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Piperine From Black Pepper Inhibits Activation-Induced Proliferation and Effector Function of T Lymphocytes

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ABSTRACT

Piperine is a major alkaloid component of black pepper (*Piper nigrum* Linn), which is a widely consumed spice. Here, we investigated the effect of piperine on mouse T lymphocyte activation. Piperine inhibited polyclonal and antigen-specific T lymphocyte proliferation without affecting cell viability. Piperine also suppressed T lymphocyte entry into the S and G_2/M phases of the cell cycle, and decreased expression of G_1-associated cyclin D3, CDK4, and CDK6. In addition, piperine inhibited CD25 expression, synthesis of interferon-γ, interleukin (IL)-2, IL-4, and IL-17A, and the generation of cytotoxic effector cells. The inhibitory effect of piperine on T lymphocytes was associated with hypophosphorylation of Akt, extracellular signal-regulated kinase, and inhibitor of κBα, but not ZAP-70. The ability of piperine to inhibit several key signaling pathways involved in T lymphocyte activation and the acquisition of effector function suggests that piperine might be useful in the management of T lymphocyte-mediated autoimmune and chronic inflammatory disorders. J. Cell. Biochem. 116: 2577–2588, 2015.

KEY WORDS: PIPERINE; T LYMPHOCYTE; CELL CYCLE; CYTOKINES; CYTOTOXICITY; SIGNAL TRANSDUCTION

T lymphocytes are key components of the adaptive cell-mediated immune response owing to the production of cytokines by $CD4^+$ T helper lymphocytes and the cytotoxic effector function of $CD8^+$ T lymphocytes [Zhang and Bevan, 2011; Zygmunt and Veldhoen, 2011]. However, inappropriate activation of T lymphocytes as a result of immune dysregulation can lead to chronic inflammatory and autoimmune conditions such as inflammatory bowel disease and multiple sclerosis [Zenewicz et al., 2009; Venken et al., 2010]. Current immunosuppressive agents used to treat these conditions include biologics, corticosteroids, and calcineurin inhibitors, all of which are costly and have adverse side effects that include lymphoproliferative disorders, bone marrow suppression, hepatotoxicity, nephrotoxicity, and cardiovascular events [Grinyó et al., 2012]. The shortcomings of these current therapies therefore demand the continued search for more affordable and better tolerated immunosuppressive agents.

Phytochemicals are plant chemicals that possess biological activity and are a source of novel immunomodulatory and anti-inflammatory compounds that have clinical potential [Aggarwal et al., 2006; Butler, 2008]. For example, the dietary phytochemicals curcumin and resveratrol are able to suppress various aspects of T lymphocyte function in vitro [Gao et al., 2001; Forward et al., 2011], as well as showing efficacy in mouse models of T lymphocyte-mediated inflammatory disease [Xuzhu et al., 2012; Castro et al.,

Abbreviations: BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IκBα, inhibitor of κBα; OVA, ovalbumin; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; WT, wild-type.

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2014]. Piperine, the alkaloid responsible for the pungency of black pepper (*Piper nigrum* Linn), is consumed in significant quantities worldwide [Koleva et al., 2011] and has a number of important physiological effects, including the ability to suppress cell-mediated immunity [Srinivasan, 2007]. For example, piperine reduces lymphocyte infiltration and inflammation in rats with carrageenan-induced arthritis and interferes with the accumulation of activated T lymphocytes and interleukin (IL)-4 production in the lungs of mice with ovalbumin (OVA)-induced asthma [Kim and Lee, 2009]. In addition, oral administration of piperine to mice inhibits phytohemagglutinin-induced T lymphocyte proliferation ex vivo [Dogra et al., 2004]. Taken together, these findings suggest that piperine down-regulates cell-mediated immunity; however, a direct effect of piperine on T lymphocyte function has not yet been demonstrated.

The aim of this study was to determine the direct effect of piperine on T lymphocyte activation and subsequent cell proliferation, cytokine production, and the generation of cytotoxic effector cells. The effect of piperine on signal transduction pathways involved in T lymphocyte activation and acquisition of effector function was also investigated.

MATERIALS AND METHODS

ANIMALS

Wild-type (WT) C57BL/6 (H-2^b) mice and OT-II mice were obtained from Charles River Canada (Lasalle, QC). TRPV$1^{-/-}$ mice (B6.129 × 1-*Trpv1*^{tm1Jul}/J) on a C57BL/6 background and age-matched C57BL/6 WT control mice were purchased from The Jackson Laboratory (Bar Harbour, ME). All mice were housed in the Carleton Animal Care Facility at Dalhousie University. Animals were fed a standard diet of rodent chow and water ad libitum. All animal protocols were approved by the Dalhousie University Committee on Laboratory Animals and were in accordance with the Canadian Council on Animal Care Guidelines.

REAGENTS

Piperine (purity ≥97%) was purchased from Sigma–Aldrich Canada (Oakville, ON). A 100 mM stock solution of piperine was prepared in DMSO and stored at −80°C. Recombinant murine IL-2 was from PeproTech Inc. (Rocky Hill, NJ) and recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) was from R&D Systems Inc. (Minneapolis, MN). Paraformaldehyde was purchased from Bio-Shop Canada Inc. (Burlington, ON). Propidium iodide (PI) was from Invitrogen Canada Inc. (Burlington, ON). Antibodies against ZAP-70, phospho-ZAP-70 (Tyr 319)/Syk (Tyr 352), Akt, phospho-Akt (Ser 473), inhibitor of κBα (IκBα), phospho-IκBα (Ser 32), cyclin D3, CDK4, and CDK6 were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibodies against actin, extracellular signal-regulated kinase (ERK), and phospho-ERK (Tyr 204), as well as horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated bovine anti-goat IgG, and HRP-conjugated donkey anti-rabbit IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD25 antibody was purchased from Cedarlane Laboratories Ltd. (Burlington, ON). Functional grade anti-mouse T cell receptor (TCR) β, phycoerythrin (PE)-conjugated anti-CD4, PE-conjugated anti-CD8a, FITC-conjugated anti-CD69, PE-conjugated rat IgG2b, PE-conjugated rat IgG2a, FITC-conjugated rat IgG1, and FITC-conjugated Armenian hamster IgG antibodies were from eBioscience Inc. (San Diego, CA). P815 mastocytoma cells were obtained from ATCC (Manassas, VA).

T LYMPHOCYTE ISOLATION AND ACTIVATION

Mice were euthanized; spleens were excised using aseptic technique, and a single cell suspension was prepared in ice-cold phosphate-buffered saline (PBS). Erythrocytes were lysed by osmotic shock. $CD3^+$ T lymphocytes were isolated by negative selection using the Pan T Cell Isolation MACS® kit (Miltenyi Biotech, Auburn, CA), as per the manufacturer's instructions. T lymphocyte purity was at least 97%. For select experiments, $CD8^+$ or $CD4^+$ T Lymphocyte Isolation MACS® kits were used. Cell viability was typically greater than 95%, as determined by trypan blue dye exclusion. Cells were cultured in RPMI 1640 medium (Sigma–Aldrich) supplemented with 5% heat-inactivated (56°C for 30 min) fetal calf serum (FCS, v/v), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5 mM HEPES (all from Invitrogen). Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Cell culture plastics were purchased from Sarstedt Inc. (Montreal, QC).

Treatments were added 15 min prior to T lymphocyte activation, unless otherwise noted. T lymphocytes were activated using Mouse T-Activator anti-CD3 and anti-CD28 antibody-coated Dynabeads® (Invitrogen), typically at a ratio of one bead for every two T lymphocytes. For select experiments, OT-II $CD4^+$ T lymphocytes were activated using bone marrow-derived dendritic cells (DC) loaded with 300 nM chicken $OVA_{323–339}$ peptide (Sigma–Aldrich). Briefly, bone marrow cells from the femur and tibia of C57BL/6 mice were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Wisent Inc., St-Bruno, QC), 200 U/ml penicillin, 200 μg/ml streptomycin, 10 mM HEPES, 5 mM 2-mercaptoethanol (Sigma–Aldrich), and 20 ng/ml GM-CSF. Fresh medium was added on day 3, and on day 6 non-adherent cells were transferred to fresh 6-well plates and cultured in fresh medium containing 10 ng/ml GM-CSF. On day 7, DCs were matured for 18 h with 500 ng/ml lipopolysaccharide (Sigma–Aldrich).

TRITIATED-THYMIDINE ([^{3}H]TDR) INCORPORATION ASSAY

T lymphocytes were plated in quadruplicate at 2.5×10^5 cells/well in 96-well round-bottom plates, treated as indicated, and activated for 24–96 h. For the last 6 h of incubation, cells were pulsed with 7.4 kBq of methyl [^{3}H]TdR (MP Biomedicals, Irvine, CA). Cells were then harvested onto fiberglass filter mats with a Titertek® Cell Harvester (both from Skatron Instruments, Sterling, VA). [^{3}H]TdR incorporation into newly synthesized DNA was measured using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON).

FLOW CYTOMETRY

Flow cytometric analysis of T lymphocytes was performed with a FACSCaliber flow cytometer using BD CellQuest™ software (version 3.3; BD Biosciences) and a minimum of 1×10^4 counts per sample.

Counts were gated on the live cell population within each sample, with the exception of Annexin-V/PI staining in which both live and dead cells were counted. Data were analyzed using FCS Express software (version 3.0; De Novo Software, Thornhill, ON). When cells were co-stained with two different fluorochromes or dyes with overlapping emission spectra, data acquisition was corrected by compensation using appropriate unlabeled and single-stained control samples.

To measure T lymphocyte division, cells were stained with 2 μM CellTrace™ Oregon Green® 488 (Invitrogen) in warm PBS for 10 min in the dark. Excess dye was inactivated with FCS and cells were centrifuged and incubated for 30 min at 37°C in warm medium to allow conjugation of the dye to proteins within the cells. T lymphocytes were then plated at 2.5×10^5 cells/well in 96-well round-bottom plates, treated as indicated, and activated for 72 h. Fluorescence intensity of T lymphocytes was determined by flow cytometry.

For cell cycle analysis, T lymphocytes were plated at 2.5×10^5 cells/well in 96-well round-bottom plates, treated as indicated, and activated for 72 h. Cells were fixed with 70% ethanol and stored at −20°C for at least 24 h, after which cells were washed and exposed to PI staining solution consisting of 0.2 mg/ml DNase-free RNase A (Qiagen Inc., Mississauga, ON), 0.02 mg/ml PI, and 0.1% Triton X-100 (v/v; Sigma–Aldrich) in PBS for 30 min in the dark. The percentage of cells in the various phases of the cell cycle was determined by flow cytometry using ModFit LT software (Verity Software House, Topsham, ME).

To measure expression of T lymphocyte surface molecules, cells were collected from 96-well round-bottom plates, washed with flow cytometry buffer consisting of 0.2% sodium azide (w/v) and 1% bovine serum albumin (BSA; w/v) in PBS, and labeled on ice with fluorochrome-conjugated antibodies or isotype-matched fluorochrome-conjugated control antibodies (10 μg/ml in flow cytometry buffer) for 45 min in the dark. Cells were then washed twice with flow cytometry buffer, fixed in 1% paraformaldehyde (w/v) solution, and analyzed by flow cytometry.

To assess T lymphocyte viability, cells were cultured for 24 h in the absence or presence of 100 μM piperine, then washed and stained for 15 min in the dark with a 1/50 dilution of Annexin-V-FLUOS (Roche Diagnostics, Laval, QC) and 1 μg/ml of PI in incubation buffer (10 mM HEPES, 140 mM sodium chloride, 5 mM calcium chloride in water). Samples were then diluted with incubation buffer and analyzed by flow cytometry. Percent cell death was calculated as the percentage of cells that stained positive for either Annexin-V-FLUOS (early apoptosis) and/or PI (late apoptosis/necrosis).

RNA ISOLATION AND RT-PCR

T lymphocytes and spinal cord homogenates were prepared by repeated passage through an 18 gauge needle and RNA was isolated with TRIzol® Reagent (Invitrogen) as per manufacturer's instructions. RNA was resuspended in pyrogen-free water and treated with 2.5 U of RNase-free DNase (Promega, Madison, WI). RNA quantity and quality was assessed by spectrophotometric analysis. Only samples with an A260/A280 ratio $\geq$1.7 were used for RT-PCR.

All reverse transcription and PCR reagents were purchased from Invitrogen unless otherwise noted. RNA was reverse transcribed to cDNA as per the manufacturer's instructions using SuperScript™ II reverse transcriptase on a Biometra® T gradient thermocycler (Whatman Canada Ltd., Toronto, ON). cDNA was amplified by PCR according to manufacturer's instructions with *Taq* DNA polymerase. A 457 bp fragment of murine TRPV1 was amplified using a Biometra® T gradient thermocycler under the following conditions: 94°C for 4 min, followed by 36 cycles of 94°C for 45 sec, 52°C for 45 sec, 72°C for 60 sec, with a final elongation step of 10 min at 72°C. TRPV1 primer sequences were 5′-TGTCCTGCATTGACACCTGT-GAGA-3′ (forward) and 5′-TCCTTGCGATGGCTGAAGTACAGT-3′ (reverse; both from Integrated DNA Technologies, Coralville, IA). A 627 bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control using the same amplification conditions and the following primer sequences: 5′-TTCACCAC-CATGGAGAAGC-3′ (forward) and 5′-GCGATGGACTGTGGT-CATGA-3′ (reverse; both from Integrated DNA Technologies). Amplicons were diluted in 10× BlueJuice™, run at 80 V for 1 h on a 2% agarose gel containing 0.5 μl/ml ethidium bromide (Sigma–Aldrich) in 1× Tris-acetate-EDTA running buffer, visualized, and photographed by ultraviolet light exposure using an Alpha Innotech Red™ Gel Imaging System (ProteinSimple, Santa Clara, CA).

CYTOKINE QUANTIFICATION

T lymphocytes were plated at 2.5×10^5 cells/well in 96-well round-bottom plates, treated as indicated, and activated for 24 h. Cell-free supernatants were harvested and analysed for cytokine content: IL-2 and interferon (IFN)-γ using sandwich-ELISA kits from BD Biosciences; IL-4 and IL-17A using sandwich-ELISA kits from eBiosciences, as per the manufacturers' instructions. Absorbance was read at 450 nm on a microplate reader. SOFTmax® PRO software (version 4.3; Molecular Devices Corp., Sunnyvale, CA) was used to quantify cytokines.

CYTOTOXICITY ASSAY

$CD8^+$ T lymphocytes were plated at 5×10^6 cells/well in 24-well flat-bottom plates, treated as indicated, and activated for 72 h in the presence of 50 U/mL of IL-2. T lymphocytes were then washed, counted, and the number of live cells was normalized between treatments. T lymphocytes and P815 target cells (labeled for 6 h with 185 kBq/mL [^{3}H]TdR; 5×10^3 cells/well) were combined at effector: target cell ratios of 50:1 and 25:1 in 96-well round-bottom plates and 1 μg/ml of anti-TCRβ antibody was added to the wells to induce redirected lysis of the P815 cells. Effector and target cells were incubated together for 4 h, harvested, and the [^{3}H]TdR content was determined by liquid scintillation counting. The loss of [^{3}H]TdR counts per min (cpm) compared to P815 target cells alone was used to determine percent cytotoxicity, i.e., percent cytotoxicity $= 1 - (\text{experimental}_{\text{cpm}}/\text{control}_{\text{cpm}}) \times 100$, where $\text{control}_{\text{cpm}}$ was the average cpm of the P815 cells cultured alone.

WESTERN BLOT ANALYSIS

T lymphocytes were treated as indicated in 1.5 ml microcentrifuge tubes (5×10^6 cells/tube) for 0–60 min or in 5 ml tubes (2.5×10^6 cells/tube) for 48 h under standard culture conditions, then lysed with ice-cold lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM sodium chloride, 50 mM disodium hydrogen phosphate, 0.25% sodium

deoxycholate [w/v], 0.1% Nonidet P-40 [v/v], 5 mM EDTA, and 5 mM EGTA) containing freshly added protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μM sodium orthovanadate, 10 μM phenylarsine oxide, and 10 μg/ml aprotinin). Samples were incubated on ice for 15–30 min and clarified by centrifugation at 10,000–14,000*g*. Total cell protein was collected and quantified using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc., Mississauga, ON) and BSA standards of known concentration, which were read at 570 nm on a microplate reader. Protein levels were equalized between samples, which were then denatured by the addition of SDS-PAGE sample loading buffer (200 mM Tris–HCl [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], 15% 2-mercaptoethanol [v/v], and 0.01% bromophenol blue [w/v]). Each sample was then heated to 95°C for 5 min and stored at −80°C until use.

Pre-stained protein markers (Bio-Rad Laboratories) and protein samples were resolved on Tris–HCl acrylamide gels (7.5 or 12% acrylamide resolving gels containing 375 mM Tris–HCl [pH 8.8], 0.1% SDS [w/v], 0.1% ammonium persulfate [w/v], and 0.15% tetramethylethylenediamin [v/v] with a 4% acrylamide stacking gel containing 125 mM Tris–HCl [pH 6.8], 0.1% SDS [w/v], 0.1% ammonium persulfate [w/v], and 0.3% tetramethylethylenediamine [v/v]). Gels were electophoresed at 200 V for 1 h in SDS-PAGE running buffer (20 mM Tris–HCl [pH 8.3], 200 mM glycine, and 0.1% SDS [w/v]) and then transferred to nitrocellulose membranes using the iBlot® dry blotting system (Invitrogen). Nitrocellulose membranes were incubated for 1 h at ambient temperature or overnight at 4°C in blocking solution containing 5% fat-free milk [w/v] in Tris-buffered saline (20 mM Tris–HCl [pH 7.6], 200 mM sodium chloride) and 0.05% Tween-20 [v/v] (TBST). Membranes were washed extensively with TBST and then incubated with the appropriate primary antibody for 1 h at ambient temperature or overnight at 4°C. Stock antibodies were typically diluted 1:1000 in TBST containing either 5% fat-free milk or 5% BSA as per the manufacturers' instructions. Following extensive washing with TBST, membranes were incubated with the appropriate HRP-conjugated secondary antibody diluted 1:1000 in TBST containing 5% fat-free milk for 1 h at ambient temperature. Membranes were reacted with enhanced chemiluminescence reagents (GE Healthcare, Baie d'Urfe, QC) for 1 min and exposed to X-ray film (Sci-Med Inc., Truro, NS), which was processed in a Kodak X-OMAT 1000A automated X-ray developer. To confirm equal protein loading, membranes were probed for actin expression. For blots of phosphorylated proteins, membranes were stripped using stripping buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS [w/v], and 100 mM β-mercaptoethanol), and reprobed to determine total protein expression. Protein bands were quantified by densitometry using AlphaEase®FC software (Protein-Simple, Santa Clara, CA) or Scion Image for Windows (Scion Corporation, Frederick, MD).

STATISTICAL ANALYSIS

Statistical analysis was performed using Student's *t*-test or one-way ANOVA with the Tukey-Kramer multiple comparisons post-test, as appropriate, and InStat® analysis software (GraphPad Software Inc., La Jolla, CA).

RESULTS

PIPERINE INHIBITS T LYMPHOCYTE PROLIFERATION

The direct effect of increasing concentrations of piperine (25, 50, and 100 μM) on polyclonal T lymphocyte proliferation induced with anti-CD3 and anti-CD28-coated Dynabeads® was determined by [^{3}H]TdR incorporation. As shown in Figure 1A, the addition of piperine 15–30 min prior to T lymphocyte stimulation (in order to allow time for piperine to enter the cell) resulted in a significant reduction in DNA synthesis by T lymphocytes over 24–96 h of culture. Because 100 μM piperine caused maximal inhibition of T lymphocyte proliferation at all time points, this dose was chosen as the maximum for use in subsequent experiments. In addition, a 100 μM concentration of piperine was well below the solubility limit for piperine in culture medium. Some of the following experiments included 50 μM piperine because this intermediate dose also inhibited DNA synthesis by T lymphocytes at all time points. Potent inhibition of T lymphocyte proliferation was also seen when T lymphocytes were stimulated 24 h prior to piperine treatment (vehicle−31,080 ± 5,160 cpm; 100 μM piperine−1,270 ± 300 cpm at 72 h culture post piperine treatment). The inhibitory effect of piperine was transient and reversible since DNA synthesis was restored when piperine was washed out after 24 h of culture (vehicle −32,650 ± 7,290 cpm; 100 μM piperine−35,200 ± 4,830 cpm at 72 h culture post piperine removal). The recovery of DNA synthesis by activated T lymphocytes following the removal of piperine indicated that piperine was not simply killing T lymphocytes. The lack of a nonspecific toxic effect of piperine on T lymphocytes was confirmed by flow cytometric analysis of Annexin V-FLUOS/PI-stained T lymphocytes, which showed 24 ± 1% cell death in vehicle-treated cultures versus 28 ± 3% cell death in cultures treated with 100 μM piperine. The anti-proliferative effect of piperine was confirmed using T lymphocytes stained with cell-permeable Oregon Green® 488 dye. As shown in Figure 1B, treatment with piperine (100 or 50 μM) inhibited T lymphocyte division induced by Dynabeads®, as indicated by increased fluorescence intensity. Within $CD3^+$ T lymphocyte cultures, $CD8^+$ T lymphocytes were more sensitive to piperine than $CD4^+$ T lymphocytes since both 100 and 50 μM piperine significantly reduced the percentage of $CD8^+$ T lymphocytes undergoing cell division compared to the vehicle control, whereas only 100 μM piperine significantly reduced the percentage of $CD4^+$ T lymphocytes undergoing division (Fig. 1C). In contrast, piperine had a significant inhibitory effect on the proliferation of purified $CD4^+$ T lymphocytes at both 100 and 50 μM, whereas only 100 μM piperine significantly inhibited the proliferation of purified $CD8^+$ T lymphocytes (Fig. 1D). Piperine also inhibited antigen-specific proliferation of OVA-specific OT-II T lymphocytes following stimulation with OVA-loaded DCs (Fig. 1E). Taken together, these data indicate that piperine inhibited polyclonal and antigen-specific proliferation of $CD4^+$ and $CD8^+$ T lymphocytes.

PIPERINE-MEDIATED INHIBITION OF T LYMPHOCYTE PROLIFERATION IS INDEPENDENT OF TRPV1

Since the cation channel TRPV1 is a cell surface receptor for piperine [McNamara et al., 2005], we examined the possible involvement of TRPV1 in piperine-mediated inhibition of

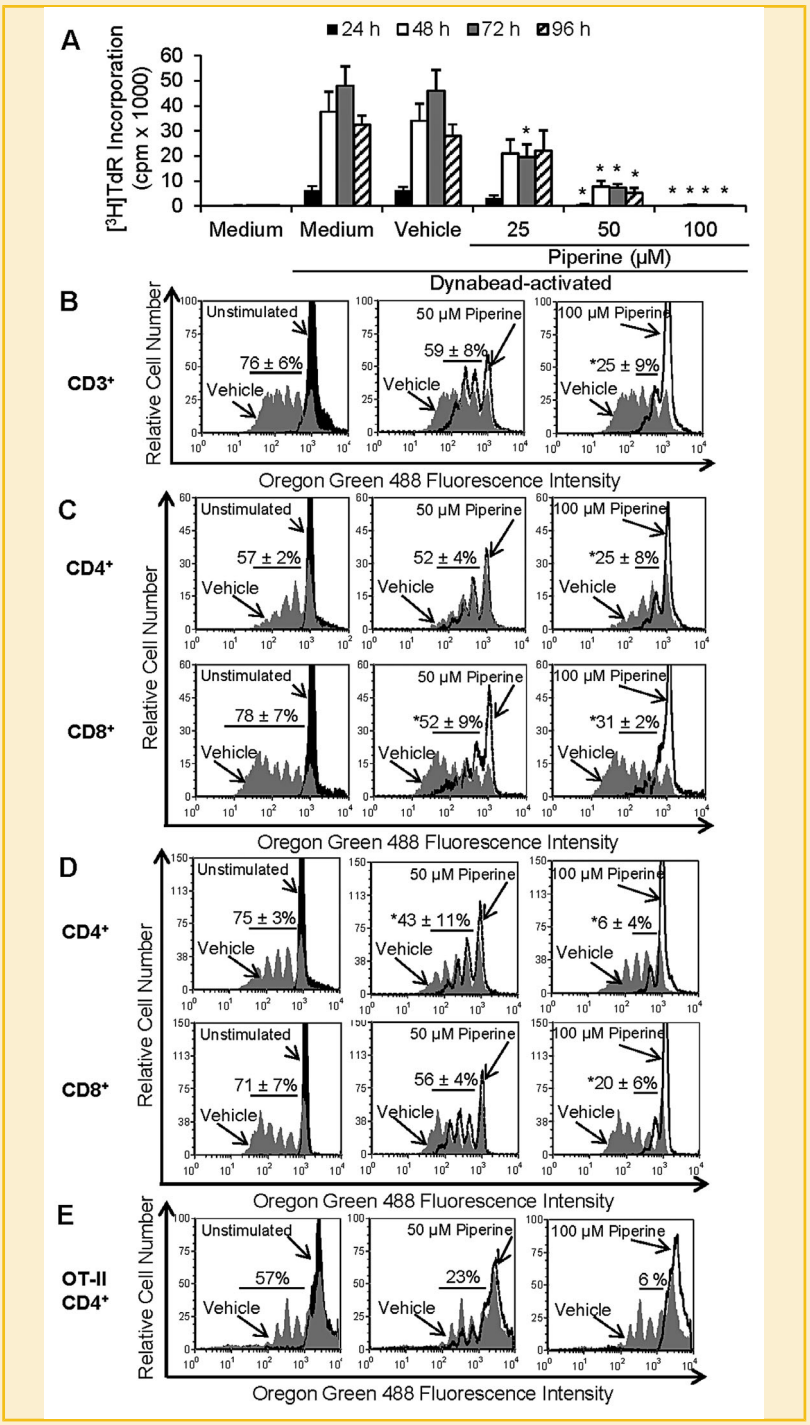

Fig. 1. Piperine inhibits T lymphocyte DNA synthesis and cell division. (A) $CD3^+$ T lymphocytes were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads® for the indicated times. T lymphocytes were pulsed with [^{3}H]TdR for the last 6 h of incubation and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of three independent experiments ± SEM. (B,C) $CD3^+$ T lymphocytes and (D) $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes were stained with Oregon Green® 488 dye, treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured for 72 h with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads®. $CD3^+$ T lymphocytes were stained with anti-CD4-PE or anti-CD8-PE antibodies or their respective isotype controls for examination of CD4 and CD8 subpopulations, respectively. (E) Oregon Green 488-labeled $CD4^+$ OT-II T lymphocytes were co-cultured with OVA-pulsed bone marrow-derived DCs for 72 h in the absence or presence of piperine (50, 100 μM). Flow cytometry was used to measure cell division by quantifying the Oregon Green® 488 fluorescence intensity. Data shown are from representative experiments comparing activated T lymphocytes treated with DMSO vehicle (closed grey peaks) to unstimulated cells (closed black peaks), activated T lymphocytes treated with 100 μM piperine (open black peaks), and activated T lymphocytes treated with 50 μM piperine (open dotted peaks). Numbers shown are the average percentage of T lymphocytes undergoing division compared to the total T lymphocyte population ± SEM of three independent experiments; * denotes $P < 0.05$ compared to the appropriate vehicle control, as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

T lymphocyte proliferation. RT-PCR analysis showed that TRPV1 mRNA was not present in naïve T lymphocytes (Fig. 2A). The spinal cord from WT C57BL/6 mice, which contains TRPV1-expressing dorsal root ganglia [Greffrath et al., 2003], was used as a positive control. The lack of TRPV1 involvement in the suppressive effect of piperine on T lymphocyte proliferation was confirmed by stimulating T lymphocytes from TRPV1$^{-/-}$ mice in the absence or presence of piperine in parallel with T lymphocytes from WT mice. As shown in Figure 2B, there was no significant difference in the inhibitory effect of piperine on T lymphocyte proliferation regardless of the TRPV1 genotype, indicating that piperine did not act via TRPV1 to inhibit T lymphocyte proliferation.

PIPERINE INHIBITS T LYMPHOCYTE ENTRY INTO THE S AND G_2/M PHASES OF THE CELL CYCLE

Cell cycle analysis revealed a decreased percentage of T lymphocytes in the S and G_2/M phases of the cell cycle, as well as a modest increase in the percentage of T lymphocytes in the G_0/G_1 phase of the cell cycle following T lymphocyte activation in the presence of 100 μM piperine (Fig. 3A). A piperine-induced G_0/G_1 blockade was confirmed by western blot analysis (Fig. 3B), which showed reduced expression of G_1-associated cell cycle proteins cyclin D3, CDK4, and CDK6 in piperine-treated T lymphocytes. Piperine therefore caused a partial block in cell cycle progression at the G_0/G_1 phase by inhibiting the expression of cell cycle regulatory proteins, which impaired T lymphocyte entry into the S and G_2/M phases of the cell cycle.

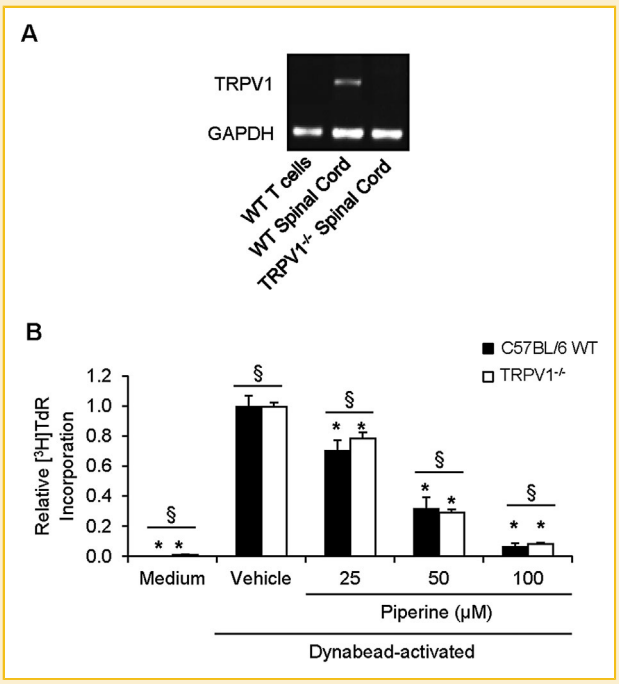

Fig. 2. Piperine does not mediate effects on T lymphocytes via TRPV1. (A) RNA was extracted from C57BL/6 WT and TRPV1$^{-/-}$ spinal cords, as well as from WT T lymphocytes. RNA was reverse transcribed into cDNA, and then amplified for TRPV1 or GAPDH. Data shown are from a representative experiment (n = 3). (B) CD3$^+$ WT and TRPV1$^{-/-}$ T lymphocytes were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured for 48 h with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads®. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean ± SEM of three independent experiments, each normalized to activated control T lymphocytes; * denotes $P < 0.05$ compared to the vehicle control, § denotes "not significant" for C57BL/6 WT versus TRPV1$^{-/-}$ comparisons, as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

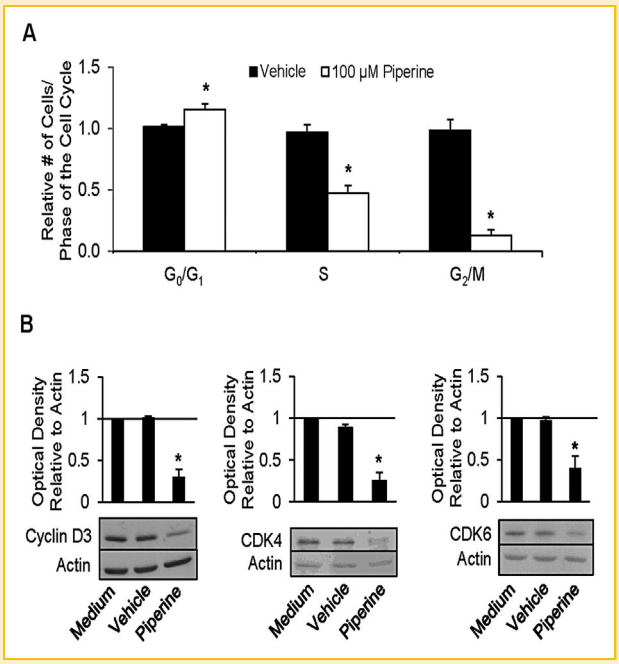

Fig. 3. Piperine inhibits cell cycle progression of T lymphocytes. (A) CD3$^+$ T lymphocytes were treated with medium, vehicle (DMSO), or 100 μM piperine and cultured with anti-CD3 and anti-CD28 antibody-coated Dynabeads® for 72 h. Cells were fixed with ice-cold 70% ethanol for at least 24 h and stained with the DNA-intercalating dye PI for cell cycle analysis by flow cytometry. The number of cells in each phase of the cell cycle was determined by ModFit software analysis and normalized to T lymphocytes activated with Dynabeads® in the presence of medium alone; * denotes $P < 0.05$ compared to the vehicle control, as determined by Student's *t*-test. (B) CD3$^+$ T lymphocytes were treated with medium, vehicle (DMSO), or 100 μM piperine and activated with anti-CD3 and anti-CD28 antibody-coated Dynabeads® for 48 h prior to lysis. Total protein was collected for western blot analysis. Membranes were probed with anti-cyclin D3, anti-CDK4, or anti-CDK6 antibodies, washed, and then probed with anti-actin antibody to confirm equal protein loading. Optical density ratio was calculated as the expression of cyclin D3, CDK4, or CDK6 relative to actin, normalized to the activated medium control. Data shown are the mean of four independent experiments ± SEM with one representative experiment; * denotes $P < 0.05$ compared to the vehicle control, as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

PIPERINE INHIBITS CD25 BUT NOT CD69 EXPRESSION

Activated T lymphocytes up-regulate cell surface expression of CD25 and CD69 [Trevillyan et al., 1990]. As shown in Figure 4A, T lymphocytes that were stimulated with Dynabeads® in the presence of 100 μM piperine showed a substantial reduction in CD25 expression but only slightly reduced CD69 expression. Additionally, Figure 4B shows that the relative number of CD25$^+$ T lymphocytes was significantly reduced following piperine treatment for 24 h;

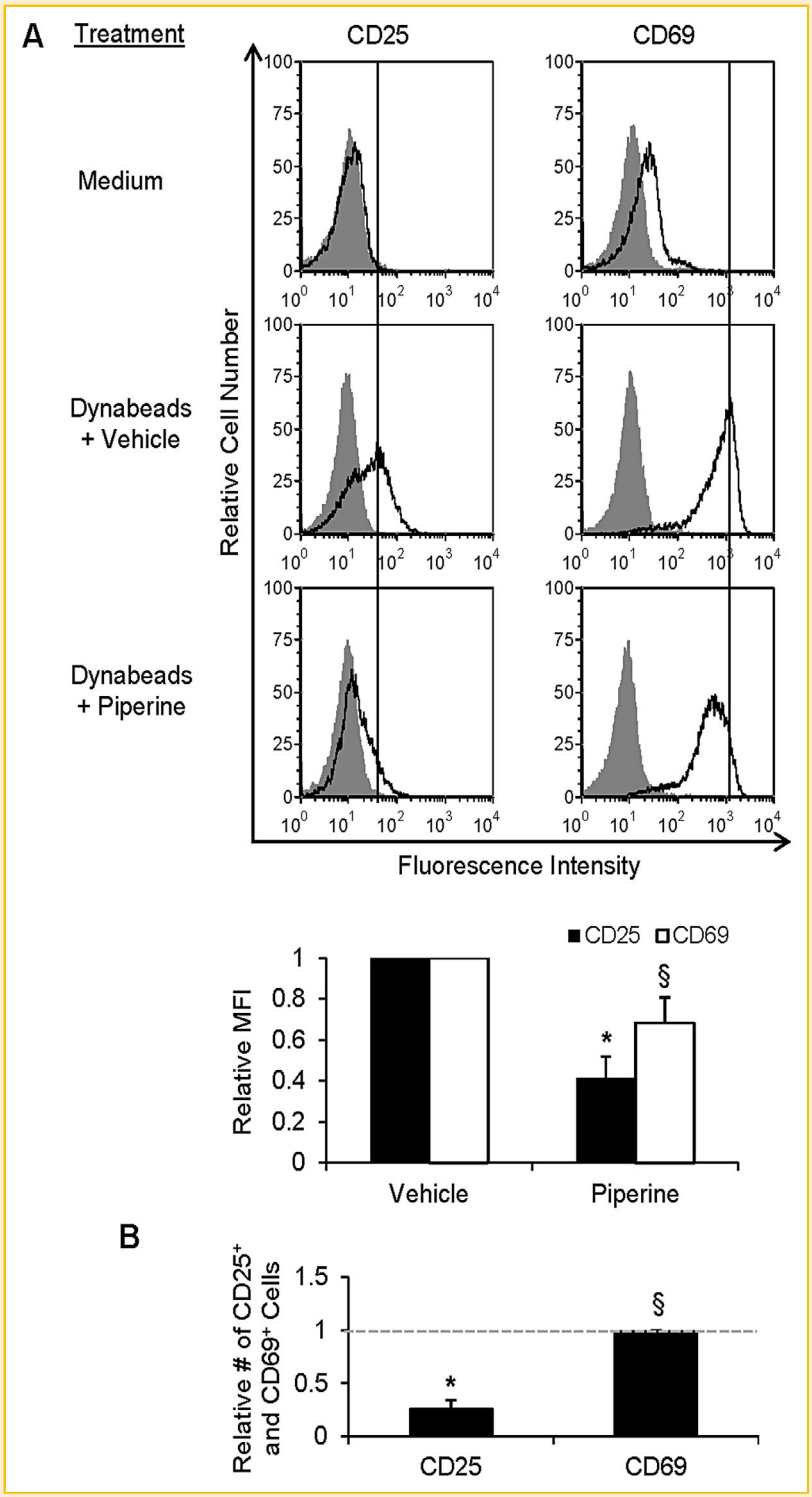

Fig. 4. Piperine inhibits T lymphocyte expression of CD25 but not CD69. $CD3^+$ T lymphocytes were treated with medium, vehicle (DMSO), or 100 μM piperine and cultured for 24 h with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads®. Cells were stained with anti-CD25-FITC, anti-CD69-FITC, or the appropriate isotype control antibodies, fixed, and analysed by flow cytometry. (A) Data shown are from one representative experiment and the mean fluorescence intensity (MFI) of three independent experiments ± SEM, normalized to the vehicle control. Closed gray peaks depict background staining by isotype control antibodies while open black peaks depict CD25 or CD69 expression. (B) The numbers of CD25- and CD69-expressing T lymphocytes following treatment with 100 μM piperine were normalized to the appropriate vehicle control (grey dotted line). Data shown are the mean of three independent experiments ± SEM; * denotes $P < 0.05$ and § denotes "not significant" when compared to the appropriate vehicle control, as determined by Student's *t*-test.

however, there was no decrease in the relative number of CD69-expressing T lymphocytes. These findings indicate that piperine did not simply inhibit all protein expression. Rather, piperine had specific effects on T lymphocytes and inhibited signaling pathways that induce CD25, but not CD69.

PIPERINE INHIBITS EFFECTOR T LYMPHOCYTE INDUCTION

Cytokine production by $CD4^+$ T helper lymphocytes is an important component of cell-mediated immunity; IFN-γ, IL-4, and IL-17A are representative of the Th1, Th2, and Th17 T helper lymphocyte subsets, respectively [Zygmunt and Veldhoen, 2011], whereas IL-2 drives T lymphocyte proliferation but is also important for immune regulation via its effect on T regulatory lymphocytes [Bayer et al., 2013]. Consistent with our finding that piperine inhibited the proliferation of $CD4^+$ T lymphocytes, we observed that T lymphocytes stimulated with Dynabeads® in the presence of 100 μM piperine synthesized significantly less IL-2, IFN-γ, IL-4, and IL-17A; however, only IL-2 and IFN-γ synthesis was inhibited by 50 μM piperine (Fig. 5A). In addition, piperine-mediated inhibition of IFN-γ production by WT and $TRPV1^{-/-}$ T lymphocytes was equivalent (Fig. 5B), which was in line with a TRPV1-independent mechanism of T lymphocyte suppression by piperine. These data indicate that higher doses of piperine caused a general inhibition of cytokine production whereas lower concentrations of piperine had a more selective effect on IL-2 and IFN-γ synthesis. Interestingly, the addition of exogenous IL-2 (50 U/ml) did not rescue the proliferation of T lymphocytes treated with 100 μM piperine (piperine—98% inhibition; piperine plus IL-2–98% inhibition, at 72 h of culture), suggesting that decreased IL-2 production and reduced IL-2 utilization due to decreased CD25 expression both contribute to the inhibitory effect of piperine on T lymphocyte proliferation.

Because IL-2 and IFN-γ are important for the induction of cytotoxic T lymphocytes [Maraskovsky et al., 1989], we used a cell-mediated cytotoxicity assay to determine the effect of piperine on the development of cytotoxic effector function. $CD8^+$ T lymphocytes that were treated with 100 μM piperine during their differentiation into cytotoxic T lymphocytes showed significantly decreased cytotoxic activity against P815 target cells (vehicle—64 ± 1% cytotoxicity; piperine—39 ± 2% cytotoxicity, at an effector to target cell ratio of 50:1, $P < 0.05$). In contrast, the addition of 100 μM piperine to the cell-mediated cytotoxicity assay did not substantially

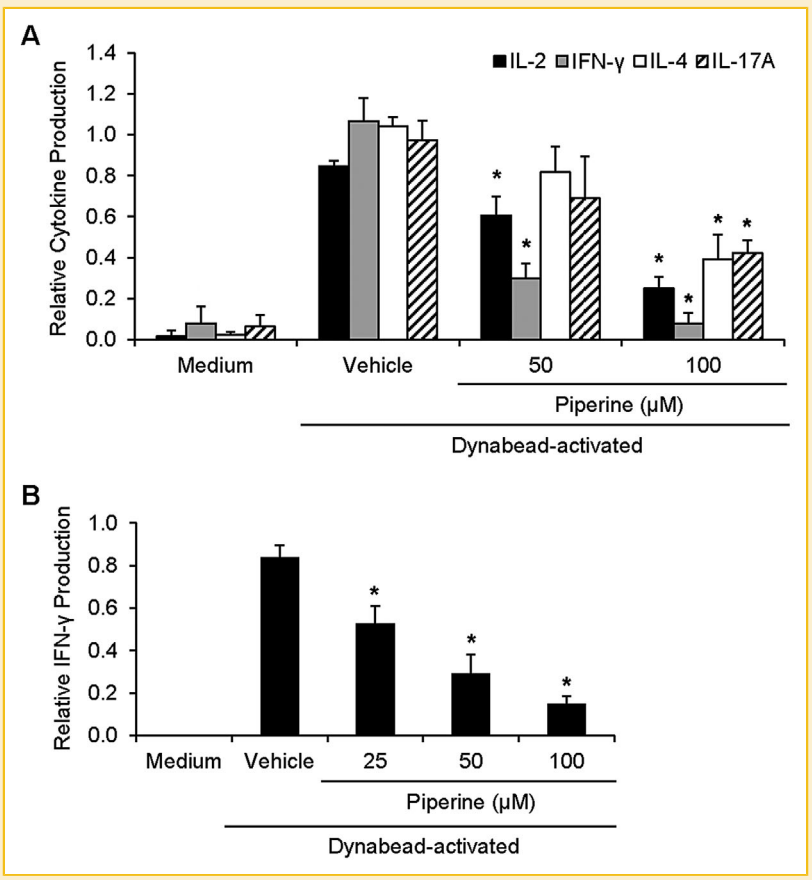

Fig. 5. Piperine inhibits T lymphocyte synthesis of IL-2, IL-4, IFN-γ, and IL-17A. $CD3^+$ T lymphocytes from (A) C57BL/6 WT mice and (B) $TRPV1^{-/-}$ mice were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured for 24 h with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads®. Supernatants were collected and analyzed for IL-2, IL-4, IFN-γ, and IL-17A cytokine content by sandwich-ELISA. Cytokine expression was normalized to the activated medium control. Data shown are the mean of three independent experiments ± SEM; * denotes $P < 0.05$ when compared to the appropriate vehicle control, as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

affect killing of target cells by cytotoxic T lymphocytes that were induced in the absence of piperine (vehicle—48 ± 6% cytotoxicity; piperine—40 ± 6% cytotoxicity, at an effector to target cell ratio of 50:1), indicating that piperine did not inhibit the release of preformed cytotoxic effector molecules and/or Fas/Fas ligand interactions between cytotoxic T lymphocytes and target cells. Piperine therefore inhibited the differentiation of naive $CD8^+$ T lymphocytes into cytotoxic T lymphocytes but did not affect pre-existing cytotoxic effector function. Piperine-mediated inhibition of cytotoxic effector cell induction is consistent with our finding that $CD8^+$ T lymphocytes were sensitive to growth inhibition by piperine.

PIPERINE INHIBITS PHOSPHORYLATION OF AKT, ERK2, AND IκBA BUT NOT ZAP-70

T lymphocyte activation involves ZAP-70 and Akt, which are proximal components of the TCR/CD3 signaling pathway, as well as ERK 1/2 and NFκB, which are distal to TCR/CD3 [Kannan et al., 2012]. Figure 6A shows that phosphorylation of ZAP-70 at Tyr 319 was not altered in T lymphocytes stimulated with Dynabeads® in the presence of 100 μM piperine. In contrast, phosphorylation of Akt at Ser 473 was significantly inhibited by 100 μM piperine (Fig. 6B). In addition, 100 μM piperine significantly inhibited the phosphorylation of ERK2 (Fig. 6C) and IκBα (Fig. 6D) at 60 min and 15 min,

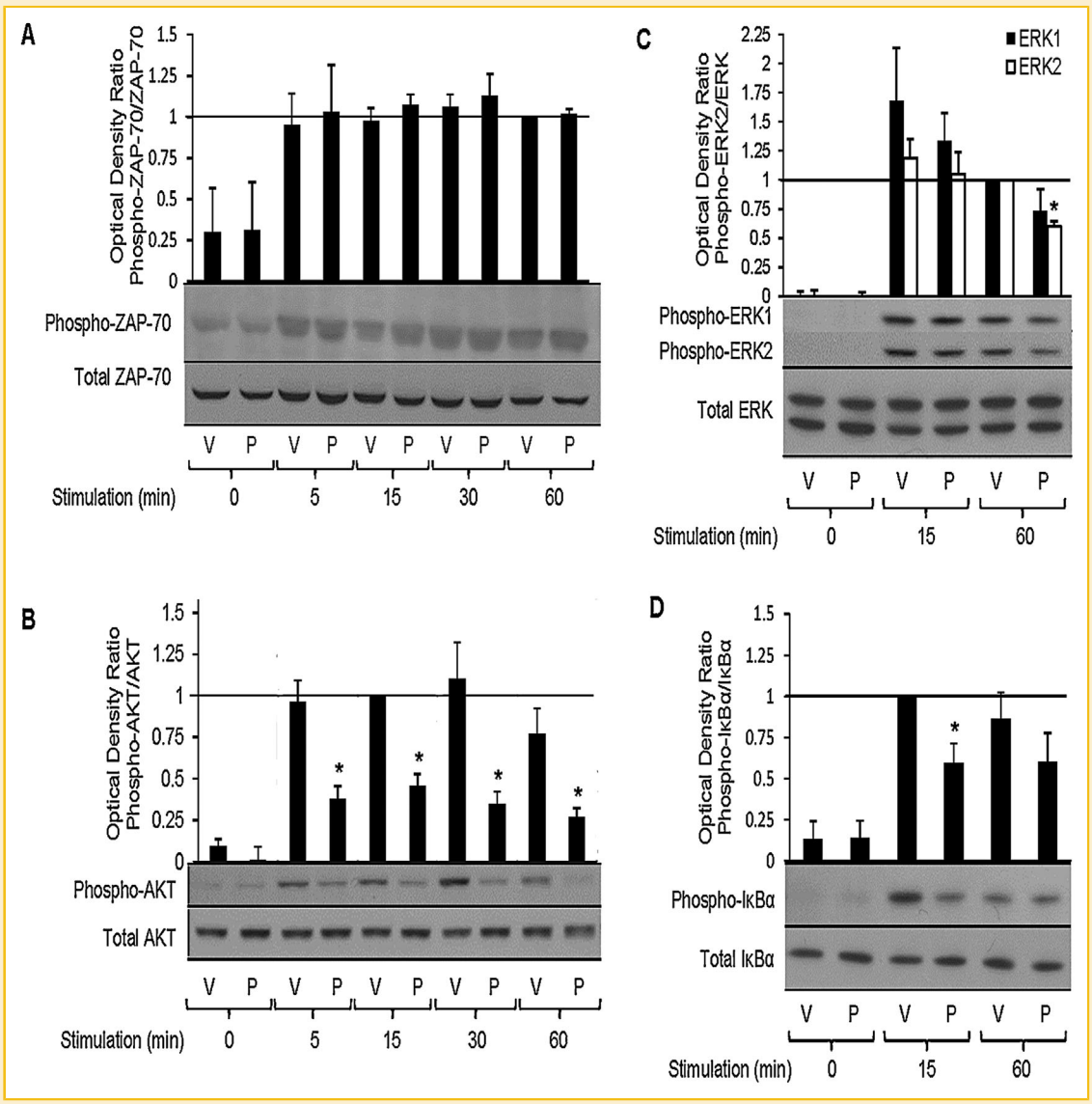

Fig. 6. Piperine has differential effects on Akt, ERK, IκBα, and ZAP-70 phosphorylation. $CD3^+$ T lymphocytes were treated with DMSO vehicle (V) or 100 μM piperine (P) for 30 min prior to culture with or without anti-CD3 and anti-CD28 antibody-coated Dynabeads® for the indicated times. Cells were then lysed, and total protein was collected for western blot analysis. Membranes were probed with antibodies for (A) phospho-ZAP-70, (B) phospho-Akt, (C) phospho-ERK1/2, or (D) phospho-IκBα, stripped, and then probed with antibodies for (A) ZAP-70, (B) Akt, (C) ERK1/2, or (D) IκBα to confirm equal protein loading. Optical density ratio was calculated as the ratio of phosphorylated protein expression to total expression, normalized to vehicle treatment at 15 or 60 min post-activation. Data shown are the mean of at least three independent experiments ± SEM with one representative experiment; * denotes $P < 0.05$ when compared to the vehicle control, as determined by Student's *t*-test.

respectively, post stimulation. Taken together, these data indicate that piperine inhibited the activation of several key signaling molecules involved in the activation of T lymphocytes.

DISCUSSION

Piperine is known to have diverse physiological effects [Srinivasan, 2007]; however, the impact of piperine on T lymphocyte function has not been explored. In this study, we show that piperine inhibited polyclonal and antigen-specific proliferation of mouse T lymphocytes, as well as cytokine synthesis and the induction of cytotoxic effector cells. These actions were not mediated via TRPV1, which is a known cell surface receptor for piperine [McNamara et al., 2005]. This latter finding is consistent with an earlier report that mouse spleen cells, lymph node cells, and EL4 T lymphoma cells do not express TRPV1 [Inada et al., 2006]. Importantly, piperine-mediated inhibition of T lymphocyte activation was not associated with any reduction in cell viability. The anti-proliferative action of piperine in T cell cultures is in line with our earlier finding that piperine prevents the proliferation of endothelial cells [Doucette et al., 2013]. In addition, as with T cells, piperine-treated endothelial cells also experience a block at the G_0/G_1 phase of the cell cycle, as well as decreased cyclin D3 expression [Doucette et al., 2013], suggesting that this effect of piperine is common to different cell types.

T cell activation in the presence of piperine resulted in a failure to upregulate CD25 expression whereas there was a near normal increase in CD69 expression. CD25 and CD69 expression is regulated by the transcription factors NFAT, NF-κB, and AP-1 [Castellanos et al., 1997; Kim and Leonard, 2002]; however, CD25 induction occurs 6–8 h after T lymphocyte activation whereas CD69 is induced as early as 30 min as a consequence of early signaling via the Ras-Raf1-ERK pathway [D′Ambrosio et al., 1994; Taylor-Fishwick and Siegel, 1995]. The rapid expression of CD69 may have prevented its inhibition by piperine whereas the slower kinetics of CD25 expression could have provided sufficient time for piperine to inhibit CD25-inducing signaling pathways. Protein kinase C (PKC) inhibition by piperine may also contribute to the selective effect of piperine on CD25 expression since PKC is more important for CD25 expression than CD69 expression [Risso et al., 1991], and piperine has been shown to inhibit PKC isoforms in HT-1080 fibrosarcoma cells [Hwang et al., 2011].

T lymphocytes that were activated in the presence of piperine synthesized significantly less IL-2, IFN-γ, IL-4, and IL-17A. Decreased IL-2 production in combination with the profound inhibition of CD25 (the α subunit of the high affinity IL-2 receptor) explains the anti-proliferative effect of piperine since IL-2 drives T lymphocyte proliferation [Bayer et al., 2013]. Piperine also decreases IL-4 levels in the bronchoalveolar lavage fluid in an OVA-induced asthma model in mice and inhibits IL-4 production by OVA-stimulated splenic T lymphocytes ex vivo, but fails to affect IFN-γ production by OVA-stimulated splenic T lymphocytes [Kim and Lee, 2009]. However, the T lymphocytes used in the study by Kim and Lee were previously polarized to a Th2 phenotype in vivo and therefore produced only low levels IFN-γ whereas we used unpolarized T lymphocytes that produce abundant IFN-γ upon stimulation. In spite of the apparent general inhibition of T lymphocyte cytokine production by piperine documented in the present study, tumor necrosis factor α production by mouse macrophages is selectively inhibited by piperine since the phytochemical does not affect IL-6 or IL-1β synthesis by macrophages [Bae et al., 2010]. Piperine may therefore inhibit the synthesis of specific cytokines under certain conditions, and/or act in a cell type-specific manner. Interestingly, IL-2 and IFN-γ synthesis was most sensitive to piperine-mediated inhibition, suggesting that the greatest impact of piperine is on overall T lymphocyte proliferation and Th1 effector function. This is consistent with the ability of piperine to interfere with the induction of cytotoxic T lymphocytes, which require both IL-2 and IFN-γ for the expression of cytotoxic effector molecules [Maraskovsky et al., 1989]. The profound inhibitory effect of piperine on IL-2 synthesis and CD25 expression suggests that piperine may also impact T regulatory lymphocytes, which require IL-2 for their development and function [de la Rosa et al., 2004; Burchill et al., 2007]. This is a potentially important issue since an inhibitory effect of piperine on regulatory T lymphocytes may diminish the overall effectiveness of piperine in suppressing T lymphocyte-mediated inflammation.

Importantly, piperine inhibited Akt, ERK, and NF-κB components of the TCR/CD3 signaling pathway but did not diminish ZAP-70 signaling. We conclude that piperine-induced suppression of Akt, ERK, and NF-κB activation likely accounts for the reduced proliferation and effector function of piperine-treated T lymphocytes since Akt, ERK, and NF-κB are important signaling intermediates involved in T lymphocyte activation, proliferation, and acquisition of effector function. Akt releases T lymphocytes from FoxO-induced cell cycle arrest and induces cell cycle progression by inhibiting $p27^{Kip1}$ via GSK-3β inhibition [Appleman et al., 2002]. The importance of the ERK pathway is indicated by the finding that pharmacologic inhibitors of this pathway decrease T lymphocyte proliferation [DeSilva et al., 1998; Dumont et al., 1998]. T lymphocytes expressing a dominant IκB mutant that constitutively inhibits NF-κB activity have a reduced proliferative capacity [Boothby et al., 1997]. Consistent with our findings, piperine also inhibits Akt phosphorylation in growth factor-stimulated endothelial cells [Doucette et al., 2013]. Since Akt phosphorylation at Ser 473 is regulated by PKC α in T lymphocytes [Yang et al., 2010], and PKC α is inhibited by piperine in HT-1080 fibrosarcoma cells [Hwang et al., 2011], it is reasonable to speculate that decreased Akt activity in piperine-treated T lymphocytes resulted from PKC α inhibition. In line with piperine-mediated inhibition of ERK2 phosphorylation in T lymphocytes that were stimulated with Dynabeads®, piperine inhibits ERK activation in other cell types, including phorbol 12-myristate 13-acetate (PMA)-stimulated HT-1080 fibrosarcoma cells and IL-1β-stimulated synoviocytes [Bang et al., 2009]. Downstream of ERK, piperine inhibits the PMA-induced transcriptional activity of AP-1 [Hwang et al., 2011], as well as, tumor necrosis factor α-induced nuclear localization of c-fos [Pradeep and Kuttan, 2004]. Interestingly, IFN-γ, IL-2, IL-4, and IL-17A synthesis is in part regulated by AP-1 [Hermann-Kleiter and Baier, 2010], suggesting that reduced ERK activation in the presence of piperine may contribute to the inhibitory effect of piperine on T lymphocyte cytokine production. Conversely, piperine increases basal levels of ERK phosphorylation in immortalized auditory cells [Choi et al.,

2007]. Piperine therefore has different effects on inducible versus constitutive ERK phosphorylation. As in T lymphocytes, piperine inhibits IκBα phosphorylation and its subsequent degradation in PMA-stimulated HT-1080 cells and tumor necrosis factor α-stimulated endothelial cells [Kumar et al., 2007]. Nuclear localization of active NF-κB, which requires phosphorylation-dependent IκBα degradation [Vancurova and Vancura, 2012], is also inhibited in piperine-treated HT-1080 fibrosarcoma cells and B16-F10 melanoma cells [Pradeep and Kuttan, 2004]. However, the effect of piperine on NF-κB transcriptional activity varies with cell type since cerulean-induced degradation of IκBα in pancreatic acinar cells and IL-1β-induced activation of NF-κB in synoviocytes are not altered by piperine [Bang et al., 2009].

The concentrations of piperine used in our in vitro study were substantially higher than could likely be obtained in the plasma by diet alone; however, a peak plasma concentration of approximately 20 μM is achievable in rats by oral gavage of piperine at 54 mg/kg [Liu et al., 2011], suggesting that oral dosing of piperine might be sufficient to affect T lymphocyte function. Indeed, oral piperine has recently been shown to ameliorate acetic acid-induced inflammatory bowel disease in mice [Gupta et al., 2015]. Importantly, concentrations of piperine as high as 100 μM have little effect on the viability of normal human endothelial cells and epithelial cells [Doucette et al., 2013; Greenshields et al., 2015], suggesting that systemic administration of piperine at inhibitory concentrations for T lymphocytes should have little toxicity in humans. Alternatively, nanoparticles could be used for the localized delivery of piperine to inflamed tissues since nanoparticles naturally accumulate in areas of high vascular permeability that characterize sites of inflammation [Clares et al., 2012]. Interestingly, pharmacokinetic studies show that lipid nanosphere-encapsulated piperine has a lower rate of clearance and increased efficacy [Priprem et al., 2011]. The use of nanotechnology to deliver piperine to sites of chronic inflammation and/or autoimmunity for reduction or prevention of tissue damage associated with excessive T lymphocyte proliferation and effector function therefore warrants further investigation.

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