlying immunodeficiency disease that prevents control of EBV-induced B cell proliferation (56). Itk-deficient patients have decreased T cells (57), which are required to control EBV infection and prevent viral reactivation from latently infected B cells (58). Detailed characterization of Itk-deficient mice reveals multiple requirements for Itk during T cell development, differentiation, and function (59, 60). Like Btk in B cells, Itk participates in proximal antigen receptor signaling and is directly phosphorylated by a Src family kinase, in this case Lck (61). Activated Itk phosphorylates PLCγ1, which induces IP_3-dependent increased intracellular Ca^{2+} levels as well as DAG-mediated signaling (59, 62, 63). Itk is required for efficient $CD4^{+}$ T cell differentiation toward the Th2 and Th17 lineages (59). Itk-deficient mice cannot generate protective Th2 responses in multiple infection models, including *Leishmania major*, *Nippostrongylus brasiliensis*, and *Schistosoma mansoni* (59, 64). Defective Th2 differentiation is accompanied by substantially reduced production of the Th2 cytokines IL-4, IL-5, and IL-13 by Itk-deficient T cells (65, 66). Itk is also required

for optimal production of the Th17 cytokine, IL-17A but not IL-17F (67). The selective requirement for Itk in IL-17A production is mechanistically linked to a requirement for the transcription factor nuclear factor of activated T cells (NFAT) in IL-17A transcription (64, 67, 68). Prolonged Itk activation maintains cytosolic Ca^{2+} levels to promote sustained calcineurin-dependent NFAT nuclear translocation. Itk deficiency or suboptimal TCR signaling restricts autoimmunity by biasing T cell differentiation from the Th17 toward the regulatory T cell lineage (69). In addition, autoimmune organ destruction can be limited by Itk-dependent control of transendothelial migration and tissue infiltration of effector T cells (70). Thus, mechanisms that regulate the magnitude and kinetics of Itk activity in T cells are important for induction of effector functions, specification of appropriate T cell lineages, and control of T cell trafficking.

SOLUBLE ANALOGS OF PIP_3 DIFFERENTIALLY REGULATE PIP_3 EFFECTORS

Some PIP_3-binding PH domains can associate with soluble PIP_3 analogs. These include the cytosolic inositol phosphates Ins(1,3,4,5)P_4 (IP_4), Ins(1,2,3,4,5,6)P_6 (IP_6), and 5-PP-I(1,2,3,4,6)P_5 (IP_7) that are generated inducibly or constitutively by distinct inositol kinases (82). The effect of IP_4, IP_6, and IP_7 binding is distinct for different PH domains and cell types (**Figure 4**).

The inositol kinases IP_3 kinase (Itpk) isoforms A, B, and C, and inositol polyphosphate multikinase (IPMK) can each generate IP_4 by phosphorylating Ins(1,4,5)P_3 (IP_3) at the D-3 hydroxyl group [reviewed in Ref. (87)]. However, mice deficient in the ubiquitously expressed ItpkC or IPMK isoforms or in the neuronally enriched ItpkA isoform have no detectable immune abnormalities. In contrast, ItpkB expression is selectively enriched in hematopoietic cells and catalytically activated by the Ca^{2+}-sensing protein calmodulin (CaM) following antigen receptor signaling. Analysis of ItpkB-deficient mice revealed a non-redundant requirement for ItpkB in lymphocyte development and activation (88–92). ItpkB deficiency results in severely reduced peripheral T cell numbers due to an absolute block in positive selection of $CD4^+CD8^+$ thymocytes (88). Defective activation of the Ras/MAP kinase pathway contributes to the T cell developmental defect (88, 89, 93). However, ItpkB-deficient $CD4^+CD8^+$ thymocytes are also defective in activation of Itk and its downstream effector PLC γ1 in response to TCR engagement (93). Itk fails to localize to the plasma membrane or assemble with the adapter protein LAT in the TCR signalosome of ItpkB-deficient thymocytes, indicating a requirement for IP_4 in promoting Itk interactions (93). Interestingly, addition of IP_4 increases binding of recombinant Itk PH domain to PIP_3-coated beads *in vitro*, suggesting that IP_4 may alter Itk PH domain conformation to enhance PIP_3 accessibility (93).

Distinct from its effect on Itk, IP_4 suppresses Akt activity by directly competing with PIP_3 for binding to the Akt PH domain (94). ItpkB-deficient mice develop profound alterations in neutrophil and NK cell functions due to enhanced Akt activity during their development and activation (94, 95). Addition of membrane permeable IP_4, but not an isomer, to the myeloid cell line HL-60 disrupts membrane localization of an Akt PH domain fused to GFP (94). In ItpkB-deficient neutrophils, Akt phosphorylation is enhanced in response to the bacterial peptide Formyl–Methionyl–Leucyl–Phenylalanine (fMLP). Enhanced Akt signaling in ItpkB-deficient neutrophils contributes to augmented anti-microbial and chemotaxis responses (94). The magnitude and kinetics of Akt phosphorylation are also increased in ItpkB-deficient NK cells

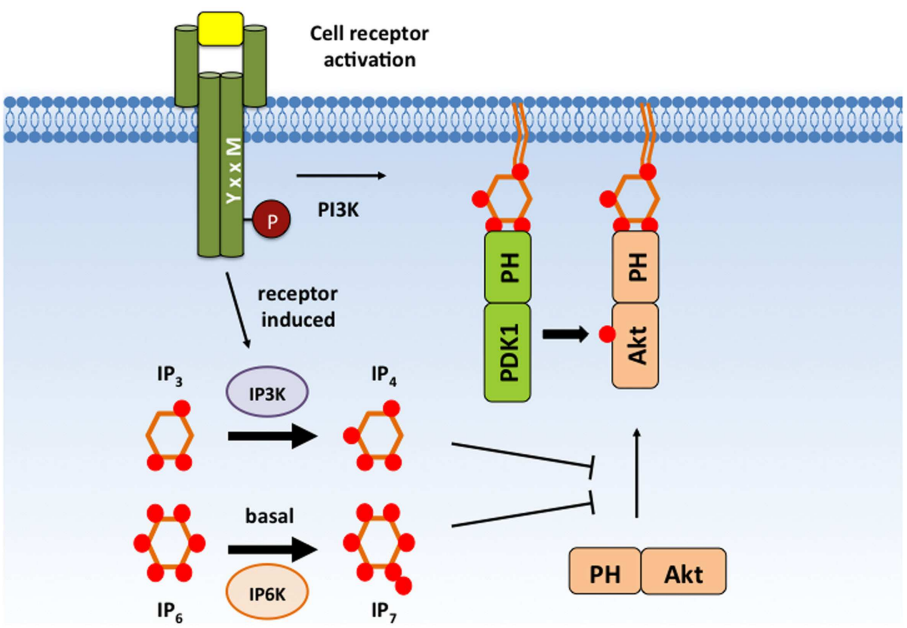

FIGURE 4 | IP_4 and IP_7 negatively regulate Akt signaling. IP_4 and IP_7 are cytosolic PIP_3 analogs that are able to associate with the Akt PH domain with high affinity and can compete with membrane PIP_3. IP_4 and IP_7 binding has been proposed to dissociate Akt from the plasma membrane to prevent Akt activation and substrate accessibility. IP_4, Ins(1,3,4,5)P_4; IP_7, 5-PP-(1,2,3,4,6)IP_5; PIP_3, PI(3,4,5)P_3.

(95). Elevated IFNγ secretion, granule exocytosis, and tumor cell lysis by ItpkB-deficient NK cells can be suppressed by Akt inhibition (95). Together, these studies indicate that IP_4 dampens Akt activity in neutrophils and NK cells to restrict effector functions. Whether this occurs to shut-off innate functions during the resolution phase of an immune response or as a check to limit inflammatory damage remains unclear.

Similar to IP_4, IP_7 also competes with PIP_3 for binding to the Akt PH domain and negatively regulates its activity (96). IP_7 is generated by pyro-phosphorylation of IP_6 at the 5-phosphate group by IP_6 family kinases, IP6Ks (97, 98). While the importance of IP6K1 in lymphocyte function remains to be determined, analysis of IP6K1-deficient neutrophils demonstrates similar functional defects as ItpkB-deficient neutrophils. Both deficiencies result in enhanced fMLP-induced chemotaxis, superoxide production, and bacterial killing (94, 99). Akt membrane localization and activation are significantly increased in IP6K1-deficient neutrophils (99). Interestingly, IP_7 is readily detectable in resting HL-60 cells but rapidly decreases upon fMLP stimulation (99). This suggests that IP_7 may act to suppress initial Akt activation while IP_4 regulates subsequent Akt activity following its induced production. Precise regulation of basal and induced IP_4 and IP_7 levels may act together to control the magnitude and kinetics of Akt activation in these innate immune cells. Future studies are required to determine the functional effects of IP_4 and IP_7 on Akt-dependent regulation of lymphocyte differentiation and effector responses. It also remains to be determined whether IP_7 acts on other PIP_3 effectors in immune cells as it does in *Dictyostelium discoideum* (100) or whether selective IP_7 binding allows regulation of a particular subset of PIP_3 effectors.

Recently, biochemical and structural analyses of Btk identified a new activating function for the inositol phosphate, IP_6 (101). As with PIP_3-containing liposomes, addition of soluble IP_6 induces Btk trans-phosphorylation and activation. However, IP_6 promotes Btk activation by an unconventional mechanism that is independent of the PIP_3-binding pocket and membrane recruitment. Analysis of the co-crystal structure of IP_6 with the Btk PH domain reveals an additional peripheral IP_6 binding site sandwiched between two PH modules, termed the Saraste dimer. Molecular dynamics simulations suggest that IP_6 neutralizes electrostatic forces in the monomer that oppose dimer formation. Mutation of the IP_6 peripheral binding site disrupts transient dimerization and significantly abrogates IP_6-dependent Btk trans-phosphorylation (101). IP_6-induced Btk activation in solution represents a new PI3K-independent mechanism for controlling Btk activity. Considering that IP_6 levels are basally high in lymphocytes, it will be important in future studies to determine whether IP_6 contributes to tonic or B cell receptor-induced Btk function.

PROTEINS INTERACT WITH AND REGULATE THE ACTIVITY OF PH DOMAIN-CONTAINING PROTEINS

Although the Akt and Itk PH domains specifically bind to PIP_3 with (nanomolar) affinities, only ~40 mammalian PH domains appear to be PIP_3-regulated according to Teruel and colleagues, who developed a prediction algorithm based on experimental analyses of 130 mouse PH domains (102). The majority of PH domains do not interact with lipids or bind lipids promiscuously or with low affinity ($K_d \geq 10\,\mu M$). Furthermore, a growing number of PH domains have been reported to participate in inter- and/or intra-molecular protein interactions (discussed below). These findings support a revised view of PH domains as diverse, multifunctional domains that bind lipids, proteins, or both to regulate the activity of their parent proteins.

T and B cells induce Ca^{2+} and DAG-mediated signaling through PLCγ1- and PLCγ2-mediated cleavage of PIP_2 (103, 104). T cell-specific ablation of PLCγ1 causes defects in thymocyte selection during T cell development, reduced T cell proliferation and cytokine secretion, and the development of autoimmunity resulting from defective regulatory T cells (104). PLCγ2 plays important roles in regulating B cells, neutrophils, mast cells, and dendritic cells (105–107). PLCγ1 and PLCγ2 both contain two PH domains. The conventional, N-terminal PH domain associates with PIP_3 (108); however, the C-terminal PH domain is interrupted by an intervening amino acid sequence comprising two tandem SH2 domains and an SH3 domain (109, 110). This split PH domain is also critical for substrate binding (111). The C-terminal half of the PLCγ1 split PH domain associates with a partial PH domain in TRPC3 (112, 113), a Ca^{2+} channel that can mediate Ca^{2+} entry in T cells. The formation of this inter-molecular PH-like domain allows PLCγ1 to bind to its substrate PIP_2 and is critical for TRPC3 membrane targeting and surface expression (113). Conversely, the split PH domain of PLCγ2 interacts with the small GTPase Rac2, which mediates PLCγ2 activation and localization to the plasma membrane (114–116).

Pleckstrin homology domains also participate in intra-molecular interactions. In resting cells, the Akt PH domain associates with the kinase domain (KD) to maintain a closed conformation in which the activation loop is blocked (117, 118). PIP_3 binding to the Akt PH domain exposes the activation loop, allowing T308 and S473 to be accessed and phosphorylated by PDK1 and mTORC2, respectively (119). Phosphorylation of T308 and S473 fully activates Akt and keeps the activation loop "open" for substrate docking (117–119). PH domain mutations that disrupt PH–KD interaction (e.g., L52R and Q79K) result in constitutive Akt activation (119).

The Dbl family RhoGEF Vav is also regulated by lipid and intra-molecular interactions involving its PH domain (**Figure 5**). Vav plays crucial roles during T cell and B cell development (120, 121) and T cell, B cell, neutrophil, and NK cell activation (9, 107, 120–123). Vav contains a Dbl homology (DH) domain that promotes the activation of the small GTPase Rac in response to PI3K activation (124, 125). In quiescent cells, Vav1 adopts an auto-inhibitory conformation, which is stabilized by interactions between its PH, acidic (Ac), and calponin homology (CH) domains (126, 127). A truncation mutation of the Vav N-terminal CH domain was shown to have oncogenic potential (128), highlighting the importance of these intra-molecular interactions in limiting Vav activity. During T cell activation, Lck phosphorylates tyrosine residues within the Ac domain to release Vav1 from auto-inhibition (127). PIP_3 binding to the PH domain significantly enhances Lck-dependent Vav1 phosphorylation *in vitro* (129) and promotes GEF activity (124, 129, 130) likely through the release of auto-inhibition (131). Interestingly, PIP_2 binding to the Vav1 PH domain inhibits GEF

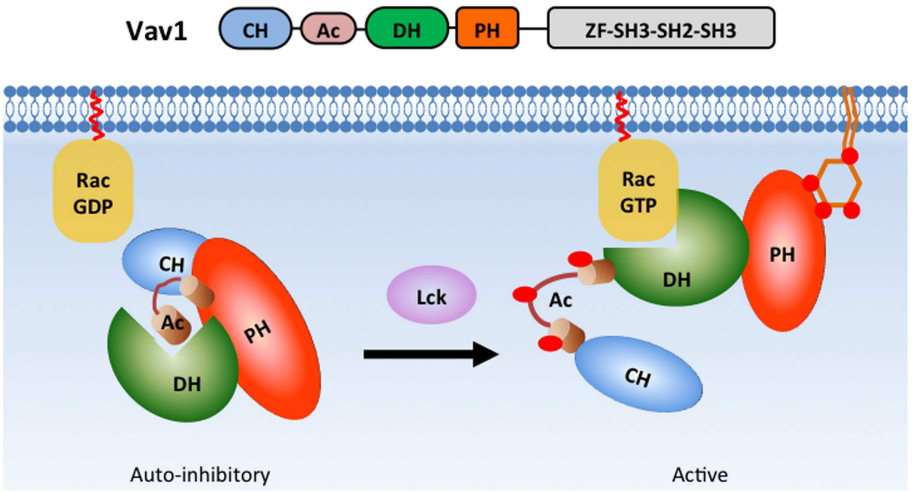

FIGURE 5 | PH domain interactions stabilize Vav1 auto-inhibition in basal state. In the basal state, Vav1 adopts an auto-inhibitory conformation in which the substrate-docking site within the DH domain is blocked by interactions with a helix region from the Ac domain. The interactions between CH, PH, and Ac domains greatly strengthen the auto-inhibitory conformation (left). During T cell activation, phosphorylation of the Ac domain by Lck releases the substrate-docking site and allows GTPase binding (right).

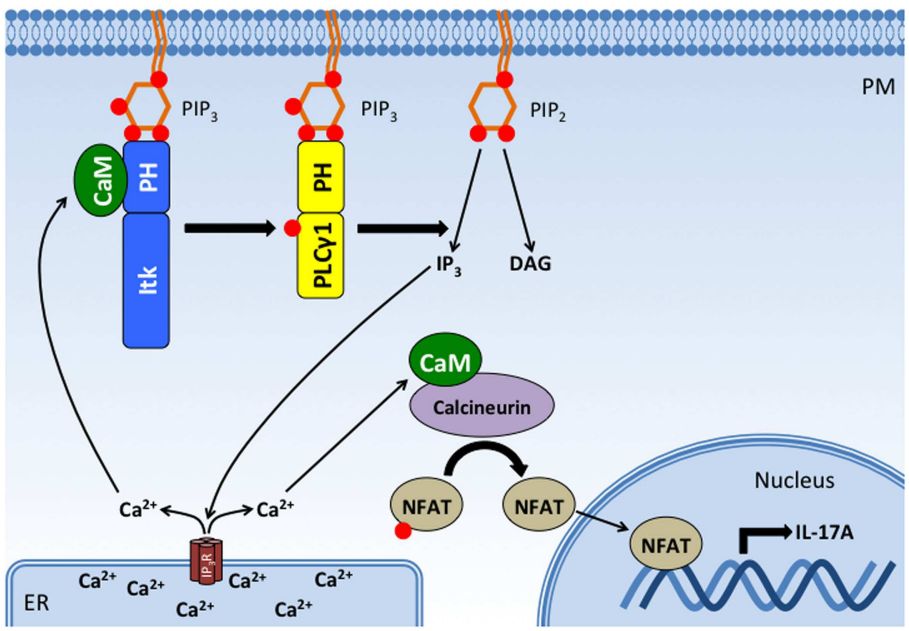

FIGURE 6 | CaM binds the Itk PH domain in a positive feedback loop that potentiates Itk activity, intracellular Ca^{2+} release, and IL-17A production. Binding of Itk to PIP_3 promotes Itk activation and the subsequent phosphorylation and activation of PLC γ1. PLCγ1 cleaves PIP_2 to produce DAG and IP_3, which binds IP_3 receptors on the ER. The IP_3 receptor is a ligand-gated Ca^{2+} channel, and its activation increases Ca^{2+} levels in the cytosol. Increased cytosolic Ca^{2+} activates CaM, which has at least two effects on T cell activation: (1) Ca^{2+}/CaM binds to Itk's PH domain, enhancing its interaction with PIP_3 and Itk activity. (2) Ca^{2+}/CaM binds to and activates calcineurin, a phophatase that dephosphorylates NFAT, allowing NFAT translocation to the nucleus where it drives the transcription of IL-17A. Thus, CaM binding to Itk's PH domain completes a positive feedback loop that potentiates the downstream effects of Itk. PM, plasma membrane; ER, endoplasmic reticulum; Itk, IL-2-inducible tyrosine/T cell kinase; PLCγ1, phospholipase C gamma 1; CaM, calmodulin; NFAT, nuclear factor of activated T cells; IP_3R, IP_3 receptor.

activity (129). Thus, distinct lipids bind to the Vav1 PH domain to promote conformational changes that either reinforce or relieve its auto-inhibitory state.

Pleckstrin homology domains can also participate in intermolecular interactions with other proteins. The PH domain of Dbs, a Cdc42/RhoGEF, associates with Cdc42 through the β3/β4

loop of its PH domain to improve substrate docking and catalysis (132). Interestingly, we recently identified the β3/β4 loop of the Itk PH domain as an important binding site for the ubiquitous Ca^{2+}-sensing protein CaM (133). The CaM C-terminal EF hands bind to the β3/β4 loop of the Itk PH domain at basal intracellular Ca^{2+} levels while the CaM N-terminal EF hands engage the β5/β6 loop upon an increase in Ca^{2+} levels. CaM and PIP_3 (but not IP_4) reciprocally enhance binding of one another to the Itk PH domain *in vitro*, suggesting that CaM and PIP_3 cooperate to regulate Itk signaling at the plasma membrane. Pharmacological inhibition of Ca^{2+}/CaM activity or mutation of the CaM-binding β3/β4 loop disrupts Itk-dependent activation of PLCγ1 and downstream Ca^{2+} responses (133), indicating that CaM participates in a positive feedback loop whereby binding of CaM to the Itk PH domain enhances further Itk activation and downstream Ca^{2+} responses. Importantly, this positive feedback is required for optimal TCR-induced, NFAT-dependent production of the pro-inflammatory cytokine, IL-17A (133). Thus, CaM represents a novel protein-binding partner for the Itk PH domain that serves an important function in potentiating T cell pro-inflammatory responses (**Figure 6**). It remains to be determined how CaM, PIP_3, and IP_4 coordinate to regulate the kinetics and magnitude of Itk activation and whether they differentially participate in Itk-dependent T cell activation, differentiation, and effector responses.

Calmodulin has also been reported by Dong and colleagues to bind the PH domain of Akt family kinases (134). Using short peptide fragments of Akt1 in a pulldown assay, this interaction was further mapped to the first 42 residues of the Akt1 PH domain. Although CaM did not directly alter Akt kinase activity, CaM was reported to reduce the ability of PIP_3 to co-precipitate Akt (134), suggesting that CaM competes with PIP_3 to dampen Akt activity. However, this finding is inconsistent with other published data demonstrating a requirement for CaM in optimal Akt phosphorylation at T308 and S473 (135, 136). Thus, further investigation is warranted to clarify the functional significance of CaM binding to the AKT PH domain and to determine the precise role of this interaction in lymphocytes.

CONCLUSION

The studies discussed herein highlight the essential yet complex functions of PH domain-containing proteins in lymphocytes and other immune cells. It is well established that a subset of PH domains modulate the function of their parent proteins by binding to membrane-bound lipids as well as soluble lipid analogs. Furthermore, proteins regulated in this manner, such as the PI3K effector kinases Akt and Itk, are indispensable for immune cell function. Indeed, mutations that disrupt the lipid-binding capacity of PH domains are known to result in human disease, a phenomenon perhaps best demonstrated by the immunologic defects associated with mutations in Tec family kinases. Analogous and unique pathological processes observed in animal models and *in vitro* experiments reinforce the critical role of PH domain-containing proteins in the immune system. However, evidence increasingly shows that PH domains also interact with non-lipid substrates, and these interactions can be cooperative, antagonistic, or completely independent of lipid-binding capacity. The breadth of these interactions must be elucidated in order to fully understand role of PH domain-containing proteins in immune cell function. Thus, future work should investigate the capacity of PH domains to interact with multiple substrates, including both lipids and proteins, and should include careful evaluation of how binding of each substrate affects the binding of others.

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Role of inositol poly-phosphatases and their targets in T cell biology

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T lymphocytes play a critical role in host defense in all anatomical sites including mucosal surfaces. This not only includes the effector arm of the immune system, but also regulation of immune responses in order to prevent autoimmunity. Genetic targeting of PI3K isoforms suggests that generation of PI(3,4,5)P_3 by PI3K plays a critical role in promoting effector T cell responses. Consequently, the 5′- and 3′-inositol poly-phosphatases SHIP1, SHIP2, and phosphatase and tensin homolog capable of targeting PI(3,4,5)P_3 are potential genetic determinants of T cell effector functions *in vivo*. In addition, the 5′-inositol poly-phosphatases SHIP1 and 2 can shunt PI(3,4,5)P_3 to the rare but potent signaling phosphoinositide species PI(3,4)P_2 and thus these SHIP1/2, and the INPP4A/B enzymes that deplete PI(3,4)P_2 may have precise roles in T cell biology to amplify or inhibit effectors of PI3K signaling that are selectively recruited to and activated by PI(3,4)P_2. Here we summarize recent genetic and chemical evidence that indicates the inositol poly-phosphatases have important roles in both the effector and regulatory functions of the T cell compartment. In addition, we will discuss future genetic studies that might be undertaken to further elaborate the role of these enzymes in T cell biology as well as potential pharmaceutical manipulation of these enzymes for therapeutic purposes in disease settings where T cell function is a key *in vivo* target.

Keywords: SHIP1, SHIP2, T cells, T lymphocytes, adoptive T cell transfer, INPP4, PTEN, PI3K

INTRODUCTION

Inositol phospholipid signaling pathway plays an integral role in development, proliferation, differentiation, and survival of lymphocytes (1–4). The principal second messenger of the PI3K pathway PtdIns(3,4,5)P_3 is generated by phosphorylation of the 3′-hydroxyl group of PtdIns(4,5)P_2 by PI3Ks. PI3Ks are grouped into three categories, Class I, II, and III on the basis of substrate specificity and structure. Only class I PI3Ks can use PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3 at the inner leaflet of plasma membrane (5). PtdIns(3,4,5)P_3 acts as binding site for several intracellular signaling molecules that containing a Pleckstrin-homology domain (PH-domain) and thus facilitates their recruitment to the plasma membrane. AKT/PKB is the most important PH-domain containing kinase required for cell growth, survival, and proliferation in most cell types and appropriately its PH-domain can bind PtdIns(3,4,5)P_3 (6, 7). In addition to AKT, the PH-domain containing Tec family tyrosine kinases ITK (IL-2-inducible T cell kinase) and BTK (Bruton agammaglobulinemia tyrosine kinase) also have specificity for PtdIns(3,4,5)P_3 and are important mediators of PI3K signaling pathway in T and B cells, respectively (**Figure 1**). Based on genetic models both class IA (p110α, p110β, and p110δ) and class IB PI3K (p110γ) play roles in thymocyte development. p110γ-knockout mice have increased apoptosis of DP thymocytes and double knockout p110δ/γ mice have significantly reduced number of thymocytes, a profound T cell lymphopenia and multiple organ inflammation (8–12). In addition to that mice with a knock-in point mutation of p110δ (p110deltaD910A/D910A) have severe defects in T cell receptor signaling and impaired Treg cell function (13–15). The T cell specific class IA PI3K deficient mice do not have defects in thymocyte and in peripheral T cell development, but they do exhibit defective TCR signaling, *in vitro* proliferation and cytokine production (16, 17). Altogether these findings demonstrate that the PI3K signaling pathway responsible for generation of PtdIns(3,4,5)P_3 plays an important role in T cell development and activation and suggest that inositol poly-phosphatases like phosphatase and tensin homolog (PTEN), SHIP1, SHIP2, and INPP4A/B may have an opposing, or in some cases, a facilitating role downstream of PI3K in T lymphocytes.

The cellular pool of inositol phospholipids is determined in part by inositol phosphatases that by dephosphorylation of PtdIns(3,4,5)P_3, can regulate PI3K-mediated signaling pathway. Three important phosphatases, which dephosphorylate PtdIns(3,4,5)P_3 are PTEN, SHIP1, and SHIP2. PTEN is 3′ polyphosphatase that converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 while the SHIP family phosphatases, SHIP1 and SHIP2, are 5′ polyphosphatases, which convert PtdIns(3,4,5)P_3 to PtdIns(3,4,)P_2 (18, 19). The importance of these phosphatases in immune cell signaling was revealed by the demonstration that SHIP1 deficiency leads to severe myeloproliferative disorder and impaired NK cell

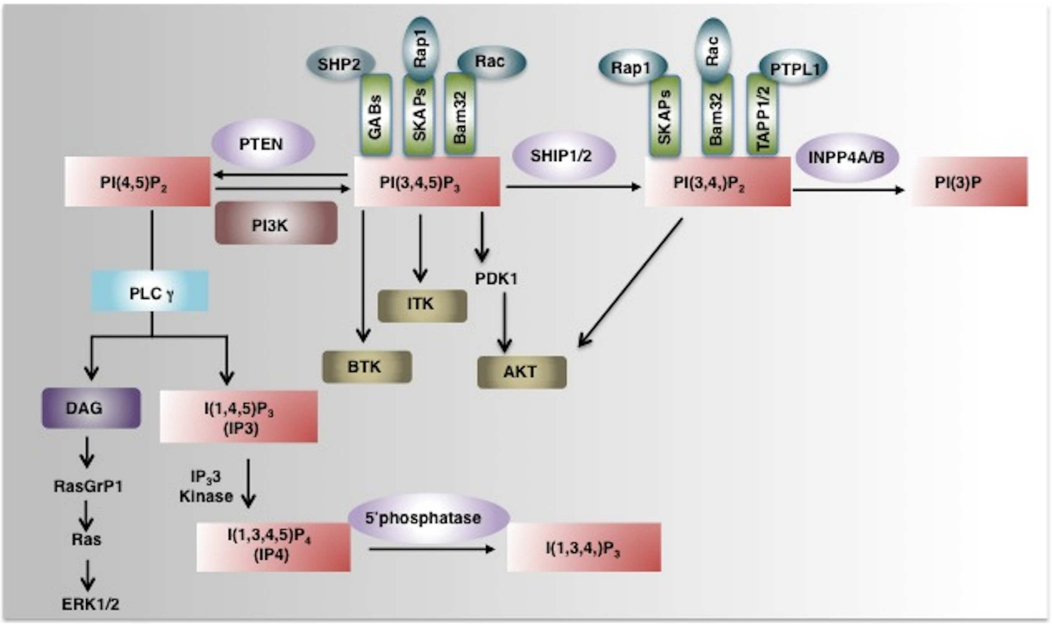

FIGURE 1 | Phosphoinositide signaling and its regulation by phosphatases. PI3K converts PI(4,5)P_2 to a key secondary messenger PI(3,4,5)P_3. Phosphatases like PTEN and SHIP1/2 regulate cellular levels of PI(3,4,5)P_3 by hydrolyzing it to PI(4,5)P_2 and PI (3,4)P_2 respectively. PLCγ converts PI(4,5)P_2 to IP_3 and DAG. IP_3 a soluble inositol phosphate is required for Ca^{2+} mobilization while DAG can activate the Ras-Raf-ERK1/2 pathway. IP_3 3-Kinases convert IP_3 to IP_4, another important soluble inositol poly-phosphate that either positively or negatively regulates the binding of PI(3,4,5)P_3 to PH-domain containing proteins. The SHIP1/2 product PI(3,4)P2 is hydrolyzed by INPP4A/B into PI(3)P by removal of the phosphate at the 4-position of the inositol ring. PI(3,4,5)P_3 and/or PI(3,4)P_2 enable recruitment to the plasma membrane of several PH-domain containing proteins including PDK1, AKT, BTK, ITK, and thus regulate pivotal cellular processes including activation, proliferation, and survival. PH-domain containing adaptor proteins (GABs, SKAPs, Bam32, and TAPP) can also bind to phosphoinositides and regulate cell signaling (indicated as green boxes). AKT, Protein Kinase B; PDK1, phosphoinositide-dependent kinase-1, PLCγ phospholipase Cγ; ITK, IL-2-inducible T cell kinase) and BTK, Bruton agammaglobulinemia tyrosine kinase.

function while mice with a conditional deletion of PTEN have impaired T cell immune responses (20–22). The present review focuses on the role of these inositol phosphatases in T cell biology.

SHIP1 IN T CELL BIOLOGY

SHIP1 (Src homology 2-containing inositol phosphatase) is a 5′-inositol poly-phosphatase that removes the 5′ phosphate from PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4, thereby regulating PI3K signaling pathway (23). SHIP1 is expressed in hematopoietic cells, mesenchymal stem cells, and osteoblasts (24) as a 145/150 kDa doublet. SHIP1 contains an N-terminal SH2 domain, a central phosphatase domain, a C-terminal NPXY motifs, a Serine residue that can be phosphorylated by PKA, proline rich sequences as well as domains adjacent to the phosphatase domain that can recognize either its substrate or its product (23). The SH2 domain mediates binding of SHIP1 to ITAM and ITIM motifs in receptor tails such as the CD3 chains that associate with the TCR (25, 26) or with various adaptor proteins (27, 28). By virtue of its enzymatic as well as its non-enzymatic functions, SHIP1 is implicated in various signaling pathways related to proliferation, apoptosis, cytokine signaling in lymphocytes and myeloid cells (23). Germline SHIP1$^{-/-}$ mice although viable after weaning develop profound infiltration of myeloid cells in the lungs and severe inflammation in the terminal ileum of the gut resembling human Crohn's disease (29) which leads to their early demise (20, 30). SHIP1$^{-/-}$ mice have increased number of myeloid cells in most of tissues, but are lymphopenic (20) and have a profound deficit of T cells in the gut (29) indicating diverse functions for SHIP1 signaling in myeloid cells and T lymphocytes.

SHIP1 IN T CELL SIGNALING

First demonstration of involvement SHIP1 in T cells came from the observation that ligation of CD3 or CD28 on T cells results in SHIP1 tyrosine phosphorylation and membrane re-localization (31). SHIP1 is thought to be a component of a signaling complex that includes LAT (linker for activation of T cells), Grb2, Dok (downstream of tyrosine kinase) 1, and Dok2 that negatively regulate TCR signaling (32). SHIP1 functions as an adaptor that is required for tyrosine phosphorylation of Dok1 and Dok2 and thus enables Dok1/2 anchoring to LAT to negatively regulate the Zap-70 and AKT kinases thus attenuating TCR signaling (32). Consistent with the proposed negative regulation of TCR signaling, SHIP1 together with adaptor Dok1 and Dok2 has also been shown to be associated with the CD4-mediated inhibitory signaling (33). SHIP1 can also negatively regulate activation and membrane localization of Tec Kinase, which plays an essential role in PLCγ activation upon TCR stimulation (34, 35). However, despite these biochemical studies suggesting SHIP1 limits TCR signaling splenic T cells isolated from germline SHIP1$^{-/-}$ mice have defective TCR signaling as shown by their poor proliferation in response

to TCR stimulation. In addition, SHIP1$^{-/-}$ T cells fail to induce IL-2 and IFNγ upon PMA/ionomycin stimulation although they have elevated levels of CD69 and CD25 and dramatically reduced expression CD62L and CD45RB expression (36). However a T cell-restrictive deletion of SHIP1 (CD4CreSHIP$^{\Delta IPflox}$) that deletes SHIP1 at double positive thymocyte stage does not exhibit the same T cell phenotype observed in the germline SHIP1$^{-/-}$ mice (37). SHIP1 deleted T cells in these mice do not regulate TCR signal strength and no difference in the phosphorylation status of AKT, ERK, Zap-70, PLCγ, or calcium influx was observed between SHIP1$^{-/-}$ and WT T cells. Also, in contrast to the poor proliferation of T cells from germline deficient mice, T cells from CD4CreSHIP$^{\Delta IPflox}$ mice proliferate normally in response to TCR stimulation. The authors argued that the observed phenotype of T cells in germline SHIP1$^{-/-}$ mice is due to pleiotropic effect of dysregulated immune system as a consequence of SHIP1-deficient environment (37, 38). However, we and others have found that the in-frame deletion strategy utilized still allows substantial expression of a near full-length version of SHIP1 that only lacks the enzyme domain (39). Because of SHIP1's ability to function in cell signaling by masking binding sites on receptor tails for other regulatory kinases and phosphatases (40, 41) confounds interpretation of results from SHIP$^{\Delta IPflox}$ strain difficult.

SHIP1 IN T CELL DEVELOPMENT

SHIP1 alone does not affect T cell development as no deficiencies in the development of T cells in the thymus was observed in either germline SHIP1$^{-/-}$ deficient mice or in CD4CreSHIP$^{\Delta IPflox}$mice (20, 36, 37). However, a double knockout of SHIP1 and adaptor protein Dok1 plays an important role in T cell development since mice with combined deficiency of SHIP1 and Dok1 have significantly reduced total thymocyte numbers, percentage of CD4^{+} CD8^{+} double positive T cells and increased CD4^{-}CD8^{-} double negative T cells (36).

SHIP1 has been shown to be required for both CD4^{+} and CD8^{+} T cell survival homeostasis at mucosal sites (29, 42). SHIP1$^{-/-}$ mice develop spontaneous intestinal inflammation, the disease is highly demarcated and confined to the terminal ileum, which resembles classical human Crohn's disease (29). The disease is characterized by severe reduction in CD4^{+} and CD8^{+} T cells in the lamina propria of SHIP1$^{-/-}$ mice suggesting that SHIP1 is required for effector T cell persistence in the small intestine. Because T cells play an important role in normal immune surveillance to both commensal microorganism and pathogens, in their absence SHIP1-deficient Neutrophils and other myeloid cells over-respond resulting in lethal inflammation in SHIP1$^{-/-}$ mice (29). The mechanism of selective loss of T cells in mucosal tissues is currently under investigation. T cell-restrictive SHIP1-deficient CD4CreSHIP$^{\Delta IPflox}$ reported by Tarasenko et al. have apparently no defect in T cell activation or T cell numbers in periphery; however, mucosal T cells were not examined in their report (37). Interestingly T cells from CD4CreSHIP$^{\Delta IPflox}$ mice show biased toward Th1 skewing and have defective production of Th2 cytokines IL-4, IL-5, and IL-13. Consistent with this T cells from CD4CreSHIP$^{\Delta IPflox}$ mice respond poorly to *in vivo* challenge to *Schistosoma mansoni* eggs, which normally induce a Th2 response. These cells also express elevated levels of T-bet which has been shown to regulate CD8 T cell function. Consistent with that CD8 T cells from CD4CreSHIP$^{\Delta IPflox}$ mice that also delete SHIP1 in CD8 T cells were more efficient in a cytotoxicity assay as compared to WT controls (37).

REGULATION OF Tregs AND TH17 CELLS BY SHIP1

SHIP1 has been shown to limit expansion both myeloid and T lymphoid immune-regulatory cell (30, 36, 43–46). Peripheral T cells from SHIP1$^{-/-}$ mice have significantly increased numbers of CD4^{+}CD25^{+}FoxP3^{+} conventional regulatory T cells (36, 45). They exhibit significantly higher levels of CD103, GITR, OX40, and FcRγII/III, which is associated with their regulatory function (45). SHIP1$^{-/-}$ regulatory T cells are equally suppressive both *in vitro* and *in vivo* when compared to SHIP1-competent T regulatory cells (45). In addition to conventional regulatory T cells, SHIP1 deficiency also promotes the accumulation of CD4^{+}CD25^{-} iTreg cells that express FoxP3 in the periphery that have suppressive function (45). Although SHIP1 deficiency seems to promote regulatory T cell expansion, the inflammatory environment brought about by SHIP1-deficient myeloid cells may also play a role in Treg cell development. An elegant study by Collazo et al. demonstrated that SHIP1 regulates Treg cell development and iTreg formation in both a T cell intrinsic and extrinsic manner. Both T cell specific deletion of SHIP1 in LckCreSHIP$^{flox/flox}$ or myeloid cell-specific deletion in LysCreSHIP$^{flox/flox}$ mice increased the peripheral pool of CD4^{+}CD25^{+}FoxP3^{+} regulatory T cells and CD4^{+}CD25^{-} iTreg cells expressing FoxP3 (44). These results indicate that SHIP1 exerts both T cell intrinsic and extrinsic control over peripheral Treg cell development and conversion in the periphery. In contrast to this, Tarasenko et al. in the CD4CreSHIP$^{\Delta IPflox}$ model reported that SHIP1 deletion had no effect on Treg cell development. However, the concern noted above regarding residual expression of a near full-length SHIP1 mutant in SHIP$^{\Delta IPflox}$ mice hampers interpretation of this negative finding. Locke et al. also reported T cell-intrinsic function of SHIP1 in iTreg development. They showed that the ability of SHIP1$^{-/-}$ CD4^{+}CD25^{-}CD45RB^{high} T cells to develop into Foxp3^{+} cells *in vitro* in presence of TGFβ alone or in combination with retinoic acid (RA) was much higher compared to WT T cells (47). Interestingly, FoxP3 can enhance the expression of miR-155 by binding to an intron within the DNA sequence of the miR-155 precursor RNA suggesting that FoxP3 could potentially maintain Treg numbers by suppressing SHIP1 expression through induction of miR-155 (48–50). Altogether the above studies suggest a potent role for SHIP1 in T cell-intrinsic control of native Treg development and iTreg formation in the periphery. In contrast to SHIP1's function in limiting Treg numbers, it has been shown to required for Th17 development. SHIP1$^{-/-}$ T cells fail to differentiate into Th17 cells and this deficiency was accompanied by reduced IL-6 mediated phosphorylation STAT3 (47). SHIP1$^{-/-}$ T cells have high basal level of T-bet, a transcription factor known to negatively regulate Th17 differentiation and lower levels of RORγt mRNA, and thus it is likely that the altered T cell differentiation are regulated by SHIP1 via its control of these transcription factors at the molecular level. **Table 1** summarizes the function SHIP1 in T cells in different genetic mouse models.

Table 1 | T cell phenotypes of inositol poly-phosphatase mutant mice.

No.	KO	Gene deletion	T cell phenotype	Reference
1	SHIP$^{-/-}$	Germline SHIP1 deletion	Poor proliferation of T cells Elevated levels of CD69, CD25 on T cells, and reduced levels of CD69L, CD45RB Increased CD4^{+}CD25^{+}FoxP3^{+} Tregs Increased CD4^{+}CD25^{-}FoxP3^{+} iTregs Reduced CD4^{+} and CD8^{+} T cells in the gut	Helgason et al. (20), Kerr et al. (29), Kashiwada et al. (36), Collazo et al.(45)
2	SHIP$^{-/-}$DOK1$^{-/-}$	Germline SHIP1 and DOK1 deletion	Reduced thymocytes Reduced CD+CD8+ T cells in thymus Reduced CD8^{+} T cells in the spleen Altered CD4:CD8 ratio Increased CD4^{+}CD25^{+}FoxP3^{+} Tregs	Kashiwada et al. (36)
3	CD4CreSHIP$^{f/f}$	SHIP1 deletion in T cells	Normal T cell development No defect in T cell activation Reduced levels of TH2 cytokines IL-4, IL-5, and IL-13 CD8^{+} T cells are more cytotoxic	Tarasenko et al. (37)
4	LckCreSHIP$^{f/f}$	SHIP1 deletion in T cells	Increased CD4^{+}CD25^{+}FoxP3^{+} Tregs Increased CD4^{+}CD25^{-}FoxP3^{+} iTregs	Collazo et al. (44)
5	LysCreSHIP$^{f/f}$	SHIP1 deletion in myeloid cells	Increased CD4^{+}CD25^{+}FoxP3^{+} Tregs Increased CD4^{+}CD25^{-}FoxP3^{+} iTregs	Collazo et al. (44)
6	PTEN$^{-/+}$	Heterozygous deletion of PTEN	Increased proliferation of T cells Reduced AICD	Di Cristofano et al. (51)
7	LckCre-PTEN$^{flox/-}$	PTEN deletion in T cells/heterozygous deletion of PTEN other tissues	CD4^{+} T cell lymphoma Defect in thymic negative selection Increased TH1/TH2 cytokines T cells are resistant to apoptosis	Suzuki et al. (22)
8	LckCre-PTEN$^{f/f}$	PTEN deletion in T cells	T cells are hyper-responsive to TCR stimulation Refracted to anergy induction Reduced expansion of Tregs Increased TH2 cytokine	Hagenbeek et al.(52), Walsh et al.(53)
9	OX40^{cre}PTEN^{flox}	PTEN deletion in mature CD4^{+}T cells	T cells are hyper-proliferative Secrete more cytokine T cells are super-helper with enhanced inflammatory antibacterial and anti-tumor responses	Soond et al. (54)

SHIP1 IN T CELL MIGRATION

PI3K-associated pathways have been implicated in regulation of chemokine signaling and migration of cell toward chemokine gradient (55, 56). In a polarized plasma membrane PI3K accumulates at the leading edge of the migratory cells leading to localized production of PI(3,4,5)P_3 and thereby regulating cell migration. Because SHIP1 regulates levels of PI(3,4,5)P_3 at PI3K signaling complexes it stands to reason that it may then regulate chemotaxis. Nishio et al. demonstrated that SHIP1-deficient neutrophils fail to polarize PI(3,4,5)P_3 at the leading edge of migrating cells resulting in the inefficient migration of neutrophils and reduced polarity in response to chemoattractants (57). In T cells enhanced chemotaxis in response to stromal cell-derived factor-1 (SDF1) has been reported with SHIP1$^{-/-}$ thymocytes and splenic CD4^{+} T cells (58). Consistent with this enforced overexpression of SHIP1 in Jurkat T cells abrogated CXCL12 mediated chemotaxis by this cell line (59). However it was also shown that the chemotaxis of SHIP1$^{-/-}$ lymphocytes with other chemokines was comparable with that of WT lymphocytes indicating that SHIP1 involvement in regulating chemotaxis may be chemokine specific (58). More recently Harris et al. by using a lentivirally expressed SHIP1-specific shRNA in human CD4^{+} T cells showed that although the directional chemotaxis toward CXCL11 was unaffected, the overall basic motility and morphology of T cells was impaired in SHIP1 knockdown (KD) primary human T cells (60). SHIP1 KD T cells exhibited increased actin polymerization and loss of microvilli projection upon stimulation with CXCL11. Formation of microvilli involves phosphorylation of ezrin/radixin/moesin (ERM) proteins and once the cell is activated microvilli are frequently lost due to Rac-mediated dephosphorylation of ERM

proteins (61). SHIP1 seems to negatively regulate Rac activation and/or ERM phosphorylation through a non-catalytic function as pretreatment with the PI3K inhibitor Ly294002 fails to rescue microvilli disassembly (60). However, a partial rescue in ERM phosphorylation by a Rac inhibitor in SHIP1 KD T cells indicates that Rac independent pathways are also involved. Additionally, the PH-domain containing adaptor protein Bam32, that can bind to both $PI(3,4,5)P_3$ and $PI(3,4)P_2$, the SHIP1 substrate and product, respectively, is required for Rac1 activation and efficient BCR-induced cell adhesion (62). Thus, it is possible that both SHIP1 catalytic and non-catalytic functions are required for chemotaxis and cytoskeletal rearrangement; however, mechanistic studies in T cell conditional SHIP1 mutants are required to better define specific functions of SHIP1 in regulating these processes in an *in vivo* setting.

SHIP1 IN T CELL APOPTOSIS

The PI3K pathway is largely associated with cellular survival and proliferation as its product $PI(3,4,5)P_3$ is known to activate molecules required for cell survival and proliferation. Because SHIP1 degrades $PI(3,4,5)P_3$, it is primarily considered a negative regulator of PI3K-mediated cell survival. Indeed, SHIP1 plays a pro-apoptotic function in myeloid, erythroid, and in some instances B cells (63–66). However, it appears to play an opposite function in T cells. For instance SHIP1 limits Fas-induced apoptosis in human primary T cells *ex vivo* and a leukemic T cell line (67). Jurkat T cells, which do not express SHIP1 at normal levels are very sensitive to FasL mediated apoptosis; however, when SHIP1 is over-expressed in Jurkat T cells they become resistant to H_2O_2 and FasL mediated apoptosis (67, 68). It is also reported that SHIP1 attenuates FcγRIIB mediated apoptosis in B cells and that the failure to recruit SHIP1 to the receptor results in enhanced apoptosis (69, 70). Importantly $SHIP1^{-/-}$ mice are lymphopenic, and have profound deficiency of both $CD4^+$ and $CD8^+$ T cells in the gut indicating that SHIP1 might be required for T cell survival (20, 29). A selective deficiency of effector T cells at these sites might result in recruitment of myeloid cells, which subsequently leads to the lethal mucosal inflammation in both the lungs and gut of $SHIP1^{-/-}$ mice (23, 29). Interestingly reconstitution of sub-lethally irradiated $SHIP1^{-/-}$ mice with SHIP1-competent T cell graft protects them from mucosal inflammation and prolongs their survival. Moreover, SHIP1 is required for persistence of mature T cells in the periphery and at mucosal surfaces as $SHIP1^{-/-}$ T cells are impaired for survival when forced to compete with $SHIP1^{+/+}$ T cells for representation in the peripheral T cell pool of either immunocompetent or SCID hosts. Our preliminary studies indicate that SHIP1 mediated protection of T cell death at mucosal surfaces involves Fas-FasL death receptor pathway (42). Unlike myeloid cells in which SHIP1 appears to promote cell death, T cells require SHIP1 for their survival and persistence. A growing body of evidence implicates $PI(3,4)P_2$, the SHIP1 product, in cell survival as it can more efficiently recruit and activate Akt (71, 72) and protects cancer cells from apoptosis induced by SHIP1 selective (43) and pan-SHIP1/2 inhibitors (73). Consistent with this role of $PI(3,4)P_2$ and SHIP1/2 in promoting cell survival, increased levels of $PI(3,4)P_2$ in INPP4A and INPP4B mutant mice promote cell transformation and tumorigenicity (74, 75). SHIP1 and SHIP2 should not only be considered terminators of PI3K-mediated survival pathway, but paradoxically also facilitators of such survival signaling. With the growing evidence of its anti-apoptotic role in T cells, and in various cancer cells, it is important to understand when and how SHIP1 promotes pro-apoptotic vs. anti-apoptotic signaling. Here both cell types and the involved receptor(s) are likely critical determinants of this positive vs. negative role for SHIP1 and SHIP2 in apoptosis.

SHIP1 AND PHOSPHOINOSITIDE-BINDING PH-DOMAIN CONTAINING ADAPTOR PROTEINS

SHIP1's role as a positive regulator of PI3K signaling pathway can also be attributed to the ability of $PI(3,4)P_2$, the SHIP1 product to mediate recruitment of PH-domain containing adaptor proteins including SKAP adaptors (SKAP55 and SKAP-hom), Bam32 (also known as DAPP1), TAPP1, and TAPP2 (76). These adaptor proteins have differential ability to bind phosphoinositides, $PI(3,4,5)P_3$ vs. $PI(3,4)P_2$ (**Figure 1**) and also exhibit differential expression across immune cell types. For instance SKAP55 expression is relatively more restricted to T cells while SKAP-hom is more widely expressed in immune cells. Although Bam32 is restricted to hematopoietic cells, it is more abundant in B cells and expressed in lower levels in T cells, dendritic cells, and macrophages. TAPP proteins are widely expressed in all the tissues; however TAPP2 is more abundant in immune cells (76). TAPP1 and TAPP2 stand out among the adaptor proteins as they can only bind to $PI(3,4)P_2$ (27, 77), while SKAP adaptors and Bam32 can bind to both $PI(3,4,5)P_3$ vs. $PI(3,4)P_2$ with equal affinity (76). Mice deficient in SKAP55, which predominantly functions in T cells have impaired TCR induced adhesion to integrin ligands suggesting a role of SKAP in PI3K-mediated integrin activation in lymphocytes (78). Bam32 has been implicated in BCR signaling of B cells as $Bam32^{-/-}$ mice have defects in various aspects of B cell activation. $Bam32^{-/-}$ B cells have impaired BCR-induced proliferation and defective T-independent antibody responses (62). Bam32 has also shown to be required for germinal center progression and antibody affinity maturation (79). $Bam32^{-/-}$ B cells are defective in cell spreading presumably due impaired cytoskeleton rearrangement (76). In T cells Bam32 is required for TCR mediated ERK activation (80, 81). Thus, SHIP1 through hydrolysis of PI(3,4,5)P3 to $PI(3,4)P_2$ could differentially regulate the recruitment of SKAP and Bam32 adaptors and thereby impact T cell signaling. This question merits further study in SHIP mutant T cells and in the mice mutants for these adaptor proteins.

SHIP1 has been shown to enhance membrane recruitment of TAPP1 and TAPP2, the only adaptor proteins known to exclusively bind $PI(3,4)P_2$ (82). Recently a knock-in mouse model that express normal endogenous level of mutant TAPP1 and TAPP2 which are incapable of binding to $PI(3,4)P_3$ has been made to understand their physiological functions (83). Interestingly, the defects observed in the B cells of TAPP KI mice showed remarkable similarities with that of $SHIP1^{-/-}$ mice (84). TAPP KI mice have elevated levels of serum immunoglobulin, autoantibody production, and they show a lupus-like phenotype. Importantly AKT phosphorylation was significantly increased upon BCR cross linking in B cells purified from these mice enhancing their proliferation (84). This indicates that in the absence of TAPP adaptor proteins, the

$PI(3,4)P_2$ is available to promote AKT recruitment and resulting in increased proliferation and survival, consistent with the proposed positive function of SHIP1 in survival and proliferation (23). The precise role(s) of TAPP as adaptor proteins in T cells is relatively uncharacterized; however, TAPP can bind to PTPL1 which, has been shown to inhibit cytokine-induced TH1/TH2 differentiation (85). Therefore TAPP may potentially play a role in cytokine signaling in T lymphocytes by promoting membrane localization or activity of PTPL1. Although much is known about Bam32 and TAPP's function in B cells, it remains to determine whether there is a physiological function for the adapters proteins that involves T cell signaling.

SHIP1 AND SOLUBLE INOSITOL PHOSPHATE IP_4

In addition to hydrolysis of $PI(3,4,5)P_3$, SHIP1 can also dephosphorylate soluble inositol-1,3,4,5 tetrakisphosphate (IP_4) *in vitro* (18, 86). IP_4 is generated by phosphorylation of $Ins(1,4,5)P_3$ (IP_3) at its 3-position by IP_3 3-Kinases ($IP_3$3K) (**Figure 1**). Mammals express four IP3Ks; ItpkA/B/C and IPMK (IP multikinase). Lymphocytes predominantly express two Iptks, IptkB, and Iptkc, while IptkC is expressed in many tissues, expression of IptkB is restricted to hematopoietic cells and brain (2, 87). IP4 is required for T cell development as *Itpkb*$^{-/-}$ mice, are severely immunocompromised and lack mature T cells because of a block at the $CD4^+CD8^+$ DP stage due to impaired positive selection in the thymus (88, 89). Interestingly, PLCγ mediated DAG-induced ERK activation which is essential for positive selection is profoundly impaired in *Itpkb*$^{-/-}$ mice (90). IP_4 strongly resembles the phosphate headgroup of $PI(3,4,5)P_3$ and therefore it can bind to PH-domain containing proteins that also bind to $PI(3,4,5)P_3$ (e.g., ITK, AKT) and perhaps several others (2). In T cells IP4 functions as a second messenger and regulates Itk membrane recruitment and activation upon TCR stimulation and therefore it is essential for full activation of ITK and its effector PLCγ (90). At physiological concentrations of IP4 in TCR stimulated T cells it promotes ITK binding to $PI(3,4,5)P_3$, whereas at high IP4 concentrations it competes with $PI(3,4,5)P_3$ for PH-domain binding (2). Because of the essential role of IP4 in T cell development and function it would be intriguing to know whether IP4 turnover at in primary T cells is regulated by SHIP1 5′phosphatase activity. This might be investigated by determining the measuring IP4 levels in SHIP1-deficient T cells (vs. WT) to provide evidence of negative regulation of IP4 by SHIP1 *in vivo*. If this appears to be the case, then it would then be interesting to test whether the increased IP4 concentration in SHIP1$^{-/-}$ T cells results in diminished $PI(3,4,5)P_3$ binding of PH-domain signaling proteins recruited to $PI(3,4,5)P_3$ (e.g., ItK or AKT) to regulate T cell function.

SHIP2

A close homolog of SHIP1 is the ubiquitously expressed 150 kDa protein SHIP2. Unlike SHIP1, whose expression is confined to hematolymphoid cells, osteoblasts (24), and mesenchymal stem cells (91). SHIP2 is expressed broadly in both hematopoietic and non-hematopoietic tissues such as brain, skeletal muscle, heart, liver, and kidney (92, 93). SHIP2 hydrolyzes the 5′ phosphate of $PI(3,4,5)P_3$ *in vitro* and *in vivo* and has also been shown to dephosphorylate $PI(4,5)P_2$ *in vitro* (94, 95). Thus, it may not be restricted to hydrolysis of the 5′PO_4 groups on 3′ PO_4-containing polyphosphates, PI(3,4,5)P3 and I(1,3,4,5)P4, like its close homolog SHIP1. SHIP2 is tyrosine phosphorylated upon stimulation with stem cell factor (SCF), interleukin-3 (IL-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which results in its association with SHC (src homologous and collagen gene). Suggesting that SHIP2, similarly to SHIP1, is linked to downstream signaling events after activation of hematopoietic growth factor receptors. SHIP2 plays a major role in negatively regulating insulin signaling in non-immune cells (93). Bruyns et al. reported that both SHIP1 and SHIP2 are expressed in human T lymphocytes with only SHIP2 protein levels increased after long-term stimulation of the TCR (96). SHIP2 has also been shown to associate with the SH3 domain of Tec kinase and inhibit Tec-mediated TCR signaling (35). However, a physiological role for SHIP2 in T cell biology and function remains to be demonstrated and defined.

In addition to SHIP1 and SHIP2, eight other 5′ phosphatases have been reported; OCRL1 (oculocerebrorenal syndrome of Lowe), synaptojanin1, synaptojanin 2, proline rich inositol poly-phosphate 5-phosphatase (PIPP), 72-5ptase/Type IV/Inpp5e, SKIP, INPP5B, and 5-phosphatase1. With the exception of 5-phosphatase1 that hydrolyzes only the soluble inositol phosphates $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, other phosphatases can dephosphorylate 5-phosphorylated phosphoinositides including $PtdIns(4,5)P_2$, $PtdIns(3,4,5)P_3$, $PtdIns(3,5)P_2$, and soluble inositol phosphates although with variable efficiency (95). Some of these phosphatases are implicated in human diseases, for instance OCRL1 mutations are associated with Lowe's syndrome and Dent 2 disease (95, 97), SKIP and 72-5ptase/Type IV/Inpp5e are implicated in insulin signaling and glucose homeostasis while Synaptogenin1 in mice is required for neuronal function. Overlapping functions for some of the 5′phosphatases have been reported for example OCRL1$^{-/-}$ mice do not develop Lowe's disease since loss of OCRL1 was compensated by a highly homologous protein Inpp5b (98). Thus far no immune phenotype has been reported in any mouse mutant of these other 5′ inositol phosphatases. However, further studies are merited to rigorously exclude a specific role in T cell signaling.

PHOSPHATASE AND TENSIN HOMOLOG (PTEN)

Phosphatase and tensin homolog deleted on chromosome 10 was originally identified as a tumor suppressor gene, which negatively regulates cell survival and proliferation and is mutated in several cancers (99, 100). PTEN germline mutations are associated with several hereditary disorders characterized by hamartomas and increased cancer risk such as Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome, collectively classified as PTEN hamartoma tumor syndrome (PHTS) (101). PTEN predominantly acts as a 3′ lipid phosphatase to oppose PI3K signaling by dephosphorylating $PI(3,4,5)P_3$, a product of PI3K, at its 3′ hydroxyl position to yield $PI(4,5)P_2$ (102). Other than its 3′ lipid phosphatase activity, PTEN also possess protein phosphatase activity and has been reported to dephosphorylate focal adhesion kinase (FAK) by direct binding (103). Homozygous PTEN$^{-/-}$ knockout mice die early during embryogenesis, precluding analysis of PTEN role in various adult tissues and organs in germline mutant mice. However a wide range

of information has been collected from studies using mice heterozygous for PTEN or lacking PTEN in various tissues using Cre-loxP models (21). **Table 1** summarizes the function PTEN in T cells in different genetic mouse models.

Phosphatase and tensin homolog heterozygous mice show high tumor incidence, impaired Fas mediated cell death, and develop autoimmune disorders. T cells from these mice show increased proliferation, reduced activation induced cell death suggesting an important role of PTEN in T cell survival and activation (51). Studies from mice lacking PTEN in T cells revealed an important role for PTEN in T cell development, function, and homeostasis (22). Mice lacking PTEN in T cells (LckCre-PTEN$^{flox/-}$) die prematurely due to CD4$^+$ T cell lymphomas and develop symptoms of autoimmunity like autoantibody production and hypergammaglobulinemia. These mice show defective lineage commitment, altered thymic selection, and impaired peripheral tolerance. T cells from these mice were hyper-proliferative, secreted increased levels of Th1/Th2 cytokines, and were autoreactive. Resistance to apoptosis, increased AKT and ERK phosphorylation, and increased Bcl-X_L expression were observed in these T cells suggesting a vital role PTEN in regulation of T cell survival and apoptosis signaling (22). Hagenbeek et al. further confirmed the role of PTEN in T cell survival and development by analyzing LckCre-PTEN$^{flox/flox}$ mice. They showed that in the absence of PTEN there is a diminished requirement for both IL-7R and pre-TCR signaling in T cell development and proliferation (52). PTEN deficient CD4$^+$ T cells show hyper-responsiveness to TCR stimulation without requirement for co-stimulation signals and are refractory to anergy induction. Moreover, PTEN$^{-/-}$ T cells show increased AKT and GSK3β phosphorylation and enhanced IL-2 production upon TCR stimulation. This suggests that by negatively regulating TCR signaling, PTEN sets a threshold for T cell activation and imposes a requirement for co-stimulation and thus regulates T cell anergy (104). PTEN regulates the response of Tregs to IL-2 and plays a negative role in IL-2R signaling in Tregs, which normally do not expand in response to IL-2 alone. However, when Treg cells are deficient in PTEN they can proliferate upon IL-2 stimulation without the requirement for TCR stimulation (53). PTEN deficient CD4 T cells also produce more Th2 cytokines (IL-4, IL-10) in response to TCR stimulation alone or in combination with CD28 suggesting a role of PTEN in regulation Th2 cytokine production (105). Thus PTEN negatively regulates the TCR signaling and the induction of key cytokines. Thus, efficient and sustained TCR signaling and cytokine responses by T cells requires down-modulation of PTEN which occurs following TCR stimulation (106). Further Cbl-b has been shown to regulate down-modulation of PTEN in response to TCR/CD28 stimulation by inhibiting PTEN association with Nedd4, which targets PTEN K13 for K63-linked polyubiquitination suggesting that multiple pathways may regulate PTEN in the context to TCR/CD28 stimulation (107).

Studies from various knockout models showed clearly that PTEN plays an important role as a tumor and autoimmunity suppressor. However, the mechanistic insights into the relationship of these two functions of PTEN in T cells revealed that these two functions of PTEN are distinct, context dependent and are mediated in T cells at different developmental stages. By using mice with deletion of PTEN in T cells (CD4CrePten$^{fl/fl}$), Liu et al. demonstrated that T cell lymphomas arise in the thymus whereas autoimmunity was mediated by mature peripheral T cells (108). Subsequently Soond et al. studied the role of PTEN in mature CD4 T_H cells by using OX40CrePten^{flox} mice. Contrary to models of thymocyte-specific PTEN deletion OX40CrePten^{flox} mice did not develop lymphomas and autoimmunity even at an advanced age suggesting that PTEN does not act as a tumor suppressor or repressor of autoimmunity in mature T cells. In fact, PTEN deficient CD4 T_H cells produced increased concentrations of cytokines and were hyper-proliferative. The authors postulated that enhanced cytokine production turned PTEN deficient T_H cells into "super-helpers" as enhanced inflammatory, antibacterial, and anti-tumor responses were observed in OX40^{cre}Pten^{flox} mice (54). Thus contrary to the prevalent view PTEN does not essentially always function as a tumor suppressor or immune-suppressor and can also, like SHIP1, have varied functions depending on cell type, developmental stage of cell, and biological context. This was further confirmed by a recent study by Locke et al. by employing a model where PTEN is deleted in post-thymic T cells. They observed enhanced cytokine production, proliferation, and activation of post-thymic PTEN deleted T cells. As observed earlier, these effects were associated with increased AKT activity. However, CD28 independence and anergy resistance were not observed (109). Enhanced cytokine production, antibacterial, and anti-tumor responses of PTEN deficient T cells argue that therapeutic strategies targeting pharmacological inhibition of PTEN may prove attractive in immunotherapeutic strategies that require enhanced T effector function. Recently small molecule inhibitors of PTEN has been identified and used *in vivo* without causing prominent toxicity (110, 111). However, further studies are required to assess the role of these inhibitors on T cell effector and regulatory functions before considering their use in immunotherapeutic approaches.

INOSITOL POLY-PHOSPHATE 4-PHOSPHATASE (INPP4)

Inositol poly-phosphate 4-phosphatases are a class of enzymes that has two isoforms INPP4A and INPP4B, that selectively remove the phosphate group at position 4 on the inositol ring to convert PI(3,4)P_2 to PI(3)P (112). In contrast to INPP4A, which is predominantly expressed in brain, INPP4B is highly expressed in skeletal muscle, heart, brain, and pancreas, epithelial cells of the breast, and prostate glands (113). INPP4A has been shown to regulate neuroexcitatory cell death whereas INPP4B has emerged as potent tumor suppressor in breast cancer (114, 115). The function of the INPP4A and INPP4B phosphatases in immune cells has not been investigated, although a prominent role for INPP4B in myeloid-derived osteoclast function and bone remodeling has been shown (116). Thus, further investigation of these 4′-phosphatases appears merited and particularly in cell types and immune contexts where SHIP1 has a positive signaling role (e.g., T cell survival the gut). Function of inositol phosphatases in T cells is summarized is **Figure 2**.

CONCLUDING REMARKS

Consistent with studies implicating class I PI3K in T cell biology, the inositol phosphatases, SHIP1 and PTEN, have been documented to be important regulators of PI3K signaling pathway in T cells. Although SHIP1 and PTEN by dephosphorylating the PI(3,4,5)P_3 negatively regulate PI3K signaling, their *in vivo*

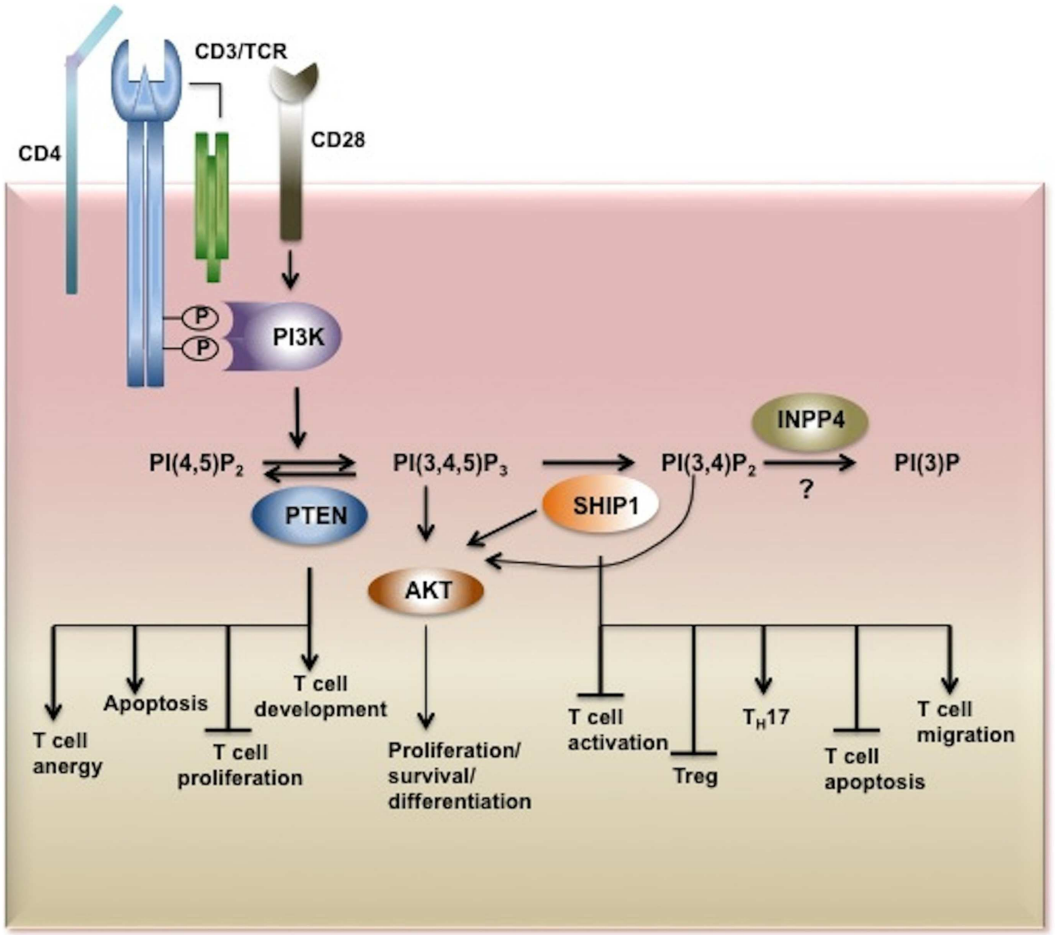

FIGURE 2 | Inositol phosphatases in T cell biology. Upon TCR stimulation PI3K is activated and recruited to the membrane through its SH2 domain where it phosphorylates its substrate $PI(4,5)P_2$ converting it to $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is bound by PH-domain containing proteins such as AKT, PDK1, BTK, ITK, Vav, and PLCγ triggering secondary signaling cascades and thus T cell activation, proliferation, survival, and cytokine production. PI3K signaling is tightly regulated by inositol phosphatases. $PI(3,4,5)P_3$ is a substrate for three inositol phosphatases, SHIP1/2 and PTEN which hydrolyze $PI(3,4,5)P_3$ to $PI(3,4,)P_2$ and $PI(4,5)P_2$, respectively. By limiting the cellular pool of the second messenger $PI(3,4,5)P_3$, PTEN and SHIP play important functions in T cell development, proliferation, and activation. The SHIP1 product $PI(3,4)P_2$ which can also recruit and activate AKT is dephosphorylated by INPP4. However the role of these 4-phosphatases in T cell biology has yet to be determined. AKT, Protein Kinase B; PDK1, phosphoinositide-dependent kinase-1, PLCγ phospholipase Cγ; ITK, IL-2-inducible T cell kinase) and BTK, Bruton agammaglobulinemia tyrosine kinase.

functions in this signaling pathway, as reveled by genetic analysis, diverge significantly. SHIP1 appears to be required for the survival of T cells *in vivo*, and particularly in the lamina propria, while PTEN inhibits T cell proliferation and prevents from lymphoproliferative syndromes. Therefore these phosphatases at the cellular level provide a fine balance of PI3K signaling necessary for the proper activation and development of T cells in order to avoid immunopathology. As SHIP1 deficiency has been shown to promote T cell apoptosis there is a significant potential for SHIP1 inhibitors, which have already shown promising results in cancer (43, 73), to be used to target autoreactive T cells in IBD conditions. Although PTEN has been shown to regulate CD4 T cell function and tolerance little is known about its role in other T cell subtypes. Further studies are therefore required to dissect PTEN signaling in T cells before therapeutic application of PTEN inhibitors in immunotherapy for cancer could be considered. In addition, the role of other lipid phosphatases SHIP2 and INNP4, which regulate the cellular pools of PtdIns $(3,4,5)P_3$ and $PI(3,4)P_2$, respectively, merit examination *in vivo* in the coming years using sophisticated genetic models that enable conditional and/or inducible ablation of their expression in specific T cell populations.

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An emerging role for PI5P in T cell biology

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Phosphoinositides are critical regulators in cell biology. Phosphatidylinositol 4,5-bisphosphate, also known as PI(4,5)P_2 or PIP_2, was the first variety of phosphoinositide to enter in the T cell signaling scene. Phosphatidylinositol bis-phosphates are the substrates for different types of enzymes such as phospholipases C (β and γ isoforms) and phosphoinositide 3-kinases (PI3K class IA and IB) that are largely involved in signal transduction. However until recently, only a few studies highlighted phosphatidylinositol monophosphates as signaling molecules. This was mostly due to the difficulty of detection of some of these phosphoinositides, such as phosphatidylinositol 5-phosphate, also known as PI5P. Some compelling evidence argues for a role of PI5P in cell signaling and/or cell trafficking. Recently, we reported the detection of a PI5P increase upon TCR triggering. Here, we describe the current knowledge of the role of PI5P in T cell signaling. The future challenges that will be important to achieve in order to fully characterize the role of PI5P in T cell biology, will be discussed.

Keywords: PI5P, PtdIns5P, phosphoinositide, T cell signaling, Dok proteins

INTRODUCTION

Phosphoinositides (PIs) are well known regulators of cell biology processes. Their polar inositol head group can be reversely phosphorylated on three different positions on the inositol ring (D3, D4, and D5). This can give rise to seven different phosphoinositides from the unphosphorylated one (PI) to the famous PI(3,4,5)P_3 or PIP_3. Phosphoinositides are anchored to cell membranes *via* two fatty acid chains inserted to the lipid bilayer. The membrane localization of phosphoinositides allows them to play a very important role in controlling protein localization within the cell, making them important players in cell signaling pathways.

From the 1950s to early 1980s, several research teams contributed to identify PI(4,5)P_2 cleavage by the phospholipase C gamma (PLCγ) into Diacylglycerol (DAG) and Inositol triphosphate (IP_3) (Berridge and Irvine, 1984). Subsequently these products lead to the activation of protein kinases C (PKC) and the release of Ca^{2+}. These studies provided the first evidence that PIs could be of great importance for cell signaling. Later, the detection of increased level of PI(3,4,5)P_3 upon oncogenic transformation and receptor tyrosine kinase (RTK) engagement led to the identification of the phosphoinositide 3 kinase (PI3K) enzymes. This introduced poly-phosphoinositides into many cell signaling pathways and identified a new common signaling pathway, PI3K/AKT, that is still under intense investigation (Whitman et al., 1988; Courtney et al., 2010; So and Fruman, 2012).

Until recently only few studies highlighted mono-PIs as signaling molecules in cell biology. This is mostly due to the difficult nature of detecting them (Pendaries et al., 2005). But several recent compelling studies argue for an important role of these mono-PIs in cell signaling. Among these mono-PIs, the phosphatidylinositol 5-phosphate PI5P has been the most recently identified PIs. Its late identification is mainly due to the difficulty in separating it from its close isomer PI4P in High-Performance-Liquid-Chromatography (HPLC) (Rameh et al., 1997; Sarkes and Rameh, 2010). Since then, several studies highlighted PI5P as a new potential important signaling molecule that could influence cell signaling pathways in epithelial cells after their activation (Pendaries et al., 2005; Wilcox and Hinchliffe, 2008; Grainger et al., 2012). Cell invasion by bacterial pathogens such as *Shigella* and *Salmonella* induce a high level of cellular PI5P. This increase is due to the Phosphoinositide 4-phosphatase activity of the virulence factors IpgD (*Shigella flexneri*) (Niebuhr et al., 2002) or SigD/SopB (*Salmonella* spp.) (Mason et al., 2007). PI5P has been localized in different subcellular compartments such as the plasma membrane, endoplasmic reticulum, Golgi apparatus, and the nucleus (Jones et al., 2006; Coronas et al., 2007; Sarkes and Rameh, 2010). PI5P was detected in T cells following ectopic expression of a PI(3,5)P_2 3-phosphatase, myotubularin-1 (MTM1) (Tronchere et al., 2004). MTM1 expression in Jurkat T cells induces a high level of cellular PI5P as detected by PI5P mass assay. Using similar methods, we were also able to detect a PI5P increase upon TCR triggering in the Hut-78 T cell line (Guittard et al., 2009). Recently, direct detection of PI5P by HPLC has been described (Sarkes and Rameh, 2010). These assays require expertise in analysis of lipids; thus these approaches are difficult to apply in cell signaling teams more familiar with protein biochemical analysis.

Here, we will discuss of the potent effects of PI5P in T cell signaling and the nature of the enzymes that could generate PI5P. We discuss identification of some direct PI5P partners (Guittard et al., 2009, 2010), and speculate about different protein domains that

bind PI5P in order to dissect the potential functional role of PI5P and to design some potential probes for PI5P as has been done for $PI(3,4,5)P_3$ detection with the Akt Pleckstrin Homology (PH) domain. Finally, increase in PI5P levels could be involved; not only in T cell signaling and gene transcription, but also in T cell chemotaxis (Konradt et al., 2011) and/or other cellular processes such as vesicular trafficking and chromatin rearrangement.

PI5P SYNTHESIS DURING T CELL ACTIVATION

Measuring PIs levels requires a large amount of cellular material. Thus, most of these experiments are performed in cell lines. Indeed, many T cell lines harbor mutations in the PTEN gene, which induces a high level of cellular $PI(3,4,5)P_3$ that can induce a bias when studying other PIs species (Astoul et al., 2001). To detect PI5P levels in T cells, we used a wild-type PTEN human T cell line, HUT-78. By stimulating this cell line with an anti-CD3 mAb, a nearly fourfold PI5P increase was detected using a lipid mass assay (Guittard et al., 2009). This fold increase is in accordance with other reported results in response to insulin stimulation in other cell types (Sbrissa et al., 2004; Sarkes and Rameh, 2010). As TCR-induced PI5P elevations appear to be rapid (peaks at 2 min) and transient (Guittard et al., 2009), we suggest that there is a rapid recruitment of a specific lipid kinase/phosphatase to the plasma membrane upon TCR engagement, as observed previously for the class IA PI3K (Fabre et al., 2005). The enzyme or the enzymatic complex involved in the PI5P increase in T cells is still unknown.

$PI(4,5)P_2$ is found at high levels at the plasma membrane. Thus, it represents a potent substrate for a $PI(4,5)P_2$ 4-phosphatase resulting in PI5P synthesis. IpgD *S. flexneri* virulence factor, has been clearly identified to be a $PI(4,5)P_2$ 4-phosphatase (Niebuhr et al., 2002). Ectopic IpgD expression has been used in several studies to access the role of PI5P in eukaryotic cells (Pendaries et al., 2006; Guittard et al., 2009, 2010; Sarkes and Rameh, 2010; Ramel et al., 2011; Oppelt et al., 2012). So far no eukaryotic enzyme has been identified that synthesizes only PI5P. Next we will discuss enzymes that can lead to production of PI5P that are expressed in T cells and may be involved in early TCR signaling. As summarized in **Figure 1**, there are three different possible routes to synthesize PI5P: the 5-kinases (PIKfyve), 3-phosphatases (MTMs family members), and type I/II $PI(4,5)P_2$ 4-phosphatases.

PHOSPHOINOSITIDE 5-KINASE, PIKfyve/PIPKIII

The simplest way to produce PI5P would be a direct phosphorylation of PI by a phosphoinositide 5-kinase. So far, only PIKfyve has been suggested to play such a role (Sbrissa et al., 1999, 2002). PIKfyve is a lipid 5-kinase that bears a FYVE domain that recognizes PI3P species. PIKfyve can act on two substrates, PI and PI3P to generate PI5P and $PI(3,5)P_2$, respectively. So far, this is the only kinase proposed to directly produce PI5P from PI *in vitro*. Moreover, PIKfyve shRNAs decrease the PI5P pool in fibroblasts from a hypomorphic gene-trap mouse mutant (Zolov et al., 2012). Interestingly, their observation of the early thymus degeneration in these mice suggests a possible role for PIKfyve in T cell development. A role in peripheral T cell functions could be possible as PIKfyve is expressed in spleen (Zolov et al., 2012). However, it is still difficult to understand if the thymic degeneration result from PI5P and/or $PI(3,5)P_2$ loss. Recently a new PIKfyve inhibitor, YM201636, has been identified. Unexpectedly, at low doses (10–25 nM), it inhibited preferentially PI5P rather than $PI(3,5)P_2$ production *in vitro*, whereas at higher doses, the generation of the two lipid products were similarly inhibited. YM201636 or potential second generation molecules may represent a possible avenue for discriminating biologic effects observed consequent to PI5P loss versus $PI(3,5)P_2$ loss (Sbrissa et al., 2012).

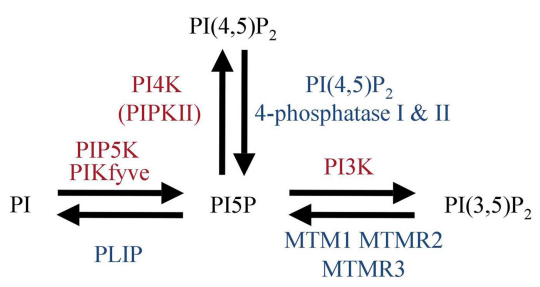

FIGURE 1 | Mammalian enzymes involved in PI5P metabolism.

PI5P synthesis in different cell types occurs mainly at the plasma membrane (Sarkes and Rameh, 2010). Our observations of a rapid PI5P increase upon TCR engagement (Guittard et al., 2009) prompted us to postulate that PI5P pools are produced at the plasma membrane. However, PIKfyve is essentially located at intracellular organelles where it plays a key role in vesicular transport (Sbrissa et al., 2012). Therefore other enzymes should be considered to regulate the PI5P level at the plasma membrane.

PHOSPHOINOSITIDE 3-PHOSPHATASES, MTM1, AND MYOTUBULARIN-RELATED PROTEINS

Enzymes from the myotubularin family are 3-phosphatases that can regulate PI3P and $PI(3,5)P_2$ pools (Tronchere et al., 2004). These enzymes are ubiquitously expressed (Laporte et al., 1996, 1998) and are able to generate PI5P from $PI(3,5)P_2$ *in vitro* (Schaletzky et al., 2003; Vaccari et al., 2011). They were the first identified eukaryotic phosphatases able to produce PI5P *in vivo* (Tronchere et al., 2004). However, here again, $PI(3,5)P_2$ is thought to be mainly localized at late endosomal membranes (De Matteis and Godi, 2004). Thus, it would be difficult to consider that MTMs enzymes are responsible for early PI5P synthesis at the plasma membrane upon TCR stimulation.

TYPE I/II $PI(4,5)P_2$ 4-PHOSPHATASES

Two human $PI(4,5)P_2$ 4-phosphatases (type I and type II isoforms) have been identified (Ungewickell et al., 2005; Zou et al., 2007). They share a CX_5R phosphatase motif with the IpgD prokaryotic $PI(4,5)P_2$ 4-phosphatase (Ungewickell et al., 2005). *In vitro*, these eukaryotic phosphatases are also able to convert $PI(4,5)P_2$ to PI5P. Both enzymes are ubiquitously expressed and localize to late endosomal/lysosomal membranes in epithelial cells (Ungewickell et al., 2005). Again this makes them less likely to be involved in early signaling from the T cell receptor. Moreover type I phosphatase has been shown to be translocated to the nucleus where it can increase PI5P levels following genotoxic stress (Zou et al., 2007). Thus, this raises the possibility that these enzymes play a role in transcriptional activity.

In conclusion, based on the claimed cellular localizations of these enzymes or their substrates (**Table 1**), it is hard to imagine a scheme inducing a major PI5P synthesis at the plasma membrane. However, some of these enzymes have a role in T cells. For example, MTMR6 could down-regulate calcium receptor KCa3.1 expressed in T and B cells, in a PI3P-dependent manner (Srivastava et al., 2005). One cannot exclude a transient and local recruitment of enzymatic complexes able to produce PI5P at the plasma membrane. Further investigations in T cell biology studying these proteins could define some new functions for PI5P especially in endosomal compartments and/or in the nucleus.

PI5P BINDING DOMAINS

As mentioned above, PIs are organized into specific subcellular compartments in order to recruit protein to a specific organelle (McCrea and De Camilli, 2009). As summarized in **Table 2**, some studies have been conducted to identify potential partners of PI5P and, therefore, to suggest potential functions for this phospholipid.

THE PHD MOTIFS OF NUCLEAR PROTEINS, ING2 AND ATX-1

The plant homeo-domain (PHD) motif is a conserved Cys4-HisCys3 orphan zinc finger domain present throughout eukaryotic proteomes. A large number of chromatin regulatory factors contain PHD fingers, including the ING family of putative tumor suppressors (Feng et al., 2002; Fyodorov and Kadonaga, 2002; Kalkhoven et al., 2002). In 2003, the PHD of ING2 protein became the first identified PI5P-binding domain. It was identified by three different *in vitro* experimental approaches by PIP-beads binding assay, by fat-blotting, and by surface plasmon resonance (SPR) analysis (Gozani et al., 2003). However, although binding to PI5P, a significant binding to other mono-PIs such as PI3P could not be excluded from this study. To strengthen this PHD motif binding affinity, a 3X PHD motif has been generated and shows a stronger PI5P binding. This 3X PHD ING2 construct has been used as a tool for PI5P investigations (Pendaries et al., 2006; Guittard et al., 2009, 2010; Ramel et al., 2011). A similar PHD motif was identified in plants. The *Arabidopsis* homolog of trithorax-1 (ATX-1) binds PI5P using its PHD domain (Alvarez-Venegas et al., 2006). Authors are suggesting a role for PI5P in inhibiting ATX-1 protein by delocalizing it from the nucleus where it can repress gene expression. Nuclear PI5P localization has been reported and PI5P can modify the function of some PHD motif containing proteins (Gozani et al., 2003; Alvarez-Venegas et al., 2006; Jones et al., 2006). A role for nuclear PI5P in regulating nuclear protein function has not yet been assessed in T cell biology.

THE PH DOMAIN OF DOK FAMILY MEMBERS

Only 10% of known PH domains bind PIs with high specificity (Lemmon, 2008). The first identified PH domain harboring some PI5P binding properties was the PH domain of the p62 subunit of the transcription factor IIH (TFIIH) (Di Lello et al., 2005). This observation is really close to what has been reported for the PI5P binding PHD domains, suggesting again a potential role for PI5P in cell transcriptional activity.

Table 1 | Enzyme expression, localization, and functions in lymphoid cells.

Enzymes	Substrates *in vitro*	Lymphoid tissues expression	Localization	Immune function	Reference
PIPK					
PIPKI/PI4P 5-kinase (α, β, γ)	PI4P, PI	Spl., LNs	Nu (α), PM (γ), PNu (β)	PIPKIγ in NK, Blast cells T cells, PIPKIγ 90 is negative regulator of T cell activation, adhesion, and proliferation	Doughman et al. (2003), Micucci et al. (2008), Bolomini-Vittori et al. (2009), Vasudevan et al. (2009), Wernimont et al. (2010)
PIPKIII/Pykfyve (5-kinase)	PI, PI3P	Spl., Thy	LE	KO: early degeneration of Thymus, role in T cell development?	Zolov et al. (2012)
3-PHOSPHATASE					
MTM1	PI3P, $PI(3,5)P_2$	LN, Spl., Thy	Cyt., PM?	Overexpression enhance PI5P pool in Jurkat T cells	Laporte et al. (1996), Tronchere et al. (2004)
MTMR2, 3, 6	PI3P, $PI(3,5)P_2$	Ubiquitous	Cyt., PM?	Not tested, but MTMR6 down-regulate KCa3.1 Ca2+ rec. expressed on B, T cells	Laporte et al. (1998), Walker et al. (2001), Berger et al. (2002), Schaletzky et al. (2003), Srivastava et al. (2005), Lorenzo et al. (2006), Vaccari et al. (2011)
4-PHOSPHATASE					
Type I, II/$PI(4,5)P_2$ phosphatase (IpgD homolog)	$PI(4,5)P_2$	Spl., BM, Thy., PBL	LE, Ly, Nu	Not tested	Ungewickell et al. (2005), Zou et al. (2007)
5-PHOSPHATASE					
PLIP/PTPM T1	PI5P?	Spl., LNs, BM	G	Not tested	Merlot et al. (2003), Pagliarini et al. (2004), Zhang et al. (2011)

Nu, nucleus; PM, plasma membrane; C, cytosol; PNu, peri-nuclear; LE, late endosomes; Ly, lysosomes; G, Golgi; Spl, spleen; BM, bone marrow; Thy, thymus; PBL, peripheral blood leukocytes.

Table 2 | Some cellular proteins containing a lipid/protein interaction domain were identified as PI5P binding partners.

Binding domain	PI binding	Experiment used	Protein role	Reference
CYTOSOL, PLASMA MEMBRANE				
PH Dok-1/Dok-2	PI4P, PI5P	Fat blot, SPR	Negative regulation T cell signaling	Guittard et al. (2009)
PH-Dok-4	PI5P > Mono-PIs	Fat blot, SPR	Negative/positive regulation T cell signaling	Guittard et al. (2010)
PH Dok-5	PI5P+++	Fat blot, SPR	Cardiomyocyte differentiation PI3K depdt	Guittard et al. (2010)
BIN1	PI5P, PI3P	SPR	Tubular invaginations of membranes, biogenesis of muscle T tubules	Nicot et al. (2007), Fugier et al. (2011)
NUCLEUS				
ATX-1-PHD	PI5P+++	Fat blot	Plant (*Arapidopsis thaliana*) chromatin modification stress induced	Alvarez-Venegas et al. (2006)
ING2 PHD	PI5P+, PI3P	Fat blot, SPR, PIP-beads	Nucleus cellular stress response	Gozani et al. (2003)
Sap30L/Sap 30	PI5P > PI3P > PI4	Fat blot	Chromatin remodeling, transcription	Viiri et al. (2009)
PH-tfb1 TFII subunit	PI5P, PI3P	Fat blot	Transcription factor	Di Lello et al. (2005)

Different experimental approaches were used to characterize this PI5P binding. These proteins are expressed in different subcellular compartments and are involved in different cell functions. PH, Pleckstrin homology domain; PHD, plant homeo-domain; SPR, surface plasmon resonance.

Dok (for downstream of kinase) proteins are adaptor proteins that are expressed in lymphocytes (Favre et al., 2003). Upon T cell stimulation, these PH domain-containing proteins are recruited to (or are in the vicinity of) the plasma membrane (Boulay et al., 2005; Gerard et al., 2009). Dok-1 and Dok-2 PH domains were shown to bind PI5P and PI4P in SPR analysis (Guittard et al., 2009). But using different enzymatic approaches, PI5P appeared to be essential for Dok proteins tyrosine phosphorylation. Moreover, PI5P binding domain expression (Dok-1 PH domain or 3X PHD ING2) block PI5P-induced Dok phosphorylation.

Among Dok family PH domains (Favre et al., 2003), the PH domain of Dok-5 revealed the highest PI5P binding affinity in SPR experiments (Guittard et al., 2009, 2010). Dok-5 PH domain expression reduced IpgD-induced IL-2 promoter activity in T cells by sequestering PI5P within the cell (Guittard et al., 2010). These observations highlighted PI5P as a newly identified actor in T cell signaling that acts by regulating cytosolic Dok proteins.

A ROLE FOR PI5P IN T CELL SIGNALING

Stimulation of membrane receptors such as the TCR on T cells induces the activation of protein tyrosine kinases (PTKs) and subsequently the phosphorylation of substrates, which contributes to the formation of a cytoplasmic multiprotein network (Smith-Garvin et al., 2009). TCR leads to the activation of several physically separated protein modules (**Figure 2**). First, Src-family protein tyrosine kinases (SFK) Lck/Fyn are activated by a yet not fully known mechanism. SFK activation is followed by tyrosine phosphorylation of TCR and CD3 chains leading to the recruitment of ZAP-70 PTK. Finally, cytoplasmic protein networks are established based on interactions with numerous adaptor proteins including LAT (Acuto et al., 2008).

Dok-5 PH expression selectively reduces some TCR-induced signaling events such as SFK activation (Lck/Fyn) and Akt phosphorylation (AKT is a PI3K effector) (Guittard et al., 2010). Independently of TCR engagement, ectopic expression of IpgD induces the phosphorylation of SFK family members and Akt (Guittard et al., 2010). Thus, PI5P could be a part of membrane signaling TCR-containing modules, such as the SFK regulation module (Acuto et al., 2008; Nika et al., 2010).

These SFKs are controlled by membrane lipid content (lipid rafts). The presence of Csk and CD45 protein tyrosine phosphatase (PTP) is involved in maintaining a balance between active and inactive SFK forms (Acuto et al., 2008). The selective inhibition of Csk activates this early SFK signaling module leading to Dok-1 tyrosine phosphorylation. This suggests that PI5P could be involved in SFK-containing lipid rafts, perhaps by modulating the dynamics of these plasma membrane structures (Schoenborn et al., 2011). Involvement of a PH containing molecule, such as SKAP-55, could also bring CD45 in close proximity to the SFK signaling module leading to its activation (Wu et al., 2002).

It has long been known that PI3K signaling is activated upon TCR triggering (Ward et al., 1992). However, it is always difficult to draw a general connection map of proximal TCR signaling pathways that integrates the PI3K/Akt pathway (Acuto et al., 2008; Smith-Garvin et al., 2009). A possible explanation would be that the PI3K/Akt signaling module is also physically independent of other proximal TCR signaling modules (Acuto et al., 2008). The full activation of Akt is dependent upon its presence in some membrane structures corresponding to lipid raft nanodomains (Lasserre et al., 2008). Several reports described PI5P acting upstream of the PI3K/Akt pathway (Carricaburu et al., 2003; Pendaries et al., 2006; Grainger et al., 2012). For instance, IpgD-produced PI5P persistently activates PI3K/Akt signaling in epithelial cells (Pendaries et al., 2006). In this condition, PI5P at the plasma membrane at the early stages of *S. flexneri* infection is rapidly enriched in endosomes and alters growth factor receptor signaling by impairing lysosomal degradation, a property used by the pathogen to favor survival of host cells. Thus far, there is no direct link between PI5P generation and PI3K activation in T cells. As it is the case for the SFK regulation module, we can hypothesize

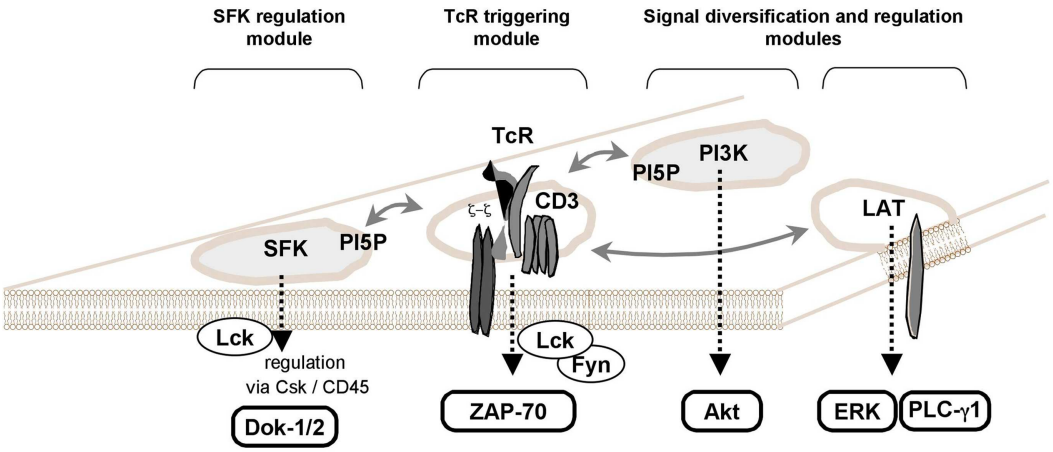

FIGURE 2 | PI5P as a new key player in TCR signaling. TCR stimulation induces PI5P increase (Guittard et al., 2009). By expressing a bacterial PI(4,5)P_2 4-phosphatase, IpgD in T cells, the PI5P elevation reveals a selective activation of signaling events such as the activation of Src-family protein tyrosine kinases (SFK) and the Ser/Thr kinase, Akt (a PI3K effector) (Guittard et al., 2010). As previously illustrated (Acuto et al., 2008), some physically independent signaling modules in the T cell membranes could be involved in establishing full TCR signals, when there are interconnected. In this scheme, we added a separated module for PI3K/Akt signaling where the Class IA PI3K could recognize a SFK or some membrane protein containing a Tyr-x-x-Met motif. Plasma membrane PI5P could participate to the lipid compounds of some of these modules such as SFK regulation module and PI3K-dependent signal diversification/regulation module (see text).

that PI5P could participate in lipid raft nanodomains dynamics where PI3K/Akt activation would take place. This potential PI3K/Akt module could also explain why PI5P elevation provokes a Dok protein tyrosine phosphorylation (Guittard et al., 2009), as the PTK Tec, a PI3K effector in T cells, phosphorylates the Dok-1 and Dok-2 proteins (Yang et al., 2001; Gerard et al., 2004).

It has been reported that PI5P and other PIs interact with high affinity to a TCR ζ basic-rich stretch (DeFord-Watts et al., 2011). The elimination of PIs-binding regions significantly impaired the ability of TCR ζ chains to be stably expressed at the plasma membrane (DeFord-Watts et al., 2011). Taken together, a role for PI5P in T cell signaling should be further investigated in these potentially physically independent modules, for instance via experiments evaluating membrane fluidity and dynamics (Lasserre et al., 2008).

OTHER PERSPECTIVES FOR A ROLE OF PI5P IN T CELL BIOLOGY

Cell fractionation has revealed that a major fraction of PI5P is in the plasma membrane (Sarkes and Rameh, 2010). As discussed above, membrane PI5P is involved in T cell signaling (Guittard et al., 2009, 2010). This lipid could also be involved in the control of T cell migration. Indeed, *S. flexneri* is able to infect activated T cells and IpgD [converting PI(4,5)P_2 into PI5P] inhibits chemokine-induced T cell migration (Konradt et al., 2011). In this study, the authors concluded that the T cell chemotaxis block was due to a PI(4,5)P_2 breakdown. But they could not exclude a role for PI5P increase in this process. However, in other cell types, PI5P increases appear to induce cell migration (Oppelt et al., 2012). These apparent discrepancies likely result from differences between lymphoid cells and other cell types in their cell migration. For instance, many cell types use a PI3K-dependent pathway in inducing cell migration, but T cell chemotaxis seems to be independent of a PI3K signaling pathway (Asperti-Boursin et al., 2007). The Rac small GTPase, probably regulated by DOCK2 RacGEF, could drive this T cell migration (Fukui et al., 2001). One exiting hypothesis would be that PI5P is involved in a Rac-dependent pathway and/or participates in the connection between plasma membrane and the cytoskeleton dynamics.

In skeletal muscle, the bridging integrator-1 (BIN1) proteins bind to membrane PI5P and is involved in tubular invaginations of membranes and is required for the biogenesis of muscle T tubules (Nicot et al., 2007; Fugier et al., 2011). PI5P can be detected in endosomes (Sarkes and Rameh, 2010) and can favor RTK signaling prolongation in early endosomes (Ramel et al., 2011). Furthermore IpgD expression induces a striking amount of IL-2 promoter activity in T cells (Guittard et al., 2010). These results could be due to sustained T cell signaling at the plasma membrane or in intracellular compartments. Thus, the role of PI5P in vesicular trafficking in T cells should be considered.

In summary, PI5P is now taking its place in T cell biology. As in other mammalian cell types, the localization of basal and inducible PI5P should be characterized by cell fractionation followed by lipid composition analysis. PI5P-specific probes should be improved to visualize phospholipid dynamics upon T cell activation. Further investigations should be performed to assess the exact role of PI5P at the plasma membrane (for T cell signaling and migration), but also in vesicular trafficking and nuclear function.

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The glycerophosphoinositols: from lipid metabolites to modulators of T-cell signaling

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Glycerophosphoinositols (GPIs) are bioactive, diffusible phosphoinositide metabolites of phospholipase A_2 that act both intracellularly and in a paracrine fashion following their uptake by specific transporters. The most representative compound, glycerophosphoinositol (GroPIns), is a ubiquitous component of eukaryotic cells that participates in central processes, including cell proliferation and survival. Moreover, glycerophosphoinositol 4-phosphate (GroPIns4*P*) controls actin dynamics in several cell systems by regulating Rho GTPases. Recently, immune cells have emerged as targets of the biological activities of the GPIs. We have shown that exogenous GroPIns4*P* enhances CXCL12-induced T-cell chemotaxis through activation of the kinase Lck in a cAMP/PKA-dependent manner. While highlighting the potential of GroPIns4*P* as an immunomodulator, this finding raises questions on the role of endogenously produced GroPIns4*P* as well as of other GPIs in the regulation of the adaptive immune responses under homeostatic and pathological settings. Here we will summarize our current understanding of the biological activities of the GPIs, with a focus on lymphocytes, highlighting open questions and potential developments in this promising new area.

Keywords: glycerophosphoinositol, T-cell chemotaxis, CXCL12, Lck

The glycerophosphoinositols (GPIs) are ubiquitous water-soluble phosphoinositide metabolites produced by all eukaryotic cells (1–3). Not surprisingly considering their central role in the orchestration of signaling cascades, among the phosphoinositides it is the inositol phosphates that have monopolized the scene. Accumulating evidence has however highlighted a role for the GPIs as modulators of important biological functions in a number of cell types, including T-lymphocytes, in both physiological and pathological settings. Here we will summarize our current understanding of the metabolic pathways that regulate GPI production and discuss their biological activities, focusing on T-cells.

BIOSYNTHESIS, TRANSPORT AND DEGRADATION OF GPIs

The GPIs, which include glycerophosphoinositol (GroPIns) and its phosphorylated derivatives glycerophosphoinositol 4-phosphate (GroPIns4*P*) and glycerophosphoinositol 4,5-bisphosphate (GroPIns4,5P_2), are generated from membrane phosphoinositides through two sequential deacylation reactions that are carried out by a phospholipase A_2 (PLA_2) and a lysophospholipase (2). Studies on the most abundant of these metabolites, GroPIns, have provided evidence that both of these reactions can be catalyzed by the same enzyme, which has been identified in thyroid cells and macrophages as the α isoform of group IV PLA_2 (PLA_2IVα) [(4, 5); **Figure 1**]. We have recently shown that the same enzymatic pathway is responsible for GroPIns4*P* production in macrophages upon treatment with a pro-inflammatory stimulus (unpublished results; **Figure 1**).

Glycerophosphoinositols can interact with intracellular targets and/or be released into the extracellular medium through specific membrane transporters, following their chemical gradient. The GroPIns transporter, which is responsible for the bidirectional transfer of GroPIns, was initially identified in yeast (6), and its human ortholog is the Glut2 permease (7). Reasonably, Glut2 represents only one of the mammalian GroPIns transporters, as it has cell-specific expression patterns, while GroPIns membrane permeation appears to be a general process. There is also evidence of GroPIns4*P* membrane transport. Although no specific transporter for GroPIns4*P* has been identified to date, several GroPIns4*P*-mediated activities show biochemical features that indicate specific, transporter-mediated mechanisms (8).

The half-life of GPIs is relatively short both inside the cell and in the extracellular milieu. This applies in particular to the phosphorylated, biologically active derivative, GroPIns4*P* which is rapidly metabolized within the cell, undergoing dephosphorylation to GroPIns through a Ca^{2+}-dependent and GroPIns4*P*-selective activity associated with the cell membrane fraction (**Figure 1**). Alternatively, a Ca^{2+}-insensitive activity leads to the phosphorylation of GroPIns4*P* to GroPIns4,5P_2 (8). GroPIns can be reacylated to phosphatidylinositol (PtdIns) both in whole cells and in membrane fractions. At variance, no detectable reacylation of GroPIns4*P* has been documented to date (8).

The glycerophosphodiesterases GDE1 and GDE3, both of which are membrane-bound ectoenzymes, catalyze the hydrolysis of extracellular GroPIns (9, 10). GDE1 is ubiquitously expressed and hydrolyzes GroPIns to produce inositol and glycerol phosphate (9). GDE1 activity is regulated by G-protein-coupled receptors, and it is stimulated by β-adrenergic receptor agonists but inhibited by α-adrenergic receptor agonists and lysophosphatidic

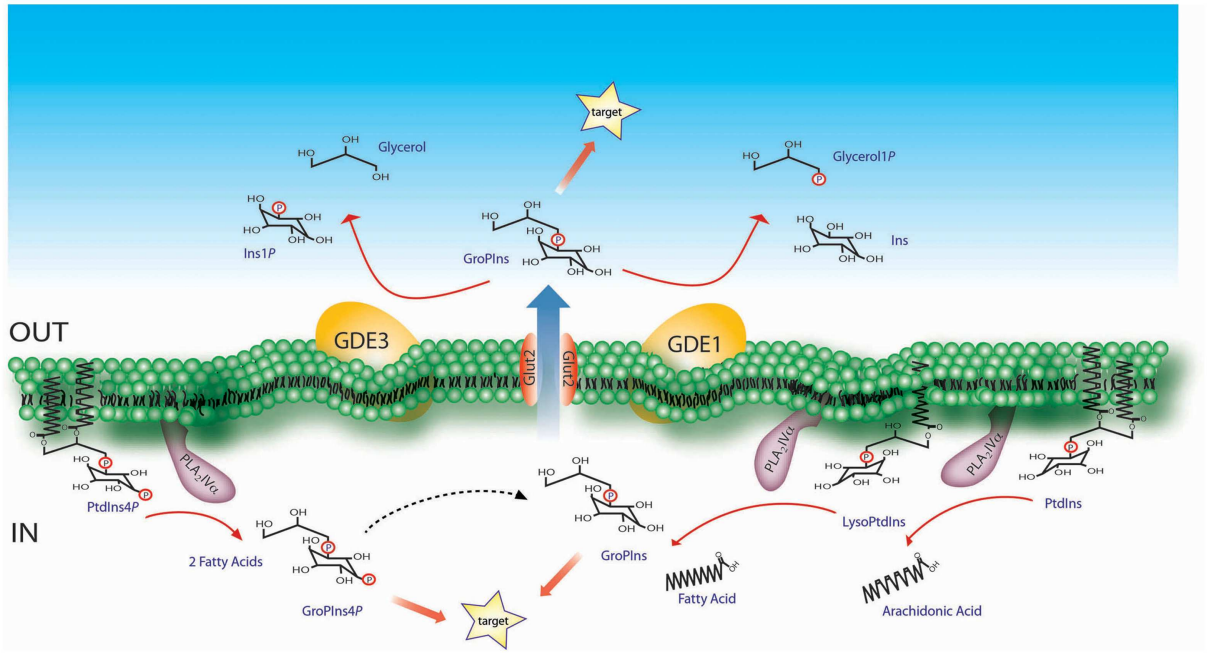

FIGURE 1 | Schematic representation of the GroPIns metabolism. The formation of GroPIns occurs from membrane phosphatidylinositol (PtdIns) via two sequential steps, both of which are catalyzed by $PLA_2IV\alpha$. The first deacylation produces lysophosphatidylinositol (LysoPtdIns) and free arachidonic acid, since $PLA_2IV\alpha$ selectively hydrolyzes phosphoinositides substituted in the *sn-2* position with arachidonic acid (49). The second deacylation releases free fatty acid and GroPIns. As indicated, $PLA_2IV\alpha$ supports both of these deacylation steps, as demonstrated in *in vitro* investigations using purified phosphoinositide and lysophosphatidylinositol substrates together with the recombinant enzyme (4). Once produced in the cytoplasm, GroPIns can be active on intracellular targets or can be released through the Glut2 transporter into the extracellular space, where it can act as a paracrine factor on nearby target cells. The subsequent catabolism of GroPIns is instead located on the extracellular side of the plasma-membrane and is mediated by the GDEs. GroPIns4*P* formation, also schematized, occurs starting from membrane phosphatidylinositol 4-phosphate which is hydrolyzed, as for GroPIns, by $PLA_2IV\alpha$ (unpublished observations, see main text for details).

acid, thus providing a further level of modulation of GroPIns metabolism (9). GDE3 is a marker of osteoblast differentiation, and is predominantly expressed in mature osteocytes (11). GDE3 hydrolyzes GroPIns with a different type of attack of the phosphodiester bond, which produces inositol phosphate and glycerol (10). GroPIns4*P* is not substrate of GDE1 or GDE3, but it can compete with GroPIns for its hydrolysis by these glycerophosphodiesterases (9, 10).

GPI PRODUCTION IN IMMUNE CELLS

GroPIns IS PRODUCED BY MACROPHAGES IN RESPONSE TO PRO-INFLAMMATORY STIMULI

A number of pharmacological and pro-inflammatory stimuli have been shown to trigger phosphoinositide hydrolysis in macrophages (2). Similar to other cell types, GroPIns production in these cells is regulated by a Ca^{2+}-dependent pathway involving the PLA_2-catalyzed deacylation of PtdIns (2). Studies on macrophages treated with cholera or pertussis toxin provided evidence that PLA_2 is activated downstream of G proteins, catalyzing the hydrolysis of PtdIns and leading to the production of arachidonic acid derivatives and GroPIns (12). A similar pathway was identified in Kupffer cells, the resident macrophages of the liver, following stimulation with inflammatory mediators produced upon bacterial endotoxin challenge (13, 14). A concerted activation of the arachidonate pathway and production of GroPIns has been reported in several other cell types (2).

$PLA_2IV\alpha$, which had been identified as the specific, Ca^{2+}-dependent PLA_2 responsible for GroPIns production in thyroid cells (4), has been recently demonstrated to carry out this function also in macrophages. Zizza and colleagues (5) showed that $PLA_2IV\alpha$, which is abundantly expressed in macrophages, is phosphorylated by the MAP kinases Erk1/2 and by the stress-activated kinases p38 and JNK and translocates to the membrane of nascent phagosomes during Fc-Receptor (FcR)-mediated phagocytosis. A selective $PLA_2IV\alpha$ activation was observed to also occur upon LPS treatment which, similar to FcR engagement, triggers arachidonic acid release (5). Moreover, pharmacological inhibition of $PLA_2IV\alpha$ completely abolished both LPS- and phagocytosis-mediated GroPIns production. Interestingly, a time course analysis of GroPIns production during FcR-mediated phagocytosis revealed a persistent increase in the levels of intracellular GroPIns over time, which was paralleled by GroPIns release into the extracellular medium (our unpublished observations). This suggests that GroPIns may participate in the inflammatory responses of macrophages by acting not only in an autocrine manner, but also as a paracrine factor.

The intracellular levels of the GPIs have also been measured in T-cells. Mass spectrometry data showed that Jurkat

T-cells are among the cell lines with low intracellular levels of GroPIns ($45 \pm 1\ \mu M$) (15, 16). Moreover, these basal levels are not increased by known pharmacological activators of $PLA_2IV\alpha$, such as Ca^{2+} ionophores, or by chemotactic stimuli, such as CXCL12, which suggests that a Ca^{2+}-independent enzyme is involved in GroPIns production in these cells. Alternatively, the concentrations of arachidonoyl-substituted PtdIns, the GPI precursor, may not be sufficient to produce significant increases in the levels of intracellular GroPIns.

MODULATION OF GroPIns PRODUCTION DURING IMMUNE CELL DIFFERENTIATION

Phospholipase A_2 activation is not only triggered by plasma-membrane receptors but also occurs during cell differentiation (17–19). Mountford and colleagues provided evidence that the levels of phosphoinositides change during the differentiation of both myeloid and lymphoid cells (20–22). Using HL60 promyelocytic cells, which can differentiate either to neutrophils in response to all-trans retinoic acid and granulocyte-colony-stimulating factor or to monocytes in response to 1α-25-dihydroxyvitamin D_3, they showed that the intracellular GroPIns levels increased in the early stages of differentiation to either lineages, eventually doubling in fully differentiated cells. Consistent with these findings, GroPIns levels increased in neutrophils that spontaneously differentiated in culture, as compared to the initial blasts (22). Similar experiments, carried out on paired cell lines representative of immature and mature states of B-lymphocytes (Ba/F3 and NSI cells) and T-lymphocytes (S49 and C8166 cells) showed substantial increases in GroPIns levels in the cells representative of the mature states (22). Although more accurate methods to quantitate GroPIns as well as more physiological differentiation conditions will be required to validate these data, the changes in the concentrations of intracellular GroPIns suggest a role for this metabolite in the regulation of both myeloid and lymphoid cell differentiation.

GroPIns4P PRODUCTION BY MACROPHAGES

In addition to GroPIns, its monophosphorylated derivative, GroPIns4*P*, has been detected in several cell types, including macrophages [(2); our unpublished observations)]. $PLA_2IV\alpha$, is responsible for the production of both GPIs (i.e., GroPIns and GroPIns4*P*), based on the relative availability of the respective lipid precursors (PtdIns and PtdIns4*P*, respectively) [(4, 5); our unpublished observations]. A conundrum in these studies are the technical limitations in the accurate measurement of these metabolites. For example, the relatively high levels of GroPIns make the detection of small increases more difficult, which might explain why increases in GroPIns4*P* (which generally represents <3% of the total GPIs) do not always appear to be paralleled by increases in GroPIns. The rapid metabolism of GroPIns4*P* is a further drawback for precise determinations of its levels, although this was partially overcome by performing GroPIns4*P* measurements in the presence of orthovanadate, a general phosphatase inhibitor (2), which made it possible to monitor GroPIns4*P* increases in macrophages exposed to LPS (our unpublished observation). At variance with macrophages, no detectable production of GroPIns4*P* can be observed in T-cells (16).

THE GPIs AS MODULATORS OF T-CELL FUNCTIONS: FACTS AND HYPOTHESES

GroPIns4P PROMOTES ACTIN POLYMERIZATION IN T-LYMPHOCYTES

Cortical actin rearrangements, which are regulated by the Rho family of small GTPases (23), are crucial for a number of processes that orchestrate T-lymphocyte activation and motility (24, 25). Exogenous administration of GroPIns4*P* to fibroblasts induces the formation of actin ruffles and stress fibers by modulating the activity of Rac and Rho (26, 27), suggesting a potential role for this phosphoinositide derivative in the regulation of the actin cytoskeleton in other cell types. Treatment of both Jurkat T-cells and peripheral blood lymphocytes from healthy donors with GroPIns4*P* induces indeed actin polymerization (16), suggesting that the processes involving F-actin dynamics, including redistribution of components associated with lymphocyte motility and immune synapse assembly might be modulated by GroPIns4*P*.

The ability of GroPIns4*P* to promote actin polymerization in T-cells stems, at least in part, from its ability to induce the phosphorylation of the GDP/GTP exchanger Vav (16), which controls the activation of Rac and Cdc42 in hematopoietic cells (28). These data are consistent with the finding that GroPIns4*P* triggers a signaling cascade in fibroblasts that leads to plasma-membrane translocation of Tiam1, a Rac-specific GDP/GTP exchanger in these cells (27). This activity provides a mechanistic explanation of the agonistic effects of GroPIns4*P* on the actin cytoskeleton dynamics.

The ability of GroPIns4*P* to promote Vav activation suggests the possibility that it may also modulate gene expression. Vav initiates indeed a pathway involving recruitment to active Rac of the serine/threonine kinase Pak1, which triggers the activation of p38 and JNK that directly or indirectly activate a number of transcription factors (29). Consistent with this notion, T-cell treatment with GroPIns4*P* resulted in activation of both p38 and JNK, with a similar time course as Vav (16).

VAV ACTIVATION BY GroPIns4P REQUIRES LCK

Tyrosine phosphorylation of Vav is mediated by the cooperative activity of Syk and Src family protein tyrosine kinases (PTKs) (28). We have shown that treatment of T-cells with GroPIns4*P* results in enhanced Lck activity [(16); **Figure 2**]. Although other mechanisms may account for Vav activation, this event is likely to be causal to the agonistic activity of GroPIns4*P* on Vav activation and the resulting actin cytoskeleton rearrangements. GroPIns4*P* fails indeed to trigger Vav phosphorylation in the Lck-deficient Jurkat T-cell variant JCaM.1 (16, 30). Moreover, GroPIns4*P* triggers Src phosphorylation in fibroblasts, which is required for plasma-membrane translocation of Tiam1 (27). Lck activation, as well as Src activation, is not a direct effect of GroPIns4*P*, at least as assessed *in vitro* (16). Unfortunately, while these studies have restricted the field, no final mechanism of action for the GPIs can be postulated, until a proteomic approach to identify direct interactors can be completed.

GroPIns4P TARGETS LCK-DEPENDENT SIGNALING BY MODULATING cAMP

In quiescent T-cells Lck is kept in an inactive state by the inhibitory kinase Csk, which becomes phosphorylated and activated by the cAMP-dependent serine/threonine protein kinase A (PKA) (31).

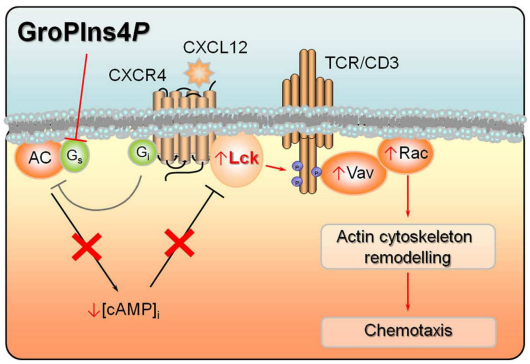

FIGURE 2 | Convergence of signals by CXCL12 and GroPIns4*P* on adenylate cyclase activity. CXCL12 binding to its cognate receptor CXCR4 leads to activation of Lck, which stably interacts with the receptor. Lck in turn phosphorylates multiple tyrosine residues in the cytosolic tails of the TCR/CD3 complex, thereby triggering a signaling cascade involving Vav activation and eventually actin cytoskeletal rearrangements. Lck activity is further potentiated by CXCR4-dependent stimulation of Gi protein which, by inhibiting adenylate cyclase, lowers the levels of intracellular cAMP, resulting in decreased PKA-dependent activation of Csk, a negative regulator of Lck. GroPIns4*P* potentiates migratory signaling by CXCL12 by blocking the activity of Gs protein, thereby further lowering adenylate cyclase activity and hence the intracellular cAMP levels and contributing to Lck activation. Phosphorylation states and events are shown as small blue circles. Activation events are shown as arrows, inhibition events as truncated lines.

We showed that treatment of Jurkat T-cells with GroPIns4*P* results in a decrease in the levels of cAMP, leading to impaired PKA activation and Csk phosphorylation. Hence the agonistic activity of GroPIns4*P* on Lck activation and downstream signaling results from its ability to inhibit cAMP production (16). These results are consistent with the finding that GroPIns4*P* (but not GroPIns or GroPIns4,5P_2) inhibits adenylate cyclase activity in other cell types (32, 33). It is noteworthy that cAMP/PKA-mediated modulation of molecules downstream of Lck may also contribute to its effects on F-actin dynamics in T-cells (34), including Rho itself, which is phosphorylated by PKA on an inhibitory residue (35, 36).

The ability of GroPIns4*P* to activate Lck, which is responsible for initiation of the T-cell receptor (TCR) signaling cascade, strongly suggests that other signaling events, in addition to the Vav/p38/JNK pathway, might be triggered by GroPIns4*P*. One of the key targets of Lck both in TCR (37) and chemokine receptor signaling (38) is the PTK ZAP-70, which couples these receptors to multiple signaling pathways, including the Ras/MAP kinase pathway (39). GroPIns4*P* induces indeed the activation of ZAP-70 and the adaptor Shc (16), which interacts with, and becomes phosphorylated by ZAP-70 in response to TCR engagement and contributes to Ras activation by recruiting the Grb2/Sos complex (40). Consistent with its ability to promote Shc phosphorylation, GroPIns4*P* activates Erk1/2. These effects of GroPIns4*P* are crucially dependent on Lck, as they fail to occur in JCaM.1 cells (16).

GroPIns4P ENHANCES CXCR4 SIGNALING

The ability of GroPIns4*P* to promote actin polymerization in T-cells profoundly influences their responses to chemokines. GroPIns4*P* (but not the other GPIs) enhances indeed CXCR4-dependent chemotaxis toward CXCL12 (16), a chemokine that regulates lymphocyte homing to secondary lymphoid organs under homeostatic conditions (39). Interestingly, the two signals may converge, at least in part, on adenylate cyclase. In fact, CXCL12-mediated activation of CXCR4 promotes the release of the α-subunit of heterotrimeric inhibitory G protein (G_i), thereby inhibiting adenylate cyclase activity (39). Moreover, in Jurkat T-cells GroPIns4*P* reverses the cAMP-elevating activity of cholera toxin, which ADP-ribosylates the α-subunit of stimulatory G proteins (G_s) resulting in their persistent activation. This indicates that GroPIns4*P* is able to decrease cAMP production and cAMP/PKA-dependent Csk activation by inhibiting G_s. These mechanisms can account for the additive effect of CXCL12 and GroPIns4*P* on Lck activation [(16); **Figure 2**].

Recent evidence supports a crosstalk between the TCR and CXCR4. The TCR is indeed transactivated by CXCR4 which then uses the TCR machinery to elicit and potentiate downstream signaling (38, 41). Similar to other G-protein-coupled receptors, CXCR4 directly interacts with and activates Lck. The association of CXCR4 with the TCR at the cell surface allows Lck to localize in close contact with the intracellular domains of the TCR/CD3 complex and phosphorylate CD3ζ, thereby triggering downstream signaling (39). By promoting Lck activation GroPIns4*P* might potentiate the ability of CXCR4 to transactivate the TCR and hence enhance the signaling cascades leading to T-cell chemotaxis, including the pathway leading to the Lck-dependent recruitment of Shc to the CXCR4/TCR dimer.

GroPIns4P PRODUCTION BY BYSTANDER CELLS: A POTENTIAL MECHANISM TO CONTROL T-CELL RESPONSES IN THE LOCAL MICROENVIRONMENT

How can the evidence obtained using exogenously added GroPIns4*P* be related to the physiological context of T-cell trafficking? Measurements of the GPI levels in Jurkat T-cells using a quantitative mass spectrometry approach revealed that these cells are among those with the lowest intracellular levels of GroPIns (15). At variance with T-cells, macrophages produce large amounts of GPIs in response to pro-inflammatory stimuli (2, 5), generating a gradient for their transporter-mediated release into the extracellular medium (2, 15, 42). We propose that these macrophage-derived GPIs may act as paracrine factors for lymphocytes. In this scenario, GPIs produced at the site of infection would enhance effector T-cell recruitment, and thereby contribute to bacterial clearance. Interestingly, among the highest GPI producers are certain tumor cells (2, 43). The release of these metabolites, combined with chemotactic signals provided by the tumor microenvironment (44), could be hypothesized to promote T-cell infiltration and activation of anti-tumor immunity.

GPIs AND T-CELL FATE: A WORKING HYPOTHESIS

By promoting the activation of Lck, exogenous administration of GroPIns4*P* to T-lymphocytes triggers a Rho-family dependent pathway that is integrated with chemokine receptor signaling to potentiate T-cell chemotaxis (16). Given the central role of Lck as the initiator kinase in TCR signaling (37), it can be hypothesized that GroPIns4*P* has the ability to modulate T-cell activation.

Lck has also been implicated in the regulation of T-cell apoptosis induced by a wide range of stimuli, including prolonged TCR stimulation (45) and treatment with sphingosine (46), and it is also an essential component of the signaling pathways that control Ca^{2+}-mediated T-cell apoptosis, which involve both the conformational activation of Bax and the expression of proapoptotic Bcl-2 family members (47). How TCR engagement can lead to cell fates as diverse as activation, anergy, and apoptosis is one of the fundamental and as yet open questions in immunology. Investigating the effects of GroPIns4*P*, as well as of the other GPIs, on these processes, may provide valuable information on how TCR signaling is fine-tuned by these phosphoinositide metabolites to elicit different biological outcomes.

CONCLUSION

The interesting scenarios opened by the studies outlined in this review using exogenously added GPIs underscore the need to address the physiological function of endogenous GPIs in the modulation of immune cell function. It has however to be underlined that the results summarized in the present review, while in part validated on normal peripheral T-cells, have been largely obtained by exogenous administration of GroPIns4*P* to Jurkat T-cells, which are known to be defective in the activity of enzymes critically involved in lipid signaling, such as PTEN (48). We have therefore to take into account a possible impact of this defect on global lipid signaling, which might also involve GPI metabolism. Since, among immune cells, the major producers of GPIs are macrophages, it will be interesting to assess the impact of this physiological source of exogenous GPIs on normal T-cells in co-culture experiments. Unfortunately the study of immune modulation by GPIs *in vivo*, which in principle could be approached using $PLA_2IV\alpha^{-/-}$ mice, is prevented by lack of effect of $PLA_2IV\alpha$ deficiency on GPI synthesis, at least in this model. This finding is likely to be accounted for by the fact that in $PLA_2IV\alpha^{-/-}$ cells GPI synthesis is taken over by the calcium-independent PLA_2VI, suggesting that a compensatory mechanism is activated under these conditions (15). A way to circumvent this problem in the future could be to generate an inducible knockout mouse lacking $PLA_2IV\alpha$ expression in macrophages and/or T-cells, or alternatively to modulate the expression of the specific GDEs.

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Regulation of Ras exchange factors and cellular localization of Ras activation by lipid messengers in T cells

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The Ras-MAPK signaling pathway is highly conserved throughout evolution and is activated downstream of a wide range of receptor stimuli. Ras guanine nucleotide exchange factors (RasGEFs) catalyze GTP loading of Ras and play a pivotal role in regulating receptor-ligand induced Ras activity. In T cells, three families of functionally important RasGEFs are expressed: RasGRF, RasGRP, and Son of Sevenless (SOS)-family GEFs. Early on it was recognized that Ras activation is critical for T cell development and that the RasGEFs play an important role herein. More recent work has revealed that nuances in Ras activation appear to significantly impact T cell development and selection. These nuances include distinct biochemical patterns of analog versus digital Ras activation, differences in cellular localization of Ras activation, and intricate interplays between the RasGEFs during distinct T cell developmental stages as revealed by various new mouse models. In many instances, the exact nature of these nuances in Ras activation or how these may result from fine-tuning of the RasGEFs is not understood. One large group of biomolecules critically involved in the control of RasGEFs functions are lipid second messengers. Multiple, yet distinct lipid products are generated following T cell receptor (TCR) stimulation and bind to different domains in the RasGRP and SOS RasGEFs to facilitate the activation of the membrane-anchored Ras GTPases. In this review we highlight how different lipid-based elements are generated by various enzymes downstream of the TCR and other receptors and how these dynamic and interrelated lipid products may fine-tune Ras activation by RasGEFs in developing T cells.

Keywords: T cell, signaling, lipids, Ras, SOS, RasGRP, LAT, P38

NON-ONCOGENIC Ras ACTIVATION FIRST OBSERVED IN T LYMPHOCYTES

Ras is a membrane-bound small GTPase that plays a pivotal role in transducing responses to diverse extracellular signals that impact various cellular processes, prominently cell proliferation, differentiation, apoptosis (1). Ras cycles between a GTP-associated active state (Ras·GTP) and GDP-bound inactive state (Ras·GDP). In both the Ras·GDP and Ras·GTP states the nucleotide is very tightly bound (2–4) and for Ras activation to occur Ras guanine nucleotide exchange factors (RasGEFs) need to loosen the grip of Ras on the bound nucleotide, stabilizing nucleotide-free Ras that stochastically but preferentially associates with GTP, because GTP is present in the cell in higher concentrations than GDP (5). Reciprocally, GTP hydrolysis is critical for inactivation from Ras·GTP to Ras·GDP and Ras' modest intrinsic rate of GTP hydrolysis requires the hydrolysis-augmenting action of RasGAPs (Ras GTPase activating proteins) (**Figure 1**).

The physiological importance of Ras' GTPase activity was recognized in the late 80s through the detection and biochemical characterization of GTPase impairing Ras mutations commonly found in various human tumor tissues (6). Ras·GTP is a potent signaling hub, connecting to many downstream effector molecules like RAF, PI3K, and RalGDS. The best-characterized signaling cascade is the Ras·GTP-RAF-MEK-ERK pathway (4, 7, 8). In cells without mutations in Ras only a small portion of the total amount of Ras is GTP-loaded following receptor stimuli, which makes detection more challenging. In the early 90s Doreen Cantrell's group first showed Ras activation (or Ras·GTP loading) in normal T lymphocytes that were stimulated with the interleukin 2 (IL2) cytokine or a phorbol ester, agents that were known to induce lymphocyte proliferation (9, 10). The physiological significance of biochemical signals transduced by an intact Ras-RAF-MEK-ERK pathway in lymphocytes was subsequently shown through transgenic expression of mutant Ras- and MEK-alleles in thymocytes; for example, expression of dominant-negative H-Ras^{S17N} under the control of *lck* promoter or catalytically inactive MEK-1 perturbs positive selection of developing thymocytes (11, 12).

Research over the past two decades has revealed many intricate ways of regulated Ras activation, not only in lymphocytes but also in other cell types. In this review we will discuss the role of lipid messengers in regulating the Son of Sevenless (SOS) and RasGRP RasGEF families. We will focus on recent findings related to lipid-RasGEF regulation, recent insights from novel mouse models, as well as on the ongoing debate of the cellular compartment or location of Ras activation. For additional information on the RasGEF family of exchange factors we refer to previous review articles (8, 13–15).

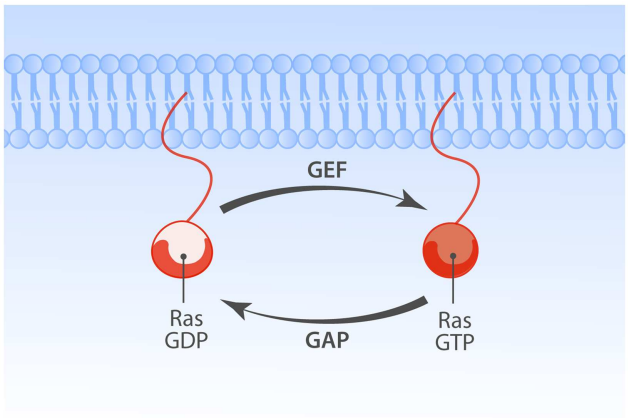

FIGURE 1 | Regulation of Ras family proteins. The Ras GTPases cycle between GDP-bound inactive and GTP-bound active forms. Activation of Ras is regulated by the balance of opposing actions of two classes of Ras regulatory enzymes. Guanine nucleotide exchange factors (GEFs) promote GTP-bound Ras state by enhancing exchange of GDP with GTP. GTPase activating proteins (GAPs) enhance slow rate of intrinsic Ras GTPase activity, promoting the inactive GDP-bound state of Ras.

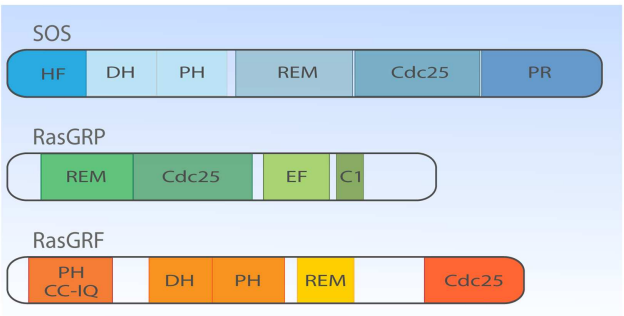

FIGURE 2 | Structural domain organization of three families of RasGEFs expressed in T cells. Cartoon highlighting the general protein domains in the three families of RasGEFs: SOS, RasGRP, and RasGRF. Cdc25, Cdc25 homology domain; DH, Dbl homology domain; HF, N-terminal histone-like fold; PH, Pleckstrin homology domain; PR, C-terminal PR domain; REM, Ras exchange motif; EF, Ca^{2+}-binding EF hand; C1, DAG-binding C1 domain; CC-IQ, coiled coil – ilimaquinone domain. Protein size is drawn to approximate scale based on SOS1, RasGRP1, and RasGRF1 (53).

THE PLAYERS; THREE FAMILIES OF Ras GUANINE NUCLEOTIDE EXCHANGE FACTORS

The earlier-mentioned dominant-negative Ras approach established a critical role for Ras in lymphocytes. Data from numerous laboratories have meanwhile demonstrated that dominant-negative Ras^{S17N} exerts its blocking action mainly by usurping and blocking RasGEFs [although other features of Ras^{S17N} probably contribute to its inhibitory action (16, 17)]. Thus, the ability of dominant-negative Ras^{S17N} to affect lymphocyte biology not only highlights the importance of Ras but points also to a critical role of GEFs.

If we fast-forward roughly two decades, we now know that lymphocytes can simultaneously express three types of RasGEF proteins (**Figure 2**). The overlapping expression profiles create the impression of seemingly redundant and unnecessary complex mechanisms to couple antigen receptor stimulation to Ras activation. However, distinct lymphocyte developmental defects in mice deficient for unique RasGEFs argue for specialized functions for each RasGEF (18–20). We will cover the mouse phenotypes in more detail in subsequent paragraphs and will first focus on the different protein domains in the three RasGEF families [also reviewed in Ref. (5, 8)].

SON OF SEVENLESS

There are two members in SOS-family RasGEFs, SOS1 and SOS2. Structurally, the SOS protein is composed of six domains that have distinct functional importance: starting from the N-terminus, the histone-like fold (HF), the Dbl homology domain (DH), the Pleckstrin homology (PH) domain, the Ras exchange motif (REM), the Cdc25 homology domain, and the proline-rich (PR) domain (**Figures 2** and **3**). The naming of HF comes from structural resemblance to histone 2 dimer H2a-H2b, and HF mediates lipid interaction with phosphatidylinositol 4,5-bis phosphate [$PI(4,5)P_2$, hereafter PIP_2] or phosphatidic acid (PA) (21). The DH domain is a functional domain commonly found in Rho family GEFs, suggesting SOS may also have Rho-specific GEF function in addition to the more established RasGEF activity (22, 23). PH domains are lipid/protein-interacting domains (24). The PH domain of SOS has an auto-inhibitory function, that is regulated by interaction with membrane lipids such as PIP_2 or PA (25–29). REM-Cdc25 domains make up the RasGEF catalytic core of SOS and all other RasGEFs. Unique to SOS, its catalytic core contains two distinct Ras-binding sites: one for GDP/GTP exchange and the other for allosteric regulation of SOS by Ras (30, 31). The C-terminal PR domain is the only segment of SOS that remains to be structured for analysis. Functionally, the PR domain contains multiple PR motifs that can bind SH3 domain-containing proteins such as the SH2-SH3-SH2 adapter Grb2 (32, 33), the p85 subunit of PI3kinase (34), PLCγ1 (35–38), and Avi1/E3b1 (39). In addition, the PR domain contains multiple documented phosphorylation sites of ERK and probably other kinases (40–44), spiked in between the PR stretches that are, at least in part, postulated to play a role in feedback control of SOS activity.

RAS GUANINE NUCLEOTIDE RELEASING PROTEINS

Much less is known about the function of the domains or even the identity of domains in the RasGRP RasGEFs. To date, there is no RasGRP structure and we are therefore limited to make predictions based on amino acid sequence. There are four RasGRP proteins, RasGRP-1 through RasGRP-4, with specific expression profiles and nuances in biochemical function. All RasGRP's contain a central catalytic core consisting of the catalytic REM-Cdc25 cassette. Sequence divergency between the RasGRP and SOS REM-Cdc25 cores predicts that RasGRPs are not regulated through an allosteric activation mechanism. Although RasGRP2 contains the REM-Cdc25 core and early studies indicated RasGEF activity (45), it is generally accepted that RasGRP2 functions as a GEF for the small GTPase Rap (46). Analogously, all four proteins are predicted to have a C1 domain positioned C-terminal of the catalytic core, but

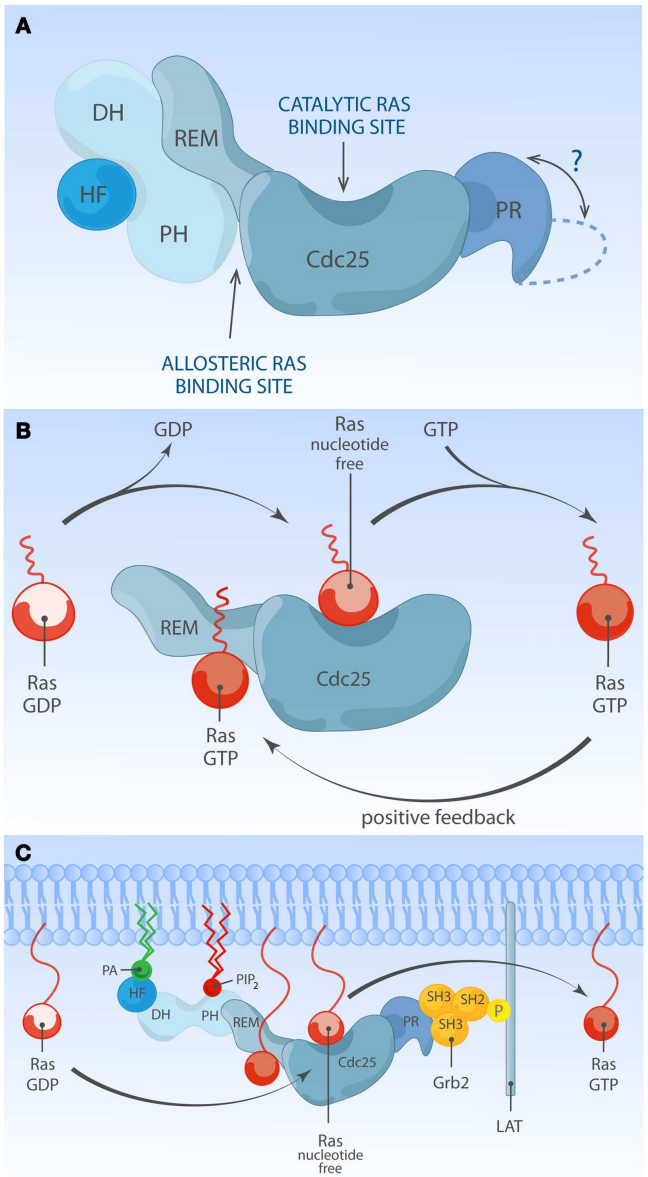

FIGURE 3 | Multiple membrane-derived signals determine the RasGEF activity of SOS. (A) Model of inactive SOS. In the inactive state, SOS's DH-PH domains obscure the allosteric Ras-binding pocket. Without engagement of the allosteric pocket by Ras·GTP, SOS only shows low reactivity for Ras·GDP at the catalytic binding site. The HF docks itself to a helical linker region (not depicted) between PH and REM domains, further stabilizing auto-inhibited state of SOS. The protein structure of the C-terminal proline-rich domain has not been determined to date. **(B)** Model of allosteric activation of SOS. Ras·GTP binding to the allosteric site enhances SOS exchange activity by increasing Ras-binding affinity for the catalytic pocket, establishing a positive feedback mechanism. Other SOS domains are omitted for simplicity. **(C)** Model of full activation of SOS. Full activation of SOS requires the integration of multiple membrane-derived signals. Grb2-mediated membrane recruitment of SOS to phosphorylated LAT is thought to be one of the initial membrane recruitment mechanisms. Membrane phospholipids such as PIP_2 and PA interact with HF and PH domains, and these interactions further relieve auto-inhibition state of SOS, allowing efficient access of Ras to both the allosteric and catalytic sites.

again, RasGRP2 appears to be most divergent in that its C1 domain does not bind diacylglycerol (DAG) (47) and RasGRP2 protein does not translocate to the membrane when cells are stimulated with DAG analogs (48). A third shared domain in all RasGRP proteins is the pair of EF hands that occupies an interesting position in the protein, sandwiched between the catalytic core and the C1 domain (**Figures 2** and **4**). EF hands typically come in pairs with each hand binding one calcium ion (49, 50). However, not all EF hands bind calcium. For instance, RasGRP1 with two predicted EF hands based on the amino acid sequence can only bind one calcium ion with one EF hand, not with both (51). Close examination of the sequence similarities and divergence in the EF hand domains of all RasGRP proteins (not shown) tells us that there are likely going to be substantial differences in the ways that the different RasGRP's are regulated by calcium. Thus, the four RasGRP proteins demonstrate specific biochemical regulatory mechanisms and activities that have likely evolved over time to establish their individual exchange functions in the specific cell types where they are expressed. In this review we will not cover the differences between the RasGRPs in much more detail, instead we refer you to an excellent review by Stone (15) and one on cancer (52).

RAS GUANINE NUCLEOTIDE RELEASING FACTOR

More closely related to SOS than RasGRP are RasGRF's; RasGRF-1 and RasGRF-2 make up this family of proteins with multiple domains [reviewed in Ref. (53)]. Similar to the two other RasGEF family proteins, RasGRF proteins contain a REM-CDC25 catalytic core domain. Uniquely, RasGRFs contain two PH domains; one at the N-terminus (PH1) and the other PH in tandem with the DH domain (PH2), similar to the configuration of the DH-PH domain of SOS-family proteins (**Figure 2**). PH1 cooperates to promote stimulation-dependent membrane localization of RasGRF in fibroblasts, probably through interaction with membrane lipid (53–55). The coiled-coil (CC) domain is known to mediate protein oligomerization (56), whereas the ilimaquinone (IQ) domain mediates calmodulin binding (57). In cooperation with the PH1 domain, CC and IQ domains notably mediate the interaction with a MAPK p38 scaffold protein IB2/JIP2 in COS7 cells (58), which is interesting because the DH-PH domain of RasGRF has GEF activity toward Rac (59, 60) indicating that RasGRF may efficiently link Rac to the p38 pathway through the IB2/JIP2 scaffold protein (58).

EXPRESSION PATTERNS OF THE EIGHT RASGEF GENES

The RasGRP and RasGRF families of exchange factors have tissue-specific expression patterns whereas SOS proteins are ubiquitously expressed (15). For instance, RasGRP1 is expressed in dynamic patterns in developing T cells (20, 61), in the brain (46), and in primary keratinocytes (62). RasGRF1 and RasGRF2 are predominantly expressed in the central nervous system (63). In addition, RasGRF2, but not RasGRF1, is expressed in T cells (64). Analyses of *rasgrf2*-deficient mice revealed that this RasGEF play a critical role in the activation of NFAT target genes in T cells (64). However, T cell development is normal in *Rasgrf2*$^{-/-}$ mice, and Rasgrf2 appears to have only limited activity toward Ras-ERK in T cells (64). We will therefore limit ourselves to the regulation of SOS and RasGRP here. Significantly, these two distinct types of RasGEFs cooperate to establish robust yet controlled activation of Ras

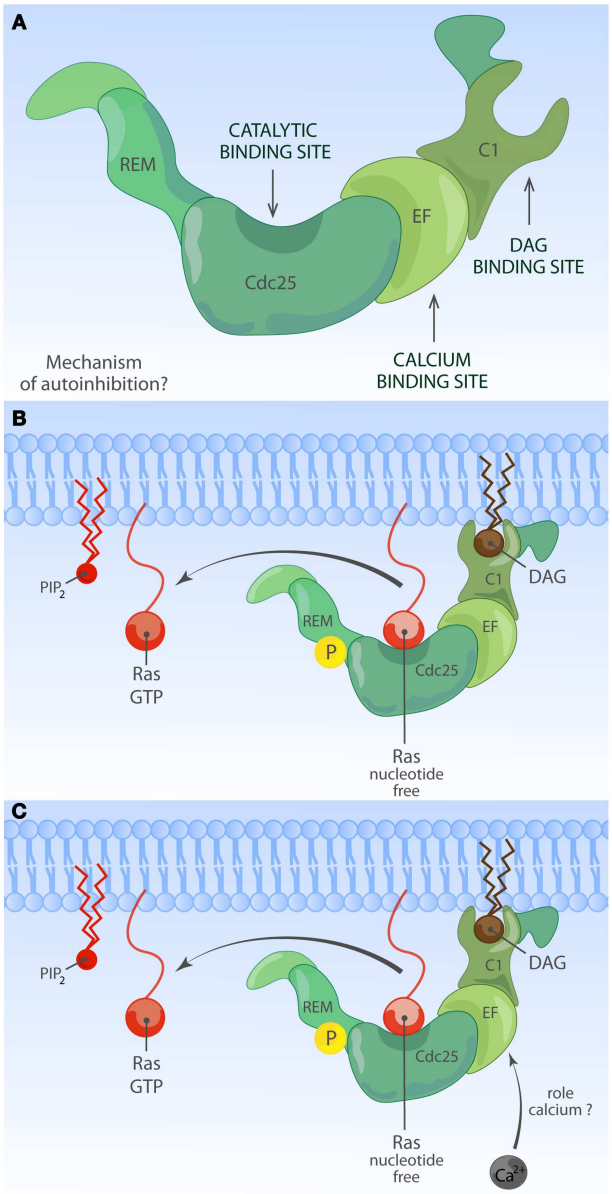

FIGURE 4 | Activation of RasGRP. **(A)** Depiction of RasGRP with its protein domains. RasGRPs must be controlled to prevent spurious Ras activation but the exact mechanism of auto-inhibition is unknown. Roles for various domains C-terminal of the Cdc25 domain to limit membrane recruitment of RasGRP have been proposed. **(B)** DAG-regulated membrane recruitment of RasGRP. Receptor-induced generation of diacylglycerol (DAG) results in efficient membrane recruitment on RasGRP1, RasGRP3, and RasGRP4 where these RasGEFs can encounter Ras·GDP to activate it to Ras·GTP. RasGRP1 and RasGRP3 are known to be phosphorylated on a conserved threonine residue at the very start of the Cdc25 domain, which enhances their catalytic activity through an unknown mechanism. RasGRP2 does not efficiently bind DAG and must have a different membrane-recruiting mechanism. **(C)** Other regulatory mechanisms for RasGRP. Amino acid sequence homologies predict that RasGRPs lack and allosteric Ras-binding pocket as the one observed for SOS. RasGRP proteins contain EF hands, structure that can often bind calcium. Calcium has been implicated in the recruitment of RasGRP1 to the membrane but nuances appear to exist in different cell types. It is not known if other lipid moieties such as PIP_2 can regulate the activity or residence time of RasGRP1 at the membrane.

and Ras' RAF-MEK-ERK effector pathway (65, 66). In response to T cell receptor (TCR) stimulation, both RasGRP1 and SOS are recruited to the membrane where they encounter membrane-anchored Ras and both convert Ras·GDP to Ras·GTP. Why is it then that knockout mouse models for SOS1 and RasGRP1 show different impairments in terms of thymocyte selection and T cell development (20, 61, 67)?

AUTO-INHIBITION OF SOS RasGEFs

Ample structural and cellular studies indicate that catalytic activity of SOS1 is self-limited by an intramolecular auto-inhibitory mechanism which involves multiple internal protein domains. Auto-inhibition can be relieved by membrane signals from proteins and lipid species. The physiological relevance of auto-inhibition of SOS1 is highlighted by a clinical condition called Noonan syndrome (NS). NS is a relatively common autosomal developmental abnormality and RASopathy, a disease that is caused by germline mutations in molecules leading to modestly increased Ras signaling (68, 69). NS is genetically heterogeneous: the majority of mutations are associated with PTPN11, K-Ras, N-Ras, SOS1, B-Raf, Raf-1, SHOC1, and CBL (69). Among eight NS-associated genes, missense mutations in SOS1 are identified in about 10% of NS cases (69–73). Most NS-associated SOS1 mutations are predicted to relieve auto-inhibiting structural constraints within SOS1, allowing increased signal output through the Ras pathway. Indeed, several NS-associated *SOS1* mutant alleles (R552G, E108K, W729L, and E846K) have been experimentally characterized *in vivo*, showing increased Ras·GTP accumulation and ERK activation at basal state or upon stimulation (70, 71, 74, 75). These findings visibly illustrate that normal SOS1 function is tightly regulated and highlight the clinical relevance of such regulation (**Figure 3A**). These observed defects in fine-tuning of Ras activity control in NS cells are also likely to impact on the patient´s immune biology, because patients with gain-of-function mutations in Ras proteins are at a higher risk of developing autoimmune disorders (76–79). In the following few sections, we will review the literature on normal SOS regulatory mechanisms and how membrane-based signals from proteins and phospholipids influence the activation status of SOS.

MEMBRANE RECRUITMENT OF SOS BY Grb2: INITIAL STEP IN SOS ACTIVATION

T cell receptor stimulation leads to rapid activation of Src family kinases and the Syk family kinase ZAP70. ZAP70 phosphorylates the adapter LAT, a key scaffold to which various downstream signal transducers are assembled, including molecules that are coupled to Ras-MAPK pathway activation (80). Prior to cell stimulation, most SOS is found in the cytoplasmic compartment, constitutively bound to the SH3-SH2-SH3 domain-containing adapter Grb2. Upon stimulation, SOS rapidly localizes to the plasma membrane (PM) (32, 33, 81, 82). SOS1 membrane targeting is an essential event for SOS-Ras activation and is mediated by binding of the SH2 domain of Grb2 (with SOS1) to phosphorylated tyrosine residues of LAT (82). A truncated SOS1 variant incapable of Grb2 binding is still functional as a RasGEF but can activate Ras only if targeted elsewise to the membrane, indicating that membrane recruitment is an essential step in ligand-dependent activation of SOS (83). Unlike Ras, lipid modification of SOS was never been reported.

Therefore, Grb2-mediated membrane anchorage has been viewed as the key regulatory mechanism of SOS GEF signal output.

However, the traditional view that Grb2 association is dominant or even essential for SOS1 membrane targeting has also been challenged. Expression of C-terminally truncated SOS1 incapable of Grb2 binding has been documented to have comparable or even better Ras-ERK signal responses compared to full-length SOS1 (84–86). Similarly, $SOS^{\Delta C}$, a C-terminally truncated SOS mutant lacking residues 1050–1333 becomes recruited to the membrane in response to serum stimulation, indicating that Grb2 is not the only mechanism for ligand-dependent SOS1 membrane targeting (29). These studies may collectively imply that Grb2 is a redundant mechanism for stimulation-dependent SOS membrane localization and subsequent SOS activation. However, little attention is given to the physiological relevance of the protein levels of the C-terminal truncated SOS1 variant examined in these studies and time kinetics of Ras-ERK response. It is very plausible that Grb2 is important and a major membrane anchorage mechanism when physiological levels of SOS1 are available to the activated ligand. Supporting this notion, structural studies and recent mouse embryonic stem cell (mESC) study demonstrate that, besides Grb2-mediated membrane recruitment, the SOS1 activity is determined by summation of weak to moderate membrane protein and lipid interactions mediated by multiple protein domains of SOS1 (87).

ALLOSTERIC ACTIVATION OF SOS; A POSITIVE FEEDBACK LOOP

The SOS1-mediated nucleotide exchange rate on Ras is 500-fold higher when Ras is membrane-bound compared to when Ras activation is measured in solution (88), supporting a view that ligand-dependent membrane recruitment of SOS1 not only exists to promote the chance of substrate encounter but is also instrumental to enhance SOS1 enzymatic activity. One hint for the existence and identity of additional membrane signals regulating SOS1 came from structural studies by the Bar-Sagi and Kuriyan groups. Unexpectedly SOS1 was found to be associated with two discrete Ras molecules, forming a 2:1 ternary complex between two Ras molecules and one SOS1 molecule. One Ras molecule serves as a substrate and is bound at its catalytic pocket within the Cdc25 domain, while the second non-substrate Ras occupies the allosteric site in the REM domain (31). Occupation of the allosteric site by Ras·GTP results in conformational change stabilizing SOS1 catalytic pocket and stimulates *in vitro* nucleotide exchange activity by ∼75-fold (89, 90). In support of this notion, a SOS1 mutant unable to bind to Ras at allosteric site (W729E) shows reduced affinity for Ras at the catalytic site and has low *in vitro* activity (89). The allosteric Ras-binding pocket shows 10-fold higher affinity for GTP-loaded Ras than Ras·GDP. This preferential affinity for Ras·GTP endows SOS1 to sense the activation status of Ras at the membrane and establishes a positive feedback regulation (**Figure 3B**) (31, 91). Ectopic expression studies provided *in vivo* evidence of allosteric regulation of SOS1 in COS-1 cells (89, 91) or Jurkat cells (65, 66). Recently, allosteric mutant-SOS1 reconstitution into SOS-deficient mESC (87) and DT40 B cells (92) provided more definitive proof of allosteric SOS1 activation regulating the output through the Ras-ERK pathway. In addition to enhancing catalytic activity of SOS, allosteric Ras·GTP binding could potentially affect SOS residence time at the PM by providing an additional membrane anchor for SOS1 other than Grb2 binding.

REGULATION OF SOS BY MEMBRANE LIPIDS

Current evidence argues that allosteric Ras binding to SOS1 is such a pivotal step that SOS stays inactive unless Ras·GTP is bound at the allosteric site (93). Then, how has SOS1 evolved to limit spontaneous signaling yet allow for controlled allosteric activation near the membrane interface? In this regard, N-terminal SOS domains play a critical role in regulating SOS1 activation in the context of membrane proximity by sensing membrane lipids.

One membrane lipid sensing N-terminal regulatory unit is the tandem DH and PH domain. *In vitro* and *in vivo* studies identified DH-PH domain being important for membrane-proximal SOS regulation (29, 84, 87, 93). DH domain is commonly found with GTP exchange factors (discussed later). In SOS, the DH domain serves as a gatekeeper preventing promiscuous access to the allosteric Ras-binding pocket. In its auto-inhibited state, SOS1 DH domain blocks the allosteric pocket from Ras binding, which has a critical impact on SOS1's catalytic pocket. Without allosteric activation the catalytic pocket is not fully receptive to accommodate Ras·GDP and the helical hairpin of SOS1 is not in the correct orientation to dislodge GDP from Ras (89, 93). PH domain is generally known for protein or lipid interactions (55). The PH domain of SOS1 was shown to have affinity for PIP_2 (25–28) or PA (29). The auto-inhibiting DH domain can be released by electrostatic interaction of membrane PIP_2 or PA with positively charged residues within the PH domain (29, 93). Therefore, lipid-DH-PH interactions facilitate re-orientation of SOS1 at the membrane interface, allowing allosteric Ras binding (**Figure 3C**). In support, addition of cell-permeable PA to COS-1 cells is sufficient to induce GTP loading of Ras, and charge-inversion mutations of H475E and R479E in SOS1 abolish PA interaction and PA-induced Ras·GTP loading response (29). Similarly, two different basic residues (K456 and R459) within the PH domain interact with PIP_2 (93). The biological significance of PIP_2-PH domain interaction during mESC differentiation was elegantly demonstrated in a recent report from Tony Pawson's group (87).

Located upstream of DH-PH domains, the HF is an evolutionarily conserved segment (residue 1–191) resembling dimerized histone (21). Based on structural studies, this HF docks itself into the helical linker region of SOS1, located between DH-PH domains and catalytic segment (REM-Cdc25), ensuring SOS autoinhibition by blocking allosteric activation and by stabilizing a closed conformation of SOS (88, 94). HF interacts with membrane lipids such as PA and PIP_2, and HF-lipid interaction reverses autoinhibitory docking, allowing allosteric and catalytic Ras binding at distal and proximal Ras-binding sites (75, 88). Electrostatic charge distribution at the phospholipid-interacting interface of HF appears to be finely tuned by charge neutralization, e.g., the negatively charged residue E108 is surrounded by patches of basic residues (75). Disturbing charge balance by offsetting positive charges leads to reduced Ras-ERK activation in COS-1 and mouse ES cells (75, 87). Additionally, a negative charge neutralization mutation (E108K) is found to be associated with a hyperactive SOS1 allele of human NS (71, 75).

There are some inconsistencies in the lipid species recognized by SOS1's N-terminal regulatory domains (29, 75, 88, 93). This discrepancy might arise from the variability in the presence of regulatory domains or post-translational modifications of the SOS1 proteins investigated. Perhaps more significant, membrane lipids are also dynamically regulated during cell activation processes (reviewed in Krishna and Zhong (95) in this Research Topic and by Sauer and Cooke (96)). Perhaps, the reported discrepancies regarding the role of lipid species may reflect heterogeneous lipid patterns in distinct cellular backgrounds and the involvement of different lipids at different stage of SOS1 activation.

Taken together, studies *in vitro* and *in vivo* support the view that N-terminal HF and DH-PH domains serve as membrane lipid sensing regulatory segments. On one hand, lipid mediated regulation of SOS1 leads to juxtaposition of SOS1 to substrate/effector. On the other hand, the regulatory domains also contribute to prevent spontaneous activation of SOS1. In this regard, it is worth noting that the second class of human NS-associated SOS1 mutations target N-terminal regulatory domains and often implicate enhanced membrane recruitment of the mutant SOS protein (73).

RasGRP AUTO-INHIBITION?

RasGRP proteins have been studied most extensively in T- and B-lymphocytes. In these lymphocytes, RasGRP1 and RasGRP3 activate Ras in a manner that is non-redundant with SOS (18, 65, 97–101). More recently, RasGRP proteins, particularly RasGRP1, have also been associated with human diseases such as autoimmune disease and cancer.

Single nucleotide variants near *RASGRP1* are associated with susceptibility to autoimmune (Type 1) diabetes and to thyroid autoantibodies in Graves disease (102, 103). At this point it is not known what effect these variants in non-coding regions of the *RasGRP1* gene have, but possible mechanisms include altered expression or RasGRP1. *RASGRP1* splice variants have been documented for patients with systemic lupus erythematosus (SLE) (104). Several of these RasGRP1 mRNA splice variants are predicted to miss portions of RasGRP1's EF hands, which may have an important regulatory role (see below). In addition, it also appeared that many splice variants resulted in lower proteins expression levels of RasGRP1 (104).

RasGRP4 was originally isolated as a Ras activator in acute myeloid leukemia (AML) (105). RasGRP3 plays a role in human melanoma (106) and in prostate cancer (107) that are distinct from those of SOS. When overexpressed from transgenes, RasGRP1 promotes the development of squamous cell carcinoma and melanoma in mouse models in conjunction with skin wounding or carcinogen painting of the skin (108–110). Transgenic over-expression of RasGRP*1* in developing T lymphocytes causes thymic lymphomas in mice (111) and several unbiased mouse model screens for leukemia genes have identified the RasGRP*1* locus as a hot-spot for leukemia virus integrations driving blood cancer (112–114). The molecular basis of these viral integrations is that these cause leukemia through the dysregulated expression of the target gene, typically through overexpression. Significantly, Oki and colleagues as well as our own group have recently shown that elevated RasGRP1 expression also occurs in T cell leukemia patients (115, 116). For more detailed reading on RasGRP1's role in cancer we refer you to a different review (52). Needless to say these studies collectively indicate that RasGRP1 requires tight regulation. Regulation occurs most definitely at the level of RasGRP1 expression since dysregulated expression of a wild-type RasGRP1 form results in leukemia (116). Extrapolating from our knowledge of SOS1, we propose that RasGRP1 also possesses an auto-inhibited state (**Figure 4A**) to prevent spurious activation and to balance the activating mechanisms of molecules like DAG, which we will discuss next.

DIACYLGLYCEROL AS A RasGRP1 ACTIVATOR

Phorbol esters such as PMA (a synthetic DAG analog produced out of the plant-derived compound phorbol) had long been known as potent stimulators of Ras activation, but it was not until 1998 when Stone and colleagues cloned the *RasGRP1* gene, that the biochemical connection between DAG and Ras activation was established (51).

In T lymphocytes that receive a TCR stimulus, PLCγ1 is recruited to the membrane and activated so that it cleaves PIP_2 into inositol-3-phosphate (IP_3) and DAG. IP3 couples to the calcium pathway (117) and we will come back to this in a moment. The increase of DAG levels in the membrane results in recruitment of RasGRP1 through its C1 domain to the membrane where is can activate Ras (**Figure 4B**) (19, 51). There is a second, indirect route from DAG to RasGRP1 and RasGRP3, which involves PKC-mediated phosphorylation of these two RasGEFs. RasGRP1 is phosphorylated on threonine 184 (T184) in TCR-stimulated T cells whereas RasGRP3 is phosphorylated on the analogous site, T133, in BCR-stimulated B cells (97, 100, 118). Mutations of T184 or T133 into alanine residues results in impaired, but not absent, stimulus-dependent Ras activation (97, 118) and incubation of cells with PKC inhibitors blocks the phosphorylation of RasGRP1 on T184 (65, 97, 100), providing a rationale for the long established observation that PKC inhibition inhibits the output through the Ras-ERK pathway in lymphocytes. How the phosphorylation of RasGRP1 and RasGRP3 enhances their RasGEF activity is not known.

Because of DAG's prominent role in RasGRP1 and RasGRP3 activation in T- and B-lymphocytes, generation of DAG by PLCγ enzymes, and turnover by DAG kinases (DGKs) should be considered. In agreement with a PLCγ1-DAG-RasGRP1 signaling axis (**Figure 5**), conditional PLCγ1 knockout mice and RasGRP1-deficient mice share a similar defect in positive selection of thymocytes and ERK activation (18, 119). On the other side of the cycle, DGK's convert DAG to PA, which is interesting because this would dampen DAG-RasGRP signals but perhaps promote PA-SOS signals. In agreement with a critical role for DGK in dampening RasGRP activity (as well as the activity of other proteins containing DAG-binding C1 domains), deletion of DGKα and DGKγ results in increased incidence of T cell lymphoma (120). In normal T cells, DGK enzymes play a critical role in controlling the balance between activation and anergy or unresponsiveness (121, 122). For a complete review of DAG metabolism and the role of DGK enzymes we refer to Krishna and Zhong (95) in this Research Topic. The role of DAG in RasGRP1 regulation is obvious but may not be

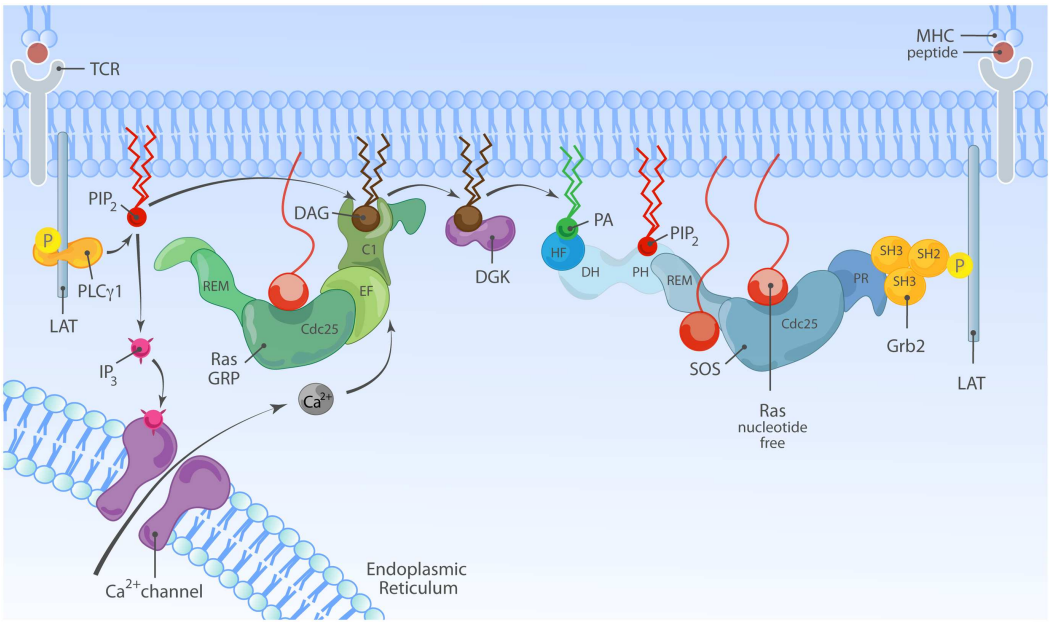

FIGURE 5 | Model of synergy between RasGRP and SOS in TCR signaling. TCR stimulation is connected to activation of RasGRP via tyrosine phosphorylation of the adapter molecule LAT and activation of PLCγ1, that metabolizes PIP_2 into IP_3 and DAG to trigger two second messenger pathways; Ca^{2+} and DAG. Activated RasGRP can enhance the full activation of SOS by providing Ras·GTP, allosterically activating SOS. In principle, the TCR-LAT-PLCγ1 pathway can also indirectly facilitate SOS activation via DAG; DGK metabolizes DAG and converts it to PA, which is a possible target for the HF and/or PH domains in SOS.

exclusive. Non-antigen receptor triggered pathways that are typically not associated with DAG production have been implicated in RasGRP1 membrane localization. Specifically, RasGRP1 but not RasGRP3 signals downstream of the CXCR4 chemokine receptor in thymocytes (123) and a heterodimer of TCR/CXCR4 has been described to recruit the PLC enzymes essential in this pathway (124). How different receptor systems couple to DAG and RasGRP and may be able to synergistically trigger this pathway is an interesting concept for future research.

Whereas RasGRP1 is expressed in various cell lineages (20, 46, 61, 62), it is most abundant in developing thymocytes, which perhaps offers an explanation for the fairly specific thymocyte developmental defect that is observed in RasGRP*1*-deficient animals (18). Reciprocally, RasGRP3 abundance is high in B lymphocytes and RasGRP*3* deficient mice demonstrate B cell defects (99), although there is a role for RasGRP1 in this lineage as well, at least in early B cell subsets (101, 125). The developmental defects in thymocytes lacking RasGRP1 are a consequence of severely impaired positive selection of these cells and biochemically visible through the impaired activation of the ERK kinases (61). A causative link between the impaired RasGRP1-Ras-ERK signaling and defective positive selection has been very nicely provided through the analyses of *ERK-1* and *ERK-2* doubly deficient mice in which the thymocytes also show a positive selection defect (126). Perhaps surprisingly, other RasGEFs, be it of the RasGRP-, Rasgrf-, or SOS-type, do not effectively compensate for the loss of RasGRP1 in thymocytes. The fact that there is only minimal compensation for loss of RasGRP1 coming from RasGRP3 or RasGRP4 (123, 127) makes one wonder about the underlying mechanism. Is it purely the relative abundance of RasGRP1 that bestows its unique function in thymocytes and would expression of RasGRP3 from the RasGRP*1* promoter be able to compensate for the loss of RasGRP1? Or, are there unique biochemical properties in the RasGRP1 protein that are lacking in other RasGEFs?

ADDITIONAL MECHANISMS OF RasGRP1 REGULATION

Only a small portion of protein flanks RasGRP1's catalytic REM-Cdc25 core on the N-terminal side (**Figure 2**). There is no predicted protein domain in this N-terminal part, but this stretch is either only 9 or 57 amino acids long, depending on the use of an alternative internal start codon in *RasGRP1* or its most N-terminal ATG codon (128). The C-terminus appears far more interesting. Not only does it contain the DAG-binding C1 domain, there are also a pair of EF hands sandwiched between the Cdc25 and C1 domains and a roughly 200-amino acid long C-terminal tail without clear domains except for a leucine zipper motif (51, 129, 130). Significantly, genetic deletion of this 200-amino acid long C-terminal tail reduces the formation of mature thymocytes in RasGRP$1^{d/d}$ mice (131), thus there are critical regulatory functions encoded on RasGRP1's C-terminus that are relevant for thymocyte function.

Not all EF hands bind calcium, but RasGRP1 has been reported to bind calcium *in vitro* (51) and the position of the pair of EF hands between the catalytic core and the membrane-recruitment C1 unit is an interesting one. EF hands usually come in pairs and are structures consisting of two α-helices connected by a loop that contain residues such as aspartic acid, which are critical for binding and positioning of a calcium ion. The calcium-binding event

induces protein conformational changes through the alteration of the directional vectors of the α-helices (50). It is very possible that calcium binding alters the structural conformation of RasGRP1 and other RasGRP family members. Deducting from cell biological assays, it appears that calcium orchestrates membrane recruitment of RasGRP together with DAG although this may vary from cell to cell type.

Kay and colleagues reported that in a chicken DT40 B cell line, the first EF hand pair enables the recruitment function of a C-terminal PT domain (PM targeting domain), which contains the leucine zipper motif (132). Mutation of the characteristic triplet of negatively charged aspartic acids in the first EF hand results in impaired enrichment of this RasGRP1-EF1μ molecule to the PM, following either BCR or G-protein coupled receptor stimuli. Whereas both of RasGRP1's EF hands contain very similar triplets of aspartic acids, mutation of these into serine in the second EF hand does not impact the membrane recruitment of the RasGRP1-EF2μ molecule (132). Intriguingly, the contribution of the PT domain toward membrane recruitment appears to differ from cell to cell type; it is substantial in BCR-stimulated B cell lines, very modest in T cell lines, and negligible in fibroblasts (129). It should also be noted that these studies relied on ectopic expression of RasGRP1 that was N-terminally tagged with GFP and that the T and B cells tested in this manner also express endogenous RasGRP1. We will discuss the relevance of overexpression of molecules in the Ras signaling pathway later. The concern of co-expressing a tagged (and mutated) RasGRP1 together with endogenous RasGRP1 is appropriate in light of the predicted leucine zipper. It is possible that the C-terminal leucine zipper motif functions as a RasGRP1 dimerization interface, which would make analysis of the individual contribution of introduced- versus endogenous-RasGRP1 molecules complex. Regardless, the Kay group studies clearly revealed for the first time that calcium-dependent regulation, while incompletely understood, plays an important role in RasGRP1 signaling (**Figure 4C**). Consistent with the notion of calcium-dependent RasGRP1 regulation, the calcium chelator BAPTA-AM and a calcium channel blocker prevented the appearance of Ras·GTP at the Golgi of activated T-cells in imaging experiments (133) (see below for spatial considerations of Ras activation). In biochemical studies, removal of all free calcium by chelators had only a modest effect on TCR-driven Ras activation (134) and RasGRP1 can activate Ras in T-cells in the absence of free calcium (19), although it is difficult to asses the efficiency of calcium chelation or to determine how much cellular calcium would be needed to couple to RasGRP1. In addition, there is an enrichment of calcium ions near the negatively charged polar headgroups of phospholipids in the PM (135), the localization to which RasGRP1 is recruited via DAG. Perhaps it is the membrane-localized calcium that is most relevant to enhance RasGRP1 function. With these biochemical and cellular experiments in mind, it is interesting to speculate on how the regulation of various of the Lupus-associated *RasGRP1* mRNA splice variants that lack portions of the EF hands may be altered (104).

Are there additional mechanisms of RasGRP membrane recruitment or retention that may rely on protein-protein interactions or phospholipids other than DAG? RasGRP1 can interact with a kinase dead version of PKCθ in transfected cells (100). Similarly, RasGRP1 appears to make contacts with DGKζ (136). It is not clear at this point if these results reflect the common intersection point of DAG or if these are true (perhaps transient) protein–protein interactions between RasGRP1 and PKCθ or DGKζ and what the biological implications of these may be for lymphocytes. SKAPP-55 is a multi-domain adapter molecule that interacts with RasGRP1 in a resting T cell line and SKAPP-55/RasGRP1 interactions become more abundant upon TCR or integrin stimulation (137). The immunological implication of SKAPP55 function and its interaction with RasGRP1 are unclear, both a positive role (138) and a negative role (137) have been proposed. Besides a C-terminal SH3 domain, SKAP-55 contains an N-terminal PH domain (just like SOS). It is highly speculative but interesting to consider that both SOS and RasGRP1 may be regulated by phospholipids like PIP_2 and PA interacting with PH domains, but that this occurs in an indirect manner for RasGRP1 through its interaction with SKAP-55. Lastly, Cornell and colleagues demonstrated that RasGRP1's PT domain harbors a basic/hydrophobic cluster of amino acids that is conserved among species and that a protein-purified PT domain can bind to phosphoinositide-containing vesicles (130). Thus, it appears that there will be multiple mechanisms of RasGRP activation and regulation, some perhaps surprisingly similar as for SOS RasGEFs.

BIOCHEMICAL SYNERGY BETWEEN SOS1 AND RasGRP1

When SOS and RasGRP's are co-expressed in a T cell, TCR stimulation can take two routes to Ras-ERK activation; one through RasGRP and the other through SOS (**Figure 5**). However, genetic studies in cell lines and mice indicate that RasGRP plays a more dominant role in antigen receptor-stimulated Ras-ERK activation (18, 61, 66, 67, 92, 139). A recent study also reports that SOS1/2 maybe inhibitory for TCR-induced ERK activation in human peripheral T cells (140), although this finding is inconsistent with several other studies showing a positive contribution of SOS in antigen receptor-stimulated ERK activation, both in lymphocyte cell lines and primary mouse and human lymphocytes (20, 65–67, 92). Reduction of SOS expression leads to moderate but consistent ERK activation impairment in human peripheral T cells, mouse DP thymocytes, and DT40 B cell line (20, 66, 67, 92, 139). Furthermore, the ERK activation defect in $SOS1^{-}2^{-}$ DT40 cells is most noticeable at low and physiological levels of antigen receptor stimulation, indicating that ranges of stimuli across multiple time points are required to conclusively analyze ERK activation defects (66, 92, 139).

Interestingly, flow cytometry-based examination of ERK activation for single cells within a population revealed that not only the quantity but also quality of phosphorylated ERK (pERK) output differs depending on RasGEFs connecting stimulated antigen receptor to Ras (66). In the DT40 model B-cell system, the pERK pattern in BCR-stimulated wild-type DT40 cells (co-expressing RasGRP1/3 and SOS1/2) demonstrates a highly thresholded and bimodal/digital pERK pattern. RasGRP1/3 double-deficiency in DT40 cells results in poor pERK response consistent with near abolished ERK activation in RasGRP1-deficient mouse lymphocytes, indicating that RasGRP play a dominant role in ERK regulation (66). In the absence of SOS1/2, RasGRP1/3 can still activate ERK downstream of BCR, albeit at reduced level. More

significantly, these flow-based assays show that RasGRP1/3-driven ERK activation gradually increases over time and displays analog/unimodal pERK patterns, but does not yield a bimodal pattern (**Figure 6**).

Multiple models at different levels of Ras/MAPK signal transduction explain the shaping of digital/bimodal ERK activation, such as Ras nano-clusters (141), dual negative feedback control by SHP-1 (142) or scaffold-mediated signal quality change (143), and subcellular location of cascade activity (144). But, none of pre-existing models explain the change in ERK activation pattern depending on the RasGEFs availability. Based on computer modeling analysis, we hypothesized that the optimal Ras-ERK response observed in wild-type cells co-expressing SOS and RasGRP involved allosteric activation of SOS primed by Ras·GTP produced by RasGRP. To test this hypothesis, we uncoupled the potential positive feedback loop between two RasGEFs by introducing W729E mutation that prevents Ras·GTP binding at the SOS1 allosteric pocket (66, 89, 92). Whereas RasGRP1 is comparably activated (measured by T184 phosphorylation), BCR-induced Ras-ERK response in cells expressing W729E mutant-SOS1 resembles that of SOS1/2-deficient cells [unpublished data (92)]. Which RasGEF generates an initial flux of Ras·GTP priming full activation of SOS? Theoretically, allosteric activating Ras·GTP can come from self (SOS) or from RasGRP. Indeed, HeLa cervix carcinoma cells that do not express RasGRP1 (Roose lab, data not shown) are able to engage the SOS-Ras·GTP-SOS loop in response to EGFR stimulation (91). However, both lymphocyte cell lines and primary lymphocytes lacking RasGRP poorly respond in terms of Ras-ERK activation, indicating that RasGRP plays an essential role in ERK activation by signaling to Ras-ERK but also to Ras-SOS, via an early surge of Ras·GTP allosterically activating SOS (18, 61, 65–67, 92, 139).

NOVEL INSIGHTS AND PUZZLES FOR THYMOCYTE SELECTION SIGNALS FROM MOUSE MODELS DEFICIENT FOR RasGEFs

In the cellular context where two RasGEFs co-exist, biochemical activation of RasGRP appears temporally ahead of activation of SOS (Roose lab, data not shown). Moreover, whereas SOS requires allosteric activation by Ras·GTP and therefore in a sense relies on RasGRP1 (65), the reverse relationship does not exist: RasGRP1 does not appear to require SOS. These relationships between RasGRP/SOS lead to the hypothesis that the differential fate of thymocytes undergoing selection might be determined by how two RasGEFs are differentially activated upon TCR stimulation (145). This hypothesis was also founded by the observation that positively selected DP thymocytes demonstrate graded (or analog) ERK activation (146, 147). In this model, weak TCR stimulation of positively selected thymocytes sub-optimally phosphorylates LAT, enough to activate PLCγ1-DAG-RasGRP1 pathway and analog ERK signals but without coupling SOS1 membrane recruitment and digital ERK signaling (**Figure 6**). Genetic support for this

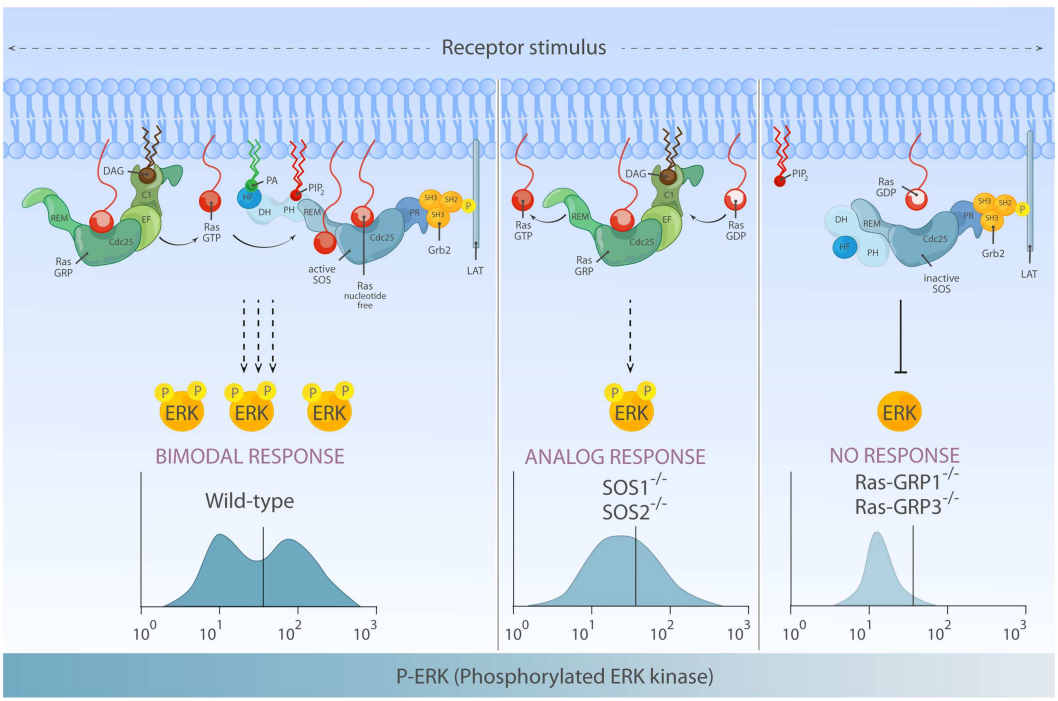

FIGURE 6 | Differential activation of RasGEF determines the quantity and quality of Ras-ERK output. Left: full activation of the ERK response requires activation of both RasGRP and SOS and can lead to bimodal (digital) ERK activation patterns. In this mode of signaling, RasGRP activation temporally precedes activation of SOS and provides initial Ras·GTP that primes full activation of SOS. Middle: in the absence of SOS, there is substantial Ras-ERK activation mediated by RasGRP alone, but the ERK activation patterns are analog and therefore differs both quantitatively and qualitatively from ERK signal generated by two RasGEFs in synergy. Right: in lymphocytes, RasGRP plays a dominant role in connecting TCR-Ras-ERK pathway. SOS alone has difficulty to prime its own allosteric activation, which results in a high threshold for Ras-ERK activation.

model comes from the observation that RasGRP1 is essential for positive selection but is not required for negative selection (61, 67). On the other hand, strong TCR stimulation during negative selection induces extensive LAT phosphorylation, enough to recruit and activate both RasGRP1 and SOS1, and enable allosteric activation of SOS, which generates strong ERK activation characteristic of negatively selected DP thymocytes (66, 147). Whereas this is a plausible model it does not address the question if digital SOS-ERK signals are negative selection cues for thymocytes. In fact, genetic deletion of *ERK-1* and *ERK-2* does not impair negative selection of DP thymocytes at all (148), perhaps arguing that the characteristic ERK activation profiles of thymocytes under negative selection conditions is only a byproduct of a different signal that causes the true negative selection (we will discuss this later). Furthermore, recent studies of a conditional *SOS1* knockout mouse model from Samelson and colleagues provided yet another puzzling insight into the different roles of RasGEFs during T cell development (20, 67), which we will discuss next.

Conditional genetic deletion of *SOS1* in thymocytes, SOS1(T)$^{-/-}$ revealed that SOS1 is dispensable for negative selection, disfavoring the previously mentioned differential-RasGEF-usage model for thymocyte fate decision [reviewed in Ref. (149)]. Instead, SOS1 expression is required for DN stage thymocytes undergoing DN to DP transition. *SOS2* deficiency alone does not significantly affect either positive or negative selection (67). The developmental block in SOS1(T)$^{-/-}$ thymocytes is accounted for by impaired proliferative expansion of DN to DP thymocytes (20). The early developmental defect in SOS1(T)$^{-/-}$ thymocytes can be explained by developmental stage-specific expression profile of different RasGEFs: protein level of SOS1 and RasGRP1 dynamically changes as thymocytes develop. SOS1 protein level is highest in DN thymocytes, while DP thymocytes only express 20% of the SOS1 levels seen in DN thymocytes. RasGRP1 protein level follows the opposite trend: little RasGRP1 is expressed in DN, RasGRP1 is most abundant in DP thymocytes (20, 61). Most puzzling is the finding that combined deletion of RasGRP1/SOS1 impairs negative selection (67). What would be the signaling components regulated by two RasGEFs for negative selection? It is unlikely to be Ras-ERK since negative selection is not affected in H-Ras^{S17N} transgenic nor in *Erk1*$^{-}$*Erk2*$^{-}$ doubly deficient thymocytes, indicating that Ras-ERK activation is dispensable for negative selection (12, 148).

One explanation could be that rather than mediating TCR-sparked responses, both GEFs provide a permissive type of input by sustaining steady-state, basal Ras and Ras-effector levels, as documented in other systems (150, 151). Another explanation may be provided by other functions of RasGEFs in addition to activation of the canonical Ras-ERK pathway. Pharmacological inhibition of the p38 MAPK impairs negative selection in fetal thymic organ culture system (152). Additionally, both Grb2 haploinsufficiency as well as complete Grb2 deletion is concomitant with reduced p38 activation and impaired negative selection (153, 154). We recently uncovered an unanticipated link between SOS and p38 (92). Significantly, SOS1 plays a critical role to connect TCR triggering to p38 activation. By contrast, RasGRP1 plays only a very minor regulatory role in TCR-induced p38 activation in human peripheral T cells and Jurkat cell line and p38 activation is unaffected in thymocytes deficient of RasGRP*1* (92). Surprisingly, SOS1's role in p38 activation is independent of allosteric activation of SOS or even of any enzymatic activity in SOS1, arguing that this is indeed a non-canonical SOS pathway [discussed in more detail later; (92)].

SPATIAL CONTROL OF Ras ACTIVATION: A ROLE FOR LIPID MESSENGERS AND GEFs IN COMPARTMENTALIZED Ras SIGNALING?

Traditionally, Ras activation in leukocytes and other cell types has been intuitively assumed to proceed at the PM based on the notion that Ras activation is bound to happen in close proximity to growth factor or antigen receptor systems that do, in their majority, operate at the cell surface. Early immunocytochemical studies confirmed the predominant presence of Ras at the PM (155–162), lending support to the view that Ras activation proceeds at the PM. However, a diffuse staining of the cytoplasm was apparent in some reports (155, 161, 163), suggesting early on that meaningful amounts of Ras proteins might also be present and signal from internal membranes (endomembranes). The concept that Ras proteins do associate with subcellular membranes was cemented in a series of studies from the 1980s documenting that Ras proteins are subject to a complex series of post-translational modifications that gradually increase their hydrophobicity and effectively govern the association of Ras with cellular membranes [for a review, see (13, 164, 165)]. Recent imaging studies have added a spatial and temporal dimension to this view by showing, firstly, that the stepwise post-translational processing of nascent Ras proteins proceeds at endomembranes *en route* to the PM (163), and second, by disclosing dynamic cycling of the two palmitoylated Ras proteins H-Ras and N-Ras between PM and endomembranes in dependency of their palmitoylation status (166–169). According to this latter "acylation cycle" model, palmitoylation at the Golgi apparatus "traps" H-Ras and N-Ras proteins at endomembranes, tagging them for exocytotic transport and accumulation at the PM. Upon depalmitoylation by the recently characterized acyl protein thioesterase 1 (APT1) (167, 170) and possibly other as yet unidentified depalmitoylating activities, Ras proteins loose their tight and inert binding to the PM, leading to a fast inter-membrane exchange of depalmitoylated Ras and, in consequence, to the tendency to distribute equally to all cellular membrane compartments. One round of the cycle is completed by the renewed palmitoylation of Ras at the Golgi apparatus, a reaction that essentially provides a vectorial component ensuring the predominant localization of Ras at the PM. In contrast to the dynamic palmitoylation-dependent cycling of H-Ras and N-Ras, the non-palmitoylated K-Ras protein is assumed to reside and function largely at the PM, although alternative modes for K-Ras internalization have also been described (171, 172). Knowing this, the intriguing question is whether compartmentalization of Ras activity represents a means of signal diversification in antigen receptor signaling and whether or not second messenger lipids coordinate spatial aspects of Ras activation.

IMAGING ACTIVE Ras·GTP IN T-CELLS

In 2003, Mark Philips and coworkers presented the first of a series of studies that reported for the first time a view of Ras activation

in real-time in lymphocytes challenged via the T-cell receptor (133, 173, 174). Ras·GTP visualization was accomplished using a genetically encoded, fluorescent reporter probe composed of EGFP and the Ras-binding domain (RBD) of the Ras-effector c-Raf. EGFP-RBD features several orders of magnitude higher affinity for Ras·GTP versus Ras·GDP causing it to redistribute and illuminate subcellular sites of Ras·GTP accumulation (162, 175, 176). However, levels of endogenous Ras·GTP are too low to be visualized by EGFP-RBD and researchers have been forced to overexpress Ras. Use of EGFP-RBD to image activation of overexpressed Ras in Jurkat T-cells challenged by clustering the CD3ε chain of the TCR alone or in combination with co-stimulatory triggers yielded an unexpected picture: a bimodal pattern of Ras activation consisting of K-Ras activation at the PM followed or paralleled by a more sustained accumulation of N-Ras·GTP at the Golgi apparatus (173). Strikingly, N-Ras became GTP-loaded only at the Golgi despite the fact that it was present in large amounts at the PM, where the same TCR stimulation induced robust GTP loading of K-Ras (174). While the precise mechanisms enabling the TCR to discriminate among Ras isoforms and subcellular platforms of activation are not fully clear, a number of factors involved in spatial control of Ras activation have been characterized. Pharmacological experiments and use of genetically engineered Jurkat lines provided evidence that the delayed Golgi activation of N-Ras occurred by means of a PLCγ1/RasGRP1 pathway acting specifically on Golgi-resident N-Ras (133, 173), whereas SOS and RasGRP1 acted in concert to load K-Ras with GTP at the PM. Intriguingly, the segregation of the Ras·GTP reporter probe to PM versus endomembranes depended on a number of stimulation parameters: first, the strength of TCR stimulation, with low-grade stimulation (achieved by applying CD3 and CD28 cross-linking antibodies at a final concentration of 1 μg/ml) causing the accumulation of the Ras·GTP reporter only at the Golgi apparatus, whilst high-grade stimulation (5 μg/ml) lead to the described b dual activation pattern (133, 173). This distinct activation pattern was attributed to the ability of low-grade TCR signals to engage the Golgi-specific PLCγ1/RasGRP1 pathway but not other pathways targeting K-Ras at the PM (173). Arguing against this scenario, other investigators have reported K-Ras activation in response to anti CD3ε Abs administered at concentrations as low as 0.15 μg/ml (19, 177), suggesting that low-grade TCR signals cannot discriminate between PM and endomembrane Ras-pools or between K-Ras and N-Ras isoforms. Interestingly, non-leukocyte cell lines like COS, MDCK, or HeLa, which do not express RasGRP1 (150, 168) (Roose, unpublished) exhibit the same segregation of EGFP-RBD to the PM and Golgi in response to growth factor stimulation (133, 169, 178, 179), evidencing that mechanisms of endomembrane Ras activation other than the RasGRP pathway do exist. Data from Bastiaens lab illustrate that (overexpressed) Ras·GTP generated at the PM of MDCK cells relocates to endomembranes following its depalmitoylation at the cell surface in the context of the acylation cycle (169, 179). This mode of endomembrane Ras activation may well operate also in T lymphocytes, but this would imply that endomembrane Ras activation should be preceded by a first "wave" of N-Ras activation at the PM, which was not reported in those studies (173, 174). In conclusion, the individual contribution of the two known modes of endomembrane Ras activation in TCR signaling in T lymphocytes still needs to be evaluated in detail.

THE ROLE OF CO-STIMULATION

Another parameter that can affect the spatial segregation of Ras·GTP is the nature of the co-stimulus provided along with the CD3-cross-linking Ab. For example, CD28 co-stimulation enhances DAG production in T-cells (121, 180) and this in turn is expected to enhance Ras activation via RasGRP1. CD28 co-stimulation is thus intuitively expected to affect the magnitude and possibly also the location of Ras·GTP formation. Somewhat unexpectedly, therefore, this turned out not to be the case, since co-stimulation with soluble CD28 antibodies does not ostensibly affect Ras·GTP levels and/or Ras·GTP localization (174, 177). Perhaps CD3/CD28 co-stimulation experiments need to be re-evaluated using immobilized rather than soluble Abs for receptor crosslinking (181). Co-stimulation via the lymphocyte function-associated antigen-1 (LFA-1), on the other hand, was reported to stimulate activation of Ras at the PM (174). Interestingly, LFA-1 facilitated Ras·GTP formation by stimulating the generation of DAG at the PM via the sequential action of PLD2 and Phosphatidic acid phosphate (PAP), a pathway that had before been linked to DAG/PA metabolism at the Golgi (182). In agreement with an important role of LFA-1 signals for Ras·GTP formation, co-stimulation via LFA-1 reportedly enhanced Ras·GTP accumulation in response to TCR-clustering (174). In opposition to that scenario, others have not observed an effect of LFA-1 on Ras·GTP levels in T-cells (177). Along the same vein, co-stimulating T-cells via SLAM, a measure that also leads to an enhanced production of DAG in T cells (180) did not further stimulate Ras·GTP production, further indicating that an elevation of DAG levels in response to particular TCR/co-receptor stimulations does not always automatically translate in elevated Ras·GTP levels.

ENDOGENOUS VERSUS OVEREXPRESSED Ras AND OTHER EXPERIMENTAL CONSIDERATIONS

The pioneering imaging studies described above have changed the way we think about Ras activation, away from the traditional, rather unilateral view of "static" Ras proteins acting at the PM to the more dynamic picture that has now emerged and has been delineated in the previous sections. It is, however, important to recall that the experimental approaches that have led to this new conception feature a number of caveats and limitations that should be borne in mind. One limitation is that stimulation with cross-linking antibodies toward the CD3ε chain and various co-receptors, as used for reasons of simplicity in most studies, may not reliably reflect the physiological setting of a T-cell challenged by an antigen-loaded APC. Secondly, overexpression of Ras proteins, as applied in most imaging experiments, is an issue worth considering.

Since Ras activation and trafficking are finely regulated processes it is arduous to judge whether or not images obtained from cells overexpressing Ras proteins do always truly reflect the behavior of endogenous Ras. Evidence arguing that this may indeed be an important fact to bear in mind comes from studies reporting on the subcellular localization of endogenous Ras·GTP

in live T cells (177, 183). Visualization of endogenous levels of Ras·GTP in T cells was achieved using refined fluorescent biosensors for Ras·GTP that consisted of three concatenated RBD modules, yielding increased avidity toward Ras·GTP (183), and three EGFP proteins, that conferred threefold higher fluorescence intensity to the probes (177). These probes redistributed only to the PM of PMA or TCR-stimulated Jurkat cells and to the immunological synapse of primary T lymphocytes conjugated to APCs (177, 183), but the probes did not illuminate the Golgi or other endomembranes, in contrast to what was observed in T cells overexpressing H-Ras or N-Ras (133, 174). This remarkable variance in experimental outcome can be interpreted in two ways: first, the trivalent EGFP × 3-RBD × 3 reporter probes do illuminate endogenous Ras·GTP formed at the PM but they are not sensitive enough to visualize Ras·GTP at the Golgi. Since the signals obtained for endogenous Ras·GTP at the PM using the EGFP × 3-RBD × 3 biosensors are clear and well visible, this interpretation would imply that Ras·GTP levels at the Golgi are markedly lower than those at the PM. The alternative explanation is that accumulation of N-Ras·GTP at the Golgi results from perturbances in Ras trafficking, processing, or activation processes as a consequence of Ras overexpression. For example, the reported relocation of GAPs to the cell surface at later stages of TCR signaling for the shutdown of PM Ras signaling (133, 184) could cause a drop in GAP activity at endomembranes that could facilitate increased Ras·GTP loading at the Golgi in a background of Ras overexpression. Also, a sheer increase in the flux of N-Ras through the acylation cycle in Ras overexpressing T-cells is expected to lead to the redistribution of more N-Ras·GTP from the PM to endomembranes. In sum, it is currently difficult to judge whether the observed accumulation of overexpressed N-Ras·GTP at the Golgi is a physiological response of T-cells to antigen stimulation or rather reflects an effect that is only seen with anomalously high levels of Ras.

COMPARTMENTALIZATION OF DAG-RasGRP1 SIGNALS

Given that Ras activation downstream of the activated TCR is largely driven by the concerted action of SOS and RasGRP1 GEFs, can knowledge about the segregation of GEFs and the lipid second messengers that regulates GEF action help us understand the spatial control of Ras activation? The subcellular distribution and TCR-dependent, spatially localized formation of DAG, as the most prominent lipid second messenger involved in the regulation of Ras activity, have been investigated in quite some detail. In addition to its presence at the PM, DAG is present in meaningful amounts at various other subcellular sites including the Golgi apparatus and the nuclear membrane (185, 186). It appears that the sources for these distinct pools of DAG are different. For example, DAG at the Golgi arises largely from Sphingosine metabolism and to some extent also from the sequential action of PLD and PAP on phospholipids (182, 187). PM-located and nuclear DAG is mostly replenished by *de novo* synthesis but is also generated to a variable extent by the action of Phospholipases of various kinds on precursor phospholipids (for comprehensive reviews on DAG metabolism see (187, 188) and in this review issue). Although lymphocytes reportedly have a pool of nuclear DAG, too (186), most attention has been devoted to the PM and Golgi-populations of DAG, since these are, arguably, the two major platforms of TCR signaling. While some subcellular sites, prominently the Golgi apparatus, are rich in steady-state levels of DAG (182), it is generally assumed that DAG-dependent signaling downstream of the TCR involves the *de novo* generation and spatially restricted accumulation of DAG in response to antigen stimulation. Since DAG can directly recruit the Ras activator RasGRP1 it appears reasonable to predict, that domains of DAG formation in response to TCR stimulation should coincide with sites of Ras·GTP accumulation.

Where does TCR-sparked DAG production occur and where within the antigen-stimulated T-cell does DAG accumulate? Several laboratories have imaged DAG in live T-cells using fluorescent reporter probes derived from DAG-binding domains including C1 domains from RasGRP1, PKCθ, or PKD (189–192). Interestingly, C1 domains from RasGRP1 or PKCθ illuminated endomembranes in unstimulated T-cells, suggesting that resting levels of DAG in T-cells are primarily found in that compartment. Upon conjugation with APCs, the same reporter probes relocalized to the IS (190, 191), illustrating that DAG accumulates at the IS. The accumulation of active PLCγ1 (assessed by phosphorylation on Y783) to PM and IS in response to TCR cross-linking or conjugation with APCs (193) is also in line with this view. Consistent with the notion that TCR-activation induces DAG formation/accumulation at the PM, the full-length versions of the DAG-effector proteins PKD and chimaerins accumulate at the PM or IS of TCR-challenged T-cells (194). DGKα and DGKζ, two enzymes that metabolize DAG by converting it to PA, also accumulate at the PM of T-lymphocytes conjugated to antigen-loaded APCs (192, 195), a step proposed to be critical for the spatial confinement of DAG to the IS (196). In the case of RasGRP1, some studies reported exclusive redistribution of RasGRP1 to the PM or IS of T-cells challenged via the TCR (122, 177, 194, 197–200) while others documented TCR-activation dependent accumulation of RasGRP1 at PM and Golgi (133, 147, 174). Importantly, while these considerations may cause the impression that DAG alone determines the subcellular distribution of many of its effector proteins, DAG is likely to be only one of various factors that coincidentally determine the spatial distribution of RasGRP1 and other DAG-target proteins. For example, the DAG-effector PKD features a transient and short-lived recruitment to the IS despite the much more prolonged presence of DAG at the IS (191).

COMPARTMENTALIZATION OF LIPID-SOS SIGNALS?

Recently, the lipid product of PLD, PA, has been reported to recruit SOS via its PH domain, thus providing yet a new link for a lipid messenger and Ras activation. Since PA is found both at the PM and endomembranes (174, 182), mechanisms for the oriented and regulated recruitment of SOS to subcellular membranes must exist. This involves probably the concerted action of PA with other upstream inputs such as PIP_2, Ras-binding, and Grb2 binding, as described extensively above (see sections on SOS regulation).

Another important second messenger lipid with relevance to SOS-driven Ras activation is the PI3K reaction product phosphatidylinositol-3,4,5 trisphosphate (PIP_3) (201, 202). The subcellular distribution of PIP_3 in lymphocytes has been visualized using fluorescent reporter proteins based on the PH-domain of Akt (203–205). These studies reported that PIP_3 was produced and accumulated at the PM, but in contrast to DAG, PIP_3 was

not restricted to the IS but expanded also to regions outside the IS (203). Indeed, a sustained accumulation of PIP_3 was even observed at the antisynapse or uropod of the T-cell (204). Remarkably, other upstream modulators or known activators of Ras like ZAP70 and ezrin, respectively, also accumulate at the antipodal pole of conjugated T-cells (206–208). Intriguingly, ezrin is an important co-factor in Sos activation in some systems (207), which raises the intriguing possibility that concerted Sos-dependent Ras activation by means of ezrin and PIP_3 and subsequent Ras-signaling (to PI3K?) may proceed at the T-cell uropod at later stages of T-cell-APC conjugation.

The subcellular distribution of PIP_3 in the course of T-cell stimulation is consistent and certainly suggestive of a role of PI3K in the control of Ras activation and/or signal propagation. However, the precise role played by PI3K and its lipid products in Ras activation is an intensively debated, and as yet not settled issue. PI3Ks [refers collectivelly to the four members of the class I family of PI3Ks (209)] were originally described and characterized as effector proteins of Ras, and a large body of experimental evidence [including the recent analysis of transgenic animals expressing PI3K variants that cannot be activated by Ras·GTP (210, 211)] has firmly established the notion that PI3Ks do function downstream of Ras [reviewed in Ref. (212)]. On the other hand, a number of studies has also documented a role for PI3K upstream of Ras (201, 202, 213), indicating that PI3K lipid products could fulfill dual roles as second messengers in the propagation of Ras-sparked signals and as modulators in the (feedback?) control of Ras activation.

How could PI3K lipid products regulate Ras activation in lymphocytes? PIP_3 interacts physically with the Ras-GAP species GAP1(m) (214) and biochemical evidence for a regulation of Ras-GAP activities by PIP_3 in leukocytes does exist (202). Beyond this largely unexplored connection with GAP proteins, PIP_3 interacts with and recruits members of the Tec family of protein kinases, prominently Bruton's tyrosine kinase, Btk, in B cells and Itk in T-cells (215), via an amino-terminal PH domain (216–218). Tec kinases, in turn, can affect Ras activation in two ways: first, Tec kinases are critically involved in antigen receptor-induced PLCγ activation (219, 220), and defective Tec activation in response to antigen receptor stimulation leads to a number of defects in pathways dependent on DAG/IP3, including PKC and ERK activation (221, 222). The latter finding suggests that Ras activation should also be affected, although this has, to our knowledge, not been directly assessed. Secondly, defects in Tec kinase function cause a decrease in PA levels (223), which could in turn result in diminished Ras·GTP loading via SOS (29). In this regard, it is probably important to consider PIP_3 in a broader context in conjunction with the fate of its precursor lipid PIP_2. Beyond serving as a substrate for PI3Ks, PIP_2 plays a critical function as the substrate of PLCγ enzymes and it is well established that the agonist-evoked activation of PI3K and PLCγ signaling can lead to a marked, acute and probably spatially restricted drop of PIP_2 levels in leukocytes (224, 225). Since PIP_2 can modulate Ras activation via the direct, PH-domain dependent interaction with SOS, the concerted and locally confined regulation of the PIP_2/PIP_3 ratio is predicted to have a large impact on the activation status of Ras. From a technical point of view, one important challenge for the years to come will be to address this aspect of Ras activation by visualizing PIP_2 and PIP_3 simultaneously with Ras·GTP in life cells, an approach that should ideally be expanded to other second messengers involved in the control of RasGEFs.

A PHYSIOLOGICAL ROLE FOR COMPARTMENTALIZED Ras SIGNALING?

Is the segregation of Ras signaling to endomembranes and possibly other subcellular sites an inherent and fundamental component of TCR signaling that provides an additional level of signal diversification? Evidence for a possible physiological role of compartmentalized Ras signaling in T-cell biology comes from provocative data reported by Ed Palmer's lab arguing that Ras localization and signaling from PM versus endomembranes could be a major fate determinant during thymic T-cell selection (147). Using a collection of agonist ovalbumin (OVA) peptide variants with graded affinities toward the TCR on transgenic OT-I T lymphocytes these investigators observed a distinct compartmentalization of Ras and its downstream effector protein c-Raf (also known as Raf-1) in dependency of agonist strength: in T-cells driven into negative selection by high-affinity antigen peptides Ras and c-Raf distributed largely to the PM whilst positive selecting, low affinity ligands induced a relocation of Ras and Raf to endomembranes. Intriguingly, localization of RasGRP1 followed a similar pattern. At first sight the relocation of Ras signaling to endomembranes by high-affinity ligands in the thymocyte selection model and by low-grade TCR stimulation of Jurkat cells in the study by Perez de Castro et al. (173) may appear hard to reconcile, although it is probably tedious to compare peptide/APC-stimulation of immature double-positive thymocytes with Jurkat cells or primary mature T cells challenged by means of cross-linking Abs. It is also important to note that Ras accumulation at endomembranes, as observed in positively selected thymocytes, must not necessarily reflect high Ras·GTP loading and Ras signaling at that organelle. In this regard, the coincident accumulation of Raf in the Golgi apparatus of positively selected thymocytes may not be a reliable marker for the presence of Ras·GTP as suggested (147). Since only about 3% of c-Raf interacts with Ras·GTP in antigen challenged T-cells at the peak of Ras·GTP formation (226), the observed quantitative relocation of c-Raf to endomembranes is unlikely to result from recruitment by Ras·GTP but could rather argue for the action of a small second messenger molecule in recruiting c-Raf. For example, c-Raf is recruited and activated by PA (227, 228), and thus PA generated by DGK-catalyzed phosphorylation of DAG or by PLD activation downstream of PKC (229, 230) is an attractive candidate in this respect. In sum, the documentation of spatial Ras segregation in the context of thymic selection provides important evidence for a role of compartmentalized Ras signaling in T-cell biology, but we need to understand more about the underlying mechanisms governing spatial control of Ras activity. Moreover, the fact that mice devoid of both palmitoylated Ras variants, H-Ras and N-Ras, live a mostly healthy life (231), have normal T-cell differentiation and feature only relatively minor defects in mature lymphocyte biology (232) evidences that the compartmentalization of Ras signaling to endomembranes is not essential or critically important for TCR-dependent signaling, at least in rodents. Perhaps the ability to compartmentalize Ras signals to endomembranes is part of a signaling repertoire for fine-tuning

of TCR responses, the physiological relevance of which has so far escaped our attention.

SOS1 AS A LIPID REGULATED ADAPTER MOLECULE

Overshadowed by its primary role as a RasGEF in the canonical SOS-Ras pathways, it is relatively underappreciated that SOS1 may function as a scaffold molecule that can potentially sense membrane lipid- and protein-originated signals. Particularly interesting is SOS's PR C-terminal segment with multiple potential SH3 binding sites (PxxP motifs) and at least four sites that bind to Grb2's SH3 domain *in vitro* (32, 233). The multiplicity of the SH3 ligand sites in the C-terminus bestows the capacity to interact with more than one interacting partner at any one time. The availability for multiple PxxP motifs opens the possibility for interacting with more than one molecule of Grb2 or other related SOS-interacting SH3-SH2-SH3 adapters such as Grap or Gads. Thus, SOS may function as a scaffold to integrate upstream membrane signals and coordinate activation of multiple downstream pathways.

Houtman and colleagues actually observed complexes of SOS1 and Grb2 in a 1:2 stoichiometry, particularly when molar concentration of Grb2 is in excess (234). The multivalent interaction between Grb2 and SOS can lead to formation of oligomeric LAT clusters, in this case, SOS-Grb2 complex functions as a cytosolic adapter cross-linking multiple LAT molecules together (234–236) (**Figure 7**). Expression of PR C-terminal SOS1 fragment in Jurkat cells decreases the size of aggregated LAT clusters and also attenuates weak TCR stimuli-induced calcium flux (234). These observations support the functional existence of SOS-Grb2-LAT clusters, which can facilitate amplification of weak TCR stimulation. SOS can also synergize with LAT clusters by stabilizing LAT signalosome components such as PLCγ1. Upon TCR stimulation, PLCγ1-SH2 is recruited and bound to tyrosine-phosphorylated (Y132; human or Y136; mouse) LAT (237, 238). In addition, the SH3 domain of PLCγ1 directly interacts with PR segments of SOS both *in vitro* and *in vivo*, including in T lymphocytes (35–38). Direct SOS-PLCγ1 binding can promote stable association of PLCγ1 within LAT signalosome by collaborating with SH2-PLCγ1 binding with phospho-LAT. Additionally, direct SOS-PLCγ1 interaction can recruit PLCγ1 to the proximity of its substrate, PIP_2, which is also a ligand for the HF and PH domains of SOS as described earlier. Thus, it is plausible that LAT and SOS together nucleate a signaling hub in lymphocytes in which many molecules and therefore pathways come together.

Our recent study indicates that SOS1 plays an important adapter function regulating p38 pathway activation independently of SOS1' catalytic activity (92). In principle, SOS1's DH domain could act as nucleotide exchange domain in a SOS-Rac-P38 pathway since DH domains are commonly shared structural modules of GEFs regulating Rho family GTPases such as Rac (23, 239). Indeed, SOS has been suggested to operate as a GEF with dual specificity: REM-Cdc25 domains targeting for Ras and DH and PH domain for Rac (240). The latter activity occurs in epithelial cells when SOS1 is coupled to EPS8 and E3b1 co-factors (22, 23, 39). Rac·GTP accumulation is thought to be upstream of classical p38 activation pathway (241, 242). Interestingly, the absence of SOS-1 and -2 profoundly impairs BCR-stimulated Rac·GTP accumulation and p38 activation (92). Combined deficiency of

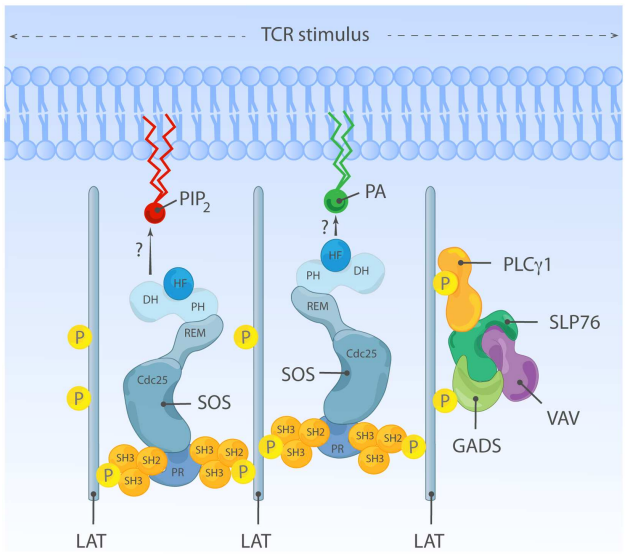

FIGURE 7 | An adapter function for SOS in oligomeric LAT clusters? Grb2-SOS complexes can serve as a cytosolic linkers and aggregate multiple LAT molecules and LAT signalosome-constituent proteins together. This SOS-containing complex may facilitate activation of other, non-canonical Ras-ERK signal transduction pathways such as activation of the MAPK p38, perhaps through a Vav-Rac·GTP connection. We found that regulation of p38 is independent of any enzymatic function of SOS, further strengthening the notion that SOS can signal as an adapter to non-canonical pathways in lymphocytes.

RasGRP-1 and -3 abolishes BCR-induced ERK activation, while its impact on p38 phosphorylation (pT180pY182) is only minimal (92). Unexpectedly, SOS1 versions with either a point mutation (F929A) within Cdc25 that cripples SOS1's RasGEF function, an allosteric pocket mutation W729E, or a mutation of seven amino acids in the DH domain (LHYFELL → IIIRDII) that would disrupt SOS1's putative RacGEF activity, all rescue BCR-induced p38 phosphorylation in SOS-deficient DT40 B cells, indicating that enzymatic activity of SOS1 is not required for p38 regulation and SOS1 is functioning as an adapter for p38 activation pathway (92). Thus, whereas the exact nature of SOS1's adapter function and the potential role of phospholipids binding to SOS1 as an adapter (**Figure 7**) remain to be further studied, p38 appears to connect to a non-canonical SOS pathway in lymphocytes.

CONCLUDING REMARKS

The need for controlled Ras activation in not only lymphocytes but also in all other cell types is clearly provided by the devastating consequences of aberrant, oncogenic Ras signals in cancer. Not all cell types express both the SOS and RasGRP types of RasGEFs and lymphocytes are perhaps somewhat unique in that these cells have developed an intricate mechanism for sensitive and robust Ras signals via both types of RasGEFs that is still under tight control. We have discussed how membrane recruitment and biochemical activation of the RasGRP and SOS RasGEF is fine-tuned through the concerted input of various mechanisms that include lipid messengers. Future research will undoubtedly further refine the model of Ras activation we sketched here and may reveal how

lipid messengers could integrate signals to RasGRP and SOS as adapters in non-canonical pathways that are distinct from Ras.

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Regulation of lipid signaling by diacylglycerol kinases during T cell development and function

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Diacylglycerol (DAG) and phosphatidic acid (PA) are bioactive lipids synthesized when the T cell receptor binds to a cognate peptide-MHC complex. DAG triggers signaling by recruiting Ras guanyl-releasing protein 1, PKCθ, and other effectors, whereas PA binds to effector molecules that include mechanistic target of rapamycin, Src homology region 2 domain-containing phosphatase 1, and Raf1. While DAG-mediated pathways have been shown to play vital roles in T cell development and function, the importance of PA-mediated signals remains less clear. The diacylglycerol kinase (DGK) family of enzymes phosphorylates DAG to produce PA, serving as a molecular switch that regulates the relative levels of these critical second messengers. Two DGK isoforms, α and ζ, are predominantly expressed in T lineage cells and play an important role in conventional αβ T cell development. In mature T cells, the activity of these DGK isoforms aids in the maintenance of self-tolerance by preventing T cell hyper-activation and promoting T cell anergy. In this review, we discuss the roles of DAG-mediated pathways, PA-effectors, and DGKs in T cell development and function. We also highlight recent work that has uncovered previously unappreciated roles for DGK activity, for instance in invariant NKT cell development, anti-tumor and anti-viral CD8 responses, and the directional secretion of soluble effectors.

Keywords: diacylglycerol kinase, phosphatidic acid, T cell development, T cell activation, T cell tolerance, T cell receptor, mast cells, macrophages

INTRODUCTION

Lipids are small hydrophobic molecules that perform a variety of cellular functions. Though best known for their role in maintaining cell structure and storing energy, lipids have gained in importance over the past few decades as signaling mediators (1, 2). While lipids that participate in signaling are thought to be much less abundant in the cell as compared to structural lipids, their levels vary dynamically in response to external signals.

In this review, we discuss the signaling functions of two key lipid second messengers, diacylglycerol (DAG) and phosphatidic acid (PA), in the context of T cell development and function. DAGs are esters of glycerol in which two of its hydroxyl groups are esterified with long-chain fatty acids. One manner of PA generation in cells is via phosphorylation of the free hydroxyl group in DAG by a family of enzymes called diacylglycerol kinases (DGKs) (3, 4). DGKs therefore act as molecular switches that simultaneously turn off DAG-mediated signaling and turn on PA-mediated signals.

While all 10 mammalian DGK isoforms contain a kinase domain and at least two cysteine-rich C1 domains, they can be grouped into five types based on the homology of their other structural features. α and ζ are the major isoforms expressed in T cells (5). DGKα is a type I DGK and contains an N-terminal recoverin homology domain and two $Ca2^{+}$-binding EF hands. DGKζ is a type IV DGK and contains a myristoylated alanine rich C kinase substrate (MARCKS) motif, four ankyrin repeats and a C-terminal PDZ-binding domain. DGKζ undergoes alternative splicing, producing a 130 kDa ζ2 isoform that is highly expressed in immature thymocytes and a 115 kDa ζ1 isoform that is predominant in mature thymocytes and peripheral T cells (6). Functional differences between the two splice variants remain unclear.

Here, we begin by discussing the various effector molecules that transduce DAG-mediated signals and PA-mediated signals. We then switch gears to review our current understanding of the role of DGKs in T cell development and function with an emphasis on recent advances that have revealed hitherto unknown functions for these enzymes. The roles of DGK activity in other immune cell lineages are also discussed briefly.

DAG-MEDIATED SIGNALING

Several enzymes contribute toward DAG production upon receptor stimulation in immune cells (7) (**Figure 1**). Phosphatidylinositol-dependent phospholipases hydrolyze membrane phosphatidylinositol bisphosphate (PIP_2) to DAG and inositol triphosphate, and phosphatidylcholine (PC)-dependent phospholipases hydrolyze PC to DAG and phosphoryl choline. In addition, sphingomyelin synthase generates DAG and sphingomyelin from PC and ceramide, while PA phosphatases such as lipins dephosphorylate PA to DAG. On the other hand, DAG is primarily removed by the activity of DGKs, which catalyze its phosphorylation to PA. DAG can recruit a variety of downstream effector molecules through their C1 domains, and thereby trigger multiple signaling pathways.

The protein kinase C (PKC) family of serine/threonine kinases consists of 10 isozymes that are activated by a number of distinct

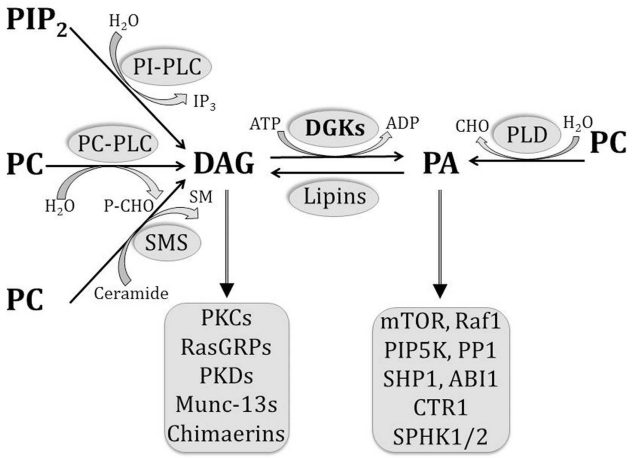

FIGURE 1 | Pathways involved in the generation and removal of DAG and PA. Multiple pathways contribute to DAG generation in the cell, including the hydrolysis of PIP_2 by PI-dependent PLCs, hydrolysis of PC by PC-dependent PLCs, dephosphorylation of PA by lipins, and sphingomyelin synthesis by SMS. On the other hand, PA is generated by PLD-mediated hydrolysis of PC and by DGK-mediated phosphorylation of DAG. As bioactive lipids, both DAG and PA are able to bind to a number of effector molecules, as listed.

mechanisms (8, 9). Upon engagement of the TCR, production of DAG by activated PLCγ1 recruits PKCθ to the plasma membrane in T cells. Co-stimulation via CD28 also plays an important role in the recruitment and spatial segregation of PKCθ at the immunological synapse (10, 11). Activation of PKCθ is indispensable for TCR-mediated NF-κB activation in mature T cells (12, 13). A role for PKCθ has also been identified in an array of key processes (14) including invariant NKT (*i*NKT) cell development and activation (15, 16), T cell survival (17), IL-2 production (18), T_H2 responses (19, 20), and T_H17 responses (21). Thus, by recruiting PKCθ, DAG regulates multiple aspects of T cell function.

Another important protein that is brought to the plasma membrane by DAG upon TCR stimulation is Ras guanyl-releasing protein 1 (RasGRP1) (22). RasGRP1 is a member of the RasGRP family of factors that help activate Ras by exchanging bound GDP for GTP (23), and is selectively expressed in T cells and a few other cell types (24). RasGRP1 plays an essential role in thymocyte development (25), and is particularly required for the selection of thymocytes that express weakly selecting TCRs (26). RasGRP1 is not critical for the development of γδ T cells, but is important for their proliferation and IL-17 production (27). Other studies have shown that RasGRP1 may play a role in promoting antigen-induced CD8 cell expansion by lowering the threshold of T cell activation (28). RasGRP1 is therefore a key effector downstream of DAG that plays a multitude of critical roles in T cell development and function.

Members of the protein kinase D (PKD) family have been identified more recently as DAG effectors (29). A unique characteristic of PKDs is that they are targets of both DAG and DAG-activated PKCs (30). PKDs are thought to be activated by a multi-step mechanism. Upon cell stimulation, inactive PKD translocates from the cytosol to the plasma membrane in response to membrane DAG production, where it is then activated by novel PKCs that are also recruited to the membrane by DAG. During T cell development, PKD has been shown to exert different effects on VDJ recombination at the TCRβ locus and on CD4 and CD8 expression, based on its localization at the cytosol or plasma membrane (31). Subsequent work has revealed that regulation of thymocyte development by membrane-localized PKD, but not cytosol-localized PKD, is dependent on the GTPase RhoA (32). Bringing PKD to the plasma membrane therefore represents another important mechanism by which DAG regulates T cell development.

Munc13 proteins are mammalian homologs of the *C. elegans* Unc13 that is localized to pre-synaptic active zones of neurons and important for neurotransmitter secretion (33). Munc13-1, Munc13-2, and Munc13-3 isoforms bind to DAG with high affinity, and translocate to the plasma membrane in response to receptor stimulation. In the immune system, the Munc13-4 isoform which lacks a C1 domain (34, 35) has been shown to be important for granule maturation and exocytosis in NK cells and cytotoxic T lymphocytes (CTLs) (36, 37), and for phagosomal maturation and killing of intracellular bacteria in neutrophils (38, 39). Further studies are required to investigate parallel roles for DAG-binding Munc13 isoforms in NK cells, CTLs, neutrophils, and other types of immune cells. Over-expressing human Munc13 in opossum renal epithelial cell lines enhanced their susceptibility to apoptosis after DAG treatment, suggesting that Munc13 proteins may transduce apoptosis-inducing signals downstream of DAG in some cell types (40). The role of Munc13 proteins in T cell development and function remain poorly understood.

Chimaerins, a family of proteins that possess Rac-specific GTPase Activating Protein (GAP) activity, contain C1 domains that bear about 40 percent homology to those of PKCs (41, 42). Chimaerin isoforms α2 and β2 are expressed at different levels in T cells and have been shown to participate in TCR signaling (43). Results from the study suggest that these chimaerin isoforms translocate to the immunological synapse upon T cell activation, but in a manner that is independent of canonical DAG-binding by the C1 domains. Catalytic activity of these chimaerins was found to play an important role in inhibiting TCR-mediated NFAT activation. Other studies have delineated a role for β2 chimaerin in mediating DAG-dependent changes in T cell adhesion and chemotaxis (44). In this study, expression of GFP-tagged β2 chimaerin revealed that active Rac and C1-dependent PMA binding could co-operate to induce sustained localization of β2 chimaerin to the plasma membrane in Jurkat T cells. Overexpression of GFP-β2 chimaerin was associated with decreased CXCL12-induced static adhesion but enhanced CXCL12-induced migration. Chimaerin proteins therefore represent an important class of DAG effectors in T cells, but further work is required to dissect aspects of their function that are dependent on and independent of DAG-binding.

PA-MEDIATED SIGNALING

Diacylglycerol kinases and enzymes of the phospholipase D (PLDs) family act as key mediators of PA production in immune cells by phosphorylating DAG and hydrolyzing PC, respectively (7, 45) (**Figure 1**). On the other hand, enzymes such as lipins that possess PA phosphatase activity play a critical role in turning off

PA-mediated signaling by removing PA (46). Cellular levels of PA have been shown to change dynamically in response to environmental stimuli, and a wealth of data has revealed a diverse array of functions for this bioactive lipid (47).

Phosphatidic acid performs its signaling functions primarily by associating with a growing number of effector molecules that include kinases such as mammalian/mechanistic target of rapamycin (mTOR) and phosphatidylinositol-4-phosphate 5-kinase (PIP5K), and phosphatases such as Src homology region 2 domain-containing phosphatase 1 (SHP1) (48). In mammalian HEK293 cells, treatment with exogenous PA was found to promote the phosphorylation of S6K1 and 4E-BP1, which are substrates of mTOR complex 1 (49). This phosphorylation was abolished by rapamycin, a bacterial macrolide that inhibits mTOR activity. Results from this study showed that mitogenic stimulation of HEK293 cells led to cellular PA accumulation within 5 min. Small unilamellar vesicles containing PA could also directly bind to the FKBP12-rapamycin binding (FRB) domain on mTOR in a manner that competed with FKBP12-rapamycin. Together, these results suggest a role for PA as a critical mediator that connects mitogenic stimuli to mTOR activation in mammalian cells (50). More recent work has revealed that PA may activate mTOR by a distinctive two-step mechanism that involves the displacement of the endogenous mTOR-inhibitor FKBP38 and allosteric activation of the kinase (51). On the other hand, studies with Rat2 fibroblasts suggest that PA may indirectly activate mTOR complex 1 via the MEK-ERK pathway (52). In this study, two structurally distinct MEK inhibitors were found to inhibit PA-mediated activation of mTOR complex 1. Other studies with human renal cell adenocarcinoma cell lines have shown that suppression of cellular PA production by treatment with a PLD inhibitor may inhibit the association of mTOR with both Raptor and Rictor, suggesting that PLD-derived PA may act as a key stabilizer of mTOR complexes 1 and 2 (53).

Diacylglycerol kinase-derived PA has also been shown to modulate mTOR complex 1 activity in HEK293 cell lines (54). Overexpression of DGKζ, but not DGKα, led to increased mTOR complex 1-dependent phosphorylation of S6K1 both in the presence and absence of serum, suggesting that DGKζ-derived PA may activate mTOR complex 1. DGKζ-induced S6K1 phosphorylation was dose-dependent, as cells expressing higher levels of DGKζ showed more intense S6K1 phosphorylation. DGKζ-mediated increase in S6K1 phosphorylation was abolished when a mutant form of mTOR (that lacked the capacity to bind PA) was co-expressed, suggesting that DGKζ-generated PA enhances S6K1 phosphorylation by directly binding to mTOR.

While it is tempting to extend these observations to suggest that PA may directly bind and activate mTOR in immune cells, only a few studies have rigorously examined the relationship between PA and mTOR in these cell types. RAW264.7 macrophage cell lines showed enhanced secretion of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β in response to PA stimulation (55). This was specifically abrogated by rapamycin, suggesting that PA-induced production of pro-inflammatory cytokines in macrophages may occur through the mTOR complex 1 pathway. Treatment of mice with PA also led to an increase in serum levels of these cytokines, suggesting that PA may promote systemic inflammatory responses in a manner that is dependent on mTOR complex 1 activation.

As we discuss later in this review, a bevy of studies provide clear genetic evidence of critical roles for DGKα and DGKζ in multiple aspects of T cell maturation and function. However, much work remains to be done in understanding the role of DGK-derived and PLD-derived PA in activating mTOR complex 1 during T cell development and function.

Another key signaling pathway that is activated by PA is the Ras/MEK/ERK signaling cascade (56). The first line of evidence for PA-mediated modulation of MAP kinase signaling came from a study which showed that a C-terminal domain of Raf1 could bind to PA in a canine kidney cell line (57). Subsequent work showed that growth factor-mediated activation of PLD and the concomitant production of PA were directly linked to the activation of the Raf1-MAP kinase pathway (58). Based on findings that insulin-dependent PLD activation was critically dependent on ADP ribosylation factor (ARF) activation, the researchers used an ARF inhibitor (brefeldin A) to block PLD activation (59). Blocking PLD activity and PA production with brefeldin A blocked insulin-induced activation of the MAPK pathway in Rat-1 fibroblasts. Results from the study also showed that PA did not directly activate Raf1, but instead enhanced its recruitment to the plasma membrane to allow for its activation by Ras. Mutations in Raf1 that disrupted Raf1-PA interaction prevented plasma membrane recruitment of Raf1 in response to insulin stimulation (60).

Kinase suppressor of Ras 1 (KSR1) is a scaffolding protein that interacts with several components of the Raf-MEK-ERK cascade to co-ordinate the formation of localized multi-protein complexes that enable efficient signal transduction (61). More recent work has shown that KSR1 contains a sequence homologous to Raf1's PA-binding domain that allows it to directly bind PA and be recruited to the plasma membrane in response to insulin stimulation in fibroblasts (62). The recruitment of KSR1 by PA was also found to be essential for its scaffolding function, as mutations in its PA-binding motif impaired insulin-induced MEK and ERK phosphorylation. Studies have shown that PA also recruits Sos, an activator of Ras, to the plasma membrane through its plextrin homology domain (63). PLD2-derived PA was found to play essential role in EGF-induced Ras activation, as mutations of Sos that impaired PA-binding prevented its membrane recruitment and subsequent activation of Ras.

Studies with Jurkat T cells suggest that PLD2 may act upstream of RasGRP1 upon TCR crosslinking and co-stimulation via the integrin lymphocyte function-associated antigen 1 (LFA1) (64). In this case, production of PA by PLD2 and subsequent dephosphorylation of PA to DAG by PA phosphatase were shown to be critical for plasma membrane Ras activation. Others have demonstrated a role for PLD-derived PA in ERK1/2 activation downstream of Galectin-8 engagement in Jurkat T cells (65). Galectins are a family of widely expressed carbohydrate-recognizing proteins, and Gal-8 has previously been shown to bind certain integrins on the T cell surface to provide co-stimulatory and proliferative signals (66). In this study, Gal-8 induced PA accumulation in Jurkat T cells within 15 min in a manner that was inhibited by treatment with a PLD inhibitor (1-butanol). Such PLD-inhibition also abrogated ERK1/2 phosphorylation, suggesting that PLD-derived PA

may play an essential role in ERK activation downstream of Gal-8 stimulation in Jurkat T cells. However, this result must be interpreted with caution, as PLD-derived PA may also activate ERK in an indirect manner involving the lipin-mediated conversion of PA to DAG.

Type I PIP5K enzymes catalyze the production of PIP_2 from phosphatidylinositol-4-phosphate (PIP). During T cell activation, PIP_2 is involved in modulating T cell rigidity but is primarily hydrolyzed to produce key second messengers DAG and inositol triphosphate (IP_3) (51). A number of studies have established that PA can bind to and activate PIP5K (67). Modulation of PIP5K activity by PA was originally shown using PIP5K purified from bovine brain membranes, where PA was shown to enhance enzymatic activity by up to 20-fold (68). Subsequent studies demonstrated that type I PIP5Ks (which phosphorylate PIP to PIP_2), but not type II PIP5Ks (which phosphorylate phosphatidylinositol 5 phosphate to PIP_2), are specifically regulated by PA (69). More recent work has suggested that PA may regulate the affinity of murine PIP5K-1β for its substrate PIP (70). In this study, PA was shown to bind specifically to the C-terminal region of PIP5K-1β and kinetic analysis revealed that the addition of PA increased the affinity of PIP-binding to the enzyme's active site by nearly 70-fold. Other studies have elegantly demonstrated a role for DGKζ-derived PA in stimulating PIP5K-1α activity to increase local PIP_2 levels and promote actin polymerization in cell lines (71). Expression of DGKζ enhanced PIP5K-1α activity in thrombin-stimulated HEK293 cells, and DGKζ and PIP5K-1α were found to co-localize and co-immunoprecipitate with each other. DGKζ and PIP5K-1α were also found to co-localize with actin at lamellipodial protrusions in epithelial cells. While there is evidence to suggest that the activity of PIP5K-1α and γ isoforms may be critical for normal human NK cell cytotoxicity (72, 73), the role of PLD-derived and DGK-derived PA in regulating PIP5K activity in immune cells remains quite poorly understood.

Protein phosphatase 1 (PP1) is a eukaryotic serine/threonine phosphatase that regulates the function of a variety of proteins in the cell. The PP1 catalytic subunit is able to interact with more than 50 different regulatory subunits in a mutually exclusive manner and this allows the enzyme to target different substrates in diverse subcellular locations depending on its binding partner (74). Initial studies identified that PA acted as a highly specific tight-binding inhibitor of the γ isoform of human PP1 *in vitro* (75). Further studies used a deletion mutagenesis approach to reveal that residues 286–296 of PP1γ were necessary and sufficient for PA-binding (76). Results from one study suggest that PP1 activity may play a role in suppressing T cell function in a rat model of alcohol intoxication and burn injury (77). While this suggests that PA-mediated inhibition of PP1 function may facilitate T cell activation, further experiments are required to better understand the role of PLD-derived and DGK-derived PA in suppressing PP1 activity in T cells.

Src homology region 2 domain-containing phosphatase 1 is a tyrosine phosphatase that plays a critical role in T cell function (78). "Moth-eaten" mice carry a spontaneous frame-shift mutation in the SHP1 gene and lack detectable SHP1 protein (79). Studies with these mice revealed a role for SHP1 in negatively regulating positive and negative thymocyte selection (80), while the use of conditional SHP1 knockout mice showed that SHP1 limits the number of short-lived effector CD8 cells produced in response to viral infection (81). Early studies showed that PA could increase the phosphatase activity of SHP1 toward the EGF receptor when the two proteins were transiently co-expressed in 293 cells (82). Subsequently, PA was shown to directly bind to recombinant SHP1, and two distinct PA-binding sites (a high affinity site on the C-terminal end and a low affinity site on the N-terminal end) were identified on SHP1 (83). Future studies are required to determine if PA modulates SHP1 activity in immune cells and if PA may serve as an effective therapeutic agent to modulate immune responses.

ROLE OF DGK ACTIVITY IN THYMOCYTE DEVELOPMENT

Bone marrow-derived early progenitor cells must go through an elaborate process of development in the thymus to become mature T cells (84, 85). Thymocytes at different stages of maturation are readily distinguished by a combination of CD4 and CD8 co-receptors expressed on their cell surface, proceeding from the earliest stage with neither CD4 nor CD8 (double negative/DN) through an intermediate stage expressing both CD4 and CD8 (double positive/DP) to a mature stage marked by the expression of either CD4 or CD8 (CD4 single positive/CD4SP or CD8 single positive/CD8SP) (86, 87). While progenitor cells enter the thymus at the cortico-medullary junction, a number of sequential chemokine/chemokine-receptor interactions help guide a developing thymocyte through the thymic cortex and medulla, facilitating its progressive relocation to appropriate micro-environments within the thymus (88, 89).

With the expression of RAG proteins, DN cells undergo VDJ recombination at the TCRβ locus, expressing a pre-TCR on the cell membrane. DN cells with a productive TCRβ rearrangement pass through the β-selection developmental checkpoint, undergoing multiple rounds of proliferation and upregulating expression of CD4 and CD8 to become DP cells. DP cells subsequently rearrange V and J genes at the TCRα locus, expressing a unique TCR on the cell surface. Subsequently, cells bearing TCRs that recognize self-peptide-MHC complexes on thymic epithelial cells receive survival signals during the so-called positive selection process, while others that fail to recognize these complexes die of "neglect" (90, 91). On the other hand, DP cells with TCRs that recognize self-peptide-MHC complexes with high affinity are eliminated by apoptosis-inducing signals during negative selection (92, 93). Together, positive and negative selection processes ensure the generation of a T cell repertoire that is both functional and self-tolerant (94, 95). DP cells that survive these selection processes mature into CD4SP and CD8SP cells that eventually migrate to secondary lymphoid organs as naïve CD4 and CD8 T cells (96, 97).

A plethora of studies have implicated DAG-dependent signaling pathways in β, positive and negative selection. For instance, early studies showed that signaling via the pre-TCR activates ERK1/2 (98), while more recent ones have demonstrated an essential role for RasGRP1 and ERK activation in efficient β-selection (27, 99). Mice with a T cell-specific deficiency of PLCγ1 show dramatically reduced numbers of mature CD4SP and CD8SP thymocytes, and defects in both positive and negative selection when crossed with HY TCR transgenic mice (100). Impairment

of thymic selection in the absence of PLCγ1 suggests that its product DAG may play an important role in the process. Lending further credence to this notion, RasGRP1-deficient mice show impaired Ras-ERK signaling in thymocytes and defective thymic selection with a 70–90% reduction of mature SP cells (25, 26). Transgenic mice expressing a dominant negative form of Ras present with defects in positive selection, but not negative selection, when crossed with HY TCR transgenic mice (101). Similar observations were made with transgenic mice that expressed a catalytically inactive form of MEK1 (K97A) under the control of the thymocyte-specific Lck proximal promoter (102). ERK1 deficiency results in a severe developmental block at the DP stage (103). Conditional deletion of ERK2 using proximal Lck-Cre partially blocked DN3 to DN4 progression, while deletion with CD4-Cre led to defective positive selection. Mice with a combined deficiency of ERK1 and ERK2 showed that ERK activity is required for proliferation and differentiation associated with β-selection, and for positive selection (104). The MAP kinase-interacting serine/threonine kinases (Mnks) 1 and 2 lie downstream of ERK1/2 and p38 (105, 106). Recent studies have shown that TCR triggering can activate Mnk1/2 via the Ras-ERK pathway in a manner that is negatively regulated by DGK αζ activity (107). Although Mnk1/2 phosphorylate EIF4E, which is thought to promote translation initiation, combined deficiency of Mnk1/2 did not lead to obvious changes in thymocyte development. The mechanisms by which ERK1/2 regulate thymocyte selection remain to be clearly defined. Together, these studies suggest that the DAG-RasGRP1-Ras-ERK pathway plays a critical role in thymocyte development.

The role of the DAG-mediated PKCθ-IKK-NF-κB pathway in T cell development has also been studied extensively. While initial studies found no obvious developmental defects in PKCθ deficient thymocytes, more recent ones have suggested that PKCθ may be required for efficient positive selection (108, 109). T cell-specific deletion of IKKγ or replacement of IKKβ with a dominant kinase-dead form results in a reduction of mature CD8SP cells (110), while transgenic models that allow for activation or inhibition of NF-κB have revealed its role in the establishment of signaling thresholds for positive and negative selection (111).

The importance of these DAG-mediated pathways suggests that their tight regulation by DGK may be critical for normal thymocyte development. Studies with mice that lack both DGKα and DGKζ (DGKαζDKO) have confirmed this hypothesis (112). DGKαζDKO thymocytes experience excessive DAG accumulation and enhanced DAG-mediated signaling after TCR engagement. This is associated with a severe developmental block at the DP stage and a marked paucity of mature CD4SP and CD8SP cells. Defects in positive, but not negative, selection were revealed using a HY TCR transgenic system. Addition of exogenous PA to fetal thymic organ cultures increased the frequency of SP cells in DGKαζDKO thymi without obvious effects on control thymi, suggesting that DGKα and DGKζ play a synergistic role in T cell development not just by dampening DAG-mediated signals but also by promoting PA-mediated signals. This DGK-induced switch from DAG-driven to PA-driven signals may also play a critical role in preventing prolonged activation of the highly oncogenic Ras-ERK and NF-κB pathways in developing thymocytes. Indeed, HY TCR transgenic mice with decreased DGK activity showed significantly enhanced thymic lymphomagenesis, suggesting an important role for DGK activity in tumor suppression (112).

More recent work from our group has uncovered a novel role for DGKs as negative regulators of mTOR activity in thymocytes (113). Results from the study showed that low concentrations of phorbol 12-myristate 13-acetate (PMA), a functional analog of DAG, were able to induce phosphorylation of mTOR complex 1 substrates S6K1 and 4E-BP1 and mTOR complex 2 substrate Akt (S473), suggesting that DAG-mediated signaling is sufficient to induce activation of both mTOR complexes in thymocytes. DGKαζDKO thymocytes showed enhanced phosphorylation of S6K1, 4E-BP1, and Akt (S473) upon TCR engagement as compared to WT counterparts, suggesting that DGK activity inhibits TCR-induced activation of mTORc1 and mTORc2. Further studies are required to determine if dysregulated mTOR signaling might contribute to the defects in T cell development and function observed in DGKαζDKO mice.

Emerging evidence also suggests that tight regulation of DAG-mediated signaling by DGK activity may be critical for the development of *i*NKT cells. *i*NKT cells are a rare but distinct lineage of αβ T cells that express a highly restricted TCR repertoire and recognize glycolipids presented on CD1d. Sometimes called the "Swiss-Army knife" of the immune system, *i*NKT cells bridge innate and adaptive immunity by performing an array of functions that include killing of infected cells and secretion of cytokines and chemokines (114). Despite their relative rarity, an important role for *i*NKT cells has been demonstrated in immune responses to pathogens, allergens, self-antigens, and cancer.

Previous work has revealed a critical role for signaling via the PKCθ-IKK-NF-κB pathway in the ontogeny of *i*NKT cells (15, 115–117). More recent studies have identified that RasGRP1-Ras-ERK signaling may also be indispensable for *i*NKT development. In one study, the absence of RasGRP1 was associated with a severe reduction of *i*NKT cell numbers in the thymus, spleen, and liver (118). The generation of bone marrow chimeras showed that the *i*NKT cell developmental defects were cell-intrinsic, and the remaining RasGRP1-deficient iNKT cell population displayed both a selective absence of $CD4^+$ cells and defects in TCR-induced proliferation. In another study, the expression of a dominant negative form of Ras dramatically hindered *i*NKT development (118, 119).

While lack of IKK-NF-κB and Ras-ERK signaling is detrimental to *i*NKT cell development, recent findings indicate that hyperactive signaling via these DAG-mediated pathways may also perturb *i*NKT development (120). In this study, T lineage specific expression of a constitutively active form of Ras resulted in a late stage block in *i*NKT cell maturation, and constitutively IKKβ activity was associated with increased cell death at multiple developmental stages. Since the maintenance of optimal levels of DAG-mediated signaling appears to be essential for normal *i*NKT development, we hypothesized that tight regulation of DAG-mediated signals by DGK activity might be essential for this process. Results from our studies showed that while *i*NKT cell numbers were unaltered in mice lacking either DGKα or DGKζ, they were dramatically diminished in DGKαζDKO counterparts, suggesting that these DGK isoforms may play a redundant role in regulating *i*NKT cell

development (120). Defective DGKαζDKO *i*NKT development was associated with enhanced cell death and co-incident with enhanced activation of the Ras-ERK and NF-κB pathways. Taken together, these results suggest that DGKα and DGKζ work synergistically to maintain an optimal level of DAG-mediated signaling that is essential for normal *i*NKT development.

ROLE OF DGK ACTIVITY IN T CELL FUNCTION

DGKα AND DGKζ IN T CELL ACTIVATION

T cell activation is a dynamic cellular process that involves the activation of multiple signaling cascades (**Figure 2**). The termination of such signaling, however, is important to prevent unrestrained immune responses and the development of autoimmunity. Research over the last decade has delineated a role for DGKα and DGKζ isoforms as molecular brakes that terminate DAG-mediated signals after TCR engagement. Early studies demonstrated that DGKζ is expressed in multiple lymphoid organs, with high levels in the T cell compartment (6). Overexpression of DGKζ in Jurkat T cells indicated that it substantially hindered TCR-induced Ras-ERK activation and upregulation of the activation marker CD69. DAG-binding and kinase domains, but not the ankyrin repeats, of DGKζ were found to be required for these inhibitory effects. Analysis of germline DGKζ knockout mice revealed no dramatic differences in T cell development or homeostasis (121). However, DGKζ-deficient T cells showed enhanced Ras-ERK activation and diminished PA production upon TCR engagement. Complementing observations from the Jurkat system, DGKζ-deficiency resulted in increased upregulation of CD69 and CD25 (markers of T cell activation) upon TCR engagement. Consistent with enhanced activation, DGKζKO T cells were hyper-proliferative in response to both antigenic stimulation and lymphopenia.

The role of DGKα in controlling T cell activation largely parallels that of its ζ counterpart. Overexpression of DGKα in Jurkat cells greatly impaired TCR-induced activation of a co-transfected AP1 driven luciferase reporter construct without obvious effects on calcium influx (122). While DGKαKO mice did not present with obvious changes in T cell development or homeostasis, enhanced Ras-ERK activation and hyper-proliferation were observed upon TCR engagement in DGKαKO T cells. Taken together, these results suggest that DGKα and DGKζ act in a non-redundant manner to restrain DAG-mediated signaling and prevent T cell hyper-activation upon TCR engagement. Further studies are required to better understand the unique mechanisms by which these isoforms act.

DGKα AND DGKζ IN T CELL ANERGY

Mechanisms of central and peripheral tolerance play a critical role in preventing the development of autoimmunity (123, 124). T cell anergy is a form of peripheral tolerance whereby T cells that

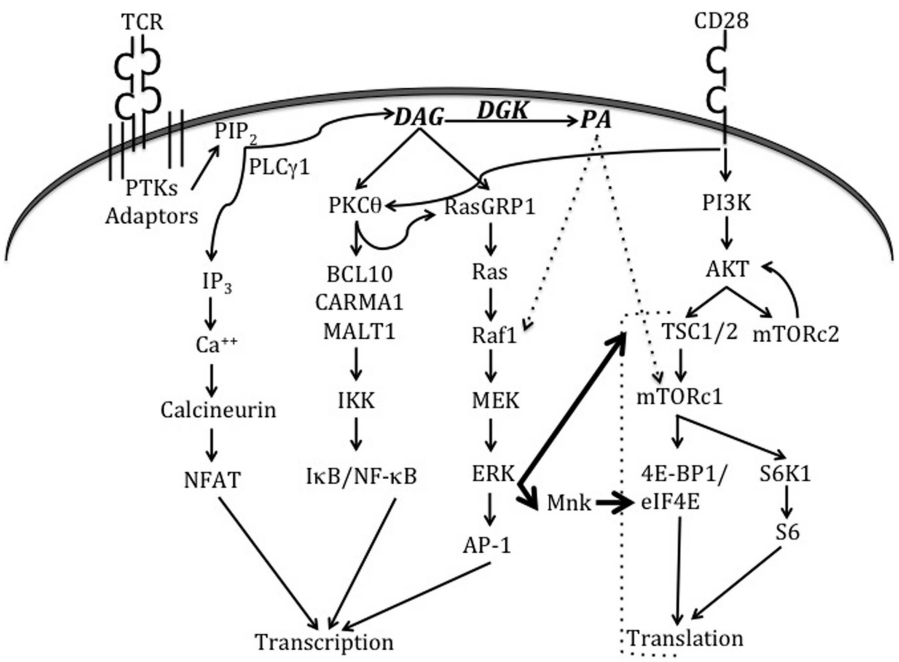

FIGURE 2 | Signaling pathways triggered by TCR and CD28 engagement. When the TCR engages a cognate peptide-MHC complex in the presence of appropriate co-stimulatory signals, this activates TCR proximal tyrosine kinases (PTKs) and results in the recruitment of a number of adaptor molecules. Eventually, the activation of PLCγ1 enables it to hydrolyze membrane PIP_2 to form second messengers IP_3 and DAG. IP_3 activates the calcineurin-NFAT pathway, while DAG activates the Ras-ERK-AP1 and NF-κB pathways. DGKs dampen DAG-mediated signals by converting DAG to PA. CD28 engagement plays an important role in the activation of PKCθ and the PI3K-Akt-mTOR axis. Recent work (indicated by thick arrows) has shown that TCR signaling can also directly activate mTOR complexes via the Ras-ERK pathway, and that such activation is negatively regulated by DGK activity. ERK can also activate Mnk1/2 kinases that phosphorylate eIF4E to promote translation. PA is produced in T cells by the action of both DGKs and PLDs (not shown in this figure). In other cell types, PA has been shown to activate Raf1 and mTORc1. Please refer to the text for more details about TCR-triggered signaling pathways and effector molecules that bind to DAG or PA.

recognize self-antigen in the absence of co-stimulatory signals are rendered functionally inactive (125–128).

While anergic T cells express elevated levels of DGKα as compared to naïve counterparts, levels of DGKζ increase or remain unchanged depending upon the experimental system (122, 129, 130). Transduction of resting Coxsackievirus and adenovirus receptor (CAR) transgenic T_H1 cells with DGKα or DGKζ-containing adenoviral constructs revealed that overexpression of DGKα, but not DGKζ, was sufficient to cause an anergy-like state (130). T_H1 cells transduced with the DGKα construct showed diminished ERK activation and IL-2 production in response to stimulation with anti-CD3 and anti-CD28. Experiments with RAG2-deficient 2C TCR transgenic cells also indicated that DGKα overexpression resulted in impaired recruitment of RasGRP1 to the plasma membrane. Brief treatment of anergic T_H1 cells with a pharmacological inhibitor of DGK activity before overnight re-stimulation with anti-CD3 and anti-CD28 led to a dose-dependent increase in IL-2 production, suggesting a causal function for high DGK activity in T cell anergy. Similar results were obtained with RAG2-deficient 2C TCR transgenic T cells that had been rendered anergic *in vivo*.

Genetic evidence for the role of DGKα activity in T cell anergy comes from an *in vivo* anergy induction model in which mice were injected with the super-antigen staphylococcal enterotoxin B (SEB) that renders $V\beta8^+$ T cells anergic (122). When re-stimulated with SEB *ex vivo*, in contrast to WT counterparts, $V\beta8^+$ T cells from DGKαKO mice retained the ability to produce IL-2 and proliferate. These findings complement the data from the adenoviral-based over-expression studies, and confirm that DGKα is essential for anergy induction *in vivo*.

Further studies also indicate that DGKα and DGKζ may play synergistic roles in anergy induction (122). When splenocytes from WT, DGKαKO, and DGKζKO mice were depleted of CD8 cells and stimulated in the presence of anti-CD3 and CTLA4-Ig (to block co-stimulatory signals), DGKαKO, and DGKζKO T cells underwent 2–3 rounds of proliferation in contrast to WT counterparts that did not divide. Proliferation of DGKζKO T cells under similar culture conditions was highly enhanced by the addition of a DGKα inhibitor, and was comparable to that of WT cells stimulated with anti-CD3 and anti-CD28. These results support the notion that α and ζ DGK isoforms may act in a synergistic manner to induce T cell anergy.

ROLE AND REGULATION OF DGKα AND DGKζ AT THE PLASMA MEMBRANE

Early studies found that in T cell lines, DGKα translocated from the cytosol to the plasma membrane in response to stimulation via an ectopically expressed muscarinic type 1 receptor as well as via the TCR (131). Examination of the redistribution of GFP-tagged DGKα revealed rapid but transient translocation of cytosolic DGKα to the plasma membrane after anti-CD3 and anti-CD28 crosslinking. Tyrosine-kinase phosphorylation, along with increases in intracellular calcium levels, was found to be essential for receptor-induced membrane translocation of DGKα. Pre-treatment of cells with the type I DGK inhibitor R59949 enhanced DGKα translocation to the plasma membrane at 2 min but also prevented DGKα dissociation from the membrane even after 60 min, suggesting that removal of DAG to produce PA may play a critical role in enzyme release. Results from this study thus showed that plasma membrane localization of DGKα is controlled not just by receptor-derived signals, but also by its own enzymatic activity.

More recent work has suggested that direct tyrosine phosphorylation of DGKα by the Src family kinase Lck may promote its membrane association in T cells (132). Results from the study showed that Lck phosphorylates DGKα at the Y335 residue in the hinge region between its C1 domains and the kinase domain. TCR triggering was found to induce rapid and transient phosphorylation of DGKα at Y335 in both Jurkat cells and primary human T cells. Fractionation analysis of Jurkat cells revealed that Y335-phosphorylation was detected only in the membrane-associated (but not cytosolic) fraction. In addition, a Y335F mutant form of DGKα failed to show plasma membrane localization in response to anti-CD3/anti-CD28 stimulation, in contrast to its WT counterpart. Immuno-precipitation experiments showed that Lck and DGKα interacted with each other, but that the pool of DGKα pulled down with Lck was not phosphorylated at Y335. The authors hypothesize that Lck-mediated phosphorylation of DGKα may induce the latter's dissociation from Lck but play a role in stabilizing DGKα at the membrane. Intriguingly, calcium flux induced by ionomycin was able to increase Y335-phosphorylation of DGKα, leading the authors to hypothesize that binding of Ca^{++} to DGKα's EF hands might induce a conformation change that increases Lck-mediated phosphorylation in the basal state.

Another study has since shown that c-Abl, a tyrosine-kinase involved in regulating cell cycle and proliferation, directly phosphorylates DGKα at Y218 in NIH 3T3 cells (133). Phosphorylation of this residue is thought to play an important role in serum-induced export of DGKα from the nucleus to the cytosol, as a Y218F mutant form was not exported from the nucleus in response to serum addition.

A role for SAP (an adaptor molecule recruited by the SLAM family of co-receptors) in inhibiting DGKα activity following TCR/CD28 stimulation has also been identified recently (134). Results from the study showed that DGKα (but not DGKζ) activity was reduced in response to TCR/CD28 or TCR/SLAM stimulation in Jurkat cells and human peripheral blood lymphocytes. However, such inhibition was not observed in Jurkat cells upon shRNA-mediated knockdown of SAP. SAP knockdown was also found to impair the recruitment of DGKα to the plasma membrane selectively upon TCR/SLAM stimulation, but not TCR/CD28 stimulation, suggesting that the enzymatic activity and localization of DGKα may be regulated by distinct mechanisms. Overexpression of SAP was sufficient to reduce DGKα activity in Jurkat cells, providing further evidence of SAP's role as a negative regulator. Such a role for SAP is also corroborated by findings from previous studies that SAP-deficient T cells showed reduced recruitment of PKCθ to the plasma membrane and diminished ERK1/2 activation upon TCR stimulation, leading to abnormal T cell differentiation and function (19). Pharmacological inhibition of DGKα activity by R59949 partially restored PKCθ membrane recruitment, ERK1/2 activation, and IL-2 production by SAP-deficient cells, suggesting that unrestrained DGKα activity might contribute to these signaling defects in the absence of SAP.

Like its α counterpart, the ζ isoform of DGK also shows dynamic changes in its subcellular localization in response to signals via the TCR. Early studies showed that GFP-tagged DGKζ rapidly translocated from the cytosol to the plasma membrane upon stimulation of an ectopically expressed muscarinic type 1 receptor, but not the TCR, in Jurkat T cells (135). Deletion of the C-terminal domain (containing the PDZ-binding domain and the ankyrin repeats), however, enabled DGKζ to translocate to the plasma membrane following TCR stimulation, suggesting that these domains may negatively regulate membrane translocation. The results also revealed that intact cysteine-rich C1 domains and PKCθ-mediated phosphorylation of the MARCKS domain are essential for DGKζ membrane translocation, while enzymatic activity is dispensable. Others have shown that DGKζ can translocate to the nucleus in COS-7 cells, and that this translocation is regulated by PKC-mediated phosphorylation of the MARCKS motif (136). Future studies should investigate a role for DGKζ in regulating nuclear DAG levels in immune cells.

When a T cell recognizes cognate peptide-MHC complexes and co-stimulatory molecules on an antigen-presenting cell (APC), this leads to the formation of a specialized junction at the T cell-APC interface. This so-called "immunological synapse" typically consists of a central cluster of T cell receptors surrounded by a ring of adhesion molecules, and synapse formation is thought to sustain robust signaling by facilitating the co-localization of kinases and adaptor proteins while excluding phosphatases (137, 138).

Previous studies have shown that DGKζ can physically associate with RasGRP1 in co-transfection experiments, but a similar function for DGKα was not tested (139). A recent study directly analyzed the recruitment of DGKα and DGKζ to the immunological synapse (140). Affinity purification of TCR complexes from Jurkat cells activated by anti-CD3 and anti-CD28 crosslinking suggested that both DGK isoforms were recruited rapidly to the TCR complex. However, video-microscopic experiments with GFP-tagged DGK proteins indicated that only DGKζ translocates rapidly to the plasma membrane at the early stages of synapse formation. These discrepant results need to be interpreted with caution, as fusion with GFP could potentially alter a protein's structure and disrupt its normal localization pattern. However, RNA interference experiments from this study showed that PA production at the TCR complex was substantially reduced by knock down of DGKζ but not DGKα, strengthening the notion of functional differences between the isoforms. The addition of PMA was found to enhance DGKζ activity upon TCR stimulation, indicating that DAG itself may regulate DGKζ activity. The use of a fluorescently tagged DAG-sensor domain showed that both plasma membrane localization and kinase activity of DGKζ were critical for DAG consumption at the immune synapse. Together, these results indicate a specific function for the DGKζ isoform in regulating DAG levels at the immunological synapse. Further studies are required to fully characterize the TCR-induced translocation of DGKα and DGKζ in primary T cells.

DAG AND T CELL SECRETION

The directed release of soluble factors is an important mechanism by which T cells kill target cells and communicate with other cell types. Early studies have shown that the microtubule-organizing center (MTOC) of a T cell reorients itself to a position just below the immunological synapse within minutes of TCR stimulation. Such polarization is though to aid in directional secretion by aligning the protein synthesis and secretion machinery of the T cell with the immune synapse (141, 142). Inhibiting MTOC translocation after TCR stimulation resulted in reduced phosphorylation of ZAP70 and LAT, disorganized immune synapse architecture and impaired IL-2 secretion, suggesting that MTOC translocation may also play a critical role in synapse formation and sustained TCR signaling (143).

More recent studies using a photoactivation system in which individual T helper cells can be activated by a pulse of ultraviolet light, have revealed a critical role for localized DAG production in MTOC polarization toward the synapse (144). MTOC polarization was abrogated in the presence of a PLCγ1 inhibitor, but unaffected in the presence of a Ca^{2+} chelator, suggesting that DAG may play a critical role in the process. Treatment with PMA, but not ionomycin, disrupted MTOC polarization providing further evidence that DAG, not Ca^{2+}, links PLCγ1 activity to MTOC polarization. Data from imaging experiments showed robust accumulation of DAG-sensor proteins at the region of photoactivation, followed by reorientation of MTOC to this region with an average delay of 13 s between the two events. Treatment with a DGK inhibitor prevented sustained C1-GFP accumulation at the irradiated region and thereby impaired MTOC polarization. Experiments with a version of DAG that could be activated by ultraviolet light showed that a localized increase in DAG concentration was sufficient to drive transient polarization of the MTOC. Taken together, these experiments indicate an important role for DAG and DGKs in directional secretion. Treatment with PMA or a type II DGK inhibitor impaired T cell-mediated killing of target cells, without affecting degranulation. This suggests that localized DAG signaling plays a critical role in CTL killing not by blocking granule release but by directing the granules toward their appropriate target. Further studies using the photoactivation system have shown that DAG recruits three distinct PKC isoforms, ε, η, and θ, to the immune synapse to promote cytoskeletal polarization following TCR stimulation (145).

A role for DGKα has also been established in the secretion of lethal FasL-bearing exosomes during activation induced cell death (AICD) (146). In this study, pre-treatment with a type I DGKα inhibitor increased the secretion of FasL-bearing exosomes upon TCR stimulation, and enhanced FasL-dependent AICD in J-HM1-2.2 cell line, suggesting that DGK may act as a negative regulator of exosome secretion. Based on the co-localization of DGKα with the trans-Golgi network and its presence in secreted exosomes, the authors proposed a model by which DAG may recruit PKD1 to the trans-Golgi network to promote vesicle budding.

Building on these results, a more recent study has identified a role for DGKα in the polarization of multi-vesicular bodies (MVBs) involved in the secretion of FasL-bearing exosomes (147). MVBs are late endosomes containing multiple exosomes/vesicles within their lumen that are formed by inward budding of the limiting membrane. In this study, inhibition of DGKα activity with a type I inhibitor was found to increase the number of mature MVBs, while overexpression of DGKα inhibited their formation, indicating that DGKα may negatively regulate the formation of

MVBs. However, siRNA-mediated inhibition of DGKα impaired the polarization of MVBs and subsequent exosome release, suggesting a positive role for DGKα in this process. Thus, DGKα plays a complex role in the secretion of FasL-bearing exosomes, impairing their formation but aiding their polarization toward the immune synapse.

DGKα AND DGKζ IN CTL RESPONSES

CD8 responses or CTL responses are critical for host defense against intracellular pathogens and tumors. CTL responses typically consist of three distinct phases – an expansion phase during which antigen-specific CD8 cells proliferate rapidly and differentiate into effector cells that kill infected target cells, a contraction phase during which 90–95 percent of these effector CD8 cells undergo apoptosis in response to diminishing antigen levels, and a memory maintenance phase in which the remaining 5–10 percent of cells are retained as a small but stable pool of fast-responding memory cells (148–150). Much effort over the recent years has focused on how signaling mechanisms in CD8 cells can be manipulated to alter the amplitude and kinetics of the CTL response. Preliminary experiments with lymphocytic choriomeningitis virus (LCMV) infection showed that mice deficient in DGKζ mounted a more robust response to the pathogen than WT counterparts (121). DGKζ-deficient mice showed a greater increase of splenic CD8 cell numbers than WT mice at day 7, with a bigger portion of $CD62L^{lo}$ $CD44^{hi}$ effector-memory (T_{EM}) cells and IFNγ-producing cells within the CD8 population. Viral titers were 50–70 percent lower in DGKζ-deficient mice than WT mice, arguing that DGKζ activity may negatively regulate CTL responses.

These results were confirmed and extended by a subsequent study, which showed that DGK activity differentially regulates primary and memory responses to LCMV (151). In this study, both DGKαKO and DGKζKO mice showed enhanced expansion and increased cytokine production upon LCMV infection, but contained fewer memory cells than WT counterparts after a 4-month period. When equal numbers of memory cells from these mice were transferred to new recipients and re-challenged with LCMV, DGK-deficient memory cells expanded significantly less than WT memory cells, indicating that DGK activity may somehow promote the expansion of memory cells. Other studies have revealed that the temporal kinetics of mTORc1 activity may play a critical role in effector versus memory differentiation of CD8 cells (152, 153). Results from these studies suggest that sustained mTORc1 activity may induce the expression of the T-box transcription factor T-bet that promotes effector differentiation. The identification of DGKs as negative regulators of mTOR activity (113) suggests the possibility that sustained mTORc1 activity in DGK-deficient CD8 cells might favor effector differentiation and mitigate memory formation. While mTORc1 activity was indeed found to be elevated in DGK-deficient CD8 cells (as measured by phosphorylation of the ribosomal protein S6) (151), further studies are required to dissect the contribution of enhanced mTORc1 activity to the dysregulation of CD8 responses seen in DGKαKO and DGKζKO mice.

DGKζ also acts as a negative regulator of anti-tumor CTL responses in an EL4-Ova lymphoma model (154). In this model, significantly smaller tumors were recovered from DGKζ-deficient mice as compared to WT mice, 3 weeks after implantation of tumor cells. Evaluation of CD8 splenocytes revealed a higher proportion of T_{EM} cells and a higher proportion of Ova-specific CD8 cells in DGKζ-deficient mice than in WT mice. An increased percentage of tumor-infiltrating CD8 cells was also found to be proliferating in DGKζ-deficient mice as compared to WT counterparts. Taken together, these results suggest that DGKζ activity may play a critical role in restraining anti-tumor responses, closely mirroring its functions during CTL responses to viral infection. When naïve WT-OT1 and DGKζKO-OT1 cells were adoptively transferred into congenically marked recipients that subsequently received EL4-Ova lymphoma cells, recipients with DGKζKO-OT1 cells developed smaller tumors. DGKζKO-OT1 cells also contained a bigger pool of $CD44^{hi}$ cells and IL-2 producing cells. Collectively, these results argue for a CD8 cell-intrinsic role for DGKζ in curtailing anti-tumor responses.

Investigation of tumor-infiltrating CD8 cells in human renal cell carcinoma patients showed increased DGKα activity and diminished signaling via MAPK pathways, as compared to CD8 cells that were present in non-tumor areas of the kidney (155). Increased DGKα activity was associated with defects in granule exocytosis and lytic function of these CD8 cells, and treatment with a DGKα inhibitor was able to increase ERK phosphorylation.

Table 1 | Biological functions of DGKs in T cells and other immune cells.

Functions regulated by DGK activity	Reference
DAG metabolism at the T cell-APC immunological synapse	Sanjuan et al. (131) Topham and Prescott (139) Santos et al. (135) Merino et al. (132) Baldanzi et al. (134) Gharbi et al. (140) Matsubara et al. (133)
Development of αβ T cells	Guo et al. (112) Gorentla et al. (113)
Development of *i*NKT cells	Shen et al. (120)
T cell activation and anergy	Zhong et al. (6) Zhong et al. (121) Olenchock et al. (158) Zha et al. (130)
CD8 T cell responses to pathogens and tumors	Zhong et al. (121) Riese et al. (154) Prinz et al. (155) Shin et al. (151)
MTOC polarization and directional secretion	Alonso et al. (146) Quann et al. (144) Alonso et al. (147)
Mast cell degranulation and cytokine production	Olenchock et al. (158)
Macrophage and DC cytokine production	Liu et al. (159)

Culturing with low-dose IL-2 reduced DGKα expression and enhanced ERK activation and degranulation. IL-2 treatment also increased the frequency of tumor-infiltrating cells that produced perforin, granzyme B, or IFNγ. Taken together, these data indicate that increased DGK activity and DAG metabolism dampen the responsiveness of tumor-infiltrating CTLs in a reversible manner. What factors in the tumor microenvironment drive increased DGK activity in CD8 cells is an important question that remains to be addressed.

ROLE OF DGK ACTIVITY IN OTHER IMMUNE CELLS

Apart from T cells, DAG and PA are critical signaling intermediates in several cell types including mast cells, dendritic cells (DCs), and macrophages. It is therefore not surprising that tight regulation of DAG and PA levels by DGK activity is essential for normal functioning of these cell types. Mast cells are abundant at the host's interface with the environment, such as the skin and mucose (156). While best known for their role in the pathogenesis of asthma, allergy, and anaphylaxis, mast cells also play a critical role in pathogen surveillance and defense against parasites (157). In contrast to observations with T cells, mast cell function *in vivo* was diminished in the absence of the DGKζ, impairment of local anaphylactic responses (158). Bone marrow-derived mast cells that lacked DGKζ showed impaired degranulation but enhanced production of cytokines such as IL-6 when stimulated *ex vivo*. Other studies have shown that both the ζ1 and ζ2 isoforms of DGKζ expressed in bone marrow-derived macrophages and DCs, with ζ1 being predominant (159). Considered "professional phagocytes," macrophages, and DCs express a diverse array of pattern recognition receptors (including toll-like receptors or TLRs) that enable them to detect the presence of pathogens and cell debris. Upon stimulation with *Toxoplasma gondii* – stable tachyzoite antigen (STAg, which activates multiple TLRs), DGKζ-deficient splenic DCs and bone marrow-derived macrophages (BMMϕ) produced less TNFα and IL-12 p40 than WT counterparts. Consistent with this impairment in cytokine production, both resistance to endotoxin shock and susceptibility to *T. gondii* infection were increased in DGKζ KO mice. While these findings provide tantalizing evidence of a role for DGKζ in regulating innate immune responses, further studies are required to gain a better understanding of the underlying molecular mechanisms. In addition, the involvement of other DGK isoforms in the regulation of innate immune responses remains to be investigated. The varied biological functions of DGK activity in T cells and other immune cells are summarized in **Table 1**.

SUMMARY

Over the past few years, a remarkable number of elegant studies that have furthered our understanding of the roles of DAG-mediated and PA-mediated signaling pathways, and their regulation by enzymes of the DGK family, in T cell development and function. A role for DGK activity has been identified in a variety of critical processes including conventional αβ T cell and *i*NKT cell development, T cell activation and anergy, directional secretion, and suppression of CD8 responses against viruses and tumors. While a multitude of interesting and fundamental questions in the field have been addressed by these recent studies, it is important to note that perhaps just as many others await answers. The roles of DGK-derived PA and PLD-derived PA in T cell development and function have proved challenging to dissect, as have differences between DGK isoforms in terms of substrate specificity and subcellular localization. Key elements such as transcription factors, microRNAs, and post-translational modifications that control the dynamic expression and function of DGKs during a T cell's lifetime also remain relatively unexplored. Applying the scientific method to answer these intriguing questions is likely to yield a better understanding of how DAG and PA signals and DGK activity regulate immune responses, enhancing our ability to modulate such responses to quell self-reactivity or generate protective immunity.

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Polyunsaturated fatty acid-derived lipid mediators and T cell function

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Fatty acids are involved in T cell biology both as nutrients important for energy production as well as signaling molecules. In particular, polyunsaturated fatty acids are known to exhibit a range of immunomodulatory properties that progress through T cell mediated events, although the molecular mechanisms of these actions have not yet been fully elucidated. Some of these immune activities are linked to polyunsaturated fatty acid-induced alteration of the composition of cellular membranes and the consequent changes in signaling pathways linked to membrane raft-associated proteins. However, significant aspects of the polyunsaturated fatty acid bioactivities are mediated through their transformation to specific lipid mediators, products of cyclooxygenase, lipoxygenase, or cytochrome P450 enzymatic reactions. Resulting bioactive metabolites including prostaglandins, leukotrienes, and endocannabinoids are produced by and/or act upon T leukocytes through cell surface receptors and have been shown to alter T cell activation and differentiation, proliferation, cytokine production, motility, and homing events. Detailed appreciation of the mode of action of these lipids presents opportunities for the design and development of therapeutic strategies aimed at regulating T cell function.

Keywords: T cells, polyunsaturated fatty acids, eicosanoids, prostaglandins, leukotrienes, cyclooxygenase, lipoxygenase, endocannabinoids

INTRODUCTION

The regulation of energy metabolism is crucial to T cell-mediated immunity including activation, proliferation, and differentiation (1). Following recognition of antigen in the lymph nodes, naïve T lymphocytes undergo massive clonal expansion and differentiation, followed by a contraction or death phase, and the establishment and maintenance of immunological memory (2, 3). Before undergoing division, T cells activate biosynthetic pathways for the production of proteins, nucleic acids, lipids, carbohydrates, and other "building blocks" necessary for the generation of new cells. Following this stage, the metabolic machinery of T cells is reprogramed, switching from the β-oxidation of fatty acids in naive T cells to the glycolytic pathways in activated T cells (4–6).

Downstream of T cell receptor (TCR) signaling, phosphatidylinositol 3′-kinase (PI3K) leads to the activation of the serine–threonine kinase AKT, which promotes glucose metabolism by stimulating the localization of the glucose transporter Glut1 to the plasma membrane, and the activity of hexokinase and phosphofructokinase, two rate-limiting enzymes of the glycolytic pathway. Increased glycolytic flux enables activated T cells to generate ATP and, at the same time, efficiently utilize carbon sources in the form of amino acids and lipids for the biosynthesis of proteins and membranes necessary for the expansion phase that characterizes the immune response (7–11). AKT also controls the activation state of mammalian target of rapamycin (mTOR), a sensor of nutritional and energetic status in cells that promotes protein synthesis.

T cell activation also initiates distinct transcriptional programs, which determine their differentiation into functional subsets depending on the context [cytokines, prostaglandins (PG), and other extracellular signals] in which they were activated (12–14). These subsets define the characteristics of the immune response. Whereas CD8+ T cells differentiate into cytotoxic T lymphocytes that kill infected host cells, CD4+ T lymphocytes differentiate into either the Th1, Th2, or Th17 subset of helper T cells (effector T cells) that mediate appropriate immune responses or into induced regulatory T cells (iTreg cells) that suppress uncontrolled immune responses (12). There is evidence that the cytokine milieu in which T cells differentiate can influence their metabolic programing. A comparison of activated T cells responding to related cytokines IL-2 and IL-15 illustrates the differential regulation of T lymphocyte metabolism by distinct cytokine environments: IL-2 promotes elevated glucose metabolism and glycolysis, while IL-15 does not maintain this metabolic state and T cells responding to IL-15 are smaller with reduced nutrient uptake and glycolysis (15, 16).

After clearance of the infection, most clonally expanded and differentiated T cells undergo apoptosis (contraction phase). The surviving antigen-specific T cells (memory T cells) are responsible for enhanced immunity after re-exposure to the same pathogen. Of these various T cell subsets, the iTreg cells and memory T cells rely on lipid oxidation as a major source of energy, whereas cytotoxic T lymphocytes and effector T cells are characterized by high glycolytic activity (17–19).

Further to oxidation for energy production, fatty acids are involved in many other aspects of T cell biology. In particular,

omega-3 polyunsaturated fatty acids (*n*-3 PUFA) are recognized as modulators of inflammation and immunity mediating their pleiotropic activity through regulation of gene expression, influencing signaling cascades, and altering the composition of the cellular membranes (20, 21). The latter has implications for the structure and function of the membrane, as well as a direct impact on the production of *n*-6 and *n*-3 PUFA-derived bioactive lipids including PG, leukotrienes (LT), resolvins (Rv), protectins (PD), endocannabinoids, and related congeners.

Although the immunomodulatory properties of PUFA have been known for many years, the molecular mechanisms underlying these properties are not fully understood. It has been shown that *n*-3 PUFA suppress antigen presentation, T cell activation and proliferation, and lower the expression of signature cytokines (21–27). Disappointingly, early studies using daily supplementation with foods rich in *n*-3 PUFA failed to show significant improvement in organ transplantation rejection (28, 29). However, recent reports indicate that administration of purified eicosapentaenoic acid (EPA; 20:5*n*-3) induces the differentiation of regulatory T cells through upregulation of peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated nuclear receptor that regulates lipid and glucose metabolism, leading to increased allograft survival (30, 31).

Following this direction, studies have explored the effect of cellular incorporation of the main *n*-3 PUFA, EPA, and docosahexaenoic acid (DHA; 22:6*n*-3). These fatty acids can alter the composition and molecular organization of membrane rafts with a consequent impact on the activity of raft-associated signaling proteins and related events. Examples include recruitment and activation of PLCγ and F-actin, impairing mitochondrial translocation necessary to maintain Ca^{+} signaling for NFκB and AP-1 activation and IL-2 secretion, and suppression of phosphatidylinositol-dependent actin remodeling, all linked to reduced CT4+ T cell activation [recently reviewed in Ref. (20)]. Importantly, many of the PUFA mediated activities are conveyed through their metabolites that tend to be produced and metabolized upon request, can act near the site of their synthesis or transported via circulation and in this way mediate systemic effects (autacoids). These families of potent mediators are intimately involved in inflammation and immunity, with pro- and/or anti-inflammatory, proliferative, and chemoattractive activities (21).

Overall, these new findings suggest that a better understanding of the molecular mechanism of action of PUFA may lead to the development of effective therapeutics. In this article, we will overview the current knowledge of the function and impact of eicosanoids and related metabolites, as well as that of endocannabinoids and their congeners on T cell function, and examine potential applications in biomedical research.

PUFA-DERIVED LIPID MEDIATORS: BIOSYNTHESIS AND METABOLISM

The cellular membrane serves as a pool of PUFA available for further metabolism to various bioactive lipids. These potent autacoids act as local hormones and are produced upon request following the activation of signaling pathways or effect of environmental and other stimuli. The arachidonic acid (AA; C20:4*n*-3)-derived eicosanoids are some of the best known and studied bioactive lipids. The term "eicosanoids" is used to describe the bioactive derivatives of three fatty acids with 20-carbon acyl chains, namely: AA, EPA, and dihomo-gamma linolenic (DGLA; 20:3*n*-6). These metabolites, although mostly linked to inflammation, are also involved in cell migration, proliferation, chemotaxis, and immune reactions (32–34). Eicosanoids and related mediators derive from the activities of cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) epoxygenases and mono-oxygenases (**Figures 1** and **2**) [reviewed in Ref. (35)]. The term "endocannabinoids" refers to endogenous lipids ligands of the cannabinoid receptors CB1 and CB2. These are also derivatives of AA, while other PUFA ethanolamides are now recognized as members of this family (36). Although endocannabinoids can be metabolized by COX and LOX, their precursor phospholipids and metabolism are different to eicosanoids (**Figure 3**).

CYCLOOXYGENASE-MEDIATED FORMATION OF PROSTANOIDS

The eicosanoid cascade starts with the activation of phospholipases (PL), predominantly PLA_2 but also PLD and diacylglycerol (DAG) lipase that release AA and other PUFA from the cellular membrane (35). The family of PLA_2 comprises a large number of enzymes with distinct characteristics in terms of their activation, cellular localization, and substrate specificity (37). There is evidence for the presence of various PLA_2 isoforms in primary T cells and the Jurkat T cell line, including $cPLA_2$, $sPLA_2$, and $iPLA_2$ (38–42). Inducible isoforms of PLC and DAG lipase have also been identified in tumor and peripheral T lymphocytes (42, 43).

Free AA is then metabolized via the constitutive and inducible COX isoforms (COX-1 and -2, respectively) to the unstable endoperoxide PGH_2 that is then transformed to PG, thromboxanes (TX), and prostacyclin (PGI_2) via tissue specific terminal prostaglandin synthases (**Figure 1**); these COX-derived mediators belong to the family of eicosanoids and are collectively known as prostanoids. Apart from AA, prostanoids are formed from the other two 20-carbon containing PUFA, DGLA, and EPA, with the resulting metabolites having different activities and being considered less-inflammatory than the AA-derived ones (35, 44).

The exact profile of prostanoids is determined by the prevalence of specific synthases in the cell type or tissue of interest. PGE_2 is produced by prostaglandin E synthase (PGES) that is found as membrane bound (mPGES-1 and -2) or cytosolic (cPGES). mPGES-1 is an inducible isoform and is frequently found co-expressed with COX-2 (45, 46). PGD_2 is produced by the hematopoietic-type (H-PGDS) or the lipocalin-type (L-PGDS) synthases (47), while further non-enzymatic hydrolysis of PGD_2 gives rise to the anti-inflammatory cyclopentanone PGs PGJ_2 and 15d-PGJ_2 (48, 49). $PGF_{2\alpha}$ is produced either directly from PGH_2 via the prostaglandin F synthase (PGFS) or through further metabolism of PGE_2 and PGD_2 by PGE 9-ketoreductase and PGD 11-ketoreductase, respectively (50). Prostacyclin (PGI_2) is produced via the prostacyclin synthase (PGIS) and is usually detected as its stable but inactive metabolite 6-keto-$PGF_{1\alpha}$ (51). Finally, thromboxane synthase (TXS) converts PGH_2 to TXA_2, an unstable prostanoid that is quickly hydrolyzed to the stable but inert metabolite TXB_2 (51). The bioactivity of prostanoids is mediated through G protein-coupled receptors for PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, designated EP, DP, FP, IP, and TP,

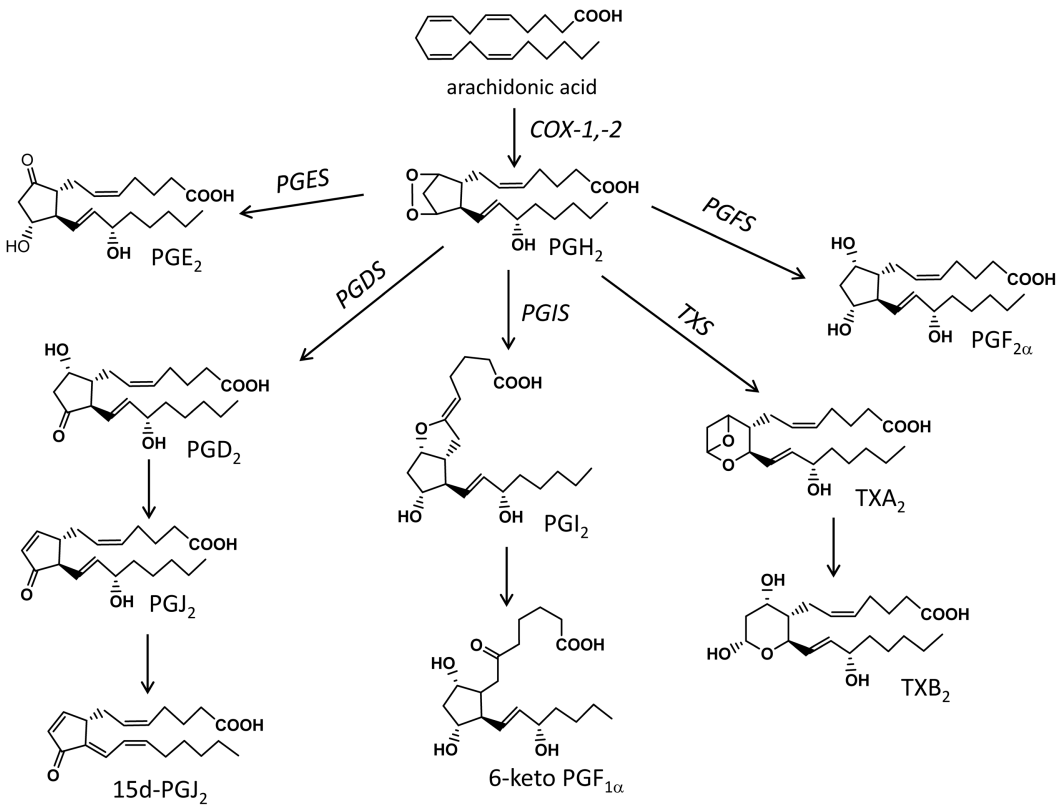

FIGURE 1 | Schematic representation of the main biochemical pathways that mediate the production of prostanoids. COX, cyclooxygenase; PGES, prostaglandin E synthase; PGDS, prostaglandin D synthase; PGFS, prostaglandin F synthase; PGIS, prostacyclin synthase; TXS, thromboxane synthase.

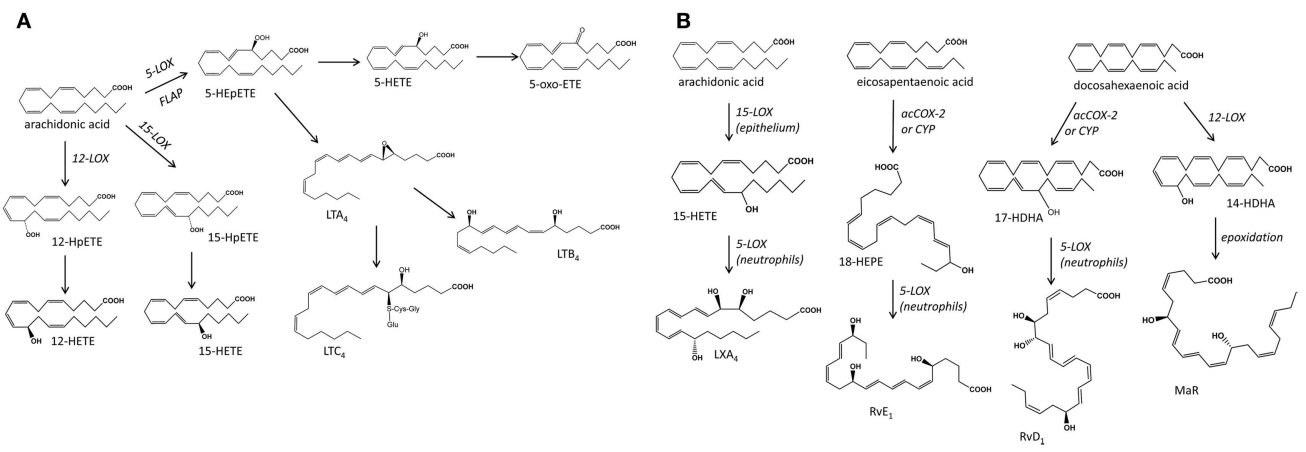

FIGURE 2 | Schematic representation of the main biochemical pathways that mediate the production of mono-hydroxy fatty acids and leukotrienes (A), and the poly-hydroxy fatty acids lipoxins, resolvins, and protectins (B), products of transcellular metabolism. LOX, lipoxygenase; HETE, eicosatetraenoic acid; HEpETE, eicosaperoxytetraenoic acid; LT, leukotriene; acCOX-2, acetylated cyclooxygenase-2; CYP, cytochrome P450; LX, lipoxin; RvE, resolving series E; RvD, resolving series D; MaR, maresin.

respectively. Pharmacological studies into their ligand-binding profiles and signal transduction pathways, and genetic analysis led to their classification into eight groups (EP1, EP2, EP3, EP4, DP1, FP, IP, and TP) although new developments have revealed the presence of a second PGD receptor, DP2, and the presence of heterodimers (52, 53). Overall, prostanoids are potent autacoids and their levels are controlled through enzymatic catabolism via dehydrogenations and reductions resulting in the formation of

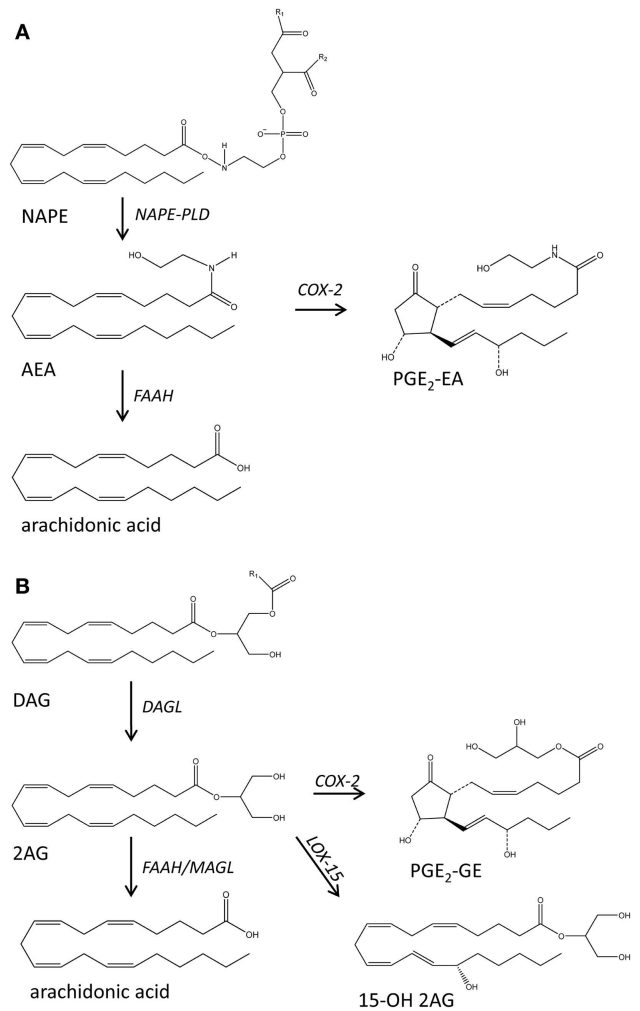

FIGURE 3 | Schematic representation of the main biochemical pathways that mediate the production of endocannabinoids anandamide (AEA) (A) and 2 arachidonoyl glycerol (2-AG) (B). DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; 15OH 2AG, 15 hydroxy-2 arachidonoyl glycerol; MAGL, monoacylglycerol lipase; NAPE, *N*-acylated ethanolamine phospholipids; PGE_2, PGE_2 ethanolamide; PGE_2-GE, PGE_2-glyceryl ester.

metabolites with significantly reduced bioactivities (e.g., 15-keto- and 13,14-dihydro-15-keto PGs) (54).

LIPOXYGENASE-MEDIATED PRODUCTION OF LEUKOTRIENES AND OTHER HYDROXY FATTY ACIDS

Lipoxygenases mediate the oxygenation of free fatty acids including AA and other PUFA. Their activities are commonly defined by their positional selectivity when they oxygenate AA and, following this system, the main mammalian LOX enzymes are defined as 5-, 12-, and 15-LOX. They catalyze the stereoselective insertion of OH in the *S* configuration, with the exception of a mammalian skin-specific enzyme 12*R*-LOX. The products of LOX reactions are unstable hydroperoxides that are then reduced to hydroxy acids (55–57) (**Figure 2**). 5-LOX acts in concert with 5-lipoxygenase activating protein (FLAP) to metabolize AA to 5*S*-hydroperoxyeicosatetraenoic acid (HPETE) that is further reduced to 5*S*-HETE or dehydrated to LTA_4, an unstable epoxide containing a conjugated triene system characteristic of all LT. LTA_4 can be metabolized to LTB_4 or form the cysteinyl LT, LTC_4, LTD_4, LTE_4 following conjugation with reduced glutathione (58). 5*S*-HETE can be also enzymatically reduced to the 5-oxo-eicosatetraenoic acid (5-oxo-ETE), a chemoattractant mediator (59). Mammalian 12- and 15-LOX isozymes oxygenate a range of PUFA, both free and esterified in membrane phospholipids and lipoproteins (57), forming a multitude of mono- and poly-hydroxy fatty acids: e.g., AA produces hydroxyeicosatetraenoic acids (HETE), EPA generates hydroxyeicosapentaenoic acids (HEPE), DHA produces docosanoids including hydroxydocosahexaenoic acids (HDHA), linoleic acid (LA; 18:2*n*-6) forms octadecanoids such as hydroxy octadecadienoic acids (HODE), DGLA forms hydroxyeicosatrienoic acids (HETrE), etc.

CYTOCHROME P450 MEDIATED FATTY ACID EPOXIDES AND THEIR DERIVATIVES

Cytochrome P450 mono-oxygenases relevant to PUFA metabolism catalyze epoxygenations and mid-chain and omega-hydroxylations producing a range of LOX-like mono-hydroxy fatty acids (e.g., HETE, HEPE, HODE) although not necessarily of the *S* configuration [reviewed in Ref. (35)]. Interestingly, partially inhibited COX-2 (e.g., acetylated COX-2 following treatment with aspirin) can also generate LOX-like products with the OH group at *R* configuration, e.g., 15*R*-HETE from AA and 18*R*-HEPE from EPA (60). These metabolites are important in aspects of transcellular metabolism where sequential LOX/LOX or acetylated COX-2/LOX or CYP/LOX reactions involving more than one cell types are involved in the formation of multi-hydroxy fatty acid species. These include the lipoxins (LX) that are tri-hydroxytetraene-products of AA, and the di- and tri-hydroxy-PUFA termed Rv, PD, and maresins (MaR) that are derivatives of EPA and DHA. All these mediators are involved in inflammation and immunity exhibiting a range of protective roles (61–63).

THE ENDOCANNABINOIDS

The endocannabinoids anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2AG) are derivatives of AA and act as endogenous ligands to the cannabinoid receptors CB1 and CB2 [reviewed in Ref. (36)]. This family of bioactive lipids has been extended to include other fatty acid ethanolamides and glycerols, while recent findings regarding their metabolism suggest a wider involvement in inflammation and immunity. The biochemical precursors of AEA and its congers are various *N*-acylated ethanolamine phospholipids (NAPE) that found in very low concentrations in the biological membranes and are hydrolyzed by NAPE-specific PLD or PLC-type lipases. 2AG production is mediated by PLC-diacylglycerol lipase. AEA and 2-AG can be deactivated via hydrolysis mediated by fatty acid amide hydrolases (FAAH) or can be metabolized by COX-2 to generated prostaglandin ethanolamides known as prostamides (e.g., PGE_2-EA) and prostaglandin glyceryl esters (e.g. PGE_2-GE) (**Figure 3**) (52). LOX isozymes can also metabolize these lipids although the

prevalence and bioactivities of the resulting mediators remain to be explored.

EICOSANOIDS AND RELATED MEDIATORS IN T CELL FUNCTION/BIOLOGY

PROSTANOIDS

It is now recognized that resting and activated T cells express the COX-1/-2 system (64–68). Although the constitutive COX-1 is not affected during T cell activation, the inducible COX-2 is upregulated as has been shown in studies with CD4+ cells, Jurkat T cells and adaptive Tregs (66–69). To date, very little is known about the exact profile of prostanoids produced by T cells with only a few studies reporting the production of PGE_2, PGD_2 and its dehydration product 15d-PGJ_2, as well as low levels of TXA_2 (67, 68, 70). There is also very little information on the type of prostanoid synthases expressed in T cells, including evidence for H-PGDS and PGES in Tregs (67, 68). However, a number of studies have explored the role of PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$, and TXA_2 on various aspects of T cell function, showing that prostanoid-mediated effects process through receptors and related signaling pathways expressed in most T cell populations and subtypes. Interestingly, it has been shown that treatment with AA upregulates the CXCR3/1 inducible chemokine receptors expressed in CD4+ T cells and increases their chemotactic responses through a COX-related pathway (71), suggesting a potential role for this pathway in the regulation of T cell migration.

PGE_2

Although considered to be a, primarily, pro-inflammatory eicosanoid, PGE_2 can also mediate anti-inflammatory signals, and is a potent immunosuppressor (72). PGE_2 is one of the best-studied bioactive lipids in T cell biology, exhibiting a multitude of effects. It is involved in the early stages of T cell development in the thymus, where it stimulates the differentiation of CD4+CD8+ thymocytes (73), while in later stages it regulates the development and balance of Th1, Th2, and Th17 subsets (74–76) and, overall, influences proliferation, differentiation, cytokine production, and apoptosis of mature T cells (14, 77–80). Interestingly, the activity of PGE_2 on T cells appears to be concentration-dependent: while at low concentrations, it is involved in homeostatic events and inhibits the activation and differentiation of T lymphocytes, at high concentrations, PGE_2 has the opposite effect, increasing T cell proliferation, and suppressing immune functions [recently reviewed in Ref. (81)]. For example, in ultraviolet radiation (UVR)-induced immunosuppression, impaired development of peripheral memory T cells can be attributed to UVR-induced PGE_2 production (82).

Antigen presenting dendritic cells (DC) and macrophages secrete PGE_2 and in this way can influence proliferation and differentiation of CD4+ and CD8+ cells, and direct the balance of Th1, Th2, and Th17 cell subtypes (14). PGE_2 can also affect the maturation of DC and alter DC-produced cytokines, thus influencing the differentiation of T cell subtypes: for example, DC cells matured in the presence of PGE_2 *in vitro* promote Th17 and inhibit Th1/Th2 polarization (78). PGE_2 can also enhance the proliferation of T cells through the induction of costimulatory molecules OX40L, CD70, and 4-1BBL on DC (83), while other studies have reported that PGE_2 inhibits the ability of DC to produce CCL19 and attract naive T cells (84). Interestingly, the ratio DC:T cells appears to be crucial in determining the overall immunogenic effect of PGE_2: it has been reported that at high DC:T cell ratios, PGE_2-maturated DC cells inhibit the proliferation of T cells, while, when this cell ratio is low, an enhanced T cell stimulation is observed (85). A dose-dependent effect has also been observed in the way PGE_2 mediates the balance Th1 to Th2 subtypes: high levels of PGE_2 suppress Th1 cell differentiation and polarization, shifting the immune response toward a Th2 phenotype (79). These observations have been confirmed *in vivo* using COX-2 inhibitors (e.g., celecoxib) and COX-2 knockout models demonstrating that when PGE_2 production is reduced, an increase in Th1 responses is observed [reviewed in Ref. (81)]. The regulation of Th2 cells by PGE_2 is likely to impact in Th2-mediated immune disorders such as atopic dermatitis and asthma (86, 87). Finally, when PGE_2 is produced by activated macrophages it reduces T cell activation and proliferation; this in turn leads to a reduction in cytokine production and consequent reduced stimulation of macrophages in a negative feed-back loop (72).

In vivo work has elucidated the role of EP receptors in mediating PGE_2 effects. PGE_2 produced by DC in the lymph node acts through the EP1 receptor to promote the differentiation of naive T cells to Th1 cells (88). Studies on the BALB/c mice, a strain showing propensity to generate Th2 responses, have shown that Th2 cells express high levels of EP2 and that PGE_2 signaling through this receptor protects Th2 cells against activation induced cell death (77). Furthermore, in a model of experimental autoimmune encephalomyelitis (EAE), PGE_2 signaling through EP4 was shown to exert a dual role: promoting immune inflammation through Th1 cell differentiation and Th17 cell expansion during the induction phase. In contrast, during the effector phase of the disease, it attenuated the access of these pathogenic T cells to the brain by protecting the blood brain barrier (89, 90).

PGE_2-induced effects mediated via the EP2/EP4 receptors are linked to cAMP concentration and related signaling (53). In cytotoxic T cells, PGE_2 and other cAMP activators trigger increased concentration of cAMP and this interferes with the cytoskeleton function and terminates cytotoxic T cell secretion and adhesion (91). Dietary interventions with *n*-3 and *n*-6 PUFA can alter the cell membrane composition with consequent changes in the concentration of PGE_2 produced, as well as the prevalence of the less-inflammatory PGE species, PGE_1 and PGE_3 (44). Although frequently cited as anti-inflammatory, these species do not always appear to be different in their immunomodulatory properties: for example, studies have shown that both PGE_2 and PGE_1 suppress mitogen-induced blastogenesis in T cells, an effect confirmed with experiments using indomethacin, a non-specific COX inhibitor (92).

PGE_2 ethanolamide appears to be also involved in the motility of T cells (93, 94) and recent work using imaging has identified PGE_2 as an antagonist of the T cell migration stop signal (95). This activity was shown to be subset specific, with Th migration in response to IL-2 inhibited at 10–100 ng/ml PGE_2 *in vitro*, although, in the same experimental conditions, the migration of cytotoxic T cells was not affected (96, 97). PGE_2 has also been suggested to inhibit the transendothelial migration of T cells through increased

calcium and cAMP concentrations (98, 99). In rats, PGE_2 was found to inhibit the migration of T cells across the microvascular retinal endothelial cells although it did not affect the expression of adhesion molecules on either endothelial or T cells (100). However, PGE_2 at nanomolar to micromolar concentrations elicited migration of T cells *in vitro* and increased secretion matrix metalloproteinases (MMP); although MMP inhibitors suppressed the transmigration, the inhibition did not affect the PGE_2-initiated T cell motility (101). Finally, overexpression of COX-2 in a mouse breast cancer model increased the recruitment of Tregs in the tumor, an effect mediated via EP2 and EP4 receptors (102).

PGD_2 and15d-PGJ_2

PGD_2 is considered an immunomodulatory prostaglandin and some of its cyclopentanone PG metabolites, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), are endowed with anti-inflammatory activities (49, 103). Production of PGD_2 has been detected in Th2 cells and this was linked to expression of H-PGDS, while L-PGDS has not been identified in any T cell subtype (67, 104, 105). The downstream product of PGD_2 dehydration, 15d-PGJ_2, has also been detected in human T cell cultures (67).

PGD_2 mediates its effects through two receptors DP1 and DP2, the latter better known as chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). DP1 belongs to the prostanoid family of receptors, signals through cAMP and has been detected in Th1, Th2, and CD8+ cells (106). DP2/CRTH2 has little similarity to prostanoid receptors and belongs to the cytokine receptor family; it signals through increased calcium and inhibition of cAMP and has been found to be preferentially expressed by activated Th2 cells mediating their recruitment and motility (106, 107). While PGD_2 can signal through either receptor, findings to date indicate that 15d-PGJ_2 activates only DP2 (103). It has been suggested that PGH_2 may also be an agonist of DP2 (108). PGD_2 and 15d-PGJ_2 are also agonists of PPARγ and can induce differentiation of fibroblasts to adipocytes; this has been shown in the case of Grave's disease where it was reported that activated T cells drive fibroblast differentiation in ocular tissue through production of PGD_2 and 15d-PGJ_2, implying that T cell infiltrates can influence fat deposition in other tissues (67).

PGD_2 can mediate different effects depending on the target receptor and related signaling events (109). DP1 can induce differentiation of Th2, whilst DP2/CRTH2 is mostly involved in their recruitment, although the two receptors may exert opposing effects, as examined in an animal model of contact hypersensitivity where DP2/CRTH2 appeared to mediate inflammatory events while DP1 was inhibitory (110). Furthermore, both receptors have been reported involved in T cell proliferation, and DP1 has been suggested to promote T cell apoptosis and downregulate immune responses, while DP2 has been reported to delay Th2 apoptosis (111). A potentially anti-inflammatory protective effect of 15-dPGJ_2 in pregnancy has been attributed to its suppression of Th1 response and promotion of Th2 immunity through DP2 (112).

Activation of Th2 cells by PGD_2 is thought to occur predominantly through DP2/CRTH2 with concomitant increase in the production of cytokines and pro-inflammatory proteins (106, 113–115). PGD_2 binding to this receptor is also very important for CD4+ T cell trafficking and motility (116, 117). When produced at high concentrations by mast cells, as seen in allergic inflammation, there is a consequent activation and recruitment of Th2 cells toward the PGD_2 producing sites (118, 119). Activated T cells can also produce PGD_2 and this may promote further accumulation of Th2 in the inflamed tissue (107, 116).

Finally, PGD_2 has been shown to affect the maturation of monocyte derived DC impacting on their ability to stimulate naive T cells and favoring their differentiation toward Th2 cells (120, 121). Interestingly, age related increase in PGD_2 levels have been associated with decreased DC migration and reduced T cell responses in a mouse model of respiratory infections, suggesting that inhibition of PGD_2 functions may be an effective therapeutic approach (122).

$PGF_{2\alpha}$

To date, there is very limited information on the contribution of this vasoactive prostaglandin on T cell function. There are no reports on the production of $PGF_{2\alpha}$ or expression of the relevant synthases on T lymphocytes. Early work exploring the involvement on PG on T cell locomotion considered the involvement of $PGF_{2\alpha}$ but this was not supported by the resulting data (93). However, a recent report on allergic lung inflammation presents evidence for the contribution of $PGF_{2\alpha}$ in Th17 cell differentiation, an autocrine effect mediated through cell surface FP receptors (123).

PGI_2

PGI_2 is best known as an inhibitor of platelet aggregation and potent vasodilator, while recent finding has shown its involvement in immune regulation with particular importance in airway inflammation. The IP receptor is expressed in a number of immune cells in the lung, including T lymphocytes of the Th1 and Th2 lineage (124, 125). However, there is very little information on the actual production of PGI_2 by T cells with only some indirect evidence for possible transcellular biosynthesis operating between platelets and lymphocytes, and some recent work showing PGIS mRNA in an animal model of contact hypersensitivity (125, 126).

Studies in various models suggest that PGI_2 is involved in regulating the balance of Th1 and Th2 responses, as well as promoting Th17 cell differentiation (13, 127). Work in a mouse model of asthma has shown that PGI_2 produced by endothelial cells and signaling through the IP receptor prevents the recruitment of Th2 in the airways (128). However, a mouse model of contact hypersensitivity shows that in cutaneous disease PGI_2-IP signaling raises intracellular cAMP concentration and promotes Th1 differentiation (125). Furthermore, PGI_2 increased the ratio of IL-23/IL-12 leading to differentiation of Th17 cells and exacerbation of EAE in mice (129). Finally, the anti-inflammatory effect of PGI_2 has been explored through analogs that reduced the production of pro-inflammatory cytokines and chemokines by DC, increased the production of anti-inflammatory IL-10, and inhibited their ability to stimulate CD4+ T cell proliferation (124).

TXA_2

Although production of TXA_2 by T cells has been reported, albeit at very low levels, the expression of the relevant synthase has not yet been shown (70, 130). However, the TP receptor has been

found in a range of T cell populations and a polymorphism identified in Th2 cells has been linked to aspirin-exacerbated respiratory disease (130–133). Work with human lymphocytes suggested that TXA_2 is involved in the inhibition of T cell proliferation and related cytokine production (134). Following production of TXA_2 by DC, stimulation in TP expression was observed and this appeared to be involved in the random movement of naive but not memory T cells, suggesting that TXA_2 can mediate DC–T cell interactions (130).

LEUKOTRIENES, HYDROXY FATTY ACIDS, LIPOXINS, RESOLVINS, AND PROTECTINS

Lipoxygenase isoforms identified in various T cell populations include 5-, 12-, and 15-LOX (135–138). Although some early studies suggested that externally provided AA could inhibit 5-LOX, recent reports have indicated that provision of substrate may be necessary for the synthesis of LTs (135, 139). There is evidence that 5-HETE, LTA_4 and LTB_4, and the cysteinyl LT LTC_4, LTD_4, and LTE_4, are produced by human and animal primary T cells and cell lines (43, 135, 138, 139). Furthermore, the presence of 5-LOX and 12/15-LOX would suggest the production of hydroperoxy- and hydroxy-PUFA by T cells. Nevertheless, there are not many studies examining the formation of such mediators and the majority of relevant reports focus on the effect of 12- and 15-HETE, LX, resolvins, and PD on T cell function.

LTB_4

The main activity attributed to LTB_4 is chemotaxis, a property mediated through the high affinity receptor BLT1 that is expressed in many CD4+ and CD8+ T cell subtypes (140–143). BLT1 is also important for homing events, as it enables the adhesion of T cells to epithelial cells, and appears of particular importance for the recruitment and direction of T cells to the airways in asthma (141, 144). Blockade of LTB_4/BLT1 pathway has also been shown to improve CD8+ T cell mediated colitis (145). Finally, LTB_4 appears involved in Th17 cell differentiation, Th1 and Th2 proliferation, and cytokine production (146–149).

LTC_4, LTD_4, LTE_4

The cysteinyl LT specific receptors CysLT1 and CysLT2 have been found to be expressed by peripheral blood T cells (150). Interestingly, it has been reported that resting Th2 cells display higher expression of the CysLT1 receptor compared to Th1 or activated Th2 cells, suggesting its involvement in Th2 cell differentiation (151, 152). Accordingly, in the presence of PGD_2, LTD_4 and LTE_4 have been shown to enhance Th2 cell activation and cytokine production, in a more than additive effect (153).

Furthermore, LTC_4 appears to induce T cell proliferation (154), while LTC_4-maturated DC appear to stimulate CD4+ responses and induce cytotoxic T cells *in vitro* without concomitant recruitment of Tregs (155).

5-HETE and 5-oxo-ETE

Oxidative stress appears to stimulate the metabolism of 5-HETE to 5-oxo-ETE in peripheral blood lymphocytes, although the role of this lipid mediator in T cell function is not clear (156, 157).

12-, 15-HETE

12-HETE has been involved in T cell function, with particular relevance to allergic disease. Although 12(S)-HETE is a neutrophil chemoattractant it does not appear to have a similar effect on T cells. Work on skin-derived lymphocytes involved in psoriasis has shown that 12(*R*)-HETE, a 12*R*-LOX product found in psoriatic skin, has modest chemotactic properties for T cells but is less potent than LTB_4 (158, 159). Furthermore, it has been shown that inhibition of 12/15-LOX enhanced the production of Th2 cytokines and attenuated the development of allergic inflammation in a mouse model of allergic lung disease, whilst delivery of 12(S)-HETE had the opposite effect (136). Increased levels of 12-HETE were also associated with metabolic changes in T cells leading to development of autoimmune disease (137).

It has been reported that 15-HETE regulates T cell division and displays anti-proliferative effects on a leukemia T cell line (160–162). Metabolism of 15-HETE through β-oxidation has been observed in blood T cells leading to the hypothesis that the resulting β-hydroxy acids and their oxidized and decarboxylated products may play a role in T cell biology (163). 15-LOX metabolites have also been involved in Th1 responses in a mouse model of Th1 allergic inflammation induced by double-stranded RNA (164).

Lipoxins

Although not directly produced by T cells, LXA4 has been shown to interact with the LTB_4 receptor expressed in T cells (165, 166). Aspirin-triggered LXA_4 and LXB_4, and stable analogs, inhibited TNFα production by human peripheral blood T cells suggesting the involvement of these metabolites in T cell mediated inflammation (167). Finally, LXA_4 appears to be involved in Treg-mediated tumor protection through the induction of myeloid suppressor cells, as shown in a murine liver cancer model (168).

Resolvins and protectins

These products of EPA and DHA are formed through transcellular metabolism and some of their anti-inflammatory and pro-resolution effects are mediated through their effects on T cells. It has been reported that PD1 is formed by Th2-skewed peripheral blood mononuclear cells and appeared to block T cell migration, inhibit TNFα and IFγ secretion, and promote apoptosis *in vivo* (169). Reduction of CD4+ and CD8+ T cell infiltrates and CD4+ T cell-produced cytokines was also observed in a mouse model of DNFB-induced atopic dermatitis treated with RvE1 (170). Furthermore, RvE1-treated bone marrow-derived DC appear to induce apoptosis of T cells, and it has been suggested that instead of migrating to the lymph nodes they remain on the inflammatory sites targeting the infiltrating effector T cells (171). RvE1 has also been shown to reduce the influx of Th1 and Th17 cells in the cornea of a mouse model of stromal keratitis, a virally induced immunopathological disease; it has been suggested that this may have contributed to a significant reduction in lesions observed (172).

ENDOCANNABINOIDS AND CONGENERS

The endocannabinoid system is considered an important regulator of the immune response with AEA, 2AG, and related enzymes and receptors being involved in T cell function (173–176). Production of AEA and 2AG have been shown in human T lymphocytes

(177, 178), while the receptors CB1 and CB2 have been identified in primary T cells and T cell lines where their expression is stimulated upon activation (179, 180). In particular, the CB2 receptor has been shown to mediate the inhibition of mixed lymphocyte reactions by cannabinoids and is of interest for the development of novel chemotherapeutic approaches to prolong graft survival (181). Furthermore, CB2 has been suggested as an important factor for the formation of T cell subsets including splenic memory CD4+ cells and natural killer T cells (182). Interestingly, a common CB2 gene polymorphism has been linked to reduced immune modulation by endocannabinoids and may be a risk factor for autoimmune disorders (183). Finally, FAAH and monoacylglycerol lipase (MAGL) are also present in human T lymphocytes (179). FAAH appears to play a protective role controlling the levels of AEA in pregnancy as well as immune-mediated liver inflammation (178, 184).

AEA and congeners

Work with activated primary human T lymphocytes has shown that AEA can suppress T cell proliferation and cytokine release in a CB2-dependent manner, without exerting cytotoxic effects (185, 186). However, other studies suggested that AEA inhibits T cell proliferation and induces apoptosis through a mechanism that may not be receptor mediated but most probably related to lipid rafts (187, 188).

The immunosuppressive effect of AEA extends to Th17 cell and this is of particular interest for the development of immunotherapeutic approaches (186). Endogenous AEA or inhibition of FAAH leading to increased AEA levels, were effective in reducing cytokine levels, decreased liver injury, and increased numbers of Treg cells in a murine model of immune-mediated liver inflammation (184). AEA inhibited the migration of CD8+ T cells in a collagen-based migration assay, again through the CB2 receptor (189). However, a study evaluating the direct anti-cancer potential of AEA, reported no effect on lymphocyte proliferation or Treg generation or cytokine production (190). In contrast, other studies have reported proinflammatory effects by AEA. In a mouse model of atherosclerosis, reduced levels of FAAH that resulted in increased AEA and its congeners, palmitoyl- and oleoyl-ethanolamide, were accompanied by reduced CD4+FoxP3+ regulatory T cells, suggesting a pro-inflammatory effect on the overall immune response (191). In addition, AEA appears to promote Th1 immunity as shown in a model of sensitization where it was reported to induce DC activation and IFNγ production (192). Finally, a recent study with bimatoprost suggested that this prostamide can induce calcium signaling in human T cells (193).

2AG

The chemotactic properties of 2AG are also mediated through the CB2 receptor and this has been shown in various immune cells including migration of splenocytes (194), homing of B cells (195), and motility of human natural killer cells (196). When this potential was assessed in activated T lymphocytes, it was reported that although 2AG did not induce T cell migration, it inhibited migratory responses toward the chemokine CXCL12, suggesting a possible regulatory role in T cell migration (179). Furthermore, 2AG can act as DC chemoattractant and indirectly shift the memory response toward a Th1 phenotype in a CB2-mediated fashion (197). 2AG can also suppress IL-2 production in Jurkat cells through PPAR-γ activation and independently of CB1 and CB2-mediated signaling (198). The contribution of a COX-2 metabolite of 2AG has also been considered by recent reports confirming that the 15-deoxy-delta (1)(2),(1)(4)-PGJ_2-glycerol ester (15d-PGJ_2-GE) is a PPAR-γ ligand that suppresses IL-2 production in activated Jurkat cells (111, 199).

CONCLUDING REMARKS

While current evidence support a key role for PUFA-derived bioactive lipids in the regulation of T cell immunity (**Table 1**), the complexity of their biological properties and the lack of a comprehensive understanding of their exact contribution to different stages of the immune response hinders the identification of mediators of interest either as markers or as target compounds for drug development. In general, it appears that lipid mediators regulate T helper cell polarization into Th1/Th2 and Th17 cells, a key event in many immune-mediated diseases. Despite the molecular mechanisms for this effect and the regulatory role of these lipids on other T cell functions have yet to be explored, an extensive number of studies in mice and humans underscore their therapeutic potential.

This concept is supported by the large number of studies using their precursor fatty acids. Of particular importance is the focus on *n*-3 PUFA that have been explored as anti-inflammatory and immune-protective agents for a range of diseases and relevant experimental models including psoriasis, rheumatoid arthritis, and atherosclerosis (32, 33, 200). A recent study has shown that dietary *n*-3 PUFA favorably modulate intestinal inflammation in part by downregulating pathogenic T cell responses (201). The Fat-1 mouse, a genetic model that synthesizes long-chain *n*-3 PUFA *de novo*, was shown to be relatively resistant to colitis induction due to a reduced differentiation of Th17 cells and related cytokines (202). The immunoregulatory potential of a number of fatty acids has been reported over the years including that of DGLA and GLA (203), stearidonic acid (204) as well as various CLA mixtures used for inflammatory bowel syndrome and human Crohn's disease (205). Parenteral administration of fatty acids has been shown to ameliorate disease via immunomodulatory effect in a model of rat sepsis (206), A randomized study in patients awaiting carotid endarterectomy showed that *n*-3 PUFA ethyl esters are incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and T cell infiltration, and increased stability following dietary supplementation with EPA (207).

Furthermore, altering the profile of lipid mediators to strengthen the responses of T cells may be of value to cancer immunotherapy and could result in the development of potent and/or less toxic therapeutics. For example, it is well-documented that most tumors express PGE_2 and this can contribute to immune suppression (103, 208). Pharmacological inhibition of PGE_2 via non-steroidal anti-inflammatory drugs or EP receptor agonists could be supported or even replaced by systemic administration of EPA, precursor of the less potent eicosanoid PGE_3 and the anti-inflammatory resolving series E (RvE) that can tone down the

Table 1 | Summary of the main immunoregulatory roles of bioactive lipid mediators related to T cell function and biology.

Lipid mediator	Receptor	Effect on T cells	Reference
PGE_2	EP1, EP2/EP4	Differentiation	(73, 78, 79, 81, 88)
	EP2/EP4	Proliferation	(72, 81, 83, 85)
	EP2/EP4	Cytokine production	(72, 77–80)
	EP2/EP4	Apoptosis	(77, 91)
	EP2/EP4	Motility of T cells	(93–95)
	EP2/EP4	Treg recruitment	(102)
	EP2/EP4	Th1, Th2, Th17 balance	(14, 74–76, 78, 79)
PGD_2	DP1	Differentiation of Th2; T cell apoptosis	(110, 120, 121)
	DP1, DP2	Recruitment, proliferation of Th2	(111)
	DP2	Activation, cytokine production, trafficking, and motility of Th2	(106, 107, 113–119)
15d-PGJ_2	DP2	Suppression of Th1 and promotion of Th2	(103, 112)
		DC–T cell interaction	(120, 121)
$PGF_{2\alpha}$	FP	Th17 differentiation	(123)
PGI_2	IP	Th1/Th2 balance	(13)
		Th1, Th17 differentiation	(125, 127, 129)
TXA_2	TP	Inhibition of T cell proliferation	(134)
		Mediation of DC-T cell interactions	(130)
LTB_4	BLT1	Homing	(141, 144)
		Differentiation, proliferation, and cytokine production	(146–149)
$CysLTD_4$ $CysLTE_4$	CysLT1	Th2 differentiation	(151–153)
12-HETE		Weak T cell chemotaxis	(158, 159)
		Metabolic changes	(137)
15-HETE		Proliferation	(160–162)
		Th1 responses	(164)
LXA4	BLT1	Cytokine production	(167)
AEA	CB2	Suppression of Th1 and Th17 proliferation and cytokine release	(185, 186)
	–	Inhibition of proliferation; apoptosis via membrane rafts	(187, 188)
		Increased Tregs	(184)
2AG	CB2	T cell migration	(179)
		Suppression of cytokine production via PPAR-γ	(189)

PGE_2-mediated effects. Finally, a large number of other investigations have reported that immunonutrition with fatty acids leads to amelioration of a variety of immune-mediated disease by targeting T cell function. Examples include studies showing that the use of *n*-3 PUFA can improve lung injury and sepsis in animal models, and reduce infectious complications in patients undergoing major surgery and following severe trauma (209–211), while other reports draw attention to the contribution of fatty acids and their mediators in vaccine-induced immunity in infants, the prevention of experimental autoimmune encephalomyelitis through inhibition of Th1/Th17 differentiation by DHA, EPA-mediated protection of cardiac allografts, and amelioration of contact dermatitis following DHA and AA supplements (212–215).

Overall, there is a strong case for further developing therapeutic approaches based on the use of bioactive lipids as immunomodulators. The unmet challenge to fully exploit their therapeutic potential will be to unravel the circuits and molecular mechanisms by which these powerful mediators impact on T cell-mediated immunity.

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Proinflammatory and immunoregulatory roles of eicosanoids in T cells

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Eicosanoids are inflammatory mediators primarily generated by hydrolysis of membrane phospholipids by phospholipase A2 to ω-3 and ω-6 C_{20} fatty acids that next are converted to leukotrienes (LTs), prostaglandins (PGs), prostacyclins (PCs), and thromboxanes (TXAs). The rate-limiting and tightly regulated lipoxygenases control synthesis of LTs while the equally well-controlled cyclooxygenases 1 and 2 generate prostanoids, including PGs, PCs, and TXAs. While many of the classical signs of inflammation such as redness, swelling, pain, and heat are caused by eicosanoid species with vasoactive, pyretic, and pain-inducing effects locally, some eicosanoids also regulate T cell functions. Here, we will review eicosanoid production in T cell subsets and the inflammatory and immunoregulatory functions of LTs, PGs, PCs, and TXAs in T cells.

Keywords: prostaglandins, leukotrienes, cyclooxygenase 2, regulatory T cells, cAMP, immunoregulation effect, inflammation mediators, inflammation

INTRODUCTION

The eicosanoids constitute a large and expanding family of lipid signaling molecules derived from ω-3 and ω-6 C_{20} fatty acids (Smith, 1989; Funk, 2001). This conversion of membrane phospholipids into potent signaling mediators provides an efficient way for cells to respond to various stimuli that require a cellular response. As part of a complex network of regulators controlling a number of important physiological properties including smooth muscle tone, vascular permeability, and platelet aggregation, eicosanoids have also been implicated in a wide array of pathophysiological processes and diseases, including inflammation, autoimmunity, allergy, HIV, and cancer (Harizi et al., 2008; Greene et al., 2011; Bertin et al., 2012). While eicosanoids, in particular prostaglandins, were originally thought of primarily as proinflammatory mediators given their high expression in inflamed tissues and ability to induce inflammatory symptoms, this picture has over time become more nuanced. It is now recognized that these lipids can have both pro- and anti-inflammatory roles by regulating the immune response (Tilley et al., 2001).

While some eicosanoids are produced from eicosapentaenoic acid (EPA, 20:5 ω-3) (Wada et al., 2007) or dihomo-γ-linolenic acid (DGLA, 20:3 ω-6), the majority arise from processing of arachidonic acid (AA, 20:4 ω-6) (Harizi et al., 2008). AA-derived eicosanoids comprise the P-450 epoxygenase-generated hydroxyeicosatetraenoic acids (HETEs) and epoxides, the lipoxygenase (LOX) – generated hydroperoxyeicosatetraenoic acids (HPETEs), lipoxins (LXs), and leukotrienes (LTs), and the cyclooxygenase (COX)-produced prostanoids (see **Figure 1** for overview of biosynthetic pathways). The prostanoids are perhaps the most well-known class of eicosanoids and include the prostaglandins (PGs) PGD_2, PGE_2, and $PGF_{2\alpha}$ as well as prostacyclin (PC/PGI_2) and thromboxane (TXA_2). Together with the leukotrienes, the AA-derived prostanoids will be the major focus of this article.

Constitutive eicosanoid production is normally low, with the rate-limiting factor being the availability of free fatty acids, in particular AA. Free fatty acids are generated from membrane glycerophospholipids by phospholipase A_2s (PLA_2s) (Kudo and Murakami, 2002; Leslie, 2004; Burke and Dennis, 2009) in response to stimuli such as increased Ca^{2+} levels or phosphorylation (Kudo and Murakami, 2002). This elevation in intracellular free fatty acid levels, in particular that of arachidonic acid, then allows eicosanoid biosynthesis to proceed. In the case of prostanoid biosynthesis, AA is converted into PGG_2 and then PGH_2 through the actions of COX-1 and COX-2 (also known as PGH synthases 1 and 2). These enzymes act first as cyclooxygenases to create PGG_2 and then as peroxidases to reduce the peroxide in PGG_2 to an alcohol in PGH_2 (Smith et al., 2000, 2011). Both PGG_2 and PGH_2 are thought to be transient intermediates and their production constitutes the committed step in prostanoid biosynthesis. PGH_2 is then converted into one of four possible downstream signaling molecules (**Figure 1**). Prostacyclin synthase (PGIS) converts PGH_2 to PGI_2, hematopoietic (H-PGDS) or lipocalin-type (L-PGDS) PGD_2 synthase convert PGH_2 into PGD_2, TXA_2 synthase (TXAS) converts PGH_2 into TXA_2, and membrane-bound (mPGES-1 or -2) or cytosolic (cPGES) PGE_2 synthases convert PGH_2 into PGE_2. $PGF_{2\alpha}$ can be synthesized through a number of different pathways (Basu, 2010; Smith et al., 2011).

On the other hand, in leukotriene biosynthesis, AA is not processed by COX enzymes, but instead by 5-LOX, which with the

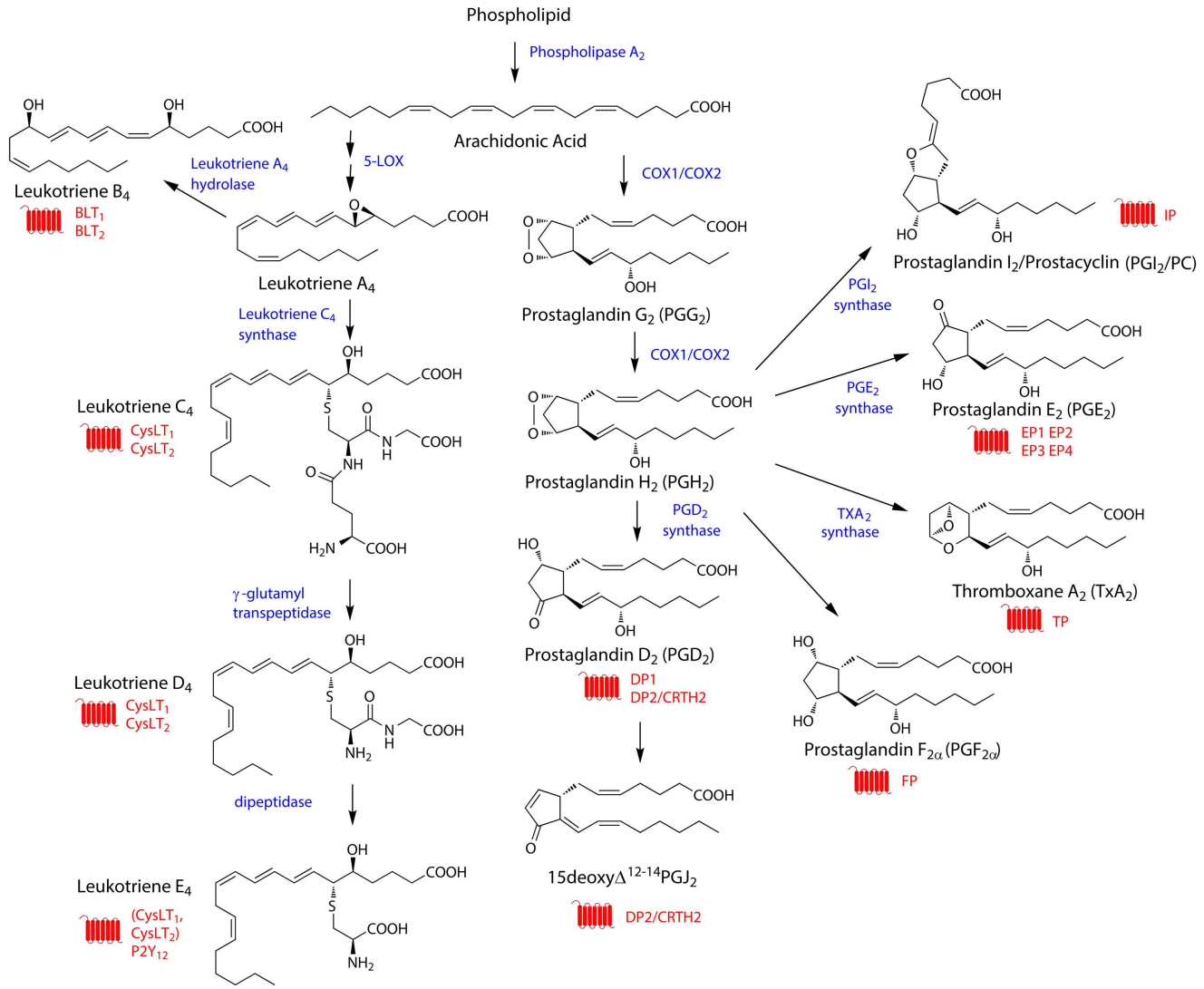

FIGURE 1 | General overview of synthesis pathways for eicosanoids. The biosynthetic pathway for the arachidonic acid-derived eicosanoids described in this article. The figure shows the structures of the relevant eicosanoids (black), and indicates the enzymes involved in their biosynthesis (blue), as well as the GPCRs through which these eicosanoids signal (red). $PGF_{2\alpha}$ can be synthesized through a number of different pathways.

help of 5-LOX -activating protein (FLAP) converts AA first into 5-HPETE and then into LTA_4, an inactive intermediate and precursor for other leukotrienes. LTA_4 can either be converted into LTB_4 by LTA_4 hydrolase or into LTC_4 by LTC_4 synthase, which conjugates a glutathione to LTA_4 (Yokomizo, 2011). LTC_4 can then be converted sequentially to LTD_4 by gamma-glutamyl transpeptidase and LTE_4 by dipeptidases (Brink et al., 2003).

By signaling through their receptors on the surface of T cells, eicosanoids have an important role in regulating many aspects of T lymphocyte function, usually through autocrine or paracrine signaling (Tilley et al., 2001; Sakata et al., 2010a). It has also recently become evident that T cells provide a source of these short-lived signaling mediators in compartments such as lymph nodes and spleen and in lymphoid infiltrates. In the present review, we will summarize the evidence for the production of and signaling by these molecules in T cells, especially in the context of the regulation of immunomodulatory or inflammatory functions.

BIOSYNTHESIS OF AND SIGNALING BY EICOSANOIDS IN T LYMPHOCYTES

PGG_2 AND PGH_2

Production of PGG_2 and PGH_2 proceeds through the actions of PLA_2 and COX-1 or COX-2. While there has been some discussion about which PLA_2 variant(s) are most relevant for eicosanoid biosynthesis, it is generally agreed that cytosolic $PLA_{2\alpha}$ ($cPLA_{2\alpha}$) plays a major role in this process, with the Ca^{2+}-independent PLA_2 ($iPLA_2$) more involved in membrane homeostasis and secreted PLA_2 ($sPLA_2$) regulating extracellular phospholipids (Murakami et al., 2011). Expression of $cPLA_{2\alpha}$ in T cells has been controversial,

with some groups finding no evidence for it in peripheral blood monocytes (Roshak et al., 2000) while others have observed mRNA but no effect of inhibiting this enzyme on AA production in Jurkat T cells (Tessier et al., 2002) and yet others observe this protein both in Jurkat T cells and peripheral blood lymphocytes (Burgermeister et al., 2003) (see **Figure 1** for overview of biosynthetic enzymes and **Table 1** for their expression in T cells).

Some groups have concluded that other PLA_2 variants may also be active in T cells, with evidence for both $iPLA_2$ (Roshak et al., 2000; Tessier et al., 2002) and $sPLA_2$ (Tessier et al., 2002) being present and active in T lymphocytes. Interestingly, an $sPLA_2$ isoform has also been shown to be expressed in and enhance the function of regulatory T cells (Treg), but this effect was found to be independent of the enzyme's catalytic activity (von Allmen et al., 2009).

Table 1 | Eicosanoid synthesis in T cells.

Synthase	Presence in T cells
PLA_2	$cPLA_{2\alpha}$: Jurkat (Tessier et al., 2002; Burgermeister et al., 2003) $iPLA_2$: Jurkat, primary T cells (Roshak et al., 2000; Tessier et al., 2002) $sPLA_2$: Jurkat (Tessier et al., 2002)
COX-1	CD3+CD4+ primary T cells, Jurkat (Iniguez et al., 1999; Pablos et al., 1999)
COX-2	CD3+CD4+ primary T cells, Jurkat (Iniguez et al., 1999; Pablos et al., 1999). Upregulated upon T cell activation (Feldon et al., 2006). Expressed also in adaptive Tregs (Mahic et al., 2006)
PGIS	Lymphocytes (Merhi-Soussi et al., 2000). No specific evidence for expression in T cells
PGDS	L-PGDS: not present in T cells H-PGDS: present in primary T cells (Feldon et al., 2006), in particular activated Th2 and Tc2 cells (Tanaka et al., 2000)
TXAS	No direct evidence for expression in T cells. However, the presence of TXAS products in some T cells indicates that it may be expressed at low levels (Genaro et al., 1992; Kabashima et al., 2003)
PGES	No direct evidence, but product is present in Tregs, implying expression (Mahic et al., 2006)
PGFS	No evidence for expression in T cells
5-LOX	Present in peripheral blood T cells, including naive and memory CD4+ and CD8+ as well as TCR–γδ cells (Cook-Moreau et al., 2007). Also T cell lines (Cook-Moreau et al., 2007)
LTC4S	Jurkat (Cook-Moreau et al., 2007), peripheral blood T cells (Cifone et al., 1995)
LTA4H	Jurkat (Cook-Moreau et al., 2007), peripheral blood T cells (Los et al., 1995)

Alternatively, arachidonic acid can be released from membrane phospholipids by phospholipase D (PLD) (Liscovitch et al., 2000; Melendez and Allen, 2002), which has been shown to be inducible in human T cells (Bacon et al., 1995, 1998; Exton, 1999). Diacylglycerol (DAG) lipase has also been shown to play a role in the release of AA in lymphocytes (Cifone et al., 1995).

COX-1 and COX-2, which are capable of converting AA into PGH_2, are both expressed in CD3+CD4+ cells and in Jurkat T cells. COX-1 is expressed constitutively in T cells and does not change in response to T cell activation (Pablos et al., 1999). In contrast, COX-2 is normally expressed at low levels but significantly upregulated in response to T cell activation (Iniguez et al., 1999; Pablos et al., 1999; Feldon et al., 2006). A study from this lab further demonstrated that during differentiation of adaptive Tregs, these cells also begin expressing COX-2 and producing PGE_2 (Mahic et al., 2006).

In the context of a discussion of the cellular localizations of PLA_2 and COX enzymes, it is worth noting that transcellular eicosanoid biosynthesis has recently been proposed as a mechanism whereby the entire biosynthetic pathway for a given eicosanoid need not be present in one particular cell. Instead, the synthesis may begin in one cell, followed by the transfer of a synthetic intermediate to a different cell where the final product is synthesized. PGH_2, LTA_4, and arachidonic acid have all been proposed as possible intermediates transported between cells, suggesting that in some cases, PLA_2 (and COX enzymes) could be present in one cell and the remaining synthases required for prostanoid or leukotriene synthesis in another (Folco and Murphy, 2006; Sala et al., 2010). For PGH_2, it has also been proposed that two distinct pathways for PGH_2 synthesis exist: one for production of PGH_2 to be converted into downstream prostanoids in the usual manner and one for production of untransformed PGH_2 to be released for signaling functions.

PGG_2 is a transient intermediate in prostanoid biosynthesis, with a half-life of about 5 min in aqueous solution at 37 °C, pH 7.4 and significantly shorter – on the order of seconds – in plasma (Corey et al., 1975). Although there have been some suggestions that PGG_2 may have a biological function (Kuehl et al., 1977; Seidel et al., 2001) it is primarily considered an ephemeral intermediate without independent signaling functions. There is no evidence for a signaling role of this species in T cells.

In the case of PGH_2, this is also an unstable endoperoxide species with comparable half-life to that of PGG_2 (Corey et al., 1975). Although no specific receptor has been identified for this species either, there is some evidence that it can interact with other prostanoid receptors, including the DP and CRTH2 receptors (Schuligoi et al., 2009) as well as the TP receptor (Saito et al., 2003). Because of the rapid conversion of both PGG_2 and PGH_2 to other prostanoid species, however, it has been challenging to unequivocally prove that there is a direct action of these intermediate species on any of the prostanoid receptors. Several of the receptors thought to be activated by PGH_2, in particular CRTH2, are known to be expressed on T cells, but so far it has not been demonstrated that PGH_2 has a biologically relevant role in activating these receptors when expressed on T cells *in vivo* (See **Figure 1** for overview of receptors and **Table 2** for overview of expression of eicosanoid receptors in T cells).

Table 2 | Eicosanoid receptors in T cells.

Receptor	Present in which T cells
IP	T lymphocytes (Tilley et al., 2001), in particular Th1 and Th2 (Zhou et al., 2007)
TP	T lymphocytes (Tilley et al., 2001). Highly expressed in immature thymocytes (CD4+CD8+ and CD4−CD8−) and present in mature CD4+ and CD8+ cells (Ushikubi et al., 1993; Kabashima et al., 2003) and in splenic T cells (Ushikubi et al., 1993)
DP1	Th1, Th2, and CD8+ (Tanaka et al., 2004), CD3+ cells in thymus and lymph nodes (Nantel et al., 2004)
DP2/CRTH2	T lymphocytes (Tilley et al., 2001), activated Th2 and Tc2 cells (Hirai et al., 2001; Tsuda et al., 2001; Tanaka et al., 2004)
EP1	T lymphocytes (Tilley et al., 2001), splenic T cells (Nataraj et al., 2001), low expression in peripheral blood naive T cells (Boniface et al., 2009)
EP2	T lymphocytes (Tilley et al., 2001), splenic T cells (Nataraj et al., 2001), peripheral blood naive T cells, upregulated upon T cell activation (Boniface et al., 2009)
EP3	T lymphocytes (Tilley et al., 2001), splenic T cells (not α, β isoforms) (Nataraj et al., 2001), low expression in peripheral blood naive T cells (Boniface et al., 2009)
EP4	T lymphocytes (Tilley et al., 2001), splenic T cells (Nataraj et al., 2001), peripheral blood naive T cells, upregulated upon T cell activation (Boniface et al., 2009)
FP	No evidence for expression in T cells
BLT_1	CD4+ and CD8+ effector T cells, particularly after activation (Tager et al., 2003; Islam et al., 2006), small fraction of peripheral blood T cells, including helper and cytotoxic T cells as well as NKT and γδ T cells (Yokomizo et al., 2001; Pettersson et al., 2003; Islam et al., 2006)
BLT_2	CD4+ and CD8+ peripheral blood T cells, downregulated upon T cell activation (Yokomizo et al., 2001)
$CysLTR_1$	Small fraction of peripheral blood T cells (Figueroa et al., 2001; Mita et al., 2001), activation induces higher expression (Prinz et al., 2005), as does IL-4 (Early et al., 2007). Significant amount in resting Th2 cells (Parmentier et al., 2012)
$CysLTR_2$	Small fraction of peripheral blood T cells (Mita et al., 2001). IL-4 and IFN-γ induce expression (Early et al., 2007)

PGI_2/PC

Prostaglandin I_2 was originally characterized as an inhibitor of platelet aggregation and a potent vasodilator (Boswell et al., 2011) and its analogs are used as treatments for pulmonary hypertension (Olschewski et al., 2004). Recently it has also been shown that this molecule has important roles in immune regulation (Boswell et al., 2011) and some studies suggest that treatment with PGI_2 analogs may improve early graft viability in liver transplant patients, partly by reducing levels of inflammatory cytokines (Barthel et al., 2012).

While PGIS is expressed in some immune cells, in particular follicular dendritic cells (FDCs) (Lee et al., 2005; Boswell et al., 2011), there is no direct evidence for expression of this synthase in T cells. It has, however, been shown that lymphocytes are able to produce PGI_2 through a transcellular mechanism when co-cultured with human vascular endothelial cells (HUVECs) (Merhi-Soussi et al., 2000) and that a similar mechanism appears to be operating between platelets and lymphocytes (Wu et al., 1987), although in neither of these cases were T cells specifically implicated.

The PGI_2 receptor, IP, can be either G_s or G_q-coupled, leading to either increases in intracellular cyclic AMP (cAMP) levels through G_s-coupling, which can trigger cAMP-PKA signaling pathways or, through G_q-coupling, to the initiation of other signaling cascades (Woodward et al., 2011). IP is expressed on T cells, in particular cells of the Th1 and Th2 lineages (Zhou et al., 2007). Signaling through the IP receptor on these cells leads to inhibited cytokine secretion – in particular, IFNγ production in Th1 cells is abrogated and Th2 cells express less IL-4, IL-10, and IL-13 after IP stimulation. These results are mirrored by studies in IP knockout mice, where IL-4 and IFNγ production by splenocytes, which includes some T cells, was significantly higher in sensitized IP KO mice than in WT mice (Takahashi et al., 2002). With the exception of IL-10, where other studies have also shown upregulation in response to IP signaling (Jaffar et al., 2002), these downregulated cytokines are proinflammatory, and PGI_2 is generally considered to be an anti-inflammatory and immune suppressive prostaglandin.

This inhibitory effect of IP signaling on cytokine production from Th1 and Th2 cells appears to be mediated by a cAMP-PKA pathway, since the PKA inhibitor Rp-8-Br-cAMPS significantly reduces the IP-stimulation induced effects on cytokine production. Further, it is accompanied by a reduction in nuclear-factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor known to enhance expression of IFNγ and IL-4 (Zhou et al., 2007). While signaling through the IP receptor has a direct negative regulatory effect on Th1 and Th2 function, it appears to promote differentiation into the Th17 lineage (Boswell et al., 2011; Truchetet et al., 2012). This effect is partially due to a reduction in IL-12 expression and/or an increase in IL-23 from dendritic cells (DCs) or monocytes, thus perturbing the IL-23 to IL-12 ratio and favoring Th17 cell differentiation (Boswell et al., 2011; Truchetet et al., 2012; Zhou et al., 2012). It appears that this pathway is IP-specific and proceeds through a PKA pathway (Truchetet et al., 2012). In addition, the favoring of the

Th17 lineage during T cell differentiation upon IP-stimulation appears also to be due to inhibited secretion of IL-4 (Zhou et al., 2012), a cytokine known to promote Th2 and antagonize Th17 development (Boswell et al., 2011).

In addition to its role in regulating T cell differentiation, PGI_2 also has an important role in mediating FDC-T cell interactions in the germinal centers. FDC-produced PGI_2 has been shown both to inhibit T cell proliferation and to protect T cells from TCR-mediated activation-induced death (AICD) (Lee et al., 2005, 2008), thus improving the current understanding of why T cells don't proliferate or undergo AICD in germinal centers.

TXA_2 AND TXB_2

Thromboxane A_2 is a proinflammatory, short-lived (half-life ∼30 s (Remuzzi et al., 1994)) prostanoid primarily produced in platelets, but also in activated monocytes, macrophages, and DCs (Narumiya, 2003) through the actions of thromboxane A_2 synthase. There is limited evidence for TXAS expression in T cells and this synthase was found to be absent in thymic lymphocytes (Ushikubi et al., 1993). However, TXA_2's stable and inactive downstream metabolite, TXB_2, is produced by helper T cells in response to isoproterenol stimulation (Genaro et al., 1992) and another study found very low levels of TXB_2 produced from CD4+ cells (Kabashima et al., 2002), suggesting that TXAS could be present at low levels in certain T cells.

By signaling through the TP receptor, which is coupled to G_q, TXA_2 activates protein kinase C (PKC) and raises intracellular calcium levels (Narumiya, 2003; Woodward et al., 2011). TXA_2 is best known for causing vasoconstriction and platelet aggregation and promotes fibrosis and scarring by regulating extracellular matrix protein levels (Thomas et al., 2003). The TP receptor is known to be highly expressed in immature thymocytes (CD4+CD8+ and CD4−CD8−) and to a lesser extent in CD4+CD8− and CD4−CD8+ thymocytes (Ushikubi et al., 1993; Kabashima et al., 2003). Splenic T cells also express lower amounts of the TP receptor. In line with this, immune regulatory functions for TXA_2 have been proposed. Signaling through the TP receptor has been shown to cause apoptosis in immature thymocytes, in particular in CD4+CD8+ cells (Ushikubi et al., 1993), suggesting a potential role in T cell maturation. In other T cell populations, it has been suggested that signaling through the TP receptor could affect T cell proliferation (Kelly et al., 1979; Ceuppens et al., 1985), with a recent study showing that TXA_2 signaling through the TP receptor inhibits anti-CD3 stimulated T cell proliferation (Thomas et al., 2003). Interestingly, proliferation in response to PMA and ionomycin, which produces a robust intracellular calcium response and bypasses the normal T cell activation mechanism, is not affected in TP-deficient cells (Thomas et al., 2003) or in cells treated with a TP agonist (Kabashima et al., 2003). These results indicate that TXA_2 signaling through the TP receptor may play an important role in the initial activation of T cells by antigen-presenting cells (APCs), in particular DCs, but not in the later downstream intracellular signaling. TP signaling further attenuates DC-T cell interactions by promoting chemokinesis of naïve T cells and inhibiting DC-T cell adhesion, thus playing an important role in adaptive immunity (Kabashima et al., 2003).

Thromboxane A_2 signaling has been implicated in anti-graft immune responses, with allografts eliciting higher levels of TXA_2 than isografts (Gibbons et al., 1987). Mice deficient in the TP receptor have been shown to display weaker anti-allograft immune responses (Thomas et al., 2003) and blocking TXA_2 synthesis pharmacologically has been shown to reduce alloreactive immune responses *in vitro* (Ruiz et al., 1992) and at least temporarily improve allograft survival and function *in vivo* by limiting cytotoxic T cell activity (Ruiz et al., 1989). On the other hand, in models of induced unresponsiveness to allografts by thymic injection of MHC allopeptides, TXA_2 signaling abrogation through synthesis inhibition or receptor antagonists blocked the unresponsive state, suggesting that TXA_2 signaling in the thymus is involved in mediating immune tolerance in this situation, possibly by leading to apoptosis of alloactivated T cells circulating through the thymus (Remuzzi et al., 1994). Together, these data suggests an important role for TXA_2-TP signaling in T cells in the thymus, in particular in T cell maturation, activation by DCs and in anti-allograft immune responses.

PGD_2 AND 15-DEOXY-$\Delta^{12,14}$-PGJ_2

PGD_2 is produced by activated mast cells in response to allergen exposure and is thought to play an important role in mediating allergic inflammation by acting as a vasodilator, recruiter of eosinophils, basophils, and Th2 cells, modulator of Th2 production, and bronchoconstrictor (Pettipher et al., 2007). It also has important roles in regulating sleep, platelet aggregation, smooth muscle contraction, and reproduction (Saito et al., 2002; Woodward et al., 2011). Beyond mast cells, a few other cell types also produce PGD_2 from PGH_2 through one of the two types of PGD synthase, L-PGDS and H-PGDS (Joo and Sadikot, 2012). The former is not known to be expressed in T cells, while the latter is expressed in certain T cells under specific conditions. In particular, activated COX-2-expressing T cells have been shown to express H-PGDS and thereby produce PGD_2 and likely the downstream PGD_2 processing product 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (Feldon et al., 2006). It appears that H-PGDS is particularly prevalent in activated Th2 and Tc2 cells but not Th1 cells (Tanaka et al., 2000; Herlong and Scott, 2006). As for the synthesis of 15d-PGJ_2, no specific synthase has been described and few details are known about the dehydration steps leading to its formation from PGD_2 (Scher and Pillinger, 2005).

PGD_2 can signal through either the DP1 or DP2/CRTH2 receptor, while 15d-PGJ_2 signals through the DP2 receptor (Harris et al., 2002a; Schuligoi et al., 2010). The DP1 receptor is G_s-coupled and its activation leads to increases in intracellular cAMP and PKA activation and can also lead to increased intracellular calcium levels (Woodward et al., 2011). This receptor was shown to be expressed in certain malignant T cell lines (Harris and Phipps, 2002), but was not detected in normal peripheral blood T cells in this study (Harris and Phipps, 2002). However, other groups have found DP1 to be constitutively expressed in both Th1, Th2 and CD8+ cells (Tanaka et al., 2004) and to be present in CD3+ cells in the thymus and lymph nodes (Nantel et al., 2004). CRTH2 has little sequence homology with other prostanoid receptors, being more closely related to the *N*-formyl peptide receptor subfamily

of receptors (Hirai et al., 2001). This receptor is G_i-coupled, leading to increases in intracellular calcium and inhibition of cAMP formation in response to signaling (Hirai et al., 2001). It is thought to be mainly expressed in activated Th2 and Tc2 cells (Tsuda et al., 2001; Tanaka et al., 2004) and has also been detected in a subset of infiltrating T cells in patients suffering from polyposis, a severe form of rhinosinusitis (Nantel et al., 2004). Interestingly, when heterologously expressed, DP and CRTH2 can form heterodimers, where DP enhances the signaling by the CRTH2 receptor. In these heterodimers, when DP signaling is pharmacologically blocked, CRTH2 function is also inhibited, but not *vice versa* (Sedej et al., 2012). In addition to signaling through the cell surface receptors DP1 and CRTH2, 15d-PGJ_2 and PGD_2 can also bind the nuclear hormone receptor transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ) (Forman et al., 1995; Kliewer et al., 1995; Harris et al., 2002a; Feldon et al., 2006). By activating PPAR-γ, these prostanoids induce differentiation of fibroblasts into fat cells, and it has been shown that this can be pathophysiologically relevant. For instance, in the case of Graves' disease, activated T cells infiltrate the eye orbit and by producing PGD_2 and 15d-PGJ_2, cause the differentiation of fibroblasts in the eye orbit to adipocytes, leading to disfiguration and sometimes blindness (Feldon et al., 2006).

Both PGD_2 and 15d-PGJ_2 affect cytokine production from T cells. In particular, 15-dPGJ2 is often thought of as an anti-inflammatory prostaglandin, in part due to its enhancement of PPARγ's anti-inflammatory effects (Harris et al., 2002a; Scher and Pillinger, 2005). However, 15-dPGJ2 can also induce secretion of IL-8, a cytokine with chemotactic and angiogenic effects, from activated T cells, suggesting a proinflammatory role of this prostaglandin as well (Harris et al., 2002b). This effect is not PPAR-γ-dependent, but instead proceeds through a mitogen-activated protein kinase (MAPK) and NF-κB pathway, possibly by first binding an extracellular receptor such as CRTH2.

PGD_2 has a well-established role in regulating cytokine secretion from Th2 cells. In particular, PGD_2 produced in mast cells stimulates IL-4, IL-5, and IL-13 secretion from Th2 cells and this process is believed to be important in the pathophysiology of allergic inflammations (Xue et al., 2009a). It has been demonstrated that phosphoinositide 3-kinase (PI3K) and Ca^{2+}/calcineurin/nuclear factor of activated T cells (NFAT) signaling pathways downstream of CRTH2 are both important in regulating PGD_2-induced cytokine production (Xue et al., 2007) and that LTE_4 enhances the PGD_2-CRTH2-mediated secretion of cytokines from Th2 cells (Xue et al., 2012). Another study confirmed the effect of PGD_2 receptor signaling on cytokine secretion and further noted that while signaling through CRTH2 increases secretion of IL-2, IL-4, IL-5, and IL-13 as well as the proinflammatory proteins CD11b and CD40L in Th2 cells, signaling through DP1 reduces the number of CD4+ and CD8+ cells expressing IFNγ and IL-2 (Tanaka et al., 2004). By thus promoting Th2 function and suppressing Th1 functions, PGD_2 signaling may have an overall effect of promoting Th2 function, which could be relevant in allergic responses, where Th2 activity is elevated. PGD_2 can have further inhibitory effects on cytokine secretion, for instance in invariant natural killer T (iNKT) cells, where PGD_2 signals through DP1 and PKA to inhibit the production of IFNγ, but not IL-4, the other major cytokine produced in this cell type (Torres et al., 2008). Thus, PGD_2 signaling also contributes to regulating the innate immune system.

While signaling through PGD_2 receptors apparently has a role in driving Th2-type processes as described above, 15-dPGJ_2 may have a role in resolving certain Th1-driven responses by inhibiting the proinflammatory NF-κB pathway (Trivedi et al., 2006). Also, 15-dPGJ_2 is able to inhibit IL-2 production in T cells by promoting an interaction between PPARγ and NFAT, a crucial transcription factor for IL-2 production, which prevents NFAT from binding to the IL-2 promoter (Yang et al., 2000).

Aside from pro- and anti-inflammatory effects of PGD_2 and 15-dPGJ_2 mediated by cytokine secretion, these prostaglandins also affect T cell function by regulating proliferation and apoptosis. Both PGD_2 and 15-dPGJ_2 are capable of inducing apoptosis in T cells through a PPARγ-dependent mechanism (Harris and Phipps, 2001, 2002; Harris et al., 2002b). It has also been reported that 15-dPGJ_2 and to a lesser extent PGD_2 can induce apoptosis in Jurkat T cells through a non-PPARγ dependent mechanism involving activation of the mitochondrial apoptosis pathway (Nencioni et al., 2003). In other situations, PGD_2 can also have anti-apoptotic effects. For instance, in the case of apoptosis induced by cytokine deprivation, PGD_2 signaling through the CRTH2 receptor inhibits apoptosis in Th2 cells, suggesting that this pathway may hinder resolution of allergic inflammation (Xue et al., 2009b). In T lymphocytes, 15-dPGJ_2 can also inhibit proliferation by acting as a PPARγ ligand (Clark et al., 2000; Yang et al., 2000; Harris and Phipps, 2001; Nencioni et al., 2003). However, only TCR-mediated and not IL-2 induced proliferation is affected by 15-dPGJ_2 treatment (Clark et al., 2000).

Signaling through the PGD_2 receptors also plays an important role in the chemotaxis of T cells. When PGD_2 acts on the CRTH2 receptor on Th2 cells, this induces chemotactic migration of the Th2 cells (Hirai et al., 2001), probably through a PI3K pathway (Xue et al., 2007), providing a possible mechanism for recruitment of Th2 cells to sites of allergic inflammation, for instance in asthma (Luster and Tager, 2004). It has been demonstrated that blocking the CRTH2 receptor pharmacologically inhibits the trafficking of lymphocytes, including T cells, to the inflamed airways in a model of chronic obstructive pulmonary disease (COPD), presenting a possible new strategy for treating this disease (Stebbins et al., 2010). Further chemoattractive effects of PGD_2 on T cells is the CRTH2-mediated recruitment of Th2 and Tc2 cells to the materno-fetal interface, where they are thought to increase in early pregnancy (Saito et al., 2002) and PGD_2's ability to promote transendothelial migration of memory T cells across blood vascular endothelial cells and lymphatic vascular endothelial cells (Ahmed et al., 2011).

PGE_2

PGE_2 is the most abundant prostanoid found in the body and has important roles in reproduction, gastro-intestinal function, the immune system, cardiovascular function, and the central nervous system (Sreeramkumar et al., 2012). It is present in large amounts in many cancers, in particular colorectal and lung cancers, where it stimulates tumor growth by inhibiting apoptosis,

inducing Tregs and promoting metastasis, cell invasion, and angiogenesis (Bergmann et al., 2007; Wang et al., 2007; Greenhough et al., 2009; Mandapathil et al., 2010; Brudvik et al., 2011; Nakanishi et al., 2011). PGE_2 is produced from PGH_2 through one of three different PGE_2 synthases – cytosolic (cPGES) or microsomal (mPGES-1 and mPGES-2). While cPGES and mPGES-2 are constitutively expressed, mPGES-1 is inducible in response to mitogenic or proinflammatory stimuli and is often upregulated in concert with COX-2 (Scher and Pillinger, 2005). In terms of expression in T cells, little is known except that it has been demonstrated that adaptive Tregs express COX-2 and produce PGE_2 upon differentiation (Mahic et al., 2006), implying that they must also express a PGES. This production of PGE_2 from adaptive Tregs has implications both in cancer and chronic infectious diseases.

PGE_2 can signal through any of its four receptors – EP1, EP2, EP3, EP4 – often with opposing effects (Breyer et al., 2001; Harris et al., 2002a; Woodward et al., 2011; Sreeramkumar et al., 2012). EP2 and EP4 receptors are G_s-coupled and lead to increased intracellular cAMP levels and PKA signaling. The EP1 receptor is G_q-coupled and results in increased intracellular calcium levels. In the case of the EP3 receptor, three main isoforms of this receptor exist – EP3 α, β, and γ – and they can signal through different G proteins, but it appears that the major pathway is through G_i, which leads to decreased intracellular cAMP levels. Messenger RNA for all the different PGE_2 receptors, with the exception of the EP3 α and β isoforms, is present in murine splenic T cells (Nataraj et al., 2001). In naive T cells isolated from peripheral blood, EP2 and EP4 receptors appear to be the most abundant and are upregulated in response to activation (Boniface et al., 2009).

Through its receptors, PGE_2 controls T cell function in a variety of ways and a number of recent reviews have addressed this topic (Harris et al., 2002a; Brudvik and Tasken, 2012; Sreeramkumar et al., 2012). First, it appears to differentially regulate apoptosis in T cells depending on the subpopulation and condition of the cells. In particular, CD4+CD8+ thymocytes undergo apoptosis when stimulated by PGE_2 *in vivo* (Mastino et al., 1992), but may also be protected against activation-induced cell death by this prostanoid (Goetzl et al., 1995). Similarly, while apoptosis is stimulated in resting mature T cells (Pica et al., 1996), activation-induced cell death is inhibited (Porter and Malek, 1999; Pace et al., 2007). PGE_2 also has other known negative regulatory functions in T cells. It is known to influence the function of CD8+ cells through the inhibitory complex CD94/NKG2A (Zeddou et al., 2005) and the cytotoxicity of gamma delta T cells through a cAMP-PKA pathway (Martinet et al., 2010). An anti-proliferative effect is also well documented. Through the EP2 (Nataraj et al., 2001) and possibly the EP4 (Kabashima et al., 2002) receptor, PGE_2 can inhibit T cell proliferation in CD4+ and CD8+ cells (Goodwin et al., 1977; Hendricks et al., 2000). It has also been shown that PGE_2 inhibits the proliferation of double-negative Tregs (Lee et al., 2009). It appears that proliferation is inhibited in these cells by a negative regulatory effect of increased intracellular cAMP levels resulting from EP2 or EP4 stimulation on IL-2 synthesis and IL-2 receptor expression, resulting in diminished IL-2-stimulated proliferation responses (Farrar et al., 1987; Mary et al., 1987; Rincon et al., 1988; Anastassiou et al., 1992). Other possible mechanisms of inhibition of proliferation include downregulation of the transferrin receptor (Chouaib et al., 1985), inhibiting intracellular Ca^{2+} increase and inositol phosphate production in response to T cell activation (Chouaib et al., 1987; Lerner et al., 1988; Choudhry et al., 1999) and preventing K^+ movements which would dampen signaling via G proteins (Bastin et al., 1990).

Recent studies have provided additional information about the intracellular signaling pathways initiated by PGE_2 through which T cell function and proliferation is affected. In particular, a combined phosphoflow/phosphoproteomics approach allowed for the collection of detailed information about phosphorylation cascades initiated in response to different amounts of PGE_2 stimulation in different T cell populations (Oberprieler et al., 2010). Furthermore, a pathway was described in effector T cells where signaling through EP2 or EP4, with its concomitant increase in cAMP levels, leads to PKA activation and, through an EBP50-Ezrin-PAG scaffolded process, phosphorylation of the C-terminal Src kinase (Csk). Phosphorylated Csk in turn inhibits Lck-mediated phosphorylation of the TCR complex, thus inhibiting TCR signaling and T cell proliferation and function (Vang et al., 2001; Ruppelt et al., 2007; Mosenden and Tasken, 2011) (see **Figure 2** for schematic depiction of the PGE_2-cAMP-PKA-Csk inhibitory pathway in T cells). This pathway is of particular relevance during inflammatory responses or cancer, where production of PGE_2 is increased. It has been shown that disrupting this pathway in cells by molecular or genetic means prevents PGE_2 – mediated inhibition of effector T cell function (Carlson et al., 2006; Ruppelt et al., 2007; Stokka et al., 2010). In mice, disrupting this pathway by overexpressing a PKA anchoring disruptor also leads to an increase in effector T cell function, as evidenced by increased signaling, enhanced IL-2 secretion, and reduced sensitivity to PGE_2-mediated inhibition of T cell function. These mice also have improved resistance to murine AIDS, an immunodeficiency disease induced by the murine leukemia virus where the PKA-Csk pathway is hyperactivated (Mosenden et al., 2011). In mice with murine AIDS, this pathway can also be targeted with COX-2 inhibitors (Rahmouni et al., 2004). Interestingly, the PKA-Csk pathway is upregulated in several immunodeficiency diseases, as well as cancer, suggesting that targeting this pathway may be of therapeutic interest (Rahmouni et al., 2004; Brudvik and Tasken, 2012; Brudvik et al., 2012). In particular, targeting this pathway with COX-2 inhibitors in patients with HIV infection appears to give significant patient benefit in clinical intervention trials as evident from regulation of surrogate parameters such as CD38 and immune function parameters such as lymphoproliferation and T cell-dependent vaccine responses (Johansson et al., 2004; Kvale et al., 2006; Pettersen et al., 2011).

As described above, PGE_2 can influence the production and secretion of IL-2 from T cells, but it also influences the production of many other cytokines and contributes to T cell differentiation. In particular, it has been proposed that PGE_2 signaling promotes a Th2 cell fate (Betz and Fox, 1991). In line with this, PGE_2 has been shown to downregulate expression of IFNγ in T cells (Aandahl et al., 2002), indicating less differentiation to a Th1 cell type, with the caveat that recent studies have shown that in

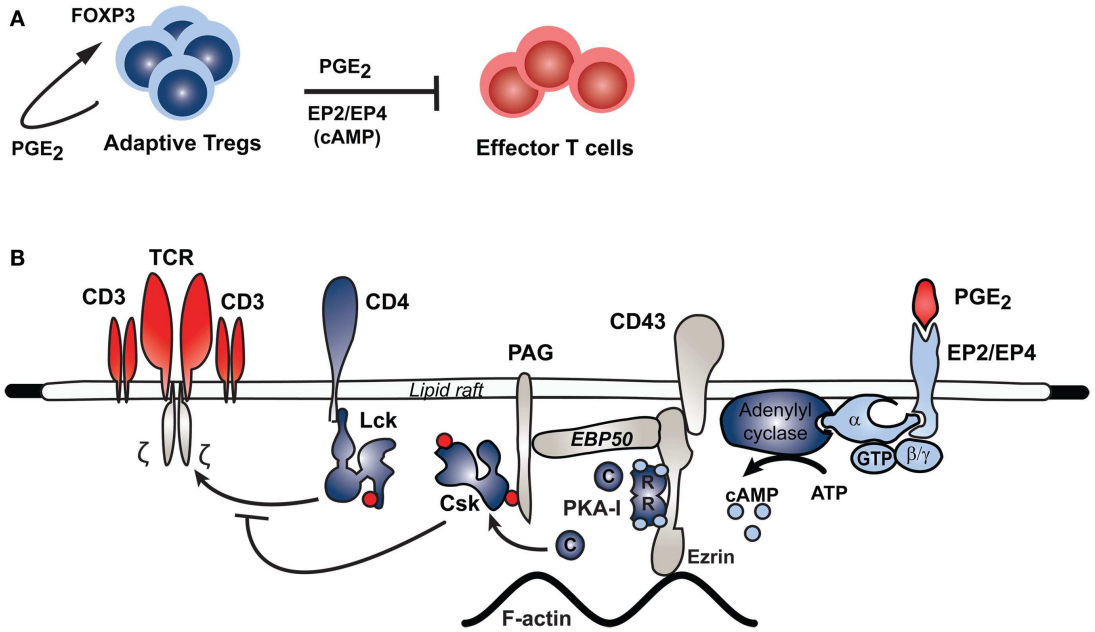

FIGURE 2 | Inhibitory pathway of PGE2 in effector T cells. PGE_2 mediates Treg inhibition of effector T cell function through a PKA-mediated pathway. **(A)** In response to continuous antigen exposure, for instance in cancer and HIV, adaptive regulatory T cells express COX-2 and produce PGE_2, which stimulates FOXP3 expression in these cells. The Treg-derived PGE_2 can signal through the EP2 and EP4 receptors on effector T cells to inhibit the function of these cells through the pathway shown in **(B)**. Binding of PGE_2 to its receptors on effector T cells stimulates adenylyl cyclase activity, which increases intracellular cAMP levels and thus activates PKA. Aided by an Ezrin-EBP50-PAG scaffold, PKA phosphorylates Csk, which in turn phosphorylates Lck to inhibit its activity. Lck normally acts to promote TCR signaling; thus Lck inhibition through this PGE_2-initiated pathway inhibits TCR signaling in effector T cells.

the presence of strengthened TCR stimulation, the Th1 cell fate can actually be promoted by PGE_2 (Yao et al., 2009). In contrast, Th2-derived cytokines including IL-4, IL-5, IL-10, and IL-13 are unaffected or upregulated in response to PGE_2 signaling (Betz and Fox, 1991; Snijdewint et al., 1993; Demeure et al., 1997). Furthermore, the IL-12 receptor is downregulated on T cells in response to PGE_2, further promoting a Th2 cell fate (Wu et al., 1998). PGE_2 has also been proposed to play a role in the differentiation of Th17 and Tregs. There is some debate about the role of PGE_2 in the differentiation and expansion of Th17 cells (Sakata et al., 2010b), with some studies finding an inhibitory role in mouse Th17 differentiation (Chen et al., 2009) and others finding a promoting role in human Th17 differentiation (Boniface et al., 2009). There seems to be general agreement that Th17 IL-23-mediated expansion is enhanced by PGE_2, however (Chizzolini et al., 2008; Boniface et al., 2009; Napolitani et al., 2009). In Treg differentiation the majority of reports seem to suggest an enhancing effect (Baratelli et al., 2005; Sharma et al., 2005; Mahic et al., 2006; Bryn et al., 2008), although some have found PGE_2 to have an inhibitory effect on this process (Chen et al., 2009). Due to its role in promoting Treg differentiation and inhibiting effector T cell function and proliferation, PGE_2 has traditionally been considered an immunosuppressant, but with recent studies showing a possible enhancing effect of this eicosanoid on Th17 and Th1 differentiation, some have argued that the picture is more nuanced (Sakata et al., 2010b; Sreeramkumar et al., 2012).

$PGF_{2\alpha}$

$PGF_{2\alpha}$ has important functions in reproduction, inflammation, cardiovascular function, and other (patho)physiological processes (Simmons et al., 2004; Basu, 2007, 2010; Woodward et al., 2011). This prostaglandin can be synthesized through a number of different pathways (Basu, 2010), but there appears to be no evidence for any $PGF_{2\alpha}$ synthesis in T cells. Evidence for a role of $PGF_{2\alpha}$ signaling in T cells is also very limited, although a recent study demonstrated a role for this prostaglandin in promoting Th17 differentiation during allergic lung inflammation (Li et al., 2011). In this study, the authors propose that $PGF_{2\alpha}$ together with PGI_2 promotes differentiation of Th17 cells – proinflammatory cells and major contributors in allergic responses – from naïve CD4+ cells by signaling through their respective receptors in an autocrine fashion.

LEUKOTRIENES LTA_4, LTB_4, LTC_4, LTD_4, LTE_4

The first step in leukotriene biosynthesis, conversion of arachidonic acid to the unstable epoxide intermediate LTA_4, is catalyzed by 5-LOX, an enzyme shown to occur in human T cell lines as well as in purified peripheral blood T cells (Cook-Moreau et al., 2007). 5-LOX expression is found across a wide range of T cells, including naive and memory helper and cytotoxic T cells as well as TCR–γδ cells (Cook-Moreau et al., 2007). However, some have noted that T lymphocytes require exogenous arachidonic acid in order to synthesize leukotrienes (Cook-Moreau et al., 2007). This is interesting in light of the proposed transcellular eicosanoid biosynthesis

mechanism, and it has also been shown that LTA_4 can act as the transferred intermediate metabolite in some systems (Folco and Murphy, 2006; Sala et al., 2010). LTB_4 synthesis, which proceeds through LTA_4 hydrolase, and LTC_4 synthesis, which proceeds through LTC_4 synthase, both occur in Jurkat T cells upon CD2, CD3, and CD28 crosslinking (Cook-Moreau et al., 2007). In primary T cells, synthesis of LTB_4 and LTC_4 was only found to occur if cells were stimulated by CD3 crosslinking and supplied with exogenous arachidonic acid (Cook-Moreau et al., 2007). Depending on the stimulation protocol, others have also detected the production of LTB_4 or the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) in various T cell lines and primary cells (Cifone et al., 1995; Los et al., 1995). It should be noted that LTD_4 and LTE_4 are typically generated extracellularly after export of LTC_4 from the producing cell.

LTB_4 can signal through either of its two receptors, the high affinity BLT_1 receptor and the low affinity BLT_2 receptor (Yokomizo et al., 1997, 2000), which couple to G_i or G_q to exert their function (Back et al., 2011). BLT_1 is expressed on CD4+ and CD8+ effector T cells, particularly shortly after activation (Tager et al., 2003; Islam et al., 2006). In peripheral blood T cells in healthy humans, BLT_1 is found on a small fraction of the population, including both helper and cytotoxic T cells as well as NKT and γδ T cells (Yokomizo et al., 2001; Pettersson et al., 2003; Islam et al., 2006), and can expand in response to acute inflammation. The BLT_2 receptor is more ubiquitously expressed across tissues, with very high expression levels in the spleen (Yokomizo et al., 2000). One study found no evidence for BLT_2 expression in naive CD4+ cells or Th0, Th1, or Th2 cells 7 days after activation (Tager et al., 2003), while others have shown it to be present on both CD4+ and CD8+ peripheral blood T cells, but downregulated in response to T cell activation (Yokomizo et al., 2001).

In T cells, LTB_4 is primarily known for its role in chemotaxis, but it has also been shown to have other functions, for instance in differentiation and proliferation. In chemotaxis, LTB_4 signals through the BLT_1 receptor on CD4+ or CD8+ cells to mediate cell movement, which is of particular relevance during T cell recruitment to airways and lungs in asthma (Tager et al., 2003; Luster and Tager, 2004; Gelfand and Dakhanna, 2006), after lung transplants (Medoff et al., 2005) and in various inflammatory settings (Goodarzi et al., 2003; Ott et al., 2003). In addition, signaling through BLT_1 appears to enable adhesion of T cells to epithelial cells (Tager et al., 2003), which is important for migration into tissues. In T cell differentiation, LTB_4 has been shown to promote Th17 and inhibit Treg generation, which may be of relevance in autoimmune diseases such as rheumatoid arthritis (Chen et al., 2009). However, it should be noted that early reports from 1985 had suggested that LTB_4 may have an immunoregulatory role by inducing so-called suppressor T cells (Yamaoka and Kolb, 1993; Morita et al., 1999) but this has not been revisited since the definition of Treg. Proliferation and cytokine production in T cells can also be affected by LTB_4. In particular, treatment with a BLT_1 antagonist was shown to inhibit cytokine (IL-2, IFN-γ, IL-4) secretion and proliferation of T cells in response to activation (Rolapleszczynski, 1985), while LTB_4 stimulation enhanced IL-5 production (Gualde et al., 1985), suggesting that LTB_4 promotes T cell activation.

For the cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4, two receptors have been discovered, $CysLT_1$ and $CysLT_2$ (Brink et al., 2003; Kanaoka and Boyce, 2004; Singh et al., 2010; Back et al., 2011; Laidlaw and Boyce, 2012). These receptors can bind all the cysteinyl leukotrienes, albeit with significantly higher affinity for LTC_4 and LTD_4 than LTE_4 (Laidlaw and Boyce, 2012). Recently, further receptors involved in cysteinyl leukotriene signaling have been identified, in particular GPR17, which is a ligand-independent negative regulator of $CysLT_1$, as well as the LTE_4-specific $P2Y_{12}$ (Austen et al., 2009; Maekawa et al., 2009; Paruchuri et al., 2009; Laidlaw and Boyce, 2012). While it is unclear whether these latter two receptors are expressed in T cells, the $CysLT_1$ and $CysLT_2$ receptors have been shown to be expressed in a small fraction of peripheral blood T cells (Figueroa et al., 2001; Mita et al., 2001). Activation of the T cells induces higher expression of the $CysLT_1$ receptor (Prinz et al., 2005), as does certain mutations in the linker for activation of T cells (LAT) (Prinz et al., 2005). It also appears that expression of this receptor is significantly higher in resting Th2 cells than in Th1 cells or activated Th2 cells (Parmentier et al., 2012). Interestingly, both receptors can also be upregulated in response to inflammatory stimuli. In particular, IL-4 induces expression of both receptors on T cells, while IFN-γ specifically upregulates expression of the $CysLT_2$ receptor (Early et al., 2007). Presumably, this upregulation has the effect of making T cells more responsive to cysteinyl leukotriene signaling in inflammatory environments.

There is some functional evidence for a role of cysteinyl leukotriene signaling in T cells. For one, these molecules appear to be important in Th2 cells where, as mentioned above, the $CysLT_1$ receptor is present in significant amounts. It has been demonstrated that LTE_4, through a montelukast-sensitive pathway, indicating $CysLT_1$ involvement, enhances PGD_2-mediated cytokine secretion in isolated Th2 cells (Xue et al., 2012). In line with this, another $CysLT_1$ antagonist, pranlukast, inhibits production of Th2 cytokines, in particular IL-3, IL-4, GM-CSF and possibly IL-5, from peripheral blood mononuclear cells of patients with bronchial asthma (Tohda et al., 1999). Further roles in Th2 cells include a demonstrated effect of LTD_4 on the induction of calcium signaling as well as chemotaxis in these cells, both processes being $CysLT_1$-specific (Parmentier et al., 2012). Cysteinyl leukotriene signaling in Th2 cells may also be involved in disease. For instance, it has been suggested that cysteinyl leukotrienes may enhance GM-CSF stimulated Th2 functions in atopic asthmatic patients *in vivo* (Faith et al., 2008). There has also been a suggested role for the cysteinyl leukotrienes in T cell-mediated late airway responses to allergen challenge, since treatment with the $CysLT_1$ antagonist pranlukast inhibits these responses (Hojo et al., 2000).

CONCLUSION

Eicosanoids are an important class of lipid signaling mediators and have long been studied for their proinflammatory functions. In recent years, however, it has become evident that these molecules not only promote inflammation, but can occasionally also act as anti-inflammatory agents and have more complex and nuanced roles in the regulation of immune and inflammatory responses. Here, we have summarized the evidence for the expression of

and signaling by some important eicosanoids, the AA-derived prostanoids and the leukotrienes, in T lymphocytes. These lipid mediators regulate a number of functions in T cells, including proliferation, apoptosis, cytokine secretion, differentiation, chemotaxis, and more. Through these processes, eicosanoids regulate a wide array of physiological processes, ranging from inflammatory processes such as asthma and allergies, to immune regulation and involvement in graft rejection, as well as diseases such as cancer and AIDS. There is significant interest in targeting some of these pathways for therapeutic gain and it is therefore crucial to develop a complete understanding of all the different physiological functions of these important signaling mediators.

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The vitamin D receptor and T cell function

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The vitamin D receptor (VDR) is a nuclear, ligand-dependent transcription factor that in complex with hormonally active vitamin D, $1,25(OH)_2D_3$, regulates the expression of more than 900 genes involved in a wide array of physiological functions. The impact of $1,25(OH)_2D_3$-VDR signaling on immune function has been the focus of many recent studies as a link between $1,25(OH)_2D_3$ and susceptibility to various infections and to development of a variety of inflammatory diseases has been suggested. It is also becoming increasingly clear that microbes slow down immune reactivity by dysregulating the VDR ultimately to increase their chance of survival. Immune modulatory therapies that enhance VDR expression and activity are therefore considered in the clinic today to a greater extent. As T cells are of great importance for both protective immunity and development of inflammatory diseases a variety of studies have been engaged investigating the impact of VDR expression in T cells and found that VDR expression and activity plays an important role in both T cell development, differentiation and effector function. In this review we will analyze current knowledge of VDR regulation and function in T cells and discuss its importance for immune activity.

Keywords: vitamin D receptor, T cell function, vitamin D, signaling, expression, activity

INTRODUCTION

The purpose of the immune system is to recognize and clear pathogens from the body. However, occasionally unwanted immune reactions against self-tissue that lead to autoimmune diseases occur. The frequency of autoimmune diseases such as type 1 diabetes mellitus (Staples et al., 2003; Sloka et al., 2010), rheumatoid arthritis (Vieira et al., 2010), multiple sclerosis (MS) (Hogancamp et al., 1997), and inflammatory bowel disease (Khalili et al., 2012) has been linked to geographic location with a higher incidence of these diseases at higher degrees of latitude. One explanation of this geographical distribution is low exposure to sunlight and hence lower levels of vitamin D ($25(OH)D_3$) at higher degrees of latitude, as confirmed by studies showing an association between low serum levels of $25(OH)D_3$ and development of autoimmune diseases (Pierrot-Deseilligny and Souberbielle, 2010; Rossini et al., 2010; Greer et al., 2012). Low serum levels of $25(OH)D_3$ have also been linked to higher susceptibility to infections such as tuberculosis (Nnoaham and Clarke, 2008), influenza (Cannell et al., 2006; Grant, 2008), HIV (Rodriguez et al., 2009), respiratory syncytial virus (Grant, 2008), and viral infections of the upper respiratory tract (Ginde et al., 2009). It is therefore apparent that vitamin D plays a role in immune modulation. A recent acknowledgment that the majority of immune cells expresses the vitamin D receptor (VDR) (Kreutz et al., 1993; Hewison et al., 2003; Baeke et al., 2010; von Essen et al., 2010; Geldmeyer-Hilt et al., 2011; Joseph et al., 2012) and also the enzyme CYP27B1 used for internal conversion of circulating $25(OH)D_3$ to the VDR-ligand $1,25(OH)_2D_3$ (Hewison et al., 2003; Baeke et al., 2010) has further strengthen this perception.

VITAMIN D, VDR, AND T CELL FUNCTION

MECHANISM OF $1,25(OH)_2D_3$ ACTION

The cellular actions of $1,25(OH)_2D_3$ are mediated by the VDR, a ligand-dependent transcription regulator molecule belonging to the superfamily of nuclear receptors. In the absence of $1,25(OH)_2D_3$-VDR is mainly distributed to the cytoplasm (Nagpal et al., 2005). Interaction of VDR with its ligand $1,25(OH)_2D_3$ induces formation of two independent protein interaction surfaces on the VDR, one that facilitates association with the retinoid X receptor (RXR) necessary for DNA binding, and one that is required for recruitment of co-regulators necessary for gene modulation (Pike et al., 2012). Following interaction with $1,25(OH)_2D_3$-VDR dimerizes with RXR and translocates to the nucleus where it binds to vitamin D response elements (VDRE) in vitamin D responsive genes. Depending on the target gene either co-activators or co-repressors are attracted to the VDR/RXR complexes to induce or repress gene transcription (Nagpal et al., 2005; Pike et al., 2012; Haussler et al., 2013). Even though details of how these co-regulatory complexes work are only slowly beginning to emerge, it is now evident that they include ATPase-containing nucleosomal remodeling capabilities, enzymes with chromatin histone modifying abilities (e.g., acetyl- or methyl-transferases) and proteins involved in recruitment of RNA polymerase II (Pike et al., 2012; Haussler et al., 2013). Besides regulation through VDRE, VDR can inhibit genes by antagonizing certain transcription factors (Alroy et al., 1995; Takeuchi et al., 1998; Towers and Freedman, 1998). One such example is VDR-dependent inhibition of the T cell cytokine IL-2. Here, VDR first competes with the transcription factor NFAT1 for binding to the enhancer motif of AP1 and subsequently VDR binds to c-Jun. This co-occupancy of

VDR-c-Jun to AP1 leads to inhibition of IL-2 expression. The VDR inhibition of the IL-2 gene requires that VDR dimerizes with RXR, illustrating a need for $1,25(OH)_2D_3$ (Alroy et al., 1995; Takeuchi et al., 1998; Towers and Freedman, 1998). Overall, the cellular action of vitamin D therefore depends on sufficient production and delivery of $1,25(OH)_2D_3$ and adequate expression of VDR and its associated proteins. Since the VDR in 1983 was reported to be expressed in immune cells (Bhalla et al., 1983; Provvedini et al., 1983) an increasing effort to elucidate the importance of vitamin D on immune function has been undertaken. It has become increasingly clear that a major mechanism to control the immune regulatory effect of vitamin D is adjustment of the expression level and activity of the VDR.

VDR EXPRESSION AND DEVELOPMENT OF T CELLS

Due to the importance of T cells in protective immunity and in development of inflammatory and autoimmune disorders, several studies have examined the impact of VDR expression on T cell development, differentiation, and function. One approach to determine the role of VDR expression in development of T cells has been to study mice lacking the VDR (VDR-KO). These mice show normal numbers of $CD4^+$ and $CD8^+$ T cells including naturally occurring $CD4^+$ $FoxP3^+$ regulatory T cells (nTreg) (Yu et al., 2008), suggesting that VDR is not required for development of either of these cell types. A study performed by Hayes and coworkers using a mouse model with defective VDR in only the T cells confirmed that VDR is not essential for development of either conventional $CD4^+$ T cells, $CD8^+$ T cells, or $CD4^+$ $FoxP3^+$ nTreg cells (Mayne et al., 2011). Even so, VDR-KO mice appear to have a more vigorous immune response as seen by their increased risk of development of autoimmune diseases (Froicu et al., 2003; Froicu and Cantorna, 2007), and the enhanced response of VDR-KO T cells in mixed lymphocyte reactions (Froicu et al., 2003). In a series of studies Cantorna and coworkers have establish that the increased immune reactivity observed in VDR-KO mice in part is caused by a failure to develop the two regulatory T cell subsets, invariant NKT (iNKT) cells and CD8αα/TCRαβ T cells (Yu and Cantorna, 2008; Yu et al., 2008; Bruce and Cantorna, 2011). iNKT cells are a subset of T cells with a regulatory role in autoimmunity and infection (Godfrey et al., 2000; Bendelac et al., 2001; Singh et al., 2001). CD8αα T cells are mainly present in the gut, where they help maintain tolerance and suppress inflammation by dampening the response to a large number of gut antigens (Poussier et al., 2002; Cheroutre, 2004). The VDR-KO mice have significant fewer iNKT cells, due to a block in development as VDR is implicated in Tbet expression and conversion to the mature NK1.1 expressing mature iNKT cell. The few iNKT cells present in the periphery are furthermore functionally defective (Yu and Cantorna, 2008; Ooi et al., 2012). Like the iNKT cells, there are also fewer CD8αα/TCRαβ precursors in the thymus of VDR-KO animals. Moreover, to complete development CD8αα/TCRαβ cells must travel from the thymus to the gastrointestinal tract where IL-15 induces proliferation and upregulation of CD8αα. Due to decreased levels of IL-15 receptor expression VDR-KO CD8αα/TCRαβ cells proliferate poorly, resulting in a diminished mature CD8αα/TCRαβ population in the VDR-KO gut (Yu et al., 2008; Bruce and Cantorna, 2011; Ooi et al., 2012). These data illustrate that in contrast to conventional T cells, VDR expression is mandatory for development of both iNKT cells and CD8αα/TCRαβ T cells.

VDR EXPRESSION AND DIFFERENTIATION OF T CELLS

Adaptive immune responses require priming and proliferation of naïve T cells followed by migration of the resulting effector T cells to the site of infection. Antigen-specific triggering of TCRs expressed on the surface of antigen-naïve T cells together with co-stimulation induces intracellular signaling events that promote upregulation of the VDR (Provvedini et al., 1983; von Essen et al., 2010; Joseph et al., 2012). This activation-induced upregulation of VDR in naïve human T cells encourages $1,25(OH)_2D_3$-VDR signaling. $1,25(OH)_2D_3$-VDR signaling induces upregulation of the VDRE containing enzyme PLC-γ1, which is a central molecule in the classical TCR signaling pathway. Following VDR-induced PLC-γ1 upregulation classical TCR signaling is established and full T cell activation accomplished (von Essen et al., 2010). VDR expression therefore contributes to priming of naïve human T cells. Interestingly, this VDR-induced PLC-γ1 upregulation is not a mechanism involved in T cell priming of mouse T cells, as naïve mouse T cells already expresses substantial amounts of PLC-γ1 (Ericsson et al., 1996). In order for T cells to proliferate they need the cytokine IL-2. IL-2 is produced and secreted by T cells in response to antigen-induced T cell stimulation. In an autocrine and paracrine fashion IL-2 binds to high affinity IL-2 receptors on the same or adjacent T cells, inducing cell proliferation and hence a clonally expanded population of antigen-specific effector T cells (Cantrell and Smith, 1984; Smith, 1988). As VDR expression has been shown to inhibit transcription of the IL-2 gene (Alroy et al., 1995; Takeuchi et al., 1998), it is likely that upregulation of VDR serves as a negative feedback mechanism to control potential overreactions of the immune system. Besides inducing the early priming phase of naïve human T cells and possibly ensuring immune integrity, Mathieu and coworkers showed that a $1,25(OH)_2D_3$ agonist drastically changed the surface expression of homing receptors on both CD4 and CD8 T cells, resulting in a profile corresponding to an increased migration ability to sites of infection (Baeke et al., 2011); and hence implying a role for VDR in all phases of T cell differentiation.

In agreement with a suggested role of VDR in preventing immune overreaction, a changed distribution of naïve and antigen-experienced T cells was observed in a VDR-KO study performed by Bruce et al. (2011). The $CD4^+$ T cells had a more activated phenotype and readily developed into the proinflammatory Th17 effector cells that produced twice as much IL-17 as their WT counterparts *in vitro* (Bruce et al., 2011). Furthermore, vitamin D has been shown to modify the phenotype of antigen presenting dendritic cells (DC) to a more tolerogenic phenotype that favors differentiation of inducible Treg (iTreg) cells instead of the inflammatory Th1 and Th17 cells (Griffin et al., 2001; Adorini et al., 2003; Adorini and Penna, 2009). In VDR-KO mice, the frequency of total DC populations were not affected, but a significant reduction in tolerogenic DCs was observed (Bruce et al., 2011). In accordance with the reduced population of tolerogenic DCs and increased population of activated inflammatory T cells, a decrease in the population of iTregs that differentiated from naïve T cells

was observed (Bruce et al., 2011). This lead to an increased pathogenic potential of the T cell population, which manifested in development of more severe experimental inflammatory bowel disease (Bruce et al., 2011). These observations emphasize the importance of VDR expression in controlling the balance between effector and tolerogenic cells.

VDR EXPRESSION AND FUNCTION OF T CELLS

Only few studies have investigated whether there is coherence between VDR expression and T cell effector function. In the iNKT cell study performed by Cantorna and coworkers, a reduction of at least fifty percent in iNKT cells that produced the effector cytokine IL-4 and IFN-γ was observed in multiple organs (Yu and Cantorna, 2008). However, as iNKT cells most likely acquire the ability to transcribe IL-4 and IFN-γ during thymic development at the stage where they diverge from conventional T cells (Bezbradica et al., 2006), it is possible that the reduced cytokine production observed is due to defects in iNKT cell development. In a study of conventional T cells from VDR-KO mice, Bruce et al. (2011) showed that VDR-KO Th17 cells induced in *in vitro* cultures overproduced IL-17 as compared to WT cells. In contrast to the study performed by Cantorna using iNKT cells from VDR-KO mice, Bruce et al. found no change in IFN-γ production in the cultured conventional VDR-KO T cells. Taking this into consideration and the fact that Th17 cells are more readily induced in the VDR-KO mice, it is likely that the increased IL-17 production observed by Bruce et al. (2011) is also a developmental defect. Conversely, an *in vitro* study in human T cells performed by Youssef and coworkers favors a direct effect of VDR on IL-17 production. Here they showed that VDR blocks binding of the transcription factor NFAT1 to the promoter of the human IL-17 gene leading to a decrease in IL-17 production (Joshi et al., 2011). This inhibitory mechanism somehow resembles VDR's control of both IL-2 and GM-CSF transcription in which VDR also inhibits NFAT1 binding to the DNA of the respective cytokine genes (**Figure 1**) (Alroy et al., 1995; Takeuchi et al., 1998; Towers and Freedman, 1998). As NFAT1 is a transcription factor involved in regulation of a wide range of genes and as VDR's inhibition of NFAT1 appears not to include a canonical VDRE sequence in the promoter regions (Towers and Freedman, 1998), the transcriptional control of VDR's target genes is likely far more widespread than first anticipated. Today, a direct effect of $1,25(OH)_2D_3$-VDR signaling on the expression of effector T cell molecules includes not only cytokines but also chemokines and chemokine homing receptors as reviewed by Peelen et al. (2011).

Studies in which either T cell conditional VDR knock-out animals or animal models of adoptive transferred T cells from a VDR knock-out animal would substantially increase our understanding of VDR's direct influence on T cell effector function. Along this line Hayes and coworkers developed a mouse model in which only the T cells included an inactive VDR gene in order to investigate the implication of T cells in development of autoimmunity. In this model T cells developed normally in thymus but peripheral T cells expressing an inactive VDR were resistant to the inhibitory effect of vitamin D on autoimmune disease development (Mayne et al., 2011). Future studies will likely elaborate on specific T cell effector functions in similar animal models.

ALLELIC VARIATIONS AND NON-FUNCTIONAL VDR IN THE HUMAN POPULATION

Allelic variants of the VDR gene occur naturally in the human population. Even though interpretation of these polymorphic variants in relation to different diseases is difficult due to the small numbers of subjects included in the performed studies, an association with disease risk has been suggested. This includes a greater susceptibility to infections and a higher incidence of autoimmune diseases and cancer. The impact of VDR polymorphism on VDR function may in part be due to reduced VDR-mRNA stability and hence of reduced VDR expression (Feldman et al., 2005). In addition to allelic variations of the VDR gene, a rare genetic disorder has been described in which the VDR gene contain mutations that renders the gene product non-functional. This includes mutations in the DNA-binding domain and in the ligand binding domain, rendering binding of VDR to DNA, RXR, or co-regulators impossible (Malloy and Feldman, 2010). Individuals with a non-functional VDR suffer from the absence of VDR signaling giving rise to the disease hereditary vitamin D resistant rickets (HVDRR). In HVDRR patients the serum level of $1,25(OH)_2D_3$ is exceedingly high and most patients are completely resistant to vitamin D therapy. As there are only very few cases of HVDRR, long-term effects of defective VDR signaling on immune function such as development of autoimmune diseases and control of cancer have not yet been documented (Malloy and Feldman, 2010). A promising model system regarding this issue is the VDR-KO mouse in which the VDR gene has been deleted. These mice show increased sensitivity to autoimmune diseases, and are more prone to oncogene- and chemocarcinogen-induced tumors (Bouillon et al., 2008) illustrating a possible *in vivo* relation between VDR expression and immune function.

REGULATION OF VDR EXPRESSION AND ACTIVITY

The studies described above have led to an understanding of the importance of VDR expression in T cell development, differentiation, and function. Even though the abundance of VDR in T cells reflects the cells responsiveness to $1,25(OH)_2D_3$, this concept likely is far more complex. Besides transcriptional regulation of the VDR, additional factors with an impact on VDR activity should be considered. This includes ligand availability, induction of intracellular signaling pathways, posttranslational modifications of VDR, nuclear translocation, and DNA binding as well as recruitment of activated co-regulators.

LIGAND AVAILABILITY

The role of the VDR-ligand $1,25(OH)_2D_3$ is to convert VDR into a functionally active protein that can bind to RXR and to specific gene sequences and co-regulators necessary for modulation of gene expression (Pike, 2011; Pike et al., 2012; Haussler et al., 2013). Availability of $1,25(OH)_2D_3$ is therefore a prerequisite for VDR activity. The circulating concentration of $1,25(OH)_2D_3$ is very low ($\leq$100 pM) compared to its metabolic inactive precursor $25(OH)D_3$ ($\leq$100 nM) (Feldman et al., 2005). During an immune reaction it is therefore most likely that the source of $1,25(OH)_2D_3$ predominantly is endogenous production from the precursor molecule $25(OH)D_3$. In support of this, several studies of immune cells have revealed that $25(OH)D_3$ can be taken up and

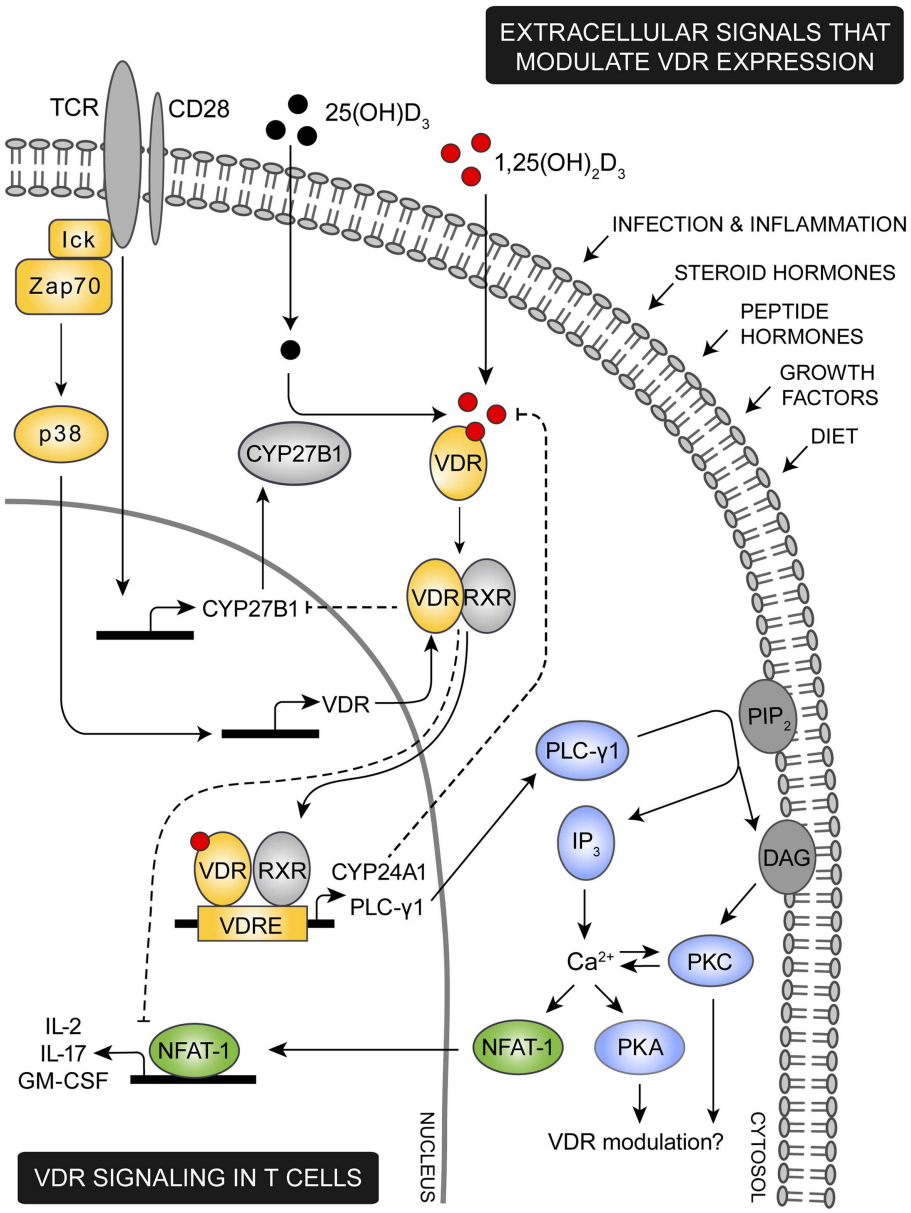

FIGURE 1 | Proposed model for VDR signaling in T cells. Various extracellular signals including infection, inflammation, steroid and peptide hormones, and diet are involved in regulation of the intracellular VDR level. During an immune response the TCR is triggered by specific antigens, inducing a cascade of intracellular signaling events. Among these, Ick and ZAP-70 are activated leading to activation of the p38 kinase which in naïve human T cells induce expression of VDR. TCR triggering also promotes expression of the $1,25(OH)_2D_3$ synthesis enzyme CYP27B1. Through intrinsic synthesis of $1,25(OH)_2D_3$ and uptake of $1,25(OH)_2D_3$ from the extracellular environment, VDR is activated and translocated into the nucleus where it either induce or suppress transcription of a variety of genes. As an example, VDR induce upregulation of PLC-γ1 in naïve human T cells. Once PLC-γ1 is expressed, TCR induced activation of PLC-γ1 leads to activation of PKA and PKC and an increase in the intracellular calcium level. In other cell types PKA and PKC has been shown to modulate expression of VDR, depending on the particular cell type and cellular differentiation state investigated. An increase in intracellular calcium concentration activates NFAT1 a necessary transcription factor for expression of IL-2 and other cytokines. IL-2 is a cytokine required for proliferation of T cells and one mechanism by which VDR adjust T cell activity is to outcompete NFAT1's binding to the IL-2 promoter and furthermore to down-regulate the actual expression of NFAT1. To control VDR activity a series of negative feedback loops exists; activated VDR both induce expression of the $1,25(OH)_2D_3$ degrading enzyme CYP24A1 and down-regulates expression of the $1,25(OH)_2D_3$ synthesizing enzyme CYP27B1.

subsequently converted into $1,25(OH)_2D_3$ through the action of the enzyme CYP27B1 (**Figure 1**) (Jeffery et al., 2012). CYP27B1 has been identified in most cells of the immune system (Fritsche et al., 2003; Liu et al., 2006; Sigmundsdottir et al., 2007; Krutzik et al., 2008; Correale et al., 2009; Baeke et al., 2010), however, it is not clear whether all cells can take up the precursor $25(OH)D_3$ and convert it. When $1,25(OH)_2D_3$ is synthesized a great part is secreted to adjacent cells, minimizing the need for endogenous

production in all immune cells (Jeffery et al., 2012). In addition, $1,25(OH)_2D_3$ holds the capacity to restrict its own synthesis by exerting a negative feedback on the vitamin D signaling system. $1,25(OH)_2D_3$ induces displacement of a key transcriptional factor responsible for CYP27B1 expression leading to a decrease in CYP27B1 (Murayama et al., 1999) and also induces a rapid binding of VDR-RXR to the promoter sequence of the $1,25(OH)_2D_3$ degrading enzyme CYP24A1 leading to an increase in CYP24A1 (**Figure 1**) (Ohyama et al., 1996; Kim et al., 2005). The net result is a reduction in endogenous $1,25(OH)_2D_3$. As illustrated by Vidal et al. this negative feedback mechanism can be partly prevented by inflammatory-induced proteins. In this study, they showed that IFN-γ induced activation of STAT1 promoted binding of STAT1 to the DNA-binding domain of VDR, preventing VDR from inducing expression of CYP24A1 (Vidal et al., 2002).

A major determinant of $25(OH)D_3$ availability is the carrier protein DBP that binds most circulating vitamin D in the serum. In immune reactions DBP restricts the availability of $25(OH)D_3$ to the immune cells (Chun et al., 2010; Jeffery et al., 2012). More than 100 genotypes of DBP have been documented but most people express the three most common variants GC1S, GC1F, and GC2 (Arnaud and Constans, 1993). These DBP variants have different properties including a difference in their affinity for $25(OH)D_3$ (Arnaud and Constans, 1993; Wood et al., 2011). *In vitro* studies performed with immune cells using different DBP genotypes in addition to $25(OH)D_3$ have shown that the particular genotype used influences the magnitude of the immune response (Chun et al., 2010; Jeffery et al., 2012). Along this line, an association between DBP genotype and development of inflammatory diseases has been described (Papiha and Pal, 1985; Speeckaert et al., 2006; Martineau et al., 2010). $1,25(OH)_2D_3$ availability therefore is the sum of the circulating $25(OH)D_3$ level, DBP genotype, CYP27B1 function, near proximity to other cells that produces and secretes $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ self-restriction.

EXTRACELLULAR SIGNALS THAT MODULATE VDR EXPRESSION

Vitamin D receptor expression can be modulated by numerous physical stimuli such as dietary composition (e.g., calcium and phosphorus), steroid hormones, growth factors, peptide hormones (Feldman et al., 2011), and inflammatory agents (Provvedini et al., 1983; Liu et al., 2006, 2009; von Essen et al., 2010; Joseph et al., 2012). For example, VDR expression is significantly regulated by the steroid hormones estrogen, glucocorticoid, and retinoids which appears to be rather cell specific (Feldman et al., 2011). The effect of glucocorticoid on VDR expression in the immune system has not been evaluated, but glucocorticoids are known to have a profound anti-inflammatory and immune suppressive effect (Miller and Ranatunga, 2012). Glucocorticoid therapy is used to suppress inflammation implicated in the pathogenesis of various inflammatory diseases (Hanaoka et al., 2012; Miller and Ranatunga, 2012), and it could be speculated that one mechanism used by glucocorticoids to suppress immune responses is by increasing the expression levels of VDR. Estrogen (Chighizola and Meroni, 2012) and retinoids (Cassani et al., 2012) also appear to have strong immunomodulatory effects, but like glucocorticoid the implication of VDR regulation as a possible mechanism to modulate immune function has not been investigated. Receptors for the peptide hormone parathyroid hormone (PTH) was recently identified on T cells (Geara et al., 2010). This renders PTH-induced modulation of VDR expression in T cells a possibility as observed for other cell types (Feldman et al., 2011). Again, this is unexplored territory even though PTH possesses an immune regulatory ability (Geara et al., 2010). The most well described hormonal effect on VDR activity and expression is that of $1,25(OH)_2D_3$ itself, as $1,25(OH)_2D_3$ directly influences the expression levels of VDR by homologous regulation. Although varying between different cell types, $1,25(OH)_2D_3$ in general increases VDR-mRNA production (McDonnell et al., 1987), stabilizes VDR-mRNA, and protects the VDR against degradation (Feldman et al., 2005), altogether increasing the total amount of the VDR.

Various inflammatory signals have also been shown to induce upregulation of VDR in immune cells. During an innate immune response, pathogen-induce activation of toll-like-receptors on human monocytes and macrophages results in upregulation of the VDR (Liu et al., 2006). Likewise, antigen-induced activation of TCR on human naïve T cells induce upregulation of the VDR (Provvedini et al., 1983; von Essen et al., 2010; Joseph et al., 2012). Furthermore, T cell cytokines induced during inflammation can modulate VDR expression (Edfeldt et al., 2010; Spanier et al., 2012), illustrating that regulation of the VDR level is a common mechanism used in the defense against pathogens.

INTRACELLULAR SIGNALING PATHWAYS THAT MODULATE VDR EXPRESSION

Modulation of VDR expression as a result of physical stimuli is mediated by various intracellular signaling pathways. Although only a sparse numbers of publications concern this issue, a few studies agree that activation of the cAMP-dependent protein kinase A (PKA) pathway leads to an increase in VDR abundance (Pols et al., 1988; Krishnan and Feldman, 1992; Song, 1996). Both cellular responses to PTH (Pols et al., 1988) and to prostaglandin (Smith et al., 1999) activate PKA causing an increase in the VDR level. In contrast, Feldman and coworkers showed that stimuli that induce protein kinase C (PKC) activity down-regulate both VDR-mRNA and VDR protein levels in fibroblastic cells (Krishnan and Feldman, 1991). Moreover, Reinhardt and Horst (1994) has shown that the impact of PKC activation on the VDR-mRNA level highly depends on the particular cellular differentiation state investigated. This suggests that other signaling pathways may cooperate to determine the final effect on VDR expression. In support of this idea, a study by Krishnan and Feldman (1992) indicated a mutual antagonism between the PKA and PKC pathways in regulation of the VDR level, an observation confirmed by others (van Leeuwen et al., 1992). Furthermore, it has been suggested that the intracellular calcium level that is known to influence and be influenced by PKC activity is implicated in PKA induced VDR upregulation (**Figure 1**) (van Leeuwen et al., 1990). A new signaling pathway which leads to VDR expression has recently been described in human naïve T cells. Here, TCR stimulation induces VDR expression through activation of the p38 mitogen activated protein kinase by ZAP-70 (**Figure 1**) (von Essen et al., 2010). In contrast, Gocek et al. (2007) showed that VDR expression was controlled by Erk and PI3K signaling in a myeloid leukemia cell

line where p38 activity appeared irrelevant. This implies that not only might different intracellular signaling pathways cooperate to regulate the expression of VDR, but also that the implicated signaling events differs between different cell types and different differentiation states of the cells.

TRANSCRIPTIONAL REGULATION OF VDR

Until recently the regulatory responses to hormones at the VDR-gene promoter were unknown. To clarify this, Zella et al. (2010) used ChIP–chip analysis to investigate the VDR gene transcription. These investigations revealed the presence of several enhancers, including the transcription factor C/EBPβ involved in basal expression of VDR as well as the transcription factor glucocorticoid receptor (GR) which mediates the action of glucocorticoids, the transcription factor retinoid acid receptor (RAR) mediating the action of retinoic acid, and the transcription factor CREB mediating the action of PTH (Zella et al., 2010). In case of VDR enhancement by $1,25(OH)_2D_3$, Zella et al. (2006, 2010) found accumulation of VDR-RXR and RNA pol II at the VDR gene together with an increase in C/EBPβ binding . They also detected a substantial increase in histon H4 acetylation associated with enhancer regions across the VDR locus (Zella et al., 2010). An induction of transcription from promoters is often associated with an increase in H4 acetylation, and the observations therefore indicated the existence of multiple enhancers in the VDR-gene locus that may contribute to $1,25(OH)_2D_3$-induced VDR expression. Transcriptional regulation of the VDR gene therefore includes the presence and activity of a wide range of enhancers induced by extracellular signals as well as induction of various epigenetic changes. In case of inflammatory-induced VDR upregulation, the regulatory responses at transcriptional level have not been investigated. As new techniques such as ChIP–chip and ChIP–seq have emerged, this topic will likely be explored in nearby future.

POSTTRANSLATIONAL MODIFICATIONS OF VDR

In addition to transcriptional regulation of VDR, several *in vitro* studies have suggested that VDR can be post-translationally modified. Studies by Haussler and coworkers revealed that $1,25(OH)_2D_3$ binding to VDR led to serine phosphorylation at multiple sites of the receptor. PKC was implicated in phosphorylation at serine 51, an event that partly inhibited VDR transcriptional activity (Hsieh et al., 1991). Although not required for VDR transcriptional activity, casein kinase II (CK II)-induced phosphorylation at serine 208 led to an enhancement of VDR transcriptional activity (Jurutka et al., 1996). As both PKC and CKII activity is induced in cells in response to various stimuli, it can be proposed that these posttranslational modifications although probably not obligatory for VDR function represents a mode to adjust the activity of VDR according to the specific signals received by the cell. Disease-induced posttranslational modifications leading to a dysfunctional VDR has also been documented. In a study by Patel et al. (1995) plasma toxins from uremic patients was shown to bind to the patients VDR, thereby disrupting binding of VDR-RXR to DNA resulting in a diminished VDR response. It so appears that posttranslational modifications of VDR adjust VDR activity in both health and disease.

RXR AND OTHER CO-REGULATORS OF VDR

The genomic actions of $1,25(OH)_2D_3$ also highly depends on the abundance and activity of proteins that interact with VDR. Binding of VDR to its ligand $1,25(OH)_2D_3$ facilitates association with RXR and in the absence of RXR, VDR is unable to bind to most VDRE in vitamin D target genes (Kliewer et al., 1992; Forman et al., 1995; Chambon, 1996). In addition to RXR binding, VDR interacts with various co-activators or co-repressors once bound to the DNA (Nagpal et al., 2005; Pike et al., 2012; Haussler et al., 2013). These co-regulatory complexes are necessary for the VDR-RXR heterodimer to either induce or suppress gene transcription and include ATPase-containing nucleosomal remodeling capabilities, enzymes with chromatin histone modifying abilities (e.g., acetyl- or methyl-transferases), and proteins involved in recruitment of RNA polymerase II (Pike et al., 2012). GRIP1 (Issa et al., 2001), RAC3 (Issa et al., 2001), SRC-1 (Masuyama et al., 1997a), TIF-1 (vom et al., 1996), ACTR (Chen et al., 1997), pCIP (Torchia et al., 1997), and Mediator (Oda et al., 2010) are some of the described co-activator proteins and co-activator complexes to date. These co-activators all regulate VDR function through co-assembling with VDR but they modulate VDR activity via distinct mechanisms. GRIP1 and RAC3 for example regulate VDR activity by modulating crosstalk between VDR and RXR (Issa et al., 2001), ACTR encompass histone acetyltransferase capacity and can recruit other nuclear factors (Chen et al., 1997), and Mediator which is a large complex composed of several MED-proteins activates transcription by direct recruitment of the RNA polymerase II transcriptional machinery (Oda et al., 2010). Although most co-activators facilitate VDR-induced transcriptional activation by binding to VDR, others are shown to be released from VDR to enable transcription, e.g., TFIIB (Masuyama et al., 1997b); illustrating the functional complexity of these co-activator complexes. Only a few co-repressor proteins involved in VDR silencing of genes have been described. As an example NcoR-1, NcoR-2, and Hairless can recruit histone deacetylase activity to VDR-target genes, leading to chromatin compaction and hence gene silencing (Nagpal et al., 2005). A recent study by Singh et al. (2012) furthermore showed that recruitment of co-repressors inappropriately can change during disease, causing a deregulation of VDR-target genes. In addition to transcriptional control of VDR, co-regulator proteins can modulate VDR abundance by enhancing degradation of VDR. Certain cellular signaling events have been shown to motivate the physical interaction of VDR-$1,25(OH)_2D_3$ with SUG1 of the proteasome complex, targeting VDR for ubiquitination and subsequent proteolysis (Masuyama and MacDonald, 1998). Therefore, it is evident that regulation of the expression level of RXR and other co-regulators are important to modulate the activity of VDR, and it could be speculated that expression of particular co-regulators are dictated by the inflammatory environment.

T CELLS MODULATE VDR EXPRESSION IN OTHER IMMUNE CELLS

A recent study by Edfeldt et al. revealed that VDR expression is not only modulated on a single cell level. Their study showed that VDR expression of innate immune cells could be regulated by nearby T cells (Edfeldt et al., 2010). In innate immunity, pathogen-induced signaling through Toll-like-receptors on human monocytes and macrophages up-regulate the expression of VDR. This in turn,

leads to VDR-induced expression of the antimicrobial peptide cathelicidin resulting in killing of microbes (Liu et al., 2006). VDR-induced cathelicidin expression by human monocytes was shown to be adjusted by cytokines produced by T cells. By modulating the level of VDR and the amount of VDR-ligand available by adjusting the CYP27B1 level, the T cell cytokine IFN-γ increases cathelicidin expression and IL-4 attenuates cathelicidin expression (Edfeldt et al., 2010). This example illustrates how interplay between innate and adaptive immunity cooperates to mount an appropriate response to infection through regulation of the VDR-system.

CONCLUDING REMARKS

This review indicates that VDR expression and activity are important for all stages of a T cells life, ranging from development to differentiation and elicitation of effector functions. In concordance, VDR expression and activity are associated with immunity against certain infections and with the prevalence of some autoimmune diseases. In animal models $1,25(OH)_2D_3$ has been shown to prevent development of autoimmune diseases. This includes experimental autoimmune encephalomyelitis (EAE), the animal model for MS (Mayne et al., 2011). EAE studies performed in VDR-KO animals (Bouillon et al., 2008) or in animals with a dysfunctional VDR (Mayne et al., 2011) illustrates the requirement of a functional VDR in $1,25(OH)_2D_3$ mediated EAE-inhibition. Furthermore, a study by Hayes and coworkers showed that VDR-gene inactivation selectively in the T cells completely eradicated the ability of $1,25(OH)_2D_3$ to inhibit EAE (Mayne et al., 2011). The biological relevance of low levels of VDR in development of MS was confirmed in a microarray analysis performed by Achiron et al. Here they compared blood mononuclear cells from healthy subjects that later developed MS with healthy subjects that remained MS-free. One of the early disease markers identified turned out to be suppressed VDR expression (Achiron et al., 2010). These observations may not only reflect a change in conventional T cells (e.g., development of more memory T cells that are predisposed to develop into Th1 and Th17 cells as observed in VDR-KO mice (Bruce et al., 2011) but also a reduced development of iNKT cells (as observed in VDR-KO mice, Yu and Cantorna, 2008; Ooi et al., 2012). iNKT cells are negative regulators of EAE (Matsuda et al., 2008) and furthermore, fewer iNKT cells can be found in the blood of MS patients (Araki et al., 2003). Along this line Araki et al. (2003) showed that an increase in iNKT cell number is associated with remission from symptoms in MS patients. Altogether, these observations emphasize a role for VDR expression in development and progression of autoimmunity.

Most experiments investigating susceptibility to a given autoimmune disease is, however, based on animal models. The question therefore remains whether these animal models which are executed in a pathogen free environment reflect the real life situation where humans continuously are bombarded with a variety of pathogens. It is slowly becoming apparent that the microbial environment has a greater influence on development of autoimmune diseases than previously anticipated. For example, certain microbes have been shown to slow innate immune defenses by dysregulating the VDR. One mechanism used by the innate immune system to clear a pathogen is VDR-induced production of the antimicrobial peptide cathelicidin which possesses antiviral, antibacterial, and antifungal activity. Therefore, any microbe capable of dysregulating expression of the VDR would enhance its chance for survival (Waterhouse et al., 2009; Proal et al., 2013). Klein and coworkers illustrated *in vitro* that Epstein-Barr virus (EBV) were able to effectively down-regulate expression of VDR in B cells (Yenamandra et al., 2009), Modlin and coworkers that *Mycobacterium leprae* inhibits VDR activity through down-regulation of CYP27B1 in monocytes (Liu et al., 2012), Wang and coworkers that *Mycobacterium tuberculosis* down-regulate expression of VDR in macrophages (Xu et al., 2003), and McElvaney and coworkers that the fungus *Aspergillus fumigates* secretes a toxin capable of down-regulating VDR in macrophages (Coughlan et al., 2012). This allows pathogens to accumulate in tissue and blood and the weakened innate defense further causes susceptibility to additional infections. As more pathogens are incorporated into this microbiome, people start to show symptoms characteristic of inflammatory and autoimmune diseases. Accumulating evidence now supports the observation that a number of autoimmune diseases can be reversed by restoring VDR function (using the VDR agonist olmesartan) along with antibiotics. This includes rheumatoid arthritis, systemic lupus erythematosis, sarcoidosis, scleroderma, psoriasis, Sjogren's syndrome, autoimmune thyroid disease, and type I and II diabetes mellitus (Waterhouse et al., 2009; Proal et al., 2013). Knowledge of the regulation of VDR abundance and activity in immune cells potentially is of great therapeutic importance, and therapeutic enhancement of VDR should therefore be considered in the clinic today.

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Role of adipokines signaling in the modulation of T cells function

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The field that links immunity and metabolism is rapidly expanding. Apparently non-immunological disorders such as obesity and type 2 diabetes have been linked to immune dysregulation, suggesting that metabolic alterations can be induced by or be consequence of an altered self-immune tolerance. In this context, adipose tissue produces and releases a variety of pro-inflammatory and anti-inflammatory factors, termed "adipokines," which can be considered as the bridge between obesity-related exogenous factors, such as nutrition and lifestyle, and the molecular events leading to metabolic syndrome, inflammatory, and/or autoimmune conditions. In obesity, increased production of most adipokines impacts on multiple functions such as appetite and energy balance, modulation of immune responses, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism, and so on. This report aims to discuss some of the recent topics of adipocytokine research and their related signaling pathways, that may be of particular importance as could lead to effective therapeutic strategies for obesity-associated diseases.

Keywords: leptin, adiponectin, adipocytokines, T cells, obesity

THE LINK BETWEEN ADIPOSE TISSUE AND CHRONIC INFLAMMATION

It is well established from literature that in more affluent countries, where increased metabolic overload is more frequent, incidence of obesity is higher and it has been associated with a series of consequences, such as increased risk of cardiovascular disorders including atherosclerosis, diabetes, fatty liver disease, inflammation, and cancer (1–5). All these pathological conditions are closely associated with chronic inflammation, as they are characterized by abnormal cytokine production, increased acute-phase reactants such as C-reactive protein (CRP) and interleukin-6 (IL-6) and activation of a network of inflammatory signaling pathways. They seem to be consequent to the long-term "low-degree" chronic inflammation typical of obesity (6, 7).

A new field of study that investigates the interface and the link among immune response, nutrition, and metabolism has recently developed and many of the interactions between the metabolic and immune systems seem to be orchestrated by a complex network of soluble mediators derived from immune cells and adipocytes (fat cells) (8). It has been found that certain genetic alterations (i.e., mutation, loss of function, among others) of leptin (Lep), leptin receptor (LepR), pro-opiomelanocortin (POMC), pro-protein convertase 1 (PCSK1), and melanocortin-4 receptor (MC4-R), can cause obesity and can also significantly affect immune responses (9–16). Therefore, the immune function in obesity has become a factor of particular interest and relevance to better understand and possibly modulate the inflammatory condition associated with this disorder.

The current view of adipose tissue is that of an active secretory organ and not merely an inert tissue devoted to energy storage. Indeed it is able to send out and respond to signals that modulate appetite, energy expenditure, insulin sensitivity, endocrine and reproductive systems, bone metabolism, and inflammation and immunity (5). Recent studies have centrally placed adipose tissue as a crucial site in the generation of inflammatory responses. In this context, the finding that tumor necrosis factor-α (TNF-α) and IL-6 are overexpressed in the adipose tissue of obese mice and humans and when administered exogenously leads to insulin resistance, provided the first clear link between obesity, diabetes, and chronic inflammation (17–19). Moreover adipocytes share with a diverse set of immune cells (including T cells, macrophages, and dendritic cells) several features, such as complement activation, production of inflammatory mediators to pathogen sensing and phagocytic properties (20–22). In addition to adipocytes, adipose tissue also contains pre-adipocytes (which are adipocytes that have not yet been loaded with lipids), endothelial cells, fibroblasts, leukocytes, and most importantly, macrophages. Macrophage infiltration of adipose tissue has recently been associated with obese conditions and it has been suggested that expanding adipocytes or neighboring pre-adipocytes might be responsible for the production of chemotactic signals, leading to macrophage recruitment in the adipose tissue (23, 24). Once macrophages are present and active in the adipose tissue, they, together with adipocytes and other cell types present in the adipose tissue, might perpetuate a vicious cycle of macrophage recruitment and production of pro-inflammatory cytokines (25, 26).

Adipose tissue is a mix of adipocytes, stromal pre-adipocytes, immune cells, and endothelium, and it can respond rapidly and dynamically to alterations in nutrient excess through adipocyte hypertrophy and hyperplasia (27). With obesity and progressive adipocyte enlargement, the blood supply to adipocytes may be reduced with consequent hypoxia (28). Hypoxia has been proposed to be an inciting etiology of necrosis and macrophage infiltration into adipose tissue, leading to an overproduction of pro-inflammatory factors like inflammatory chemokines. This results in a localized inflammation in adipose tissue which propagates an overall systemic inflammation associated with the development of obesity-related co-morbidities (28).

There is increasing evidence that besides macrophages other immune cells, such as T cells, might infiltrate adipose tissue (29). Wu and co-workers recently presented evidence that, at least in mice, adipose tissue from diet-induced obese insulin-resistant mice is infiltrated by T cells and that this infiltration was accompanied by an increased expression of the T-cell chemoattractant RANTES (29).

The presence of an abundant immune cell infiltrate in adipose tissue of obese subjects is considered one of the classical pathologic lesions present in obesity. The real significance of these infiltrates is still unknown and has been until now, considered directly or indirectly the result of a massive attraction exerted by adipocytes toward immune cells, particularly of the natural immunity compartment (i.e., macrophages, neutrophils, natural killer cells, dendritic cells) through the secretion of adipocytokines and chemokines (30–32). Strikingly, a series of recent studies have shown in mice that T cells in the adipose tissue show specific T cell receptor (TCR) rearrangements suggesting that there are clonal T cell populations infiltrating adipose tissue. These data along with extensive macrophage infiltration and Th1 cytokine secretion account for the consequent insulin resistance in adipocytes and chronic inflammation typical of obesity (33). Taken together these data can lead to the hypothesis to consider obesity as an autoimmune disorder. Typically, criteria to consider a pathological condition as "autoimmune" include: (1) infiltration by immune cells of self-target organ and its consequent tissue damage; (2) the presence of circulating autoantibodies that react against self-antigens and subsequent complement system activation; (3) the clonality of TCRs from infiltrating T cells; (4) secretion of pro-inflammatory Th1 cytokines; (5) quantitative or qualitative alterations of regulatory T (Treg) cells; (6) association with other autoimmune disease. In the case of obesity, most of the above-mentioned points have been detected (34, 35). However the self-antigen present in the adipose tissue is still unknown. Identifying these antigens and the corresponding antigen-presenting cells in fat is clearly the next challenge for the field.

The discovery of leptin and other adipocytokines has provided a further link among adipose tissue and immune cells. These molecules, indeed, function as hormones to influence energy homeostasis and to regulate neuroendocrine function, but acting as cytokines, adipocytokines are able to module immune functions and inflammatory processes throughout the body. In this review, we provide an overview of recent advances on the role of adipocytokines and their signaling pathways in the modulation of immune cells function, with particular emphasis on T cells subsets.

LEPTIN

Leptin, a cytokine-like hormone product of the obesity (*ob*) gene, belongs to the family of long-chain helical cytokines (characterized by a four a-helix bundle) and is mainly produced by adipose tissue, indeed its levels directly correlate with body fat mass and adipocyte size. However, it is produced, at lower levels, also by other tissues such as the stomach, skeletal muscle, placenta, and bone marrow (36–39). In the hypothalamus, leptin regulates appetite, autonomic nervous system outflow, bone mass, and the secretion of HPA hormones (36). Although an important role of leptin is to regulate body weight through the inhibition of food intake and stimulation of energy expenditure by increased thermogenesis, recent evidence has indicated that leptin is much more than a "fat sensor" (40). Indeed, leptin-deficient (*ob/ob*) mice and leptin-receptor-deficient (*db/db*) mice are not only severely obese, but also have a series of marked abnormalities that are secondary to the effects of leptin on reproduction (41), hematopoiesis (42), angiogenesis (43, 44), metabolism of bone (45), lipids and glucose (36), and last but not least, innate and adaptive immunity (46–48).

Leptin signaling

Leptin mediates its effects by the binding with the its specific LepR, a member of the class I cytokine receptor family (which includes receptors for IL-6, IL-12, OSM, and prolactin) and the pleiotropic biological effects of leptin can be partly explained by the wide distribution of LepRs on different types of cells, including those in extraneural tissues. Alternative splicing of LepR results in six receptor isoforms with different length of cytoplasmic domains, known as LepRa, LepRb, LepRc, LepRd, LepRe, and LepRf (49). Among all the LepR isoforms, only full-length isoform (LepRb) is able to fully transduce activation signals into the cell, as its cytoplasmic region contains several motifs required for signal transduction. The other LepR isoforms lack some or all of these motifs and their function is still unclear, even though several data suggest that they could be involved in the transport of leptin across the blood-brain barrier or in its degradation. Intracellularly, the LepR does not have an intrinsic tyrosine kinase domain, therefore it binds cytoplasmic kinases – mainly Janus tyrosine kinase 2 (Jak2) (50). LepR contains a highly conserved, proline-rich box 1 (51) and two putative, less conserved, box2 motifs (52, 53). Box 1 and box 2 motifs are considered important in recruiting and binding Jaks (54, 55) for full Jak activation (56). Recent studies indicate that, under physiological conditions, only Jak2 is activated during LepR signaling (53). Once activated, Jaks proteins trans-phosphorylate each other, as well as other tyrosine residues (Tyr985, Tyr1138, and Tyr 1077) of the LepR (57, 58), providing docking sites for downstream molecules such as signal transducer and activation of transcriptions (STATs). These proteins dissociate from the receptor and form homo- or hetero-dimers, which translocate into the nucleus and act as transcription factors by binding specific response elements in the promoter region of their target genes, such as sis-inducible-element (SIE), acute-phase-response-element (APRE), and GAS-like elements (59, 60) (**Figure 1**).

In response to leptin, STAT3 binds to phospho-Tyr1138, allowing Jak2 to phosphorylate and activate STAT3. Confirming the importance of this site of phosphorylation, mutation of Tyr1138

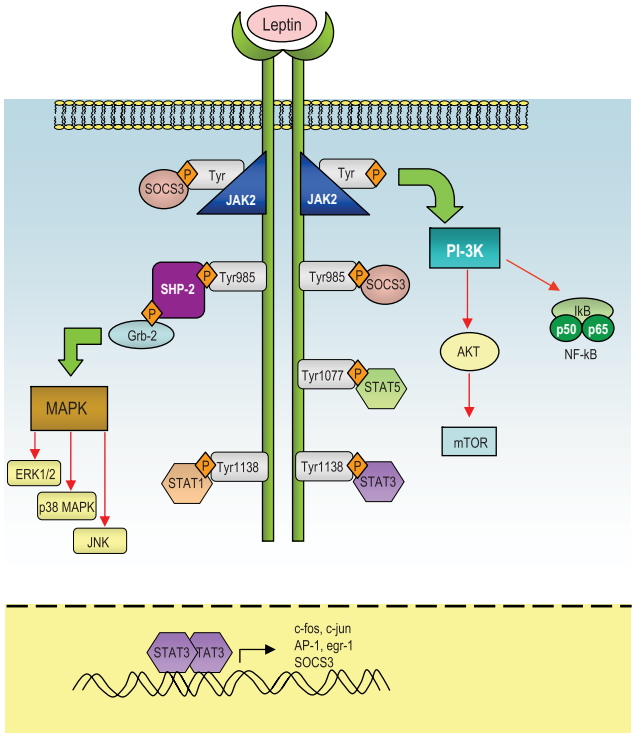

FIGURE 1 | Schematic representation of the leptin-induced pathways. After leptin binds to the long isoform of the leptin receptor (LepRb), Jak2 is activated at the box 1 motif, resulting in the autophosphorylation of tyrosine residues and phosphorylation of tyrosines that provide docking sites for signaling proteins containing src homology 2 (SH-2) domains. The autophosphorylated Jak2 at the box 1 motif can lead to activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Akt can regulate a wide range of targets including FOXO1 and NF-κB. Both Tyr1077 and Tyr1138 bind to STAT5, whereas only Tyr1138 recruits STAT1 and STAT3. STAT3 proteins form dimers and translocate to the nucleus to induce expression of genes such as c-fos, c-jun, egr-1, activator protein-1 (AP-1), and suppressors of cytokine signaling 3 (SOCS3). SOCS3 negatively regulates signal transduction by leptin by binding to phosphorylated tyrosines on the receptor, to inhibit the binding of STAT proteins and the SH-2 domain-containing phosphatase-2 (SHP-2). SHP-2 activates the mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and p42/44 MAPK through an interaction with the adaptor protein growth factor receptor-bound protein 2 (GRB2), to induce cytokine and chemokine expression in immune cells.

abolishes the ability of leptin to activate STAT3, resulting in severe hyperphagia and morbid obesity (61–63). Leptin stimulates also phosphorylation of LepRb on Tyr1077, which binds to STAT5 and subsequently mediates STAT5 phosphorylation (64, 65).

The Jak/STAT pathway is under the negative-feedback control of suppressors of cytokine signaling (SOCS) proteins, which are induced upon cytokine stimulation and act as negative regulators of signaling by binding to phosphorylated Jak proteins or by direct interaction with tyrosine-phosphorylated receptors (66, 67). Structurally, SOCS proteins are characterized by a central SH-2 domain, an N-terminal preSH-2 domain, in some cases a kinase inhibitory region (KIR) domain, which abolishes the kinase activity of the Jaks, and a more conserved C-terminal SOCS-box – which represents a key mediator of proteasomal degradation (by linking ubiquitin to the substrate) (66). Only SOCS1 and 3 carry a KIR domain in their N-terminal region and it is involved in the inhibition of the Jak activity and thus leptin signaling. Recent data showed that SOCS3 inhibits kinase activity through its KIR domain after the binding through its SH-2 domain with phosphotyrosine motifs in the receptor in the proximity of the Jaks. Interestingly, leptin can induce SOCS3 expression (68–71) and the Tyr985 of LepRb is a high-affinity binding site for SOCS3 (57, 70). In this context, the participation of SOCS3 in the negative-feedback mechanism of leptin signaling has been proposed to underlie the development of leptin resistance in relation to the hyperleptinemia observed during obesity (69).

Another negative regulator of leptin signaling is represented by the SH-2 domain-containing phosphatase-2 (SHP-2), which is a constitutively expressed tyrosine phosphatase involved in the dephosphorylation of Jak2 (72). SHP-2 carries two tandem SH-2 domains followed by a tyrosine phosphatase catalytic domain. When one SH-2 domain interacts with a tyrosine-phosphorylated ligand, a conformational change occurs and brings this phosphatase to activation of LepR at position Y985 (73). This specific site has an important role in leptin-induced extracellular signal-regulated kinases (ERK) activation (57). More specifically, as a result of leptin administration, Tyr985 becomes phosphorylated by recruited Jaks (mainly Jak2 and Jak1), and provides a docking site for SHP-2. After binding to that specific tyrosine residue, SHP-2 is phosphorylated at the C-terminus and together with its adapter molecule Grb2, it activates downstream signaling, leading to the activation of the p21Ras/ERK signaling cascade (57), with the final induction of specific target genes expression, such as c-fos or egr-1, a zinc-finger transcription factor that influences the initiation of growth and differentiation (74) (**Figure 1**).

Leptin can activate also another member of the MAP kinase family, p38 MAPK (75) and stress-activated protein kinase c-Jun N-terminal kinase (JNK). Among the possible downstream targets of leptin-induced activation of p38 and JNK MAPK pathways, the regulation of the transcription factor nuclear factor-κB (NF-κB) appears to be crucial for the transcriptional regulation of pro-inflammatory cytokines such as TNFα and IL-1β.

In addition, leptin is able to regulate phosphoinositide 3-kinase (PI3K) activity, indeed the binding of PI3K regulatory subunit to tyrosine-phosphorylated proteins induces a conformational change allowing the activation of its catalytic subunit and consequent full activation of PI3K, whose products typically stimulate protein kinases such as Akt, also called protein kinase B (PKB), protein kinase C (PKC) (76), and Forkhead box O1 (FOXO1), a transcriptional factor that is phosphorylated and inactivated by Akt (77–80). Leptin inhibits both the activity and expression of hypothalamic FOXO1 through the PI 3-kinase pathway (77). Indeed, overexpression of a constitutively active FOXO1 mutant decreases leptin sensitivity in mice with consequent increase in food intake and body weight, whereas small interfering RNA-mediated knockdown of FOXO1 increases leptin sensitivity and decreases food intake and body weight (77, 78).

Finally, leptin stimulates phosphorylation of ribosomal S6 kinase (S6K), a major physiological substrate of the mammalian target of rapamycin (mTOR) kinase in the hypothalamus. Indeed,

rapamycin, a specific inhibitor of mTOR attenuates leptin's anorexigenic effects (81), conversely, activation of S6K enhances leptin sensitivity (82) (**Figure 1**). mTOR binds to raptor and GβL to form the mTOR complex 1 (mTORC1), which directly phosphorylates and activates S6K (83). mTORC1 is inhibited by the TSC1/TSC2 complex (84–86). Akt phosphorylates TSC2 and inactivates the TSC1/TSC2 complex (85). Therefore, the mTOR/S6K pathway is likely to be a downstream target of the PI 3-kinase/Akt pathway in leptin-stimulated neurons.

Leptin and T cells

Leptin stimulates and promotes the proliferation of human peripheral blood mononuclear cells (PBMC) (40, 48), as the presence of LepR on monocytes and lymphocytes has been shown in mice (46, 87) and confirmed in human peripheral blood T-lymphocytes (both CD4 and CD8) (88).

In PBMCs, leptin stimulation induces tyrosine phosphorylation and translocation of STAT3 molecules to the nucleus (89–91) and the phosphorylation of the STAT3-associated RNA binding protein Sam68 (a tyrosine-phosphorylated adaptor protein in TCR activation, which is associated with the SH2 and SH3 domains of Src and other signaling molecules, such as Grb2, PLC-γ-1, and PI3K) (92–95).

Recent evidence has shown that leptin induces tyrosine phosphorylation of Sam68 and Insulin receptor substrate 1 (IRS-1), which associate with p85 (96, 97), the regulatory subunit of PI3K via the SH-2 domain, recruiting and leading to stimulation of PI3K activity (98). In this context, leptin has been shown to inhibit apoptosis of thymocytes through an IRS-1/PI3K-dependent pathway since this effect was inhibited by the PI3K inhibitor LY294002 (99). Moreover, Martín-Romero et al. have shown that both ERK-1 and ERK-2 were found phosphorylated in a dose-dependent fashion in PBMC after incubation with human leptin (98).

It was also found that leptin could induce sustained phosphorylation of p38 MAPK in human PBMCs and the phosphorylation of the ribosomal protein S6 – the only protein in the large 40S subunit that has been shown to be phosphorylated in response to growth factors and mitogens (100). One route of leptin-induced S6 phosphorylation in human PBMCs is via MEK and p42/p44 MAPK (101–103), which activate MAPK-dependent S6 Kinase p90 RSK and S6. The other way seems to be mediated via activation of p70 S6 kinase, since it has been shown that leptin phosphorylates p70 S6 kinase at Thr389 (104). Accordingly, pre-treatment of cells with rapamycin abolished this phosphorylation (104). Strikingly, the MEK inhibitor PD98059 has been shown to inhibit not only p90 RSK phosphorylation, as expected, but also p70 S6 Kinase and S6 phosphorylation, thus suggesting an essential role of MEK activation in a full induction of p70 S6 kinase activity in human PBMC (105, 106).

In $CD4^+CD25^-$ effector T cells (Teff), De Rosa et al. have shown that leptin-induced strong STAT3 phosphorylation, while stimulation of $CD4^+CD25^+$ Treg cells was not associated with a marked increase of phosphorylated STAT3 (107). SOCS3, a negative regulator of cytokine signaling, was activated by leptin blockade in Treg cells, in which the stimulation with anti-CD3/28 induced phosphorylation of ERK1/2 and subsequent cell proliferation (107). In the same subset of cells, the cyclin-dependent kinase inhibitor p27 (p27kip1, a molecule involved in the control of cell cycle and T cell anergy) was elevated before and after anti-CD3/28 stimulation, and leptin neutralization induced degradation of this molecule, partly explaining the reversal of the anergic state and proliferation of these cells.

Recently, the contribution of leptin to mTOR activation in human Teffs has been well defined. Indeed, it has been shown that leptin treatment had little effect on mTOR phosphorylation, but it induced a significant increase in p70S6K and S6 phosphorylation, concomitant with a consistent increase in AKT phosphorylation. The induction of mTOR, as well as AKT phosphorylation induced by TCR engagement, was significantly reduced by leptin blockade and this inhibition was partially reversed by the addition of recombinant leptin to cultures, thus suggesting suggest a link between autocrine secretion of leptin and mTOR activation in Teffs through an AKT-dependent mechanism (108). A recent study by Galgani et al. shows that nutritional status, through leptin, directly affects survival and proliferation of autoreactive T cells, modulating the activity of the survival protein Bcl-2, the Th1/Th17 cytokines, and the nutrient/energy-sensing AKT-mTOR pathway (109). Moreover, a paper by the same group has shown that leptin activates the mTOR pathway to control also Treg cells responsiveness (110, 111). More specifically leptin inhibited rapamycin-induced proliferation of Tregs, by increasing activation of the mTOR pathway. In addition, under normal conditions, Tregs secreted leptin, which activated mTOR in an autocrine manner to maintain their state of hyporesponsiveness. Finally, Tregs from db/db mice exhibited a decreased mTOR activity and increased proliferation compared with that of wild-type cells (110, 111). Together, these data suggest that the leptin-mTOR axis sets the threshold for the responsiveness of Tregs and that this pathway might integrate cellular energy status with metabolic-related signaling in Treg cells that use this information to control immune tolerance.

ADIPONECTIN

Human adiponectin is encoded by ADIPOQ gene localized on the chromosome locus 3q27. It has a sequence homology with a family of proteins characterized by an amino-terminal collagen-like sequence and a carboxy-terminal complement 1q-like globular region and shares homologies with collagens, complement factors, TNF-α, and brain specific factor cerebellin (112, 113). Two different forms of this molecule exist: a full-length protein, which is present in the plasma, and a globular adiponectin which consists of the globular C-terminal domain resulting from a photolytic cleavage mediated by a leukocyte elastase secreted by monocytes and/or neutrophils. After cleavage the globular form can trimerize, while the full length can exist as a trimer low molecular weight (LMW) adiponectin, as an hexamer, that consists of two trimers bound through a disulfide bond middle molecular weight (MMW) adiponectin and as a 12- to 18-mer high molecular weight (HMW) adiponectin. Adiponectin is mainly produced in white adipose tissue (WAT) by mature adipocytes, with increasing expression and secretion during adipocyte differentiation, but it can be also found in skeletal muscle cells, cardiac myocytes, and endothelial cells. Its levels inversely correlate with visceral obesity and insulin resistance and in this context weight loss is considered a potent inducer of adiponectin synthesis, thus suggesting a key role exerted by

adiponectin in protection against obesity and obesity-related disorders. Indeed TNF as well as other pro-inflammatory cytokines such as IL-6 suppress adiponectin secretion in adipocyte (114, 115). Adiponectin acts thought the interaction with two different receptors: ADIPOR1 and ADIPOR2, which differ both in localization and binding affinity since ADIPOR1 is expressed mainly in skeletal muscle and binds globular adiponectin while ADIPOR2 is expressed mainly in the liver and engages the full-length adiponectin (116). Expression of ADIPORs has been reported on human monocytes, B-cells, and NK cells, but only a small percentage of T cells express these molecules (117). The binding of adiponectin to ADIPOR1 and/or ADIPOR2 results in the activation of peroxisome-proliferator-activated receptor-α (PPAR-α), AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase (MAPK). More specifically, AMPK acts as a major downstream component of adiponectin signaling, since it represents the cellular energy sensor in the body and it is normally activated when there is an increase in the intracellular AMP/ATP ratio (118, 119).

Over the past 5 years, several interacting and adapter proteins for ADIPORs have been discovered. The adaptor protein containing a pleckstrin homology domain, a phosphotyrosine domain and a leucine zipper motif (APPL1) has been shown to bind to ADIPORs (120, 121) and is required for adiponectin-induced activation of AMPK, p38 MAPK, and ERK1/2–MAPK pathways. In addition, the regulatory subunit of the protein kinase casein kinase (CK) 2 or the receptors for activated C-kinase-I (RACK-I) and the endoplasmic reticulum protein 46 (ERp46) have been reported as other potential binding partners for ADIPOR1.

Initial studies suggested that adiponectin could act as an anti-inflammatory adipocytokine, as it exerted its anti-inflammatory effects on endothelial cells through the inhibition of TNF-α-induced adhesion molecule expression (122). Adiponectin-deficient mice had higher levels of TNF-α expression in adipose tissue and higher plasma levels compared with wild-type mice (114). Adiponectin inhibited NF-κB activation in endothelial cells and interfered with the function of macrophages (122, 123), as testified by the finding showing that treatment of cultured macrophages with adiponectin markedly inhibited their phagocytic activity and their production of TNF-α in response to lipopolysaccharide (LPS) stimulation (123). Adiponectin increases the secretion of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1Ra) by human monocytes, macrophages, and DCs and suppresses the production of IFN-γ by LPS-stimulated human macrophages (124) and Toll-like receptor (TLR)-induced NF-κB activation (125).

In addition adiponectin has been shown to be a negative regulator of NK cell function (77), since it suppressed IL-2-enhanced cytotoxic activity of NK cells through the AMPK-mediated inhibition of NF-κB activation and down-regulated IFN-γ-inducible TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand expression on these cells. Contrasting results have recently shown that adiponectin can also act as a pro-inflammatory cytokine. Indeed it has been shown that its levels are high in arthritis, preeclampsia, and end-stage renal diseases (126–130). Also, adiponectin was shown to induce production of the pro-inflammatory mediator IL-6 and activation of NF-κB in human synovial fibroblasts and adhesion molecule expression in endothelial cells (131–133). One possible explanation for the pleiotropic effects exerted by adiponectin could be the presence of various circulating oligomers of adiponectin. Although HMW multimers appear to be the most bioactive form of adiponectin in the circulation, other isomeric forms of adiponectin like hexamers could differently modulate intracellular signaling pathways in several anatomical districts, thus exerting quite different effects (134, 135). Thus, the question of whether adiponectin might be considered an anti- or pro-inflammatory adipocytokine still needs to be clarified.

Adiponectin and T cells

Little is know about the effect of adiponectin on T cell function. Several data suggest that adiponectin is a negative regulator of T cell activity. In particular, although a small percentage of T cells express ADIPOR on their surface, a great amount of T cells store ADIPORs within clathrin-coated vesicles and these receptors colocalized with Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) molecules. After stimulation of T cells, the expression of both ADIPORs and CTLA-4 has been shown to be upregulated. Interestingly, it has observed that the addition of adiponectin results in a significant decrease of antigen-specific T cell proliferation and cytokines production, through the enhancement of T cells apoptosis. Confirming these findings *in vivo*, adiponectin-deficient mice had higher frequencies of $CD137^+$ T cells upon Coxsackie B virus infection, thus suggesting that adiponectin is a novel negative T-cell regulator (136).

Adiponectin has been shown to inhibit allograft rejection in murine cardiac transplantation, indeed Okamoto et al. have shown that allografts transplanted to $APN^{-/-}$ mice showed severe acute rejection to transplants in $APN^{+/+}$ hosts accompanied by increased accumulation of $CD4^+$ and $CD8^+$ T cells and macrophages (137). A recent paper by Tsang et al. suggests that the immunomodulatory effect of adiponectin on immune response could be at least in part mediated by its ability to alter dendritic cell functions (138). Indeed, adiponectin-treated dendritic cells show a lower production of IL-12p40 and a lower expression of CD80, CD86, and histocompatibility complex class II (MHCII). Moreover, in co-culture experiments of T cells and adiponectin-treated dendritic cells, a reduction in T cells proliferation and IL-2 production and an higher percentage of $CD4^+CD25^+Foxp3^+$ Treg cells was observed (138) suggesting that adiponectin could also control regulatory T cell homeostasis. Moreover adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and consequently reduces T-lymphocyte recruitment and accumulation during atherogenesis (139).

On the contrary, Cheng et al. have recently shown that addition of adiponectin to polyclonally activated $CD4^+$ T cells induced secretion of IFN-γ and IL-6, increased phosphorylation of p38 MAPK and STAT4 and augmented T-bet expression, indicating that adiponectin enhances Th1 differentiation (140). In the same direction, the paper by Jung et al. has shown that adiponectin-induced maturation and activation of DCs, as demonstrated by the increased expression of MHC class II, co-stimulatory molecules in both mouse and human DCs, and it significantly enhanced production of pro-inflammatory cytokines. moreover, adiponectin-treated DCs significantly induced both Th1 and Th17 responses

in allogeneic T cells, leading to enhanced pro-inflammatory responses (141).

RESISTIN

Resistin is a 114-amino-acid polypeptide, originally shown to induce insulin resistance in mice (142). It belongs to the family of resistin-like molecules (RELMs), also known as "found in inflammatory zone (FIZZ)," a family of molecules that has been implicated in the regulation of inflammatory process (143). Resistin was shown to circulate in two distinct forms: a more prevalent HMW hexamer and a substantially more bioactive, but less prevalent, LMW complex (144). Initially, resistin has been shown to be predominantly expressed by adipocytes but recent evidence has suggested that macrophages, rather than adipocytes, appear to be the most important source of resistin in human subjects (145) and mRNA encoding resistin can be found in mice and humans in various tissues, including the hypothalamus, adrenal gland, spleen, skeletal muscle, pancreas, and gastrointestinal tract (146).

Contradictory findings have shown that resistin levels can be either increased, unchanged, or decreased in murine and human obesity and type II diabetes, however, recent data indicate that in human PBMCs, expression of resistin mRNA is markedly increased by the pro-inflammatory cytokines IL-1, IL-6, and TNF, and by LPS (147). Also, resistin levels are mutually correlated with those of cell-adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) in patients with obstructive sleep apnea, and in atherosclerotic patients are positively associated with other markers of inflammation, such as soluble TNF-R type II and lipoprotein-associated phospholipase A2 (148, 149). Similarly, stimulation of human macrophages with LPS led to increased resistin mRNA expression, via a cascade involving the secretion of pro-inflammatory cytokines and administration of LPS to human volunteers is associated with dramatically increased circulating resistin levels (150), thus suggesting that this molecule can act as a critical mediator of the insulin resistance associated with sepsis and possibly other inflammatory conditions. In further support of its pro-inflammatory profile, resistin also up-regulates the expression of vascular cell-adhesion molecule 1 (VCAM1), ICAM-1, and CCL2 by human endothelial cells and induces these cells to release endothelin-1 (151).

Resistin and T cells

A small number of studies have been performed to address the role of resistin in T cell functions, but recent evidence has showed that resistin strongly up-regulates the expression of TNF and IL-6 by human PBMCs and induces arthritis after injection into the joints of healthy mice (152). These pro-inflammatory properties of resistin were abrogated by an NF-κB inhibitor, thus showing the key role of NF-κB in resistin-induced modulation of inflammatory reactions. Moreover Son et al. have recently shown that resistin induces expansion of functional Tregs, as testified by increased protein and mRNA expression of FoxP3, only when $CD4^+$ T cells are co-cultured with DCs (153).

VISFATIN

Another protein clearly representing an additional link between adipose tissue and inflammation is Visfatin [also known as pre-B-cell colony-enhancing factor (PBEF)] which has recently been identified as an adipocytokine secreted primarily by adipocytes in visceral fat and able to decrease insulin resistance (154). This molecule is an insulin-mimetic adipokine, being able to bind and activate the insulin receptor without competing with insulin. Visfatin mRNA levels increase in the course of adipocyte differentiation, and visfatin synthesis is regulated by several factors, including glucocorticoids, TNF, IL-6, and growth hormone. Originally it has been identified as a growth factor for B lymphocyte precursors PBEF (155) and since its discovery it has been associated with several inflammatory disease states such as acute lung injury (156, 157). Indeed the presence of specific single nucleotide polymorphisms in the visfatin/PBEF gene, which decrease gene transcription rate, highly increases the risk of development of acute lung injury in septic patients (157).

Furthermore, expression of visfatin has been shown to be upregulated in activated neutrophils from septic patients (155, 157) and to inhibit the apoptosis of neutrophils, through a caspase 3- and caspase 8-mediated mechanism (155). On monocytes, visfatin is able to induce their chemotaxis and their ability to induce allo-proliferative responses in lymphocytes, through a p38 and MEK-dependent mechanism. More specifically, it ha been shown that visfatin up-regulates the production of the pro-inflammatory cytokines IL-1b, IL-6, and TNF-α (158), the expression of the co-stimulatory molecules CD80 (B7-1), CD40, and also of ICAM-1 and other co-stimulatory ligand that binds to LFA-1 (lymphocyte function-associated antigen-1), thereby promoting the activation of T cells (159). In this context, Moschen et al. have also shown that PBEF/visfatin is a potent chemotactic factor particularly for $CD14^+$ monocytes and $CD19^+$ B-cells (158).

ADIPSIN

Adipsin (which in human subjects corresponds to complement factor D46) is the rate-limiting enzyme in the alternative pathway of complement activation (160). Adipsin, together with several other components of both the classical and alternative complement cascade, is primarily expressed by adipocytes in mice and by both adipocytes and monocytes-macrophages in human subjects (161). Adipsin levels are reduced in murine models of obesity but either increased or unchanged in obese human subjects (162).

INTRACELLULAR METABOLIC PATHWAYS IN THE CONTROL OF IMMUNE FUNCTIONS

Recent evidence shows that the intracellular metabolic pathways, that sense environmental signals, such as nutrient availability, are able to control T cell function and differentiation, including Treg cell activity and immune tolerance pathways. This might represent a mechanism that allows immune cells to finely tune their response according to their metabolic competence.

In particular, mTOR, a serine-threonine kinase that can integrate signals from environmental nutrients and growth factors to control T cell proliferation and differentiation (163, 164), together with AMPK, its activator LKB1, the NAD^+-dependent deacetylase Sirtuin 1 (SIRT1), and the Forkhead-box-o-family (Foxo) proteins, have been described as the dominant intracellular elements linking metabolism and self-tolerance. mTOR kinase, which can operate in two distinct signaling complexes (mTORC1 and 2) (165, 166), regulates different aspects of helper T (Th) cell differentiation and

fate. Differentiation of naive CD4 T cells into Th1 and Th17 subsets is controlled in part by mTORC1 signaling an event dependent on the small GTPase Ras homolog enriched-in-brain (Rheb) (167). In contrast, conditional deletion of mTORC2 adaptor rictor protein impairs Th1 and Th2 cell differentiation, without altering Th17 differentiation or frequency of Treg cells, by promoting phosphorylation of PKB or Akt, PKC, and NF-κB (168). In Treg cells, mTOR is a negative regulator of TCR-dependent FoxP3 expression (169), of *de novo* Treg cell differentiation (170), and of Treg cell lineage commitment (171).

In this context, several biological molecules have been associated to the control of intracellular metabolic pathways; among these the adipocyte-derived hormone leptin has been shown to bring the gap between metabolism and immune cell tolerance. We have previously demonstrated that leptin can be produced by, and inhibits, the proliferation of Treg cells (107). Indeed, genetic deficiency of leptin (*ob/ob* mice) is associated with an increased percentage of peripheral Treg cells as compared to WT mice. These data are in agreement with recent reports showing that adipose tissue in normal individuals is a preferential site of accumulation of Treg (34). Their precise role in this tissue is still object of extensive investigation but what is clear is that in mice, diet-induced obesity (DIO) is associated with a body mass-dependent, progressive decline in the proportions of Treg cells in the visceral adipose tissue (VAT). In contrast, therapy with CD3-specific antibody (which promotes T cell self-tolerance through global, transient T cell depletion) normalized insulin resistance and glucose homeostasis, and selectively restored $CD4^+Foxp3^+$ T cell pools in VAT (74), by increasing IL-10 and Th2/regulatory-type cytokines (34, 35). Moreover Cipolletta et al. identified peroxisome proliferator-activated receptor (PPAR)-γ, the "master regulator" of adipocyte differentiation, as a crucial molecular orchestrator of VAT Treg cell accumulation, phenotype, and function (172). All these data indicate that leptin could represent the molecular link between obesity and reduced number/function of Treg observed in this condition and on the basis of these data, one could predict that leptin might interact with the mTOR pathway. Supporting this hypothesis, leptin increases mTOR activation and blocks proliferation of cultured TCR-activated rapamycin-treated Treg cells and Teffs (108, 110), thus modulating immune tolerance.

CONCLUDING REMARKS

During the last decade, there has been a growing understanding of how host nutritional status and metabolism can affect the immune response. In this context, several adipocytokines, are able to participate in a wide range of biological functions that include glucose metabolism and $CD4^+$ T-lymphocyte proliferation, cytokine secretion, and apoptosis, underlining the link among immune function/homeostasis, metabolism, and nutritional state.

The notion that adipose tissue was considered as "passive" source of energy in time of famine and starvation has been completely revisited and its major role in the control of "dominant" functions, such as immunity and metabolism, is providing novel insights into the pathogenesis of metabolic and autoimmune disorders.

Although many effects of these adipocytokines have been elucidated in recent times, the details of their signaling pathways need further investigation to understand how they are ultimately integrated. It will be also worthwhile to focus, in the future, on how adipocytokines signaling integrates with the intracellular cascades activated by other factors in the immune cells, since understanding the mechanism of action of these adipocytokines will soon be pivotal to the development of novel therapeutic approaches to obesity-induced inflammatory diseases.

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