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Protein Kinase B/Akt Signals Impair Th17 Differentiation and Support Natural Regulatory T Cell Function and Induced Regulatory T Cell Formation

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Protein kinase B (PKB)/Akt signals control T cell proliferation and differentiation but their effect on the generation and function of regulatory T cells (Treg) and Th17 cells is not well understood. In this study, we show that elevated PKB signals antagonize the immunosuppressive effect of TGF-β1 on cell size, CD25 and CD98 expression, and proliferation of CD3-stimulated naïve CD4+ T cells from wild-type and CD28-deficient mice. Conventional CD4+ T cells expressing active PKB are less susceptible to suppression by natural regulatory T cells. Although PKB signals do not affect the development of natural regulatory T cells, they enhance their suppressor capacity. Upon TCR triggering and TGF-β1 costimulation, wild-type and CD28-deficient CD4+ T cells transgenic for PKB readily express Foxp3, thereby acquiring suppressor capacity. These effects of elevated PKB signals on T cell function involve a marked and sustained activation of STAT5 and Foxp3 and reduction in nuclear NFATc1 levels. In contrast, PKB signals impair TGF-β1/IL-6-mediated differentiation of naïve CD4+ T cells into the Th17 lineage. This correlates with an increased signaling of ERK, STAT5, and STAT6. Finally, elevated PKB signals reduced the severity of experimental autoimmune encephalomyelitis in wild-type mice but induced experimental autoimmune encephalomyelitis in mice deficient for CD28. Altogether, these data indicate an important role of PKB signals on control of TGF-β-mediated T cell responses and, thereby, on tolerizing and inflammatory immune processes. The Journal of Immunology, 2009, 183: 6124–6134.
homeostasis of peripheral Tregs (24–26). PI3Kp110αΔ/ΔΔ α knock-in mice expressing an inactive PI3Kδ isoform showed an enhanced generation of nTregs in thymus, but peripheral p110αΔ/ΔΔ α nTregs were less abundant (27). For peripheral human nTregs, a reduced capacity to activate PKB was described previously (28) and retroviral expression of active PKB in human Treg cell lines inhibited their suppressive capacity. Expression of phosphatase and tensin homologue (PTEN), the negative regulator of PI3K-activated PKB signaling, was found to be down-regulated after T cell activation, whereas relatively higher levels were detected in CD4⁺ CD25⁺ Tregs (15, 29). However, in mice bearing inactive PTEN alleles in the T cell compartment, nTregs developed normally and showed a normal suppressor function (30). More recently, the retroviral expression of an active version of PKB was reported to impair de novo Foxp3 expression and nTreg development in the thymus (31). Likewise, by applying pharmacological inhibitors, a reduced PI3K/PKB signaling enhanced the induction of Foxp3 in TCR-primed T cells (32) and the immunosuppressant rapamycin, which inhibits mTOR activity and is a downstream target of PKB signaling, fostered Foxp3 expression, and Treg expansion (33).

In view of the emerging developmental and functional plasticity of Th17 and Treg cells (34, 35) and that CD4⁺ T cells expressing a constitutively active version of PKBα, myristoylated PKB (myrPKB), show increased TCR responsiveness and are less susceptible to suppression by the immunosuppressant cyclosporine A (36–38), we studied the effect of myrPKB on TGF-β signaling and effector function, i.e., the generation and function of Tregs and Th17 cells. We demonstrate that elevated PKB signals reduce the susceptibility of conventional CD4⁺ T cells to nTreg-mediated suppression, enhance nTreg suppressor function, and replace the requirement for CD28 signals for induction of peripheral nTregs from CD4⁺ CD25⁻ cells. Furthermore, elevated PKB signals impair TGF-β/IL-6-mediated Th17 differentiation and alter the severity of EAE disease in wild-type (wt) and CD4⁺ CD25⁻ mice. Taken together, the results reveal an important function for PKB in balancing autoimmune and inflammatory immune responses.

Materials and Methods

Mice

Heterozygous myrPKB transgenic (PKB tg) mice (on C57/BL6 background) expressing constitutively active hulPKBα (37) and Foxp3-GFP knock-in (Foxp3-GFP) mice (39) were described previously. CD28-deficient (CD28⁻/⁻) mice were provided by Dr. F. Lüthner (University of Göttgen, Göttgen, Germany). Wild-type, PKB tg, CD28⁻/⁻, and PKB tg CD28⁻/⁻ mice were crossed with Foxp3-GFP mice, provided by Dr. A. Rudensky (University of Seattle, Seattle, WA), to obtain mice homozygous for Foxp3-GFP alleles. Mice used for experiments were 6–10 wk old and in case of EAE experiments 10–12 wk.

Abs, reagents, and intracellular staining

For cell stimulation, cell isolation, and flow cytometry were obtained from BD Bioscience: CD4 (GK1.5), CD25 (7D4), and CTLA4 (UC10-4B9); Cell Signaling: pERK (Thr202/Tyr204); eBioscience: IL-10:PE (JE55-16E3), IL-17A:PE (ebio 17B7), IFN-γ:FITC (XM11.2), IL-4:FITC (BV6D-24G2), and CD98:FITC (RL38); or Miltenyi Biotec: GITR:PE (D7A-1). For Foxp3 staining, an intracellular Foxp3 (IFK-16E5) staining kit from eBioscience was used as described in the manufacturer’s protocol. Recombinant human TGF-β1 was purchased from R&D Systems. Flow cytometric measurement of ERK activation, lymph node cells from wt and PKB tg mice were restimulated for 2 h at 4°C in RPMI 1640 medium; cells were activated with biotinylated CD3 (10 μg/ml) and CD28 (10 μg/ml) Abs and streptavidin (50 μg/ml; Jackson ImmunoResearch Laboratories/Dianova). At the indicated time points, cells were fixed and lyzed with the Fix Perm buffer from eBioscience and stained for Foxp3, phosphorylated ERK (pERK), and CD4. Expression of pERK in gated CD4⁺ Foxp3⁻ and CD4⁺ Foxp3⁺ cells was analyzed by flow cytometry using a FACS Calibur and CellQuest Pro software (BD Biosciences).

Proliferation assay

CD4⁺CD25⁻ T cells were isolated from pooled lymph nodes of several mice by negative selection using a mixture of biotinylated Abs: CD25 (PC61), NK1.1 (PK136), Ter-119, CD8a (53-6.7), I-A/ E-2(G9), CD45R/B220 (RA-6/BD2), CD11b (MI/70), and streptavidin magnetic beads (Miltenyi Biotec) according to the manufacturer’s protocol. Purity of CD4⁺CD25⁻ T cells was above 95%. CD4⁺ CD25⁻ T cells were cultured in triplicates on plates coated with CD3 Ab (145.2C11) or CD3 plus CD28 (37.51) Abs in concentrations as indicated in complete RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 1% penicillin/streptomycin. Proliferation was measured at 24 h of culture by [³H]thymidine incorporation (0.2 μCi/well; MP Biomedicals) for 16 h. For nTreg proliferation assays, CD4⁺CD25⁺ Foxp3⁻ and CD4⁺CD25⁺ Foxp3⁺ cells from wt and PKB tg Foxp3-GFP mice were sorted by FACS; 1 × 10⁴ cells were stimulated with plate-bound CD3/CD28 Abs (2 and 5 μg/ml) for 3 days in the presence or absence of IL-2 (100 ng/ml; Biochem) when [³H]thymidine incorporation was measured for 8 h. Data give the mean of triplicates ± SD. For cell cycle analysis, CD4⁺ CD25⁻ T cells were labeled with 2 μM CFSE (Invitrogen) in RPMI 1640 medium for 5 min at room temperature, washed twice with RPMI 1640/10% FCS and cultured in supplemented RPMI 1640/10% FCS medium. CFSE dilution was measured on day 3 by flow cytometry.

Suppression assay

CD4⁺CD25⁻ (T effector cells (Teffs)) and CD4⁺CD25⁺ (nTreg) cells from lymph node of wt or PKB tg mice were isolated with a Treg isolation kit from Miltenyi Biotec following the manufacturer’s instructions. The purity of isolated Tregs usually was above 90%. For suppression assays, 1 × 10⁵ Teffs were cocultured with CD4⁺CD25⁺ cells at the indicated ratio and 1 × 10⁵ irradiated (30 Gy) wt splenocytes in the presence of soluble CD3 Ab (2 μg/ml) for 3 days, when proliferation was determined by [³H]thymidine incorporation.

Treg and Th17 induction

CD4⁺CD25⁻ T cells from wt, PKB tg, CD28⁻/⁻, and PKB tg CD28⁻/⁻ mice were isolated to above 95% purity. Three × 10⁴ CD4⁺ CD25⁻ T cells were cultured on CD3 Ab (2 μg/ml)- or CD3/CD28 Ab (2 and 5 μg/ml)-coated plates with or without TGF-β1 (10 ng/ml). On day 3, cells were harvested and analyzed for the expression of Foxp3, CD25, and glucocorticoid-induced TNFR-related protein (GITR) by flow cytometry. For the induction of Th17 cells, CD4⁺ CD25⁻ cells were cultured with CD3/CD28 Abs and TGF-β1 (10 ng/ml) in the presence or absence of IL-6 (20 ng/ml; eBioscience). On day 3, cells were restimulated with PMA and ionomycin (100/800 ng/ml; Calbiochem) in the presence of brefeldin A (2 μg/ml; Calbiochem) for 4 h and analyzed for the expression of Foxp3, IL-17, IFN-γ, IL-4, or IL-10. For suppression assays with iTregs, CD4⁺CD25⁻ T cells from wt and PKB tg Foxp3-GFP mice were stimulated with CD3 Ab (2 μg/ml) or CD3/CD28 Abs (2 and 5 μg/ml) and TGF-β1 (10 ng/ml) for 3 days. Induced Foxp3⁺ wt and PKB tg cells were sorted by FACS and cocultured for 3 days with 1 × 10⁵ wt CD4⁺ CD25⁻ Teff cells at the indicated ratio, CD3 Ab (2 μg/ml) and 2 × 10⁶ irradiated splenocytes. Proliferation was determined by [³H]thymidine incorporation.

EAE induction

Wild-type, PKB tg, CD28⁻/⁻, and PKB tg CD28⁻/⁻ mice were immunized with 200 μg of myelin oligodendrocyte (MOG) peptide p35–55 (MOG₃₅–₅₅) peptide in CFA (Sigma-Aldrich) containing 800 μg of heat-killed Mycobacterium tuberculosis (Difco Laboratories). A volume of 50 μl was injected s.c. at four sites over the flanks; 200 ng of pertussis toxin (List Biological Laboratories) dissolved in 200 μl of PBS was injected i.p. on the day of immunization and 2 days later. Clinical signs of EAE were monitored daily and scored by the following criteria: 0, no signs of disease; 0.5, partial loss of tail tone; 1, limp tail; 1.5, limp tail and slight slowing of righting; 2, partial paresis of one hind limb and/or marked slowing of righting; 2.5, dragging of hind limb(s) without total paralysis; 3, complete paralysis of at least one hind limb; 4, severe forelimb weakness; and 5, moribund or dead (40). Animal experiments were approved by the local state authorities; mice with a clinical score above 2.5 were killed according to the animal statutes.

Western blot analysis

Five × 10⁶ CD4⁺CD25⁻ T cells were stimulated for the indicated time points on plates coated with CD3 Ab (3 μg/ml) or CD3/CD28 Abs (2 and 5 μg/ml) in the presence or absence of TGF-β1 (10 ng/ml) and IL-6 (20 ng/ml). Harvested cells were lysed as described previously (38) to obtain...
cytoplasmic and nuclear protein extracts. Protein concentration was determined by bicinchoninic acid reagent (Pierce) and 10–25 μg of protein lysate was run on 8–10% SDS-PAGE. After transfer to nitrocellulose membranes, expression of the indicated proteins was analyzed with the following primary Abs: pSmaa3/1 (Smad3 Ser423/425, Smad1 Ser463/465), pSmaa2 (Ser245/246), Smad4, pSTAT3 (Tyr 705), pSTAT1 (Tyr 701), pSTAT6 (Tyr 694), pERK (Thr202/Tyr204), pAkt (Ser473), panAkt (C67E7), pS6 (S240/244), pGSK3β (Tyr 308), Akt (AA.128-267; Santa Cruz Biotechnology). Primary Abs were detected with HRP-coupled goat anti-mouse, mouse anti-rabbit, or donkey anti-goat Abs (Jackson ImmunoResearch Laboratories/Dianova) and the ECL detection reagent (Amersham/GE Healthcare). 

Results

PKB signals abrogate TGF-β1-induced suppression of proliferation of naive CD4+ T cells

Whereas TGF-β, as a negative regulator of immune function, inhibits TCR-mediated signaling and proliferation, PKB signals enhance cell cycle progression, cytokine production, and cell growth. Due to this functional antagonism, we analyzed the effect of a constitutively active form of PKB (myrPKB) on TGF-β1-mediated suppression of proliferation of CD4+ T cells from wt and myrPKB tg (PKB tg) mice (37). Naive cells from those mice were activated with CD3 Ab in the presence of 0.1–10 ng TGF-β1 and their proliferation was measured after 24 h. Intriguingly, whereas wt T cells were inhibited by low doses of TGF-β1 (0.1–1.0 ng), CD3-induced proliferation of PKB tk T cells remained unaffected, even by high TGF-β1 doses (10 ng; Fig. 1A). This shows that PKB signals can abrogate the inhibitory proliferative signals generated by TGF-β1 after TCR ligation. Proliferation of wt T cells costimulated by CD3/CD28 Abs was refractory to TGF-β1 inhibition, similar to PKB tk T cells stimulated in the same manner (Fig. 1B). Analysis of cell cycle progression by CFSE dilution assays extended these results. Treatment with CD3 Ab for 3 days induced vigorous cell cycling and four cell divisions in wt and PKB tg T cells (Fig. 1C). In the presence of TGF-β1 only a small fraction (18%) of wt T cells cycled once, whereas cell division of PKB tk T cells was hardly affected. Again, division of wt cells costimulated by CD28 Abs was refractory to TGF-β1 inhibition and similar to PKB tk cells. Although having extensively cycled, wt and PKB tk cells showed a strong reduction in forward side scatter in the presence of TGF-β1 (Fig. 1C, left panels), suggesting that TGF-β1 signals lead to a down-regulation of cell metabolism affecting cell size. Whereas wt T cells treated with CD3 Ab plus TGF-β1 were extremely small, PKB tk cells were of moderate cell size, indicating that PKB signals, like CD28 costimulation in wt cells, maintain cell growth at a reduced level in the presence of TGF-β1. In this context, we analyzed the expression of CD25 and CD98, indicators for the extent of T cell activation and amino acid uptake (42, 43). Pronounced differences between wt and PUB tk cells became apparent at day 2 of culture (Fig. 1D). Cells treated with TGF-β1 showed markedly lower CD25 expression than untreated cells, but CD25 expression on CD3/CD28-costimulated cells was significantly higher than that on cells treated with CD3 Ab only. Under...
all stimulatory conditions, PKB tg cells showed higher CD25 expression, which was less affected by TGF-β1. Concerning CD98 expression, the highest levels were observed in CD3/CD28-costimulated T cells and expression was similar for wt and PKB tg cells treated either with CD3 or CD3 plus CD28 Abs. TGF-β1 led to a reduction in CD98 expression, but levels were found to be significantly higher in PKB tg cells. These data indicate that similar to CD28 costimulation, elevated PKB signals keep CD25 and CD98 expression at intermediate levels and override TGF-β1-mediated inhibition of cell division after stimulation with CD3 Ab.

Since CD28-deficient (CD28−/−) T cells are less responsive to TCR stimulation (44), we asked whether PKB signals can replace CD28 costimulatory signals and antagonize TGF-β1-mediated inhibition. To answer this question, CD4+ T cells from CD28−/− and PKB tg CD28−/− mice were stimulated with CD3 Ab plus TGF-β1 and DNA synthesis and cell cycle progression were measured at day 1 or day 3, respectively. As shown in Fig. 1E, proliferation of CD28−/− CD4+ T cells (measured at 24 h) was inhibited by low doses of TGF-β1 (0.1 ng), whereas PKB tg CD28−/− CD4+ T cells significantly proliferated even in the presence of 10 ng of TGF-β1. Likewise, analysis of cell division by CFSE dilution (Fig. 1F) revealed that in response to CD3 Ab stimulation PKB tg CD28−/− cells not only proliferated better than CD28−/− cells but, importantly, they showed a significant expansion and progression through three cell cycles in the presence of TGF-β1, comparable to CD28−/− cells stimulated with CD3 Ab. In contrast, cell division of CD28−/− cells was completely inhibited by TGF-β1/CD3 treatment. CD3/CD28 costimulation served as a control for CD28 deficiency and CFSE profiles as expected were identical to CD3-treated cells. These data indicate that PKB signals can alter the outcome of TGF-β1 signaling in the absence of CD28 signaling and, therefore, can replace missing CD28 signals.

Elevated PKB signals foster expansion of nTregs along with CD4+ T cells but cannot fully replace CD28 signals for the generation of nTregs

Since the generation and homeostasis of natural regulatory CD4+CD25+ T cells (nTregs) is supported by CD28 signaling (23, 24), the data presented above suggest that elevated PKB signals might support nTreg formation. Using mice bearing Foxp3-GFP alleles (3), we analyzed nTreg development in wt, PKB tg, as well as CD28−/− and PKB tg CD28−/− mice. As shown in Fig. 2A, the majority of CD25+ cells in wt and PKB tg mice were positive for Foxp3, a marker for murine Tregs (2–4). However, the percentage of CD25+Foxp3+ nTregs within the CD4+ population of thymus, lymph node, and spleen from wt, CD28−/−, and PKB tg CD28−/− Foxp3-GFP mice (n = 4 mice each); *, p < 0.05 and ***, p < 0.001.

PKB signals enhance the suppressive capacity of nTregs

To examine the effect of active PKB on nTreg function, we performed cocultures of conventional CD4+CD25− T cells and nTregs from wt or PKB tg mice in various combinations and ratios for the determination of proliferation by [3H]thymidine incorporation. Two remarkable effects of enhanced PKB signals on nTreg-mediated suppression were observed: 1) PKB tg nTregs inhibited the proliferation of wt effector cells more efficiently than wt nTregs (Fig. 3A, □ and 2) CD4+CD25− effector cells from PKB tg mice were more resistant to suppression than those from wt mice by both wt or PKB tg nTregs (Fig. 3A, cf □ with □). At ratios of 5:1 and 10:1 of effector to suppressor cells, PKB tg effector cells proliferated 1.5–4 times better than wt effector cells, indicating that the former are less inhibited by nTregs. In contrast, we did not observe a marked difference in suppression of PKB tg effector cells by either wt or tg nTregs. These experiments allow the conclusion that elevated PKB signals enhance 1) the suppressor function of...
tg mice were cocultured with wt or PKB tg CD4/H11001/CD4/H9004/CD4/H11569/CD4/H11001/corporation. Data are representative of two experiments.

Foxp3-GFP mice were stimulated with CD3/CD28 Ab. In the presence of irradiated splenic cells and stimulated to proliferation and enhances Treg function. Active PKB decreases the susceptibility to Treg suppression and enhances Treg formation.

Values from three experiments were determined by flow cytometry. Gray lines show staining with isotype control Ab. MFI values for pERK in the experiment shown were for wt cells: wt, 20 ± 2; PKB tg, 9 ± 3; *, p = 0.03.

nTregs and 2) the resistance of Teff cells against nTreg suppression.

Since one hallmark of Treg cells is their low responsiveness against antigenic stimuli in vitro (1), we asked whether this is altered in nTregs from PKB tg mice. Similar to wt nTregs, nTregs from PKB tg mice did not significantly respond to IL-2 or CD3/CD28 Ab stimulation alone, but upon addition of exogenous IL-2 to cultures stimulated by CD3/CD28 Abs, both nTreg populations proliferated robustly and equally well (Fig. 3B). The corresponding CD4/CD25+ cells reacted to CD3/CD28 Ab stimulation alone and IL-2 addition did not significantly enhance this effect. Hence, nTregs from PKB tg mice retain their hyporesponsiveness and dependence on CD3/CD28 plus IL-2 signals for their activation. To clarify to what extent tg PKB conveys downstream signals in nTregs, we analyzed the expression of the Treg markers GITR and CTLA4 and found similar expression of GITR for nTregs from wt and PKB tg mice (Fig. 3C), but levels of CTLA4 were reduced on PKB tg nTregs (mean fluorescence intensity [MFI] wt 75 ± 7.5 vs MFI PKB tg 40 ± 3.3), indicating specific effects of elevated PKB signals on the nTreg phenotype. Western blot analysis showed that nTregs from PKB tg mice express phosphorylated tg PKB and have higher levels of pGSK3β and pTSC2, but not of pS6, than wt nTregs (Fig. 3D). However, in comparison to the corresponding CD4/CD25− cells, expression and signaling of tg PKB was reduced, indicating that in nTregs tg PKB signaling was attenuated. Also, after CD3/CD28 Ab activation the levels of pERK were found to be increased in PKB tg nTregs compared with wt nTregs (Fig. 3E).

PKB signals enhance Foxp3 expression and iTreg formation

We investigated next whether TCR plus TGF-β1 signals could drive PKB tg T cells into Treg development in the absence of CD28 costimulatory signals. In these assays, we stimulated wt and PKB tg CD4/CD25− T cells with CD3 Ab plus TGF-β1 alone or along with CD28 Ab. When these cultures were analyzed for CD25 and Foxp3 expression on day 3, we observed 14% CD25 Foxp3+ cells in wt cultures but a substantial increase of CD25 Foxp3+ cells, 57%, in PKB tg cell cultures. After CD3 plus CD28 Ab stimulation in the presence of TGF-β1, 62% of wt and 50% of PKB tg cells were CD25 Foxp3+ (Fig. 4, A and B). This indicates that in concert with CD3 plus TGF-β1 signals PKB supports Foxp3 expression. Accordingly, after CD3 plus TGF-β1 stimulation, PKB tg CD25 Foxp3+ cells showed a higher expression of the Treg markers CD25 and GTR compared with wt cells but similar to cells costimulated with CD3/CD28 Abs (Fig. 4A), indicating a strong delay in differentiation toward Tregs in wt cells. The number of CD25 Foxp3+ cells recovered after 3 days in culture under CD3 plus TGF-β1 conditions was very low: compared with wt cells (0.7 × 105), an ~40-fold higher number of PKB tg cells (27.3 × 105) was found. After CD3 plus CD28 plus TGF-β1 treatment, cell numbers were 23.4 × 105 for wt and 37 × 105 for PKB tg cultures. Thus, although the percentage of Foxp3+ cells in CD3/CD28 costimulated PKB tg cultures was reduced by ~12%, cell numbers for Foxp3+ cells were above those from wt cultures.

To extend these results, we analyzed differentiation of CD4/CD25− T cells from CD28−/− and PKB tg CD28−/− mice under identical culture conditions (Fig. 4, A and B). The percentage and cell number of CD28−/− CD25 Foxp3+ cells induced by CD3 plus TGF-β1 was similar to those of wt cells. Strikingly, PKB signals enabled the efficient differentiation of CD28−/− CD4/CD25− T cells into the Treg lineage. These data show that PKB signals are as potent as CD28 costimulation in iTreg differentiation driven by CD3 plus TGF-β1 signals in vitro.
When we tested the suppressor activity of PKB tg Treg cells induced by CD3 plus TGF-β1, indeed the cells had acquired suppressor function: they inhibited the proliferation of cocultured wt CD4^+CD25^- cells as effectively as CD3/CD28 Ab-induced wt or PKB tg CD28^-/- cells (Fig. 4C). Thus, along with TCR/TGF-β1 triggering, elevated PKB signals are sufficient to induce a suppressive capacity in CD4^+CD25^- T cells and, therefore, are able to generate iTregs.

PKB signals sustain STAT5 signaling in naive CD4^+ T cells

To elucidate molecular mechanisms through which PKB signals might affect TGF-β1 signaling, we investigated whether the expression of Smad proteins is affected in PKB tg T cells. Surprisingly, we did not observe substantial differences in the levels of cytosolic and nuclear pSmad2, pSmad3, and Smad4 proteins (Fig. 5) between wt and PKB tg T cells upon CD3/CD4^-/- TGF-β1 treatment, suggesting that PKB signals may act downstream of Smad activation or through other signaling pathways.

In these studies, we made the interesting observation that CD3 Ab stimulation alone led to phosphorylation of Smad2 and nuclear Smad4, whereas activation and nuclear localization of pSmad3 was strictly coupled to TGF-β1 signals, suggesting that Smad2 and Smad4 exert important functions in T cell activation. Furthermore, the Western blot assays confirmed that, in concert with TGF-β1 and CD3-only signals, elevated PKB signals foster the induction of Foxp3: at 40 h of stimulation by CD3 Ab plus TGF-β1, a strong expression of Foxp3 was detectable in PKB tg but not wt T cells (see Fig. 5).

Since Foxp3 induction was reported to be regulated by Smad3, NFAT, and STAT5 (45), we also investigated whether CD3 plus elevated PKB signals would affect the expression of these transcription factors. Whereas TGF-β1 treatment of wt T cells stimulated with CD3 Ab only inhibited the nuclear accumulation of pSTAT5, PKB tg cells showed a pronounced expression of both cytoplasmic and nuclear pSTAT5, with levels above those seen after CD3 stimulation alone. Similarly, TGF-β1, in relation to CD3 Ab stimulation, strongly diminished nuclear NFATc1 levels in wt cells, but to a lower degree in PKB tg cells, which overall show a reduced NFATc1 expression (Fig. 5A) (38). These data show that PKB plus TGFβ1 signals enhance STAT5 and reduce NFATc1 activation.
PKB/Akt INFLUENCES Th17 AND Treg PHYSIOLOGY

Because active PKB could replace CD28 signals in proliferation of CD4+ T cells (Fig. 1), we analyzed the expression of transcription factors in CD28−/− and PKB tg CD28−/− CD4+CD25− T cells. Again, compared with CD28−/− T cells and for the analyzed time points until 40 h, enhanced PKB signals in concert with TGF-β1 signals and CD3 Ab stimulation only supported STAT5 phosphorylation and Foxp3 induction (Fig. 5B). This indicates that the activation of STAT5 and Foxp3, i.e., of two signaling molecules critical in the development of Treg cells, is inhibited or inefficient after CD3 plus TGF-β1 stimulation of wt and CD28−/− CD4+ T cells but can be compensated or strongly enhanced by elevated PKB signaling.

PKB signals impair Th17 differentiation

Since elevated PKB signals enhance Foxp3 expression, we asked whether PKB signals affect the production of IL-17 and the development of Th17 cells. When conventional CD4+ T cells induced by CD3/CD28 Abs were cultured with TGF-β1 plus IL-6 for 3 days, IL-17 was produced in ~13% of wt cells but surprisingly only in ~3–4% of PKB tg cells. Conversely, in TGF-β1 plus IL-6 cultures, Foxp3 expression was found in 30% of PKB tg and in 12% of wt cells (Fig. 6A). In cell number, this translated into half the number of Th17 cells and 4.5 times more CD25+ Foxp3+ cells in PKB tg vs wt cultures. Without IL-6 neither of the two induced cell types produced IL-17, instead both differentiated into Foxp3+ cells. Therefore, elevated PKB signals impair the formation of Th17 cells and foster differentiation into Foxp3+ cells. Whether elevated PKB signals also affect the production of other cytokines was analyzed by intracellular staining for IFN-γ, IL-4, and IL-10 (Fig. 6B). A low percentage of wt cells produced IFN-γ (1.1%), IL-4 (1.5%), or IL-10 (3.8%). For PKB tg cells, the percentage of these cytokine-producing cells was increased by 2-fold and cell numbers 2- to 4-fold. Thus, the generation of cells producing IFN-γ, IL-10, or IL-4 was not markedly affected by PKB signals, but led to a slight increase in cell number.

To get insight into the signaling events involved in the impaired Th17 differentiation of PKB tg T cells, we analyzed the expression of RORγt and IRF4, which control Th17 differentiation (46, 47), as well as the activation of STAT factors and ERK kinases under conditions which induce either Th17 (CD3/CD28/TGF-β1 plus IL-6) or Treg (CD3/CD28/TGF-β1) differentiation (Fig. 6C). IL-6 enhanced nuclear expression of pSTAT3 and RORγt but not of IRF4. Notably, expression of RORγt was similar and expression of IRF4 was even slightly enhanced in PKB tg cells, although they showed a reduced IL-17 production. In IL-6-treated cultures, wt cells showed the highest nuclear pSTAT3 at day 1 and declining levels until day 3, whereas in PKB tg cells pSTAT3 was strongest at day 3. Such altered kinetics between wt and PKB tg cells were also observed for pSTAT1. Under conditions supporting Th17 cell differentiation, nuclear pSTAT5 levels were elevated at day 3 in PKB tg cells. A striking change in expression was detected for pSTAT6, which was up-regulated in PKB tg cultures under Th17 conditions on days 2 and 3. In addition, we repeatedly detected pERK in wt cells under Treg but not under Th17 differentiation conditions. In contrast, in PKB tg cells, pERK was detectable under both culture conditions. From three experiments we concluded that under Th17 conditions (on day 3), PKB tg cells showed enhanced expression of pSTAT3, pSTAT1, pSTAT5, pSTAT6, and pERK but overall similar expression of RORγt and IRF4. Thus, the impaired IL-17 production of PKB tg cells is not correlated to a reduction in nuclear RORγt or IRF4 levels, but accompanied with an increased phosphorylation of ERK kinases and STAT factors.

PKB signals influence the severity of EAE in wt and CD28−/− mice

Considering the effects of PKB on iTreg vs Th17 differentiation in vitro, we investigated whether PKB signals influence the development of EAE, a mouse model for human multiple sclerosis, in which Th17 cells play a vital role for disease initiation and progression (48). Therefore, we immunized wt and PKB tg mice with MOG35–55 peptide in CFA, and the development of EAE was scored until day 34 (40). PKB tg mice developed disease symptoms with a similar onset as wt mice (days 8–9), but, interestingly, the severity of EAE was markedly milder as shown in Fig. 7A. Since PKB tg CD28−/− T cells responded more strongly to TCR signals than CD28−/− T cells and expanded in the presence of TGF-β1 (Fig. 1, E and F), we also analyzed EAE in PKB tg CD28−/− mice. Although CD28−/− mice did not show overt clinical signs of neurodegeneration (49), importantly, PKB tg CD28−/− mice developed severe EAE symptoms, albeit their clinical scores were lower than in wt and similar to those in PKB tg mice.
Thus, elevated PKB signals influence the outcome of EAE, leading to an amelioration or exacerbation of disease.

**Discussion**

We show in this study that elevated PKB signals have a profound impact on the capacity of TGF-β1 to modulate the differentiation and function of peripheral Treg and Th17 effector cells. Depending on the presence of costimulatory signals and cytokines, TGF-β1 is able to promote the differentiation of conventional CD4⁺ T cells either to Tregs or Th17 cells. Elevated PKB signaling exerted two notable effects on this lineage antagonism: 1) It promoted the induction of the Treg marker Foxp3 and 2) impaired the IL-6/TGF-β1-mediated generation of Th17 cells. By supporting Foxp3 induction and the proliferative capacity, PKB signals conferred a suppressor function to conventional T cells from wt and CD28⁻/⁻ mice stimulated by TGF-β1 and CD3 Ab only and, thereby, converted those cells to iTregs. This indicates that TCR plus PKB plus TGF-β1 signals are sufficient to drive CD4⁺CD25⁺ T cells into the Treg lineage. Thus, PKB signaling can replace CD28 signals, which otherwise are required for the de novo differentiation of iTregs from naive T cells (9, 50). Therefore, we assume that the increase in number of peripheral nTregs in PKB tg and PKB tg CD28⁻/⁻ mice can arise by two mechanisms, i.e., by a PKB-mediated promotion of homeostatic expansion/survival of nTregs or by facilitating iTreg induction.

The second remarkable feature of elevated PKB signaling on TGF-β1-mediated lineage decision is that it strongly reduced TGF-β1/IL-6-mediated differentiation of CD4⁺ T cells into the Th17 lineage and supported the generation of Foxp3⁺ cells. Mechanisms of PKB-mediated inhibition of IL-17 production could involve direct or indirect mechanisms. Cytokines like IFN-γ, IL-4 (14, 51), and metabolites like retinoic acid (52) oppose Th17 differentiation, whereas IRF4 or RORγt reinforce the differentiation and function of Th17 cells (53). Also, in several reports, an important role for IL-2 in enhancing Foxp3 and restraining Th17 production has been demonstrated (54, 55). Mice expressing a constitutively active STAT5 transgene have increased nTreg numbers in the thymus and periphery (56, 57) and STAT5, STAT3, Smad3, and...
and NFAT are key signals in Foxp3 expression (58–50). Therefore, induction of Smad proteins, NFAT, and STAT5 at appropriate levels and over a sustained time period, as resulting from enhanced PKB signaling in concert with CD3 plus TGF-β1 signals, seems to be critical for the maintenance of Foxp3 expression and differentiation into Tregs. Interestingly, IRF4 or RORγt expression were similar in CD3/CD28 plus TGF-β1 stimulated wt and PKB tg cells cultured in the presence of IL-6, i.e., under Th17-inducing conditions, but nuclear pSTAT5 and pSTAT3 and pSTAT1 (at day 3) were enhanced in PKB tg cells, which would contribute to promoting differentiation into the PKB lineage in PKB tg cells (61, 62). Furthermore, we show that in contrast to Th17 differentiation iTreg formation involves stronger ERK signaling and that PKB signals maintain strong nuclear pSTAT6. Thus, enhanced pERK and pSTAT6 signals detected in PKB tg cells under Th17 culture conditions may impose additional limitations on IL-17 and instead support iTreg formation.

Although elevated PKB signals markedly affected the Treg/Th17 lineage decision, they appeared to be dispensable for the development of nTregs: tg PKB signals did neither influence the generation of nTregs in wt mice nor were they sufficient to fully compensate for CD28 deficiency. This shows that CD28 signals are required for optimal development of nTregs in thymus. T cell-specific deletion of the phosphatase Pten, which enhances PI3K/PKB signaling, did not affect thymic development of CD4+ Foxp3+ Tregs either (30), supporting our finding that elevated PKB signals leave nTreg development unaffected. However, whereas peripheral nTregs deficient for Pten expanded by IL-2 alone, myrPKB tg nTregs did not expand in response to IL-2 and proliferated as well as wt nTregs upon CD3/CD28 plus IL-2 stimulation. Reduced numbers of peripheral nTregs, with an enhanced response to IL-2 but a compromised suppressor activity, were found in Pten−/− kinase inactive mice (27), suggesting that signaling events downstream of PI3K require for establishing an effective suppressor capacity in nTregs. In this line, we found that nTregs from PKB tg mice are not compromised in suppressor activity but showed an enhanced suppressive capacity. This may involve altered TCR sensitivity and/or enhanced activation of various TCR downstream signaling molecules as we described previously for PKB tg CD4+ T cells (37, 38). Although the precise mechanisms underlying the elevated suppressive capacity of PKB signals in nTregs are presently unclear, our data indicate a specific role for PKB in enhancing Treg-mediated suppression.

Some of our results appear to be in conflict with reports published recently. Upon retroviral transfer of myrPKB or using pharmacological inhibitors, it has been reported that elevated PKB signals inhibit thymic development and function of nTregs (28, 31, 32). It seems difficult to reconcile these results, but they might arise from differences in the level and timing of PKB expression and the experimental systems used. Since we showed previously that myrPKB signals affected thymic selection processes (37, 63), differences in PKB activity in the two model systems investigated could drive double-positive thymocytes with high-affinity TCRs either into apoptosis or nTreg differentiation. Haxhinasto et al. (31) showed that inhibition of Foxp3 by retrovirally transduced myrPKB was most pronounced early after T cell activation. PKB tg CD4+ T cells may have passed or have lower PKB signals at that critical time window of possible inhibition. In this line, we find that resting nTregs from PKB tg mice have reduced levels of CTLA4 and show a reduced PKB signaling compared with PKB tg conventional T cells. Therefore, compensatory mechanisms and down-regulation of PKB activity to thresholds appropriate for nTreg development seem to occur in PKB tg cells. Indeed, limited PKB signaling in PKB tg nTregs might be the reason that they retain IL-2 dependence for proliferation and show enhanced suppressor activity. Interestingly, Delgoffe et al. (64) recently showed that T cells deficient in the GTPase Rheb retained nTORC2 signaling and differentiated into Tregs when stimulated in the presence of TGF-β despite increased phosphorylation of PKB. Therefore, it is conceivable that PKB signals exert differential effects, either enhancing or inhibiting Treg formation, depending on the timing and strength of TCR, PKB, and TGF-β signals induced during T cell activation.

Another major finding of the present study is that conventional T cells with elevated PKB activity are less susceptible to inhibition by TGF-β1 with regard to proliferation as well as suppression by wt and PKB tg nTregs, which inhibited conventional wt T cells more strongly than wt nTregs. This emphasizes that the activation status of PKB in conventional T cells is a primary determinant of T cell sensitivity to Treg-mediated suppression and that elevated PKB signals confer resistance within a range of suppressive capacity. The resistance of conventional T cells to Treg-mediated suppression is considered as a mechanism that plays a significant role in autoimmune development (65). For instance, mice deficient for the E3 ubiquitin ligases Cbl-b or TNFR-associated factor 6 develop autoimmunity, although the number or function of Tregs remained unaffected, but conventional T cells are resistant to Treg-mediated suppression in vitro. Furthermore, conventional T cells from NFATc2 and NFATc3 double-deficient mice (66), T cells deficient in SHIP (67), or cultured in the presence of IL-4 (68) are less inhibited by nTregs. The resistance of PKB tg cells stimulated with CD3 Ab only to TGF-β1-mediated inhibition of proliferation was linked to the maintenance of STAT5 signaling. The underlying mechanisms for this are presently unresolved, but since STAT5 activation is critically dependent on the phosphorylation of three key tyrosines within the IL-2Rβ chain (56, 57, 69), the activation of JAK kinases and negative regulators of the suppressor of cytokine signaling family (70), it is possible that PKB signals might modulate these events.

Finally, PKB tg mice showed an amelioration of EAE disease, which could correlate, among other factors, with the inhibitory effects of elevated PKB signals on the differentiation of Th17 cells in vivo. Otherwise, in the case of CD28−/− mice, elevated PKB signals led to the induction of EAE, suggesting that elevated PKB signals override missing CD28 costimulation. Since induction of EAE in CD28−/− mice was observed in various models (71–74), elevated PKB signals might result in enhanced activation, expansion, or survival of autoreactive T cells and an overall shift of the Th17:Treg ratio toward Th17 cells. Thus, PKB signals can exert beneficial effects on immune tolerance but also abrogate tolerogenic mechanisms and, therefore, enhance autoimmune diseases, uncovering a so far unrecognized role of PKB in influencing the balance between immune activation and suppression that is responsible for maintaining immune homeostasis.

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