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The tryptophan metabolite picolinic acid suppresses proliferation and metabolic activity of CD4$^+$ T cells and inhibits c-Myc activation

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ABSTRACT

Tryptophan metabolites, including kynurenine, 3-hydroxyanthranilic acid, and picolinic acid, are key mediators of immunosuppression by cells expressing the tryptophan-catabolizing enzyme indoleamine2,3-dioxygenase. In this study, we assessed the influence of picolinic acid on cell viability and effector functions of CD4$^+$ T cells following in vitro activation with agonistic anti-CD3/anti-CD28 antibodies. In contrast to kynurenine and 3-hydroxyanthranilic acid, exposure of T cells with picolinic acid did not affect cell viability, whereas proliferation and metabolic activity were suppressed in a dose-dependent manner. On the other hand, cytokine secretion and up-regulation of cell surface activation markers were not or only weakly inhibited by picolinic acid. Picolinic acid exposure induced a state of deep anergy that could not be overcome by the addition of exogenous IL-2 and inhibited Th cell polarization. On the molecular level, important upstream signaling molecules, such as the MAPKs ERK and p38 and the mammalian target of rapamycin target protein S6 ribosomal protein, were not affected by picolinic acid. Likewise, NFAT, NF-κB, and AP-1 promoter activity in Jurkat T cells was not influenced by exposure to picolinic acid. Whereas transcriptional levels of v-myc avian myelocytomatosis viral oncogene homolog were not affected by picolinic acid, phosphorylation at Ser62 was strongly reduced in picolinic acid-exposed T cells following activation. In conclusion, picolinic acid mediates a unique immunosuppressive program in T cells, mainly inhibiting cell cycle and metabolic activity, while leaving other effector functions intact. These functional features are accompanied by reduced phosphorylation of v-myc avian myelocytomatosis viral oncogene homolog. It remains to be determined whether this effect is mediated by direct inhibition of ERK activity or whether indirect mechanisms apply. *J. Leukoc. Biol.* 99: 583–594; 2016.

Introduction

Multiple regulatory mechanisms govern the onset, magnitude, quality, and termination of adaptive T cell responses. DCs are important regulators of T cell responses and can promote both activating and suppressive functions [1]. A key mechanism in the down-regulation of T cell responses by DCs is the up-regulation of the enzyme IDO, which acts as the rate-limiting enzyme in the catabolism of the essential amino acid tryptophan [2]. Up-regulation of IDO in DCs generates an immunosuppressive milieu, which is involved in crucial processes of immune tolerance. These include maternal tolerance toward the fetus during pregnancy [3] but also tumor-induced immunosuppression [4–6]. DCs up-regulate IDO expression in response to various stimuli, including proinflammatory signals, such as IFN-γ [7], but also backward signaling via the CD80/CD86 molecules [8]. Thus, IDO expression may serve as a feedback mechanism to down-regulate ongoing immune responses but may also generate a suppressive milieu before potentially harmful T cell responses can arise. Upon expression, IDO catalyzes tryptophan catabolism, leading to lowered levels of tryptophan and accumulation of downstream metabolites, such as KYN, 3-OH-AA, QA, and PA [9].

Activation of CD4$^+$ T cells induces a multitude of functional effects, all of which require major changes within the cellular machinery. This includes the initiation of the cell cycle, a metabolic switch from fatty acid β-oxidation to glycolysis and glutaminolysis [10], the up-regulation of surface proteins, and the production and secretion of cytokines. These processes are

Abbreviations: 3-OH-AA = 3-hydroxyanthranilic acid, c-Myc = v-myc avian myelocytomatosis viral oncogene homolog, CD = cluster of differentiation, CPD = Cell Proliferation Dyes, DC = dendritic cell, DI = division index, ECAR = extracellular acidification rate, eGFP = enhanced GFP, FCCP = carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, IDO = indoleamine 2,3-dioxygenase, KYN = kynurenine, MFI = mean fluorescence intensity,

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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governed by intracellular signaling pathways, transmitting signals from the extracellular immunologic milieu to the nucleus. These signaling pathways also provide potential targets for endogenous immunomodulatory agents, as well immunosuppressive drugs. Interestingly, blockade of certain signaling pathways, such as the mTOR pathway [11, 12] and the calmodulin-calcineurin-NFAT pathway [13–15], is sufficient to abrogate completely the full scope of the T cell response, suggesting nonredundant roles.

The cellular c-Myc transcription factor is a central regulator of proliferation and cellular metabolism [16, 17]. Its activation is regulated at multiple levels, including its transcription rate [17], as well as phosphorylation at Ser62 by ERK signaling. Following phosphorylation, c-Myc can associate with the cofactor Max, thus forming an active transcription complex. On the other hand, phosphorylation at Ser62 also licenses c-Myc to be phosphorylated at Thr58 by glycogen synthetase kinase 3, which subsequently leads to its degradation [18]. So far, the effects of c-Myc on cell cycle control and metabolism have been characterized extensively in malignant cells [19]. It is only recently that first studies have addressed its role in T cell activation [20]; a comprehensive evaluation is yet to be attempted.

Likewise, the exact functional and molecular consequences of IDO-catalyzed tryptophan depletion and metabolite accumulation have been defined only incompletely. Low tryptophan concentrations lead to a surplus of uncharged tryptophanyl tRNAs, which activate the general control nonderepressible 2 kinase, a stress response program in murine T cells [21, 22]. At the same time, a lack of essential amino acids inhibits signaling via the mTOR pathway [23, 24]. The summative effect of these signaling modifications of tryptophan depletion is the suppression of T cell activation. The effects of tryptophan metabolite accumulation have been assessed in 2 seminal studies, which both show that downstream metabolites, such as KYN and AA but not QA, inhibit T cell activation [25, 26]. This effect was mainly attributed to the induction of apoptosis in T cells, which was further substantiated later in various in vitro and in vivo models [27–29]. Interestingly, the effects of PA on $CD4^+$ T cells have not been fully investigated. In one study from the 1980s, the metal chelating properties of PA were used to study regulation of iron metabolism in activated T cells [30]. Furthermore, the functional effects of PA on $CD4^+$ T cells are mentioned briefly in the study by Frumento et al. [25], although more detailed effects were only assessed for the other tryptophan metabolites.

As the full understanding of IDO-mediated effects on T cells requires knowledge about the contribution of all tryptophan metabolites, we assessed the effects of PA on purified human $CD4^+$ T cells. For that purpose, a broad range of T cell effector functions, including proliferation, oxidative and anaerobic metabolism, cytokine secretion, and restimulation capacity, was measured in PA-exposed T cells following anti-CD3/anti-CD28 stimulation. Furthermore, the effects of PA exposure on intracellular signaling pathways were assessed by use of reporter cell lines, intracellular flow cytometry, and quantitative PCR.

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mTOR = mammalian target of rapamycin, OCR = oxygen consumption rate, p = phosphorylated, PA = picolinic acid, PKC = protein kinase C, QA = quinolinic acid, S6RP = S6 ribosomal protein

MATERIALS AND METHODS

Cell isolation and culture

Blood was taken from healthy volunteers with their written, informed consent in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of the Medical University of Vienna (EK2012/1724). PBMCs were isolated by standard density centrifugation by use of the Lymphocyte Separation Medium from Life Technologies (Carlsbad, CA, USA). $CD4^+$ T cells were isolated by use of the Human $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Jurkat reporter cell lines [31], as well as BW 5147 T cell stimulator cells [32], were maintained as described. All experiments were performed in IMDM, supplemented with 10% FCS and 15 μg/ml gentamycin sulfate (all Life Technologies).

Cell viability assay

$CD4^+$ T cells (1×10^6) were exposed to the indicated concentrations of KYN, 3-OH-AA, and PA (all Sigma-Aldrich, St. Louis, MO, USA) in 24-well plates for 60 min, followed by activation by use of 5×10^5 anti-CD3/anti-CD28-coated microbeads. After 72 h, cells were harvested and washed in Annexin staining buffer (1 mM HEPES, 140 mM NaCl, and 25 μM calcium chloride, pH 7.4; all Sigma-Aldrich). After addition of 5 μl Annexin V FITC (eBioscience, San Diego, CA, USA), cells were incubated at room temperature for 10 min. After the cells were washed in Annexin staining buffer, propidium iodide (50 ng/ml; Sigma-Aldrich) was added, and fluorescence was determined on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Annexin V-negative/propidium iodide-negative cells were identified as viable cells.

Proliferation assays

eFluor670 CPD (1×10^6; 1.5 μM; eBioscience) -labeled $CD4^+$ T cells were activated with 5×10^5 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the indicated concentrations of PA in 24-well plates. After 96 h, cells were harvested and washed in PBS, and CPD dilution was determined by flow cytometry. For comparison of proliferation data from CPD-labeled effector T cells, a DI was calculated, according to the following formula: [log(MFI unstimulated cells)/log(MFI stimulated cells)]/log(2) [33]. For time-course analyses, 1×10^5 $CD4^+$ T cells were activated with 5×10^4 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the indicated concentrations of PA in 96-well, flat-bottom plates in triplicates. After 6, 30, 54, and 78 h, 1 μCI methyl[^{3}H] thymidine/well was added for an additional 18 h, and thymidine incorporation was measured on a PerkinElmer scintillation counter (PerkinElmer, Waltham, MA, USA), as described [34]. Some experiments were conducted in tryptophan-free IMDM (Life Technologies), supplemented with 10% dialyzed FCS. For control experiments, 76 μM tryptophan (Sigma-Aldrich) was added, which amounts to the tryptophan concentration in the original IMDM formulation. Furthermore, in some experiments, ferric chloride hexahydrate (Sigma-Aldrich) was added at the indicated concentrations, and proliferation was measured after 96 h, as described above.

For restimulation assays, 1×10^6 $CD4^+$ T cells were activated with 5×10^5 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the indicated concentrations of PA in 24-well plates. After 7 d, cells were harvested and washed 3 times in PA-free IMDM, and the number of viable cells was determined on a Sysmex CA5100 hematocytometer (Sysmex, Kobe, Japan). Subsequently, 1×10^5 $CD4^+$ T cells from each condition were activated with 5×10^4 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the human rIL-2 (100 U/ml; PeproTech, London, United Kingdom) in 96-well, flat-bottom plates in triplicates, and thymidine incorporation was measured as above.

Bioenergetic assays

Analysis of OCRs and ECAR and calculation of metabolic parameters were performed by use of the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA), as reported previously [35, 36]. In brief, T cells were activated in 6-well plates in the absence or presence of the indicated concentrations of PA. After 2 d, cells were harvested, washed once in PBS, and seeded into XF 24-well cell culture microplates (1×10^6/well) precoated with Cell-Tak (Seahorse Bioscience) for 2 h. A final volume of 600 μl buffer-free assay medium (Seahorse Bioscience) was added to each well before the experimental protocol. Cells were then transferred to a CO_2-free incubator and maintained at 37°C for 1 h before the start of the assay. The measurement protocol consisted of 2 min mixture, 2 min wait, and 4 min OCR/ECAR measurement times. For the mitochondrial stress test, ATP synthase was inhibited by injection of 1 μM oligomycin, followed by 2 μM FCCP-induced mitochondrial uncoupling to determine the spare/maximal respiratory capacity. Nonmitochondrial respiration was determined after rotenone/antimycin A injection (1 μM each).

Determination of cytokine secretion

$CD4^+$ T cells (1×10^6) were activated with 5×10^5 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the indicated concentrations of PA in 24-well plates. After 24 and 72 h, cell-free supernatant was removed from the cultures, and concentrations of IL-2 (after 24 h) and IL-4, IL-10, IL-13, IL-17, and IFN-γ were determined by multiplex analysis (Luminex 100IS; Biomedica, Vienna, Austria). Values were normalized for cell number at the day of supernatant collection.

Quantitative PCR

For relative quantification of cytokine RNA production, T cells were activated in the presence or absence of the indicated concentrations of PA as above. After 24 h, RNA was isolated by use of a QIAcube RNA isolation station (Qiagen, Hilden, Germany), and cDNA was generated by random hexamer-primed reverse transcription. Relative transcriptional levels of IL-4, IL-10, IFN-γ, IL-13, IL-17, and c-Myc were quantified by use of the iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Transcriptional levels of the Abl kinase, which shows robust expression in T cells and is not modulated following T cell activation [37], were used as reference. The following primers were used: IL-4 forward 5′-ACTTTGAACA-GCCTCACAGAG-3′, IL-4 reverse 5′-TTGGAGGCAGCAAAGATGTC-3′; IL-10 forward 5′-GCCTAACATGCTTCGAGATC-3′, IL-10 reverse 5′-TGAT-GTCTGGGTCTTGGTTC-3′; IL-13 forward 5′-TGAGGAGCTGGTCA-ACATCA-3′, IL-13 reverse 5′-CAGGTTGATGCTCCATACCAT-3′; IL-17 forward 5′-TCAACCCGATTGTCCACCAT-3′, IL-17 reverse 5′-GAGTTTAGTCCG-AAATGAGGCTG-3′; IFN-γ forward 5′-CTAATTATTCGGTAACTGACTTGA-3′, IFN-γ reverse 5′-ACAGTTCAGCCATCACTTGGA-3′; c-Myc forward 5′-CTGGT-GCTCCATGAGGAGA-3′, c-Myc reverse 5′-CCTGCCTCTTTTCCACAGAA-3′; Abl forward 5′-TGTATGATTTTGTGGCCAGTGGAG-3′, Abl reverse 5′-GCCTAAGACCCGGAGCTTTTCA-3′.

Determination of activation marker up-regulation

$CD4^+$ T cells (1×10^6) were activated with 5×10^5 anti-CD3/anti-CD28-coated microbeads in the presence or absence of the indicated concentrations of PA in 24-well plates. After 24 h, cells were washed in PBS + 0.5% BSA + 0.05% NaN_3 and stained by use of the following antibodies (all eBioscience): anti-human CD25 allophycocyanin-eFluor780 (clone BC96), anti-human CD69 eFluor450 (clone FN50), anti-human CD71 PE (clone OKT9), and anti-human CD98hc FITC (clone 5E5). Surface expression was then quantified on a BD Biosciences FACSCanto II flow cytometer.

Determination of Th cell subset polarization

FACS-sorted $CD4^+CD45RO^-$ T cells (1×10^6) were activated in the presence or absence of the indicated concentrations of PA with 5×10^5 anti-CD3/anti-CD28-coated microbeads. For Th1 polarization, cultures were supplemented with human rIL-2 (100 U/ml; PeproTech) and human rIL-12 (50 ng/ml); for Th2 polarization, cultures were supplemented with human rIL-4 (50 ng/ml), anti-human IFN-γ (10 μg/ml; rabbit polyclonal), and anti-human IL-12 (10 μg/ml; goat polyclonal; all PeproTech). After 7 d, cells were harvested, and intracellular expression of human T-bet and GATA-3 were measured by intracellular flow cytometry by use of the True Nuclear Transcription Factor Buffer Set (BioLegend, San Diego, CA, USA). In short, cells were fixed for 30 min in the fixation buffer and then washed twice in permeabilization buffer. Cells were then incubated with anti-human GATA-3 allophycocyanin (clone 16E10A23) and anti-human T-bet Brilliant Violet 421 (clone 4B10; all BioLegend) for 60 min. Expression of these transcription factors was then quantified on a BD Biosciences FACSCanto II flow cytometer.

Determination of intracellular signaling

$CD4^+$ T cells (1×10^6) were activated with PMA (100 nM; Sigma-Aldrich) for 30 min or with 5×10^5 anti-CD3/anti-CD28-coated microbeads for 24 h in the presence or absence of the indicated concentrations of PA, rapamycin (100 nM; Sigma-Aldrich), cyclosporine A (1 μM; Sigma-Aldrich), or GÖ6983 (10 μM; Sigma-Aldrich). For staining of c-Myc phosphorylation at Ser62 and total c-Myc expression levels, cells were fixated in 80% methanol for 5 min and then permeabilized in PBS + 0.1% Tween for 20 min. Nonspecific protein–protein interactions were then blocked by incubation in PBS + 10% FCS + 0.3 M glycine for 15 min, followed by addition of an anti-c-Myc p-Ser62-specific antibody (1 μg; clone 33A12E10; abcam, Cambridge, United Kingdom) or a total c-Myc-specific antibody (clone 9E10; abcam) for 30 min at room temperature. Binding was visualized by adding a goat anti-mouse Fcγ-specific antibody for 30 min at room temperature.

For staining of p-ERK, p-p38, and p-S6RP, cells were harvested and fixated for 10 min in Fixation Buffer I (BD Phosflow; BD Biosciences) at 37°C. After the cells were washed in PBS + 0.5% BSA + 0.05% NaN_3, they were resuspended in prechilled (−20°C) Permeabilization Buffer III (BD Phosflow; BD Biosciences), kept on ice for 30 min, and then washed twice in PBS + 0.5% BSA + 0.05% NaN_3. The cells were then incubated with 20 μl specific mAb against p-ERK (T202/Y204; Pacific Blue conjugated; clone 20A), p-p38 (T180/Y182; PE conjugated; clone 36/p38), and p-S6RP (S240; Alexa Fluor 647 conjugated; clone N4-41; all BD Phosflow; BD Biosciences) or isotype-matched control antibodies for 1 h. After cells were washed in PBS + 0.5% BSA + 0.05% NaN_3, they were analyzed on a FACSCanto II flow cytometer and analyzed by use of FlowJo software (Tree Star, Ashland, OR, USA).

Immunoblotting

$CD4^+$ T cells (4×10^6) were activated in the presence or absence of the indicated concentrations of PA for 24 h by use of 2×10^6 anti-CD3/anti-CD28-coated microbeads. Cells were then harvested and lysed in radioimmunoprecipitation assay buffer, supplemented with protease inhibitors (Sigma-Aldrich). Cellular debris was removed by centrifugation at 25,000 *g* and 4°C for 15 min. Samples were normalized, according to their protein content, and were resolved by SDS-PAGE on 4–12% gradient gels under reducing conditions (Life Technologies), followed by transfer onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, United Kingdom). Samples were then subjected to immunoblotting by use of the following antibodies: rabbit anti-S6 (clone 5G10), rabbit anti-p38 (clone D13E1), rabbit anti-ERK (polyclonal), and rabbit anti-actin (clone D18C11; all New England Biolabs, Ipswich, MA, USA). After incubation with a secondary anti-rabbit HRP-conjugated antibody, binding was visualized by use of the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

NFAT, NF-κB, and AP-1 reporter assays

Jurkat T cells (1×10^6) expressing a GFP, under control of an NFAT, NF-κB, or AP-1 promoter element, were stimulated with 2×10^5 BW 5147 T cell stimulator cells transduced with a membrane-bound OKT3 single-chain fragment variable and human CD80 (TCS CD80) [32] in the presence of PA (2000 and 500 μM), rapamycin (100 nM), cyclosporine A (1 μM), or GÖ6983 (10 μM). After 24 h, cells were harvested and washed in PBS, and the number

of GFP-positive cells was established by flow cytometry. Promoter activity was calculated relative to the percentage of positive cells from samples activated in substance-free medium, as described previously [31].

Statistical analyses

Statistical analyses were performed by use of the SPSS 19 software (IBM, Chicago, IL, USA). Data are given as means ± SD. In some experimental series, data were normalized to the corresponding values of T cells activated in PA-free medium and are given as mean percentages. For multiple group comparison, a linear mixed-model ANOVA was used and corrected for multiple testing by use of Dunnett's post hoc test. Statistically significant values are denoted in figure legends.

RESULTS

PA does not induce apoptosis in CD4$^+$ T cells

Preceding studies have established that the tryptophan metabolites KYN and 3-OH-AA act as potent inducers of apoptosis in CD4$^+$ T cells [25, 26]. In accordance, we found that addition of KYN and 3-OH-AA led to a marked decrease in cell viability following anti-CD3/anti-CD28-mediated activation. At highly suppressive concentrations (1000 μM for KYN and 250 μM for 3-OH-AA), cell viability was consistently reduced to <5% compared with T cells activated in the absence of tryptophan metabolites ($n = 6$; $P < 0.001$, respectively; **Fig. 1A**). In contrast, treatment of T cells with PA had no significant effect on T cell viability (Fig. 1A and B). Even at concentrations of 2000 and 1000 μM PA, which highly suppressed activation-mediated T cell proliferation (see below), relative cellular viability compared with T cells activated in the absence of PA reached 0.95 ± 0.22 and 0.90 ± 0.10, respectively ($P = 0.649$ and $P = 0.237$; Fig. 1B). Likewise, lower concentrations of PA did not influence T cell viability in these assays (1.00 ± 0.09 for 500 μM PA and 1.03 ± 0.04 for 250 μM PA; $P = 0.999$ and $P = 0.968$, respectively; Fig. 1B).

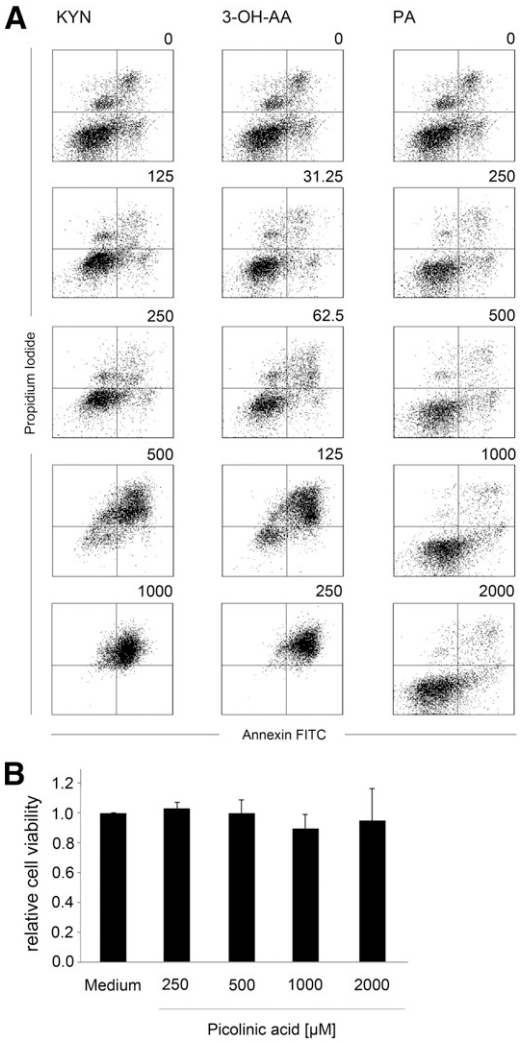

Figure 1. PA does not induce apoptosis in CD4$^+$ T cells. (A) CD4$^+$ T cells were activated in the presence of the indicated concentrations (μM) of KYN, 3-OH-AA, and PA. After 72 h, apoptosis was assessed by staining with Annexin V-FITC (*x*-axis) and propidium iodide (*y*-axis). Dot blots from 1 representative donor ($n = 6$) are shown. (B) Cumulative data from cell viability assays from 6 healthy donors. Percentages of viable (double-negative) cells of T cells activated in PA-free medium were taken as a reference.

PA suppresses T cell proliferation in a dose-dependent manner

As a first functional read-out to define immunosuppressive properties of PA on T cells, we assessed proliferation rates of CD4$^+$ T cells activated with agonistic anti-CD3/anti-CD28 antibodies. Compared with T cells activated in PA-free medium (DI: 4.18 ± 0.13; $n = 4$; **Fig. 2A** and **B**), a dose-dependent inhibition of proliferation was found in cultures supplemented with PA. A slight reduction in proliferation was already evident when T cells were exposed to 250 μM PA (DI: 3.89 ± 0.16; $P = 0.053$; Fig. 2A and B). At a PA concentration of 500 μM, a statistically significant reduction of proliferation (DI: 2.27 ± 0.57; $P < 0.01$; Fig.2A and B) was observed, whereas higher concentrations (1000 and 2000 μM) almost completely abrogated T cell proliferation (DI: 0.43 ± 0.13 and 0.18 ± 0.07, respectively; $P < 0.001$, Fig. 2A and B). From these assays, we could calculate an IC_{50} for the suppression of proliferation of 481 μM. In line with previous reports [25], the absence of tryptophan further enhanced these effects, especially at PA concentrations ranging around the IC_{50} (Fig. 2C). The immunosuppressive function of PA was also followed in a time-course analysis. After 48 h, a first robust induction of proliferation could be observed in T cells activated in PA-free medium. At this time point, all PA concentrations assessed (500–2000 μM) showed suppressive effects on the proliferation of CD4$^+$ T cells. This was even more pronounced at later time points (72 and 96 h), when proliferation in control cultures reached its maximum (Supplemental Fig. 1).

PA suppresses metabolic activity of CD4$^+$ T cells

Activation-induced proliferation of T cells is accompanied by a marked change in cellular metabolism from the use of fatty acid β-oxidation to glycolysis [10]. Similar to the proliferation rate, this metabolic activity reaches its peak 48–72 h after activation (ref. [38] and reviewed in ref. [39]). Consequently, we also assessed the effects of PA on key metabolic parameters of oxidative respiration, 48 h after activation. Exposure of

T cells to PA led to a marked decrease in the basal mitochondrial respiration rate (reduction: 54.6 ± 2.9% for 2000 μM, $P < 0.01$, and 35.6 ± 1.5% for 500 μM, $P < 0.01$; $n = 3$; **Fig. 3A**) and the ECAR, which serves as a read-out for the glycolytic activity (reduction: 61.7 ± 5.2% for 2000 μM, $P < 0.001$, and 31.2 ± 4.5% for 500 μM, $P < 0.001$; $n = 3$; Fig. 3A). Down-regulation of metabolic activity by PA did not lead to a change in the OCR/ECAR ratio, suggesting a similar inhibitory potential of PA for mitochondrial respiration, as well as for anaerobic glycolysis. The influence of PA on parameters of mitochondrial respiration was assessed further in a mitochondrial

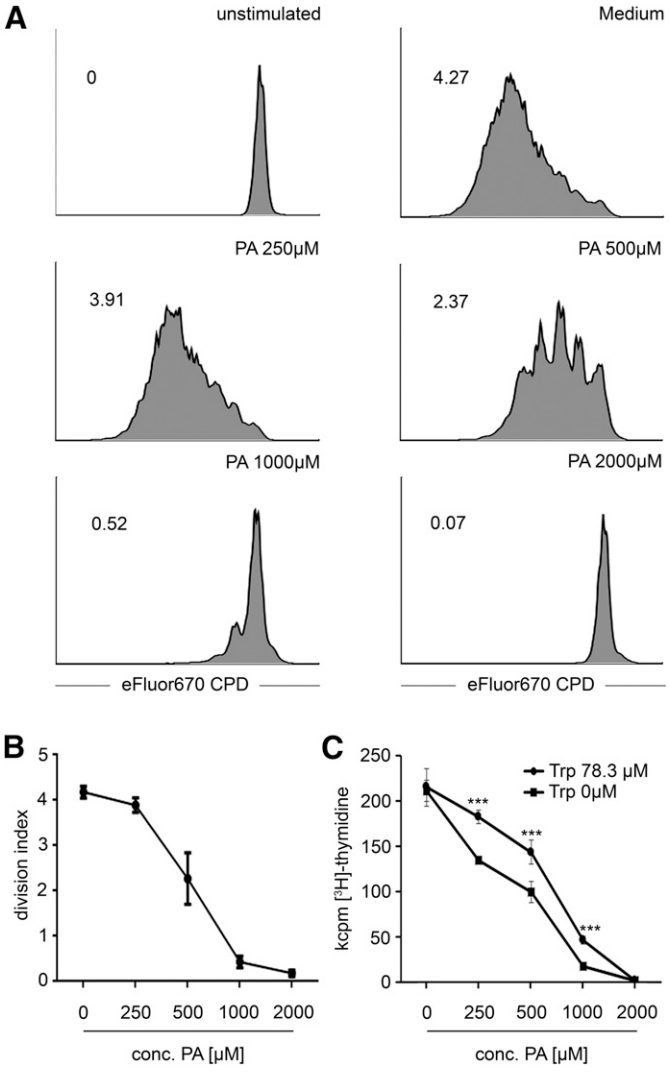

Figure 2. PA suppresses proliferation of CD4⁺ T cells. (A) Dilution of eFluor670 CPD in T cells that were left untreated or activated in the presence or absence (Medium) of the indicated concentrations (conc.) of PA. One representative experiment ($n = 4$) is depicted. Numbers in the blot indicate DI. (B) Cumulative data from 4 experiments. (C) T cells were activated in the presence or absence (medium) of PA in tryptophan (Trp)-free medium (squares) or medium resupplemented with tryptophan (circles). After 72 h of stimulation, cultures were pulsed with [^{3}H] thymidine for another 18 h. kcpm, Kilocounts/min. Data shown depict means ± SD from triplicate cultures from 1 representative donor ($n = 4$); ***$P < 0.001$.

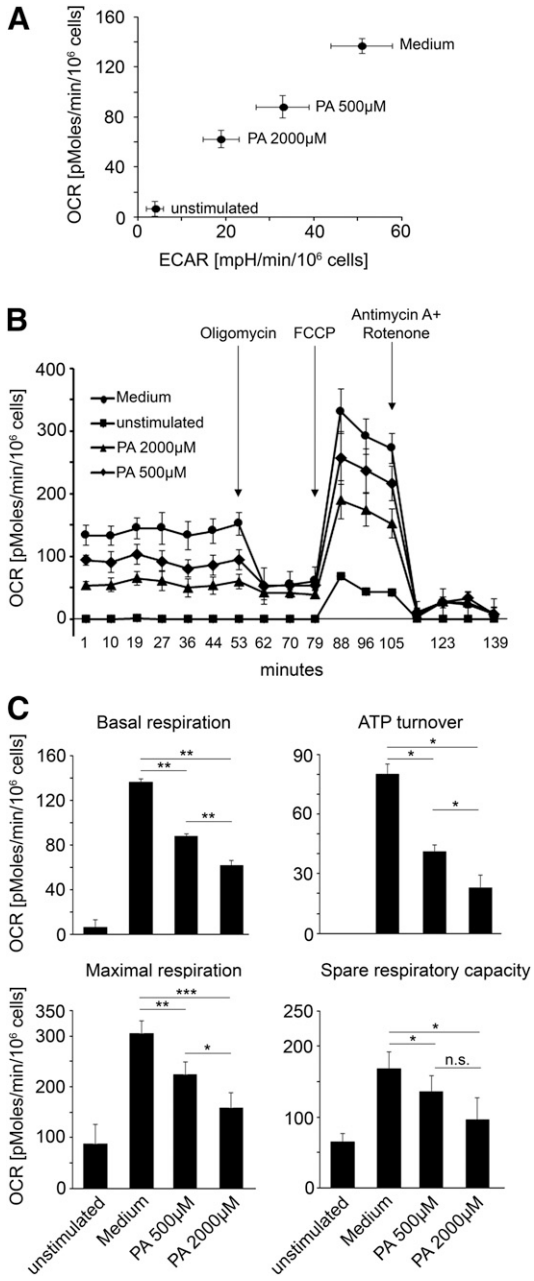

Figure 3. PA suppresses metabolic activity of CD4⁺ T cells. (A) CD4⁺ T cells were left unstimulated or activated in the absence (Medium) or presence of the indicated concentrations of PA. After 48 h, the ECAR (*x*-axis) and the OCR (*y*-axis) were determined. Cumulative data from quintuplet measurements from 3 experiments are depicted as means ± SD. (B) Mitochondrial stress test. CD4⁺ T cells were activated in the presence or absence (Medium) of the indicated concentrations of PA. After 48 h, the OCR (*y*-axis) was measured in a time course of 140 min (*x*-axis). At the indicated time points (arrows), the ATP coupling reagent oligomycin, the electron transfer chain uncoupling reagent FCCP, and the mitochondrial inhibitors rotenone/antimycin A were added. Data points depict means ± SD from quintuplet measurements from 1 representative donor ($n = 3$). (C) Cumulative calculated parameters of mitochondrial respiration from 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

stress test (Fig. 3B). Exposure to PA also resulted in a significant and dose-dependent inhibition of all major parameters of mitochondrial respiration, including the basal respiration rate, the maximal respiratory capacity (reduction: 67.3 ± 13.4% for 2000 μM, $P < 0.001$, and 37.4 ± 11.1% for 500 μM, $P < 0.01$; $n = 3$), the spare respiratory capacity (reduction: 69.4 ± 15.0% for 2000 μM, $P < 0.001$, and 31.5 ± 10.0% for 500 μM, $P < 0.001$; $n = 3$), and ATP turnover (reduction: 71.4 ± 2.3% for 2000 μM, $P < 0.001$, and 49.0 ± 1.5% for 500 μM, $P < 0.001$; $n = 3$; Fig. 3B and C).

PA does not suppress cytokine production by CD4$^+$ T cells

In a next step, we assessed the secretion of cytokines from supernatants of CD4$^+$ T cells activated in the absence or presence of PA. To account for the difference in cellular proliferation under immunosuppression by PA, all values were normalized to the cell number at the day of supernatant collection. At all concentrations tested, PA did not significantly inhibit the secretion of IL-2, IL-4, IL-10, IL-13, IL-17, and IFN-γ by anti-CD3/anti-CD28-activated CD4$^+$ T cells (**Fig. 4A**). Normalized supernatant levels of IL-4 were even increased by 68 ± 29% and 85 ± 31% in T cells exposed to 1000 μM PA and 2000 μM PA ($P < 0.05$; Fig. 4A). As cytokine levels in the cell culture supernatant represent an equilibrium of production, secretion, and consumption, we further assessed these observations about the influence of PA on production of effector cytokines by quantitative PCR. Similarly to the results from supernatant levels, no reduction in mRNA up-regulation after 24 h was observed in T cells exposed to PA compared with T cells activated in PA-free medium. For IL-10, IL-13, and IFN-γ, we even found a slight, nonsignificant up-regulation of mRNA levels at 2000 μM PA (Fig. 4B).

PA does not influence early up-regulation of cell surface activation markers in CD4$^+$ T cells

Activation-induced up-regulation of cell surface markers on CD4$^+$ T cells is dependent on the cell surface translocation of presynthesized proteins (e.g., CD69) [40] or their de novo synthesis (e.g., CD25, CD71, CD98hc) [41]. Consequently, we measured up-regulation of these markers to assess the influence of PA on these processes. As controls, we included the well-described, immunosuppressive substances rapamycin (an inhibitor of the mTOR pathway) and cyclosporine (an inhibitor of the calcineurin-NFAT pathway). We could observe that exposure of CD4$^+$ T cells to PA (2000 and 500 μM) had no effects on the up-regulation of CD69, CD71, and CD98 (**Fig. 5** and Supplemental Table 1), 24 h after activation. For CD25, a slight reduction in expression compared with T cells activated in PA-free medium was found at a concentration of 2000 μM PA (MFI: 6623 ± 471 vs. 5793 ± 115; $P < 0.05$, $n = 4$; Fig. 5 and Supplemental Table 1). At a PA concentration of 500 μM, which approximately corresponds to the IC_{50} for the inhibition of proliferation, no effect on CD25 expression was found. In contrast, cyclosporine decreased up-regulation of all assessed activation markers, whereas rapamycin partially inhibited up-regulation of CD25, CD71, and CD98 (Supplemental Table 1).

PA induces deep anergy and inhibits Th subset polarization in CD4$^+$ T cells

To assess whether activation of T cells in the presence of PA had long-lasting functional effects, CD4$^+$ T cells were activated in the presence or absence of PA and cultured for 7 d. After extensive washing to remove residual PA, equal numbers of viable cells were restimulated with agonistic anti-CD3/anti-CD28 antibodies in PA-free culture medium or in culture medium supplemented with IL-2. Compared with T cells that had been preactivated in

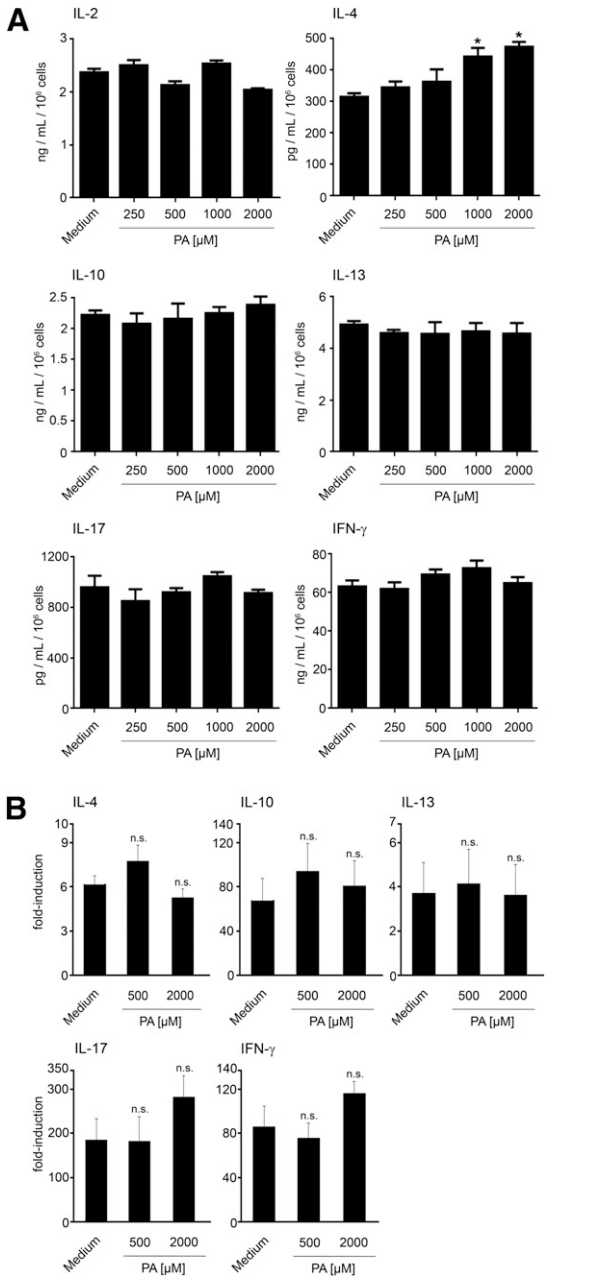

Figure 4. Cytokine secretion and production of CD4$^+$ T cells upon exposure to PA. (A) CD4$^+$ T cells were activated in the presence or absence (Medium) of the indicated concentrations of PA. After 24 h (IL-2) and 72 h (IL-4, IL-10, IL-13, IL-17, and IFN-γ), supernatant levels of the indicated cytokines were measured and normalized to cell number. Data depict means ± SD from triplicate cultures from 1 representative donor ($n = 4$); *$P < 0.05$. (B) Relative induction of mRNA levels of IL-4, IL-10, IL-13, IL-17, and IFN-γ in T cells activated in the presence or absence of the indicated concentrations of PA. Data are depicted as mean fold inductions + SD over unstimulated T cells from duplicate measurements from 3 donors; n.s., not significant.

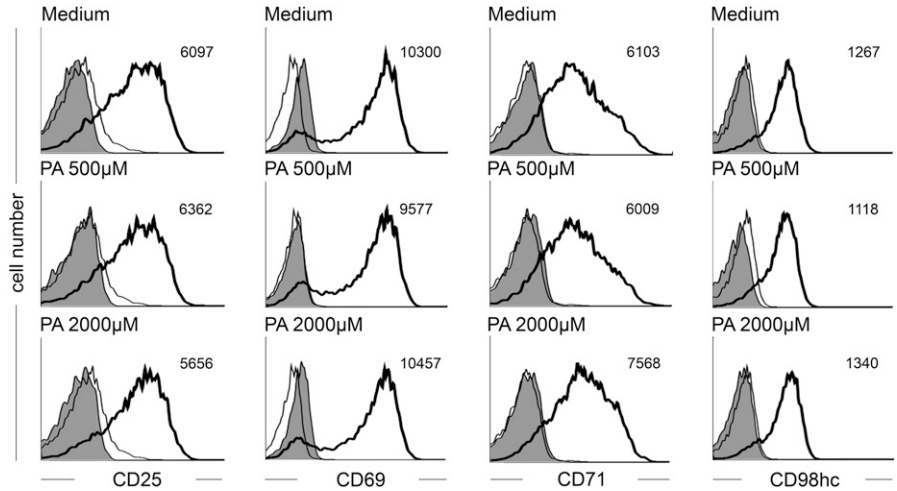

Figure 5. Up-regulation of cell surface markers of CD4⁺ T cells upon exposure to PA. CD4⁺ T cells were activated in the presence or absence (Medium) of the indicated concentrations of PA. After 24 h, surface expression of the indicated molecules was analyzed by flow cytometry. Bold black lines, Staining with indicated antibody; fine black lines, staining of unstimulated T cells with indicated antibody; gray histograms, isotype antibody staining. Numbers indicate MFI of the respective samples. One representative experiment out of 4 is depicted.

PA-free culture medium, a dose-dependent reduction of proliferation was found in the restimulation cultures that had been exposed to PA during primary stimulation. T cells preactivated in PA at concentrations of 250 or 500 μM showed a slight reduction in the restimulation cultures, which was not statistically significant, however (**Fig. 6A**). In contrast, T cells preactivated in the presence of 1000 μM PA presented with a reduction in proliferation during restimulation of 50 ± 13% ($P < 0.001$). In T cells preactivated in the presence of 2000 μM PA, this effect was even more pronounced and amounted to 72 ± 12% ($P < 0.001$). Supplementation of rIL-2, which has been shown to break classic anergy in CD4⁺ T cells, had no impact on restimulation (Fig. 6A). In a next step, we evaluated the effects of PA on Th subset polarization. $CD4^+CD45RO^-$ T cells were activated under Th1 or Th2 polarizing conditions in the presence or absence of PA. After 7 d, a robust expression of the transcription factor T-bet (91.2 ± 4.7% positive cells; $n = 4$) was found in Th1-polarized T cells, whereas no GATA-3 expression was observed. Addition of PA during polarization led to a dose-dependent decrease in the expression of T-bet in Th1-polarized T cells. Whereas this effect did not reach statistical significance in the presence of 500 μM PA (89.3 ± 3.2% positive cells; $P = 0.593$), 2000 μM PA induced a strong decrease in T-bet expression (41.1 ± 4.8% positive cells; $P < 0.001$). Likewise, the expression of GATA-3 in Th2-polarized T cells (52.9 ± 6.9% positive cells) was also inhibited by exposure to PA. Addition of 500 μM PA to the Th2 polarization cultures already induced a significant reduction in GATA-3⁺ T cells (43.6 ± 2.3%; $P < 0.05$), which was pronounced further in T cells polarized in the presence of 2000 μM PA (29.8 ± 3.3%; $P < 0.01$).

PA inhibits c-Myc activation in CD4⁺ T cells

In a final step, we aimed to elucidate the molecular mechanisms underlying the immunosuppressive properties of PA. In a first step, we examined whether the iron chelating properties of PA were responsible for the immunosuppressive effects observed [30]. In our experimental set-up, supplementation of ferric iron to the cultures had no effect on the inhibition of T cell proliferation by PA (**Fig. 7A**). Consequently, we assessed the effects of PA on intracellular signaling pathways of T cell activation. As a first screening step, we used Jurkat reporter cell lines transfected with an NFAT::eGFP, an NF-κB::eGFP, or an AP-1::eGFP reporter construct [31]. In all 3 cell lines, promoter activity was not affected by PA (2000 and 500 μM), following stimulation with BW anti-CD3/CD80-transduced T cell stimulator cells (Fig. 7B). To control the specificity of this read-out, the well-defined inhibitors rapamycin and cyclosporine A and the PKC inhibitor GÖ6983 were included in these assays, as described previously [31]. Furthermore, flow cytometric analyses in primary CD4⁺ T cells demonstrated that p-p38 and p-ERK MAPKs and p-S6RP, the mTOR downstream target, were not affected by PA (Fig. 7C). Likewise, total protein levels of these 3 signaling proteins were also not affected by PA (Supplemental Fig. 2A). In these assays, the PKC inhibitor GÖ6983 was used as an inhibitor control. As expected, a strong suppression of anti-CD3/anti-CD28-mediated as well as PMA-induced p-ERK and p-S6RP could be observed (Supplemental Fig. 2B). For p38, this could not be validated, as GÖ6983 showed strong autofluorescence in the PE channel used to detect p-p38 (not shown).

Given that PA mainly affected proliferation and metabolic activity, we hypothesized that c-Myc might constitute a molecular target of PA-mediated suppression [20]. Activity of c-Myc is regulated at several levels, including transcription and phosphorylation at Ser62 [17, 18]. Time-course analyses in CD4⁺ T cells, activated in the presence or absence of PA, showed no differences in c-Myc transcription (**Fig. 8A**). Likewise, intracellular flow cytometry for total c-Myc protein revealed no differences in CD4⁺ T cells activated for 24 h in the presence or absence of PA (not shown). Following activation of CD4⁺ T cells with PMA, rapid up-regulation of phosphorylation at Ser62 of c-Myc was observed (Fig. 8B). In contrast, a dose-dependent reduction of p-c-Myc-positive cells was found in T cells exposed to PA (reduction: 47.3 ± 4.7% for 2000 μM and 26.0 ± 3.4% for 500 μM; $n = 4$, $P < 0.01$. To assess this effect in a more physiologic stimulation context, T cells were activated with agonistic anti-CD3/anti-CD28 antibodies for 24 h, and Ser62 p-c-Myc was measured. As above, PA strongly decreased the number of p-c-Myc-positive T cells compared with cells activated in PA-free medium (reduction: 59.0 ± 1.0% for 2000 μM, $P < 0.001$, and 26.5 ± 11.5% for 500 μM, $P < 0.05$; $n = 4$; Fig. 8C). In

these assays, we also included the immunosuppressive substances rapamycin and cyclosporin as controls. Whereas treatment with rapamycin showed inhibitory effects on c-Myc activation (reduction: 39.8 ± 2.7%, $P < 0.001$), cyclosporine did not affect p-c-Myc (Fig. 8C), ruling out that reduced p-c-Myc Ser62 is a general observation in T cells exposed to immunosuppressive substances.

DISCUSSION

Tryptophan metabolites are key mediators of IDO-mediated suppression of T cell responses. Although the exact sequence of

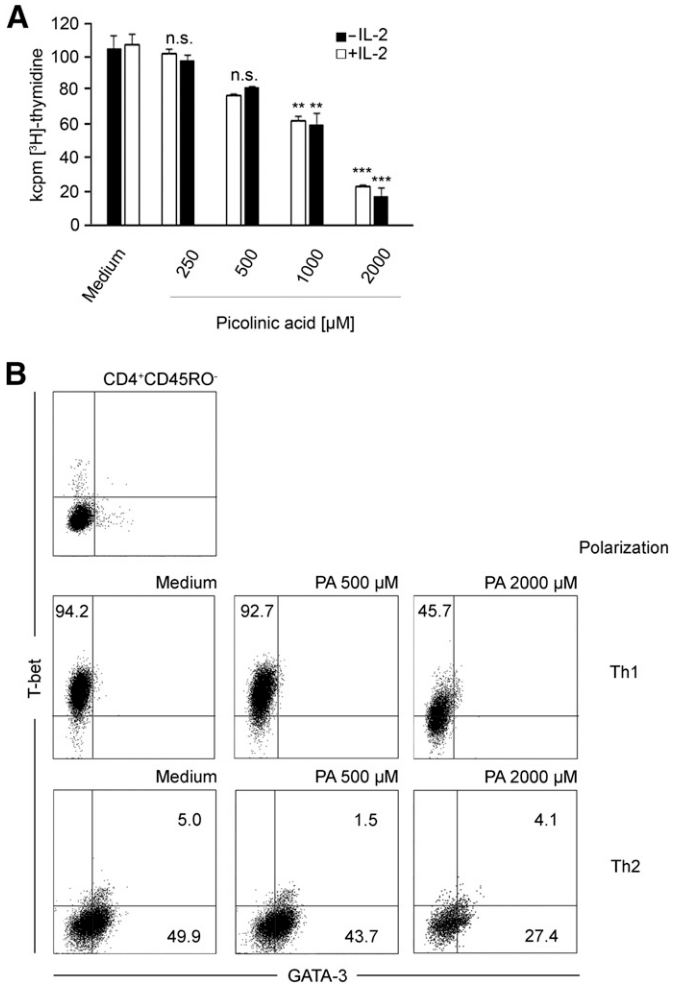

Figure 6. PA induces IL-2-resistant anergy in T cells and inhibits Th cell polarization. (A) $CD4^+$ T cells were activated in the presence or absence (Medium) of the indicated concentrations of PA. After 7 d, cells were restimulated in PA-free medium in the absence (black bars) or presence (white bars) of rIL-2 (100 U/ml). Proliferation was measured after 96 h. Data depict means + SD from triplicate cultures from 1 representative donor ($n = 4$); $^{**}P < 0.01$, $^{***}P < 0.001$. (B) $CD4^+CD45RO^-$ T cells were activated under Th1 (middle) and Th2 (bottom) polarizing conditions in the presence or absence (Medium) of the indicated concentrations of PA for 7 d. Expression of the transcription factors GATA-3 (*x*-axis) and T-bet (*y*-axis) was determined by intracellular flow cytometry. Numbers indicate the percentage of positive cells in the respective quadrants. One representative experiment ($n = 4$) is depicted.

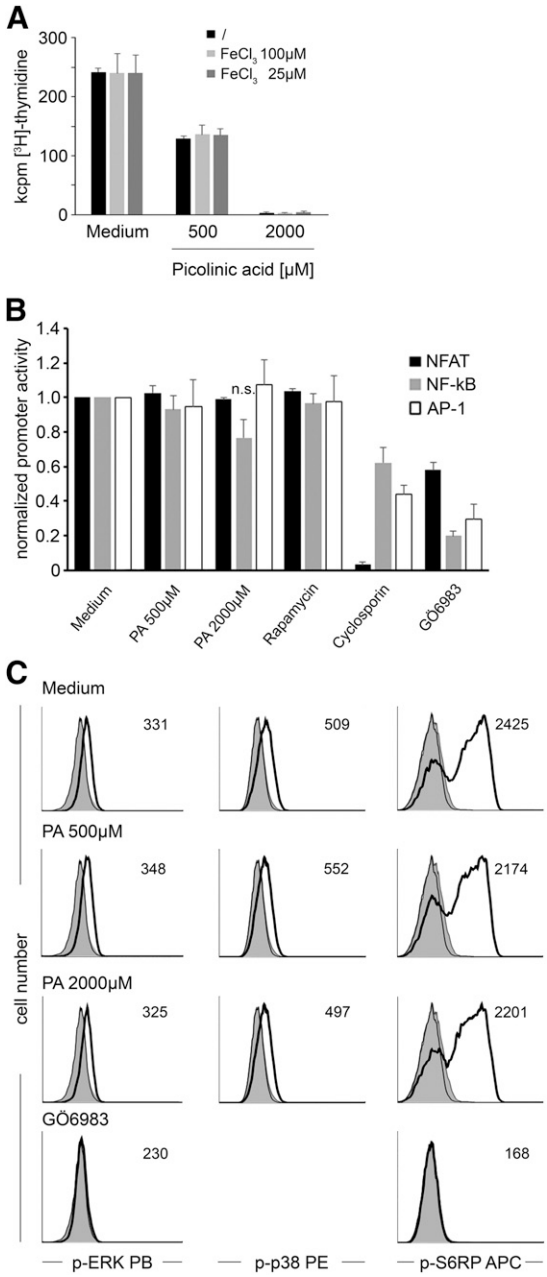

Figure 7. Mechanistic evaluation of T cells exposed to PA. (A) $CD4^+$ T cells were activated in the absence (Medium) or presence of PA. Before activation, the indicated amounts of ferric chloride were supplemented. After 96 h, thymidine incorporation was measured. Data depict means + SD from 1 representative donor ($n = 4$). (B) Jurkat T cells expressing an NFAT::eGFP, NF-κB::eGFP, or AP-1::eGFP reporter construct were activated with anti-CD3/CD80-expressing T cell stimulator cells in the presence or absence of the indicated substances. After 24 h, GFP fluorescence was determined as a read-out for promoter activity and normalized to reporter gene expression in cells activated in plain medium. Cumulative data (means ± SD) from 4 independent experiments are depicted. (C) p-ERK, p-p38, and p-S6RP in primary $CD4^+$ T cells activated with agonistic anti-CD3/anti-CD28 antibodies in the presence or absence of the indicated substances for 24 h. PB, Pacific Blue; APC, allophycocyanin. Bold black lines, Specific staining; fine black lines, specific staining in unstimulated T cells; gray histograms, isotype control. Numbers indicate MFI. Histograms from 1 representative experiment ($n = 4$) are shown.

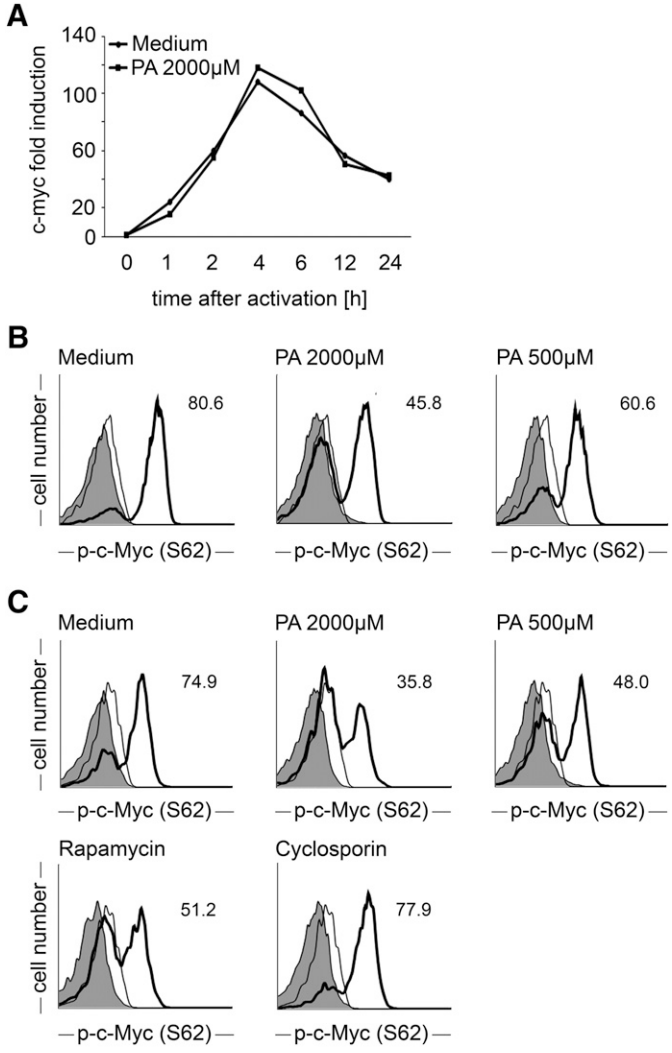

Figure 8. Reduced p-c-Myc in T cells exposed to PA. (A) $CD4^+$ T cells were activated in the presence or absence of PA by use of agonistic anti-CD3/anti-CD28 antibodies. At the indicated time points, c-Myc expression was measured by quantitative PCR. Data are representative of mean values of duplicate measurements and are depicted as fold levels relative to unstimulated cells. One representative experiment ($n = 3$) is depicted. (B and C) p-c-Myc at Ser62. $CD4^+$ T cells were activated with PMA (B) or agonistic anti-CD3/anti-CD28 antibodies (C) in the presence or absence (Medium) of the indicated substances. After 30 min (B) or 24 h (C), p-c-Myc at Ser62 was measured by flow cytometry. Bold black lines, Specific staining; fine black lines, specific staining in unstimulated T cells; gray histograms, isotype control. Numbers indicate percentage of positive cells. Histograms from 1 representative experiment ($n = 4$) are shown.

tryptophan catabolism is well characterized, so far, most studies have focused on the effects of the upstream metabolites KYN and 3-OH-AA, describing them as potent inductors of apoptosis in lymphocytes [25–27, 29]. We found that in contrast to these metabolites, PA leaves the viability of T cells intact and specifically inhibits activation-induced T cell proliferation, while having no significant influence on other activation-induced effector functions. On the molecular level, major activating signaling pathways, such as the calcineurin-NFAT, the MAPK, and the mTOR pathways, were not affected by PA. At the same time, PA-exposed T cells presented with a reduced p-c-Myc cell cycle regulator.

In the mold of typical immunosuppressive substances, PA strongly suppressed T cell proliferation in a dose-dependent manner. Concomitantly, T cells exposed to PA also presented with significantly lower metabolic activity. In this respect, dose-dependent suppression by PA resulted in a linear decrease in the ECAR/OCR ratio, indicating that PA affects anaerobic glycolysis and basal mitochondrial respiration to a similar degree. Furthermore, mitochondrial stress test analyses revealed that all parameters of mitochondrial respiration were inhibited in PA-exposed T cells. Whereas important functional consequences of T cell activation were thus strongly inhibited by PA, effector functions, which mostly rely on protein production, were largely unaffected. It was especially striking that secretion of IL-2, which is a very early consequence of T cell activation, was fully intact in PA-exposed T cells. This was not a result of a delayed suppressive effect of PA, as time-course analyses showed that PA immediately exerted suppressive effects on T cell proliferation.

Likewise, secretion of Th subset-specific cytokines was not affected by PA. For IL-4, IL-13, IL-17, and IFN-γ, even slight enhancements of supernatant levels could be observed at high PA concentrations. Given that cytokine levels in supernatants represent an equilibrium of production, secretion, and consumption, it is possible that these effects are not directly a result of modulation of cytokine production. Along those lines, PA did not significantly affect the transcription of these cytokines, 24 h after activation. Exposure to PA also had minimal effects on the up-regulation of activation-induced cell surface markers, which is also dependent on protein synthesis and intracellular transport. In these assays, no effects were observed for up-regulation of CD69, CD71, and CD98hc, whereas up-regulation of CD25 was decreased slightly at high PA concentrations. Taken together, these findings suggest that PA has no major influence on activation parameters that are dependent on protein production in T cells.

Activation of T cells in the presence of PA had long-lasting functional consequences even after its removal. In restimulation experiments, we could show that primary stimulation in the presence of PA led to a block in proliferation during restimulation. These findings suggest that PA induces an anergy-like state in T cells. However, the PA-induced state of hyporesponsiveness could not be overcome by the addition of exogenous IL-2, which has been demonstrated to break "classic" T cell anergy [42]. In this respect, recent works have indicated that states of IL-2 resistant anergy might exist in T cells [43]. It remains to be explored further whether a specific molecular signature is induced by pretreatment with PA and whether anergy-associated genes, such as Cbl-b [44–46], are involved in this hyporesponsive state. Likewise, PA effectively inhibited expression of the Th subset-specific transcription factors T-bet and GATA-3 under Th1 and Th2 polarizing conditions. Taken together, these functional experiments indicate that immunosuppression by PA is permissive for some immediate effector functions following activation. However, PA strongly affects the long-term functionality of T cells, reduces the capacity of T cells

to mount secondary responses, and inhibits Th cell polarization in naïve T cells.

In search of the mechanism underlying the immunosuppression by PA, we first focused on its metal chelating properties, as depletion of iron has antiproliferative effects in lymphocytic cells [47]. Hence, iron depletion by PA has been used to study expression of the transferrin receptor in T cells [30]. In our experimental setting, supplementation of ferric iron had no effect on immunosuppression by PA. Thus, we hypothesized that iron depletion does not constitute the main suppressive mechanism of PA in $CD4^+$ T cells. Consequently, we analyzed its effects on major intracellular signaling pathways of T cell activation. This was performed by specific assessment of signaling protein phosphorylation in primary $CD4^+$ T cells and by the use of NFAT, NF-κB, and AP-1 promoter reporter cell lines. These reporter cell lines integrate signals from several key signaling pathways and thus, permit assessment of intracellular signaling on a more general scale [31]. With the use of these approaches, we could show that PA does not affect MAPK signaling, the mTOR pathway, or the calcineurin-NFAT signaling module. Given that PA-treated T cells presented with a unique pattern of functional suppression, we hypothesized that activation of c-Myc might act as a target of PA. The signaling activity of c-Myc has been studied extensively in tumor cell lines [19]; there, it could be established that c-Myc activity is regulated at several levels, including its transcriptional rate and post-translational modifications. One key mechanism is ERK-mediated p-Ser62, which allows association of c-Myc with cofactors, such as Max [18]. Consequently, this complex is translocated to the nucleus, where it acts as a transcription factor. Active c-Myc plays an integral role in the control of the cell cycle and is also strongly involved in the regulation of cellular metabolism [20]. Although these functions are strongly affected by T cell activation, so far, the role of c-Myc in T cells in this respect has been addressed only incompletely. We found that induction of c-Myc transcription following activation was not affected by PA. In contrast, p-c-Myc at Ser62 was inhibited by PA, following PMA and anti-CD3/anti-CD28 stimulation. As p-c-Myc, in response to PMA, could already be measured as early as 30 min after stimulation, it seems conceivable that PA directly inhibits c-Myc activation. Importantly, exposure to cyclosporine did not alter p-c-Myc, ruling out the possibility that reduced p-c-Myc is a general observation in T cells exposed to immunosuppressive substances. In contrast, exposure to rapamycin showed inhibitory effects on c-Myc activation. These results are in line with recent reports showing that mTOR signaling and c-Myc activity might be interconnected processes [48, 49].

Taken together, these data indicate that PA interferes with p-c-Myc, while leaving more upstream signals intact. The MAPK ERK seems to play a major role in the activating p-c-Myc. Interestingly, PA had no influence on NF-κB and AP-1 activity, which should, at least partially, depend on phosphorylation by ERK [50, 51]. In this respect, it has not been fully investigated to which degree different MAPKs contribute to the promoter activities and whether different MAPKs can compensate for each other. Thus, it remains to be determined whether PA inhibits c-Myc activation via inhibition of ERK or whether indirect mechanisms apply.

These findings also shed substantial light on the relevance of different intracellular signaling pathways in T cells. Our data indicate that cell cycle control and control of protein synthesis are differentially and independently regulated processes. Full T cell activation requires the activation of several intracellular signaling cascades, such as the calcineurin-NFAT axis and the mTOR pathway, which non-redundantly regulate effector functions. If 1 of these pathways is blocked, T cells fail to initiate effector functions, even though the signaling capacity of other pathways is fully intact. On the other hand, c-Myc represents a signaling module that is indispensable for proliferation and metabolic activity but does not affect other effector functions, such as cytokine production and up-regulation of cell surface proteins. These observations also comply with recent reports that different effector functions, such as proliferation and cytokine secretion, are driven by separable TCR-triggered pathways and that c-Myc is an important regulatory factor for T cell proliferation [52].

As PA treatment does not induce apoptosis in T cells but rather suppresses effector functions in these cells, it is tempting to speculate that IDO-expressing, tolerogenic APCs might selectively shape the T cell response by accumulation of different tryptophan metabolites. Whereas high levels of upstream metabolites, such as KYN and 3-OH-AA, lead to the deletion of T cells, accumulation of PA limits T cell expansion while leaving some effector functions intact. As a third option, the degradation of tryptophan to QA does not affect T cell responses and might thus leave APCs fully stimulatory, even if they express high levels of IDO [25, 26]. On the other hand, it is possible that IDO activity in APCs creates a distinct microenvironment at the immunologic synapse, combining low tryptophan levels with a mixture of downstream metabolites. Given that low tryptophan levels enhance the suppressive effects of PA, tryptophan depletion, as well as the presence of other metabolites, such as KYN and 3-OH-AA, may alter the outcome of PA exposure on T cell activation. It thus remains to be determined which settings best mimic the in vivo situation and which stimuli regulate the expression of distinct tryptophan-metabolite catabolizing enzymes in APCs.

Finally, the unique immunomodulatory properties of PA may also be harnessed for therapeutic purposes to dampen the development of pathologic immune responses without killing protective T cells. Given that systemic application of PA in mice has shown no major adverse effects [53, 54], it might be envisioned that PA could indeed be used as a therapeutic agent.

In conclusion, we provide for the first time a comprehensive overview about the functional effects of PA on T cells. We describe how PA, in contrast to more upstream tryptophan metabolites, does not induce apoptosis in T cells. Furthermore, PA selectively inhibits activation-induced T cell proliferation and concomitant metabolic activity, whereas other effector functions are left intact. We describe how these effects are associated specifically with the inhibition of p-c-Myc, thus giving first evidence about a so-far not-described regulatory principle in T cells.

AUTHORSHIP

J.P., L.J.L., R.S., and F.R. designed experiments, isolated cells, and performed functional and cell signaling experiments. G.G., N.W., and G.H. established and performed cellular metabolism experiments. S.J. and P.S. designed, validated, and performed assays with Jurkat reporter cell lines. W.F.P. performed thymidine incorporation assays, supplied reagents, and was involved in project design. R.M. supplied reagents and was involved in project design and data interpretation. K.G.S. designed and supervised experimental work, performed data interpretation, and provided research funding. All authors were involved in the preparation of the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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