THE ROLE OF NKT CELLS IN IMMUNE EVASION OF LIVER METASTASES ARISING FROM INTRAOCULAR TUMORS

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by

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DEDICATIONS

To my lovely grandmother, Eshi, whom I love the most.

To my aunt, Mahvash, for all the love and support she has given me.

To Jamshid, the best brother and the most intellectual person I know.

To my husband, Mohammadreza, whom his love, presence and support is the best thing that has happened to me.

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Uveal melanoma (UM) is the most common intraocular tumor in adults and liver metastasis is the leading cause of death in UM patients. We have previously shown that NKT cell-deficient mice develop significantly fewer liver metastases from intraocular melanomas than do wild-type (WT) mice. Here, we examine the interplay between liver NKT cells and NK cells in resistance to liver metastases from intraocular melanomas. NKT cell-deficient $CD1d^{-/-}$ mice and WT C57BL/6

mice treated with anti-CD1d antibody developed significantly fewer liver metastases than WT mice following either intraocular or intrasplenic injection of B16LS9 melanoma cells. The increased number of metastases in WT mice was associated with reduced liver NK cytotoxicity and decreased production of IFN-γ. However, liver NK cell-mediated cytotoxic activity was identical in non-tumor-bearing NKT cell-deficient mice and WT mice, indicating that liver metastases were crucial for the suppression of liver NK cells. Depressed liver NK cytotoxicity in WT mice was associated with production of IL-10 by bone marrow-derived liver cells and increased IL-10 receptor expression on liver NK cells. IL-$10^{-/-}$ mice had significantly fewer liver metastases than WT mice, but were not significantly different from NKT cell-deficient mice. Thus, development of melanoma liver metastases is associated with upregulation of IL-10 in the liver and an elevated expression of IL-10 receptor on liver NK cells. This impairment of liver NK activity is NKT cell-dependent and only occurs in hosts with melanoma liver metastases.

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LIST OF ABBREVIATIONS

AC	Anterior chamber
ACAID	Anterior chamber-associated immune deviation
Ad5E1	Adenovirus type 5 early region 1
AH	Aqueous humor
AKT	Protein kinase B
APCs	Antigen presenting cells
Arg1	Arginase-1
ATCC	American Type Culture Collection
BMDC	Bone marrow-derived cell
CD	Cluster of differentiation
CM	Cutaneous melanoma
c-MeT	MNNG HOS transforming gene
CR1	Complement receptor type 1
CRPs	Complement regulatory proteins
CTL	Cytotoxic T-lymphocyte
CXCL	C-X-C Chemokine ligand
CXCR	C-X-C Chemokine receptor
DAF	Decay-accelerating factor
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalitis
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal regulated kinases
FasL	Fas ligand
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GITRL	Glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand
GR-1	Granulocyte receptor-1
HBSS	Hanks' balanced salt solution
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
ICAM1	Intracellular adhesion molecule-1
IGF-IR	Insulin-like growth factor I receptor
i.p.	Intraperitoneal
i.v.	Intravenously
IDO	Indoleamine 2,3 dioxygenase
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
iGb3	Isoglobotrihexosylceramide
IGF	Insulin-like growth factor

IL	Interleukin
iNKT	Invariant NKT
INOS	Inducible nitric oxide synthase
kDa	Kilo Dalton
KIRs	Killer inhibitory receptors
KO	Knockout
LDH	Lactate dehydrogenase
LMN	Liver mononuclear
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal endothelial cells
MAGE	Melanoma-associated antigen
MBL	Mannose-binding lectin
MCA	Methylcholanthrene
MCP	Membrane cofactor protein
MDSCs	Myeloid derived suppressor cells
MEK	Mitogen activated protein kinase kinase
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MiRNA	Micro RNA
NCRs	Natural cytotoxicity receptors
NK	Natural killer
NKT	Natural killer T

NO	Nitric oxide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PDGF	Platelet derived growth factor
PD-L1	Programmed death ligand 1
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RAF	Raf kinase
RASSF1	RAS association domain family 1
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
RPMI	Roswell Park Memorial Institute medium
SC	Subcutaneously
SLE	Systemic lupus erythematosus
TAM	Tumor associated macrophages
TGF-β	Transforming growth factor β
TILs	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Treg	T regulatory

UM	Uveal melanoma
UV	Ultra violet
VCAM1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WT	Wild type
α-GalCer	α-galactosylceramide
α-MSH	Alpha melanocyte-stimulating hormone
ζ-chain	Zeta-chain
ºC	Degrees Celsius

CHAPTER ONE:

INTRODUCTION

Anatomy of the Eye

The eye is a highly specialized organ that detects light and converts the light energy into electro-chemical impulses in neurons. In mammals, the eye is composed of three distinguished layers: the outer scleral layer, the intermediate uveal layer and the inner retinal layer (**Figure 1**). The sclera forms an opaque tough, fibro-elastic capsule which supports the eye. The epithelium that covers the exposed part of the sclera and inner surface of the eyelids is called conjunctiva. The anterior portion of the sclera is transparent and is called the cornea. The cornea focuses light through the aqueous humor, lens, and vitreous onto the retina. The middle layer of the eye is the uvea or the uveal tract. The uveal tract is a highly vascular layer which is made up three components. The choroid, which lies between the sclera and retina, is highly vascularized and therefore provides nutrition for the retina. The ciliary body is the second component of the uveal tract and represents the forward continuation of the choroid layer. The ciliary body is attached to the lens and contains smooth muscles, which controls the shape of the lens. The ciliary body facilitates the focusing of light rays onto the retina and also allows accommodation for near or distance vision. The convex lens is a transparent structure; by changing its shape the lens provides a fine focus of the corneal image on the retina. Although the function of the lens is vital for focusing light on the retina, the cornea provides about 80% of the refracting power of the eye. The iris, the third component of the uveal tract, forms a diaphragm in front of the lens and divides the anterior compartment into anterior and posterior chambers.

The anterior and posterior chambers contain a fluid called aqueous humor. Aqueous humor is a source of nutrient for the lens and cornea. The iris regulates the amount of light reaching the retina through the pupil. The uveal tract is highly pigmented which is generated by resident melanocytes resulting in the absorption of light that has passed through the retina. The large cavity posterior to the lens contains a gelatinous fluid known as vitreous humor, which supports the lens and retina inside the eye. The innermost layer of the eye is the retina which consists of ten layers composed of three cell types: neurons, pigmented epithelial cells and ganglion cells. The photoreceptors are specialized type of neurons that convert light into neuronal action potential and send the signal via optic nerves to the occipital lobe of the brain, the visual processing center of the mammalian brain. There is a highly pigmented area near the center of the retina called the macula. A conical depression in the center of macula is the fovea which is responsible for the sharp central vision and contains the largest concentration of photoreceptors.

Embryologically, the eye and the brain are derived from the neural tube. Eye cells are similar to brain cells in having restricted regenerative capacity. Consequently, they are vulnerable to potential damage resulting from immunogenic inflammation. The eye and the brain are protected from the immune-mediated inflammatory injury and destruction via a biological phenomenon called immune privilege, which will be explained later in this chapter.

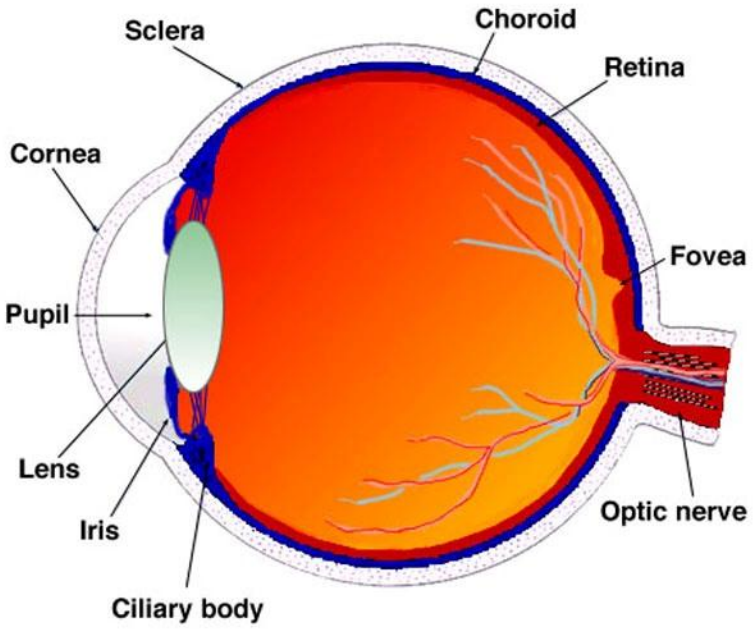


Figure 1. Vertical section of the adult human eye. The eye has three main layers: the outer sclera, the intermediate uvea and the inner retina. The anterior segment of the eye is filled with aqueous humor and includes the cornea, iris and ciliary body. The posterior segment of the eye is composed of the vitreous humor and is bound posteriorly by the retina. The lens separates both chambers. Image from http://www.webvision.med.utah.edu

Immunology of the Eye

A combination of anatomical, structural and immunoregulatory features of the eye contribute to the generation of the phenomenon called ‘‘immune privilege’’, by which the eye is protected from detrimental blinding effects of immune inflammation (1). Immune privileged sites in the body, such as the brain, testes and eyes, tolerate the introduction of antigen without eliciting inflammatory immune responses (2, 3).The term immune privilege was first described

by Peter Medawar who observed the long-term survival of allogeneic tissue grafts in the anterior chamber (AC) of the eye while the graft was promptly rejected if placed into the skin (4). Later research has revealed several major mechanisms are involved in sustaining ocular immune privilege: (a) anatomical and structural characteristics of the eye. For instance, the ocular-blood barrier and the limited presence of lymphatic drainage in the eye restrict the entrance of immune cells into the eye and trafficking of the antigen presenting cells (APCs) from the eye to the regional lymphoid tissues (5). (b) the reduced expression of major histocompatibility complex (MHC) class I molecules on ocular cells. In order to maintain the vision during an inflammatory condition, corneal endothelial and retinal cells which have limited regenerative capacity need to remain intact. The expression of MHC class I antigens is required for cells to be susceptible to killing by the cytotoxic T lymphocyte (CTL) of the adaptive immune system (5). Corneal endothelial and retinal cells evade CTL-mediated killing by reducing the expression of MHC class I molecules and therefore lessen the possibility of blindness in case of CTL attack (5). On the other hand, the expression of little or no MHC class I molecules makes ocular cells vulnerable to cytolysis by natural killer (NK) cells, the effector cells of the innate immune system. However, corneal endothelial and retinal cells are protected from NK cell-mediated lysis via expression of non-classical MHC class I molecules which act as inhibitory ligands for NK cells (5). (c) immunosuppressive micro-environment of the eye, which is the result of the expression of membrane-bound molecules that decorate many of the cells inside of the eye and the presence of soluble factors in the aqueous humor that suppress both the innate and adaptive immune responses (**Table 1**), and (d) the induction of T regulatory (Treg) cells that suppress antigen-specific immune responses, a phenomenon termed anterior chamber-associated immune deviation" (ACAID). ACAID is initiated when antigens enter the eye and culminates in a unique

form of systemic suppression of antigen specific immune responses, which in turn deviates the immune response away from a tissue-injuring inflammatory event and thus preserves the integrity of the eye, especially tissues in the eye which do not have the capacity to undergo proliferation (6, 7).

Table 1. Immunosuppressive molecules in the eye that contribute to the maintenance of immune privilege. TGF-β, transforming growth factor-beta; VIP, vasoactive intestinal peptide; sFasL, Fas ligand; IDO, indoleamine 2,3-dioxygenase; CGRP, calcitonin gene-related peptide; α-MSH; alpha-melanocyte stimulating hormone; MIF, macrophage migration inhibitory factor; CRP, complement regulatory protein; GITRL, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand; TRAIL, tumor necrosis factor related-apoptosis inducing ligand; PD-L1, programmed cell death ligand-1.

Molecule	Function
TGF-β	Suppresses T cells, NK cells, and macrophages activation and effector function, induce regulatory APCs
VIP	Suppresses T cell activation and clonal expansion
sFasL	Suppresses neutrophil recruitment and activation
IDO	Inhibiting T cell and NK cell proliferation by depleting tryptophan
CGRP	Inhibits macrophages from producing nitric oxide
α-MSH	Inhibits T cell from cytokine secretion ; suppresses neutrophils function, induce Tregs
MIF	Suppress NK cell cytotoxicity
CRP	Restrict complement activation
mTGF-β	Suppresses T cells, NK cells, and macrophages activation
GITRL	Stimulate proliferation of regulatory T cells
TRAIL	Induces apoptosis of inflammatory cells
FasL	Promotes apoptosis of lymphocytes and neutrophils
PD-L1	Induces apoptosis in T cells and inhibits their proliferation

Uveal Melanoma (UM)

Melanoma is a malignancy of melanocytes which are predominantly located in the skin, but are also found in the uveal tract (8). Only 5% of melanomas occur in the eye, of which 85% have a uveal origin (8). Tumors can form anywhere in the uveal track but they mostly occurs in the choroid (80%) and the ciliary body (15%) (**Figure 2**) (9). Thus, UM is the most common intraocular malignancy in adults (10), affecting up to 10 individuals per million Caucasians per year (8, 11). Despite its low incidence, UM accounts for 13% of the mortalities from all types of melanomas (12). The average age of patients at the time of diagnosis is 60 years (13) with the incidence among race being 196 white people affected for every black person (14).

Compared to cutaneous melanoma (CM), the etiology of UM is less understood. Even though both CM and UM are derived from the neural crest, they express significant differences in their genetic abnormalities, metastatic behaviors, responses to treatments and immune responses(15). For instance, CM can occur anywhere on the body but is mostly observed in sun-exposed area. Therefore, ultraviolet light (UV) is a major risk factor in CM but there is insufficient evidence to support the same effect in UM (16). Molecular pathogenesis of CM and UM is also different with monosomy 3 seen in the majority of patients with UM, while being rare in patients with CM (9). In CM, the $P16^{INK4A}$-CDK4 tumor suppressor pathway is frequently mutated and genetic aberration in apoptotic pathways such as protein kinase B- phosphoinositide 3-kinase (AKT-PI3K) pathway and phosphatase and tensin homolog (PTEN) pathway are seen in 30-40% of CM cell lines (17). On the other hand, in almost all of UM patients the retinoblastoma tumor suppressor pathway is mutated and the Raf kinase-Mitogen activated protein kinase kinase-Extracellular signal regulated kinases (RAF-MEK-ERK) pathway is constitutively active (18).

Histopathologically, UM are composed of spindle, epithelioid cells, or both (19). Tumors that are mainly composed of spindle cells have a better prognosis and less chance to metastasize (19). In contrast to CM, UM metastasize almost exclusively by hematogenous dissemination due to the lack of or a severely restricted presence of lymphatics in the eye (11). Primary UM metastasize in approximately 50% of patients (18). Unlike CM, which most commonly metastasize to the lungs (20), the most frequent site of metastases in UM is the liver (95%), followed by the lungs (24%), bone (16%) and skin (11%) (21).

UM is usually diagnosed in a routine eye exam or when patients present with symptoms such as blurry vision or seeing flashing lights (13). Multiple options are available for the treatment of ocular melanomas. Depending on the size or localization of the tumor, radiation therapy or enucleation are the main treatment options (11). Despite significant advances in the diagnosis and treatment of primary uveal melanoma, the survival rate of patients has not changed in the past 30 years (22). This is because in many patients, metastases have already developed prior to the detection of the ocular tumor (11). Different local or systemic therapeutic approaches have been used to manage metastatic UM including surgical resection, hepatic perfusion and/or chemotherapy (21, 23). Sadly, the 5-year survival rate for UM patients has not improved in over 30 years and at the present time there is no therapeutic modality that has been proven to be effective in the management of liver metastases of UM (22).

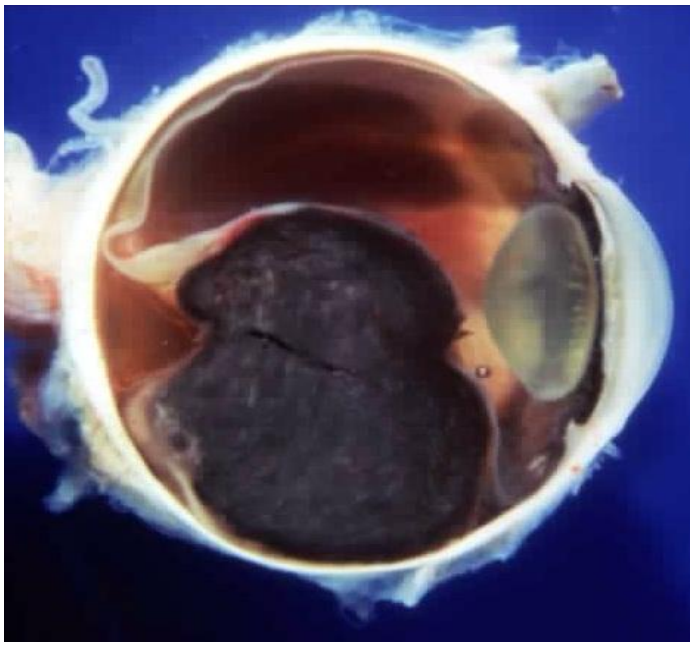


Figure 2. Cross-section of the human eye with a primary uveal melanoma. UM is the most common intraocular malignancy in adults. Image from http://www.kimmelcancercenter.org

Immunology of UM

The immune system is conventionally divided into innate and adaptive immunity, with wide-ranging crosstalk between the two. Innate immunity provides the first line of defense and employs rapid and non-specific defense mechanisms and does not confer long-lasting memory or protective immunity to the host. The cellular components of the innate immune system include macrophages, dendritic cells (DCs), natural killer (NK) cells and granulocytes. Adaptive immunity consists of a slower, antigen-specific immune response. Adaptive immune response takes several days to become protective but provides a long-lasting memory that allows for the rapid elimination of a specific pathogens after the second exposure. Adaptive immunity is mediated by T cells, B cells, and NKT cells. Inflammatory immune responses, either innate or

adaptive, may cause irreversible damage to the eye. However, as mentioned previously, the eye is an immune-privileged site and is protected from the potential immune-mediated injuries (24).

Although the eye is an immune privileged site, multiple studies have shown that intraocular tumors are vulnerable to anti-tumor immune responses (25-28). The immune-mediated rejection of intraocular tumors requires the circumvention of ocular immune privilege (29). Tumor-infiltrating lymphocytes (TILs) are present in up to 20% of primary UM (30). Several studies have suggested that intraocular tumors undergo immune rejection in a T cell-dependent manner (29). Human UM cells express tumor-associated antigens such as gp100 and melanoma-associated antigen (MAGE), which are able to induce CTL immune responses (31, 32). It has also been shown that CTLs that are isolated from UM patients are capable of killing melanoma cells (25, 33). However, unlike many types of cancers, T cell infiltration in UM is associated with an unfavorable prognosis (34, 35).

The association between TILs and a poor prognosis in UM seems counterintuitive and at odds with observations with CM in which the presence of TIL carries an improved prognosis(5). One explanation to this paradox might lie in the immune privilege of the eye and the immunomodulatory properties of the intraocular milieu. As mentioned earlier, antigens (e.g., melanoma antigens) introduced into the eye induce an antigen-specific down-regulation of T cell responses (5). Likewise, due to the presence of numerous immunosuppressive molecules in the ocular microenvironment, T cells that enter the eye are converted to Tregs which are a subpopulation of T cells that mediate immune tolerance by suppressing the immune response (1). Yoshida et al. demonstrated that pigmented epithelial cells of the iris and ciliary body suppress the proliferation and cytokine production by T cells and induced the generation of Tregs through a direct cell-cell contact dependent mechanisms (36, 37). In addition, the presence of

immunosuppressive soluble factors in the aqueous humor, such as alpha-melanocyte stimulating hormone (α-MSH), induces the local generation of Tregs (1). In the mouse model of experimental autoimmune uveitis the *in situ* generation of Tregs suppresses the delayed-type hypersensitivity (DTH) responses and terminates the immune-mediated inflammation associate with this disease (38).

It has been demonstrated that Tregs are present in 25% of primary UM (39). Mougiakakos et al. showed that the expression of cyclooxygenases (COX) is associated with a high prevalence of Tregs in primary UM which is associated with low overall survival of the patients (39). Thus, the effect of adaptive immunity in restricting primary UM is yet to be elucidated.

The effect of innate immune responses in controlling primary UM is mostly studied in the context of macrophages and NK cells which will be explained in the following sections.

Macrophages

Macrophages are professional phagocytes of the innate immune system. Macrophages are bone marrow-derived cells which differentiate from myeloid progenitor cells and play a vital role in both innate and adaptive immunity (40). In pathological conditions, macrophages are polarized into two subtypes, M1 and M2, which have inflammatory and anti-inflammatory functions respectively (41). Macrophages are polarized to the M1 phenotype in response to interferon-gamma (IFN-γ), in addition to signaling from the toll like receptors (TLRs), namely TLR4 which is triggered by microbial products such as LPS (42). M1 macrophages are generally pro-inflammatory and produce copious amounts of IL-1β, IL-15, IL-18, TNF-α, IL-23, IL-6 and IL-12 (43). M1 macrophages also recruit NK cells and Th1 cells to inflammatory sites via

production of chemokines such as CCL15, CCL20, and CXCL11 (44). When activated, M1 macrophages gain the ability to kill intracellular pathogens and tumors. This microbial killing and tumoricidal ability is facilitated by production of toxic intermediates such as nitric oxide (NO) and reactive oxygen intermediates (ROI) (42).

In contrast, macrophages in the presence of IL-4, IL-10, IL-13 and immunosuppressive agents, such as corticosteroids, are polarized to the alternatively activated phenotype, called M2 macrophages (42). The cytokine network expressed at the tumor microenvironment shifts the differentiation of recruited mononuclear cells. M2 macrophages are actively recruited to the tumor sites and are referred to as tumor-associated macrophages (TAM). TAMs express high levels of immunosuppressive factors such as IL-10 and TGF-β and indoleamine 2,3-dioxigenase (IDO) which dampens pro-inflammatory Th1 cells responses and promotes the induction of Treg (45). In addition, TAMs promote tumor growth by producing angiogenic factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet derived growth factor (PDGF) (46). In contrast with M1 macrophages which express high levels of inducible nitric oxide synthase (iNOS), TAMs express high levels of arginase-1 (Arg1) which is involved in the production of polyamines, leading to down-regulation of NO synthesis (42).

In a murine intraocular tumor model, Coursey et al. demonstrated that macrophages induce apoptosis in tumor cells resulting in rejection of the intraocular tumor (26). In UM, these macrophages are mostly M2 macrophages with immunosuppressive properties. The increased frequency of TAMs in UM patients correlates with poor survival (47).

NK cells

NK cells are the effector lymphocytes of the innate immune system. Although NK cells are bone marrow-derived lymphocytes, they are distinct from B cells and T cells since they do not require clonal proliferation or antigen receptor gene rearrangement. Unlike lymphocytes of adaptive immunity, NK cells have a fixed set of receptors and are able to kill a target cell promptly and without prior immunization (48). NK cells were initially discovered due to their ability to spontaneously kill tumor cells (49, 50). However, the effector functions of NK cells are not restricted to the recognition and elimination of malignant, infected, stressed or damaged cells. NK cells can also play vital roles in the regulation of adaptive and innate immune systems by boosting the maturation and activation of APCs, macrophages and T and B cells (51). NK cells are also able to act as a regulatory cells by killing immature DCs and T cells (52). NK cells distinguish normal healthy cells from transformed or foreign cells through a concept termed the 'Missing Self Hypothesis' (53). This hypothesis proposes that NK activity is triggered by a decrease or lack of expression of MHC class I or human leukocyte antigen (HLA) molecules on the surface of a target cell in mice or humans respectively (53). NK cell-mediated cytotoxicity is regulated by the expression of an array of cell surface activating and inhibitory receptors (53). The activating pathway of NK cells includes receptors such as the activating killer lectin-like receptor, NKG2D and natural cytotoxicity receptors (NCRs) that detect the presence of stress-induced ligands on target cells like MIC molecules in humans and RAE1 in mice (53). NK cell inhibitory receptors such as killer inhibitory receptors (KIRs) or Ly49 bind to HLA molecules or MHC class I molecules in humans and mice respectively, preventing NK cell activation (53). Transformed or infected cells that down-regulate the expression of these inhibitory ligands are prone to attack by NK cells. The NK cells' activation state is the result of the tightly regulated

balance between the engagement of inhibitory and activation receptors. In order for NK cells to exert their effector function, they require not only a lack of MHC class I expression but also the ligation of activating ligand on target cells (53).

When activated, NK cells kill target cells by various mechanisms. There are three major mechanisms involved in the destruction of tumor cells by NK cells: a) The granule exocytosis pathway (54), b) the death receptor pathway (55-57) and c) by cytokine production (58).

a) Perforin/granzyme -mediated cytotoxicity is the major pathway used by NK cells to kill target cells. The importance of perforin in the elimination of tumor cells is well documented (59, 60). Mice that are deficient in the production of perforin have limited capacity to kill tumor cells (61). It appears that perforin-mediated killing is more important in the lung and spleen than in the liver (53). Although the role of granzymes is less studied, it has been shown that the presence of granzymes is dispensable for the rejection of tumors (62).

b) The death receptor pathway is mediated by cell membrane-bound members of the tumor necrosis factor (TNF) family such as Fas ligand (FasL) and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) (53). The death receptor pathway is a slower and less efficient pathway compared to perforin-mediated cytolysis (63). In mice, 25-35% of liver NK cells express TRAIL whose expression is regulated by IFN-γ (64). Several studies have strongly suggested that TRAIL is an important effector molecule in preventing tumor formation and clearing tumor metastases both *in vivo* and *in vitro* (56, 64, 65).

   FasL is a type II transmembrane protein of TNF family that induces cell death in cells expressing its receptor, Fas (CD95/APO-1), leading to apoptosis through activation of

members of the caspase family (66). FasL is expressed in the eyes and testes to protect these organs from the damaging effects of inflammation by inducing apoptosis of inflammatory cells (67). FasL is also expressed in T cells, NK cells and various tumors such as melanoma, lymphoma and carcinoma (66). Fas receptor is not commonly expressed by tumor cells, but NK cells are able to induce its expression and therefore kill the target cell in a Fas-dependent manner causing the suppression of tumor growth (57, 68).

c) The activated NK cells can produce large amounts of cytokines and chemokines (e.g. IFN-γ, TNF-α, IL-5, IL-10, IL-13, and granulocyte- macrophage colony-stimulating factor (GM-CSF)) which can directly and indirectly affect tumor growth, anti-viral immune responses and the regulation of innate and adaptive immune responses (69). However, mature NK cells lose their competence to produce the Th2 cytokines and gain the ability to only secret Th1 cytokines (53). Undeniably, production of IFN-γ by NK cells contributes to diminishing tumor growth and metastasis by inhibiting tumor proliferation and angiogenesis (53). In addition, NK cells enhance the sensitivity of tumor cells to pro-apoptotic proteins by upregulating the expression of caspases within the tumor cells (69). Finally, by producing IFN-γ, NK cells activate DCs which contribute to the induction of an anti-tumor immune response by the adaptive immune system through their function as APCs (70).

Cytokine regulation of NK cells

In addition to engagement of activation and inhibitory cell surface receptors, the activation of NK cells is regulated by factors secreted mainly by DCs and macrophages (53, 71). Many cytokines and chemokines are involved in differentiation and activation of NK cells including IL-2, IL-12, IL-15, IL-18, IL-21, IL-23, IFNs and GM-CSF (**Table 2**) (71-73). These proinflammatory cytokines induce the expression of activation markers and receptors such as CD25, CD69 and NKG2D on NK cells, which stimulate the secretion of IFN-γ by NK cells and promotes NK cell-mediated cytotoxicity (71). In turn, IFN-γ produced by NK cells induces the maturation of DCs and causes macrophages to secrete higher levels of IL-12, IL-15 and IFN-α/β, thereby promoting more vigorous activation of NK cells (74). Interestingly, DC-activated NK cells attain the capacity to lyse DCs to prevent the onset of an adaptive immune response (74).

Multiple molecules can suppress NK cell activity. However, three cytokines in particular come to mind: IL-10, TGF-β, and macrophage migration inhibitory factor (MIF) (75-79), which are described in further detail in the following sections.

IL-10

Interleukin (IL-10) was first described by Fiorentino et al. in late 1980s as a cytokine that is produced by Th2 cells and inhibits the production of Th1-related cytokines (80). Further studies revealed that IL-10 is an 18 kDa (kilo Dalton), homodimeric and pleiotropic immune-modulatory cytokine that is produced by a variety of cells such as DCs, macrophages, Tregs, NKT cells, and hepatocytes (81-83). IL-10 mediates its biological effects by binding to a two-subunit cell surface IL-10 receptor (84). IL-10 is an anti-angiogenic factor that leads to tumor regression (85). In contrast, IL-10 can promote cancer development by stimulating tumor cell

proliferation and inhibiting apoptosis (86). In the context of tumor immunology, the general notion is that IL-10 is an immunosuppressive molecule secreted by tumors or tumor-induced immune cells allowing malignant cells to escape immune surveillance. However, multiple findings have reported that IL-10 can promote adaptive immunity, which challenges the concept of IL-10 being a solely immunoregulatory cytokine (87). The effect of IL-10 on NK cells is also controversial. Several reports have shown that IL-10 down-regulates NK cell function (88, 89). However, there is also evidence suggesting that IL-10 is a potent activator of NK cells (90, 91).

Macrophage Migration Inhibitory Factor (MIF)

MIF was originally identified in 1966 as a chemokine secreted by lymphocytes that prevents the migration of macrophages *in vitro* (92, 93). Currently, it is believed that MIF is a pleiotropic cytokine that is involved in many inflammatory diseases and cancer (94-97). In addition to epithelial and endothelial cells, most cells of the immune system produce MIF (98). When exposed to the antigen, immune cells rapidly release MIF. MIF in turn induces the production of proinflammatory cytokines such as TNF-α, IFN-β , IL-1β, IL-2 and IL-6, which results in the activation and proliferation of T cells (99). However, in malignancies, MIF plays multiple roles that favor tumor growth and invasiveness by the inhibition of the tumor suppressor gene p53, suppression of apoptosis and induction of angiogenesis (100). Apte and colleagues reported that aqueous humor contains a high concentration of MIF which causes a rapid inhibition of NK cell cytotoxicity, protecting corneal endothelial cells from NK cell mediated-lysis (75). Recently, Arcuri and colleagues showed that MIF participates in maintenance of the immune privilege of the uterus by suppressing NK cell mediated-cytotoxicity (101, 102).

Transforming Growth Factor β (TGF-β)

TGF-β is a member of a pluripotent family of growth factors with potent growth-inhibitory activity (103). In the early 1980s, Todaro and De Larco isolated a protein from virally transformed cells and named it the sarcoma growth factor (SGF) (104). Soon after, SGF was called TGF-β and was shown to be capable of both stimulating and inhibiting the cell growth *in vitro* (105). Likewise, in tumors, TGF-β can play a role as either a pro-oncogenic or a tumor-suppressor (106). TGF-β produced by tumor cells can regulate the activation and differentiation of both innate and adaptive immune cells including NK cells, DCs, macrophages and T cells (103). UM cells are able to produce TGF-β, which can cause the down-regulation of MHC class I molecules and therefore increase their susceptibility to NK cell-mediated cytotoxicity (107, 108). However, TGF-β is a potent inhibitor of NK cell activity by suppressing the expression of IFN-γ by activated NK cells and inhibiting the expression of activating receptors on NK cells (103). Alongside other immunosuppressive factors, the presence of an ample amount of TGF-β in the AH leads to the strong suppression of NK cell activity and therefore protects UM cells from NK cell-mediated cytolysis (5).

Table 2. Chemokines and cytokines that contribute to activation and suppression of NK cells

Cytokine	Source	Effect on NK cells
IL-2	T cells	Stimulate proliferation and differentiation
IL-12	DCs, Macrophages	Stimulate activation, proliferation and differentiation
IL-15	DCs, Macrophages	Stimulate activation proliferation, Enhance cytotoxicity
IL-18	DCs, Macrophages	Stimulate maturation, cytokine production and cytotoxicity
IL-21	Macrophages	Enhance cytotoxic activity
IL-23	DCs, Macrophages	Stimulate activation and differentiation
IFN-α/β	DCs, Macrophages	Enhance cytotoxic activity
IFN-γ	T cells, NK cells	Enhance cytotoxic activity
IL-10	Immune cells, Tumor	Suppress cytotoxic activity
MIF	Immune cells	Suppress cytotoxic activity
TGF-β	Tregs, Tumors	Suppress cytotoxic activity

The role of NK cells in the immune surveillance of UM

The immune surveillance theory was first proposed by Burnet and Thomas in the mid-1960s, which hypothesized that the immune system protects the host from cancer by recognizing and eradicating malignant cells (109, 110). Currently, the significant body of research supports this hypothesis and therefore the theory of immune surveillance is widely accepted. Studies in humans and mice revealed that incompetency in the immune system results in an enhanced incidence of spontaneous tumor formation and acceleration of tumor growth (5, 111, 112).

As mentioned previously, NK cells are the critical members of the innate immune system in tumor cell surveillance. The importance of NK cells in killing UM cells has also been shown in both humans and in mice. In patients with primary UM, NK cells comprise up to 40% of tumor infiltrating lymphocytes (5). Moreover, many melanoma cell lines isolated from primary UM are susceptible to NK cell-mediated cytolysis (5, 107, 113). Ksander et al. reported that NK cells that are isolated from primary UM are capable of killing melanoma cells *in vitro* (114). Studies in nude mice, which cannot mount a T cell-dependent adaptive immune response but have an intact NK cell repertoire, revealed that the depletion of NK cells *in vivo* results in a significant increase in the number of liver metastases arising from human uveal melanoma cells transplanted into the eye (115, 116).

A noteworthy phenomenon in UM is related to the expression of MHC class I molecules. The cytotoxicity of NK cells is inversely correlated with the expression of MHC class I molecules on target cells (52). In several reported cases from patients with breast, skin or laryngeal cancer, a higher expression of MHC class I molecules is associated with better prognosis, presumably due to the enhanced susceptibility of the tumor cells to CTLs (117-119). In contrast, the increased expression of MHC class I molecules in UM patients is correlated with decreased survival (120-122). This is because UM cells with high expression of MHC class I molecules are more resistant to NK cell-mediated cytotoxicity (123). It is noteworthy to mention that within the eye, UM cells are protected from lysis by NK cells due to the large amount of immunosuppressive factors that are present in the AH such as MIF and TGF-β (1). Apte at al., demonstrated that competent NK cells that are incubated with AH lose their cytotoxic ability (75). Further studies support the notion that NK cell-mediated cytotoxicity is prohibited in the eye. NK cell-sensitive UM cells were swiftly rejected when injected subcutaneously (SC) into

nude mice, but were able to grow in the eyes, despite the fact that tumor cell doses were fifty times higher when injected SC (5). Moreover, *in vivo* depletion of NK cells, prevented nude mice from rejecting subcutaneously injected UM cells, thereby supporting the notion that the progressive growth of these tumors in the eye was due to the local suppression of NK cells activity (124). However, once UM cells leave the sanctuary of the eye and enter the liver, the organ with the highest concentration of NK cells in the body, they are vulnerable to NK cell-mediated immune surveillance (5). In the liver, UM cells with low MHC class I molecule expression are removed by NK cells, which makes UM cells with high expression of MHC class I molecule expression favored to survive (5). Specimens collected from patients with primary and metastatic UM showed that the expression of MHC class I molecules are nine times higher in tumor cells isolated from metastatic UM compared to that with primary UM (125). This may explain why patients with high expression of MHC class I molecules on their primary UM cells have a lower survival rate.

Metastatic UM

The etiology for the preferential metastatic spread to the liver is poorly understood. Previous studies have tried to explain the metastatic process of UM cells. The prevalence of liver metastases in UM is not due to the blood circulation because the lung, not the liver, capillary beds are the first location that melanoma cells encounter when they leave the eye (126). It has been shown that UM cells have an up-regulation of molecules such as MNNG HOS transforming gene (c-Met), insulin-like growth factor I receptor (IGF-IR) and C-X-C chemokine receptor 4 (CXCR4) (127-130). The liver is the only organ that highly expresses ligands for these proteins, which are hepatocyte growth factor (HGF), IGF and CXC ligand 12 (CXCL12) respectively. This suggests that these molecular pathways are involved in the liver-specific metastasis in UM

(127-130). For instance, Li et al. demonstrated that the blockade of CXCR4/CXCL12 interaction significantly inhibits the chemotactic responses of human UM cell lines toward liver-derived chemoattractants (130). In the mouse model of experimental liver metastases, mice that were challenged with CXCR4-siRNA treated UM cells formed fewer liver metastases than control groups, which highlighted the importance of CXCR4 expression in homing of UM cells to the liver (130).

Recently, the role of micro-RNA (miRNA) and epigenetic regulations has been revealed in UM pathogenesis and metastases (131, 132). MiRNAs are non-coding small RNAs with the ability to regulate gene expression transcriptionally or post-translationally (132). Micro-RNA-137 (miR-137) acts as a tumor suppressor gene in tumorigenesis but it is epigenetically silenced in UM cells lines (132). Chen and colleagues suggested that the ocular microenvironment induces the down-regulation of CXCR4 expression via epigenetic mechanisms through methylation of the CXCR4 promoter (133). It has also been reported that the silencing of a tumor suppressor gene, RAS association domain family 1 (RASSF1), by hyper-methylation is a common epigenetic event in UM patients and it is positively correlated with the development of metastatic disease (134).

In addition to the properties of UM itself, a significant body of research suggests that the liver's unique immunoregulatory micro-environment might foster the growth of tumors that metastasize to the liver (135) which will be discussed in the following section.

Liver: an organ with a tolerogenic micro-environment

The liver is an organ with a distinct anatomical location in which blood enters it both through the systemic circulation and the hepatic portal vein (136). More than 80% of the blood

reaches the liver via the portal circulation, which carries the blood from the intestines and contains intestinal derived-microbial products such as lipopolysaccharide (LPS) (135). Although in the liver, the cells of the innate immune system express the LPS receptor and constantly exposed to the gut-derived LPS, yet they elicit a distinctive set of mechanisms to prevent the induction of an immune response and instead, maintain immune tolerance (137). However, unlike an immune privileged organ such as eye, the liver is able to eliminate microbial infections by eliciting a pro-inflammatory immune response, mostly through its innate immune components (137).

The liver is composed of parenchymal (i.e., hepatocytes) and non-parenchymal cells, including stellate cells, liver sinusoidal endothelial cells (LSECs) which are unique microvascular endothelial cells, Kupffer cells which are the resident macrophages of the liver and lymphocytes (138). Alongside conventional APCs such as DCs and Kupffer cells, hepatocytes and several non-parenchymal liver cells are able to act as APCs. For instance, hepatocytes, LSECs, and stellate cells present antigens to T cells by expressing low levels of MHC class II molecules (135, 138). However, under steady-state conditions, the constant exposure to LPS and production of HGFs by liver stromal cells induce a tolerogenic phenotype in the liver APCs resulting in the promotion of the immune tolerance (135, 138-140). For example, HGF induces IL-10 and TGF-β production and stimulates the generation of tolerogenic dendritic cells (139). Liver DCs have a lower expression of MHC class II and co-stimulatory molecules, secrete less IL-12 and produce high amounts of IL-10, all which favor the generation of immune tolerance (141). Liver DCs promote Th2 polarization and induce production of Treg (142). Multiple studies indicate that Kupffer cells are also immunosuppressive (135). Kupffer cells are major APCs in the liver (137) and account for 20% of non-parenchymal liver cells

(136). Like liver DCs, Kupffer cells express low levels of MHC class II and co-stimulatory molecules and prevent DC activation via production of prostaglandin E2 (PGE2) (143). Additionally, Kupffer cells induce T-cell suppression by their secretion of IL-10 and TGF-β (144, 145). By expressing FasL and programmed death ligand 1 (PD-L1), Kupffer cells lead to the induction of T-cell apoptosis (146, 147). LSECs express several adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which cause the retention of T-cells in the liver (148). However, instead of promoting the conversion of naïve T-cells to T helper cells, LSECs produce high levels of IL-10, which induces the development of Treg cells (149, 150). Hepatocytes and stellate cells can also induce apoptosis of T cells and promote the generation of regulatory T-cells (19-21). Taken together, these findings show that different cell types and cytokines, in collaboration with the innate immune elements in the liver, provide a unique micro-environment that induces T-cell tolerance and systemic immune regulation.

The tolerogenic characteristics of the liver are demonstrated by its role in oral and systemic tolerance, and the maintenance of microbial infection and liver metastases. For instance, the unique immunological properties of the liver lead to immune tolerance of certain categories of allografts, which are otherwise rejected if transplanted in any other organ (151). Because of the partial suppression of an adaptive T cell response in the liver, innate immune elements play an important role in the elimination of infectious microorganisms and malignant cells in this organ (135, 152).The liver is enriched with Kupffer cells, NK cells and NKT cells which are key components of innate immunity (152). The abundance of NK cells and NKT cells in the liver suggests that they have important roles in the immunity of the liver. Many studies have shown the anti-tumor and anti-viral effects of liver NK and NKT cells (153-156).

NKT cells

NKT cells are a distinct population of T cells with the characteristics of both innate and adaptive immunity (157). NKT cells are involved in a wide spectrum of immune responses ranging from anti-tumor responses and resistance to the regulation of autoimmune and allergic responses (158). The relative frequency of these cells varies from organ to organ. Murine NKT cells are found at the highest frequency in the liver (10 - 45% of liver lymphocytes) and with lower frequencies (<2%) in the thymus, bone marrow, spleen, lymph node, and blood (151). The distribution of human NKT cells in different tissues is not well-defined, but NKT cells are significantly less frequent in human livers than in the livers of mice (159, 160).

Two populations of NKT cells have been described. Type I NKT cells express a T cell receptor and accordingly are described as invariant NKT (iNKT) cells and encompass 80% of total NKT cells (158). Murine iNKT cells have an invariant Vα14Jα18 chain paired with a limited set of β chains (Vβ2, Vβ7 and Vβ8). Human iNKT cells bear a TCR with an invariant Vα24Jα18 with diverse Vβ11 chains (158). Type II NKT cells express more diverse TCRs. Unlike conventional T-cells that react to peptides presented by MHC molecules, NKT cells are activated via the non-classical MHC class I molecule, CD1d, which presents endogenous lipid antigens such as isoglobotrihexosylceramide (iGb3) and/or exogenous lipid antigen such as α-galactosylceramide (α-GalCer) (158). Activated NKT cells rapidly produce large amounts of both Th1 (IFN-γ) and Th2 (IL-4, IL-10 and IL-13) associated cytokines upon stimulation (81, 161, 162). The simultaneous expression of Th1 and Th2 associated cytokines by the same cell is a unique property of NKT cells. Due to the production of Th1 and Th2 cytokines, NKT cells are able to exert either pro-inflammatory or anti-inflammatory immune responses depending on the environmental conditions in which the NKT cells reside (163). Therefore, they play roles in the

suppression of tissue destruction, autoimmunity, anti-tumor responses, host defense, allergy, inflammation and protection from protozoan parasites (164-167)

The role of NKT cells in cancer

The role of NKT cells in tumor immunity has been controversial. In murine models, it is widely believed that type I NKT cells have an anti-tumor function whereas type II NKT cells contribute to the suppression of anti-tumor immune responses (157). The protective role of iNKT cells in anti-tumor immune responses was first detected when it was discovered that the activation of iNKT cells with αGalCer leads to a strong anti-tumor immune response in mice (168, 169). Smyth et al. showed that in iNKT cell deficient mice, methylcholanthrene (MCA)-induced fibrosarcomas grew faster indicating that iNKT cells play a protective role (170). Since then, numerous studies in murine models confirmed that iNKT cells have a protective role in the immune surveillance against tumors either with or without αGalCer or IL-12 stimulation (171, 172). Several studies showed that NKT cells are able to directly lyse tumor cells (173-175) in a CD1d-dependent manner (176, 177). However, most tumor cells do not express or down-regulate CD1d expression and are therefore unrecognizable by NKT cells (178).Thus, the protective role of iNKT cells is mostly indirect and depends on the secondary response which is initiated in part by IL-12 expression by myeloid cells. In turn, IL-12 induces the production of IFN-γ by the iNKT cells and consequently affects the downstream activation of NK cells and $CD8^+$ T cells (179-182).

The notion that iNKT cells are solely protective in tumor immunity was challenged by several findings showing that a lack of iNKT cells in mice results in enhanced anti-tumor immune responses. In the mouse model of renal carcinoma, Subleski et al. found that CD1d

deficient mice, which lack both type I and type II NKT cells, had reduced numbers of liver metastases compared with WT mice (155). Other studies showed that there are significantly fewer lung metastases and improved survival in mice lacking iNKT cells (183, 184). Several studies have suggested that soluble factors produced by iNKT cells that are isolated from healthy individuals induce a regulatory phenotype in DCs and macrophages causing the suppression of adaptive immunity via induction of tolerogenic APCs (185, 186). Additionally, iNKT cells are able to suppress anti-tumor immune response via expression of IL-2 which is essential for maintenance of Tregs (187). Terabe et al. also showed that IL-13 released by NKT cells induces TGF-β production by myeloid cells that eventually inhibits the effector function of $CD8^+$ T-cells (188). The role of NKT cells in the formation of primary and metastatic UM has not been sufficiently investigated. Therefore, in the present study, we investigate the role of NKT cells in the development of liver metastases in mice bearing intraocular melanoma.

Hepatic NK and NKT cells

As mentioned previously, NK and NKT cells are critical components of the immune system in the liver, enabling the host to mount a prompt immune response against pathogens while maintaining the tolerogenic state of the liver. The residence of hepatic NK and NKT cells in the liver sinusoids, a specific type of low pressure blood vessel with a discontinuous endothelium, allows them to efficiently kill transformed or infected cells (135). At the same time, the unique immunological micro-environment of the liver contributes to the distinct phenotype of liver NK and NKT cells in comparison to those residing in other organs. Multiple studies showed that liver NK cells have enhanced anti-tumor activity compared to splenic NK cells (189-191). It has been suggested that the higher liver NK cell-mediated cytotoxicity is due to the higher expression of TRAIL and perforin/granzyme and the lower expression of Ly-49

inhibitory receptor (56, 64, 190). Additionally, Crowe et al., reported that relative to the splenic and thymic NKT cells, liver NKT cells have augmented tumoricidal properties (192).

Mechanisms of immune escape

Cancer is the second leading cause of death in the United States (193). This challenges the immune surveillance hypothesis, which proposes that malignant cells are eliminated by the immune system (194). Multiple studies suggest that chronic inflammation is positively correlated with an increased risk of cancer (195, 196). This leads to the generation of a more recent concept called the "immune editing" hypothesis (194). The immune editing hypothesis proposes that the immune system applies a selective pressure on the tumor cells, which leads to the emergence of tumor cell populations that are resistant or invisible to the innate and adaptive immune responses (111). The recognition of transformed cells by the immune system has three stages. First, the elimination process in which immune surveillance occurs and the immune system is capable of successfully deleting malignant cells. Second, the equilibrium phase wherein a tumor cell variant that has endured immune surveillance enters into a dormancy stage. Finally, the escape stage in which the remaining tumor cells that are insensitive to the immune attack begin to grow unrestrained (194).

Like all other malignant cells, UM cells utilize a number of mechanisms to evade or suppress the immune response (**Table 3).** Some of these mechanisms include: 1) up-regulation of MHC class I molecules; 2) down-regulation of NK cell activating ligands; 3) up-regulation of IDO; 4) expression of PD-L1; 5) expression of the complement regulatory proteins (CRPs); 6) resistance to FasL-induced apoptosis; 7) expression of MIF and TGF-β; 8) resistance to perforin and granzyme; and 9) induction of MDSCs.

Primary UM cells are protected from NK cell-mediated cytolysis while residing in the eye due to the immunosuppressive environment of the eye. However, in the liver, UM cells are susceptible to NK cell-mediated killing. As mentioned previously, it has been shown that the expression of MHC class I molecules on metastatic UM cells is significantly higher than that on primary UM cells, which results in the suppression of NK cell anti-tumor functions in the liver (125).

Down-regulation of NK cell activating ligands, such as NKG2D, is another mechanism that UM cells exploit to evade NK cell-mediated cytolysis (5). NKG2D is an activation receptor expressed by NK cells which plays a vital role in NK cell-mediated immunosurveillance. The engagement of NKG2D with its ligands, MIC-A/B, induces calcium flux, cytokine release, and cytotoxicity of NK cells (197). MIC-A/B are poorly expressed by healthy cells but are frequently up-regulated in tumor cells (198). The NKG2D ligands are expressed on half of the primary UM cells (5). However, metastatic UM cells do not express the MIC-A/B ligands and therefore fail to activate NK cells (199). These findings indicate that the population of UM cells that survive in the liver undergo a selection process through which they gain resistance to NK cell-meditated killing.

In addition, UM cells utilize IDO as an effective escape mechanism for eluding adaptive and innate immune responses. IDO is an enzyme that catalyzes the degradation of tryptophan, which is an essential amino acid for T cell growth and differentiation (200). Thus, IDO can promote tumor escape by depleting T cells residing in and around the tumor nodules. In addition to inhibiting T cell immunity, IDO impairs NK cells activity (5). Chen et al. showed that the treatment of UM cells with exogenous IFN-γ resulted in the expression of IDO in both primary and metastatic UM cell lines (201). In inflammatory conditions, T cells and NK cells are capable

of producing significant amount of IFN-γ which can promptly induce the expression of IDO in UM cells. Consequently, the production of IDO by UM cells helps tumor cells escape the anti-tumor immune response by suppressing T cells and NK proliferation and cytotoxic ability (1).

PD-L1 is another molecule that can contribute to the tumor's capacity to avoid immune rejection. PD-L1 is a type 1 transmembrane glycoprotein and a member of B7 family (202). PD-L1 is expressed on a variety of cells including 50% of primary and 20% of metastatic UM cells (5). The ligation of PD-L1 and its receptor PD-1, which is expressed on T cells and myeloid cells, leads to suppression of T cell proliferation, reduction of cytokine production and induction of apoptosis in T cells (203). In concert with these findings, Yang et al. reported that the blockade of PD-1/PD-L1 interaction restores the production of pro-inflammatory cytokines by T cells (202). Like IDO, exposure to IFN-γ induces the expression of PD-L1 on all the primary and metastatic UM cells and facilitate the incapacitation of the immune system by UM cells (5).

The complement system is a part of the innate immune system and is composed of small plasma proteins. When activated, the complement system induces an inflammatory response that helps fight infections through activation of phagocytes and granulocytes (5). Activation of the complement system occurs through three different pathways named the mannose-binding lectin (MBL), classical and the alternative pathways (204). All three pathways produce an intermediate complement called C3 convertase. Upon activation of C3 convertase, the downstream signaling cascade is activated which results in the generation of the membrane attack complex (MAC), recruitment of inflammatory cells, opsonization and killing of the infected cells (204). The complement system is regulated by CRPs which suppress complement-mediated cell lysis (5). There are several CPRs with different mechanisms of action including decay-accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor type 1 (CR1) and CD59. DAF,

MCP, and CR1 prevent the activation of C3 and CD59 prevents the formation of the MAC at the terminal step of the complement activation cascade (205). Multiple ocular tissues express CRPs in both soluble and membrane bound forms to protect the eye from complement-mediated injuries (5). UM cells have also been shown to express DAF, MCP and CD59, which help them evade the complement-related anti-tumor immune response (206). Goslings et al. reported that *in vitro* removal of CPRs make UM cells vulnerable to complement-mediated cytolysis (206).

As mentioned previously, effector lymphocytes use FasL to induce apoptosis in malignant cells. On the other hand, FasL expressing tumor cells counterattack to kill tumor-infiltrating lymphocytes (66). Although UM cells express both Fas and FasL (207), they are not susceptible to FasL-mediated killing by either NK cells or CTLs (5). The membrane-bound FasL that is expressed by UM cells is cleaved by proteases to form soluble FasL. The soluble FasL binds to the Fas expressed on UM cells, however the binding is not strong enough to induce apoptosis. The binding of soluble FasL to Fas on UM cells protects the tumor cells from apoptosis by preventing the binding of membrane-bound FasL that is expressed on NK cells or CTLs (5, 208).

Production of cytokines is another strategy used by UM cells to evade the anti-tumor immune response. As described above, cytokines such as MIF and TGF-β that suppress both the innate and adaptive immune responses are produced by metastatic UM cells (1). These cytokines are specifically able to suppress NK cell mediated-cytolysis and protect UM melanoma cells from the harsh environment of the liver which is enriched with NK cells (108, 209).

Interestingly, UM cells take advantage of the presence of bystander IFN-γ which is one the pro-inflammatory cytokines that is present in the UM microenvironment (210). IFN-γ not

only induces the expression of MHC class I molecules, IDO and PD-L1 on UM cells, which makes them resistance to NK cell-mediated killing but it also protects UM cells from perforin/granzyme-mediated cytolysis by NK cells (5, 211). Following treatment with IFN-γ, otherwise susceptible UM cells show compelling resistance to perforin/granzyme-mediated cytolysis. IFN-γ potently diminishes the binding ability of granzyme to UM cells and therefore prevents granzyme-induced apoptosis (5). Thus, IFN-γ facilitates the evasion of UM cells from NK cell-mediated surveillance via three different pathways.

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of bone marrow-derived myeloid cells that fail to mature (212). In healthy individuals, MDSCs comprise approximately 0.5% of circulating peripheral blood mononuclear cells (PBMCs) (213). However, in cancer and other pathological conditions such as parasitic infection, traumatic stress or autoimmunity, MDSCs proliferate and promote the suppression of immune responses (213-215). In humans, MDSCs are mostly defined as $CD14^{-}CD11b^{+}CD33^{+}$ cells. In mice, MDSCs are characterized by the expression of both myeloid-cell lineage differentiation Gr1 and CD11b. (212). The Gr1 detection antibody binds to two different epitopes, Ly6C and Ly6G. Based on the expression of these epitopes, two distinct populations of MDSCs have been identified: granulocytic ($CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$) and monocytic ($CD11b^{+}$ $Ly6G^{-}Ly6C^{+}$) MDSCs (216). Because the expansion of MDSCs is influenced by a diverse set of factors secreted by tumors, MDSCs display even more diverse phenotypes within these two subsets (217). MDSCs suppress both the innate and adaptive immunity through a variety of mechanisms. These immunosuppressive activities of MDSCs appear to require direct contact with the target cell, suggesting that these suppressive activities function through cell-surface receptors and/or through the release of short-lived soluble mediators (212). Factors implicated in the suppression

of T cell and NK cell function and proliferation include production of inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), TGF-β, and through the induction of Tregs and depletion of cysteine and arginine which are two essential amino acids for T cells survival (212, 218, 219). MDSCs deplete arginine via expression of arginase1 which is an enzyme that is responsible for the catabolism of arginine (212). In addition, arginase1 inhibits T cell activation by suppressing the expression of TCR-associated CD3zeta-chain (ζ-chain) (220). It has been reported that in UM patients an increase in the number of MDSCs is correlated with a significant reduction in the expression of CD3 ζ-chain on T cells and poor survival (221, 222).

Table 3. Mechanisms used by UM cells to evade the anti-tumor immune response.

Mechanism	**Effect**
Up-regulation of MHC class I	Inhibits NK cells
Down-regulation of MIC-A/B	Reduces NK cell-mediated cytotoxicity
Up-regulation of IDO	Depletes T cells
Expression of Trail, PD-L and Fas-L	Suppresses T cell proliferation and induce apoptosis
Expression of MIF and TGF-β	Suppresses T cell and NK cells
Induction of MDSC and Tregs	Suppresses anti-tumor immune response
Expression of CRPs	Inactivates complement pathway

Rationale and objective of the dissertation

Uveal melanoma is the most common intraocular malignancy in adults and one the most lethal types of cancers. Half of the patients with primary UM develop metastases to other organs, with the liver being the most frequently affected organ. The current median survival time of patients with liver metastases is less than one year (223). The clinical prognosis of patients diagnosed with UM is highly dependent on the disease progression in the liver. Unfortunately, there is currently no therapy for metastatic UM.

NK cells are a critical part of the innate immune system and their presence and importance in the immunity of the liver and infighting metastatic UM is evident (29). NKT cells are another distinct population of immune cells that are more abundant in the liver than any other organ (224). Unlike NK cells, the role of NKT cells in the development of metastatic UM has not been sufficiently investigated. Preliminary data from our laboratory showed that NKT cells play a pro-tumorigenic role. The first aim of this study is focused on investigating the role of liver NKT cells in the formation of liver metastases in mice bearing intraocular melanoma and determining whether the immunosuppressive effect of liver NKT cells is NK cell-dependent. The second aim of this dissertation is to determine the mechanisms by which NKT cells suppress NK cell-mediated cytotoxicity in melanoma-bearing livers. IL-10 is recognized as a cytokine that profoundly inhibits a broad range of immune responses (84). Multiple studies have reported that IL-10 expression is up-regulated in various cancers (84). We therefore seek to determine whether NKT cell-induced suppression of NK cells is IL-10 dependent.

NK cells exert their effector functions through a variety of mechanisms including the production of pro-inflammatory cytokines, such as IFN-γ and TNF-α, secretion of perforin, and expression of FasL and TRAIL (53). The last aim of the study is to determine how the effector

functions of liver NK cells are affected in melanoma-bearing NKT cell-deficient mice. The overall goal of this study is to elucidate the potential mechanisms of immune evasion employed by UM cells by inducing a regulatory phenotype in NKT cells.

CHAPTER TWO:

Materials and Methods

Cells

B16LS9 cutaneous murine melanoma cell line was kindly provided by Hans E. Grossniklaus (Emory University School of Medicine, Atlanta, GA). B16LS9 cells were derived from hepatic metastases originating from posterior compartment inoculation of B16-F1 cutaneous melanoma cells in C57BL/6 mice (225). P815 murine mastocytoma cells were obtained from American Type Culture Collection (ATCC) and were used as a control target cell in some of the in vitro assays. Tumor cells were maintained in complete RPMI 1640 medium containing 10% FBS (HyClone), 100 U/ml of penicillin, 50 ng/ml of streptomycin, 0.1% Fungizone (BioWhittaker), 2.0 mM glutamine (BioWhittaker), 0.01 M HEPES buffer (BioWhittaker), and 0.5% 2-Mercaptoethanol (Sigma-Aldrich). Ad5E1 tumor cells were kindly provided by Dr. Rene E.M. Toes (Leiden University Medical Center, Leiden, The Netherlands). The tumor cells were generated by the transformation of C57BL/6 mouse embryo cells with a plasmid encoding the human adenovirus type 5 early region 1 (Ad5E1) and propagated as previously described (226). Ad5E1 tumor cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL, Grand Island, NY). The RAW 264.7 macrophages were obtained from National Center for Cell Science (NCCS, Pune, India) and maintained in complete DMEM. Corneal endothelial cells were generated as described previously (227). Briefly, freshly dissected corneal cells from NZB and C3H/Hej mice were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum. The cells were then immortalized with human papilloma virus genes E6 and E7 using the disabled recombinant retroviral vector pLXSNl 6E6/E7.

Mice

Female 8-12 week old C57BL/6 mice were purchased from the Wakeland Animal Colony at the University of Texas Southwestern Medical Center (Dallas, TX). Breeding pairs of CD1d$^{-/-}$ mice (C57BL/6 background), which lack both type I and type II NKT cells, were kindly provided by Mark Exley (Beth Israel Deaconess Medical Center, Boston, MA) and had been backcrossed to C57BL/6 mice for 12 generations (Exley M, personal communication August 26, 2010). IL-10$^{-/-}$ mice (B6129P2-^{il10tm1Cgn}/J), TNF-$\alpha^{-/-}$ mice (B6.129S6-TNF^{tm1Gk1}/J), GLD mice (B6Smn.C3-Tnfsf6^{gld}/J) and perforin$^{-/-}$ mice (C57BL/6-Pfp^{tmlSd2}) were obtained from The Jackson Laboratory (Bar Harbor, ME). All the mice were maintained in a dedicated pathogen-free environment and used for experiments between 8 and 12 weeks of age. All the animals were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center and the Association for Research in Vision and Ophthalmology (ARVO) statement about the Use of Animals in Ophthalmic and Vision Research.

Generation of liver metastases

Intravitreal Injection

Intraocular tumor inoculation was performed as described previously (228). Intraocular melanoma that forms after tumor inoculation invades the retina and choroid and causes the formation of liver metastases (115, 229). The mice were deeply anesthetized using an intraperitoneal (i.p.) injection of ketamine hydrochloride (115 mg/kg) (Vetalar; Parke-Davis and Co., Detroit, MI) and xylazine hydrochloride (5.6 mg/kg). Additionally, the eye was desensitized with a drop of 0.5% proparacaine hydrochloride. Intravitreal injections were performed with a sterile 30-gauge needle and a 0.1-ml Hamilton syringe (Hamilton, Whittier, CA) at the

corneoscleral junction. Two microliter of a mono-cellular suspension of B16LS9 tumor cells (4 x 10^5 cells/2 μl) was inoculated into the vitreous cavity. Eyes were examined three times per week, and the tumor volume was measured using calipers. Tumor-bearing eyes were enucleated when the eyes reached 4 mm in diameter. After enucleation, the upper and lower eyelids were sutured together using a 7-0 nylon suture (Ethicon, Somerville NJ). Mice received buprenorphine (0.05/kg) as an analgesic after the procedure was done. The mice were euthanized two weeks after enucleation and their livers were collected for histological analysis.

Intrasplenic Injection

Intrasplenic tumor cell injection is an alternative method to produce liver metastases by facilitating the dissemination of tumor cells to the liver via the splenic portal route (230, 231). The mice were anesthetized as mentioned previously. A small left abdominal flank incision was created, and the spleen was exteriorized. Melanoma cells ($5x10^4$/50ul HBSS) were injected beneath the spleen capsule slowly and carefully to avoid tumor leakage using a 30-gauge needle. The spleen was returned to the abdomen, and the wound was closed with wound clips. Mice were euthanized 14 days later and their livers collected for further analyses.

Subcutaneous injection

B16LS9 melanoma cells ($5x10^4$/50ul HBSS) were injected subcutaneously (SC) in the right flank. Inoculation sites were palpated two times per week to assess SC tumor growth. Tumors were surgically removed when they reached 4 mm in diameter. Mice were euthanized two weeks after tumor removal and the livers were collected for histological analysis and for NK cell-mediated cytotoxicity assays.

Assessment of liver metastases

Liver metastases of B16LS9 melanomas arising from intraocular or SC injections were assessed by histology. The left lobes of 10% formalin-fixed livers were sectioned by a microtome at 100 μm intervals, stained with hematoxylin and eosin (H&E), and examined in a blinded fashion by three independent observers. The results were reported as the mean number of metastases per 10 random low-power fields (10X) objective lens.

Liver metastases arising from intrasplenic B16LS9 melanoma injection were assessed by counting surface tumor nodules on the liver using a dissecting microscope or naked eyes (155). If the number of liver surface nodules exceeded 150, then the number of metastases was considered as ≥ 150.

Splenectomy

The mice underwent splenectomy 5 minutes after intraocular tumor injection, as described previously (7). Briefly, the mice were anesthetized and their spleens were exteriorized through an incision in the abdominal wall. The splenic pedicle was cut with scissors. Hemorrhage was arrested by tamponade of the splenic blood vessels. Wounds were closed with stainless-steel wound clips. Splenectomy procedures were only used in experiments in which we assessed the role of spleen in the production of IL-10.

Isolation of liver leukocytes

After euthanizing the mice, livers were perfused through the hepatic portal vein with 5 ml HBSS containing 2% FCS (FBS, Hyclone, Logan, UT) and 10 U/ml heparin (MP Biomedicals, Solon, OH). Livers were cut into small pieces around 1 mm^3 in size and put into a Stomacher strainer bag (Seward, Bohemia, NY), along with 20 ml of cold HBSS containing 2% fetal bovine serum and 10 U/ml heparin. The tissue was disturbed using a stomacher 80 for 2 minutes

(Seward, Bohemia, NY) to obtain a homogenous single cell suspension. The cells were collected and centrifuged at 800*g* for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was suspended in 40% percoll (GE Health care, Pittsburgh, PA) and centrifuged at 800*g* for 30 minutes at room temperature. The supernatant was discarded and the cell pellet re-suspended in the hemolytic agent- ACT- at room temperature for 5 minutes. Leukocytes were washed twice with HBSS for 5 minutes at 800*g* at 4 °C.

NK cell isolation

Liver NK cells were enriched by using the EasySep Mouse NK Cell Enrichment Kit (Stemcell technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, the mouse CD49b positive selection kit isolates $CD49b^+$ cells from single cell suspensions using an anti-CD49b antibody which binds to the CD49b expressed on the surface of NK cells. Antibody-bound cells are retained in the column by magnetic particles present on the captured antibody, and unwanted cells are eluted. NK cells were also isolated by cell sorting using a FACSAria II (BD Biosciences). NK cells were defined as $NK1.1^+$ TCR-β^- populations using anti-mouse PE-NK1.1 and APC-TCR-β (BD Biosciences, San Jose, CA). The purity of sorted NK cells was consistently ≥ 95%.

NKT cell isolation

Liver NKT cells were isolated as described elsewhere (232). Briefly, the isolated leukocytes were washed twice with HBSS for 5 minutes at 800*g* at 4 °C. Liver NKT cells were then enriched by cell sorting using a FACSAria II (BD Biosciences). NKT cells were defined either as $NK1.1^+$ TCR-β^+ populations using anti-mouse PE- NK1.1 and APC-TCR-β antibodies

(BD Biosciences, San Jose, CA) or as $CD1d^{+}$ $CD3^{+}$ $CD19^{-}$ population using anti-mouse PE-CD19, FITC -CD3 (BD Biosciences, San Jose, CA) and APC-CD1d tetramer (NIH tetramer core facility, Atlanta, GA) . The purity of sorted NKT cells was consistently ≥ 95%.

***In vitro* NK cell cytotoxicity assay**

Splenic and liver cells were isolated and enriched for NK cells as mentioned above. Purified NK cells were co-cultured with B16LS9 cells using an effector to target cell ratio of 25:1 for 18 hours, and cytotoxicity was assessed with a CytoTox 96 NonRadioactive Cytotoxicity Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured in a colorimetric coupled enzymatic assay in which the amount of color formed is proportional to the number of lysed cells. The wavelength absorbance data at 490 nm were collected using a standard 96-well plate reader (Biotek Instrument, Winooski, VT).

***In vitro* NK cell suppression assay**

NK cells were collected from spleens or livers of the naïve non-tumor-bearing WT mice. NKT cells were collected from livers of both naïve non-tumor-bearing and B16LS9 melanoma-bearing WT mice. The ability of NKT cells to directly suppress NK cell-mediated cytotoxicity by producing soluble factors was determined by a transwell system (Costar Corning, Tewksbury, MA) with a 0.4um pore size which was placed in a 48-well microtiter plate. NKT cells were activated with 10ug/ml anti-CD3 and 10ug/ml anti-CD28 (BD Biosciences, San Jose, CA) and placed in the upper chamber of the transwell. NK cells were placed in the lower chamber using several different NKT cell to NK cell ratios (1:5, 2:5, 1:2 and 1:1). Both types of cells were co-cultured for 24 hours in the RPMI 1640 supplemented with 10% FBS and IL-2 (10 ng/mL). The

cytotoxicity of NK cells was measured against B16LS9 melanoma cells via LDH assay, using an effector to target cell ratio of 25:1. The ability of NKT cells to directly suppress NK cell-mediated cytotoxicity in a contact dependent manner was determined with a similar experimental design but without using the transwell system. Briefly, 1 x 10^4 B16LS9 melanoma cells were incubated in 48-well plates with NK cells and either naïve or melanoma-induced liver NKT cells for 24 hours at different NKT cell to NK cell ratios. The cytotoxicity of NK cells was measured against B16LS9 via LDH assay.

Generation of bone marrow chimera mice

C57BL/6 WT mice were given a lethal dose of gamma-irradiation (900 Gy) and reconstituted with bone marrow cells from the femurs and tibias of either C57BL/6 WT or C57BL/6 IL-$10^{-/-}$ mice. Bone marrow cells (1×10^6/100ul HBSS) from donor mice were injected intravenously (i.v.) into each recipient mouse. Mice were kept on drinking water supplemented with 2 mg/ml of neomycin sulfate (Durvet, Blue Spring, MO) for the duration of the experiments. Chimeric animals were allowed to recover for eight weeks before the injection of B16LS9 tumor as described above.

Antibody treatment

To inactivate NKT cells, mice were injected i.p. with 200 µg of anti-CD1d antibody (ATCC, Clone HB323) three times per week starting one week prior to tumor inoculation and continuing for the duration of the experiment. To neutralize IFN-γ *in vivo*, mice were injected i.p. twice a week with 500µg of anti-IFN-γ antibody (ATCC, Clone HB170) starting on the day of tumor injection (233). To deplete myeloid derived suppressor cells (MDSCs) *in vivo*, mice were injected i.p. every three days with 250 µg/mouse of anti-Gr1 antibody starting on the day of tumor injection (UCSF, Clone RB6-8C5) (234). An equivalent amount of normal rat IgG isotype

control (Sigma-Aldrich St. Louis, MO) was administered as an isotype control. To neutralize IFN-γ *in vitro*, NK cells were incubated with 20μg/ml of anti-mouse IFN-γ antibody or isotype control (Biolegend, San Diego, CA) (235). To neutralize TRAIL *in vitro*, NK cells were incubated with 50ng/ml of anti-mouse TRAIL antibody or isotype control (Abcam, Cambridge, MA)(236, 237).

Flow cytometry analysis

Cells were stained by standard protocols using the following antibodies: Anti-mouse PE-NK1.1 , APC-TCR-β, PE-CD95 and PE-anti DR5, FITC-IL-10, PE-CD11b, APC-Streptavidin, PE-CD45 and FITC-NKG2D (BD Biosciences, San Jose, CA), anti-mouse APC-perforin, APC-TRAIL, APC-FasL (eBioscience, San Diego, CA), Biotin anti-mouse CD210 (IL-10RA), FITC-GR1 (Biolegend, San Diego, CA) and isotype control monoclonal antibodies. For assessing the expression of CD1d molecule on B16LS9 melanoma cells, tumor cells were incubated with primary anti-mouse CD1d antibody (ATCC, Clone HB323) for 30 minutes at 4°C. The cells were washed three times and re-suspended in FACS buffer solution containing fluorochrome-conjugated secondary antibody, Alexa fluor 488 goat anti-Rat IgG (Invitrogen, Grand Island, NY) for 30 minutes at 4°C. Cells were washed twice and analyzed by flow cytometry on a FACScan (BD Biosciences).

For intracellular cytokine staining, harvested cells were stimulated with PMA (50 ng/μl) and ionomycin (1 μM) (Sigma-Aldrich St. Louis, MO) for 4 hours in the presence of Brefeldin A (5ug/mL) (Sigma-Aldrich, St. Louis, MO) at 37°C. The cells were re-suspended in 100μl PBS with 1% FCS and incubated with 1μg BD Fc Block CD16/CD32 monoclonal antibody (BD Biosciences, San Jose, CA) at 4°C for 15 minutes. Cells were washed twice and intracellular

cytokine staining performed according to the manufacturer's recommendations (BD Biosciences, San Jose, CA), and analyzed by flow cytometry on a FACScan (BD Biosciences). The results were analyzed with allied software (CellQuest ver. 3.1f; BD Biosciences).

Serum sample collection

Mice were bled via brachial artery incision and blood was taken from animals under anesthesia as described previously (238). The mice were then sacrificed by cervical dislocation without recovering consciousness. Blood was allowed to clot at room temperature for 30 minutes followed by centrifugation for 30 minutes at 12,000 *g* at room temperature. At least 250 µl of serum was collected from each mouse and stored at -20°C. The serum from each mouse was aliquoted to avoid multiple freeze/thaw cycles. The concentrations of IL-10 in serum were measured by enzyme-linked immunosorbant assay (ELISA) as recommended by the manufacturer (R&D Systems, Minneapolis, Minn.)

Cytokine quantification by ELISA

Isolated liver NK cells from WT or $CD1d^{-/-}$ mice were cultured in triplicate in 96-well U-bottom plates in 200 µl of complete RPMI. The cells were either left untreated or stimulated with PMA (50 ng/µl) and ionomycin (1 µM) (Sigma-Aldrich St. Louis, MO) for 24 hours. Purified NKT cells from naïve and tumor-bearing WT mice were cultured in complete RPMI containing 10ug/ml anti-CD3, 10ug/ml anti-CD28 (BD Biosciences, San Jose, CA) for 48 hours (55). Supernatants from these cultures were assessed by sandwich ELISA for mouse IL-10, IFN-γ or TNF-α (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Cytokines levels were quantified by comparison to standards supplied by the manufacturer.

Real-Time PCR

Total RNA was extracted from harvested cells using an RNA isolation kit (RNeasy Mini Kit) purchased from Qiagen (Valencia, CA). The quality (260/280 ratio) and the concentration of RNA was evaluated using spectrophotometry. Briefly, 0.01-1.0 μg of total RNA was converted into first-strand cDNA using the Qiagen RT^2 First Strand Kit (Valencia, CA) according to the manufacturer's conditions. The PCR amplification reactions contained 1.0 μl of first-strand cDNA mixed with 12.5 μl of RT^2 qPCR Master Mix Qiagen (Valencia , CA), 10.5 μl ddH_2O, and RT^2 qPCR primers (Qiagen, Valencia , CA) in a final reaction volume of 25 μl. All reactions were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and performed in duplicate. Primers used in real-time RT-PCR that are specific for mouse IFN-γ (PPM03121A), TNF-α (PPM03113G), IL-10 (PPM03017B), IL-10RA (PPM03031E), perforin (PPM34456B), FasL (PPM02926E), TRAIL (PPM02925B), MIF (PPM02985G), TGF-β (PPM02992A) and mouse GAPDH (PPM02946E-200) were purchased from Qiagen (Valencia, CA). Real-time RT-PCR amplifications were performed on an RT-PCR detection system (iCycler MyIQ; Bio-Rad, Hercules, CA) according to a standard protocol (95°C, 10 minutes, followed by 40 cycles of: 95°C, 15 seconds and 60°C, 1 minute). We used the comparative Ct method to compute relative expression values according to the manufacturer's instructions Qiagen (Valencia, CA).

Statistical analyses

The Student's *t*-test or Rank-Sum *t*-test was used to assess the statistical significance of the differences between experimental and control groups (sigma-plot). A *P*-value of < 0.05 was considered significant. Results are shown with the mean +/- the standard error.

Chapter 3

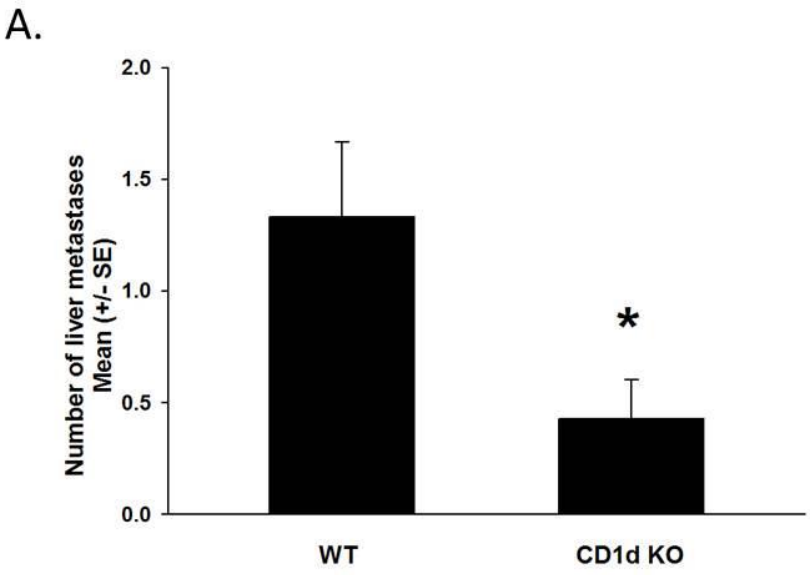
Results

I. The effect of NKT cells on the formation of liver metastases

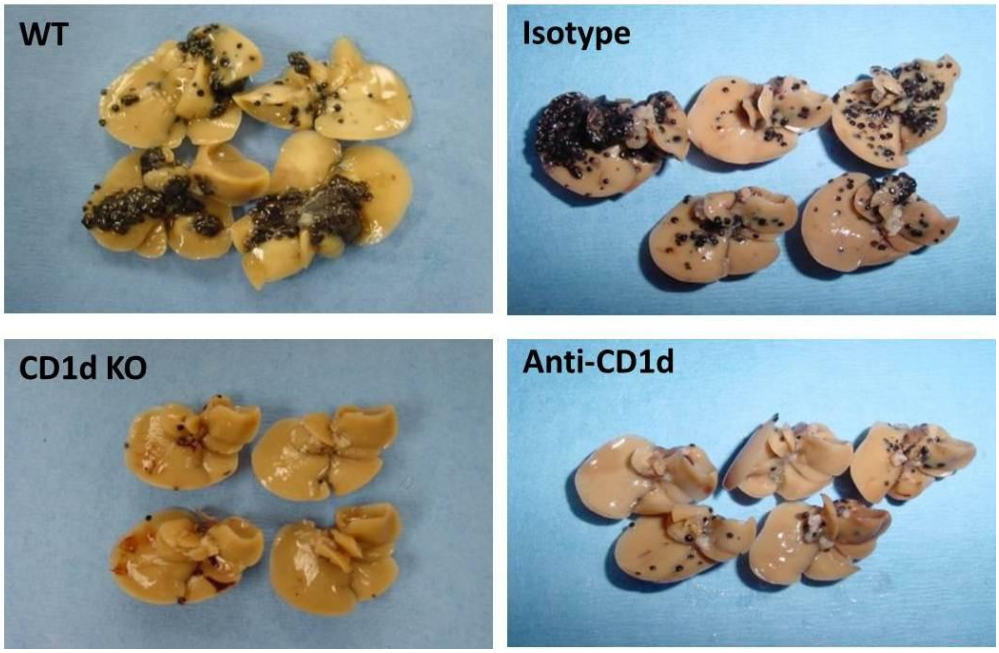
NKT cell-deficient mice have reduced numbers of liver metastases.

To examine the role of NKT cells in the formation of liver metastases arising from intraocular tumors, we injected B16LS9 melanoma cells into the vitreous cavities of C57BL/6 WT and CD1d$^{-/-}$ mice. Tumor-containing eyes were removed when they reached 4mm in diameter. Two weeks after enucleation, mice were euthanatized and livers were processed for histological analysis. The number of micro-metastases in the livers was determined by staining the liver samples with H&E. The results of multiple experiments revealed that NKT cell-deficient mice developed significantly fewer liver metastases than WT mice (**Figure 3A**). In order to facilitate the formation of liver metastases, we used an established intrasplenic tumor injection model for producing liver metastases (231). Unlike intravitreal injection, when tumor cells were injected intrasplenically, large metastatic colonies were easily observed on the liver surface, which could be discerned either with the naked eye or a dissecting microscope (**Figure 3B and 3C**). The results indicated that regardless of the route of tumor inoculation, NKT cell-deficient mice formed less liver metastases than WT mice. Mice treated with anti-CD1d antibody also showed reduced numbers of liver metastases compared to mice treated with the isotype control antibody (**Figures 3C**). Therefore, depletion of NKT cells, either by gene deletion or *in vivo* treatment with antibody, results in a steep reduction in the formation of liver metastases arising from either intraocular tumors or via intrasplenic injections.

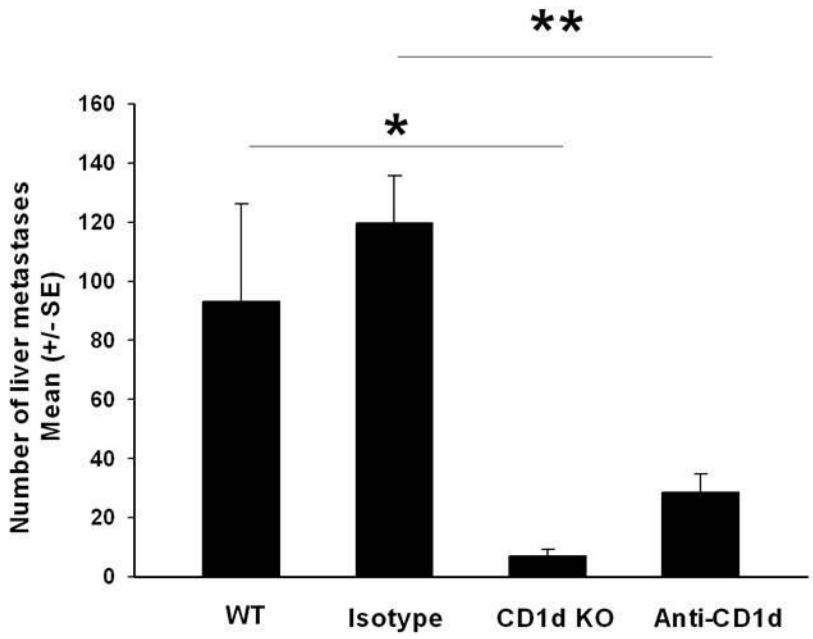
Figure 3. NKT cells promote the formation of liver metastases.(A) Number of micro-metastases, determined by H&E staining, in the livers of B16LS9 melanoma-bearing WT or CD1d$^{-/-}$ mice three weeks after intravitreal tumor cell injection. (B) Surface liver metastases in WT and NKT cell-deficient mice two weeks after intrasplenic tumor injection. (C) Number of surface liver metastases in WT and NKT cell-deficient mice or anti-CD1d antibody or rat IgG isotype-treated mice two weeks after intrasplenic tumor injection. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). * P<0.05 or **P<0.01.



B.


WT
Isotype
CD1d KO
Anti-CD1d

C.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*
Number of liver metastases
Mean (+/- SE)
160
140
120
100
80
60
40
20
0
WT
Isotype
CD1d KO
Anti-CD1d

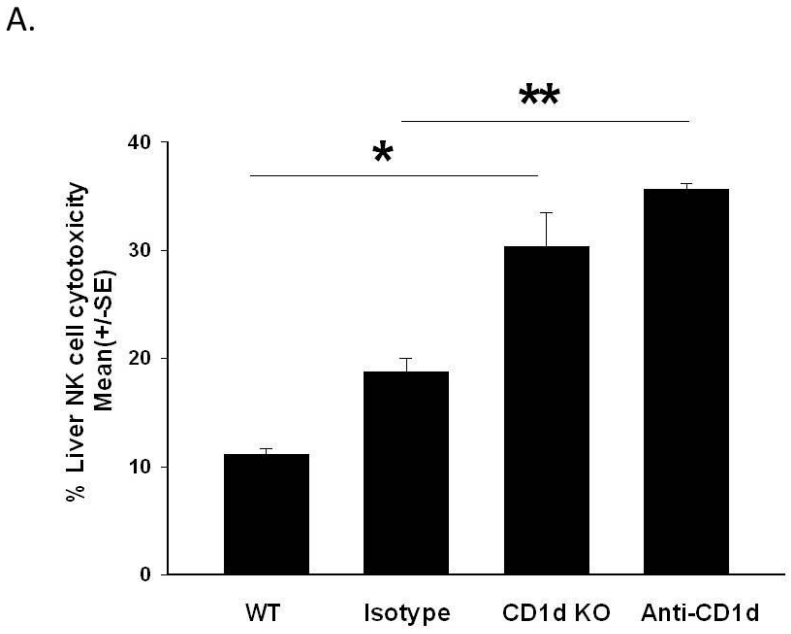
Liver NK cell-mediated cytotoxicity is elevated in NKT cell-deficient mice.

The liver, in both humans and mice, is enriched with NK cells (239). NK cells participate in anti-tumor immune responses and contribute to the resistance to the formation of liver metastases in various cancers including uveal melanoma (5, 151). The importance of NK cells in resistance against uveal melanoma has been shown by NK cell-depleting antibodies (115, 116). NK cell-depleted mice showed a significant increase in the number of liver metastases compared with isotype control antibody-treated mice. Based on these data, we hypothesized that NKT cells promote the development of liver metastases by suppressing NK cell anti-tumor immune responses. To test this hypothesis, WT, $CD1d^{-/-}$, and anti-CD1d-treated or isotype-treated WT mice were injected with B16LS9 melanoma cells in the vitreous compartment of the eye. As mentioned previously, eyes were enucleated when they reached 4mm in diameter. Two weeks later, liver NK cells were isolated and the cytotoxicity of liver NK cells was assessed against B16LS9 melanoma cells (**Figure 4A**). We also tested the cytotoxicity of liver NK cells in WT and $CD1d^{-/-}$ mice or anti-CD1d-treated and isotype control antibody-treated WT mice following intrasplenic injection of B16LS9 melanoma cells (**Figure 4B**). Regardless of the route of tumor inoculation, liver NK cells from NKT cell-deficient mice had elevated cytotoxicity against B16LS9 melanoma cells compared to WT mice with an intact NK cell repertoire (**Figures 4A and 4B**). The results indicated that melanoma-induced liver NKT cells suppress NK cell-mediated cytotoxicity in the liver.

Next, we tested whether the increase in the cytotoxicity of liver NK cells in NKT cell-deficient mice was simply due to an increase in the number of liver NK cells in $CD1d^{-/-}$ mice. WT and $CD1d^{-/-}$ mice were injected intrasplenically with B16LS9 cells. After two weeks, mice were necropsied and liver leukocytes were isolated. The number of NK cells was determined by

flow cytometry and revealed that there was no significant difference in the number liver NK cells in tumor-bearing WT and CD1d$^{-/-}$ mice (**Figure 5**). These results indicated that NK cells in NKT cell-deficient mice have more cytolytic activity on a per cell basis and NKT cells suppress NK cell-mediated cytotoxicity in tumor-bearing mice.

Figure 4. NKT cells suppress NK cell cytotoxicity in tumor-bearing mice. (A) Cytotoxicity of liver NK cells against B16LS9 melanoma cells in mice harboring liver metastases arising from intravitreal tumor inoculation. (B) Cytotoxicity of liver NK cells against B16LS9 melanoma cells in mice harboring liver metastases arising from intrasplenic tumor inoculation. Freshly isolated liver NK cells were incubated with B16LS9 melanoma cells at an E:T ratio of 25:1 for 18 hours in a non-radioactive cytotoxicity assay. Results are expressed as the Mean +/- SE and are representative of three independent experiments (N=10). *P<0.05 or **P<0.01.



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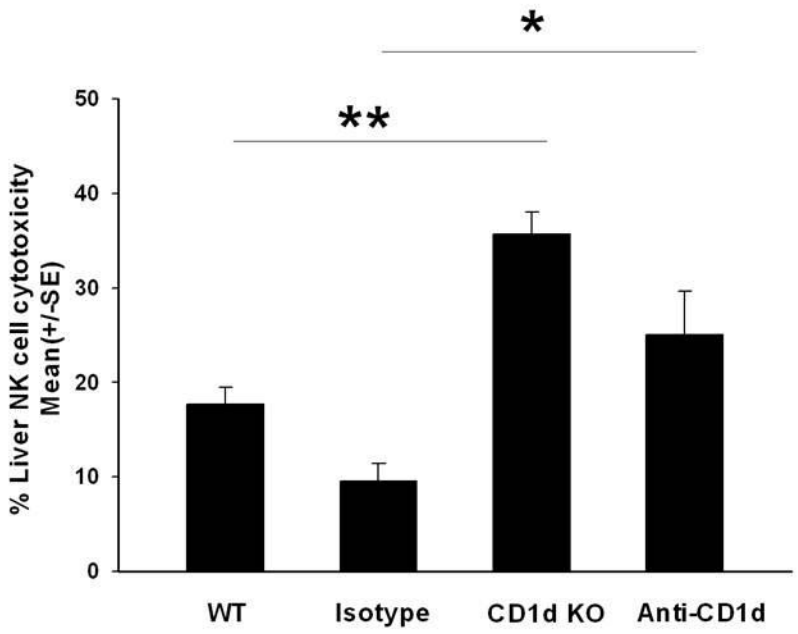
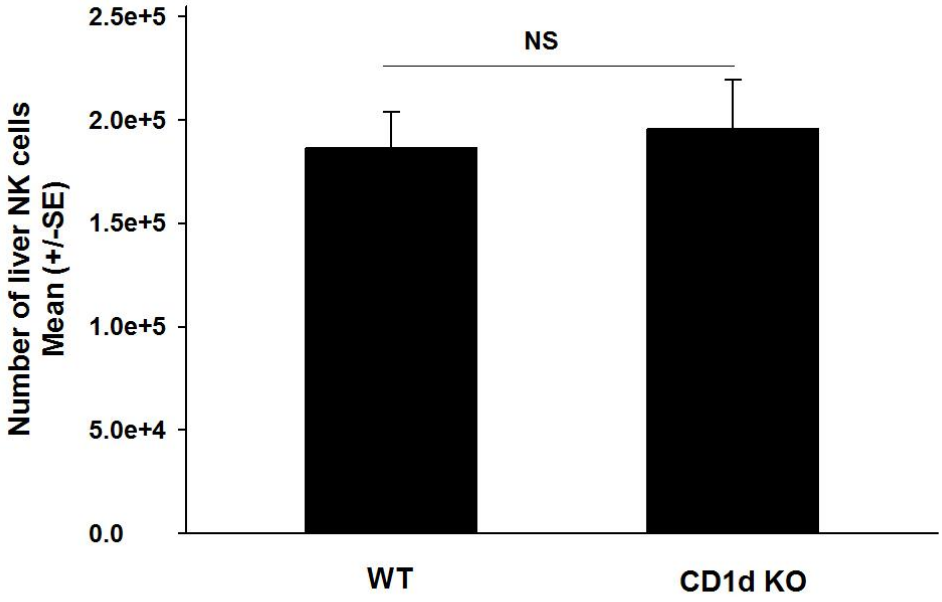

*
**
50
40
30
20
10
0
% Liver NK cell cytotoxicity
Mean(+/-SE)
WT
Isotype
CD1d KO
Anti-CD1d

Figure 5. Similar numbers of liver NK cells in melanoma-bearing WT and NKT cell deficient mice. NK cells were isolated from the livers of tumor-bearing WT and NKT cell deficient mice using the EasySep Mouse NK Cell Enrichment Kit. The number of NK cells was determined by flow cytometry. NK cells were defined as $NK1.1^{+}$ $TCR\beta^{-}$ cells. Results are expressed as the Mean +/- SE and are representative of three independent experiments. NS= Not significant.



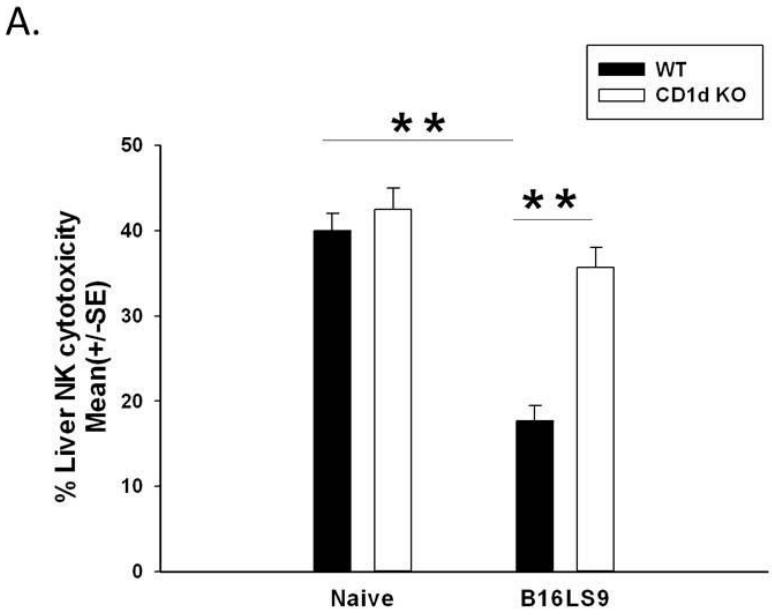
NKT cell-dependent suppression of liver NK anti-tumor function depends on the presence of tumor in the liver

We next investigated whether enhanced liver NK cell-mediated cytotoxicity in tumor-bearing CD1d$^{-/-}$ mice depended on the presence of tumor in the liver or whether NKT cells were capable of suppressing NK cell cytotoxicity in naïve, non-tumor-bearing WT mice. We compared liver NK cell-mediated cytotoxicity of non-tumor-bearing naive WT and CD1d$^{-/-}$ mice. Liver NK cells isolated from these mice showed no difference in cytotoxicity against B16LS9 melanoma cells (**Figure 6A**), implying that the presence of tumor was required for NKT cells to suppress NK cell-mediated cytotoxicity in WT mice. It has been shown that the liver has a unique immunosuppressive micro-environment and is able to induce systemic tolerance (135, 240). We therefore investigated whether the suppression of NK cell cytotoxicity is a liver-specific phenomenon or a systemic effect. WT and CD1d$^{-/-}$ mice were injected intravitreally with B16LS9 melanoma cells. The cytotoxicity of liver and splenic NK cells was compared and revealed that liver NK cell cytotoxicity was suppressed in WT mice compared to NKT cell-deficient mice (**Figure 6A**). However, splenic NK cells in tumor-bearing WT and CD1d$^{-/-}$ mice displayed similar cytotoxicity against B16LS9 melanoma cells (**Figure 6B**).

In other experiments, WT and CD1d$^{-/-}$ mice were injected subcutaneously with B16LS9 melanoma cells. Subcutaneous tumors were removed when they reached 4 mm in diameter. Mice were euthanized 14 days later and liver NK cell-mediated cytotoxicity was assessed. We found no microscopic evidence of liver metastases in either WT mice or CD1d$^{-/-}$ mice. Moreover, the liver NK cell-mediated cytotoxicity in WT mice was not significantly different from that found in CD1d$^{-/-}$ mice (**Figure 6C**). These data suggest that the suppression of liver NKT cell-induced

NK cell-mediated cytotoxicity is unique to hosts with liver metastases and is not a systemic phenomenon related to the growth of melanoma cells at sites other than the liver.

Figure 6. The presence of tumors in the liver is required for the NKT cell-dependent suppression of liver NK cell mediated-cytotoxicity. (A) Cytotoxicity of liver NK cells against B16LS9 melanoma cells in non-tumor-bearing and tumor-bearing WT and NKT cell-deficient mice. Liver metastases were generated via intrasplenic tumor inoculation. (B) Liver and splenic NK cell cytotoxicity against B16LS9 melanoma cells in tumor-bearing WT and NKT cell-deficient mice arising from intravitreal tumor inoculation. (C) Cytotoxicity of liver NK cells in anti-CD1d or isotype treated mice that were injected subcutaneously with B16LS9 melanoma cells. Freshly isolated liver and splenic NK cells were incubated with B16LS9 melanoma cell at an E:T ratio of 25:1 for 18 hours in a non-radioactive cytotoxicity assay. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). **P<0.01 or NS= Not significant.



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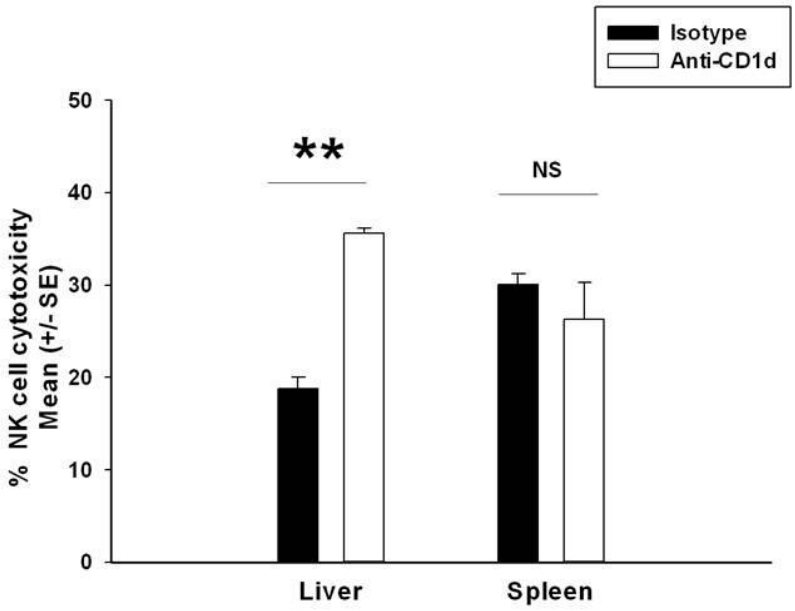
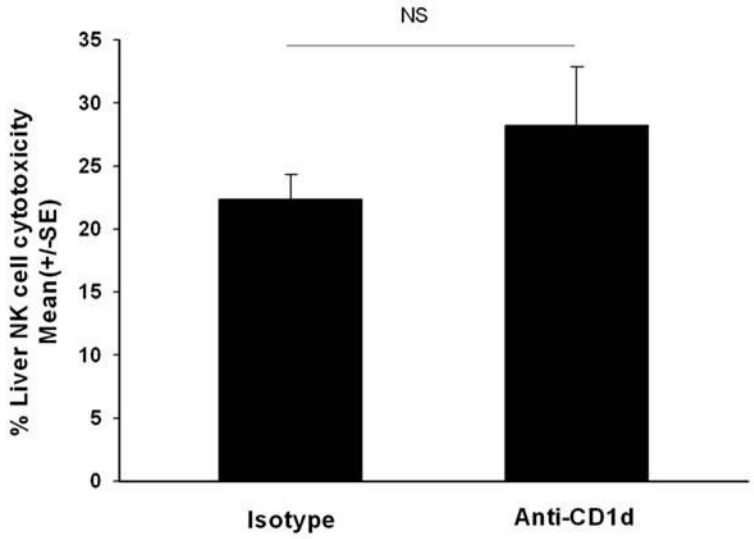

Isotype
Anti-CD1d
% NK cell cytotoxicity Mean (+/- SE)
50
40
30
20
10
0
**
NS
Liver
Spleen

C.


% Liver NK cell cytotoxicity Mean(+/-SE)
35
30
25
20
15
10
5
0
NS
Isotype
Anti-CD1d

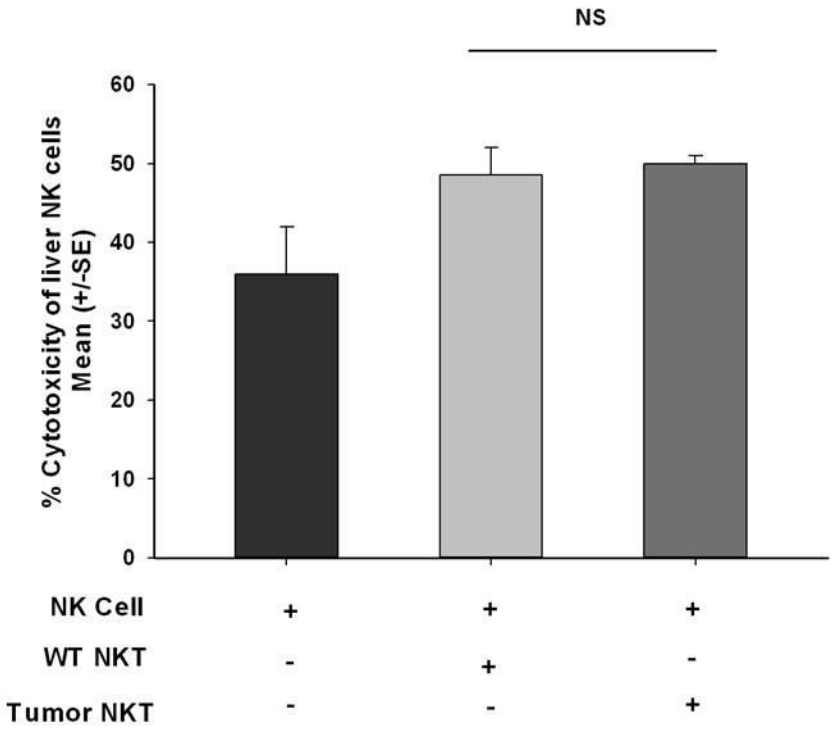
II. The effect of NKT cells on liver NK cell-mediated cytotoxicity

Liver NKT cells are unable to suppress NK cell-mediated cytotoxicity *in vitro*

In a *Chlamydia* infection model, Zheo et al. suggested that NKT cells can have inhibitory effects on the cytolytic function of NK cells (241). In order to elucidate the mechanisms by which NKT cells suppress NK cell-mediated cytotoxicity in B16LS9 melanoma model, we designed an *in vitro* experiment to determine whether melanoma-induced NKT cells suppress NK cell anti-tumor function directly and in a contact-dependent manner or by production of soluble factors. NK cells were collected from either spleens or livers of naïve non-tumor-bearing WT mice. NKT cells were collected from livers of both naïve non-tumor-bearing and B16LS9 melanoma-bearing WT mice. The ability of NKT cells to directly suppress NK cell-mediated cytotoxicity by producing soluble factors was determined by using a transwell culture system. NKT cells were activated with anti-CD3/CD28 and placed in the upper chamber of the transwell and NK cells were placed in the lower chamber using several different NKT cell to NK cell ratios (1:5, 2:5, 1:2 and 1:1). The cells were co-cultured for either 24 or 48 hours. NK cells were then used in a non-radioactive cytotoxicity assay against B16LS9 melanoma cells. Regardless of what permutation was used in the assay, there was no significant difference in NK cell-mediated cytotoxicity when NK cells were incubated with either naïve or melanoma-induced liver NKT cells (**Figure 7**). Based on these experimental approaches, the results suggested that NKT cells were unable to suppress NK cell anti-tumor function through the production of soluble factors.

Figure 7. The soluble factors produced by liver NKT cells are unable to suppress NK cell cytotoxicity. Anti-CD3 and anti-CD28 activated liver NKT cells were placed in the upper chamber of a transwell, and splenic NK cells were placed in the lower chamber of the transwell.

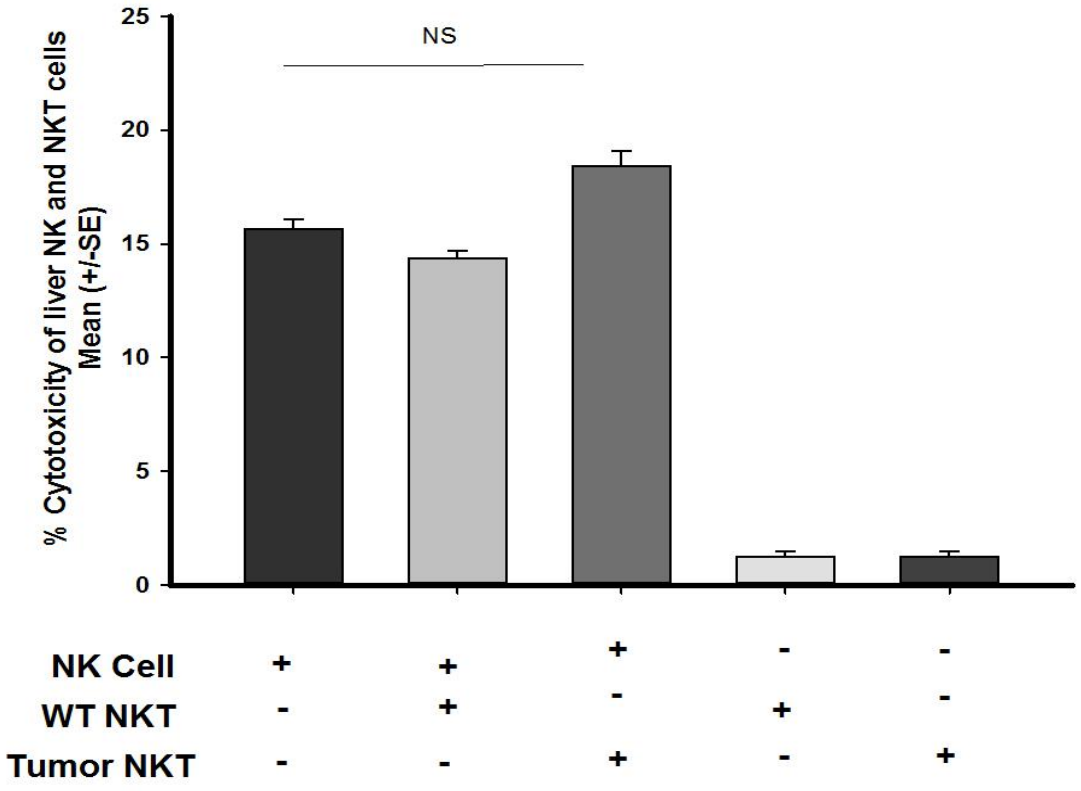
Cells were incubated for 24 hours in the presence of 10 ng/mL of IL-2. The cytotoxicity of NK cells was measured in a non-radioactive cytotoxicity assay against B16LS9 melanoma cells. NKT: NK cell ratio of 1:1. NK: B16LS9 ratio of 25:1. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). NS= Not significant.



The ability of NKT cells to directly suppress NK cell-mediated cytotoxicity in a contact-dependent manner was determined with a similar experimental design but without using the transwell system. Briefly, liver or splenic NK cells were incubated with either naïve or melanoma-induced liver NKT cells at different NKT cell to NK cell ratios for 24 or 48 hours. The cytotoxicity of NK cells was measured in an 18 hour non-radioactive cytotoxicity assay against B16LS9 melanoma cells. Once again, regardless of which permutation was used in these experiments, no inhibition of NK cell anti-tumor function was observed. There were no significant changes in the cytotoxicity of NK cells when they were incubated with target cells alone or with naïve or melanoma-induced liver NKT cells (**Figure 8**). These data indicate that melanoma-induced liver NKT cells were unable to suppress NK cell-mediated cytotoxicity in a contact-dependent manner *in vitro*.

It has been reported that NKT cells can directly lyse tumor cells (176). We therefore tested the cytotoxicity of liver NKT cells against B16LS9 melanoma cells *in vitro* (**Figure 8**). The incubation of liver NKT cells with B16LS9 melanoma cells did not result in the lysis of melanoma cells *in vitro*. Although NKT cells were incubated with NK cells and B16LS9 melanoma cells simultaneously, since the NKT cells were unable to lyse B16LS9 melanoma cells, the percentage cytotoxicity against B16LS9 melanoma cells in figure 8 is merely a result of melanoma cell lysis by NK cells.

Figure 8. Liver NKT cells are unable to suppress NK cell cytotoxicity in a contact-dependent manner. Anti-CD3 and anti-CD28 activated liver NKT cells were incubated with splenic NK cells and B16LS9 melanoma cell for 24 hours in the presence of IL-2. The cytotoxicity of NK and/or NKT cells was measured in a non-radioactive cytotoxicity assay. NKT: NK cell ratio of 1:1. NK: B16LS9 and NKT: B16LS9 ratio of 25:1. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). NS= Not significant.

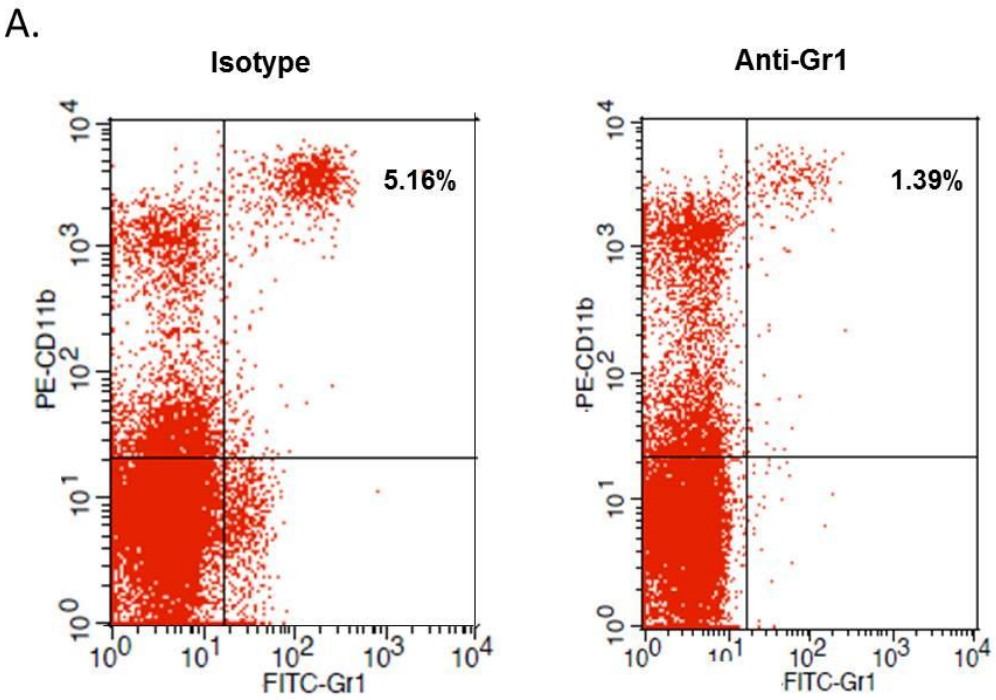


Melanoma induced-NKT cell suppression of NK cells is independent of MDSCs

Since NKT cells were unable to directly suppress NK cell-mediated cytotoxicity *in vitro*, we hypothesized that the presence of a third party cell is required for the NKT cell-induced NK cell suppression. One of the mechanisms that malignant cells use to evade the anti-tumor immune response is through the induction of MDSCs (213). We therefore hypothesized that the presence of MDSCs promotes the formation of liver metastases in B16LS9 melanoma-bearing mice. The number of liver metastases was examined in MDSC-depleted mice and in mice with an intact MDSC repertoire. To deplete MDSCs *in vivo*, mice were injected i.p. every 72 hours with 250 µg/mouse of anti-Gr1 antibody starting on the day of tumor injection (234). Treatment with anti-Gr1 antibody resulted in a 73% reduction in the number of MDSCs in the liver of tumor-bearing WT mice which were identified as $CD11b^+$ $Gr1^+$ cells (**Figure 9A**) (242). Surprisingly, MDSC depletion did not reduce the number of liver metastases but instead enhanced the formation of liver metastases (**Figure 9B and 9C**). These data indicated that $CD11b^+$ $Gr1^+$ cells were unable to promote the formation of liver metastases in melanoma-bearing mice. To investigate the role of other third party cell candidates in the NKT cell-induced NK cell suppression in the ocular melanoma model is beyond the scope of this study and has been addressed elsewhere.

Figure 9. MDSCs are unable to promote the formation of liver metastases. (A) Percentages of MDSCs present in the liver of B16LS9 melanoma-bearing WT mice treated with anti-Gr1 or rat IgG isotype control antibody. Freshly isolated liver leukocytes were stained with PE-CD11b and FITC-Gr1 anti-mouse antibodies and analyzed by flow cytometry. (B) Surface liver metastases in WT mice treated with anti-Gr1 or rat IgG isotype control antibody two weeks after

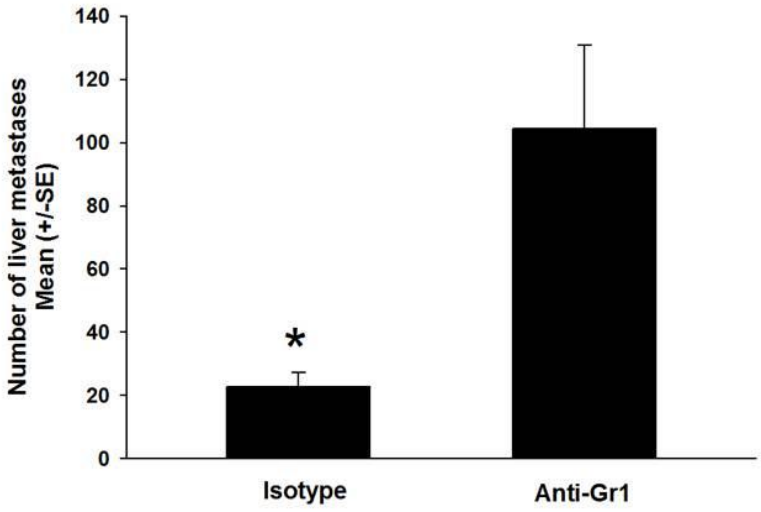
intrasplenic tumor injection. (C) Number of surface liver metastases in WT mice, treated with anti-Gr1 or rat IgG isotype control antibody two weeks after intrasplenic tumor injection. Results are expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). * P<0.05 or NS= Not significant.



B.


Isotype
Anti-Gr1

C.

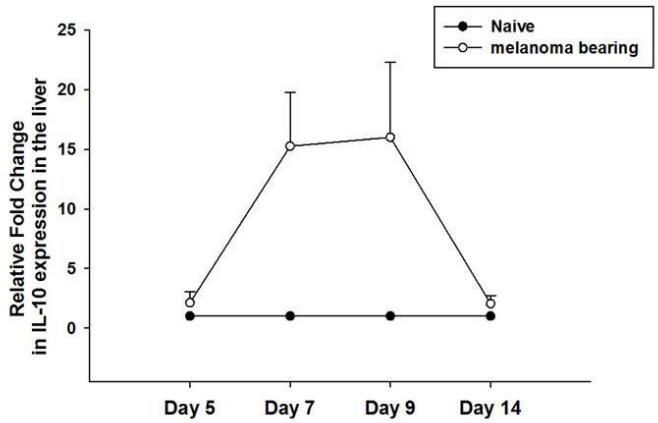

Number of liver metastases
Mean (+/-SE)
140
120
100
80
60
40
20
0
*
Isotype
Anti-Gr1

Elevated expression of IL-10, MIF and TGF-β in melanoma-bearing mice

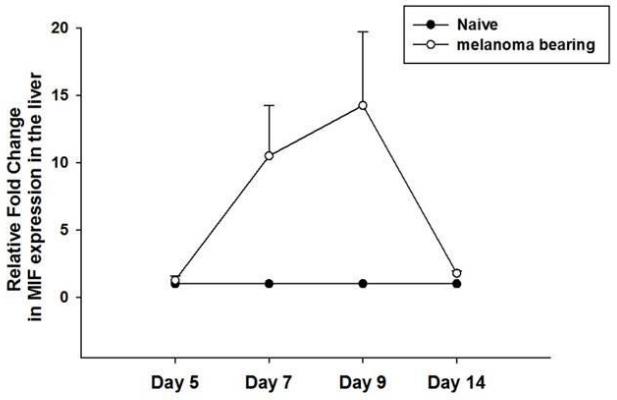
IL-10, MIF and TGF-β are three important cytokines that suppress NK cell-mediated killing (76, 77, 79). Like many tumor models, data from our laboratory indicated that the NK cell cytotoxicity is severely reduced in WT mice harboring B16LS9 melanoma cells in comparison with naïve-non-tumor-bearing mice (**Figure 6A**) (243). Therefore experiments were performed to determine whether the expression of these immunosuppressive cytokines is elevated in B16LS9 melanoma-bearing mice. WT mice were injected with B16LS9 melanoma cells intrasplenically. RNA was extracted from single cell suspension of the liver of naïve, non-tumor-bearing and melanoma-bearing mice on days 5, 7, 9 and 14 post tumor inoculations. mRNA levels of IL-10, MIF and TGF-β were assessed by real-time PCR. The expression of all three cytokines was significantly elevated starting at day 7 and peaked at day 9 after tumor injection (**Figure 10**). The mRNA levels of IL-10, MIF and TGF-β in non-tumor-bearing and melanoma-bearing mice were comparable on day 14 post tumor injection (**Figure 10**). These results suggested that the presence of tumor induces the production of immunoregulatory cytokines in the liver and possibly contributed to impaired NK cell-mediated anti-tumor immune response.

Figure 10. The expression of IL-10, MIF and TGF-β is elevated in tumor-bearing mice. RNA was extracted from the livers of WT non-tumor-bearing and melanoma-bearing mice 5, 7, 9 and 14 days after intrasplenic injection of B16LS9 melanoma cells. mRNA levels assessed by quantitative RT-PCR. (A) IL-10 (B) MIF (C) TGF-β. The results are representative of four independent experiments (N=5).

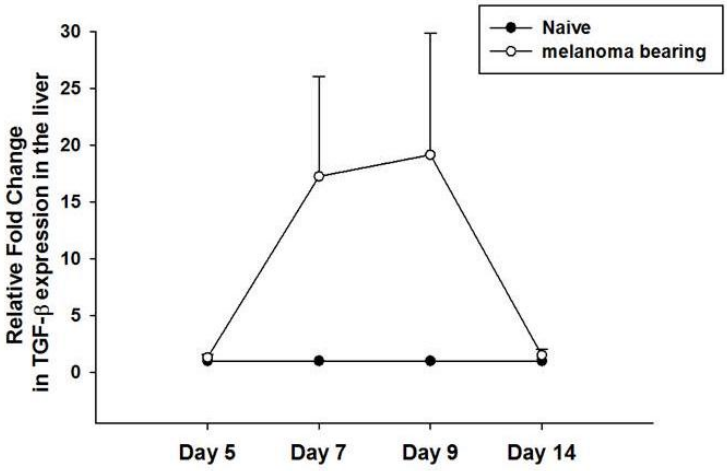
A.


Naive
melanoma bearing
Relative Fold Change
in IL-10 expression in the liver
25
20
15
10
5
0
Day 5
Day 7
Day 9
Day 14

B.


Naive
melanoma bearing
Relative Fold Change
in MIF expression in the liver
20
15
10
5
0
Day 5
Day 7
Day 9
Day 14

C.

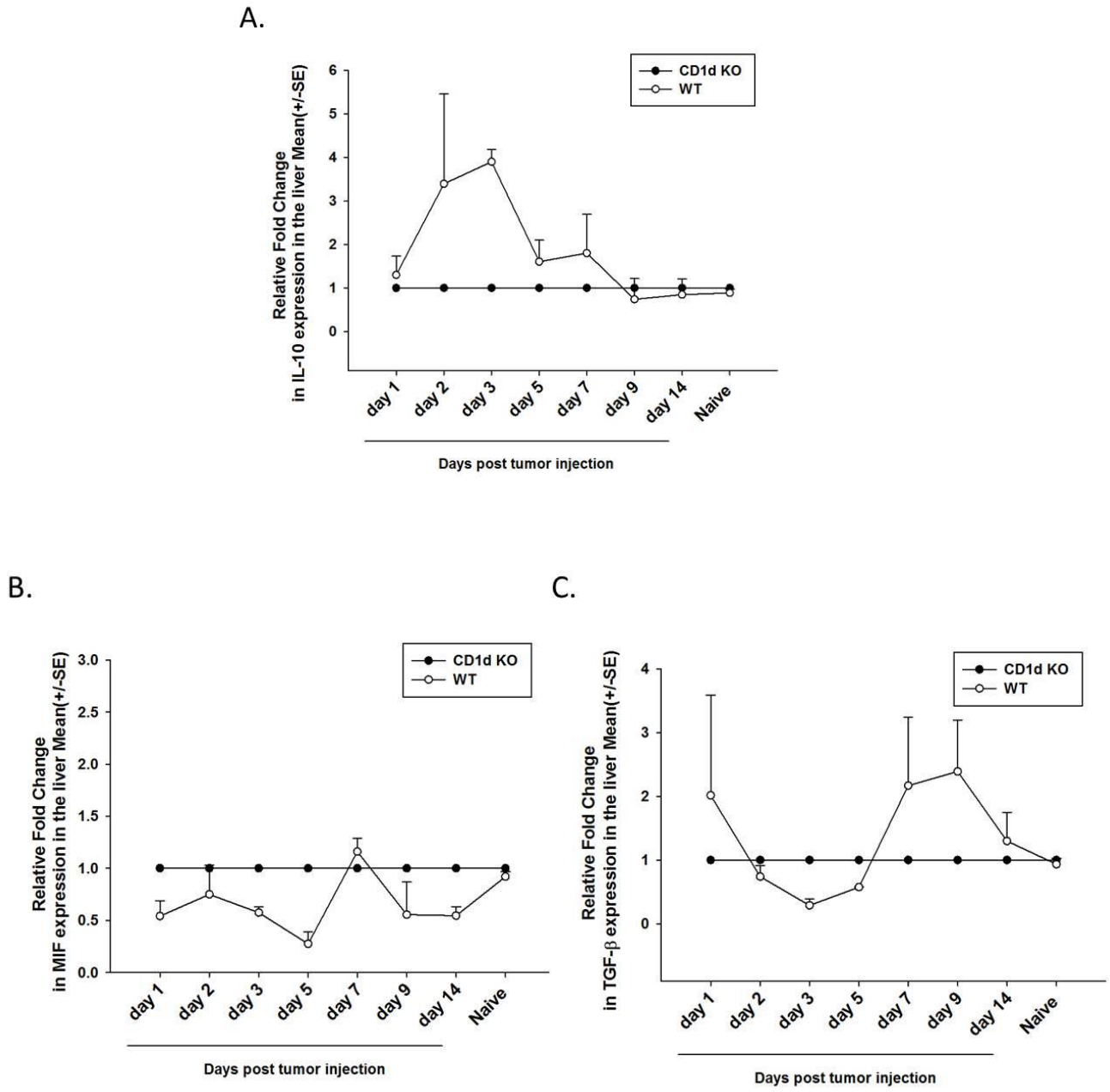

Naive
melanoma bearing
Relative Fold Change
in TGF-β expression in the liver
30
25
20
15
10
5
0
Day 5
Day 7
Day 9
Day 14

Since tumor-bearing WT mice have higher number of liver metastases and reduced NK cell-mediated cytotoxicity compared to NKT cell-deficient mice, we hypothesized that the IL-10, MIF and TGF-β are up-regulated in melanoma-bearing WT mice relative to melanoma-bearing $CD1d^{-/-}$ mice. To address this hypothesis, RNA was extracted from single cell suspensions of livers of naïve-non-tumor-bearing and melanoma-bearing WT and $CD1d^{-/-}$ mice on days 5, 7, 9 and 14 post tumor inoculations. mRNA levels of IL-10, MIF and TGF-β were assessed by real-time PCR. None of the cytokines of interest displayed significant elevation of expression on day 5 and onward after tumor inoculation (**Figure 11**). Slightly higher expression of IL-10 (on days 5 and 7 post injections) and TGF-β (on days 7 and 9 post injections) was observed in WT mice relative to NKT cell-deficient mice (**Figure 11A and 11C**). The expression of MIF was moderately reduced in WT mice on days 5, 9, and 14 post injections (**Figure 11B**).

In a zymosan induced-granuloma model, Kobayashi at al. reported that zymosan treated WT mice have higher expression of IL-10 than NKT cell-deficient mice (244). Interestingly, the enhanced levels of IL-10 in the sera of WT mice were detected hours after zymosan administration but were similar to that in NKT cell-deficient mice only 24 hours after zymosan challenge and remained the same for 28 days (244). Therefore, we sought to determine the expression of IL-10, MIF and TGF-β at earlier time points (days 1, 2 and 3) post tumor inoculation. IL-10 was the only cytokine with significantly increased expression in WT mice in comparison to $CD1d^{-/-}$ mice in the first week post tumor injection (**Figure 11**). These data suggested that IL-10 is potentially a key cytokine in NKT cell-induced NK cells suppression which exerts its immunosuppressive effects during the early phase of liver metastases.

Figure 11. IL-10 expression is elevated in the livers of tumor-bearing mice WT. mice relative to $CD1d^{-/-}$ mice. RNA was extracted from the liver of naïve-non-tumor-bearing and melanoma-

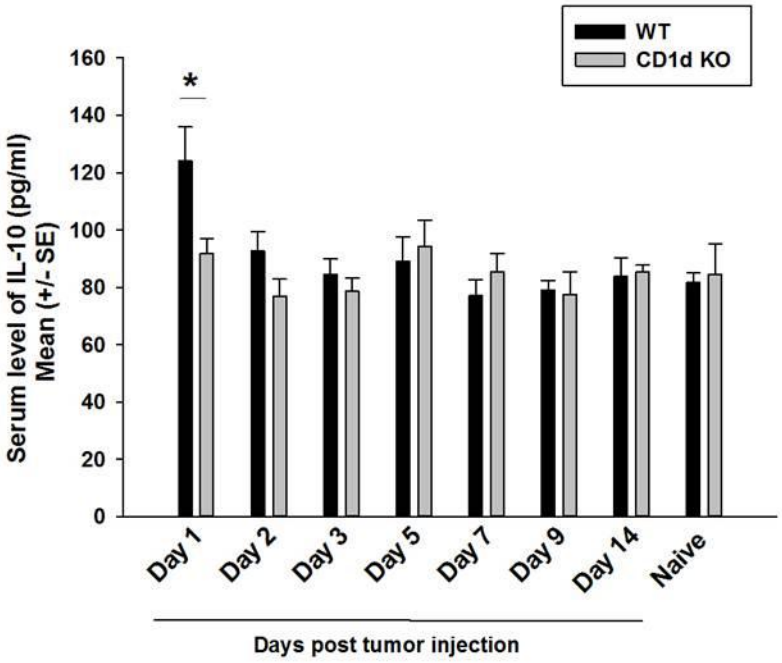
bearing WT and CD1d$^{-/-}$ mice 1, 2, 3, 5, 7, 9 and 14 days after intrasplenic injection of B16LS9 melanoma cells. mRNA levels assessed by quantitative RT-PCR. (A) IL-10 (B) MIF (C) TGF-β. The results are representative of two independent experiments (N=5).



NKT cells are unable to induce the systemic production of IL-10

Since the unique immunological properties of the liver lead to systemic tolerance (151), we hypothesized that there is an increase in the serum levels of IL-10 in melanoma-bearing mice. However, there was no significant difference in the serum levels of IL-10 in naive and melanoma-bearing WT or NKT cell-deficient mice, with the exception of day one post tumor injection (**Figure 12**). This indicated that suppression of liver NK cell-mediated cytotoxicity is not due to systemic IL-10 production, but is most likely due to local effects of IL-10 produced in the liver.

Figure 12. Serum levels of IL-10. Levels of IL-10 in the serum of naïve or B16LS9 melanoma-bearing WT and CD1d$^{-/-}$ mice measured at different time points after intrasplenic injection of B16LS9 melanoma cells. IL-10 was quantified by sandwich ELISA. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=5). *P<0.05.

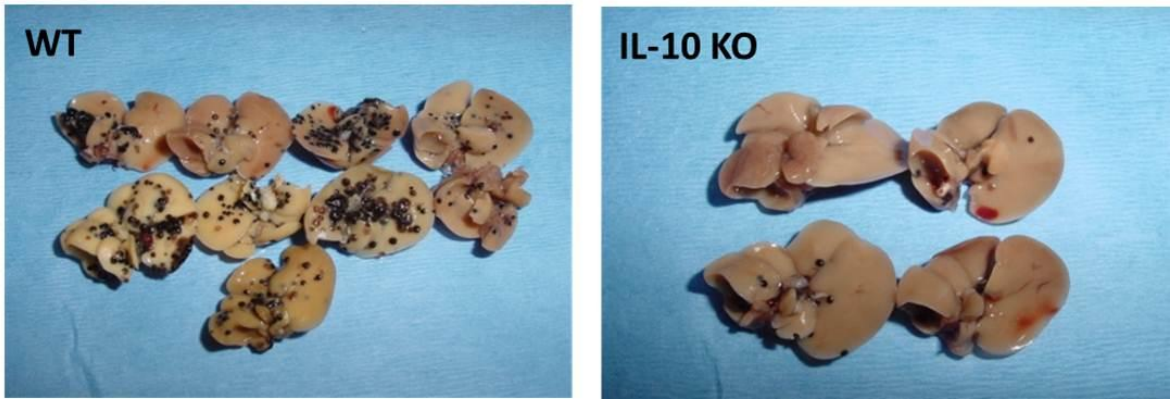


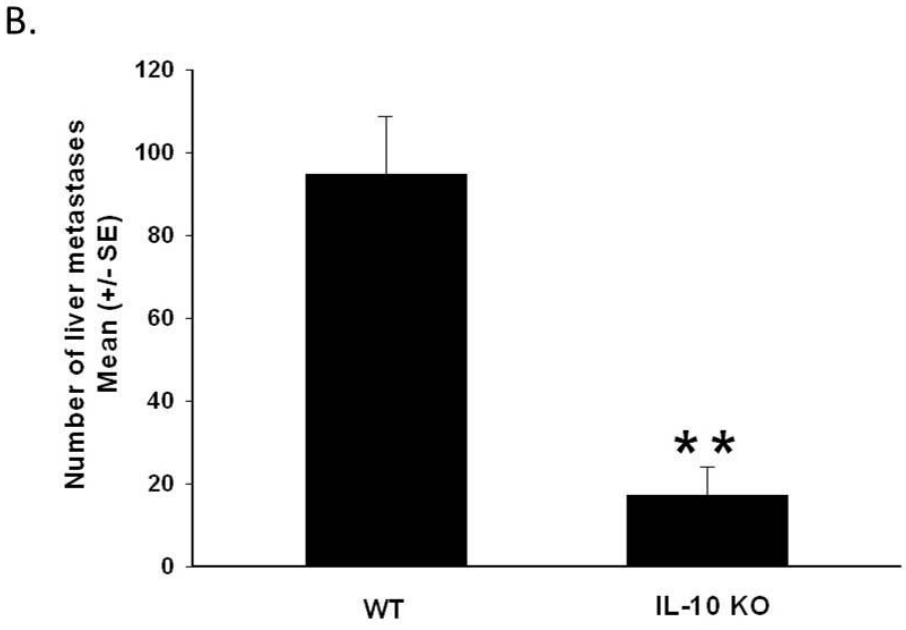
IL-10 promotes the formation of liver metastases

As mentioned previously, IL-10 is recognized as a cytokine that profoundly inhibits a broad range of immune responses (84). Multiple studies have reported that IL-10 expression is up-regulated in various cancers including the B16LS9 intraocular melanoma model (245, 246). We therefore investigated the role of IL-10 in the formation of liver metastases in B16LS9 melanoma-bearing mice. We assessed the number of liver metastases in IL-$10^{-/-}$ and WT mice after intrasplenic tumor injection (**Figures 13A and 13B**). There was a four-fold reduction in the number of liver metastases in IL-$10^{-/-}$ mice in comparison with WT mice, indicating the important role of IL-10 in the suppression of the immune response in melanoma-bearing mice.

Figure 13. IL-10 promotes the exacerbation of liver metastases. (A) Surface liver metastases in WT and IL-$10^{-/-}$ mice injected intrasplenically with B16LS9 melanoma cells. (B) Number of surface liver metastases in WT and IL-$10^{-/-}$ mice injected intrasplenically with B16LS9 melanoma cells. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10 for WT mice and N=5 for IL-$10^{-/-}$ mice). **P<0.01

A.



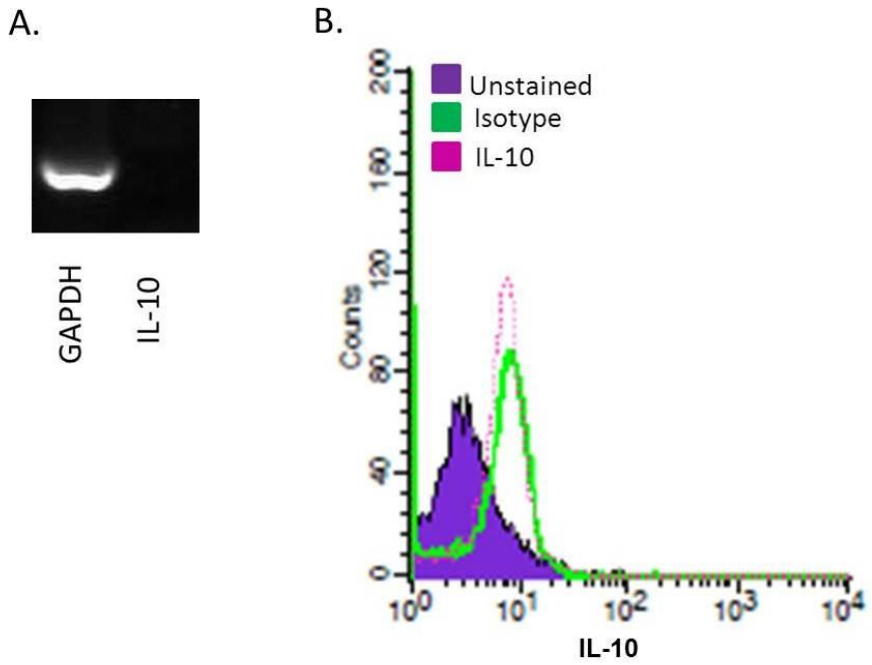


Bone marrow-derived cells are the source of IL-10 production

Since IL-10 plays a substantial role in the formation of liver metastases in B16LS9 melanoma-bearing mice, we sought to determine the major source of IL-10 producing cells. Various tumor cells express IL-10 which further promotes tumor growth and metastases (247). Therefore, the production of IL-10 by B16LS9 melanoma cells was assessed. IL-10 gene and protein expression in B16LS9 melanoma cells were assessed with reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometry analysis, respectively. No IL-10 expression was detected in B19LS9 melanoma (**Figures 14A and 14B)**.

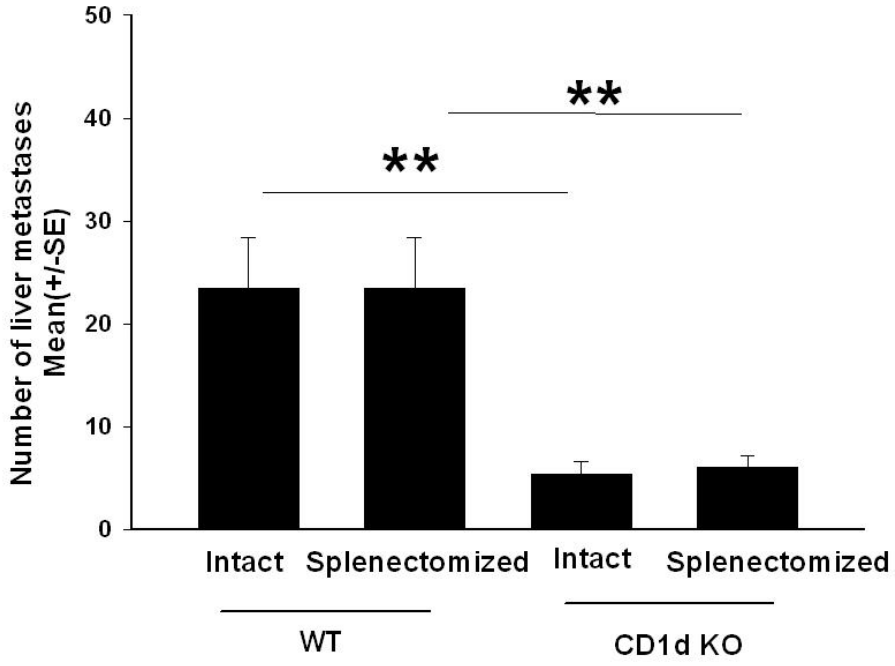
Figure 14. B16LS9 melanoma cells do not express IL-10. (A) Expression of IL-10 mRNA in B16LS9 melanoma cells measured via quantitative RT-PCR. (B). Expression of IL-10 in

B16LS9 melanoma cells followed by staining for IL-10 and interrogation by flow cytometry. The results are representative of two independent experiments.



Given that liver metastases are generated via intrasplenic injections of B19LS9 melanoma cells and tumor nodules form in the spleen, we hypothesized that splenic cells are the source of IL-10 production in melanoma-bearing mice. To address this hypothesis, WT and CD1d$^{-/-}$ mice were splenectomized five minutes after intrasplenic injection of B16LS9 melanoma cells or had their spleen left intact. The absence or presence of the spleen had no impact on the number of liver metastases in WT or CD1d$^{-/-}$ mice (**Figures 15**). These data indicated that the spleen is not the major source of IL-10 in melanoma-bearing mice.

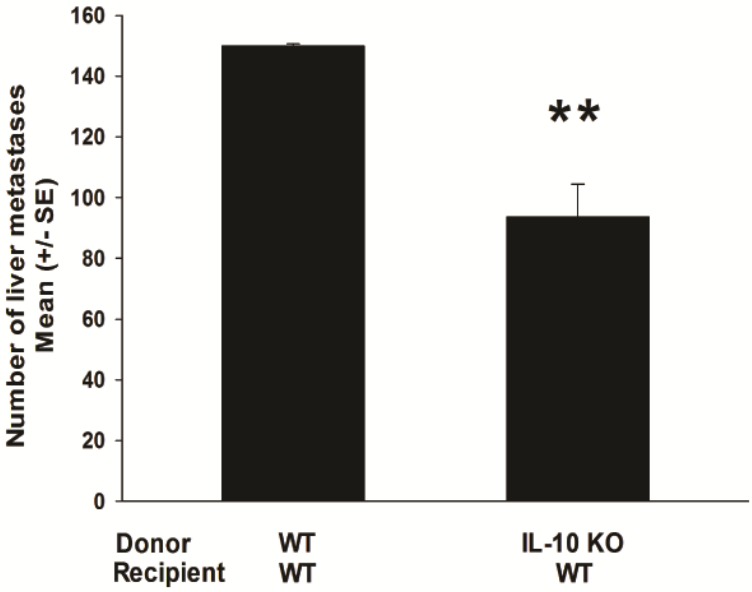
Figure 15. The spleen is not the major source of IL-10 production. Number of surface liver metastases in WT and CD1d$^{-/-}$ mice injected intrasplenically with B16LS9 melanoma cells. Mice were left intact or splenectomized five minutes after intrasplenic injection of B16LS9 melanoma cells. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). ** P<0.01



In order to examine whether IL-10-producing cells were bone marrow-derived cells or from liver parenchymal cells, we generated bone marrow chimeric mice in which bone marrow-derived cells from either IL-10$^{-/-}$ or WT mice were adoptively transferred into irradiated WT recipient mice. Eight weeks post bone marrow reconstitution, mice were injected intrasplenically with B16LS9 melanoma cells and the formation of liver metastases was examined (**Figure 16**). Mice that received bone marrow cells from IL-10$^{-/-}$ mice developed significantly fewer liver

metastases than mice receiving bone marrow cells from WT mice. These results indicated that bone marrow-derived cells are the major source of IL-10 production in the livers of melanoma-bearing mice.

Figure 16. Liver bone marrow-derived cells are the source of IL-10 production. Number of liver metastases in WT or IL-$10^{-/-}$ bone marrow chimeric mice injected intrasplenically with B16LS9 melanoma cells. WT mice were γ-irradiated and reconstituted with bone marrow cells from WT or IL-$10^{-/-}$ mice. Eight weeks after receiving bone marrow cells, recipient mice were injected intrasplenically with B16LS9 melanoma cells. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=15). ** P<0.01

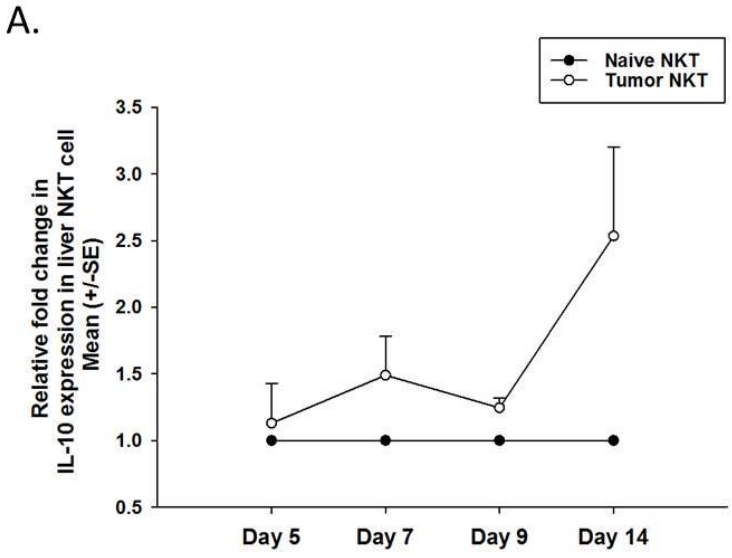


NKT cells are not responsible for the excessive production of IL-10 in the liver

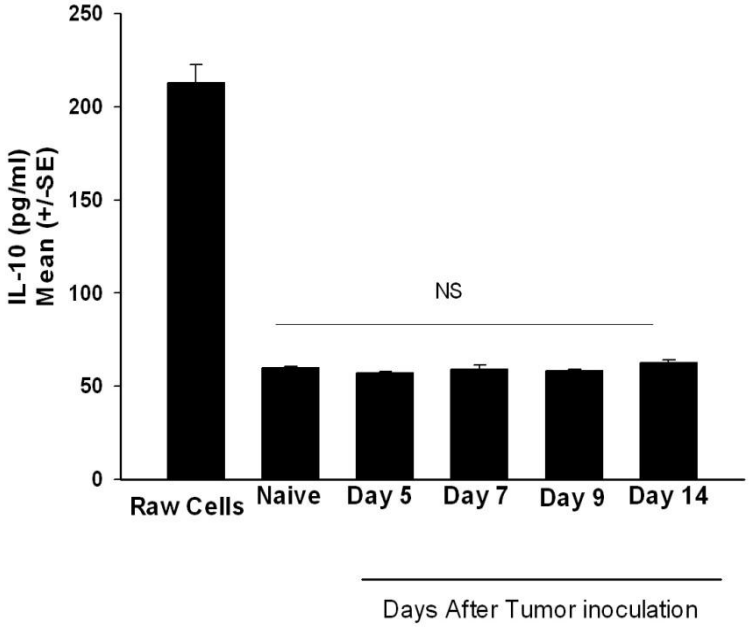
Activated NKT cells are capable of IL-10 production (55, 248) and IL-10 suppresses production of IFN-γ and TNF-α by NK cells (84). As shown in Figure 10, there is elevated expression of IL-10 in the livers of tumor-bearing mice compared to non-tumor-bearing mice. Therefore, we hypothesized that IL-10 produced by tumor-induced NKT cells is responsible for the higher amounts of IL-10 in the livers of melanoma-bearing mice and the suppression of liver NK cells cytotoxicity in these mice. We assessed the production of IL-10 mRNA and protein by liver NKT cells isolated from naïve or melanoma-bearing 5, 7, 9 and 14 days after tumor inoculation. NKT cells were identified as either $NK1.1^+$ $TCR\text{-}\beta^+$ cells or as $CD1d^+CD3^+CD19^-$ cells and sorted by flow cytometry. RNA was extracted from freshly isolated liver NKT cells for quantitative RT-PCR analyses or NKT cells were cultured in the presence anti-CD3/CD28 and supernatant were collected for analyses by ELISA. Liver NKT cells in tumor-bearing and non-tumor-bearing mice produced similar amounts of IL-10 mRNA and protein on days 5, 7 and 9 post tumor injections as compared to naïve mice (**Figures 17A, 17B**). On day 14 post tumor inoculation, there was slightly higher expression of IL-10 in the tumor-induced NKT cells than naïve NKT cells (**Figures 17A, 17B**). Therefore, we assessed the expression of IL-10 by liver NKT cells 14 days after tumor injections by flow cytometry. There was no significant difference in the expression of IL-10 protein between naïve and tumor-induced liver NKT cells (**Figures 17C)**. These data suggest that liver NKT cells are not the source of IL-10 producing cells in melanoma-bearing mice.

Figure 17. The expression of IL-10 in naïve and tumor-induced NKT cells is comparable. (A) IL-10 mRNA expression in liver NKT cells of naïve non-tumor-bearing and B16LS9 melanoma-

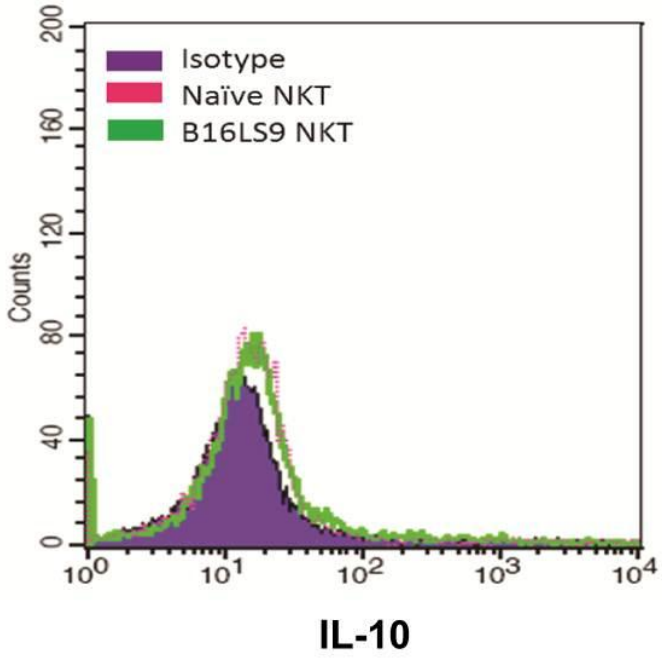
bearing WT mice harvested at different time points following intrasplenic tumor injection. (B) IL-10 production by liver NKT cells which were isolated from naïve non-tumor-bearing and tumor-bearing WT mice at day 5, 7, 9 and 14 after tumor inoculation. Raw cells used as a positive control. Purified NKT cells were cultured in media containing anti-CD3 and anti-CD28 for 48 hours. Supernatants from these cultures were assessed by ELISA for mouse IL-10. (C) Cytoplasmic expression of IL-10 in freshly isolated liver NKT cells from WT and $CD1d^{-/-}$ mice 14 days after intrasplenic injection of B16LS9 melanoma cells. Anti-CD3 and anti-CD28 activated NKT cells were cultured and stimulated with PMA and ionomycin in the presence of Brefeldin A for 4 hours, followed by staining for IL-10 and interrogation by flow cytometry. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). NS= Not significant.



B.


250
200
150
100
50
0
IL-10 (pg/ml)
Mean (+/-SE)
NS
Raw Cells
Naive
Day 5
Day 7
Day 9
Day 14
Days After Tumor inoculation

C.

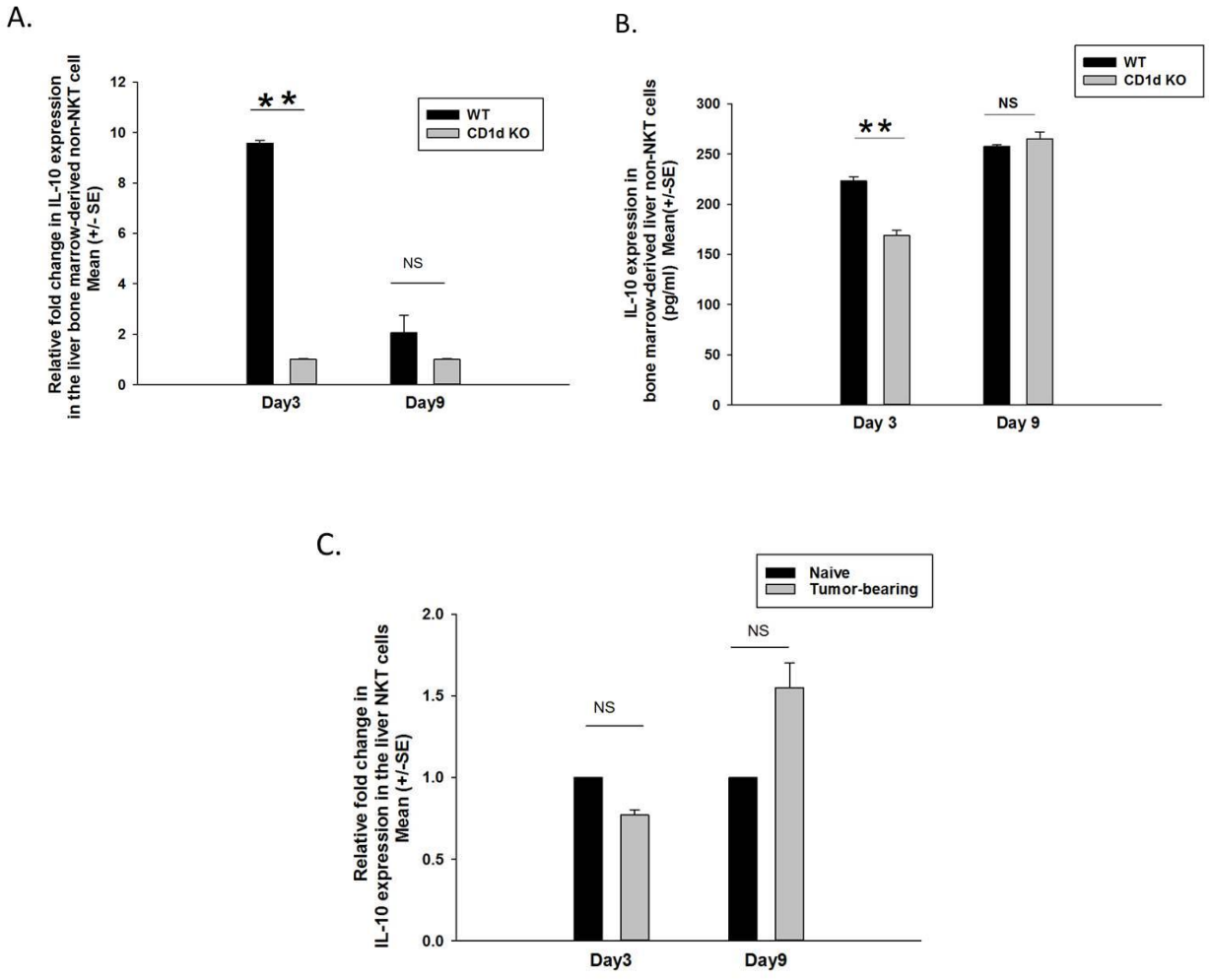

Isotype
Naïve NKT
B16LS9 NKT
Counts
200
160
120
80
40
0
10^0
10^1
10^2
10^3
10^4
IL-10

NKT cells induce the production of IL-10 in bone marrow-derived non-NKT cells

As shown above, it is very unlikely that liver NKT cells are the major source of IL-10 production in melanoma-bearing. Thus, we tested an alternative hypothesis that NKT cells induced the production of IL-10 in other cells in the liver and found that IL-10 is mainly produced by bone marrow-derived cells in B16LS9 melanoma-bearing mice (**Figure 16)**. Therefore we assessed the production of IL-10 mRNA and protein in bone marrow-derived non-NKT cells. Non NKT cells were identified as $CD1d^{-}CD3^{+}CD19^{+}$ cells. Freshly isolated liver non-NKT cells were isolated from melanoma-bearing WT and $CD1d^{-/-}$ mice 3 and 9 day after intrasplenic injection of B16LS9 melanoma cells. IL-10 mRNA levels were assessed by qPCR (**Figure 18A)** and protein levels were detected by ELISA (**Figure 18B)**. As indicated in Figure 11, the maximal and minimal expression of IL-10 in melanoma-bearing WT mice relative to $CD1d^{-/-}$ mice were observed on days 3 and 9 post injection, respectively. We chose these days to assess the expression of IL-10 in liver bone marrow-derived non-NKT cells. As expected, the expression of IL-10 was up-regulated in bone marrow-derived non-NKT cells and not NKT cells, in WT mice compared to NKT-cell deficient mice on day 3 post tumor inoculation and it was similar on day 9 (**Figure 18A and 18B and 18C)**. These results suggested that NKT cells induced the production of IL-10 in bone marrow-derived non-NKT cells in the liver of WT mice during the initial stage of liver metastases.

The bone marrow-derived non-NKT cells were also assessed for the expression of CD45 which is known as Leukocytes Common Antigen (LCA) (249). CD45 is expressed by all the hematopoietic cells excluding mature erythrocytes and platelets (249) and therefore is used as a marker for bone marrow-derived cells. 99.6% of liver bone marrow-derived non-NKT cells expressed CD45 (Data not shown).

Figure 18. Melanoma-induce liver NKT cells stimulate the production of IL-10 in bone marrow-derived non-NKT cells. Bone marrow-derived $CD45^+$ $CD1d^-CD3^+CD19^+$ non-NKT cells were isolated from the livers of WT and $CD1d^{-/-}$ mice 3 and 9 days after intrasplenic injection of B16LS9 melanoma cells. (A) IL-10 mRNA levels assessed by qPCR. (B) IL-10 protein detected by ELISA. (C) IL-10 mRNA levels of liver NKT cells assessed by qPCR. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=5). ** P<0.01. NS= Not significant.



NKT cells induce down-regulation of NKG2D activation receptor in liver NK cells

NKG2D is a C-type lectin-like receptor that is expressed on almost all NK cells (250, 251). NKG2D plays a crucial role in activating NK anti-tumor function and consequently in the immune surveillance of tumors (252, 253). Recently, Hansen et al. reported that the expression of NKG2D is pronouncedly increased in intestinal epithelial cells (IECs) in IL-10-deficient mice (254). Since the expression of IL-10 is elevated in WT mice compared to NKT cell-deficient mice, we examined the surface expression of NKG2D on liver NK cells in naïve non-tumor-bearing and tumor-bearing WT and $CD1d^{-/-}$ mice 3, 9, and 14 days following intrasplenic injection of B16LS9 melanoma cells. NKG2D expression was the same in naive WT and $CD1d^{-/-}$ mice on days 3 post tumor injection **(Figure 19A and 19B)**, but was significantly decreased in WT mice on day 9 post tumor injection **(Figure 19C).** At day 14 post injection, the expression of NKG2D was comparable in WT and $CD1d^{-/-}$ mice but was significantly reduced relative to non-tumor-bearing mice and mice harboring tumor for only three days **(Figure 19D)**.

Multiple studies have reported that the expression of NKG2D is correlated with NK cell cytotoxicity in cancer patients (255, 256). Thus, we assessed the cytotoxicity of liver NK cells in naïve non-tumor-bearing and melanoma-bearing WT and $CD1d^{-/-}$ mice 3, 9, and 14 days following intrasplenic injection of B16LS9 melanoma cells. As expected, the maximum depression of liver NK cell activity in WT mice relative to $CD1d^{-/-}$ mice coincide with the least expression of NKG2D receptor in WT mice **(Figure 20)**.

Figure 19. Enhanced expression of NKG2D on liver NK cells in WT mice. Liver NK cells from isolated from (A) Naïve WT mice or naive CD1d$^{-/-}$ mice or from WT mice or CD1d$^{-/-}$ mice (B) 3, (C) 9, or (D) 14 days after intrasplenic injection of B16LS9 melanoma cells. Cells were assessed for surface expression of the NK cell activating receptor NKG2D by flow cytometry. The results are representative of two independent experiments (N=10).

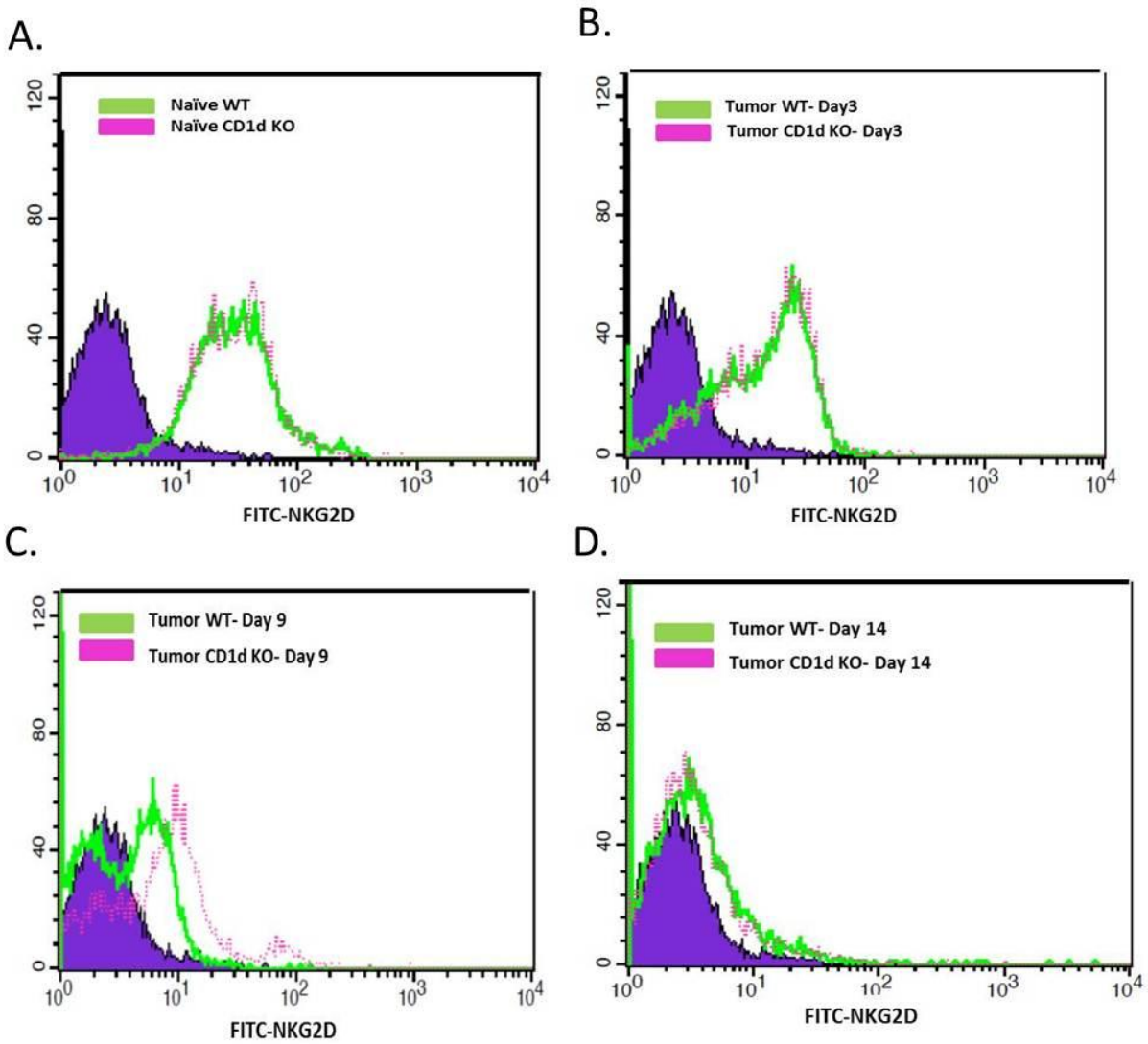
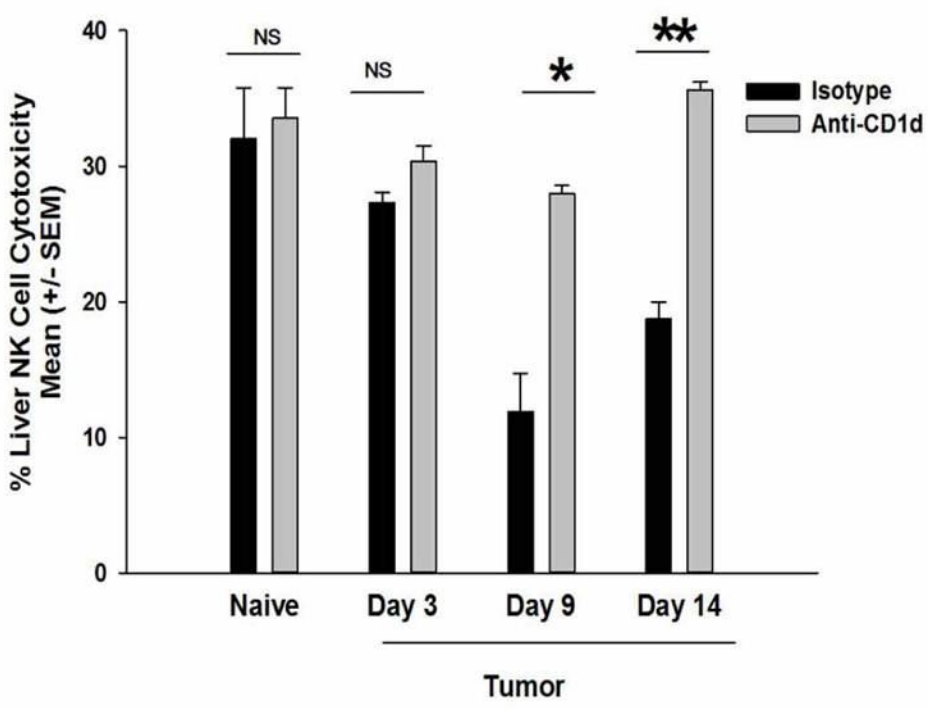


Figure 20. NKT cells suppress NK cell cytotoxicity in tumor-bearing mice. Cytotoxicity of liver NK cells from mice harboring liver metastases arising from intrasplenic tumor inoculation against B16LS9 melanoma target cells. Freshly isolated liver NK cells were incubated with B16LS9 melanoma target cells at an E:T ratio of 25:1 for 18 hours in a non-radioactive cytotoxicity assay. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). *P<0.05 or **P<0.01.

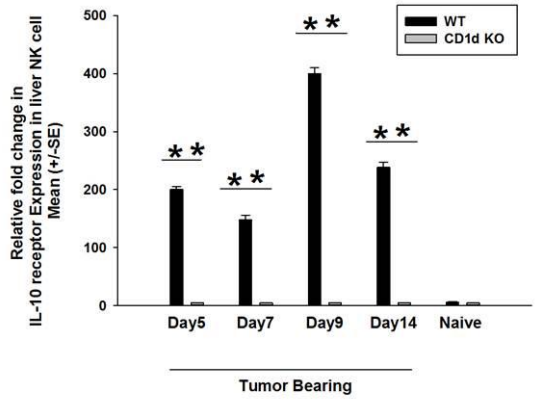


Liver NK cells in tumor-bearing NKT cell-deficient mice are hyporesponsive to the regulatory effects of IL-10

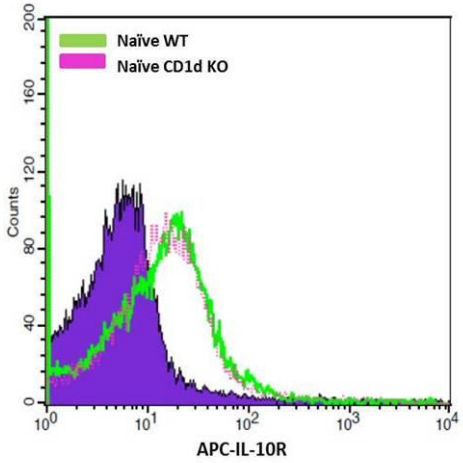
The regulatory functions of IL-10 are exerted by signaling through the IL-10 receptors (84). Here, we sought to determine the expression of IL-10 receptor on liver NK cells in tumor-bearing WT and NKT cell-deficient mice. The expression of IL-10 receptor was comparable in naïve mice but consistently higher in tumor-bearing WT mice relative to $CD1d^{-/-}$ mice with a 400-fold increase in the expression of IL-10 receptor message occurring at day 9 post tumor injection (**Figures 21A**). The expression of IL-10 receptor protein in WT mice was constantly expressed in melanoma-bearing mice, with the exception with a reduction on day 14 post injection. On the other hand, the expression of IL-10 receptor was significantly diminished in melanoma-bearing $CD1d^{-/-}$ mice as early as 5 days post tumor injection (**Figures 21B-21F**). Thus, one of the mechanisms that NKT cells use to suppress NK cell anti-tumor functions is by inducing the up-regulation of IL-10 receptor expression in NK cells.

Figure 21. Reduced expression of IL-10 receptor in liver NK cells in NKT cell-deficient mice. (A) Expression of IL-10 receptor mRNA in NK cells isolated from the livers of naïve and B16LS9 melanoma-bearing WT and $CD1d^{-/-}$ mice 5, 7, 9 and 14 days following intrasplenic injection of B16LS9 melanoma cells by quantitative RT-PCR. Expression of IL-10 receptor protein on NK cells isolated from the livers of (B) naïve mice or day (C) 5, (D) 7, (E) 9, (F) 14 post intrasplenic tumor cell injection by flow cytometry. The results are representative of two independent experiments (N=10).

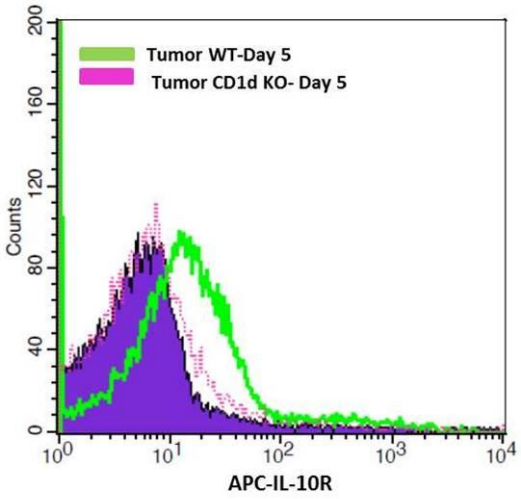
A.


Relative fold change in IL-10 receptor Expression in liver NK cell Mean (+/-SE)
500
400
300
200
100
0
WT
CD1d KO
**
**
**
**
Day5
Day7
Day9
Day14
Naive
Tumor Bearing

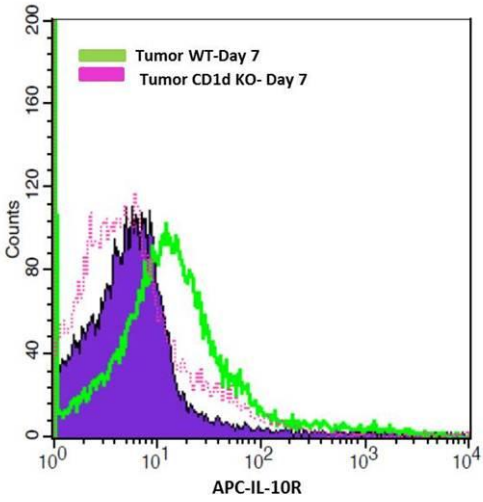
B.


Naïve WT
Naïve CD1d KO
Counts
APC-IL-10R

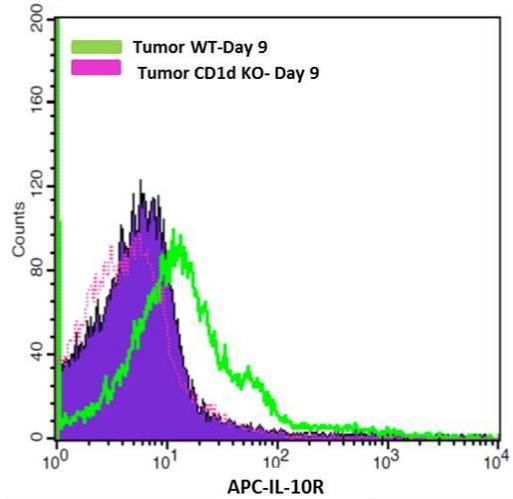
C.


Tumor WT-Day 5
Tumor CD1d KO- Day 5
Counts
APC-IL-10R

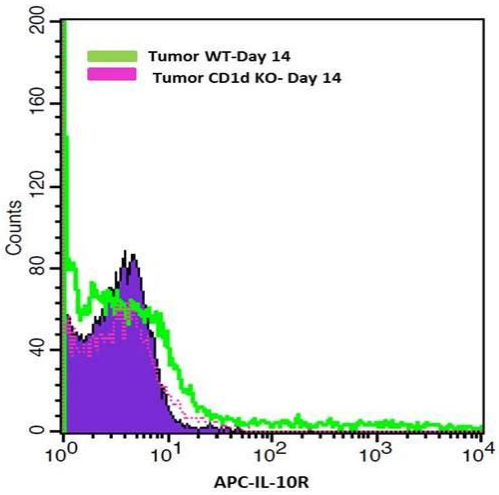
D.


Tumor WT-Day 7
Tumor CD1d KO- Day 7
Counts
APC-IL-10R

E.


Tumor WT-Day 9
Tumor CD1d KO- Day 9
Counts
APC-IL-10R

F.


Tumor WT-Day 14
Tumor CD1d KO- Day 14
Counts
APC-IL-10R

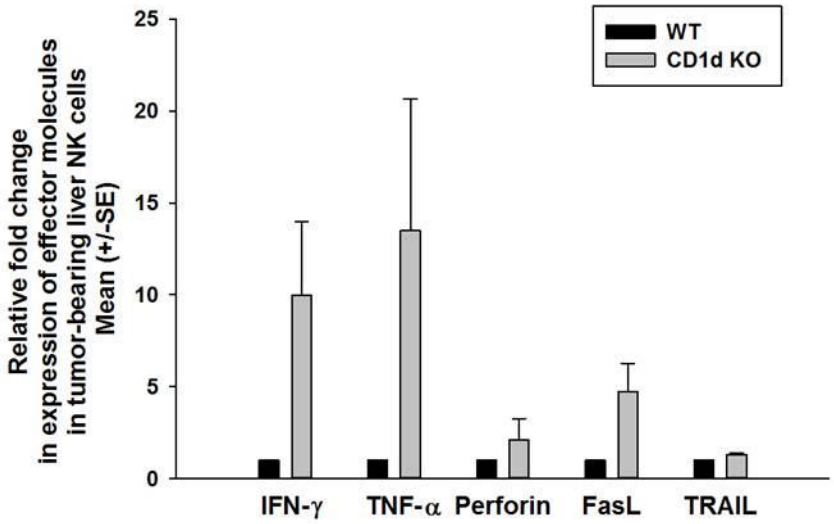
III. The effector functions of liver NK cells in NKT cell-deficient mice

Elevated expression of IFN-γ and TNF-α in liver NK cells of NKT cells-deficient mice

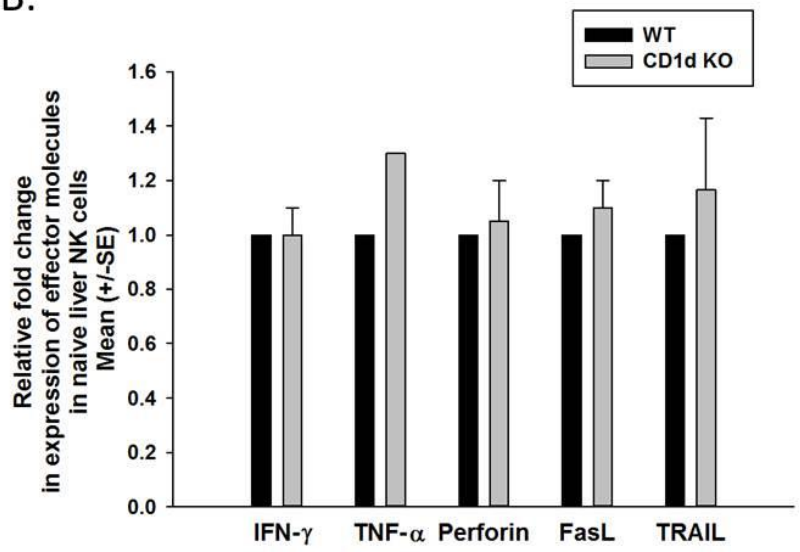
NK cells exert their effector functions through a variety of mechanisms including the production of pro-inflammatory cytokines, such as IFN-γ and TNF-α, secretion of perforin/granzyme, and expression of FasL and TRAIL (53). To elucidate the mechanisms by which liver NK cells in tumor-bearing CD1d$^{-/-}$ mice display enhanced NK cytolytic activity, we assessed the gene expression of IFN-γ, TNF-α, perforin, TRAIL and FasL on liver NK cells of WT and CD1d$^{-/-}$ mice. The mRNA levels for IFN-γ, TNF-α, and FasL were elevated in freshly isolated liver NK cells in tumor-bearing CD1d$^{-/-}$ mice compared to WT mice, however mRNA levels were expressed to a similar degree for perforin and TRAIL in these mice (**Figure 22A**). The expression of IFN-γ, TNF-α, perforin, TRAIL and FasL in the liver NK cells of naïve non-tumor-bearing WT and CD1d$^{-/-}$ mice were comparable (**Figure 22B**).

Figure 22. Enhanced mRNA levels of IFN-γ and TNF-α in tumor-bearing NKT cell-deficient mice. (A) Liver NK cell expression of IFN-γ, TNF-α, perforin, FasL and TRAIL in tumor-bearing mice WT and CD1d$^{-/-}$. (B) In naive non-tumor-bearing mice. RNA was harvested from freshly isolated liver NK cells from WT and CD1d$^{-/-}$ mice 14 days after intrasplenic injection of B16LS9 melanoma cells or from non-tumor-bearing mice and quantitative RT-PCR was performed. The results are representative of two independent experiments (N=10).

A.


WT
CD1d KO
Relative fold change in expression of effector molecules in tumor-bearing liver NK cells Mean (+/-SE)
25
20
15
10
5
0
IFN-γ
TNF-α
Perforin
FasL
TRAIL

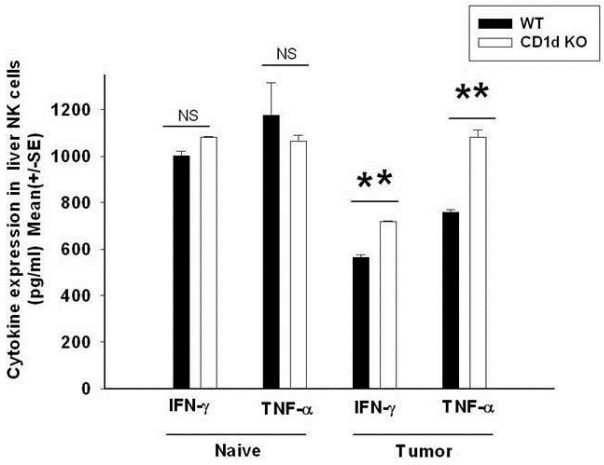
B.


WT
CD1d KO
Relative fold change in expression of effector molecules in naive liver NK cells Mean (+/-SE)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
IFN-γ
TNF-α
Perforin
FasL
TRAIL

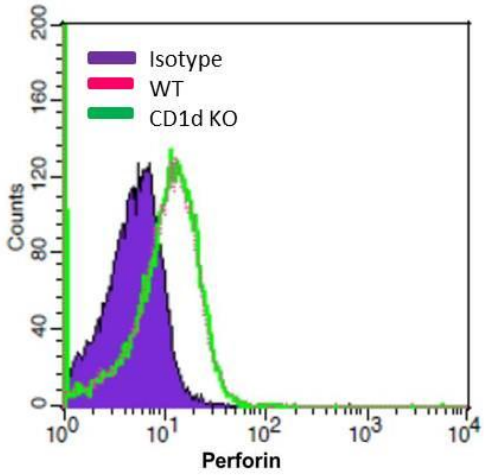
In addition, we assessed protein expression of these effector molecules in liver NK cells in tumor-bearing WT and CD1d$^{-/-}$ mice. The production of IFN-γ and TNF-α was significantly increased in tumor-bearing CD1d$^{-/-}$ mice compared to WT mice 14 days after tumor inoculation (**Figure 23A**). It is important to note that there was also a significant reduction in the production of IFN-γ and TNF-α in tumor-bearing WT mice in comparison with naïve WT mice ($p<0.001$), indicating that presence of liver metastases induces suppression of liver NK cell activity (**Figure 23A**). The expression of perforin, FasL and TRAIL in liver NK cells in NKT cell-deficient mice and WT mice harboring liver metastases was comparable (**Figure 23B-23D**). Therefore we concluded that the enhanced liver NK cell cytotoxicity was not due to increased expression perforin, FasL or TRAIL.

Figure 23. Enhanced protein levels of IFN-γ and TNF-α in tumor-bearing NKT cell-deficient mice. (A) Freshly isolated liver NK cell were stimulated with PMA and ionomycin for 24 hours and cell supernatants from these cultures were assessed by ELISA for mouse IFN-γ and TNF-α. (B) For the expression of perforin, isolated NK cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for 4 hours, followed by intra-cellular cytokine staining for perforin. For the cell surface expression of (C) FasL and (D) TRAIL, isolated NK cells were stained for FasL and TRAIL with no stimulation and followed by follow cytometry analysis. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). ** P<0.01or NS= Not significant.

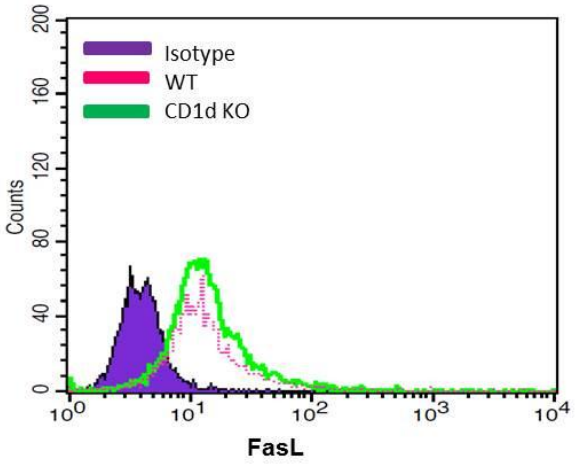
A.


WT
CD1d KO
Cytokine expression in liver NK cells (pg/ml) Mean(+/-SE)
0
200
400
600
800
1000
1200
NS
NS
**
**
IFN-γ
TNF-α
IFN-γ
TNF-α
Naive
Tumor

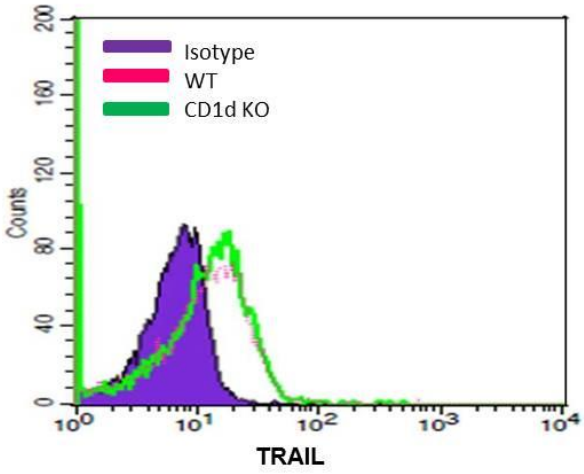
B.


Isotype
WT
CD1d KO
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
Perforin

C.


Isotype
WT
CD1d KO
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FasL

D.


Isotype
WT
CD1d KO
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
TRAIL

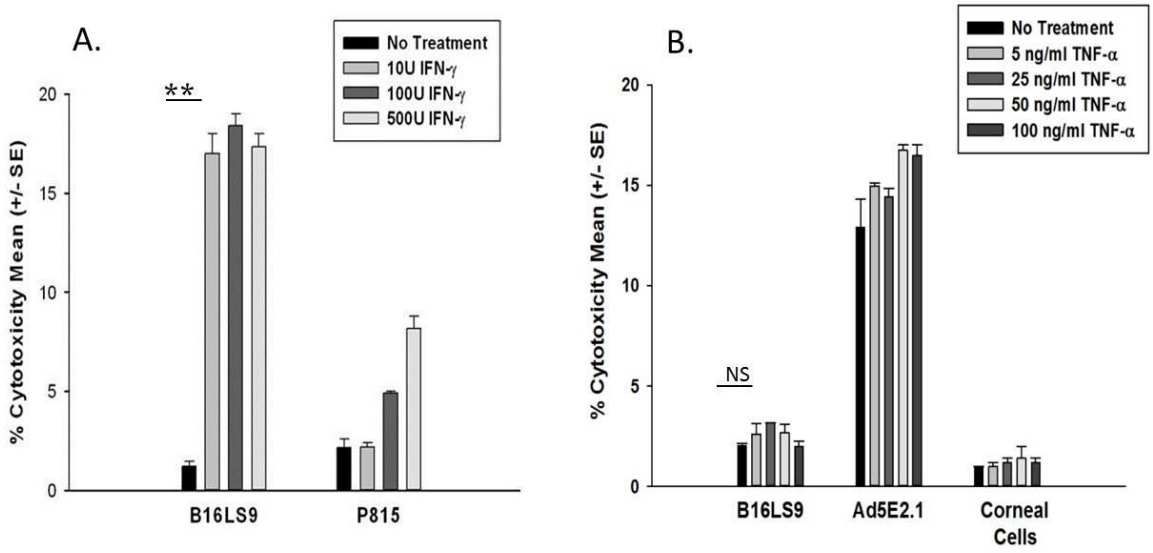
B16LS9 melanoma cells are susceptible to NK cell-mediated killing

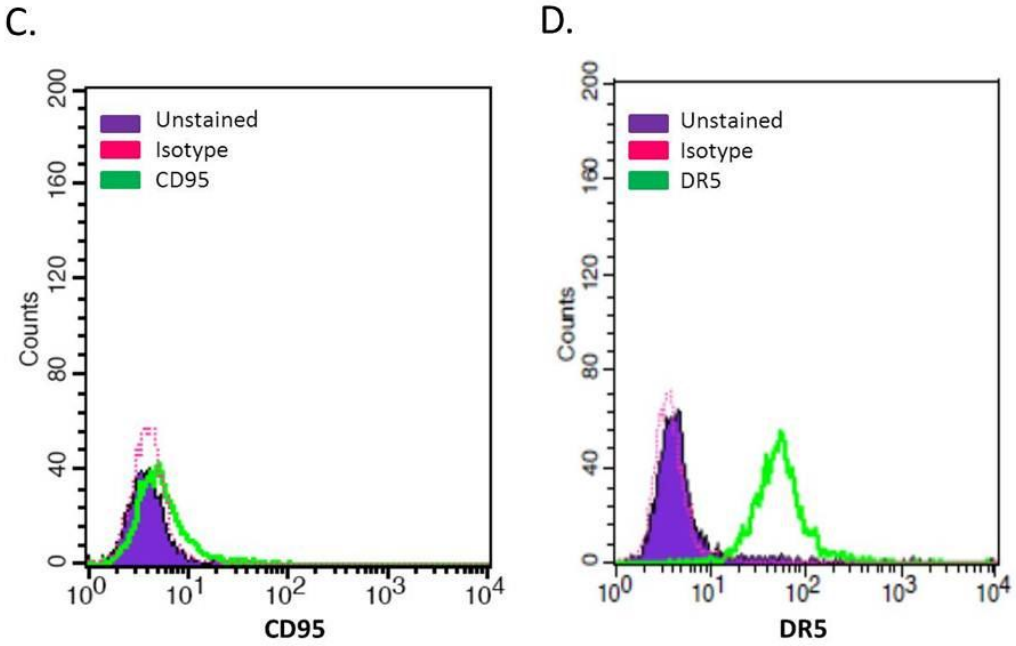
We further investigated whether B19LS9 melanoma cells were susceptible to killing by IFN-γ or TNF-α, by treating the tumor cells *in vitro* with either IFN-γ or TNF-α. B16LS9 melanoma cells were incubated for 24 hours with increasing concentration of TNF-α or IFN-γ and the percentage of killing was measured in a non-radioactive cytotoxicity assay. Unlike TNF-α, IFN-γ was able to directly kill B16LS9 melanoma cells (**Figure 24A and 24B**).

Engagement of Fas receptor (CD95/APO1), a transmembrane protein which belongs to TNF receptor family with FasL triggers apoptosis in variety of cells (257). TRAIL also induces program cell death via binding to other members of TNF receptor family such as DR4 and DR5 (258). Therefore, we assessed whether B16LS9 melanoma cells were susceptible to FasL and TRAIL killing via expression of Fas and TRAIL receptor, DR5. The expression of Fas and TRAIL receptors was detected on B16LS9 melanoma cells (**Figure 24C and 24D**). Although B19LS9 melanoma cells are vulnerable to perforin, FasL and TRAIL killing, there was no difference in the expression of perforin, FasL and TRAIL observed in tumor-induced liver NK cell in NKT cell-deficient mice and WT mice. Thus, the elevated liver NK cell cytotoxicity in NKT cell-deficient mice was not due through B16LS9 melanoma cells being susceptible to enhanced killing via perforin, FasL or TRAIL.

Figure 24. B16LS9 melanoma cells are susceptible to NK cells-mediated cytotoxicity. Susceptibility of B16LS9 melanoma cells to killing by (A) IFN-γ or (B) TNF-α was tested *in vitro* in a non-radioactive cytotoxicity assay. 2 x 10^4 B16LS9 melanoma cells were incubated for 24 hours with increasing concentration of TNF-α (5ng/ml, 25ng/ml, 50ng/ml, 100ng/ml), IFN-γ (10U, 100 U, 500U) or left untreated. Ad5E1 tumor cells were used as a positive control and corneal endothelial cells and P815 cells were used as a negative control. The percentage of

killing by cytokines was measured in a non-radioactive cytotoxicity assay. B16LS9 melanoma cells were analyzed for the expression of (C) Fas receptor (CD95) or (D) TRAIL receptor (DR5) by flow cytometry. Results were expressed as the Mean +/- the SE and are representative of two independent experiments (N=10). ** P<0.01or NS= Not significant.





NKT cells impair IFN-γ-dependent killing of melanoma cells by liver NK cells

To confirm the effect of IFN-γ on the killing of B16LS9 cells *in vivo,* we treated tumor-bearing $CD1d^{-/-}$ mice with either anti-IFN-γ antibody or rat IgG isotype control. *In vivo* blockade of IFN-γ in NKT cell-deficient mice resulted in a significant increase in the number of liver metastases compared to isotype control antibody-treated mice (**Figures 25A and 25B**). To further establish that the IFN-γ produced by liver NK cells was responsible for the killing of B16LS9 melanoma cells in NKT cell-deficient mice, freshly isolated liver NK cells from $CD1d^{-/-}$ mice were treated with anti-IFN-γ antibody or rat isotype control *in vitro* and the cytotoxicity of NK cells was assessed against B16LS9 melanoma cells (**Figure 26A**). Neutralizing IFN-γ significantly reduced the ability of liver NK cells to kill B16LS9 melanoma cells. These results suggest that NK cells play an essential role in the resistance to liver metastases in mice deficient in NKT cells through the production of IFN-γ.

To further assessed the effect of TRAIL that is expressed by liver NK cells on the killing of B16LS9 cells *in vitro,* freshly isolated liver NK cells from $CD1d^{-/-}$ mice were treated with anti-TRAIL-antibody or isotype control and the cytotoxicity of NK cells was assessed against B16LS9 melanoma cells (**Figure 26B**). Neutralizing TRAIL had no effect on liver NK cell mediated-cytotoxicity, which indicated that the resistance to liver metastases in NKT cell-deficient mice is independent of TRAIL.

Figure 25. Resistance to liver metastases in NKT cell-deficient mice is IFN-γ-dependent. (A) Surface liver metastases in NKT cell-deficient mice 14 days after intrasplenic injection with B16LS9 melanoma cells. Mice were treated with i.p. 500 ug of anti-IFN-γ antibody twice a week. (B) Number of liver metastases in NKT cell-deficient mice 14 days after intrasplenic

injection with B16LS9 melanoma cells and treated with anti-IFN-γ antibody. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). *P<0.05

A.

B.

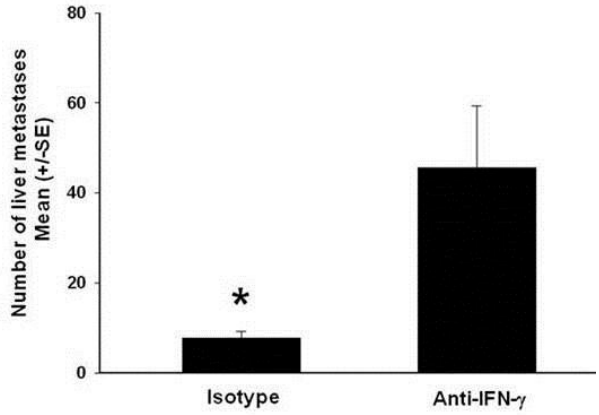
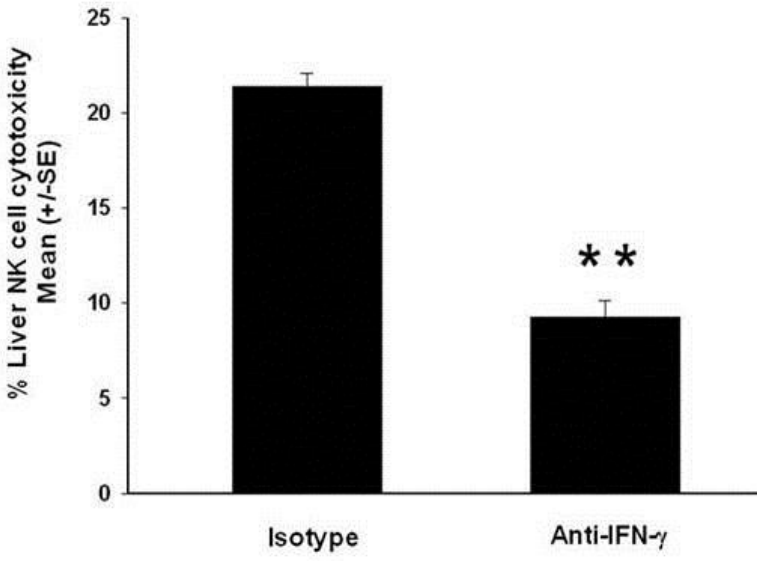
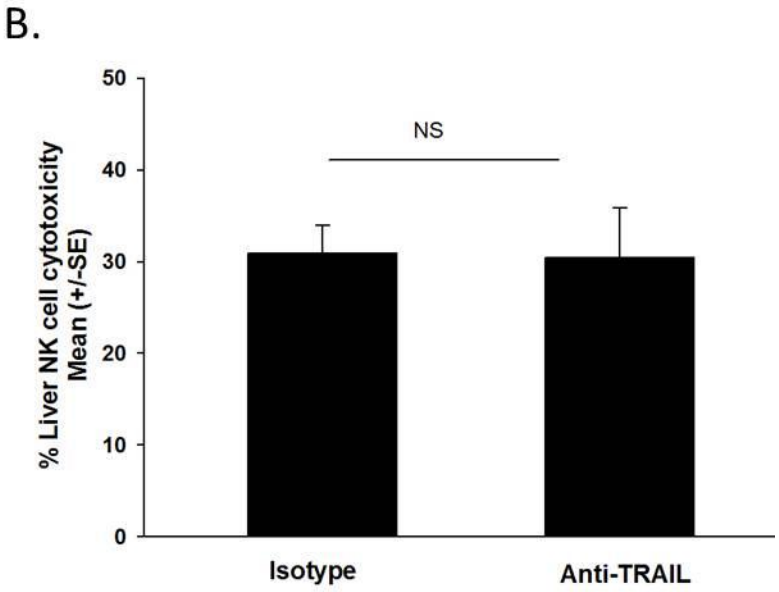


Figure 26. Neutralizing IFN-γ and not TRAIL reduces liver NK cell-mediated cytotoxicity against B16LS9 melanoma cells. Cytolytic activity of liver NK cells isolated from NKT cell-deficient mice and tested *in vitro* against B16LS9 melanoma cell at an E:T ratio of 25:1 for 18 hours in the presence of either (A) 20 ug/ml of anti-mouse IFN-γ or (B) 50ng/ml of anti-TRAIL or isotype control antibody. Results are expressed as the Mean +/- SE and are representative of two independent experiments (N=10). **P<0.01

A.



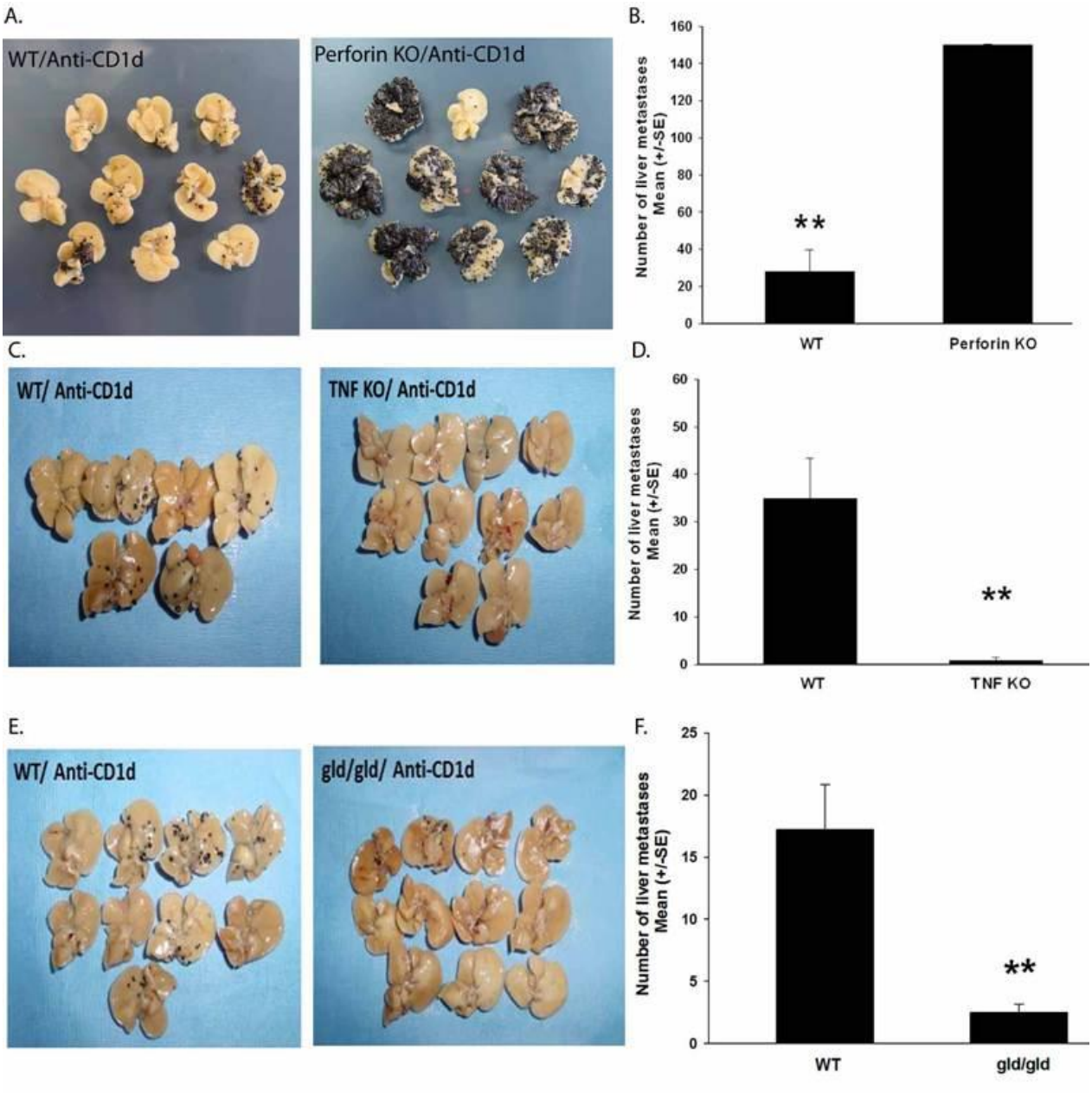


Enhanced liver metastases in NKT cell-deficient mice is perforin-dependent, but TNF-α- and FasL-independent

We next turned our attention to the role of perforin in NK cell-mediated cytotoxicity of B16LS9 melanoma cells in CD1d$^{-/-}$ mice. To do so, we assessed the formation of liver metastases in anti-CD1d antibody-treated perforin$^{-/-}$ mice and WT mice after intrasplenic injection of B16LS9 melanoma cells. A significant increase in the number of liver metastases was observed in NKT cell-deficient perforin$^{-/-}$ compared to WT mice (**Figures 27A and 27B**). These data suggested that perforin is a crucial molecule for inhibiting metastasis in NKT cell-deficient mice. A similar approach was used to assess the role of TNF-α and FasL in resistance to liver metastases in NKT cell-deficient mice. The number of liver metastases was determined in anti-CD1d antibody-treated WT, TNF-α$^{-/-}$ mice (**Figures 27C and 27D**) or FasL defective (gld/gld) mice (**Figures 27E and 27F**). Surprisingly, formation of liver metastases in NKT cell-deficient

mice that were depleted of either TNF-α or expressed non-functional FasL was not enhanced, but instead, the removal of these two molecular pathways of cytotoxicity noticeably diminished, rather than enhanced the number of liver metastases. Therefore, we concluded that NK cell-mediated cytotoxicity of melanoma cells in NKT cell-deficient mice was independent of TNF-α and FasL.

Figure 27. Perforin but not TNF-α or FasL is required for liver NK cells to kill B16LS9 melanoma cells in NKT cell-deficient mice. (A) Surface liver metastases in WT and perforin-deficient mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. (B) Number of surface liver metastases in WT and perforin-deficient mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. (C) Surface liver metastases in WT and TNF-$\alpha^{-/-}$ mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. (D) Number of surface liver metastases in liver in WT and TNF-$\alpha^{-/-}$ mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. (E) Surface liver metastases in WT and FasL-defective (gld/gld) mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. (F) Number of surface liver metastases in liver in WT and FasL-defective (gld/gld) mice treated with anti-CD1d antibody and challenged with an intrasplenic injection of B16LS9 melanoma cells. Results were expressed as the Mean +/- SE (N=10). *$P<0.05$ or **$P<0.01$.


A.
WT/Anti-CD1d
Perforin KO/Anti-CD1d
B.
Number of liver metastases Mean (+/-SE)
160
140
120
100
80
60
40
20
0
**
WT
Perforin KO
C.
WT/ Anti-CD1d
TNF KO/ Anti-CD1d
D.
Number of liver metastases Mean (+/-SE)
60
50
40
30
20
10
0
**
WT
TNF KO
E.
WT/ Anti-CD1d
gld/gld/ Anti-CD1d
F.
Number of liver metastases Mean (+/-SE)
25
20
15
10
5
0
**
WT
gld/gld

CHAPTER FOUR

Discussion

Clinical relevance of this study

UM is the most common intraocular malignancy in adults (15, 223) . Half of the patients with primary UM develop metastases to other organs, with the liver being the most frequently affected organ (223). The current median survival time of patients with liver metastases is less than a year (259). The clinical prognosis of patients diagnosed with UM is highly dependent on the disease progression in the liver. Unfortunately, there is currently no therapy for metastatic UM (260).

The etiology for the preferential metastatic spread to the liver is poorly understood. Chemokine receptors, such as CXCR4, are widely expressed on UM and facilitate the chemotactic responses of UM cells to liver cells that express their complementary ligand, CXCL12, which presumably facilitates the preferential migration of blood-borne UM cells to the liver (261, 262). A significant body of research suggests that the liver's unique immunoregulatory micro-environment might foster the growth of tumors that metastasize to the liver through the suppression of adaptive immunity (135). The liver is endowed with immunosuppressive cytokines such as IL-10 and TGF-β which are known to promote the induction of tolerogenic APCs and T-cells (137). By contrast, the innate immune system has a strong presence in the liver and plays a vital protective role against pathogens and malignant tumors (263).

NK cells, the effector cells of the innate immunity, are more abundant in the liver than in any other organ (135). The importance of NK cells in controlling metastases of UM has been

shown in both humans and mice. Like NK cells, NKT cells are abundant in the liver and account for up to 25% and 40 % of human and mouse liver lymphocytes, respectively (224). However, the role of NKT cells in the development of liver metastases has not been sufficiently investigated. In the present study, we investigated the role of NKT cells in the development of liver metastases in mice bearing intraocular melanoma. Unique properties and functional abilities of NK and NKT cells within the liver microenvironment make them appealing objectives of immunotherapeutic approaches with the goal of restricting tumor metastasis in the liver.

NKT cells promote the formation of liver metastases

NKT cells display paradoxical pro-inflammatory and anti-tumor functions. The general notion is that iNKT cells promote anti-tumor immunity and type II NKT cells impair it (157). However, there is compelling evidence that in some conditions, activated iNKT cells enhance tumor immunity by generating a cytokine cascade that primes anti-tumor immune responses (264-267). iNKT cells are also able to directly kill CD1d-expressing tumor cells (176). However, most tumor cells, including B16LS9 melanoma cells, do not express CD1d (unpublished data) (178). It has been suggested that CD1d-dependent cross presentation of endogenous tumor antigen by dendritic cells can activate iNKT cell anti-tumor function (268). Additionally, it has been reported that murine NKT cells can exert regulatory effects on tumor immune surveillance (183, 269, 270). Bricard at al. showed that the frequency of $CD4^+$ iNKT cells with regulatory properties significantly increased in the patients with intrahepatic malignancies (271). Interestingly, the same study reported that patients with UM had the highest number of hepatic iNKT cells compared with hepatocellular carcinoma patients (271). In the present study, we found that NKT cells play a regulatory rather than an anti-tumor role and promote the formation

of liver metastases arising from either intraocular melanomas or following intrasplenic tumor injections. CD1d$^{-/-}$ mice which lack both iNKT and type II NKT cells form significantly fewer liver metastases than WT mice. We previously showed that the development of liver metastases in Jα18$^{-/-}$ mice, which only lack iNKT cells, was the same as in CD1d$^{-/-}$ mice (116), indicating that iNKT cells are responsible for the resistance to the liver metastases in the current study.

Liver NK cell killing is augmented in NKT cell-deficient mice

The resistance to liver metastases arising from intraocular melanomas in NKT cell-deficient mice is T-cell-independent but NK cell-dependent (116). SCID mice, which lack both T and B cells but have functional NK cells, are resistant to liver metastases and have higher numbers of liver-infiltrating NK cells, which express elevated cell-mediated cytotoxicity against B16LS9 melanoma cells (116). These findings are consistent with other reports showing that NK cells play a vital role in limiting liver metastases arising from intraocular melanomas in both humans and mice (5, 115, 228). In a murine model of intraocular melanoma, Dithmar and colleagues reported that the depletion of the host's NK cells results in a significant increase in the number of hepatic micro-metastases (115). Although, the number of liver NK cells in melanoma-bearing WT and CD1d$^{-/-}$ mice was comparable, we found that the cytolytic activity of liver NK cells was elevated in melanoma-bearing NKT cell-deficient CD1d$^{-/-}$ mice and anti-CD1d-treated mice. Regardless of the route of tumor cell dissemination (intrasplenic or intraocular), the enhanced liver NK cell-mediated cytotoxicity was intimately associated with the resistance to the formation of liver metastases.

Liver metastases are required for NKT cell-dependent suppression of liver NK cells

Tumor cells utilize many different strategies to evade immune surveillance by NK and NKT cells (272-275). Multiple studies have shown that the anti-tumor effector functions of NK and NKT cells are suppressed in the tumor microenvironment (175, 243, 276). In this study, we showed that the presence of melanoma metastases in the livers of WT mice resulted in the suppression of liver NK cell-mediated cytotoxicity. Interestingly, in the absence of NKT cells, the presence of liver metastases does not lead to enhanced suppression of liver NK cell cytolytic activity. This in turn, strongly suggests that NKT cells enhance, rather than inhibit, the development of liver metastases by suppressing NK cell activity in the liver. In addition, the comparable levels of NK cell mediated-cytotoxicity in non-tumor-bearing WT and $CD1d^{-/-}$ mice suggest that the presence of tumor is required for the NKT cell-dependent suppression of liver NK cell anti-tumor function. In addition, impaired NK cell activity in WT mice compared to NKT cell-deficient mice appears to be restricted to the liver, as splenic NK activity is the same in NKT cell-deficient mice and WT mice harboring liver metastases.

Although the liver with its unique immunosuppressive micro-environment is capable of inducing immune tolerance (135), similar intensities of liver NK cell anti-tumor activity in WT and anti-CD1d treated mice bearing subcutaneous B16LS9 melanoma which forms no liver metastases, suggest that in this model the suppression of NKT cell-induced NK cell-mediated cytotoxicity is exclusive to hosts with liver metastases and is not a systemic phenomenon.

NKT cells are unable to suppress NK cells in vitro

The crosstalk between NKT cells and NK cells has been reported by multiple research groups. Subleski et al. found that the removal of immunosuppressive NKT cells along with activation of NK cells resulted in significant reduction in the number of liver metastases produced by renal carcinoma cells (155). By contrast, it has also been reported that activated NKT cells stimulate cytolytic activity of NK cells (179, 277). Given that NKT cells can produce IL-10, which plays an immunosuppressive role in the present model of intraocular melanoma, we hypothesized that NKT cells were responsible for the suppression of liver NK cell anti-tumor function by secreting immunosuppressive cytokines. In spite of testing multiple permutations, the data we obtained from the transwell assays suggest that NKT cells are unable to directly suppress NK cell-mediated cytotoxicity by producing soluble factors.

NK cells often need to be primed by APCs to gain optimal effector functions. (278). In most cases, the activation of NK cells by accessory cells requires close proximity of the two cell types, even though the activation is facilitated by cytokines. For instance, Borg et al. reported that physical contact between DCs and NK cells is necessary for IL-12-induced NK cell activation and IFN-γ production (279). This notion was used as a rationale for the experiment in which we tested the hypothesis that tumor-induced NKT cells suppress NK cells in a contact dependent manner. Once again, regardless of the various permutations we used, the co-incubation of NKT cells and NK cells did not result in the suppression of NK cell-mediated cytotoxicity. However, based on the outcomes of these experiments, we cannot draw a final conclusion that NKT cells are unable to directly suppress NK cells. The result may be a reflection of the significant differences between the *in vitro* and *in vivo* conditions, for instance, the ratio of NKT to NK cell, the duration of the co-incubation time between NKT and NK cells,

the absence of other cells such as APCs and hepatocytes and the various cytokines and soluble factors that normally exist in the tumor microenvironment in the liver. There are several studies reporting that NKT cells are capable of lysing tumor cells upon recognition of CD1d (173, 174). However, in our study, NKT cells were unable to lyse B16LS9 melanoma cells *in vitro*, which might be explained by the lack of CD1d expression by B16LS9 melanoma cells.

MDSCs are unable to promote the formation of liver metastases

The impact of MDSCs on tumor progression is well documented in humans and mice (212). Berzofsky's group and others conducted a series of elegant experiments which suggested that the activation of NKT cells by endogenous ligands lead to the production of IL-13 by NKT cells, which acted on MDSCs that expressed IL-13 receptor (188, 269). IL-13 induces the production of TGF-β in MDSCs, which subsequently impairs CTL and NK cells functions (188, 219, 269, 280, 281). Since liver NKT cells are unable to directly suppress NK cell-mediated cytotoxicity, we hypothesized that the presence of MDSC is required for NKT-induced NK cell suppression. We suspected that MDSCs promoted the formation of liver metastases in melanoma-bearing mice. We tested this by depleting MDSCs *in vivo* using a widely accepted protocol involving anti-Gr1 antibody treatment (234). Surprisingly, the depletion of MDSCs enhanced, rather than reduced, the formation of liver metastases in B16LS9 melanoma-bearing mice. This suggests that MDSCs are incapable of promoting the formation of liver metastases in the mouse model of intraocular melanoma. One possible mechanism for this phenomenon has been explained by Ko et al. who reported that activated iNKT cells can convert immunosuppressive MDSCs to highly immunogenic APCs. These reprogrammed MDSCs

induced the generation of antigen-specific CTLs without increasing the number of Tregs (282). In another study Manjili's group reported that activated iNKT cells rendered CTLs resistant to the immunosuppressive actions of MDSCs (283). Since iNKT cells play an immunoregulatory role in our model, these are unlikely mechanisms for the enhancement of liver metastases in MDSC depleted mice. The question yet to be answered is why higher numbers of liver metastases form in MDSC-depleted mice.

The mouse anti-granulocyte receptor-1 (Gr-1) monoclonal antibody reacts with Gr1 antigen, which is identified as a member of the Ly6 gene family (217). Gr1 antigen binds with high affinity to mouse Ly-6G molecules and, to a lesser extent to Ly-6C molecules, which are expressed by several sub-populations of myeloid-derived cells other than MDSCs such as DCs, macrophages and neutrophils (217, 284). It is likely that *in vivo* administration of anti-Gr1 antibody not only depletes MDSCs, but also depletes neutrophils, macrophages and other myeloid-derived cells. DCs, macrophages and neutrophils activate NK cells (285, 286). A plausible explanation for the enhanced number of liver metastases in anti-Gr1 antibody treated mice is that the depletion of accessory cells, which activate NK cells, affects NK cell-mediated cytotoxicity against B16LS9 melanoma cells, resulting in an increase in the number of liver metastases. Our investigations rule out the role of neutrophils in the enhancement of liver metastases in anti-Gr1 antibody treated mice. Depletion of neutrophils with anti-Ly6G antibody, which specifically depletes neutrophils (284), does not result in an increased number of liver metastases. On the other hand, depletion of Kupffer cells with clodronate liposomes (287) significantly augments the formation of liver metastases, indicating that Kupffer cells are responsible for the activation of NK cells and their depletion with anti-Gr1 antibody results in enhanced liver metastases.

IL-10 promotes the formation of liver metastases

IL-10, TGF-β, and MIF are able to suppress NK cell activity via distinct pathways (75-79, 209). It has been suggested that IL-10 suppresses the production of IFN-γ in NK cells indirectly by blocking the expression of IL-2 and IL-12 in accessory cells such as monocytes (78, 288). Likewise, TGF-β, and MIF restrain NK cell anti-tumor function by inhibiting NK cell proliferation and IFN-γ production or by preventing the release of perforin, respectively (76, 289). We found that the expression of all three cytokines is up-regulated in the livers of tumor-bearing WT mice in comparison with naïve mice which can explain why NK cell-mediated cytotoxicity is suppressed in melanoma-bearing mice. However, when the expressions of these cytokines were compared in melanoma-bearing WT and CD1d$^{-/-}$ mice, IL-10 was the only cytokine with up-regulated expression in WT mice, indicating that the role of TGF-β, and MIF signaling pathways are dispensable in this ocular melanoma model. In addition, Yang et al. reported that anti-TGF-β treated B16LS9 melanoma-bearing mice developed a similar number of liver metastases as isotype treated mice (116). Therefore, we focused our investigation on dissecting the role of IL-10 in the formation liver metastases in melanoma-bearing mice.

IL-10 is a pleiotropic cytokine that is produced by variety of cells in the liver such as DCs, Kupffer cells, NKT cells, hepatocytes and hepatic stellate cells (81, 82). IL-10 acts as a regulator of the pro-inflammatory response in numerous infections, and if it is overexpressed, it exerts its immunosuppressive role by inhibiting the production of proinflammatory cytokines, suppressing the expression of the co-stimulatory molecules MHC class II antigens, as well as by suppressing the function of APCs (290). The effect of IL-10 on NK cells is controversial. Several reports have shown that IL-10 down-regulates NK cell function (88, 89). However, there is also evidence suggesting that IL-10 is a potent activator of NK cells (90, 91). In the present study, we

report that IL-10$^{-/-}$ mice are resistant to the development of liver metastases, which supports the notion that IL-10 plays a pro-tumorigenic role in the formation of metastatic intraocular melanomas. This finding is in agreement with previous results showing that the susceptibility of mice to melanoma is concomitant with elevated production of IL-10 by immune cells, and that the neutralization of IL-10 by gene therapy results in enhancement of tumor rejection *in vivo* (291).

Systemic levels of IL-10 are not reduced in NKT cell-deficient mice

The crosstalk between the liver and other organs regulates the immune responses outside of the liver that may lead to systemic tolerance. For instance, liver allografts can survive without the use of immunosuppressive drugs even after other transplanted organs are rejected (292). A recent study suggested that Tregs and NKT cells are involved in the induction of systemic tolerance by producing IL-4 and IL-10 (293). Furthermore, Sonoda *et al.* suggested that the presence of iNKT cells is required for the induction of systemic tolerance by ACAID (294). Similarly, it has been suggested that the NKT cells play a vital role in the induction of tolerance to xenogeneic islet grafts and vascularized cardiac allografts (167, 295). On a different note, it has been reported that the serum level of IL-10 is a useful marker for melanoma progression (296). IL-10 levels are elevated in 73% of melanoma patients with metastatic spread (297). Given these observations, we speculated that there was an increase in the serum levels of IL-10 in melanoma-bearing mice. In contrast to the findings which suggest NKT cells are involved in the induction of systemic tolerance, we failed to detect any difference in the serum levels of IL-10 in melanoma-bearing WT and NKT cell-deficient mice. These data suggest that NKT cell-induced suppression of NK cell-mediated cytotoxicity in melanoma-bearing mice is associated with increased concentration of IL-10 in the liver but is independent of systemic levels of IL-10.

NKT cells suppress NK cells by inducing the production of IL-10 in bone marrow-derived cells in the liver

Due to the importance of IL-10 in the formation of liver metastases, we sought to determine the source of the IL-10-producing cells in melanoma-bearing mice. Our findings indicated that neither B16LS9 melanoma cells nor splenic cells are the source of IL-10 production. However, results from bone marrow chimera experiments revealed that bone marrow-derived cells are the major source of IL-10 that suppresses liver NK cell activity in mice with melanoma liver metastases.

Given that NKT cells and IL-10 play an immunosuppressive role in the present model of intraocular melanoma, we hypothesized that NKT cells, which are derived from bone marrow, are responsible for the suppression of liver NK cell anti-tumor function by producing IL-10. However, we failed to detect increased production of IL-10 message or protein by liver NKT cells in WT mice harboring liver metastases. However, there was a significant elevation of IL-10 expression in the livers of WT mice compared to $CD1d^{-/-}$ mice during the first seven days following intrasplenic tumor injection. This indicates that although NKT cells were not the major source of IL-10 production, they induced the production of IL-10 in other cells in the liver, which consequently resulted in the suppression of NK cell-mediated cytotoxicity. Indeed, our results demonstrate that $CD45^{+}$ bone marrow-derived cells that not NKT cells have produced significantly more IL-10 in WT mice compared to NKT cell-deficient mice 3 days after tumor inoculation. The increased production of IL-10 by bone marrow-derived non-NKT cells was not observed at later time points after tumor challenge, which indicates that NKT cells induced the production of IL-10 in the liver bone marrow-derived non-NKT cells during the initial stage of liver metastases. These data are in contrast with the results of a previously published report by

Yang et al. showing that NKT cells and no other bone marrow-derived cells, are the cells responsible for the production of IL-10 (116). The exact reasons for these contrasts are unknown. However, it can be speculated that the modifications in experimental procedures used to obtain the data are the cause of observed disparities.

NKT cells suppress NK cell functions by enhancing IL-10 receptor expression on liver NK cells

IL-10 mediates its biological effects by binding to a cell surface IL-10 receptor which is crucial for IL-10-mediated immune regulation (84). Functional IL-10 receptor is a tetrameric complex comprised of two ligand-binding alpha chains and two accessory beta chains (84). Recently, Cui et al. reported that the expression of IL-10 receptor on $CD4^+$ and $CD8^+$ T cells in systemic lupus erythematosus (SLE) patients is negatively correlated with the severity of the disease suggesting that IL-10 and its receptor have a regulatory role in the pathogenesis of SLE (298). We used these data as a rationale to hypothesize that NKT cells suppress the expression of IL-10 receptor on liver NK cells. Our results demonstrate an enhancement in the expression of IL-10 receptor on tumor-induced liver NK cells in WT but not NKT cell-deficient mice. Thus, NKT cells increase the susceptibility of NK cells to the immunosuppressive effects of IL-10 by the induction of IL-10 receptor on liver NK cells.

Decreased expression of NKG2D activation receptor correlates with depressed liver NK cell cytotoxicity

NKG2D is an activation receptor that is expressed on NK cells and CTLs and its expression directly correlate with NK cell-mediated cytotoxicity (250). Our results revealed that

in the absence of NKT cells, the activating receptor on NK cells, NKG2D, was up-regulated on liver NK cells. It is noteworthy that, this up-regulation of liver NK cell-mediated cytotoxicity in NKT cell-deficient mice occurred at day 9 post tumor injection, which coincided with the peak up-regulation of IL-10R on liver NK cells and the maximum down-regulation of liver NK cytolytic activity in WT mice. Thus, we propose that in WT mice, the combination of increased production of IL-10 in the liver along with up-regulation of IL-10R on liver NK cells conspire to depress NK cell-mediated resistance to liver metastases. The coincidental up-regulation of the NK cell activation receptor NKG2D on NK cells in $CD1d^{-/-}$ mice further accentuates these effects. These data are in concert with several findings indicating that there is a negative correlation between the amounts of IL-10 and the expression of NKG2D receptor on NK cells (254, 299).

NKT cells impair IFN-γ-dependent killing of melanoma cells by liver NK cells

There are several mechanisms by which NK cells kill malignant cells. Activated NK cells exert their cytotoxic functions by secreting granules that contain perforin/granzyme or by producing cytokines such as IFN-γ and TNF-α. NK cells can also induce apoptosis in target cells by expressing death receptor pathway molecules such as FasL and TRAIL (53).

Our data indicate that resistance to liver metastases and enhanced liver NK cell-mediated cytotoxicity in NKT cell-deficient mice is associated with the production of IFN-γ and not TNF-α by liver NK cells. Liver NK cells in melanoma-bearing NKT cell-deficient mice produce measurably higher amounts of IFN-γ compared to amounts produced in WT mice. B16LS9 melanoma cells are also highly susceptible to IFN-γ-mediated cytolysis. In addition, liver NK cells collected from $CD1d^{-/-}$ mice and treated *in vitro* with anti-IFN-γ antibody resulted in the

impairment of NK cell-mediated melanoma killing. Finally, neutralizing IFN-γ *in vivo* resulted in a significant enhancement of liver metastases in NKT cell-deficient mice. Together, these data indicate that IFN-γ plays a vital role in resistance to the spread of metastatic melanoma. These findings are also in concert with a substantial amount of evidence that demonstrates the critical role of IFN-γ in promoting host responses to tumors (300).

Although liver NK cells in tumor-bearing $CD1d^{-/-}$ mice produce higher levels of TNF-α than their WT counterparts, B16LS9 melanoma cells are not sensitive to TNF-α killing. Therefore, we conclude that TNF-α is dispensable in the battle against metastatic ocular melanoma. Moreover, a deficiency in TNF-α did not lead to an increase in the number of liver metastases in NKT cell-deficient mice but surprisingly resulted in a mitigation of metastases compared to WT mice. These data suggest that TNF-α plays a pro-tumorigenic role in the formation liver metastases in NKT cell-deficient mice. The pro-inflammatory activities of TNF-α are very well established. Recently though, several mechanisms for immunosuppressive effects of TNF-α have been proposed. It has been shown that TNF-α can exert immunoregulatory effects by inhibiting T-cell receptor signaling, inducing apoptosis in CD8+ T-cells or by impairing antigen presentation by dendritic cells (301). However, we have previously shown that the reduced development of liver metastases in NKT cell-deficient mice is T cell-independent (302).

Resistance to liver metastases in NKT cell-deficient mice is perforin-dependent, FasL- or TRAIL-independent

The role of perforin which is exclusively expressed by NK cells and CTLs and is used in lysing tumor cells and inhibiting tumor growth is well-established (303). Perforin is also needed for NK cell mediated-cytotoxicity against B16LS9 melanoma cells. However, the comparable

expression of perforin in melanoma-bearing WT and NKT cell-deficient mice suggests that the increased liver NK cell-mediated cytotoxicity in NKT cell-deficient mice is not the result of increased production of perforin in the NKT cell-deficient host.

Surprisingly, a deficiency in FasL, did not result in an increase in the number of liver metastases in NKT cell-deficient mice, but instead caused the reduction of metastases formation compared to WT mice, suggesting that FasL has an immunoregulatory role in the formation of liver metastases in NKT cell-deficient mice. Recently, a number of studies have reported that FasL can dampen immune response by transmitting signals into FasL-bearing cells as well as Fas receptor expressing target cells via a mechanism called "reverse signaling" (304, 305). Sub populations of NK cells express Fas receptor on their surface (306, 307). Therefore, another possible explanation for the observed immunoregulatory effect of FasL in this melanoma model is that FasL expressing liver NK cells can kill Fas receptor expressing NK cells resulting in a depletion of liver NK cells. However, we found no evidence of attrition of liver NK cells and there were no differences in the number of liver NK cells in $CD1d^{-/-}$ mice and WT mice.

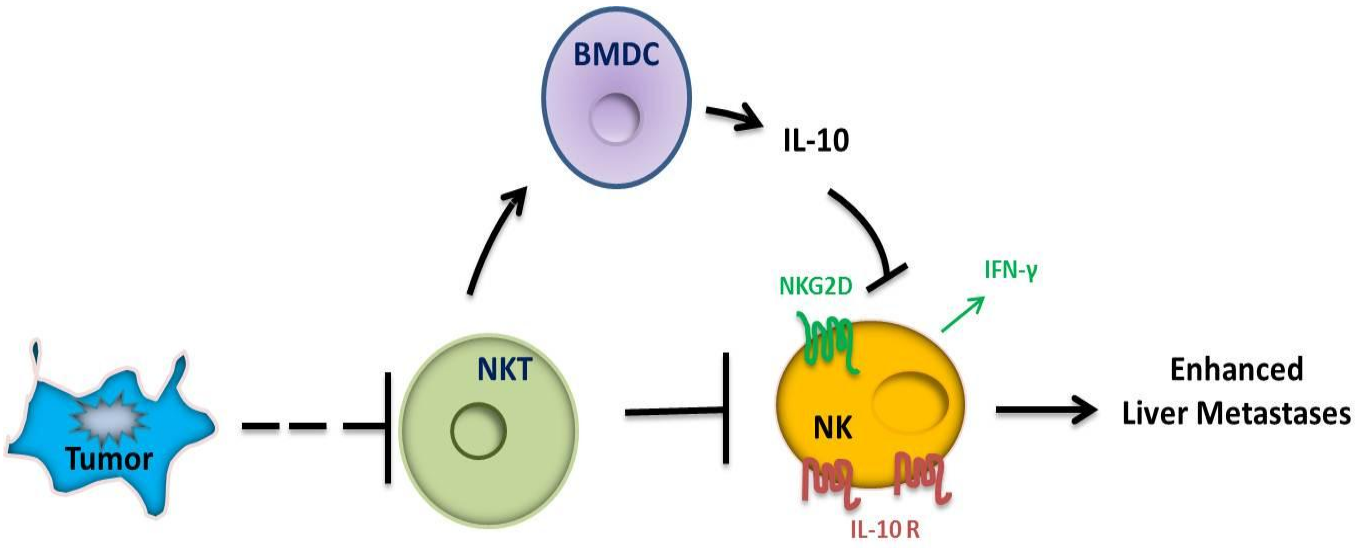
Finally, we showed that despite the expression of TRAIL receptor on B16LS9 melanoma cells, neutralization of TRAIL had no measurable impact on liver NK cell cytotoxicity in $CD1d^{-/-}$ mice. These data indicate that the resistance to liver metastases in NKT cell-deficient mice is also independent of TRAIL.

Proposed model of liver NK cell suppression by melanoma-induced NKT cells

The results of this study indicate that the presence of melanoma liver metastases induces a regulatory phenotype in liver NKT cells, which is associated with the elevated production of IL-10 by bone marrow-derived cells (BMDCs) in the liver. The excessive amounts of IL-10

produced by BMDCs down-regulate the expression of NKG2D activating receptors and the production of IFN-γ on liver NK cells. Simultaneously, the expression of IL-10 receptors on liver NK cells is elevated, which makes them more sensitive to the immunoregulatory effects of IL-10. These conditions in turn eventuate in depressed liver NK cell-mediated tumor immunity and a commensurate increase in the severity of melanoma metastases in the liver (**Figure 28**).

Figure 28. A model for the suppression of liver NK cells anti-tumor function by melanoma-induced NKT cells. Tumor-induced NKT cells stimulate the production of IL-10 in the liver, which suppresses the expression of NKG2D on NK cells and reduces their secretion of and IFN-γ. Additionally, liver NK cells up-regulate the expression of IL-10 receptors on their surface resulting in diminished NK cell-mediated cytotoxicity and the enhanced formation of liver metastases.



Immunotherapy of UM

As mentioned earlier, there are multiple options available for the treatment of primary UM that restrain the growth of primary tumor within the eye. However, none of these therapies are capable of preventing the dissemination of UM cells into the liver. The development of liver metastases is the major cause of death in UM patients and no conventional treatment exist to prevent it. Immunotherapy is one of the novel approaches that is being explored for its effectiveness against UM. UM cells express melanoma specific antigens that can stimulate the adaptive immune system and serve as targets for T cell-based immunotherapy. Recently, administration of Ipilimumab, which is a human monoclonal antibody that blocks cytotoxic T lymphocytes-associated antigen 4 (CTLA-4), has shown to improve overall survival of patients with metastatic UM (308). CTLA-4 acts as a regulatory molecule to prevent T cell activation (308). However, most UM cells are weak immunogens and are unable to induce a robust immune response (309).

Most of the NKT-based immunotherapeutic approaches used for cancer treatment are based on the anti-tumor activity of iNKT cells. However, it is becoming evident that NKT cells perform both effector and regulatory functions in cancer. Thus, this study as well as several others, indicates that iNKT cells are able to acquire regulatory functions (310). The functional heterogeneity of NKT cell subsets partially explain some of the opposing roles NKT cells play in tumor immunity. This study suggests that tumor microenvironment has profound effects on inducing regulatory phenotype in iNKT cells and has to be taken into consideration when designing immunotherapy for the management of liver metastases in UM patients. After identifying whether NKT cells play an anti-tumorigenic or regulatory role, the goal of future anti-tumor immunotherapy, should be to block the activation of regulatory NKT cells for

instance by blocking CD1d or to enhance the activity of effector NKT cells by infusing α-GalCer-loaded DCs into the patients.

There has been considerable interest in employing NK-based immunotherapy for various malignancies such as renal cell carcinoma, malignant melanoma and hepatic cancers (53). Numerous studies have been conducted to enhance the anti-tumor functions of NK cells by administration of cytokines that activate NK cells such as IL-2, IL-12, IL-15 and IFN-α (53). Patients with metastatic UM have been treated with IL-2 or IFN-α, however the efficacy of these NK cell-based tumor therapies has not been confirmed (311). The results reported here suggest that the profound effect of NKT cells on NK anti-tumor activity, as well as the organ specific factors that may affect the function of NK cells should be considered when such therapies are designed and implemented.

Future directions

In this study we investigated the interplay between liver NKT cells and NK cells in resistance to liver metastases. The present findings shed light on the mechanisms by which melanoma-induced iNKT cells inhibit the effector functions of liver NK cells. Our *in vitro* data suggested that NKT cells are unable to suppress NK cell-mediated cytotoxicity directly which implies a possible involvement of a third party cell in the NKT cell-induced NK cell suppression in melanoma-bearing mice. Our investigations rule out the role of cancer-induced immunosuppressive MDSCs in promoting the formation of liver metastases in mice harboring intraocular melanoma. Therefore, further experiments need to be conducted to determine what cells are involved in NKT-dependent NK cell suppression. The liver is composed of many different cell types such as Kupffer cells, DCs, hepatic stellate cells, LSECs and even hepatocytes that can affect NKT cells activation and function in a CD1d-dependent manner (137). All of these CD1d expressing liver cells have tolerogenic properties and therefore can play a role as a third party cell to induce a regulatory phenotype of liver NKT cells (137). Our preliminary experiments suggest that $Gr1^+$ Kupffer cells are the most plausible candidate for NKT cell-induced NK cell suppression. We showed that the depletion of Kupffer cells significantly enhanced the number of liver metastases (unpublished data). Kupffer cells are the most abundant APCs in the liver and have the ability to present lipid antigen to NKT cells in a CD1d-dependent manner (137). Cytokines that are secreted by macrophages cells such as IL-2, IL-12, IL-15, IL-18, and type I IFN are potent activators of NK cells (312). Therefore, our current hypothesis proposes that tumor-induced NKT cells suppress the production of pro-inflammatory cytokines by Kupffer cells which prevents the Kupffer cell-induced activation of NK cells. Therefore, in NKT cell-deficient mice, NK cells are activated to their full potential

which results in the elimination of liver metastases in these mice. Investigating these possibilities was beyond the scope of this dissertation but needs to be addressed in the future.

As noted earlier, IL-10 plays a key role in the formation of liver metastases in melanoma-bearing mice. Our data indicated that WT mice have elevated levels of IL-10 in their liver because NKT cells induce the production of IL-10 bone marrow-derived non-NKT cells. A variety of liver bone marrow-derived cells can produce IL-10 in the livers of tumor-bearing mice, including Tregs, MDSCs, and M2 macrophages. Future studies can examine which one of these cells is responsible for the enhanced production of IL-10 in melanoma-bearing mice. In addition to the presence of the higher amounts of IL-10 in the liver of melanoma-bearing WT mice, the expression of IL-10 receptor is up-regulated in liver NK cells of NKT cell-competent mice when compared to NKT cell-deficient mice. These data indicated that NKT cells exert their regulatory functions through IL-10 via a variety of mechanisms. Schreibe's group reported that LPS induces the expression of IL-10 receptor on fibroblast (313). Unfortunately, other factors that modulate the expression of IL-10 receptors are poorly understood. Future experiments will elucidate the molecular mechanisms by which NKT cells induce the expression of IL-10 receptor on liver NK cells in melanoma-bearing mice.

A balance of signals from activating receptors and inhibitory receptors control NK cell activation (52). In mice, NK cells express multiple activating receptors including NKp46, NKG2C, Ly49D and Ly49H as well as inhibitory receptors such as NKG2A (53). In this study we assessed the expression of one of the activating receptors, NKG2D on liver NK cells. However, in order to fully understand the mechanisms underlying NKT cell-induced NK cell suppression, the expression of other receptors also needs to be examined.

Finally, while investigating the role of TNF-α and FasL in the formation of liver metastases in NKT cell-deficient mice, we were surprised to observe that the absence of these pro-inflammatory factors resulted in the mitigation of liver metastases. The regulatory roles of TNF-α have been documented in several autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) or suppression of DTH responses (314). Masli et al. suggested that TNF-α exerts its anti-inflammatory effects through signaling via TNF-receptor 2 (314). Some reports indicate that FasL is capable of diminishing the immune response (304), however, its molecular mechanisms have not yet been fully understood. Thus, further investigations are required to reveal the mechanisms underpinning the pro-tumorigenic roles of TNF-α and FasL in NKT cell-deficient mice.

In conclusion, this study has improved our understanding of molecular and cellular mechanisms of cross-talk between NK cells and regulatory NKT cells in a tumor microenvironment. Hopefully these findings will provide helpful hints for designing immunotherapeutic modalities for the treatment of metastatic UM.

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