MECHANISMS OF TGF β-INDUCED INHIBITION OF CD1D-MEDIATED ANTIGEN PRESENTATION

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ABSTRACT

Jennifer Carrie Ryan

MECHANISMS OF TGF β-INDUCED INHIBITION OF CD1D-MEDIATED ANTIGEN PRESENTATION

CD1d is a cell surface glycolipid that, like Major Histocompatibility Complex (MHC) class I and MHC class II molecules, presents antigen. However, instead of peptides, CD1d presents lipids to Natural Killer (NK) T cells, a subset of T cells that express both NK cell markers and the T cell receptor and produces both T helper (Th) 1 and Th2 cytokines. Our lab focuses on the regulation CD1d-mediated antigen presentation. TGF β is a known regulator of the immune system, such as controlling MHC class II antigen presentation. Further, TGF β can activate the mitogen activated protein kinase (MAPK) p38, a known negative regulator of CD1d-mediated antigen presentation. Therefore, we hypothesized that TGF β would be a negative regulator of CD1d-mediated antigen presentation, and our results showed a decrease in antigen presentation by CD1d in response to TGF β treatment. However, this inhibition was not through p38 activation, as indicated by the absence of a rescue of CD1d-mediated antigen presentation in, TGF β-treated, p38 dominant negative-expressing cells. Alternatively, the Smad pathway, the canonical pathway activated by TGF β, was investigated through a lentivirus shRNA-mediated knockdown of Smad2, Smad3 and Smad4 proteins. Smad2 shRNA-expressing cells showed in an increase in CD1d-mediated antigen presentation, suggesting an

inhibitory role for Smad2. In contrast, Smad3 shRNA-expressing cells did not differ from control cells. However, as in the case of Smad2, $CD1d^{+}$ cells in which Smad4 was knocked down, were substantially better at CD1d-mediated antigen presentation than control cells, suggesting that it also negatively regulates antigen presentation. Overall, these studies demonstrate that the canonical TGF β/Smad pathway regulates an important part of the host's innate immune response, vis-à-vis CD1d-mediated antigen presentation.

Randy R. Brutkiewicz, Ph.D., Chair

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

α-GalCer	alpha galactosylceramide
BGS	bovine growth serum
BMDC	bone marrow-derived dendritic cell
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DN	dominant negative
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FOXP3	fork head box P3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEF	guanine exchange factor
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycosylphospatidylinositol
GSL	glycosphingolipid
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IFN-γ	interferon gamma
iGb3	isoglobotrihexosylceramide
IMDM	Iscove's modified Dulbecco's medium
iNKT	invariant natural killer T cells
JNK	c-jun N-terminal kinase
LAMP-1	lysosome-associated membrane protein 1
LAP	latency-associated protein
LCMV	lymphocytic choriomeningitis virus
LIMK	LIM domain kinase
LMTK	LM thymidine kinase
LTBP	latent TGF β binding protein

MAPK	mitogen activated protein kinase
MH1	MAD homology 1
MHC	major histocompatibility complex
MKK	mitogen activated protein kinase kinase
NK	natural killer
NKT	natural killer T cells
PKC δ	protein kinase C delta
RBD	Rho-binding domain
ROCK	Rho-associated kinase
RPMI	Roswell Park Memorial Institute
SARA	smad anchor for receptor activation
TAK1	TGF β activated kinase 1
TCR	T cell receptor
TGF β	transforming growth factor beta
TGFβR	transforming growth factor beta receptor
Th	T helper
TNF α	tumor necrosis factor alpha
Treg	T regulatory cell
VV	vaccinia virus
WT	wild-type

Introduction

CD1d-Mediated Antigen Presentation

A eukaryotic host needs to distinguish between self and non-self to avoid improper immune responses and to enhance pathogen and tumor immunity. To accomplish this, three different antigen presenting pathways that include major histocompatibility complex (MHC) class I, MHC class II and CD1d molecules are used. The central focus of the research described here is on the CD1d cell surface glycoprotein [1-3]. It is mainly expressed on hematopoietic cells, such as lymphocytes, macrophages, B cells and dendritic cells [4]. Functionally, CD1d is a lipid antigen presenting molecule, important in innate immunity [5]. Like MHC class I molecules, CD1d presents antigen and is comprised of a heavy chain with three domains (α1, α2, and α3) and is noncovalently associated with β2-microglobulin. However, unlike either MHC class I or class II that presents peptide antigens, the CD1d molecule presents lipids [6]. CD1d has two deep hydrophobic grooves, designated A' and F' pockets, in which the fatty acid chains of the lipid is inserted [7]. The immune cells that are capable of recognizing antigen presentation by CD1d are Natural Killer T (NKT) cells [8, 9]. NKT cells express Natural Killer (NK) cell markers Such as CD161 or NK1.1 and a T cell receptor (TCR) [10-12]. Upon recognition of CD1d and bound lipid, NKT cells promptly produce both T helper (Th) 1 and Th2, cytokines such as the mediators IL-4 [13] and IFN-γ [14], which activate

immune cells such as NK cells or B cells as a means of furthering the innate antitumor immune response.

Upon synthesis, CD1d associates with the chaperones calrexin and calreticulum in the endoplasmic reticulum (ER) [15, 16]. In addition to (or concomitant with) β2-microglobulin association [17], CD1d acquires glycosylphosphatidylinositol (GPI) as a temporary ligand in the ER [18]. This complex is then transported to the cell surface, but is unable to be recognized by NKT cells. Upon internalization into the cell from the surface, which is facilitated by an endosomal targeting tyrosine-based motif in its cytoplasmic tail, CD1d traffics to intracellular compartments (e.g. late endosomes, lysosomes) [19, 20]. It is likely here that GPI is exchanged for a different ligand through the assistance of saposins [21]. Saposin is capable of plucking out the temporary ligand from CD1d and replacing it with a new ligand. The saposin primarily responsible for exchanging CD1d bound lipids is saposin B [22]. These newly-loaded CD1d molecules are then re-expressed on the cell surface where they can be recognized by NKT cells (Fig 1).

CD1d Lipid Ligands

A plethora of molecules (predominantly lipids) have been found to serve as ligands for CD1d, and the search continues for even more [23]. The most sought after ligand is the natural endogenous antigen that stimulates NKT cells. CD1d can activate NKT cells without the presence of externally added lipids, suggesting the presence of such a self antigen [9]. Phospholipid antigens such as GPIs which bind CD1d with high affinity, were eluted from CD1d and may be a natural ligand [18, 24]. This affinity and notion of

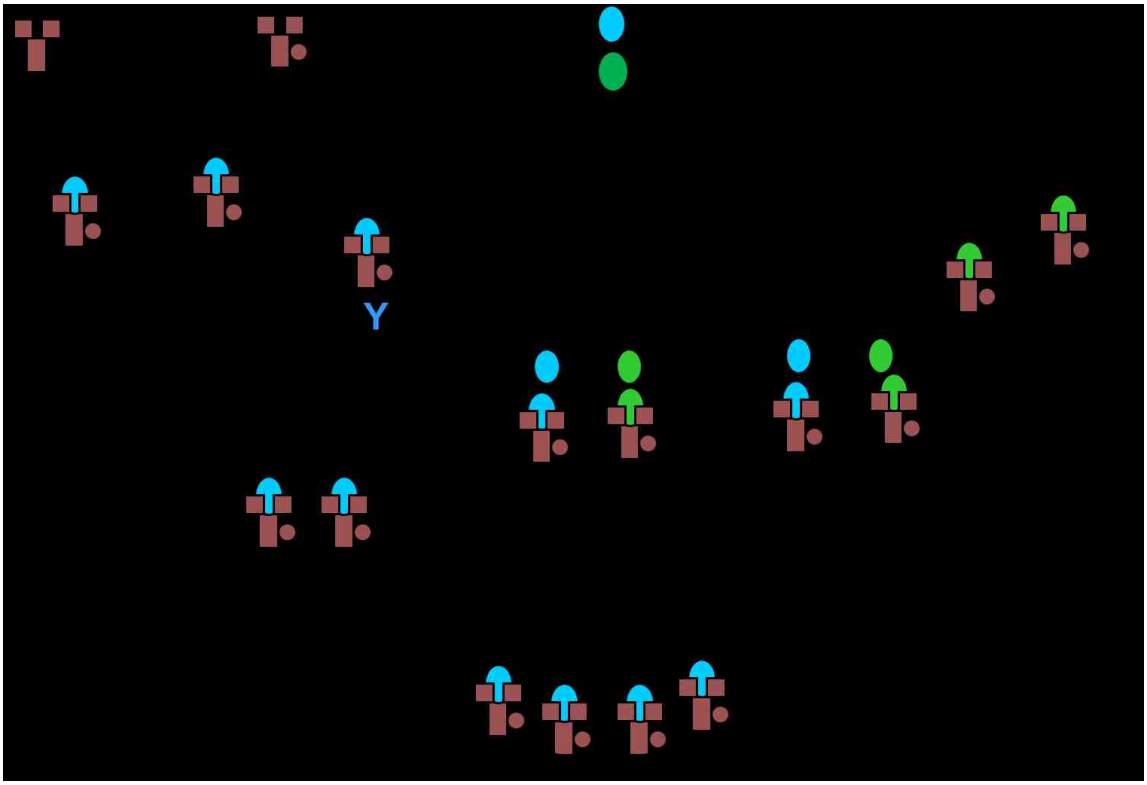


Figure 1. CD1d recycles through the late endosome and lysosome to acquire a lipid antigen. Once protein synthesis of CD1d has occurred within the ER, CD1d, bound to a lipid ligand, trafficks to the cell surface. Through a tyrosine-based motif in the cytoplasmic tail, CD1d can be recycled within the cell and traffic from the early endosome (EE) to either the recycling endosome (RE) or the late endosome (LE) and lysosome (L). Once in the late endosome or lysosome, the natural lipid ligand bound to CD1d is exchanged by saposin B for the lipid antigen that will be expressed on the cell surface and activate NKT cells.

GPI as the natural ligand emerged from the analysis of lipids bound to purified CD1d proteins by mass spectroscopy and metabolic radiolabeling [18]. Using similar methods, another group found phosphatidylcholine and sphingomyelin as CD1d-associated lipids [25].

In addition to self phospholipids, glycosphingolipids are possible natural ligands of CD1d. As evidence for this potential relationship, a cell line with a mutated β-glucosylceramide synthase gene was defective in invariant NKT (iNKT) cell activation, suggesting that lysosomal glycosphingolipids are CD1d natural ligands [26]. Isoglobotrihexosylceramide (iGb3) was found to be a candidate natural lipid ligand for CD1d, after the analysis of mouse mutants that have defects in glycosphingolipid synthesis and degradation pathways [27]. Most importantly, iGb3 was shown to activate iNKT cells when bound to CD1d [27]. Also, NKT cell development is reduced in mice lacking β-hexoaminidase B; this enzyme generates a precursor of iGb3 biosynthesis [28]. These findings support the glycosphingolipid iGb3 as a natural ligand presented by CD1d to NKT cells. However, despite this strong evidence for iGb3 as a natural ligand, recent reports refute its relevance in the development of NKT cells. For instance, iGb3-synthase deficient mice have normal NKT cell development [29]. Also, further investigation indicated that humans and mice lack iGb3, although it was detectable in murine dorsal root ganglia [30]. Despite this, iGb3 remains an NKT cell activating lipid ligand for CD1d (at least *in vitro*). In addition to iGb3, other glycosphingolipids such as the ganglioside GD3, a lipid expressed by tumors of neuroectodermal origin, have been found to be CD1d lipid ligands [31]. Sulfatide (3'-sulfogalactosyl ceramide) is a CD1d glycolipid ligand found in myelin that is specifically recognized by and activates type II

NKT cells [32, 33]. These are all examples of glycolipids that can serve as lipid antigens for CD1d.

It is expected that for CD1d-activation of NKT cells to have relevance in an immune response, microbial invasion of a host should lead to NKT cell activation. Therefore, investigations into potential microbial glycolipids as ligands for CD1d began. The first NKT cell antigen of microbial origin identified was PIM4, a phosphatidylinositol purified from *Mycobacterium bovis*, which caused activated NKT cells to produce IFN-γ, but not IL-4 [34]. From the spirochete *Borrelia burgdorferi*, a diacylglycerol-based glycolipid was shown to activate mouse and human iNKT cells; these also preferentially secreted IFN-γ [35]. Further, lipids from *Sphingomonas* species of bacteria, such as GSL-1 and GSL-4 (after processing into GSL-1) have been shown to activate NKT cells [36-38].

α-galactosylceramide (α-GalCer) is a potent NKT cell activating antigen that leads to elevated NKT cell secretion of IFN-γ and IL-4 [5, 39]. Extracted from the marine sponge, *Agelas mauritianus* is found off the coast of Japan; α-GalCer was part of an investigation into natural immune-potentiating compounds that would combat tumor development *in vivo* [40].

Due to the complexities of the immune system, there are instances when it is preferred to only elicit a Th1 or Th2 response. Because α-GalCer causes iNKT cell to secrete of both Th1 and Th2 cytokines, synthetic analogues of α-GalCer have been genereated that induce the preferential secretion of either Th1 or Th2 cytokines. OCH, the first synthetically modified version of α-GalCer, caused a Th2 bias towards NKT cell secretion of IL-4 but not IFN-γ [41]. Therefore, it is thought that truncation of the phytosphingosine chain, as in OCH, leads to an increased release of IL-4 [42].

Alternatively, the C-glycosidic analogue of α-GalCer, α-C-GalCer, which is created by exchanging the glycosidic oxygen, a polar hydrogen bond acceptor, with a non-polar CH2 group, causes a sustained Th1 response [43].

NKT cells

As mentioned above, NKT cells are a special subset of T lymphocytes that are activated specifically by CD1d presenting its lipid antigen. NKT cells express both NK cell markers such as NK1.1 (in the mouse) or CD161 (in humans) and the T cell receptor (TCR) [44]. The TCR divides NKT cells into two groups: invariant (or type I) NKT cells and variant (or type II) NKT cells. Type I NKT cells express a specific TCR arrangement consisting of Vα14Jα18 paired with Vβ2, 7, or 8.2 (in mice) or Vα24Jα18 with Vβ11 in humans. As their name suggests, variant or type II NKT cells express a diverse range of TCR usage. iNKT cells can express the CD4 and/or CD8 T cell co-receptors and are typically identified as either CD4-CD8- or $CD4^+$ whereas, a small fraction can be $CD8\alpha^+$ [10, 11].

Steady state distribution studies on iNKT cells in mice have shown that iNKT cells comprise about 1% of lymphocytes in the thymus. Interestingly, iNKT cell frequency is highest in the liver, where it makes up approximately 30% of the liver mononuclear cells. iNKT cells also express a high level of activation markers such as CD44 and CD69 on the cell surface and low expression of CD62L [12]. Unlike conventional $CD4^+$ or $CD8^+$ T cells, NKT cells do not always require classical costimulatory signals to be activated after TCR engagement [45]; costimulatory molecules have been shown to be important in NKT cell activation [46]. Once activated by TCR engagement, NKT cells are capable of

rapidly producing a high level of Th1 cytokines, such as IFN-γ, IL-2 and TNF-α as well as Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13 [10, 39]. This rapid secretion of cytokines may be explained by data suggesting the presence of preformed cytokine-encoding mRNA transcripts in the iNKT cell cytoplasm, which would allow for a quick release of cytokines and is therefore a rapid effector response [47]. In addition to these cytokines, hematopoietic growth factors such as IL-3 and GM-CSF have been reported to be secreted by NKT cells upon activation [48]. Recently, IL-17 [49, 50], a potent proinflammatory cytokine associated with autoimmune disease, and IL-21 [51], was shown to be produced by activated NKT cells. In addition to cytokine release, NKT cells possess cytotoxic functions through the upregulation of FasL, granzyme B, and perforin [50, 52-54]. Interestingly, some reports suggest that cytokines alone, such as IL-12 secreted by LPS-activated dendritic cells, may be adequate to activate NKT cells without TCR engagement of lipid-loaded CD1d molecules [55].

As suggested by the many cytokines produced, NKT cells can have effects on many other immune cells. For instance, NKT cells play a role in promoting the development of peripheral monocytes into dendritic cells (DCs) through the secretion of IL-13 and GM-CSF [56, 57]. Also, activated NKT cells induce DC production of IL-12 which promotes Th1 polarization, in addition to upregulating MHC class II and costimulatory molecules on the DC cell surface [58, 59]. Finally, activated NKT cells enhance the migration of DCs to lymph nodes [60]. In addition to affecting DCs, NKT cells have been shown to transactivate NK cells. This is thought to be through NKT secretion of IFN-γ as well as IL-2 and IL-12 [14, 61, 62]. After α-GalCer-induced activation of NKT cells, NK cells have increased cell surface expression of the activation marker CD69 and secrete vast

amounts of IFN-γ [14]. Activated NKT cells also provide help to B cells through cell surface upregulation of the costimulatory molecule B7.2 and MHC class II molecules on splenic B cells [14]. This activation of B cells has been shown to be through NKT cell secretion of the Th2 cytokine, IL-4 [63, 64]. However, others have shown that NKT cells directly interact with B cells via CD1d lipid presentation; further, CD40L, B7.1, and B7.2 interactions between NKT and B cells leads to an antibody class-switch [65, 66]. Macrophages and other antigen presenting cells like dendritic cells and B cells have enhanced phagocytic activity upon activation by NKT cells [67]. Finally, through the secretion of cytokines such as IL-2 and IFN-γ, activated NKT cells increase the proliferation of $CD4^+$ and $CD8^+$ T cells and may induce the upregulation of the activation marker CD69 [14, 68]. As mentioned above, NKT cells enhance DC maturation, which can enhance the priming of naïve T cells. All these observations indicate that NKT cells have a great influence in generating an immune response.

NKT cells and Viral Infections

CD1d and NKT cells are important to the antiviral immune response. Viruses have developed the means to avoid activation of NKT cells upon infection. For example, herpes simplex virus (HSV)-infected cells are able to evade NKT cell recognition by preventing endocytosed CD1d from recycling back to the cell surface [69]. NKT cells seem to be of further importance in an HSV infection due to a low survival weight in HSV-infected NKT cell-deficient mice [70]. The HIV Nef protein is also able to inhibit NKT cell activation by downregulating CD1d from the cell surface [71, 72]. It is thought that NKT cells themselves are a target of HIV, due to their expression of CD4 and CCR5,

with a depletion of the NKT cell population as long as one year after infection. This is reversed through treatment with IL-2 or highly active antiretroviral therapy (HAART) [73, 74]. Activation of NKT cells also seems to be important for immunity against influenza A virus, due to the finding that NKT cell-deficient mice are more susceptible to this virus [75]. Consistent with this, adoptive transfer of iNKT cells reduced influenza A virus titers *in vivo* [75]. Additionally, our laboratory has shown that Vaccinia virus (VV), through the function of the VV-encoded molecules, B1R and H5R, inhibits CD1d-mediated antigen presentation, as another means by which viruses alter NKT cell activity [76]. Overall, these data indicate that NKT cells are important for the antiviral immune response due their direct (or indirect) effect on a variety of viruses.

NKT cells and Cancer

NKT cells play a dual role in cancer by their ability to both promote and inhibit antitumor immunity. Upon activation of NKT cells by α-GalCer when injected into mice, protection against a variety of tumors was observed [40, 77]. This protective activity was dependent on IFN-γ production by NKT cells and subsequent activation of NK cells, and possibly CTLs [78]. The NKT cells responsible for protection against tumors were $CD4^-$ and $CD8^-$ and predominantly present in the liver [79]. Compared to the injection of free α-GalCer, *in vivo* inoculation of dendritic cells pulsed with α-GalCer is capable of inducing more potent NKT cell protection against tumors; this was due to an elevated secretion of IL-12 and IL-15 by the DCs [80]. These studies led to human clinical trials in which α-GalCer was tested as anti-cancer therapy. Although the use of both free α-GalCer and α-GalCer pulsed DCs were tested and some immune responses

were obtained, no significant clinical benefit was achieved [81, 82]. In contrast to the protective role observed, NKT cells, through IL-13 secretion, induce myeloid cell secretion of transforming growth factor (TGF) β which ultimately inhibits CTL function [83]. Overall, NKT cells are important for anti-tumor immunity, yet in other instances can work to negate such immunity.

TGF β and the Immune System

Because CD1d/NKT cell interactions play an important role in eliciting an immune response against viral infections and cancer, our interests centered on regulation of CD1d-mediated antigen presentation and NKT cell activation. This brought the focus TGF β, a known immune regulator. In mammals, TGF β has been identified as having three isoforms, TGF β1, β2, and β3. However, TGF β1 is the isoform predominantly expressed by cells of the immune system and is believed therefore to be a cytokine with immuno-modulating roles. In fact, mice deficient in TGF β1 develop a multiorgan autoimmune inflammatory disease and usually die within a few weeks after birth [84]. This finding suggests that TGF β plays many roles in immune control and could be a potent immune regulator.

Early studies showed the inhibitory effects of TGF β on NK cells. NK cells are innate lymphoid cells that can lyse virus-infected and transformed cells, as well as produce chemokines and cytokines during an immune response. Rook et al., showed suppressed cytolytic capabilities by NK cells after treatment with TGF β, in addition to a TGF β-dependent reversal of IFN-α activation of NK cells [85]. Subsequently, others showed that this inhibition of NK cell cytolytic activity was due to TGF β inhibiting NKp46 and

NKG2D, receptors essential for target cell killing by NK cells [86]. Specifically, TGF β inhibited NKG2D expression on NK cells and $CD8^+$ T cells in individuals with glioma [87]. Also, in patients with lung and colorectal cancer, elevated levels of TGF β in the serum have been associated with a downregulation of NKG2D on NK cells [88]. Important for the control of pathogen invasion, NK cells produce IFN-γ, a cytokine that promotes the development of Th1 T cells. TGF β suppresses NK cell production of IFN-γ which leads to a decreased Th1 response against *Leishmania major*. By signaling through Smad3, TGF β suppresses the production of IFN-γ. Downstream, Smad3 suppresses T-bet, a transcription factor necessary for IFN-γ production [89]. All of these findings provide evidence that TGF β regulates NK cell immune function.

Dendritic cells (DCs) are vital to the immune system as professional antigen presenting cells. DCs can activate T cell-mediated adaptive immunity, as well as NK cells. Activation of immature DCs occurs after exposure to pathogens, and involves the upregulation of MHC and co-stimulatory molecules. Although important in the immune response, TGF β can modulate DCs in several ways as discussed below. TGF β can immobilize DCs, which can interfere with their *in vivo* trafficking and ability to present antigen to immune cells in lymph nodes [90]. Also, TGF β may induce apoptosis in DCs [91]. Further, DCs in the tumor microenvironment can secrete TGF β. This TGF β can downregulate the expression of MHC class II molecules and the co-stimulatory molecules CD40, CD80 and CD86 on DCs [92]. Antigen presentation without costimulation can result in T cell tolerance. Also, DCs through the secretion of TGF β, can promote the formation of T regulatory (Treg) cells which inhibit the function of other T cells [93]. These observations point to TGF β-mediated control of DC function.

The evidence discussed above indicates that TGF β plays an important role in immune tolerance, so it is expected that TGF β would also affect effector T cells. A decreased number of tumor-specific cytotoxic T lymphocyte (CTL) in the tumor microenvironment with impaired function is caused by TGF β-induced signaling within these CTLs [94]. This can be detrimental to tumor clearance, because there is a direct correlation between the frequency of CTLs and the overall survival of cancer patients [95]. This is supported by evidence showing that blocking of TGF β signaling in CTLs results in tumor clearance and increased numbers of CTLs at the site of the tumor [96]. Also, $CD8^+$ T cells with blocked TGF β signaling using a TGFβRII dominant-negative construct were more successful in infiltrating the tumor, secreting cytokines such as IFN-γ, and killing tumor cells upon adoptive transfer [97, 98]. In fact, systematic blockade of TGF β using a monoclonal antibody prevents tumor recurrence and increases the level of CTL cytotoxic activity [99]. This inhibition of CTL activity by TGF β has been shown to be through the regulation of specific cytolytic genes encoding granzyme A, granzyme B, IFN-γ and FAS ligand [94]. TGF β can also influence T cell differentiation due to its ability to inhibit T-bet and GATA3 expression [100]. In addition to this, TGF β can also suppress the Th1 response by shifting T cells to a Th2 response [101]. These findings show TGF β is a regulator of T cell function and differentiation.

The previous sections focused on TGF β regulation of $CD8^+$ T cells, but TGF β can also influence $CD4^+$ T cells, specifically, T regulatory cells (Tregs). Tregs are a special subset of T cells that play a role in regulating T cell activation. Tregs are identified by the fork head box P3 (FOXP3) and CD25 markers that are upregulated in $CD4^+$ T cells upon TGF β signaling and differentiation into Tregs [102]. Two types of T regs exist: natural

Tregs (nTregs) and induced Tregs (iTregs) [103]. TGF β is responsible for inducing the development of iTregs from naïve T cells. In support of this, TGF β blockade results in fewer Tregs in tumor-bearing mice [104].

Another subset of immune cells that is regulated by TGF β is Th17 cells, a population of $CD4^+$ T cells identified by their production of the cytokine IL-17 [105-107]. Th17 cells are pro-inflammatory cells found mostly in the digestive tract and are connected to the autoimmune disease, experimental autoimmune encephalomyelitis. Similar to the upregulation of FOXP3 to generate Tregs, TGF β upregulates the transcription factor retinoic acid receptor-related orphan receptor-γt (RORγt) through the Smad2 pathway, to generate Th17 cells [108]. Veldhoen et al. found that the combination of TGF β and IL-6 causes the differentiation of T cells into Th17 cells [107, 109]. Unlike the effects on the other T cell subsets mentioned above, in this case, TGF β acts to promote an immune response through its differentiation of Th17 cells, rather than negatively regulating immune responses.

TGF β and Cancer

Like NKT cells, TGF β also plays a role in cancer. TGF β is involved in cancer progression as both a tumor suppressor in the early stages and as a tumor promoter in the later stages of cancer development. TGF β was first shown to be a tumor suppressor when it was observed that mutations in the genes encoding TGFβRI and TGFβRII were reported in cancer [110]. Specifically, mutations in TGFβRII are very frequent in tumors [110]. In addition to this, decreased levels of Smads are associated with a poor prognosis in cancer patients [111]. Further, downregulated Smad pathway activation by TGF β

resulted in more malignant phenotypes of tumors in mice [112]. Also in support of all this evidence of TGF β as a tumor suppressor, it was shown that the abrogation of TGF β signaling leads to the spontaneous development of carcinomas [113]. These data support a role for TGF β as a tumor suppressor.

Alternatively, TGF β can act as a strong promoter of tumor progression. Using a mouse model, constitutively active TGFβRI and TGFβRII expressed in mammary epithelial cells led to increases in metastases, whereas the inhibition of TGF β signaling resulted in a decrease [114]. In line with these data, a mutation in the TGFβRII gene is correlated with a positive prognosis in individuals with colon cancer [115]. As further evidence of TGF β as a tumor promotor, it has been shown that elevated TGF β-dependent activation of the Smad pathway is present in aggressive gliomas and associated with a poor prognosis in patients [116]. All of this evidence supports the conclusion that TGF β can act as a tumor promotor as well as a tumor suppressor.

TGF β and Viral Infection

TGF β plays a role in viral immunity. TGF β has been shown to be elevated in response to a viral infection. In fact, it has been shown that a chronic infection with hepatitis C virus (HCV) induces the upregulation of TGF β via the HCV NS4 protein [117]. The Th1 immune response is important for control of a HCV infection, yet the upregulated TGF β was shown to inhibit this Th1 response, as well as suppress Th17 cells [118]. Another virus to upregulate TGF β is human cytomegalovirus, which induces the transcription and secretion of TGF β by infected cells [119]. Also, TGF β was shown to enhance rhinovirus replication as determined by increased viral RNA, viral

protein synthesis, and eventually higher levels of cellular virus release [120]. The persistent infection caused by LCMV clone 13 is due to upregulated TGF β, which inhibits the function of virus-clearing $CD8^+$ T cells, through the upregulation of Smad2 activation [121]. These same $CD8^+$ T cells are induced to produce TGF β by human immunodeficiency virus (HIV), which leads to inhibited IFN-γ responses [122]. These data support the idea that viruses induce the production of TGF β, which in most cases leads to a suppressed immune response.

TGF β and NKT cells

With such evidence as described above, demonstrating that TGF β has a regulatory role in effector T cell function, it would seem likely that TGF β would also play some role in NKT cell function or differentiation. In fact, it has been found that TGF β suppresses NKT cells in cancer patients. Treatment of α-GalCer-activated iNKT cells with 10 ng/mL TGF β for 24 hours resulted in decreased iNKT cell proliferation, IFN-γ production and CD25 expression [123]. More interestingly, this control by TGF β possibly influences NKT cell differentiation. Early work in this area showed that knockout of the TGFβRII under a CD4 promotor using a cre-lox system resulted in the depletion of α-GalCer-reactive type I NKT cells in the thymus and spleen [124]. These results suggest that TGF β signaling is required for thymic development of type I NKT cells. To support this, another group also using a CD4 promotor-driven knockout of TGFβRII produced similar results; however, they defined the deficiency of NKT cells further. Mechanistically, Smad4 signaling via TGF β was required for the promotion of

NKT cell development [125]. In conclusion, TGF β can also regulate the activation, function, and development of NKT cells.

TGF β and Antigen Presentation

Due to having essential roles in immune control, it is conceivable that TGF β may regulate antigen presentation and ultimately, CD1d-mediated antigen presentation. It has been found that TGF β can downregulate IFN-γ-induced MHC class II cell surface expression [126]. Other groups also found that TGF β actually inhibits antigen presentation by MHC class II [127, 128]. Due to the similarities between MHC class II and CD1d as antigen presenting molecules, it seems likely that TGF β may also alter CD1d-mediated antigen presentation. In fact, Ronger-Savle et al., found that when they treated human Langherhan cells with TGF β, they saw a downregulation of CD1d on the cell surface as determined by flow cytometry [129]. Also, TGF β treatment caused a decrease in CD1d mRNA expression [129]. In addition to this, tumor-bearing mice with elevated levels of serum TGF β had lower levels of CD1d expression on DCs [130]. Moreover, dominant negative (DN) TGF β receptor (TGFβR) II transgenic mice have elevated levels of NKT cells in the liver [131]. All these observations provide evidence that TGF β has a negative effect on the functional expression of CD1d. Therefore, the studies presented here were begun under the hypothesis that TGF β inhibits CD1d-mediated antigen presentation. Another connection between CD1d-mediated antigen presentation and TGF β is p38 activation. TGF β can phosphorylate and activate p38 [132]; interestingly, CD1d-mediated antigen presentation is inhibited by p38 activation [133]. Therefore, we hypothesized that if TGF β inhibits CD1d-mediated antigen

presentation, it is likely through the activation of p38. The hypothesis that signaling cascades may be mechanistically involved in TGF β inhibition of CD1d-mediated antigen presentation, brought into consideration other pathways associated with TGF β signaling. Thus, the Smad pathway, the primary pathway associated with TGF β, and the Rho/ROCK pathway remain considerations and will be discussed further.

TGF β Signaling

TGF β is a potent cytokine capable of activating many pathways [134, 135]. The pre-pro-TGF β precursor is the early, inactive form of synthesized TGF β [136]. This pre-pro-TGF β is a dimeric proprotein consisting of mature TGF β bound to a latency-associated peptide [137] [138]. This LAP/TGF β combination then binds to the latent TGF β-binding protein (LTBP) [139]. This trimeric complex of LTBP/LAP/TGF β is secreted by cells [140]. Low pH or proteolysis is required to liberate mature TGF β, which is then able to bind TGF β receptor II (TGFβRII) [141]. The complex of TGF β bound to TGFβRII recruits and binds TGFβRI [141, 142]. A TGF β receptor III, known as betaglycan, binds to the two receptors and has no known function other than to stabilize the receptor/ligand complex [141, 143]. Once formed, this ligand and receptor complex is internalized [144]. TGFβRI and TGFβRII are structurally similar serine/threonine kinases; however, the TGFβRI has a conserved Gly/Ser-rich "GS sequence" immediately upstream from the kinase domain [145]. Ligand binding allows for the stable formation of a ligand/receptor complex. This complex leads to TGFβRII

phosphorylation of TGFβRI GS sequences [146], and activates the TGFβRI kinase [147], resulting in eventual phosphorylation of the Smad proteins.

The Smads are the main and best-studied effectors of TGF β signaling [148]. Smads can be subdivided into three groups: R-Smads (receptor Smads); (Smad2 [149] and Smad3), Co-Smads (Smad4), and inhibitory Smads (I-Smad: Smad 7). R-Smads and Smad4 contain a MAD homology 1 (MH1) (N-terminus) and an MH2 (C-terminus) domain linked by a conserved linker region. The MH1 domain is responsible for DNA binding and interaction with transcription factors [150], whereas the MH2 domain is responsible for receptor interaction and Smad oligomerization [151]. The R-Smads have a C-terminal SXS motif in which both serines are targeted for direct phosphorylation by the TGFβRI. Upon ligand binding, TGFβRI recruits and phosphorylates both Smad2 and Smad3 [152]. Facilitating this interaction of Smad2 with the TGF β receptor complex is the Smad Anchor for Receptor activation (SARA), which recruits Smad2 to TGFβRI at both the cell surface and in early endosomes [153-155]. Once the TGFβR complex forms and binds Smad2 through SARA, this complex can be internalized into the early endosome as shown by the association of SARA with the early endosome associated protein 1 (EEA-1) [156]. C-terminal SXS phosphorylation then leads to conformational changes of the R-Smads, their disassociation from the TGFβRI and the formation of a complex consisting of Smad2, Smad3 and Smad4 [157]. This trimeric complex translocates to the nucleus and acts as a transcription factor in association with co-activators [158, 159]. Smad7, an I-Smad, is capable of inhibiting TGFβRI activation of Smad2 and Smad3 by binding to the TGFβRI, which inhibits the recruitment and

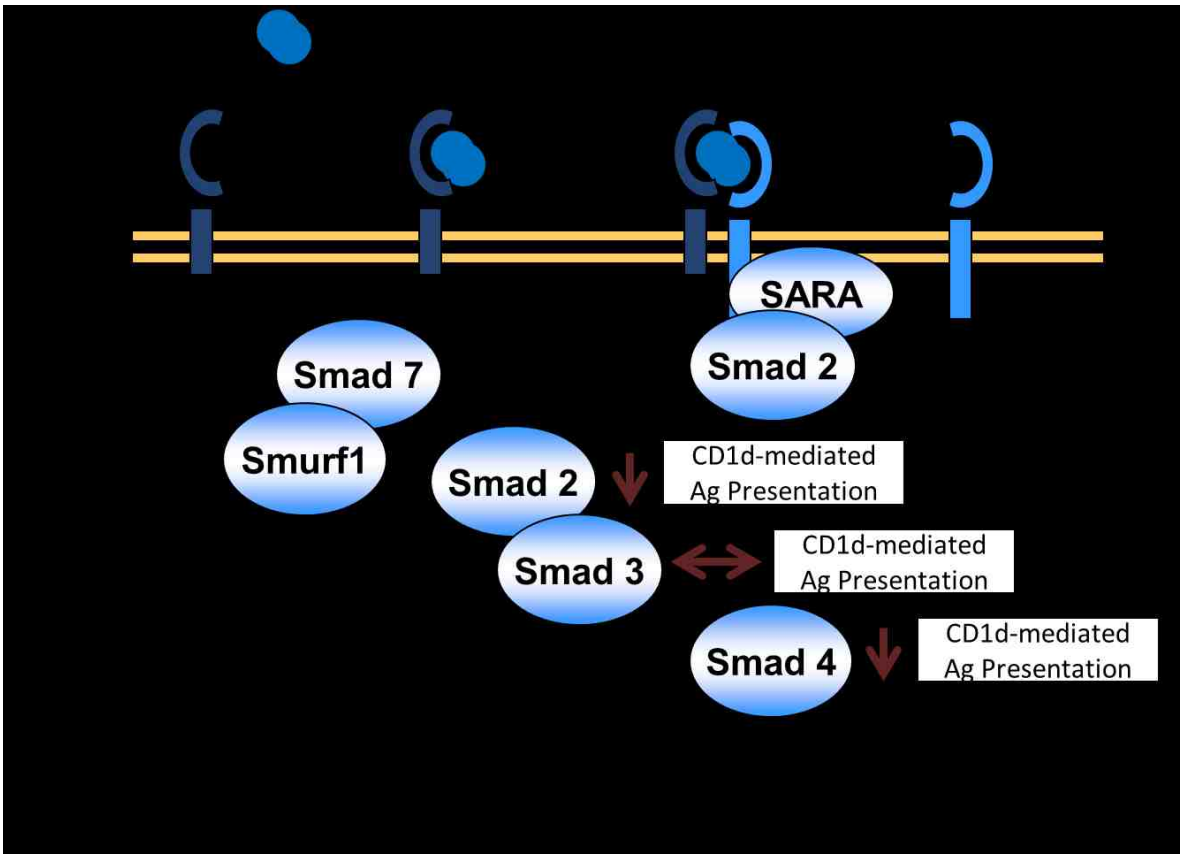


Figure 2. TGF β activates the Smad pathway. Activated TGF β receptor I, a serine/threonine kinase, recruits Smad 2 through SARA and phosphorylates Smad2 and Smad3 which allows the formation of the Smad2/Smad3 complex. Once bound, Smad4 will bind Smad2 and Smad3 and this trimeric Smad complex will move into the nucleus to upregulate genes. Smad7 is an inhibitor of Smad2 and Smad3 phosphorylation that bind TGF β receptor I and recruits Smurf1, an E3 ubiquitin ligase that targets the TGF β receptor complex for degradation. The effects of Smad2, 3 and 4 on CD1d-mediated antigen presentation is indicated as well.

phosphorylation of Smad2 and Smad3 [160]. As another means of inhibiting TGF β signaling through the Smad pathway, Smad 7 can recruit Smurf1 or Smurf2 proteins, ubiquitin ligases that target the TGF β receptor complex for degradation by ubiquitination [161, 162] (Fig 2).

In addition to activating Smads, TGF β can activate mitogen-activated protein kinase (MAPK) signaling. Specifically, TGF β has been shown to activate the stress-induced MAPK, p38. As a MAPK, p38 is typically activated by MAPKK, such as MKK3, MKK4, or MKK6 [163-165]. These MAPKKs can be phosphorylated and activated by TAK1 [132]. It is at the cell surface that TAK1 is activated by the TGF β receptor I (TGFβRI). TGF β, an immune regulatory cytokine, activates TGFβRI by binding to it and TGF β receptor II (TGFβRII) [166]. p38, like Smads, can be activated by TGF β signaling [132] (Fig 3).

The cytokine TGF β causes the formation of stress fibers, long parallel bundles of actin chains. Vardouli et al., have shown that this formation of stress fibers is through the TGF β-dependent activation of the Rho GTPases and the resulting pathways [167]. Activation of Rho-GTPases is achieved through the function of guanine exchange factors (GEFs) [168]. GEFs facilitate the exchange of GDP bound to Rho for GTP. Rho-GTP signals downstream by binding to the Rho-associated kinase (ROCK) at the Rho binding domain (RBD) [169] and changing the protein conformation of ROCK [170]. As an activated kinase, ROCK phosphorylates LIM domain kinase (LIMK) 1 and LIMK2 [171, 172]. These LIMKs then phosphorylate cofilin [173], a regulator of actin polymerization [174]. In the unphosphorylated state, cofilin prevents actin from polymerizing into long

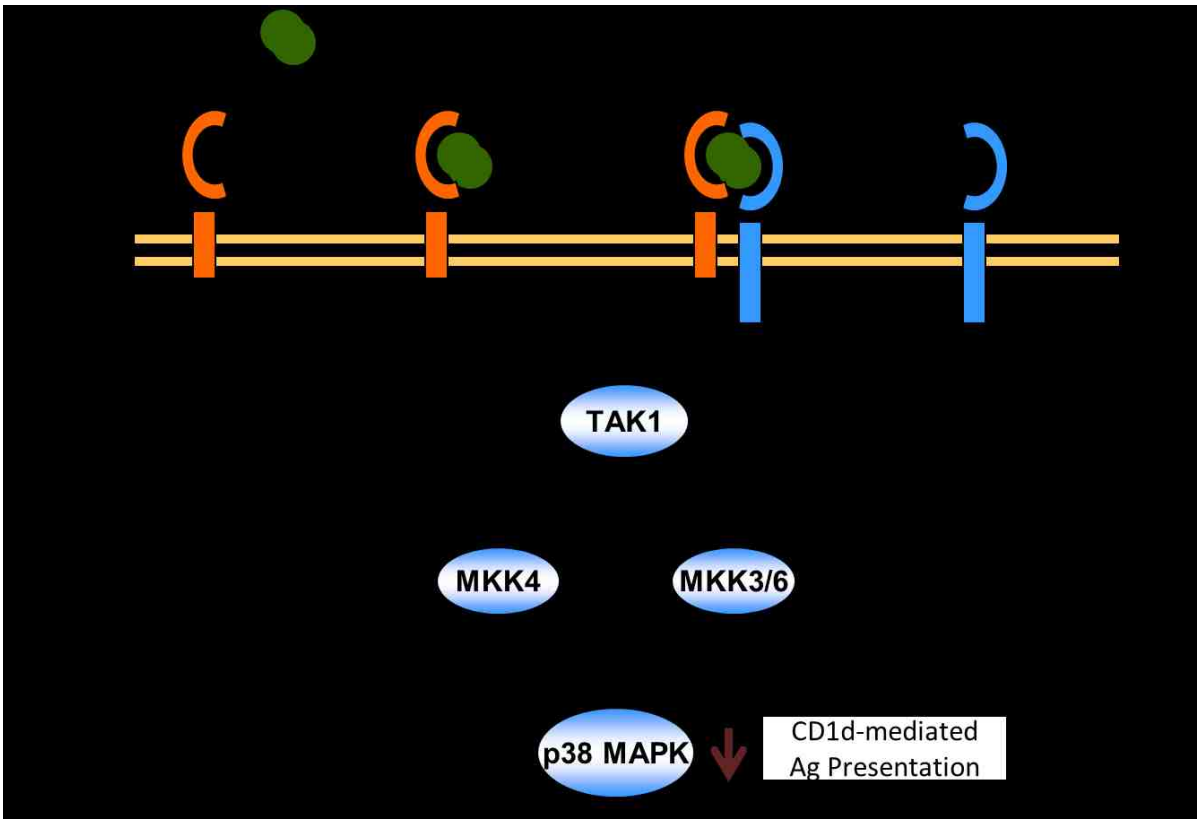


Figure 3. TGF β activates the p38 pathway. Once activated by TGF β ligand binding, TGF β receptor I phosphorylates TAK1. Activated TAK1 is capable of phosphorylating and activating the MAPKK's, MKK4, MKK3, or MKK6. All three of the MAPKKs phosphorylate and activate the MAPK, p38. The effect of p38 MAPK on CD1d-mediated antigen presentation is also indicated.

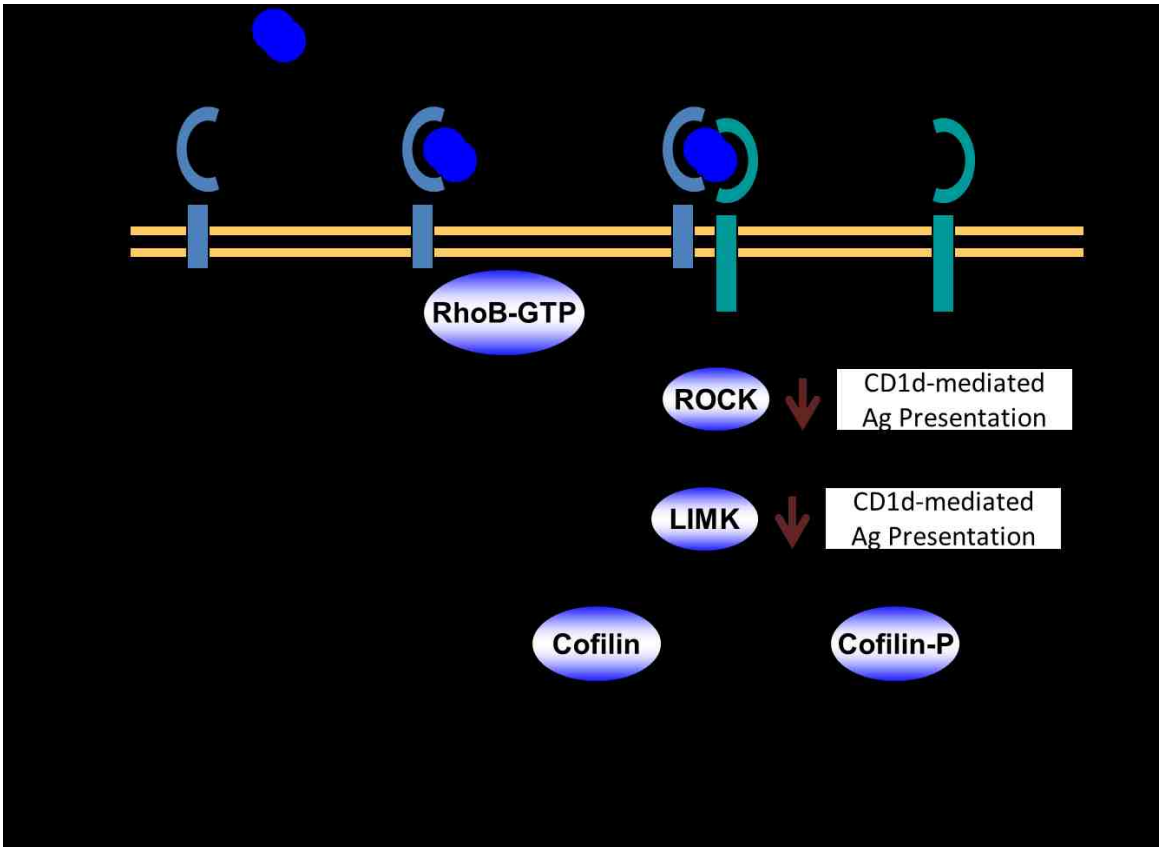


Figure 4. TGF β activates the ROCK pathway. TGF β receptor induces the activated of RhoB. RhoB binds ROCK, changes its conformation and causes the activation of ROCK. As a kinase, ROCK phosphorylates and activates another kinase, LIMK. Continuing the pathway, LIMK phosphorylates cofilin, a regulator of actin polymerization. Once phosphorylated, cofilin activity is inhibited which leads to uncontrolled actin polymerization and thus, stress fiber formation [177]. The effect of ROCK and LIMK on CD1d-mediated antigen presentation is indicated.

chains [137, 175]. When phosphorylated by LIMK, cofilin is inactivated and F-actin, known as stress fibers, will form [176] (Fig 4).

Interestingly, several of these pathways that are activated by TGF β overlap and can regulate each other. In support of this, a follow-up publication by Vardouli et al., provides evidence that activation of the Smad pathway, specifically Smad2 and Smad3, leads to the upregulation of RhoB gene expression [178]. Others have shown that gene regulation by the Smad pathway can cause the upregulation of RhoA, RhoB, and RhoC expression. Also, these pathways can cooperate with one another, as the p38 and Smad2 pathway do in the induction of collagen synthesis by TGF β [179]. Additionally, the Smad2 pathway can cross-talk with the p38, ERK1/2 and JNK pathways to induce collagen IV synthesis through MAPK phosphorylation of the linker between the MH1 and MH2 regions of Smad2. Moreover, these investigators found that expression of a Smad2 dominant negative construct led to less TGF β-induced phosphorylation of JNK and ERK1/2, suggesting that the regulation of these pathways can be bidirectional [179]. In conclusion, the TGF β pathway can be more complex than simple single directional signaling.

Mechanisms of Regulating CD1d

The encompassing hypothesis of the studies described here is that TGF β will inhibit CD1d-mediated antigen presentation. Regarding, a signaling mechanism behind this inhibition, the p38, Smad, and Rho/ROCK pathways will be considered and investigated. However, if TGF β does inhibit CD1d-mediated antigen presentation, there are non-signaling mechanisms to consider. The first is CD1d cell surface expression. A decrease

in CD1d cell surface expression caused by TGF β could account for a change in antigen presentation. Additionally, CD1d may not be getting to the late endosome or lysosomes, where lipid exchange and loading occurs (Fig 1). Also, there is the rate at which CD1d recycles from the late endosome and lysosome to the cell surface that should be taken into consideration. If the rate of CD1d recycling is slowed then following then following TGF β treatment, antigen-loaded CD1d may have trouble moving to the cell surface, which could reduce NKT cell activation. Alternatively, if the rate of recycling is enhanced, proper lipid loading of CD1d may not occur. Finally, if endogenous antigen presentation is altered by TGF β, this raises the question of whether exogenous antigen presentation is regulated; this can be evaluated through the addition of an exogenous antigen such as α-GalCer. In the course of testing the hypothesis that TGF β inhibits CD1d-mediated antigen presentation and determining a signaling mechanism, these non-signaling mechanisms will also be investigated.

Materials and Methods

Cell lines and other reagents

Murine LM thymidine kinase-deficient (LMTK)-CD1d cells (mouse LMTK cells transfected with the murine *cd1d1* cDNA) were cultured in Dulbecco's modified eagle medium (DMEM) (BioWhittaker/Lonza) supplemented with 10% fetal bovine serium (FBS) (Hyclone), 2 mM L-glutamine, and 500 μg/ml G418 (D^+ G418). Transforming Growth Factor β was purchased from Peprotech, Inc. The mouse CD1d-specific NKT cell hybridomas, DN32.D3, N38-2C12, and N37-1A12 were cultured in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker/Lonza) supplemented with 5% FBS and 2mM L-glutamine. Purified and biotinylated mAbs specific for murine IL-2 were purchased from BD Biosciences. Recombinant IL-2 used as a standard in enzyme-linked immunosorbent assay (ELISA) assays and recombinant IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) was obtained from Peprotech, Inc (Rocky Hill, NJ). Antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), p38, phosphorylated Smad2, as well as total Smad2, Smad3, and Smad4 used in Western blot were purchased from Cell Signaling Technologies, Inc. The compounds Y-27632 and cytochalasin D were purchased from Sigma.

Generati[o]n of bone marrow-derived dendritic cells (BMDCs)

Female C57BL/6 mice were purchased from The Jackson Laboratory. All procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. After sacrificing 15 mice, femurs and tibias were removed by cutting into the hip bone and below the ankle. Next, the muscle was carefully removed to prevent bone damage. The bones were sterilized with 70% ethanol and washed with Roswell Park Memorial Institute medium (RPMI) 1640 (BioWhittaker) containing 1% FBS. Finally, the bone tips were snipped and the bones were flushed with 10 mL RPMI 1640 containing 1% FBS using a 27 gauge needle attached to a 10 mL syringe. Subsequently, bone marrow cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 50 mM 2-ME, 10% FBS and antibiotics, as well as 10 ng/ml each of murine GM-CSF and IL-4. On day 6, BMDCs were treated with 10 ng/ml LPS for 24 hours. On day 7, the plates were gently flushed (three or four times) to remove the loosely adherent cells which were subsequently used in analyses as BMDCs.

TGF β Treatment

A stock solution of TGF β was generated by reconstituting 5 mg lyophilized TGF β-1 (Peprotech, Inc.) in 100 μL 10 mM citric acid. Subsequently this solution was diluted further as 10 μL in 240 μL of 2 mg/mL BGS/PBS to make a stock solution of with a final concentration of 2 μg/mL TGF β. The vehicle control stock consisted of 10 μl of citric acid in 2 mg/mL BGS/PBS. LMTK-CD1d cells [untransfected, shRNA negative control (NC), or shRNA] were pre-plated overnight at 2.5 x 10^5 cells per well in a 96 well flat-bottom plate (Costar) with 200 μL of D^+G418. TGF β was diluted in Assay Buffer

(IMDM supplemented with 10% FBS (Hyclone), 2 mM L-glutamine, and Penicillin/Streptomycin or Serum Free Media supplemented with 2mM L-glutamine to make concentrations of 0 ng/mL (Control), 1, 5, 10 and 20 ng/mL. TGF β and vehicle were added to the appropriate wells containing the cells for 24 hours. Alternatively, $1x10^7$ cells (sufficient cells for both an NKT cell assay and FACS analyses) were added to 7 mL of medium containing 0, 1, 5, 10, and 20 ng/mL TGF β in 50 mL conical tubes and incubated at 37°C for 24 hours in a water bath. (Note: both methods of treatment produced similar results). Following treatment, the cells were used as targets in an NKT cell co-culture assay and for FACS analysis.

NKT cell coculture

LMTK-CD1d1 cells or BMDCs were treated with different concentrations (0, 1, 5, 10, and 20 ng/ml) of TGF β for 24 hours or for the indicated time course. The cells were then washed twice with PBS, fixed in 0.05% paraformaldehyde for 15 min on ice and washed twice again with PBS. To pre-plated cells, 100 mL of Assay Buffer was added to each well. LMTK-CD1d cells treated in conical tubes were distributed into well of a 96 well plate at $5x10^5$ cells per well in 100 μl. NKT hybridoma cells were harvested and plated in triplicate at $5x10^4$ cells in 100 μl per well. The plates were incubated at 37°C for 22 to 24 hours and the supernatants were harvested. To measure exogenous antigen presentation, cells were treated with 100 ng α-galactosylceramide (α-GalCer) for one hour in the presence of TGF β following the initial 24 hour TGF β treatment. The cells were washed, fixed, washed again, and cocultured with the indicated NKT cell

hybridomas as above. IL-2 secreted by the NKT hybridoma cells served as an indicator of CD1d-mediated antigen presentation, and was measured by ELISA.

IL-2 ELISA

96 well ELISA NUNC high protein-binding plates were coated with 50 μL per well of 20 mg/mL purified rat anti-mouse IL-2 antibody (BD Biosciences) and incubated overnight at 4^{o}C. The plates were washed twice with PBS-Tween (5%) and blocked for 2 hours at RT with 200 μL per well of 10% bovine growth serum in PBS. After washing the plates twice with PBS-Tween, 50 mL of the NKT cell co-culture supernatant and the IL-2 standards were added. To make IL-2 standards (Peprotech, Inc.), stock IL-2 (2 μg/mL) was first diluted initially in Assay Buffer for a final concentration of 2000 pg/mL and then two-fold serially diluted to 31.5 pg/mL. Samples and standards were incubated overnight at 4^{o}C. Plates were washed four times and 50 μL of 20 mg/mL of biotin-labeled rat anti-mouse IL-2 antibody (BD Biosciences) was added and incubated at room temperature for 30 minutes. After washing the plates four times, 50 μL of avidin peroxidase (Sigma) was added for 30 minutes at room temperature. Plates were washed again four times, 50 μl of hydrogen peroxide (1:1000) in 2,2'Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Sigma) was added, incubated for 10 minutes at room temperature, and absorbance was recorded at 405 nm in a microplate reader. IL-2 concentrations in the supernatant were determined by BioRad Microplate reader software.

Smad2 short-hairpin RNA (shRNA) plasmids and stable cell lines.

To generate cell lines that stably expressed shRNA targeted against Smad2 or a control (nonspecific) sequence, we used the lentiviral-based pLKO.1 vector (Sigma). Only one shRNA plasmid was able to successfully knockdown Smad2 expression, as determined by Western Blot. This plasmid targets the mouse DNA coding sequence number 926 (5' CCGGTGGTGTTCAATCGCATACTATCTCGAGATAGTATGCGATTGAACACCAT TTTTG 3'). The purified plasmid was transformed into DH5α *Escherichia coli.* Ampicillin selected clones were propagated and a large plasmid preparation was generated (Qiagen). The plasmid was transfected into LMTK-CD1d and grown in the presence of 3 μg/mL of puromycin.

Smad3, Smad4, ROCK1, LIMK1, and LIMK2 short-hairpin RNA (shRNA) plasmids and stable cell lines.

To generate cell lines that stably expressed shRNA targeting Smad3, Smad4 or a control (nonspecific) sequence, we used the pLKO.1 vector. The negative control shRNA construct was 5'-TCAGTCACGTTAATGGTCGTT-3'. The shRNA construct used to target ROCK1 mRNA was 5'-GCTCGAATTACATCTTTACAA-3'. The shRNA construct for LIMK1 was 5'-GATGGTGATGAAGGAACTTAT-3' and for LIMK2 it was 5'-GATGCACATCAGTCCCAACAA-3'. For Smad3 and Smad4, only the validated constructs were purchased, which consisted of constructs that targeted the mouse DNA coding sequence 1137 (Smad3; 5'CCGGCTGTCCAATGTCAACCGGAATCTCGAGATTCCGGTTGACATTGGACA

GTTTTTG 3'), 1416 (Smad4;
5'CCGGGCTTACTTTGAAATGGACGTTCTCGAGAACGTCCATTTCAAAGTAAG
CTTTTT 3'), and 1925 (Smad4;
5'CCGGGCGATTGTGCATTCTCAGGATCTCGAGATCCTGAGAATGCACAATCG
CTTTTT). Ampicillin-resistant glycerol stocks were purchased, propagated and large plasmid preps were generated. These plasmids were tranfected into cells using lentiviral infection as described below.

Lentivirus-mediated transduction of LMTK-CD1d cells

A lentivirus infection was used to quicken and increase the efficiency of Smad3 and Smad4 shRNA-mediated knockdown. On day one, 1.5 x 10^6 human 293T cells were plated into 100 mm plates. The following day, the vial master mix was prepared by adding 6 mg VSV-G Lenti, 5 mg pRSV-Rev, and 10 mg pMDLg/pRRE. Then 500 μL calcium phosphate reactions were prepared with 20 μg of shRNA (NC, Smad3 1137, Smad4 1416 or Smad4 1925), 1 reaction's worth of viral master mix, 61 μL 2M $CaCl_2$, and water. The reaction mixtures were then added to the 293T cells and incubated overnight. The medium was removed and placed into bleach to inactivate the lentivirus particles, and 6 mL of medium was added to each plate of cells. On the same day, 2.5 x 10^5 LMTK-CD1d cells were plated in 60 cm dishes to prepare for infection (along with an uninfected control dish). On day four, the viral supernatants were harvested and filtered (.45 μm syringe filter). 6 μL of polybrene were added to the viral supernatant which was added to the LMTK-CD1d target cell dishes. Fresh $DMEM^+$ medium was

added to the cells on day five; and on day six, the selection of the cells began following the addition of 15 μg/mL of puromycin.

Western Blotting

LMTK-CD1d1 (NC, p38 wild-type, p38 dominant negative, and Smad shRNA-expressing) cells were treated with vehicle or the indicated concentrations of TGF β. Lysis buffer (10% glycerol, 50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 25 mM sodium fluoride, 10 mM sodium orthovanadate) containing Complete protease inhibitor tablets (Roche Diagnostics) was added to pre-plated cells in 100 mm plates, the cells were scraped, and incubated on ice for 20 minutes. Samples were centrifuged for 10 minutes at 10,000 rpm to remove cell nucleus and the supernatants were collected and stored at -80^{o}C. The amount of protein in the clarified cell lysates was estimated using Bio-Rad Bradford protein assay reagents by measuring absorbance at 595 nm. Equal amounts of protein were loaded into each well and resolved on an 8% SDS-PAGE gel at 35 mAmps. In the case of determining knockdown of protein by shRNA expression, 25, 12.5, 6.25 and 3.12 μg of protein was loaded. The gel was subsequently transferred to a polyvinylidene difluoride membrane (Millipore) at 75 mAmps overnight at 4^{o}C. The blot was blocked for 1 hour at room temperature in PBS-Tween (5%) containing 5% milk, washed four times for five minutes shaking in PBS-Tween, and then incubated overnight with antibodies specific for the indicated proteins diluted 1:1000 in PBS-Tween containing 5% BSA at 4^{o}C. Next, the blot was washed again, and incubated for 1 hour at room temperature with a 1:2000 dilution of HRP-conjugated goat anti-rat antibody (Bio-Rad) in PBS-Tween with 5% BSA. After a final washing, the bands were developed

using chemiluminescence before exposure on film. After stripping the blot with 2% SDS/1 mM Tris for 25 minutes at 55^{o}C, equal loading was determined by probing for GAPDH using specific antibodies

Immunofluorescence

Cell analysis by confocal microscopy consisted of treatment with or without TGF β (20 ng/ml), the ROCK inhibitor Y-27632 (25 and 100 μM), Cytochaslasin D (2 μM), or vehicle (2 mg/ml BSA/PBS and DMSO) for 24 hours in 35 mm collagen-coated glass bottom dishes (MatTek). After a 24 hour treatment, the cells were washed twice in PBS and fixed for 20 min on ice with 4% paraformaldehyde. The cells were then quenched with 1 mM Glycine-PBS for 10 min on ice and washed in HBSS-BSA. After treatment, the cells were permeabilized at room temperature using 0.1% saponin in HBSS-BSA for 5 min and blocked with 5% BSA/.1% Saponin/1mM Glycine-PBS for 5 min. To visualize the actin cytoskeleton, the cells were stained for one hour with 0.14 mM Rhodamine-Phalloidin (Cytoskeleton Inc.), washed, and placed in PBS for analysis by a Carl Zeiss-UV confocal microscope using the 63x water objective. Permeabilized LMTK-CD1d (NC and shRNA) cells were stained for CD1d using neat 1H6 antibody hybridoma supernatant followed by an Alexa488 anti-mouse IgG antibody (Invitrogen). For LAMP-1 specific staining, neat 1D4B antibody hybridoma supernatant was followed by a Texas Red anti-rat IgG antibody (Jackson Laboratories). Colocalization of CD1d and LAMP-1 was determined from images collected from an Olympus 2 confocal microscope with the 60x water objective by Metamorph (Molecular Devices) analysis. All images were collected as a series of slices into z-stacks. Slice sizes for images

collected from the Carl Zeiss confocal microscope was 0 .5 μm; for the Olympus 2, 0.2 μm approximately.

CD1d ELISA

LMTK-CD1d cells were plated 5 x 104 per well in a 96-well flat-bottom plate overnight. These cells were treated with vehicle and TGF β as described above. Once treated, the cells were washed in cold PBS and fixed with 1% paraformaldehyde for 15 minutes at 4^{o}C. The cells were washed again and permeabilized for intracellular staining with HBSS/BSA containing 0.1% saposin for 15 minutes at room temperature. To stain intracellularly and/or extracellularly, cells were incubated with CD1d-specific neat TIB126, 1H6, 1A8, 1E2, 9E4, 6F7, and 9G1 neat antibody hybridoma supernatants for 1 hour at room temperature. The cells were washed again with cold HBSS/BSA (containing 0.1% saposin for intracellular staining). As secondary stain, cells were incubated with goat anti-mouse IgG alkaline phosphatase conjugated antibody (BD Biosciences) (1:200) for 1 hour at room temperature. After the cells were washed again, a colorimetric reaction was produced by adding 1 mg/mL PnPP in 0.1 M Glycine and incubate 1 hour in the dark at room temperature. Finally, the yellow color change indicating staining of CD1d is measured at 405 nm by the microplate reader.

Flow Cytometry

LMTK-CD1d cells treated with vehicle or TGF β were washed with PBS. In 96 well round-bottom plates, the cells were stained for CD1d with 50 μL neat 1H6 antibody hybridoma supernatants and for MHC class I with 50 μL neat TIB126 antibody

hybridoma supernatants. The cells were incubated with these primary antibodies for 30 minutes on ice. Then the cells were washed with cold HBSS/BSA and stained with PE-conjugated anti-mouse antibody (for CD1d) and PE-conjugated anti-rat antibody (for MHC class I) for 30 minutes on ice. Next, the cells were fixed with 100 mL of 1% paraformaldehyde per well for 20 minutes on ice. Finally, the cells were washed again and placed in 400 mL of HBSS/BSA for analysis by flow cytometry.

CD1d Recycling Assay

To begin, LMTK-CD1d cells were treated in triplicate with 0 and 20 ng/mL of TGF β for 24 hours in 50 mL conical tubes at 37^{o}C. After treatment, the cells were washed twice with cold PBS and incubated with 25 μg/mL of cyclohexamide (Sigma) in 2 mL assay buffer for 30 minutes at 37^{o}C to block CD1d protein synthesis. Next, the cells were washed twice again with cold PBS and treated with 5 mg/mL of CD1d-specific 1B1 antibody in 200 μL in assay buffer for 30 minutes on ice. After being washed twice again with cold PBS, the cells were incubated with 25 μg/mL cyclohexamide and TGF β (0 and 20 ng/mL). Over a time course, aliquots of 5 x10^{5} cells were collected from each tube and placed on ice in 1 mL of PBS. Following harvesting of the last aliquot, the cells were washed again with cold PBS and fixed with 1% paraformaldehyde for 15 minutes on ice and then stained for CD1d with PE-conjugated 1B1. As a control, unblocked cells were stained for CD1d as described above as well as stained with isotype control antibody and for MHC class I. Cells were then analyzed by flow cytometry.

Statistical analysis

The data were analyzed with an unpaired two-tailed Student's t-test using GraphPad Prism software (version 5.0 for Windows;GraphPad Software). A p value < 0.05 was considered significiant. The error bars in the bar graphs show the SE from the mean of triplicate samples. In order to exclude false positives in determining significance (the 'p' value), the Bonferroni correction was used in the Student's t-test

Chapter 1

TGF β-dependent inhibition of CD1d-mediated antigen presentation

In recent years, it has become an area of interest to discover and define the mechanisms by which CD1d antigen presentation is controlled. Specifically, the role of signaling proteins such as mitogen activated protein kinases, p38 and ERK1/2. p38 and ERK1/2 reciprocally regulate CD1d-mediated antigen presentation when activated by phosphorylation [180]. When p38 is activated, antigen presentation by CD1d is reduced, whereas when ERK1/2 is activated, antigen presentation is increased. However, it is unknown how p38 is activated to induce this regulation of CD1d-mediated antigen presentation. As a stress-activated protein kinase (SAPK), p38 can be activated by UV radiation exposure and osmotic stress [181]. Ultimately, as a MAPK, p38 is part of a signaling pathway that leads to its activation. Generically, this involves a MAPKKK activating a MAPKK which finally phosphorylates a MAPK, such as p38 (Fig 3). The upstream signaling molecules that control p38 activation in the context of this inhibitory regulation of CD1d-mediated antigen presentation are unknown. It is known that p38 is typically activated by MAPKK, such as MKK3, MKK4, or MKK6 [163-165]. These MAPKKs can be phosphorylated and thereby activated by TAK1 (Fig 3) [132]. Besides stress, the activation of p38 can be receptor-mediated, such as through the pro-inflammatory cytokines IL-1 and epidermal growth factor (EGF) [181]. Interestingly, TGF β has been shown to activate p38. This process begins with TAK1 phosphorylation

at the cell surface by the TGF β receptor I (TGFβRI) [5] which binds TGF β and is activated by TGF β receptor II (TGFβRII) [166]. TGF β is a potent immune regulator that exerts its effects on many types of immune cells such as inhibition of Natural Killer cell cytolytic activity and IFN-γ production [85, 89], dendritic cells maturation and migration [90, 92], and decreased T cell activity [94]. In addition to regulating specific immune cells, TGF β can regulate MHC class II antigen presentation [127]. Possibly through cell surface expression downregulation, TGF β has been shown to inhibit/downregulate antigen presentation by MHC class II molecules [128]. With TGF β's connection to p38, an inhibitor of CD1d-mediated antigen presentation, and its known role as a negative regulator of the immune system, specifically antigen presentation, it seemed conceivable that TGF β may play some regulatory role on the functional expression of CD1d. Some evidence indeed exists to support such a hypothesis. For example, knockout of TGF β gene expression in CD4 positive cells resulted in decreased levels of NKT cells [124]. Also, TGF β decreases NKT cell proliferation and IFN-γ secretion. As another connection, TGF β-treated human Langerhan cells resulted in a diminished level of CD1d on the cell surface [129]. Similar to these findings, it has been shown that the decreased level of CD1d expression in the liver is related to increased levels of TGF β in the serum induced by tumor progression [130]. All this evidence, which suggests that TGF β has a connection to CD1d, led us to hypothesize that TGF β may inhibit CD1d-mediated antigen presentation.

Due to the solid evidence that TGF β can alter NKT cell activation, CD1d expression and p38 activation, we hypothesized that TGF β can negatively affect CD1d-mediated antigen presentation. In order to determine whether TGF β influences antigen

presentation by CD1d, LMTK-CD1d cells, mouse LMTK fibroblasts transfected with the pcDNA3.1 vector expressing the mouse *cd1d1* cDNA [182], were treated with increasing doses of TGF β for 24 hours. After treatment, the cells were fixed in paraformaldehyde, and co-cultured with NKT cell hybridomas for 22 to 24 hours. The supernatants were harvested and the IL-2 produced by CD1d-stimulated NKT hybridomas was determined by ELISA. Thus, CD1d-mediated antigen presentation is measured by the level of IL-2. It was found that TGF β was able to decrease CD1d-mediated antigen presentation in a dose-dependent manner (Fig 5). Next, we set out to define the extent of this TGF β-dependent inhibition. To do this, LMTK-CD1d cells were treated with two concentrations of TGF β: 5 and 10 ng/mL in a kinetics experiment (0, 3, 6, and 24 hours) in order to determine the optimum timeframe under which TGF β inhibits antigen presentation by CD1d. In as early as 3 hours, but at each time point tested, we observed that TGF β inhibited CD1d-mediated antigen presentation (Fig 6). Considering that this inhibition was shown in LMTK cells, a cultured cell line in which mouse CD1d was transfected, it was important to demonstrate that TGF β had a similar effect on primary $CD1d^+$ cells, specifically, bone marrow-derived dendritic cells (BMDCs). These bone marrow cells were taken from the femurs and tibia of C57BL/6 mice and cultured in the presence of IL-4 and GM-CSF for six days and then matured with LPS on day 7. After treating these cells with TGF β for 24 hours, they were co-cultured with NKT hybridoma cells and CD1d-mediated antigen presentation measured by an IL-2 ELISA. As observed with LMTK-CD1d1 cells, a dose-dependent decrease in CD1d-mediated antigen presentation in response to TGF β was found (Fig 7). Therefore, these results suggest that TGF β can inhibit endogenous antigen presentation by CD1d. However, as CD1d

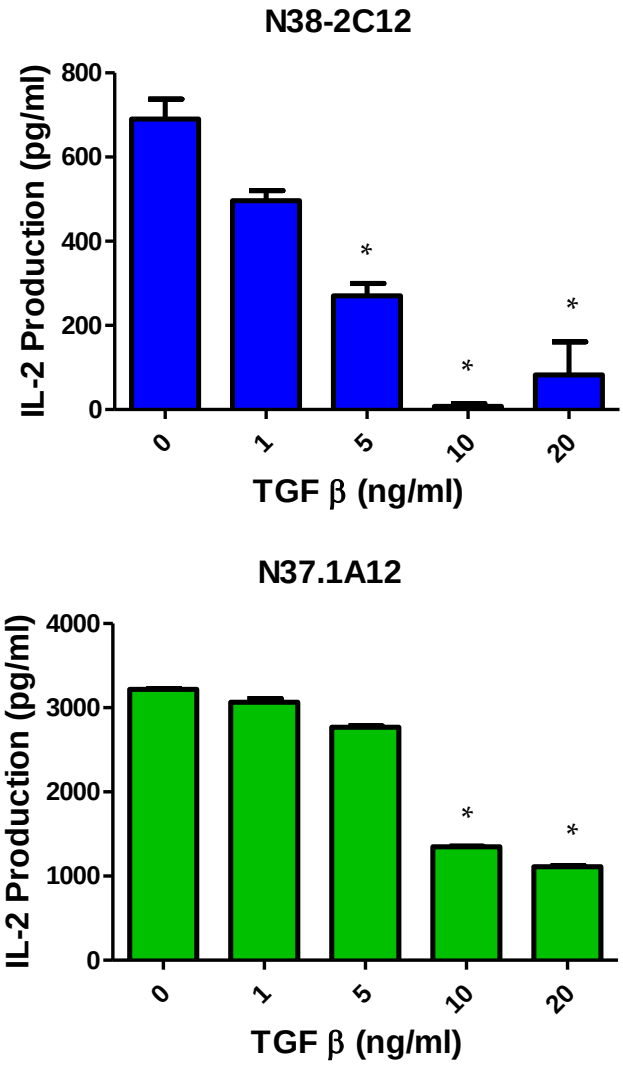
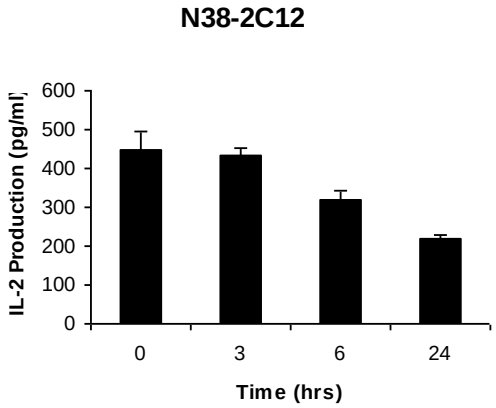


Figure 5. TGF β inhibits CD1d-mediated antigen presentation. LMTK-CD1d cells were treated with 0, 1, 5, 10, and 20 ng/mL of TGF β for 24 hours and then co-cultured with the indicated NKT cell hybridomas. IL-2 secreted by CD1d-activated NKT hybridomas was measured by ELISA. (One representative experiment; n = 10; *$p < 0.01$)

A.



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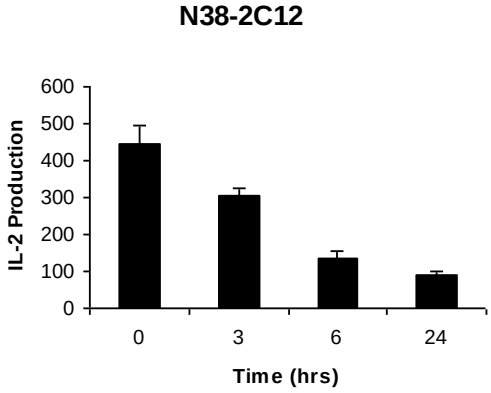

Figure 6. TGF β inhibits CD1d-mediated antigen presentation over time. LMTK-CD1d cells were treated with 0 or 5 ng/mL (A), or 10 ng/mL (B) of TGF β for 0, 3, 6, and 24 hours, washed, and then co-cultured with the NKT hybridoma cells, N38-2C12 for 22-24 hours. IL-2 secreted by CD1d-activated NKT hybridoma cells into the supernatant was determined by ELISA.

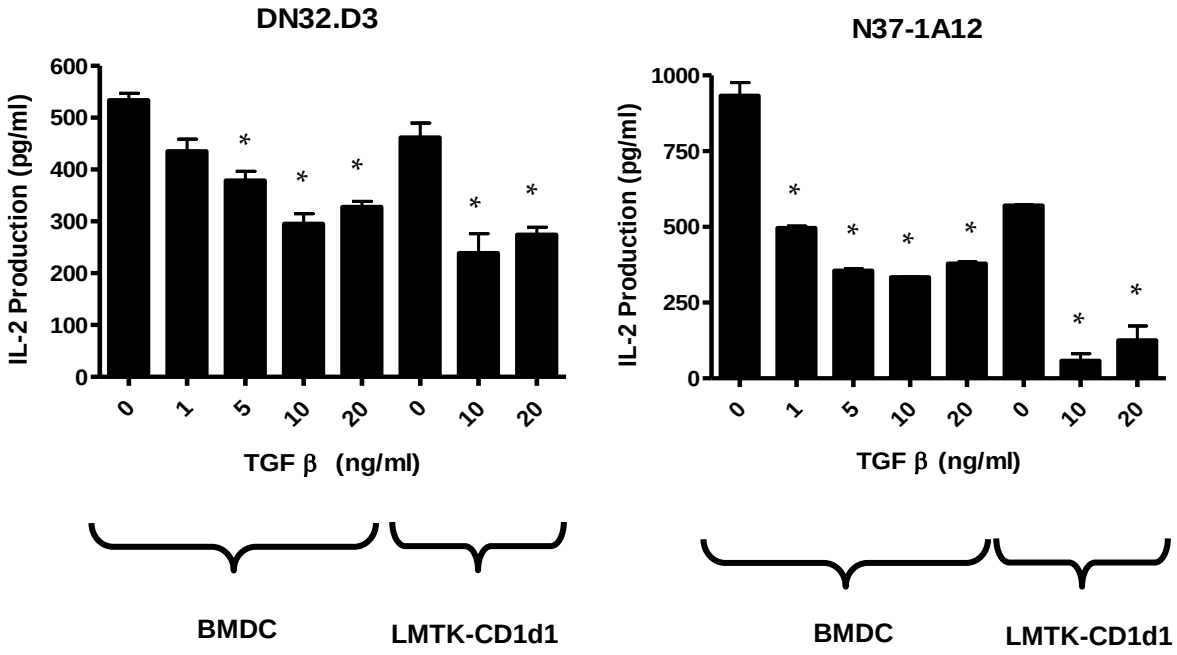

Figure 7. TGF β inhibits CD1d-mediated antigen presentation in BMDCs. Bone marrow cells were harvested from the femurs and tibias of C57BL/6 mice and cultured in 10 ng/mL each of IL-4 and GM-CSF for 6 days. On day 7 the BMDCs were matured with 10 ng/mL of LPS for 24 hours. These BMDCs were then treated with 0, 1, 5, 10, or 20 ng/mL of TGF β for 24 hours, and co-cultured with the indicated NKT hybridomas. IL-2 secreted into the supernatant by CD1d-activated NKT hybridoma cells was measured by ELISA. As a control, LMTK-CD1d cells were treated in the presence or absence of TGF β and co-cultured with the NKT hybridomas as above. (One representative experiment; n = 3; *$p < 0.01$)

can also present exogenous lipid antigens to NKT cells [183], it was important to analyze what would happen with an exogenous antigen such as α-galactosylceramide (α-GalCer), the most common exogenous lipid antigen used in antigen presenting studies. To address this question, 24 hours following TGF β treatment, LMTK-CD1d cells were pulsed with the potent CD1d-presented glycolipid, α-GalCer, for one hour prior to co-culture with NKT cell hybridomas as above. As expected, the invariant NKT cell hybridoma, N37-1A12, did not increase production of IL-2 in response to α-GalCer which has been shown previously by others [20]. It was found that α-GalCer could effectively rescue the decrease in CD1d-mediated antigen presentation caused by TGF β treatment (Fig 8).

After defining this inhibition caused by TGF β, it became necessary to determine the mechanism by which TGF β is causing this decrease in antigen presentation by CD1d. TGF β has been shown to lower the cell surface expression of CD1d, which might have caused the decrease in antigen presentation [129]. To address this possible mechanism, the level of CD1d cell surface expression was analyzed by flow cytometry. LMTK-CD1d cells were treated with various doses of TGF β for 24 hours. The cells were then stained for CD1d and MHC class I (control) molecules. In contrast to prior findings, we observed no difference in CD1d cell surface expression when LMTK-CD1d cells or BMDCs were treated with TGF β (Fig 9).

CD1d is transported to the late endosome and lysosome in order to acquire its lipid antigen for presentation on the cell surface (Fig 1) [20]. While in these compartments, the exchange of lipids bound to CD1d is facilitated by the lipid transfer protein, saponin B [21, 22]. Then, CD1d is recycled back to the cell surface to present these lipids to NKT cells. Therefore, localization to late endosomes and lysosomes is necessary fo

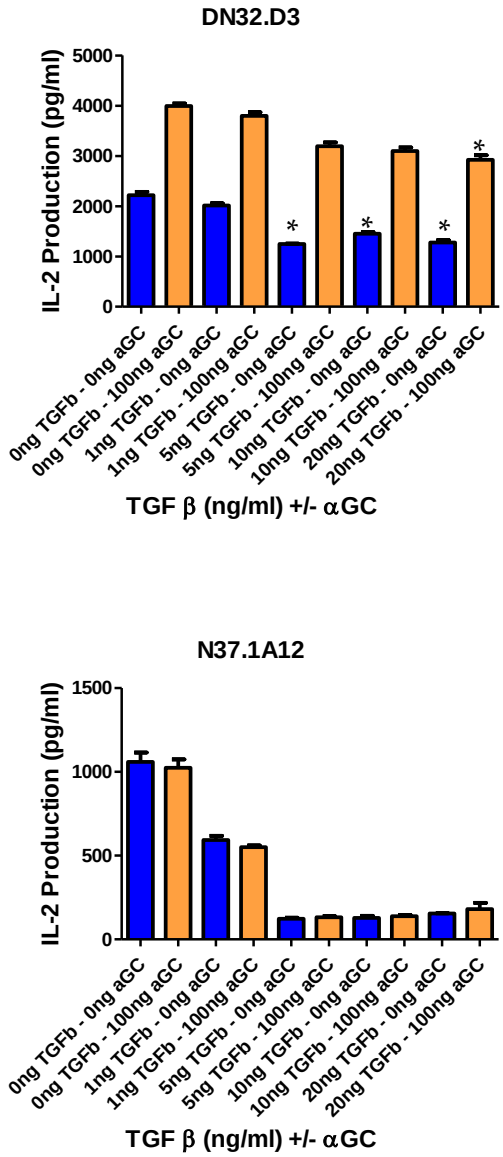

Figure 8. α-GalCer rescues TGF β-induced inhibition of CD1d-mediated antigen presentation. LMTK-CD1d cells were treated with 0, 1, 5, 10, or 20 ng/mL of TGF β for 24 hours and then pulsed with 100 ng/mL of α-GalCer for 1 hour. The cells were then co-cultured with the indicated NKT cell hybridomas 22-24 hours. IL-2 secreted into the supernatant by the NKT cell hybridomas was measured by ELISA. (One representative experiment; n = 3; *$p < 0.01$)

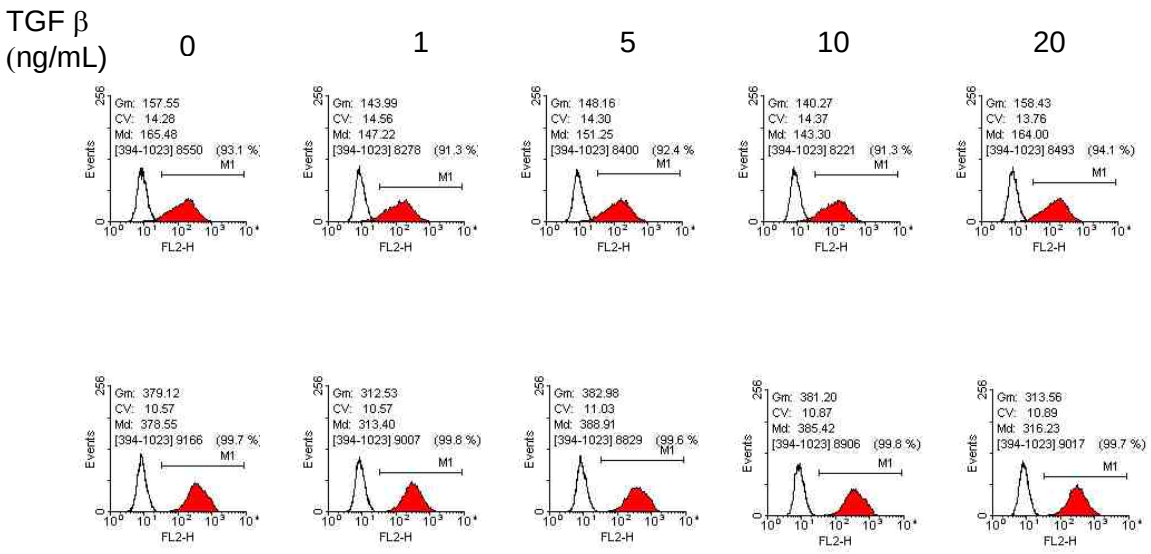

Figure 9. TGF β treatment does not alter CD1d cell surface expression. LMTK-CD1d cells treated with 0, 1, 5, 10 and 20 ng/mL of TGF β for 24 hours were stained for CD1d (top row) and MHC class I molecules (second row) (positive control). The stained cells were analyzed for cell surface expression by flow cytometry. (n = 10)

proper lipid loading and antigen presentation by CD1d (Fig 1). There is precedence for inhibition of CD1d antigen presentation being related to changes in CD1d localization, such as when CD1d-mediated antigen presentation is inhibited by the activation of p38; it causes a change in CD1d localization to LAMP-1 positive compartments, specifically late endosomes and lysosomes [180]. Therefore, because TGF β can activate p38 [132] and p38 alters CD1d localization, it was hypothesized that the mechanism behind TGF β inhibition of antigen presentation was due to reduced trafficking of CD1d to these late endocytic compartments. To test this hypothesis, LMTK-CD1d cells were treated with TGF β for 24 hours and stained with fluorescent antibodies specific for CD1d and LAMP-1. The cells were then analyzed by confocal microscopy. It was observed that the co-localization of CD1d and LAMP-1 in LMTK-CD1d cells treated with TGF β was indistinguishable from that found in control cells. Therefore, these results indicate that TGF β does not substantially alter the intracellular localization of CD1d (Fig 10).

Recycling of CD1d to the cell surface can be affected by events such as an HSV infection [69]. Such a reduction in CD1d recycling could explain the TGF β-induced inhibition of antigen presentation by CD1d. A recycling assay can measure the return CD1d. Therefore, only the existing CD1d pool within the cell will be measured. After blocking cellular protein synthesis, all CD1d on the cell surface is blocked so that only the intracellular CD1d returning to the cell surface are recorded in the assay. Once cell surface CD1d is blocked, the cells are incubated at 37^{o}C for different time points and then of recycling CD1d to the cell surface over time. This is accomplished by first treating the cells with cyclohexamide, which blocks all new synthesis of new proteins, including

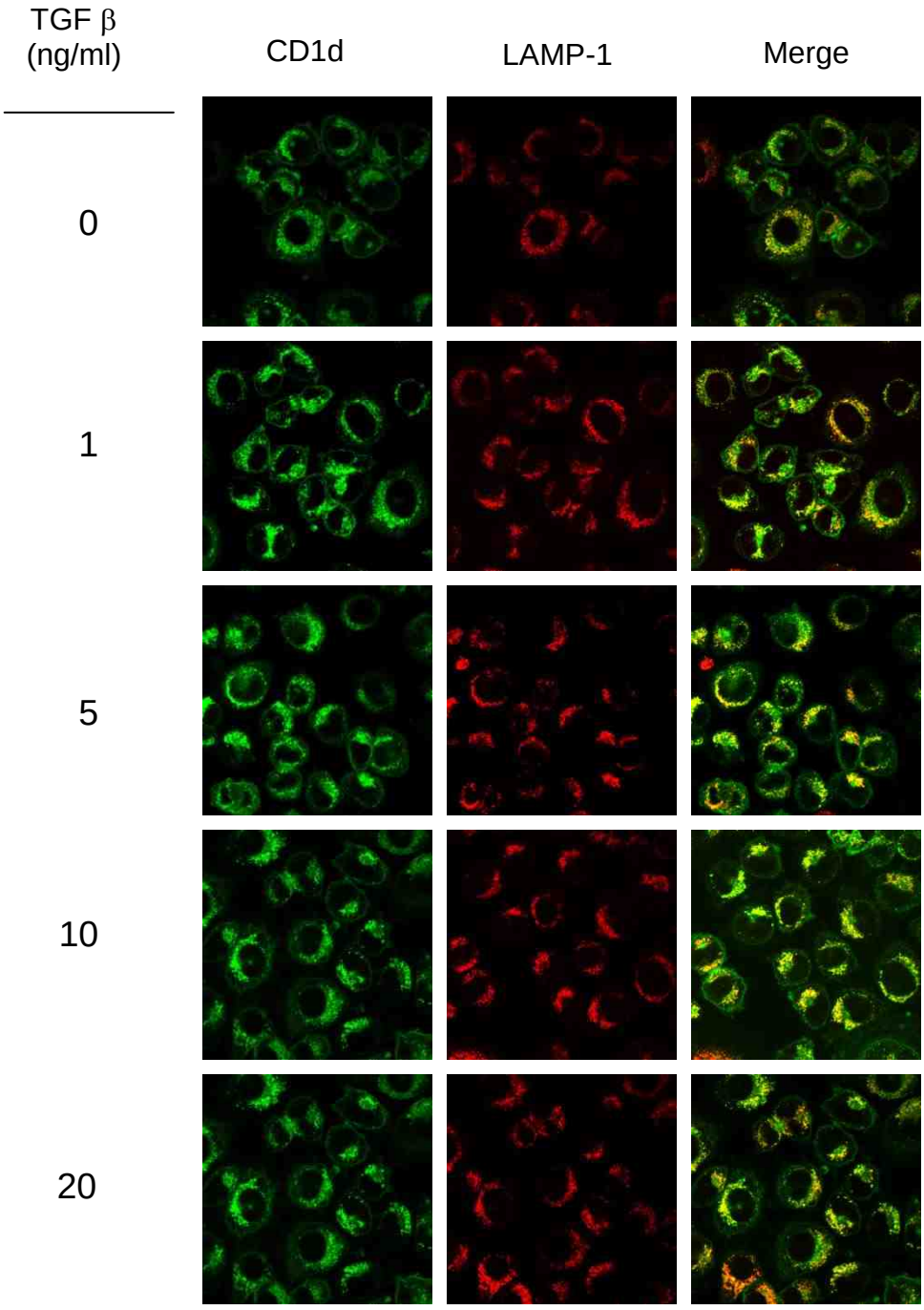

Figure 10. TGF β does not alter the intracellular localization of CD1d. LMTK-CD1d cells were treated with 0, 1, 5, 10 and 20 ng/mL of TGF β for 24 hours and then stained for CD1d and LAMP-1, a marker for late endosomes and lysosomes. The stained cells were analyzed by confocal microscopy. Metamorph analysis showed that there was no difference in CD1d/LAMP-1 co-localization in the presence of TGF β as compared to control cells.

fixed with paraformaldehyde. The new CD1d that recycled to the cell surface is stained with FITC-conjugated antibodies on the cell surface and analyzed by flow cytometry. TGF β-treated LMTK-CD1d cells were tested in a recycling assay as described above. In cells treated with 0 and 20 ng/mL of TGF β, CD1d recycling in LMTK-CD1d cells was comparable to that in untreated cells. Therefore, TGF β did not appear to inhibit antigen presentation by CD1d through influencing its cell surface recycling (Fig 11).

Although we found no difference in CD1d cell surface expression when cells were treated with TGF β, it was thought that a more sensitive assay could potentially show minute differences post-TGF β treatment. This assay was a CD1d ELISA [184]. This ELISA consisted of using a panel of available antibodies specific for various and distinct epitopes on CD1d. It was hypothesized that TGF β might cause a conformational change in CD1d, which ultimately could affect CD1d-mediated antigen presentation. Such a conformational change could be detected by a CD1d ELISA as an increase or decrease in antibody binding by this assay method. Thus, LMTK-CD1d1 cells were treated with or without TGF β for 24 hours and then stained (intracellular and extracellular) for CD1d with the panel of different CD1d-specific antibodies. Finally, secondary antibodies were applied and the cells were analyzed by flow cytometry. The 1H6 antibody produced an interesting result as shown by an increase in CD1d binding at the lower levels of TGF β treatment. However, flow cytometry with this antibody did not show any increases in CD1d binding by the 1H6 antibody. Also, the enhanced antibody binding was at the low concentrations of TGF β, but at high concentrations of TGF β, a significant decrease in CD1d-mediated antigen presentation is observed (e.g. Fig 5). In these experiments, no

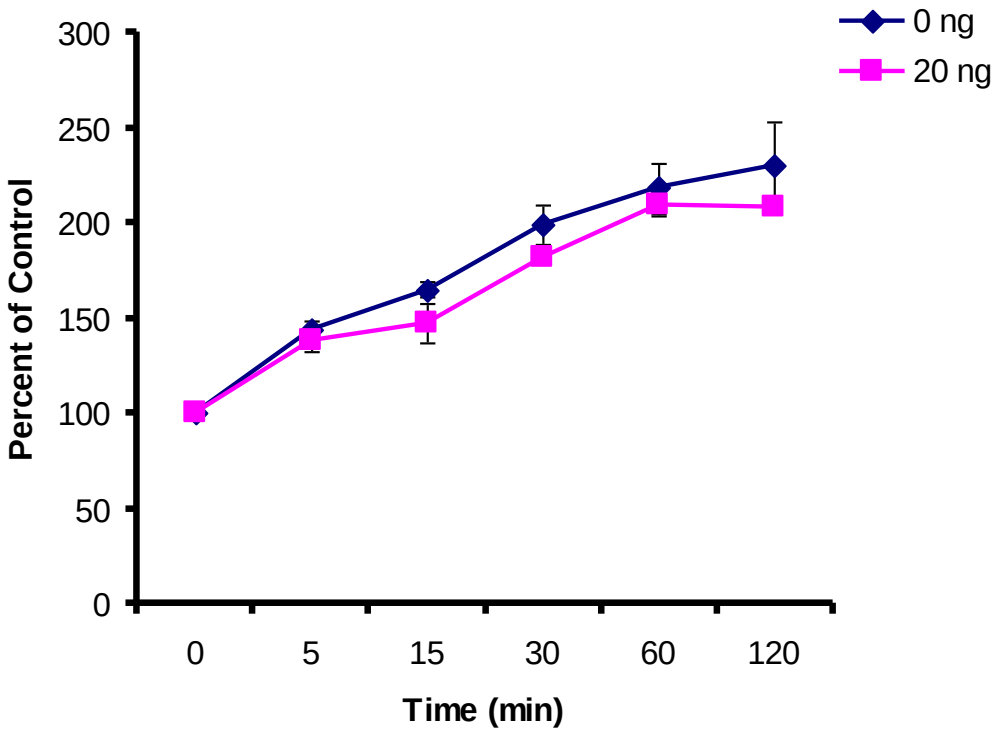

Figure 11. TGF β does not alter CD1d recycling to the cell surface. LMTK-CD1d cells were treated with 0 and 20 ng/mL of TGF β for 24 hours. New protein synthesis of the TGF β-treated cells was blocked using cyclohexamide and existing cell surface CD1d was blocked with unconjugated CD1d-specific antibodies. During incubation at 37°C, aliquots removed at different time points (0, 5, 15, 30, 60, and 120 minutes) and stained with a PE-conjugated anti-mouse Ig antibody to detect newly recycled CD1d on the cell surface. The cells were analyzed by flow cytometry.

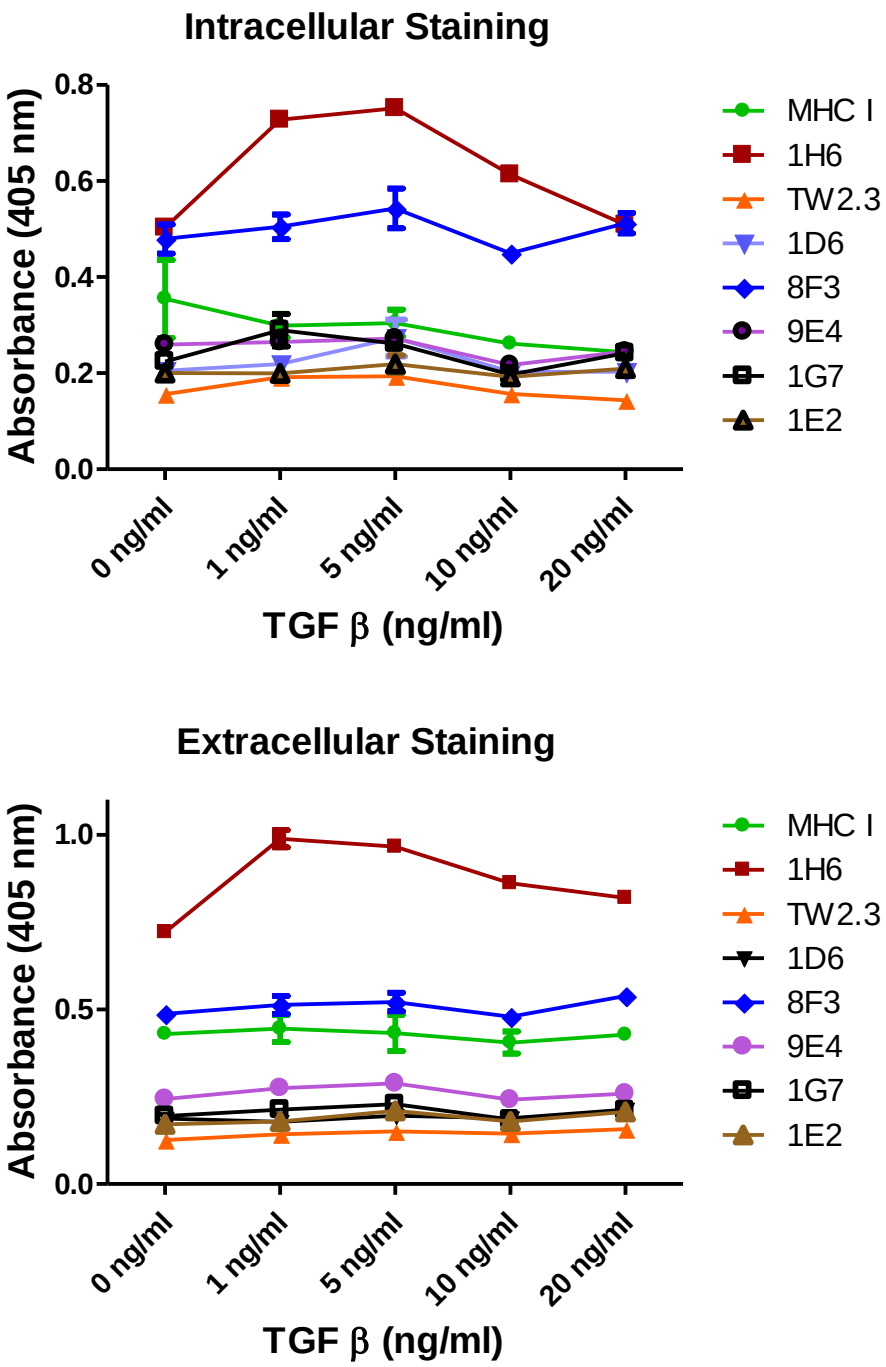

Figure 12. TGF β does not alter the conformation of CD1d. LMTK-CD1d cells were treated with 0, 1, 5, 10 or 20 ng/mL of TGF β for 24 hours and the binding of a panel of CD1d-specific antibodies was measured by ELISA [24]. Antibodies for MHC class I were used as a positive control and TW2.3, an antibody for the vaccinia virus E3L protein, served as a negative control.

change in CD1d-specific antibody binding was detected with all other CD1d specific antibodies (Fig 12). Therefore, TGF β did not appear to affect the conformation or cell surface expression of CD1d, as a means to explain its ability to reduce antigen presentation by CD1d.

In continuing our studies to determine the mechanism behind the TGF β-mediated inhibition of CD1d-mediated antigen presentation, p38 activation and signaling became the focus as an hypothesized mechanism. Others have shown that TGF β can activate the p38 MAPK [132]. Because both TGF β and p38 inhibit CD1d-mediated antigen presentation, our hypothesis was that TGF β activation of p38 leads to inhibition of antigen presentation by CD1d. Thus, the first step was to confirm the phosphorylation of p38 by TGF β in our LMTK-CD1d cells. To do this, LMTK-CD1d cells treated with 0 and 20 ng/mL of TGF β for different time points were lysed in 1% NP40 lysis buffer. The lysates were analyzed by Western blot for the level of phosphorylated p38 and total p38. It was found that p38 was activated by TGF β treatment in as early as 30 minutes (Fig 13). As a means to determine if TGF β impairs antigen presentation by CD1d via p38 activation, LMTK-CD1d cells transfected with plasmids expressing wild-type (WT) or dominant negative (DN) p38 cDNA (or empty vector control), were mock-treated or treated with 0, 1, 5, 10, and 20 ng/mL of TGF β for 24 hours. It was observed that TGF β was capable of inhibiting antigen presentation in each of these cells lines, especially in the p38 DN expressing cells (Fig 14). These results indicate that p38 activation is not the mechanism by which TGF β is inhibiting CD1d-mediated antigen presentation. These data were confirmed when LMTK-CD1d cells were treated with the p38 inhibitor SB203580 and TGF β (data not shown). SB203580-treated cells showed enhanced

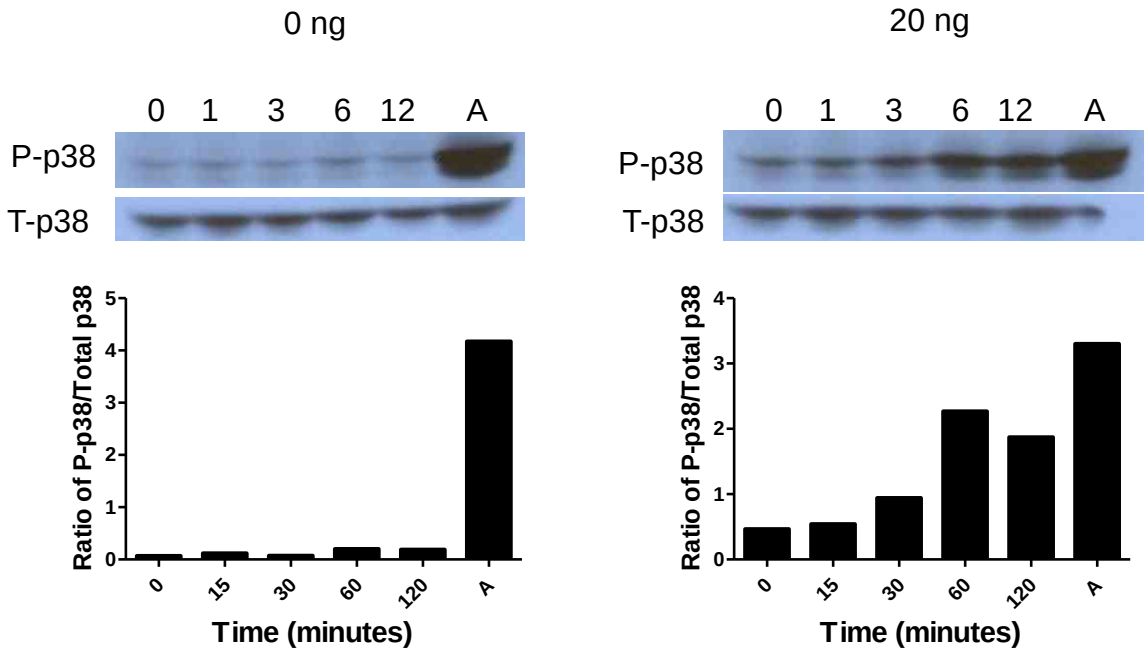

Figure 13. TGF β activates p38 in LMTK-CD1d cells. LMTK-CD1d cells were treated with 0 and 20 ng/mL of TGF β for 0, 15, 30, 60 and 120 minutes. As a control, cells were treated with 10 ng/mL of anisomycin (A), which served as a positive control that activates p38. For the Western Blot, the cells were lysed and 300 μg of cell lysates were loaded onto a 10% SDS-PAGE gel and transferred to a PDVF membrane. The membrane was probed with an anti-phospho-p38 antibody to detect p38 (P-p38). The blot was then stripped and reprobed using an antibody specific for total p38 (T-p38). The bands were visualized using densitometry and developed on film. The ratio of phosphorylated to total p38 was determined using ImageJ analysis.

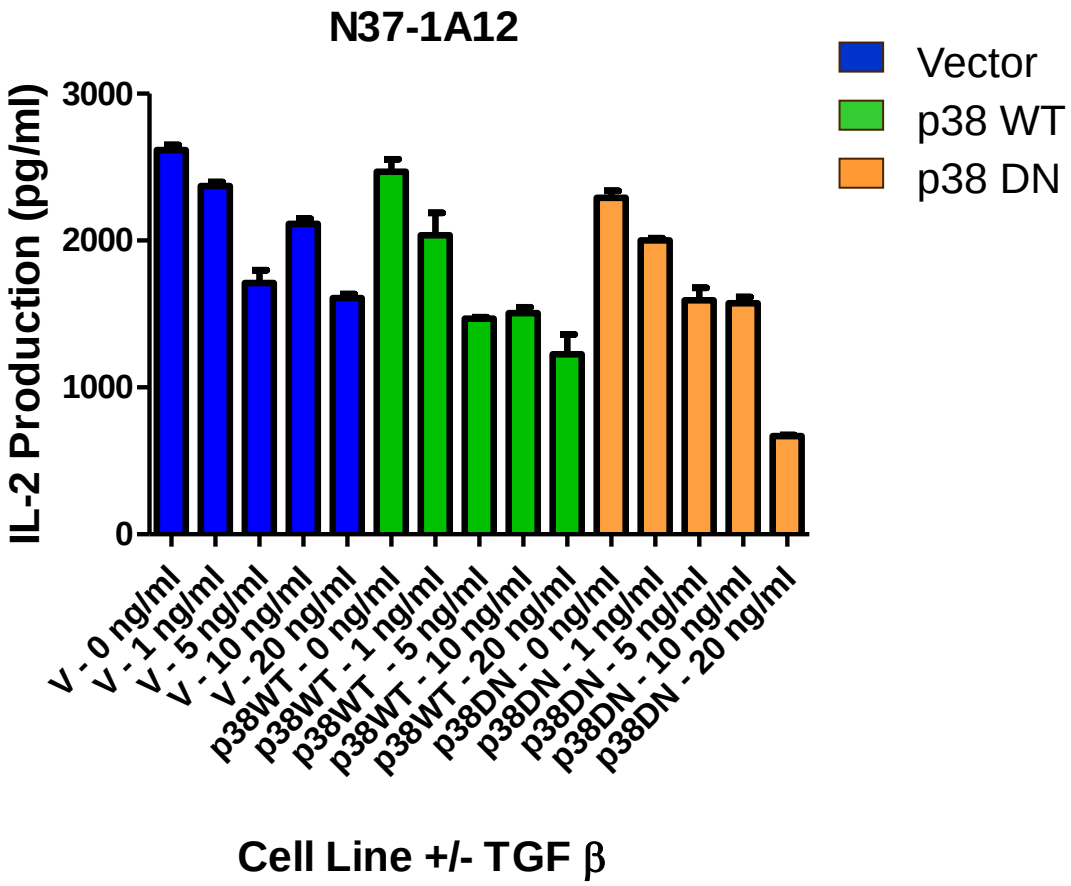

Figure 14. TGF β does not inhibit CD1d-mediated antigen presentation through activation of the p38 MAPK. LMTK-CD1d cells transfected with empty vector (V), p38 WT, or p38 DN cDNA were treated with 0, 1, 5, 10 and 20 ng/mL of TGF β for 24 hours and co-cultured with an NKT cell hybridoma. IL-2 secretion by the CD1d-activated NKT cells was measured by ELISA.

CD1d-mediated antigen presentation as we reported previously. However, TGF β inhibition of CD1d was not rescued by SB203580 treatment as would be expected if p38 were involved in TGF β-dependent inhibition of CD1d- mediated antigen presentation. Therefore, chemically inhibiting p38 activation also did not alter the ability of TGF β to decrease CD1d-mediated antigen presentation.

In testing the hypothesis that TGF β may alter CD1d-mediated antigen presentation it was found that treatment of $CD1d^+$ cells with TGF β led to the inhibition of antigen presentation by CD1d. However, determining the mechanism behind this inhibition proved to be difficult. The cell surface levels of CD1d were not affected by TGF β as determined by flow cytometry and a CD1d ELISA. Furthermore, neither CD1d localization nor CD1d recycling were altered by TGF β treatment, suggesting that TGF β does not alter CD1d trafficking through the recycling pathway. Finally, p38, a MAPK signaling protein known to be activated by TGF β and that negatively regulates CD1d-mediated antigen presentation, was determined to not play a role in the TGF β-mediated inhibition of antigen presentation by CD1d. Therefore, alternative pathways such as the Smad pathway, becomes the focus of the next chapter as a possible mechanism to explain our results.

Chapter 2

Regulation of CD1d-mediated antigen presentation by Smad2 and Smad4

TGF β is a cytokine capable of activating many signaling pathways [134, 135]. The canonical pathway most often associated with TGF β is the Smad pathway (Fig 2). TGF β binds to the constitutively active TGF β receptor II, which then binds TGF β receptor I, forming a trimeric complex [185]. The formation of this complex brings TGFβRII in close proximity to TGFβRI, which allows TGFβRII to phosphorylate and activate TGFβRI [141]. TGFβRI is a serine threonine kinase that phosphorylates "Receptor-Smads" such as Smad2 and Smad3 [149, 152, 186, 187]. Smad2 and Smad3 are similar in structure with MH1 and MH2 domains and TGFβRI phosphorylates both within the MH2 domain. Once phosphorylated, Smad2 and Smad3 will bind to each other to form a heterodimer. Smad4, a "Co-Smad", is essential to the Smad complex moving into the nucleus. Once the Smad2-Smad3 complex forms, it binds to Smad4, and this trimolecular complex traffics to the nucleus [157]. It is in the nucleus that the Smad2-Smad3-Smad4 complex will bind to co-activators or co-repressors to cause gene up-regulation or down-regulation, respectively [158, 188, 189].

As mentioned in the previous chapter, TGF β inhibits CD1d-mediated antigen presentation (Fig 5). MAPK p38 activation was initially suspected to be the mechanism by which TGF β is causing this inhibition. However, a dominant-negative p38 expressing LMTK-CD1d1 cell line and p38 inhibitor, SB203580, were incapable of

rescuing the inhibition of antigen presentation after TGF β treatment. Therefore, the Smad pathway, the major pathway specifically associated with TGF β signaling, was the next candidate as the potential mechanism behind inhibition of CD1d-mediated antigen presentation by TGF β. As addressed previously, TGF β is a potent regulator of the immune system and now has been shown to also regulate CD1d-mediated antigen presentation. In other systems, TGF β has been historically shown to exert immune control through the Smad pathway. For example, the regulation of natural killer cell IFN-γ production has been shown to be through Smad3 activation [89]. Further, TGF β control of T cell function is through the induction of Tregs and promotion of Th1 development, which has been shown to be through the activation of Smad2 and Smad3 [190]. Another group found that a Smad3 deficiency resulted in lower expression of a Treg specific gene, Foxp3, in T cells [191]. In the case of B cells, Smad3 and Smad4 phosphorylation by TGF β results in induced production of IgA [192]. Therefore, it is possible that Smad pathway activation is the means by which TGF β inhibits CD1d-mediated antigen presentation, and thus, this is the new hypothesis tested in this chapter. The strategy to investigate this potential mechanism was to use shRNA constructs to specifically knockdown Smad2, Smad3, and Smad4 protein expression.

These studies began by testing the new hypothesis that TGF β is inhibiting CD1d-mediated antigen presentation through activation of the Smad pathway, specifically phosphorylation and activation of Smad2. First, it was important to determine whether Smad2 is activated by TGF β in LMTK-CD1d cells, the same cell line originally used to show a TGF β-dependent decrease in CD1d antigen presentation. Thus, LMTK-CD1d cells were treated with 0 and 20 ng/mL of TGF β for different lengths of time and the

level of phosphorylated Smad2 was determined by Western Blot. The results demonstrated a strong and prompt activation of Smad2 by phosphorylation on Serine 465 at 15 minutes that was consistent over 2 hours (Fig 15). In conclusion, TGF β was capable of initiating activation of the Smad pathway in LMTK-CD1d cells.

The next objective was to determine whether this phosphorylation of Smad2 and activation of the Smad pathway is the mechanism by which TGF β is inhibiting CD1d antigen presentation. To determine the necessity of Smad2, pLKO.1 vectors encoding shRNA constructs specifically targeting Smad2 mRNA were transfected into LMTK-CD1d cells. As a negative control, a pLKO.1 vector expressing a scrambled, nonspecific shRNA sequence was also transfected into LMTK-CD1d cells (NC). Smad2 knockdown was analyzed by Western Blot. Different concentrations (25, 12.5, 6.25, and 3.12 μg) of lysates from LMTK-CD1d NC and Smad2 shRNA cells were analyzed by Western Blot for the level of total Smad2 protein present in the cells. Compared to LMTK-CD1d NC, Smad2 shRNA-expressing cells had reduced levels of Smad2 protein expression by 55%, demonstrating that the Smad2 shRNA 926 construct successfully knocked down Smad2 (Fig 16).

To verify the role of Smad2 in TGF β-induced inhibition of CD1d-mediated antigen presentation, LMTK-CD1d NC and LMTK-CD1d Smad2 shRNA were treated with increasing concentrations of TGF β for 24 hours. These treated cells were co-cultured with NKT cells, and CD1d-dependent NKT cell activation was determined.
TGF β inhibited CD1d-mediated antigen presentation in both NC and Smad2 shRNA cells, which might, on initial inspection, indicate that Smad2 may not be the signaling

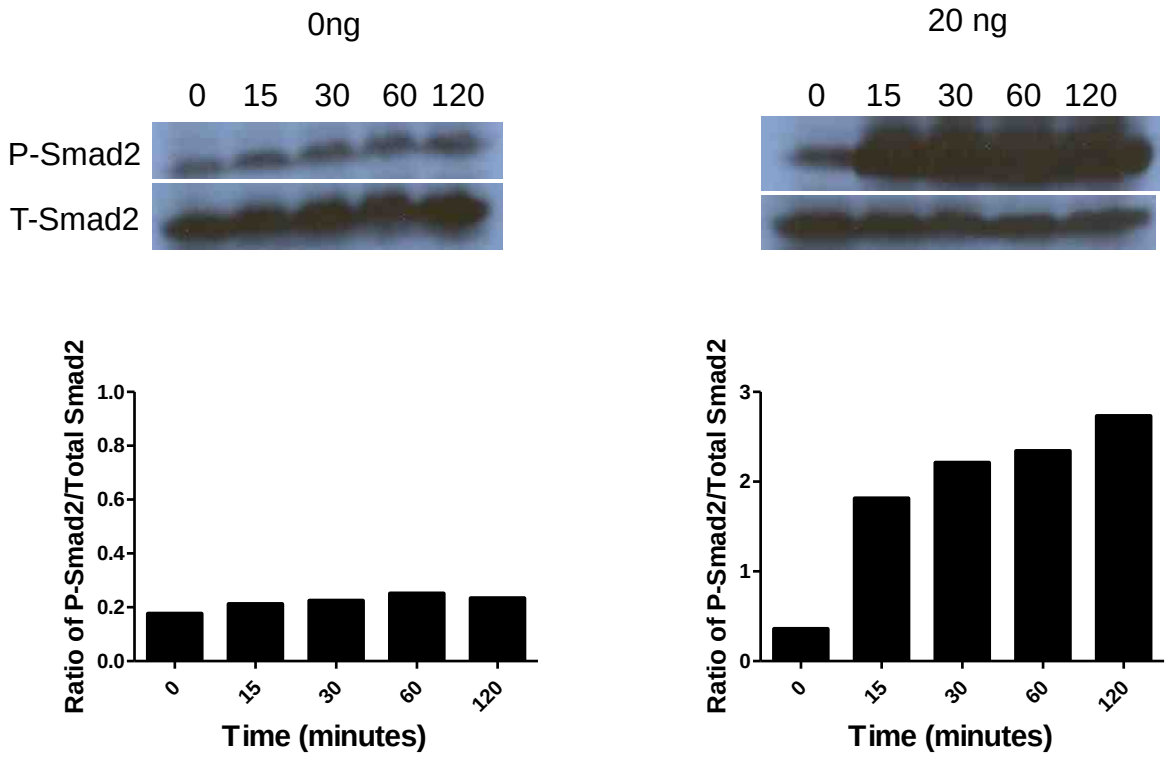

Figure 15. TGF β activates Smad2 by the phosphorylation of Serine 465 in LMTK-CD1d cells. LMTK-CD1d cells were treated with two concentrations of TGF β; 0 and 20 ng/mL, for 24 hours. After treatment, the cells were lysed, protein concentrations in lysates were determined, and 150 μg of protein was loaded onto a 10% SDS-PAGE gel. After gel transfer onto a PVDF membrane, blots were probed for Ser465 phospho-Smad2 and total Smad2. ImageJ was used to determine band intensity and the ratio of phospho-Smad2 (P-Smad2) to total Smad2 (T-Smad2) is displayed above. (One representative experiment; n = 3)

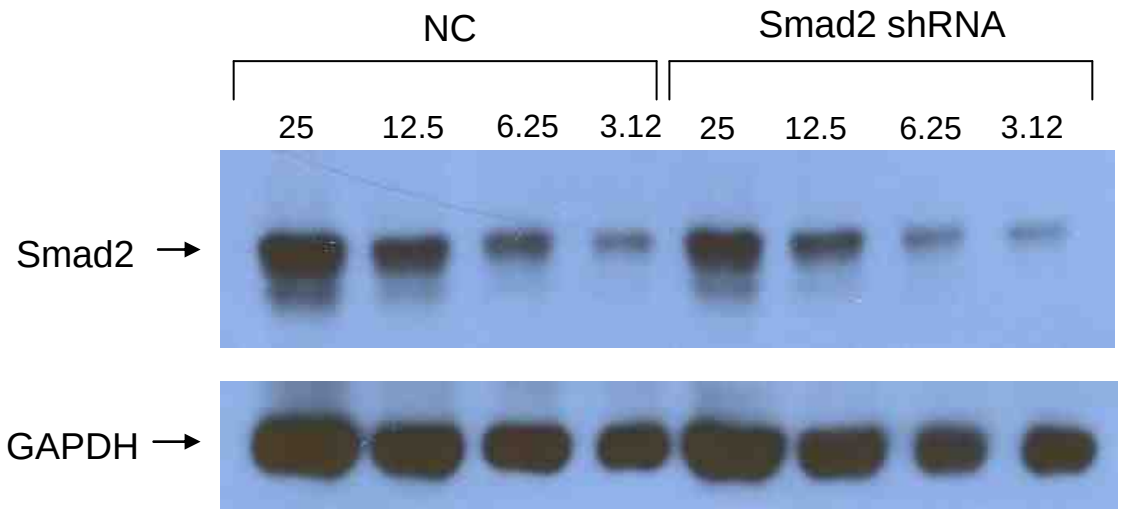

Figure 16. Smad2 is knocked down by the pLKO.1 vector expressing Smad2 shRNA in LMTK-CD1d cells. The lentiviral vector pLKO.1 expressing a shRNA targeting coding sequence 926 of the Smad2 mRNA was transfected into LMTK-CD1d cells. After puromycin selection, the cells were lysed, and 25, 12.5, 6.25, and 3.125 μg from both the control cells, LMTK-CD1d NC, and Smad2 shRNA-expressing cells were loaded onto an 8% SDS-PAGE gel. After transfer to a PVDF membrane, the blot was probed for total Smad2 and for GAPDH as a loading control. (n = 5)

pathway by which TGF β is inhibiting CD1d-mediated antigen presentation (Fig 17). It is arguable that the lack of inhibition rescue may be due to lack of a 100% knockdown of Smad2 and the small amount of Smad2 left in the cells is sufficient to continue the TGF β-dependent inhibition. Despite this, an interesting result was observed. At baseline, LMTK-CD1d Smad2 shRNA cells had increased levels of CD1d-mediated antigen presentation compared to the NC cells, suggesting that Smad2 activation indeed inhibits CD1d-mediated antigen presentation. This inhibition is functional due to no observed change in CD1d cell surface expression in Smad2 shRNA-expressing cells compared to NC shRNA-expressing cells.

As shown in the previous chapter, TGF β was analyzed for its influence on CD1d and LAMP-1 intracellular co-localization and found to have no effect as determined by confocal microscopy. Due to this, it was predicted that Smad2 knockdown would similarly not affect CD1d localization. LMTK-CD1d NC and Smad2 shRNA cells were treated with 0 and 20 ng/mL of TGF β for 24 hours. After treatment, cells were stained for CD1d and LAMP-1 and analyzed by confocal microscopy. As shown before, TGF β did not affect CD1d co-localization to LAMP-1^{+} compartments (Fig 18). Upon comparison of NC cells to Smad2 shRNA cells it was apparent that Smad2 knockdown did not alter CD1d and LAMP-1 co-localization.

As mentioned above, Smad2 cooperates with two other Smads, Smad3 and Smad4, to enable the Smad pathway. Therefore, if Smad2 knockdown could not reverse TGF β's inhibitory effects, then it is plausible that the knockdown of Smad3 could accomplish this. Similar to Smad2, Smad3 was knocked down in LMTK-CD1d cells through

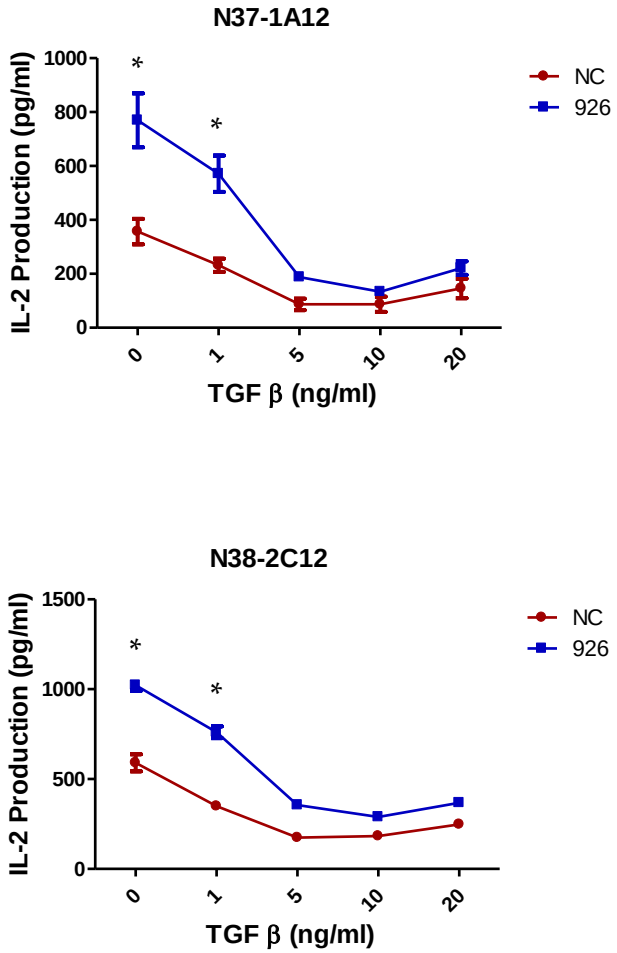

Figure 17. Smad2 knockdown increases CD1d-mediated antigen presentation. LMTK-CD1d NC and Smad2 shRNA cells were treated for 24 hours with 0, 1, 5, 10, and 20 ng/mL of TGF β. After treatment, cells were co-cultured with NKT cell hybridomas for 22-24 hours at 37°C. Supernatants from the co-culture were analyzed for IL-2 concentration by an ELISA. (One representative experiment; n = 3, *$p < 0.01$)

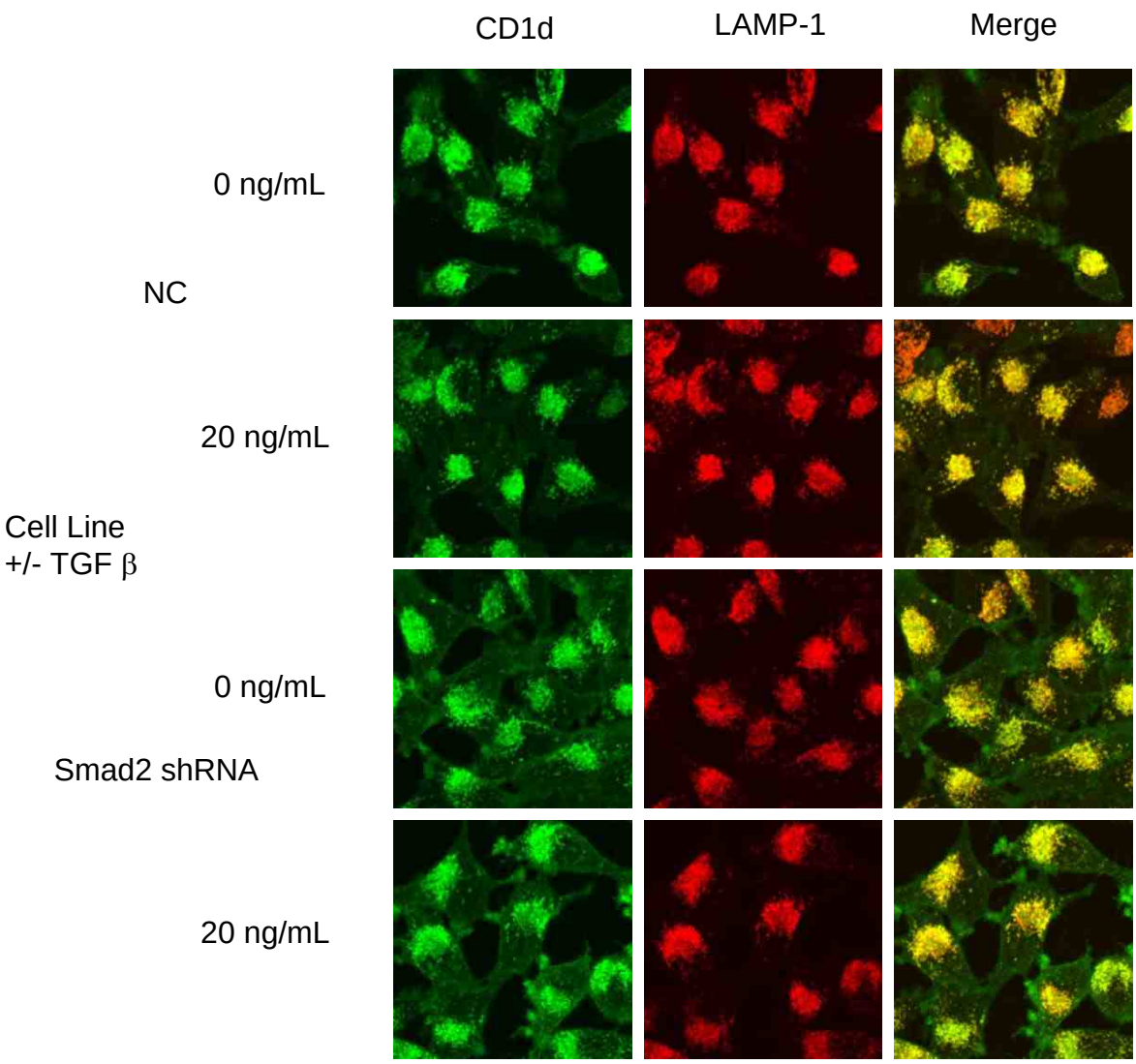

Figure 18. Smad2 knockdown does not alter CD1d co-localization with LAMP-1. LMTK-CD1d NC and Smad2 shRNA cells were treated with 0 and 20 ng/mL for 24 hours. After treatments, cells were fixed, stained for CD1d and LAMP-1 and analyzed by confocal microscopy. (n = 5)

transfection of Smad3 shRNA- expressing pLKO.1 vector by lentivirus infection. First, the level of Smad3 protein knockdown was determined by Western blot. Different concentrations of LMTK-CD1d NC and Smad3 shRNA cell lysates were compared. It was found that Smad3 was significantly knocked down (80%) by the Smad3-specific shRNA (Fig 19). The cells were then tested for their ability to activate NKT cells through CD1d-mediated antigen presentation. After treatment with increasing concentrations of TGF β, LMTK-CD1d NC and Smad3 shRNA cells were co-cultured with NKT hybridomas. Antigen presentation to NKT cells was determined by an IL-2 ELISA. Unfortunately, TGF β was unable to inhibit antigen presentation in either of these cell lines, which was suspected to be due to the lentivirus infection. However, a comparison of the untreated cells showed that knockdown of Smad3 by shRNA did not alter CD1d-mediated antigen presentation (Fig 20). Therefore, these data indicate that Smad3 does not influence antigen presentation by CD1d.

As the Co-Smad, Smad4 is the final addition to the Smad2/Smad3 complex and is essential for the Smad pathway specific upregulation or downregulation of target genes. Therefore, decreasing the level of Smad4 expression is one means of inhibiting the Smad pathway. To determine the potential role of Smad4 in the TGF β-induced inhibition of CD1d antigen presentation, Smad4 shRNA expressed in the pLKO.1 vector was infected into LMTK-CD1d cells. For Smad4, two different shRNA were used. The first shRNA construct targeted the Smad4 coding sequence 1416 and the other targeted 1925. The efficiency of knockdown achieved by these shRNA constructs was determined by Western blot. Comparison of NC to Smad4 shRNA 1416 and 1925 cells shows a

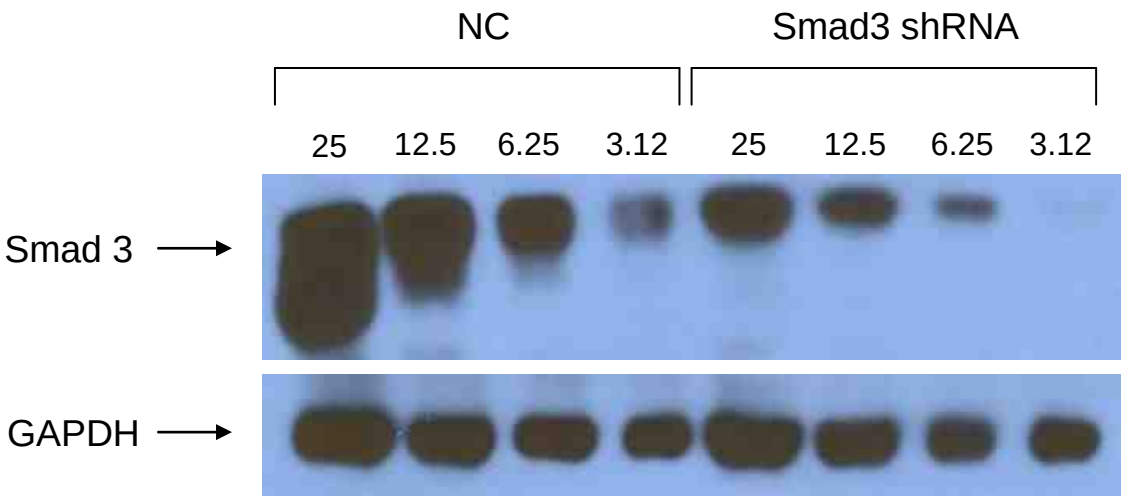

Figure 19. Smad3 is knocked down by the pLKO.1 vector expressing Smad3 shRNA in LMTK-CD1d cells. The lentiviral vector pLKO.1 expressing an shRNA sequence targeting coding sequence 1137 of the Smad3 mRNA was transfected into LMTK-CD1d cells. After puromycin selection, cells were lysed, and 25, 12.5, 6.25, and 3.125 μg from both the control cells, LMTK-CD1d NC, and Smad3 shRNA cells were loaded onto an 8% SDS-PAGE gel. After transfer to a PVDF membrane, the blot was probed for total Smad3 and for GAPDH as a loading control. (n = 3)

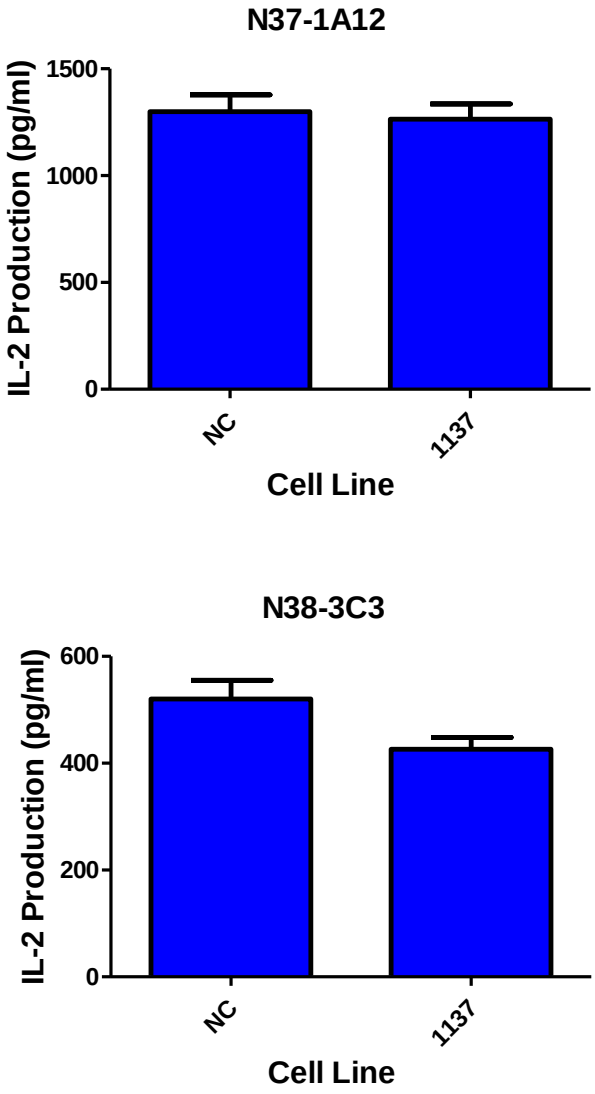

Figure 20. Smad3 knockdown does not alter CD1d-mediated antigen presentation. LMTK-CD1d NC and Smad3 shRNA cells were treated for 24 hours with 0, 1, 5, 10, and 20 ng/mL of TGF β. After treatment, the cells were co-cultured with the indicated NKT cell hybridomas for 22-24 hours at 37^{o}C. Supernatants from the co-culture were analyzed for IL-2 concentration by ELISA. (One representative experiment; n = 6, N.S.)

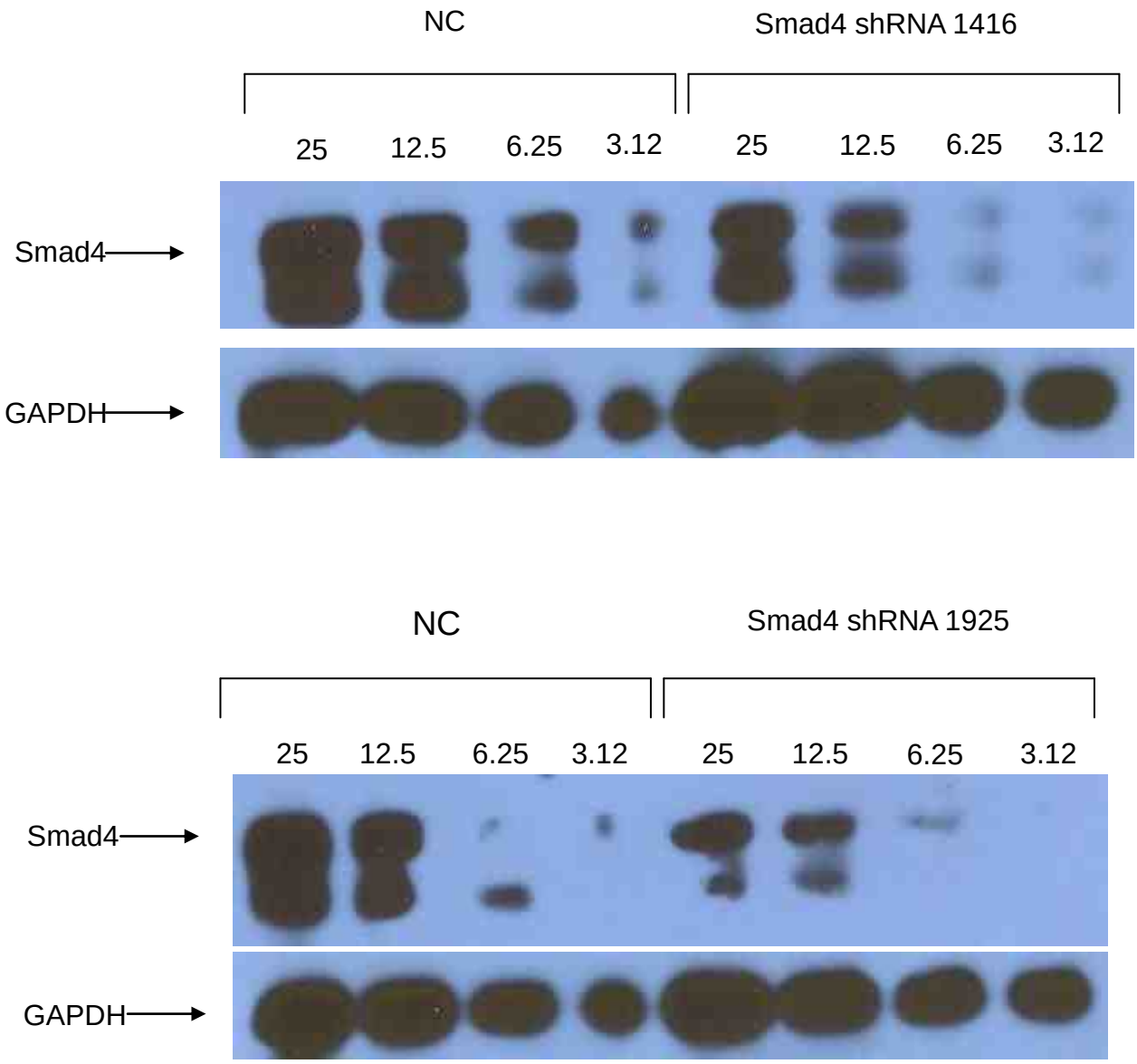

Figure 21. Smad4 is knocked down by pLKO.1 vector expressing Smad4 shRNA. The lentiviral vector pLKO.1 expressing an shRNA sequence targeting coding sequence 1416 and 1925 of the Smad4 mRNA was transfected into LMTK-CD1d cells. After puromycin selection, the cells were lysed, and 25, 12.5, 6.25, and 3.125 μg from both the control cells, LMTK-CD1d NC, and Smad4 shRNA cells were loaded onto an 8% SDS-PAGE gel. After transfer to a PVDF membrane, the blot was probed for total Smad4 and for GAPDH as a loading control. (n = 3)

significant reduction in the level of Smad4 protein expression which was measured to be 85% knockdown in Smad4 shRNA 1416 cells and 70% in Smad4 shRNA 1925 cells (Fig 21). Therefore, both shRNA constructs were successful in knocking down Smad4 protein expression.

To determine the influence of Smad4 on CD1d-mediated antigen presentation, LMTK-CD1d NC, and Smad4 shRNA 1416 and 1925 cells were treated for 24 hours with increasing concentrations of TGF β. These cells were co-cultured with NKT cell hybridomas and the IL-2 secreted into the supernatants was determined by an ELISA. It was found that antigen presentation by the Smad4 shRNA 1416 and 1925 cells was substantially increased relative to the LMTK-CD1d NC cells (Fig 22). These data suggest that Smad4 activity inhibits antigen presentation by CD1d. Like Smad2, this inhibition is functional due to no observed changed in CD1d cell surface expression in Smad4 shRNA-expressing cells compared to NC-expressing cells as analyzed by flow cytometry.

Because neither a Smad2 knockdown nor TGF β treatment caused a change in CD1d co-localization with LAMP-1, it was hypothesized that knockdown of Smad3 and Smad4 would also not change the intracellular localization of CD1d. To test this, LMTK-CD1d NC, Smad3 shRNA, and Smad4 shRNA cells were treated for 24 hours with TGF β, stained for CD1d and LAMP-1, and analyzed by confocal microscopy. As expected, TGF β treatment alone did not alter CD1d co-localization with LAMP-1. Also, comparisons of NC cells with Smad3 shRNA (Fig 23), Smad4 shRNA 1416 (Fig 24), and Smad4 shRNA 1925 (Fig 25) –expressing LMTK-CD1d cells showed no difference in

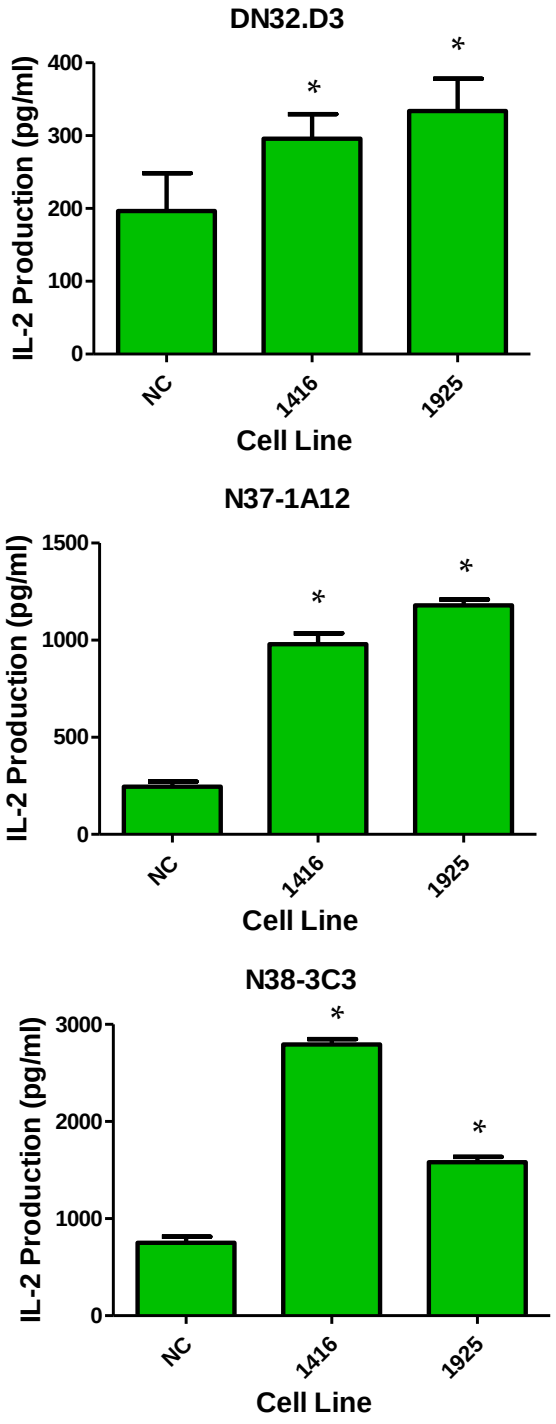

Figure 22. Smad4 knockdown increases CD1d-mediated antigen presentation. LMTK-CD1d NC and Smad4 shRNA cells were treated for 24 hours with 0, 1, 5, 10, and 20 ng/mL of TGF β. After treatment, the cells were co-cultured with the indicated NKT cell hybridomas for 22-24 hours at 37^{o}C. Supernatants from the co-culture were analyzed for IL-2 concentration by ELISA. (One representative experiment; n = 6, *$p < 0.01$)

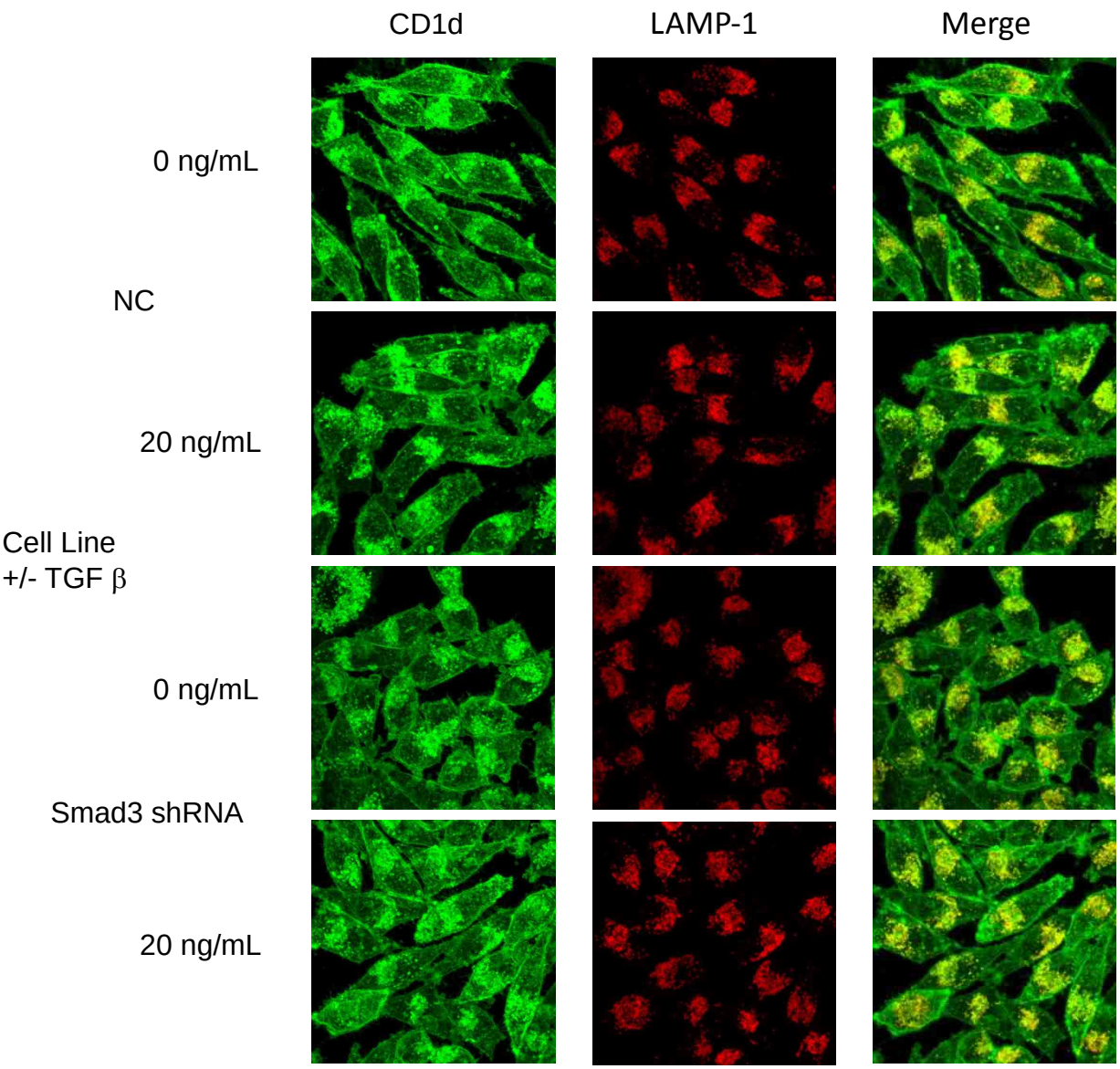

Figure 23. Smad3 knockdown did not alter CD1d co-localization with LAMP-1. LMTK-CD1d NC and Smad3 shRNA cells were treated with 0 and 20 ng/mL TGF β for 24 hours. After treatment, the cells were fixed, stained for CD1d and LAMP-1, and analyzed by confocal microscopy. (n = 3)

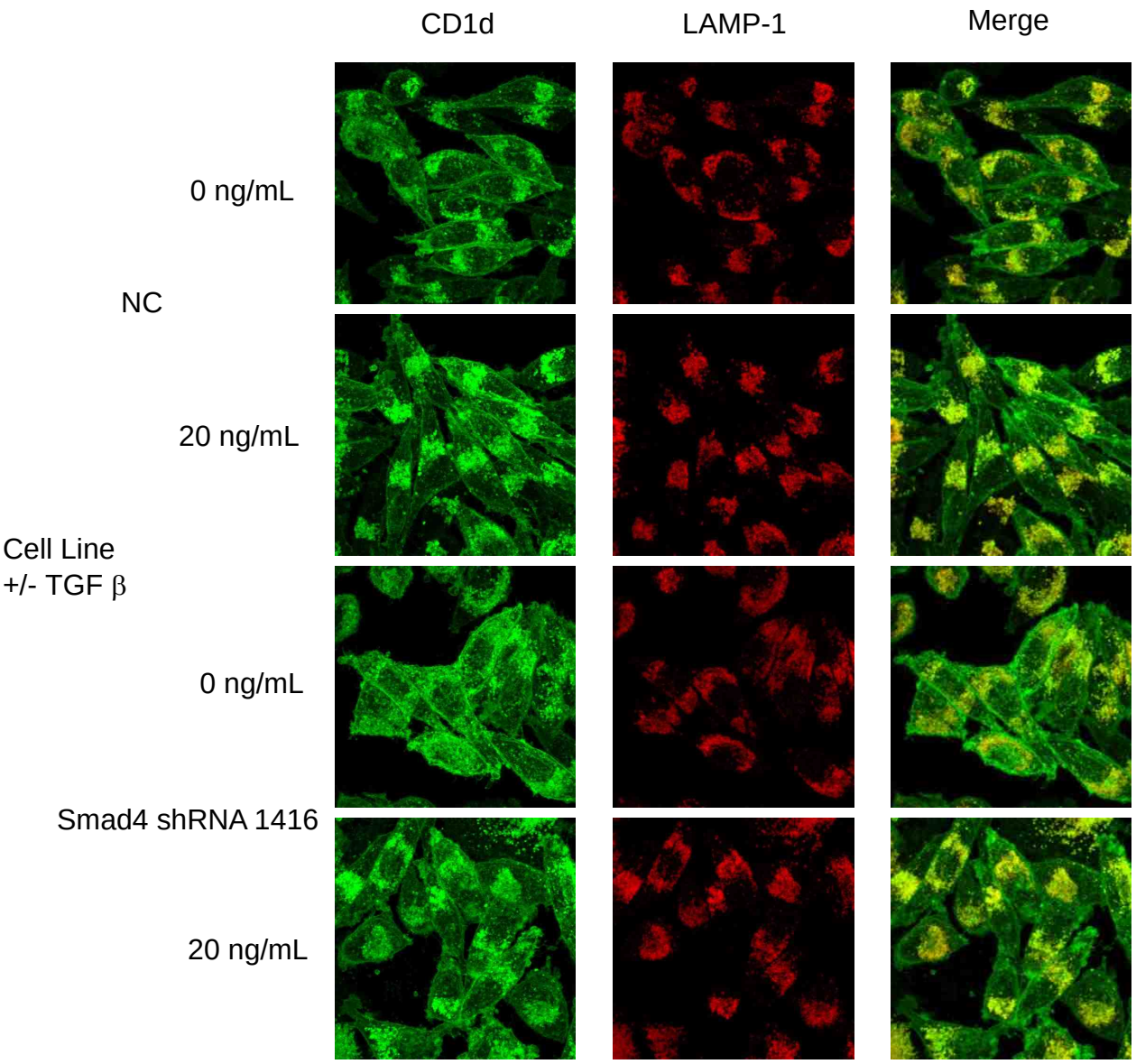

Figure 24. Smad4 knockdown did not alter CD1d co-localization with LAMP-1. LMTK-CD1d NC and Smad4 shRNA 1416 cells were treated with 0 and 20 ng/mL TGF β for 24 hours. After treatment, the cells were fixed, stained for CD1d and LAMP-1, and analyzed by confocal microscopy. (n = 3)

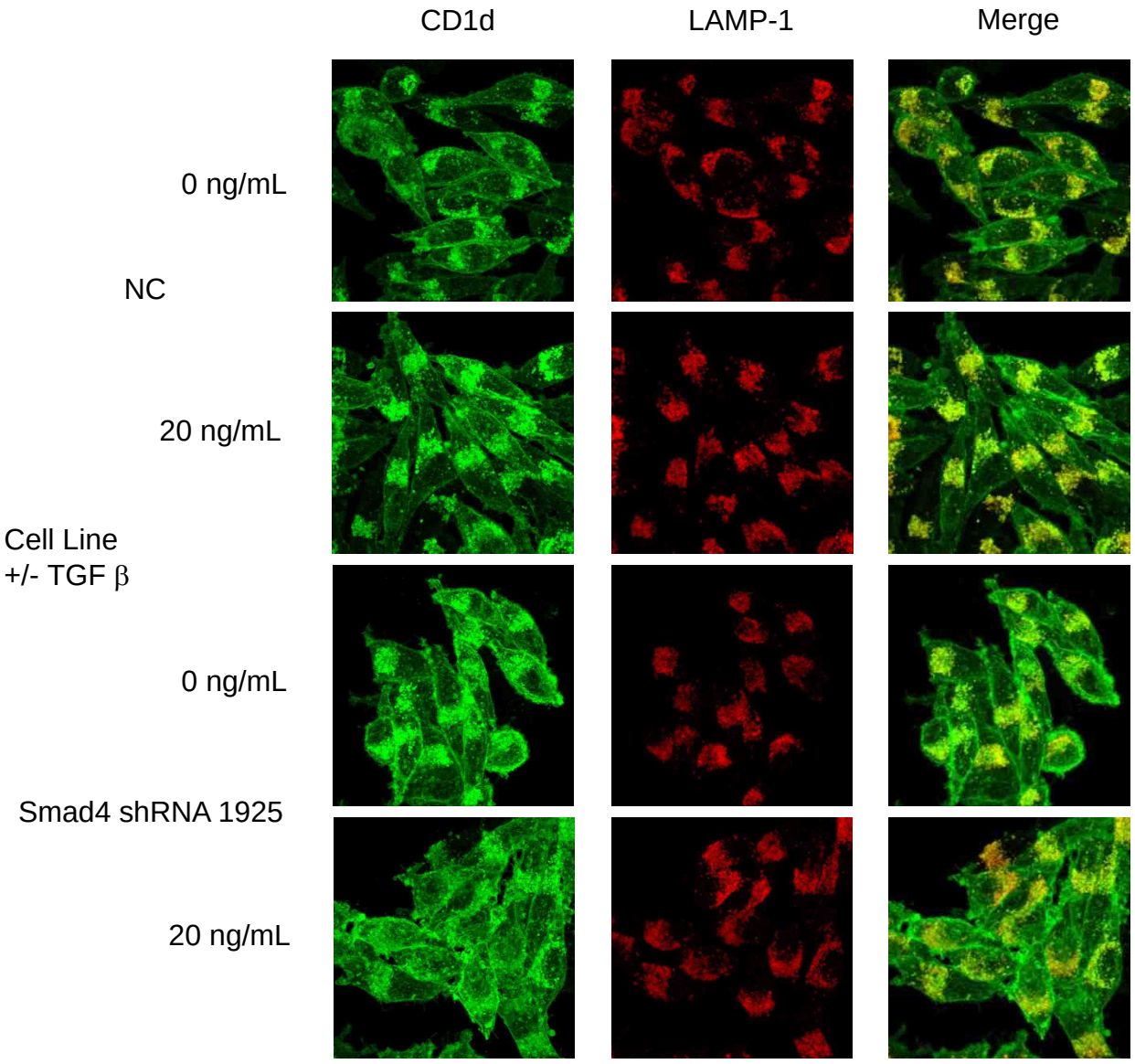

Figure 25. Smad4 knockdown did not alter CD1d co-localization with LAMP-1. LMTK-CD1d NC and Smad4 shRNA 1925 cells were treated with 0 and 20 ng/mL TGF β for 24 hours. After treatment, the cells were fixed, stained for CD1d and LAMP-1, and analyzed by confocal microscopy. (n = 3)

CD1d co-localization in LAMP-1^{+} compartments (e.g. the lysosomes and late endosomes). Therefore, neither TGF β nor the Smad pathway influences CD1d localization to the late endocytic compartments within the cell.

In conclusion, the Smad pathway plays a strong role in regulating CD1d-mediated antigen presentation. Two Smads, Smad2 and Smad4, are inhibitors of antigen presentation by CD1d, whereas Smad3 appears to have no effect. This is indicative of the Smad pathway being the mechanism behind TGF β's inhibitory effects.

Chapter 3

ROCK and LIMK inhibit CD1d-mediated antigen presentation.

The cytokine TGF β causes the formation of stress fibers, long parallel bundles of actin chains. Vardouli et al have shown that this formation of stress fibers is through TGF β-dependent activation of the Rho/ROCK/LIMK/cofilin pathway [167]. Activation of Rho-GTPases is achieved through the function of guanine exchange factors (GEFs) [168]. GEFs facilitate the exchange of GDP bound to Rho for GTP. Rho-GTP signals downstream by binding to the Rho-associated kinase (ROCK) at the Rho binding domain (RBD) [169] and changing the protein conformation of ROCK [170]. As an activated kinase, ROCK phosphorylates LIMK1 and 2 [171, 172]. These LIMKs then phosphorylate cofilin [173], a regulator of actin polymerization [174]. In the unphosphorylated state, cofilin prevents actin from polymerizing into long chains [137, 175]. When phosphorylated by LIMK, cofilin is inactive [177]. As a result of cofilin inactivity, long bundled parallel chains of polymerized bundles, known as stress fibers, form [176] (Fig 4).

What is unknown is whether the Rho pathway is part of TGF β-dependent inhibition of CD1d-mediated antigen presentation. A follow-up publication by Vardouli et al gives evidence that activation of the Smad pathway, specifically activation of Smad2 and Smad3, leads to gene upregulation of RhoB [178]. Others have shown that gene regulation by the Smad pathway can cause the upregulation of RhoA, RhoB, and RhoC.

Therefore, it was hypthosized that this pathway may possibly be connected to inhibition of CD1d-mediated antigen presentation by TGF β. Potentially, TGF β signals through the Smad pathway which upregulates RhoB expression. In addition to this, TGF β activation of TGFβRI may lead to more RhoA bound to GTPase, more stress fiber formation and ultimately, less CD1d-mediated antigen presentation. However, such a connection is unproven and therefore, the Rho-ROCK pathway and its connection to CD1d-mediated antigen presentation becomes the focus of this chapter.

Several groups have shown that TGF β causes the formation of stress fibers which are many long chains of G-actin monomers polymerized into F-actin [193]. These long fibers are bundled together and lie across the breadth and width of the cell [194]. As mentioned previously, these stress fibers are caused by TGF β signaling through the Rho/ROCK pathway. Therefore, the initial objective in investigating this pathway was to confirm the observation of TGF β inducing the formation of stress fibers. LMTK-CD1d cells were treated with increasing concentrations of TGF β for 24 hours. After treatment, the cells were stained with rhodamine phalloidin, a flourophore-conjugated fungal toxin known to bind F-actin. Cell imaging was performed by confocal microscopy. The results showed an increase in stress fiber formation as the concentration of TGF β increased (Fig 26). At 1 ng/mL, no stress fiber formation was detectable, but a 5 ng/mL treatment showed the faint development of stress fibers. Compared to untreated cells (0 ng/mL), 20 ng/mL of TGF β caused substantial stress fibers formation. These results confirm that TGF β induces stress fiber formation.

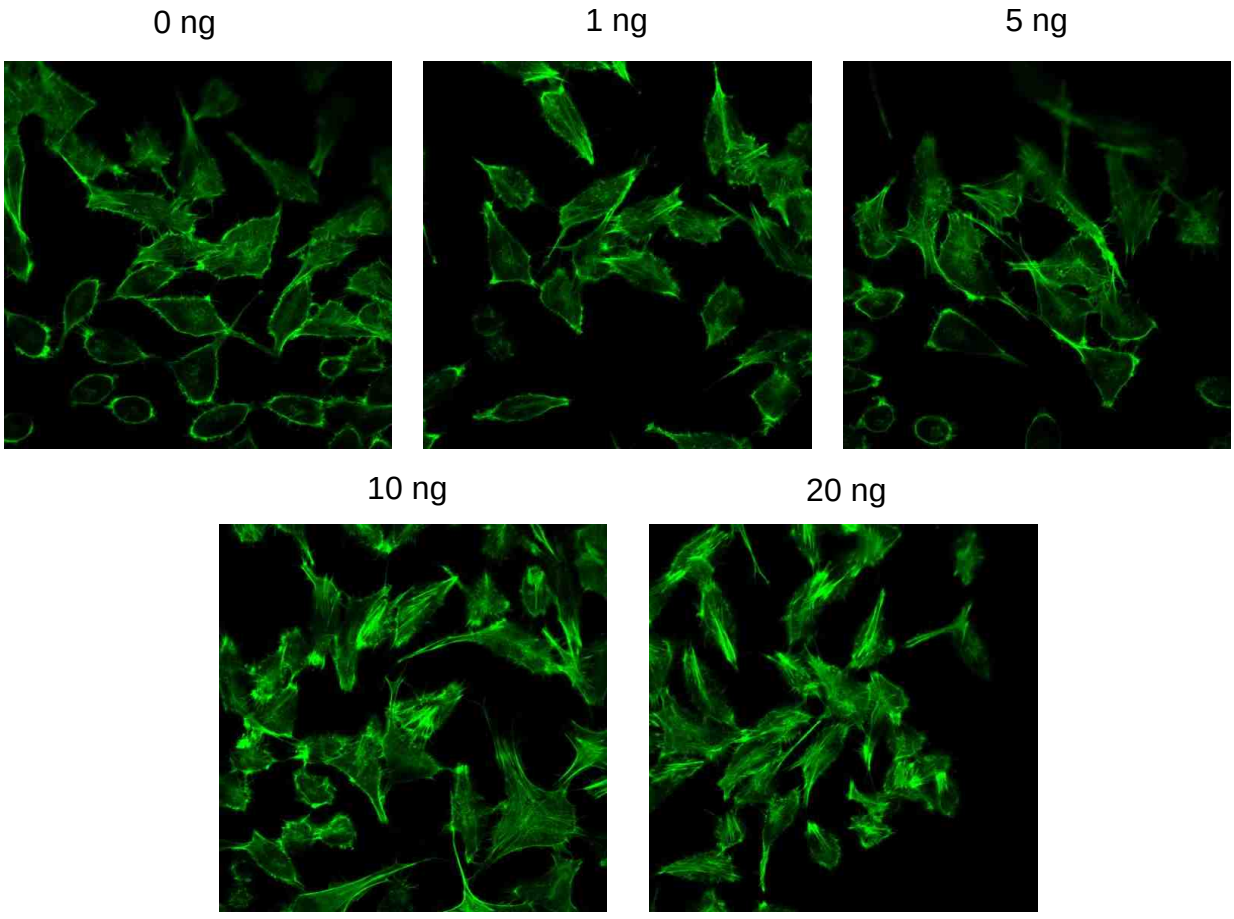

Figure 26. TGF β induces stress fiber formation. LMTK-CD1d cells were treated with 0, 1, 5, 10 and 20 ng/mL of TGF β for 24 hours. After treatment, the cells were stained for actin with rhodamine-phalloidin. The stained actin was visualized by confocal microscopy. (n = 5)

In the previous chapter, Smad2, Smad3 and Smad4 shRNA were utilized to determine the role of Smads in CD1d-mediated antigen presentation. Vardouli's prior work showed that the overexpression of Smad2 and Smad3 increased stress fiber formation [13]. Therefore, it was hypothesized that knockdown of the various Smads would result in a decrease in stress fibers after TGF β treatment. To test this, LMTK-CD1d negative control (NC) and Smad2 shRNA-expressing cells were treated for 24 hours with 0 and 20 ng/mL of TGF β. As expected, TGF β induced stress fiber formation in the negative control cells. However, in the LMTK-CD1d Smad2 shRNA-expressing cells, TGF β was unable to induce stress fiber formation (Fig 27). The same results were observed in TGF β-treated LMTK-CD1d Smad4 shRNA cells (Fig 28). Unlike the NC cells, TGF β was not able to induce stress fiber formation in the Smad4 shRNA-expressing cells. Again, the same was observed in LMTK-CD1d Smad3 shRNA cells but with a slightly different result (Fig 29). Although TGF β was not able to cause stress fiber formation in cells expressing Smad3 shRNA, very large clusters of actin polymerization were detected by confocal microscopy, suggesting a further connection between Smad3 and actin regulation. These results are consistent with Vardouli's prior study.

It is just an hypothesis that altering the actin cytoskeleton could affect CD1d and its ability to present antigen. Therefore, TGF β-induced stress fibers may not alter antigen presentation by CD1d. To know more about actin and its potential role in antigen presentation, an inhibitor of actin polymerization was used. Cytochalasin D is a fungal toxin that competes with actin cap-binding proteins and binds to the F-actin cap [195]. F-actin works as a treadmill, in that G-actin monomers polymerize with F-actin at its cap and the depolymerization of F-actin occurs at the opposite end [196]. By binding to the

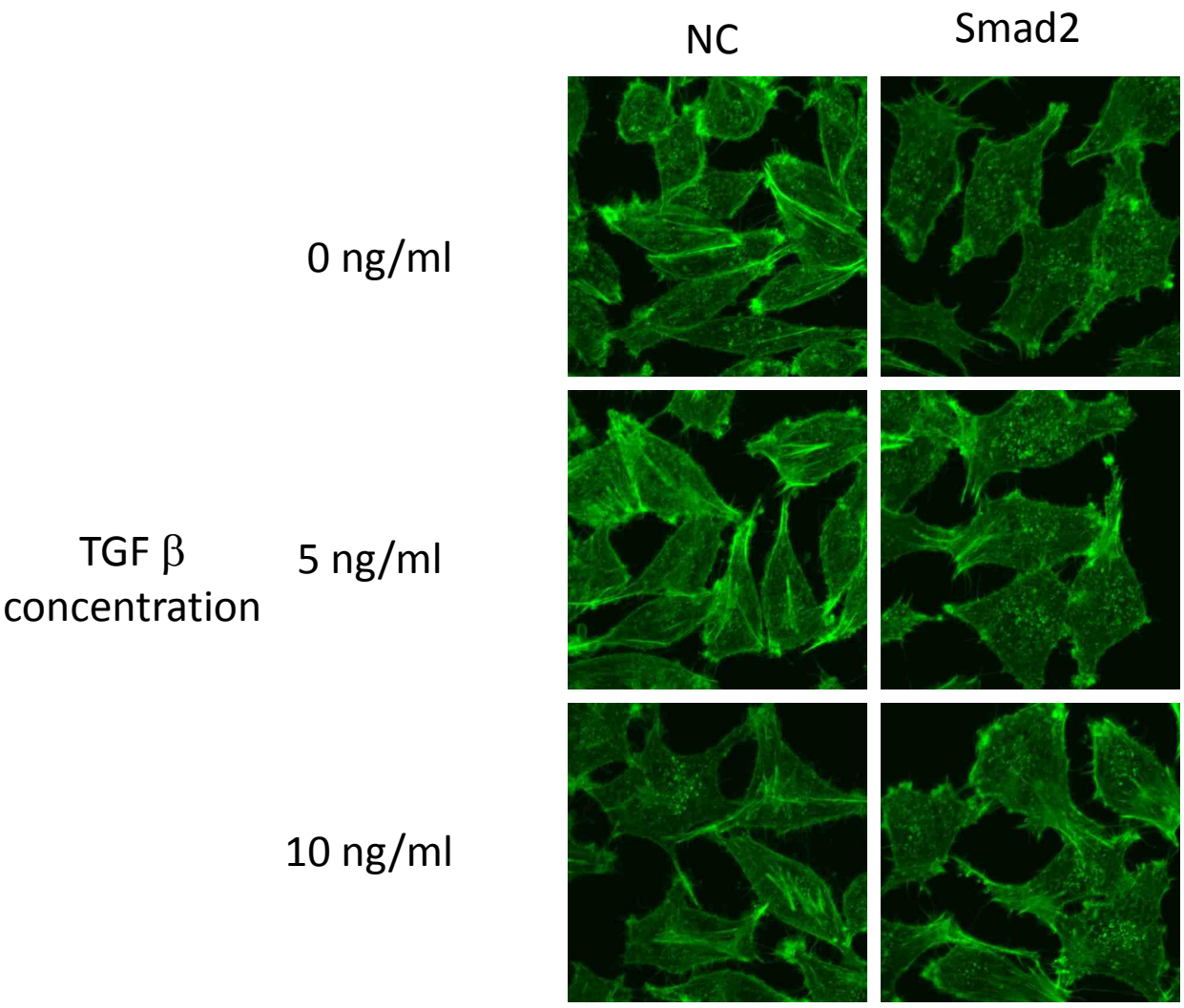

Figure 27. Smad2 knockdown inhibits TGF β-induced stress fiber formation. LMTK-CD1d Smad2 shRNA-expressing cells were treated with 0, 5 and 10 ng/mL TGF β for 24 hours. The cells were stained with rhodamine-phalloidin and the actin was visualized by confocal microscopy. (n = 3)

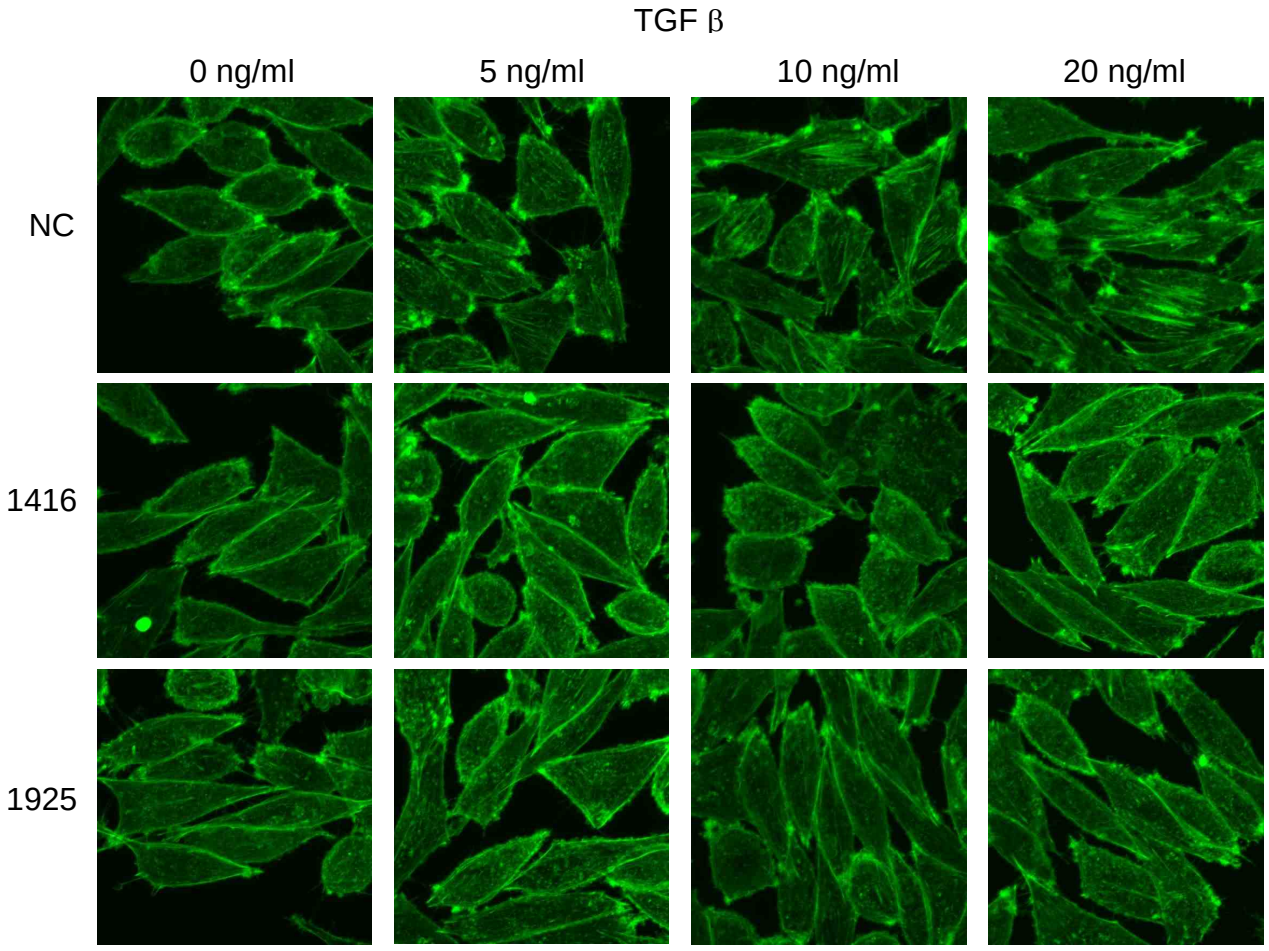

Figure 28. Smad4 knockdown inhibits TGF β-induced stress fiber formation. LMTK-CD1d Smad4 shRNA-expressing cells were treated with 0, 5, 10 and 20 ng/mL TGF β for 24 hours. The cells were stained with rhodamine-phalloidin and the actin was visualized by confocal microscopy. (n = 3)

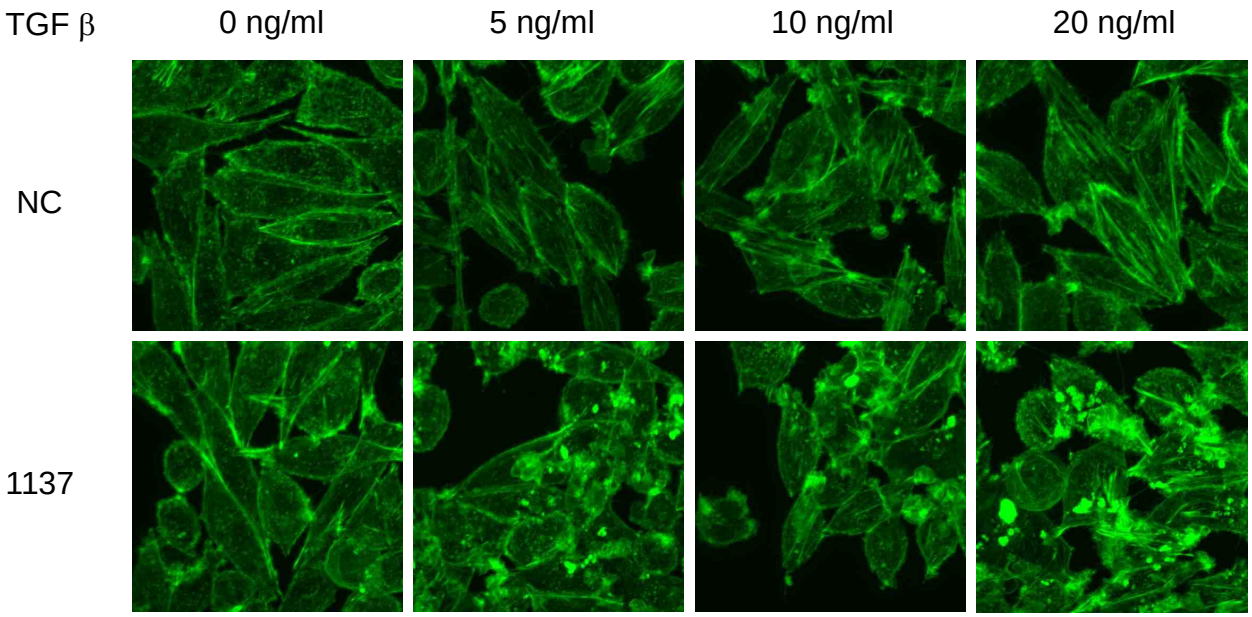

Figure 29. Smad3 knockdown inhibits TGF β-induced stress fiber formation. LMTK-CD1d Smad3 shRNA-expressing cells were treated with 0, 5, 10 and 20 ng/mL TGF β for 24 hours. The cells were stained with rhodamine-phalloidin and the actin was visualized by confocal microscopy. (n = 3)

cap of F-actin, Cytochaslasin D inhibits F-actin formation. Therefore Cytochalasin D should inhibit stress fiber formation in our system. To test this, untreated and TGF β-treated LMTK-CD1d cells were stained with rhodamine-phalloidin and analyzed by confocal microscopy. As seen before, treatment of 20 ng/mL TGF β for 24 hours caused the formation of stress fibers. However, treatment with 2 μM Cytochalasin D for 6 hours disrupted all actin formation including the stress fibers caused by TGF β (Fig 30). What did result were large focal aggregates of F-actin that remained after Cytochalasin D-mediated inhibition of actin polymerization. These focal aggregates have been previously observed by others in different systems [197].

If TGF β-dependent inhibition of CD1d-mediated antigen presentation was related to stress fiber formation, then disruption of actin polymerization by Cytochalasin D should increase antigen presentation by CD1d. To test this, LMTK-CD1d cells were treated with increasing concentrations of Cytochalasin D for 6 hours and co-cultured with NKT hybridomas for 22-24 hours. The production of IL-2 by the NKT cells, a measure of CD1d-mediated antigen presentation in the co-culture supernatant, was determined by an ELISA. The results showed that Cytochalasin D treatment at all concentrations increased CD1d-mediated antigen presentation (Fig 31). Therefore, cellular disruption of actin polymerization by Cytochalasin D caused an increase in CD1d-mediated antigen presentation. Cytochalasin D did not alter CD1d cell surface expression as determined by flow cytometry. If the actin structure was altered, then the trafficking and therefore the co-localization of CD1d may be changed. To determine this, LMTK-CD1d cells seeded into 35 mm glass bottom plates were treated with 1 and 2 μM of Cytochalasin D for 6 hours, stained for CD1d and Lamp-1, and analyzed by confocal microscopy. As

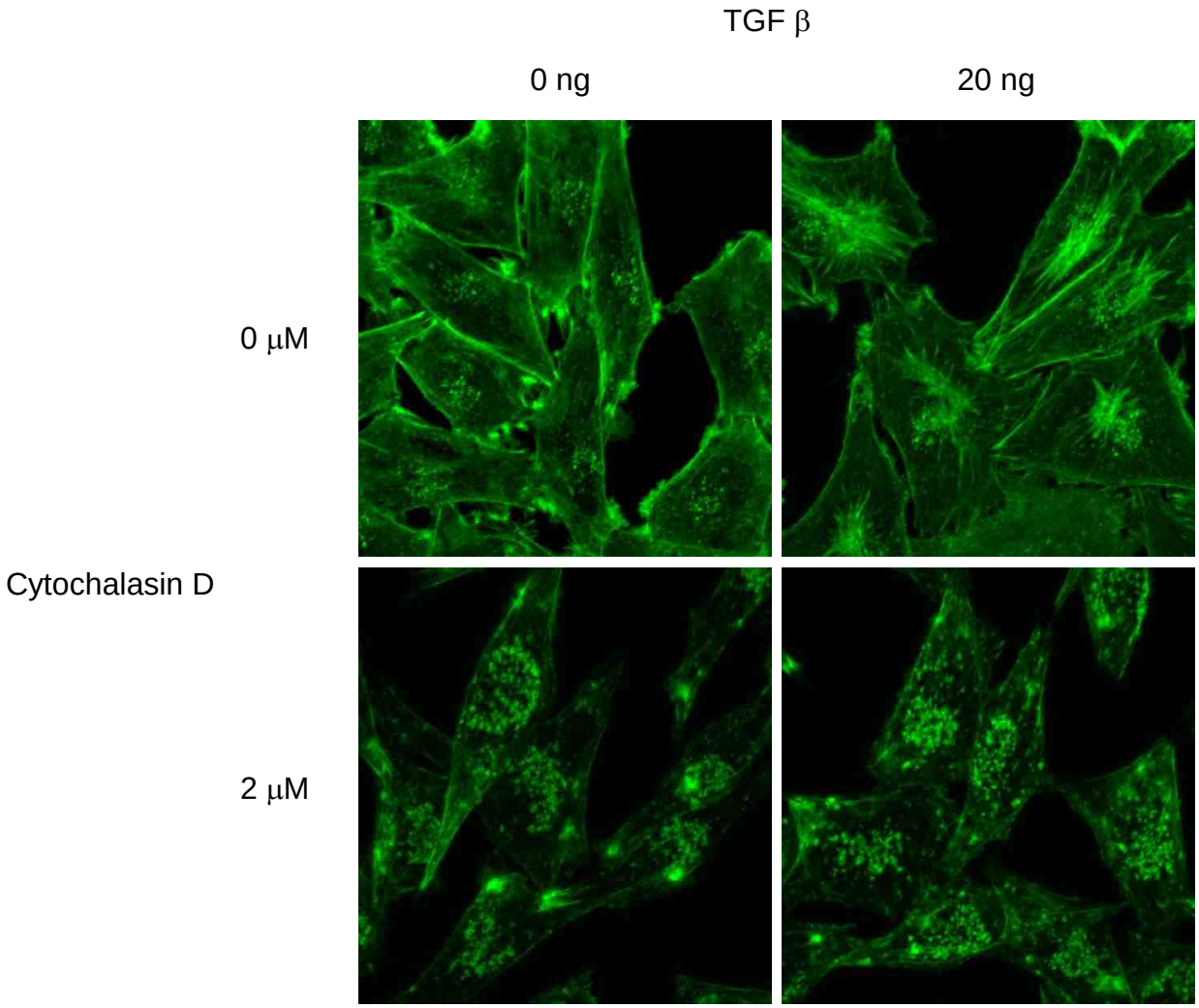

Figure 30. Cytochalasin D disrupts stress fiber formation induced by TGF β. LMTK-CD1d cells were treated with 0 and 20 ng/mL TGF β and 0 and 2 μM Cytochalasin D for 6 hours. After treatment the cells were stained with rhodamine-phalloidin and the actin was visualized by confocal microscopy. (n = 3)

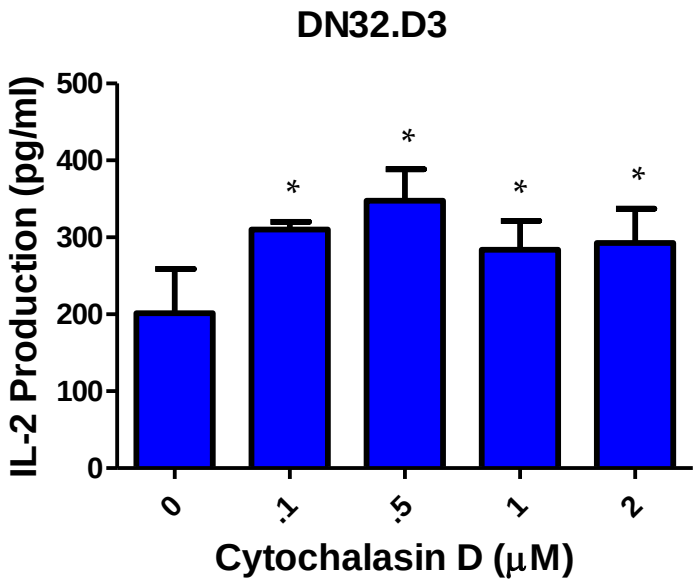

Figure 31. Cytochalasin D increases CD1d-mediated antigen presentation. LMTK-CD1d cells were treated with 0, 0.1, 0.5, 1 and 2 μM of Cytochalasin D for 24 hours. After treatment, these cells were co-cultured with the indicated NKT cell hybridoma cells for 22-24 hours. The resulting supernatants were analyzed by ELISA for IL-2 production. (One representative experiment; n = 3; *$p < 0.01$)

determined by a correlation coefficient analysis (Metamorph), no difference in CD1d localization to LAMP-1^+ compartments was observed (Fig 32). Therefore, Cytochalasin D does not alter the intracellular localization of CD1d.

TGF β leads to the activation of Rho through binding of GTP. Once bound to GTP and activated, Rho binds ROCK through the latter's Rho binding domain. This binding changes the protein conformation and activates ROCK. As part of the TGF β/stress fiber pathway, ROCK should influence the actin cytoskeleton. This was tested by blocking ROCK activity with its inhibitor, Y-27632, at 100 μM in TGF β-treated and untreated LMTK-CD1d cells for 24 hours. The actin in these cells was stained with rhodamine-phalloidin. Confocal microscopic analysis revealed a significant disruption of TGF β-induced stress fiber formation by the ROCK inhibitor, in addition to a significant change in cell morphology (Fig 33). It was hypothesized that inhibiting ROCK would increase CD1d-mediated antigen presentation which was tested by treating LMTK-CD1d cells with 0, 25, 50, and 100 μM of Y-27632 for 24 hours. These cells were then co-cultured with NKT hybridomas for 22-24 hours and the IL-2 produced by the NKT cells was measured by ELISA. As the concentration of Y-27632 increased, the level of IL-2 and therefore CD1d-mediated antigen presentation, also increased (Fig 34). The increase in antigen presentation is functional due to flow cytometry revealing no change in CD1d cell surface expression caused by Y-27632. This is consistent with the hypothesis that preventing actin polymerization promotes CD1d-mediated antigen presentation. Also, ROCK function being inhibitory to antigen presentation by CD1d further supports the putative role of ROCK in TGF β-dependent inhibition of CD1d-mediated antigen presentation.

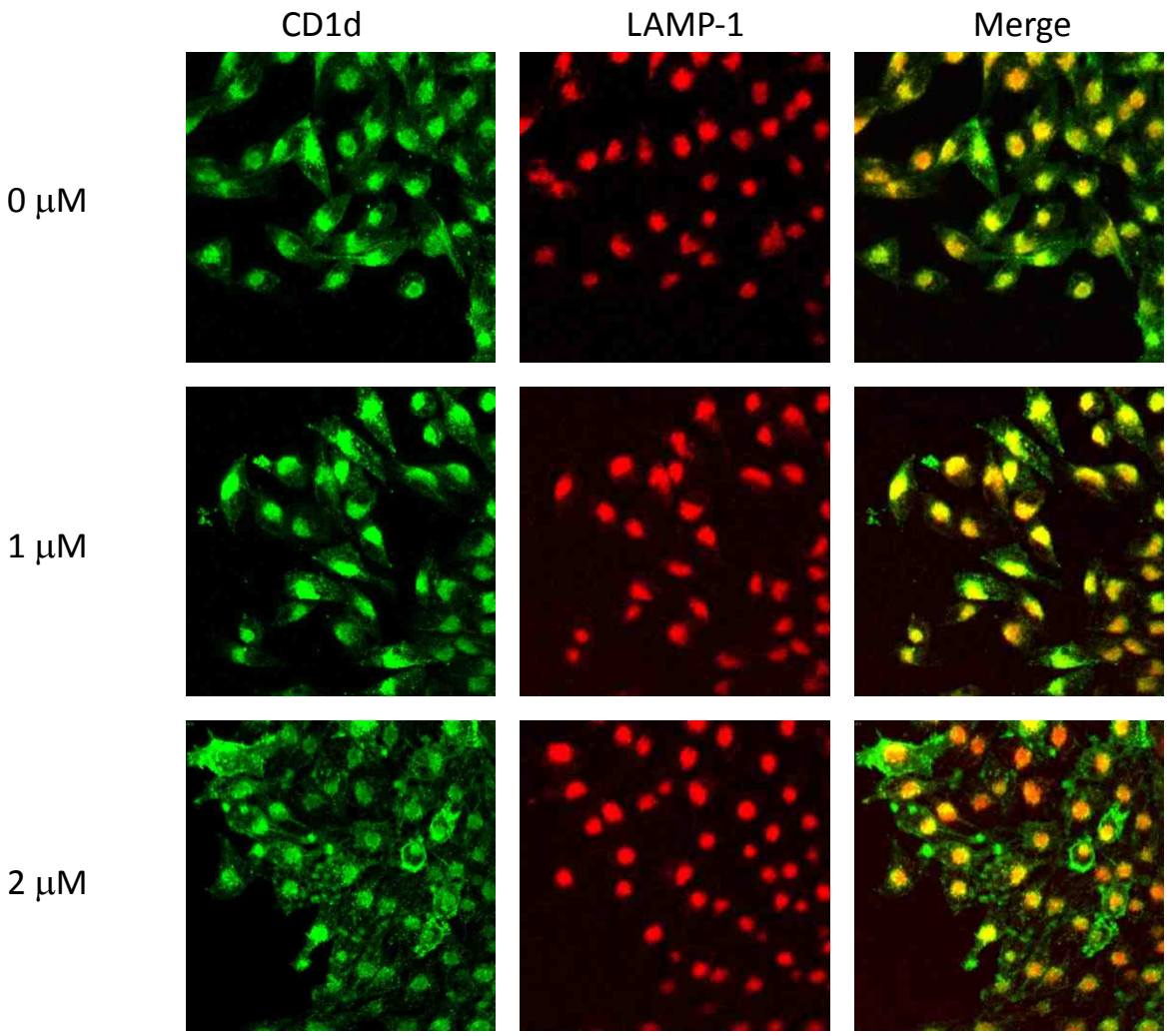

Figure 32. Cytochalasin D does not alter CD1d intracellular localization. LMTK-CD1d cells were treated with 0, 1 and 2 μM of Cytochalasin D for 24 hours. After treatment, the cells were stained for CD1d and LAMP-1 and co-localization was visualized by confocal microscopy. (n = 3)

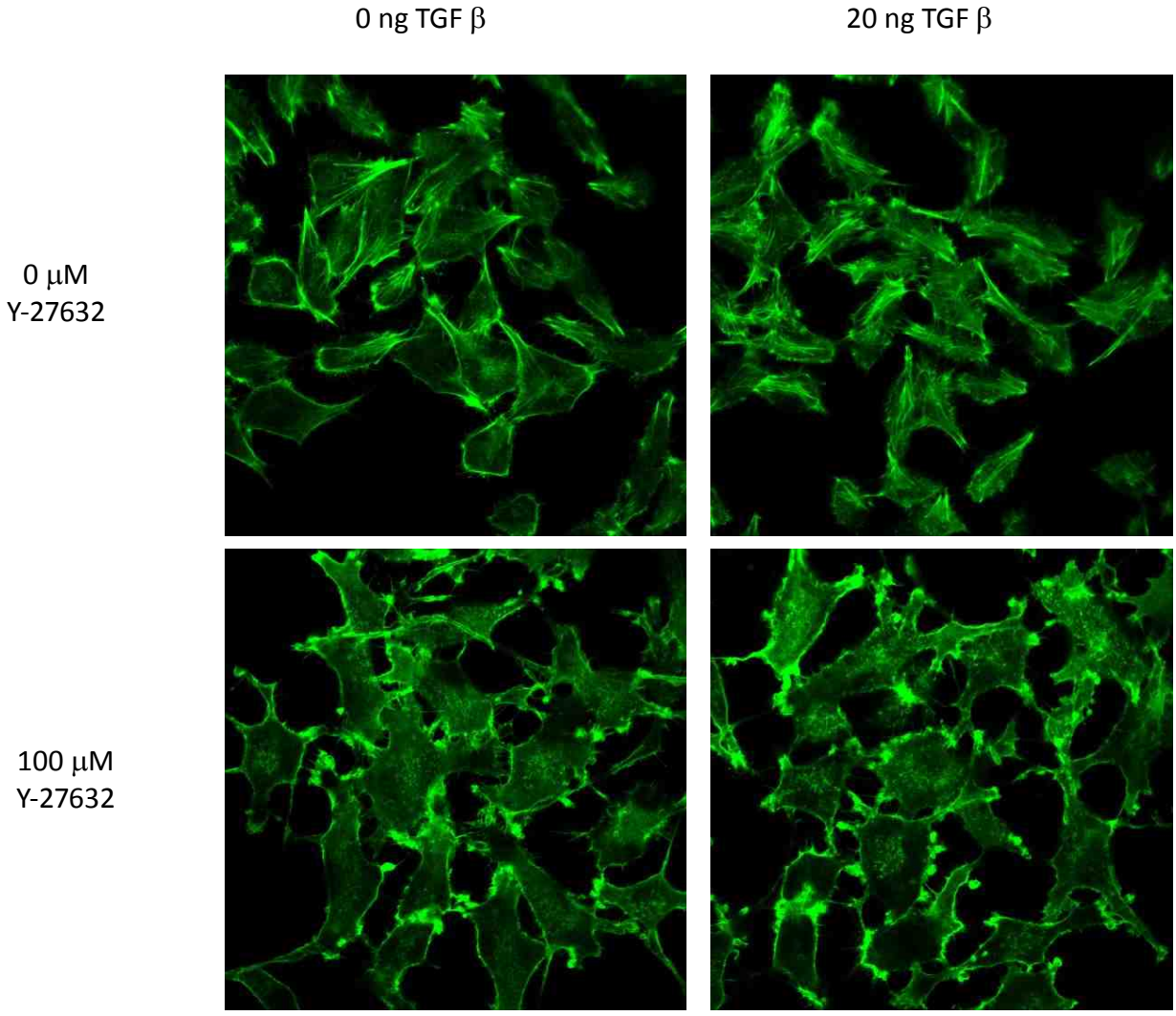

Figure 33. ROCK inhibitor, Y-27632, disrupts stress fiber formation by TGF β. LMTK-CD1d cells were treated with 0 and 20 ng/mL of TGF β as well as 0 and 100 μM of Y-27632 for 24 hours. After treatment, the actin was stained with rhodamine phalloidin and was visualized by confocal microscopy. (n = 3)

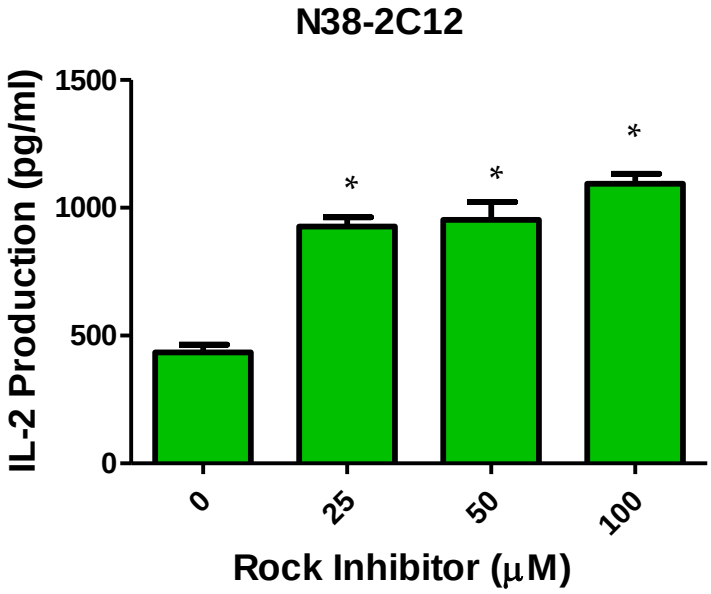

Figure 34. ROCK inhibitor, Y-27632, increased CD1d-mediated antigen presentation. LMTK-CD1d cells were treated with 0, 25, 50, and 100 μM of Y-27632 for 24 hours. After treatment, the cells were co-cultured with N38-2C12 for 22-24 hours. The resulting supernatants were analyzed by ELISA for IL-2 production. (One representative experiment; n = 3; *$p < 0.01$)

Although able to inhibit both ROCK1 and ROCK2, the two isoforms of ROCK [198], like any drug, Y-27632 could potentially have unexpected alternative targets and effects. In order to determine the importance of the two isoforms and to eliminate the possibility of the drug affecting non-ROCK targets, an shRNA construct specifically targeting ROCK1 was expressed by the pLKO.1 vector transfected into LMTK-CD1d cells. Like what we observed with Y-27632, it was suspected that the knockdown of ROCK1 expression would cause a decrease in TGF β-induced stress fibers. To determine the ROCK1 shRNA effects on the actin cytoskeleton, these cells were plated onto glass-bottom 35 mm plates, treated with 0 and 20 ng/mL of TGF β for 24 hours and stained with rhodamine-phalloidin. Confocal microscopy revealed that the knockdown of ROCK1 prevented the development of TGF β-induced stress fibers. This supported our previous evidence that ROCK plays a role in stress fiber formation in LMTK-CD1d cells (Fig 35). Also, because treatment of LMTK-CD1d cells with Y-27632 produced an increase in CD1d-mediated antigen presentation, it was likely that ROCK plays an inhibitory role in our system. Therefore, it was hypothesized that ROCK shRNA would produce similar results as Y-27632 and further support the findings that ROCK negatively regulates CD1d-mediated antigen presentation. To test this, LMTK-CD1d ROCK1 shRNA cells and LMTK-CD1d NC, which were control cells transfected with a pLKO.1 vector expressing a non-specific shRNA sequence, were co-cultured with NKT hybridoma cells for 22-24 hours at 37^{o}C. Supernatants were collected and analyzed by an ELISA for IL-2 production. As expected, the cells with a knockdown of ROCK shRNA caused increased levels of IL-2 production by NKT cells (Fig 36). The

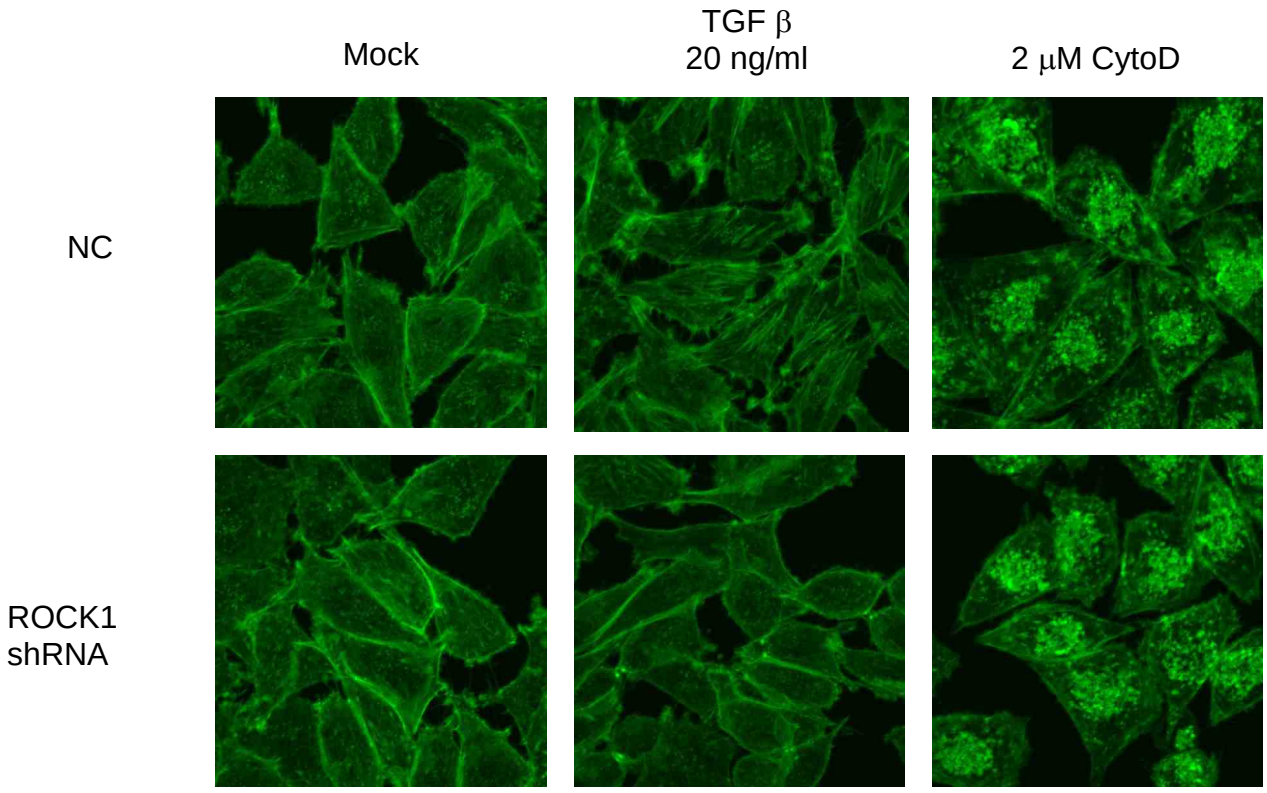

Figure 35. ROCK1 knockdown disrupts TGF β-induced stress fiber formation. LMTK-CD1d ROCK1 shRNA and control shRNA expressing cells were untreated or treated with 20 ng/mL of TGF β and 2 μM of cytochalasin D for 24 hours. The cells were then stained with rhodamine phalloidin and the actin was visualized by confocal microscopy. (n = 3)

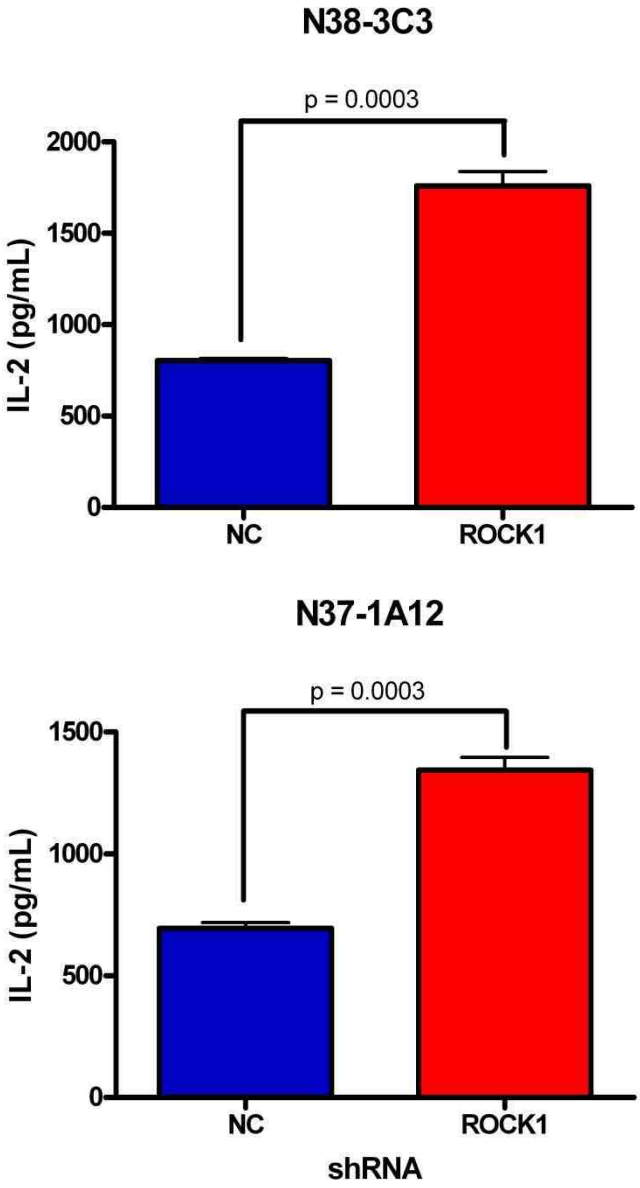

Figure 36. ROCK1 knockdown increases CD1d-mediated antigen presentation. LMTK-CD1d ROCK1 shRNA-expressing cells were co-cultured with the indicated NKT cell hybridomas for 22-24 hours. The resulting supernatants were analyzed by ELISA for the level of IL2 production. (Experiment performed by Richard Gallo)

knockdown of ROCK1 was verified previously by Western blot (Richard Gallo; data not shown). Also, ROCK1 knockdown did not alter CD1d cell surface expression as determined by flow cytometry. Therefore, these data with the ROCK1 shRNA further support the finding that ROCK inhibits CD1d-mediated antigen presentation.

It was originally thought that the knockdown of ROCK and the effect of decreased actin polymerization may cause a change in the localization of CD1d to late endocytic compartments. However, Cytochalasin D, an inhibitor of actin polymerization, did not alter CD1d localization to the late endosome and lysosome as shown above. Therefore it seemed unlikely that the knockdown of ROCK1 would alter this localization. This was found to be correct upon staining LMTK-CD1d NC and ROCK1 shRNA for CD1d and LAMP-1 and analysis by confocal microscopy (Fig 37).

ROCK, as a kinase, will phosphorylate and activate LIM kinase, the next protein in the pathway (Fig 4). LIMK exists in two isoforms, LIMK1 and LIMK2. Therefore, the importance of LIMK1 and LIMK2 in CD1d-mediated antigen presentation was investigated through targeting LIMK1/2 mRNA with shRNA expressed by the pLKO.1 vector. As shown above, ROCK inhibitor Y-27632 and ROCK shRNA were able to inhibit stress fiber formation caused by TGF β. As part of the ROCK pathway, LIMK shRNA was expected to also inhibit stress fibers in cells treated with TGF β. Subsequently, LMTK-CD1d NC, LIMK1 shRNA and LIMK2 shRNA were treated with 20 ng/mL TGF β for 24 hours, stained with rhodamine-phalloidin and analyzed by confocal microscopy. The results showed reduced stress fiber formation in cells expressing LIMK shRNAs when compared to the negative control cells (Fig 38). This

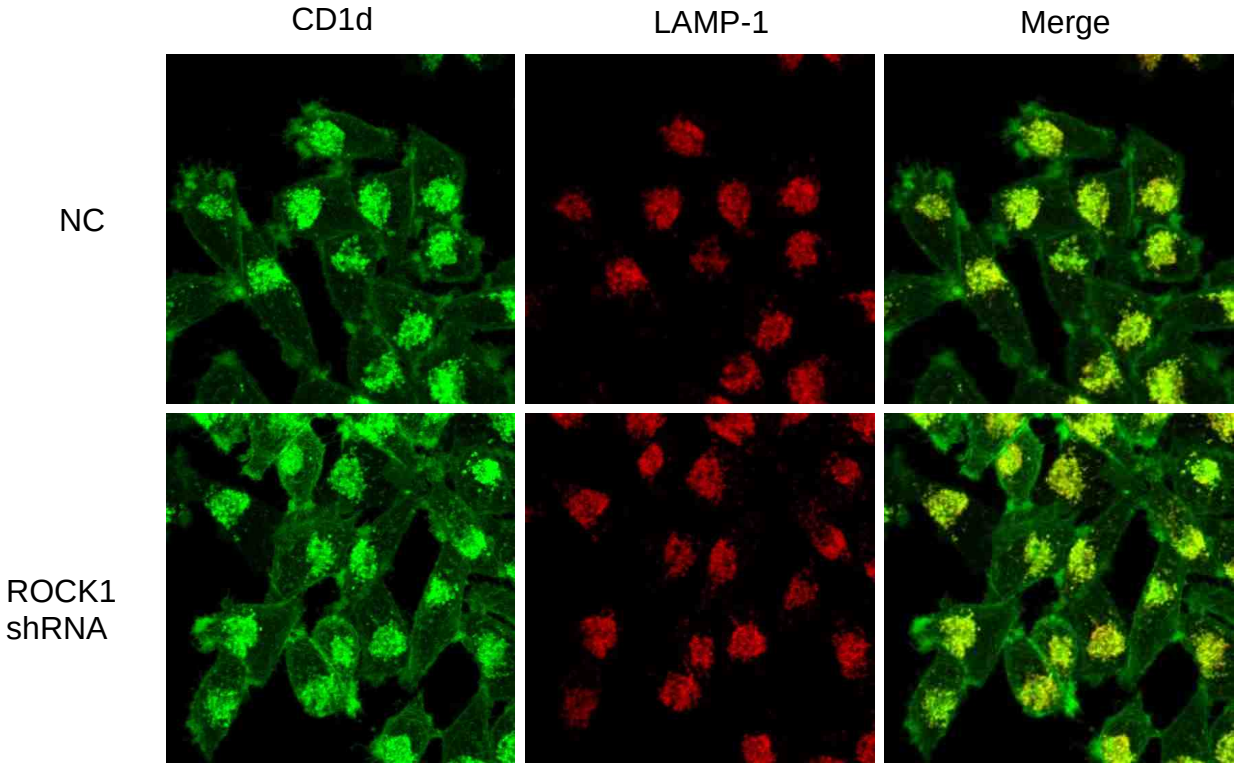

Figure 37. ROCK1 knockdown does not alter CD1d intracellular localization. LMTK-CD1d control and ROCK1 shRNA-expressing cells were stained for CD1d and LAMP-1. Co-localization was visualized by confocal microscopy and measured by Metamorph. (n = 3)

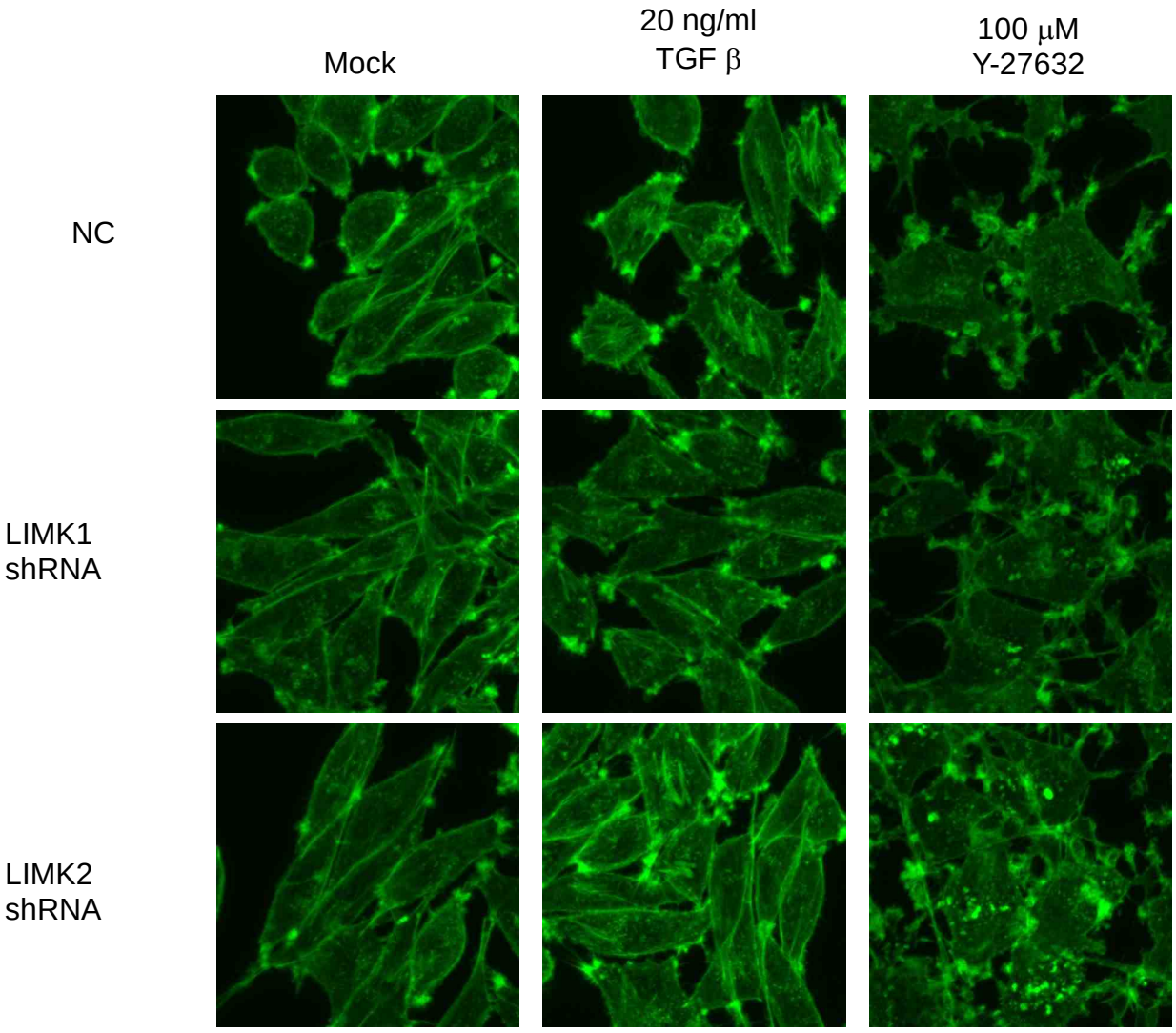

Figure 38. LIMK knockdown inhibits TGF β-induced stress fiber formation. LMTK-CD1d control, LIMK1 and LIMK2 shRNA-expressing cells were treated with 0 and 20 ng/mL of TGF β and 100 μM Y-27632 as a control for 24 hours. The actin was stained with rhodamine phalloidin and was visualized by confocal microscopy. (n = 3)

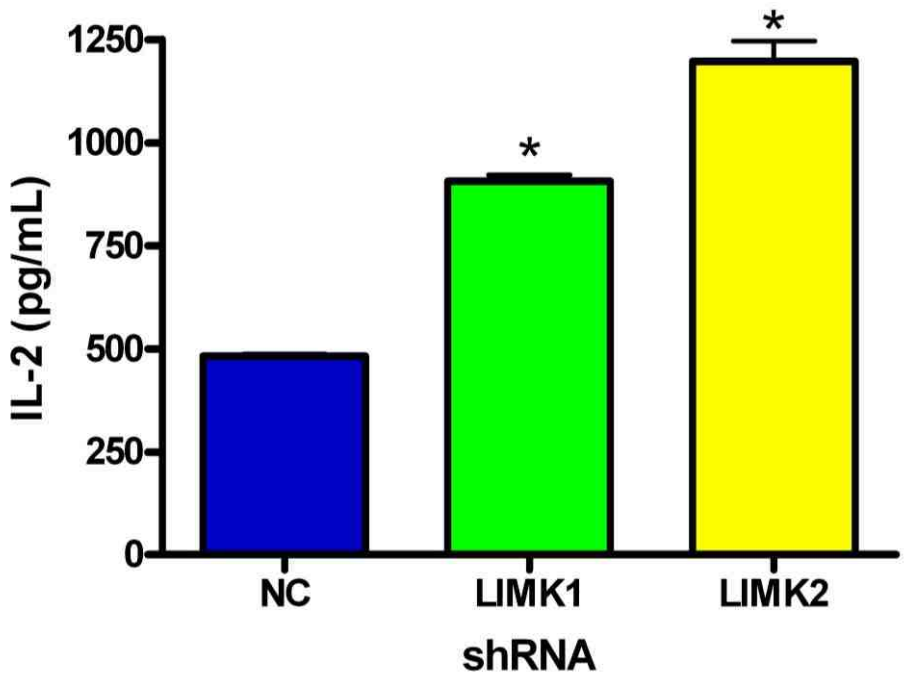

Figure 39. LIMK1 and LIMK2 knockdown increases CD1d-mediated antigen presentation. LMTK-CD1d negative control, LIMK1, and LIMK2 shRNA-expressing cells were co-cultured with an NKT hybridoma for 22-24 hours. The resulting supernatants were analyzed by ELISA for the level of IL-2 production. (*$p < 0.01$) (Experiment performed by Richard Gallo)

result supports what was previously known about the role of LIMK in stress fiber formation by TGF β.

Because the knockdown of ROCK1 increased antigen presentation by CD1d, it was hypothesized that the knockdown of LIMK would also lead to an increase in antigen presentation via CD1d. Therefore, LMTK-CD1d NC, LIMK1 shRNA and LIMK2 shRNA cells were co-cultured with NKT cells for 22-24 hours. Co-culture supernatants were collected and IL-2 production was determined by ELISA. The results showed a significant increase in CD1d-mediated antigen presentation by LMTK-CD1d LIMK1 and LIMK2 shRNA-expressing cells compared to the negative control (Fig 39). The knockdown of LIMK1 and LIMK2 by shRNA was verified by Western blot (Richard Gallo; data not shown). Also, flow cytometry revealed no change in CD1d cell surface expression in LIMK1 and LIMK2 shRNA-expressing cells. These results support the hypothesis that the ROCK/LIMK/cofilin pathway inhibits CD1d-mediated antigen presentation.

Prior studies have shown that LIMK can alter the endocytic pathway, specifically internalization of the EGF receptor and its trafficking to the late endosome and lysosome. Therefore, it was hypothesized that knockdown of LIMK1 or LIMK 2 would cause changes to CD1d trafficking as a means to inhibit CD1d-mediated antigen presentation. This hypothesis was tested by staining LMTK-CD1d NC, LIMK1 shRNA, and LIMK2 shRNA-expressing cells for CD1d and LAMP-1 and analyzing these cells by confocal microscopy. The results showed no difference in CD1d co-localization with LAMP-1 in the LIMK shRNA-expressing LMTK-CD1d cells compared to the negative control (Fig

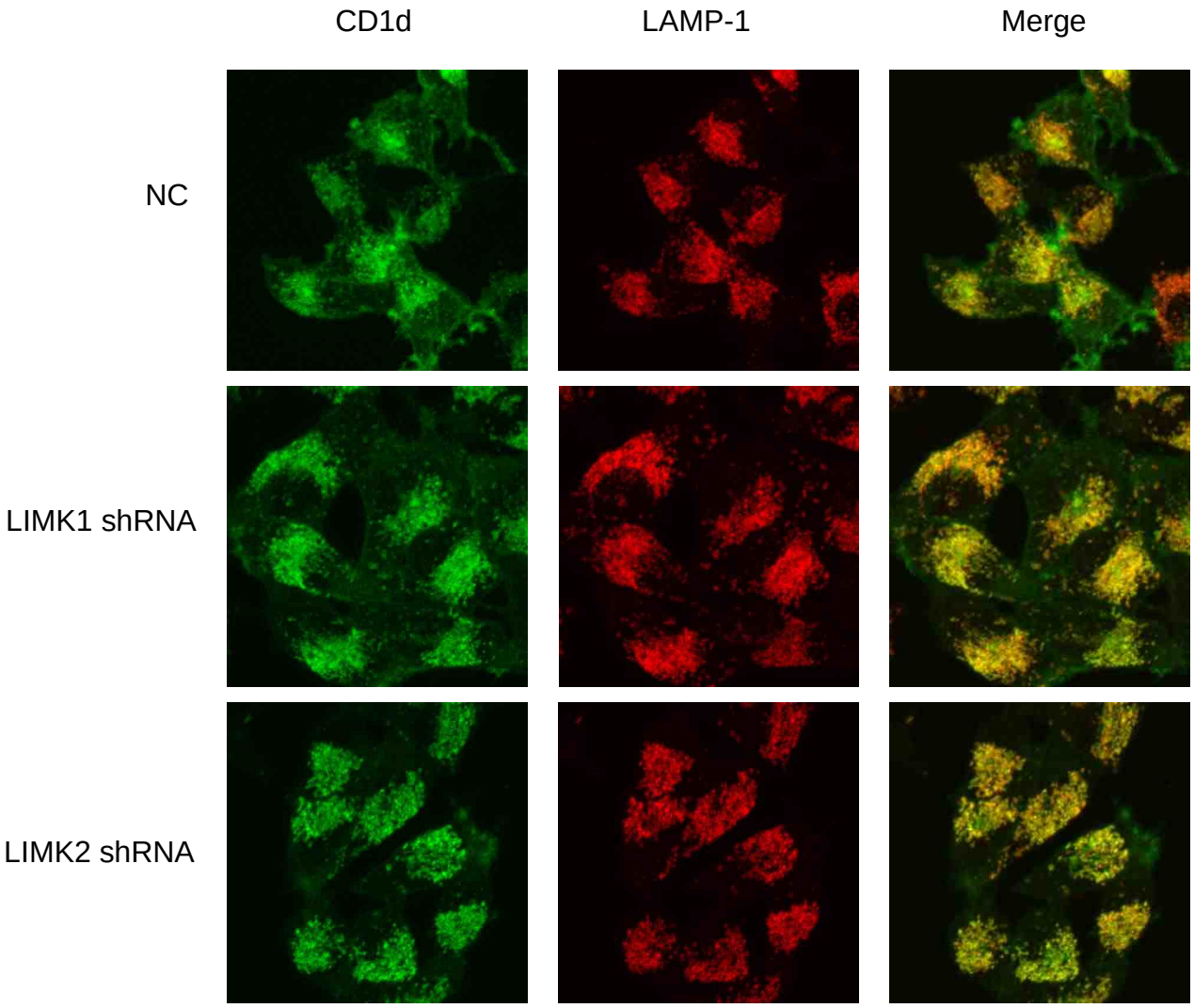

Figure 40. LIMK knockdown does not alter CD1d intracellular localization. LMTK-CD1d control, LIMK1, and LIMK2 shRNA-expressing cells were stained for CD1d and LAMP-1. The cells were visualized by confocal microscopy and co-localization was measured by Metamorph. (n = 3)

40). This is consistent with our results showing that ROCK1 knockdown also does not affect CD1d/LAMP-1 co-localization.

In continuing to investigate the Rho/ROCK signaling pathway, the next protein to consider is cofilin. Unphosphorylated cofilin is a potent regulator of actin polymerization. F-actin works as a treadmill in that G-actin monomers through ATP hydrolysis polymerize onto the positive end of actin referred to as the barbed end. Meanwhile, depolymerization occurs, albeit at a slower rate, at the negative end referred to often as the "pointed end" or "cap". Cofilin in the unphosphorylated state will bind to the negative end of F-actin and promote an increased rate of actin depolymerization. Additionally, cofilin may bind F-actin further up and sever F-actin into smaller fragments. All this allows cofilin to negatively regulate actin polymerization. However, when phosphorylated by LIMK, cofilin function is inhibited. Without cofilin activity, actin polymerization is unregulated which causes uncontrolled long bundles of actin to form known as stress fibers, which explains how the RhoA/ROCK pathway causes stress fiber formation. Therefore, as the last step in the Rho/ROCK pathway, the effects of cofilin on CD1d-mediated antigen presentation was investigated next. As with ROCK and LIMK, cofilin-specific shRNA constructs were expressed in the pLKO.1 vector, and after transfection, were expressed in LMTK-CD1d cells. Due to the essential role of cofilin in preventing stress fibers, the cofilin shRNA-expressing cells were analyzed for the expected absence of actin regulation. It was hypothesized that the knockdown of cofilin would result in increased levels of stress fibers. To test this, LMTK-CD1d NC and -cofilin shRNA cells were treated with 0 and 20 ng/mL of TGF β for 24 hours.

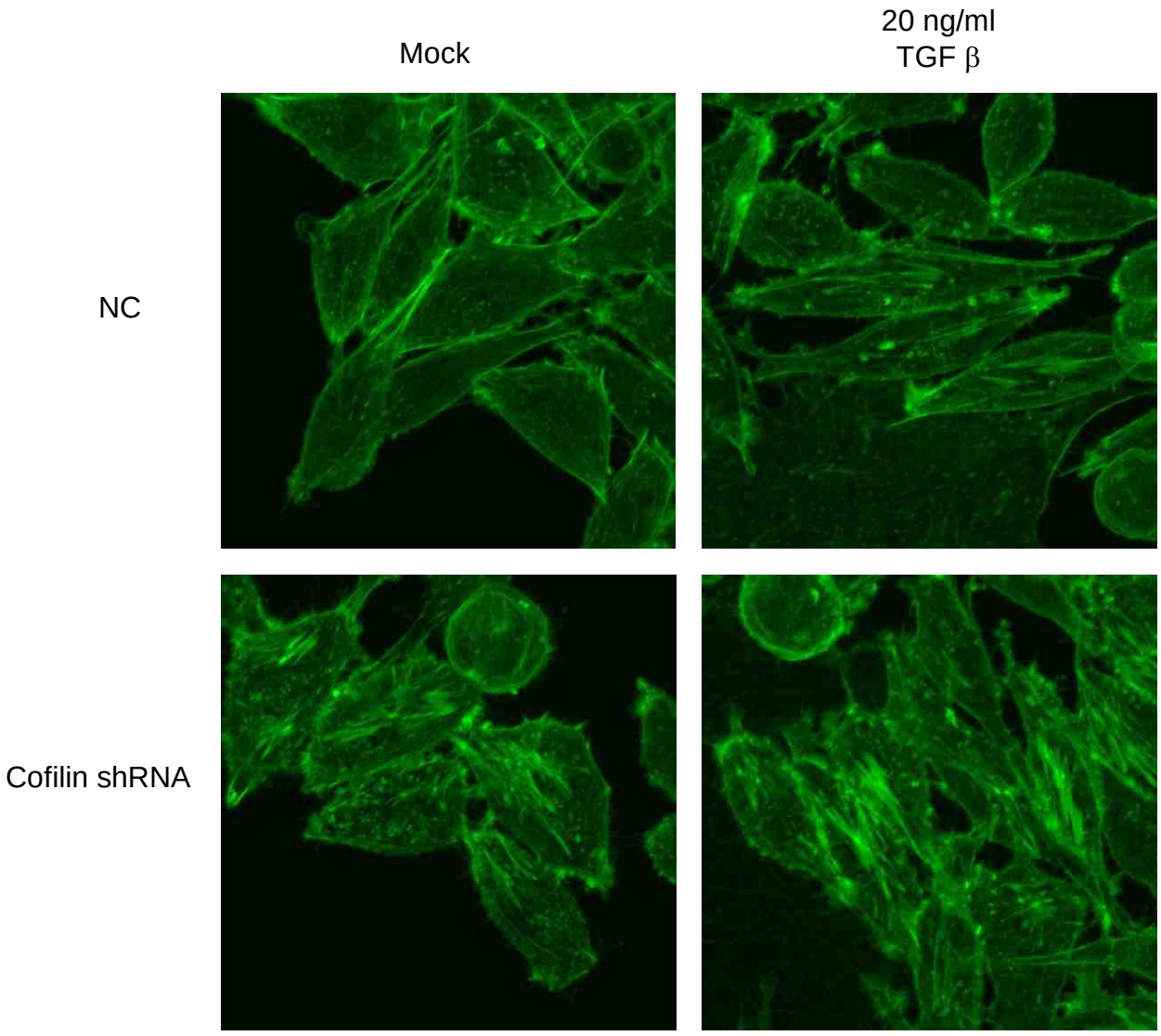

Figure 41. Cofilin knockdown increases TGF β-induced stress fiber formation. LMTK-CD1d control and cofilin shRNA-expressing cells were treated with 0 and 20 ng/mL of TGF β for 24 hours. The actin was stained with rhodamine phalloidin and visualized by confocal microscopy. (n = 3)

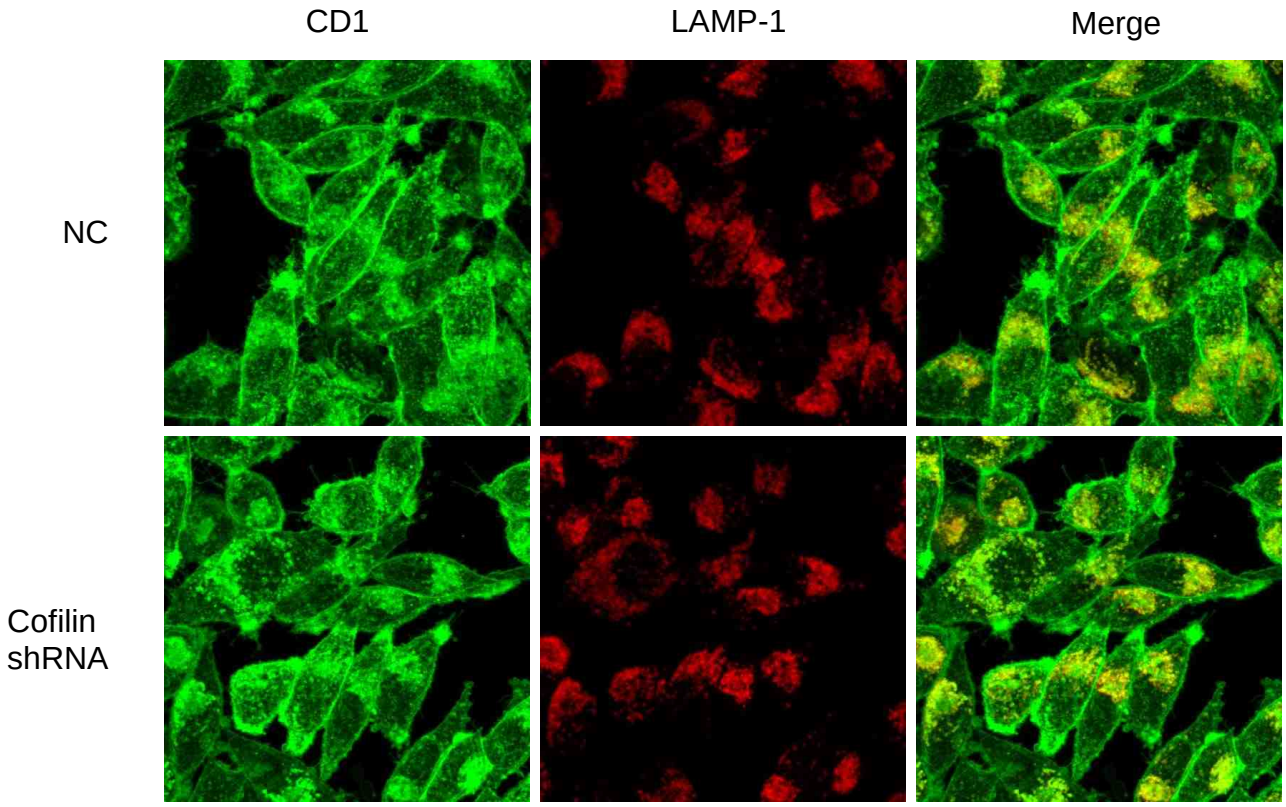

Figure 42. Cofilin knockdown does not alter CD1d intracellular localization. LMTK-CD1d control and cofilin shRNA-expressing cells were stained for CD1d and LAMP-1. The cells were analyzed by confocal microscopy and co-localization was measured by Metamorph. (n = 3)

After treatment, the actin in the cells was stained with rhodamine-phalloidin and analyzed by confocal microscopy. As expected, the untreated negative control cells had no stress fibers, whereas TGF β-treated cells exhibited stress fiber formation (Fig 41). However, untreated cofilin shRNA-expressing LMTK-CD1d cells had formed stress fibers similar to the TGF β treated negative control cells. TGF β treatment of the LMTK-CD1d cofilin shRNA cells had even more stress fibers when compared to the untreated cofilin shRNA cells. Therefore, these results support what is already known about cofilin regulation of actin polymerization.

As discussed above, the knockdown of ROCK1, LIMK1 and LIMK2 resulted in increased levels of CD1d-mediated antigen presentation. Therefore, it was hypothesized that knocking down of cofilin would have the same results - an increase in antigen presentation. Unfortunately, cofilin knockdown leads to unhealthy cells trying to adapt to the absence of a very important protein. These conditions led to variable and uninterpretable results when CD1d-mediated antigen presentation was analyzed in these cells. However, these cofilin shRNA-expressing LMTK-CD1d cells were analyzed for the effect of cofilin on CD1d localization to late endosomes and lysosomes. Because ROCK and LIMK knockdown did not alter CD1d/LAMP-1 co-localization, it was hypothesized that cofilin knockdown would also not affect CD1d. This was proven true when LMTK-CD1d NC and cofilin shRNA cells were stained for CD1d and LAMP-1. Confocal microscopy analysis of these stained cells showed no difference in CD1d localization to LAMP-1^{+} compartments in the cofilin shRNA-expressing LMTK-CD1d cells when compared to the negative control (Fig 42). Therefore, cofilin does not alter the intracellular trafficking of CD1d.

In conclusion, the results in this chapter support the findings that TGF β causes stress fiber formation via Smad pathway activation, as shown by the inhibition of stress fiber formation through the knockdown of Smad2, Smad3, and Smad4. Also, further supported here is the connection of TGF β signaling through ROCK, LIMK and cofilin, to cause the formation of stress fibers. Interestingly, the knockdown of ROCK and LIMK caused an increase in CD1d-mediated antigen presentation, which suggests that these proteins are negative regulators of CD1d. Also, the knockdown of ROCK, LIMK and cofilin did not alter CD1d intracelluar localization. Although important findings for understanding the regulation of CD1d-mediated antigen presentation, neither ROCK, LIMK nor cofilin were determined to be the mechanism by which TGF β inhibits antigen presentation by CD1d.

Discussion

TGF β-dependent inhibition of CD1d-mediated antigen presentation

TGF β is involved in multiple ways in regulating both the adaptive and innate immune response. Our studies have provided yet another example of TGF β regulation of an immune response via inhibition of CD1d-mediated antigen presentation. However, the very act of inhibiting antigen presentation by CD1d, which decreases the activation of NKT cells, can also have substantial effects throughout the course of an immune response. As discussed previously, NKT cells are capable of rapidly producing high levels of Th1 cytokines, such as IFN-γ, IL-2, and TNF-α and Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13 [39]. The many cytokines secreted by NKT cells can activate other immune cells (Fig 43). For example, the IL-4 secreted by NKT cells is responsible for the activation of B cells [63, 64]. NKT cells have been shown to play an important role in the clearance of influenza virus as indicated by higher mortality of NKT cell deficient mice that were infected with the influenza virus [75]. In relation to this, it is the IFN-γ produced by activated NKT cells that leads to improved cytolytic capabilities of NK cells and $CD8^+$ T cells [14, 61]. Also, activated NKT cells secrete IL-12, which contributes to the maturation of dendritic cells; these mature dendritic cells induce enhanced $CD4^+$ and $CD8^+$ T cell activation, due to their superior antigen presentation capabilities [58, 60]. This evidence that NKT can be potent stimulators of an immune

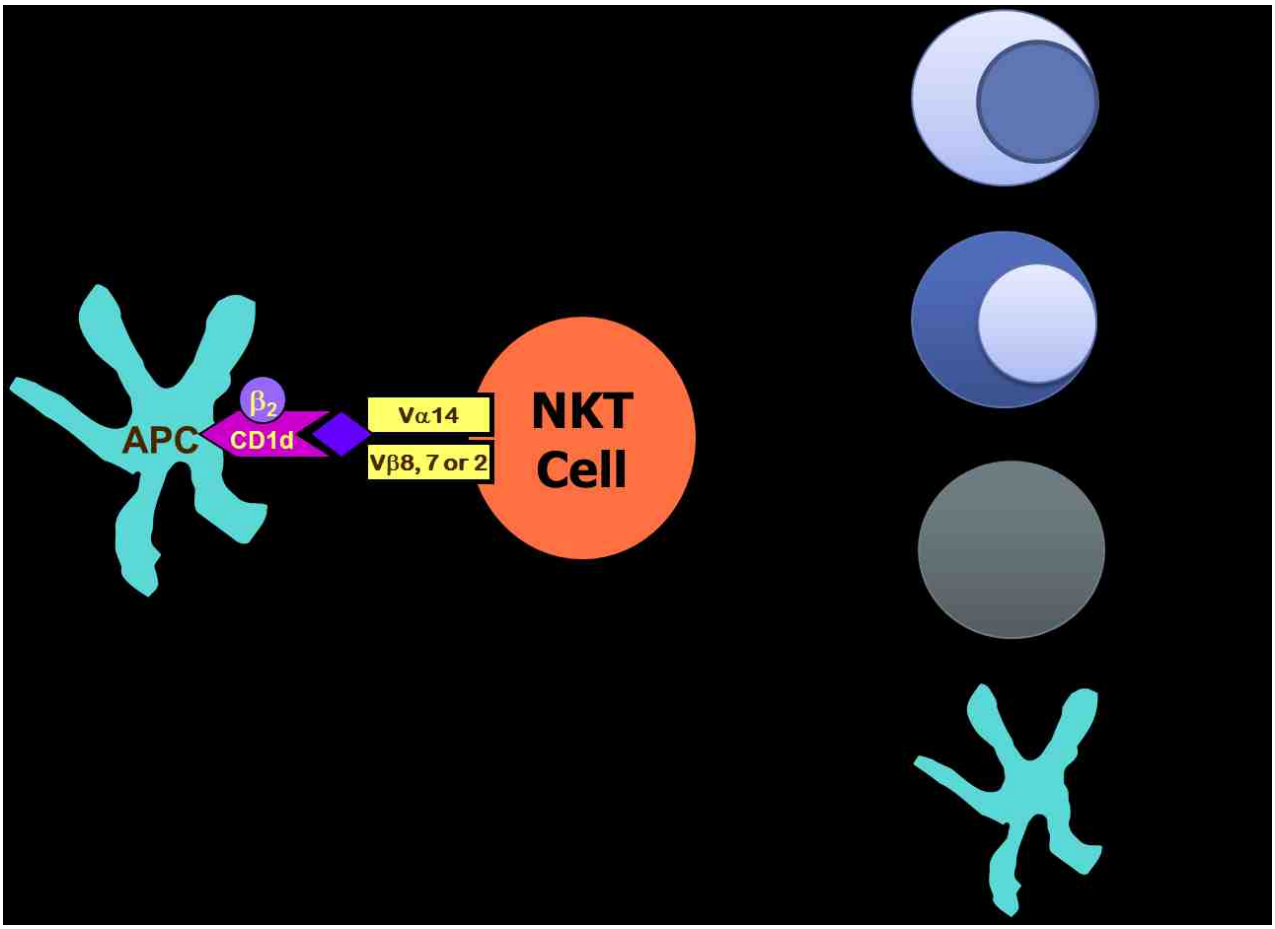

Figure 43. Implications of TGF β-induced inhibition of CD1d-mediated antigen presentation. Inhibition of antigen presentation by CD1d results in decreased NKT cell activation and cytokine release. Reduced levels of cytokine release such as IL-4, IFN-γ, and IL-12 can result in a diminished immune response. This includes affecting B cell antibody release, cytolytic function of $CD8^+$ T cells and NK cells, and dendritic cells maturation.

response due to its rapid and abundant cytokine release, can have many implications in both antiviral and antitumor immunity. Therefore, there can be implications to the immune response by the inhibition of CD1d-mediated antigen presentation by TGF β and thus, reduced activation of NKT cells. In the case of a virus infection, TGF β has been shown to be elevated in serum. In addition to this, TGF β is also elevated in the serum of tumor-bearing mice and is present in the tumor microenvironment [130]. In fact, one group showed that the introduction of a subcutaneous tumor in mice led to elevated levels of TGF β in the serum, which they suggested coincided with lower levels of CD1d cell surface expression on liver mononuclear cells [130]. Therefore, the implications of TGF β inhibition of CD1d-mediated antigen presentation is yet another means by which TGF β can regulate the innate immune response. Also, TGF β potentially regulates the adaptive immune response as the ultimate effect of inhibiting NKT cells and their cytokine production.

Mechanism of TGF β-dependent inhibition of CD1d-mediated antigen presentation

Once we established that TGF β can inhibit CD1d-mediated antigen presentation, we began to look for the mechanism through which this inhibition was occurring. To begin, we first verified that this lowered level of antigen presentation was not due to a simple decrease in cell surface expression of CD1d as other groups have shown (Fig 9). We found that the decrease in CD1d-mediated antigen presentation was not caused by a reduction in CD1d cell surface expression. These results were further supported by the CD1d ELISA, arguably a more sensitive assay for showing changes in CD1d cell surface expression, using cells treated with TGF β (Fig 12). This same ELISA also established

that TGF β does not change the protein conformation of CD1d as indicated by no difference in binding by a panel of CD1d-specific antibodies following TGF β treatment.

With the knowledge that CD1d recycling is critical for acquiring the ligand that will be presented on the cell surface for recognition by NKT cells, we hypothesized that the means by which TGF β is negatively regulating antigen presentation by CD1d is through altering CD1d recycling to late endocytic compartments. Therefore, we used confocal microscopy to demonstrate that CD1d localization to LAMP-1^{+} late endosomes and lysosomes was not changed by TGF β (Fig 10). However, this is not a comprehensive look at potential alterations in CD1d bound ligands caused by TGF β. Within the late endosome and lysosome, exchange of lipids bound to CD1d is facilitated by the lipid transfer protein saposin, specifically saposin B [21, 22]. It is possible that although CD1d still recycles to the late endosome and lysosome in cells treated by TGF β as our results show, the exchange of lipids bound could be altered by TGF β. For example, TGF β could alter function of saposins in the late endosome and lysosomes. To begin an investigation into whether TGF β can affect saposins, the experiment would consist of determining whether saposin B is reaching the late endosomes and lysosome in TGF β-treated cells. To determine this, confocal microscopy analysis would be utilized on TGF β-treated cells stained for both saposins and LAMP-1 which would show localization of saposins. Therefore, this study would tell us whether TGF β affects the localization of saposin.

Results that show no difference in saposin co-localization to the late endosomes and lysosomes in TGF β-treated cells compared to control cells would not mean that TGF β is not altering the function of saposins. If the function of saposins is altered by TGF β, then

TGF β treatment would result in no change in the lipid bound to CD1d. Alternatively, TGF β could alter saposin function which could lead to saposins exchanging the natural lipid for a lipid ligand that is a poor stimulator of NKT cells. Such changes in saposin function would be determined by analyzing the lipids bound to CD1d by Mass Spectrometry using cells treated and untreated with TGF β. However, if TGF β is altering what lipid ligands are bound to CD1d, it is possibly via saposin. Ultimately, any change in the lipid bound to CD1d is presumably the end result of TGF β-induced signaling.

MAPK and other signaling pathways

Our original hypothesis was that TGF β signals through the MAPK p38 to inhibit CD1d-mediated antigen presentation. Despite confirming phosphorylation of p38 by TGF β, treatment of p38 dominant negative cells with TGF β did not result in a rescue of antigen presentation by CD1d (Fig 14). This refuted our hypothesis and thus, p38 activation was not the mechanism involved in TGF β-induced inhibition. At this point, the focus became centered on the Smad pathway. However, TGF β is capable of activating other known regulators of CD1d-mediated antigen presentation that are important to discuss. Positive regulators such as ERK1/2 and PKC δ and the negative regulator JNK are known to be activated by TGF β [180, 199]. However, attempts to show that TGF β activates these signaling proteins through Western blot were inconclusive. Since TGF β is an inhibitor of CD1d-mediated antigen presentation, it is unlikely that the signaling mechanism would be through ERK1/2 or PKC δ, unless TGF β was able to inhibit these signaling proteins. However, no evidence exists to suggest

such a relationship. Therefore, JNK must be considered as a possible part of the mechanism behind TGF β-induced inhibition of antigen presentation. To investigate this further, $CD1d^+$ cells would be treated with TGF β and the JNK inhibitor, SP600125, and analyzed by an NKT cell assay to determine whether inhibition by TGF β is rescued. Similarly, the BMDCs from JNK-deficient mice could be treated with TGF β and analyzed in an NKT cell assay. Despite these alternative pathways, the Smad pathway continues to be the hypothesized pathway through which TGF β inhibits CD1d-mediated antigen presentation.

Smad pathway

After excluding p38, we hypothesized that TGF β signals through the Smad pathway to inhibit CD1d-mediated antigen presentation. In order to investigate the potential role of the Smad pathway, the strategy was to use shRNA which would target Smad2, Smad3 and Smad4, to determine whether deficiencies in these proteins would disable TGF β-induced inhibition. LMTK-CD1d cells expressing Smad2 shRNA when treated with TGF β, displayed an increase in CD1d-mediated antigen presentation (Fig 17) . However, inhibition of antigen presentation caused by TGF β was not rescued by Smad2 knockdown which is likely due to an incomplete knockdown of Smad2 or compensation for the knockdown by the other co-Smad, Smad3. However, Smad3 knockdown did not result in a change in the level of antigen presentation by CD1d (Fig 20). It was thought that targeting Smad4 would completely block the Smad pathway due to Smad4 being necessary for movement of the Smad complex into the nucleus and upregulation of gene targets. Smad4 knockdown resulted in a substantial increase in CD1d-mediated antigen

presentation (Fig 22). However, in the case of Smad3, Smad4, and control shRNA-expresssing LMTK-CD1d cells, TGF β was no longer able to inhibit CD1d-mediated antigen presentation as observed numerous times before. This can be potentially explained in two ways. First, these cells, unlike the Smad2 shRNA-expressing cells, were infected with the lentivirus to transfect the pLKO.1 vector expressing shRNA constructs targeting Smad3 and Smad4 into the cells. It is possible this virus infection somehow altered the cell in such a way that TGF β could no longer inhibit CD1d-mediated antigen presentation. Although these experiments were repeated five to six times for each Smad3 and Smad4, it is possible that experimental error was involved because TGF β was still able to cause stress fiber formation in the control shRNA-expressing cells. This indicates that the TGF β receptor is functional and capable of downstream signaling. In the end, what can be deduced from these results is that Smad2 and Smad4 are negative regulators, whereas Smad3 plays no role in CD1d-mediated antigen presentation. However, the hypothesis remains the same, which is that TGF β signals through the Smad pathway to inhibit antigen presentation by CD1d. In future, it will need to be determined whether this pathway is the mechanism by which TGF β induces inhibition. To do this without potential problems caused by lentivirus infection, the pLKO.1 encoding the shRNA constructs specific for Smad3 and Smad4 could be transfected into cells without the use of a lentivirus infection. Then these cells should be treated with TGF β and analyzed by an NKT cell analysis for a rescue in inhibition caused by TGF β. This should make it clear whether the Smad pathway is the mechanism.

Since we continue to hypothesize that TGF β inhibits CD1d-mediated antigen presentation through the Smad pathway, and Smad2 and Smad4 are negative regulators of antigen presentation, other players in the Smad pathway are important to investigate. The phosphorylation of Smad2 by TGFβRI is dependent upon Smad2 being brought into the vicinity of the TGF β receptor complex. This is facilitated by the FYVE domain containing the Smad anchor for receptor activation (SARA), which can bind both Smad2 and Smad3 [153]. By binding TGFβRII, SARA binds the MH1 domain of Smad2 and brings Smad2 to the TGFβRI to be phosphorylated [155]. However, this is not important for Smad3. Later, it was found that although Smad3 can bind SARA, interrupting this binding did not decrease Smad3 phosphorylation by TGFβRI and therefore Smad3 phosphorylation is independent of SARA [200]. This further implicates SARA in playing a role in Smad2-induced inhibition of CD1d-mediated antigen presentation as Smad2 is an inhibitor of antigen presentation, whereas Smad3 plays no role. To further investigate SARA, shRNA constructs specifically targeting SARA could be used to knockdown protein expression. The cells expressing SARA shRNA should be then treated with TGF β and analyzed in an NKT cell assay to measure CD1d-mediated antigen presentation. The hypothesized result is that SARA knockdown would result in decreased levels of Smad2 phosphorylation, and therefore an increase in antigen presentation by CD1d - similar to Smad2 protein knockdown. Since the knockdown of SARA would reduce phosphorylation of Smad2 by TGF β, then under the Smad pathway hypothesis, SARA shRNA expression should cause a rescue in TGF β-induced inhibition of CD1d-mediated antigen presentation. Therefore, using SARA shRNA would not only demonstrate its involvement in CD1d-mediated antigen presentation, but would also

serve to determine whether TGF β signals through the Smad pathway to inhibit antigen presentation.

Although ubiquitination is typically associated with the inhibition of TGF β/Smad pathway signaling, it can also positively regulate it. Once phosphorylated by the TGFβRI, Smad2 can form a complex with Smurf2, an E3 ubiquitin ligase that is more commonly known to associate with Smad7 to ubiquitinate the TGF β receptor complex, causing its degradation [201]. However, formation of this complex mediates the recruitment of Smurf2 to SnoN. SnoN is a transcriptional co-repressor that represses the genes targeted for upregulation by the Smad pathway [202]. Therefore, it is necessary to remove SnoN for the Smad pathway to function. This is accomplished by Smurf2 ubiquitination of SnoN which targets it for degradation. There are two strategies for determining whether Smad initiated Smurf2 ubiquination of SnoN controls CD1d-mediated antigen presentation. The first is to knockdown Smurf2 protein expression by shRNA. In this model, Smurf2 knockdown should result in an increase of CD1d-mediated antigen presentation, as the actions of Smad2 leads to decreased levels of antigen presentation. The second method is to overexpress SnoN to overcompensate for degradation of SnoN caused by Smurf2. This should also lead to increased levels of CD1d-mediated antigen presentation as determined by an NKT cell assay. In the end, the action of Smad2 binding Smurf2 and causing the degradation of SnoN, may play some role in regulating antigen presentation by CD1d.

Ultimately, the Smad pathway via Smad2 and Smad4 phosphorylation results in the upregulation of gene expression of certain target proteins. Therefore, to determine how CD1d is being inhibited by the Smad pathway it will be necessary to discover what genes

- and therefore what proteins are being upregulated that could possible affect CD1d-mediated antigen presentation. A thorough search of the literature has revealed some possible candidates. However, an RNA microarray with TGF β-treated and untreated cells, in addition to Smad2 and Smad4 shRNA cells would confirm within our system what changes there are in gene expression caused by the Smad pathway. This could lead to the mechanism of how the TGF β/Smad pathway is causing inhibition of CD1d-mediated antigen presentation.

ROCK pathway

Vardouli et al found that overexpression of Smad2 and Smad3 by an adenovirus infection led to upregulation of the RhoB gene, a known target gene of the Smad pathway [178]. These findings, along with the previous observation that TGF β can activate the Rho GTPases, RhoA and RhoB [167], led us to look more closely at the Rho pathway. Because we are interested in the genes upregulated by the Smad pathway, we considered Rho as a possible mechanism by which Smads can inhibit CD1d-mediated antigen presentation. Additionally, the knowledge that TGF β can activate the Rho GTPase pathway provides us with an alternative or cooperative pathway by which TGF β can inhibit CD1d-mediated antigen presentation. As discussed in Chapter 3, the Rho GTPase/ROCK pathway results in stress fiber formation [167] (Fig 4). We confirmed that TGF β could cause stress fibers (Fig 26), and we established that stress fiber formation has negative effects on CD1d-mediated antigen presentation as determined by the cytochalasin D-mediated reduction of stress fibers and increase in antigen presentation by CD1d (Fig 31). Rho GTPases bind to ROCK, which changes the

conformation of and activates ROCK [170]. Drug inhibition and knockdown of ROCK results in an increase in antigen presentation by CD1d (Fig 34, 36), which indicates that ROCK is a negative regulator of CD1d-mediated antigen presentation. LIMK, which is then phosphorylated and activated by ROCK, phosphorylates cofilin [171, 172, 174]. The phosphorylation of cofilin, which in its unphosphorylated form inhibits actin polymerization, leads to stress fiber formation. Knockdown of LIMK also resulted in an increase in antigen presentation, which suggests that, like ROCK, LIMK is an inhibitor of CD1d-mediated antigen presentation. Overall, the Rho/ROCK pathway inhibits CD1d-mediated antigen presentation which is consistent with TGF β-induced inhibition of antigen presentation by CD1d. However, recent observations in the lab have shown that the knockdown of RhoA and RhoB result in a decrease in CD1d-mediated antigen presentation, suggesting a positive role for the Rho GTPases. Therefore, this suggests that the Smad pathway is not inhibiting antigen presentation through upregulation of Rho GTPase gene expression. It also refutes the hypothesis that TGF β signals through Rho GTPases to inhibit antigen presentation. Therefore, the Rho GTPases connection to TGF β and the Smad pathway is independent of the effects of TGF β on antigen presentation by CD1d.

Although Rho no longer fits the model, the ROCK/LIMK pathway does fit. It has been shown that ROCK can be activated in a Rho-independent manner. Apoptosis associated with activated caspases cleaves ROCK, which causes its activation [203, 204]. Interestingly, TGF β, an initiator of apoptosis, is known to elevate the level of activated caspases [205, 206]. Therefore, it is conceivable that TGF β-induced an increased level of activated caspases leading to cleaved and activated ROCK, an inhibitor of CD1d-

mediated antigen presentation. Our lab has shown that through treatment with apoptosis-inducing drugs, activated caspases are elevated and CD1d-mediated antigen presentation is inhibited [207]. This inhibition was rescued through treatment with the pan-caspase inhibitor, Z-VAD-fmk. In order to verify whether TGF β signals through caspase activation to stimulate the ROCK/LIMK pathway to inhibit antigen presentation by CD1d, TGF β-treated cells would be treated with Z-VAD-fmk, and analyzed by an NKT cell assay for the level of CD1d-mediated antigen presentation. Based upon the model, it is expected that inhibiting caspases would cause a rescue of CD1d-mediated antigen presentation inhibition. Before determining this connection, it is important to first show that TGF β can elevate caspase activation. To do this, lysates from TGF β-treated cells would be analyzed by Western blot for increased levels in the cleaved forms of caspase 3 as predicted. Also, Western blot analysis would be used to show the predicted increase in cleaved ROCK, indicating TGF β-induced caspase activation. In the end, this model of TGF β signaling through ROCK activation, independent of Rho GTPases, provides a possible alternative or cooperative pathway to the Smad pathway.

Smad7 and Smurf1

One important gene upregulated by the Smad pathway in response to TGF β is *smad7* [160]. Smad7 is similar to the other Smads in structure and has both an MH1 and MH2 domain. Whereas the R-Smads, Smad2 and Smad3, serve as effectors phosphorylated by the TGF β receptor complex bound to the soluble homodimer TGF β cytokine, Smad7 is an inhibitor of TGF β Smad pathway [208]. To inhibit TGF β signaling, Smad7 binds to TGF β receptor I, the serine/threonine kinase responsible for downstream

phosphorylation and signaling initiation [208]. Once bound, Smad7 prevents Smad2 and Smad3 phosphorylation which stops the Smad pathway. Another method of inhibition of the TGF β/Smad pathway is the recruitment by Smad7 of Smurf1 and Smurf2 to the TGF β receptor complex [161, 162]. Smurfs are E3 ubiquitin ligases that ubiquinate TGF β receptor I which targets it for degradation by the proteasome. In our studies, Smad7 became a focus in trying to discern whether TGF β was signaling through the Smad pathway to inhibit CD1d-mediated antigen presentation. If the Smad pathway was important for this inhibition, then knockdown of the Smad7 protein would result in aberrant Smad pathway signaling, resulting in increased levels of Smad2 phosphorylation. Our hypothesis was that knockdown of Smad7 would result in an even greater increase in the inhibition of CD1d-mediated antigen presentation caused by TGF β. Therefore, we used the pSuppressor plasmid to express Smad7 shRNA in LMTK-CD1d cells. After treatment with increasing doses of TGF β we saw a slightly lower level of CD1d-mediated antigen presentation in Smad7 shRNA-expressing cells compared to the control TGF β-treated cells which confirmed our hypothesis. Initial Western blot analysis in which protein lysates from TGF β-treated Smad7 shRNA-expressing and control cells were probed for Smad2 phosphorylation indicated that Smad7 shRNA cells had an increased level of Smad2 phosphorylation when treated with TGF β as compared to controls. This is consistent with what is expected when Smad7 is knocked down. However, these results were difficult to repeat and the results from attempts at using a Smad7 antibody to verify knockdown in these cell by Western Blot were inconclusive. However, due to these promising results, Smad7 should be analyzed

further in the future as part of determining a more precise mechanism of the inhibition of CD1d-mediated antigen presentation by TGF β.

Smurf1 and Smurf2 are proteins of interest in the regulation of CD1d-mediated antigen presentation due to their association with Smad7. Interestingly, as a more convincing possible connection, a TAP-TAG purification of CD1d revealed Smurf1 as a protein bound to the CD1d tail. The binding of Smurf1 to the CD1d tail should be confirmed by an immunoprecipitation assay. However, it is possible that Smurf1 plays some role in regulating CD1d-mediated antigen presentation. Verification of a role would consist of knocking down the Smurf1 protein through Smurf1 targeting shRNA expressed by the pLKO.1 vector, as was used to knockdown Smad2, Smad3, and Smad4. The cells with Smurf1 knockdown would be treated with a series of doses of TGF β and used in an NKT cell assay to determine whether the absence of Smurf1 would reduce CD1d-mediated antigen presentation, as would be predicted.

Upon ligation of TGF β to its receptors, Smad7 is upregulated in the nucleus. Smad7 will then bind Smurf1 within the nucleus which activates the nuclear export abilities of Smurf1 [161]. The Smad7/Smurf1 complex then moves to the plasma membrane. It has been shown that Smurf1 is capable of ubiquitinating the GTPase RhoA [209]. As discussed previously, the Rho family GTPases are positive regulators of the immune system. Our lab has just recently shown that shRNA-dependent knockdown of RhoA and RhoB leads to decreased levels of CD1d-mediated antigen presentation. This was confirmed by the treatment of $CD1d^+$ cells with the C3 toxin, an inhibitor of Rho GTPases, which led to decreased levels of antigen presentation by CD1d. This supports the conclusion that Rho GTPases promote CD1d-mediated antigen presentation.

Therefore, the ability of Smurf1 to cause degradation of RhoA through ubiquitination fit the model of TGF β as a negative regulator of CD1d-mediated antigen presentation. Potentially, TGF β could activate the Smad pathway, specifically Smad2 and Smad4 which would lead to the upregulation of Smad7. Smad7 would bind Smurf1, which would then facilitate the movement of Smad7 and Smurf1 from the nucleus to the plasma membrane. Once there, Smurf1 is in the vicinity of RhoA and could target it for degradation, which would ultimately lead to decreased levels of CD1d-mediated antigen presentation. A few experiments would be necessary to indicate whether this model is correct. First, TGF β-treated $CD1d^+$ cells would be analyzed by Western Blot for a change in RhoA, RhoB, and RhoC levels within the cell. The prediction would be that TGF β leads to decreased levels of RhoA and possibly RhoB and RhoC. To determine whether this is through TGF β-induced relocalization of Smurf1 to the plasma membrane, Smurf1 shRNA-expressing and control cells would be treated with TGF β and analyzed by Western Blot to show changes in protein levels of the Rho GTPases. Cells expressing Smurf1 shRNA should result in high levels of Rho GTPases. However, these experiments would only confirm in our cells and system what has already been shown. To relate this model to CD1d-mediated antigen presentation, we would do as suggested previously. Smurf1 and control shRNA-expressing $CD1d^+$ cells would be treated with TGF β and these cells analyzed by an NKT cell assay which should result in a rescue of TGF β-induced inhibition of antigen presentation by CD1d. Next, this experiment would be repeated with one alteration, which is the inhibition of Rho GTPases through the C3 toxin or shRNA targeting of RhoA. With a knockdown of Smurf1, RhoA would be present to promote CD1d-mediated antigen presentation. Therefore, inhibition of Rho

GTPases, specifically RhoA, in cells with Smurf1 knockdown, should result in rescue reversal and renewed inhibition caused by TGF β. It is important to note that RhoA is not the only positive regulator of CD1d-mediated antigen presentation. ERK1/2, a MAPK, and PKC δ when activated by phosphorylation have been shown by us to increase antigen presentation by CD1d. Despite the absence in the literature indicating a connection between Smurf1 and either ERK1/2 or PKC δ, it is worth investigating whether Smurf1 could ubiquitinate these enhancers of CD1d-mediated antigen presentation. In conclusion, RhoA may play a role after all as a target for degradation.

Another model exists to explain how Smurf1 could be bound to CD1d and what other interactions this might suggest are occurring. It has been shown that SARA-facilitated TGF β receptor complex signaling through Smad2 is localized to clathrin positive areas of the plasma membrane. Importantly, the receptor complex, upon signaling through the Smad pathway, is internalized in clathrin-coated pits to the early endosome. In constrast, Smad7 association with the receptor complex is localized at the plasma membrane to lipid rafts. Interestingly, CD1d localization to lipid rafts enhances its antigen presentation to NKT cells, resulting in increased activation of NKT cells. Therefore, when the Smad7/Smurf1 complex moves to the cell surface and Smad7 binds to TGFβRI, this is occurring within the vicinity of abundant CD1d. This may be how Smurf1 binding to CD1d is facilitated. Because Smurf1 may bind CD1d and is known to bind TGF β through Smad7, this raises the question of whether CD1d my bind or form a complex with the TGF β receptor. The TGF β receptor may bind CD1d through Smad7 and Smurf1, which could be determined through an immunoprecipitation assay. This is

another proposed mechanism through which the Smad pathway inhibits CD1d-mediated antigen presentation.

However, there is an inconsistency in hypothesizing a role for Smurf1. As mentioned above, a Smad7 deficiency seems to cause a further decrease in CD1d-mediated antigen presentation that ranges around 5-10%, seemingly not significant. This is in direct conflict to the model that requires Smad7 for transporting Smurf1 from the nucleus to the plasma membrane. Smad7 knockdown would inhibit this necessary transport. Although the data may indicate this, it is not flawless. It is possible Smad7 is not knocked down in these cells and the observed differences were natural variations in knockdown of TGF β. At this point, more experimentation is required.

Model of TGF β-induced inhibition of CD1d-mediated antigen presentation

The final working model consists of finding the pathway by which TGF β inhibits CD1d-mediated antigen presentation (Fig 44). The two pathways that may play a cooperative or exclusive role are the Smad pathway and the ROCK/LIMK pathway. Both pathways have been shown to inhibit CD1d-mediated antigen presentation. However, it has yet to be determined whether these pathways are the mechanism by which TGF β inhibits CD1d-mediated antigen presentation. Caspases activated by TGF β ligation cleave and activate ROCK, an inhibitor of CD1d-mediated antigen presentation. The ROCK pathway leads to the phosphorylation of LIMK and cofilin which ultimately results in stress fiber formation. The alternative pathway, the Smad pathway consists of Smad2 and Smad4 which are inhibitors of CD1d-mediated antigen presentation. However, other players are involved in Smad2 signaling. SARA is

responsible for bringing Smad2 to the TGFβRI for phosphorylation and Smad pathway activation. Additionally, Smad2 associates with Smurf2 to ubiquitinate SnoN, a repressor of Smad pathway gene upregulation. Smad pathway activation ultimately results in the upregulation of genes such as Smad7, an inhibitor of the Smad pathway. Smad7 is transported from the nucleus to the cell surface by binding to Smurf1 in the nucleus. Once at the cell surface, Smad7 binds TGFβRI which blocks the receptor's ability to phosphorylate Smad2. Also, Smad7 brings Smurf1 to the TGF β receptor which leads to ubiquitin-mediated degradation of the TGF β receptor. Because degradation of TGF β by Smad7 and Smurf1 occurs in lipid rafts, this brings Smurf1 near CD1d, which is also present in lipid rafts. This possibly explains how Smurf1 may be bound to CD1d. Additionally, Smurf1 localization to the plasma membrane facilitates Smurf1 ubiquination of Rho GTPases, promotors of CD1d-mediated antigen presentation. Degradation of Rho GTPases is a potential means by which TGF β and/or the Smad pathway inhibits CD1d-mediated antigen presentation. In conclusion, there are multiple facets to explain TGF β inhibition of CD1d-mediated antigen presentation.

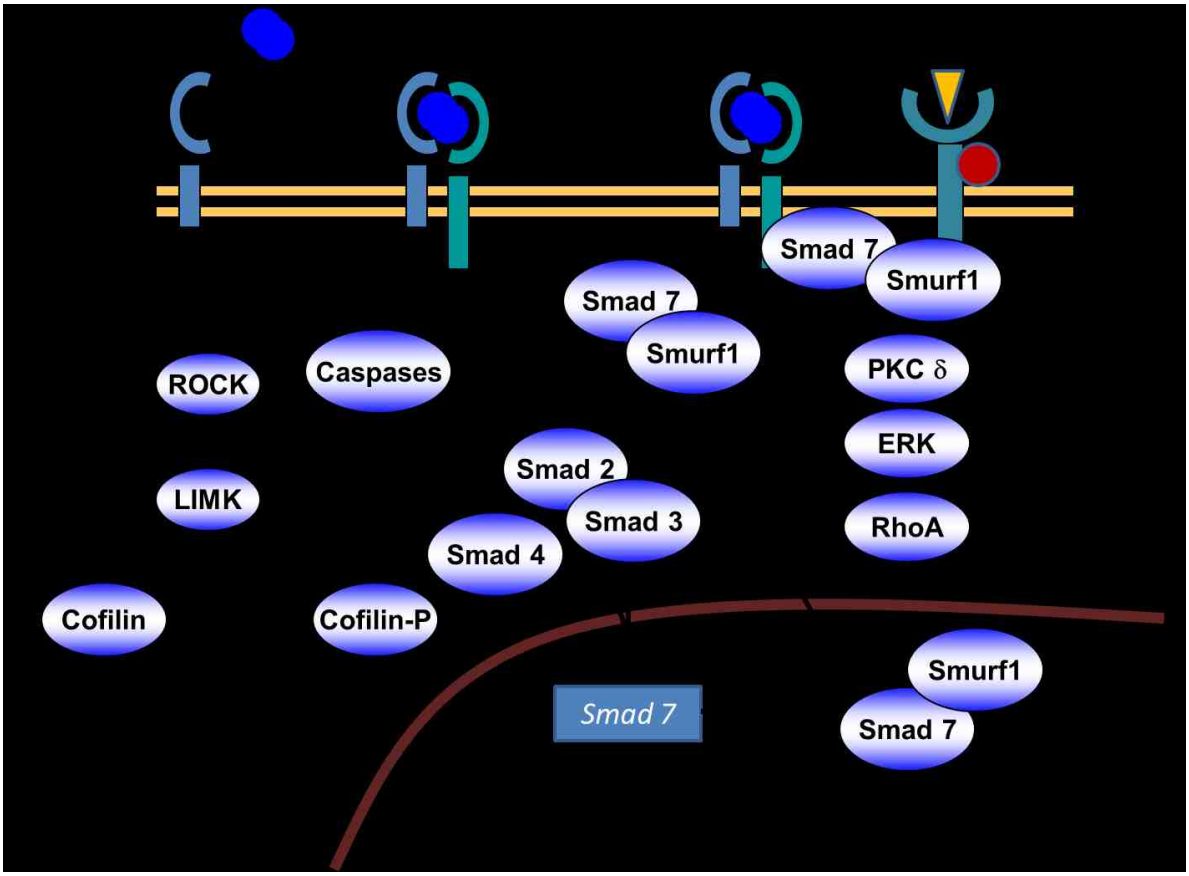

Figure 44. Model of TGF β-induced inhibition of CD1d-mediated antigen presentation. The proposed pathway(s) through which TGF β is signaling to inhibit antigen presentation by CD1d is the ROCK/LIMK and Smad pathways, two pathways that have been shown also inhibit CD1d-mediated antigen presentation. In considering the Smad pathway the proposed mechanism is through the upregulation of Smad7 expression, which binds Smurf1. The Smad7/Smurf1 complex moves to the plasma membrane and binds TGF β receptor I. Once at the cell surface, Smurf1 can ubiquitinate RhoA, targeting it for degradation, and Smurf1 can potentially bind CD1d.

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CURRICULUM VITAE

JENNIFER CARRIE RYAN

EDUCATION

2000 - 2004	Hanover College, B.A. in Biology Hanover, Indiana Honors Thesis title – "Isolation of *Escherichia coli* Resistant to S-Adenosylmethionine Hydrolase-Induced Cell Filamentation."
2004 - 2011	Indiana University, Ph.D. in Immunology Indianapolis, Indiana Ph.D. Dissertation title – "Mechanisms of TGF β-induced inhibition of CD1d-mediated antigen presentation."

RESEARCH ACTIVITIES

2002 - 2004	The role of S-Adenosylmethionine in *Escherichia coli* cell division.
2005 - 2010	Investigations into the mechanisms involved in the intracellular trafficking of (and antigen presentation by) murine CD1d molecules.

RESEARCH PRESENTATIONS

1. Bailey, J.C., Hughes, J. Development of a Non-Radioactive Assay for S-Adenosylmethionine. Butler Undergraduate Research Conference. April 2003.

2. Bailey, J.C., Hughes, J. Investigation of *Escherichia coli* Resistant to S-Adenosylmethionine Hydrolase-Induced Cell Filamentation. Butler Undergraduate Research Conference. April 2004.

3. Bailey, J.C., Hughes, J. Investigation of *Escherichia coli* Resistant to S-Adenosylmethionine Hydrolase-Induced Cell Filamentation. NCUR: National Conference on Undergraduate Research. April 2004.

ABSTRACTS

1. Bailey, J.C., Genukaradhya, G.J., Brutkiewicz, R.R. Transforming Growth Factor β-Dependent Inhibition of CD1d-Mediated Antigen Presentation. American Association of Immunologists Annual Meeting. Miami Beach, FL. May 2007.

2. Bailey, J.C., Genukaradhya, G.J., Brutkiewicz, R.R. Transforming Growth Factor β-Dependent Inhibition of CD1d-Mediated Antigen Presentation. Cancer Day at Indiana University School of Medicine. May 2006, 2007, 2008, 2009, 2010.

3. Bailey, J.C., Genukaradhya, G.J., Brutkiewicz, R.R. Transforming Growth Factor β-Dependent Inhibition of CD1d-Mediated Antigen Presentation. Autumn Immunology Conference. Chicago, IL. November 2008.

4. Bailey, J.C., Genukaradhya, G.J., Brutkiewicz, R.R. Transforming Growth Factor β-Dependent Inhibition of CD1d-Mediated Antigen Presentation. American Association of Immunologists Annual Meeting. Seattle, WA. May 2009.

PUBLICATIONS

1. Brutkiewicz, R.R., Willard, C.A., Gillett-Heacock, K.K., Pawlak, M.R., Bailey, J.C., Khan, M.A., Nagala, M., Du, W., Gervay-Haque, J., Renukaradhya, G.J. Protein kinase C δ is a critical regulator of CD1d-mediated antigen presentation. *Eur. J. Immunol.* 37:2390-2395, 2007.

2. Gallo R.M., Khan, M.A., Shi, J., Kapur, K., Wei, L., Bailey, J.C., Liu, J., Brutkiewicz, R.R. Regulation of the actin cytoskeleton by Rho kinase controls antigen presentation by CD1d. Submitted.

3. Bailey, J.C., Renukaradhya, G.J, Nguyen, H., Brutkiewicz R.R. Smad2 and Smad4-dependent inhibition of CD1d-mediated antigen presentation. Submitted.

HONORS AND AWARDS

1. Honorable mention at Cancer Day at Indiana University School of Medicine.