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Vitamin D controls T cell antigen receptor signaling and activation of human T cells

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Phospholipase C (PLC) isozymes are key signaling proteins downstream of many extracellular stimuli. Here we show that naive human T cells had very low expression of PLC-γ1 and that this correlated with low T cell antigen receptor (TCR) responsiveness in naive T cells. However, TCR triggering led to an upregulation of ~75-fold in PLC-γ1 expression, which correlated with greater TCR responsiveness. Induction of PLC-γ1 was dependent on vitamin D and expression of the vitamin D receptor (VDR). Naive T cells did not express VDR, but VDR expression was induced by TCR signaling via the alternative mitogen-activated protein kinase p38 pathway. Thus, initial TCR signaling via p38 leads to successive induction of VDR and PLC-γ1, which are required for subsequent classical TCR signaling and T cell activation.

Recognition of antigens by T lymphocytes and B lymphocytes occurs via two distinct sets of receptors: the T cell antigen receptor (TCR) and the B cell antigen receptor. Antigen-primed B cells increase their antigen responsiveness in part by affinity maturation mediated by somatic hypermutation of the B cell antigen receptor genes and selection of B cell clones of higher affinity[1]. Unlike the B cell antigen receptor, the TCR cannot undergo affinity maturation. Nevertheless, antigen-primed T cells substantially increase their antigen responsiveness compared with antigen-inexperienced (naive) T cells by a process called 'functional avidity maturation'[2,3]. Phenotypic and functional studies of naive and antigen-primed human T cells have found that antigen-primed T cells show much greater proliferation and cytokine production than do naive T cells after TCR stimulation[4–7]. Subsequent studies have confirmed functional avidity maturation in wild-type and TCR-transgenic mice[8–12]. Impaired calcium mobilization in naive T cells relative to that of primed T cells after TCR triggering has been described, which suggests that the coupling of the TCR to its signaling pathway is more efficient in antigen-primed T cells than in naive T cells[13,14]. In support of that idea, the 50-fold increase in T cell responsiveness to antigen found during the early stages of viral infection in mice correlates with an increase in expression of the tyrosine kinase Lck[3].

The present model for TCR signaling[15,16] postulates that after TCR ligation, Lck is activated, which results in phosphorylation of the CD3 coreceptor complex and ζ-chains of the TCR and activation of the ζ-chain-associated protein Zap70. Activated Zap70 phosphorylates the membrane adaptor Lat, which subsequently recruits several Src homology–containing proteins, including phospholipase C-γ1 (PLC-γ1). Activation of PLC-γ1 results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-3,4,5-triphosphate and diacylglycerol. Inositol-3,4,5-triphosphate regulates intracellular calcium mobilization, and diacylglycerol regulates the activation of protein kinase C and RasGRP, an activator of the GTPase Ras. By contributing to Ras activation, PLC-γ1 indirectly controls the mitogen-activated protein kinase (MAPK) cascades and the ensuing production of transcription factors, which leads to gene expression and cell-cycle entry. Thus, in the classical TCR signaling pathway, PLC-γ1 is a central, indispensable molecule.

An alternative TCR signaling pathway leading to activation of the MAPK p38 has been described[17,18]. In this pathway, after Zap70 is activated, it directly phosphorylates and activates p38. This makes the alternative TCR signaling pathway independent of Lat and thus of PLC-γ1 and the classical Ras-MAPK cascades[17,19,20].

Here we show that naive human T cells had very low expression of PLC-γ1. TCR signaling via the classical PLC-γ1-dependent pathway was consequently greatly impaired in these cells. However, TCR signaling via the alternative p38 pathway was intact in naive T cells, and it induced expression of the vitamin D receptor (VDR). Together with vitamin D, VDR subsequently activated the gene encoding PLC-γ1, which resulted in upregulation of PLC-γ1 protein expression by ~75-fold. This much higher PLC-γ1 expression allowed TCR signaling via the classical PLC-γ1-dependent pathway and explains the higher sensitivity of primed T cells to antigen stimulation.

RESULTS

Lower sensitivity of naive T cells to TCR triggering

To analyze antigen sensitivity in human T cells during their differentiation from naive to primed T cells, we purified naive T cells from freshly drawn human peripheral blood and propagated them with beads coated with antibody to CD3 (anti-CD3) and anti-CD28. We allowed the resulting primed T cells to 'rest' for at least 24 h before

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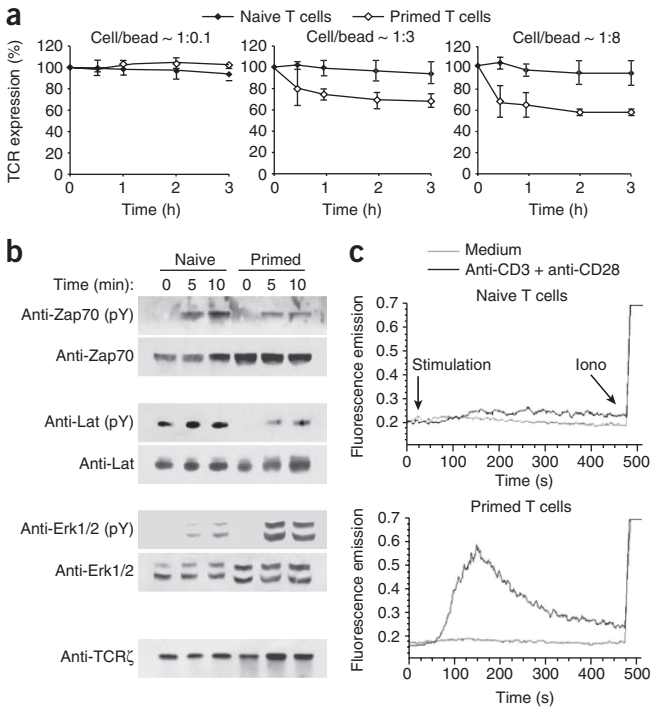

Figure 1 Lower sensitivity of naive T cells to TCR triggering. (**a**) TCR expression in naive and primed T cells after stimulation with beads coated with anti-CD3 and anti-CD28 (cell/bead ratio, above graphs), presented relative to that of untreated cells. Data are from five independent experiments (error bars, s.d.). (**b**) Immunoblot analysis of tyrosine phosphorylation (pY) and expression of Zap70, Lat, Erk and TCRζ (loading control) in naive and primed T cells stimulated for 0–10 min with beads coated with anti-CD3 and anti-CD28. Each vertically aligned lane represents the same cell sample. Data are representative of three independent experiments. (**c**) Calcium flux in naive and primed T cells left unstimulated (Medium) or stimulated at 30 s with a mixture of antibody to CD3 (anti-CD3) and anti-CD28, with ionomycin added at 465 s. Results are for gated $CD8^+$ T cells; $CD4^+$ T cells produced the same results. Data are representative of three independent experiments.

restimulation. We then determined the antigen sensitivity of naive and primed T cells by measuring TCR downregulation, tyrosine phosphorylation of signaling molecules and calcium mobilization. We did not observe TCR downregulation in naive T cells even after 3 h of maximal stimulation, in contrast to primed T cells, which downregulated their TCR expression by approximately 40% (**Fig. 1a**). As TCR downregulation is dependent on the activation of kinases downstream of the TCR[21], we assessed the tyrosine phosphorylation of key signaling molecules. We consistently found that tyrosine phosphorylation of Zap70 and Lat was greater in naive T cells than in primed T cells, whereas tyrosine phosphorylation of the kinase Erk was much lower in naive T cells than in primed T cells (**Fig. 1b**). In addition, compared with primed T cells, naive T cells were severely impaired in their ability to flux calcium (**Fig. 1c**). This indicates that the initial steps in the classical signaling pathway are intact in naive T cells but that a block is present between Lat and Erk, probably at the level of PLC-γ1.

PLC-γ1 activation and expression in naive T cells

We next analyzed PLC-γ1 activation in naive and primed T cells after TCR triggering. We did not detect phosphorylation of PLC-γ1 in naive T cells, whereas there was PLC-γ1 phosphorylation in primed T cells as early as 2 min after TCR triggering. Reblotting of the membranes showed that naive T cells had almost no expression of PLC-γ1 (**Fig. 2a**). We further examined the expression of PLC-γ1 mRNA and protein in T cells stimulated for 0–9 d. Using expression of Glut1 (the glucose transporter in hematopoietic lineage cells) as a marker of T cell activation[22], we found significant upregulation of the expression of both PLC-γ1 mRNA and protein during T cell differentiation (**Fig. 2b–e**). At 2 d after stimulation, PLC-γ1 protein expression was upregulated more than 50-fold in primed T cells relative to its expression in naive T cells. At its peak, PLC-γ1 protein was upregulated 75- to 80-fold in primed T cells relative to its expression in naive T cells (**Fig. 2d,e**). This demonstrates that naive human T cells have very low expression of PLC-γ1 and explains their impaired ability to flux calcium.

VDR induction precedes PLC-γ1 upregulation

Regulation of the human gene encoding PLC-γ1 remains unclear. Treatment with the active form of vitamin D (1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)) results in higher PLC-γ1 expression in human keratinocytes[23], and a DR6-type vitamin D–responsive element has been identified in the promoter of the human PLC-γ1 gene[24]. In addition, VDR is not expressed in naive human T cells but begins to be expressed after TCR stimulation[25]. We hypothesized that initial TCR signaling in naive human T cells induces expression of VDR independently of PLC-γ1 and that subsequent signaling through

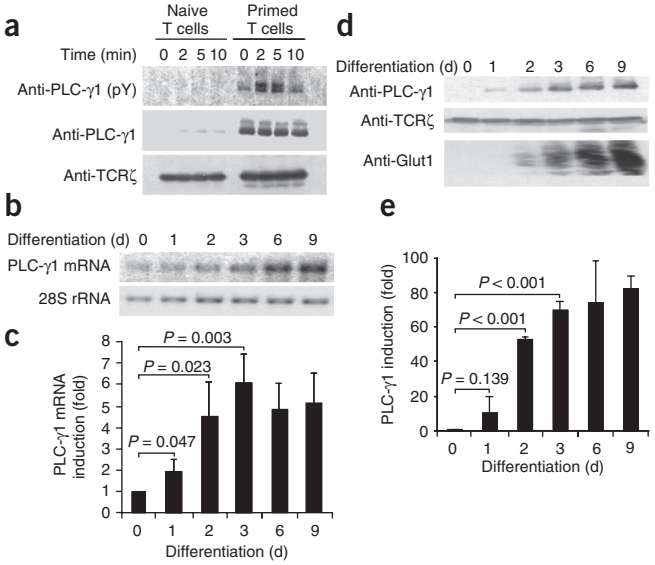

Figure 2 Phosphorylation and expression of PLC-γ1 in naive and primed T cells. (**a**) Immunoblot analysis of tyrosine phosphorylation and expression of PLC-γ1 and TCRζ (loading control) in naive and primed T cells stimulated for 0–10 min with beads coated with anti-CD3 and anti-CD28 (cell/bead ratio, 1:3). (**b**) RNA-hybridization analysis of the expression of PLC-γ1 mRNA and 28S rRNA (loading control) in T cells stimulated for 0–9 d. (**c**) Quantification of PLC-γ1 mRNA expression during differentiation, normalized to the loading control and presented (per T cell) relative to basal expression in naive T cells. (**d**) Immunoblot analysis of the expression of PLC-γ1, TCRζ (loading control) and Glut1 (activation marker[22]) in T cells stimulated for 0–9 d. (**e**) Quantification of PLC-γ1 expression during differentiation, normalized to the loading control and presented (per T cell) relative to basal expression in naive T cells. Data are representative of three separate experiments (**a**,**b**,**d**) or are from three experiments (**c**,**e**; error bars, s.d.).

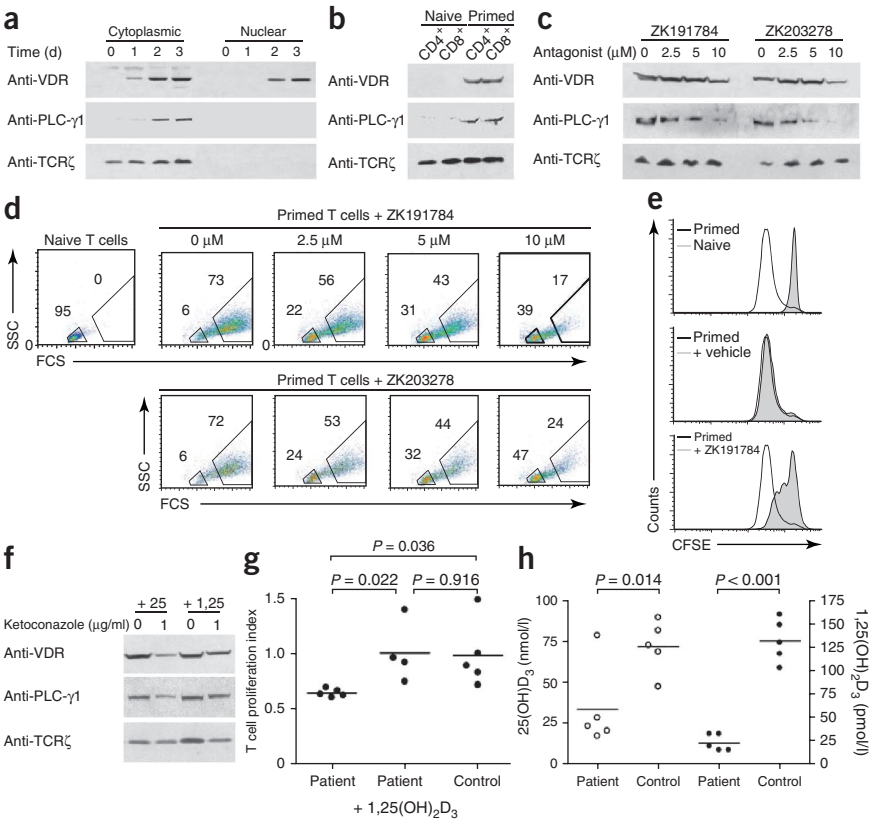

Figure 3 VDR induction precedes PLC-γ1 upregulation. (**a**) Immunoblot analysis of VDR, PLC-γ1 and TCRζ (loading control) in the cytoplasmic and nuclear fractions of T cells stimulated for 0–3 d with beads coated with anti-CD3 and anti-CD28 (cell/bead ratio, 1:3). (**b**) Immunoblot analysis of the expression of VDR, PLC-γ1 and TCRζ in purified naive and primed $CD4^+$ and $CD8^+$ T cells. (**c**) Immunoblot analysis of the expression of VDR, PLC-γ1 and TCRζ in T cells stimulated for 3 d in the presence of the VDR antagonists ZK191784 and ZK203278 (concentration, above lanes). (**d**) Flow cytometry of naive T cells (far left, top row) and T cells stimulated for 3 d in the presence of various concentrations (above plots) of ZK191784 (other plots, top row) or ZK203278 (bottom row). Numbers adjacent to outlined areas indicate percent cells in each. SSC, side scatter; FSC, forward scatter. (**e**) CFSE profiles of naive T cells (top row; gray filled histogram) and primed T cells stimulated for 3 d without further additions (black lines) or in the presence of vehicle (middle; gray filled histogram) or ZK191784 (bottom row; gray filled histogram). (**f**) Immunoblot analysis of the expression of VDR, PLC-γ1 and TCRζ in T cells stimulated for 3 d in the presence of ketoconazole (0 or 1 μg/ml) and either $25(OH)D_3$ (25; 0.5 μM) or $1,25(OH)_2D_3$ (1,25; 0.5 μM). (**g**) Proliferation index of T cells from patients with chronic deficiency of $25(OH)D_3$ and $1,25(OH)_2D_3$ and controls with normal serum concentration of $25(OH)D_3$ and $1,25(OH)_2D_3$, stimulated for 3 d. (**h**) Concentration of $25(OH)D_3$ (left vertical axis; open circles) and $1,25(OH)_2D_3$ (right vertical axis; filled circles) in patient and control sera. Each symbol represents an individual serum sample; small horizontal lines indicate the mean. Data are representative of three separate experiments.

the VDR induces expression of the gene encoding PLC-γ1. To test our hypothesis, we measured expression of VDR and PLC-γ1 in the cytoplasmic and nuclear fraction of T cells stimulated for 0–3 d. We found that naive T cells did not express VDR. We detected VDR in the cytoplasmic fraction but not in the nuclear fraction after 1 d of stimulation. We detected VDR in both the cytoplasmic and nuclear fraction after 2 d of stimulation, a time at which we also detected PLC-γ1 in the cytoplasmic fraction (**Fig. 3a**). There was expression of VDR and PLC-γ1 in both purified $CD4^+$ T cells and purified $CD8^+$ T cells after TCR stimulation (**Fig. 3b**).

Binding of $1,25(OH)_2D_3$ to the VDR induces a conformational change in the VDR that allows the VDR to bind to the vitamin D–responsive element in the promoter of VDR target genes[26]. The VDR antagonists ZK191784 (ref. 27) and ZK203278 (ref. 28) competitively bind to the VDR with an affinity close to that of $1,25(OH)_2D_3$ and most probably impede the conformational change required for the interaction of VDR with the vitamin D–responsive element[29]. At concentrations shown before to inhibit the proliferation of mixed-lymphocyte cultures[28], VDR antagonists substantially inhibited activation-induced expression of PLC-γ1, whereas they resulted in only slightly lower VDR expression (**Fig. 3c**). Cells duplicate their contents and double their size (cell growth) before cell division, and this process is central to the expansion phase of activated T cells[30]. VDR antagonists inhibited cell growth and proliferation in a dose-dependent manner (**Fig. 3d**,**e**).

To further investigate whether the induction of PLC-γ1 is dependent on $1,25(OH)_2D_3$ and VDR, we stimulated T cells in the presence or absence of ketoconazole, an antagonist of the hydroxylase CYB27B1 (ref. 31). CYP27B1 is required for the intracellular conversion of the inactive vitamin D precursor $25(OH)D_3$ into active $1,25(OH)_2D_3$ (ref. 32). In turn, $1,25(OH)_2D_3$ stabilizes the VDR and inhibits its degradation[33]. Thus, inhibition of CYP27B1 should result in less $1,25(OH)_2D_3$ and VDR. We found that treatment of T cells with ketoconazole inhibited VDR expression as well as PLC-γ1 induction. This inhibition was reversed by the exogenous addition of $1,25(OH)_2D_3$ but not by the addition of $25(OH)D_3$ (**Fig. 3f**), which supported the idea that $1,25(OH)_2D_3$-VDR signaling is required for PLC-γ1 induction. Finally, we analyzed whether chronic deficiency in $25(OH)D_3$ and $1,25(OH)_2D_3$ affected the proliferative capacity of T cells. We found that T cells isolated from patients with low serum concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ had a lower proliferation index after stimulation than that of T cells from healthy controls with normal concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ (**Fig. 3g**,**h**). The impaired T cell proliferation observed was reversed by the exogenous addition of $1,25(OH)_2D_3$ (**Fig. 3g**). These observations support the idea that $1,25(OH)_2D_3$-VDR is required for the induction of PLC-γ1 and full activation of human T cells.

The alternative TCR signaling pathway induces VDR expression

Our data indicated that the classical PLC-γ1-dependent TCR signaling pathway is not operational in naive T cells. An alternative PLC-γ1-independent TCR signaling pathway leading to p38 activation after Zap70 phosphorylation has been described in T cells[17,18], and p38 is known to have a role in activating the gene encoding VDR in human breast cancer cells and kidney cells[34,35]. To determine whether the p38 pathway is functional in naive human T cells, we stimulated naive and

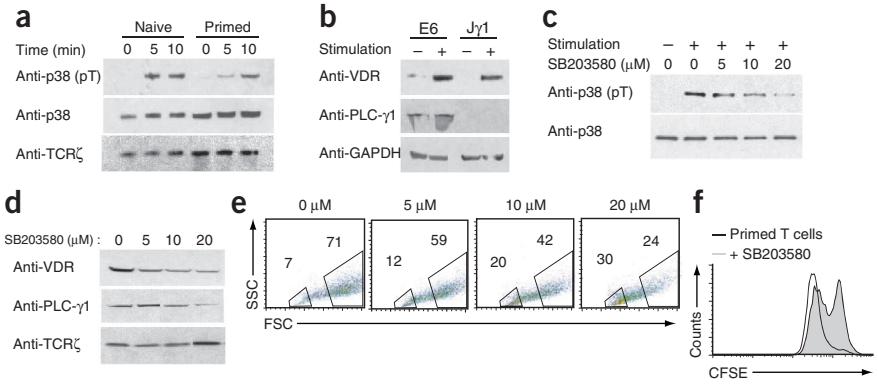

Figure 4 The alternative TCR signaling pathway induces VDR expression. (**a**) Immunoblot analysis of Thr180 phosphorylation (pT) and expression of p38 and TCRζ (loading control) in naive and primed T cells stimulated for 0–10 min with beads coated with anti-CD3 and anti-CD28 (cell/bead ratio, 1:3). (**b**) Immunoblot analysis of the expression of VDR, PLC-γ1 and GAPDH (glyceraldehyde phosphate dehydrogenase; loading control) in E6-1 (E6) and J.gamma1 (Jγ1) cells stimulated for 24 h with anti-CD3 and anti-CD28. (**c**) Immunoblot analysis of Thr180 phosphorylation and expression of p38 in unstimulated naive T cells and in naive T cells stimulated for 30 min with magnetic beads coated with anti-CD3 and anti-CD28 (cell/bead ratio, 1:3) in the presence or absence of the p38 inhibitor SB203580 (concentration, above lanes). (**d**) Immunoblot analysis of the expression of VDR, PLC-γ1 and TCRζ in T cells stimulated for 3 d in the presence or absence of SB203580 (concentration, above lanes). (**e**) Flow cytometry of T cells stimulated for 3 d in the presence or absence of SB203580 (concentration, above plots). Numbers adjacent to outlined areas indicate percent cells in each. (**f**) CFSE profiles of primed T cells stimulated for 3 d with beads coated with anti-CD3 and anti-CD28 (black line) or beads plus 10 μM SB203580 (gray filled histogram).

primed T cells and assessed p38 phosphorylation. We found that p38 phosphorylation was intact in naive T cells (**Fig. 4a**). Furthermore, to determine whether VDR expression could be induced independently of PLC-γ1, we stimulated PLC-γ1-deficient J.gamma1 and wild-type E6-1 Jurkat human T cell lines[36]. We found that VDR expression was as readily induced in J.gamma1 cells as it was in E6-1 cells (**Fig. 4b**), which indicated that the alternative p38-activating TCR signaling pathway is functional in naive T cells and that VDR expression can be induced independently of PLC-γ1.

Alternative p38 activation relies on autophosphorylation of p38 Thr180. In contrast, classical MAPK cascade–induced activation of p38 leads to dual phosphorylation of p38 Thr180 and Tyr182 independently of p38 activity[17,18,20]. To further analyze whether p38 was indeed activated by the alternative pathway in naive T cells, we used the p38 inhibitor SB203580. SB203580 inhibited phosphorylation of p38 Thr180 in naive T cells in a dose-dependent manner (**Fig. 4c**), which suggested that p38 is activated by the alternative pathway in human naive T cells. We next studied the effect of the p38 inhibitor on the induction of VDR and PLC-γ1. SB203580 diminished the expression of VDR and PLC-γ1 in a dose-dependent manner (**Fig. 4d**). The concentrations of SB203580 that inhibited VDR and PLC-γ1 induction were equal to the concentrations of SB203580 that inhibited the autophosphorylation of p38 Thr180. Likewise, SB203580 inhibited T cell growth and proliferation in a dose-dependent manner (**Fig. 4e**,**f**). These observations support the idea that naive human T cells use the alternative TCR signaling pathway to induce VDR expression.

DISCUSSION

In this study we have shown that naive human T cells had very low expression of VDR and PLC-γ1. However, TCR signals through the alternative p38 pathway induced VDR expression. VDR binds $1,25(OH)_2D_3$, translocates to the nucleus and activates the gene encoding PLC-γ1, which results in the accumulation of PLC-γ1 protein in the cytoplasm of primed T cells approximately 48 h after the initial activation signals. Because PLC-γ1 has a central role in classical TCR signaling and T cell activation[15,16], the differences in PLC-γ1 expression in naive and primed T cells might explain the process of functional avidity maturation observed in T cells.

The PLC family consists of at least 13 divergent PLC isozymes with key roles in mediating the physiological effects of various extracellular stimuli[37]. They all share the ability to convert phosphatidylinositol-4,5-bisphosphate to inositol-3,4,5-triphosphate and diacylglycerol after activation; however, their requirements for activation differ. The PLC-γ subfamily contains two members, PLC-γ1 and PLC-γ2, both of which bear structural motifs that allow regulation by protein tyrosine kinases. Human T cell lines express mainly PLC-γ1, which is largely responsible for calcium mobilization and activation of the transcription factors NFAT and NF-κB after TCR triggering[36,38,39]. Many of the fundamental insights into TCR signaling and PLC-γ1 in human T cells have been obtained from studies of activated or transformed T cell lines, such as the Jurkat T cell line[15]. Jurkat T cells can be compared with primed T cells, and they constitutively express PLC-γ1 (ref. 36). To our knowledge, this is the first report to describe PLC-γ1 activation and expression in freshly isolated, naive human T cells. However, our results are in agreement with published studies describing severe impairment in the ability of naive T cells to flux calcium[13,14].

As seen with human T cells, antigen-primed mouse T cells have much greater antigen responsiveness than do naive T cells, as measured by their proliferative capacity and cytokine production[3,8–12]. Furthermore, like naive human T cells, naive mouse T cells are considerably impaired in PLC-γ1 phosphorylation and calcium mobilization compared with primed mouse T cells[13,14]. However, the molecular mechanisms behind the impaired PLC-γ1 activation seem to differ in mouse and human T cells. Thus, in contrast to naive human T cells, naive mouse T cells have substantial PLC-γ1 expression[14], and therefore VDR-induced regulation of PLC-γ1 expression does not seem to serve a role in functional avidity maturation in mouse T cells. In agreement with those observations, naive mouse T cells have VDR expression similar to that of primed mouse T cells[40]. In addition, although the development of natural killer T cells[41] and $CD8\alpha\alpha^+$ intraepithelial lymphocytes[42] is disturbed in VDR-knockout mice, T cell activation does not seem to be affected much[43]. The evolution of different mechanisms for the regulation of PLC-γ1 activity in human and mouse T cells parallels the development of divergent VDR-dependent and VDR-independent antimicrobial pathways in human and mouse macrophages[31], respectively, and may reflect the fact that mice are nocturnal animals with fur and humans are daytime creatures that synthesize vitamin D in the skin after exposure to ultraviolet light.

Although we observed phosphorylation of Zap70, Lat and p38 in primed T cells after stimulation, the phosphorylation of these molecules was consistently greater in naive T cells. In contrast, phosphorylation

of Erk was consistently greater in primed T cells than in naive T cells. These observations indicate that the action of negative feedback pathways, such as Dok2 (ref. 44), on the phosphorylation of early signaling molecules may be activated differently in naive and primed T cells.

According to the basic paradigm for TCR signaling, PLC-γ1 has a central role in TCR signal transduction and T cell activation[16]. In mutant T cells lacking Lat, there is no effect on TCR-induced CD3 phosphorylation or Zap70 activation, but all the classical signaling steps distal to Lat are inhibited: PLC-γ1 activation, calcium flux, Erk activation and induction of transcriptions factors are lost[45,46]. However, an alternative TCR signaling pathway leading to p38 activation has been described[17,18]. In this pathway, Zap70 directly phosphorylates and activates p38. Consequently, the alternative TCR signaling pathway is independent of Lat and thus of PLC-γ1 and the classical Ras-MAPK cascades[17,19]. Our observations have indicated that p38 is activated independently of PLC-γ1 by the alternative pathway in naive human T cells, which is in agreement with a published study of p38 activation in T cells[20]. The idea that p38 is activated by the alternative pathway in naive T cells was further confirmed by the observation that the p38 inhibitor SB203580 inhibited p38 Thr180 autophosphorylation[17].

A connection between p38 and VDR induction has been demonstrated in other cell systems[34,35]. It has been shown that p38 can mediate activation of transcription factors such as AP-1 and ATF2, which presumably interact with their corresponding elements in the promoter of the gene encoding VDR[34,35]. We suggest that similar activation of this promoter takes place in T cells after p38 activation. The induction of PLC-γ1 expression occurs approximately 48 h after initial TCR signaling, and this might explain the lag phase of 48–72 h normally seen from initial TCR triggering to the initiation of T cell division.

An evolutionary benefit for the lag phase between antigen recognition and antigen-specific T cell division has been suggested[47]. This lag phase might allow the innate immune response the opportunity to quickly control infection. If this is successful, the onset of T cell division takes place in a relatively uninflammatory microenvironment with scarce amounts of antigen and proceeds to a limited extent. Conversely, if the innate immune system fails to control the infection or if the lag phase did not exist, T cell division would occur in a more proinflammatory microenvironment with more antigen, and the T cell response would be driven to a much higher peak. Given that T cells are capable of explosive proliferation, the lag phase imposed by the vitamin D–VDR 'prelude' may diminish the risk of unwanted immunopathology.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

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AUTHOR CONTRIBUTIONS

M.R.v.E. did most of the experiments, analyzed data and contributed to the writing of the manuscript; M.K. and P.S. contributed to the ketoconazole and mRNA experiments; K.O. contributed to the planning and analyses of studies involving patients; N.Ø. contributed to the design and analysis of some of the experiments; and C.G. conceptualized the research, directed the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cells and patients. Blood mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Nycomed). Naive T cells were subsequently isolated by negative selection with a Dynal Negative Isolation kit for naive T cells according to the manufacturer's instructions (Invitrogen). Primed T cells were generated by the culture of 1×10^6 mononuclear cells per ml in X-VIVO 15 serum-free medium (Lonza) supplemented with 1×10^3 units of recombinant human IL-2 per ml (Proleukin) and 2×10^5 Dynabeads Human T-activator CD3/CD28 beads per ml (Invitrogen) for up to 9 d at 37 °C in 5% CO_2. Beads were removed at least 24 h before the final experiments. Wild-type (E6-1) and PLC-γ1-deficient (J.gamma1)[36] Jurkat cell lines were from American Type Culture Collection. For analysis of the effect of chronic deficiency in $25(OH)D_3$ and $1,25(OH)_2D_3$ on T cell function, blood was obtained from five patients on chronic hemodialysis (permission approved by The Danish National Committee on Biomedical Research Ethics); they had been on dialysis between 6 and 18 months. Their chronic kidney disease was due to previous thrombotic thrombocytopenic purpura (one patient), chronic glomerulonephritis (two patients), hypertensive nephropathy (one patient) and nephropathy of unknown origin (one patient).

Reagents. Antibodies used included anti-CD3 (F101.01)[48]; anti-TCRζ (6B10.2; Santa Cruz Biotechnology); anti-Glut1 (provided by the University of Copenhagen); anti-PLC-γ1 (05-163) and anti-Lat (06-807; both from Upstate Biotechnology); antibody to PLC-γ1 phosphorylated at Tyr783 (44-696; Biosource); anti-Zap70 (99F2; 2705), antibody to Zap70 phosphorylated at Tyr319 (2701), antibody to Lat phosphorylated at Tyr171 (3581), anti-Erk1/2 (9102), antibody to Erk phosphorylated at T202 and Tyr204 (9101), anti-p38 (9212) and antibody to p38 phosphorylated at Thr180 and Tyr182 (9211; all from Cell Signaling Technology); and anti-CD4 (RM4-5), anti-CD8 (53-6.7) and anti-CD28 (L293; all from BD Pharmingen). Phycoerythrin-conjugated goat anti–mouse immunoglobulin G, horseradish peroxidase–conjugated swine anti–rabbit immunoglobulin and horseradish peroxidase–conjugated rabbit anti–mouse immunoglobulin were from Dako. Phycoerythrin-conjugated goat anti–mouse immunoglobulin G, horseradish peroxidase–conjugated swine anti–rabbit immunoglobulin and horseradish peroxidase–conjugated rabbit anti–mouse immunoglobulin were from Dako. The VDR antagonists ZK191784 and ZK203278 were provided by Bayer Schering Pharma AG; $25(OH)D_3$ and $1,25(OH)_2D_3$ were from BIOMOL International; and ketoconazole and SB203580 were from Sigma.

TCR downregulation, immunoblot analysis and calcium flux. Naive and primed T cells were washed twice, were adjusted to a density of 0.5 × 106 cells per ml X-VIVO 15 serum-free medium and were incubated at 37 °C with Dynabeads Human T-activator CD3/CD28 beads (Invitrogen). Cells were then stained with anti-CD3 (F101.01) plus phycoerythrin-conjugated goat anti–mouse immunoglobulin G and mean fluorescence intensity (MFI) was determined by flow cytometry (FACSCalibur; BD Biosciences). TCR downregulation was calculated as (MFI of treated cells / MFI of untreated cells) × 100. For immunoblot analysis, cells were stimulated at 37 °C with Dynabeads Human T-activator CD3/CD28 beads, then were lysed in lysis buffer (50 mM Tris base, pH 7.5, 150 mM NaCl and 1 mM Mg_2Cl) supplemented with 1% (vol/vol) Triton X-100, Pefabloc SC (1 mg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), EDTA (5 mM), NaF (10 mM) and Na_3VO_4 (1 mM) and were separated by electrophoresis through 10% polyacrylamide gels. For specific detection of proteins in the cytosol and the nucleus, reagents from the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit were used according to the manufacturer's instructions (Thermo Fisher Scientific). An equal number of cells per lane (1×10^6) was used for immunoblot analysis throughout the study whether naive or primed cells were studied. Proteins were transferred to nitrocellulose membranes (Amersham Biosciences) and were visualized with primary antibodies plus horseradish peroxidase–conjugated swine anti–rabbit immunoglobulin or horseradish peroxidase–conjugated rabbit anti–mouse immunoglobulin through the use of enhanced chemiluminescence technology (Amersham Biotechnology). For band intensity quantification, enhanced chemiluminescence–exposed films were scanned into a computer with an Arcus II scanner (Agfa) and the intensity of the bands was quantified with the program Quantity One (Bio-Rad). Induction of PLC-γ1 expression normalized to TCRζ (relative values) was calculated as follows: PLC-γ1 on day x / TCRζ on day x, with PLC-γ1 on day 0 / TCRζ on day 0 set as 1.

For calcium-flux analysis, T cells were loaded for 30 min at 37 °C with 5 μM Fura Red-AM and 2 μM Fluo-3-AM in serum-free medium with Pluronic F-127 (all from Molecular Probes). Cells were plated in aliquots of 1×10^6 cells per well in 96-well plates, washed twice in serum-free medium, labeled with anti-CD4 and anti-CD8 and analyzed by flow cytometry. Each sample was heated to 37 °C before analysis. Baseline measurements were achieved by running the sample without stimulation for 30 s. At 30 s, anti-CD3 (5 μg/ml; F101.01) plus anti-CD28 (400 ng/ml) was added. Data were collected for 512 s. At 465 s, ionomycin (500 ng/ml) was added as a calcium-flux control. Samples were maintained at 37 °C during the entire flow cytometry through the use of a coil-heated Falcon tube. FlowJo software (TreeStar) was used for ratiometric analysis.

RNA hybridization. Total RNA was isolated by phenol extraction (TriReagent; Molecular Research Center) and analyzed as described[49]. The PLC-γ1 probe was made from cloned PCR products with the sense primer 5′-GACGCAACCCTGGCTTCTATG-3′ and antisense primer 5′-GTGAGT CGGCAGCAACATCC-3′. The 28S probe was made by 5′ phosphorylation of an oligonucleotide complementary to 28S rRNA (5′-TCGCCGTTACTG AGGGAATCCTGGTTAGTTTCTTT-3′) with T4 polynucleotide kinase and [γ-^{32}P]ATP. Signals were detected and quantified on a phosphorimager (Bio-Rad). The PLC-γ1-hybridized membrane was stripped and rehybridized with a 28S rRNA oligonucleotide for normalization. The induction of PLC-γ1 mRNA was normalized to 28S rRNA expression and is presented relative to expression at day 0, set as 1. Because an equal amount of total RNA was loaded, each loading contained RNA from a varying number of cells. The induction of PLC-γ1 mRNA is presented per cell; thus, a correction factor for cell numbers (cell factor) was included in the calculation as follows: (PLC-γ1 on day x / 28S on day x) × cell factor.

T cell growth, division and proliferation index. T cell growth was determined by flow cytometry of forward and side scatter. Cell division was analyzed by labeling of cells with CFSE (carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Invitrogen) as described[50]. Proliferation index was calculated by division of the fraction of CFSE-labeled cells that had undergone four or more cell divisions by the fraction of CFSE-labeled cells that had not undergone cell division.

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