STUDIES ON THE T CELL SUPPRESSIVE AND ANTI-ANGIOGENIC ACTIVITIES OF THE DIETARY PHYTOCHEMICAL PIPERINE

by

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DEPARTMENT OF PATHOLOGY

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For my amazing husband

Were I to await perfection, it would never be finished.

-- Chinese Proverb

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ABSTRACT

Piperine, a pungent alkaloid found in the fruits of long and black pepper plants, has diverse physiological effects, including anti-inflammatory and anti-cancer activities. The effect of piperine on the function of T cells and endothelial cells, two important elements of inflammation, have not been examined previously and were the focus of this study. Piperine inhibited the proliferation of human endothelial cells, murine T cells, and IL-2-dependent CTLL-2 T cells, without affecting cell viability. Progression into the S phase of the cell cycle was inhibited in all three cell types. In T cells, piperine inhibited expression of the early activation marker CD25, production of IFN-γ, IL-2, IL-4, and IL-17A, and the generation of cytotoxic effector cells. In endothelial cells, piperine inhibited migration and tubule formation *in vitro* and *ex vivo*, as well as breast cancer cell-induced angiogenesis in chick embryos. Piperine inhibited Akt phosphorylation in signaling pathways associated with growth factor receptors on endothelial cells, T cell receptor and CD28 on T cells, and IL-2 receptor on CTLL-2 cells. Additionally, piperine inhibited ERK1/2 and IκB phosphorylation in activated T cells, as well as STAT3, STAT5, and ERK1/2 phosphorylation in IL-2-stimulated CTLL-2 cells. However, piperine is not a broad-spectrum inhibitor of phosphorylation as it did not inhibit ZAP-70 phosphorylation in activated T cells or phosphorylation of JAK1 and JAK3 in IL-2-stimulated CTLL-2 cells. Piperine-mediated inhibition of T cell activation and IL-2 receptor signaling suppresses T cell proliferation and effector cell differentiation, suggesting possible utility in treating T cell-mediated autoimmune and chronic inflammatory conditions. Additionally, the potent anti-angiogenic activity of piperine warrants further study for the prevention of inflammation- and cancer-promoting angiogenesis.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$[^3H]TdR$	Tritiated-Thymidine
α	Alpha
Ab	Antibody
Abs	Antibodies
Ang	Angiopoietin
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
APS	Ammonium Persulfate
ATCC	American Tissue Culture Collection
β	Beta
β-ME	Beta-Mercaptoethanol
BaP	Benzo(a)pyrene
Bcl	B-Cell Lymphoma
BCRP	Breast Cancer Resistant Protein
BCTC	N-(4-t-Butylphenyl)-4-(3-Chloropyridin-2-yl)Tetrahydropyrazine-1(2H)-Carboxamide
bp	Base Pair
BSA	Bovine Serum Albumin
°C	Degrees Celsius
c	Complete
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CAM	Chorioallantoic Membrane
CARMA	Caspase Recruitment Domain-Containing Membrane-Associated Guanylate Kinase Protein
CBM	CARMA1-Bcl10-MALT1 Complex
CD	Cluster of Differentiation
CD25	IL-2Rα Chain
CD40L	CD40 Ligand

CDC25	Cell Division Cycle 25
CDK	Cyclin-Dependent Kinase
cDNA	Complementary DNA
CHX	Cycloheximide
CIS	Cytokine Inducible SH2 Protein
CMFDA	5-Chloromethylfluorescein Diacetate
CNH	N′-((4-Oxo-4H-Chromen-3-yl)Methylene)Nicotinohydrazide
cpm	Counts Per Minute
CsA	Cyclosporin A
CTL	Cytotoxic T Lymphocyte
CTLL-2	Cytotoxic Lymphoid Line-2
CYP	Cytochrome P450
δ	Delta
DAG	Diacylglycerol
DC	Dendritic Cell
DEPC	Diethylpyrocarbonate
DLA	Dalton's Lymphoma Ascites
DMBA	Dimethylbenz-α-Anthracene
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DSS	Dextran Sulfate Sodium
DTH	Delayed-Type Hypersensitivity
DTT	Dithiothreitol
ε	Epsilon
EAC	Ehrlich Ascites Carcinoma
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbant Assay

eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal-Regulated Kinase
EtOH	Ethanol
F	Forward
FACS	Fluorescence-Activated Cell Sorting
FasL	Fas Ligand
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FGFR	FGF Receptor
FITC	Fluorescein Isothiocyanate
FK506	Tacrolimus
Fox	Forkhead Box
γ	Gamma
g	Gravity
Gab2	Grb2-Associated Binding Protein 2
Gads	Grb2-related Adaptor Downstream of Shc
GEF	Guanine Exchange Factor
Grb2	Growth Factor Receptor-Bound Protein 2
GSK	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate
h	Hour
HCl	Hydrogen Chloride
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HIF	Hypoxia-Inducible Factor
HMC-1	Human Mastocytoma Cell Line
HRP	Horseradish Peroxidase
HTLV	Human T-Lymphotropic Virus
hTRPV1	Human TRPV1
HUVECs	Human Umbilical Vein Endothelial Cells
IAPs	Inhibitor of Apoptosis Proteins
IBD	Inflammatory Bowel Disease

ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IFNAR	IFN-α Receptor
Ig	Immunoglobulin
IGF	Insulin-Like Growth Factor
IκB	Inhibitor of κB
IKK	IκB Kinase
IL	Interleukin
IL-2R	IL-2 Receptor
IL-4R	IL-4 Receptor
IL-6R	IL-6 Receptor
IL-7R	IL-7 Receptor
IL-23R	IL-23 Receptor
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol Trisphosphate
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
Itk	IL-2-Inducible T cell Kinase
iTregs	Inducible T Regulatory Cells
JAK	Janus Kinase
JNK	c-Jun N-Terminal Kinase
κ	Kappa
kDa	Kilodaltons
LAT	Linker of Activated T Cells
LCMV	Lymphocytic Choriomeningitis Virus
LDH	Lactate Dehydrogenase
LO	Lipoxygenase
LPS	Lipopolysaccharide
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MALT	Mucosa-Associated Lymphoid Tissue
MAPK	Mitogen-Activated Protein Kinase

MEK	MAPK/ERK Kinase
MCF	Mean Channel Fluorescence
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Complex
min	Minute
MMPs	Matrix Metalloproteinases
mRNA	Messenger RNA
MRP	Multidrug Resistant Protein
MS	Multiple Sclerosis
mTOR	Mammalian Target of Rapamycin
mTRPV1	Murine TRPV1
NaCl	Sodium Chloride
Na_2HPO_4	Disodium Hydrogen Phosphate
NaF	Sodium Fluoride
NaN_3	Sodium Azide
Na_3VO_4	Sodium Orthovanadate
Nck	Non-Catalytic Region of Tyrosine Kinase Adaptor Protein
NF-κB	Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells
NFAT	Nuclear Factor of Activated T cells
NK	Natural Killer
NO	Nitric Oxide
NP-40	Nonidet P-40
nTregs	Natural T Regulatory Cells
OVA	Ovalbumin
p	Phosphorylated
P-gp	P-Glycoprotein
PAGE	Polyacrylamide Gel Electrophoresis
PAMPs	Pattern-associated Molecular Patterns
PAO	Phenylarsine Oxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PDGF	Platelet-Derived Growth Factor
PDK	Phosphoinositide-Dependent Kinase
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
PH	Pleckstrin Homology
PHA	Phytohemagglutinin
PI	Propidium Iodide
PI3K	Phosphoinositide-3 Kinase
PIAS	Protein Inhibitors of Activated STAT
PIP_2	Phosphatidylinositol Bisphosphate
PIP_3	Phosphatidylinositol Triphosphate
PKC	Protein Kinase C
PKD	Protein Kinase D
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
pMHC	Peptide-Major Histocompatibility Complex
PMSF	Phenylmethylsulfonyl Fluoride
PRR	Pattern Recognition Receptors
PTK	Protein Tyrosine Kinase
R	Reverse
RasGRP	Ras Guanyl Nucleotude-Releasing Protein
Rb	Retinoblastoma protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
SDS	Sodium Dodecyl Sulphate
sec	Second
SEM	Standard Error of the Mean
Ser	Serine

SH2	Src Homology 2
Shc	Src Homology and Collagen Protein
SHP	SH2 Domain-Containing Protein
SLP-76	SH2 Domain-Containing Leukocyte Protein of 76 kDa
SOCS	Suppressors of Cytokine Signaling
SOS	Son of Sevenless
STAT	Signal Transducer and Activator of Transcription
θ	Theta
TAE	Tris-Acetate-EDTA
TBST	Tris-Buffered Saline and Tween-20
TCR	T Cell Receptor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming Growth Factor
Th	T Helper Cell
Thr	Threonine
TMB	Tetromethylbenzidine
TNF	Tumor Necrosis Factor
Tregs	T Regulatory Cells
TRPV	Transient Receptor Potential Vanilloid
Tyr	Tyrosine
UDP	Uridine Diphosphate
UV	Ultraviolet
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor
WT	Wildtype
ζ	Zeta
ZAP-70	Zeta-Chain-Associated Protein Kinase 70 kDa

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CHAPTER 1

INTRODUCTION

1.1.1 Overview of the Immune System

Inflammation is a localized reaction to irritation, injury, or infection and is mediated by the immune system as a protective mechanism for the host [1]. The immune system is made up of a variety of cell types that work together to protect the host against anything that is considered "non-self", including foreign substances, pathogens, and cancer. The immune system has two main arms; the first arm is innate immunity, which is the host's first line of defense against pathogens and is made up of physical and physiological barriers, recruitment and activation of phagocytic cells, and the production of soluble mediators of inflammation [2]. The innate immune response is very rapid and identifies pathogens by pattern recognition receptors (PRR) on the innate immune cells that bind to conserved pathogen-associated molecular patterns (PAMPs, referred to in more recent literature as microbe-associated molecular patterns or MAMPs), allowing the innate immune system to distinguish "non-self" from "self" [2,3]. While the innate immune system recognizes pathogens, it does not generate an immune response that is specific to the pathogen. The second arm of the immune system is adaptive immunity, which generates a highly specific immune response directed against a particular pathogen [4]. It is slow to develop upon primary challenge, but possesses immunological memory resulting in long-lasting specific immunity that allows for a more rapid and robust response upon recurrent exposure.

The innate immune system is able to both activate and modulate the adaptive immune response through specialized cells known as antigen presenting cells (APCs), which, upon PRR activation, phagocytose pathogens, process, and degrade the pathogens to small antigenic peptides that are bound within the cleft of a major histocompatibility complex (MHC) molecule and presented on the surface of the APC [3]. These peptide-MHC (pMHC) complexes are presented to and activate a specific cell of the adaptive immune system known as the T cell, named for the organ in which they mature, the thymus [4]. As well as activating an adaptive immune response via T cells, innate

immune cells produce cytokines and chemokines that modulate the T cell-mediated immune response [3]. Chemokines help the activated T cells migrate to the source of the antigenic pathogen, such as the site of infection [5,6]. Cytokines modulate the type of immune response that is generated by polarizing the T cells towards different phenotypes, which results in T cell production of cytokines and effector molecules that are effective against that specific pathogen [3].

T cells are referred to as the conductors of the immune orchestra because the cytokines produced by T cells modulate and direct various other cells in the immune system [7]. T cells express on their surface a specific antigen receptor, known as the T cell receptor (TCR). During T cell development, the TCR genes undergo rearrangement, resulting in the generation of an almost infinite number of possible polymorphic TCRs that can recognize an equally large number of different antigens [8]. In order for the TCR to recognize its cognate antigen, the antigen must be presented to the T cell bound in an MHC molecule on the surface of an APC as discussed above [4]. In the case of naïve T cells, which have not encountered their specific antigen before, the APC that most effectively induces primary T cell activation is the dendritic cell (DC) [9]. External antigens, or antigens originating outside the cell, are presented in MHC class II molecules, and the expression of MHC class II molecules is restricted to APCs [10]. In contrast, internal antigens are processed differently in that the intracellular contents of all cells, not just APCs, are sampled, processed, and peptides are expressed on the surface of the cell in the context of MHC class I molecules [10]. Different subsets of T cells are selective in their recognition of MHC molecules. T cells expressing the coreceptor cluster of differentiation (CD)4 selectively bind to pMHC class II while T cells expressing the coreceptor CD8 selectively bind to pMHC class I, although there are exceptions to this rule [4]. Moreover, DCs, as well as macrophages, through the process of cross-presentation, are able to express internal antigens in the context of either MHC class I or MHC class II, allowing for potent activation of both CD8$^+$ and CD4$^+$ T cells expressing a TCR specific for that antigen [11]. Upon binding of the TCR complex to the pMHC, the resulting intracellular signaling within the T cell initiates T cell activation and associated responses, although the mechanism by which this occurs is not completely understood [12].

1.1.2 T Cell Activation

The TCR complex is made up of a cluster of proteins. The antigenic specificity of the TCR is conferred by the α and β chains, which contain variable regions that differ from one T cell to another based on TCR gene rearrangement as described above. Not all T cells express α and β chains, and for these cells, antigenic specificity is conferred by the γ and δ chains. T cells of the γδ type recognize non-peptide antigen and have a limited number of possible γ and δ chains [13]. These T cells possess characteristics of both the innate and adaptive immune system, are found in mucosal sites in the body, and were not the focus of this study.

The TCR is associated non-covalently with various CD3 proteins, which in contrast to the α and β chains of the TCR, are not polymorphic [12]. The CD3 complex is made up of a γε dimer, a δε dimer, and a ζζ homodimer, which all associate with the α and β chains of the TCR to form the complete TCR complex [14]. The CD3 complex association with the TCR is a requirement for T cell signaling since the α and β chains have short cytoplasmic tails that are unable to activate intracellular signaling pathways [14]. The CD3 proteins all contain specific motifs, known as immunoreceptor tyrosine (Tyr)-based activation motifs (ITAMs), which have a consensus sequence that includes two Tyr residues [14]. Upon stimulation of the TCR/CD3 complex by pMHC or antibody (Ab) crosslinking, the Tyr residues of the ITAMs become phosphorylated, activating intracellular signaling pathways [15], which in the right context cumulates in T cell activation.

Phosphorylation of the ITAMs is induced by the Src protein Tyr kinase (PTK) Lck [16] (Figure 1.1). These PTKs associate with the cytoplasmic domains of the CD4 and CD8 coreceptors, which bind the constant regions of the MHC molecule [17]. Upon TCR-pMHC interaction, Lck is dephosphorylated at Tyr 505 by CD45, resulting in Lck autophosphorylating itself at Tyr 394 and inducing its kinase activity [18,19]. Lck is then responsible for phosphorylating the Tyr residues within the ITAMs on the CD3 complex proteins [16]. These phosphorylation events create docking sites on the ITAMS for

proteins containing an Src homology (SH)2 domain, which recognizes the phosphorylated Tyr residue. The major molecule recruited to the phosphorylated ITAMs is the Syk PTK, zeta-chain-associated protein kinase 70 kilodalton [kDa] (ZAP-70) [20]. Upon binding to the phosphorylated ITAMs, ZAP-70 is phosphorylated by Lck, thereby inducing the kinase activity of ZAP-70 [16]. ZAP-70 activation begins a phosphorylation cascade resulting in the stimulation of key pathways that initiate transcription factors, thereby conveying the activation signal from the membrane to the nucleus [20].

ZAP-70 activation results in the formation of a molecular signaling complex centered around key adapter proteins that are recruited to the membrane and form the backbone of the signaling complex [12]. These adaptor proteins are vital for maintaining the right proteins in the right configuration at the right time to induce the appropriate signaling cascades. One such adapter protein is Linker of Activated T cells (LAT), which is a transmembrane protein that is phosphorylated on its Tyr residues by ZAP-70, creating docking sites for various proteins containing SH2 domains, which recognize and bind to the phosphorylated Tyr residues [21]. SH2-domain containing proteins that bind to LAT include phospholipase C (PLC)γ, as well as adaptor proteins such as growth factor receptor-bound protein (Grb)2 and Grb2-related adaptor downstream of Shc (Gads) [22-24]. Grb2 is constitutively bound to the effector protein SOS (son of sevenless), which is a guanine exchange factor (GEF) [25]. Gads binds another key adaptor molecule, SH2 domain containing leukocyte protein of 76 kDa (SLP-76), and brings it into the signaling complex [24]. SLP-76 is then phosphorylated on its Tyr residues by ZAP-70 [26], creating docking sites for the effector proteins phosphatidylinositol 3-kinase (PI3K) p85 regulatory subunit [27], Vav [28], non-catalytic region of tyrosine kinase adaptor protein (Nck) [29], and IL-2-inducible T-cell kinase (Itk) [30] via their SH2 domains. Formation of the signaling complex is not a linear progression, but rather a combination of various interactions that impact each other at multiple levels, making each protein within the signaling complex integral, not only to its own downstream signaling pathway, but also to the proper assembly and conformation of the signaling complex itself [12].

When proper formation of the signaling complex occurs, various effector molecules are activated, triggering multiple downstream signaling pathways required for

T cell activation. The signaling pathways that will be focused on for their pertinence to the current study are PI3K, PLCγ, Akt, protein kinase C (PKC)θ, and Ras guanyl nucleotide-releasing protein (RasGRP), and are discussed below.

PI3K is made up of two subunits, p85 and p110 [31]. p85 is a regulatory subunit while p110 is the catalytic subunit. Activation of PI3K occurs upon membrane localization through binding of the p85 subunit to the phosphorylated SLP-76 adaptor protein [27]. PI3K phosphorylates phosphatidylinositol to phosphatidylinositol bisphosphate (PIP_2) and triphosphate (PIP_3) [32]. These membrane-associated lipid second messengers result in membrane localization of proteins containing pleckstrin homology (PH) domains, which bind to PIP_2 and PIP_3. PH-domain-containing proteins activated in T cell stimulation include phosphoinositide-dependent kinase (PDK)1, Akt [31], and Itk [33]. Membrane localization of PDK1 results in its activation and phosphorylation of threonine (Thr) 308 on Akt [34], as well as other kinases important to T cell activation that will be discussed shortly.

PLCγ is phosphorylated by Itk, inducing its activation [35]. PLCγ cleaves PIP_2 to form the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) [36]. IP_3 binds to the IP_3 receptor on the endoplasmic reticulum and induces calcium (Ca^{2+}) release into the cytoplasm [37]. The increase in cytoplasmic calcium results in the opening of Ca^{2+} release activated Ca^{2+} channels on the plasma membrane, further increasing the concentration of cytoplasmic Ca^{2+}. Ca^{2+} binds to calmodulin and induces its interaction with the phosphatase calcineurin. Calcineurin then dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), unmasking a nuclear localization sequence, and resulting in the translocation of NFAT to the nucleus where it induces gene transcription [37]. DAG induces the membrane localization of PKC isoforms, such as PKCθ and PKCα, where they can then be phosphorylated by PDK1, initiating conformational changes and autophosphorylation events that culminate in PKC isoform activation [38].

PKCα, which requires DAG as well as Ca^{2+} and interaction with phospholipids in the plasma membrane for its activation [38], is the kinase responsible for full activation of Akt by phosphorylating it at serine (Ser) 473 [39]. Activation of Akt results in its

phosphorylation of various signaling molecules involved in T cell proliferation, including glycogen synthase kinase (GSK)-3β, p27^{Kip1}, and forkhead box (Fox)O transcription factors [40]. GSK-3β phosphorylates NFAT, resulting in its exclusion from the nucleus and preventing its transcriptional activity. Phosphorylation of GSK-3β by Akt results in inhibition of GSK-3β activity, thereby maintaining NFAT nuclear localization [41]. Additionally, GSK-3β phosphoryles the cell cycle protein cyclin D1, inducing its nuclear export, and inhibiting cell cycle progression [42,43]. Inactivation of GSK-3β also results in decreased expression of p27^{Kip1} and promotes cell cycle progression [44]. Akt phosphorylation of the FoxO transcription factors results in their nuclear exclusion, preventing them from inducing the transcription of cell cycle arrest proteins such as p27^{Kip1}, thereby allowing for cell cycle progression.

In addition to PKCα, PKCθ is important for T cell activation [38]. Activation of PKCθ by PDK1 and DAG results in PKCθ-induced phosphorylation of caspase recruitment domain-containing membrane-associated guanylate kinase protein (CARMA)1, allowing it to associate with B-cell lymphoma (Bcl)10 and mucosa-associated lymphoid tissue (MALT)1 to form the CBM complex, which is then responsible for activation of the canonical NF-κB pathway [45]. Downstream of the CBM complex, the inhibitor of κB kinase (IKK) complex, which is composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ, is activated by CBM-induced ubiquitination and degradation of IKKγ. In the absence of IKKγ, IKKα and IKKβ are active and are able to phosphorylate the inhibitor of κB (IκB), which is normally bound to nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and sequesters it from the nucleus [46]. IκB phosphorylation results in its ubiquitination and degradation, thereby unmasking a nuclear localization sequence of NF-κB and allowing its translocation to the nucleus where it induces gene transcription.

In addition to CARMA1, PKCθ also phosphorylates RasGRP, which is localized to the plasma membrane by DAG [47]. RasGRP is a GEF, which is able to induce the activation of the guanosine triphosphate (GTP)ase, Ras. The GEF SOS, which is also localized to the membrane by its association with Grb2 that binds to phosphorylated LAT, also induces Ras activation [25]. Interestingly, SOS-induced Ras activation is dependent on RasGRP function, indicating that RasGRP is the primary GEF involved in

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Ras activation in T cells [48]. Active Ras phosphorylates the mitogen-activated protein kinase (MAPK) kinase kinase known as Raf, which phosphorylates and activates MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK), resulting in phosphorylation and activation of ERK [49]. ERK phosphorylates the transcription factor Elk-1, resulting in expression of the transcription factor c-fos, which is a component of the transcription factor activator protein (AP)-1 [50]. Additionally, PKCθ synergizes with Vav-1 to induce c-Jun N-terminal kinase (JNK) activation [51], which results in the phosphorylation of the transcription factor c-jun [52], which upregulates its own expression and induces association of c-jun with c-fos to form AP-1 [53].

1.1.3 T Cell Costimulation

In addition to signaling by the TCR/CD3 complex, T cells also require a second stimulus, known as costimulation, which can be provided by the triggering of various T cell costimulatory molecules by APCs [54]. The most studied T cell costimulatory molecule is CD28 [55,56]. While CD28 is able to induce intracellular signaling on its own, many of the signaling pathways downstream of CD28 overlap with the pathways downstream of the TCR/CD3 complex, hence CD28 costimulation augments the strength of TCR/CD3 complex signaling [57]. CD28 costimulation decreases the duration of T cell/APC contact that is required for T cell activation [58] and reduces the number of antigen-stimulated TCRs required to induce proliferation and interferon (IFN)-γ production, thereby reducing the activation threshold of the T cell [59]. Additionally, CD28 costimulation contributes to the reorganization of membrane receptors at the T cell/APC interface and promotes lipid raft redistribution in the cell membrane [60]. Furthermore, CD28 costimulation augments and prolongs calcium influx [61] and extends the duration of Tyr phosphorylation events [57]. Interleukin (IL)-2 and IFN-γ mRNA stability is increased in the presence of CD28 costimulation, resulting in increased cytokine production [62]. In the absence of CD28 costimulation, the activation of a variety of costimulatory molecules is reduced, including the phosphorylation of ZAP-70, SLP-76, Vav, and ERK, NFAT and AP-1 activity, and the production of IL-2 [57]. CD28 costimulation also augments the expression of various cell cycle proteins associated with cell cycle progression, including cyclin D3, cyclin E, and cyclin A, as

well as inhibiting the expression of the cell cycle inhibitor p27^{Kip1}, thereby augmenting cell proliferation [44]. CD28 costimulation is also associated with increased T cell survival [63,64] and the prevention of T cell anergy, which is a state of T cell unresponsiveness upon restimulation [65,66].

1.1.4 Outcomes of T Cell Activation and Costimulation

1.1.4.1 Cell Cycle Progression

Successful T cell activation results in the upregulated expression of proteins involved in cell cycle progression [67]. The cell cycle is made up of multiple stages [68]. Quiescent naïve T cells are in the G_0 phase of the cell cycle but transition into the G_1 phase upon stimulation, where they increase in size and synthesize proteins necessary for nucleic acid replication in preparation for cell cycle progression. In S phase, cells synthesize a duplicate copy of their deoxyribonucleic acids (DNA) that will be split between the two daughter cells upon cell division. The cell continues to increase in size and protein content during the G_2 phase and then enters into M phase and undergoes mitosis. Upon T cell activation, cyclin D2 and D3 are upregulated [69] and bind to the cyclin-dependent kinases (CDK), CDK4 and CDK6, inducing their activation [70]. Cyclin D-CDK4/6 complexes phosphorylate the retinoblastoma (Rb) protein and induce the disassociation of histone deacetylases from Rb, which allows for E2F induction of cyclin E expression. Cyclin E binds to and activates CDK2, but the activity of cyclin E-CDK2 complexes is inhibited by the CDK inhibitor p27^{Kip1}. The excess production of cyclin D-CDK4/6 complexes sequesters p27^{Kip1} away from cyclin E/CDK2 complexes, resulting in their activation and further phosphorylation of Rb [70]. Hyperphosporylation of Rb results in its dissociation from its binding partner, E2F transcription factors, which are responsible for inducing the expression of various proteins involved in S phase transition, such as cyclin A and DNA polymerases. During the S phase, CDK2 binds to cyclin A and together the complex promotes DNA synthesis [68]. Upon completion of DNA synthesis, cells progress through the G_2 phase of the cell cycle where they undergo preparation for mitosis. Upon late G_2/early M phase, cyclin A associates with CDK1, whereas later in M phase, cyclin B associates with CDK1 [68]. In M phase, cell division cycle (CDC)25 dephosphorylates and activates cyclin-CDK1 complexes, which initiates

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nuclear membrane breakdown, chromosomal condensation, and the beginning of mitosis [71].

1.1.4.2 Differentiation into Effector Cells

CD4$^+$ T Helper Cells

Activation results in differentiation of naïve CD4$^+$ T cells into T helper (Th) cells that shape immune reactions through their production of cytokines [72]. Different Th cell subsets are defined based on the specific cytokines produced, ie., different groups of cytokines promote different immune responses to pathogens [72]. Antigen dose, antigen affinity, and strength of signal all shape the differentiation of T cells, as well as cytokines produced by APCs, which polarize T cells towards a specific T cell subset [7,73]. Cytokine production by APCs is mediated by PRRs on innate immune cells, which recognize specific PAMPs [3,7]. The combined signaling of various PRRs results in the production of specific cytokines, which drive the differentiation of T cells towards the Th subtype that is best suited for clearance of that type of pathogen. These polarizing cytokines induce the production of specific transcription factors that result in the commitment of the T cells to a specific Th lineage [7,72].

Th1

In response to intracellular bacteria and viral infections, T cells are polarized towards a Th1 phenotype by IL-12 and IFN-γ, which induce the expression of the transcription factor T-bet [72]. Th1 cells are characterized by the production of IL-2, tumor necrosis factor (TNF)-α, and IFN-γ [74,75]. IL-2 is a cytokine that promotes T cell proliferation and will be discussed in detail in a later section. TNF-α and IFN-γ are pro-inflammatory cytokines with various functions, including activation of the endothelium and promoting leukocyte extravasation into the inflamed tissued [76]. IFN-γ promotes natural killer (NK) cell and macrophage activation, resulting in increased cytotoxic activity of these cells against intracellular pathogens [77-79]. IFN-γ also signals in an autocrine and paracrine fashion to further promote commitment to the Th1 lineage [72], as well as promote the differentiation of CD8$^+$ T effector cells and their clearance of virally-infected cells [80]. IFN-γ also increases antigen presentation to T cells by increasing MHC class II expression on APCs [81] and MHC class I expression on most

cell types [82], and increasing the expression of proteins involved in antigen processing/transport [82], thereby promoting further antigen-induced T cell activation [83]. Th1 cells also support B cells to class switch to Ab subtypes that promote the opsonization and phagocytosis of microbes, as well as Ab-dependent cell-mediated cytotoxicity [84].

Adverse Th1 Responses

While T cell-mediated clearance of viral and intracellular bacteria are protective for the host, these same T cell processes result in tissue damage when activated inappropriately such as in delayed-type hypersensitivity (DTH) reactions [85]. Some DTH reactions are induced by the binding of haptens to host proteins, resulting in a T cell-mediated immune response to the hapten-protein complex [86]. In DTH reactions, T cell activation results in inflammation that involves IFN-γ-induced activation of macrophages, which produce various tissue damaging compounds such as reactive oxygen species (ROS), nitric oxide (NO), lysosomal enzymes, and pro-inflammatory cytokines, such as TNF-α [79,87]. Additionally, stimulation of CD8^{+} T cells induces direct cytolysis of tissue cells expressing their cognate antigen in the context of MHC class I, resulting in further tissue damage [86]. DTH reactions also contribute to transplant rejection [88]. In direct allorecognition, graft APCs either migrate to lymph nodes or stay in the graft and present antigen to the graft recipient's T cells [89]. The MHC class I and II molecules on the graft APCs are recognized as non-self, resulting in the activation of the recipient's CD4^{+} and CD8^{+} T cells. IFN-γ production by activated CD4^{+} T cells results in increased MHC class I expression by the cells of the graft [90], allowing for CD8^{+} cytotoxic T lymphocytes (CTLs) to recognize the pMHC class I on the graft and induce direct cytolysis [91]. IFN-γ production also increases macrophage activation, resulting in the release of tissue damaging molecules and graft injury [92]. Additionally, TNF-α production by both activated T cells and macrophages results in increased vascular permeability and leukocyte accumulation [93,94]. In indirect allorecognition, the graft recipient's APCs also infiltrate the graft where graft antigens are picked up, processed, and presented to the recipient's T cells, resulting in further T cell activation and graft damage [89].

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Th2

Extracellular parasitic infections, including helminth infections, induce Th2 differentiation [7,72]. Th2 polarization occurs in the presence of relatively low antigen dose and weaker TCR engagement. Th2 cells are characterized by the production of IL-4, which signals in an autocrine and paracrine fashion to further promote Th2 differentiation [7,72]. IL-4 receptor (IL-4R) signaling induces the transcription factor GATA3, which promotes the production of IL-4, IL-5, and IL-13. IL-5 promotes Ab production by B cells and eosinophil maturation and effector function [95], while IL-13 promotes immunoglobulin (Ig)E Ab production and mediates gut parasite expulsion [96]. Eosinophil activation results in degranulation and release of molecules that are cytotoxic to the parasites [97]. IgE binds to the Fcε receptors on mast cells, which in the presence of specific antigens, leads to mast cell degranulation and damage to the parasite [98]. Together, these mediators of Th2 immune responses are important in the clearance of extracellular parasites and helminths.

Adverse Th2 Responses

Although Th2 responses generate an immune response to clear extracellular parasitic infections, inappropriate activation of Th2 responses can result in tissue damage to the host, such as in allergic asthma [99]. In this case, Th2 cells are inappropriately activated by antigens within the host's environment, such as inhaled plant pollen and dust [100]. Th2 activation to these usually inert antigens, known as allergens, results in the production of IL-4 and the induction of IgE synthesis by B cells [101]. IgE binds to the Fcε receptors on mast cells and upon re-exposure to the antigen, induces mast cell degranulation. The release of mast cell granule contents such as histamine near the host's bronchial airway results in increased vascular permeability, bronchoconstriction, inflammation, and airway remodeling, contributing to airway obstruction [102]. Additionally, eosinophils are recruited and activated by IL-5 production by Th2 cells [103]. Eosinophil degranulation further promotes inflammation and tissue damage. IL-13 production also increases mucus production and airway hyperreactivity, which causes further airway obstruction [104].

Th17

The most recent Th subset to be identified is Th17, which are important in the clearance of extracellular bacteria and fungal infections [7,72]. Th17 polarization is induced by TGF-β in combination with IL-6 and/or IL-21, which induces the transcription factor RORγt [105]. Additionally, APC production of IL-23 maintains the population of Th17 cells. Th17 cells are characterized by their production of IL-17A and IL-17F, which promote recruitment, migration, and activation of neutrophils [105-108], as well as IL-21, which further promotes Th17 differentiation [109]. In addition, Th17 differentiation is promoted by the IL-17-induced production of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β by macrophages and other stromal cells [110,111]. Monocyte recruitment is also induced by Th17 responses [112]. Along with IL-17A and IL-17F, Th17 cells also produce IL-22 and promote the production of anti-microbial peptides [113], which together with neutrophil and macrophage recruitment and activation, participate in a potent immune response aimed at clearing extracellular bacteria and fungal infections [107].

Adverse Th17 Responses

In addition to clearing extracellular bacteria and fungal infections, Th17 cells are also associated with chronic inflammation and autoimmune diseases [114]. Inflammatory bowel disease (IBD) largely consists of ulcerative colitis and Crohn's disease, both of which are chronic inflammatory conditions of the gastrointestinal system [115]. Various factors mediate IBD development, including defects in specific PRRs, defects in the function of the mucosal barrier, and inappropriate activation of T cells [116]. Th17 cells and their associated cytokines have recently been associated with IBD pathogenenesis [117]. In IL-10 knockout mice, which spontaneously develop colitis, IL-23 is essential for disease development [118]. Furthermore, anti-IL-23 Ab treatment cures murine IBD [119] and IL-23p19 knockout mice have less severe disease [120,121]. In humans, an Ab against the shared p40 subunit of IL-12 and IL-23 also ameliorates Crohn's disease [122]. A specific polymorphism in the IL-23R is protective against Crohn's disease [123], indicating that the IL-23 pathway is important in the human disease, as well as the murine models. Different cytokines produced by Th17 cells may have differential effects on IBD

since IL-17F expression is increased in active Crohn's disease [124] and IL-17A is protective in the dextran sulfate sodium (DSS)-induced model of colitis [125]. This trend was also observed in IL-17F knockout mice, which had less severe disease compared to IL-17A knockout mice that were more severely affected [126].

In addition to chronic inflammatory conditions, such as IBD, Th17 cells are also associated with autoimmune diseases such as psoriasis [105]. Psoriasis results from inappropriate T cell activation and cytokine production in the skin, leading to increased angiogenesis, epidermal proliferation, and plaque formation [127]. Th17 cells are the predominant T cell subtype isolated from skin samples of psoriasis patients [128,129]. Additionally, IL-23 is involved in psoriasis and induces hyperkeratosis in mice [130,131], and IL-23R variants are protective against psoriasis [132-134]. IL-22 is also required for psoriatic symptoms [135] and promotes keratinocyte proliferation [130,136]. Furthermore, inhibition of the transcription factor signal transducer and activator of transcription (STAT)3, which signals downstream of the IL-6, IL-23, and IL-21 receptors, is therapeutic in mouse models of psoriasis, as well as in human patients [137].

T Regulatory Cells (Tregs)

Upon T cell activation in the presence of transforming growth factor (TGF)-β and in the absence of the pro-inflammatory cytokines IL-6 and IL-21, $CD4^+$ T cells are polarized to become inducible Tregs (iTregs) through expression of the transcription factor FoxP3 [138]. In addition to iTregs, which are generated in the periphery, Tregs and FoxP3 expression are also induced in the thymus through high avidity TCR interactions during selection, and these cells are referred to as natural Tregs (nTregs). Tregs dampen immune responses and T cell activation through various mechanisms such as production of the anti-inflammatory cytokines TGF-β and/or IL-10 [139]. Furthermore, peripheral tolerance and inhibition of self-reactive T cells that have escaped thymic selection are also mediated by Tregs.

$CD8^+$ *CTLs*

Activation of $CD8^+$ T cells results in their differentiation into CTLs, which are able to induce apoptosis of malignant cells or cells infected with viruses or intracellular bacteria through multiple mechanisms [140,141]. CTLs express high levels of Fas ligand

(FasL), which binds to the Fas receptor on target cells upon antigen recognition by the CTL [142]. The Fas receptor is a member of the TNF receptor family and activation of this receptor by FasL causes the formation of a death-inducing signaling complex that leads to caspase activation and apoptosis induction [142]. Additionally, CTLs produce cytotoxic effector molecules such as perforin and granzyme, which are released from granules into the intercellular space between the CTL and its target cell [143]. Perforin causes membrane damage in target cells through the formation of pores, which promotes membrane disruption and loss of osmotic regulation [144]. Granzyme binds to the mannose-6-phosphate receptor and perforin triggers endocytosis of receptor-bound granzyme [145], leading to apoptosis by activating caspases and cleavage of Bid to the pro-apoptotic truncated Bid, which results in mitochondrial membrane destabilization [146,147].

1.1.4.3 Differentiation into Memory Cells

Following T cell activation, proliferation, and effector differentiation, approximately 90% of these effector cells will die by apoptosis following antigen clearance in a process known as contraction [148]. The CD4$^+$ and CD8$^+$ T cells that survive develop into long-lived memory T cells [149,150]. These memory cells constitute the immunological memory of the T cell arm of the adaptive immune system, and upon antigenic rechallenge, respond more rapidly compared to naïve cells, and repopulate the pool of antigen-specific T effector cells [149,150]. Folowing clearance of the pathogen, the cytokines IL-7 and IL-15 contribute to the continued survival and maintenance of these CD4$^+$ and CD8$^+$ memory T cells [148].

1.2 IL-2

IL-2 is a cytokine that is important for T cell function both *in vitro* and *in vivo* [151]. It is a 15 kDa monomeric glycoprotein that is produced primarily by activated T cells and signals in an autocrine and paracrine fashion [151]. IL-2 signaling is critical for T cell proliferation *in vitro* [152].

1.2.1 Signaling by the IL-2R

IL-2 signals through the heterotrimeric IL-2 receptor (IL-2R) made up of an α (CD25), β, and γ subunits [153] (Figure 1.2). The expression of CD25 on T cells is induced by TCR/CD3 and costimulatory molecule signaling [154]. The β and γ chains are expressed on naïve T cells, and their expression is further upregulated by TCR/CD3 and costimulatory molecule signaling [153]. The receptor subunits are not preassembled and are only brought together by the binding of IL-2 [151]. Although CD25 is not involved in intracellular signaling, it greatly increases the affinity of the IL-2R for IL-2, therefore lowering the activation threshold of the receptor [151]. The β and γ chains, which without CD25 form the intermediate affinity IL-2R, are responsible for the intracellular signaling events initiated by IL-2 binding [155]. While the IL-2R does not have any intrinsic kinase activity, the β and γ chains are constitutively associated with Janus kinase (JAK)1 and JAK3, respectively [156], and rely on these non-receptor Tyr kinases to activate intracellular signaling pathways [157-159]. IL-2 binding to the IL-2R results in assembly of the heterotrimeric receptor subunits, bringing JAK1 and JAK3 into close proximity with each other [155]. A recent study by Haan *et al.* [157] has helped to elucidate the events that follow IL-2R assembly. Using knockdown experiments and transfection with kinase inactive and constitutively active mutants, Haan *et al.* [157] propose a model in which IL-2R assembly induces a conformational change in JAK1 and JAK3, resulting in their activation in the absence of phosphorylation. Following conformational activation, JAK1 and JAK3 transphosphorylate each other, resulting in full activation of the kinases. JAK activation results in the phosphorylation of three Tyr residues of the IL-2Rβ chain that are important in IL-2R signaling, Tyr 338, 392, and 510 in humans [160] or Tyr 341, 395, and 498 in mice [161,151] (numbers from here on will correspond to mice). JAK1 has been shown to directly phosphorylate these Tyr residues in response to IL-2 [160], and IL-2-dependent proliferation can be induced through JAK3 in the absence of JAK1 [158,162]. Other PTKs are also activated by IL-2R signaling, including Syk and Lck, which have been linked to the IL-2-induced expression of c-myc and c-fos, respectively [163-165]. Interestingly, neither one of these proteins is required for normal IL-2R

signaling [166-168], suggesting that IL-2-induced activation of these proteins may be redundant.

Phosphorylation of the IL-2Rβ chain at Tyr residues creates binding sites for proteins with SH2 domains [160]. The two major SH2-containing proteins involved in IL-2R signaling are Src homology and collagen (Shc) and STATs [151]. Phosphorylated Tyr 341 forms a binding site for the adaptor protein Shc, which then becomes phosphorylated and interacts with the adaptor protein Grb2 [169,170]. Grb2 associates with the guanine exchange factor SOS, which activates Ras and the downstream MAPK pathway (see section 1.2 for details). Phosphorylated Grb2 can also bind the adaptor protein Grb2-associated binding protein 2 (Gab2), which becomes phosphorylated and associates with the p85 subunit of PI3K [171], resulting in activation of PI3K and the downstream signaling molecule Akt (see section 1.1.2 for details). Although alternative pathways have been suggested for IL-2-induced PI3K activation [172-175], these studies used protein overexpression, making it possible that the PI3K activation observed was an artefact of the overexpression and that these alternative pathways would not induce PI3K activation under physiological conditions. Additionally, Gu *et al.* [171] showed that mutation of the Shc binding site on the IL-2Rβ chain completely ablated IL-2-induced Akt phosphorylation, indicating that Shc is crucial to IL-2-induced activation of the PI3K/Akt pathway. Phosphorylated Tyr 395 and 498 are the primary binding sites for STAT5 proteins [160], of which there are two isoforms, STAT5a and STAT5b [176] that will be referred to as STAT5 from here on. Additionally, phosphorylated Tyr 341, which primarily associates with Shc [160], is a minor binding site for STAT5, which remained underappreciated for many years [177]. As a result, signaling downstream of Tyr 341 was attributed exclusively to Shc, resulting in an overexaggeration of the activities of Shc [160,178]. Upon binding to the phosphorylated IL-2Rβ chain, STAT5 is phosphorylated by JAK1 and JAK3, although there are conflicting studies showing whether JAK1 or JAK3 is predominately responsible [157,159]. In addition to STAT5, STAT3 has also been shown to be involved in IL-2R signaling, although not as potently as STAT5 [179]. Interestingly, STAT3 has been shown to be constitutively associated with the IL-2Rβ chain in a different region of the receptor from the major STAT5 binding sites [179]. Additionally, the IL-2Rβ chain does not need to be phosphorylated for STAT3 to

associate with it [179]. Regardless of this unusual association, STAT3 only becomes active upon IL-2R ligation [179]. Once phosphorylated, STAT5 and STAT3 homo- or hetero-dimerize through the interaction of the SH2 domain of one STAT protein with the phosphorylated Tyr residue on the other STAT protein [180]. The STAT dimers then translocate to the nucleus where they act as transcription factors [180]. STATs act in concert with other transcription factors, resulting in the upregulation of various genes involved in cell survival and proliferation [181].

1.2.2 Regulation of IL-2R Signaling

The three major families of proteins that negatively regulate the activation of the JAK-STAT pathway are Tyr phosphatases, suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STAT (PIAS) [182]. Upon IL-2 stimulation of the IL-2R, constitutively expressed Tyr phosphatases are rapidly recruited [183]. SH2 domain-containing phosphatase (SHP)-1 dephosphorylates JAK1, JAK3, and the IL-2Rβ chain in response to IL-2 stimulation [184]. SHP-2 is also activated in response to IL-2 stimulation [185] and directly dephosphorylates STAT5 [186,187]. In contrast to Tyr phosphatases, SOCS protein expression is induced upon IL-2R signaling and STAT activation, allowing for transient activation of the IL-2R signaling [183]. Three SOCS proteins are involved in regulating IL-2R signaling. Cytokine Inducible SH2 Protein (CIS) associates with the IL-2Rβ chain and inhibits STAT5 phosphorylation without inhibiting the phosphorylation of the IL-2Rβ chain [188]. SOCS-1 associates with JAK1 and JAK3, but inhibits the phosphorylation and kinase activity of JAK1 more potently than JAK3 [189]. In contrast, SOCS-3 has no inhibitory activity against JAK3 but inhibits the kinase activity of JAK1, and partially inhibits the phosphorylation of JAK1 and the IL-2Rβ chain [190]. Induction of CIS, SOCS-1, and SOCS-3 all result in a decrease in STAT5 phosphorylation and its downstream transcriptional activity [188-190]. The third protein family involved in regulating JAK-STAT signaling is PIAS, which interacts with STAT dimers, blocking their DNA binding activity [191]. PIAS proteins are constitutively expressed and act as a buffer, regulating the amount of activated STAT present within the T cell [183]. PIAS3 binds to and inhibits STAT3 transcriptional activity downstream of IL-6 receptor (IL-6R) signaling [192], however, the importance of this pathway in IL-2R signaling has not been confirmed.

1.2.3 IL-2 Production

Upon initial activation, T cells produce high levels of IL-2, but following T cell differentiation, Th1 cells are the major T cell subset responsible for IL-2 production [193]. APC production of small amounts of IL-2 is believed to help the APCs promote initial T cell activation. A recent study showed that DCs secrete IL-2 at the DC-T cell interface [194]. Furthermore, DCs also express CD25, which together with DC-produced IL-2, is transpresented to T cells to initiate IL-2R signaling prior to T cell expression of IL-2 and the CD25, thereby facilitating early T cell proliferation [194].

1.2.4 Functions of IL-2 *In Vitro*

Originally, IL-2 was identified as a T cell growth factor that was able to induce T cell proliferation *in vitro* [151,152]. The deletion of the CD25 gene or addition of anti-CD25 antibodies (Abs) dramatically inhibits the proliferation of T cells in culture [195,196], indicating that IL-2 production and receptor signaling are crucial for T cell proliferation *in vitro*. Additionally, IFN-γ production [197] and the differentiation of CD8$^+$ T cells into functional CTLs is dependent on IL-2 stimulation *in vitro* since IL-2R signaling promotes the expression of the cytotoxic effector molecules granzyme [198] and perforin [199]. IL-2 also influences Th cell differentiation by inducing expression of the cytokine receptors for IL-12, IL-4, and IL-6, which are involved in polarizing T cells towards Th1, Th2, and Th17 differentiation, respectively [200,201]. IL-2 is therefore an important cytokine for *in vitro* T cell responses.

1.2.5 Functions of IL-2 *In Vivo*

Due to the requirement of IL-2 production and signaling for the proliferation of T cells *in vitro*, it is surprising that IL-2 and IL-2R knockout mice develop fatal T cell-mediated autoimmunity, thereby confounding the function of IL-2 *in vivo* [151,202]. The discovery that Tregs constitutively express CD25 and require IL-2R signaling for their maintenance, shed light on the reason for development of autoimmunity in the absence of IL-2R signaling [202]. Interestingly, IL-2- and IL-2R-deficient T cells have functional immune responses *in vivo*, further calling into question the importance of IL-2 for T cell processes *in vivo* [151,202]. It appears that other cytokines are able to compensate for the loss of IL-2 or IL-2R expression and allow for relatively normal T cell responses *in vivo*

[151,202]. Several research groups are currently attempting to clarify the role and relative importance of IL-2 in the generation of T cell responses *in vivo*, including its impact on proliferation, effector cell function, and memory cell generation. To circumvent the problem of autoimmunity in IL-2$^{-/-}$ and IL-2R$^{-/-}$ mice, IL-2 or IL-2R-deficient mice have been crossed with TCR transgenic mice [203,204]. Alternatively, other research groups have generated chimeric mice by adoptively transferring a mixture of WT and CD25-deficient bone marrow cells into lethally-irradiated WT mice [205-207].

D'Souza *et al.* [203,204] demonstrated that IL-2 was of differential importance in non-lymphoid tissues such as liver, lung, and lamina propria of the intestines, compared to lymphoid tissues, such as lymph nodes and spleen. Thus, IL-2 is required to sustain T cell proliferation in non-lymphoid tissues while it is dispensable for T cell proliferation in lymphoid tissues, possibly due to the differences in anatomy between lymphoid and non-lymphoid tissues [203,204]. Lymphoid tissue has a defined structural organization that promotes cell-cell interactions, whereas this organization is not present in non-lymphoid tissues. Therefore, in non-lymphoid tissues where cell-cell interactions are not supported by the tissue architecture and there is a lower number of growth-promoting stimuli compared to lymphoid tissues, IL-2 becomes an important growth factor for supporting T cell responses *in vivo* [203,204]. D'Souza *et al.* [203,204] suggest a model in which APC-T cell interactions result in T cell activation in the lymph node, leading to IL-2-independent T cell expansion. These primed T cells then migrate to the tertiary tissues where they undergo IL-2-regulated T cell expansion [203,204].

IL-2 has also been shown by various groups to be important for the differentiation of functional CD8$^+$ memory T cells that are able to respond to rechallenge [206-208]. Primary CD8$^+$ T cell-mediated immune responses in response to lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* are only modestly inhibited by the absence of IL-2 signals, and memory T cell differentiation appears normal until rechallenge at which point the T memory cells are unable to differentiation into secondary effector T cells [206-208]. Williams *et al.* [206] demonstrated that IL-2R signaling during the secondary response is unable to restore memory T cell responses upon rechallenge, indicating that the presence of IL-2 during the primary immune response is critical for memory T cell programming for functional responses upon

secondary challenge. Mitchell *et al.* [207] suggest that the presence of IL-2 during the primary response may induce epigenetic imprinting of memory T cells, which allows for secondary effector responses upon rechallenge. This dependence of memory cells on IL-2R signals may depend on the infection and experimental conditions examined, since Obar *et al.* [209] did not find the same defects in secondary responses to *Listeria monocytogenes* or *Vaccinia virus* infection.

IL-2 is also important for CD4^{+} memory T cell differentiation, as demonstrated by Dooms *et al.* [210]. Without IL-2-induced signals during the initial primary response, CD4^{+} T cells do not express IL-7 receptor (IL-7R)α later on in the response. Expression of this cytokine receptor is necessary for IL-7-induced survival signals and CD4^{+} memory T cell generation. The absence of the IL-7R due to the loss of IL-2 signaling results in poor development of long-lived CD4^{+} memory T cells, although responses of these mice to secondary challenge was not examined [210].

The effect of IL-2 on T cell apoptosis *in vivo* is currently unclear. D'Souza *et al.* [203,204] found that IL-2 is not required for the initial proliferation of CD8^{+} T cells following antigen challenge, but is critical for sustaining their expansion. D'Souza *et al.* [203,204] also determined that the proliferation of CD8^{+} T cells in non-lymphoid tissues is differentially regulated based on the origin of the IL-2. Paracrine IL-2 signaling promotes the expansion of the CD8^{+} T cell population, whereas autocrine IL-2 signaling promotes T cell contraction and apoptosis. This indicates that IL-2 plays a dual role in both promoting and limiting T cell expansion. In contrast to D'Souza's findings, Blattman *et al.* [211] found that IL-2 administration during the expansion phase of the LCMV-induced immune response does not alter the CD8^{+} T cell response but dramatically inhibits the CD4^{+} T cell response, indicating that IL-2 differentially regulates the expansion of these two T cell subsets. In contrast, IL-2 administration during the contraction phase of the immune response results in increased CD8^{+} and CD4^{+} T cell survival [211]. Therefore, IL-2 plays a complex role in regulating T cell proliferation, expansion, and survival *in vivo* that has not been fully elucidated.

IL-2 is also required for the induction and maintenance of Treg populations *in vivo* [151,202]. Interestingly, mutation of the 3 critical Tyr residues of the IL-2Rβ chain

still allows for sufficient IL-2R signal transduction to support the development and maintenance of nTregs despite having impaired T effector cell proliferation and impaired induction of iTregs [212]. Thus, nTregs require a lower thesis of IL-2R signaling compared to iTregs and T effector cells [212]. nTregs are unable to make IL-2 themselves and inhibit IL-2 production by other T cells, thus creating an IL-2-poor environment [212]. Therefore, the low threshold for IL-2R signaling in nTregs potentially allows for the continued maintenance of nTregs in IL-2-poor environments [212].

1.2.6 Clinical Inhibition of IL-2 Signaling

As discussed above, IL-2 has an important role in T cell responses *in vivo* through memory T cell generation and responsiveness and T cell function in non-lymphoid tissues [203,204,206,207,211]. It is not surprising that clinical inhibition of IL-2R signaling using anti-CD25 Abs such as daclizumab has shown great promise in the treatment of various T cell-mediated conditions, including non-infectious uveitis, multiple sclerosis (MS), psoriasis, human T-lymphotropic virus (HTLV)-1 associated adult T cell leukemia, and transplant rejection [213,214]. Non-infectious uveitis patients on daclizumab have been able to reduce or eliminate their use of other systemic immunosuppressive agents, such as corticosteroids, resulting in a reduction in adverse side effects that include increased blood pressure and high serum cholesterol levels [213,215]. Daclizumab induced a 78% reduction in new lesions in IFN-β-refractory MS patients [216] and a 30% reduction in psoriasis severity [217]. There has been a reduction in the number of acute rejection episodes and an increased graft survival rate with daclizumab treatment of renal, cardiac, and liver transplant recipients [214]. In addition, IL-2 production is also effectively inhibited by the immunosuppressants cyclosporin A (CsA) and tacrolimus (FK506), which are commonly used to prevent transplant rejection [218]. CsA and FK506 target calcineurin and prevent its activation, leading to inhibition of NFAT-induced IL-2 gene transcription. Another immunosuppressant that interfers with IL-2 is rapamycin, which targets mammalian target of rapamycin (mTOR) and prevents its activation [219]. mTOR is activated downstream of both TCR and CD28 as well as the IL-2R, resulting in increased protein translation, CDK inhibitor degradation, and IL-2 and CD25 expression [220]. Therefore, inhibitors of IL-2 and IL-2R signaling have great

potential as clinically effective therapeutic agents for the treatment of various T cell-mediated conditions, including autoimmunity and transplant rejection [213,214].

1.3 Angiogenesis

Angiogenesis is the formation of new blood vessels from previously existing blood vessels and is a critical component of various physiological processes such as wound healing, embryogenesis, and the menstrual cycle [221,222]. Under normal conditions, angiogenesis is a complex process that is tightly regulated by both pro- and anti-angiogenic factors [221]. Pathological angiogenesis results from dysregulation of the balance between pro- and anti-angiogenic factors in diseases such as cancer and those caused by chronic inflammation [223,224].

1.3.1 The Process of Angiogenesis

The normal vascular is maintained in a quiescent state with only approximately 0.04-0.67% of the endothelial cells within an adult mouse undergoing proliferation at a given time [225]. Various stimuli, including tissue hypoxia and inflammation, can cause angiogenesis by promoting the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [223]. These pro-angiogenic factors activate receptors on the endothelium and induce increased vascular relaxation and permeability, resulting in the entrance of plasma proteins into the tissue (Figure 1.3) [223,226]. With the loss of the normal tight junctions between cells, the endothelial cells are no longer restricted by close contact with their neighboring endothelial cells and are more responsive to pro-angiogenic stimulation [227]. At the beginning of the angiogenic process, the supporting cells of the vasculature, such as pericytes, detach from the endothelium [223]. Proangiogenic factors such as VEGF increase the production of degradative enzymes, such as plasminogen activators and matrix metalloproteinases (MMPs) by the endothelium, resulting in the degradation of the basement membrane and extracellular matrix (ECM) [223]. MMPs are secreted in a pro-form that requires cleavage for their activation. One of the enzymes involved in MMP cleavage and activation is plasmin, which is cleaved from the plasma protein plasminogen by plasminogen activators [228]. MMP activity allows for the movement of endothelial cells through the degraded basement membrane and also causes release of growth factors, such as fibroblast growth

factor (FGF), that are embedded in the ECM [229]. Growth factors further promote the angiogenic response and induce the endothelial cells to proliferate [230]. The pro-angiogenic factors also act as chemotactic factors and promote endothelial cell migration towards the source of the angiogenic stimuli [229]. As the endothelial cells begin to migrate through the extracellular matrix towards the angiogenic stimuli, they configure themselves in a bipolar alignment, forming capillary tubules with a central lumen [231]. Upon arrival at the angiogenic stimulus, the tips of the adjacent capillary sprouts connect, forming a capillary loop. These newly formed vessels are immature and leaky until they undergo vessel maturation and differentiation, which involves the formation of a new basement membrane, recruitment of supporting cells such as pericytes and smooth muscle cells to line the vessel, and the reestablishment of endothelial tight junctions and the permeability barrier [231]. These supporting cells inhibit endothelial cell proliferation and are important in returning the vasculature to its formerly quiescent state [230].

1.3.2 Regulation of Angiogenesis

Angiogenesis is regulated by the balance of pro- and anti-angiogenic factors in the vicinity of the vasculature [221]. Because angiogenesis is so tightly regulated, there must be increased production of pro-angiogenic factors, decreased production of anti-angiogenic factors, or a combination of the two in order for angiogenesis to be initiated [232].

1.3.2.1 Pro-Angiogenic Factors

There are a multitude of pro-angiogenic factors that can have direct effects on endothelial cells, such as VEGF and FGF-2 [230], as well as factors that act indirectly to promote angiogenesis by inducing the release of pro-angiogenic factors from other cells [233]. Moreover, the effect of certain factors on angiogenesis depends on the context and the dose, further complicating the influence of these factors on the angiogenic process [234]. The major factors involved in promoting angiogenesis are outlined below.

VEGF

VEGF, also known as VEGF-A, is one of the most potent pro-angiogenic factors [223]. VEGF binds to homo- or hetero-dimers of VEGF receptor (VEGFR)1 and

VEGFR2, which are expressed on the vascular endothelium [222]. Other members of the VEGF family include VEGF-C and VEGF-D, which bind to VEGFR3 on the lymphatic endothelium [235]. VEGFR1 has a limited signaling response to VEGF binding and is in some circumstances believed to be a negative regulator of VEGFR2 through competition for VEGF-A binding [231,236]. VEGFR2 is the main pro-angiogenic receptor for VEGF [223], and activation of this receptor subunit promotes various processes of angiogenesis, including vascular permeability, endothelial survival and proliferation, ECM degradation and migration, and vessel maturation and differentiation [236]. Intracellular signaling is induced downstream of VEGFR homo- or hetero-dimerization, which results in the autophosphorylation of the cytoplasmic portion of the receptor, and creates binding sites for signaling molecules containing SH2 domains [235,236]. VEGF is also responsible for activating the enzyme endothelial nitric oxide synthase (eNOS), which promotes an increase in NO production [237] and augments the proliferation-inducing effect of VEGF on endothelial cells [238]. NO also potentiates increased vascular relaxation and permeability that is seen in the early stages of angiogenesis [239].

FGF

Another potent pro-angiogenic factor is FGF, which induces the survival, proliferation, migration, and differentiation of endothelial cells [236]. Binding of FGF to FGF receptor (FGFR) involves heparin and induces receptor dimerization that leads to autophosphorylation and binding site formation for SH2 containing signaling molecules, similar to VEGFR signaling [240].

Angiopoietin (Ang)-2

The role of Ang-2 in promoting angiogenesis is not clear cut. Ang-2 binds to the Tie2 receptor and can have either a stimulatory or blocking effect on this receptor, depending on the context [234]. Some of the main pro-angiogenic outcomes of Ang-2 are loosening of the endothelial tight junctions and detachment of endothelial supporting cells, such as pericytes and smooth muscle cells, which are very early and critical steps in the initiation of angiogenesis [230,234]. Additionally, Ang-2 promotes MMP production and degradation of the ECM [241], which allows for endothelial cell migration. The role of Ang-2 is angiogenesis is further complicated by the requirement of additional pro-

angiogenic factors such as VEGF. In the absence of additional pro-angiogenic factors, Ang-2 signaling results in endothelial cell death and blood vessel regression [242,243]. The role of Ang-2 in promoting angiogenesis is therefore very complex and context dependent.

1.3.2.2 Vessel Maturation Factors

Various factors are involved in promoting the maturation of newly formed endothelial vessels and the completion of angiogenesis rather than promotion of the early events in angiogenesis initiation [230]. These factors include Ang-1 and platelet-derived growth factor (PDGF)-BB, as well as many others. Ang-1 activates the Tie-2 receptor and stimulates stabilization of capillary networks, as well as endothelial survival, but has no mitogenic effect, indicating that Ang-1 does not promote the early stages of endothelial cell proliferation during angiogenesis [244]. Ang-1 also increases the tightness of the tight junctions between endothelial cells by increasing the expression of tight junction proteins such as zonula occludens-2 [245] and inhibiting vascular leakage [246], thereby promoting vessel maturation and quiescence [230]. Interestingly, since the critical first step in the angiogenic process is the loss of normal tight junction function, Ang-1 in certain contexts can be anti-angiogenic [234]. Conversely, Ang-1 can also be pro-angiogenic by increasing the recruitment of endothelial progenitor cells that can differentiate into new vessels [247]. PDGF-BB is important in recruiting pericytes and smooth muscle cells, allowing for newly formed endothelial vessels to mature and have normal permeability and perfusion [230].

1.3.2.3 Anti-Angiogenic Factors

Other endogenous factors have an anti-angiogenic role, including angiostatin and endostatin. Angiostatin is a fragment of plasminogen [248], which can bind to the integrin $\alpha_v\beta_3$ [249] and inhibit endothelial cell proliferation [248,250], as well as induce apoptosis [251]. Endostatin is a proteolytic fragment of collagen XVIII [250], which increases endothelial cell apoptosis by altering the balance of pro- and anti-apoptotic proteins of the Bcl-2 family [252]. Endostatin binds to the integrin $\alpha_5\beta_1$ and inhibits the endothelial cell from binding to the ECM, which is required for endothelial migration and angiogenesis [253]. Endostatin also blocks VEGF binding by interacting with VEGFR2

[254]. Therefore, endostatin not only interferes with integrin function but also interferes with pro-angiogenic signaling pathways.

1.3.3 Signaling Pathways

The signaling pathways initiated by pro-angiogenic factors that induce phenotypic changes in endothelial cells are not completely elucidated; however, two main pathways have been shown to be of particular importance in the angiogenic process and will be highlighted.

1.3.3.1 ERK

In endothelial cells, ERK is activated downstream of the VEGFR and FGFR [240]. VEGF and FGF binding causes dimerization and autophosphorylation of their receptors, resulting in activation of the adaptor proteins Shc and Crk, respectively [255,256]. Shc and Crk associate with Grb2 and its binding partner SOS, a guanine exchange factor that promotes the activation of the Ras-ERK pathway [256], although this is a minor pathway in VEGFR signaling [257]. Additionally, VEGFR2 and FGFR signaling induces the activation of PLCγ and PKC isoforms [258,259], which are able to activate protein kinase D (PKD) and promote ERK activation by a mechanism that is not yet fully elucidated [260,261]. Pharmacological inhibition of ERK reduces VEGF- and FGF-2-induced endothelial proliferation [262] and FGF-2-induced angiogenesis [263], indicating the importance of this pathway to the pro-angiogenic effects of VEGF and FGF-2.

1.3.3.2 Akt

In endothelial cells, downstream signaling from VEGFR2, FGFR, and Tie-2 all result in the activation of PI3K and Akt [236]. Akt activation is required for VEGF-induced endothelial cell migration and the formation of endothelial sprouts *in vitro* and *ex vivo* [264]. Constitutive activation of Akt induces excessive endothelial cell proliferation and angiogenesis in the chorioallantoic membrane assay [265], indicating that Akt plays an important role in endothelial cell function and angiogenesis. Another major outcome of Akt activation is increased endothelial cell survival through the direct phosphorylation and inactivation of pro-apoptotic molecules, including Forkhead transcription factors,

Bad, Bax, and caspase-9 [40,266-268]. Akt further promotes endothelial cell survival by activating IKK, leading to NF-κB-mediated expression of the inhibitor of apoptosis proteins (IAPs) [266]. eNOS is also stimulated by Akt signaling, leading to increased NO production [269], which inhibits caspase activation [270,271] and promotes vascular relaxation and permeability [272]. Thus, Akt is important not only in various angiogenic processes, but also in the maintenance of endothelial cell viability during angiogenesis.

1.3.4 Angiogenesis in Chronic Inflammation

Pathogenic angiogenesis has been linked to a variety of chronic inflammatory conditions [273], including IBD, psoriasis, and rheumatoid arthritis. In IBD, increased mucosal vessel density has been identified in active lesions of both Crohn's disease and ulcerative colitis patients [274]. Additionally, anti-angiogenic therapy reduces disease severity in IL-10$^{-/-}$ [275], DSS, and CD4$^+$CD45^{hi} transfer models of IBD [276], suggesting that angiogenesis plays an active role in IBD pathogenesis [274]. Psoriasis patients have abnormally high serum levels of VEGF that return to normal upon disease regression [277]. Furthermore, abnormal blood vessel growth predicts the spread of the psoriatic plaque, indicating that angiogenesis is involved in the early stages of disease pathogenesis [278]. Immature vasculature and high serum levels of VEGF are also present in rheumatoid arthritis patients [279]. While the mechanism of the relationship between inflammation and angiogenesis is only partially elucidated, there is a clear and definite connection between angiogenesis and chronic inflammation that appears to be co-dependent.

1.3.4.1 Inflammation Initiates Angiogenesis

Inflammation results in the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IFN-γ, all of which increase the vascular permeability of the endothelium [280,281]. Increased vascular permeability is one of the first steps in the angiogenic process that makes the endothelium more responsive to stimulation by pro-angiogenic factors such as VEGF [230,231]. As well as increased vascular permeability, inflammatory cytokines also increase the endothelial cell expression of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin [282], resulting in a large influx of inflammatory

cells into the tissue [283]. This excess of metabolically active inflammatory cells depletes the normal nutrient supply provided by the vasculature to the tissue, creating a hypoxic environment and further inducing the expression of proangiogenic factors [273,284]. Under hypoxic or inflammatory conditions, a variety of inflammatory cells, including keratinocytes, fibroblasts, monocytes, and particularly macrophages, can be sources of pro-angiogenic factors [273]. In addition to the production of well-known pro-angiogenic factors such as VEGF and FGF, inflammatory cells also produce pro-inflammatory cytokines and chemokines that have pro-angiogenic activity. For example, IL-8 is a chemotactic factor for neutrophils that is also chemotactic and mitogenic for endothelial cells, resulting in increased angiogenesis [273,285]. Large amounts of IL-8 and VEGF are produced by TNF-α-stimulated human intestinal fibroblasts [286]. Furthermore, IL-1β and low doses of TNF-α increase corneal angiogenesis in rabbits [287,288]. TNF-α also has direct pro-angiogenic effects on endothelial cell migration and tubule formation *in vitro* [289]. The pro-inflammatory mediators prostaglandin (PG)E$_2$, IL-6, and IL-1 indirectly promote angiogenesis by increasing VEGF mRNA expression [290,291]. Additionally, T cells play a role in promoting angiogenesis in inflammation. Activated T cells not only make VEGF [292], but are a major source of VEGF in atherosclerotic arteries [293]. Activated T cells also express CD40 ligand (CD40L), which upon engagement of CD40 on intestinal fibroblasts induces the expression of VEGF and IL-8 [294]. T cell interaction with endothelial cells via CD40L-CD40 binding increases the expression of MMPs by endothelial cells, resulting in increased tubule formation [244]. Inflammation is thus a potent inducer of angiogenesis and increases the blood vessel density in inflamed tissues.

The role of the immune system in angiogenesis is complex and context-dependent since not all cytokines and chemokines are pro-angiogenic. For example, IL-12 and the C-X-C motif chemokine ligands 4, 9, 10, and 11 all have anti-angiogenic activity [285,233]. Additionally, IFN-γ promotes the production of anti-angiogenic chemokines [295]. Therefore, in a normal inflammatory reaction there are mediators present that limit angiogenesis and induce vascular maturation upon resolution of the inflammatory reaction [285]. Unfortunately, in chronic inflammation, dysregulation of inflammation

and angiogenesis occurs, resulting in a perpetual loop of inflammation-induced angiogenesis, which supports and maintains pathological inflammation [284].

1.3.4.2 Angiogenesis Sustains Chronic Inflammation

During chronic inflammation, angiogenesis is also dysregulated and the new inflammation-induced vasculature is abnormal and immature, and has increased permeability and a torturous structure [274]. This is due to an imbalance in pro- and anti-angiogenic factors which interfers with pericyte recruitment and vessel maturation [274,296]. The vessels remain in a highly active state in the inflammatory milieu, resulting in high vascular permeability and increased expression of adhesion molecules. Anatomical expansion of the highly activated endothelium allows for more efficient extravasation of leukocytes, maintaining an influx of inflammatory cells into the tissues [284]. Furthermore, the increased vasculature supports the nutrient and oxygen needs of the inflamed tissue, maintaining the survival of the infiltrating and proliferating leukocytes. The endothelium itself can contribute to the inflammation through the production of NO, inflammatory cytokines, and chemokines [273]. Chronic inflammation and angiogenesis become mutually co-dependent on one another, since inhibition of inflammation diminishes angiogenesis and inhibition of angiogenesis attenuates inflammation [274]. Consequently, the inhibition of abnormal angiogenesis associated with psoriasis and rheumatoid arthritis has proven to be a promising treatment option [279,297,298]. Furthermore, inhibition of angiogenesis in murine models of IBD reduces the disease severity [275,276], indicating that anti-angiogenic therapy is a new potential treatment option for IBD patients which warrants clinical investigation.

1.3.5 Angiogenesis in Cancer

During carcinogenesis, tumors can only grow to a size of 1-2 mm in diameter before they outgrow the nutrient and oxygen supply provided by passive diffusion [299]. The lack of oxygen and nutrients, as well as the accumulation of waste products, results in hypoxia, which induces the transcription factor hypoxia-inducible factor (HIF) and leads to the transcription of pro-angiogenic factors such as VEGF [300]. The hypoxic conditions also cause the necrosis of cancer cells at the centre of the developing tumor, which results in the production of inflammatory cytokines [301]. Additionally, tumor

cells also produce chemotactic factors that recruit macrophages to the tumor, which provide an additional source of pro-angiogenic factors [302]. These angiogenic factors, together with the inflammatory cytokines from the necrotic core, result in activation of the endothelium and induction of angiogenesis [301]. Tumors that do not acquire a new blood supply remain dormant, while vascularization of tumors results in logarithmic growth, making angiogenesis the rate-limiting step in tumorigenesis and a critical turning point in the development of clinical disease [299].

1.4 Phytochemicals

Natural products are an underappreciated source of novel pharmacological agents for therapeutic use [303,304]. Previously, many immunosuppressive agents in use today for the treatment of transplant rejection were originally discovered as fungal metabolites, including the calcineurin-inhibitors cyclosporin and tacrolimus, the mTOR inhibitor rapamycin, and mycophenolate from which the *de novo* purine synthesis inhibitor mycophenolate mofetil is derived [305]. Therefore, it should not be surprising that a myriad of phytochemicals also demonstrate potent immunosuppressive potential in *in vitro* and pre-clinical studies, e.g., curcumin, resveratrol, epigallocatechin-3-gallate, and capsaicin to name a few [306-310]. Additionally, many of these phytochemicals also possess anti-angiogenic activity [311-314], which highlights the potential of these agents for controling both inflammation and inflammation-induced angiogenesis associated with chronic inflammation.

1.5 Piperine

1.5.1 Properties of Piperine

Piperine is a dietary phytochemical from the fruits of black and long pepper plants, *Piper nigrum* and *Piper longum*, respectively. Both plants are used in Ayurvedic medicine [315] to treat pain and gastrointestinal disorders [316]. Piperine is the alkaloid responsible for the pungent, burning flavor of black pepper, due to its activation of the transient receptor potential vanilloid (TRPV)1 [317]. Piperine constitutes approximately 5-7% of black pepper [318], has a molecular formula of $C_{17}H_{19}O_3N$, and its full chemical name is 1-[(2E,4E)-5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-piperidine. Piperine

is derived from the amino acid lysine [319] and contains several functional groups, including an α,β-unsaturated carbonyl group, a piperidine group, and a methylenedioxyphenyl group [320] (Figure 1.4).

A variety of physiological effects have been observed with piperine [321], including anti-bacterial [322], anti-fungal [323], anti-parasitic [324], and insecticidal [325] activities. Piperine also has various neurophysiological properties. For example, the mood-enhancing and anti-depressant effects associated with piperine [326] are correlated with the inhibition of monoamine oxidase enzymes by piperine [327]. Additionally, piperine inhibits memory impairment and neurodegeneration in an animal model of Alzheimer's disease [328]. These neuroprotective properties of piperine demonstrate the potential of this phytochemical for the treatment of depression and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. The cardioprotective activity of piperine has also been demonstrated through inhibition of platelet aggregation [329], reduction in blood pressure [330], and inhibition of lipid accumulation in macrophages [331]. Furthermore, piperine acts as an anti-oxidant [332] and increases the ratio of high-density lipoprotein to low-density lipoprotein in animals on a high-fat diet [333], making piperine a potential protective agent against heart disease and atherosclerosis.

Piperine has various effects on the gastrointestinal system that aid in digestion [321]. For example, piperine increases the secretion of gastric acid [334], while protecting against gastric ulcer formation [335]. Within the duodenum, the enzymatic activity of pancreatic lipase and amylase is increased following piperine treatment [336], aiding in digestion. Furthermore, piperine reduces gastrointestinal motility [337,338], fluid accumulation [339], and has anti-diarrheal activity [340]. Collectively, these beneficial effects of piperine make it a potential therapeutic agent for the treatment of diarrhea in conditions such as irritable bowel syndrome and IBD.

Piperine is a potential novel therapy for vitiligo, a disfiguring autoimmune condition of the skin that is caused by T cell-mediated destruction of pigmented melanocytes [341]. Melanocyte proliferation is stimulated by piperine [342], which has

been effective in treating vitiligo in multiple animal models [343,344]. Piperine is currently patented for use as a vitiligo therapy (Patent # CA 2337205) [345].

1.5.2 Modes by which Piperine Mediates its Effects

TRPV1

Binding of piperine to its known receptor, TRPV1, results in short-term activation of the receptor, followed by receptor desensitization [317]. TRPV1 is a non-selective cation channel expressed on a variety of tissues, including nociceptive sensory neurons, thereby linking receptor activation to pain sensation [346]. TRPV1 is activated by a variety of noxious stimuli such as heat and acidic conditions [346]. Desensitization of TRPV1 following piperine exposure prevents further activation of the receptor [317], contributing to the analgesic activity of piperine [347,348]. Capsaicin, like piperine, is an agonist of TRPV1 [349], but also mediates its effects via TRPV6 [350], indicating receptor promiscuity among TRPV agonists; therefore, TRPV1 may not be the only TRPV receptor that is activated by piperine.

Passive Diffusion

In addition to altering cellular function by inducing cation flux and desensitization of TRPV1, piperine is also extremely lipophilic, allowing it to be efficiently absorbed by passive diffusion in the small intestine [351]. Piperine is apolar and easily penetrates the hydrophobic core of cell membranes [352], suggesting that entry of piperine into cells by passive diffusion may not be limited to cells of the small intestine. Orally administered piperine has a high bioavailability with 97% being absorbed within the digestive tract and only approximately 3% excreted in the feces of rats [353,354]. Piperine distribution 6 h following oral administration resulted in physiologically active levels of piperine in the serum, although piperine primarily accumulated in the spleen, liver, and kidneys, and was cleared after 24 h [353]. Piperine is not found in the urine or bile in its native form but is metabolized by glucuronidation and sulphation and is excreted as the biliary metabolite piperic acid and various urinary metabolites, including vanillic acid, piperonylic acid, piperonyl alcohol, and piperonal [353]. The physiological activity of these metabolites remains poorly understood.

1.5.3 Effect of Piperine on Bioavailability

Piperine is patented for its ability to increase the bioavailability of various drugs and nutrients (Patent # CA 2247467) [355] and is available commercially from Sabinsa Corporation as BioPerine®. Piperine influences the bioavailability of other chemicals by increasing their absorption in the small intestine [351,352] and by inhibiting their metabolism [356-358]. For example, it is thought that piperine increases the membrane fluidity of intestinal epithelial cells [352] by lowering the cholesterol to phospholipid ratio in the lipid membrane [359], as well as by increasing the length and perimeter of the intestinal microvilli, which cumulatively results in increased absorptive surface area [352]. Additionally, piperine inhibits the expression of various drug efflux pumps such as P-glycoprotein (P-gp), multidrug resistant protein (MRP)1, and breast cancer resistant protein (BCRP), which are able to pump various xenobiotics out of cells [360]. Bhardwaj *et al.* [357] showed that piperine administration results in higher absorption and serum concentrations of P-gp-targeted xenobiotics, as a direct result of diminished P-gp activity in the intestine due to piperine. Piperine also inhibits the activity of various enzymes involved in xenobiotic metabolism such as cytochrome P450 (CYP)3A4 [357] and CYP1A1 [361], as well as uridine diphosphate (UDP)-glucoronsyltransferase [362]. The inhibitory effect of piperine on the metabolism of various drugs and nutrients by these enzymes results in higher plasma concentrations of various xenobiotics in humans, such as propanolol and theophylline [363]. Piperine therefore increases the plasma concentrations of certain drugs and nutrients by increasing their absorption as well as decreasing their metabolism.

1.5.4 Anti-Inflammatory Activity of Piperine

The anti-inflammatory activity of piperine was first discovered by Lee *et al.* [364] using a carrageenan-induced paw edema model in rats. Mujumdar *et al.* [365] expanded on the findings of Lee *et al.* [364] and found that piperine inhibited paw edema induced not only by carrageenan but also by histamine and formalin. Interestingly, piperine did not affect PGE_1-induced paw edema, indicating that the inhibitory activity of piperine on paw edema was specific to certain inflammatory stimuli and involves a mechanism that is independent of PGE_1 signaling [365]. In addition to its inhibitory activity on acute

edema, piperine also inhibited chronic granuloma formation in rats [365]. These early *in vivo* studies were mostly observational and no examination of piperine's mechanism was performed; however, more recent *in vitro* work has shed some light on how piperine mediates its anti-inflammatory effects. For example, piperine inhibits TNF-α-induced expression of the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin, resulting in reduced neutrophil-endothelial cell interactions [366,367]. This suggests that piperine may inhibit the extravasation of inflammatory cells from the vasculature into sites of inflammation by inhibiting their attachment to the endothelium. Piperine also inhibits production of the chemokine monocyte chemotactic protein (MCP)-1 by macrophages, as well as macrophage migration stimulated by adipose tissue-conditioned medium [368], which is high in pro-inflammatory cytokines and chemokines [369,370]. This suggests that piperine may also inhibit leukocyte infiltration into inflamed tissue by inhibiting chemokine synthesis and chemotactic migration. Additionally, piperine inhibits the *in vitro* production of the pro-inflammatory cytokine TNF-α by macrophages [371,372], as well as IL-1β and IL-6 synthesis by melanoma cells [373]. Recent *in vivo* studies have also shown piperine to inhibit LPS-induced NO and TNF-α production by macrophages [371,372] and increase the survival of lipopolysaccharide (LPS)-treated mice [372], suggesting that inhibition of pro-inflammatory cytokine production contributes to the anti-inflammatory activity of piperine. Interestingly, piperine not only inhibits pro-inflammatory cytokine production, but also inhibits downstream mediators activated by pro-inflammatory cytokines. For example, expression of the pro-inflammatory enzyme cyclooxygenase-2, as well as one of its downstream products, PGE_2, were inhibited by piperine treatment of IL-1β-stimulated synoviocytes [348]. Piperine also inhibits the enzyme 5-lipoxygenase (LO) [374], which is a key enzyme in the synthesis of leukotrienes, pro-inflammatory mediators of neutrophil chemoattraction [375] and bronchoconstriction in asthma [376]. Piperine-induced inhibition of 5-LO activity, pro-inflammatory cytokine production, and leukocyte migration and extravasation *in vitro* may all contribute to the *in vivo* inhibitory activity of piperine in various inflammatory models. For example, piperine inhibits ovalbumin (OVA)-induced airway inflammation and hyperresponsiveness in a mouse model of asthma [377]. In this asthma model, mice treated with 4.5 mg/kg of piperine showed a reduction in the numbers of eosinophils,

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CD4$^+$, CD8$^+$, and CD3$^+$CD69$^+$ T cells in the lungs compared to control animals, suggesting an inhibition of eosinophil and T cell infiltration and/or proliferation by piperine [377]. Additionally, piperine-treated mice had reduced bronchoalveolar lavage fluid levels of IL-4 and IL-5 and serum levels of histamine and anti-OVA IgE, indicating an inhibitory effect of piperine on the OVA-induced Th2 response [377]. Additionally, piperine was as effective in inhibiting cellular infiltration and bronchial edema as the well established anti-inflammatory agent, cyclosporin [377]. In a carrageenan-induced arthritis model in mice, piperine reduced paw swelling and pain, as well as the histological inflammation score, showing comparable anti-inflammatory potency to the steroid prednisone [348]. Piperine-treated mice also had greater limb function and smaller areas of lymphocytic infiltration into the joint [348]. Finally, piperine inhibited pancreatitis induced in mice by administration of cerulein, a cholecystokinin analogue [378]. Bae *et al.* [378] demostrated that piperine reduced cerulein-induced edema, inflammation, vacuolization, and necrosis of the pancreas by histological examination, which was attributed to reduced serum and pancreatic levels of TNF-α, IL-1β, and IL-6, and diminished neutrophil infiltration.

Despite the established anti-inflammatory properties of piperine and our understanding of the key roles that lymphocytes play in shaping inflammatory responses [4], little research has focused on the direct effects of piperine on lymphocytes. A recent *in vitro* analysis showed that piperine directly inhibits B cell proliferation, cytokine production, and B cell-mediated T cell stimulation [379]; moreover, piperine inhibits T cell proliferation induced by DC antigen presentation [380]. The direct effects of piperine on DCs include inhibition of chemokine receptor and costimulatory molecule expression, cytokine production, migration, and T cell stimulatory activity [380]. The current study investigates the direct effect of piperine on T cells.

1.5.5 Chemopreventative Activity of Piperine

There is a considerable body of evidence connecting inflammation and cancer [301,381,382], so it is not surprising that piperine also possesses chemopreventative activities. For example, in the dimethylbenz-α-anthracene (DMBA)-induced hamster buccal pouch carcinogenesis model, oral administration of 50 mg/kg of piperine reduced

the development of hyperkeratosis, hyperplasia, and dysplasia and completely prevented the development of squamous cell carcinoma [383,384]. In this model, piperine treatment of DMBA-treated mice increased the levels of the phase II detoxification enzymes, glutathione-S-transferase and glutathione reductase, and various antioxidants such as glutathione, as well as decreasing lipid peroxidation compared to DMBA-treated mice alone [384]. This finding is supported by Khajuria *et al*. [385], who report that piperine inhibited oxidative stress and lipid peroxidation induced in rat intestinal mucosa by the carcinogens DMBA and methycholenthrene. In another *in vivo* mouse model, the incidence of benzo(a)pyrene (BaP)-induced lung carcinogenesis was decreased when 50 mg/kg of piperine was given orally before or after carcinogen exposure [386], and is attributed to piperine preventing BaP-induced increases in lipid peroxidation and antioxidant depletion [387]. Taken together, these observations suggest that piperine possesses chemopreventative activity. Interestingly, the chemical carcinogens used in the above carcinogenesis models require metabolic activation, primarily by the enzymes CYP1A1 and CYP1B1, to acquire their mutagenic and carcinogenic activities [388,389]. Since piperine inhibits CYP1A1 activity [361], the chemopreventative activity of piperine in these chemically-induced carcinogenesis models may be related to piperine-mediated inhibition of CYP1A1 bioactivation of the procarcinogens DMBA and BaP. However, the chemopreventative activity of piperine is not limited to inhibition of carcinogen activation, as piperine also prevents oxidative stress, antioxidant depletion, and lipid peroxidation induced by ethanol [390] and high-fat diet [332], which do not directly involve CYP1A1 or CYP1B1 activation. Furthermore, Kakarala *et al*. [391] showed that piperine inhibits mammosphere formation by primary breast epithelial cells and selectively inhibits the proliferation of undifferentiated mammary stem and progenitor cells, which are thought to initiate carcinogenesis upon dysregulation of their proliferation and subsequent malignant transformation [392]. Therefore, the chemoprotective qualities of piperine are diverse, involving antioxidant activity, inhibition of carcinogen activation, and inhibition of mammosphere formation and proliferation of progenitor and stem cells.

1.5.6 Chemotherapeutic Activity of Piperine

Piperine also has chemotherapeutic activity against a variety of cancer types *in vitro* and *in vivo*. Piperine is cytotoxic to B16-F10 melanoma cells [393], Dalton's lymphoma ascites (DLA) cells, and Ehrlich ascites carcinoma (EAC) cells [394], and inhibits the *in vitro* proliferation of DLD-1 colon cancer cells [395]. In addition to cytotoxic and anti-proliferative activities, piperine also inhibits the production of MMPs as well as collagen invasion by melanoma [373] and HT-1080 fibrosarcoma cells [396]. *In vivo*, piperine inhibits the growth of Sarcoma 180 [397,398] and DLA cells [394]. Piperine-treated mice with EAC tumors have increased life spans compared to mice with untreated EAC tumors [394]. Furthermore, piperine inhibits the metastasis of tail vein-injected B16-F10 melanoma cells, as demonstrated by a reduction in lung nodules, tumor burden on the lungs, and an increased life span of the mice [393].

Interestingly, piperine enhances the effects of other chemotherapeutic agents. For example, piperine increases the efficacy of the chemotherapeutic agent 5-fluorouracil on MDA-MB-435 breast cancer and HL-60 leukemia cells *in vitro* [398]. Moreover, piperine not only inhibits tumor growth in PC-3 prostate tumor xenografts in mice, but this inhibitory effect was amplified in combination with the chemotherapeutic agent docetaxel due to a piperine-mediated reduction in docetaxel metabolism by CYP3A4 [399]. Therefore, piperine has potential use as a chemotherapeutic agent alone or as an adjunct therapy for current chemotherapeutic regiments.

1.5.7 Mechanism of Piperine's Activities

Although the mechanism by which piperine exerts its various physiological effects is not fully elucidated, studies on various cell types have identified several intracellular signaling molecules that are altered in the presence of piperine. Various studies have shown that the IκB/NF-κB pathway is inhibited in the presence of piperine. Piperine inhibits the phosphorylation and/or degradation of the inhibitory protein IκBα in endothelial cells [367] and HT-1080 fibrosarcoma cells [396]. IκBα phosphorylation and degradation leads to the release and nuclear translocation of the transcription factor NF-κB, an important transcription factor for cell proliferation and survival [400]. Downstream of IκB, nuclear localization of the NF-κB subunit RelA (also known as p65)

is inhibited by piperine in HT-1080 fibrosarcoma [396], B16-F10 melanoma [373], and endothelial cells [367]. In addition to the RelA subunit, nuclear localization of the p50 and cRel NF-κB subunits is also inhibited by piperine in melanoma cells [373]. Piperine also inhibits the transcriptional activity of NF-κB in fibrosarcoma cells [396] and endothelial cells [367]. In contrast to these studies, no change in IκBα degradation or NF-κB RelA nuclear localization is seen in synoviocytes cultured in the presence of piperine [348], suggesting that the effect of piperine on intracellular signaling pathways may be dependent on the type of stimuli. Phosphorylation of the MAPK ERK is also inhibited in the presence of piperine in various cell types, including fibrosarcoma cells [396], synoviocytes [348], and pancreatic cells [378]. Conversely, basal ERK phosphorylation is increased in piperine-treated auditory cells [401]. ERK activation is important in cell proliferation and leads to the downstream expression of the transcription factor c-fos [50]. Nuclear localization of c-fos is inhibited by piperine in melanoma cells [373], synoviocytes [348], and fibrosarcoma cells [396]. The transcriptional activity of AP-1, a heterodimeric transcription factor traditionally composed of members of the c-fos and jun transcription factor families [53], was also inhibited by piperine in fibrosarcoma cells [396]. In contrast to ERK, piperine has differential effects on the MAPKs JNK and p38. Piperine inhibits JNK and p38 phosphorylation in pancreatic cells [378], increases basal levels of JNK and p38 phosphorylation in auditory cells [401], and has no effect on JNK and p38 phosphorylation in synoviocytes or fibrosarcoma cells [396]. Conversely, the nuclear localization and phosphorylation of c-jun, the transcription factor that is phosphorylated by JNK [52] is inhibited by piperine in fibrosarcoma cells [396]. Thus, the differential effect of piperine on JNK and p38 activation in different cell types is poorly delineated and requires further evaluation. Upstream of MAPK and IκB/NF-κB activation in various signaling pathways is activation of the signaling molecule PKC [402,403]. Interestingly, piperine treatment inhibits the phosphorylation and membrane localization of PKCα but not PKCδ in fibrosarcoma cells [396]. Inhibition of specific PKC isoforms may be upstream of the piperine-induced inhibition of ERK and IκB/NF-κB activation seen in various studies, although this requires further study in multiple cell types. Based on these observations, the signaling mechanism of piperine involves inhibition of various signaling pathways, including ERK and IκBα and their downstream

transcription factors c-fos and NF-κB, although the effect of piperine on these pathways differs in different cell types and stimulation conditions. Furthermore, the effect of piperine on JNK, p38, and PKC pathways is poorly delineated and requires further evaluation.

1.6 Rationale and Objectives

T cells are important mediators of immunity against pathogens and cancer [4]. When dysregulation of the immune system occurs and T cells are inappropriately activated, chronic inflammation and autoimmunity can result and lead to such conditions as IBD [404], MS [405], and rheumatoid arthritis [406]. Angiogenesis also plays a critical role in the pathogenesis of many of these chronic inflammatory and autoimmune conditions [407-409]. Current therapies for the treatment of chronic inflammation are costly and are associated with a myriad of undesirable side effects [410], prompting an ongoing search for less expensive and less toxic agents with T cell inhibitory and/or anti-angiogenic activities.

The anti-inflammatory properties of piperine have been demonstrated both *in vitro* and *in vivo* [348,365,371,372,378]. *In vivo* administration of piperine inhibits T cell proliferation and cytokine production *ex vivo* [377,411], but the direct effects of piperine on T cell function remain largely unknown. Furthermore, piperine inhibits endothelial cell activation marker expression [366,367], but the effect of piperine on endothelial cell function and angiogenesis involved in inflammation has not yet been examined. Based on the previously identified anti-inflammatory properties of piperine, I hypothesize that piperine will have inhibitory activity on the function of T cells and endothelial cells, as well as the process of angiogenesis.

The objectives of this study are:

1) To determine the effect of piperine on *in vitro* T cell activation and function, including proliferation, activation marker expression, cytokine production, and CTL activity (Chapter 3, Manuscript 1).

2) To determine the effect of piperine on IL-2-induced proliferation and IL-2R signaling in an IL-2-dependent T cell line *in vitro* (Chapter 4, Manuscript 2).

3) To determine the effect of piperine on endothelial cell proliferation, migration, and angiogenesis *in vitro, ex vivo*, and *in vivo* (Chapter 5, Manuscript 3).

Figure 1.1 T cell receptor signaling. See text for details.
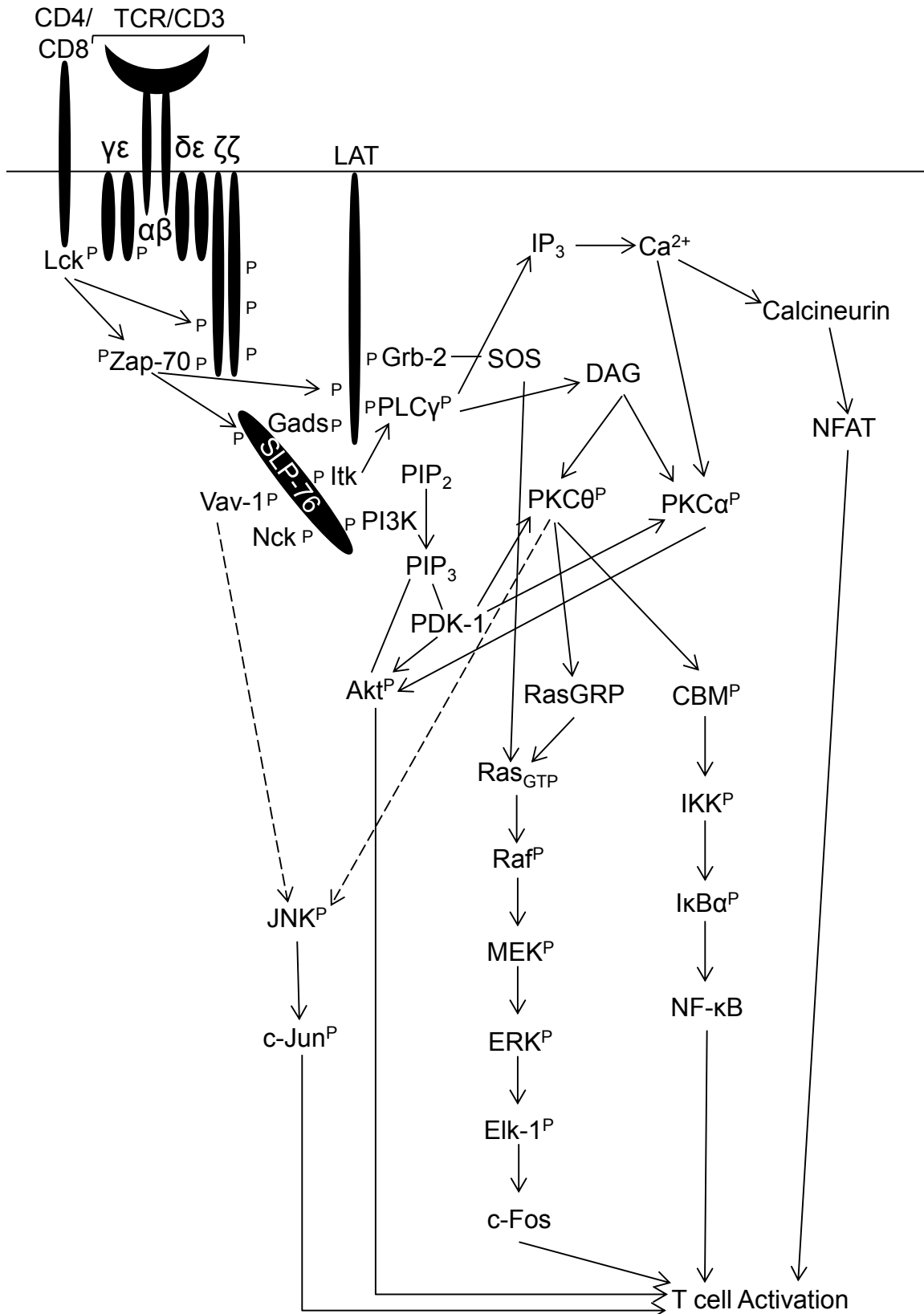
Figure 1.1

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Figure 1.2 IL-2R signaling. See text for details.
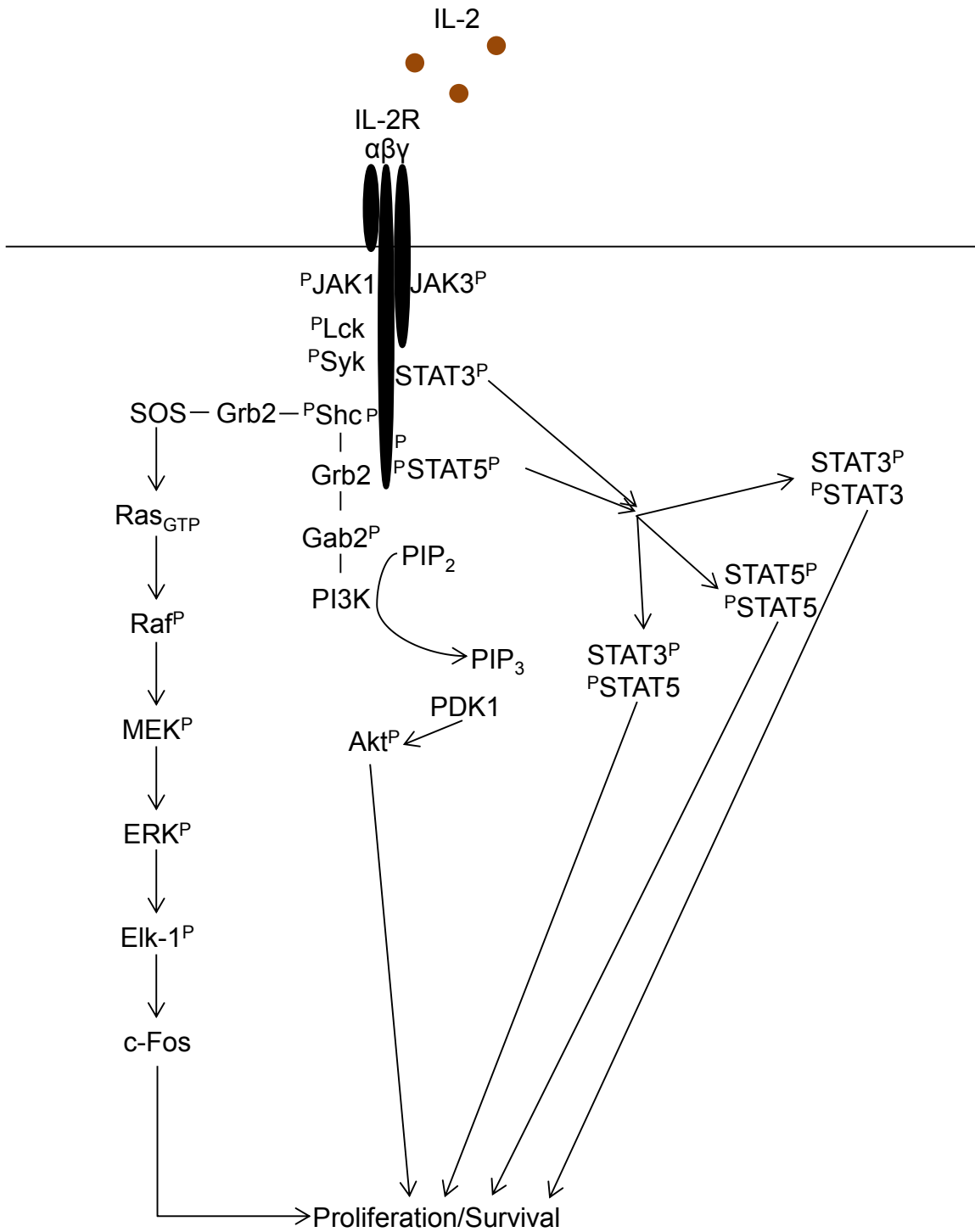
Figure 1.2

Figure 1.3 Process of Angiogenesis. Modified from [230]. See text for details.

Angiogenic Stimulation

Endothelial Activation

Basement Membrane Degradation

Endothelial Proliferation

VEGF

Endothelial Migration

Vascular Maturation
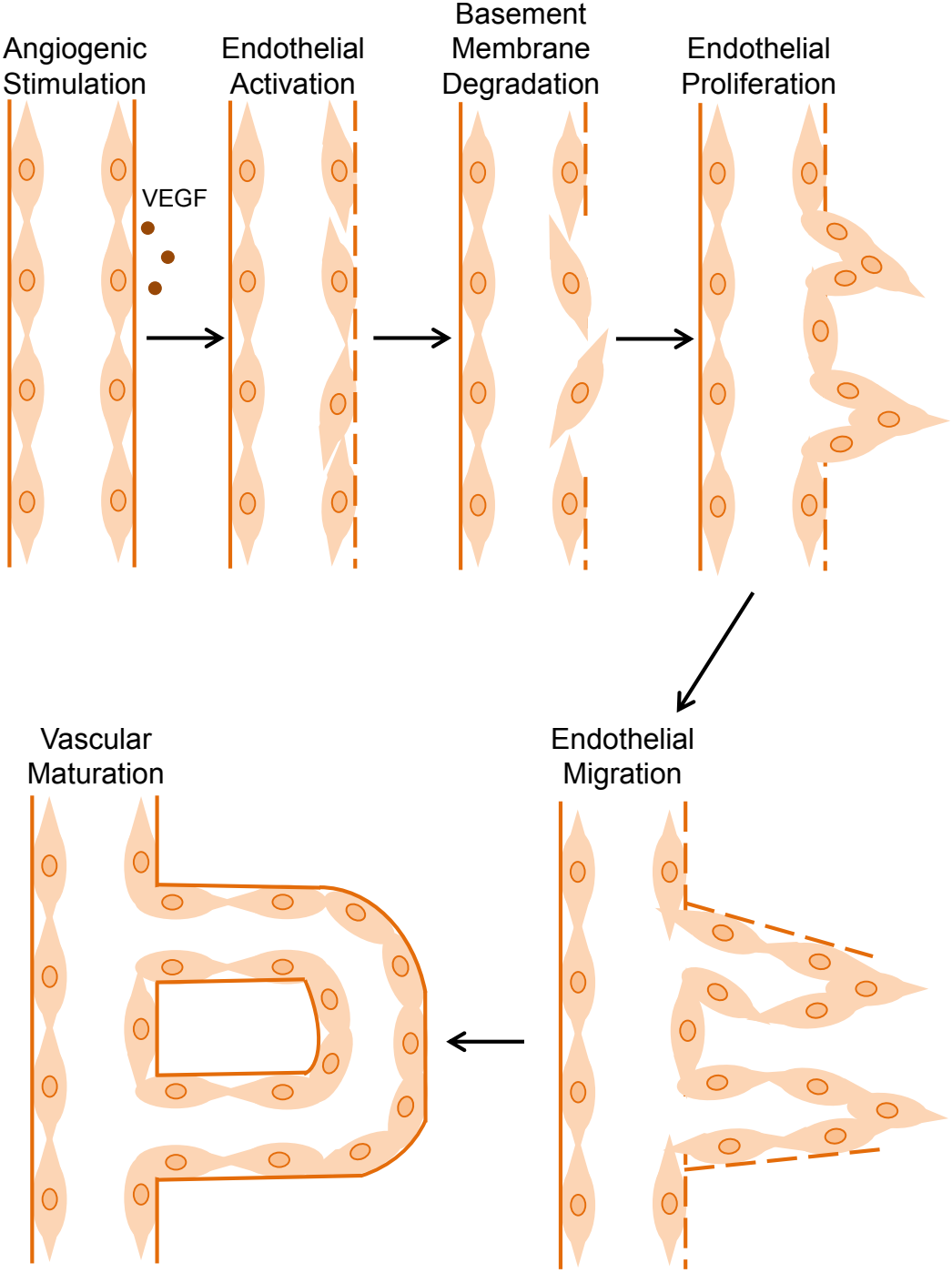
Figure 1.3

Figure 1.4 Structure of Piperine.
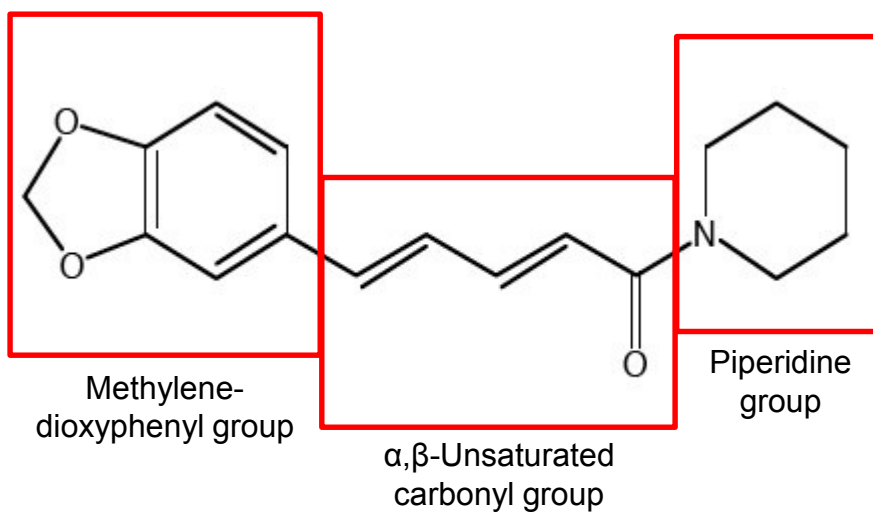
Figure 1.4

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

C57BL/6 (H-2^b) mice of 6-8 weeks of age were purchased from Charles River Canada (Lasalle, QC) and were used for all primary T cell experiments unless otherwise noted. TRPV1$^{-/-}$ mice (B6.129X1-*Trpv1^{tm1Jul}*/J) on a C57BL/6 background and age-matched C57BL/6J wildtype (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.

2.2 Reagents

Aprotinin, bovine serum albumin (BSA), bromophenol blue, cycloheximide (CHX), dimethyl sulfoxide (DMSO), isopropanol, leupeptin, β-mercaptoethanol (β-ME), nonidet P-40 (NP-40), pepstatin A, phenylarsine oxide (PAO), phenylmethylsulfonyl fluoride (PMSF), phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI)-1640 medium, SB-366791, sodium azide (NaN$_3$), sodium deoxycholate, sodium fluoride (NaF), Triton X-100, and piperine (purity ≥ 97%) were all purchased from Sigma-Aldrich Canada (Oakville, ON). Polymyxin B, which binds to and inactivates endotoxin [412], was added to some cell proliferation assays to confirm the endotoxin-free status of piperine. Fetal calf serum (FCS), 200 mM L-glutamine, 10,000 units/mL penicillin/10,000 μg/mL streptomycin, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 0.4% trypan blue dye solution, 0.25% trypsin-EDTA were purchased from Invitrogen Canada Inc. (Burlington, ON). Sodium orthovanadate (Na$_3$VO$_4$), PD 98059, STAT5 inhibitor ([N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide]; CNH), and calcium chloride (CaCl$_2$) were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Disodium hydrogen phosphate (Na$_2$HPO$_4$) and ethylene diamine tetraacetic acid (EDTA) were purchased from EM

Industries Inc. (Hawthorne, NY). Acrylamide/bis-acrylamide (29:1, 30% solution), ammonium persulfate (APS), diethylpyrocarbonate (DEPC), dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), glycine, paraformaldehyde (PFA), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), Tris base and Tween-20 were purchased from Bio-Shop Canada Inc. (Burlington, ON). LY-294002, N-(4-t-Butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC), and capsazepine were purchased from Enzo Life Sciences (Plymouth Meeting, PA). Concentrated hydrochloric acid (HCl) and concentrated sulfuric acid were purchased from Fisher Scientific Canada (Ottawa, ON). Chloroform, glacial acetic acid, and glycerol were purchased from BDH Inc. (Toronto, ON). Pyrogen-free water was purchased from Baxter Corporation (Toronto, ON). Anhydrous ethyl alcohol (ethanol; EtOH) was purchased from Commercial Alcohols (Brampton, ON). All cell culture plastics were purchased from Sarstedt Inc. (Montreal, QC) unless otherwise noted.

2.3 Stock Solutions

A 100 mM stock solution of piperine was prepared in DMSO and stored at -80°C. To compare vehicles, a 100 mM stock of piperine in EtOH was prepared and stored at -20°C. Unless otherwise noted, cells were treated with piperine made up in DMSO, and DMSO was used as the vehicle control. Stock concentrations of 40 mM PD 98059, 50 mM STAT5 inhibitor (CNH), 13 mg/mL SB-366791, 50 mM BCTC, and 25 mg/mL capsazepine were all prepared in DMSO and stored at -80°C. A 80 mM stock solution of LY-294002 was prepared in DMSO and stored at -20°C. CHX was prepared in EtOH at a stock concentration of 50 μg/mL and stored at -20°C. A 1 M stock of Na_3VO_4 was made up in water and stored at -20°C. Working solutions of drugs were made up in medium immediately prior to use and individual treatments contained a final DMSO concentration of 0.2% or less.

2.4 Antibodies

Antibodies (Abs) against phospho-ZAP-70 (Tyr 319)/Syk (Tyr 352), total ZAP-70 (clone 99F2), phospho-JAK1 (Tyr 1022/1023), phospho- STAT5 (Tyr 694), phospho-

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STAT3 (Tyr 705), total STAT3, phospho-Akt (Ser 473), total Akt, cyclin D2 (clone D52F9), cyclin D3 (clone DCS22), CDK4 (clone DCS156), and CDK6 (clone DCS83) were all purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-total STAT5a and anti-phospho-Tyr (clone 4G10) Abs were purchased from Millipore (Billerica, MA). Abs against actin, phospho-JAK3 (Tyr 980), phospho-ERK (Tyr 204; clone E-4), total ERK (clone K-23), and CD25 (clone M-19), as well as horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated bovine anti-goat IgG, and HRP-conjugated donkey anti-rabbit IgG Abs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phycoerythrin (PE)-conjugated anti-CD25 (clone 3C7) and fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (clone PC61.5.3) Abs were purchased from BD Biosciences (Mississauga, ON) and Cedarlane Laboratories Ltd. (Burlington, ON), respectively. Functional grade anti-mouse TCRβ (clone H57-597), PE-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-CD8a (clone 53-6.7), FITC-conjugated anti-CD69 (clone H1.2F3), PE-conjugated rat IgG2b, PE-conjugated rat IgG2a, FITC-conjugated rat IgG1, and FITC-conjugated Armenian hamster IgG, Ab were purchased from eBioscience, Inc. (San Diego, CA).

2.4 Cell Cultures

All centrifugations involving whole cells occurred at 500 gravity (*g)* for 5 minutes (min) unless otherwise noted. Viability of cell cultures was determined by trypan blue dye exclusion with a 0.1% trypan blue dye solution (v/v) in PBS, based on the principle that live cells are able to extrude the dye, whereas dead cells, which are no longer able to extrude the dye, turn blue when viewed under a light microscrope. All cells used for experiments were typically ≥95% viable. All incubations and cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2 unless otherwise noted.

2.4.1 Primary Murine T Cells

Highly purified primary $CD3^+$ T cells were isolated by negative selection using the Pan T Cell Isolation MACS® kit (Miltenyi Biotech, Auburn, CA) as per the manufacturer's instructions with slight modifications. C57BL/6 WT mice were used for all primary T cell isolations unless otherwise noted. Mice were euthanized by cervical dislocation and spleens were excised using aseptic technique. All isolation procedures

were performed on ice and all centrifugations took place at 4°C. Spleens were processed in ice-cold PBS with a tissue homogenizer, and tissue debris were removed to obtain a single cell suspension. Erythrocytes were lysed by hypo-osmotic shock using a 20 second (sec) exposure to a hypotonic 0.2% NaCl solution before the addition of a hypertonic 1.6% NaCl solution to restore the solution to an isotonic state. Cell debris were removed, cells were centrifuged, and resuspended in 1 mL of MACS® buffer (2% BSA [w/v], 2 mM EDTA in PBS, pH 7.2). Cells were run through a MACS® Pre-Separation Filter (30 µm nylon mesh; Miltenyi Biotech) to remove any remaining debris and obtain a single cell suspension. Cells were then centrifuged, resuspended in MACS® buffer, and counted using a hemocytometer and trypan blue dye exclusion. 1.35×10^8 live splenocytes were diluted in 450 µL of MACS® buffer and incubated with 50 µL of biotin-conjugated Ab cocktail for 10 min at 4°C. This cocktail contained Abs against CD11b (Mac-1), CD45R (B220), CD49b (DX5), and Ter-119, which will collectively bind to B cells, NK cells, dendritic cells, macrophages, granulocytes, and erythroid cells. The cells were further diluted with 400 µL of MACS® buffer and 100 µL of microbeads coated with anti-biotin Ab was added to the cell mixture and incubated for 15 min at 4°C. Cells were diluted with 9 mL of MACS® buffer and centrifuged to remove excess antibodies and microbeads. Cells were resuspended in 500 µL of MACS® buffer and run through an activated MACS® LS column placed in the magnetic field of a MACS® separator magnet (Miltenyi Biotech). The column was washed with 12 mL of MACS® buffer. Any cells bound by the biotin-conjugated Ab cocktail and anti-biotin microbeads remained in the column due to the magnetic field, allowing for the elution of highly purified negatively-selected $CD3^+$ T cells. T cells were washed with 10 mL of RPMI-1640 medium supplemented with 5% heat-inactivated (56°C for 30 min) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 5 mM HEPES, hereafter referred to as complete (c) RPMI. Cell pellets were resuspended in cRPMI, and cells were counted using a hemocytometer and trypan blue dye exclusion. T cell purity using this isolation procedure was ≥97% based on CD3 expression by flow cytometry.

2.4.2 P815 Cells

The murine mastocytoma cell line P815 $(H-2^d)$ was obtained from American Tissue Culture Collection (ATCC; Manassas, VA) and cultured in cRPMI.

2.4.3 CTLL-2 Cells

Cytotoxic Lymphoid Line 2 (CTLL-2) cells were obtained from ATCC and were cultured in HEPES-free cRPMI supplemented with 10% heat-inactivated FCS and 30 U/mL (6 ng/mL) of recombinant murine IL-2 (PeproTech Inc., Rocky Hill, NJ).

2.4.4 HUVECs

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Inc. (Walkersville, MD) and were cultured in Clonetics® EGM®-2 Endothelial Cell Growth Medium (Lonza). EGM®-2 medium, which contains a proprietary mix of endothelial cell growth factors, including epidermal growth factor (EGF), VEGF, FGF-2, and insulin-like growth factor (IGF), was used as the culture medium for all experiments unless otherwise noted. HUVECs from passages 2-6 were used in all experiments. Cells were grown to a confluency of approximately 80% in T75 vented flasks. To lift cells from the cell culture plastic, cells were washed with HEPES-PBS buffer (5 mM HEPES in PBS) and incubated with 3 mL of 0.25% Trypsin-EDTA for 2 min at ambient temperature. Trypsin was diluted and inactivated by the addition of EGM®-2 medium, cells were centrifuged, and excess trypsin was poured off of the cell pellet.

2.4.5 HMC-1

Human mastocytoma cells (HMC)-1, which were a generous gift from Dr. Jean Marshall (Dalhousie University, Halifax, NS), were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 5 mM HEPES.

2.5 Cell Seeding Conditions

For all T cell and CTLL-2 cell experiments, treatments were added 15 min prior to cell activation, unless otherwise noted. Experiment volumes varied based on the type of tissue culture plasticware, where 200 μL was the final volume for all experiments in 96-well flat- or round-bottom plates. For 24 well plates and 1.5 mL microcentrifuge tubes (PROgene®, St-Laurent, QC), the final volume for experiments was 1 mL. For 6 well plates and 5 mL round-bottom polystyrene tubes (BD Biosciences), the final volume for experiments was 2 mL. The final volume for experiments in T75 flasks was 10 mL.

2.5.1 T Cells

T cells for tritiated-thymidine ([^{3}H]TdR) incorporation (24-96 h), cytometric analysis of cell division (72 h), Annexin-V/propidium iodide (PI) staining (24 h), cell surface staining with fluorescent Ab (24 h), cell cycle analysis (72 h), and enzyme-linked immunosorbant assay (ELISA; 24h) were cultured for the times shown in parentheses at 2.5×10^5 cells/well in 96-well round-bottom plates with a minimum of quadruplicate wells per treatment. T cells were cultured at 5×10^6 cells/well in 24-well flat-bottom plates for 72 h for the generation of CTLs. For western blot analysis, T cells were cultured at 5×10^6 cells/tube in 1.5 mL microcentrifuge tubes for 0-24 h timepoints, or at 2.5×10^6 cells/tube in 5 mL tubes for 48 h timepoints.

2.5.2 CTLL-2 Cells

For the [^{3}H]TdR incorporation assay, CTLL-2 cells were plated at 1×10^4 cells/well in 96-well flat-bottom plates with quadruplicate wells per treatment and incubated for 48 h. For Annexin-V/PI staining and cytometric analysis of cell division, CTLL-2 cells were plated at 1×10^5 cells/well in 24-well flat-bottom plates and incubated for 24 and 72 h, respectively. CTLL-2 cells were plated at 5×10^5 cells/well in 24-well flat-bottom plates and incubated for 24 h for cell surface staining using fluorescent Abs. For western blot analysis, CTLL-2 cells were cultured at 1×10^6 cells/tube in 1.5 mL microcentrifuge tubes for 0-1 h timepoints or at 5×10^5 cells/well in 24-well flat-bottom plates for 24 h timepoints.

2.5.3 HUVECs

For the [^{3}H]TdR incorporation assay and lactate dehydrogenase (LDH) assay, HUVECs were plated at 3×10^3 and 5×10^3 cells/well, respectively, in 96-well flat-bottom plates with quadruplicate wells per treatment and incubated for 24 h following treatment. For cytometric analysis of cell division and cell cycle analysis, HUVECs were plated at 2.5×10^4 cells/well in 6-well flat-bottom plates and incubated for 72 h following treatment. HUVECs were plated at 6×10^4 cells/well or at 2.5×10^5 cells/well in 6-well flat-bottom plates and incubated for 24 h following treatment for Annexin-V staining and the *in vitro* migration assay, respectively. For western blot analysis, HUVECs were plated at 3×10^5 cells/T75 flask and incubated for 24 h following treatment. At the end of

their respective incubation periods, HUVECs were collected by trypsinization and remained in suspension for the duration of the following experiments: cytometric analysis of cell division, cell cycle analysis, Annexin-V staining, and western blot analysis. HUVECs were plated at 5 x 10^3 cells/well in a 96-well flat-bottom plate for the *in vitro* angiogenesis assay and incubated for 18-24 h. After plating, HUVECs were always allowed to adhere overnight before treatment, with the exception of the *in vitro* angiogenesis assay.

2.6 T Cell Activation

Freshly isolated primary T cells were activated using Dynabeads® Mouse T-Activator CD3/CD28 Ab-coated beads (Invitrogen) at a ratio of 1 bead for every 2 T cells. These beads were coated with anti-CD3 and anti-CD28 Abs by the manufacturer and are designed to act as surrogate APCs, resulting in the activation and expansion of murine T cells.

2.7 [^{3}H]TdR Incorporation Assay

For the last 6 h of incubation, cells were pulsed with 0.2 μCi of methyl [^{3}H]TdR (MP Biomedicals, Irvine, CA). Cells then underwent one freeze-thaw cycle prior to harvesting (HUVECs) or were harvested immediately (T cells and CTLL-2 cells) 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).

2.8 Flow Cytometry

Fluorescence analysis of cells was performed with a FACSCaliber flow cytometer using BD CellQuest™ software (version 3.3; BD Biosciences) and a minimum of 1 x 10^4 counts per sample. Counts were gated on the live cell population within each sample, with the exception of Annexin-V/PI staining where both live and dead cells were counted. Acquired data were analysed 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.

2.9 Cytometric Analysis of Cell Division

2.9.1 Suspension Cells

T cells and CTLL-2 cells were stained with 2 μM CellTrace™ Oregon Green® 488 (Invitrogen) in warm PBS for 10 min in the dark at ambient temperature to allow uptake of the dye into the cells. Excess dye was inactivated by the addition of 4 mL of FCS, and cells were centrifuged and then incubated for 30 min at 37°C in 10 mL of warm cRPMI to allow conjugation of the dye within the cells. Cells were then plated as described previously, treated as indicated, and incubated for 72 h. At the end of the timepoint, cells were transferred to 5 mL tubes and the mean channel fluorescence (MCF) of each sample was analysed by flow cytometry. Decreased fluorescence intensity was indicative of cellular division on the basis of the dye quantity being approximately symmetrically divided between two daughter cells, resulting in a sequential halving of the fluorescence intensity with each cellular division.

For T cells, the number of cell divisions was analysed using ModFit LT software (Verity Software House, Topsham, ME). Additionally, percent responding cells, which is the percentage of the original T cell population that underwent proliferation, was calculated using the formula:

$$\% \text{ Responding cells} = 100 \times \left(\frac{\sum_1^n \left(\frac{X_n}{2^n} \right)}{\sum_0^n \left(\frac{X_n}{2^n} \right)} \right)$$, where X_n is the number of cells in the division

peak n, and where the non-proliferative peak is numbered 0. In brief, the number of daughter cells within each division was divided by the precursor extrapolation (2^n) to estimate the number of precursor T cells from the original population that proliferated, as is previously described [413]. Following extrapolation, precursor numbers were summed and the number of precursors that proliferated was divided by the total number of precursors and multiplied by 100 to generate the percent responding cells, also known as the division precursor frequency, of the original T cell population.

For CTLL-2 cells, the number of cell divisions (n) was calculated using the formula $MCF_{control} = 2^n \times MCF_{sample}$, where $MCF_{control}$ is the MCF of CTLL-2 cells that were not stimulated with IL-2 for the duration of the experiment (non-proliferative control).

2.9.2 Adherent Cells

Staining of HUVECs for the analysis of cell division by flow cytometry occurred after the cells were plated in a 6-well flat-bottom plate and allowed to adhere overnight. Medium was removed, and wells were washed with warm PBS. Warm serum-free RPMI-1640 medium (1 mL) containing 5 μM CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) was added to each well and cells were incubated for 45 min at 37°C to allow uptake of the dye into the cells. Staining medium was then removed and wells were washed three times with warm HEPES-free cRPMI containing 10% FCS. Warm EGM®-2 medium was added to each well, and cells were incubated for 2-3 h at 37°C to allow conjugation of the dye within the cells. To act as a non-proliferative baseline, control cells were trypsinized, fixed with PFA solution (1% PFA (w/v) in 1x PBS), and stored at 4°C until analysis at the end of the experiment. The remaining cells were treated as indicated and incubated for 72 h. At the end of the timepoint, cells were trypsinized, fixed with PFA solution, and the MCF of each sample was analysed by flow cytometry. Decreased fluorescence intensity was indicative of cellular division as described above. The number of cell divisions (n) was calculated using the formula $MCF_{control} = 2^n \times MCF_{sample}$, where $MCF_{control}$ is the MCF of the non-proliferative control.

2.10 Cell Surface Staining with Fluorescent Abs

Cells were collected and washed with PBS and then with fluorescence-activated cell sorting (FACS) buffer (0.2% NaN_3, 1% BSA, in 1x PBS), and labeled on ice with fluorochrome-conjugated Abs or isotype-matched fluorochrome-conjugated control Abs at a concentration of 0.5 μg in 50 μL of FACS buffer for 45 min in the dark. Cells were then washed twice with FACS buffer, fixed in PFA solution, and analysed by flow cytometry.

2.11 Annexin-V/PI Staining

Cells were collected, centrifuged, washed with 1 mL of PBS if trypsinized, and resuspended in 50 μL of incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl$_2$). For HUVECs, the incubation buffer contained 1 μL of Annexin-V-FLUOS labeling reagent (Roche Diagnostics, Laval, QC), whereas for T cells and CTLL-2 cells, the incubation buffer contained 1 μL of Annexin-V-FLUOS labeling reagent and 1 μg/mL of PI. Samples were incubated in the dark at ambient temperature for 10-15 min, diluted with 450 μL of incubation buffer, and analysed immediately by flow cytometry. For T cells and CTLL-2 cells, percent cell death was calculated as the percentage of cells that stained positive for either PI and/or Annexin-V-FLUOS. For HUVECs, percent viability was calculated as the percentage of cells that stained negative for Annexin-V-FLUOS.

2.12 Cell Cycle Analysis

Cells were collected at the end of the 72 h incubation, resuspended in 500 μL of PBS, and then fixed by the drop-wise addition of 4.5 mL of ice-cold 70% EtOH while vortexing. Cells were stored at -20°C for at least 24 h. Samples were then centrifuged, washed with 5 mL of ice-cold PBS, and stained in 300-1000 μL of PI staining solution containing 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) in PBS. Samples were stained in the dark at ambient temperature for 30 min prior to analysis by flow cytometry. Samples were gated on the single cell population to eliminate the acquisition of debris and cell aggregates that could confound the analysis. The percentage of cells in the various phases of the cell cycle was determined using ModFit LT software.

2.13 Cell Lysate Preparation

Cells were collected, washed with 1mL of ice-cold PBS, and lysed with ice-cold lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 50 mM Na$_2$HPO$_4$, 0.25% sodium deoxycholate [w/v], 0.1% NP-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 NaF, 1 mM PMSF, 1 mM DTT, 100 μM Na$_3$VO$_4$, 10 μM PAO, and 10 μg/ml

aprotinin). Samples were incubated on ice for 15-30 min and clarified by centrifugation at 10-14,000 g for 10 min. Total cell protein was collected and quantified by colorometric assay 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 ELx800 UV universal microplate reader (BioTek Instruments, Inc., Winooski, VT) using KCJunior software (version 1.17; BioTek Instruments, Inc.). Protein levels were equalized between samples, which were then denatured by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (200 mM Tris–HCl [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], 15% β-ME [v/v], and 0.01% bromophenol blue [w/v]). Each sample was then heated to 95°C for 5 min and frozen at -80°C until use.

2.14 Western Blot Analysis

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% APS [w/v], and 0.15% TEMED [v/v] with a 4% acrylamide stacking gel containing 125 mM Tris–HCl [pH 6.8], 0.1% SDS [w/v], 0.1% APS [w/v], and 0.3% TEMED [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 [v/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 NaCl) and 0.05% Tween-20 [v/v] (TBST). Membranes were washed extensively with TBST and then incubated with the appropriate primary Ab for 1 h at ambient temperature or overnight at 4°C. Stock Abs 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 Ab 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 reprobed for actin expression, stripped using stripping buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS [w/v], and 100 mM β-ME), and reprobed for total protein expression when phosphorylated proteins were examined. Protein bands were quantified by densitometry using AlphaEase®FC software (ProteinSimple, Santa Clara, CA) or Scion Image for Windows (Scion Corporation, Frederick, MD).

2.15 Cytokine Quantification by ELISA

T cells were plated at 2.5 x 10^5 cells/well, treated with medium, vehicle, 100, or 50 µM piperine, and activated with Dynabeads® for 24 h. Supernatants were harvested and analysed for cytokine content by sandwich-ELISA kits. IL-2 and IFN-γ were analysed using sandwich-ELISA kits from BD Biosciences, while IL-4 and IL-17A were analysed using sandwich-ELISA kits from eBiosciences, as per the manufacturers' instructions. In brief, Costar® 96-well flat-bottom high binding chemistry enzyme immunoassay plates (Corning Inc., Corning, NY) were coated overnight at 4°C with capture Ab diluted in coating buffer (0.1 M Na_2CO_3 [pH 9.5] for IL-2 and IFN-γ; reconstituted ELISA/ELISPOT Coating Buffer from kit for IL-4 and IL-17A). Plates were then washed 5 times with wash buffer (0.05% Tween-20 [v/v] in PBS), and blocked with assay diluent (10% FCS [v/v] in PBS) for 1 h at ambient temperature. Plates were then washed 5 times with wash buffer and supernatants and recombinant cytokine standards were added and incubated overnight at 4°C. Plates were washed as before, and incubated with biotinylated detection Ab and streptavidin-HRP together for 1 h for the IL-2 and IFN-γ kits or incubated with biotinylated detection Ab for 1 h, washed 5 times, and then incubated with streptavidin-HRP for 30 min for the IL-4 and IL-17A kits. Plates then underwent seven 1 min washes prior to the addition of the substrate solution (tetromethylbenzidine [TMB]). After sufficient color had developed (15-30 min), stop solution (0.3 M sulfuric acid) was added to the wells, and the absorbance of the plates was read at 450 nm on a ELx800 UV universal microplate reader using KCJunior software (version 1.17; BioTek Instruments, Inc.). Further analysis of the absorbance readings was performed using SOFTmax® PRO software (version 4.3; Molecular

Devices Corp., Sunnyvale, CA) to quantify the cytokine concentrations according to the experimental standards.

2.16 Cytotoxicity Assay

To determine the effect of piperine on CTL function, freshly isolated $CD3^+$ T cells were plated at 5×10^6 cells/well in a 24-well flat-bottom plate and cultured in the presence of Dynabeads® for 72 h. Cells were then washed and the number of live cells was normalized between treatments by cell counting using trypan blue dye exclusion. Cells were then replated at effector:target cell ratios of 100:1 (5×10^5 T cells/well), 50:1 (2.5×10^5 T cells/well), or 25:1 (1.25×10^5 T cells/well) in 96-well round-bottom plates and treated with medium, vehicle, 50, or 100 μM piperine.

To determine the effect of piperine on CTL generation, T cells were plated at 5×10^6 cells/well in 24-well flat-bottom plates, treated with medium, vehicle, 50, or 100 μM piperine, and cultured in the presence of Dynabeads® for 72 h. Cells were then washed 3 times and the number of live cells was normalized between treatments by trypan blue dye exclusion. Cells were then replated at effector:target cell ratios of 100:1 (5×10^5 T cells/well), 50:1 (2.5×10^5 T cells/well), or 25:1 (1.25×10^5 T cells/well) in 96-well round-bottom plates.

P815 target cells were pulsed for 6 h with 5 μCi/mL of $[^3H]TdR$, washed 3 times, and added to the CTLs at 5×10^3 cells/well. 1 μg/mL of anti-TCR IgG Ab was added to the wells to induce redirected lysis of the P815s by the CTLs, on the basis that P815 cells express Fcγ receptors that will bind the Fc portion of the anti-TCR Ab, allowing the Fab portion of the Ab to stimulate the T cells. P815s were also plated alone to serve as a negative control for cytotoxicity. Effector and target cells were incubated together for 4 h, harvested, and the $[^3H]TdR$ content was determined by liquid scintillation counting. Percent cytotoxicity was measured as the loss of $[^3H]TdR$ counts per min (cpm) compared to P815 target cells alone using the formula: Percent cytotoxicity = 1 - (Experimental$_{cpm}$/Control$_{cpm}$) x 100, where Control$_{cpm}$ was the average cpm of the P815 cells cultured alone.

2.17 Spinal Cord Isolation

In order to confirm the genotype of the TRPV1$^{-/-}$ mice and to obtain a positive control for *TRPV1* messenger ribonucleic acid (mRNA) expression, TRPV1$^{-/-}$ and C57BL/6 WT control mice, respectively, were euthanized by cervical dislocation and their vertebral columns were excised and cut open using aseptic technique. The spinal cord, containing TRPV1-expressing dorsal root ganglia, was removed and homogenized using a syringe and 18 gauge needle.

2.18 RNA Isolation

Cells and spinal cord homogenates were lysed with TRIzol® Reagent (Invitrogen) as per manufacturer's instructions. In brief, TRIzol® was added to the cells for 5 min at ambient temperature before the addition of chloroform for 3 min. Lysates were then centrifuged at 12,000 g at 4°C for 15 min. The aqueous phase was collected, and isopropanol was added to precipitate the RNA. The RNA was pelleted by centrifugation at 12,000 g at 4°C for 10 min and the pellet was washed with ice-cold 75% EtOH [v/v] in water containing 0.1% DEPC [v/v]. RNA was centrifuged at 7,500 g at 4°C for 10 min and the pellet was dried by evaporation. RNA was resuspended in 35 µL of pyrogen-free water. 20 µL of RNA samples were treated with 2.5 U of RNase-free DNase and 2.5 µL of 10x reaction buffer using the RQ1-DNAse Kit (Promega, Madison, WI) and RNA quantity and quality was assessed by spectrophotometric analysis using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Ottawa, ON). Only samples with an A260/A280 ratio $\geq$ 1.7 were used for reverse transcription (RT)-polymerase chain reaction (PCR) to analyse *TRPV1* expression.

2.19 RT-PCR for *TRPV1* expression

All reverse transcription and PCR reagents were purchased from Invitrogen unless otherwise noted.

2.19.1 Murine *TRPV1* expression

RNA was reverse transcribed to complementary DNA (cDNA) as per the manufacturer's instructions using SuperScript™ II reverse transcriptase. In brief, 2 µg of total RNA was combined with 2 µL of 50 µM oligo-dT primers, 2 µL of 10 mM

deoxyribonucleotide triphosphate (dNTP) mix, which was topped up to 26 μL with pyrogen-free water, and heated to 65°C for 5 min and then chilled on ice. Following a brief centrifugation to collect condensation, 8 μL of 5x First-Strand Buffer, 2 μL of 0.1 M DTT, 2 μL of 40 U/μL RNaseOUT™ recombinant ribonuclease inhibitor, and 2 μL of 200 U/μL Superscript™ II reverse transcriptase were added to each RNA sample. Samples were heated to 50°C for 50 min and inactivated at 70°C for 15 min 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. In brief, 2 μL of cDNA was mixed with 5 μL of 10x PCR Buffer, 2 μL of 50 mM MgCl$_2$, 1 μL of 10 mM dNTP mix, 2 μL each of 5 μM forward (F) and reverse (R) primers, 0.4 μL of 5 U/μL *Taq* DNA polymerase, and 35.6 μL of pyrogen-free water. A 457 base pair (bp) fragment of murine *TRPV1* (m*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. m*TRPV1* primer sequences were 5'-TGTCCTGCATTGACACCTGTGAGA-3' (F) and 5'-TCCTTGCGATGGCTGAAGTACAGT-3' (R; 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 as m*TRPV1* and the following primer sequences: 5'-TTCACCACCATGGAGAAGC-3' (F) and 5'-GCGATGGACTGTGGTCATGA-3' (R; both from Integrated DNA Technologies). Amplicons were diluted in 10x BlueJuice™, run at 80 V for 1 h on a 2% agarose gel containing 0.5 μL/mL ethidium bromide (Sigma-Aldrich Canada) in 1x Tris-acetate-EDTA (TAE) running buffer, visualized and photographed by ultraviolet (UV) light exposure using an Alpha Innotech Red™ Gel Imaging System (ProteinSimple, Santa Clara, CA).

2.19.2 Human *TRPV1* expression

RNA was reverse transcribed as per the manufacturer's instructions using Moloney murine leukemia virus reverse transcriptase (M-MLV RT). In brief, 1 μg of total RNA was combined with 1 μg of 3 μg/μL random primers and 1 μL of 10 mM

dNTP mix, heated to 65°C for 5 min, and then chilled on ice. Following a brief centrifugation to collect condensation, 4 μL of 5x First-Strand Buffer, 2 μL of 0.1 M DTT, and 1 μL of pyrogen-free water were added to each RNA sample. Following an incubation at 37°C for 2 min, 1 μL (200 U) of M-MLV RT was added and the samples were heated to 25°C for 10 min, 37°C for 50 min, and inactivated at 70°C for 15 min on an PTC-100™ automatic thermal cycler (MJ Research Inc., Watertown, MA).

cDNA was amplified by PCR according to the manufacturer's instructions with *Taq* DNA polymerase. In brief, 2 μL of cDNA was mixed with 5 μL of 10x PCR Buffer, 1.5 μL of 50 mM MgCl$_2$, 1 μL of 10 mM dNTP Mix, 1 μL each of 10 μM forward and reverse primers, 0.4 μL of 5 U/μL *Taq* DNA polymerase, and 38.1 μL of pyrogen-free water. Approximately 50 μL of mineral oil was added on top of the reaction solution. A 680 bp fragment of human *TRPV1* (h*TRPV1*) was amplified as previously described [414] using an PTC-100™ automatic thermal cycler under the following conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec, with a final elongation step of 10 min at 72°C. h*TRPV1* primer sequences were 5'-AAGGCCCAGTGTTGACAGTG-3' (F) and 5'-CTCCTACAACAGCCTGTAC-3' (R). A 238 bp fragment of *GAPDH* was also amplified as a control using the same amplification conditions as h*TRPV1* and the following primer sequences: 5'-GAGTCAACGGATTTGGTCGT-3' (F) and 5'-TTGATTTTGGAGGGATCTCG-3' (R). Amplicons were diluted in 10x BlueJuice™, run at 100V for 1 h on a 1.5% agarose gel containing ethidium bromide (both from Bio-shop Canada) in 1x TAE (40 mM Tris base, 20 mM glacial acetic acid, and 1 mM EDTA [pH 8.0]), visualized by UV light, and photographed by a Polaroid DS34 camera (Bio/Can Scientific Inc., Mississauga, ON).

2.20 LDH Assay

A CytoTox 96® non-radioactive cytotoxicity assay kit purchased from Promega (Madison, WI) was used as per the manufacturer's instructions. In brief, following a 24 h incubation of HUVECs with treatment, the plate was centrifuged at 500 g for 5 min, and 50 μL of the supernatant from each well was transferred to a new plate and stored at 4°C, with the exception of the positive control wells. The original plate containing the positive control wells was frozen at -80°C and thawed at 37°C, 3 times to induce maximum cell

lysis and LDH release. Following the final freeze-thaw cycle, the original plate was centrifuged at 500 g for 5 min, and 50 μL of the supernatant from the positive control wells was transferred to the new plate. From the kit, substrate mix was reconstituted using assay buffer and 50 μL was added to each well. The plate was incubated in the dark for 30 min at ambient temperature, after which 50 μL of stop solution was added to each well. The spectrometric absorbance was read at 490 nm on an ELx800 microplate reader. Percent cytotoxicity was calculated as follows: Percent cytotoxicity = 100 x ([experimental LDH release – spontaneous LDH release]/[maximum LDH release – spontaneous LDH release]), where spontaneous LDH release was the average absorbance from the supernatents of the medium-treated cells and maximum LDH release was the average absorbance of the supernatants of the positive control cells.

2.21 *In Vitro* Migration Assay

HUVECS were plated at 2.5 x 10^5 cells/well in 6-well flat-bottom plates and allowed to adhere overnight. As previously described [415], a p10 pipette tip was used to scratch the confluent monolayer of cells in each well, then wells were washed once with medium, treated as indicated, and photographed (time 0). The migration of cells into the area that was devoid of cells was monitored for the next 24 h and photographed at various time points. HUVEC migration was quantified using ImageJ software (National Institutes of Health, USA); percent migration equaled the percentage of the original void that was repopulated by migrating HUVECs.

2.22 *In Vitro* Angiogenesis Assay

An *in vitro* angiogenesis assay kit was purchased from Chemicon International (Temecula, CA) and used as per the manufacturer's instructions. In brief, thawed ECMatrix™ was diluted with 10x Diluent Buffer and 50 μL/well of the liquefied gel solution was added to a 96-well flat-bottom plate and allowed to solidify for at least 1 h at 37°C. HUVECs were harvested by trypsinization and pretreated as indicated for 15 min before being added to the solidified gel matrix at 5 x 10^3 cells/well. Wells were photographed 18-24 h after the addition of the cells. The complexity of tubule formation

was scored using the numerical grading scale provided by the manufacturer, as indicated below.

Pattern	Value
Individual cells, well separated.	0
Cells begin to migrate and align themselves.	1
Capillary tubes visible. No sprouting.	2
Sprouting of new capillary tubes visible.	3
Closed polygons begin to form.	4
Complex mesh-like structures develop.	5

2.23 *Ex Vivo* Angiogenesis Assay

As previously described [416], excised thoracic aortas from 3 month old male Wistar rats were turned inside-out and cut into 1.5 mm segments. Each segment was placed in one well of a 12-well flat-bottom plate (BD Biosciences). Collagen matrix was prepared using a collagen cell culturing kit as per the manufacturer's instructions (Wako Chemicals USA, Inc., Richmond, VA). In brief, collagen was diluted (8 parts collagen, 1 part 10x Eagle's Minimum Essential Medium, 1 part reconstitution buffer, all from the kit) and aorta segments were covered with 500 µL/well. Collagen solution was allowed to solidify for 20 min at 37°C with 5% CO_2. RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 1% ITS+ Premix ([v/v]; BD Biosciences) was added at a volume of 2 mL/well, either alone or in the presence of vehicle control or the indicated doses of piperine or LY-294002. Aortas were monitored and photographed over a period of 7 days to document the migration of cells from the aorta (mm) and the number of tubules developing.

2.24 *In Vivo* Chick Embryo CAM Assay

In vivo chick embryo chorioallantoic membrane (CAM) assays were performed by Innovascreen Inc. (Halifax, NS), as described previously [417]. In brief, mesh onplants containing MDA-MB-231 breast cancer cells treated with the vehicle alone or 50 μM piperine were embedded in collagen and placed on 10 day old chick embryos (4 onplants/embryo, 10 embryos per treatment). Following incubation for 72 h, onplants were photographed and the presence or absence of blood vessels in each grid square of the mesh onplant was determined. Percent angiogenesis was calculated as the percentage of positive grid squares divided by the total number of grid squares per onplant.

2.25 Statistical Analyses

Statistical analyses were performed using Student's *t*-test, one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test, or the Kruskal-Wallis nonparametric ANOVA test with Dunn's multiple comparisons post-test, as appropriate, with InStat® analysis software (GraphPad Software Inc., La Jolla, CA). Differences were considered statistically significant when the *p* value was less than 0.05 and are denoted by *; ns denotes "not significant" when the *p* value is greater than 0.05.

CHAPTER 3

PIPERINE INHIBITS T CELL PROLIFERATION, CYTOKINE PRODUCTION, AND CYTOTOXIC ACTIVITY

3.1 Introduction

T cells are key modulators of immune responses, owing to CD4^{+} Th cell production of cytokines [7] and the cytotoxic activity of CD8^{+} T effector cells [140]. Immune dysregulation can result in the inappropriate activation of T cells, which is responsible for various chronic inflammatory and autoimmune conditions, such as IBD [404], MS [405], rheumatoid arthritis [406], and psoriasis [418]. Current immunosuppressive agents include biologics, corticosteroids, and calcineurin inhibitors, which are costly and have adverse side effects, including neutralizing Ab production, gastrointestinal toxicity, nephrotoxicity, and cardiovascular events [213,214]. The shortcomings of these current therapies therefore demands the continued search for more affordable therapeutic agents with fewer toxic side effects.

Phytochemicals are plant chemicals that possess biological activity and are now recognized as a source for novel immune-modulating and anti-inflammatory compounds [315,419]. Phytochemicals such as curcumin [420,421], resveratrol [422], capsaicin [423], and piperine [411] have T cell suppressive activity. Piperine is a phytochemical from the fruits of black and long pepper plants, which impairs both adhesion molecule expression and neutrophil attachment on endothelial cells, suggesting that it inhibits leukocyte extravasation from the blood [367]. Piperine ameliorates carrageenan-induced arthritis in rats [348] and increases the survival of mice with endotoxin shock, partially by inhibiting TNF-α production [372]. Furthermore, administration of piperine *in vivo* inhibits production of the Th2 cytokine IL-4 and accumulation of CD4^{+}, CD8^{+}, and CD3^{+}CD69^{+} cells in the lungs of mice with OVA-induced asthma [377]. Additionally, oral administration of piperine to mice inhibits phytohemagglutinin (PHA)-induced T cell proliferation *ex vivo* [411]. Taken together, these studies suggest that piperine has immunomodulatory effects on T cells. The purpose of this study was therefore to examine the direct effect of piperine on T cell function *in vitro*. The current study

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demonstrates that piperine inhibits various aspects of T cell function, including activation, proliferation, cytokine production, and cytotoxic effector function. Taken together, these findings indicate that piperine possesses T cell inhibitory activity that warrants further investigation of piperine as a therapeutic agent in the treatment of T cell-mediated pathologies, such as chronic inflammation, autoimmunity, and transplant rejection.

3.2 Results

3.2.1 Piperine Inhibits T Cell Proliferation

The anti-inflammatory effects of piperine on various cell types, particularly the inhibition of T cell proliferation induced by piperine-treated dendritic cells [380], led to the hypothesis that piperine may have direct inhibitory effects on T cells as well. To examine the effect of piperine on T cells, I first investigated the effect of piperine on T cell proliferation using the $[^3H]$TdR incorporation assay, which measures DNA synthesis. T cell activation was induced using Dynabeads®, which stimulate the CD3 and CD28 pathways and act as surrogate antigen presenting cells in delivering signal 1 and signal 2 to the T cells. As shown in Figure 3.1A, addition of the Dynabeads® induced strong proliferation of $CD3^+$ T cells for 24-96 h following stimulation in the presence of medium or the vehicle control, with proliferation peaking at 72 h. In contrast, the addition of piperine 15 min prior to bead stimulation significantly and dose-dependently inhibited the proliferation of $CD3^+$ T cells stimulated with Dynabeads® for 24-96 h (Figure 3.1A; $p < 0.05$). Additionally, 100 and 50 μM piperine treatments significantly inhibited T cell proliferation over the entire time course (24-96 h; Figure 3.1A; $p < 0.05$). Since the $[^3H]$TdR incorporation assay captures only a snapshot of DNA synthesis occurring during the last 6 h of each time point, the anti-proliferative effect of piperine on T cells was confirmed using the cell-permeable fluorescent dye, Oregon Green® 488, which can be used to quantify cell proliferation over the entire time course because the fluorescence intensity of the dye is halved with each round of cellular division. As shown in Figure 3.1Bi, T cells stimulated with Dynabeads® in the presence of vehicle control (black peaks) underwent multiple rounds of division compared to unstimulated T cells (grey peaks). Proliferative response was similar in stimulated T cells treated with the vehicle

control or with medium (data not shown). Treatment with 100 μM (Figure 3.1Bii) and 50 μM piperine (Figure 3.1Biii) inhibited T cell division, as shown by the fewer number of peaks and the higher number of cells present in the rightmost peak (parent peak) that is composed of the unproliferated cells. Quantification of the percentage of total cells within the peaks showed that only $25 \pm 9\%$ of the analysed cells had undergone division following 100 μM piperine treatment during the 72 h culture, compared to $76 \pm 6\%$ of vehicle-treated cells ($p < 0.05$). Additionally, quantification of the number of cells within each cell division peak allowed the percentage of T cells from the original population that responded to stimulation and underwent division to be extrapolated. Treatment with 100 μM piperine significantly inhibited the percentage of responding cells, compared to the vehicle control (Figure 3.1C; $p < 0.05$). Treatment with 100 μM piperine also significantly inhibited the maximum number of cell divisions that T cells underwent within the 72 h culture period compared to vehicle-treated T cells (2.3 ± 0.3 vs. 4.3 ± 0.3, respectively; Figure 3.1D; $p < 0.05$). Taken together, these data indicate that piperine inhibits T cell proliferation in a dose-dependent manner by reducing the number of T cells that divide in response to stimulation and the maximum number of cell divisions that occur over 72 h.

The T cell population examined in Figure 3.1 was a mixture of both CD4$^+$ and CD8$^+$ T cells. To determine whether piperine had differential effects on the proliferation of the two T cell subpopulations, PE-labelled Abs were used to differentiate between the CD4$^+$ and CD8$^+$ T cell subsets within Oregon Green® 488-stained CD3$^+$ T cell cultures that were activated with Dynabeads®. Consistent with the literature [424], more CD8$^+$ T cells treated with vehicle underwent cellular division than CD4$^+$ T cells ($78 \pm 7\%$ [Figure 3.2Bi] vs. $57 \pm 2\%$ [Figure 3.2Ai], respectively) when activated with Dynabeads®. Interestingly, both 100 and 50 μM piperine significantly inhibited the percentage of CD8$^+$ T cells undergoing cell division compared to the vehicle control ($31 \pm 2\%$ [Figure 3.2Bii] and $52 \pm 9\%$ of cells [Figure 3.2Biii], respectively; $p < 0.05$), whereas only 100 μM piperine significantly inhibited the percentage of CD4$^+$ T cells undergoing division compared to the vehicle control ($25 \pm 8\%$ [Figure 3.2Aii]; $p < 0.05$). These data suggest that CD8$^+$ T cells are more sensitive to piperine than CD4$^+$ T cells. Extrapolation of the number of original T cells that divided in response to stimulation showed that a higher

percentage of vehicle-treated $CD8^+$ T cells responded to stimulation and divided than vehicle-treated $CD4^+$ T cells (41 ± 6% vs. 29 ± 2%; Figure 3.2C). Additionally, when the percentage of the responding cells that divided more than once was examined, significantly more vehicle-treated $CD8^+$ T cells underwent multiple cellular divisions compared to vehicle-treated $CD4^+$ T cells (24 ± 5% vs. 11 ± 1%; Figure 3.2D; $p < 0.05$), confirming that $CD8^+$ T cells proliferate more in response to stimulation than $CD4^+$ cells. Treatment with 100 μM piperine significantly inhibited the percentage of responding cells within both the $CD4^+$ and $CD8^+$ T cell subsets compared to the appropriate vehicle control ($CD4^+$: 13 ±3%; $CD8^+$: 15 ± 2%; Figure 3.2C; $p < 0.05$). Additionally, 100 μM piperine treatment significantly inhibited the percentage of $CD4^+$ and $CD8^+$ T cells that underwent multiple rounds of cell division in response to stimulation compared to the respective vehicle control ($CD4^+$: 2 ± 1%; $CD8^+$: 3 ± 1%; Figure 3.2D; $p < 0.05$). While 50 μM piperine did not significantly inhibit the percentage of responding cells of either $CD4^+$ or $CD8^+$ T cells (Figure 3.2C), 50 μM piperine significantly inhibited the percentage of responding $CD8^+$ T cells that underwent multiple rounds of cell division compared to the vehicle control (9 ± 3%; Figure 3.2D; $p < 0.05$). These data indicate that both 100 and 50 μM piperine inhibited the percentage of $CD8^+$ T cells that divided more than once, whereas in the $CD4^+$ subset, only 100 μM piperine inhibited the percentage of cells that divided multiple times. Treatment with 100 or 50 μM piperine also significantly inhibited the maximum number of cellular divisions that both the $CD4^+$ and $CD8^+$ T cells underwent within the 72 h culture period compared to the respective vehicle control (Figure 3.2E; $p < 0.05$). These data suggest that 100 μM piperine treatment inhibits the number of $CD4^+$ and $CD8^+$ T cells that divide in response to stimulation, the percentage of these cells that divide multiple times, as well as the maximum number of cellular divisions that occur. In comparison, 50 μM piperine does not inhibit the percentage of cells of either T cell subset that divide in response to stimulation, but it does inhibit the percentage of $CD8^+$ T cells that divide multiple times, as well as inhibiting the maximum number of cellular divisions that occur in both $CD8^+$ and $CD4^+$ T cell subsets.

3.2.2 Inhibition of T cell Proliferation by Piperine is Independent of the Vehicle

Since the anti-proliferative effect of piperine on T cells could have been the result of the combined activity of piperine and its DMSO vehicle, the role of the DMSO vehicle

was examined by treating T cells activated with Dynabeads® with piperine made up in EtOH in parallel with T cells treated with piperine made up in DMSO. As seen in Figure 3.3, there was no significant difference in the inhibitory effect of piperine on T cell proliferation over 72 h, regardless of which vehicle piperine was made up in ($p > 0.05$). The same dose-dependent inhibition of T cell proliferation was seen with both vehicles ($p < 0.05$), as determined by [^{3}H]TdR incorporation. These data suggest that the vehicle is not contributing to the anti-proliferative effect of piperine on T cells.

3.2.3 Piperine does not Induce T Cell Death

As seen in Figure 3.1 and 3.2, piperine inhibits T cell proliferation, but neither of the two proliferation assays that were employed to examine the effect of piperine on T cell proliferation can differentiate between a cytostatic effect and a cytotoxic effect. To determine if the inhibitory effect of piperine on T cell proliferation was the result of cytotoxicity, the viability of T cells activated for 24 h in the presence of piperine was examined by Annexin-V/PI staining, which measures the phosphatidylserine translocation and membrane integrity loss, respectively, that occur during cell death. Piperine treatment did not significantly increase the amount of T cell death compared to vehicle control treatment (Figure 3.4; $p > 0.05$). In contrast, T cells that did not receive stimulation for 24 h (unstim.) had significantly more cell death than activated vehicle-treated cells and activated piperine-treated cells ($p < 0.05$). Taken together, these data indicate that piperine inhibits T cell proliferation in a cytostatic manner without causing T cell death. Additionally, these data indicate that piperine does not inhibit the survival signals that are provided by CD3/CD28 stimulation, as activated piperine-treated cells were significantly more viable than unstimulated cells.

3.2.4 Piperine Inhibits the Proliferation of Pre-Activated T Cells

As demonstrated in Figure 3.1 and 3.2, piperine inhibits the proliferation of T cells when added 15 min prior to stimulation. However, clinically, a valuable pharmacological inhibitor of T cell-mediated immune responses is one that can also inhibit a T cell response after it has already been initiated. Therefore, the ability of piperine to inhibit a T cell response that is previously initiated was examined. T cells were stimulated with Dynabeads® alone for 24 h to allow activation to proceed normally

before the addition of vehicle control or piperine treatments. T cell proliferation was measured using the [^{3}H]TdR incorporation assay for the following 24-72 h after treatment (48-96 h after initial activation). As shown in Figure 3.5, piperine significantly inhibited the proliferation of T cells that had been previously activated for 24 h at all of the time points examined ($p < 0.05$). These data indicate that piperine inhibits T cell proliferation after as well as before *in vitro* activation.

3.2.5 The Inhibitory Effect of Piperine on T Cell Proliferation is Reversible

Piperine potently inhibits T cell proliferation while the chemical is present, but it was not known whether this effect was reversible or irreversible. The clinical use of piperine as an immunosuppressive agent could be limited if the potent T cell inhibitory effect is irreversible due to the potential contraindications such as *Cytomegalovirus* and *Epstein–Barr virus* reactivation and malignancy in the presence of excessive immunosuppression, as is often seen with post-transplant immunosuppression [425]. Therefore, to determine if the inhibitory effect of piperine on T cells was reversible, T cells were treated with piperine and activated with Dynabeads® for 24 h before excess piperine was removed by thorough washing. After normalizing cell numbers between treatments, T cells were restimulated with Dynabeads® and proliferation was measured using the [^{3}H]TdR incorporation assay for the following 24-72 h after treatment wash-out (48-96 h after initial treatment and activation). There was no significant difference in T cell proliferation between vehicle treatment and piperine treatment over the time course examined (Figure 3.6; p > 0.05), indicating that the inhibition of T cell proliferation by piperine is reversible.

3.2.6 Piperine Inhibits Entry of T Cells into the S phase of the Cell Cycle

The inhibitory effect of piperine on T cell proliferation, as shown in Figure 3.1, suggested that piperine was preventing T cells from entering the S phase of the cell cycle when new DNA synthesis occurs. To confirm the effect of piperine on cell cycle progression, cell cycle analysis was performed with the DNA-intercalating dye PI on T cells pretreated with medium, vehicle control, or 100 µM piperine and activated with Dynabeads® for 72 h. With this assay, the fluorescence intensity of PI is proportional to the quantity of DNA within the cell, therefore indicating whether the cell is in the G_0/G_1,

S, or G_2/M phase of the cell cycle. As shown in Figure 3.7A, 100 μM piperine treatment significantly inhibited the relative number of cells in the S and G_2/M phases of the cell cycle compared to vehicle control ($p < 0.05$), indicating that piperine induces a block at the G_1 phase that does not allow cells to enter into S phase and progress through the cell cycle. Additionally, 100 μM piperine treatment significantly inhibited the percent of cycling cells in the S and G_2/M phases of the cell cycle, compared to vehicle control (Figure 3.7B; $p < 0.05$). To confirm the cell cycle analysis results, T cells were treated with medium, vehicle, or 100 μM piperine and activated with Dynabeads® for 48 h. Then western blot analysis was performed to examine the expression of cyclin D3, CDK4, and CDK6 cell cycle proteins associated with progression through the G_1 phase of the cell cycle [426]. As shown in Figure 3.7C-E, 100 μM piperine treatment significantly inhibited the expression of all three of these G_1-associated cell cycle proteins, compared to vehicle control ($p < 0.05$). Taken together, these data confirm that piperine induced a partial block in cell cycle progression at the G_1 phase and inhibited entry of T cells into the S phase. Additionally, these data suggest that the mechanism by which piperine inhibits cell cycle progression lies upstream of the G_1-associated proteins cyclin D3, CDK4, and CDK6.

3.2.7 Piperine Inhibits Expression of the Early Activation Marker CD25 but not CD69

Prior to expressing cell cycle proteins, activated T cells upregulate the expression of early activation markers such as CD25 and CD69 on their cell surface [427]. To determine the effect of piperine on early activation marker expression induced by CD3/CD28 stimulation, T cells were treated with vehicle or 100 μM piperine for 24 h in the presence or absence of Dynabeads®. Cells were then stained for the expression of CD25 and CD69, and analysed by flow cytometry. As shown in Figure 3.8A, 100 μM piperine treatment inhibited CD25 surface expression following T cell activation, compared to vehicle control. Additionally, there was a significant reduction in the relative number of CD25$^+$ T cells following piperine treatment for 24 h post-activation, compared to the vehicle control (Figure 3.8B; $p < 0.05$). This result was confirmed by western blot analysis showing a significant reduction in CD25 expression in piperine-treated T cells compared to vehicle-treated T cells (Figure 3.8C; $p < 0.05$). The induction of CD25

expression was not completely ablated by piperine treatment compared to unstimulated T cells (Figure 3.8A&C). Interestingly, the relative number of T cells expressing another early activation marker, CD69, was not affected by piperine treatment (Figure 3.8A&B; $p > 0.05$). Taken together, these findings indicate that piperine has specific effects on T cells and is not inhibiting general protein expression. Additionally, these data suggest that piperine is inhibiting, but not completely ablating, the stimulatory signals that induce CD25 expression. Furthermore, these data suggest that piperine inhibits the signaling pathways that induce CD25 expression but not the signaling pathways that induce CD69 expression.

3.2.8 Piperine Inhibits Expression of the Cytokines IL-2, IL-4, IFN-γ, and IL-17A

An important function of Th cells is the production of cytokines, which regulate and direct the development of an immune response [7]. Therefore, the effect of piperine on cytokine production by T cells was investigated. As the different T helper cell subsets are characterized by the production of specific cytokines, a cytokine that is associated with each of the major Th cell phenotypes was examined. IFN-γ, IL-4, and IL-17A are representative of the Th1, Th2, and Th17 subsets, respectively [7]. Additionally, IL-2, which is an important cytokine for driving T cell proliferation *in vitro* [152], was also examined. T cells were treated with medium, vehicle, 100, or 50 μM piperine for 24 h in the presence or absence of Dynabeads®, and cytokine production was quantified by sandwich ELISA of the cell supernatants. Because cytokine production was examined at 24 h before substantial DNA synthesis had occurred (Figure 3.1A), the cell numbers between the different treatments should be similar at the time of supernatant collection. As shown in Figure 3.9, 100 μM piperine treatment significantly inhibited the production of IL-2, IFN-γ, IL-4, and IL-17A, while 50 μM piperine treatment only significantly inhibited the production of IL-2 and IFN-γ ($p < 0.05$). The greatest inhibitory effect was seen on IFN-γ synthesis with both concentrations of piperine. These data suggest that piperine may inhibit general T cell cytokine production but may be more selective for IFN-γ production.

3.2.9 Exogenous IL-2 does not Reverse the Inhibition of T Cell Proliferation Induced by Piperine

IL-2 is an important cytokine for driving T cell proliferation *in vitro* [152]. Since piperine treatment reduced the production of IL-2 by activated T cells (Figure 3.9), it was possible that the reduction in IL-2 levels in piperine-treated T cell cultures was contributing to the anti-proliferative effect of piperine. Consequently, exogenous recombinant mouse IL-2 was added to cultures to determine if additional IL-2 would reverse piperine-induced inhibition of T cell proliferation. It was previously determined that 50 U/mL of exogenous IL-2 induces optimal proliferation of IL-2-dependent CTLL-2 cells (data not shown). This concentration of IL-2 is also greater than the amount of IL-2 that is normally present in supernatants from T cell cultures activated for 24 h, as quantified by sandwich ELISA or in T cell cultures activated for 48 h, as determined by IL-2 bioassay using CTLL-2 cells (data not shown). T cell proliferation was measured by [^{3}H]TdR incorporation assay after 72 h of treatment with Dynabeads® and the indicated concentrations of piperine or vehicle in the presence or absence of 50 U/mL of exogenous IL-2. As shown in Figure 3.10, the addition of exogenous IL-2 did not rescue the proliferation of piperine-treated T cells, suggesting that piperine-induced inhibition of T cell proliferation is not completely due to the reduced production of IL-2. The piperine-induced reduction in CD25 expression may contribute to the lack of proliferation of piperine-treated T cells in the presence of excess exogenous IL-2. While piperine treatment resulted in low levels of CD25 expression, it did not ablate CD25 expression, suggesting the presence of some functional high affinity IL-2R. Additionally, the intermediate affinity IL-2R made up of the β and γ subunits may also induce signaling downstream of the IL-2R in the absence of CD25. Taken together, these findings suggest that piperine may be inhibiting signaling downstream of the IL-2R.

3.2.10 Piperine Inhibits the Generation of CTLs but does not Inhibit the Killing Ability of Previously Generated CTLs

Another important function of T cells is the generation of CTLs and the ability of these effector cells to lyse target cells [428]. Therefore, the effect of piperine on CTL generation and lytic activity was examined. CTLs with potent effector activity can be

induced by stimulating CD3$^+$ T cells *in vitro* with anti-CD3/anti-CD28 Ab-coated beads for 72 h [429]. CTL cytotoxic activity was therefore determined by the co-culture of differentiated CTLs and [^{3}H]TdR-pulsed and anti-TCR Ab-labeled P815 target cells. At the end of the 4 h co-culture, the decrease in [^{3}H]TdR counts was measured as an indicator of DNA fragmentation, i.e., P815 cell death. The addition of piperine to the CTL and P815 co-cultures resulted in no significant difference in the cytotoxic activity of piperine-treated CTLs compared to vehicle control (Figure 3.11A; $p > 0.05$). This indicated that piperine did not inhibit the release of cytotoxic effector molecules (eg. perforin, granzyme) by the CTLs and/or Fas/FasL interactions between the effector cells and the target cells. P815 viability in the absence of CTLs was unaffected by piperine treatment (data not shown).

In contrast, when piperine (100 or 50 μM) was added to the T cells 15 min prior to stimulation with Dynabeads® for 72 h, and washed off before the CTLs were added to P815 target cells, there was a significant decrease in the cytotoxic activity of CTLs (Figure 3.11B; $p < 0.05$). Since piperine had no effect on the lytic activity of fully differentiated CTLs (Figure 3.11A), these findings indicate that piperine inhibits the differentiation of naive T cells into functional CTLs and suggest that piperine may inhibit the expression of cytotoxic effector molecules during CTL differentiation.

3.2.11 Piperine does not Act on T Cells via TRPV1

The cation channel, TRPV1, is a known receptor of piperine [317]. The involvement of this receptor in piperine-mediated inhibition of T cell proliferation and effector function was therefore examined. Using RT-PCR, *TRPV1* mRNA expression was not found in naive T cells (Figure 3.12A). The spinal cord from C57BL/6 mice, which contains many dorsal root ganglia that are known to express TRPV1 [430] was used as a positive control. As it was possible that *TRPV1* expression by T cells may be below the detectable limit of the PCR analysis, TRPV1 involvement in the inhibitory effect of piperine on T cells was further examined by isolating T cells from TRPV1$^{-/-}$ mice and stimulating them in the presence or absence of piperine in parallel with T cells from WT mice. As shown in Figure 3.12B, there was no significant difference in the inhibitory effect of piperine on

T cell proliferation regardless of the *TRPV1* genotype of the T cells ($p > 0.05$). These data confirmed that piperine does not inhibit T cells via TRPV1.

3.2.12 Piperine Inhibits Akt Phosphorylation but has no Effect on ZAP-70 Phosphorylation

Since TRPV1 was not involved in the anti-proliferative effect of piperine on T cells, the mechanism by which piperine inhibits T cell proliferation and function was further investigated using western blot analysis. To determine whether piperine inhibited T cell intracellular signaling, the signaling pathways downstream of CD3 and CD28 were examined. ZAP-70 is a signaling molecule that undergoes early activation following CD3 ligation [431], while Akt is a signaling molecule that undergoes early activation following CD3 and/or CD28 ligation [432,433]. Therefore, these two molecules were selected to be examined by western blot analysis. As shown in Figure 3.13A, ZAP-70 phosphorylation increases as early as 5 min following T cell stimulation with Dynabeads®. Interestingly, the presence of 100 µM piperine had no significant effect on the phosphorylation of ZAP-70 at Tyr 319 following CD3/CD28 stimulation, when compared to the vehicle control ($p > 0.05$). Since the phosphorylation of Tyr 319 is critical for ZAP-70 activation [434], this finding suggests that piperine does not inhibit ZAP-70 activation downstream of CD3 stimulation.

Similar to ZAP-70, Akt phosphorylation was also increased as early as 5 min following T cell stimulation with Dynabeads® (Figure 3.13B). In contrast to ZAP-70, however, the phosphorylation of Akt at Ser 473 was significantly inhibited by piperine at 5, 15, 30, and 60 min following CD3/CD28 stimulation ($p < 0.05$). The observation that piperine inhibited Akt phosphorylation but not ZAP-70 phosphorylation following stimulation of CD3 and CD28 indicates that piperine is affecting specific intracellular signaling pathways within T cells and is not a general inhibitor of phosphorylation events. Moreover, since ZAP-70 is activated by CD3 signaling alone [20] and Akt is activated by both TCR and CD28 signaling [435], the selective inhibition of Akt phosphorylation suggests that piperine is inhibiting TCR/CD3 signaling downstream of ZAP-70 and/or inhibiting CD28 signaling independent of TCR/CD3 signaling. Additionally, given the importance of the Akt signaling pathway to T cell activation,

these data suggest that the inhibition of Akt phosphorylation may be the mechanism by which piperine inhibits T cell activation, proliferation, and function.

3.2.13 Piperine Inhibits ERK and IκBα Phosphorylation

Phosphorylation of ERK and IκB is required for the downstream activation of the transcription factors AP-1 and NF-κB, respectively, which are two crucial transcription factors for T cell activation [45,436]. Therefore, the effect of piperine on the phosphorylation of ERK and IκB during T cell activation was examined by western blot analysis. As shown in Figure 3.14A, piperine significantly inhibited ERK phosphorylation at 60 min following T cell stimulation as compared to vehicle control ($p < 0.05$). Piperine also significantly inhibited the phosphorylation of IκBα at 15 min following T cell stimulation as compared to vehicle control (Figure 3.14B; $p < 0.05$). Taken together, these data indicate that piperine inhibits multiple intracellular signaling pathways within activated T cells.

Figure 3.1 Piperine inhibits murine T cell proliferation. (A) Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and were cultured with or without Dynabeads®. T cells were incubated for the indicated times and pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. * denotes p < 0.05 when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (B) Highly purified CD3$^+$ T cells were stained with Oregon Green® 488, treated with medium, vehicle (DMSO), 100, or 50 μM piperine, and cultured for 72 h with or without anti-CD3/anti-CD28 Ab-coated beads. Flow cytometry was used to measure cell proliferation by quantifying the Oregon Green® 488 fluorescence intensity, which halved with the subsequent divisions of stained cells. Data shown are from a representative experiment comparing activated cells treated with DMSO vehicle (Veh.; black peaks in i, ii, and iii) to unstim. cells (i. grey peak), activated cells treated with 100 μM piperine (ii. 100 μM Pip., blue peaks), and activated cells treated with 50 μM piperine (iii. 50 μM Pip., red peaks). Numbers shown are the average percentage of cells undergoing division compared to the total cell population ± standard error of the mean (SEM) of 3 independent experiments as determined by ModFit software analysis. (C) Percent responding cells is the percentage of the original T cell population that underwent proliferation and was calculated by extrapolation of the number of cells in each division peak relative to the number of cell divisions associated with that peak. (D) The maximum (max.) number (#) of cell divisions within each treatment group was calculated using ModFit software analysis. Data shown are the mean of at least 3 independent experiments ± SEM; * denotes *p* < 0.05 when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
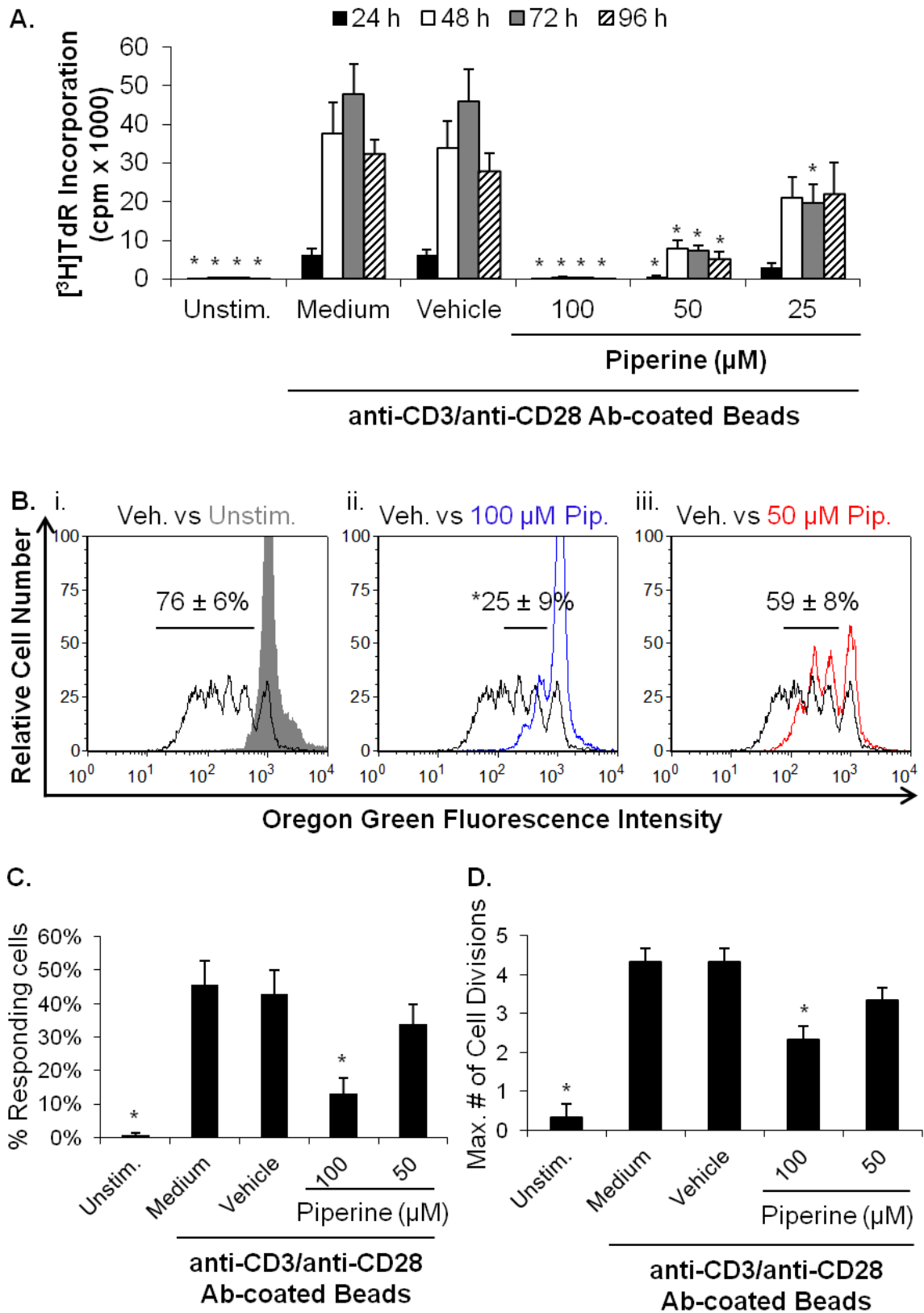
Figure 3.1

Figure 3.2 Piperine inhibits CD4$^+$ and CD8$^+$ T cell proliferation. Highly purified CD3$^+$ T cells were stained with Oregon Green® 488, treated with medium, vehicle (DMSO), 100, or 50 μM piperine, and cultured for 72 h with or without anti-CD3/anti-CD28 Ab-coated beads. Cells were then stained with anti-CD4-PE or anti-CD8-PE Ab or their respective isotype controls and fixed. Flow cytometry was used to measure the proliferation of CD4$^+$ and CD8$^+$ T cells by quantifying the Oregon Green ® 488 fluorescence intensity, which halved with the subsequent divisions of stained cells. Histograms generated in FCS Express (A & B) were normalized to the vehicle control for the respective T cell subset using the software analysis. Data shown are from a representative experiment comparing activated cells treated with DMSO vehicle (Veh.; black peaks in i, ii, and iii) to unstim. cells (i. grey peak), activated cells treated with 100 μM piperine (ii. 100 μM Pip.; blue peaks), and activated cells treated with 50 μM piperine (iii. 50 μM Pip.; red peaks) of CD4$^+$ T cells (A) and CD8$^+$ T cells (B). Numbers shown are the average percentage of cells undergoing division compared to the total cell population ± SEM of 3 independent experiments as determined by ModFit software analysis. (C) Percent responding cells is the percentage of the original T cell population that underwent proliferation and was calculated by extrapolation of the number of cells in each division peak relative to the number of cell divisions associated with that peak. (D) Percent responding cells that divided more than once is the percent of responding cells from (C) that underwent more than one cellular division. (E) The maximum (max.) number of cell divisions within each treatment group was calculated using ModFit software analysis. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the respective vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

A. CD4+

i. Veh. vs Unstim.   ii. Veh. vs 100 µM Pip.   iii. Veh. vs 50 µM Pip.

57 ± 2%   *25 ± 8%   52 ± 4%

Relative Cell Number

Oregon Green Fluorescence Intensity

B. CD8+

i. Veh. vs Unstim.   ii. Veh. vs 100 µM Pip.   iii. Veh. vs 50 µM Pip.

78 ± 7%   *31 ± 2%   *52 ± 9%

Relative Cell Number

Oregon Green Fluorescence Intensity

Figure 3.2

79
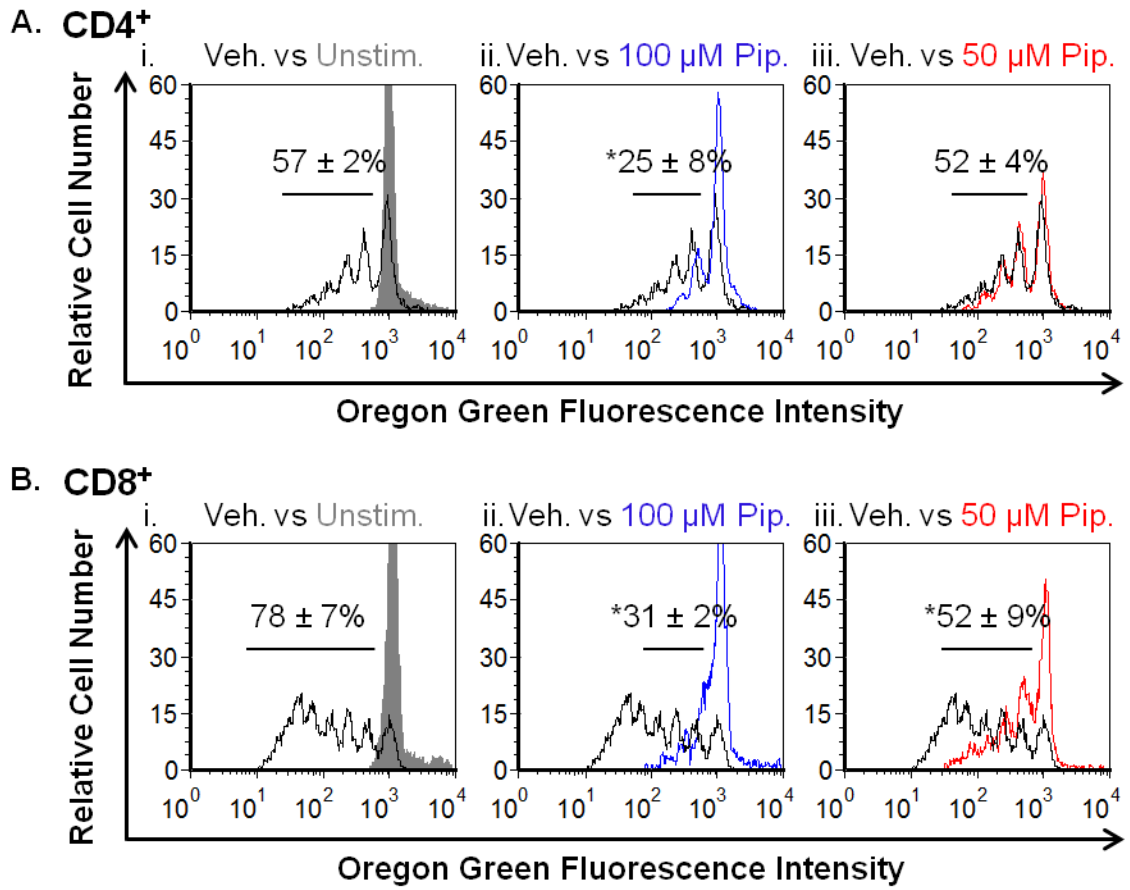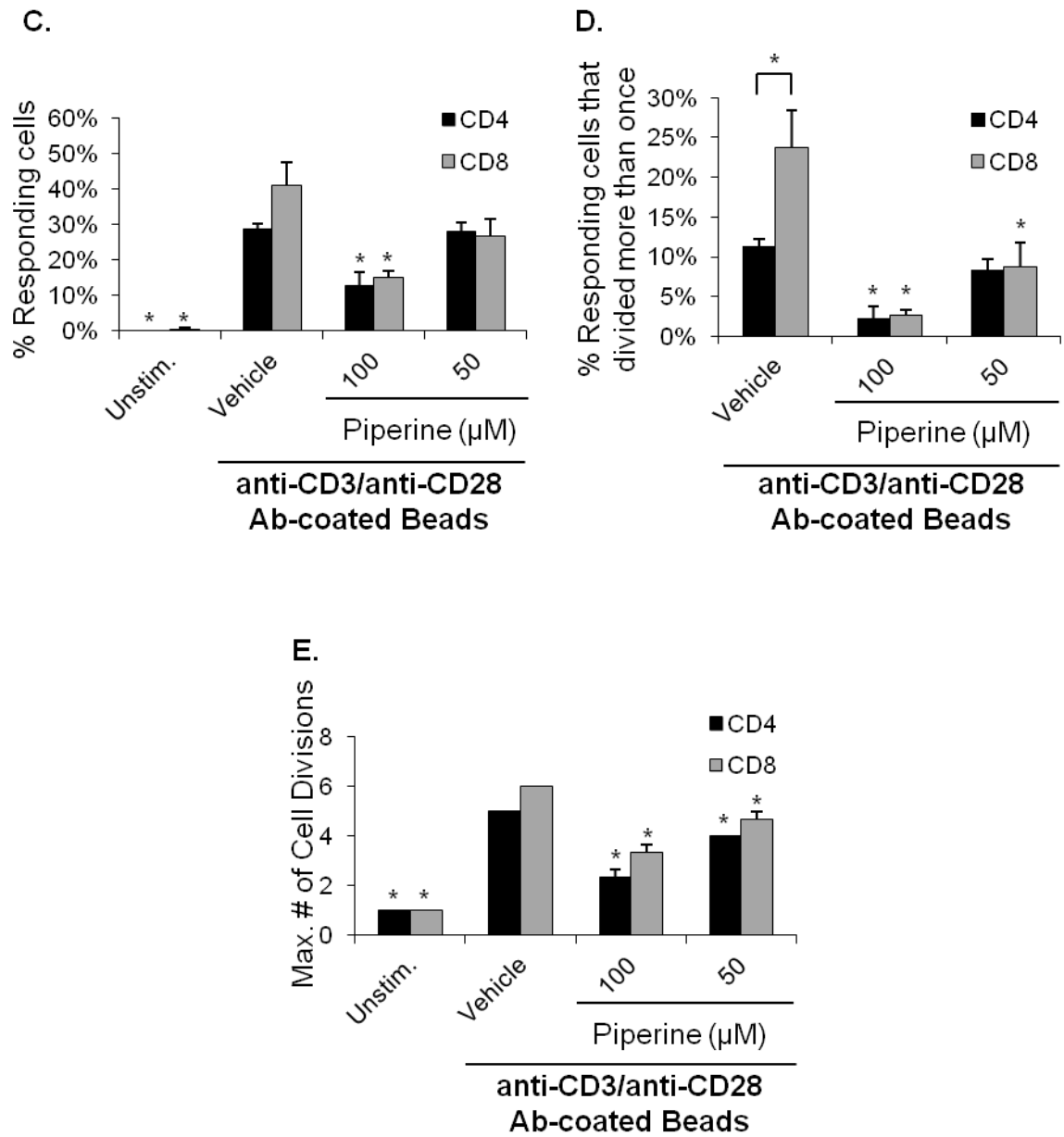
Figure 3.2 (continued)

Figure 3.3 Inhibition of T cell proliferation by piperine is independent of the vehicle.
Highly purified CD3$^+$ T cells were treated with medium, the appropriate vehicle, or the
indicated concentrations of piperine made up in either dimethyl sulfoxide (DMSO; black
bars) or ethanol (EtOH; grey bars). T cells were then activated with anti-CD3/anti-CD28
Ab-coated beads for 72 h. T cells were pulsed with [^{3}H]TdR for the last 6 h of incubation,
harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting.
Data shown are the mean of at least 3 independent experiments ± SEM; * denotes $p <$
0.05 when compared to the respective vehicle control and ns denotes "not significant"
when comparing DMSO vehicle to EtOH vehicle for each treatment as determined by
ANOVA with the Tukey-Kramer multiple comparisons post-test.
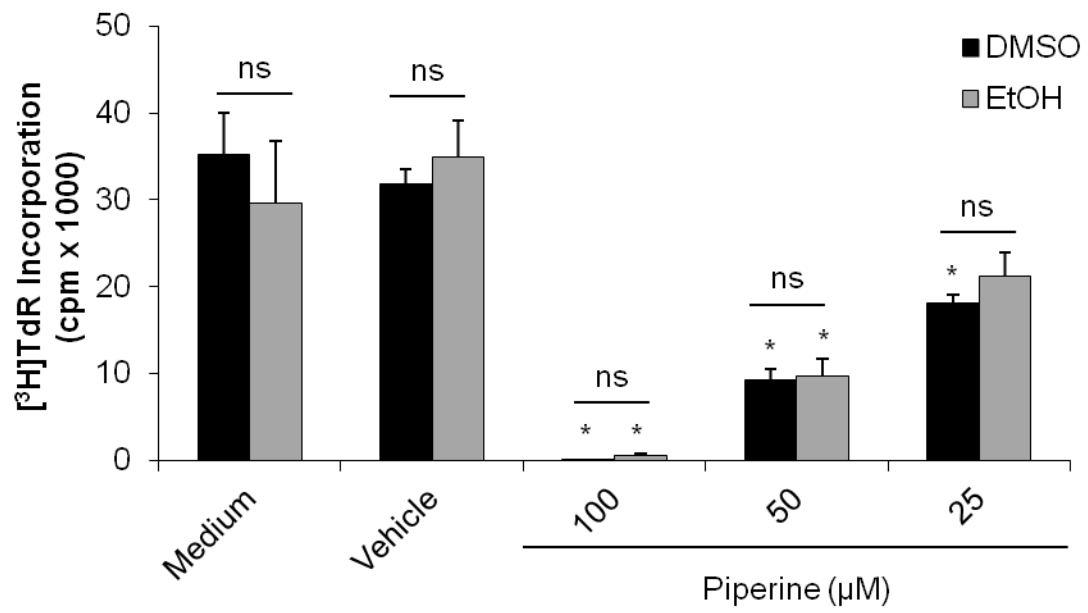
Figure 3.3

Figure 3.4 Piperine does not significantly increase T cell death. Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured with or without (unstim.) anti-CD3/anti-CD28 Ab-coated beads for 24 h. Cells were then stained with Annexin-V/PI to determine phosphatidylserine translocation and membrane integrity loss as a measure of cell death by flow cytometry. Cell death was calculated as the percentage of cells that were Annexin-V$^+$ and Annexin-V$^+$PI$^+$. (A) Data shown are the mean of 3 independent experiments ± SEM; * denotes $p <$ 0.05 and ns denotes "not significant" when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (B) Data shown are from a representative experiment.

A.

(Bar chart) y-axis: % Cell Death (0% to 60%). x-axis categories: Unstim, Medium, Vehicle, 100, 50, 25. "ns" bracket over 100, 50, 25 (Piperine (μM)). "*" above Unstim. Label: Piperine (μM). anti-CD3/anti-CD28 Ab-coated Beads

B.

Vehicle | 100 μM Piperine

Vehicle plot: 0.81% | 20.06% ; 76.19% | 2.94%

100 μM Piperine plot: 0.76% | 21.19% ; 75.99% | 2.06%

y-axis: PI, x-axis: Annexin-V
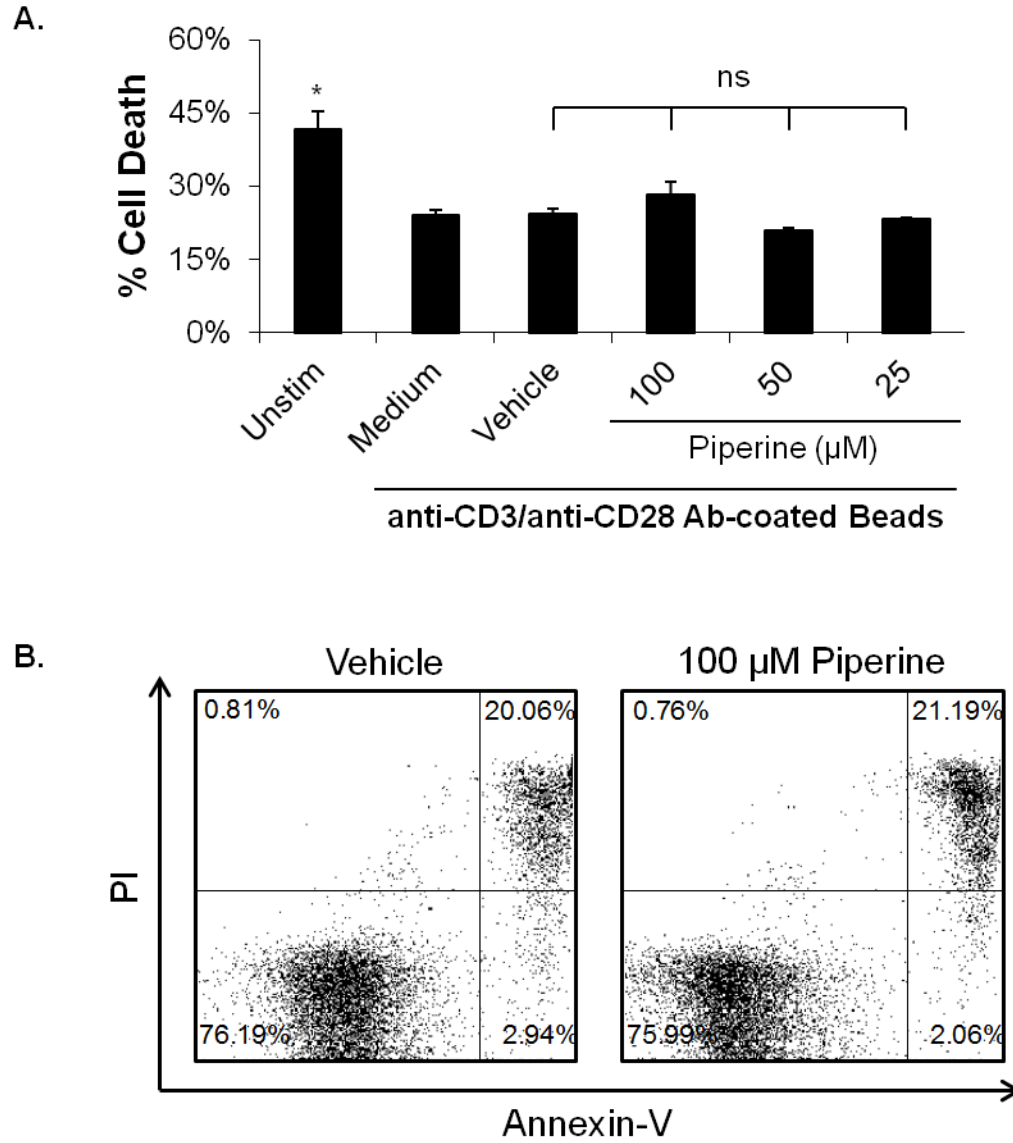
Figure 3.4

Figure 3.5 Piperine inhibits the proliferation of pre-activated T cells. Highly purified CD3$^+$ T cells were pre-activated with anti-CD3/anti-CD28 Ab-coated beads for 24 h. Pre-activated T cells were then treated with medium, vehicle (DMSO), or the indicated concentrations of piperine for the indicated times. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
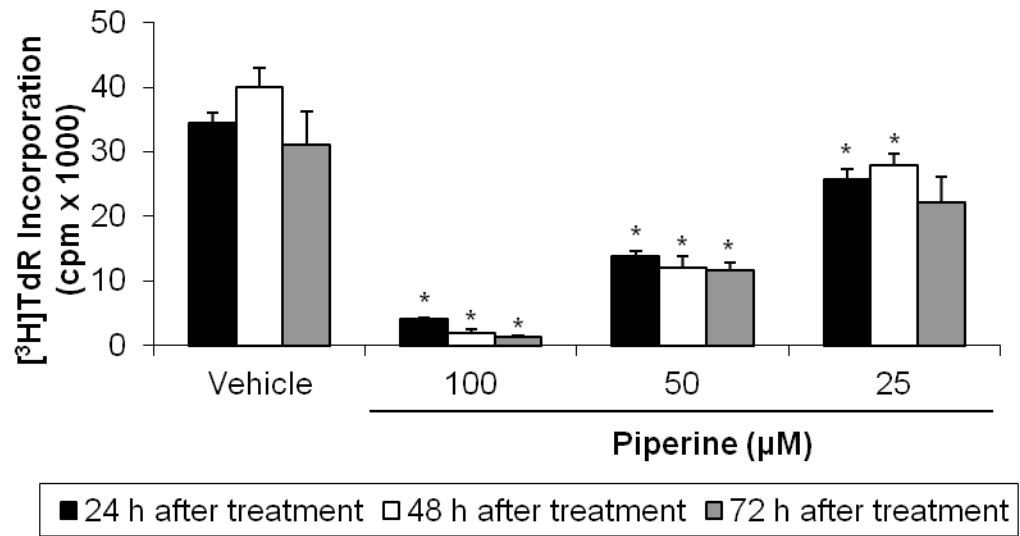
Figure 3.5

Figure 3.6 The inhibitory effect of piperine on T cell proliferation is reversible.
Highly purified CD3^{+} T cells were treated with vehicle (DMSO), 100, or 50 μM piperine
and cultured with anti-CD3/anti-CD28 Ab-coated beads for 24 h. Cells were washed, the
number of viable cells in each treatment was normalized by trypan blue counts, and the
cells were then incubated for the indicated time points. Cells were pulsed with [^{3}H]TdR
for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by
liquid scintillation counting. Data shown are the mean of 3 independent experiments ±
SEM; ns denotes "not significant" when compared to the vehicle control for the
respective time point as determined by ANOVA with the Tukey-Kramer multiple
comparisons post-test.
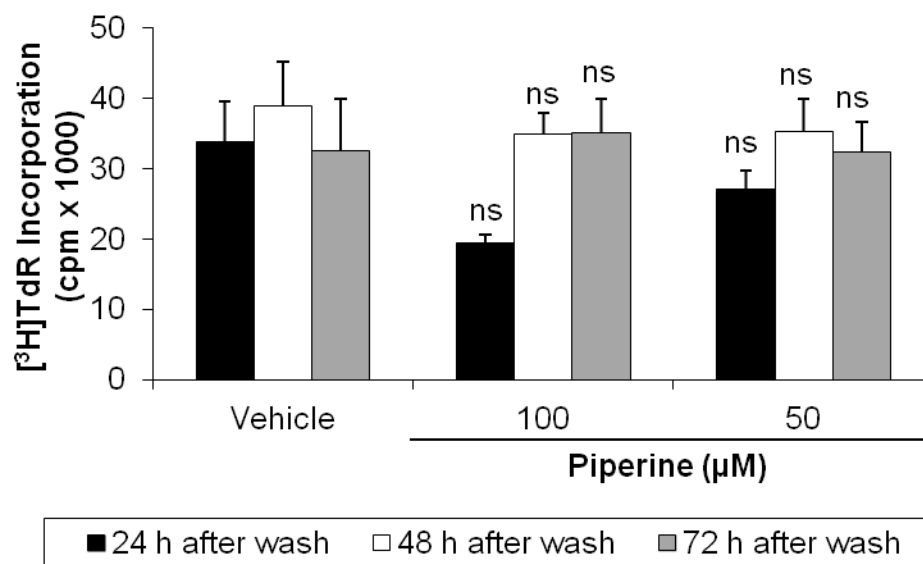
Figure 3.6

Figure 3.7 Piperine inhibits entry of T cells into the S phase of the cell cycle. (A & B) Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), or 100 μM piperine and cultured with anti-CD3/anti-CD28 Ab-coated beads for 72 h. Cells were fixed with ice-cold 70% ethanol for at least 24 h and then stained with the DNA-intercalating dye PI for 30 min prior to analysis by flow cytometry. (A) The number of cells in each phase of the cell cycle was determined by ModFit software analysis and normalized to activated T cells treated with medium; * denotes $p < 0.05$ when compared to the vehicle control for the respective cell cycle phase as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (B) The percent of cycling cells was calculated as the percentage of cells in either the S, G2, or M phases of the cell cycle using ModFit software analysis. (C, D, & E) T cells were treated with medium, vehicle (DMSO), or 100 μM piperine and activated with anti-CD3/anti-CD28 Ab-coated beads for 48 h prior to lysis. Total protein was collected for western blot analysis. Membranes were probed with anti-cyclin D3 Ab (C), anti-CDK4 Ab (D), or anti-CDK6 Ab (E), washed, and then probed with anti-actin Ab to confirm equal protein loading. Optical density ratio was calculated as the ratio of cyclin D3, CDK4, or CDK6 expression to actin expression, and was then normalized to medium control. Data shown are the mean of 4 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
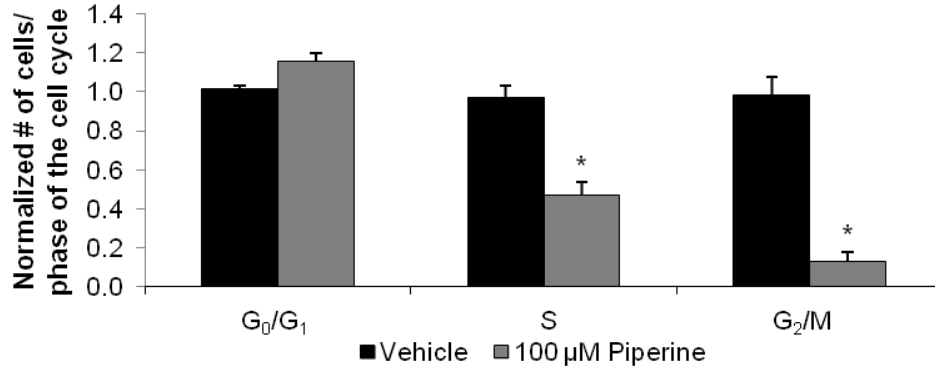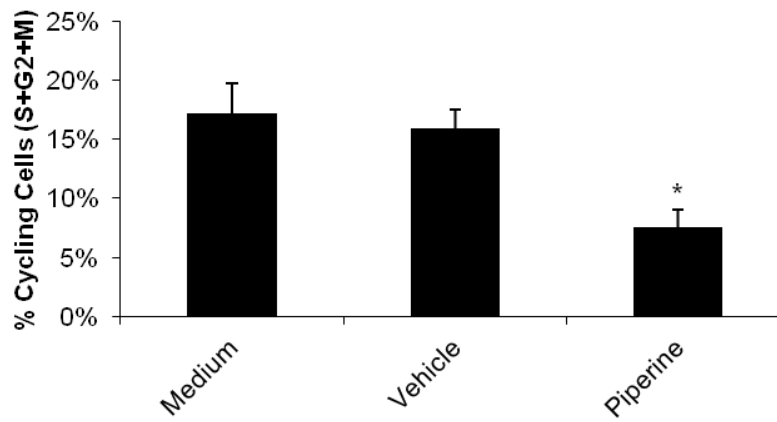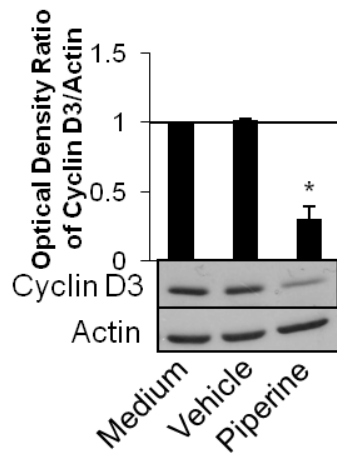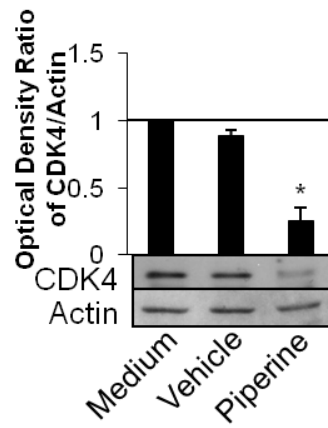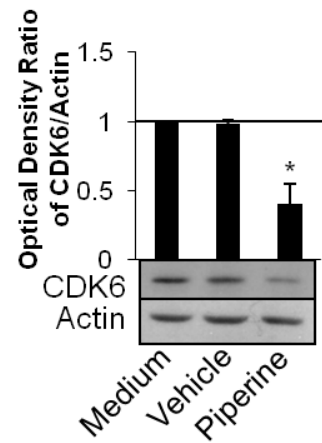
Figure 3.7

Figure 3.8 Piperine significantly inhibits the expression of the early activation marker CD25 but not CD69. Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), or 100 μM piperine and cultured for 24h with or without anti-CD3/anti-CD28 Ab-coated beads. Cells were stained with anti-CD25-FITC Ab, anti-CD69-FITC Ab, or their respective isotype controls, fixed, and analysed by flow cytometry. (A) Data shown are from representative experiments (n = 3) with isotype depicted in the grey peak and CD25 or CD69 expression depicted by the black peak. (B) The numbers of CD25 and CD69 positive (+) T cells treated with 100 μM piperine were normalized to their respective activated vehicle controls (grey line). Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the respective vehicle control as determined by one sample t-test. (C) T cells were treated with medium, vehicle (DMSO), or 100 μM piperine and cultured for with or without anti-CD3/anti-CD28 Ab-coated beads for 24 h prior to lysis. Total protein was collected for western blot analysis. Membranes were probed with anti-CD25 Ab, washed, and then probed with anti-actin Ab to confirm equal protein loading. Relative expression was calculated as the ratio of CD25 expression compared to actin expression and expressed in arbitrary densitometric units. Data shown are the mean of 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

A.

CD25 | CD69

Unstim.

Activated + Vehicle

Activated + Piperine

Relative Cell Number

Fluorescence Intensity

B.

Relative # of + Cells (Normalized to Vehicle)

ns

*

CD25 | CD69

C.

Optical Density

*

*

CD25

ACTIN

Unstim. | Vehicle | Piperine

anti-CD3/anti-CD28 Ab-coated Beads
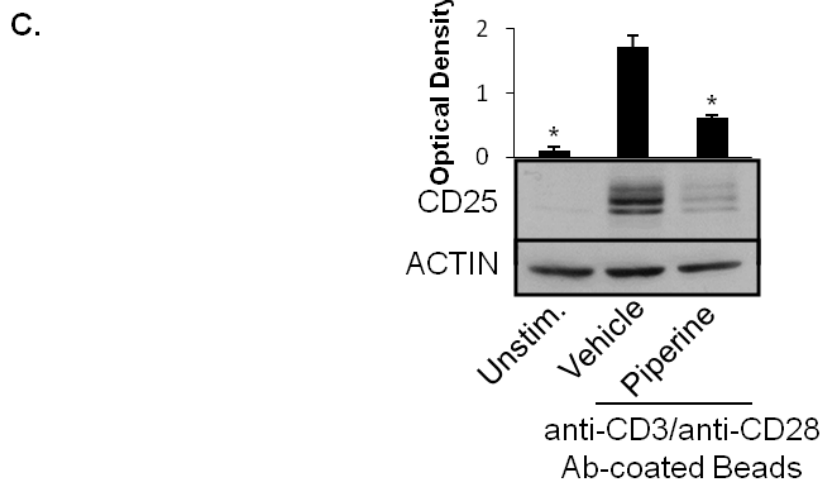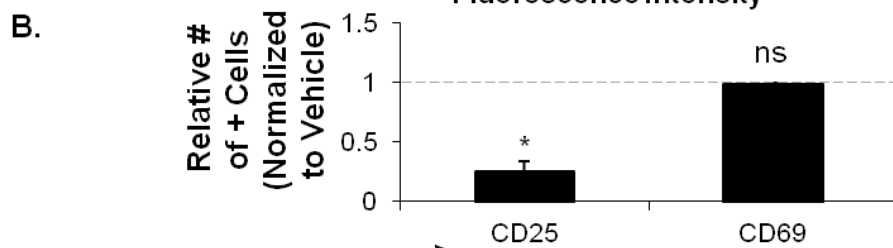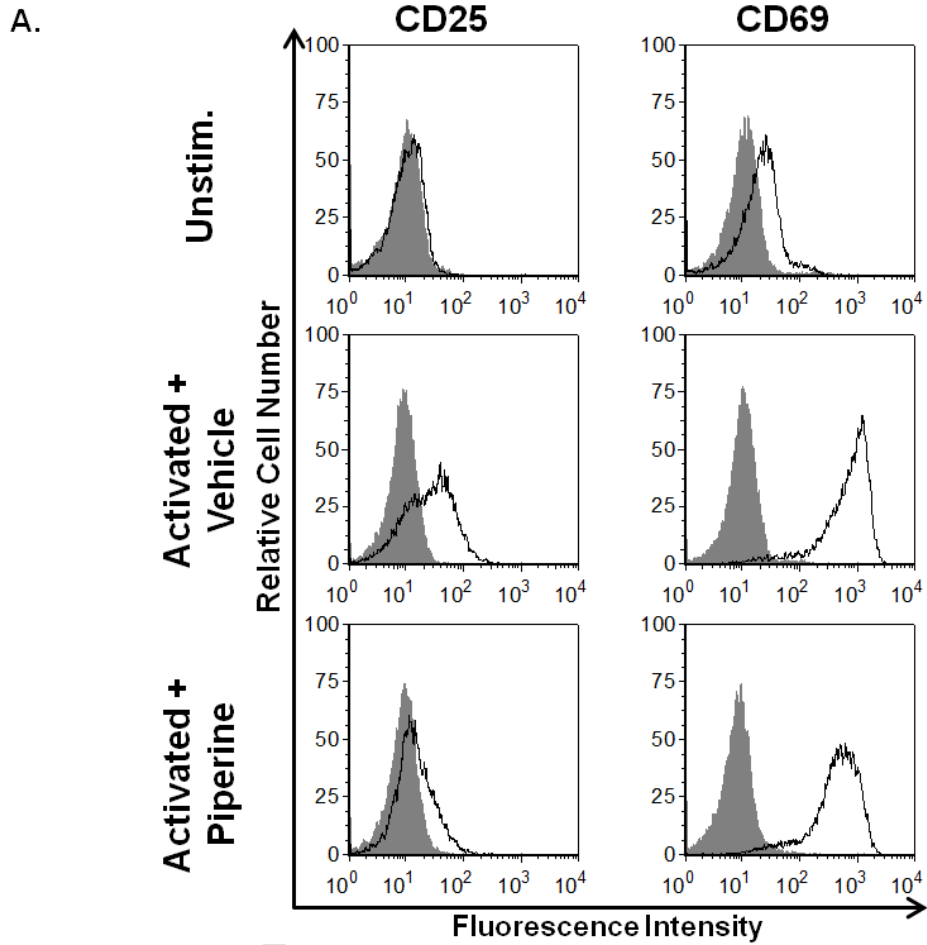
Figure 3.8

Figure 3.9 Piperine significantly inhibits the expression of the cytokines IL-2, IL-4, IFN-γ, and IL-17A. Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), 100, or 50 µM piperine and cultured for 24 h with or without anti-CD3/anti-CD28 Ab-coated beads. Supernatants were collected and analysed for cytokine content by sandwich-ELISA specific for IL-2, IL-4, IFN-γ, or IL-17A. Cytokine expression was normalized to activated T cells treated with medium. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the respective vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
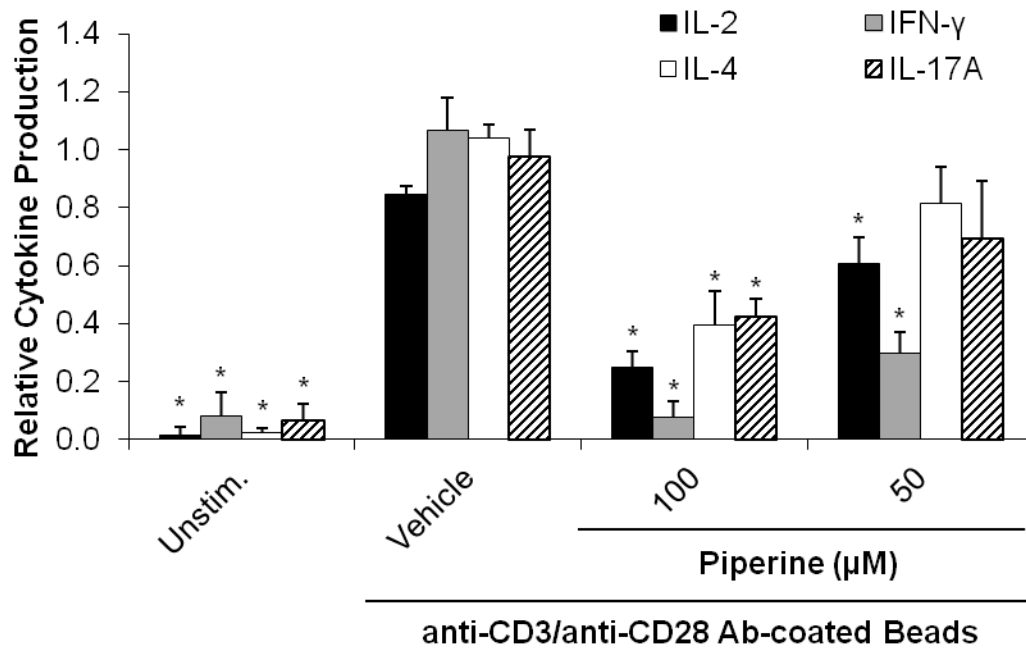
Figure 3.9

Figure 3.10 Exogenous IL-2 does not reverse the inhibition of T cell proliferation induced by piperine. Highly purified CD3$^+$ T cells were pretreated for 15 min with medium or with 50 U/mL IL-2, which is a dose that induces optimal proliferation of the IL-2-dependent cell line CTLL-2. Cells were then treated with vehicle (DMSO) or the indicated concentrations of piperine and cultured with anti-CD3/anti-CD28 Ab-coated beads for 72 h. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the respective vehicle control and ns denotes "not significant" when comparing medium to IL-2 for each treatment group, as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
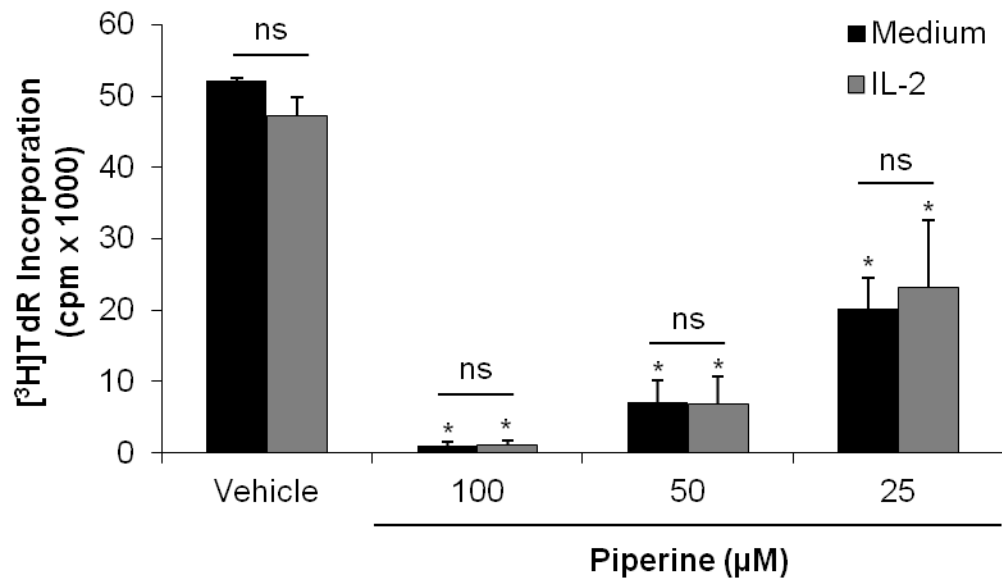
Figure 3.10

Figure 3.11 Piperine inhibits the generation of CTLs but does not inhibit the ability of previously generated CTLs to kill. (A) Highly purified CD3$^+$ T cells were activated with anti-CD3/anti-CD28 Ab-coated beads for 72 h. CTLs were then treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and incubated with anti-TCR Ab and [^{3}H]TdR-pulsed P815 target cells for 4 h at the indicated effector:target cell ratios. (B) Highly purified CD3$^+$ T cells were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and activated with anti-CD3/anti-CD28 Ab-coated beads for 72 h. Cells were then washed and the number of viable cells in each treatment was normalized by trypan blue counts. CTLs were incubated with anti-TCR Ab and [^{3}H]TdR-pulsed P815 target cells for 4 h at the indicated effector:target cell ratios. Percent cytotoxicity was measured as the loss of [^{3}H]TdR$_{cpm}$ in the presence of effector cells compared to P815 target cells alone, as determined by liquid scintillation counting. Data shown are the mean of at least 3 independent experiments ± SEM; * denotes $p <$ 0.05 and ns denotes "not significant" when compared to the vehicle control for the respective effector:target cell ratio as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

A. CTLs treated during co-culture with target cells
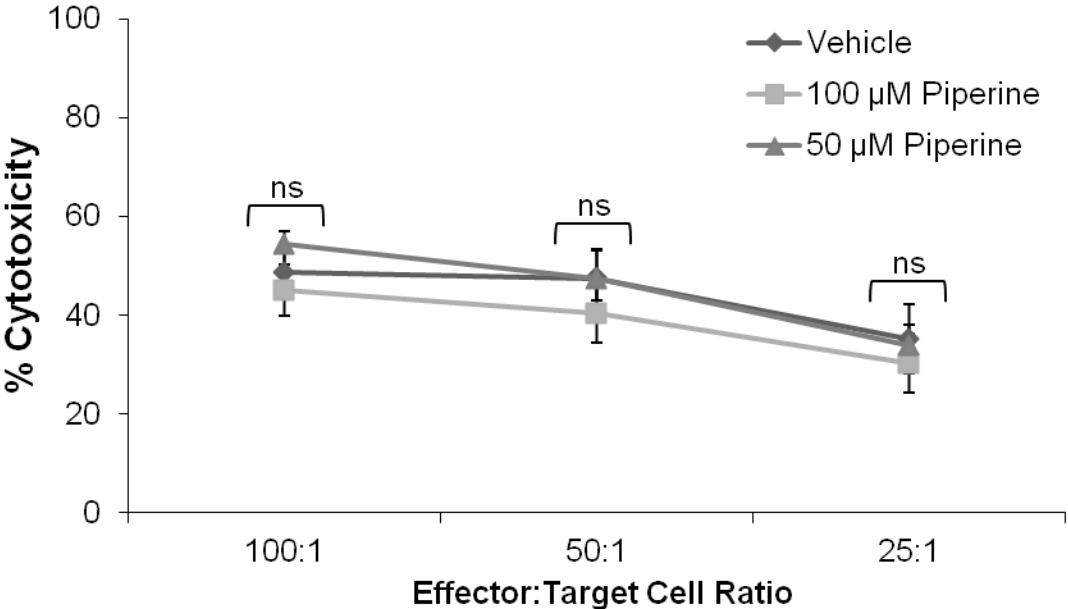
B. CTLs treated during differentiation before co-culture with target cells
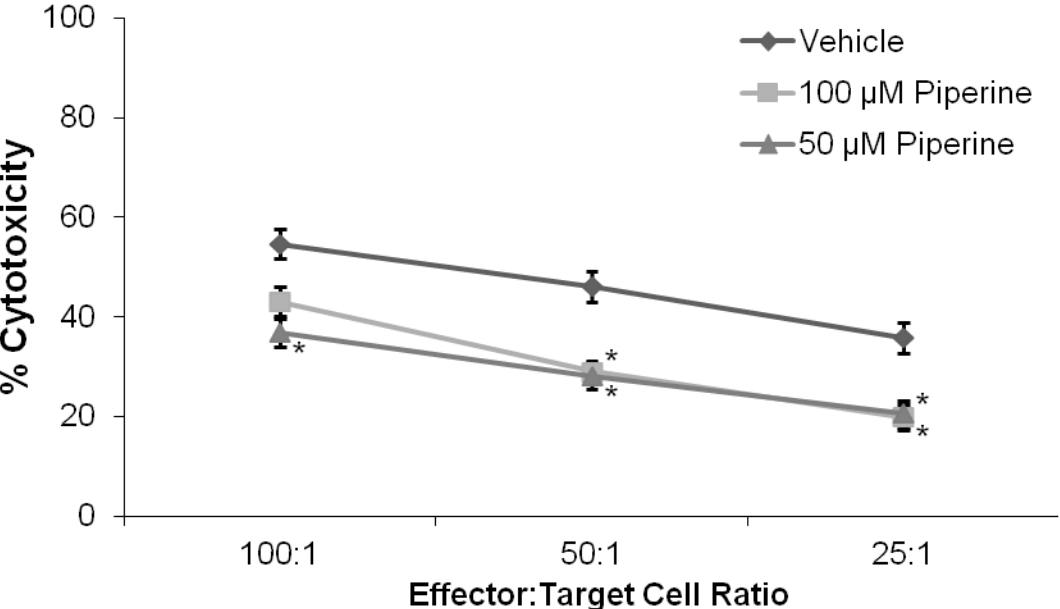
Figure 3.11

Figure 3.12 Piperine does not act on T cells via TRPV1. (A) RNA was extracted from C57BL/6 wild-type (WT) and TRPV1$^{-/-}$ spinal cords, as well as from WT T cells. RNA was reverse transcribed into cDNA, and then amplified for *TRPV1* or *GAPDH*, as a loading control. Data shown are from a representative experiment where I extracted RNA and RT-PCR was performed by Gemma Rodgers (n = 3). (B) Highly purified CD3$^+$ WT and TRPV1$^{-/-}$ T cells were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and cultured for 48 h with or without anti-CD3/anti-CD28 Ab-coated beads. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of quadruplicate wells ± standard deviation from one representative experiment (n = 2); * denotes $p < 0.05$ when compared to respective vehicle control and ns denotes "not significant" when comparing C57BL/6 WT to TRPV1$^{-/-}$ for each treatment group as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

A.
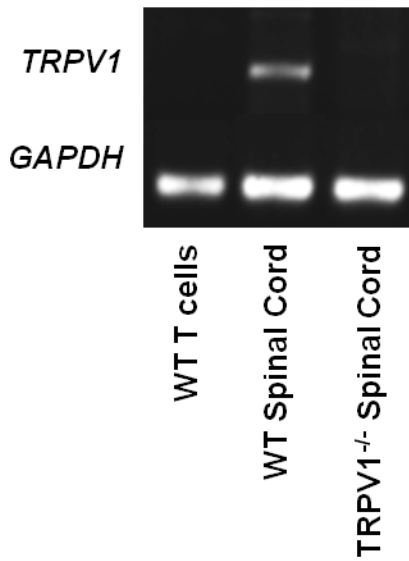
B.
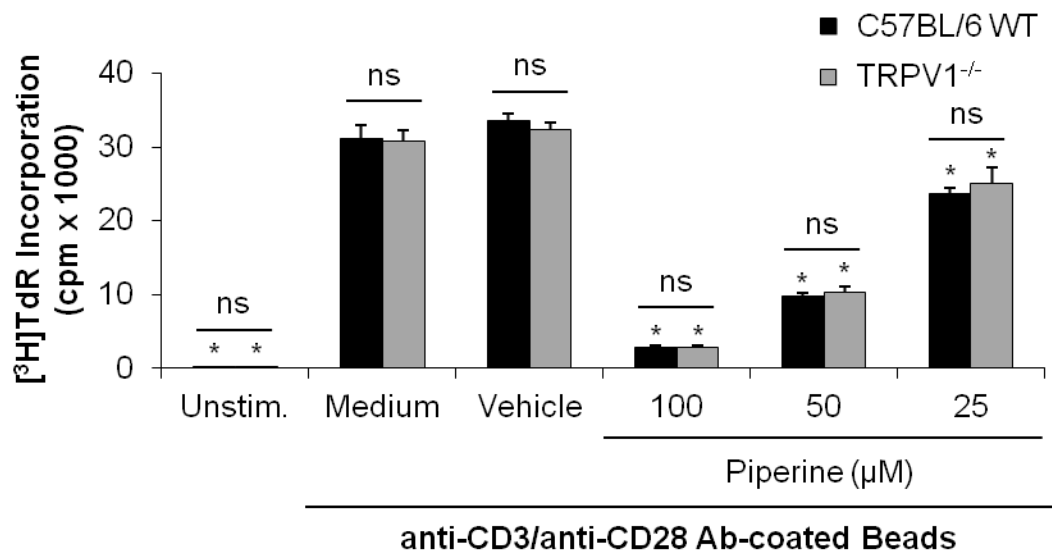
Figure 3.12

Figure 3.13 Piperine inhibits Akt phosphorylation but has no effect on ZAP-70 phosphorylation. Highly purified CD3$^+$ T cells were treated with DMSO vehicle (V) or 100 μM piperine (P) for 30 min prior to culture with or without anti-CD3/anti-CD28 Ab-coated beads for the indicated times. Cells were then lysed, and total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-ZAP-70 (A) or p-Akt (B), stripped, and then probed with Ab for total ZAP-70 (A) or total Akt (B) to confirm equal protein loading. Optical density ratio was calculated as the ratio of phospho-protein expression to total expression, normalized to vehicle treatment at 60 min post-activation. Data shown are the mean of at least 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ and ns denotes "not significant" when compared to the vehicle control for the respective time point as determined by Student's *t*-test.

A.
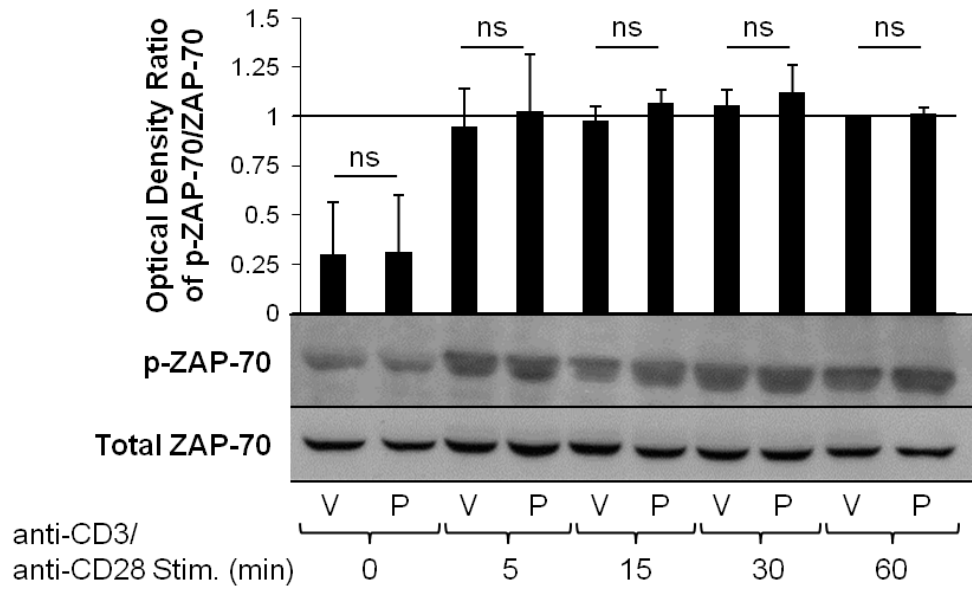
B.
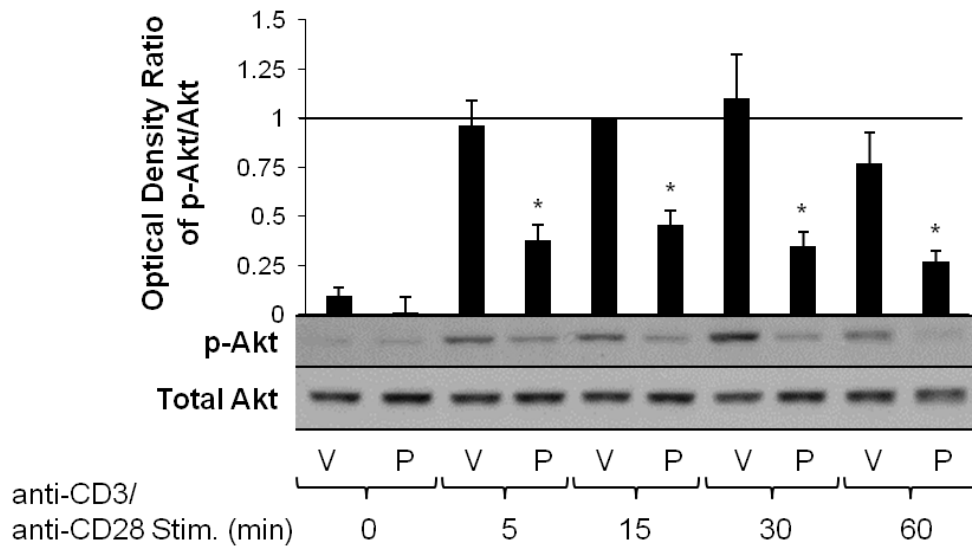
Figure 3.13

Figure 3.14 Piperine inhibits ERK and IκBα phosphorylation. Highly purified CD3^{+} T cells were treated with DMSO vehicle (V) or 100 μM piperine (P) for 30 min prior to culture with or without anti-CD3/anti-CD28 Ab-coated beads for the indicated times. Cells were then lysed, and total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-ERK (A) or p-IκBα (B), washed, and then probed for total ERK (A) or total IκBα (B) to confirm equal protein loading. Optical density ratio was calculated as the ratio of phospho-protein expression to total expression, normalized to vehicle treatment at 60 min post-activation. Data shown are the mean of at least 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control for the respective time point as determined by Student's *t*-test.

A.

B.
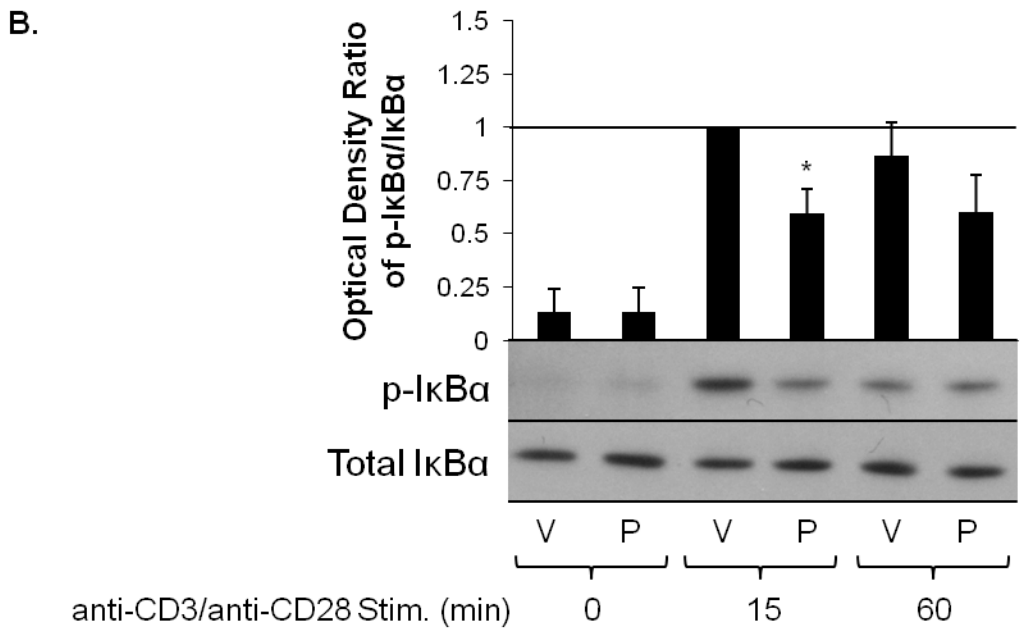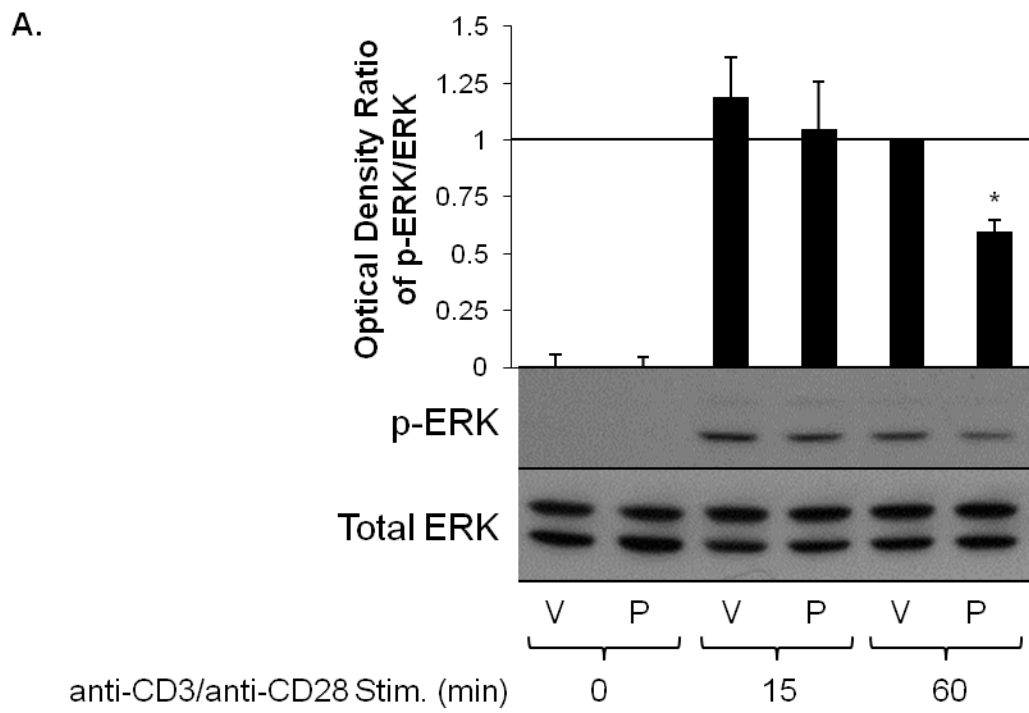
Figure 3.14

3.3 Discussion

In the present investigation, I show direct inhibitory activity of the phytochemical piperine on T cells *in vitro*. This observation is consistent with previous studies in which *in vivo* administration of piperine inhibited PHA-stimulated T cell proliferation *ex vivo* [411] and also inhibited the production of IL-4 and the number of CD4^{+}, CD8^{+} and CD3^{+}CD69^{+} cells in the lungs of OVA-induced asthmatic mice [377]. Taken together, these previous studies suggest a direct effect of piperine on T cell function. Our finding that piperine inhibits the proliferation of T cells stimulated with anti-CD3/anti-CD28 Ab-coated beads confirms that piperine has direct inhibitory effects on T cells. This anti-proliferative activity is consistent with reports that piperine has anti-proliferative effects on human breast [391] and colon [395] cancer cells and endothelial cells (Chapter 5, Manuscript 3). Consistent with the piperine-induced inhibition of cyclin D3, CDK4, and CDK6 and inhibition of T cells entering into the S phase of the cell cycle demonstrated in the current study, piperine-treated endothelial cells also displayed a block at the G_1 phase of the cell cycle and decreased cyclin D3 expression (Chapter 5, Manuscript 3). Our data contradicts a study by Pathak and Krandewal [437], which showed no effect of 175 μM piperine on ConA-induced splenocyte proliferation. However, Pathak and Krandewal [437] reported very high background splenocyte proliferation in the absence of stimulation, as well as proliferation that only doubled in the presence of ConA, and ConA-stimulation that induced very little production of IL-2 and IFN-γ (< 5 pg/mL), suggesting a problem with their experimental design. Additionally, CD8^{+} T cells were more sensitive than CD4^{+} T cells to inhibition by piperine at lower doses. The differential effect of piperine treatment on CD4^{+} and CD8^{+} T cells may be due to the higher proliferative capacity of CD8^{+} T cells compared to CD4^{+} T cells [424].

Annexin-V/PI staining confirmed that the piperine-induced inhibitory effect on T cell proliferation was not due to piperine-induced cell death. Additionally, unstimulated T cells underwent significantly more cell death than T cells activated in the presence of piperine, suggesting that the survival signals induced by CD3/CD28 stimulation are being transmitted even in the presence of piperine. Two major survival pathways that are transmitted upon CD3/CD28 stimulation are Bcl-xL upregulation through CD3- and CD28-induced NF-κB [438] and Akt activation [439]. Interestingly, in the current study,

both the Akt and the NF-κB pathways were inhibited in piperine-treated T cells, which may suggest that the inhibitory activity of piperine on these two pathways is incomplete and allows for sufficient NF-κB and/or Akt signaling to promote T cell survival. Alternatively, piperine may stimulate pro-survival signaling pathways independently of Akt/NF-κB pathways since piperine treatment also inhibits cell death in other cell types, e.g., cadmium-induced splenocyte cell death [437], cisplatin-induced apoptosis of auditory cells [401], and glutamate-induced apoptosis of hippocampal neurons [440].

T cells activated in the presence of piperine showed normal upregulation of CD69 surface expression while upregulation of CD25 surface expression was inhibited. This finding is surprising since CD69 and CD25 expression in T cells are both regulated by similar transcription factors that include NFAT, NF-κB, and AP-1 [441,442]. A possible explanation for the differential effect of piperine on CD69 and CD25 expression in T cells is that CD69 expression is induced much earlier (30 min-1 h; [427]) than CD25 expression (6-8 h) [443]. CD69 expression is primarily induced downstream of the Ras-Raf1 pathway [444,445], which leads to ERK phosphorylation and the induction of the transcription factor AP-1 [446]. ERK-dependent transcription of the AP-1 component c-fos is induced within minutes of CD3 stimulation [447], leading to the expression of CD69 within 30 min-1 h of T cell stimulation [427]. Although we show that ERK phosphorylation in activated T cells was inhibited by piperine treatment, this inhibition was not significant until 1 h following stimulation. Therefore, it is likely that the induction of CD69 expression occurred before ERK inhibition by piperine treatment. Conversely, the upregulation of CD25 expression on activated T cells occurs later than CD69 expression, with CD25 mRNA appearing approximately 3 h following T cell stimulation [448] and CD25 protein appearing approximately 6-8 h post-stimulation [443]. The slower kinetics of CD25 expression upon T cell stimulation may provide sufficient time for piperine-induced inhibition of signaling pathways, resulting in inhibition of CD25 expression. A higher level of PKC activity is also required for CD25 expression in comparison to CD69 expression. This has previously been demonstrated by the more potent inhibition of CD25 expression compared to CD69 expression in phorbol 12-myristate 13-acetate (PMA)-stimulated T cells treated with low doses of the PKC inhibitors H7 and staurosporine [449]. CD25 expression may therefore be more tightly

regulated than CD69 expression and require a stronger activation signal to induce CD25 expression. It is therefore possible that piperine may be inducing sufficient inhibition of pathways downstream of PKC such as IκB and ERK in order to inhibit CD25 expression while having no effect on CD69 expression.

We showed that piperine inhibited the production of IL-2, IFN-γ, IL-4, and IL-17A by activated T cells, suggesting that piperine has potential as a therapeutic agent for T cell-mediated inflammatory and autoimmune conditions. The inhibition of all four cytokines suggests that piperine may mediate a general inhibition of cytokine production by T cells. However, although not examined in our study, piperine has previously been shown to selectively inhibit LPS-, IFN-α-, and IFN-β-induced TNF-α production by murine peritoneal macrophages, while having no effect on IL-6 or IL-1β production [372]. It is therefore possible that piperine inhibits the synthesis of specific cytokines and not general cytokine release under certain circumstances and/or in a cell-specific manner. All four cytokines examined in the present study are transcriptionally regulated by the transcription factors AP-1 and NFAT [450]. AP-1 is activated downstream of ERK phosphorylation [446], which is inhibited in piperine-treated T cells, suggesting that the inhibition of ERK phosphorylation in the presence of piperine may contribute to the inhibition of cytokine production seen in piperine-treated T cells. Additionally, Bird *et al.* [451] determined that epigenetic modifications that occur during cell cycle progression are required to induce production of IFN-γ and IL-4, but not IL-2 in T cells. Therefore, the inhibition of IFN-γ and IL-4 production may be a consequence of the anti-proliferative activity of piperine on T cells, although this does not account for the piperine-induced inhibition of IL-2 or IL-17A production. Consistent with our data, Kim and Lee [377] showed that piperine inhibits the level of IL-4 in the bronchoalveolar lavage fluid in an OVA-induced asthma model in mice, as well as the level of IL-4 production by OVA-stimulated splenic T cells *ex vivo*. In contrast to our study, Kim and Lee [377] showed that piperine has no effect on IFN-γ production by OVA-stimulated splenic T cells. The T cells used in their study, however, were previously polarized to Th2 cells *in vivo* and therefore produced only low levels IFN-γ. Since we used resting, unpolarized T cells, the different effect of piperine on IFN-γ production in our study versus that of Kim and Lee [377] is likely due to the different experimental conditions.

In our study, the cytotoxic activity of CTLs differentiated in the presence of piperine was inhibited while piperine had no direct effect on the cytotoxic activity of previously differentiated CTLs when added to target cells. This finding suggests that piperine may inhibit CTL differentiation *in vivo*, making it a potential therapeutic agent in the treatment of CTL-mediated autoimmune disease as well as transplant rejection. The inhibition of CTL differentiation in the presence of piperine may be due to the reduction in IL-2 production by piperine-treated T cells since CTLs are dependent on IL-2 production and IL-2R signaling to express granzymes [198] and perforin [199].

In an attempt to determine the mechanism by which piperine mediates its inhibitory effects on T cell activation and effector function, we examined the involvement of the known receptor for piperine, TRPV1 [317]. Surprisingly, TRPV1 was not involved in the inhibitory activity of piperine on T cells, as determined by direct examination of TRPV1 expression in splenic T cells, as well as piperine treatment of T cells from TRPV1$^{-/-}$ mice. TRPV1, also known as VR1, has previously been detected in human peripheral blood mononuclear cells from 6 of 8 subjects [452] but not in murine splenocytes, lymph node immunocytes, or EL4 T lymphoma cells [453]. Our finding that TRPV1 is not expressed on murine splenic T cells is therefore in agreement with Inada *et al.* [453]. The lack of TRPV1 expression and the ability of piperine to inhibit the proliferation of T cells from WT and TRPV1$^{-/-}$ mice leads us to conclude that TRPV1 is not involved in the piperine-mediated inhibition of T cell function.

We determined that piperine did not inhibit all signaling pathways induced by CD3/CD28 stimulation, since ZAP-70 phosphorylation at Tyr 319 was not affected by piperine treatment. Given the normal expression of CD69 and activation-induced survival with piperine treatment, our results would suggest that ZAP-70 is able to transmit intracellular signals that are strong enough to induce these effects.

In contrast to ZAP-70, piperine inhibited the phosphorylation of the intracellular signaling molecules Akt, ERK, and IκBα, all of which are important in CD3/CD28-induced T cell proliferation. Activation of Akt following T cell stimulation results in direct phosphorylation and inactivation of FoxO transcription factors, releasing the cell from FoxO-induced cell cycle arrest [454]. Constitutively active FoxO1 inhibits cell

cycle progression by T cells stimulated with superantigen-pulsed transformed B cells [455], indicating the importance of this downstream target of Akt in regulating T cell proliferation. Akt also phosphorylates and inactivates GSK-3β, resulting in decreased expression of the cyclin-dependent kinase inhibitor p27^{Kip1}, and cell cycle progression [44]. ERK phosphorylation is important for T cell proliferation, as demonstrated by the inhibition of T cell proliferation in the presence of the pharmacological inhibitor U0126 that inhibits MEK phosphorylation of ERK [456]. T cells expressing a dominant IκB mutant that constitutively inhibits NF-κB activity show large proliferation deficits, indicating that the IκB/NF-κB pathway is also important in T cell proliferation [457]. The importance of normal functioning of Akt, ERK, and IκBα in T cell proliferation suggests that the piperine-induced inhibition of Akt, ERK, and IκBα phosphorylation contributes to the observed inhibition of T cell activation.

The inhibitory effect of piperine on T cell signaling shares similarities with its previously documented effects of ERK and IκBα inhibition in other cell types. Piperine inhibits ERK phosphorylation in PMA-stimulated HT-1080 fibrosarcoma cells [396], IL-1β-stimulated fibroblast-like synoviocytes [348], and cerulin-stimulated pancreatic cells [378]. Downstream of ERK, piperine also has been shown to inhibit PMA-induced AP-1 transcriptional activity [396], as well as TNF-α-induced c-fos nuclear localization [373]. Conversely, piperine increases basal levels of ERK phosphorylation in immortalized auditory cells [401]. These findings suggest that piperine may have differential effects on basal levels of ERK phosphorylation, compared to the levels of ERK phosphorylation induced by a stimulus such as CD3/CD28 activation or treatment with PMA, IL-1β, or cerulin. The ability of piperine to inhibit IκBα phosphorylation in activated T cells is consistent with previous studies showing that piperine inhibits IκBα phosphorylation and its subsequent degradation in TNF-α-stimulated endothelial cells [367], as well as in PMA-stimulated HT-1080 cells [396]. Consistent with IκB inhibition, piperine also inhibits the nuclear localization of NF-κB in B16-F10 melanoma cells [373] and HT-1080 cells [396], which occurs immediately downstream of IκBα degradation. In contrast to our study, however, piperine does not inhibit cerulin-induced IκBα degradation in pancreatic cells [378] or IL-1β-induced NF-κB transcriptional activity in synoviocytes [348]. The conflicting findings on the effect of piperine on IκBα phosphorylation may be

a result of piperine having cell type- and stimuli-specific effects on the IκB/NF-κB pathway.

Our finding that piperine inhibited Akt phosphorylation in stimulated T cells is consistent with a previous study by our lab, in which piperine inhibited Akt phosphorylation in growth factor-stimulated endothelial cells (Chapter 5, Manuscript 3). In T cells, phosphorylation of Akt at Ser 473 is regulated by PKCα [39]. Interestingly, Hwang *et al.* [396] showed that piperine inhibits the PMA-induced phosphorylation of PKCα in HT-1080 cells. It is therefore possible that the mechanism by which piperine inhibits Akt phosphorylation in stimulated T cells may involve the inhibition of PKCα. Additionally, ERK and IκBα phosphorylation lie downstream of PKCθ activation [47,458]. Since piperine has been previously shown to inhibit certain PKC isoforms in other cell types [396], it is possible that piperine is mediating its inhibitory effects on T cell proliferation and function by inhibiting the activation of PKC isoforms.

Taken together, our study demonstrates the ability of piperine to inhibit various aspects of T cell function, including proliferation, cytokine production, and CTL differentiation *in vitro* through inhibition of early signaling events involved in T cell activation. Combined with the previously documented anti-inflammatory activities of piperine, our findings suggest that piperine may be a potent immunosuppressive agent for the treatment of T cell-mediated autoimmunity, chronic inflammatory conditions, and transplant rejection. Piperine therefore warrants further investigation in an *in vivo* model of T cell-mediated inflammation.

CHAPTER 4

PIPERINE INHIBITS INTERLEUKIN-2 RECEPTOR SIGNALING AND

PROLIFERATION OF IL-2-DEPENDENT T CELLS

4.1 Introduction

IL-2, originally named T cell growth factor, is an important cytokine for *in vitro* T cell proliferation and effector function [151]. Results from *in vivo* studies indicate that IL-2 is involved in the differentiation of CTLs [205]. Others have shown that in the absence of IL-2 signaling, $CD8^+$ T cells receive improper programming during a primary immune response, resulting in the inability of $CD8^+$ memory T cells to generate a robust recall response and expand in response to rechallenge or persistent infection [206-208]. Additionally, T cells have a greater dependence on IL-2 signaling in non-lymphoid tissues compared to lymphoid tissues, possibly due to higher cytokine levels and defined structural organization that promotes cell-cell interactions in lymphoid tissues, which is lacking in non-lymphoid tissues [203,204]. Because IL-2 plays such important roles in shaping T cell responses, it is an excellent therapeutic target for suppressing inappropriate T cell activation that results in transplant rejection [459], autoimmune diseases such as MS [405] and rheumatoid arthritis [406], and chronic inflammatory conditions such as IBD [404]. IL-2 has been successfully targeted *in vivo* using the humanized monoclonal Ab daclizumab, which binds to and blocks the α chain of the IL-2R, CD25 [213]. Daclizumab is currently used to prevent transplant rejection [214] and has shown promise in the treatment of MS [460]. Because daclizumab is a monoclonal Ab, it is costly and has to be administered by injection, and there is a risk that the host will generate neutralizing Abs that render it inactive [461,462]. Therefore, the search for new agents targeting IL-2 signaling is ongoing.

IL-2 induces the heterotrimerization of the IL-2R, leading to activation of JAK1 and JAK3 and phosphorylation of multiple Tyr residues on the IL-2Rβ chain, which form docking sites for a variety of signaling molecules, such as Shc and STAT5 [151]. STAT5 and STAT3 are phosphorylated by JAK1 [157] and JAK3 [159], inducing the STATs to homo- or heterodimerize and translocate to the nucleus [463]. STAT5 is the major STAT

activated by IL-2R signaling [179]; STAT3 is also activated, although to a lesser degree than STAT5 [179]. STAT5 promotes IL-2-induced proliferation and transcription of various genes involved in cell cycle progression (eg. *c-myc, cyclin D2*) and cell survival (eg. *bcl-2* and *bcl-x*) [464], making it an important molecule in IL-2R signaling.

Bioactive phytochemicals provide a promising new resource of immunosuppressive agents that can inhibit IL-2 signaling. One such phytochemical, curcumin, was previously identified by our laboratory as an inhibitor of IL-2R signaling *in vitro* [421]. Studies are currently ongoing to identify other phytochemicals with activity against IL-2. Piperine is the major alkaloid found in the fruits of black pepper plants and is known for its pungent flavor [321]. Many of the physiological effects associated with black pepper in traditional Indian and Chinese medicines have been attributed to piperine [465]. We have previously shown that piperine has direct effects on T cells *in vitro* (Chapter 3, Manuscript 1), which could account for some of its *in vivo* anti-inflammatory activity [348,372,377]. Piperine inhibits the proliferation of T cells in response to CD3/CD28 stimulation and also inhibits T cell responsiveness to exogenous IL-2 (Chapter 3, Manuscript 1). Since IL-2 is an important cytokine for T cell function [151] and piperine has previously been shown to inhibit T cell responsiveness to IL-2 (Chapter 3, Manuscript 1), we wanted to examine the effect of piperine on IL-2R signaling using IL-2-dependent CTLL-2 cells. Our findings indicate that piperine warrants further study as an inhibitor of IL-2R signaling and a potential therapeutic agent for the prevention of transplant rejection and the treatment of T cell-mediated autoimmune and chronic inflammatory conditions.

4.2 Results

4.2.1 Piperine Inhibits the IL-2-Dependent Proliferation of CTLL-2 Cells without Inducing Cell Death

Inhibition of the proliferation of primary T cells by piperine was not reversed by the addition of exogenous IL-2 (Figure 3.10), suggesting that piperine might inhibit IL-2R signaling. To test this hypothesis, the murine cytotoxic T cell line, CTLL-2, which is dependent on IL-2 for proliferation and survival [466], was cultured in the absence or presence of piperine. The cell permeable dye Oregon Green® 488 and flow cytometric

analysis was used to quantify cell proliferation. Piperine treatment of IL-2-stimulated CTLL-2 cells for 72 h resulted in fewer cell divisions compared to medium or vehicle treatment (Figure 4.1A). The presence of 100 µM piperine in IL-2-stimulated CTLL-2 cultures inhibited cellular division similar to unstimulated control cells, while 50 µM piperine treatment inhibited the number of cell divisions by half compared to vehicle-treated cells (1.2 ± 0.3 vs 2.5 ± 0.2, respectively; Figure 4.1B; $p < 0.05$). The anti-proliferative effect of piperine on IL-2-stimulated CTLL-2 cells was confirmed by measuring [^{3}H]TdR incorporation. Treatments of 100 µM or 50 µM piperine for 48 h significantly inhibited CTLL-2 proliferation in a dose-dependent manner compared to the vehicle control (Figure 4.1C; $p < 0.05$) as determined by the [^{3}H]TdR incorporation assay, which measures DNA synthesis. To determine if the anti-proliferative effect of piperine was due to cytotoxicity, the viability of IL-2-starved and IL-2-stimulated CTLL-2 cells treated with medium, vehicle, or piperine for 24 h was measured using Annexin-V/PI staining. There was no significant increase in CTLL-2 cell death with piperine treatment compared to the vehicle control (Figure 4.1D; $p > 0.05$), suggesting that piperine is not cytotoxic to CTLL-2 cells. Annexin-V/PI staining also confirmed the dependence of the CTLL-2 line on IL-2 for survival, as there was a significant increase in cell death in IL-2-starved CTLL-2s compared to the vehicle control (Figure 4.1D; $p < 0.05$). Taken together, these findings indicate that piperine inhibits the IL-2-dependent proliferation of CTLL-2 cells in a dose-dependent manner without inducing cell death, suggesting that the survival signal induced by IL-2 is occurring in the presence of piperine.

4.2.2 The Inhibition of CTLL-2 Proliferation by Piperine is Reversible

To determine whether the inhibitory effect of piperine on CTLL-2 cells was reversible, CTLL-2 cells were pre-treated with medium, vehicle, 100, or 50 µM piperine during their 3 h starvation of IL-2, washed 3 times to remove excess piperine, normalized by trypan blue exclusion, and stimulated with IL-2 for 48 h. When the piperine pretreatment was removed, the cells proliferated normally compared to vehicle pretreated cells, as measured by [^{3}H]TdR incorporation (Figure 4.2; $p > 0.05$). These data indicate that the effect of piperine on CTLL-2 cells is reversible.

4.2.3 Increasing Concentrations of IL-2 do not Rescue CTLL-2 Cells from Inhibition by Piperine

Piperine inhibited IL-2-induced CTLL-2 proliferation only when it was added to cultures at the same time as IL-2; therefore, it was possible that piperine was either interfering with the ability of IL-2 to bind to the IL-2R, or with the amount of stimulation that IL-2 was delivering to the CTLL-2 cells. To investigate this, increasing concentrations of IL-2 were added to CTLL-2 cells in order to attempt to reverse the piperine-mediated inhibition of CTLL-2 proliferation. However, IL-2 concentrations as high as 500 U/mL, a full 10-fold higher than the normal concentration required for optimal CTLL-2 proliferation, were unable to rescue the proliferation of piperine-treated CTLL-2 cells (Figure 4.3; $p > 0.05$). This finding suggests that the inhibitory effect of piperine on the IL-2-dependent proliferation of CTLL-2 cells cannot be overcome by increasing IL-2 concentrations. Rather, thes data suggest that piperine is inhibiting CTLL-2 cell stimulation by interferring with IL-2R signaling.

4.2.4 Piperine does not Inhibit CTLL-2 Expression of CD25, the α chain of the IL-2R

Expression of the IL-2R α chain is transiently induced upon T cell activation, resulting in the formation of the tripartite high affinity receptor. The α chain of the IL-2R, also known as CD25, is important for T cell proliferation and survival [195,196]. CTLL-2 proliferation is dependent on the interaction of CD25 with the β and γ chains [467], and increasing IL-2 concentrations did not rescue the proliferation of piperine-treated CTLL-2 cells (Figure 4.3), therefore piperine may be inhibiting the expression of CD25, which is normally constitutively expressed by CTLL-2 cells [467]. To test this hypothesis, CD25 expression was examined by flow cytometry 24 h after piperine treatment of CTLL-2 cells. Neither 100 nor 50 μM piperine treatment had a significant effect on CD25 surface expression on IL-2-stimulated CTLL-2 cells compared to the vehicle control (Figure 4.4; $p > 0.05$), indicating that piperine does not inhibit IL-2-dependent proliferation of CTLL-2 cells by preventing the expression of CD25.

4.2.5 Piperine Inhibits Certain Tyr Phosphorylation Events in IL-2-Stimulated CTLL-2 Cells

The downstream cascade of intracellular Tyr phosphorylation events that are induced by IL-2 binding to the IL-2R were examined because piperine did not affect CD25 expression. As shown in Figure 4.5, piperine-treated CTLL-2 cells stimulated with IL-2 for 15 min showed inhibition of certain Tyr phosphorylation events compared to cells stimulated in the presence of the medium or vehicle control (eg. Figure 4.5 boxes 1 and 3). Interestingly, not all Tyr phosphorylation events upregulated by IL-2 were inhibited by piperine treatment (eg. Figure 4.5 boxes 2 and 4). Additionally, piperine appears to inhibit the Tyr phosphorylation of some proteins in the absence of IL-2 stimulation (eg. Figure 4.5 boxes 5 and 6). Taken together, these data suggest that piperine does not have a global inhibitory effect on Tyr phosphorylation in CTLL-2 cells. Instead, piperine inhibits the baseline level of Tyr phosphorylation of specific proteins in IL-2-starved CTLL-2 cells, as well as upregulated Tyr phosphorylation of specific proteins in IL-2-stimulated CTLL-2 cells.

4.2.6 Piperine does not Inhibit the Phosphorylation of JAK1 or JAK3

The IL-2R subunits do not have intrinsic kinase activity [468] and therefore rely on their association with the non-receptor Tyr kinases, JAK1 and JAK3, which are constitutively associated with the γ-chain and the β-chain of the IL-2R, respectively, to induce intracellular signaling [469]. Binding of IL-2 to the IL-2R induces in the phosphorylation of JAK1 and JAK3 [468]. Since piperine inhibited the IL-2-induced Tyr phosphorylation of certain intracellular proteins (Figure 4.5), the effect of piperine on IL-2-induced JAK1 and JAK3 phosphorylation was examined by western blot analysis at both early (5 min) and later (30 and 60 min) time points. Piperine did not effect IL-2-induced JAK1 or JAK3 phosphorylation at any of the time points examined (Figure 4.6A and B, respectively; $p > 0.05$). The finding that JAK1 and JAK3 phosphorylation was unaffected by piperine treatment at their respective activation sites suggests that piperine does not inhibit the kinase activity of JAK1 and JAK3. Additionally, because JAK1 and JAK3 phosphorylation are the proximal signaling events associated with IL-2R signaling [159,155], my finding that their phosphorylation status was unaffected by piperine

treatment suggests that piperine does not sequester or degrade IL-2, inhibit the binding of IL-2 to its receptor, or inhibit the normal assembly of the IL-2R subunits.

4.2.7 Piperine Inhibits the Phosphorylation of STAT5 and STAT3

Since STAT5 is an important signaling molecule involved in IL-2-induced proliferation and is directly phosphorylated by JAK1 and JAK3, the effect of piperine on STAT5 phosphorylation was examined at both early (5 min) and later (30 and 60 min) timepoints following IL-2 stimulation. Interestingly, treatment with both 100 and 50 μM piperine significantly inhibited IL-2-induced STAT5 phosphorylation at all time points examined following IL-2 stimulation (Figure 4.7A; $p < 0.05$), suggesting that piperine is a potent inhibitor of IL-2-induced STAT5 phosphorylation.

STAT3 also plays a role in T cell proliferation. Downstream of the IL-6 receptor subunit gp130, STAT3 induces G_1 to S phase transition of the cell cycle by the upregulation of the cell cycle proteins cyclin D2 and D3 and the downregulation of CDK inhibitors p21^{Cip} and p27^{Kip1} [470]. T cells in which STAT3 is conditionally deleted show reduced proliferation in response to IL-2 stimulation, indicating that STAT3 plays a role in IL-2R signaling [471]. Similar to STAT5, 100 μM piperine significantly inhibited the phosphorylation of STAT3 at Tyr 705 following 5, 30, and 60 min of IL-2 stimulation compared to the vehicle control (Figure 4.7B; $p < 0.05$). In contrast to STAT5, however, 50 μM piperine did not significantly inhibit the phosphorylation of STAT3. These data indicate that piperine inhibits the IL-2-induced phosphorylation of STAT5 and STAT3. Since both 100 μM and 50 μM piperine treatments were able to significantly inhibit STAT5 phosphorylation, while STAT3 phosphorylation was only significantly inhibited with 100 μM piperine treatment, piperine may inhibit the phosphorylation of STAT5 more potently than the phosphorylation of STAT3.

4.2.8 Piperine does not Inhibit STAT5 Phosphorylation by Activating a Phosphatase

STAT5 phosphorylation is regulated by phosphatases, such as SHP-2 [186,187], raising the possibility that piperine inhibited STAT5 phosphorylation by activating phosphatases involved in the negative regulation of STAT5. The phosphatase inhibitor, Na_3VO_4, which has previously been shown to inhibit SHP-2 activity in CTLL-2 cells

[472], was used to determine if the inhibitory effect of piperine on STAT5 phosphorylation involved the activation of phosphatases. IL-2-starved CTLL-2 cells were pretreated with EtOH vehicle or 1 mM Na_3VO_4 prior to piperine treatment and IL-2 stimulation, but the inhibitory effect of piperine on STAT5 phosphorylation was not reduced (Figure 4.8A; $p > 0.05$). Tyr phosphorylation events induced by IL-2 stimulation were elevated in Na_3VO_4 pretreated cells compared to EtOH vehicle pretreated cells (Figure 4.8B), confirming that the concentration of Na_3VO_4 used was sufficient to inhibit phosphatase activity. Additionally, Na_3VO_4 pretreatment was sufficient to induce low levels of STAT5 phosphorylation in the absence of IL-2 stimulation (Figure 4.8A bar graph), in accordance with a previous report [473]. The inhibitory effect of piperine on STAT5 phosphorylation, regardless of Na_3VO_4 pretreatment, indicated that phosphatases do not mediate the inhibitory activity of piperine on STAT5 phosphorylation.

4.2.9 Piperine does not Require *De Novo* Protein Synthesis to Inhibit STAT5 Phosphorylation in IL-2-Stimulated CTLL-2 Cells

The activation of STAT5 and STAT3 is also regulated by a family of proteins known as SOCS. Three members of this family, CIS [188] and SOCS1 [189], and SOCS3 [190], are induced by IL-2R signaling in T cells as part of a negative feedback loop. To determine if piperine induced the expression of these or any other proteins to mediate the anti-proliferative effect on CTLL-2 cells, CTLL-2 cells were pretreated with 1 µg/mL of the protein translation inhibitor CHX or EtOH vehicle control before treatment with piperine and stimulation with IL-2. As shown in Figure 4.9A, CHX pretreatment did not affect the inhibitory action of piperine on STAT5 phosphorylation, indicating that *de novo* protein synthesis is not needed for piperine-mediated inhibition of IL-2-induced STAT5 phosphorylation in CTLL-2 cells. Cyclin D2 expression induced by IL-2 stimulation was inhibited in CHX-pretreated cells (Figure 4.9B), confirming that the concentration of CHX used was sufficient to inhibit *de novo* protein translation. Thus, piperine does not inhibit STAT5 phosphorylation by inducing the *de novo* expression of the STAT5 negative regulators CIS, SOCS1, or SOCS3, or any other proteins.

4.2.10 Piperine Inhibits the Phosphorylation of ERK and Akt

Another important signaling molecule activated by IL-2 stimulation is Shc, which binds to the phosphorylated IL-2Rβ chain, becomes phosphorylated [474], and induces early activation of the ERK and Akt pathways through recruitment of the adapter proteins Grb2-Sos [169] and Grb2-Gab2 [171], respectively. Therefore, we examined the effect of piperine on the phosphorylation of ERK1/2 at Tyr 204 and Akt at Ser 473 at both early (5 min) and later (30 and 60 min) time points following IL-2 stimulation. As shown in Figure 4.10A, 100 μM piperine treatment significantly inhibited IL-2-induced ERK1/2 phosphorylation at Tyr 204 following 5 and 30 min of IL-2 stimulation as compared to the vehicle control ($p < 0.05$). Similarly, 100 μM piperine treatment also inhibited Akt phosphorylation at Ser 473 following 30 min of IL-2 stimulation compared to the vehicle control, as shown in Figure 4.10B ($p < 0.05$). Interestingly, by 1 h after the addition of IL-2, piperine no longer significantly inhibited ERK1/2 or Akt phosphorylation, indicating that piperine-mediated inhibition of ERK1/2 and Akt phosphorylation is transient. These data suggest that piperine inhibits early IL-2-induced phosphorylation of ERK1/2 and Akt and may therefore at least partially inhibit the activation of Shc, which lies upstream of both the ERK1/2 and Akt pathways in IL-2R signaling [169,171].

4.2.11 STAT5, ERK, and Akt are Important Signaling Pathways Involved in the Proliferation of CTLL-2 Cells

Piperine inhibits the phosphorylation of STAT5, STAT3, ERK, and Akt, as shown in Figures 4.7 and 4.10. To confirm the importance of the STAT, ERK, and Akt pathways in IL-2-induced CTLL-2 proliferation, CTLL-2 cells were treated with the pharmacological inhibitors CNH (STAT5 inhibitor; [475]), PD 98059 (an inhibitor of MEK1, which is upstream of ERK1/2; [476]), and LY-294002 (an inhibitor of PI3K, which lies upstream of Akt; [477]). CTLL-2 proliferation was then measured by [³H]TdR incorporation. CNH, PD 98059, and LY-294002 all significantly inhibited the IL-2-induced proliferation of CTLL-2 cells in a dose-dependent manner (Figure 4.11; $p < 0.05$), confirming that the STAT5, ERK, and Akt signaling molecules and their associated signaling pathways are all important for the proliferation of CTLL-2 cells.

Inhibition of these molecules may therefore contribute to the anti-proliferative effect of piperine on IL-2-stimulated CTLL-2 cells.

4.2.12 Piperine Inhibits Cell Cycle Protein Expression, Including Expression of the STAT5-Inducible Proteins, Cyclin D2 and CDK6

STAT5 is required for the expression of the G_1-associated cell cycle proteins cyclin D2 and CDK6, but not CDK4, in T cells [478]. Additionally, STAT5 binds to the *cyclin D2* promoter, inducing its expression in response to IL-2 stimulation [479]. Western blot analysis was performed to determine if the inhibition of STAT5 phosphorylation by piperine treatment was sufficient to inhibit cyclin D2 and CDK6 expression in CTLL-2 cells. As shown in Figure 4.12, treatment with either 100 or 50 μM piperine for 24 h significantly inhibited the IL-2-induced expression of cyclin D2 and CDK6 (Figure 4.12A & B, respectively; $p < 0.05$), suggesting that the inhibition of STAT5 phosphorylation seen with piperine treatment impacts downstream transcription events. Additionally, treatment with either 100 or 50 μM piperine for 24 h significantly inhibited the IL-2-induced expression of CDK4 (Figure 4.12C; $p < 0.05$). Taken together, these findings indicate that piperine inhibits the expression of the G_1-associated cell cycle proteins cyclin D2, CDK6, and CDK4, consistent with piperine inhibiting the cell cycle progression of IL-2-stimulated CTLL-2 cells. Additionally, these data further support STAT5 inhibition as a potential mechanism for the anti-proliferative effect of piperine on CTLL-2 cells and also suggest that STAT5 inhibition is not the only potential mechanism.

Figure 4.1 Piperine inhibits IL-2-dependent proliferation of CTLL-2 cells without inducing cell death. (A & B) IL-2-starved CTLL-2 cells were stained with Oregon Green® 488 dye and then treated with medium, vehicle (DMSO; black peak), 100 (blue peak), or 50 µM piperine (red peak) in the presence of IL-2 (50 U/mL) or were left unstimulated as a non-proliferative control (grey peak). Cells were cultured for 72 h and then flow cytometry was used to measure cell proliferation by quantifying the Oregon Green® 488 fluorescence intensity, which halved with subsequent cellular divisions. (A) Data shown are from a representative experiment. (B) The number of cellular divisions was calculated from the MCF of Oregon Green® 488-labeled CTLL-2 cells. (C) IL-2-starved CTLL-2 cells were incubated with vehicle (DMSO) or the indicated concentrations of piperine in the presence of IL-2 (50 U/mL) for 48 h. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. (D) IL-2-starved CTLL-2 cells were treated with medium (Med.), DMSO vehicle (Veh.), or the indicated concentrations of piperine with IL-2 (50 U/mL) or left untreated (no IL-2) for 24 h. Cells were then stained with Annexin-V/PI to determine phosphatidylserine translocation and membrane integrity loss as a measure of cell death by flow cytometry. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ and ns denotes "not significant" when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
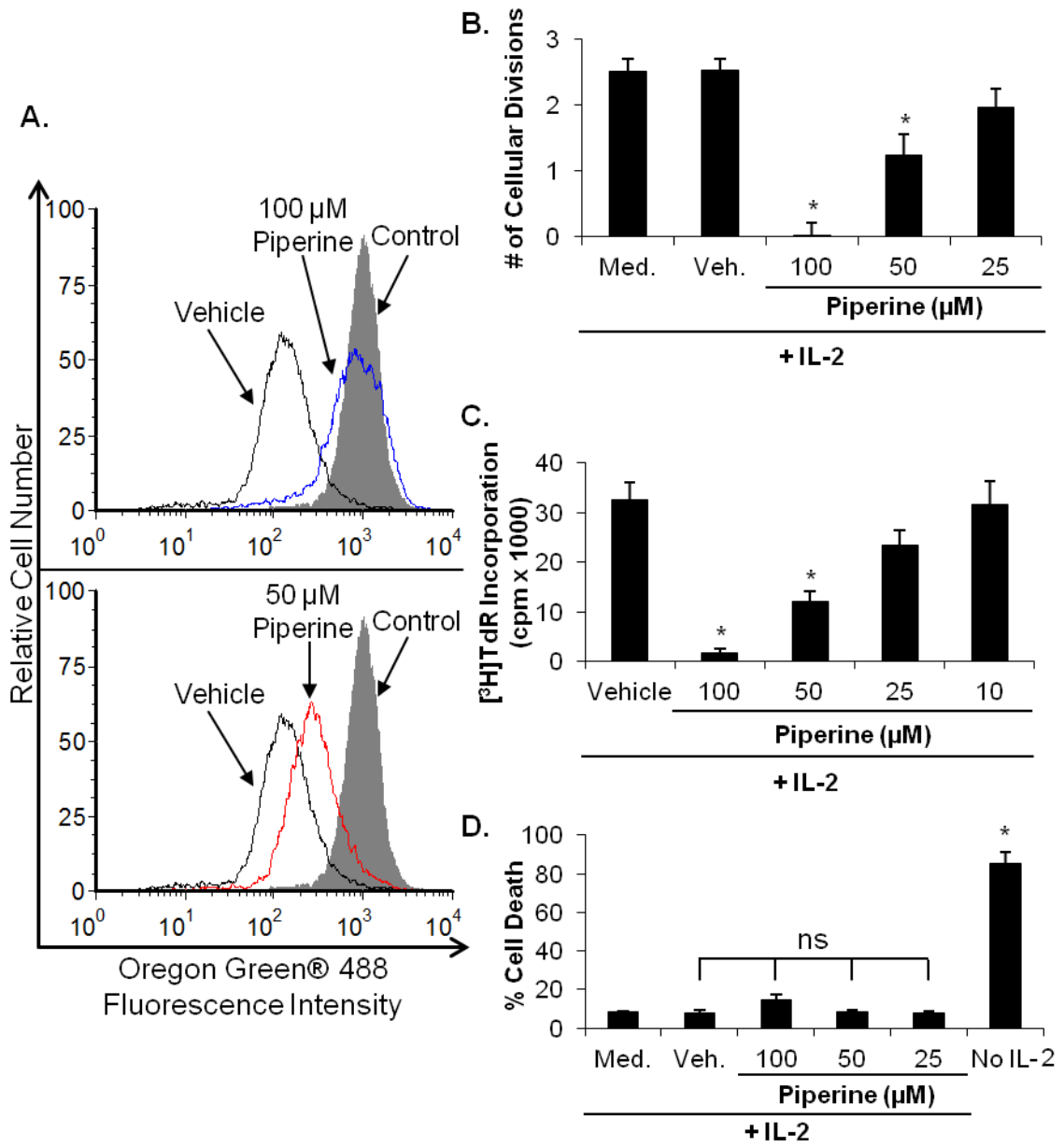
Figure 4.1

Figure 4.2 The inhibition of CTLL-2 proliferation by piperine is reversible. CTLL-2 cells received the indicated treatments for 3 h during IL-2 -starvation. Cells were washed, the number of viable cells in each treatment was normalized by trypan blue counts, and cells were stimulated with IL-2 (50 U/mL) for 48 h. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; ns denotes "not significant" when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
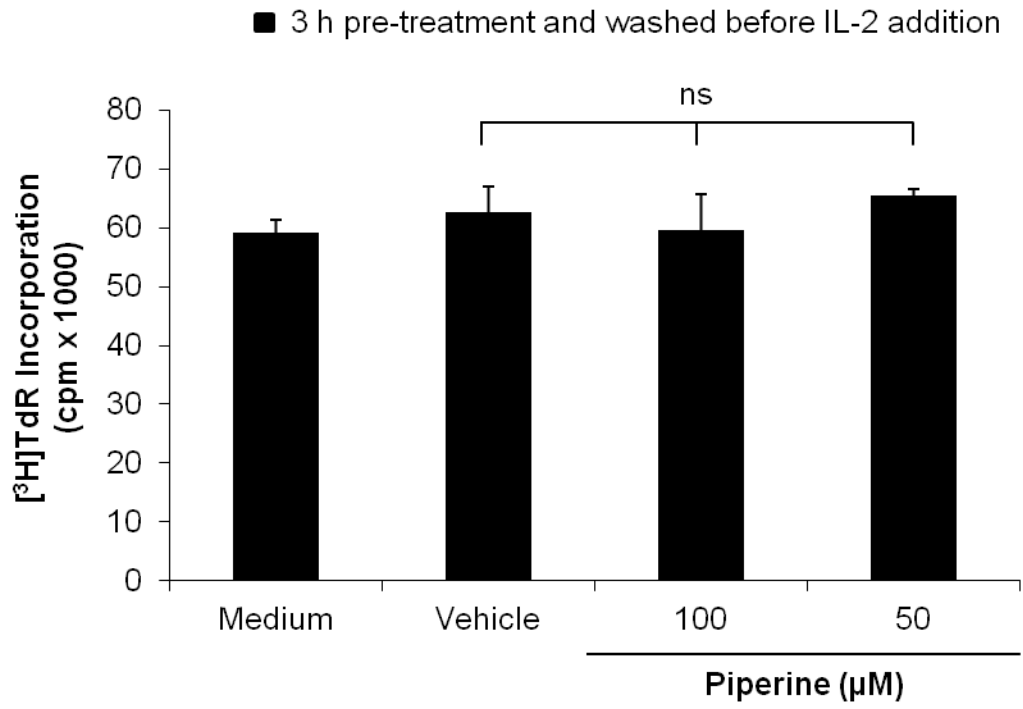
Figure 4.2

Figure 4.3 Increased concentrations of IL-2 do not rescue CTLL-2 cells from inhibition by piperine. IL-2-starved CTLL-2 cells were incubated with medium, vehicle (DMSO), 100, or 50 µM piperine for 48 h in the presence of the indicated doses of IL-2. Cells were pulsed with [³H]TdR for the last 6 h of incubation, harvested, and [³H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the respective vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. ns denotes "not significant" when compared to the 50 U/mL IL-2 treatment as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
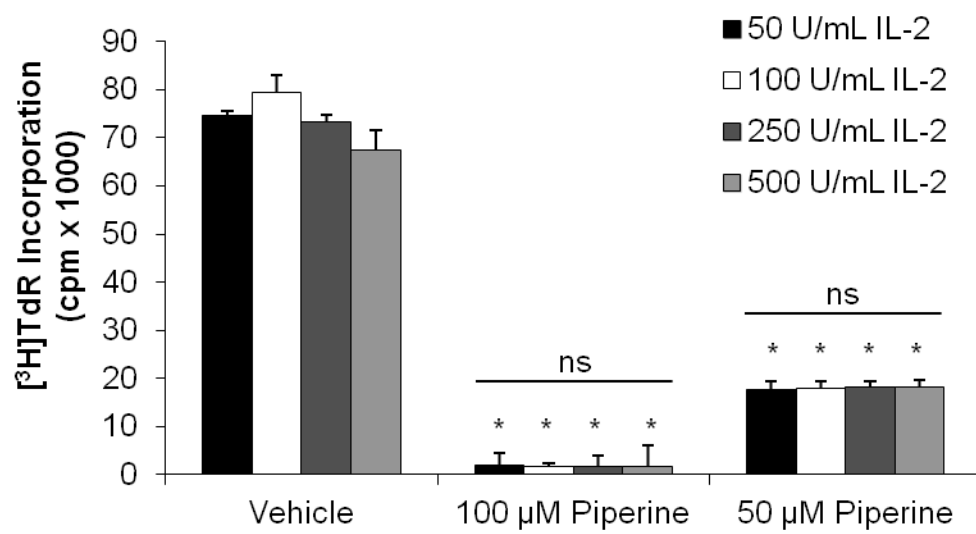
Figure 4.3

Figure 4.4 Piperine does not inhibit constitutive expression of the IL-2R α chain (CD25). IL-2-starved CTLL-2 cells were treated with vehicle (DMSO), 100, or 50 µM piperine for 24 h in the presence of 50 U/mL of IL-2. Cells were then stained with Ab specific for CD25 or isotype control, fixed, and analysed by flow cytometry. (A) Data shown are from a representative experiment with isotype control depicted by the grey peak and CD25 expression depicted by the black peak. (B) CD25 expression was calculated from the MCF. Data shown are the mean of 3 independent experiments ± SEM; ns denotes "not significant" when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
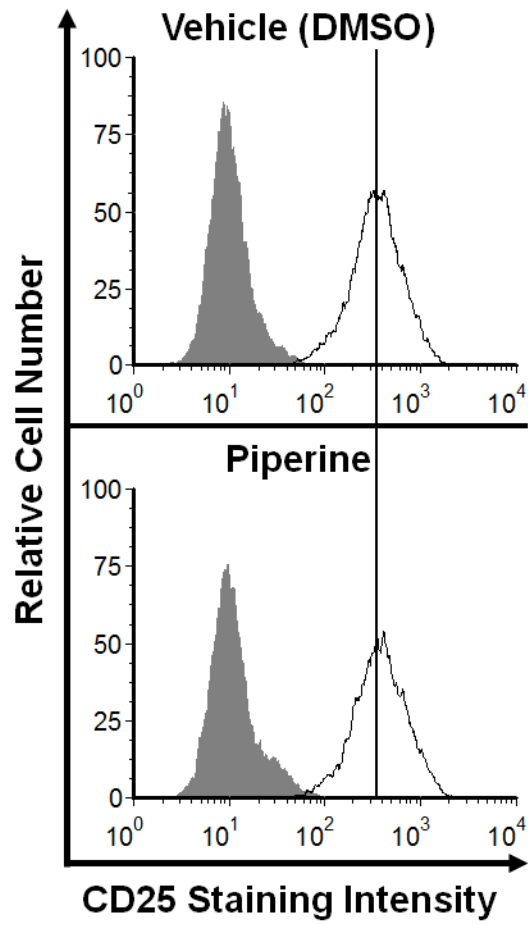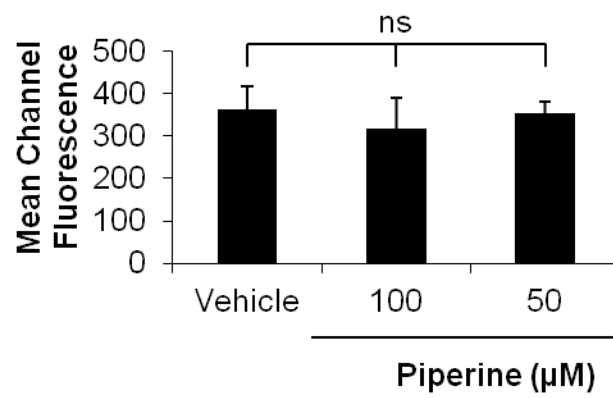
Figure 4.4

Figure 4.5 Piperine inhibits some but not all tyrosine (Tyr) phosphorylation events in IL-2-stimulated CTLL-2 cells. IL-2-starved CTLL-2 cells were pretreated for 30 min with medium (M), vehicle (DMSO; V), 100, or 50 µM piperine. Cells were then stimulated with IL-2 (50 U/mL) for 15 min prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with anti-phosphorylated (p)-Tyr Ab, washed, and then probed with anti-actin Ab to confirm equal protein loading. Data shown are from a representative experiment (n = 3). Numbered boxes indicate areas of interest as referenced in the text.

Figure 4.5

Figure 4.6 Piperine does not inhibit JAK1 or JAK3 phosphorylation. IL-2-starved CTLL-2 cells were pretreated for 30 min with medium (Med), DMSO vehicle (Veh), 100, or 50 µM piperine (Pip.). Cells were then stimulated with IL-2 (50 U/mL) for the indicated times prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-JAK1 (A) or p-JAK3 (B), washed, and then probed with Ab for actin to confirm equal protein loading. Optical density ratio was calculated as the ratio of p-JAK expression to actin expression. Ratios were normalized to medium-treated cells for each respective time point of IL-2 stimulation or to medium-treated cells stimulated with IL-2 for 5 min in the case of unstimulated cells. Data shown are the mean of at least 3 independent experiments $\pm$ SEM with one representative experiment; ns denotes "not significant" when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
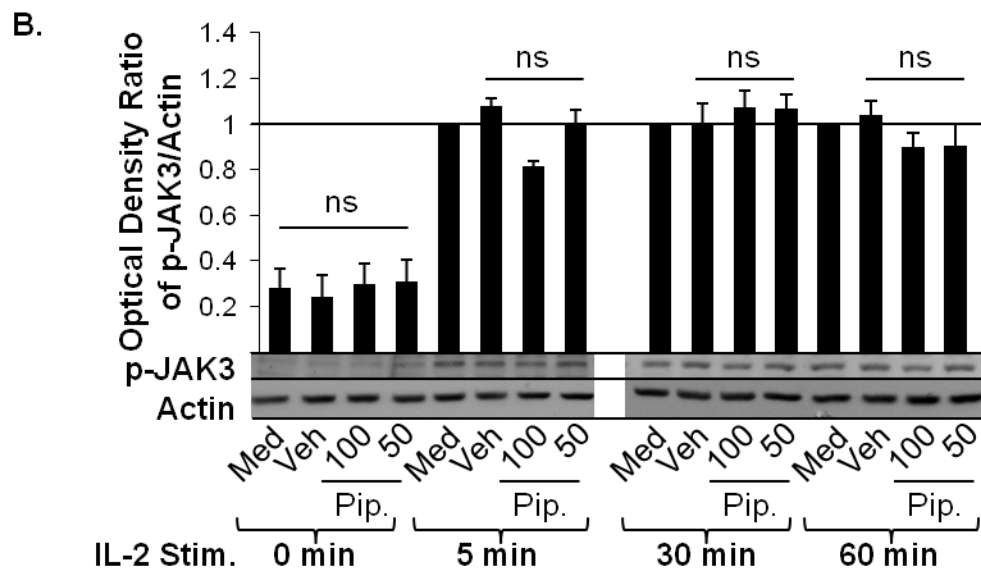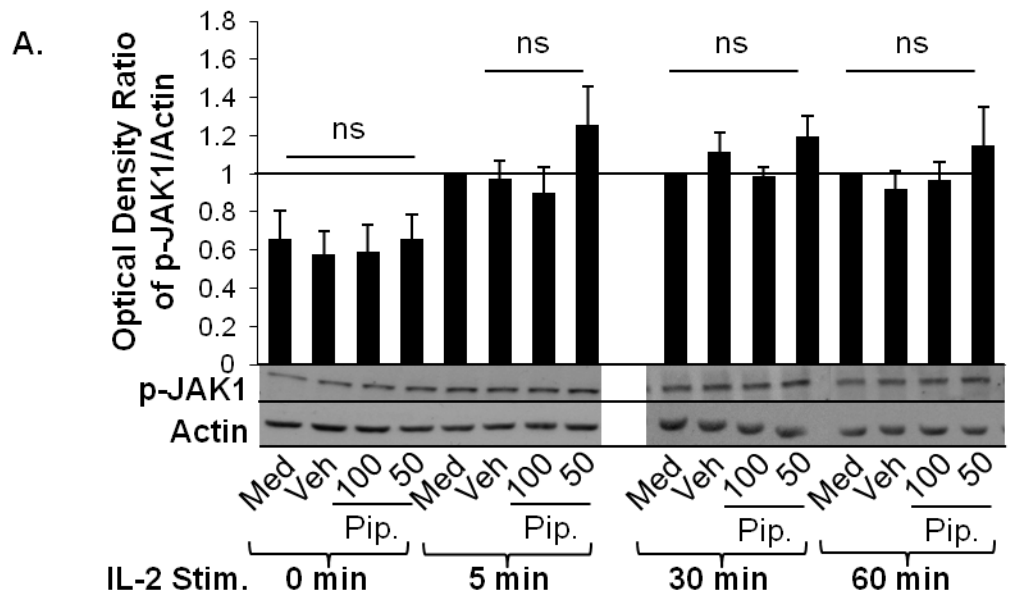
Figure 4.6

Figure 4.7 Piperine inhibits STAT5 and STAT3 phosphorylation. IL-2-starved CTLL-2 cells were pretreated for 30 min with medium (Med), vehicle (Veh), 100 or 50 μM piperine (Pip.). Cells were then stimulated with IL-2 (50 U/mL) for the indicated times prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-STAT5 (A) or p-STAT3 (B), stripped, and then probed with Ab for total STAT5 (A) or STAT3 (B) as well as actin to confirm equal protein loading. Optical density ratio was calculated as the ratio of p-STAT expression to total STAT expression. Ratios were normalized to medium-treated cells for each respective time point of IL-2 stimulation or to medium-treated cells stimulated with IL-2 for 5 min, in the case of unstimulated cells. Data shown are the mean of at least 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
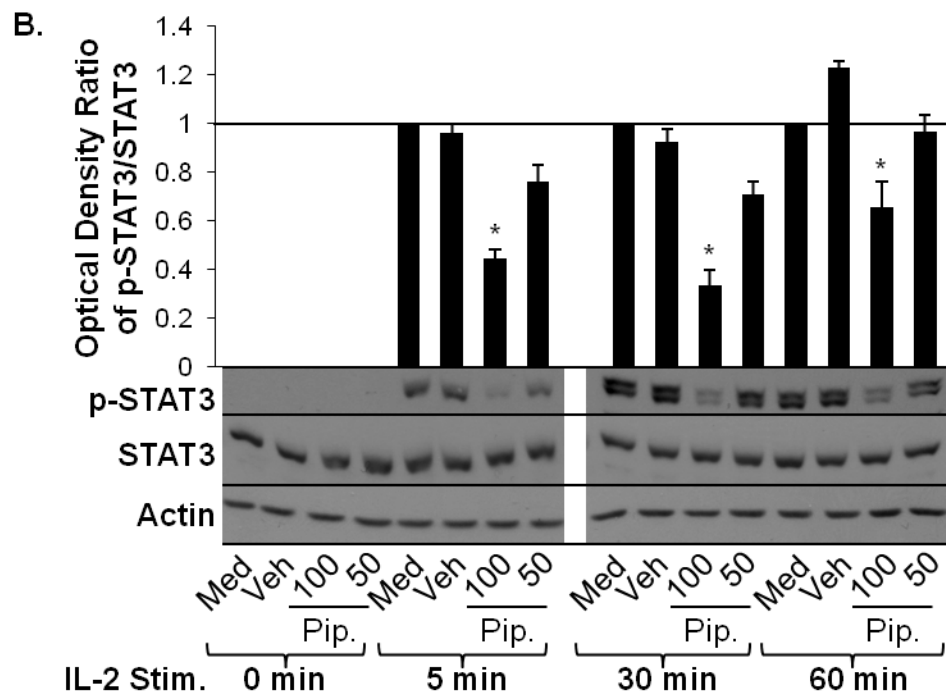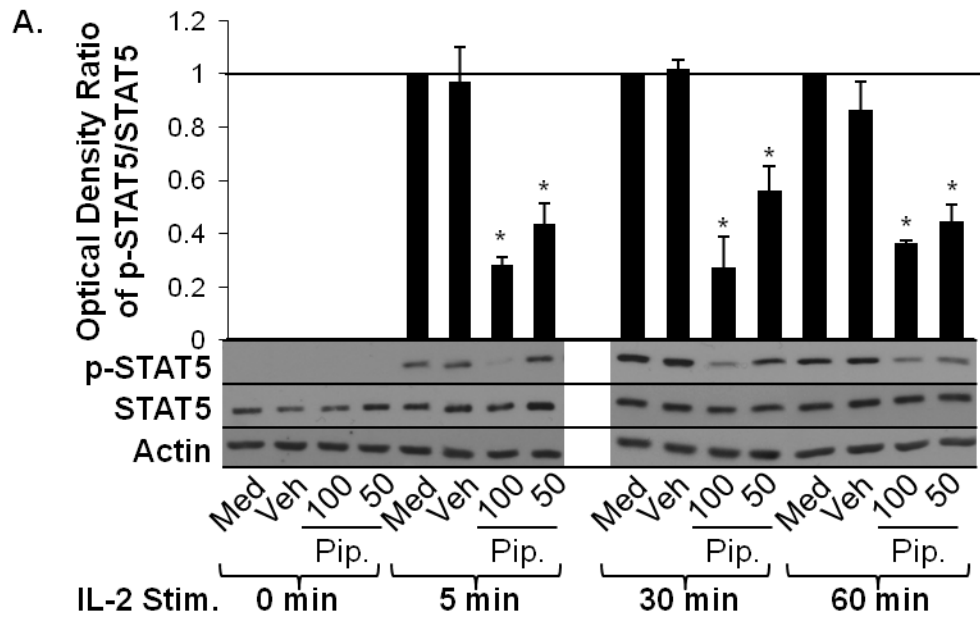
Figure 4.7

Figure 4.8 Piperine does not inhibit STAT5 phosphorylation by activating a phosphatase. (A) IL-2-starved CTLL-2 cells were pretreated with ethanol vehicle (EtOH Veh; black bars) or 1 mM of the phosphatase inhibitor Na_3VO_4 (grey bars) for 15 min, followed by treatment with DMSO vehicle (Veh), 100, or 50 µM piperine (Pip.) for 15 min. Cells were then stimulated with IL-2 (50 U/mL) for the indicated times prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-STAT5, stripped, and then probed with Ab for total STAT5 and actin to confirm equal protein loading. Optical density ratio was calculated as the ratio of p-STAT5 expression to total STAT5 expression. Ratios were normalized to medium-treated cells for each respective time point of IL-2 stimulation or to medium-treated cells stimulated with IL-2 for 5 min, in the case of unstimulated cells. Data shown are the mean of 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test; $p > 0.05$ when comparing EtOH vehicle-pretreated to Na_3VO_4-pretreated samples as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (B) To confirm the activity of Na_3VO_4, IL-2-starved CTLL-2 cells were pretreated with EtOH vehicle or 1 mM Na_3VO_4 for 15 min followed by treatment with vehicle (Veh), 100, or 50 µM piperine for 15 min. Cells were then stimulated with IL-2 (50 U/mL) for 60 min prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for p-Tyr, washed, and then probed with Ab for actin, to confirm equal protein loading.

A.
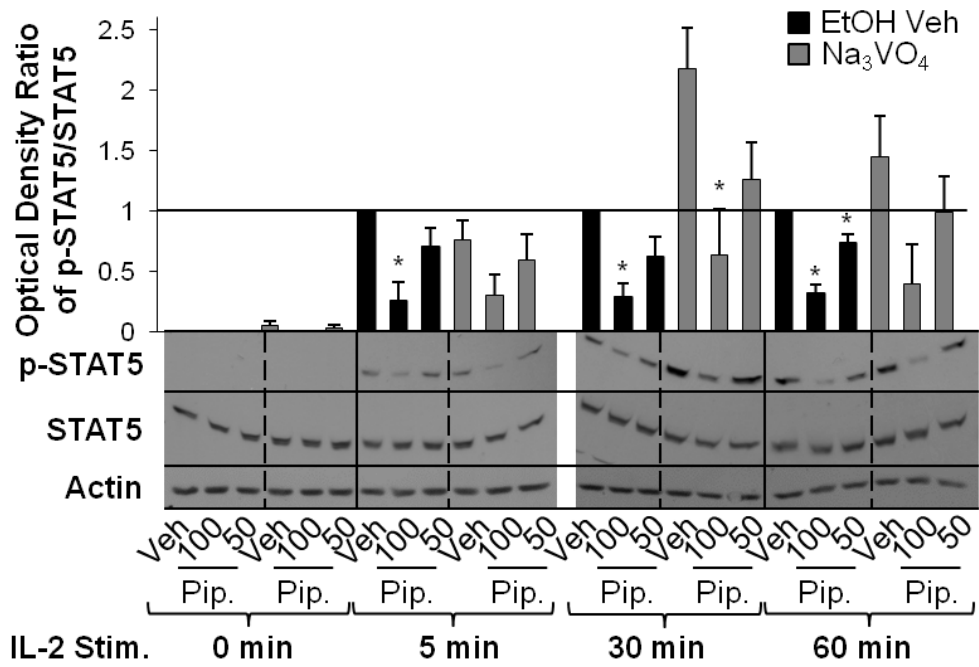
B.
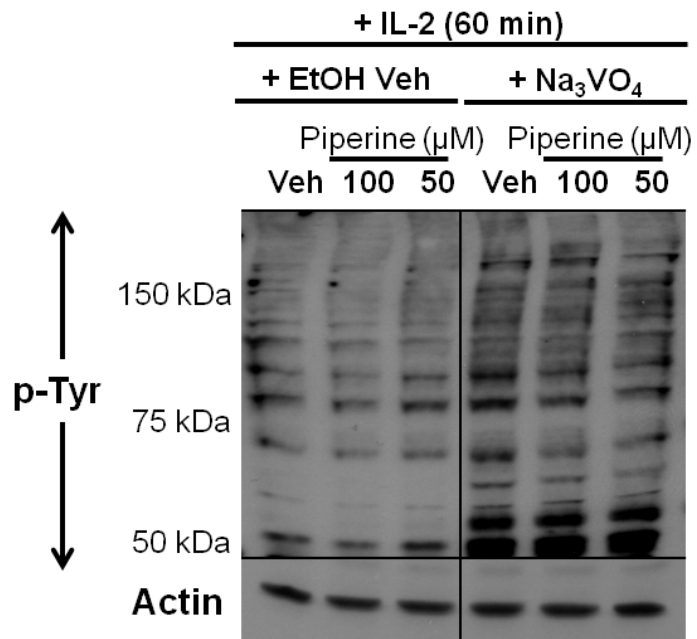
Figure 4.8

Figure 4.9 Piperine does not require *de novo* protein synthesis to inhibit STAT5 phosphorylation in IL-2-stimulated CTLL-2 cells. (A) IL-2-starved CTLL-2 cells were pretreated with ethanol vehicle (EtOH Veh) or 1 µg/mL of the protein translation inhibitor CHX for 15 min followed by treatment with DMSO vehicle (Veh), 100, or 50 µM piperine (Pip.) for 15 min. Cells were then stimulated with IL-2 (50 U/mL) for the indicated times prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-STAT5, stripped, and then probed with Ab for total STAT5 and actin to confirm equal protein loading. Data shown are from a representative experiment (n = 2). (B) To confirm the activity of CHX, IL-2-starved CTLL-2 cells were pretreated with medium (Med), EtOH Veh, or 1 µg/mL CHX for 30 min followed by culture with or without IL-2 (50 U/mL) for 24 h prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for cyclin D2, washed, and then probed with Ab for actin, to confirm equal protein loading. Data shown are from a representative experiment (n = 2).
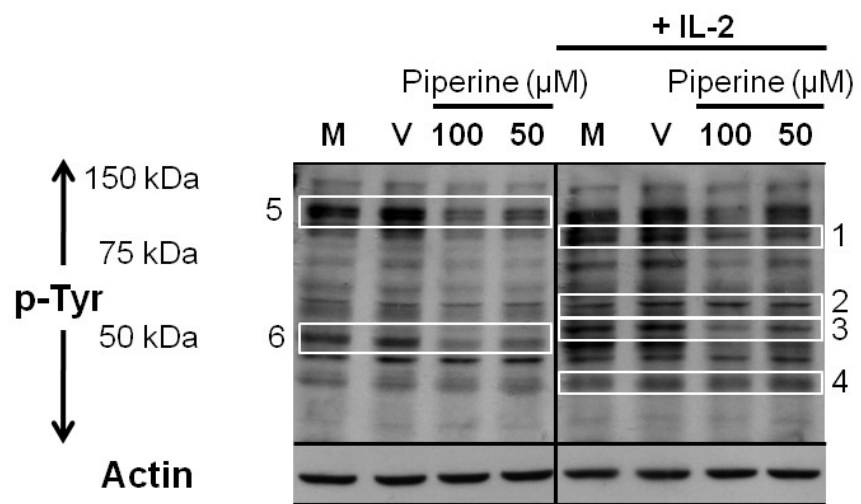
A.
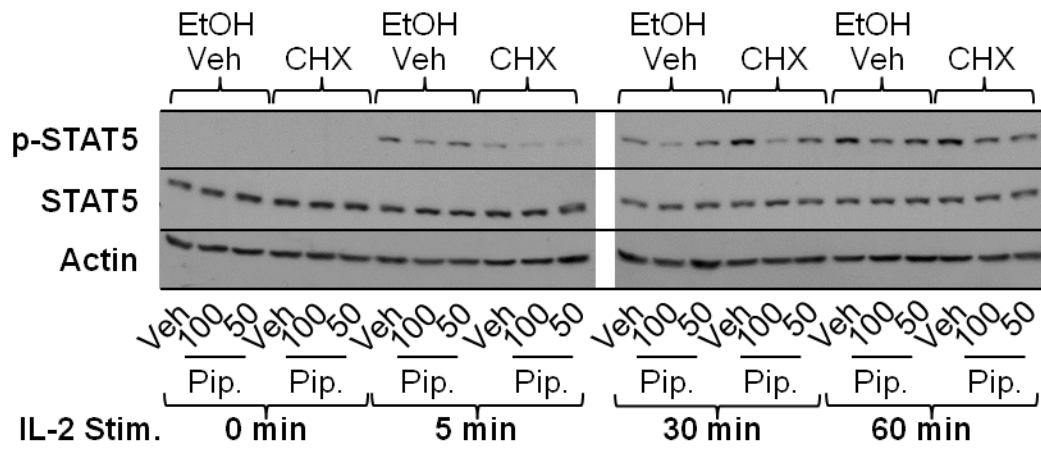
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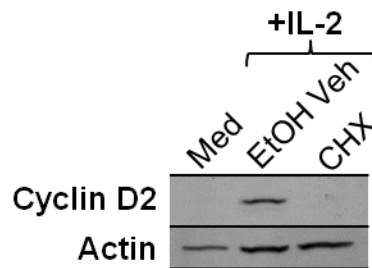
Figure 4.9

Figure 4.10 Piperine inhibits early ERK and Akt phosphorylation. IL-2-starved CTLL-2 cells were pretreated for 30 min with medium (Med), DMSO vehicle (Veh), 100, or 50 µM piperine (Pip.). Cells were then stimulated with IL-2 (50 U/mL) for the indicated times prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for phosphorylated (p)-ERK1/2 (A) or p-Akt (B), stripped, and then probed with Ab for total ERK1/2 (A) or total Akt (B) and actin to confirm equal protein loading. Optical density ratio was calculated as the ratio of p-ERK (A) or p-Akt (B) expression to total ERK (A) or total Akt (B) expression. Ratios were normalized to medium-treated cells for each respective time point of IL-2 stimulation or to medium-treated cells stimulated with IL-2 for 5 min in the case of unstimulated cells. Data shown are the mean of at least 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control for the respective time point as determined by ANOVA with the Tukey-Kramer multiple comparisons test.
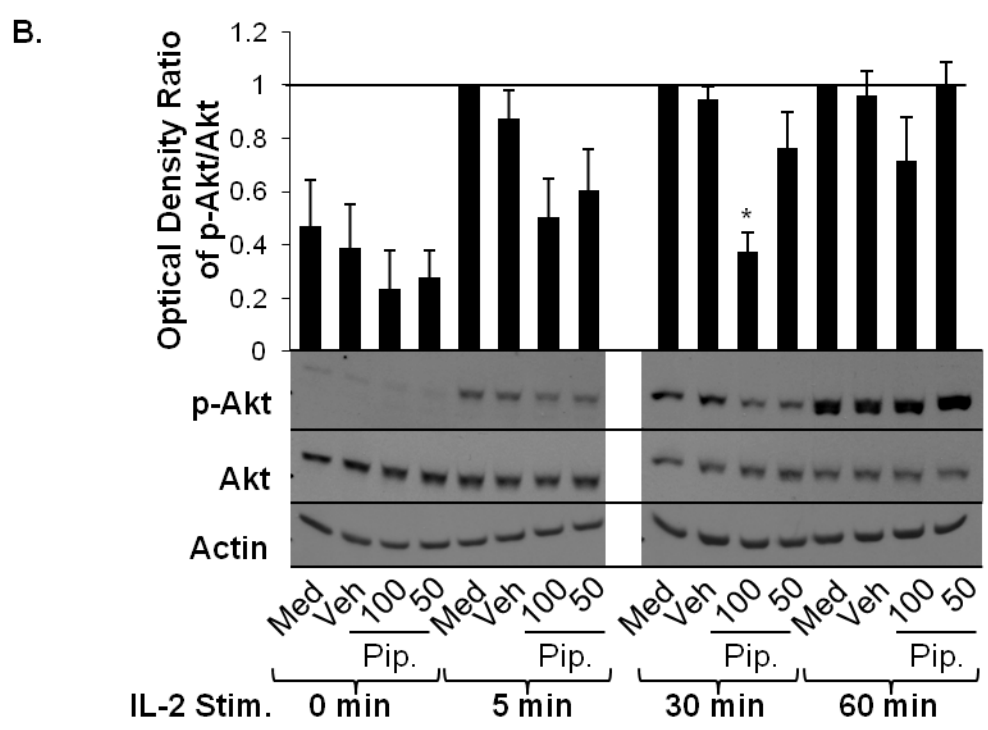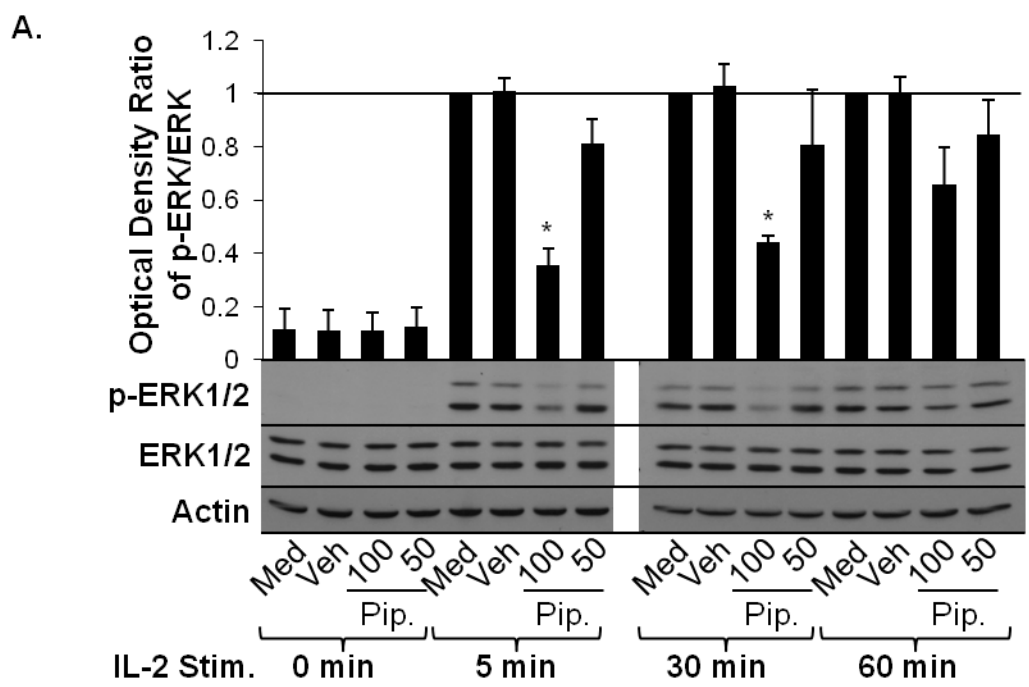
Figure 4.10

Figure 4.11 STAT5, ERK, and Akt are important signaling pathways involved in the proliferation of CTLL-2 cells. IL-2-starved CTLL-2 cells were treated with medium, vehicle (DMSO), or the indicated concentrations of (A) STAT5 inhibitor (CNH), (B) ERK inhibitor (PD 98059), or PI3K inhibitor (LY-294002) for 48 h in the presence of IL-2 (50 U/mL). Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p <$ 0.05 when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.
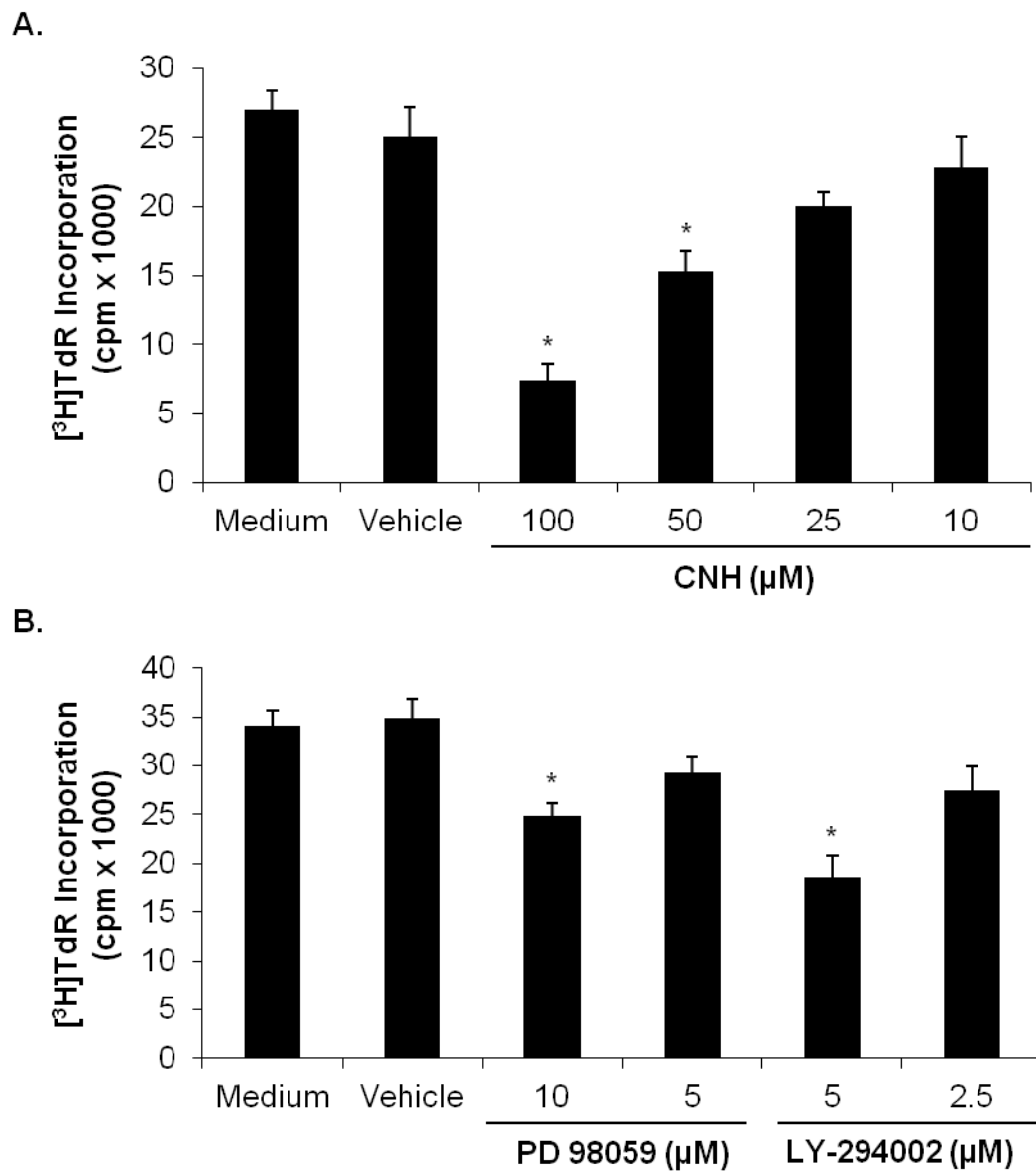
Figure 4.11

Figure 4.12 Piperine inhibits cell cycle protein expression. IL-2-starved CTLL-2 cells were pretreated for 30 min with medium (Med), DMSO vehicle (Veh), 100, or 50 μM piperine. Cells were then cultured in the presence or absence of IL-2 (50 U/mL) for 24 h prior to cell lysis. Total protein was collected for western blot analysis. Membranes were probed with Ab for cyclin D2 (A), CDK6 (B), or CDK4 (C), washed, and then probed with Ab for actin to confirm equal protein loading. Optical density ratio was calculated as the ratio of the expression of the protein of interest compared to actin expression. Ratios were normalized to medium-treated cells stimulated for 24 h. Data shown are the mean of at least 3 independent experiments ± SEM with one representative experiment; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test.

A.
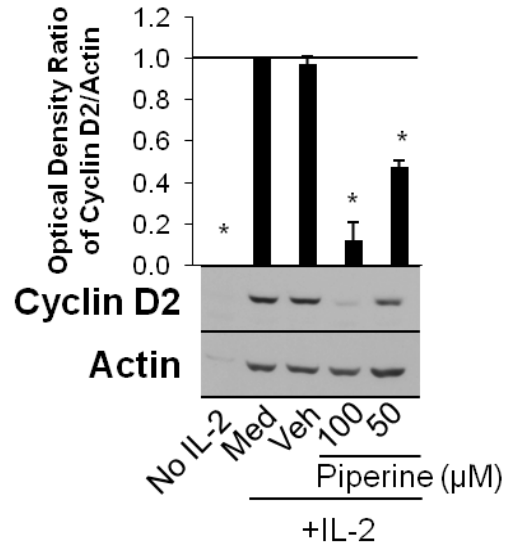
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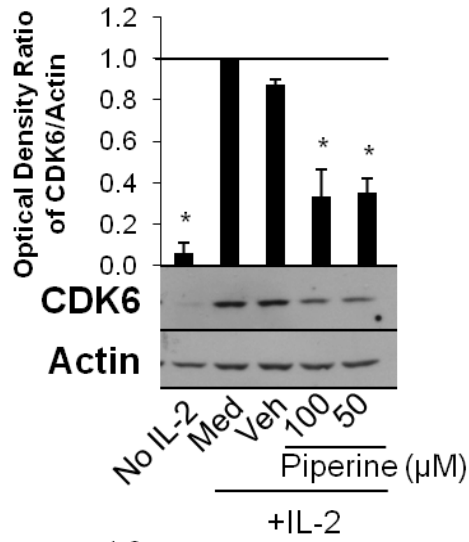
C.
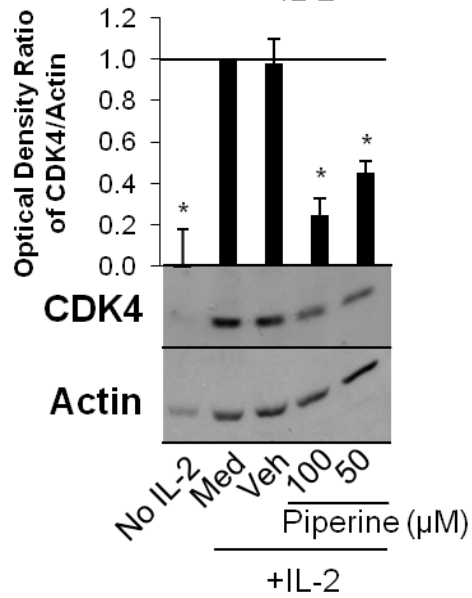
Figure 4.12

4.3 Discussion

IL-2 is an important cytokine for the development of T cell-mediated immunity [151], making IL-2 a viable target to achieve immunosuppression. In this regard, anti-IL-2Rα Ab therapy has shown great success in preventing transplant rejection [213,214]. Piperine was shown in my studies to inhibit the proliferation of CD3/CD28-stimulated primary T cells, as well as T cell responsiveness to exogenous IL-2 (Chapter 3, Manuscript 1), suggesting that piperine may inhibit IL-2R signaling. Therefore, the purpose of the current study was to examine the effect of piperine on IL-2R signaling in the IL-2-dependent CTLL-2 T cell line. Piperine potently inhibited the IL-2-dependent proliferation of CTLL-2 cells without causing cell death, suggesting that piperine allows sufficient signaling downstream of the IL-2R to maintain cell survival. Piperine did not alter CD25 expression or the phosphorylation and activation of JAK1 or JAK3, the two kinases that are directly associated with the IL-2R, indicating that piperine did not inhibit normal IL-2 binding or IL-2R assembly. Downstream of JAK1 and JAK3, piperine inhibited phosphorylation of the transcription factors STAT5 and STAT3, as well as the phosphorylation of Akt and ERK. Pharmacological inhibitors showed that STAT5, Akt, and ERK are all important pathways in IL-2-induced CTLL-2 proliferation. Consistently, cell cycle progression and the expression of the G_1-associated cell cycle proteins, CDK4, CDK6, and cyclin D2, were also inhibited by piperine treatment. Therefore, piperine was a potent inhibitor of IL-2R signaling and proliferation in the IL-2-dependent T cell line, CTLL-2.

In the presence of piperine, CTLL-2 cell survival was maintained, despite the inhibition of STAT5 and Akt phosphorylation, which are known to promote IL-2-induced survival through the expression of pro-survival proteins such as Bcl-xL and Bcl-2, respectively [464,480]. Continued survival of piperine-treated CTLL-2 cells may be due to the normal initial IL-2-induced signaling events, such as JAK1 or JAK3 phosphorylation, in the presence of piperine. In addition to STAT5, JAK1 and JAK3 phosphorylate and activate other target proteins such as Syk [159]. The Src kinase Lck is also activated by IL-2R heterotrimerization [165], although constitutive Lck activation alone is not sufficient or required to sustain CTLL-2 survival [165,166], suggesting that additional signaling pathway(s) contribute to the survival of piperine-treated CTLL-2

cells. Piceatannol, an inhibitor of Syk and Lck [481,482], inhibits the survival of an IL-2-dependent NK cell line [483], suggesting that Syk and Lck are involved in IL-2-induced survival signals. Thus it is possible that Syk and Lck activation is unaffected by piperine treatment and that these molecules can activate signaling pathways that provide a pro-survival signal to CTLL-2 cells in the absence of normal STAT5, STAT3, Akt and ERK activation. Alternatively, the piperine-induced inhibition of STAT5 and/or Akt phosphorylation may be sufficient to inhibit proliferation while allowing for adequate pro-survival signals to prevent apoptosis.

IL-2-induced activation of ERK and Akt occurs downstream of the signaling molecule Shc [151,169,171], which like STAT5, binds to the phosphorylated IL-2Rβ chain [169,170] and is phosphorylated by JAK1 [160]. Both early ERK and Akt phosphorylation were inhibited by piperine treatment, suggesting that piperine may inhibit Shc activation as well as STAT activation downstream of the IL-2R. The inhibitory effect of piperine on Akt phosphorylation has previously been demonstrated in activated T cells and endothelial cells by our laboratory (Chapter 3, Manuscript 1 and Chapter 5, Manuscript 3, respectively). Inhibition of ERK phosphorylation following piperine treatment has been demonstrated previously upon stimulation of various cell types, including HT-1080 fibrosarcoma cells [396], synoviocytes [348], and pancreatic cells [378], as well as in activated T cells (Chapter 3, Manuscript 1). Interestingly, piperine increases basal ERK phosphorylation in immortalized auditory cells [401], suggesting that piperine can have differential effects on ERK phosphorylation depending on which signaling pathways are induced. The piperine-induced inhibition of ERK and Akt phosphorylation amongst various studies and cell types suggests that these signaling pathways are common targets influenced by piperine, although the mechanism by which piperine affects ERK and Akt phosphorylation requires further investigation. Both ERK and Akt pathways are important for cell proliferation [484,485], including IL-2-induced proliferation as determined in this study (Figure 4.11). Piperine-induced inhibition of ERK and Akt phosphorylation may therefore be responsible at least in part for the inhibitory effect of piperine on IL-2-induced CTLL-2 proliferation.

Piperine inhibited the IL-2-induced expression of the G_1-associated cell cycle proteins cyclin D2, CDK6, and CDK4 in CTLL-2 cells, indicating a block in cell cycle

progression and proliferation. STAT5 is an important signaling molecule for IL-2-induced T cell proliferation [160,464,478] (Figure 4.11A) and is involved in the expression of cyclin D2 and CDK6. A STAT5 binding site has been identified in the *cyclin D2* promoter [479] and the expression of cyclin D2 and CDK6 is inhibited in splenocytes from STAT5$^{-/-}$ mice [478], suggesting that piperine-induced inhibition of STAT5 phosphorylation may contribute to the observed inhibition of cyclin D2 and CDK6 expression. Conversely, STAT5 does not appear to be involved the expression of CDK4, as CDK4 is expressed at normal levels in splenocytes from STAT5$^{-/-}$ mice [478]. Therefore, the inhibition of CDK4 expression is presumably independent of the effect that piperine has on STAT5 phosphorylation. Rather, it is due to some other mechanism, suggesting that the inhibition of STAT5 is not the only important pathway in the piperine-induced inhibition of IL-2-dependent proliferation. The importance of STAT5 inhibition to the anti-proliferative effect of piperine in IL-2-stimulated CTLL-2 cells requires further study of piperine-treated CTLL-2 cells transfected with a constitutively active STAT5 mutant that is constitutively phosphorylated and localized in the nucleus [486]. The mechanism by which piperine inhibits STAT5 phosphorylation also requires further investigation. Our finding that the piperine-induced inhibition of STAT5 phosphorylation was unaffected by treatment with the phosphatase inhibitor, Na$_3$VO$_4$, or the protein translation inhibitor, CHX, indicates that neither phosphatases or *de novo* protein translation are required for piperine-induced STAT5 inhibition. This eliminates the involvement of the phosphatase SHP-2 [186] and the IL-2-induced SOCS family of proteins [188-190] in the inhibitory activity of piperine on STAT5 phosphorylation. Although the IL-2-induced phosphorylation of JAK1 and JAK3 was not inhibited by piperine treatment, phosphorylation does not necessarily dictate kinase activity. A kinase assay is therefore needed to confirm the activity of JAK1 and JAK3 in the presence of piperine. It is possible that piperine is interfering with the phosphorylation of the IL-2Rβ chain, the interaction of STAT5 with the phosphorylated IL-2Rβ chain, or the interaction of JAK1 and/or JAK3 with STAT5, any one of which would result in inhibited STAT5 phosphorylation. Additionally, piperine demonstrated inhibitory activity on STAT1 phosphorylation in peritoneal macrophages activated with LPS, Poly (I:C), CpG-ODN,

and IFN-α and -β [372], suggesting that piperine has inhibitory activity on STAT activation in multiple signaling pathways.

In conclusion, our findings demonstrate that piperine prevents the proliferation of IL-2-dependent CTLL-2 cells by inhibiting critical IL-2R signaling events, including STAT5, STAT3, ERK1/2, and Akt phosphorylation. Piperine also inhibited the IL-2-induced expression of the G_1-associated cell cycle proteins cyclin D2, CDK4, and CDK6. Inhibiting IL-2R signaling has been beneficial in preventing transplant rejection and in the treatment of MS [213,462]. Piperine therefore warrants further investigation as a potential therapeutic agent for controlling transplant rejection, T cell-mediated autoimmune and chronic inflammatory conditions.

CHAPTER 5

PIPERINE, A DIETARY PHYTOCHEMICAL, INHIBITS ANGIOGENESIS

5.1 Introduction

Angiogenesis is normally a tightly regulated and complex process that involves endothelial cell proliferation, migration, alignment, and tubule formation in response to various growth factors [234]. Importantly, angiogenesis is essential for tumor progression since tumors cannot grow to a diameter greater than 1-2 mm before their oxygen and nutrient demands, as well as waste product elimination, can no longer be met by diffusion alone [299]. Lack of oxygen and nutrients in the tumor microenvironment causes tumor cells to express angiogenesis-promoting mediators such as VEGF. These proangiogenic factors initiate new blood vessel development from existing nearby endothelium. Because angiogenesis is the rate-limiting step in the growth of a solid tumor, the angiogenic process is considered to be an important target for cancer therapy [487]. Interestingly, anti-angiogenic therapy, when timed correctly, greatly increases the effectiveness of conventional chemotherapy and radiotherapy by normalizing the tumor vasculature [488]. The resulting increase in tumor oxygenation and decreased interstitial fluid pressure enhances the sensitivity of tumor cells to ionizing radiation and facilitates tumor penetration by chemotherapeutic drugs, respectively.

There is currently widespread interest in developing new and less toxic anticancer agents from natural sources, including spices [303]. Well known for its pungency and diverse physiological effects, black pepper obtained from the dried berries of the black pepper plant (*Piper nigrum*) has been used in traditional Indian and Chinese medicine to treat conditions ranging from gastrointestinal ailments to epilepsy [465]. The medicinal value of black pepper is attributed to the alkaloid piperine, which exerts anti-inflammatory [367,377], neuroprotective [326], and cardiovascular protective effects [330], and is used to increase the bioavailability of drugs and nutrients by increasing intestinal fluidity and the surface area of the intestinal microvilli [352], as well as by inhibiting drug-metabolizing enzymes and drug efflux pumps [357]. Recent studies show that piperine is cytotoxic for and prevents the *in vivo* growth of cancer cells, including

B16-F10 melanoma cells [393], DLA cells, and EAC cells [394]. Piperine also inhibits HT-1080 fibrosarcoma cell expression of MMP-9, thereby interfering with tumor cell migration and invasion [396]. However, the effect of piperine on angiogenesis has not yet been investigated.

In this study, we determined the effect of piperine on individual aspects of the angiogenic process, including *in vitro* proliferation, migration, and tubule formation by HUVECs, as well as the effect of piperine on collagen-induced angiogenic activity by rat aorta explants and breast cancer cell-induced angiogenesis in chick embryos. Piperine inhibited all aspects of the angiogenic process, as well as collagen-induced blood vessel outgrowth *ex vivo* and breast cancer cell-induced angiogenesis *in vivo*. The inhibitory effects of piperine on angiogenesis were not the result of piperine-mediated activation of the cation channel TRPV1 [317]; rather, piperine inhibited the PI3K/Akt signaling cascade that promotes angiogenesis [489]. Our findings indicate that piperine warrants further study as a potential anti-angiogenic agent for the prevention and/or treatment of cancer.

5.2 Results

5.2.1 Piperine Inhibits Endothelial Cell Proliferation without Causing Cell Death

We first determined the effect of piperine on the *in vitro* proliferation of HUVECs using the cell-permeable fluorescent dye, CMFDA, which can quantify cell proliferation as the dye's fluorescence is halved with each round of cell division. As shown in Figure 5.1A, HUVECs treated with 100 μM piperine for 72 h underwent fewer rounds of proliferation compared to cells treated with medium or vehicle alone. MCF values indicated that the average number of cell divisions was 6.3 ± 0.3 for both medium and vehicle treatments compared to 3.7 ± 0.7 for HUVECs treated with 100 μM piperine (Figure 5.1B; $p < 0.05$). To confirm the anti-proliferative effect of piperine treatment, a [^{3}H]TdR incorporation assay was used to measure the amount of DNA synthesis occurring in piperine-treated HUVECs. As shown in Figure 5.1C, exposure to 100 μM piperine for 24 h resulted in an 87% inhibition of DNA synthesis ($p < 0.05$). To determine whether piperine's anti-proliferative effect was due to cytotoxicity, the viability of piperine-treated HUVECs was assessed by LDH-release and Annexin-V

staining. As shown in Figure 1D, both assays indicated that piperine did not adversely affect HUVEC viability, indicating that the inhibitory effect of piperine on HUVEC proliferation was not due to cell death.

5.2.2 Piperine Inhibits Endothelial Cell Entry into the S phase of the Cell Cycle

The anti-proliferative effect of piperine on HUVECs was further examined by cell cycle analysis using the DNA-intercalating dye PI. As shown in Figure 5.2A, in comparison to vehicle-treated cells, the percentage of HUVECs in the G_0/G_1 phase of the cell cycle was significantly increased following piperine treatment ($p < 0.05$), indicating that piperine caused a block at the G_0/G_1 phase of the cell cycle and prevented cells from entering the S phase. Additionally, western blot analysis determined that piperine inhibited the expression of cyclin D3 (Figure 5.2B), which is a cell cycle protein that is normally upregulated during the G_1/S transition. Densitometric analysis showed that cyclin D3 protein expression was significantly reduced in piperine-treated HUVECs (Figure 5.2C; $p < 0.05$).

5.2.3 Piperine Attenuates Endothelial Cell Migration

We next used an *in vitro* cell migration assay to determine the effect of piperine on endothelial cell motility. Following wounding of the cell monolayer, Figure 3A shows that in the presence of 100 μM piperine, HUVEC migration was reduced by 30% and 38% at 10 h and 20 h, respectively (Figure 5.3B; $p < 0.05$), compared to vehicle-treated cells.

5.2.4 Piperine Inhibits *In Vitro* Tubule Formation

The effect of piperine on tubule formation was investigated by culturing HUVECs on growth factor-enriched gel matrix, which induces endothelial cell alignment, the development of offshoots between neighboring cells, and the formation of tubules. As shown in Figure 5.4A, piperine inhibited tubule formation by HUVECs in a dose-dependent manner. Only small clusters of cells and offshoots were visible in piperine-treated cultures while vehicle-treated cultures showed a complex mesh of tubules with the formation of numerous closed polygons. Semi-quantitative scoring of tubule complexity

revealed that 100 μM piperine significantly inhibited tubule formation (Figure 5.4B; $p < 0.05$).

5.2.5 Piperine Inhibits *Ex Vivo* Angiogenesis

We next used the *ex vivo* rat aorta angiogenesis model to confirm the anti-angiogenic activity of piperine. Figure 5.5A shows that collagen-induced angiogenesis and tubule formation by the excised and inverted aortas was inhibited in the presence of 100 μM piperine. Compared to the vehicle control, there was significant reduction in the migration of piperine-treated cells from the aorta on day 4 (55% decrease, $p < 0.05$; Figure 5.5B), as well as a near complete absence of tubule formation on day 7 (93% decrease, $p < 0.05$; Figure 5.5C).

5.2.6 TRPV1 is not Involved in the Anti-Angiogenic Activity of Piperine

Since piperine binds to and desensitizes the cation channel TRPV1 [317], we employed 3 different TRPV1 antagonists (SB-366791, capzasepine, and BCTC) to determine whether TRPV1 mediated the anti-angiogenic activity of piperine. Figure 5.6 shows that none of the TRPV1 antagonists prevented piperine from inhibiting HUVEC proliferation (Figs. 5.6A-C). In addition, HUVECs failed to express *TRPV1* mRNA, which was abundant in HMC-1 mastocytoma cells that were used as a positive control (Figure 5.6D).

5.2.7 Piperine Inhibits Akt Phosphorylation in Endothelial Cells

We next determined the effect of piperine on the phosphorylation of Akt, which is an important pro-angiogenic signaling molecule [489]. Western blot analysis showed that exposure of HUVECs to 100 μM piperine resulted in a 50% reduction in Akt phosphorylation (Figure 5.7A and B). LY-294002, a selective inhibitor of PI3K, which is upstream of Akt in growth factor receptor signaling, was used to confirm the importance of the PI3K/Akt pathway in HUVEC proliferation. Like piperine, LY-294002 inhibited HUVEC proliferation (Figure 5.7C) and also inhibited tubule development in the *ex vivo* rat aorta angiogenesis model (Figure 5.7D).

5.2.8 Piperine Inhibits Breast Cancer Cell-Induced Angiogenesis *In Vivo*

Piperine-mediated inhibition of various aspects of the angiogenic process *in vitro* and *ex vivo* led us to employ the chick embryo CAM assay to confirm the anti-angiogenic activity of piperine *in vivo*. MDA-MB-231 human breast cancer cells, which are a source of pro-angiogenic VEGF [490], were used in the embryo onplants as the angiogenic stimulus. As shown in Figure 5.8, treatment with 50 μM piperine caused a greater than 50% reduction in breast cancer-induced angiogenesis in the CAM compared to the vehicle control ($p < 0.05$). The concentration of piperine used in this experiment did not affect the proliferation or viability of MDA-MB-231 cells (data not shown).

Figure 5.1 Piperine is a non-cytotoxic inhibitor of endothelial cell proliferation. (A) HUVECs were stained with CMFDA prior to treatment with medium, vehicle (DMSO), or 100 μM piperine for 72 h. Fluorescence was quantified by flow cytometry to measure cell proliferation. Data shown are from a representative experiment (n = 3). (B) The number of cell divisions was calculated from the mean channel fluorescence of CMFDA-labeled HUVECs. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as deteremined by ANOVA with Tukey-Kramer multiple comparisons post-test. (C) HUVECs were treated with medium, vehicle (DMSO), or the indicated concentrations of piperine for 24 h. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with Tukey-Kramer multiple comparisons post-test. (D) HUVECs were incubated with medium, vehicle (DMSO), or the indicated concentrations of piperine for 24 h prior to Annexin-V staining and flow cytometry to determine phosphatidylserine translocation or detection of LDH-release by colorometric assay. Percent viability was calculated relative to a positive control. Data shown are the mean of 3 independent experiments ± SEM; $p > 0.05$ when compared to the vehicle control from the respective assay as determined by ANOVA with Tukey-Kramer multiple comparisons post-test.

A.

Medium/Vehicle

Piperine

Control

Relative Cell Number

CMFDA Fluorescence Intensity

B.

Number of Cell Divisions

Medium   Vehicle   Piperine

*

C.

[³H]TdR Incorporation (cpm x 1000)

Medium   Vehicle   100   50   25   10

Piperine (µM)

*   *

D.

Percent Viability

Medium   Vehicle   100   50

Piperine (µM)

■ Annexin-V Staining   ■ LDH Release Assay

Figure 5.1

Figure 5.2 Piperine inhibits endothelial cell entry into the S phase of the cell cycle.
(A) Medium, vehicle (DMSO), or 100 μM piperine were added to HUVECs at 0 and 48 h. Cells were harvested after 72 h of culture, fixed with 70% ethanol for 24 h, then stained with PI for 30 min prior to analysis by flow cytometry. Data shown are the mean of 4 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with Tukey-Kramer multiple comparisons post-test. (B) HUVECs cultured in the presence of medium, vehicle (DMSO) or 100 μM piperine for 24 h were lysed and total protein was collected for western blot analysis. Membranes were probed with anti-cyclin D3 Ab, then washed and probed with anti-actin Ab to confirm equal protein loading. Results from a representative experiment are shown (n = 3). Relative expression of cyclin D3 was calculated as the ratio of cyclin D3 to actin expression, as determined by densitometric analysis. Ratios were normalized to the medium control. Results shown are the mean ± SEM of 3 independent experiments; * denotes $p < 0.05$ when compared to the vehicle control as determined by Student's t-test.

A.

B.
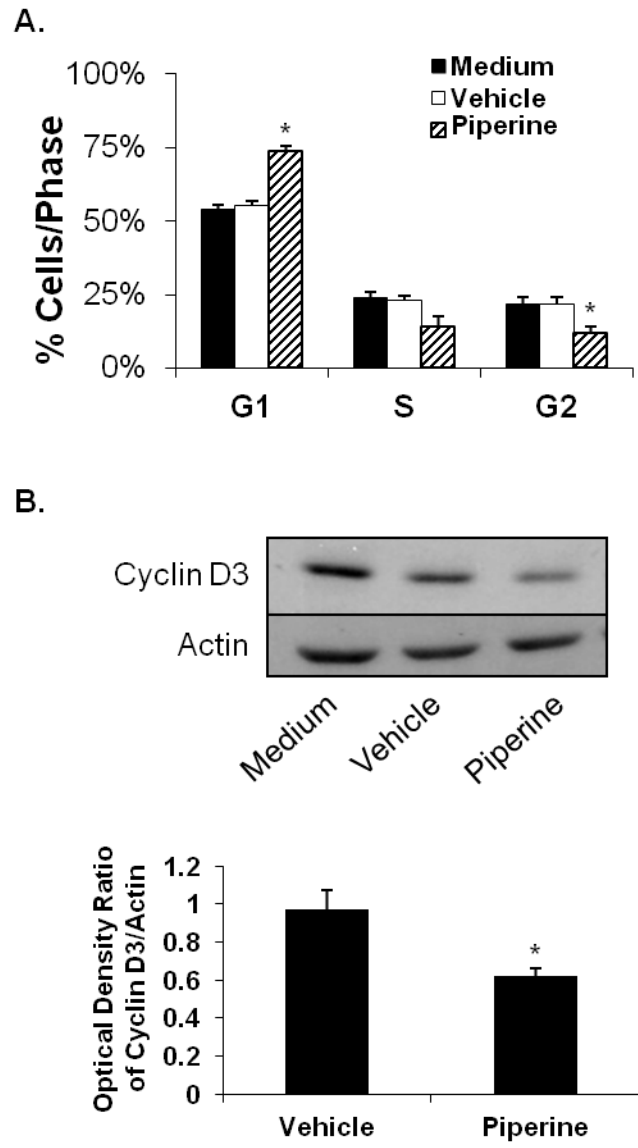
Figure 5.2

Figure 5.3 Piperine attenuates endothelial cell migration. (A) A confluent monolayer of HUVECs was wounded with a p10 pipette tip, then HUVEC cultures were washed with fresh medium, treated with vehicle (DMSO) or 100 μM piperine and photographed at 0 h under an inverted microscope (20x magnification). The wounded area was monitored for repopulation of the void and photographed at 10 h and 20 h post-wounding. Vertical lines indicate the approximate boundary of the original wound. Data shown are from a representative experiment. (B) The percentage of the wounded area that was repopulated was calculated. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as determined by Student's *t*-test.

A.

Vehicle | Piperine

0 h

10 h

20 h

B.
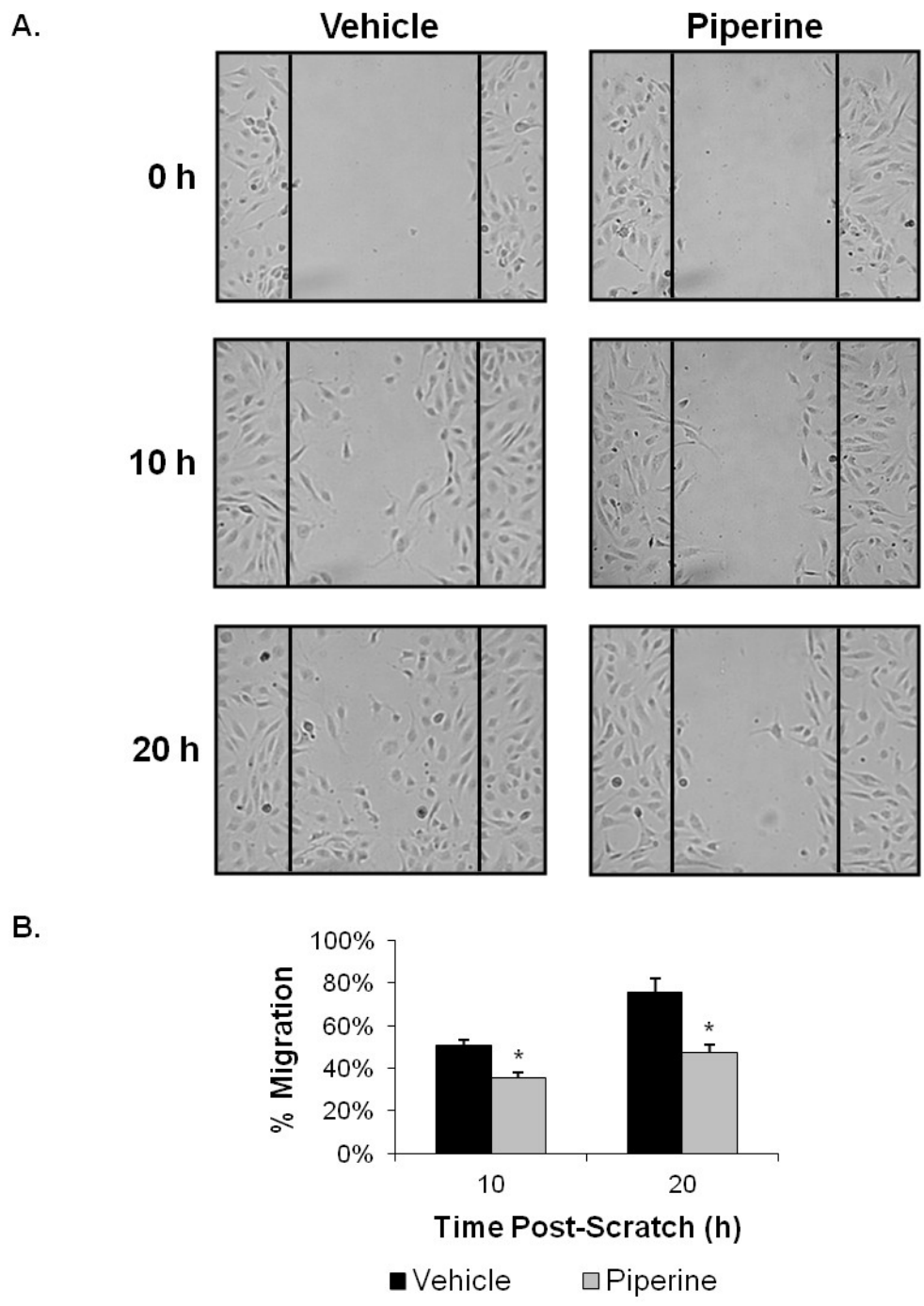
Figure 5.3

Figure 5.4 Piperine inhibits tubule formation by endothelial cells. (A) HUVECs were treated with the vehicle (DMSO) or the indicated concentrations of piperine for 15 min, plated on top of a solidified extra-cellular matrix, incubated for 18-24 h, and photographed under an inverted microscope (4x magnification). Photographs are from a representative experiment. (B) Tubule formation was graded by numerical score. Data shown are the mean ± SEM of 3 independent experiments; * denotes $p < 0.05$ when compared to the vehicle control as determined by the Kruskal-Wallis nonparametric ANOVA test with Dunn's multiple comparisons post-test.

A.

0.1% DMSO

100 µM Piperine

50 µM Piperine

25 µM Piperine

B.
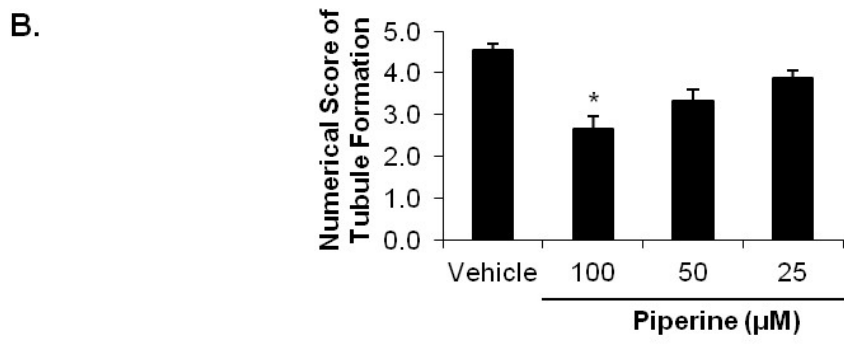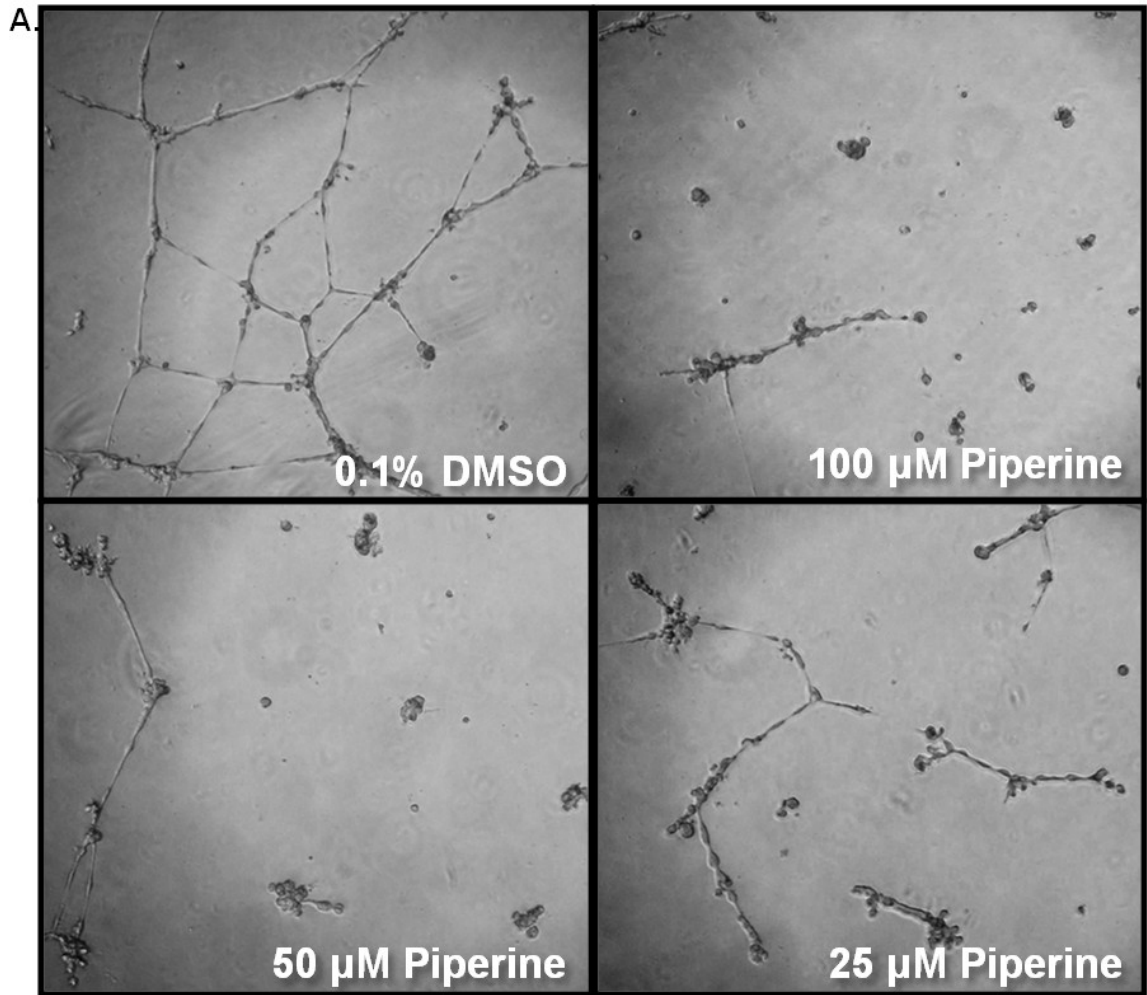
Figure 5.4

Figure 5.5 Piperine inhibits *ex vivo* angiogenesis. Rat aortas were excised, inverted, cut into segments, and incubated in a supplemented collagen matrix in the presence of the vehicle (DMSO) or 100 µM piperine. (A) Blood vessel development was monitored and photographed under an inverted microscope (4x magnification) after 7 days. Images of representative aortas (n = 4) are shown. (B) Cell migration was measured and (C) tubules were counted on days 4 and 7, respectively. Data shown are the mean of 4 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as determined by Student's *t*-test.

A.
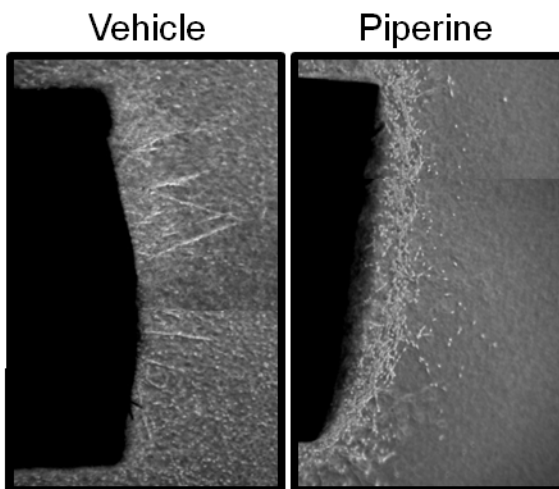
Vehicle   Piperine

B.
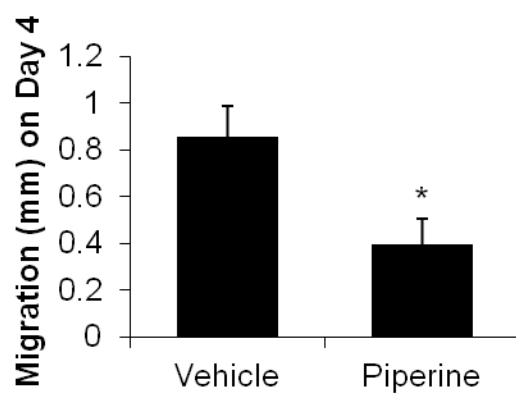
C.
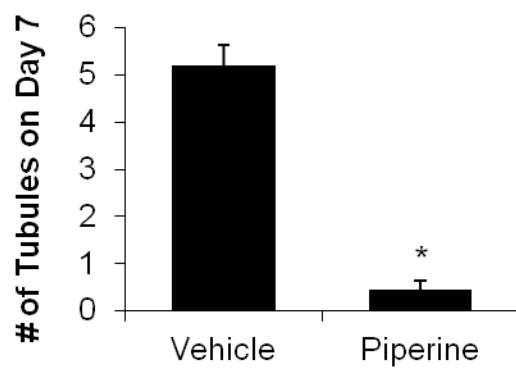
Figure 5.5

Figure 5.6 TRPV1 does not mediate the anti-angiogenic effect of piperine. HUVECs were exposed to 1 µM of the TRPV1 antagonists (A) SB-366791, (B) capsazepine (capz), or (C) BCTC for 30 min prior to the addition of medium, vehicle (DMSO), or the indicated concentrations of piperine. HUVECs were cultured for 24 h and then pulsed with [³H]TdR for the last 6 h of incubation, harvested, and [³H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to respective vehicle control and ns denotes "not significant" when comparing the antagonist vehicle control to the antagonist for each treatment as determined by ANOVA with Tukey-Kramer multiple comparisons post-test. (D) RNA was extracted from HUVECs and HMC-1 cells, reverse transcribed into cDNA, and then amplified for *TRPV1* or *GAPDH*, as a loading control.
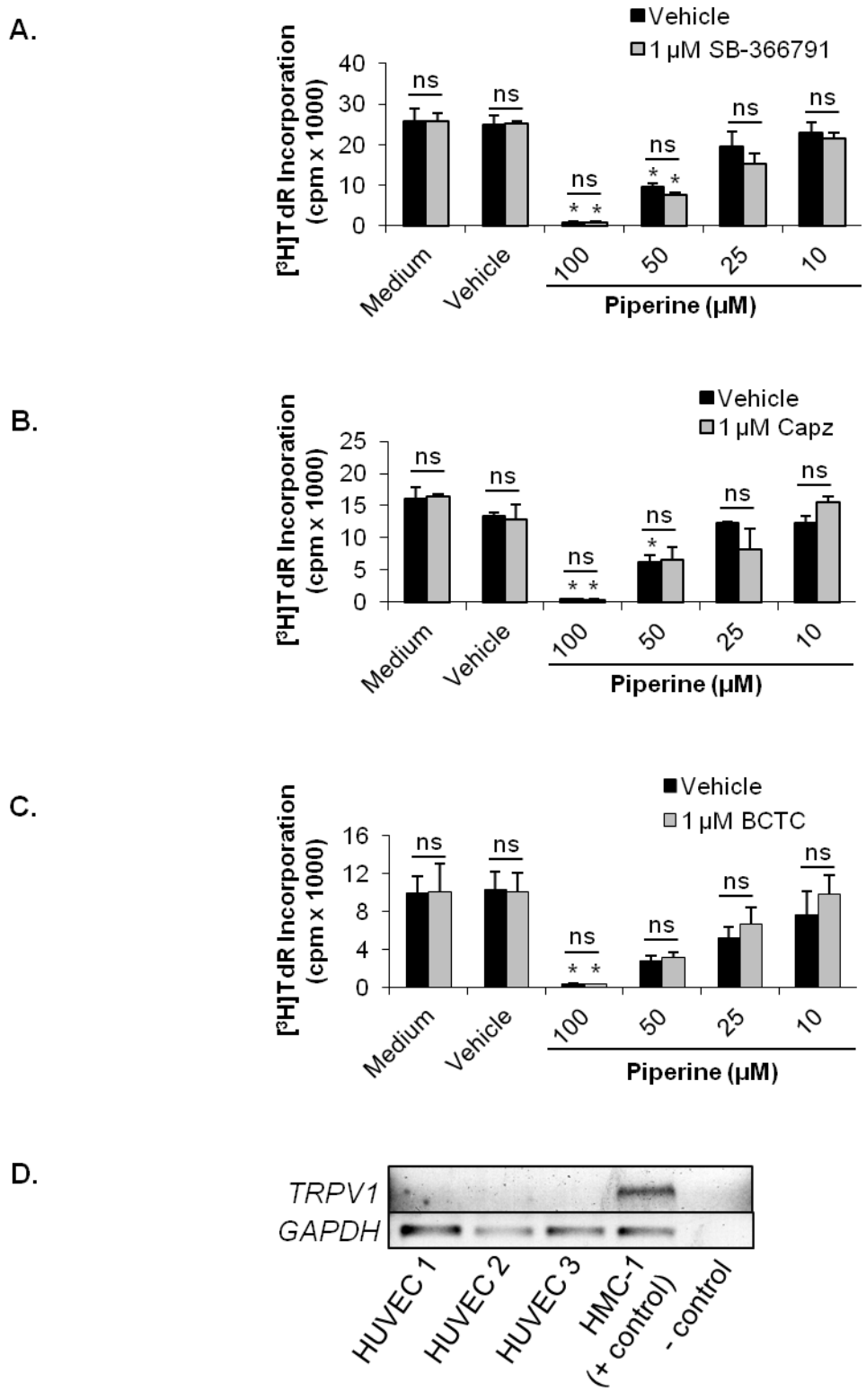
Figure 5.6

Figure 5.7 Piperine inhibits Akt phosphorylation. (A) HUVECs cultured for 24 h in the presence of medium, vehicle (DMSO), or the indicated concentrations of piperine were lysed and total protein was collected for western blot analysis. Membranes were probed with Ab against phosphorylated (p)-Akt or total Akt, then washed, and probed with anti-actin Ab to confirm equal protein loading. Results from a representative experiment (n = 4) are shown. (B) Relative expression of p-Akt was calculated as the ratio of p-Akt to total Akt expression after each band was normalized to actin, as determined by densitometric analysis. Expression was then normalized to the medium control. Results shown are the mean ± SEM of 3 independent experiments; * denotes $p <$ 0.05 when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (C) HUVECs were cultured for 24 h in the presence of medium, vehicle (DMSO), or the indicated concentrations of LY-294002. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation, harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of 3 independent experiments ± SEM; * denotes $p < 0.05$ when compared to the vehicle control as determined by ANOVA with the Tukey-Kramer multiple comparisons post-test. (D) Rat aortas were ex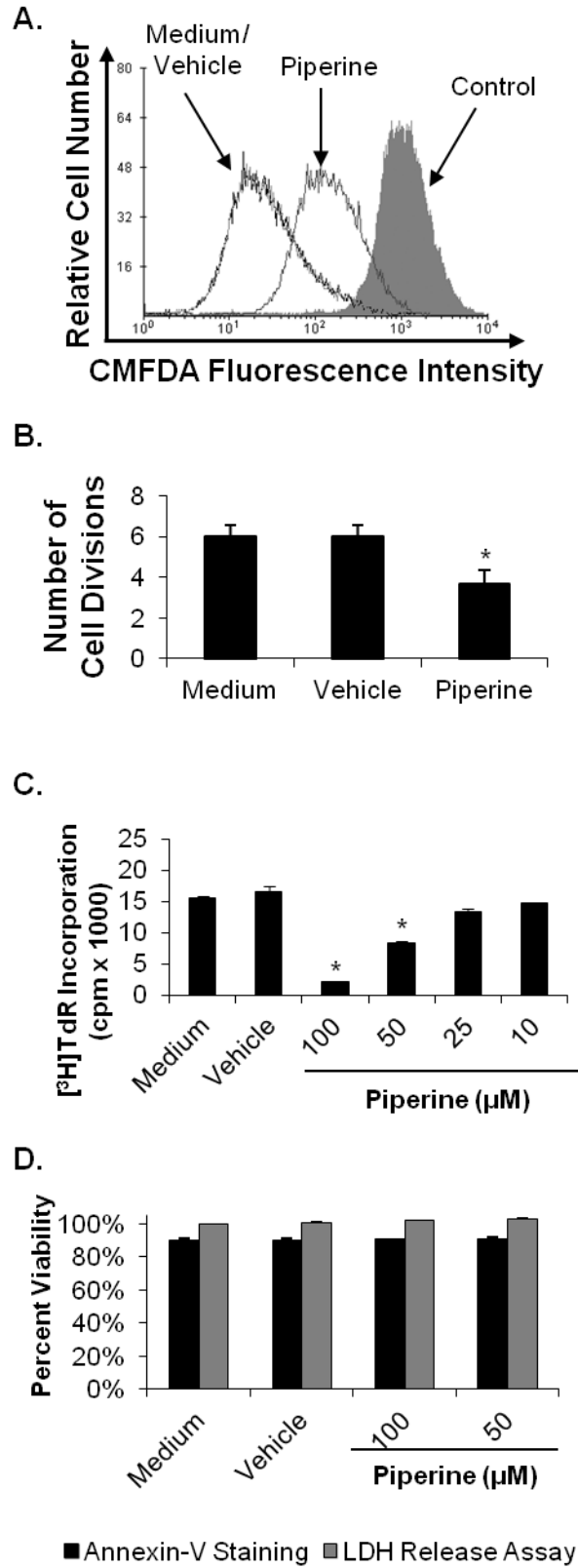cised, inverted, cut into segments, and incubated in a supplemented collagen matrix with vehicle (DMSO) or 10 μM LY-294002, a PI3K inhibitor. Blood vessel development was monitored and photographed (4x magnification) after 7 days. Images of representative aortas (n = 2) are shown.

A.

p-Akt

Akt

Actin

Medium　Vehicle　100　50

Piperine (μM)

B.

D.

Vehicle　　　　LY-294002
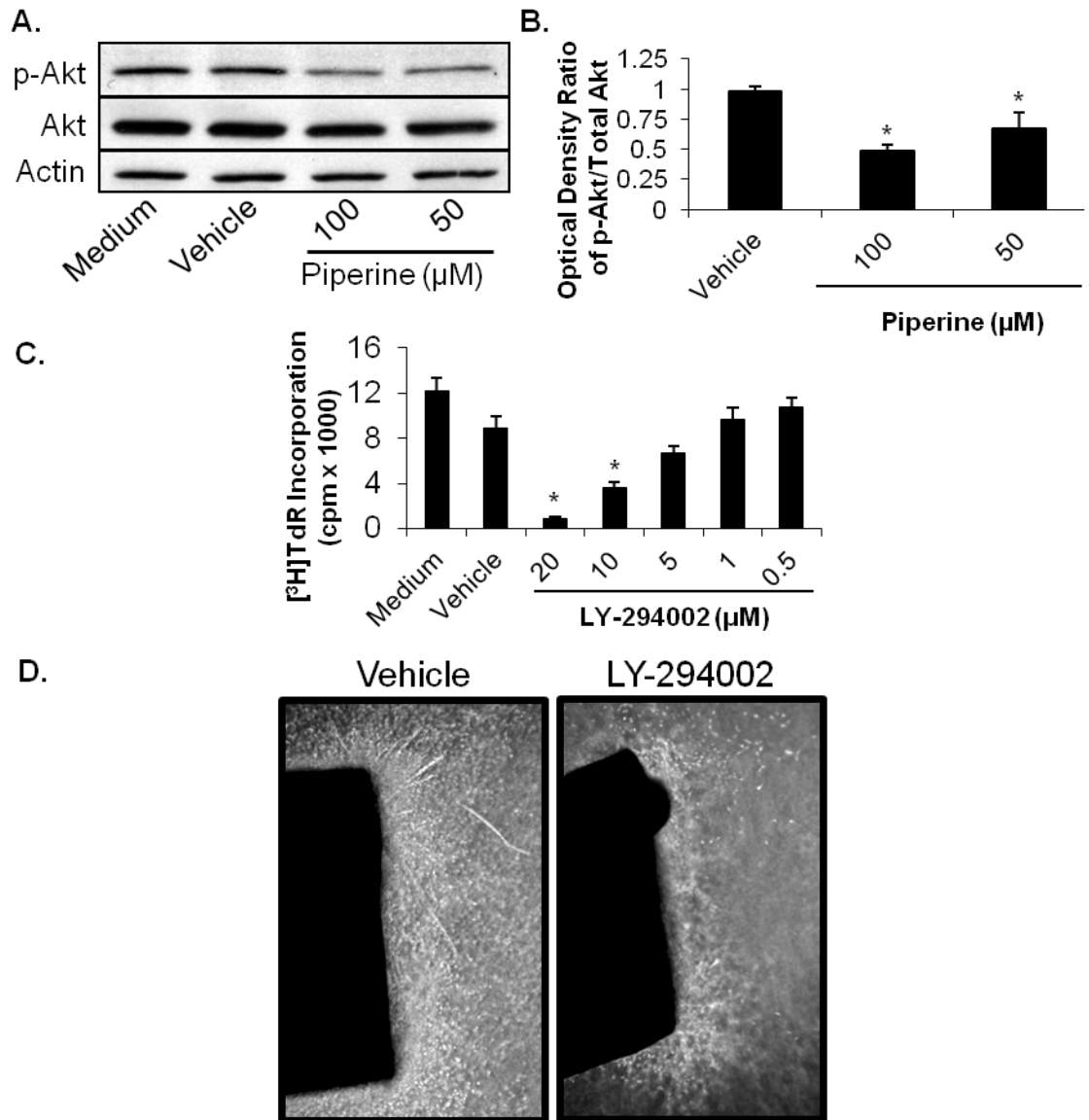
Figure 5.7

Figure 5.8 Piperine inhibits breast cancer cell-induced angiogenesis *in vivo*. Mesh onplants containing MDA-MB-231 breast cancer cells and either vehicle (DMSO) or 50 μM piperine were embedded in collagen, placed on 10 day-old chick embryos, and incubated for 3 days. Onplants were then photographed and the percent angiogenesis was calculated; * denotes $p < 0.05$ when compared to the vehicle control as determined by Student's *t*-test.
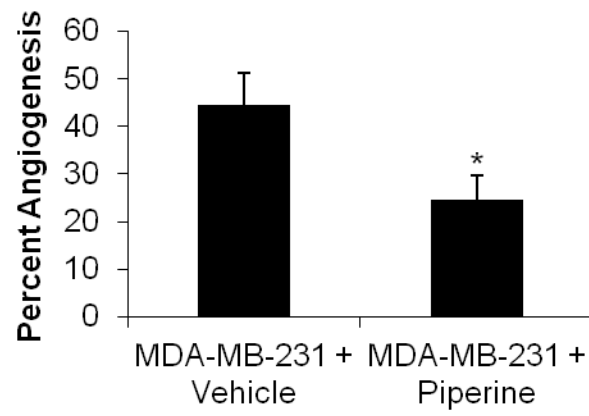
Figure 5.8

5.3 Discussion

Angiogenesis is an important component of tumor progression [299] and, as such, is considered to be a pivotal target for cancer treatment [487]. The dietary phytochemical piperine has previously been reported to kill several different types of cancer cells [393,394,396]; however, to our knowledge, we are the first to show anti-angiogenic activity by piperine. Importantly, LDH-release and Annexin-V staining established that piperine was not simply cytotoxic for HUVECs but potently inhibited *in vitro* proliferation and migration of endothelial cells, as well as tubule formation *in vitro* and *ex vivo*, and breast cancer cell-induced angiogenesis *in vivo*. Surprisingly, the anti-angiogenic activity of piperine was not mediated through TRPV1, which functions as a receptor for piperine [317]. Since different TRPV1 antagonists exhibit different potencies for inhibiting piperine-induced oral aversiveness in rats [491], we used three different TRPV1 antagonists to rule out differences in antagonist potency and receptor affinity as possible confounding factors. All three TRPV1 antagonists failed to interfere with the inhibitory effect of piperine on HUVEC proliferation. Moreover, *TRPV1* mRNA was not detected in HUVECs, although TRPV1 expression has been reported on cerebromicrovascular [492] and pulmonary arterial endothelial cells [493], as well as on immortalized HUVEC-12 cells [494]. It is possible that TRPV1 may be differentially expressed on various types of endothelial cells, as is the case for other TRP channels such as TRPP2, which is expressed on human renal artery endothelial cells but not on HUVECs [495]. In addition, we cannot rule out the possibility that TRPV1 may be differentially expressed depending on the culture conditions. Nevertheless, the absence of *TRPV1* mRNA in our HUVECs combined with the failure of TRPV1 antagonists to relieve piperine-mediated inhibition of HUVEC proliferation leads us to conclude that piperine does not mediate its anti-angiogenic activities through TRPV1.

The molecular basis for the myriad of physiological effects of piperine is poorly understood. Piperine has been reported to inhibit the translocation of nuclear transcription factors NF-κB, c-fos, activating transcription factor 2, and cyclic adenosine monophosphate response element-binding protein in B16-F10 mouse melanoma cells [373], as well as Wnt signaling in MCF7 breast cancer cells [391], and STAT1 phosphorylation in IFN-α-, IFN-β-, or LPS-treated macrophages [372]. In endothelial

cells, piperine inhibits TNF-α-induced IκBα phosphorylation but not baseline phosphorylation of IκBα [367]. Our findings indicate that piperine also interferes with Akt activation in endothelial cells in response to angiogenesis-promoting growth factors contained in EGM®-2 medium, although further investigation is required to determine whether this effect is direct or occurs upstream of Akt phosphorylation. Since Akt is an important signaling molecule during angiogenesis [489], it is reasonable to conclude that inhibition of Akt phosphorylation by piperine is likely responsible for the inhibitory effect of piperine on angiogenesis. This conclusion is in line with evidence that Akt activation is required for cell cycle progression [496], as well as endothelial cell migration and tubule formation [497]. Moreover, we confirmed the importance of Akt to HUVEC proliferation and tubule formation by rat endothelial cells by demonstrating inhibition of these angiogenesis-related processes in the presence of LY-294002, which is a selective inhibitor of PI3K that is known to function upstream of Akt in endothelial cells [498]. Inhibition of Akt activation could also be responsible for the cell cycle block and decrease in cyclin D3 expression observed in piperine-treated HUVECs since Akt signaling prevents G_1 arrest by inhibiting the nuclear import of p27^{Kip1} [496], a cell cycle inhibitor protein that inhibits the activity of cyclin-cyclin-dependent kinase complexes [499]. Additionally, Akt signaling inhibits the degradation of D-type cyclins [496,500] and thereby regulates cyclin D3 expression. While we cannot rule out piperine-mediated effects on other signaling pathways within endothelial cells, taken together, our data suggest that inhibition of Akt activation is an important component of the anti-angiogenic action of piperine.

Interestingly, despite Akt activation being important for endothelial cell survival [489], HUVECs did not show decreased viability following piperine treatment, which suggests that residual Akt function in piperine-treated cells was sufficient to maintain cell survival even though other cellular functions involved in angiogenesis were inhibited. Alternatively, other pro-survival signaling pathways in endothelial cells may be refractory to piperine, therefore allowing sufficient pro-survival signaling to prevent the death of piperine-treated HUVECs.

In addition to its anti-angiogenic properties, piperine shows *in vitro* cytotoxic activity against a range of cancer cell types, including B16-F10 melanoma cells [393],

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DLA, and EAC [394]. Nevertheless, inhibition of angiogenesis is likely to be an important component of piperine-mediated reduction of tumor progression *in vivo* [393]. Piperine may also be useful for cancer chemoprevention since anti-angiogenic agents have the capacity to inhibit the development of tumor vasculature, thereby limiting the growth of hyperplastic foci and possibly preventing the development of clinically significant disease [501]. Chronic inflammation of tissues, which has been linked to carcinogenesis, is characterized by a sustained infiltrate of inflammatory cells that secrete both inflammation- and angiogenesis-promoting factors [502]. Interestingly, piperine inhibits endothelial cell expression of adhesion molecules and the subsequent adhesion of neutrophils to activated endothelial cells [366,367], suggesting that piperine might reduce the migration of inflammatory cells into inflamed tissues. In addition, piperine blocks macrophage activation and production of the pro-inflammatory factors TNF-α and nitric oxide, both *in vitro* and *in vivo* [371]. Piperine also exhibits chemopreventive anti-oxidant activity *in vivo* in carcinogen-induced intestinal damage [385] and chemically-induced lung carcinogenesis models [386], as well as reducing lipid peroxidation and anti-oxidant loss in a high fat diet-induced oxidative stress model [332]. The combination of anti-angiogenic, anti-inflammatory and anti-oxidant activities argue that piperine may function as an effective chemopreventive agent.

In conclusion, our data show that piperine inhibits multiple aspects of the angiogenic process *in vitro* and *ex vivo*, as well as suppressing breast cancer cell-induced angiogenesis *in vivo*. These effects are independent of TRPV1 but likely involve piperine-mediated inhibition of Akt phosphorylation. Piperine is therefore identified as a novel inhibitor of angiogenesis that warrants further study as a nutraceutical with possible application in the prevention and/or treatment of cancer, as well as other angiogenesis-dependent diseases.

CHAPTER 6

DISCUSSION

6.1 Summary of the Major Findings and Comparison to Current Literature

6.1.1 Piperine has Inhibitory Effects on T Cells

This study examined the effects of the dietary phytochemical piperine on T cells and determined that piperine inhibited the activation and function of primary murine T cells, as demonstrated by piperine-mediated inhibition of proliferation, activation marker expression, cytokine production, CTL differentiation, and intracellular signaling events downstream of CD3/CD28. Additionally, piperine inhibited the proliferation of the IL-2-dependent T cell line, CTLL-2, as well as intracellular signaling events downstream of the IL-2R. Prior to the current investigation, a study of piperine by Pathak and Khandlewal [437] on cadmium-induced toxicity in splenocytes indicated that piperine has no effect on ConA-induced T cell proliferation or production of IL-2 and IFN-γ. Interpretation of Pathak and Khandlewal's data, however, is compromised by the high background proliferation of the splenocytes in the absence of stimulation and the minimal cytokine production that was observed, which likely indicated a problem with the experimental design [437]. In contrast to the study by Pathak and Khandlewal [437], Dogra *et al.* [411] found that *in vivo* administration of 4.5 mg/kg of piperine inhibited PHA-induced T cell proliferation *ex vivo*. Furthermore, Kim and Lee [377] showed that piperine inhibits Th2 cytokine production and the accumulation of CD4$^+$, CD8$^+$, and CD3$^+$CD69$^+$ T cells obtained by bronchoalveolar lavage of asthmatic mice. The current study is the first in-depth *in vitro* study evaluating the direct effects of piperine on T cells and the only study to examine the effect of piperine on IL-2R signaling.

6.1.2 Piperine has Inhibitory Effects on Endothelial Cells and Angiogenesis

Prior to the current investigation, two studies have demonstrated that piperine inhibits activation marker expression on TNF-α-stimulated endothelial cells [366,367]; however, little is known concerning the effects of piperine on endothelial cell function. Therefore, this study examined the direct effects of piperine on endothelial cells and determined that piperine inhibits endothelial cell proliferation, migration, and tubule

formation of endothelial cells *in vitro* as well as inhibiting angiogenesis *ex vivo* and *in vivo*. The various processes of endothelial cell function, such as proliferation, migration, and tubule formation are promoted and regulated by multiple factors such as VEGF and FGF-2 [503]. Signaling downstream of the VEGFR and FGFR is critical for the induction of these endothelial processes, such as ERK and Akt activation [504,505]. Pharmacological inhibition of the ERK and Akt pathways inhibits endothelial proliferation, migration, and tubule formation [262,263,504,506]. Therefore, the inhibition of Akt phosphorylation in piperine-treated endothelial cells may be involved in the anti-angiogenic activity of piperine. Additional signaling pathways may be involved in the inhibition of angiogenesis by piperine, but this requires further investigation as will be discussed in a later section.

6.1.3 Piperine-Induced Inhibition of T Cells and Endothelial Cells does not Involve Cell Death

One major finding of the current study is that piperine did not induce T cell or endothelial cell death at doses that were capable of inhibiting a variety of cellular processes and signaling pathways. This finding is consistent with previous reports that piperine protects against cadmium-induced splenocyte cell death [437], cisplatin-induced apoptosis of auditory cells [401], and glutamate-induced apoptosis of hippocampal neurons [440]. However, as Akt is considered a major promoter of prosurvival pathways, as well as proliferation [40,268,496], and the current study demonstrated an inhibition of Akt phosphorylation in all three cell types examined, the lack of apoptosis of piperine-treated cells was surprising.

Interestingly, while piperine consistently inhibited Akt phosphorylation in all three cell types examined, it never completely ablated Akt phosphorylation. This finding indicates that a level of Akt phosphorylation persisted in the presence of piperine and provides a potential explanation for the continued survival of cells in spite of piperine-induced Akt inhibition. Additionally, results from our study suggest that proliferation and survival require different thresholds of signaling pathway activation, in that a minimal level of Akt signaling maintains survival but is not sufficient to support proliferation. This was true not only for piperine, but also for the PI3K inhibitor, LY-294002, which

significantly inhibited endothelial cell proliferation without affecting cell viability at a dose of 10 μM, although cell death did occur at higher doses (data not shown). Similarly, 10 μM LY-294002 inhibits IL-2-dependent T cell proliferation but does not affect cell viability [69]. These findings suggest that the differential thresholds of signaling pathway activation between proliferation and viability are not unique to piperine, but is also seen at lower doses of other inhibitors of the PI3K-Akt axis.

Interestingly, piperine causes apoptosis in various cancer cell lines, both *in vitro* and *in vivo* [393,394]. The survival disparity between piperine-treated healthy cells and cancerous cells may be explained through oncogene addiction, in which cancer cells acquire dependence on specific signaling pathways through mutations [507]. In contrast, normal cells have unmutated signaling pathways and, in comparison, are not as dependent on one specific pathway for survival as cancer cells.

6.1.4 Mechanism of the Inhibitory Activity of Piperine

6.1.4.1 ERK

Consistent with the effect of piperine in other cell types [348,378,396], this study demonstrated that piperine inhibited ERK activation in primary T cells and CTLL-2 cells. ERK is an important signaling molecule for cell cycle progression [484]. Additionally, T cell proliferation is inhibited by pharmacological inhibitors of MEK, the upstream kinase of ERK, which results in a decrease in ERK phosphorylation [456]. Therefore, the piperine-induced inhibition of ERK phosphorylation may be responsible, at least in part, for the anti-proliferative effect of piperine on T cells and CTLL-2 cells. Previously, piperine was shown to inhibit ERK phosphorylation in HT-1080 fibrosarcoma cells [396], fibroblast-like synoviocytes [348], and pancreatic cells [378], induced by PMA, IL-1β, and cerulein, respectively. Downstream of ERK activation, piperine inhibits PMA-induced AP-1 transcriptional activity [396], as well as TNF-α-induced c-fos nuclear localization [373]. Conversely, piperine was found to increase basal levels of ERK phosphorylation in immortalized auditory cells [401]. These data suggest that piperine may have differential effects on the basal level of ERK phosphorylation, compared to the levels of ERK phosphorylation induced by a stimulus such as CD3/CD28 activation, PMA, IL-1β, or cerulein.

6.1.4.2 IκBα

The inhibition of IκBα phosphorylation that we observed in piperine-treated T cells is consistent with other reports that piperine inhibits IκBα phosphorylation and subsequent degradation in TNF-α-activated endothelial cells [367] and PMA-activated HT-1080 cells [396]. The IκB/NF-κB pathway is important for T cell proliferation, as demonstrated by the large deficit in the proliferation of T cells expressing a dominant negative mutant of IκB, which constitutively inhibits NF-κB [457]. Therefore, the inhibitory effect of piperine on T cell proliferation may be due in part to the inhibition of IκBα phosphorylation. Consistent with an inhibitory effect on IκBα, piperine inhibits the nuclear localization of NF-κB, which is immediately downstream of IκBα degradation, in both B16-F10 melanoma cells [373] and HT-1080 cells [396]. In contrast, piperine does not inhibit cerulein-induced IκBα degradation in pancreatic cells [378] or IL-1β-induced NF-κB transcriptional activity in synoviocytes [348], suggesting that IκBα phosphorylation was not inhibited. The contrasting results of these studies compared to ours may be the consequence of the specific cell types and activation stimuli that were used, suggesting that the effect of piperine on IκBα phosphorylation may be cell type- and stimuli-specific, although this requires further investigation.

6.1.4.3 Akt

A number of studies have demonstrated the molecular effects of piperine in multiple cell types [348,367,373,378,396]. To my knowledge, my study is the first to show piperine-induced inhibition of Akt phosphorylation. Akt is an important signaling molecule for cell proliferation [40,496] since it phosphorylates and inhibits FoxO transcription factors and GSK-3β, both of which promote p27[Kip1] expression and cell cycle arrest [44,455]. Inhibition of FoxO and GSK-3β results in decreased p27[Kip1] expression and cell cycle progression [44,455]. Therefore, inhibition of Akt could result in the piperine-induced anti-proliferative effect that I observed in T cells, CTLL-2 cells, and HUVECs. The conserved inhibition of Akt in piperine-treated primary T cells, the CTLL-2 T cell line, and primary HUVECs indicates that the inhibition of Akt phosphorylation by piperine is not limited to one cell type. In T cells and endothelial cells, phosphorylation of Akt at Ser 473 is regulated by PKCα [39,508]. Hwang *et al.*

[396] showed that PMA-induced phosphorylation and membrane localization of PKCα are inhibited by piperine in HT-1080 cells. Taken together, these data suggest that inhibition of PKCα and its phosphorylation of Akt at Ser 473 may account for the inhibitory effect of piperine.

6.1.4.4 STAT

My study also identified piperine as an inhibitor of STAT5 and STAT3 phosphorylation downstream of IL-2R signaling. Both STAT5 and STAT3 are important in IL-2-induced T cell proliferation (Figure 4.11A) [160,464,471], and both STAT5 and STAT3 induce the expression of G_1-associated cell cycle proteins involved in cell cycle progression [470,479,509]. The anti-proliferative effect of piperine on CTLL-2 cells may therefore be due to piperine-induced inhibition of STAT5 and STAT3 phosphorylation. Previously, piperine was found to inhibit STAT1 phosphorylation in IFN-α- and IFN-β-stimulated peritoneal macrophages [372], suggesting that piperine can also inhibit STAT activation downstream of the type I IFN receptor. Type I IFNs signal through the IFN-α receptor (IFNAR), which is a heterodimer of IFNAR1 and IFNAR2 subunits that associate with JAK kinases Tyk2 and JAK1, respectively [510]. IFNAR1 phosphorylation by JAKs creates binding sites for STAT1, STAT3, and STAT5, which are then phosphorylated by JAK1 and Tyk2 [511]. Therefore, IFNAR-associated STAT phosphorylation follows a similar mechanism as IL-2R-associated STAT phosphorylation, suggesting that piperine may inhibit IFN-induced STAT1 phosphorylation and IL-2-induced STAT5 and STAT3 phosphorylation by a similar mechanism, although this requires further investigation. In addition to their role in the IL-2R signaling pathway, STAT5 and STAT3 are important in various cancers [512-514], including HTLV-1-associated T cell leukemia [515,516]. Therefore, it will be of interest to determine if the inhibitory effect of piperine on STAT5 and STAT3 phosphorylation is limited to IL-2R signaling in T cells or also extends to additional cell types and signaling pathways, such as IL-6R signaling in multiple myeloma cells.

6.1.4.5 TRPV1

In an attempt to determine the mechanism behind the inhibitory effect of piperine on T cells, I investigated a known receptor for piperine, TRPV1 [317], for expression and

functional involvement. TRPV1 has been detected in human peripheral blood mononuclear cells from 6 of 8 human subjects [452]. In contrast, TRPV1 has not been detected in murine splenocytes, lymph node immunocytes, or EL4 T lymphoma cells [453]. In the current study, *TRPV1* mRNA was not detected in murine T cells, which is consistent with the findings of Inada *et al.* [453]. Furthermore, there was no difference in the inhibitory activity of piperine on T cells from WT or TRPV1$^{-/-}$ mice. Taken together, these data indicate that TRPV1 is not required for the inhibitory mechanism of piperine on T cells.

The involvement of TRPV1 in the inhibitory effect of piperine on HUVECs was also investigated. Although *TRPV1* mRNA was not found in HUVECs, TRPV1 expression has been identified on cerebromicrovascular [492] and pulmonary arterial endothelial cells [493], as well as on immortalized HUVEC-12 cells [494]. Other TRP channels, such as TRPP2, are expressed on human renal artery endothelial cells but not on HUVECs [495], suggesting that TRPV1 may be differentially expressed on various types of endothelial cells and/or under different culture conditions. Furthermore, three TRPV1 antagonists did not interfere with the inhibitory effect of piperine on HUVEC proliferation. Taken together, these findings indicate that the anti-angiogenic activity of piperine is not mediated through TRPV1. Alternatively, piperine may be interacting with another receptor such as TRPA1 which is activated by piperine [517], or TRPV6, which is also activated by the TRPV1-ligand capsaicin [350]. The possibility that other TRPV receptors are involved in the mechanism of piperine-mediated inhibitory activity requires further investigation. It is likely that piperine enters cells by passive diffusion due to its highly lipophilic nature [352]. Passive diffusion is the main mode of piperine absorption into the small intestine [351]. Additionally, the current study found that the anti-proliferative activity of piperine in T cells was reversible upon removal of excess piperine in the culture media, suggesting that piperine may leave the cell by passive diffusion and be washed away. This may explain why high doses of piperine are needed to induce the effects identified in this study, since the passive diffusion gradient of piperine from the culture medium needs to be maintained to retain piperine within the cells. Alternatively, upon removal of the excess piperine from the culture media,

intracellular piperine may be segregated within the cell, preventing it from interacting with its molecular targets.

6.2 Model of the Mechanism of Piperine-Induced Inhibition of T Cells and Endothelial Cells

The mechanism by which piperine exerts its inhibitory effects is not completely elucidated. A confounding factor in determining the mechanism is that studies show different effects of piperine in different cell lines under different stimulation conditions. Therefore, the model of piperine activity that I propose was established by taking into account the most common effects of piperine on a specific signaling molecule over multiple studies, including the current investigation (Figure 6.1).

In the current study, piperine inhibited Akt phosphorylation at Ser 473 downstream of growth factor-stimulation in endothelial cells and CD3/CD28-stimulation in T cells. PKCα is responsible for the phosphorylation of Akt at Ser 473 in both T cells and endothelial cells [39,508] and is activated downstream of the TCR [39] as well as the receptors for VEGF, EGF, IGF, and FGF-2 [258,508,518-522], which were used to stimulate HUVEC growth in the current study. Autophosphorylation and membrane localization of PKCα is also inhibited by piperine in PMA-stimulated HT-1080 cells [396]. It is therefore reasonable to speculate that piperine inhibited Akt phosphorylation in T cells and endothelial cells by preventing PKCα activation. PKCα autophosphorylation occurs following transphosphorylation at Thr 500 by PDK1 and binding of PKC cofactors such as Ca^{2+}, phosphatidylserine, and DAG [523]. The inhibition of PKCα autophosphorylation seen in PMA-stimulated HT-1080 cells could be due to either direct inhibition of PKCα kinase activity, inhibition of PKCα interaction with its cofactors, or from inhibition of PDK1 phosphorylation of Thr 500 prior to autophosphorylation. Piperine may therefore interfere with Ca^{2+} flux into the cell, inhibit the PI3K-mediated generation of PIP_3, which is required for PDK1 membrane localization and kinase activity, or may directly interfere with PKCα kinase activity.

Piperine also inhibited ERK and IκBα phosphorylation downstream of CD3/CD28-stimulation in T cells. PKCθ is a common upstream activator of both the ERK and IκBα pathways in T cells via RasGRP and CBM activation, respectively

[45,47,458]. However, the effect of piperine on PKCθ activity has not been examined. In T cells, PKCα and PKCθ have cooperative roles in T cell function and activation as PKCα/PKCθ double knockout T cells proliferate less and are less able to reject allografts compared to single knockout animals [524]. Additionally, in Jurkat T leukemia cells, PKCα acts upstream of PKCθ in the phosphorylation of IκBα [525], suggesting that the piperine-induced inhibition of Akt and IκBα phosphorylation may be linked to a reduction in PKCα activity. PKCα activity has been linked to activation of the ERK pathway by direct phosphorylation of Raf-1 in fibroblasts [526] and ERK phosphorylation is inhibited in T cells treated with the conventional PKC isozyme inhibitor Go6976 [48], suggesting that PKCα is involved in ERK activation. PKCα localizes to the cell membrane upon stimulation [38]. Since the cell membrane is believed to be the passive entry point of piperine into the cell [351,352], a direct effect of piperine on PKCα function is possible. In contrast, PKCα$^{-/-}$ T cells proliferate normally and produce normal levels of IL-2 [524], while proliferation and IL-2 production are severely impaired in piperine-treated T cells. Therefore, while Akt (Ser 473), ERK, and IκBα phosphorylation occur downstream of PKCα activation, the functional difference between PKCα$^{-/-}$ T cells and piperine-treated T cells suggests that PKCα cannot be the only T cell molecule affected by piperine.

Akt (Ser 473), ERK, and IκBα are all regulated by PKC isoforms, which in turn are controlled by the second messengers, DAG and Ca^{2+} [38,432]. This suggests that the inhibitory effect of piperine on ERK and IκBα phosphorylation in T cells, as well as Akt phosphorylation in T cells and endothelial cells likely involves the inhibition of other PKC isoforms, in addition to PKCα, and/or the inhibition of the production of upstream second messengers that regulate PKC activation.

6.3 Model of the Mechanism of Piperine-Induced Inhibition of IL-2R Signaling

While IL-2R signaling does activate PKC isoforms [527], PKC activation is not required for normal IL-2R signaling [528,529], suggesting that the inhibitory effect of piperine on IL-2R signaling occurs by a mechanism that is different from the proposed mechanism for piperine inhibition of CD3/CD28 and endothelial growth factor signaling. This study has demonstrated that piperine inhibited the phosphorylation of STAT5,

STAT3, Akt, and ERK in response to IL-2 stimulation (Figure 6.2). STAT5 phosphorylation occurs following binding to specific Tyr residues on the IL-2Rβ chain that have been phosphorylated by JAK1 and possibly JAK3 [158,160,162]. Upon binding to the phosphorylated IL-2Rβ chain, STAT5 is phosphorylated by JAK1 and/or JAK3 [157,159]. The piperine-induced inhibition of STAT5 phosphorylation seen in this study was independent of phosphatase activity or *de novo* protein synthesis and occurred in the presence of normal JAK3 and JAK1 phosphorylation. Inhibition of STAT5 phosphorylation by piperine may therefore be due to interference with either the phosphorylation of the IL-2Rβ chain, the interaction of STAT5 with the phosphorylated IL-2Rβ chain, or the interaction of JAK1 and/or JAK3 with STAT5, any of which would result in the inhibition of STAT5 phosphorylation in the presence of piperine observed in this study. In contrast to STAT5, STAT3 binds to a region of the acidic domain of the IL-2Rβ chain and this interaction does not require IL-2Rβ chain phosphorylation [179]. Piperine-mediated inhibition of IL-2Rβ chain phosphorylation would therefore be insufficient to inhibit STAT3 phosphorylation, which suggests that piperine may inhibit STAT3 phosphorylation by inhibiting the kinase activity of JAK1 and/or JAK3, or that piperine is preventing JAK1 and/or JAK3 from accessing STAT3.

Piperine also inhibited IL-2-induced Akt and ERK phosphorylation. In IL-2R signaling, both Akt and ERK phosphorylation are downstream of Shc, which binds to a specific phosphorylated Tyr residue on the IL-2Rβ chain [160,170]. Once bound to the IL-2Rβ chain, Shc is phosphorylated on three Tyr residues, which form binding sites for adapter proteins [530]. One such adapter protein is Grb2, which is associated with SOS, a guanine exchange factor that induces the activation of Ras, leading to ERK phosphorylation [25]. In addition to SOS, Grb2 can also bind Gab2 [531], which becomes phosphorylated in response to IL-2R signaling [532]. The three Tyr phosphorylation sites on Gab2 are all located within YXXM sequences, which is the consensus sequence capable of binding the p85 regulatory subunit of PI3K [171]. Mutation of the Shc binding site on the IL-2Rβ chain ablates the ability of the IL-2Rβ chain to induce phosphorylation of Akt at Ser 473 [171]. Since Shc is critical for both ERK and Akt phosphorylation downstream of IL-2R signaling, piperine-induced inhibition of ERK and Akt phosphorylation may involve inhibition of the IL-2-induced formation of the Shc

signaling complexes Shc-Grb2-SOS and Shc-Grb2-Gab2-PI3K, respectively. Potential mechanisms by which piperine may inhibit Shc signaling complex formation are the inhibition of JAK kinase activity and phosphorylation of the IL-2Rβ chain, inhibition of Shc binding to the phosphorylated IL-2Rβ chain, or inhibition of the phosphorylation of any of the signaling complex components, such as Shc, Grb2, and Gab2. The restoration of normal ERK and Akt phosphorylation after 1 h of IL-2 stimulation indicates that any potential inhibition of Shc signaling complexes by piperine is only transient. Consequently, restoration of ERK and Akt phosphorylation suggests that the phosphorylation of IL-2Rβ chain is normal in piperine-treated CTLL-2 cells within 1 hour of IL-2 stimulation, which suggests that JAK kinase activity may not be inhibited by piperine treatment, at least at this time point. In contrast, STAT5 phosphorylation is not restored after 1 h of IL-2 stimulation. Taken together, these findings suggest that piperine may inhibit the interaction of STAT5 and Shc with the phosphorylated IL-2Rβ chain rather than preventing IL-2Rβ chain phosphorylation.

In summary, piperine-mediated inhibition of IL-2R signaling likely involves piperine interfering with the interaction of STAT5 and/or Shc with the phosphoryated IL-2R, which could be further investigated by co-immunoprecipitating the IL-2Rβ chain and examining its binding partners by western blotting. Alternatively, piperine may be interfering with the interaction of JAK1 and/or JAK3 with their target signaling molecules, including STAT5, STAT3, and Shc, which could be further investigated by using an *in vitro* kinase assay to directly examine the ability of JAK1 and JAK3 to phosphorylate STAT5 in the presence of piperine.

6.4 Limitations of the Current Study

6.4.1 Limitations of Using *In Vitro* Cultures

The majority of the findings from the current study were obtained *in vitro* using one cell type in isolation, a situation that is not representative of the diverse and complex integration and interdependence of different cell types and whole tissues that is seen *in vivo*. For example, T cells are dependent on APCs to present antigen and induce T cell activation [3]. In addition to APC-T cell direct interactions, T cells also receive stimulatory signals from cytokines such as IL-6, IL-1β, and TNF-α produced by

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inflammatory cells [533]. Furthermore, Th cell polarization is regulated by inflammatory cell-produced cytokines, such as IL-12 for Th1, and IL-6 and TGF-β for Th17 differentiation [7]. Since *in vitro* results do not always correlate with *in vivo* findings [534], the piperine-induced effects on T cells identified in this current study should be further confirmed *in vivo*.

Just as T cell activation *in vivo* involves the actions of multiple cell types and cellular products, angiogenesis also involves not only endothelial cells *in vivo* but also the ECM and the supporting cells such as pericytes, smooth muscle cells, and fibroblasts [535,536]. These additional cell types were not present in the *in vitro* HUVEC experiments and none of the *in vitro* experiments performed with HUVECs in the current study simulate the complexity of the vascular environment and the process of angiogenesis [537]. Because of these limitations of *in vitro* angiogenesis assays using HUVECs, the rat aorta assay and the CAM assay were used to confirm that the *in vitro* inhibitory effects of piperine on HUVECs were also applicable to the overall angiogenic process *ex vivo* and *in vivo*. However, one limitation of the rat aorta and CAM assays is that these experiments were performed using non-human tissues, and differences have been identified in endothelial cells from different species [537]. Nevertheless, the different angiogenesis and endothelial cell assays used in the current study complement each other and confirm that piperine inhibits both *in vitro* and *in vivo* angiogenesis. Taken together, this current study suggests that the anti-angiogenic effect of piperine is translational into *in vivo* assays with human tissues.

6.4.2 Limitations of Cells Used in this Study

6.4.2.1 T Cells

Mouse T cells from spleen are similar to human T cells in their activation and function, but do not completely mirror human T cells. Th polarization in response to the parasite *Schistosoma* results in a Th2 response in humans, while a Th1 response is induced in mice [538]. Expression of the costimulatory molecule CD28 also differs between humans and mice, with almost 100% of CD4$^+$ and CD8$^+$ murine T cells expressing CD28 compared to only 80% of CD4$^+$ and 50% of CD8$^+$ T cells in humans [538]. Additionally, findings from *in vivo* mouse experiments do not always translate to

humans. For example, therapies that worked in mice have not been as successful in human disease, such as anti-TNF-α therapy for the treatment of MS [539]. Thus, the inhibitory effects of piperine on mouse T cells need to be confirmed in human T cells.

6.4.2.2 CTLL-2 Cells

CTLL-2 cells are a murine T cell line that is dependent on IL-2 for their proliferation and survival. Since normal T cells receive survival and proliferation signals through other cytokine receptors, as well as via the TCR and co-stimulatory molecules, CTLL-2 cells are not representative of normal T cells. Despite this limitation, CTLL-2 cells made an excellent model for examining the effect of piperine on IL-2R signaling without the involvement of other confounding signaling pathways, thereby allowing for direct examination of the effects of piperine on this pathway.

6.4.2.3 HUVECs

HUVECs are human primary endothelial cells isolated from the umbilical vein due to the ease of isolation and the availability of umbilical cords compared to other tissues [540,541]. Although HUVECs are commonly used to examine endothelial cell function and angiogenesis, the umbilical vein is a large vessel whereas the majority of angiogenesis that takes place *in vivo* involves the microvasculature [537]. Additionally, HUVECs are venous endothelial cells of fetal origin and differences have been identified between the responses of arterial and venous endothelial cells [542], as well as fetal and adult endothelial cells [543,544]. Thus, the use of HUVECs as a model for angiogenesis may limit the interpretation and relevance of my findings; however, this limitation was overcome in the current study by using the *ex vivo* rat aorta and *in vivo* CAM assays to show that the anti-angiogenic effect of piperine is not limited to an *in vitro* effect on HUVECs. Another drawback of using HUVECs is the need for substantial growth factor stimulation in order to maintain these normally quiescent cells in culture. These growth factors maintain the cells in a highly active state, which over time induces phenotypic changes and thus limits their prolonged use [545,546]. The variety of growth factors required for maintenance of *in vitro* HUVEC cultures also results in the concurrent activation of various intracellular signaling pathways [547], which makes analysis of the molecular mechanism of piperine difficult due to overlapping growth factor signaling

pathways. Nevertheless, HUVEC stimulation with multiple growth factors is more physiologically relevant since endothelial cells participating in angiogenesis *in vivo* will be integrating signals from various growth factors receptors.

6.4.3 Limitations of Activating T Cells with Ab-Coated Beads

To study the effect of piperine on T cell processes in isolation from other cell types, anti-CD3/anti-CD28 Ab-coated beads were used as surrogate APCs to provide signal 1 and signal 2 to T cells. Signal 1 is provided by the anti-CD3 Ab cross-linking and aggregating CD3 molecules, which induces downstream signaling by the TCR/CD3 complex in the absence of specific antigenic recognition by the TCR. *In vitro* activation of T cells regardless of their antigenic specificity results in greater numbers of responding T cells than would normally be found *in vivo* responding to a specific pathogen. In addition to signal 1, these beads also provide signal 2 through the costimulatory molecule CD28, thereby restricting and simplifying signal 2 to only the CD28 molecule. In comparison, T cells activated with APCs are exposed to a number of different costimulatory molecules on the APC surface, resulting in the activation of additional costimulatory intracellular signaling events, resulting in greater activation [548]. Despite the Ab-coated beads only providing co-stimulation through anti-CD28, the beads are able to induce T cell activation and proliferation, as well as effector function, that is similar to DC-induced activation *in vitro* [549]. Since Rodgers [380], found that piperine also inhibits the activation of OT-I transgenic T cells by OVA-pulsed DCs, it is apparent that the simplified costimulatory signal associated with bead stimulation did not impact the inhibitory activity of piperine on T cell proliferation.

6.4.4 Limitations of the CTL Assay

In the current study, piperine did not affect the lytic activity of fully differentiated CTLs but inhibited the differentiation of resting $CD8^+$ T cells into functional CTLs. The reduction in IL-2 production following piperine treatment may contribute to the inhibition of CTL differentiation, as CTLs are dependent on IL-2 for expression of the cytotoxic effector molecules granzyme [198] and perforin [199]. A potential confounding factor in this experiment is the changing ratio of $CD4^+$:$CD8^+$ T cells following T cell activation and the effect that piperine treatment has on this ratio. This difference in the

ratio of $CD4^+$:$CD8^+$ T cells between vehicle- and piperine-differentiated CTLs resulted in a higher proportion of vehicle-treated $CD8^+$ T cells being added to the CTL assay compared to the number of piperine-treated $CD8^+$ T cells. A reduction in the proportion of CTLs induced in the presence of piperine could produce an apparent decrease in cytotoxic activity. The approximate number of $CD8^+$ T cells present in the CTL assay for each effector cell:target cell ratio and for each treatment was calculated, plotted, and compared between treatments on the basis of the number of $CD3^+$ T cells added to the CTL assay and the percent of those that were $CD8^+$ from previous flow cytometric analysis for cellular division (Appendix Figure 1). Even when differential $CD8^+$ T cell numbers were accounted for, CTLs that differentiated in the presence of piperine exhibited reduced cytotoxicity compared to CTLs generated in the presence of the vehicle. This suggested that piperine inhibited CTL differentiation, despite the limitation of the CTL experiment. This finding, however, will need to be confirmed by isolating $CD8^+$ T cells instead of pan $CD3^+$ T cells for CTL differentiation, and using exogenous IL-2 to support functional CTL differentiation in the absence of $CD4^+$ T cell help. Additionally, measuring cytotoxic effector molecule production by $CD8^+$ T cells that differentiate into CTLs in the presence or absence of piperine would also help to evaluate the effect piperine on CTL differentiation and cytotoxic activity. Nevertheless, the ratio of $CD4^+$:$CD8^+$ T cells exposed to piperine is physiologically relevant since these two T subpopulations interact *in vivo*.

6.4.5 Limitations of the Endothelial Migration Assay

A potential limitation of the migration assay that was performed with the HUVECs is the length of the time course. Because HUVECS were cultured for 20 h, it is possible that proliferation may have occurred in the absence of piperine-treatment and skewed the results. However, similar results were observed at 10 h before HUVEC proliferation would have occurred, indicating that piperine inhibited the migration of HUVECs. The inhibitory effect of piperine on endothelial cell migration was also confirmed in the *ex vivo* rat aorta model, supporting the conclusion that piperine inhibits endothelial cell migration.

6.4.6 Limitations of Using Phosphorylation as an Indicator of Signaling Molecule Activation

The examination of phosphorylation sites as an indicator of signaling molecule activation was another possible limitation of this study. For all of the molecules examined, phosphorylation suggests but does not directly show activation of that molecule [20,550-556]. Therefore, while piperine did not inhibit the phosphorylation of ZAP-70, JAK1, or JAK3 at their activation sites, the effect of piperine on the kinase activity of these molecules should be confirmed by a direct kinase assay or indirectly by examining the phosphorylation status of target proteins of these three kinases. Additionally, while my study shows a decrease in STAT5, STAT3, Akt, ERK, and IκBα phosphorylation following piperine treatment, additional phosphorylation sites also regulate the function of these proteins [557-560]. Therefore, the activity of these signaling pathways in the presence of piperine should be confirmed. The activity of Akt and ERK in the presence of piperine could be measured directly using a kinase assay [561,562]. The effect of piperine on the transcriptional activity of STAT5, STAT3, Elk-1 (the transcription factor directly phosphorylated by ERK), and NF-κB could be directly measured using a luciferase reporter assay [563-566]. The luciferase reporter assay would also confirm that the piperine-induced inhibition of STAT5, STAT3, ERK and IκBα phosphorylation is sufficient to inhibit the downstream transcriptional activity initiated by these signaling molecules and support the involvement of these molecules in the inhibitory action of piperine.

6.5 Future Studies

6.5.1 Continuation of Current *In Vitro* Studies

6.5.1.1 The Mechanism by which Piperine Inhibits T Cell Function

T Cell Polarization

The commitment of T cells to a specific Th subset through polarization results in production of specific cytokines that regulate and shape an immune response [7,72,73]. The effect of piperine on T cell polarization to specific Th subsets is currently unknown. In the current study, piperine inhibited various aspects of T cell function, including

proliferation, CD25 expression, cytokine production, and CTL differentiation. The production of IL-4 and IFN-γ are linked to cell cycle progression, suggesting that the inhibitory effect of piperine on T cell proliferation may contribute to decreased cytokine production [451]. Additionally, IL-2R stimulation affects Th cell differentiation by altering the expression of other T cell cytokine receptors, including IL-12β2, IL-6Rα, gp130, and IL-4Rα [200,201]. IL-12, IL-6, and IL-4 that bind to these receptors are important for Th1, Th17, and Th2 cell development, respectively. Therefore, the piperine-induced inhibition of IL-2 production and IL-2R signaling would be expected to cause reduced expression of cytokine receptors that are crucial for inducing Th1, Th17, and Th2 polarization. Furthermore, in the current study, piperine directly inhibited the production of the Th1 and Th2 polarizing cytokines IFN-γ and IL-4, respectively, thereby presumably inhibiting Th1 and Th2 polarization. Taken together, these findings suggest that piperine may inhibit the polarization and differentiation of Th cell subpopulations through multiple mechanisms; however, further investigation into the effect of piperine on Th cell differentiation under polarizing conditions as well as the effect of piperine on cytokine production by previously polarized Th cells is warranted.

Effect of Piperine on Tregs

Preliminary experiments show that piperine treatment for 1 h does not inhibit the suppressive activity of Tregs (data not shown). Treatment of Tregs with piperine was followed by thorough washing, which may have removed piperine from the Tregs and may affect any influence piperine had on the Tregs. IL-2 and STAT5 are very important for Treg function [151], suggesting that the inhibitory activity of piperine on IL-2R signaling and STAT5 activation may inhibit Tregs. The low threshold of Tregs for IL-2 may allow for their survival and maintenance in the presence of piperine [212]. Alternatively, Akt signaling inhibits Treg differentiation [567] and in juvenile idiopathic arthritis, T effector cells are more resistant to Treg suppression due to high levels of Akt activation [568], suggesting that the inhibitory activity of piperine on Akt activation could promote Treg development and their ability to suppress T effector cells. Tregs are instrumental in restraining inflammation and preventing autoimmunity [139], therefore the effect of piperine on Tregs is of interest. The expression of the classical Treg transcription factor FoxP3 in the presence of piperine could be examined by western

blotting to determine the effect of piperine on Treg maintenance. Furthermore, the addition of piperine to T cell cultures under iTreg-inducing conditions could determine the effect of piperine on iTreg differentiation.

6.5.1.2 The Mechanism by which Piperine Inhibits the Phosphorylation of Various Signaling Molecules

The Relative Importance of Inhibition of STAT5, STAT3, Akt, ERK and IκBα Phosphorylation to the Inhibitory Activity of Piperine

The current study shows the piperine-induced the inhibition of various signaling molecules in T cells and endothelial cells, including STAT5, STAT3, Akt, ERK, and IκBα. While the phosphorylation of these molecules was reduced following piperine treatment, the relative importance of these changes to the inhibitory activity of piperine remains undetermined. To determine whether inhibition of these signaling molecules is responsible for the anti-proliferative activity of piperine, constitutively active mutants of these molecules/pathways could be transfected into cells [486,569-571] and proliferation measured in the presence or absence of piperine. This experiment would determine if the signaling molecules shown to be inhibited by piperine in the current study are critical, irrelevant, or partial contributors to the inhibitory activity of piperine on cell proliferation. Note that a positive control would need to be included to control for a possible direct inhibitory action of piperine on the mutant proteins.

Potential Interaction of Piperine with PKCα

As discussed in section 6.2, piperine may be directly affecting the function of PKCα. The potential direct interaction between piperine and PKCα could be further examined using an *in vitro* kinase assay [572], where the enzymatic activity of PKCα could be measured in the presence or absence of piperine in the absence of cells. Both piperine and PKCα are known to be located in the cell membrane as PKCα interacts with phosphatidylserine residues in the inner cell membrane when active [38], and diffusion into the cell membrane is the probable entry point of piperine, as discussed in section 6.1.4.5 [351,352], thus allowing for a potential interaction.

STAT activation is common to various cytokine, hormone, and growth factor receptors [512]. Since piperine inhibits the phosphorylation of STAT5 and STAT3 downstream of the IL-2R, the effect of piperine on the activation of STAT5 and/or STAT3 downstream of other signaling pathways is also of interest. Constitutive activation of STAT3 and STAT5 in cancer cells results in a resistance to apoptosis [512]. For example, multiple myeloma cells have constitutively active STAT3 due to autocrine production of IL-6, resulting in increased Bcl-xL expression [573]. Disruption of STAT3 activation decreased Bcl-xL expression and increased apoptosis, indicating that these cells are dependent on STAT3 for their continued survival [573]. It has been suggested that the anti-apoptotic phenotype of cancers with constitutively active STAT3 or STAT5 results in resistance to chemotherapy-induced apoptosis and that combination therapy using STAT3 or STAT5 inhibitors plus chemotherapeutic agents could overcome the resistance to apoptosis, resulting in re-sensitization of tumor cells to chemotherapy [512]. Furthermore, inhibition of STAT3 phosphorylation by the JAK2 inhibitor SD-1008 increases the sensitivity of ovarian cancer cells to paclitaxel [574]. To determine the effect of piperine on STAT3 activation in cancer cells, the constitutive phosphorylation of STAT3 downstream of the IL-6R could be examined in multiple myeloma cells. If piperine inhibits STAT activation downstream of additional receptors, it would broaden the potential therapeutic uses of piperine to include the treatment of cancers with constitutively active STAT3 and/or STAT5.

6.5.2 Examination of the Inhibitory Activity of Piperine *In Vivo*

6.5.2.1 Drawbacks of Oral and Systemic Administration of Piperine

Piperine is a known agonist of TRPV1, which causes the strong burning sensation associated with ingesting black pepper [317]. The high level of TRPV1 expression in the nervous and digestive systems [575], together with this unpleasant sensation, limit the feasibility of oral administration of piperine, as demonstrated by the aversion of mice and rats to oral dosing with piperine [491] (data not shown). Another potential obstacle to the therapeutic use of piperine is the high doses that are required for the T cell suppression and anti-angiogenic activity identified in the current study. Serum levels in the range of

28-39 μM can be achieved in rats following an oral dose of 170 mg/kg of piperine [353,354], which is in the lower levels of piperine determined to be therapeutically active in this study. In order to provide the high range of therapeutically active piperine levels at sites of inflammation or angiogenesis after systemic administration, the required dose of piperine would be very high and might have adverse side effects due to the multitude of physiological actions of piperine in various cells and tissues [321]. Furthermore, the inhibitory activity of piperine on CYP450 enzymes and drug efflux pumps [357,360,361], limits its systemic use due to effects on the bioavailability and metabolism of various nutrients and pharmacological agents. While this increase in bioavailability can be beneficial when drugs and nutrients are poorly absorbed or rapidly cleared, such as the bronchodilator theophylline [363], the beta blocker propranolol [363], curcumin [576], and beta-carotene [577], not all medications will benefit from decreased metabolism, especially medications that are administered as a prodrug and require metabolism by CYP450 to generate the active metabolite. Additionally, decreased metabolism could result in adverse side effects and toxicity of drugs with a narrow therapeutic window such as the anti-convulsant phenytoin, the heart medication digoxin, and the sedative pentobarbitone [358,578,579].

6.5.2.2 Potential of Piperine as a Topical Therapeutic Agent

One possible solution to the above mentioned obstacles to systemic piperine therapy is localized administration by topical application. Piperine has shown promise as a topical treatment for the depigmentation condition vitiligo in mice [343] and rabbits [344] and has been patented for this use in humans (Patent # CA 2337205) [345]. Using the topical application method that has already been shown effective by Faas *et al*. [343], piperine could be tested for T cell inhibitory activity using an *in vivo* DTH reaction induced by oxazolone or picryl chloride [580,581]. In this model, mice are sensitized to oxazolone or picryl chloride by topical application on the shaved abdomen. Mice are then challenged with oxazolone or picryl chloride 5-7 days later by application to one ear, while the other ear serves as a negative control. The thickness and/or weight of the ear are measured as indicators of edema and inflammation. With this model, the anti-inflammatory activity of topically-administered compounds can be determined. Histology performed on the challenged ear tissues indicates the level of inflammatory infiltrate;

moreover, edema, and vascular congestion can be scored and compared between different treatments [582]. Using this model, the inhibitory activity of topical piperine on a T cell-mediated delayed-type hypersensitivity reaction can be determined.

6.5.2.3 Potential of Piperine for Targeted Delivery in Nanoparticles

Targeted delivery of piperine in nanoparticles is a mode of *in vivo* administration that is predicted to lack the negative side effects associated with systemic delivery of the phytochemical. Nanoparticles naturally accumulate in areas of high vascular permeability and activated endothelium such as sites of inflammation and angiogenesis [583]. Accumulation at these sites localizes drug delivery to inflamed tissues or vascularized tumors, allowing for passive targeting of the nanoparticles to these specific sites. For example, glucocorticoid-loaded lipsomes accumulate and are retained in the inflamed paws of arthritic rats, reducing the dose that is required for therapeutic activity [584]. Similarly, nanoparticles preferentially accumulate in inflamed colonic tissue in comparison to healthy colonic tissue [585]. Importantly, splenic uptake of nanoparticles by the mononuclear phagocyte system can be minimized and delivery to inflamed tissues maximized by using nanoparticles of 90-100 nm size that are coated with inert groups such as PEG [584]. Nanoparticles also reduced the dosing needed to achieve a therapeutic effect due to the sustained release of the active agent [584], as well as reducing adverse effects of drugs in comparison to oral delivery [585]. Moreover, further targeting of the nanoparticles can be achieved by coupling the nanoparticles to Abs against a specific cell surface antigen such as E-selectin on activated endothelial cells or CD3 on T cells.

Based on passive targeting to sites of inflammation and angiogenesis, a reduction in the required therapeutic dose, and a reduction in adverse side effects, nanoparticles show great promise for *in vivo* administration of piperine for the treatment of inflammatory conditions and cancer. Piperine has already successfully been encapsulated in liposomes [586-590], as well as encased with biodegradable polymers [591]. Encapsulation of piperine also reduces the clearance time [588] and the dose of piperine required for therapeutic benefit [590]. Piperine has a variety of anti-inflammatory effects in addition to anti-cancer activity and inhibiting angiogenesis and T cell function. Therefore, passive targeting of nanoparticles provides a delivery mechanism by which

piperine can be selectively released at locations of inflammation and vascularized tumors *in vivo*. Based on the anti-angiogenic and T cell inhibitory activity of piperine identified in the current study, piperine-encapsulated nanoparticles warrant further investigation in the treatment of vascularized tumors, as well as T cell-mediated inflammatory conditions, including transplant rejection and IBD.

Since piperine is associated with gastrointestinal protective effects [337-340] and low dose oral administration of piperine would allow for localized delivery to the gastrointestinal system without requiring high serum concentrations, piperine has been examined by our lab as a possible therapeutic agent in an animal model of IBD. Both T cells [404] and angiogenesis [274,592] play important roles in the pathogenesis of IBD, suggesting that the anti-angiogenic and T cell inhibitory activities of piperine demonstrated in my research would be beneficial in the treatment of IBD. Specifically, piperine inhibits IL-2 production and IL-2R signaling by T cells, and another IL-2 targeted therapy, the anti-IL-2Rα Ab basoliximab, promotes remission of steroid-resistant ulcerative colitis [593,594], suggesting that the inhibition of IL-2 production and IL-2R signaling by piperine may be beneficial in IBD. Piperine also inhibits angiogenesis, which is a potential therapeutic target in IBD [296,409] as demonstrated by the decrease in disease severity in animal models of IBD receiving anti-angiogenic therapies [275,276]. Other studies also support the potential of piperine as a therapeutic agent for IBD. Piperine inhibits P-gp/MDR-1 expression [360] and activity [357], and high expression of this protein is associated with IBD patients who do not respond to glucocorticoid therapy [595]. Therefore, piperine may resensitize glucocorticoid-resistant IBD patients through its inhibition of P-gp, supporting its use a therapeutic agent for IBD. Additionally, piperine inhibits NO production by LPS-treated macrophages *in vitro* and *in vivo* [371], likely through inhibiting LPS-stimulated inducible nitric oxide synthase (iNOS) expression [596]. iNOS is upregulated in ulcerative colitis [597] and overproduction of NO is associated with IBD [598]. Therefore, inhibition of NO production by piperine may be beneficial in IBD therapy. Furthermore, TNF-α production is also inhibited by piperine *in vitro* and *in vivo* [371,372]. TNF-α is a key cytokine involved in IBD and therapies currently targeting TNF-α, such as the anti-TNF-α Abs, infliximab and adalimumab, are currently used in the treatment of ulcerative

colitis or Crohn's disease [599]. Therefore, the inhibition of TNF-α production by piperine suggests that piperine may have a therapeutic activity in IBD. Taken together, findings from my research and others support the application of piperine as a potent anti-inflammatory and gastrointestinal protective agent for the treatment of IBD.

Unfortunately, piperine has a very high bioavailability and is therefore absorbed primarily in the small intestine with only approximately 3% being absorbed in the large bowel [353]. Therefore, the oral administration of native piperine as a therapeutic agent for the treatment of IBD is not likely to be effective. One possible solution involves the use of nanoparticles that are designed to release their contents only under specific physiological conditions, such as a pH > ~7 [600]. There is a higher pH in the terminal ileum and colon compared to other regions of the gastrointestinal tract, which allows for targeting of the large bowel by orally-administered pH-sensitive nanoparticles [601,602]. Such pH-sensitive nanoparticles would release piperine specifically in the large bowel, which is the site of inflammation and increased angiogenesis in IBD. In addition to pH-sensitive nanoparticles, other strategies have allowed for nanoparticle targeting of the large bowel. One such strategy involves nanoparticle delivery systems that are synthesized from polymers that require degradation by the colonic microflora, resulting in localized release of the nanoparticle contents within the colon [600]. Another type of nanoparticle contains specific cellulose-based polymers that degrade and release their contents after a specific time in gastrointestinal transit [600]. New nanoparticle technologies are combining several of these colon-targeting strategies to allow for greater drug localization and to overcome the variability between individuals of colonic pH and microflora, as well as gastrointestinal transit times [600,603]. Targeting of the large bowel can also be accomplished by rectal administration of medications in the form of suppositories or enemas, as is used to administer the first-line IBD drug 5-aminosalicylic acid [604]. Similarly, rectal administration of piperine, either in its native form in an appropriate vehicle or encapsulated in nanoparticles, would allow for passive targeting of the large bowel. The potential application of piperine as an anti-inflammatory and anti-angiogenic agent for the treatment of IBD warrants further investigation through utilization of colon-targeting nanoparticles in angiogenesis- and T cell-mediated animal

models of IBD such as CD4$^+$CD45RB^{hi} T cell transfer into severe combined immunodeficiency mice [605] or IL-10 knockout mice [606].

6.6 Implications of this Study and Concluding Remarks

In summary, the current study has established that piperine has direct inhibitory activity on T cells and endothelial cells. Piperine inhibits various T cell functions, including activation, proliferation, activation marker expression, cytokine production, and CTL differentiation *in vitro*. These inhibitory effects are mediated by the piperine-induced inhibition of various intracellular signaling events downstream of the TCR, CD28, and the IL-2R, including phosphorylation of Akt, ERK, IκBα, STAT5, and STAT3. Additionally, piperine inhibits various endothelial cell processes, such as proliferation, migration, tubule formation, and angiogenesis *in vitro*, *ex vivo*, and *in vivo*. These inhibitory effects are mediated by the piperine-induced inhibition of Akt signaling.

Since dysregulated T cell functions and angiogenesis contribute to the pathogenesis of various chronic inflammatory and autoimmune conditions [128,279,297,404,406,409], my findings indicate that piperine warrants further investigation for *in vivo* use in the context of T cell-mediated and angiogenesis-mediated chronic inflammatory conditions and autoimmune diseases. In addition, the current study determined that piperine has potent anti-angiogenic activity, indicating the potential of piperine to be developed as an anti-angiogenic agent for the treatment cancer. The T cell inhibitory and anti-angiogenic activity identified in the current study complement the previously identified anti-inflammatory and anti-cancer activities of piperine and further support the possible use of piperine as an immunosuppressive and chemopreventative agent.

Figure 6.1 Model of the inhibitory activity of piperine on T cells and endothelial cells. Proteins inhibited by piperine in the current study or in the literature are depicted in red and green, respectively.
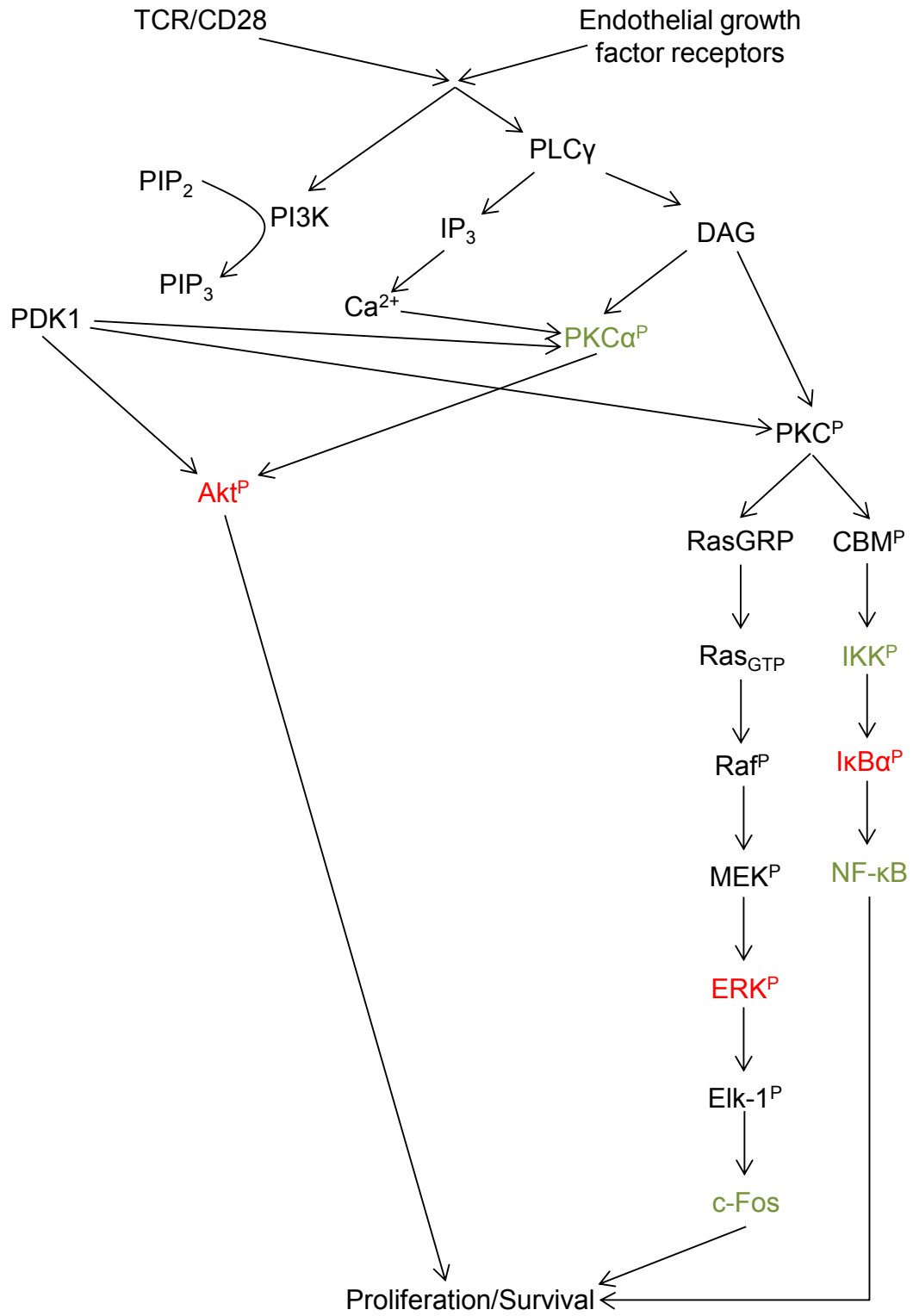
Figure 6.1

Figure 6.2 Model of the inhibitory activity of piperine on CTLL-2 cells.
Proteins inhibited by piperine in the current study or in the literature are
depicted in red and green, respectively.
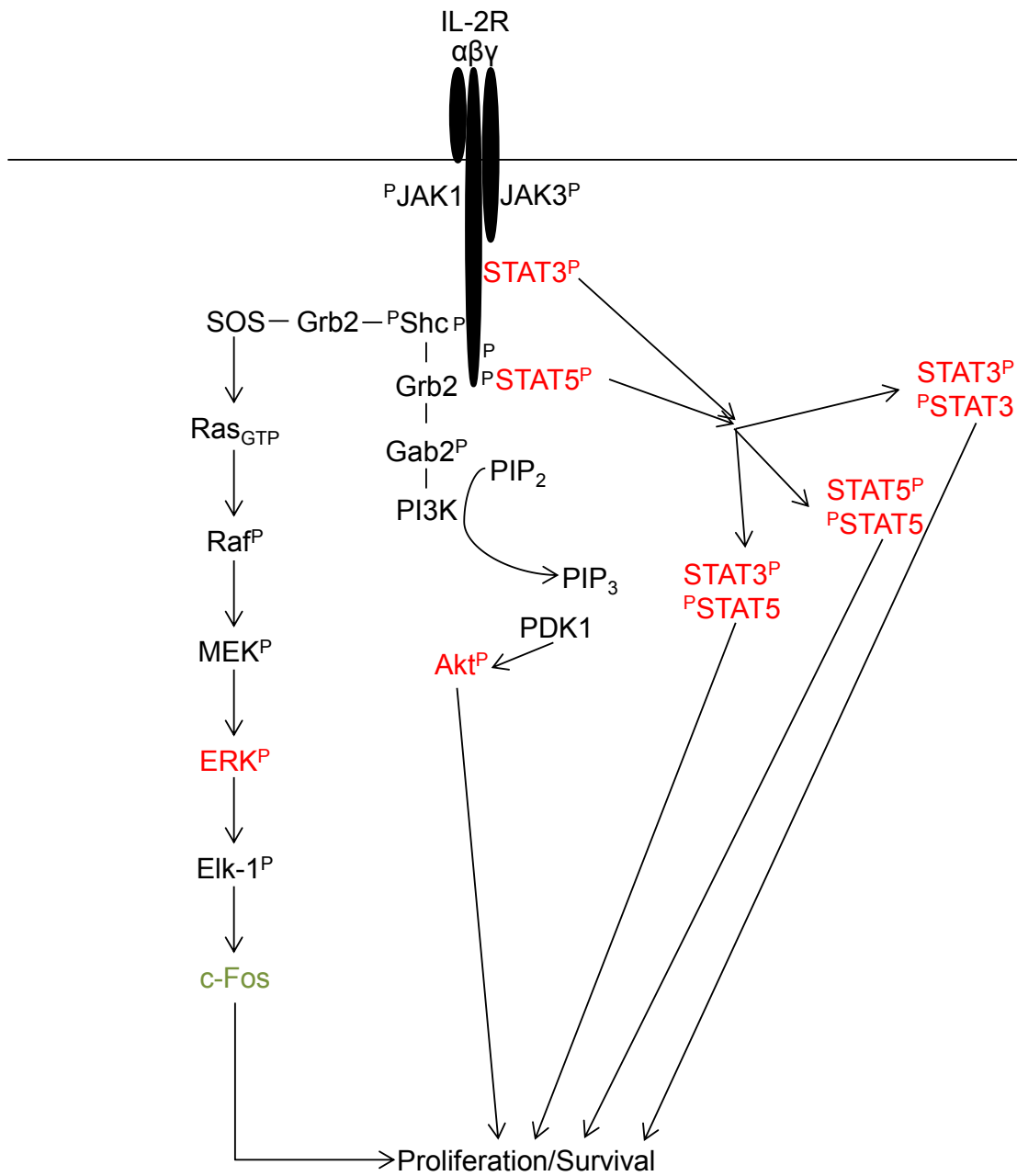
Figure 6.2

APPENDIX 1

SUPPLEMENTARY FIGURES

Appendix Figure 1. Piperine inhibits the cytotoxic activity of previously generated CTLs. Highly purified $CD3^+$ T cells were activated with anti-CD3/anti-CD28 Ab-coated beads for 72 h. CTLs were then treated with medium, vehicle (DMSO), or the indicated concentrations of piperine and incubated with anti-TCR Ab and $[^3H]$TdR-pulsed P815 target cells for 4 hours at the indicated effector:target cell ratios. Percent cytotoxicity was measured as the loss of $[^3H]$TdR$_{cpm}$ in the presence of effector cells compared to P815 target cells alone, as determined by liquid scintillation counting. Data were then normalized to the average number of $CD8^+$ T cells present within each treatment at the end of a 72 h culture as determined by cell surface staining with fluorescent Abs. Data shown are the mean of at least 3 independent experiments ± SEM.
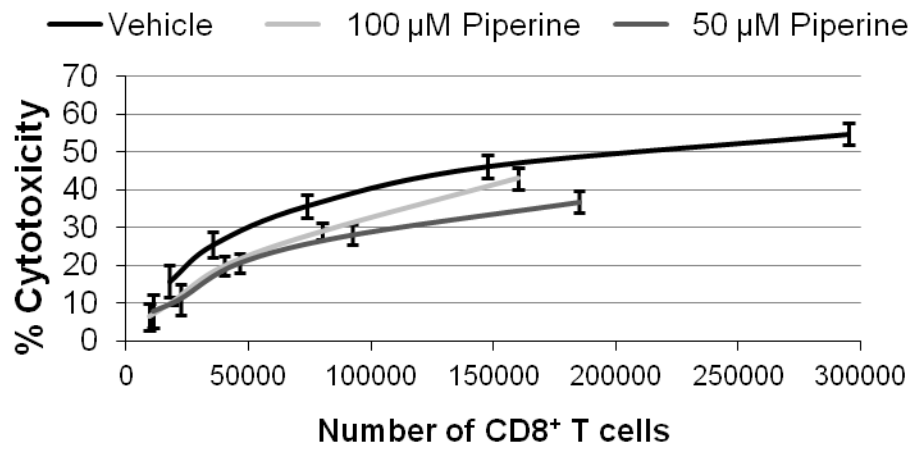
Appendix Figure 1

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