Immunoregulation By HCV-generated Myeloid-Derived Suppressor Cells

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A Dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

Department of Microbiology, Immunology, and Cancer Biology

University of Virginia
January 2016

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Abstract

The hepatitis C virus (HCV) infects more than 200 million people worldwide and establishes chronic infection in 80% of these patients, resulting in chronic persistent liver inflammation and liver disease including most notably cirrhosis and hepatocellular carcinoma. HCV's ability to establish persistent infection can be partly attributed to its immune evasion strategies, including the induction of a myeloid-derived suppressor cell (MDSCs) response.

As innate immune effectors, NK cells play an important role in controlling HCV infection and therefore suppressing chronic disease progression. IFN-γ production by liver NK cells likely suppresses virus replication in exposed hepatocytes and is important for the initiation of the adaptive immune response. However, following HCV infection, NK function is significantly impaired in chronic HCV patients compared to HCV patients who clear the infection. The cellular and molecular mechanisms to account for impaired NK function are not established.

In my research, I analyzed the interaction of human NK cells in vitro with $CD33^+$ PBMCs following prior exposure of the $CD33^+$ cells to HCV. We found that following co-culture of NK cells with HCV-conditioned $CD33^+$ PBMCs, NK cell IFN-γ production is significantly inhibited while granzyme release by stimulated NK cells is normal. This suppression of NK cell-derived IFN-γ production is mediated by $CD33^+CD11b^{lo}HLA\text{-}DR^{lo}Arg\text{-}1^+$ MDSCs. The suppression of IFN-γ production is reversed by L-arginine

supplementation, consistent with L-arginine depletion as a result of MDSC arginse-1 activity. Moreover, unlike IFN-γ, granzyme B production was not affected by depletion of L-arginine. In addition, I have determined that the mTOR pathway is responsible for decreased IFN-γ translation. Taken together, these results suggest that HCV employs an immunoregulatory mechanism to alter NK cell metabolic state, which in turn inhibits one of their main effector functions, through the generation of suppressive myeloid cells. This further implies that the blockade of MDSCs' suppressive effect on NK cells may be a potential therapeutic target to prevent chronic HCV infection and development of chronic liver diseases.

Dedication

This dissertation is dedicated to my husband, Jason Yergin,
and my puggle, Lachlan Jake.

Acknowledgements

First and foremost, I would like to thank my mentor, Dr. Young Hahn, without whom none of this is possible. I was very lucky to have such a patient and thoughtful mentor who guided me but allowed me to grow as a scientist and as a person. I would also like to thank my committee members, Drs. Amy Bouton, Jeff Saucerman, Roger Abounader, Loren Erickson, and Thomas Braciale for their insight into my project and for challenging me to become a more thorough and creative member of the scientific community.

I would also like to thank the members of the Hahn lab for their help, especially Sowmya Narayanan, for their help, both intellectually and physically, in the development of my thesis project. I'm fortunate to have worked in such a collaborative and supportive environment. In the Hahn lab, we had the privilege of working with the Braciale, Tung, Brown, and Bender labs, and I would like to thank them for their contributions to my project.

Last but not least, I'd like to thank my friends and family for their support, and the Biotechnology Training Program who made my life as a graduate student more interesting and exciting. Here's a final shout out to the UVa Gymnastics Club who kept me healthy and sane during my time at Uva.

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List of Abbreviations

4EBP1	4E-Binding Protein 1
APC	Antigen presenting cell
Arg	Arginase
ATRA	All-trans retinoic acid
Cat	Catalase
COX	Cyclooxygenase
CSF	Colony stimulating factor
DC	Dendritic cell
eIF4e	Eukaryotic translation initiation factor 4E-binding protein 1
ELISA	Enzyme
FACS	Fluorescence-Activated Cell Sorter
FLIP	FLICE-like inhibitory protein
FMO	Fluorescence minus one
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCV	Hepatitis C Virus
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
ICCS	Intracellular cytokine staining
IDO	Indolamine 2,3-dioxygenase
IFN	Interferon
IFNG	Interferon gamma gene
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JFH-1	Japanese Fulminant Hepatitis 1
L-NMMA	L-NG-monomethyl Arginine citrate
m.o.i.	Multiplicity of infection
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence Intensity
MHC	Major histocompatibility complex
miRNA	mircroRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKT	Natural Killer T
NO	Nitric Oxide
Nor-NOHA	Nω-hydroxy-nor-Arginine
NTP	Nucleoside triphosphate
PBMC	Peripheral blood mononuclear cells

PGE	Prostaglandin E
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	Regulatory T cell
UTR	Untranslated region

Chapter 1: Introduction

I. Hepatitis C Virus

Hepatitis C virus (HCV) is a member of the flaviviridae family, approximately 40-70 nm in diameter (1-3). Its positive-stranded RNA genome is approximately 9.6kb, including non-translated regions at the 5' and 3' termini and a single open reading frame encoding a large polyprotein (4-6). The viral particle is formed from the core protein and the envelope glycoproteins E1 and E2. The viral genome also encodes nonstructural proteins— p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (7), many of which are targets for HCV-treatment. The core protein is a highly basic, non-glycosylated nucleocapsid protein that binds viral RNA (8, 9). It can be found in both the complete HCV virion and as an RNA-free protein in the serum of HCV patients (10, 11). The HCV core protein serves more than one purpose. Firstly, the core protein forms the capsid shell that houses and protects the HCV genomic RNA while the virus infects a hepatocyte. More relevant to immunology, HCV core protein modulates several host pathways by interacting with a variety of cellular factors (12). Of note, core binding to gC1qR, a cell surface receptor needed for viral entry, on host immune cells has been reported to generate myeloid-derived suppressor cells or MDSCs (13).

HCV is a hepatotropic virus that is known to infect only humans and chimpanzees (14).

The lack of small animal models makes the study of the virus particularly challenging; even artificial expression of human proteins in mouse does not make the mouse susceptible to the virus (15). Chimpanzees are not only expensive, but also fail as an animal model, because they are able to clear HCV infection, unlike most humans (16, 17). Given these limitations, the majority of research investigations on chronic hepatitis C are performed *ex vivo* or *in vitro*.

Until the early 2000s, scientists were unable to grow the virus efficiently using standard cell culture techniques (18, 19). Virus grown in cell culture was not infectious (20), perhaps due to missing host factors (21), negating its use in drug development studies. In 2005, Kato and Wakita successfully developed a system where the JFH1 strain of HCV could produce infective virus particles when grown in Huh7, a human hepatoma cell line (22). The JFH1 virus (genotype 2a) has been shown to be neutralized by CD81 specific antibodies and antibodies from the serum of chronically infected patients (3). The development of this system has advanced the study of HCV and paved the way for the development of potential therapies in the last seven years.

HCV can cause significant liver damage to many infected patients because about 70% of HCV-infected patients cannot resolve the infection and become chronically infected (23). Chronically-infected patients typically develop severe liver disease including fibrosis and cirrhosis (24). Extensive fibrosis impairs liver function while cirrhosis can lead to hepatocellular carcinoma and liver failure (25). The development of fibrosis and cirrhosis is partly attributed to oxidative stress, as HCV infection is characterized by a massive

upregulation of reactive oxygen species (ROS) in the liver. While ROS benefits the host by suppressing HCV replication through disruption of HCV replication complexes (26), it also non-specifically oxidizes essential biological macromolecules, leading to acute cell damage and loss of cellular functions (25). Furthermore, the persistence of the virus leads to chronic inflammation, generating additional ROS (27) through activation of the inflammosomes by the virus in the hepatocytes (28). Hence, identifying strategies that resolve the acute infection is a prudent way in preventing chronic infection by HCV, and as such reduce the morbidity and mortality of the disease.

The innate branch of the immune system may play a detrimental role in determining if a patient clear an acute infection. Of note, the Natural Killer (NK) cell and its effector functions have been implicated in chronic hepatitis C. A strong NK cell response is needed for the activation of a robust adaptive response (29). Chronic HCV patients have been reported to have dysfunctional NK cells with impaired effector function or even altered phenotypes (18, 29).

II. Natural Killer cells (NK cells)

NK cells are innate lymphoid cells that comprise 10-20% of lymphocytes in human peripheral blood (18). In healthy livers, they exist in even greater abundance, making up 25-30% of lymphocytes (30). This high abundance of hepatic NK cells suggests that NK play an important role in liver immune responses, both in fighting infections and in maintaining liver function.

NK cells are the early responders in any viral infection (31). They are known to secrete Granzyme B and perforin to lyse virally infected cells (32), induce apoptosis of virally infected cells, and secrete cytokines such as TNF-α and IFN-γ (29). The NK cell responses, especially IFN-γ production, is detrimental to a good adaptive response (29, 32). As part of the acute response, IFN-γ produced by NK have direct cytotoxic effects on virally infected cells (18), elicit an anti-viral state in the uninfected cells (33), and induce chemotaxis to recruit cells of the adaptive immune response (34). Not coincidentally, patients who develop chronic hepatitis C have poor NK cell responses (35, 36). Therefore, good IFN-γ production might be prognostic of viral clearance.

NK cells are categorized into different subsets based on the expression of surface markers, which is thought to correlate with their effector functions. The $CD56^{high}$ NK cells are usually the cytokine producers, while $CD56^{low}$ NK cells are more cytotoxic, but produce less IFN-γ (1, 4). Some groups report that chronic HCV patients' NK cells shift from the IFN-γ-producing to the cytotoxic subtypes (37, 38), while other groups have

reported a decrease in both cytotoxicity and cytokine production (39). Others have yet reported that NK cells from hepatitis C patients can be stimulated to produce IFN-γ just as well as those from healthy individuals, but cytotoxicity was increased (40). With so many conflicting reports, understanding NK cells in chronic hepatitis C is of great importance. While increased cytotoxicity may appear more favorable than cytokine production, it may, in fact, be detrimental to clearing the infection, because IFN-γ-mediated viral clearance can act on many cells at once, while direct killing of infected hepatocytes requires 1:1 cell-to-cell contact (18, 41). Moreover, chronic HCV patients have a subset of $CD56^-$ NK cells that are impaired in both their IFN-γ production as well as their cytotoxicity (18, 35).

Furthermore, in chronically infected liver, only 1-2% of T cells are HCV specific (42) and this cell-mediated response is often too weak to match the viral loads generated by the time the adaptive response is activated (18). Because the T cell response often fails to effectively clear HCV, NK cells present an alternative, and perhaps more powerful tool to combat HCV infection. Supporting my theory, NK cells have been implicated in direct cytotoxicity of infected hepatocytes (18) and IFN-α treatment rapidly activates NK cells, enabling them to participate more vigorously in viral clearance (16).

There are numerous reasons why NK cells and their functions are important in the prevention of chronic HCV infection. Thus, it is important to elucidate the mechanism of impaired NK function during HCV infection, in hope of designing of vaccines against HCV to boost NK cell responses.

In vitro, NK cells produce IFN-γ in response to IL-12 and IL-18 stimulation (43, 44) and IFN-γ plays an important role in controlling HCV replication (45). As such, this cocktail is used throughout my experiments.

III. Myeloid-Derived Suppressor Cells (MDSCs)

Chronic viral infections are an ongoing battle between viruses and the immune response, characterized by the sustained recruitment of immune cells matched by an equally stubborn and unrelenting presence of virus. At the forefront, among the many reasons for viral persistence, are myeloid derived suppressor cells (MDSCs), a heterogeneous population of myeloid progenitors and immature myeloid cells that share the ability to suppress immune responses. MDSCs were first described in 1987 in a mouse model of lung cancer as bone marrow derived cells that inhibited T cell proliferation (46). Over the next two decades, these cells have transformed the field of cancer research, even serving as a marker of disease progression in human cancers (47, 48). Given that tumors are characterized by a constant, albeit dysfunctional immune response, it is not surprising that recent studies have begun to identify a parallel role for MDSCs in other chronic inflammatory states. In this review, we describe recent studies on MDSCs in viral infections, paying particular attention to the molecular mechanisms that aid in the recruitment and function of this immunosuppressive population.

MDSCs can be broadly classified into two groups—granulocytic and monocytic. As the name suggests, monocytic MDSCs appear similar to monocytes in that they have a single, large, round nucleus, while granulocytic MDSCs have multi-lobed nuclei resembling those of polymorphonuclear cells (49). Since they are morphologically similar to mature immune cells, MDSCs are also distinguished by the expression of surface markers and distinct mechanisms of suppression. Murine MDSCs differ in their

expression of Gr-1, a myeloid lineage marker that is recognized by antibodies to Ly6G and Ly6C: granulocytic MDSCs are defined as $CD11b^{+}Ly6G^{+}Ly6C^{low}$ while their monocytic counterparts are $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ (50). Different phenotypic markers characterize human MDSCs. It is generally accepted that MDSCs are $CD33^{+}$ $CD11b^{+}HLA\text{-}DR^{lo/-}$ (51). $CD14^{+}$ MDSCs are considered the monocytic MDSCs while their $CD14^{-}CD15^{+}$ counterparts are considered the granulocytic MDSCs (52-54). Notably, the hallmark of all MDSCs is their ability to suppress immune responses, which provides a further understanding their roles in normal physiology and disease.

MDSCs exert a range of effects. They can affect other cells via antigen-specific suppression, by the alteration of cell-surface molecules, or via a host of soluble mediators (55, 56). The three key mechanisms that MDSCs employ are via the production of reactive oxygen species (ROS), the effects inducible nitric oxide synthase (iNOS), and the effects of arginase-1(57). Generally, murine granulocytic MDSCs are thought to exert their immunosuppressive effects via ROS, usually generated by NADPH oxidase; monocytic MDSCs produce nitric oxide (NO) via iNOS (49, 58, 59). ROS, particularly hydrogen peroxide, can act on immature myeloid cells to reduce their ability to differentiate into macrophages and dendritic cells (DCs) (60) and catalyzes the nitration of the TCR, thereby preventing T cell-peptide-MHC interactions. Similarly, iNOS, in conjunction with arginase-1 or limiting concentrations of L-arginine, generates reactive nitrogen-oxide species, which also nitrosylate the TCR, thus resulting in T cell suppression or apoptosis (61). Both granulocytic and monocytic MDSCs were also reported to deplete L-arginine, which is particularly important for the survival and

function of T cells, through the action of arginase-1 (62, 63). Interestingly, the suppression of T cells by MDSCs may represent a mechanism of controlling inflammation, as evidenced by studies where treatment with IL-2, a cytokine that is essential for T cell proliferation and function, increased the numbers of arginase-positive MDSCs in cancer patients (64). Similarly, the effect of Th1 and Th2 cytokines on arginase-1 further validates the crosstalk between MDSCs and T cells: arginase-1 expression and activity are enhanced by the Th2 cytokine, interleukin-4 (IL-4), both by itself (65) or in conjunction with IL-13 (66), whereas the Th1 cytokine, interferon-gamma (IFN-γ), upregulates arginase-1 expression (67).

In contrast to murine MDSCs, human MDSCs are not characterized by such clear differences. Moreover, phenotypically distinct human MDSCs have been reported to use the same mechanisms of suppression, negating the use of cell surface markers to distinguish between subsets. In general, among the MDSCs defined as $CD33^{+}CD11b^{+}HLA\text{-}DR^{lo/-}$, the $CD14^{+}$ MDSCs are thought to be monocytic MDSCs, while the $CD14^{-}CD15^{+}$ MDSCs are considered granulocytic subtype (68, 69). Continuing this comparison to the murine paradigm, one would expect that the $CD14^{+}$ monocytic MDSCs would use iNOS and arginase-1, while the $CD14^{-}$ granulocytic MDSCs would use ROS and arginase-1. However, unlike their mouse counterparts, both $CD14^{+}$ and $CD14^{-}$ MDSCs have been reported to share the same mechanism of suppression as they both suppress T cell proliferation and IFN-γ production using ROS generated by NADPH oxidase (13, 70, 71). Human MDSCs are also capable of producing arginase, as $CD14^{+}HLA\text{-}DR^{lo/-}$ MDSCs from hepatocellular carcinoma patients displayed elevated

arginase activity that not only inhibited T-cell proliferation, but also induced IL-10 producing Tregs, which further dampen the immune response (72).

Although several studies report that MDSCs inhibit natural killer (NK) cell activity, the mechanisms underlying MDSC-mediated inhibition of NK cell responses are elusive. $CD14^{+}HLA\text{-}DR^{low/-}$ MDSCs from hepatoceullar carcinoma patients were found to suppress NK cell cytotoxicity and IFN-γ release (73). This suppression was an arginase-1 independent, contact-dependent effect that required the expression of the NK cell receptor, NKp30. In murine studies, MDSCs expanded in tumor bearing mice inhibited NK cell cytotoxicity, IFN-γ production, and expression of the activating receptor, NKG2D, through membrane-bound TGF-β1 (74). Surprisingly, MDSCs were also reported to activate NK cells by inducing the expression of Rae-1, a ligand for NKG2D (75). In this study, the interaction of NK cells with MDSCs from RMA-S tumor bearing mice resulted in NK cell activation and copious production of IFN-γ. Moreover, it is important to note that NK cell development and function are highly dependent on the expression of MHC class I. Therefore, it is likely that the interaction of NK cells with MDSCs from RMA-S tumor bearing mice would alter NK cell function, leading to MDSC-mediated activation rather than suppression. Thus, our current understanding of MDSC biology indicates that MDSCs suppress NK cells just as readily as they suppress T cells.

Not surprisingly, in addition to interacting with T cells and NK cells, MDSCs have also been reported to hamper antigen presenting cell (APC) functions. The accumulation of

MDSCs in cancers is often accompanied by the lack of DC maturation (76, 77). In the tumor microenvironment, MDSCs prevent DCs' ability to uptake antigen, thereby limiting a key contribution of DCs to the immune response (78). MDSCs also reduce the efficacy of DC vaccines by inhibiting the migration and T cell activation capacity of DCs (79), consistent with their ability to reduce DC and T cell stimulating activity (80).

Conflicting reports of the need for cell-cell contact for MDSC-mediated suppression further complicate our understanding of this population. A cursory overview of the literature suggests that several suppressor functions of MDSCs are dependent on cell-cell contact (65, 81). Considering that ROS and NO are soluble, short-lived mediators, such a need for cell-cell contact is not unexpected, as it reduces the distance between these effector molecules and their target cells. However, these cells also produce factors that remain stable in blood and act at long distances, hinting at the existence of cell-contact independent effector functions. Such ability to use multiple mechanisms of suppression makes MDSCs a versatile and potent population of cells, allowing them to effectively hinder the immune system's efforts against tumors (Fig. 1.1).

Factors involved in promoting the generation and accumulation of MDSCs

Studies examining the pathogenesis of tumor development have elucidated several mechanisms that direct the generation, recruitment, and accumulation of MDSCs. The tumor microenvironment is a dynamic participant in the recruitment of suppressive immune cells including MDSCs. Tumor and stroma-derived factors induce the generation of MDSCs, their chemotaxis to the tumor site, and their survival within the tumor (82).

Moreover, upon their initial appearance at the tumor site, MDSCs then further propagate and contribute to tumor growth by recruiting additional MDSCs and other immunosuppressive populations. Given the similarities in the microenvironments of tumors and of chronic inflammatory sites, it is likely that viruses that establish chronic infections also change the local inflammatory response, thus recruiting MDSCs in a manner similar, if not identical, to tumors.

Generation of MDSCs

As a collection of myeloid progenitors and immature myeloid cells, MDSCs are induced by factors, including soluble mediators and transcription factors that affect the activation and differentiation of myeloid populations. I here present a list of mediators and factors that have been known to induce the generation of MDSCs.

Prostaglandin E2 (PGE2). A key mediator of inflammatory responses, PGE2 is derived from the common arachadonic acid metabolite, prostaglandin H2, through a reaction catalyzed by the enzyme prostaglandin synthase. PGE2 has a number of biological actions, both anti- and pro-inflammatory, including a prominent role in the generation of MDSCs. Recent studies demonstrate that administration of exogenous PGE2 can block the differentiation of DCs and redirect myeloid progenitors to adopt features characteristic of MDSCs (83). Additionally, a positive-feedback loop between PGE2 and COX-2 induces the transcription of NOS2, indolamine-2,3-deoxygenase, and IL-10, all of which are classically immunosuppressive molecules (84-86). The importance of the PGE2-COX-2 axis to the generation of MDSCs is further accentuated by the significant

improvement in prognosis with the administration of COX2 inhibitors in numerous cancers, including colon and ovarian carcinomas (87, 88). Since several tumors have been reported to express high levels of PGE2 (89), it seems likely that the effectiveness of COX2 inhibition on tumor growth is in part due to the decrease in the generation of MDSCs.

SCF, HIF-1α, IL-6, and M-CSF. While the PGE2-COX-2 loop presents an attractive and readily available target for potential therapies, other factors that trigger the generation of MDSCs may not be as easily inhibited given their roles in normal physiology. For instance, stem-cell factor (SCF), like PGE2, is also expressed by many human and murine cancers. Blocking SCF/c-kit signaling with anti-SCF siRNA or anti-c-kit blocking antibodies resulted in fewer MDSCs at the tumor sites in mice, which corresponded with decreased tumor-specific T cell anergy, Treg generation, and tumor angiogenesis (90). However, given that SCF is a necessary component of hematopoietic homeostasis, it presents a less attractive target than more redundant targets such as PGE2. In addition, MDSCs express high levels of HIF-1α (51). HIF-1α, whose expression is triggered by hypoxia, directly affects the functions of MDSCs in the tumor microenvironment, upregulating their iNOS and arginase-1 activities (91).

Tumors are also known to secrete copious amounts of IL-6 and M-CSF (92, 93), both of which play a role in myeloid cell development, yet also inhibit myeloid progenitors from differentiating into DCs (94). Multiple tumor cell lines were shown to upregulate expression of M-CSF (95), while IL-6 levels directly correlated with numbers of MDSCs

in vivo (96). The influence of immune mediators in the generation of MDSCs is further evident in a study where blocking of IL-6 signaling significantly slowed tumor growth (97). This effect is explained in part by a decrease in activated STAT3, a key player in MDSC accumulation, as described below.

STAT3. A member of the STAT family of transcription factors, STAT3 is a stronghold of cellular function, as it is downstream of several receptors, including a variety of anti- and pro-inflammatory cytokines. A number of reports pinpoint STAT3 hyperactivity as the culprit in arresting the differentiation of myeloid progenitor cells, particularly DCs, veering them instead towards an MDSC phenotype (98, 99). STAT3 signaling upregulates myeloid-related protein S100A9, which not only prevents DC differentiation, but also contributes to the accumulation of MDSCs (76). Furthermore, STAT3 enhances the immunosuppressive activity of MDSCs by upregulating NADPH oxidase, leading to increased ROS production (70). Not surprisingly, inhibition of STAT3 reduces the presence of MDSCs in tumors (100). Interestingly, the hepatic gp130 protein, an acute phase reactant that signals through STAT3, induces the accumulation of MDSCs as a mechanism of limiting inflammation (101). Thus, as with other strategies of immune evasion, STAT3's role in inducing the accumulation of MDSCs is a physiologically important process that is hijacked by tumors and very likely, by chronic viral infections, in order to evade an effective immune response.

IL-1β and S100A8/A9. While the factors described thus far originate from the tumor itself, MDSC-generated mediators also appear to propagate the accumulation of MDSCs.

Tumors produce copious amounts of IL-1β which initiates the generation of MDSCs (102). In a murine model of IL-1β secreting breast cancer, surgical removal of the tumor alone did not curtail recruitment of additional MDSCs (103). In addition, the MDSCs generated during tumor development continued to synthesize IL-1β even in the absence of the tumor, further propagating their recruitment. Similarly, the S100A8/A9 pro-inflammatory proteins, which also stimulate MDSC recruitment (104), are another class of molecules that are both tumor and MDSC-derived (69). S100A9 is a member of the S100 family of calcium-binding proteins and is expressed in granulocytes, monocytes, and macrophages during acute and chronic inflammation. Binding of S100A9 to its receptor RAGE enhances arginase expression in an NF-κB-dependent manner, increasing the suppressive capacity of MDSCs (55, 69, 104). Thus, the multitudes of factors capable of triggering MDSC generation indicate that MDSCs themselves play a prominent role in propagating their accumulation.

Chemotaxis of MDSCs

Considering that a large number of mediators produced by MDSCs act at short distances, it is necessary for these cells to migrate to the site of an ongoing immune response in order to fully exercise their immunosuppressive effects. MDSCs, or rather immature myeloid cells, are thought to originate in the bone marrow (55) and are increased in the blood, lymph nodes, and tumor sites of cancer patients (49). Egress from the blood to the tumor is dependent on CXCR4, which, not surprisingly, is also necessary for the chemotaxis of mature myeloid cells.

Several tumor-derived factors, such as TGF-β and PGE2, increase expression of chemokine receptors on MDSCs (105-107). TGF-β upregulates miR-494 in MDSCs, leading to a degradation of PTEN and concurrent increase in the CXCR4 expression (108). PGE2 can also induce the expression of CXCR4 and its ligand, CXCL12, in a COX-2 dependent manner, allowing the influx of MDSCs to the tumor microenvironment (109). Moreover, among its many MDSC-related functions, IL-1β also affects MDSC mobilization and recruitment (110, 111). As MDSCs themselves are able to produce IL-1β (112), these studies are evidence of self-propagated and self-sustained mechanisms of generating and recruiting MDSCs to the tumor and presumably, to other sites of chronic inflammation.

Survival of MDSCs

While the genetic abnormalities of malignant cells allow them to endure the harshness of tumor microenvironments, accessory cells, including MDSCs, have developed mechanisms that aid their survival without resorting to transformation. As mentioned above, TGF-β-mediated increase in miR-494 degraded PTEN in MDSCs, which, in turn, activated the PI3K/Akt pathway, leading to enhanced activity of mTOR and NF-κB, both of which promote cell survival (108). TNF has similar pro-survival properties in that signaling via the TNFR2 on MDSCs upregulates c-FLIP and consequently, inhibits caspase-8 activity (113). Moreover, TNF also activates NF-κB and upregulates expression of COX-2 and PGE-2 (114), emphasizing the importance of this cytokine to MDSC biology. Yet again, IL-1β has been implicated in the survival of MDSCs as it promoted the accumulation of a subset of MDSCs lacking Ly6C (102). Although the

exact signals mediating IL-1β dependent survival of MDSCs are not known, it is likely that they are similar to the enhanced survival effect of IL-1β on polymorphonuclear cells (115), especially since low levels of Ly6C are characteristic of granulocytic MDSCs. Clearly, the mechanisms orchestrating the generation and chemotaxis of MDSCs are just as heterogeneous and numerous as MDSCs themselves (Fig. 1.2). As more studies examining the appearance of MDSCs and their immunosuppressive function in non-cancer systems begin to emerge, it will be important to confirm if the same mechanisms are responsible for the recruitment of MDSCs in these settings. Findings from such studies will not only inform our understanding of the pathophysiology of malignant and non-malignant diseases, but may also provide the basis for the development of targeted treatments with numerous clinical applications.

Epigenetic Control of MDSCs

Considering that MDSCs are derived from the same pool of cells that give rise to non-immunosuppressive populations, their distinct ability to suppress other immune cells raises the possibility of changes in epigenetic signatures. A recent study reported that the histone deacetylase-2 (HDAC-2) was instrumental in repressing expression of the retinoblastoma (Rb) gene, which, in turn, converted monocytic MDSCs into granulocytic MDSCs (116). The results of this study present a mechanism for the generation of granulocytic MDSCs, which is especially promising, since HDAC inhibitors have long been explored as therapeutic agents for cancer treatment. However, the granulocytic MDSCs generated in the models used in this study did not acquire an immunosuppressive phenotype by mere repression of Rb; as the authors note, acquisition of suppressive

properties was a product of the tumor microenvironment, once again emphasizing the crosstalk between tumors and MDSCs.

Consequently, the results of this study prompt a slew of questions regarding other epigenetic mechanisms that control MDSC function. In particular, what are the changes in chromosomal modifications brought about by the action of tumor-derived factors on infiltrating MDSCs? For instance, HDAC-11 was found to repress expression of IL-10, a key immunosuppressive cytokine that is copiously produced by MDSCs; conversely, inhibition of HDAC-11 upregulated IL-10 expression (117, 118). Tumor-derived factors could therefore inhibit the expression or activity of HDAC-11, allowing MDSCs to adopt their characteristic immunosuppressive phenotypes. On the other hand, low doses of the DNA methylation inhibitor, Zebularine, were shown to decrease expression of indolamine 2,3-dioxygenase (IDO), a potent immunosuppressive mediator employed by MDSCs (119). Accordingly, be it methylation or acetylation, epigenetic changes may dictate both the morphology and suppressive functions of MDSCs, highlighting the various levels of regulation that distinguish this population.

MDSCs in Viral Infection

The molecular events dictating the role of MDSCs in cancer have been extensively studied, as described above, and are detailed in other reviews (120-122). Recently, MDSCs have been reported in a variety of non-tumor pathologies, including bacterial (123), parasitic (124), fungal (125) and viral (13) infections. MDSCs generated during viral infections are particularly interesting, because many viruses are not only oncogenic,

but are also capable of establishing chronic infections that result in a dysfunctional inflammatory environment similar to that of tumors. Potent pro-inflammatory cytokines, such as TNF-α and IL-1β are elevated in chronic viral infections and as mentioned above, promote the survival and accumulation of MDSCs (110, 113). Moreover, oncogenic viruses, such as hepatitis B virus, human papillomavirus, and Epstein-Barr virus establish cancers that have a documented increase in MDSCs (51, 80, 126). Whether the influx of MDSCs in these tumors is due to viral-derived factors, chronic inflammation, or simply a consequence of the crosstalk between the tumor and the immune system is an exciting question that remains to be explored.

Similar to the variety of MDSC-recruiting mechanisms employed by cancers, viral infections also utilize diverse pathways to induce local and peripheral accumulation of MDSCs. In addition, more than one subset of MDSCs with different mechanisms of suppression can be found in the same type of cancer or viral infections (13, 127, 128). Figure 1.2 presents a summary of known factors involved in the generation and accumulation of MDSCs during viral infection.

MDSCs in Hepatitis C Virus (HCV) Infection. The blood borne pathogen, HCV, is remarkably efficient at persisting in the presence of an ongoing immune response, as it establishes chronic infection in nearly 80% of infected individuals, putting them at an increased risk of developing fibrosis, cirrhosis, and hepatocellular carcinoma. We and others have reported that the HCV core protein plays a critical role in the pathogenesis of hepatitis C as it can inhibit T cell activation and proliferation (13, 129, 130), IL-12

production by macrophages (131), and apoptosis of infected hepatocytes (132). We had previously demonstrated that the core protein is also able to activate the STAT3 pathway in APCs (133). As discussed above, STAT3 is known to stimulate generation of MDSCs, prompting us to consider if this were also the case in HCV infection. In fact, we discovered that the core protein is a potent inducer of MDSCs: addition of core to healthy human peripheral blood mononuclear cells (PBMCs) produced a distinct population of $CD33^{+}CD11b^{+}HLA\text{-}DR^{lo/-}CD14^{+}$ cells, which effectively suppressed CD4 and CD8 T cell proliferation and IFN-γ production in a ROS-dependent manner (13). Furthermore, this population of MDSCs was also found in the blood of hepatitis C patients, confirming that core is able to induce production of MDSCs *in vivo*. Besides the core protein, it is quite likely that other components of HCV that contribute to immune evasion (134) are also able to generate MDSCs in an effort to subvert the immune response. This is further supported by the recent discovery that MDSCs in the blood of chronically infected HCV patients decrease transiently during antiviral therapy (135). Notably, the MDSCs reported in this study suppress T cell proliferation via an arginase-1-dependent mechanism in contrast to the ROS-dependent suppression we observed in our studies. One explanation for this discrepancy is that slight differences in viral factors from different viral isolates can direct the development of specific MDSC subsets. Nonetheless, our findings thus far indicate that virally derived factors play a direct role in recruiting MDSCs over the course of infection.

Alternatively, inflammatory mediators generated during infection could also contribute to the recruitment of MDSCs. IL-1β, which is increased not only upon infection with HCV

in vitro (28), but also in the blood and livers of HCV infected patients (136), promotes survival of MDSCs in the tumor microenvironment (102). Consequently, it may play a similar role in generating MDSCs during HCV infection. However, given that IL-1β is present is nearly every immune response, this effect is unlikely to be specific to HCV and may instead represent a more general mechanism of MDSC generation. Also noteworthy is that HCV-infected patients with genetic polymorphisms in the COX-2 promoter region show different levels of hepatic inflammation and fibrosis (137). Given that COX-2 affects MDSC accumulation (138), it is plausible that COX-2 plays a role in the generation of MDSCs in HCV infection, perhaps contributing to the transformation of an acute infection into a chronic disease.

MDSCs as therapeutic targets

The breadth of studies demonstrating the benefit of eliminating MDSCs in tumors cannot be contested. From chemotherapies that increase anti-tumor immunity by countering MDSC-mediated suppression of T cells (139, 140) to tumor vaccines that convert MDSCs to pro-inflammatory cells that limit tumor growth (141), the reduction of MDSCs in the setting of cancer is undoubtedly beneficial. Besides traditional cancer treatments, there are several existing pharmaceutical agents that show promise in targeting MDSCs. Foremost among these, as discussed above, are COX-2 inhibitors, as the importance of the PGE2-COX-2 axis to MDSC generation cannot be understated. COX-2 inhibitors are well known to reduce the incidence of numerous cancers (142, 143) via transcriptional changes that can downregulate MDSC trafficking to the tumor site (144). While these agents inhibit the generation and recruitment of MDSCs, inhibitors of

phosphodiesterase-5 disable the functional machinery of this population by decreasing expression of arginase-1 and iNOS2 (145). Similarly, all-trans retinoic acid (ATRA) originally used in the treatment of acute promyelocytic leukemia, stimulates MDSCs to complete maturation, a process that may be aided by NKT cells; ATRA also induces expression of glutathione synthase, which produces glutathione, a ROS neutralizing agent(146). Differentiation of MDSCs into mature myeloid cells can also be achieved by paclitaxel (147) or potent pathogen associated molecular patterns, such as CpG oligonucleotides (148). This small, but diverse list of agents that can manipulate MDSC populations may rise to a more prominent role as we improve our understanding of the role of MDSCs in cancer and chronic inflammatory conditions.

IV. Arginse-1 and arginine

Arginine is a conditionally essential amino acid depending on the developmental stage and health of an individual (149). The human body can generate arginine *de novo* but this occurs only in the small intestine and kidney (150). It is usually converted from proline, glutamine, glutamate, or citrulline (151-153). Arginine is required for many cellular functions such as T cell effector function (154) (155, 156) and most possibly those of NK cells.

Arginase exists in two isoforms— arginase-1 and arginate-2. They are structurally similar, catalyze the same reaction, but differ in their tissue distribution, subcellular localization, immunologic cross-reactivity and physiologic function (157, 158). Arginase-1, the predominant form in the liver (158), is expressed in several cell types like neutrophils (159) and hepatocytes (160) and has recently been reported to be expressed in MDSCs (53, 64, 155). On the other hand, arginase-2 expression is restricted to extra-hepatic tissues, like the kidney, and sublocalized in the mitochondria (159). Arginase catalyzes the hydrolysis of arginine to ornithine and urea. As such, high levels of arginase activity would affect the bioavailability of arginine to cells, affecting their functions (161) and even their metabolism (155). As such, we looked at how arginase-1 produced by MDSCs affected NK cell IFN-γ production.

V. MicroRNAs

MicroRNAs are small non-coding RNAs that are able to regulate protein production at the translational level. Usually 20-22 nucleotides long, miRNAs bind specific 3'UTR of mRNA to suppress mRNA translation and are stabilized by an RNA-induced silencing complex (RISC)(162). The bound mRNA is degraded if the miRNA is almost perfectly complementary to the mRNA sequence (163). If the sequence is less complementary, translation is inhibited, but the mRNA is not degraded (164).

Recently, Ma *et al* showed that miR-29 can bind the 3'UTR of IFN-γ mRNA to inhibit its translation. The study proceeded to identify that miR-29 levels decrease following *Listeria* infection, allowing an increase in the production of IFN-γ protein (165). miR-29 exists in 3 isoforms, miR-29a, miR-29b and miR-29c, all three of which have an identical sequence that binds the IFN-γ mRNA (Fig. 1.3). However, these isoforms have been reported to have different subcellular distribution and mechanisms of regulation (166). There are two transcriptional promoters for the three isoforms of miR-29: miR-29b-1-miR-29a and miR-29b2-miR-29c. miR-29a and miR-29b levels can be suppressed by c-Myc, hedgehog and NF-κB at the miR-29b-1-miR-29a promoter (167). CEBPA was also found to selectively upregulate miR-29b at the miR-29b-1-miR-29a promoter (168).

Since MDSC generated during HCV infection have been shown to suppress NK cell IFN-γ production, we hypothesize that MDSC induce transcriptional changes in NK cells,

which result in altered levels of miRNAs that skew the functional phenotype of NK cells, disabling them as effectors in viral clearance.

VI. The mTOR pathway

Studies on cellular metabolism involving significant problems such as severe human metabolic diseases, including obesity, are of great interest. Importantly, mTOR pathway has been reported to play a pivotal role in adapting to changes in environmental conditions (169).

The mTOR pathway plays an important role in every cell. It acts as a starvation sensor (170) that regulates mRNA translation, cell growth, and proliferation in response to amino acid, growth factors, and hormones (171), and is quite often deregulated in human disease (172). The mTOR (mammalian target of rapamycin) protein is the 289-kDa serine-threonine kinase mTOR and is a member of the phospoinositide 3-kinase (PI3K)-related kinase family (173). This serine/threonine kinase is an essential component in two distinct complexes— mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Theses two complexes phosphorylate different substrates to affect different cellular pathways and are made up of different subunits: mTORC1 comprises of mTOR, Raptor, GβL, and DEPTOR, while mTORC2 comprises of mTOR, Rictor, GβL, Sin1, PRR5/Protor-1, and DEPTOR (174).

Unlike most triggers, amino acids activate mTORC1 requires the coordination of several protein completes such as the v-ATPase, Ragulator, the Rag GTPases, and GATOR1/2 to promote the translocation of mTORC1 to the lysosomal surface to be activated by Rhed (175). Arginine has been known to be required of the phosphorylation and activation of

mTORC1 (171, 176). This activation leads to the phosphorylation and inactivation of mRNA translation inhibitor 4EBP1 (171), freeing eukaryotic translation initiation factor 4E (eIF4E) (177), allowing it to be mediate the recruitment of ribosomes to mRNA to allow translation (178). In addition, mTOR is also known to phosphorylate ribosomal protein 6 kinase (S6K), which in turns phosphorylates other substrates like S6 small ribosomal subunit, which plays an important role in protein synthesis (179, 180) (See Fig. 1.4).

Currently, not much known about the effects of arginine-deprivation on primary human NK cell IFN-γ, but it has been reported that human $CD8^+$ T cells display impaired IFN-γ production when starved of arginine (181). Work on human NK cell line, NK-92 showed that arginine-deprivation resulted in a lower percentage of cells producing IFN-γ when stimulated by K-562 cells or PMA and ionomycin. When stimulated with K-562 cells, the IFN-γ-producing cells produced similar amounts of IFN-γ compared to their arginine-sufficient counterparts. Those stimulated with PMA and ionomycin showed decreased IFN-γ production (182). However, we criticize that this system is highly artificial. In addition to us using primary NK cells from PBMCs, our IL-12/IL-18 stimulation is more pathologically relevant.

Thus we hypothesized that when NK cells that encounter MDSCs and are deprived of arginine, they have impaired mTOR signaling.

Figure 1.1. Granulocytic and Monocytic MDSCs. MDSCs can be categorized into two subtypes— granulocytic and monocytic. In Mice, monocytic MDSCs (m-MDSCs) are $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ and often utilize iNOS and arginase-1 as their mechanisms of suppression. Granulocytic MDSCs (g-MDSCs) are $CD11b^{+}Ly6G^{+}Ly6C^{low}$ and often utilize ROS and arginase-1. In humans, m-MDSCs are $CD11b^{+}HLA\text{-}DR^{lo/-}CD14^{+}$ while g-MDSCs are $CD11b^{+}HLA\text{-}DR^{lo/-}CD15^{+}$. Both subsets have been reported to utilize arginase-1, however whether they use ROS and iNOS is disease or system specific.

Figure 1.1

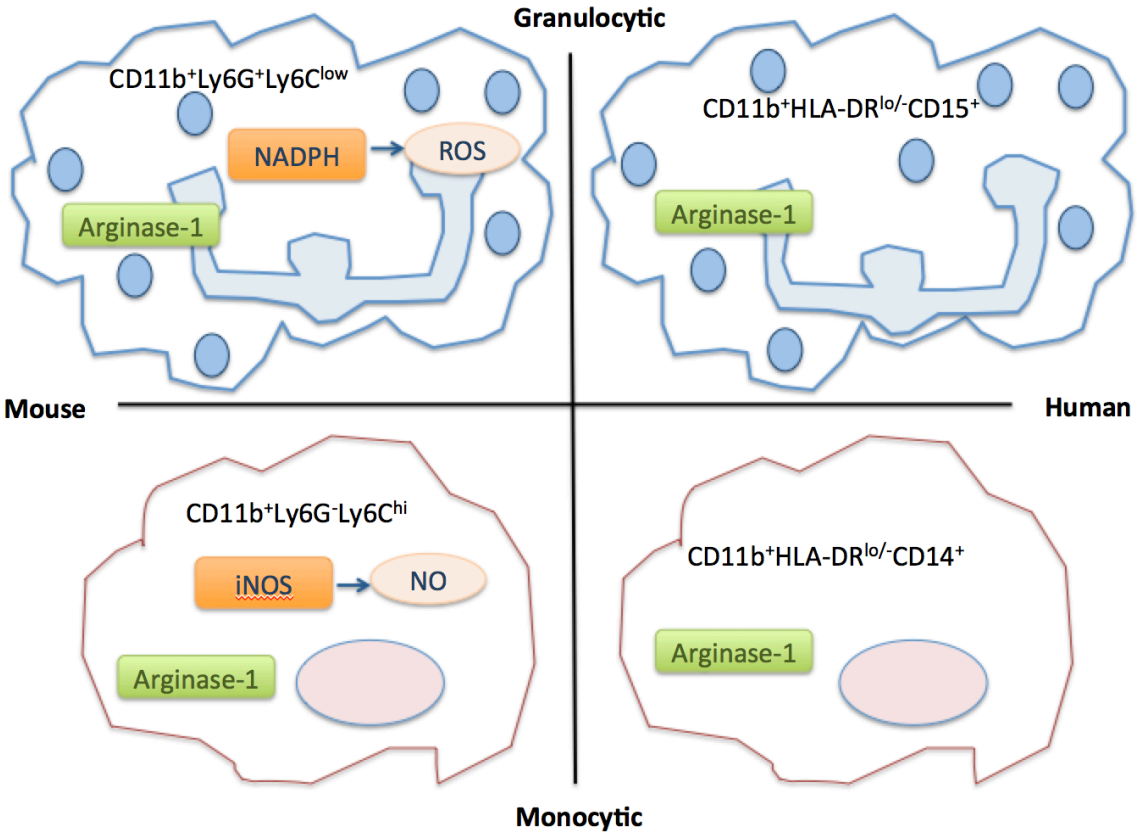

Granulocytic
CD11b+Ly6G+Ly6Clow
NADPH
ROS
Arginase-1
CD11b+HLA-DRlo/-CD15+
Arginase-1
Mouse
Human
CD11b+Ly6G-Ly6Chi
iNOS
NO
Arginase-1
CD11b+HLA-DRlo/-CD14+
Arginase-1
Monocytic

Figure 1.2. Factors involved in the generation and accumulation of MDSCs during viral infection. Upon viral infection, infected cells produce factors, such as IL-6, M-CSF, and PGE2, which prevent the differentiation of MDSCs into mature macrophages and DCs. In particular, PGE2 upregulates COX-2, which increases the production of IDO and IL-10. In addition, COX-2 acts in a positive feedback loop, generating more PGE2 that propagates immunosuppression. Under hypoxic conditions, upregulation of HIF-1α augments the immunosuppressive effect of MDSCs by increasing NOS and arginase-1. As a key transcription factor involved in the differentiation of MDSCs, STAT3 is also found to play a role in increasing ROS production by MDSCs. Apart from suppressing other immune cells, ROS prevents MDSC differentiation into mature myeloid cells. Lastly, recruitment of MDSCs to the site of infection is directed by TGF-β produced by virally infected cells, which increases the expression of CXCR4 and its ligand CXCL12. MDSCs also produce factors such as IL-1β that increase their accumulation. MDSCs, myeloid derived suppressor cells; IL, interleukin; M-CSF, macrophage colony-stimulating factor; PGE2, prostaglandin E2; COX-2, cyclooxygenase-2; IDO, indoleamine 2,3-dioxygenase; HIF-1α, hypoxia-inducible factor-1α; NOS, nitric oxide synthase; STAT3, signal transducer and activator of transcription 3; ROS, reactive oxygen species; TGF-β, transforming growth factor-β.

Figure 1.2

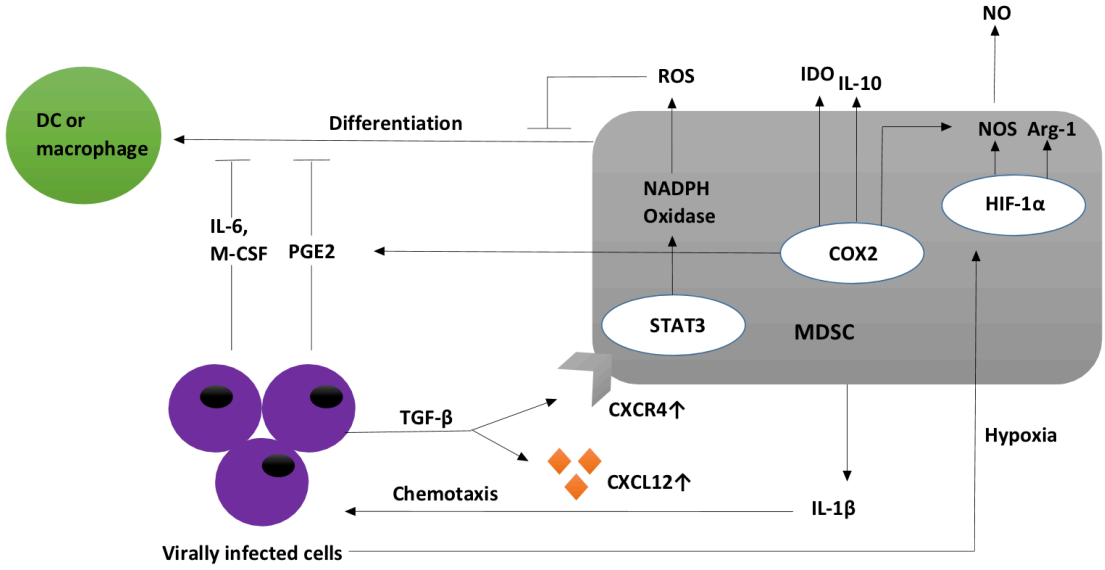
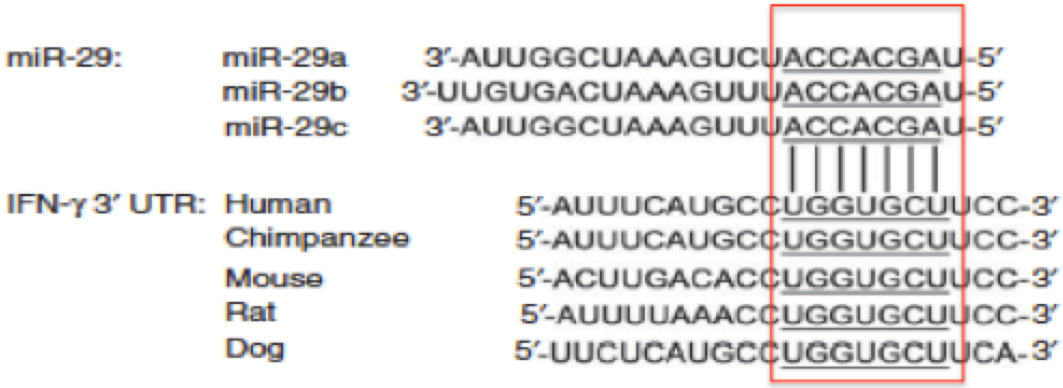

NO
ROS
IDO
IL-10
DC or macrophage
Differentiation
NOS
Arg-1
NADPH Oxidase
HIF-1α
IL-6, M-CSF
PGE2
COX2
STAT3
MDSC
TGF-β
CXCR4↑
Hypoxia
CXCL12↑
Chemotaxis
IL-1β
Virally infected cells

Figure 1.3. Binding sequences of miR-29 to IFN-γ 3'UTR. The sequences of the 3 miR-29 isoforms bind the 3'UTR of IFN-γ mRNA of 5 different species.

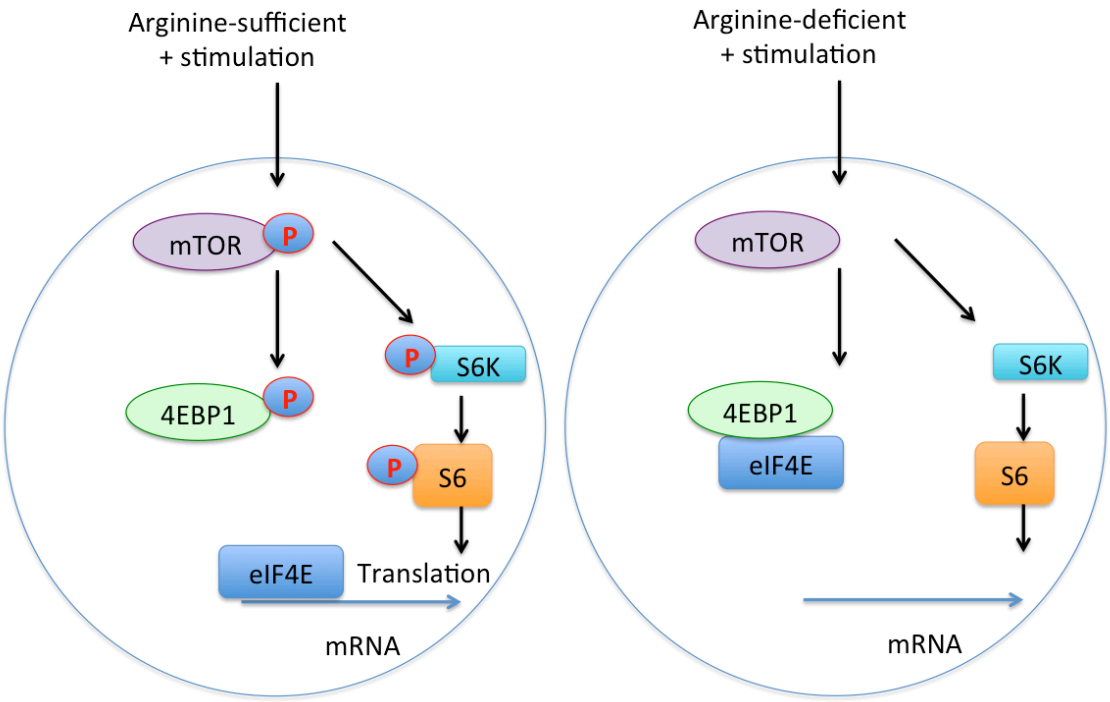
Figure 1.3



Targetscan Database

Figure 1.4. Brief summary of mTOR signaling upon IL-12/IL-18 stimulation in the presence and absence of arginine. When NK cells are stimulated with IL-12/IL-18 in the presence of amino acids, mTOR becomes phosphorylated and becomes part of the mTORC1 complex, which phosphorylates 4EBP1. Phosphorylated 4EBP1 is activated and frees eIF4E, allowing it to form the translation initiation complex, triggering the start of protein translation. mTORC1 also phosphorylates S6 kinase, which in turn phosphorylates other substrates involved in translation including S6 small ribosomal subunit.

Figure 1.4


Arginine-sufficient
+ stimulation
mTOR
P
4EBP1
P
P
S6K
P
S6
eIF4E
Translation
mRNA
Arginine-deficient
+ stimulation
mTOR
4EBP1
eIF4E
S6K
S6
mRNA

Chapter 2: Research Rationale

HCV is extremely efficient in establishing a persistent infection in the liver. This can be attributed to its many immune evasion strategies. Of note, HCV is able to generate MDSCs that go on to suppress the immune system, allowing the virus to establish a stronghold in the liver. Our lab was the first lab to discover MDSCs in a viral system. We showed that HCV causes an accumulation of MDSCs, and these MDSCS are able to suppress T cell function.

Looking at the time course of viral infections, NK cells are the first responders and therefore it's very plausible that the magnitude of their response would determine if an acute infection can be cleared, or if that infection would turn chronic. This is because NK cells play important roles in the innate branch of the immune system. They secrete cytokines that cause inflammation, trigger the production of chemokines, which causes the recruitment of other immune cells, both of the innate and adaptive branches of the immune system; they are also able to directly kill infected cells either by causing apoptosis or through the actions of preforin and granzymes. Not coincidentally, chronic HCV patients have been reported to have dysfunctional NK cells. Hence, this dissertation sought to investigate if HCV-generated MDSCs affect NK cells and their functions.

Of the multitude of NK cells functions, the IFN-γ production is of utmost importance. IFN-γ causes an anti-viral state in hepatocytes, participate in crosstalk with myeloid cells, induces the production of chemokines, causing the recruitment of other immune cells,

and also induces the adaptive immune response. Hence, after finding that HCV-induced MDSCs greatly reduce NK cell IFN-γ production, this dissertation investigated the molecular mechanisms for this suppression: the effects of MDSCs on the NK cells, as well as intracellular events within the NK cells.

The first part of my dissertation investigates how the MDSCs are exerting their effects on the NK cells. I looked into contact-dependence, soluble factors, and other known mechanisms of MDSC action. After successfully restoring the IFN-γ production by means of an arginase-1 inhibitor, I looked into reversing the effects of arginase-1 by replenishing L-arginine, an important amino acid for NK cell function, which was depleted by MDSC-produced arginase-1. I also went on to show that the MDSCs expressed arginase-1 comparing them with uninfected-conditioned myeloid cells.

The second part of my dissertation focuses on how the NK cells are impaired. After finding that IFN-γ production was not affected at the transcriptional level, I looked at post-transcriptional mechanisms of suppression. Two potential mechanisms—microRNAs and the mTOR pathway were investigated. miR-29 has been reported to suppress IFN-γ translation in other systems, while arginine-deficiency has been reported to affect the mTOR pathway, which is an essential part of translation initiation.

In summary, this dissertation sought to investigate the effects of MDSCs on NK cell IFN-γ because of the importance of NK cell-derived IFN-γ and the lack of NK cell IFN-γ production by NK cells from chronic HCV patients.

Chapter 3: Immunoregulation of HCV-induced MDSCs on NK cell functions

Hepatitis C virus-induced MDSCs Suppress NK Cell Interferon-gamma Production by Altering Cellular Metabolism via Arginase-1

Abstract

The hepatitis C virus (HCV) infects ~200 million people worldwide. The majority of infected individuals develop persistent infection, resulting in chronic inflammation and liver disease, including cirrhosis and hepatocellular carcinoma. HCV's ability to establish persistent infection is partly due to its ability to evade the immune response through multiple mechanisms, including the suppression of natural killer (NK) cells. NK cells control HCV replication during the early phase of infection and regulate the progression to chronic disease. In particular, IFN-γ produced by NK cells limits viral replication in hepatocytes and is important for the initiation of adaptive immune responses. However, NK cell function is significantly impaired in chronic HCV patients. The cellular and molecular mechanisms responsible for impairing NK cell function in HCV infection are not well defined. Here, we analyzed the interaction of human NK cells with $CD33^+$ PBMCs that were exposed to HCV. We found that NK cells co-cultured with HCV-

conditioned $CD33^+$ PBMCs produced lower amounts of IFN-γ, with no effect on granzyme B production or cell viability. Importantly, this suppression of NK cell-derived IFN-γ production was mediated by $CD33^+CD11b^{lo}HLA\text{-}DR^{lo}$ myeloid derived suppressor cells (MDSCs) via an arginase-1-dependent inhibition of mTOR activation. Suppression of IFN-γ production was reversed by L-arginine supplementation, consistent with increased MDSC arginase-1 activity. These results identify the induction of MDSCs in HCV infection as a potent immune evasion strategy that suppresses anti-viral NK cell responses. They further implicate that blockade of MDSCs may be a potential therapeutic approach to ameliorate chronic viral infections in the liver.

Introduction

Hepatitis C virus (HCV), the causative agent of hepatitis C, infects over 200 million people worldwide. The majority of infected patients are unable to clear the infection (183, 184), and consequently develop liver fibrosis, cirrhosis and hepatocellular carcinoma (185). The discovery and use of viral protease and polymerase inhibitors have dramatically improved treatment outcomes and prognoses for HCV patients. However, the high cost of these inhibitors precludes benefit to many patients infected with HCV. Furthermore, there is no vaccine available to prevent HCV infection and the subsequent spread of virus. As such, there is a continued need to find alternative, more cost-effective prophylactic and therapeutic treatments.

One of the main challenges to developing a vaccine against HCV is the virus's ability to evade immune responses (133, 186). HCV infection dysregulates both innate and adaptive immunity by hampering interferon (IFN) production, skewing the differentiation of CD4 T cells towards unfavorable Th2, Th17, and Treg subsets, and impairing the function of cytotoxic CD8 T cells (187-189). HCV is also known to suppress the function of natural kill (NK) cells (190), which play an important role in viral clearance as they comprise 20-30% of hepatic lymphocytes in humans (30, 191). Indeed, NK cells are key players in orchestrating effective immune responses, as they can directly lyse infected cells (192) and crosstalk with Kupffer cells and dendritic cells through production of IFN-γ (193, 194), leading to regulation of T cell responses (16, 18). Notably, production of IFN-γ by NK cells correlates with their expression of CD56. In healthy individuals, $CD56^{high}$ NK cells produce cytokines, while $CD56^{low}$ NK cells are both cytotoxic and

capable of producing cytokines (1, 4). In contrast, chronic HCV patients have a subset of $CD56^-$ NK cells that are impaired in both IFN-γ production and cytotoxicity through an unknown mechanism (18, 35). Given the central role of NK cells in regulating adaptive immune responses, it is important to understand how NK cell functions are impaired during HCV infection as it may aid in the design of vaccines against the virus.

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population defined by their ability to suppress proinflammatory immune responses. While the generation of MDSCs was originally described in tumor development, the immunosuppressive function of MDSCs has been reported in a variety of pathological conditions, including viral infections (195). MDSCs suppress the effector function of target cells through a number of mechanisms including the production of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and arginase-1 (Arg-1). We previously reported that HCV infection generates MDSCs, which effectively suppress T cell responses through the production of ROS (13). However, despite the pivotal role of NK cells in controlling HCV infection through inhibition of viral replication and regulation of adaptive immunity, it is not known whether MDSCs generated during HCV infection regulate the effector function of NK cells.

In recent years, there have been a number of reports demonstrating the importance of metabolic pathways to immune cell function (196-198). The mTOR (mammalian target of rapamycin) pathway is central to both cellular metabolism and immune activation as it integrates environmental cues such as nutrient availability with the growth, proliferation,

and production of effector cytokines in immune cells (169, 199, 200). Signaling through the PI3K-Akt axis stimulates the serine-threonine kinase activity of the mTOR complex, which activates protein production via phosphorylation of 4EBP1 (201). 4EBP1 is inactivated upon phosphorylation and released from the elongation initiation factor 4E (eIF4E) (202), allowing recruitment of ribosomes to the 5' cap of mRNAs to initiate protein translation (177). Amino acids play a major role in triggering mTOR activation (176, 203, 204) given that protein synthesis is one of the primary outcomes of mTOR signaling (205). In particular, the amino acid L-arginine can induce the phosphorylation and activation of mTOR (171, 176). As L-arginine availability is reduced by the production of arginase-1 by MDSCs, we hypothesized that inhibition of mTOR activation may be a key mechanism by which MDSCs regulate NK cell function.

Here we show that HCV-induced MDSCs suppress NK cell IFN-γ production by reducing the bioavailability of L-arginine via arginase-1. The suppression of NK cell IFN-γ production is due to a block in protein translation as there was no difference in the ability of NK cells to transcribe *IFNG* gene. The defect in translation of IFN-γ transcript appears likely due to a deficiency in mTOR activation, as NK cells exposed to HCV-induced MDSCs displayed decreased phosphorylation of mTOR and its substrates.

Materials and Methods

Cell lines and virus

Huh7.5.1 were grown in DMEM containing 10% FBS, penicillin/streptomycin (100μg/mL), L-glutamine (2mM), and 1x NEAA and infected with the JFH-1 strain of HCV at an m.o.i. of 0.1 for 5 days. JFH-1 was kindly provided by Dr. Wakita (Tokyo Metropolitan Institute) and grown as previously described (187).

$CD33^+$ cells and NK cell co-cultures

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (Virginia Blood Services, Richmond, VA) using Sepmate^{TM}-50 (Stemcell Technologies) and frozen in 90% FBS/10% Dimethyl Sulfoxide (DMSO). $CD45^+$, $CD33^+$, or NK cells were purified from cell mixtures using EasySep selection kits (Stemcell Technologies). $CD45^+$ cells were purified from co-culture of PBMCs with uninfected/infected Huh7.5.1 cells after 7 days and stained for MDSC markers by flow cytometry. In parallel experiments, $CD33^+$ cells were obtained from co-culture of PBMCs and uninfected/infected Huh7.5.1 cells and were subsequently co-cultured for 2 days with autologous NK cells in RPMI1640 containing 10% FBS, penicillin/streptomycin (10μg/mL), and L-glutamine (2mM). Purity of autologous NK cells was confirmed via flow cytometry as >82% $CD56^+$ cells and <2.5% $CD3^+$ cells. NK cells were stimulated with IL-12 (10ng/mL, PeproTech), IL-18 (10ng/mL, R&D Systems), and IL-2 (4μg/mL, eBioscience). The ROS scavenger catalase (100U/mL, Sigma-Aldrich, St. Louis, MO), L NG-monomethyl-L-arginineacetate (500 μM, Sigma-Aldrich), or N(ω)-hydroxy-nor-L-arginine (500μM, Cayman Chemicals, Ann Arbor, MI) was added during the 2-day co-

culture of $CD33^+$ cells and NK cells.

ELISA

IFN-γ and granzyme B in culture supernatants were measured using IFN-γ ready-set-go ELISA kit (eBioscience) and Granzyme B Platinum ELISA kit (eBioscience), respectively.

Flow cytometry for MDSCs

For identifying MDSCs, $CD45^+$ cells magnetically sorted from the co-culture of PBMCs with uninfected/infected Huh7.5.1 cells were blocked with FcR blocking reagent (Miltenyi) and stained with the live/dead marker DAPI (Life Technologies), anti-CD33, -CD11b, and -HLA-DR (all from BD Pharmingen). For detecting intracellular arginase-1 production, $CD33^+$ cells were magnetically sorted from co-cultures with NK cells and stained for MDSC surface markers. The cells were then fixed and permeabilized by Cytofix/Cytoperm (BD biosciences) and stained with the MDSC markers described above and anti-Arginase-1 (R&D Systems). Aqua live/dead stain (Life Technologies) was included to analyze cell viability. All stained cells were run on BD FACSCantoII (BD Biosciences) and analyzed using FlowJo software.

Flow cytometry for NK cells

Following co-culture with mock/HCV-conditioned $CD33^+$ cells, NK cells were magnetically sorted and replated in fresh media containing IL-12 (10ng/mL) and IL-18 (10ng/mL) in the presence of Golgi Plug (eBioscience) for 5 hours. After blocking Fc

receptor using the FcR blocking reagent (Miltenyi), the cells were stained with Aqua Live/Dead (Life Technologies), anti-CD56, -CD16, and -CD33 (all from BD Pharmingen). The cells were then permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with anti-IFN-γ (BD Pharmingen). For intracellular mTOR staining, NK cells were recovered following co-culture with mock- or HCV-conditioned CD33 cells separated by a 0.45μm transwell insert and restimulated with IL-12 (10ng/mL) and IL-18 (10ng/mL) for 2 days. The recovered cells were fixed in Cytofix (BD Biosciences), permeabilized using BD Phosflow Perm Buffer (III), and stained with rat anti-mTOR (R&D systems) and mouse anti-phospho-mTOR (BD Phosflow™), or mouse anti-phospho-4EBP1 (pT69) (BD Phosflow™). All cells were run on BD FACSCantoII (BD Biosciences) and analyzed using FlowJo software.

mRNA qRT-PCR

RNA was extracted from magnetically sorted NK cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA was made using the High Capacity RNA-to-cDNA kit (Applied Biosystems) and qRT-PCR was performed using Fast SYBR® Green master mix (Applied Biosystems). Gene expression was quantified on the StepOne Real Time PCR system (Applied Biosystems). Results were first normalized to *GAPDH* and then set relative to mock-conditioned controls. The following primers were purchased from Eurofins MWG Operon: *IFNG* forward 5'-TCGGTAACTGACTTGAATGTCCA-3' and reverse 5'-TCGCTTCCCTGTTTTAGCTGC-3', *GAPDH* forward 5'-

TGCACCACCAACTGCTTAGC-3', and reverse 5'-GCATGGACTGTGGTCATGAG-3'.

MicroRNA Assay

$CD33^+$ cells were co-cultured with autologous NK cells supplemented with IL-2 (4μg/mL), and stimulated with IL-12 (10ng/mL) and IL-18 (10ng/mL) for 48h. The $CD33^+$ cells were recovered by removing the media by centrifugation. Detachin treatment was applied to the plate to recover any adherent cells and the cell pellets were combined. RNA was extracted from magnetically sorted NK cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). TaqMan® Small RNA Assays for miR-29a, miR-29b, miR-29c, and RNU6B (Applied Biosystems) were performed according to manufacturer's instructions.

Arginase assay

$CD33^+$ cells were co-cultured with autologous NK cells separated by a 0.45μm transwell insert in complete media in the absence of phenol red. The cells were supplemented with IL-2 (4μg/mL) and stimulated with IL-12 (10ng/mL) and IL-18 (10ng/mL) for 48h. The $CD33^+$ cells were recovered and the arginase activity assay (Sigma-Aldrich) was performed according to manufacturer's instructions.

Luminex assay

$CD33^+$ cells were obtained from co-cultures of PBMCs with uninfected or HCV-infected Huh7.5.1 cells. Cells were either lysed by sonication in ice-cold lysis buffer (PBS/0.2%

Triton X-100/protease inhibitor cocktail) or replated for 24h in PBMC media, after which the cell culture media was obtained. Samples were run on the Luminex 100 IS system and the innate cytokine panel was used.

Sucrose purified JFH-1 virus

Sucrose purified JFH-1 virus was obtained from Dr. Lucy Golden-Mason. Sucrose density-gradient ultracentrifugation purification and concentration of virus was performed on pooled supernatants of JFH1 (Takaji Wakita, National Institute of Infectious Diseases, Japan) infected Huh7.5.1 cells (Francis Chisari, Scripps Research Institute, La Jolla, CA) and multiplicity of infection (m.o.i) determined by titration on Huh7.5.1 cells as previously described (206). Purified virions were used to infect Huh7.5.1 cells at m.o.i.s of 0.01, 0.1, and 1.0 for 5 days before the addition of PBMCs. After 7 days, $CD45^+$ cells were obtained by magnetic selection (Stemcell Technology) and stained for MDSC markers as shown above. In addition, $CD33^+$ cells were selected from the co-culture and cultured with autologous NK cells and stimulated for 2 days. The resulting cell culture media was obtained and tested for IFN-γ by ELISA (eBioscience).

Statistical analysis

Experimental results were analyzed for statistical significance using Wilcoxon matched pairs test, two-tailed paired *t* test, Mann-Whitney test, or Kruskal-Wallis test (One-way ANOVA) with Dunn's post-test, as appropriate. p values of <0.05 were considered significant and are indicated in the figures.

Results

NK cells exhibit impaired IFN-γ production upon co-culture with HCV-conditioned myeloid cells.

NK cells play a pivotal role in limiting virus replication via direct killing of infected cells and production of IFN-γ, which in turn augments anti-viral adaptive immunity. While NK cells from patients with acute HCV infection have intact effector function, those from chronically infected patients produce significantly lower amounts of IFN-γ (207). MDSCs are known to dampen immune responses of lymphocytes in acute and chronic viral infections (195). We therefore investigated whether myeloid cells exposed to HCV infection suppress NK effector function. To this end, we cultured peripheral blood mononuclear cells (PBMCs) with the hepatocyte cell line Huh7.5.1 uninfected or infected with HCV. Following 7 days of co-culture, we magnetically sorted CD33+ (myeloid) cells with several wash steps (hereafter referred to as uninfected-conditioned CD33+ cells or HCV-conditioned CD33+ cells) and added them to NK cells from autologous donors (See Fig. 3.1A). After 48 hours of stimulation with IL-12 and IL-18, NK cells co-cultured with HCV-conditioned CD33+ cells produced less IFN-γ (Fig. 3.1B-C). In contrast, there was no difference in granzyme B production between NK cells co-cultured with HCV-conditioned CD33+ cells and those cultured with uninfected-conditioned CD33+ cells (Fig. 3.1D-E). The decrease in IFN-γ production was not due to a loss of cell viability as the number of NK cells was comparable upon co-culture with uninfected- or HCV-conditioned CD33+ cells (Fig. 3.1F). Lastly, we confirmed that NK cells were the primary source of IFN-γ in the co-culture as only a negligible proportion of CD33+ myeloid cells stained for IFN-γ (Fig. 3.2A). To verify that the JFH-1 virus is responsible

for the MDSC-mediated suppression, sucrose gradient-purified virions were used to infect Huh cells. This too yielded the suppression of NK cell IFN-γ production (Fig. 3.1G). Together, these results show that HCV-conditioned myeloid cells specifically interfere with IFN-γ production by NK cells.

Reduction in NK cell IFN-γ production is post-transcriptionally regulated.

Since the defect in IFN-γ production was not due to differences in the number of total NK cells, the reduction in NK cell IFN-γ secretion following co-culture with HCV-conditioned myeloid cells likely reflected a defect in overall NK cell activation or a selective deficiency in IFN-γ production. To explore these possibilities, we first assessed the ability of NK cells to produce IFN-γ at the single cell level by intracellular cytokine staining. As shown in Fig. 3.3A-B, the frequency of IFN-γ producing NK cells were comparable upon co-culture with uninfected- or HCV-conditioned $CD33^+$ cells. However, the mean fluorescence intensity (MFI) of the IFN-γ-producing NK cells was reduced in NK cells that encountered HCV-conditioned $CD33^+$ cells, indicating that these cells were producing less IFN-γ on a per cell basis (Fig. 3.3C). However, there was no significant difference in the expression of the human NK differentiation marker CD56 in total NK cells when cultured with HCV-conditioned myeloid cells (Fig. 3.3A, D-E). These results suggest that the differentiation status of NK cells was not altered in the presence of HCV-conditioned $CD33^+$ cells. To further investigate the molecular basis of the decrease in IFN-γ production by NK cells, we assessed the transcriptional status of the *IFNG* gene. Quantitative PCR analysis revealed that *IFNG* mRNA was found in similar levels in NK cells co-cultured with mock- or HCV-conditioned $CD33^+$ cells (Fig.

3.3F). Thus, the deficit in IFN-γ production by NK cells was likely due to regulation of post-transcriptional events by HCV-conditioned $CD33^+$ cells.

HCV-conditioned MDSCs suppress NK cell IFN- γ production via arginase-1.

We next sought to identify the molecular determinants originating from HCV-conditioned $CD33^+$ cells that enabled regulation of IFN-γ production by NK cells. We have previously demonstrated that PBMCs co-cultured with HCV-infected hepatocytes exhibit immunosuppressive functions characteristic of myeloid derived suppressor cells (MDSCs) (13). Given that HCV-conditioned $CD33^+$ cells potently inhibited NK cell effector function, we investigated whether the $CD33^+$ population included MDSCs with immunosuppressive capabilities. Indeed, co-culture of PBMCs with HCV-infected hepatocytes produced a distinct population of $CD33^+CD11b^{lo}HLA\text{-}DR^{lo}$ cells (Fig. 3.4A-B). Importantly, this population of MDSCs was minimally represented among PBMCs cultured with uninfected hepatocytes (Fig. 3.4C-D), indicating that HCV-infection specifically induces a distinct subset of myeloid cells. To verify HCV infection in hepatocytes, we determined the quantity of HCV RNA and the level of core protein by qRT-PCR and Western blot analysis, respectively (Fig. 3.5A-B) as well as the ability of sucrose purified virus to infect hepatocytes (Fig. 3.5C). We further examined the relationship between viral dose and the frequency of MDSC by assessing MDSC accumulation after the co-culture of PBMC with various m.o.i. of JFH1 virus (Fig. 3.5D), suggesting the increased frequency of MDSC detectable in high virus dose. In addition, there is a trend where chronic HCV patients with high virus titer (>800,000IU/mL) have more MDSCs than patients with low virus titer (Fig. 3.5E), while there is no correlation

between the frequency of MDSC and ALT level (Fig. 3.5F). These results suggest that HCV is capable of inducing MDSC, which plays a role in controlling infection rather than hepatic inflammation.

MDSCs are known to inhibit immune cells through contact-dependent and contact-independent mechanisms (55). Therefore, we determined if the crosstalk between $CD33^+$ cells and NK cells required cell-cell contact by employing 0.45μm transwell inserts to physically separate the two populations during co-culture (Fig. 3.6A). HCV-dependent suppression of NK cell IFN-γ production occurred even in the absence of cell contact between myeloid and NK cells (Fig. 3.6B). Given these findings, we sought to identify soluble immunosuppressive mediators produced by HCV-conditioned MDSCs.

First, we performed a luminex assay on the cell lysates of the uninfected- and HCV-conditioned $CD33^+$ cells to see if there were any marked differences in innate cytokine levels. Even though some cytokines like IFN-α2, IL-12P40, IL-9, and VEGF showed downward trends in cytokine production (Fig. 3.7A-D), we did not obtain any significant results as these cytokines were produced at such a low level. In addition, we performed the innate panel of the luminex assay on 24h culture media. Disappointingly, IL-6 was the only cytokine that showed a downward trend (Fig. 3.7E), but that too was not significant. Thus, we looked into other known mechanisms of suppression by MDSCs.

MDSCs employ numerous soluble factors to inhibit host immune responses, including the production of reactive oxygen species (ROS), nitric oxide synthase (NOS), and arginase-

1. ROS regulates immune responses through activation of apoptosis in immune cells (208), while nitric oxide produced by NOS nitrosylates and dissociates protein complexes involved in immune activation (209, 210). Arginase-1 depletes local supplies of L-arginine and can cause inefficient proliferation of activated lymphocytes (211). Considering their potent immunosuppressive effects, we investigated if ROS, NOS, or arginase-1 may be responsible for dampening NK cell IFN-γ production using pharmacologic inhibitors of each of these factors during co-culture of NK cells with $CD33^+$ cells: catalase scavenges ROS, L-NG-monomethyl L-arginineacetate (L-NMMA) inhibits NOS, and N_ω-hydroxy-L-arginine (Nor-NOHA) inhibits arginase-1. Addition of catalase and L-NMMA failed to reverse the suppressive effect of HCV-conditioned $CD33^+$ cells on NK cell IFN-γ production (Fig. 3.5C-D). In contrast, Nor-NOHA restored IFN-γ production in NK cells exposed to HCV-conditioned $CD33^+$ cells (Fig. 3.5E). Importantly, Nor-NOHA did not change IFN-γ production when added to NK cells co-cultured with mock-conditioned $CD33^+$ cells (Fig. 3.2B). In addition, we verified our positive controls for the addition of catalase and L-NMMA (Fig. 3.8). Hence, we conclude that HCV-induced $CD33^+$ cells appear to be MDSCs that suppress NK cell IFN-γ production via arginase-1.

Inhibition of NK cell IFN-γ production by HCV-conditioned myeloid cells is reversed by L-arginine supplementation.

Arginase-1 catabolizes L-arginine into urea and ornithine. Although L-arginine is nonessential in most individuals, it is possible that an acute loss of L-arginine due to arginase-1 activity could affect the function of local immune cells. Therefore, we cultured

NK cells in complete media or media depleted of L-arginine. Consistent with the increase in IFN-γ production upon inhibition of arginase-1 (Fig. 3.5E), NK cells grown in complete media produced more IFN-γ than those grown in L-arginine-deficient conditions (Fig. 3.9A). We further confirmed the requirement for arginase-1 in suppressing NK cell IFN-γ production by replenishing L-arginine in L-arginine-depleted co-cultures of $CD33^+$ cells and NK cells. Addition of 1mM L-arginine, which approximates L-arginine levels found in standard RPMI formulations, reversed the inhibition of NK cell IFN-γ production (Fig. 3.9B). Notably, supplementing L-arginine in co-cultures of NK cells and uninfected-conditioned $CD33^+$ cells did not change IFN-γ production (Fig. 3.2C), verifying that L-arginine was specifically counteracting the enhanced arginase-1 activity of HCV-conditioned $CD33^+$ cells. Indeed, direct assessment of arginase-1 function revealed that HCV-conditioned $CD33^+$ cells had increased arginase-1 activity when compared to mock-conditioned $CD33^+$ cells (Fig. 3.9C).

We next validated the increased arginase-1 activity by intracellular staining of arginase-1. As seen in Fig. 3.9D-F, both the frequency and level of arginase-1 expression was increased in HCV-conditioned $CD33^+$ cells. Collectively, these results demonstrate that HCV-conditioned $CD33^+$ cells suppress NK cell IFN-γ production via a contact-independent, arginase-1-dependent mechanism, which is distinct from the production of ROS that is used to suppress T cells (13). Arginase-1 produced by HCV-conditioned MDSCs is thus most likely responsible for reducing IFN-γ synthesis in NK cells.

L-arginine depletion selectively affects IFN-γ production in NK cells.

To further dissect our findings on arginase-1 in MDSC-mediated suppression of NK cell IFN-γ production, we assessed the effects of L-arginine deprivation on other NK cell functions. Accordingly, we evaluated cell viability and granzyme B production in NK cells cultured in the presence of the IL-2/IL-12/IL-18 stimulatory cocktail in L-arginine-deficient media. In contrast to the decrease in IFN-γ production seen in NK cells grown in L-arginine-deficient media (Fig. 3.9A), there was no difference in NK cell viability or granzyme B production (3.10A-B), suggesting that the absence of L-arginine does not result in a global insufficiency in NK activity. Instead, the availability of L-arginine regulates specific effector functions, namely IFN-γ production, in NK cells.

MDSC-mediated suppression of IFN- γ production is not mediated by microRNA-29.

MicroRNAs bind the 3'UTR of mRNA to inhibit translation (212) and miR-29 has been reported to bind the 3'UTR of *IFNG* mRNA (165) or the mRNA of transcription factors important for IFN-γ production, Tbet and Eomes (213). Hence, we sought to see if a decrease in IFN-γ production our NK cells correlated with an increase in miR-29 levels. To this end, we examined the levels of miR-29 in the NK cells that have been exposed to MDSCs after verifying by ELISA that their IFN-γ production were impaired. Unfortunately, miR-29a, miR-29b, and miR-29c levels in both NK cells that were co-cultured with HCV-conditioned $CD33^{+}$ cells and NK cells grown in L-arginine free media did not correlate with IFN-γ production (Fig. 3.11). As such, we conclude that miR-29 did not play a role in suppression IFN-γ in our system.

MDSC-mediated suppression of IFN- γ production is mediated by reduced mTOR signaling.

L-arginine and other amino acids are a primary stimulus for activating the mTOR pathway (214, 215). The mTOR pathway integrates complex environmental cues such as nutrient availability with protein translation and higher order cellular functions, including proliferation and cytokine production (216). Considering that the defect in NK cell IFN-γ production in our system was post-transcriptional, we reasoned that the mTOR pathway would be inefficiently activated in NK cells cultured with HCV-conditioned $CD33^+$ cells. To test this hypothesis, we analyzed activation of the mTOR pathway in NK cells grown in L-arginine deficient media. As shown in Fig. 3.12A, NK cells that were cultured with HCV-conditioned $CD33^+$ cells expressed reduced levels of phosphorylated mTOR when compared to those cultured with uninfected-conditioned $CD33^+$ cells. Similarly, NK cells grown in L-arginine-free media also expressed reduced levels of phosphorylated mTOR compared to their counterparts grown in complete media (Fig. 3.13A-B).

4EBP1, a downstream target of the mTOR complex, is a translational repressor that is inactivated upon phosphorylation. To further examine the effect of arginase-1 producing cells on the mTOR pathway in NK cells, we examined the phosphorylation status of 4EBP1 in NK cells co-cultured with HCV-conditioned $CD33^+$ cells. Not surprisingly, a lower percentage of NK cells recovered after co-culture with HCV-conditioned $CD33^+$ cells expressed phosphorylated 4EBP1 compared to those isolated after co-culture with uninfected-conditioned $CD33^+$ cells (Fig. 3.12B). Complementing these results, phosphorylation of 4EBP1 was also decreased in NK cells grown in L-arginine-deficient

media (Fig. 13C-D). Lastly, we treated NK cells with the mTOR inhibitor, rapamycin, to verify the importance of the mTOR pathway to NK cell IFN-γ production. Indeed, IFN-γ production by NK cells treated with rapamycin was comparable to IFN-γ produced by NK cells grown in L-arginine-depleted media (Fig. 3.12C). Taken together, our results suggest that HCV-induced $CD33^{+}$ MDSCs exhaust local supplies of L-arginine, causing insufficient mTOR activation, which likely decreases translation of IFN-γ transcript in NK cells.

As we have established that NK cells deprived of arginine as a result of exposure to MDSCs have impaired IFN-γ production due to impairment in mTOR signaling, we sought to determine if NK cells from chronic HCV patients exhibited similar traits. NK cells obtained from chronic HCV patients exhibited impaired IFN-γ response compared to NK cells from healthy donors upon stimulation (Fig. 3.14A). Further analysis on mTOR activation by NK cells from chronic HCV patient revealed that there was a slight decrease in mTOR activation by chronic HCV patients' NK cells compared to healthy individuals with no statistical significance (Fig. 3.14B). Moreover NK cells from chronic HCV patients display lower levels of 4EBP1 activation (Fig. 3.14C), suggesting that the mTOR signaling pathway may be impaired in NK cells during chronic HCV patients.

Discussion

In this report, we demonstrate that HCV infection induces $CD33^{+}CD11b^{lo}HLA\text{-}DR^{lo}$ MDSCs, which suppress NK cell IFN-γ production by depleting L-arginine via an arginase-1-dependent mechanism. The loss of L-arginine subsequently fails to drive efficient activation of the mTOR pathway, which is necessary for translating IFN-γ mRNA into secreted protein. In contrast, L-arginine deprivation or the presence of HCV-conditioned $CD33^{+}$ cells does not affect granzyme B production or NK cell viability. These results underscore the multitude of immune evasion strategies employed by HCV and identify a specific target that can be manipulated in chronic viral infections in the liver.

MDSCs have been reported to suppress other immune cells through a variety of mechanisms. We have shown that HCV from infected cell culture media generates MDSCs (Fig 3.4, Fig 3.9). In addition, we have confirmed our findings with sucrose gradient-purified JFH1 virus, by showing that MDSCs accumulated in an m.o.i.-dependent manner (Fig 3.5D). We previously reported that extracellular HCV core protein triggers the generation of MDSCs (13). These MDSCs upregulated NADPH oxidase to increase ROS production, which subsequently suppressed CD4 and CD8 T cell IFN-γ production. As NK cells are also a significant source of IFN-γ during the early phase of viral infections, it was important to identify the influence of HCV-induced MDSCs on NK cell effector function. Indeed, the decrease in NK cell IFN-γ production by HCV-induced $CD33^{+}$ cells (Fig. 3.1B) reiterates the potent immunoregulatory role of MDSCs in inhibiting IFN-γ production during HCV infection. In contrast to ROS-

dependent suppression of T cells, however, the mechanism of suppression of NK cells required increased levels of arginase-1 in HCV-conditioned MDSCs (Fig. 3.9). It is worthwhile to point out that arginase-1 activity is increased in HCV-infected hepatocytes (217), however in our system, the contribution of hepatocyte arginase-1 has been eliminated because they have been eliminated from the culture with NK cells. Furthermore, it has been reported that the presence of arginase-1-producing MDSCs in HCV patients is associated with a poor prognosis and patients undergoing anti-viral therapy have fewer circulating arginase-1 producing myeloid cells (135). These observations suggest that L-arginine availability likely decreases over the course of infection. It is therefore tempting to speculate whether the difference in mechanism of suppression between T cells and NK cells reflects differences in nutrient utilization by responding immune cells over the duration of HCV infection; perhaps early-responders such as NK cells utilize L-arginine to a greater extent than late-arriving T cells. Conversely, a recent study reported the presence of arginase-1 producing MDSCs that dampened T cell responses in hepatitis B virus infection (155). Differences in pathogens may thus also regulate the mechanisms and target cells of MDSC-mediated suppression in chronic viral infections. Consequently, MDSCs generated *in vivo* during chronic viral infections probably change their suppression mechanisms to target various immune populations at specific stages of infection with distinct pathogens. Nevertheless, HCV-induced MDSCs must play an important role in disease progression as two reports have shown that increased MDSC frequencies in treatment-naïve HCV patients compared to healthy individuals and patients undergoing anti-viral treatment, and that the MDSC frequencies correlated positively to HCV RNA loads (135, 218).

Arginase-1 converts L-arginine into ornithine and urea, thus reducing local levels of L-arginine. As L-arginine can directly regulate NK cell phenotype and function, the acute loss of L-arginine can profoundly affect NK cell responses (182). The effect on NK cell responses likely corresponds to the number of MDSCs and hence arginase-1 in the system (Fig. 3.15). Specifically, granulocyte-derived arginase was shown to decrease NK cell IFN-γ production with little to no effect on IFN-γ transcription, NK cell viability, or granule release (219). Our results expand on these findings and identify a defect in mTOR activation as a potential mechanism by which arginase-1 activity impairs NK cell IFN-γ production. It is interesting that the percentages of cells expressing phospho-mTOR are not significantly different, but the MFIs are, and on the other hand, the percentages of cells expressing phosphor-4EBP1 are different but the MFI isn't. We attribute these observations to the fact that most cells would express some level of phospho-mTOR when stimulated by IL-12/IL-18, but when the NK cells have sufficient arginine, more mTOR molecules become activated. We propose two reasons why there is no difference in MFI of phosphor-4EBP1. On the other hand, activated mTOR can complexed into mTORC1 and mTORC2, and mTORC2 does not activate 4EBP1. Another possibility would be due to the effects seen at various time points. At this 48h time point, we observed no difference in MFI, however, it doesn't exclude the possibility that the MFI would be different over a longer suppression of the mTOR pathway. The importance of the mTOR pathway to NK cell activation was also recently demonstrated in mice, where the absence of mTOR signaling impaired nutrient uptake and acquisition of effector function, particularly IFN-γ, in NK cells (199). Given that the liver is essential for amino acid metabolism, it is intriguing to contemplate how the effect of MDSCs on NK cells would be further compounded by the metabolic status of the liver during HCV infection.

In fact, postprandial increases in viral titers are well documented in HCV patients (220). However, the specific contribution of amino acids to virus production and metabolic regulation of hepatic immune responses is largely unknown and may prove useful in preventing liver damage and promoting recovery following tissue injury.

Importantly, arginase-1 mediated inhibition of NK cell effector function was limited to IFN-γ as granzyme B production was unaffected in the presence of HCV-induced MDSCs (Fig. 3.1D-E). Differences in processing and storage of granzyme B and IFN-γ may explain the IFN- γ -specific defect in NK cells cultured with HCV-conditioned $CD33^+$ cells. Granzyme B, a serine protease that induces apoptosis in target cells, is stored in preformed granules in human peripheral blood lymphocytes (221). In contrast, NK cells store IFN-γ as transcript, which is then translated upon stimulation (222). Our results demonstrate that IFN-γ production in NK cells is inhibited at the post-transcriptional stage, as IFN-γ mRNA was present in equal amounts in NK cells cultured with mock- or HCV-conditioned $CD33^+$ cells (Fig. 3.3F). Because granzyme B release by NK cells is not dependent on translation, it could explain why the effect of HCV-induced MDSCs on NK cells was limited to IFN-γ production.

Surprisingly, the specific defect in IFN-γ production in NK cells was not due to changes in differentiation status. As shown in Fig. 3.3D-E, expression of CD56 was not significantly altered in NK cells cultured with HCV-conditioned $CD33^+$ cells. CD56 is a marker of differentiation in human NK cells: $CD56^{bright}$ cells readily produce cytokines, have minimal cytolytic activity, and are thought to give rise to the more mature, $CD56^{dim}$

population that is both cytolytic and capable of producing cytokines (223). Although both hepatic and peripheral blood NK cells express CD56, liver-resident NK cells are defined as $CD56^{+}$ while 90% of NK cells found in blood are $CD56^{dim}$ (18). Because our analysis was performed on NK cells derived from PBMCs of healthy individuals, they most likely represent the responses of mature $CD56^{dim}$ NK cells that would infiltrate the liver during infection (224). It would be interesting to compare our results to those of hepatic NK cells in humans, as liver-resident NK cells have been distinguished from conventional NK cells in mice both developmentally and in effector function (225).

Indeed, the importance of NK cells to hepatic inflammation cannot be understated as they are a major lymphocytic subset in the liver (30). Hepatic NK cells are essential not only for conventional immune responses against pathogens, but also for maintenance of tissue homeostasis. For example, NK cells regulate the development of liver fibrosis by killing hepatic stellate cells, which are the major source of matrix deposition during liver injury (226). NK cells also control hepatic inflammation by stimulating the production of IL-6 by Kupffer cells (227) and inducing apoptosis in activated NKT and T cells (228). These findings are particularly informative when considering that dysregulated activation of macrophages and lymphocytes drives immunopathology in chronic inflammatory diseases. Given that chronic inflammation is a hallmark of numerous liver diseases, such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis, autoimmune hepatitis, and hepatocellular carcinoma, the extensive crosstalk between NK cells and other hepatic cells may play a critical role in both removing the inflammatory insult and in restoring tissue homeostasis. Consequently, our findings describing a role for suppressive myeloid

populations in controlling NK cell responses may be equally beneficial in shifting chronic inflammation into an anti-inflammatory response.

In conclusion, we show that HCV-induced MDSCs suppress NK cell IFN-γ production via an arginase-1-dependent loss of L-arginine, resulting in defective mTOR signaling (Fig. 3.16). Given that NK cells play a crucial role in anti-viral immunity via cytolysis of infected cells and cytokine production, understanding how MDSCs affect NK cells provides novel insight into mechanisms that regulate NK cell function. Moreover, these results challenge us to consider the effect of MDSCs on other cells in the liver including Kupffer cells, hepatocytes, and stellate cells, all of which are key players in the progression of chronic liver diseases. Further exploration of the interplay between myeloid cells and other hepatic immune cells may thus help identify key molecular regulators that can resolve chronic inflammation and restore immune homeostasis.

Figure 3.1. NK Cells display impaired IFN-γ production when co-cultured with HCV-conditioned myeloid cells. (A-F) PBMCs were cultured with Huh7.5.1 cells uninfected or infected with HCV for 7 days, after which $CD33^+$ cells were positively selected by magnetic separation. Autologous NK cells were purified by negative selection and co-cultured with $CD33^+$ cells at a 2:1 ratio in the presence of IL-2/IL-12/IL-18 for 2 days. Culture supernatants were used for (B) IFN-γ and (D) granzyme B quantification by ELISA. Relative (C) IFN-γ and (E) granzyme B production was calculated by normalizing to values of NK cells co-cultured with mock-conditioned $CD33^+$ cells. (F) Cell viability of NK cells recovered after co-culture was assessed by flow cytometry. NK cells were gated on live cells, singlets, forward and side scatter, and $CD33^-$ cells to determine the numbers of NK cells. (G) PBMCs were cultured with Huh7.5.1 cells uninfected or infected with purified HCV virions for 7 days, after which $CD33^+$ cells were positively selected by magnetic separation. Autologous NK cells were purified by negative selection and co-cultured with $CD33^+$ cells at a 2:1 ratio in the presence of IL-2/IL-12/IL-18 for 2 days. Culture supernatants were used for IFN-γ quantification by ELISA and normalized to the uninfected control. Results are the mean or representative of 6-11 independent experiments. ns denotes not significant, ***$p<0.001$, (B, D, F) Wilcoxon signed rank test or (C, E, G) two-tailed paired *t* test.

Figure 3.1

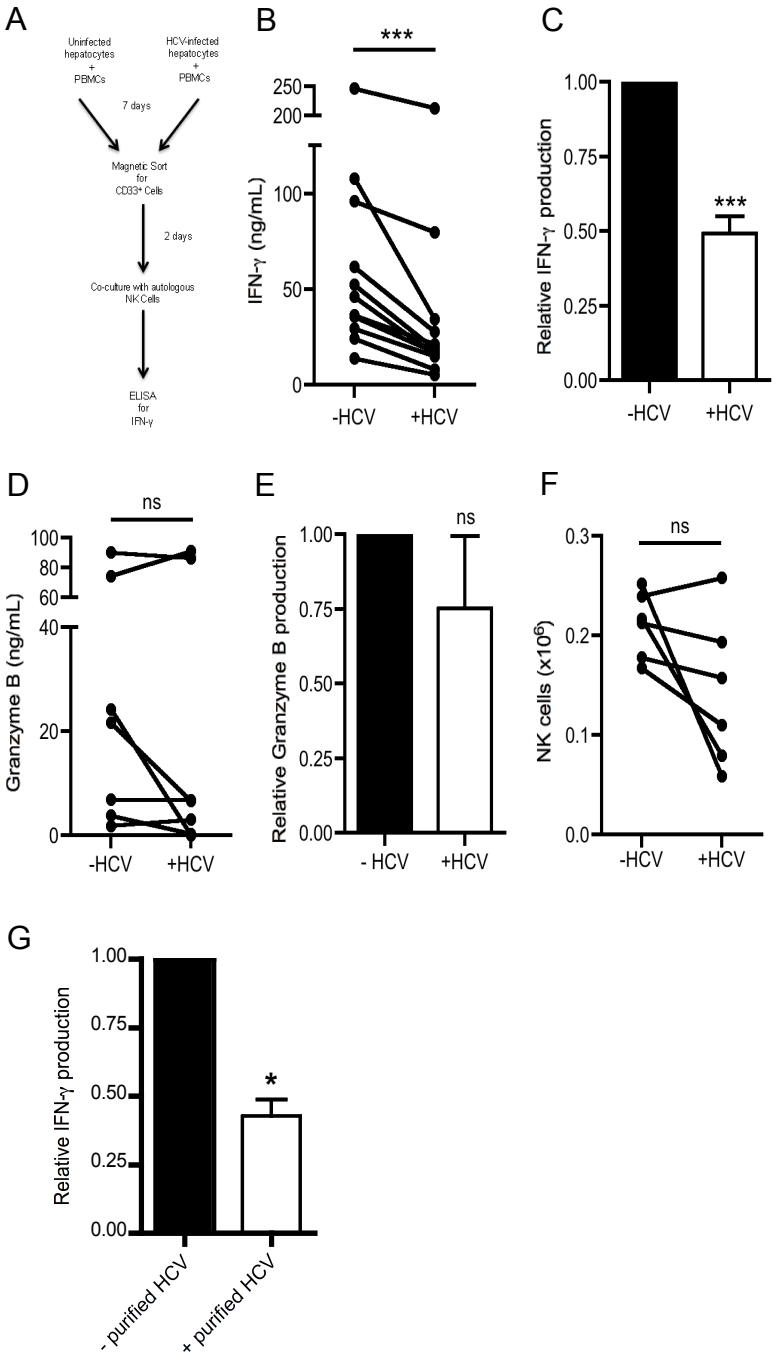

A
Uninfected hepatocytes + PBMCs
HCV-infected hepatocytes + PBMCs
7 days
Magnetic Sort for CD33+ Cells
2 days
Co-culture with autologous NK Cells
ELISA for IFN-γ
B
***
IFN-γ (ng/mL)
250
200
100
50
0
-HCV
+HCV
C
Relative IFN-γ production
1.00
0.75
0.50
0.25
0.00
***
-HCV
+HCV
D
ns
Granzyme B (ng/mL)
100
80
60
40
20
0
-HCV
+HCV
E
ns
Relative Granzyme B production
1.00
0.75
0.50
0.25
0.00
- HCV
+HCV
F
ns
NK cells (x10^6)
0.3
0.2
0.1
0.0
-HCV
+HCV
G
Relative IFN-γ production
1.00
0.75
0.50
0.25
0.00
*
- purified HCV
+ purified HCV

Figure 3.2. IFN-γ production by $CD33^+$ cells is negligible and unaffected in NK cells cultured with mock-conditioned $CD33^+$ cells treated with an arginase-1 inhibitor or arginine.

Mock- or HCV-conditioned $CD33^+$ cells were cultured with autologous NK cells for 2 days. Adherent cells were treated with detachin and cultured with IL-2/IL-12/IL-18. (A) Restimulated cultures were treated with Golgi stop for 5h and IFN-γ was detected in $CD33^+$ cells by intracellular γ staining. (B and C) Mock-conditioned $CD33^+$ cells were co-cultured with autologous NK cells for 2 days, and stimulated with IL-2/IL-12/IL-18 in the presence of (B) 0.5mM Nor-NOHA or (C) 1mM L-arginine. IFN-γ was detected in culture supernatants by ELISA. Results are mean of 4 independent experiments.

Figure 3.2

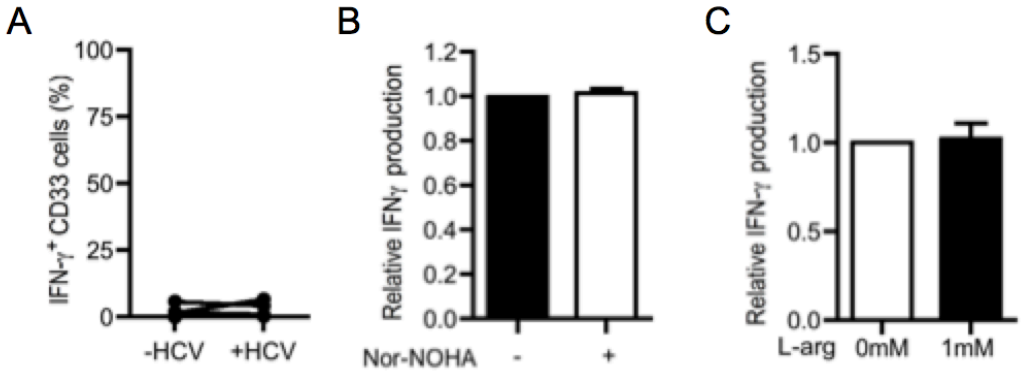

A
IFN-γ+ CD33 cells (%)
100
75
50
25
0
-HCV
+HCV
B
Relative IFNγ production
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Nor-NOHA
-
+
C
Relative IFN-γ production
1.5
1.0
0.5
0.0
L-arg
0mM
1mM

Figure 3.3. Decrease in IFN-γ production is independent of *IFNG* transcription and differentiation status of NK cells. NK cells were co-cultured with mock- or HCV-conditioned $CD33^+$ cells in the presence of IL-2/IL-12/IL-18 for 2 days. The cells were then harvested by detachin treatment and incubated with IL-12/IL-18 in the presence of GolgiPlug for 5h and stained for surface markers and intracellular expression of IFN-γ. NK cells were gated on live cells, singlets, forward and side scatter, and $CD33^-$ cells. (A) Representative dot plots of IFN-γ and CD56 expression in NK cells co-cultured with mock- or HCV-conditioned $CD33^+$ cells. (B) Frequency of IFN-γ^+ NK cells (C) Relative mean fluorescence intensity (MFI) of IFN-γ in NK cells. (D) Frequency and (E) relative MFI of $CD56^+$ NK cells was calculated from cell treated as described in (A). (F) Mock- or HCV-conditioned $CD33^+$ cells were separated from NK cells with a 0.45μm transwell insert and stimulated with IL2/IL12/IL18. After 2 days, NK cells were recovered, lysed and the expression of *IFNG* was assessed relative to *GAPDH* expression by qRT-PCR. *IFNG* expression was normalized to NK cells co-cultured with mock-conditioned $CD33^+$ cells. Results are the mean or representative of 6-8 independent experiments. ns denotes not significant, *$p<0.05$, (B-D) two-tailed paired *t* test or (E) Wilcoxon signed rank test.

Figure 3.3

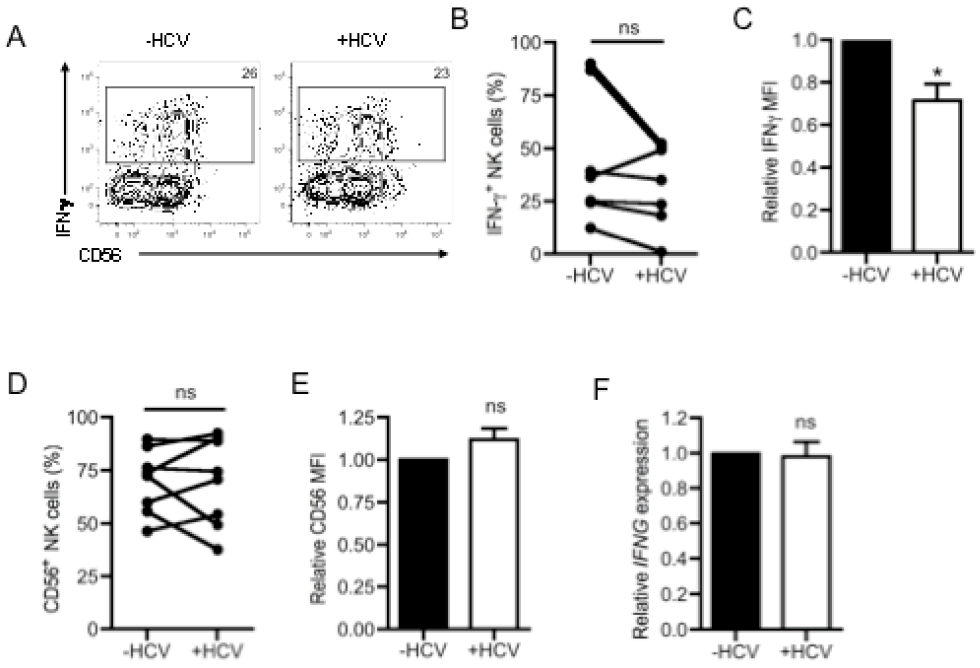

A
-HCV
+HCV
26
23
IFNγ
CD56
B
ns
IFN-γ+ NK cells (%)
100
75
50
25
0
-HCV
+HCV
C
Relative IFNγ MFI
1.0
0.8
0.6
0.4
0.2
0.0
*
-HCV
+HCV
D
ns
CD56+ NK cells (%)
100
75
50
25
0
-HCV
+HCV
E
ns
Relative CD56 MFI
1.25
1.00
0.75
0.50
0.25
0.00
-HCV
+HCV
F
ns
Relative IFNG expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-HCV
+HCV

Figure 3.4. HCV-conditioned $CD33^+$ cells include MDSCs. PBMCs were cultured with Huh7.5.1 cells uninfected or infected with HCV for 7 days after which $CD45^+$ cells were positively selected by magnetic separation. (A and B) MDSCs were defined as $CD33^+CD11b^{lo}HLA\text{-}DR^{lo}$ gated on forward and side scatter, live cells, $HLA\text{-}DR^{lo}$, CD33 and CD11b. (C) Frequency and (D) number of MDSCs was calculated from the gating strategy described in A and B. Results are the mean or representative of 6 independent experiments. *$p<0.05$, (C and D) Wilcoxon signed rank test.

Figure 3.4

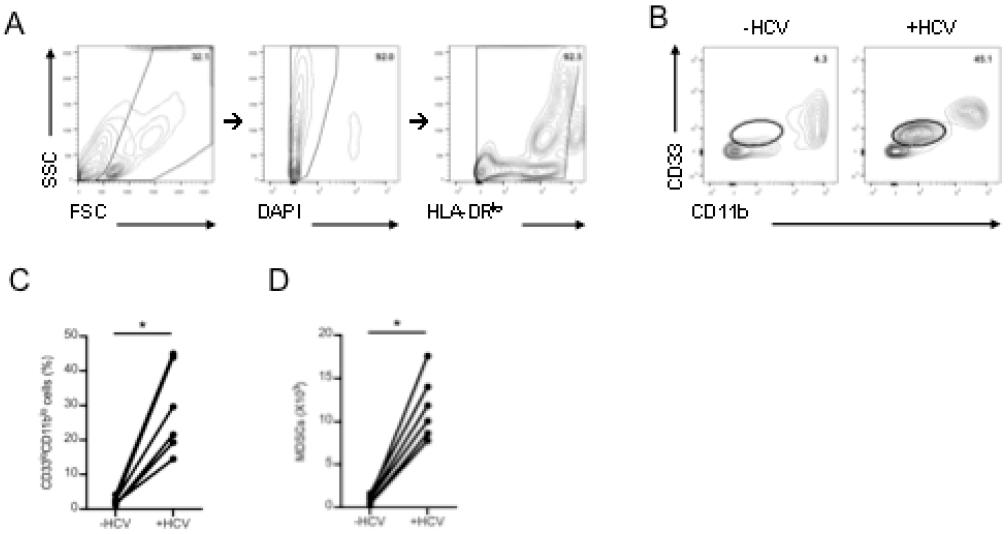


Figure 3.5. HCV-infected hepatocytes induce the accumulation of MDSCs in virus dose-dependent manner. (A) Huh7.5.1 cells were infected with JFH-1 at various m.o.i. Cells were collected at day 4 post-infection for qRT-PCR analysis to detect HCV RNA. (B) JFH-1-infected Huh7.5.1 cells at various time points were examined to detect intracellular core protein by immunoblotting. (C) Huh-7.5.1 cells grown on cover slips were infected with 3 dilutions of the sucrose-purified virus stock to achieve a level of infection to calculate the percentage of total infected cells. The level of HCV infection was determined 3 days post infection by immunofluorescence staining for HCV Core. Total number of cells and infected (HCV-core^{+}) cells are counted in 6 random fields to calculate the % infection (total cell number / infected cell number). (D) PBMCs were cultured with uninfected or HCV-infected (0.01 or 0.1 m.o.i) Huh7.5.1 cells for 7 days and CD45^{+} cells were then positively selected by magnetic separation. MDSCs were defined as CD33^{+}CD11b^{+}HLA-DR^{lo} gated on forward and side scatter, live cells, HLA-DR^{lo}, CD33 and CD11b. The percentages of MDSCs were plotted. Each line represents data from the same donor. *p<0.05, **p<0.01, Kruskal-Wallis test (One-way ANOVA) with Dunn's post-test, as appropriate (E) Patients were divided in to two groups: high virus titer (>800,000IU/mL) and low virus titer (<800,00IU/mL). PBMCs from chronic HCV patients were stained for MDSCs described as above. The percentages of MDSCs were plotted. ns denotes not significant, Mann Whitney two-tailed test. (F) The percentages of MDSCs were plotted against ALT levels and linear regression was calculated.

Figure 3.5

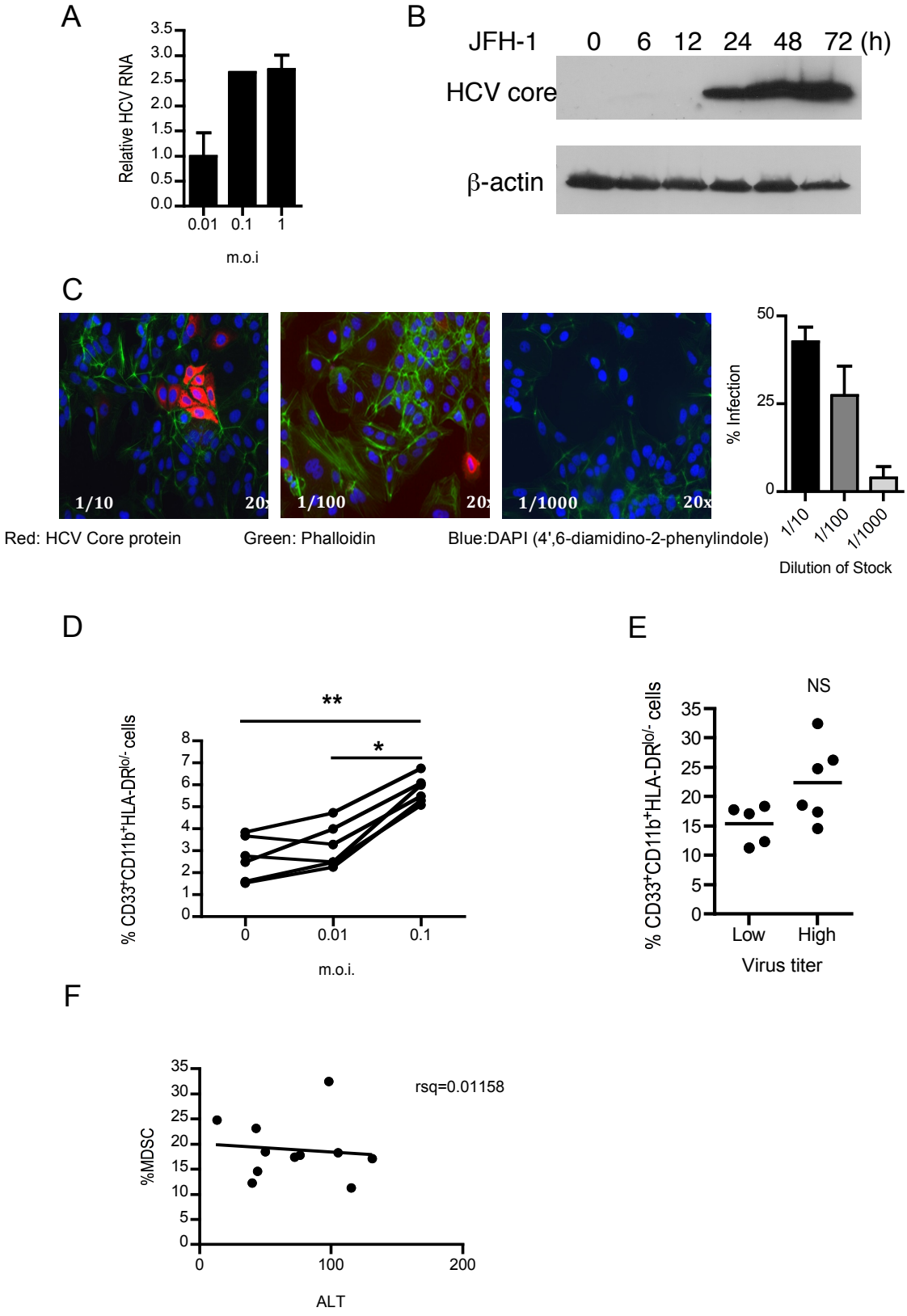

A
Relative HCV RNA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.01
0.1
1
m.o.i
B
JFH-1
0
6
12
24
48
72 (h)
HCV core
β-actin
C
1/10
1/100
1/1000
% Infection
50
25
0
1/10
1/100
1/1000
Dilution of Stock
Red: HCV Core protein
Green: Phalloidin
Blue:DAPI (4',6-diamidino-2-phenylindole)
D
% CD33+CD11b+HLA-DRlo/- cells
**
*
8
7
6
5
4
3
2
1
0
0
0.01
0.1
m.o.i.
E
% CD33+CD11b+HLA-DRlo/- cells
NS
35
30
25
20
15
10
5
0
Low
High
Virus titer
F
%MDSC
rsq=0.01158
35
30
25
20
15
10
5
0
0
100
200
ALT

Figure 3.6. HCV-induced MDSCs suppress NK cell IFN-γ production via contact-independent production of arginase-1. (A) Mock- or HCV-conditioned $CD33^+$ cells were co-cultured with NK cells in the presence of a 0.45μm transwell insert. (B) After 2 days in culture, IFN-γ production by NK cells was assessed by intracellular staining. (C-E) Mock- or HCV-conditioned $CD33^+$ cells were co-cultured with IL-12/IL-18 stimulated NK cells for 2 days in the presence or absence of (C) Catalase which decomposes ROS, (D) L-NMMA which inhibits iNOS, or E) nor-NOHA which inhibits arginase-1. IFN-γ release into culture supernatants was assessed by ELISA. Results are the mean or representative of 6-8 independent experiments. ns denotes not significant, $*p<0.05$, $**p<0.01$, (B) Wilcoxon signed rank test, (B) two-tailed paired *t* test, or (C-E) Kruskal-Wallis test with Dunn's post-test.

Figure 3.6

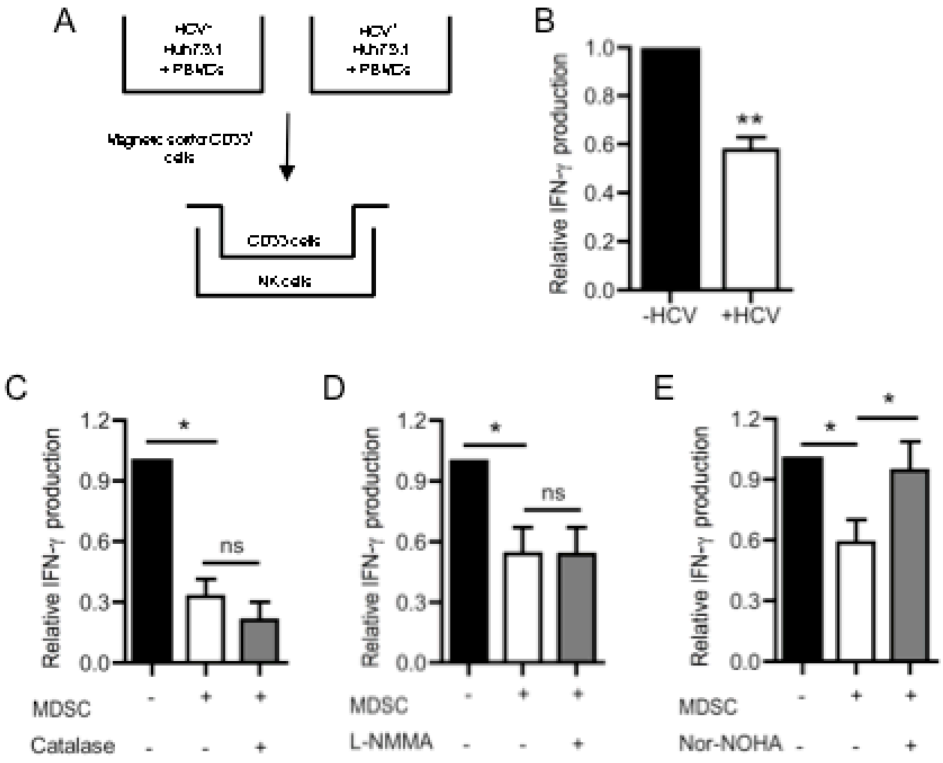

A
HCV+ Huh7.5.1 + PBMCs
HCV- Huh7.5.1 + PBMCs
Magnetic sorted CD33+ cells
CD33+cells
NK cells
B
Relative IFN-γ production
1.0
0.8
0.6
0.4
0.2
0.0
**
-HCV
+HCV
C
Relative IFN-γ production
1.2
0.9
0.6
0.3
0.0
*
ns
MDSC - + +
Catalase - - +
D
Relative IFN-γ production
1.2
0.9
0.6
0.3
0.0
*
ns
MDSC - + +
L-NMMA - - +
E
Relative IFN-γ production
1.2
0.9
0.6
0.3
0.0
*
*
MDSC - + +
Nor-NOHA - - +

Figure 3.7. Non-significant changes in innate cytokine production by HCV-conditioned CD33$^+$ compared to uninfected CD33$^+$ cells. PBMCs were cultured with Huh7.5.1 cells uninfected or infected with HCV for 7 days after which CD33$^+$ cells were positively selected by magnetic separation. (A-D) CD33$^+$ cells were lysed by sonication and an innate panel luminex assay was performed on the cell lysates. Cytokine production was normalized to the uninfected-conditioned CD33$^+$ cell controls. (E) CD33$^+$ cells were grown in PBMC media for 24 hours. The media was collects and spun to remove cells and an innate panel luminex assay was performed on the media. Cytokine production was normalized to the uninfected-conditioned CD33$^+$ cell controls. Results represent 4 different donors. *p<0.05, **p<0.01, ***p<0.001, ns denotes not significant. (A-E) Wilcoxon signed rank test.

Figure 3.7

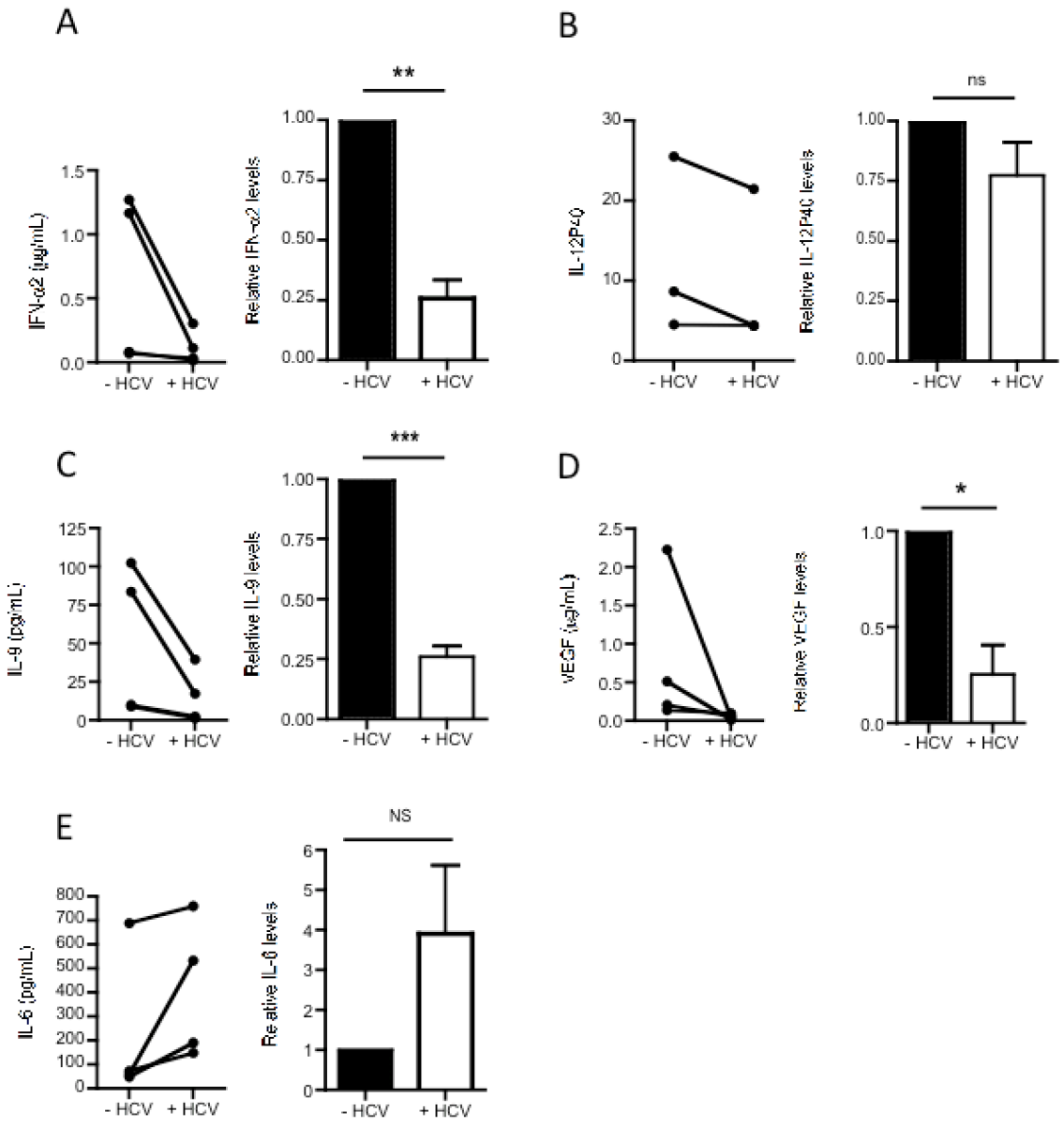

A
IFN-α2 (pg/mL)
- HCV
+ HCV
**
Relative IFN-α2 levels
B
IL-12P40
ns
Relative IL-12P40 levels
C
IL-9 (pg/mL)
***
Relative IL-9 levels
D
VEGF (pg/mL)
*
Relative VEGF levels
E
IL-6 (pg/mL)
NS
Relative IL-6 levels

Figure 3.8. Catalase reverses MDSC suppression of CD4 T cell IFN-γ production while L-NMMA suppresses iNOS production of nitric oxide. (A) PBMCs were cultured with Huh7.5.1 cells uninfected or infected with HCV for 7 days after which $CD33^+$ cells were positively selected by magnetic separation. CD4 T cells were selected by magnetic sorting and co-cultured with the $CD33^+$ cells and stimulated with plate-bound CD3/CD28 antibodies. Catalase (100U/mL) was added during co-cultures for the catalase condition. After 48, the cell culture media was obtained and IFN-γ production was measured by ELISA. (B) Raw264.7 cells were stimulated with 100ng/mL LPS for 24h with the addition of L-NMMA. The amount of NO was measured using the Greiss Assay.

Figure 3.8

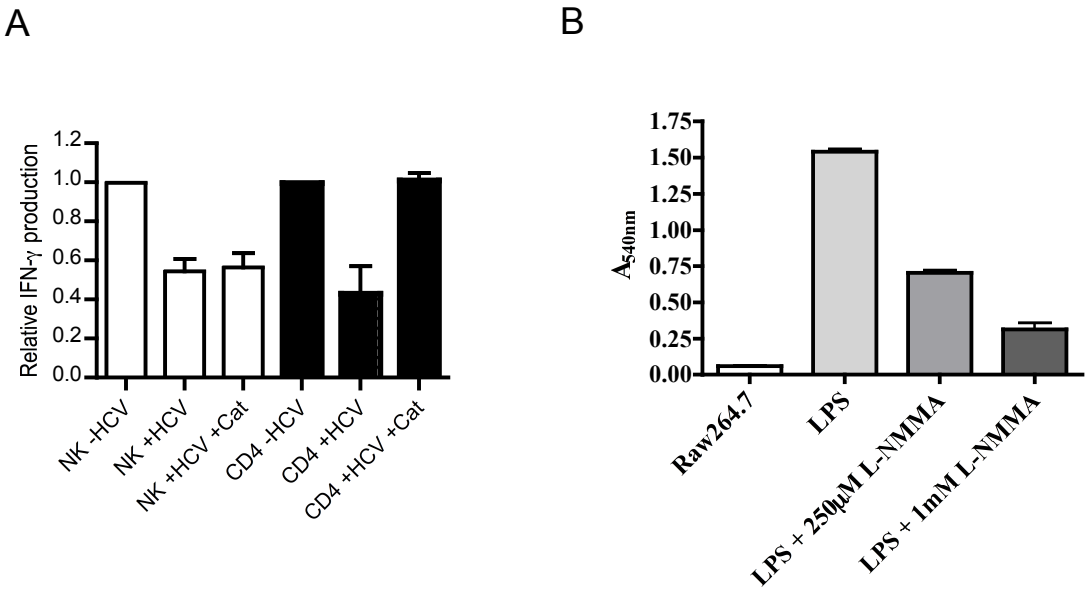

A
B
Relative IFN-γ production
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NK -HCV
NK +HCV
NK +HCV +Cat
CD4 -HCV
CD4 +HCV
CD4 +HCV +Cat
A540nm
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Raw264.7
LPS
LPS + 250μM L-NMMA
LPS + 1mM L-NMMA

Figure 3.9. Increased arginase-1 expression in HCV-conditioned CD33$^+$ cells depletes arginine levels required for NK cell IFN-γ production. (A) NK cells were grown in complete media or L-arginine-deficient media and were stimulated with IL-12/IL-18 for 2 days. IFN-γ in the supernatant was measured by ELISA. (B) NK cells cultured with mock- or HCV-conditioned CD33$^+$ cells were stimulated with IL-2/IL-12/IL-18 and supplemented with 0, 0.01, 0.1, or 1mM L-arginine for 2 days. IFN-γ in the supernatant was measured by ELISA. (C) Mock- or HCV-conditioned CD33$^+$ cells were cultured with NK cells and separated a 0.45μm transwell insert. Following 2 days of co-culture, arginase activity in the CD33$^+$ cells was determined using an arginase assay kit. (D) Mock- or HCV-conditioned CD33$^+$ cells were cultured with NK cells for 2 days. CD33$^+$ cells were then recovered by magnetic selection and intracellular arginase-1 expression was assessed on MDSCs gated on forward and side scatter, live cells, singlets, CD33$^+$, HLA-DR^{lo} and CD11b^{lo}. (E) Frequency of Arginase-1$^+$ MDSCs and (F) MFI of Arginase-1 expression was quantified from the experiment described in D. Results are the mean or representative of 3-8 independent experiments. *p<0.05, **p<0.01, (A, C, F) two-tailed paired t test, (B) Kruskal-Wallis test with Dunn's post-test. or (E) Wilcoxon signed rank test.

Figure 3.9

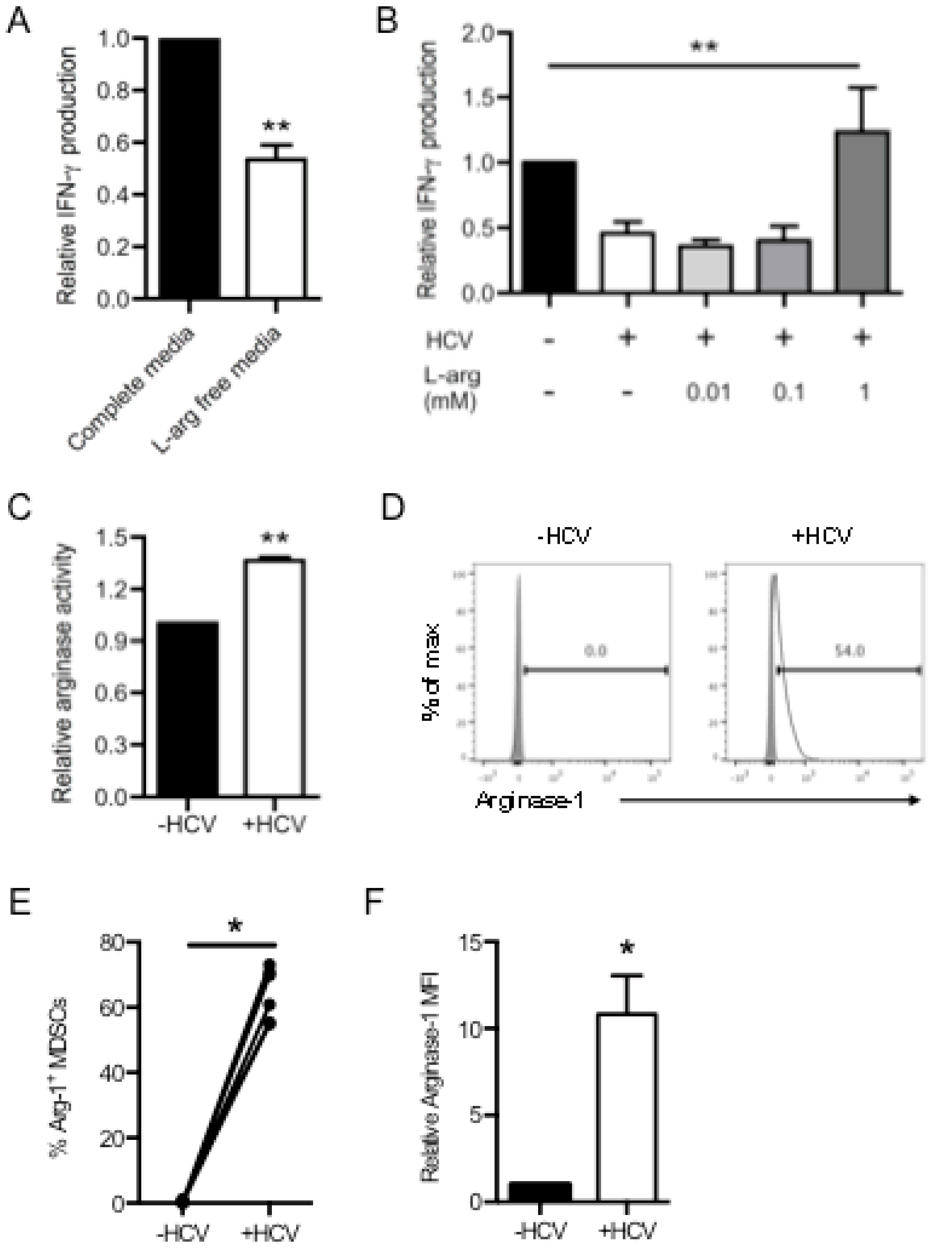

A
Relative IFN-γ production
1.0
0.8
0.6
0.4
0.2
0.0
**
Complete media
L-arg free media
B
Relative IFN-γ production
2.0
1.5
1.0
0.5
0.0
**
HCV - + + + +
L-arg (mM) - - 0.01 0.1 1
C
Relative arginase activity
1.5
1.2
0.9
0.6
0.3
0.0
**
-HCV +HCV
D
-HCV
+HCV
% of max
0.0
54.0
Arginase-1
E
% Arg-1+ MDSCs
80
60
40
20
0
*
-HCV +HCV
F
Relative Arginase-1 MFI
15
10
5
0
*
-HCV +HCV

Figure 3.10. NK cell viability and granzyme B production are unaffected by L-arginine availability.

NK cells were grown in complete media or L-arginine-deficient media and were stimulated with IL-12/IL-18 for 2 days. (A) Cell viability was assessed by flow cytometry. (B**)** Granzyme B in the supernatant was measured by ELISA. Results are representative or mean of 3-6 independent experiments. ns denotes not significant, two-tailed paired *t* test.

Figure 3.10

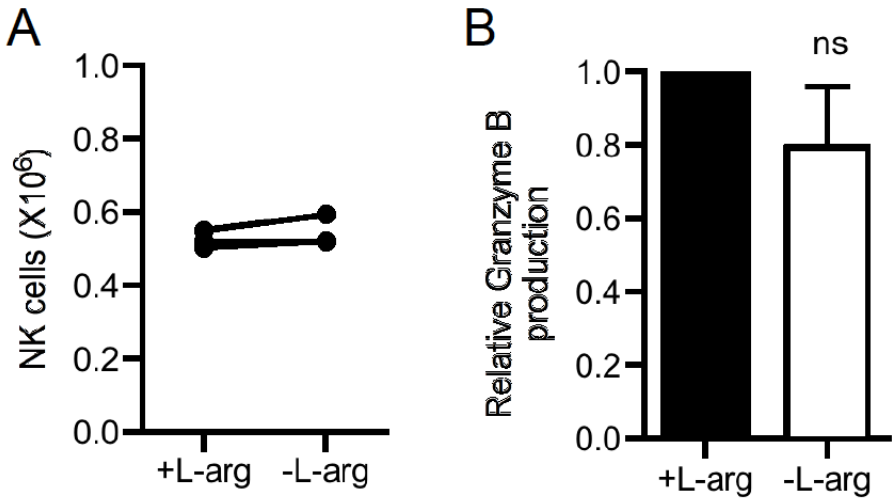

A
NK cells (X10^6)
1.0
0.8
0.6
0.4
0.2
0.0
+L-arg
-L-arg
B
Relative Granzyme B production
ns
1.0
0.8
0.6
0.4
0.2
0.0
+L-arg
-L-arg

Figure 3.11. miRNA-29 levels do not correlate with IFN-γ production in NK cells that are deprived of L-arginine or experienced MDSCs. NK cells were (A) grown in complete media or L-arginine-deficient media or (B) co-cultured with uninfected- or HCV-conditioned $CD33^+$ cells and stimulated with IL-12/IL-18 for 2 days. The NK cells were recovered and lysed. TaqMan® small RNA assays for miR-29a, miR-29b, and miR-29c were performed to determine expression levels. The expression levels were normalized to small nuclear U6 gene, RNU6B and then to the miR-29 expression levels of NK cells that were grown (A) in complete media or (B) co-cultured with uninfected-conditioned $CD33^+$ cells. The relative expression of miR-29a, miR-29b, and miR-29c from NK cells (A) grown in L-arginine free and (B) co-cultured with HCV-conditioned $CD33^+$ cells were plotted.

Figure 3.11

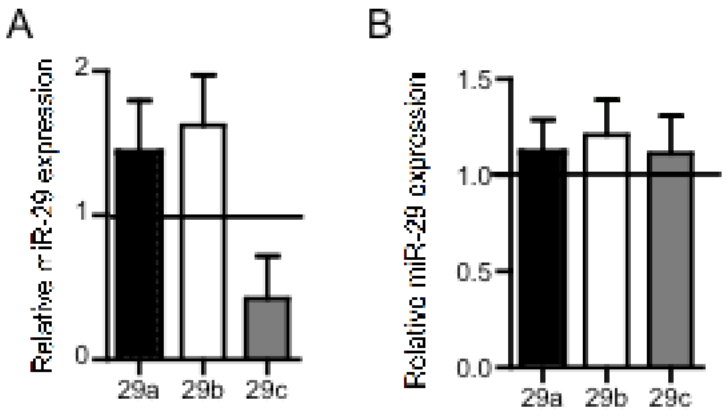

A
Relative miR-29 expression
2
1
0
29a
29b
29c
B
Relative miR-29 expression
1.5
1.0
0.5
0.0
29a
29b
29c

Figure 3.12. Suppression of NK cell IFN-γ production is mediated by reduced mTOR signaling. NK cells were co-cultured with mock- or HCV-conditioned $CD33^+$ cells separated by a 0.45 μm transwell insert. Following 2 days in culture, NK cells were recovered and phosphorylation of mTOR was assessed by flow cytometry. (A) Cells were gated on singlets, forward and side scatter, $mTOR^+$ cells, and phospho-$mTOR^+$. mTOR and phospho-mTOR positive cells were gated based on FMOs. MFI of phospho-mTOR expression was quantified from the experiment. (B) Activation of the mTOR substrate, 4EBP1, was assessed measuring the phosphorylation of 4EBP1 at residue T69 by flow cytometry. As above, cells were gated on singlets, forward and side scatter, and phosphor-$4EBP1^+$ cells based on FMOs. The MFI of p-4EBP1 of the p-$4EBP1^+$ cells was computed. (C) NK cells were grown in complete media with and without the mTOR inhibitor rapamycin or L-arginine deficient media for 2 days. IFN-γ in the supernatant was measured by ELISA. Results are representative or mean of 8-10 independent experiments. *p<0.01, **p<0.01, ns denotes not significant, (A, B) Wilcoxon signed rank test. **p<0.01, ***p<0.001, (C) Kruskal-Wallis test with Dunn's post-test.

Figure 3.12

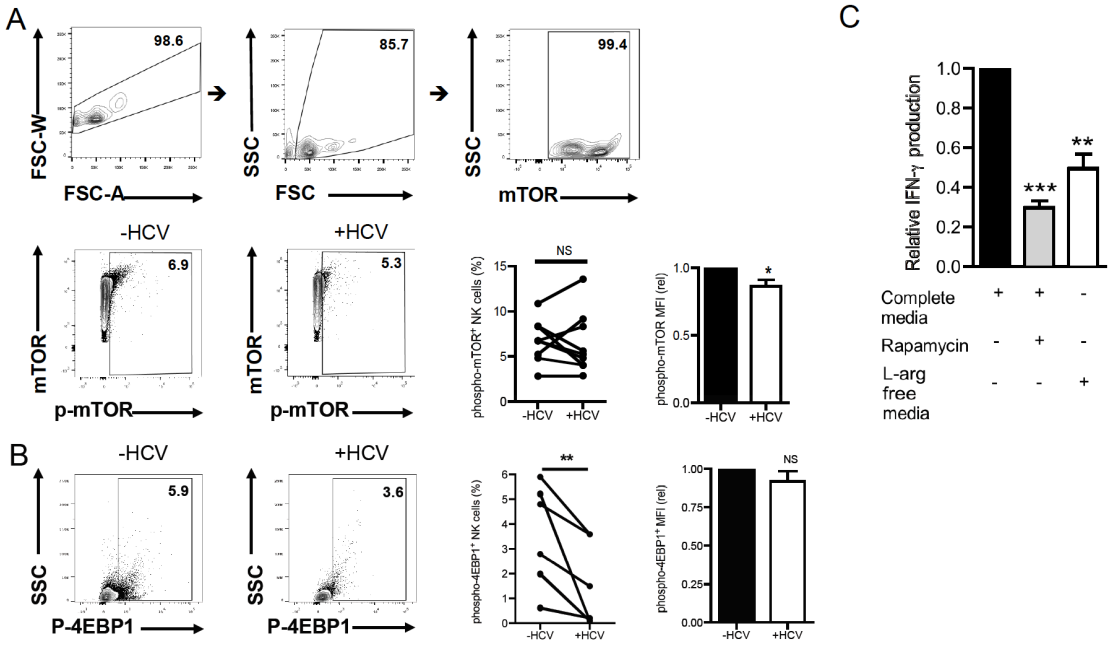

A
98.6
FSC-W
FSC-A
85.7
SSC
FSC
99.4
SSC
mTOR
-HCV
+HCV
6.9
5.3
mTOR
p-mTOR
NS
phospho-mTOR+ NK cells (%)
-HCV
+HCV
phospho-mTOR MFI (rel)
*
B
5.9
3.6
SSC
P-4EBP1
**
phospho-4EBP1+ NK cells (%)
NS
phospho-4EBP1+ MFI (rel)
C
Relative IFN-γ production
***
**
Complete media
Rapamycin
L-arg free media

Figure 3.13. Arginine deprivation results in reduced mTOR signaling in NK cells. NK cells were grown in complete media or arginine-free media and stimulated with IL-12/IL-18 for 48h. The cell media was collected, spun to obtain cell bodies, while more cells were recovered by detachin treatment and combined with the existing cell pellet. (A) The phosphorylation levels of mTOR and 4EBP1 were assessed by flow cytometry. Cells were gated on singlets, forward and side scatter, $mTOR^+$ cells. (B) MFIs of phospho-mTOR expression were quantified from the experiment. mTOR and phospho-mTOR positive cells were gated based on FMOs as shown above. (C) The level of phosphorylated 4EBP1 (pT69) was assessed. As above, cells were gated on singlets, forward and side scatter, and phosphor-$4EBP1^+$ cells based on FMOs. (D) The MFI of p-4EBP1 of the p-$4EBP1^+$ cells was computed.

Figure 3.13

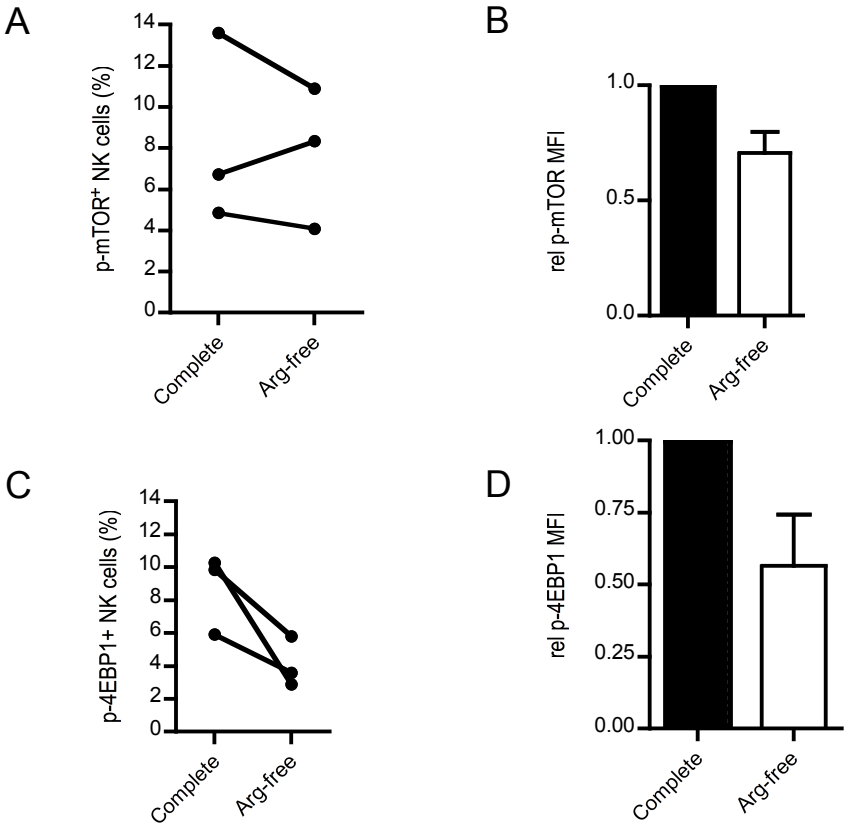

A
p-mTOR+ NK cells (%)
14
12
10
8
6
4
2
0
Complete
Arg-free
B
rel p-mTOR MFI
1.0
0.5
0.0
Complete
Arg-free
C
p-4EBP1+ NK cells (%)
14
12
10
8
6
4
2
0
Complete
Arg-free
D
rel p-4EBP1 MFI
1.00
0.75
0.50
0.25
0.00
Complete
Arg-free

Figure 3.14. Chronic HCV patients' NK cells display impaired IFN-γ production in response to IL-12/IL-18 stimulation. PBMCs were obtained from chronic 7 HCV patients and 7 healthy donors and then frozen in 90% FBS/10% DMSO. NK cells were magnetically sorted from thawed PBMCs and cultured for 2 days with IL-2/IL-12/IL-18. (A) The cell media was collected, spun to remove cell bodies, and IFN-γ in the culture media was determined by ELISA. (B, C) More cells were recovered by detachin treatment, combined with the existing cell pellet, and then phosphorylation levels of mTOR and 4EBP1 were assessed by flow cytometry. (B) Cells were gated on singlets, forward and side scatter, $mTOR^+$ cells. MFIs of phospho-mTOR and total mTOR expression were quantified from the experiment. mTOR and phospho-mTOR positive cells were gated based on FMOs as shown in Fig. 6. (C) The level of phosphorylated 4EBP1 (pT69) was assessed. As above, cells were gated on singlets, forward and side scatter, and phosphor-$4EBP1^+$ cells based on FMOs. The MFI of p-4EBP1 of the p-$4EBP1^+$ cells was computed. ***$p<0.001$, ns denotes not significant, (A-C) Mann-Whitney Test.

Figure 3.14

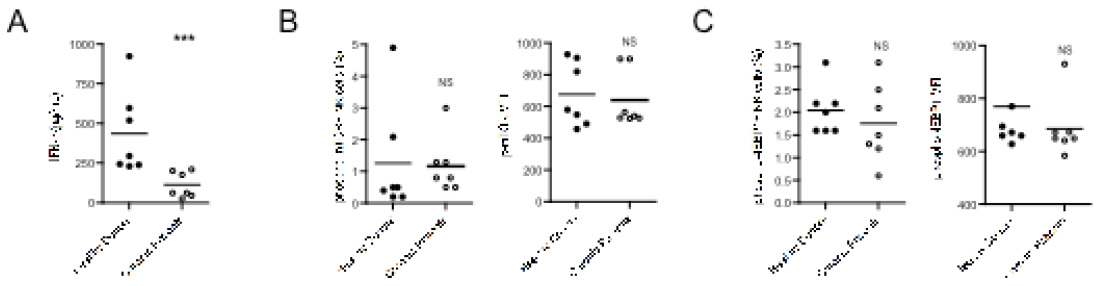


Figure 3.15. Increased CD33+ cells to NK cell ratio correlates positively with suppressive effects. Uninfected- or HCV-conditioned CD33+ cells were cultured with autologous NK cells at the ratios of 1:1, 1:2, 1:4, and 1:8. The cells were stimulated with IL-12/IL-18 for 2 days. Cell culture supernatants were recovered and IFN-γ production was measured by ELISA. The IFN-γ production was normalized to the corresponding uninfected-conditioned sample. Results are mean of 3 independent experiments.

Figure 3.15

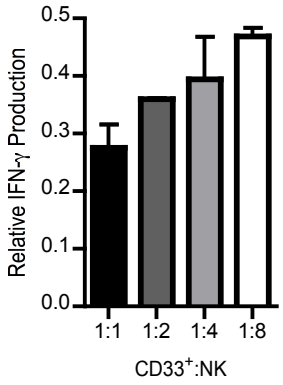
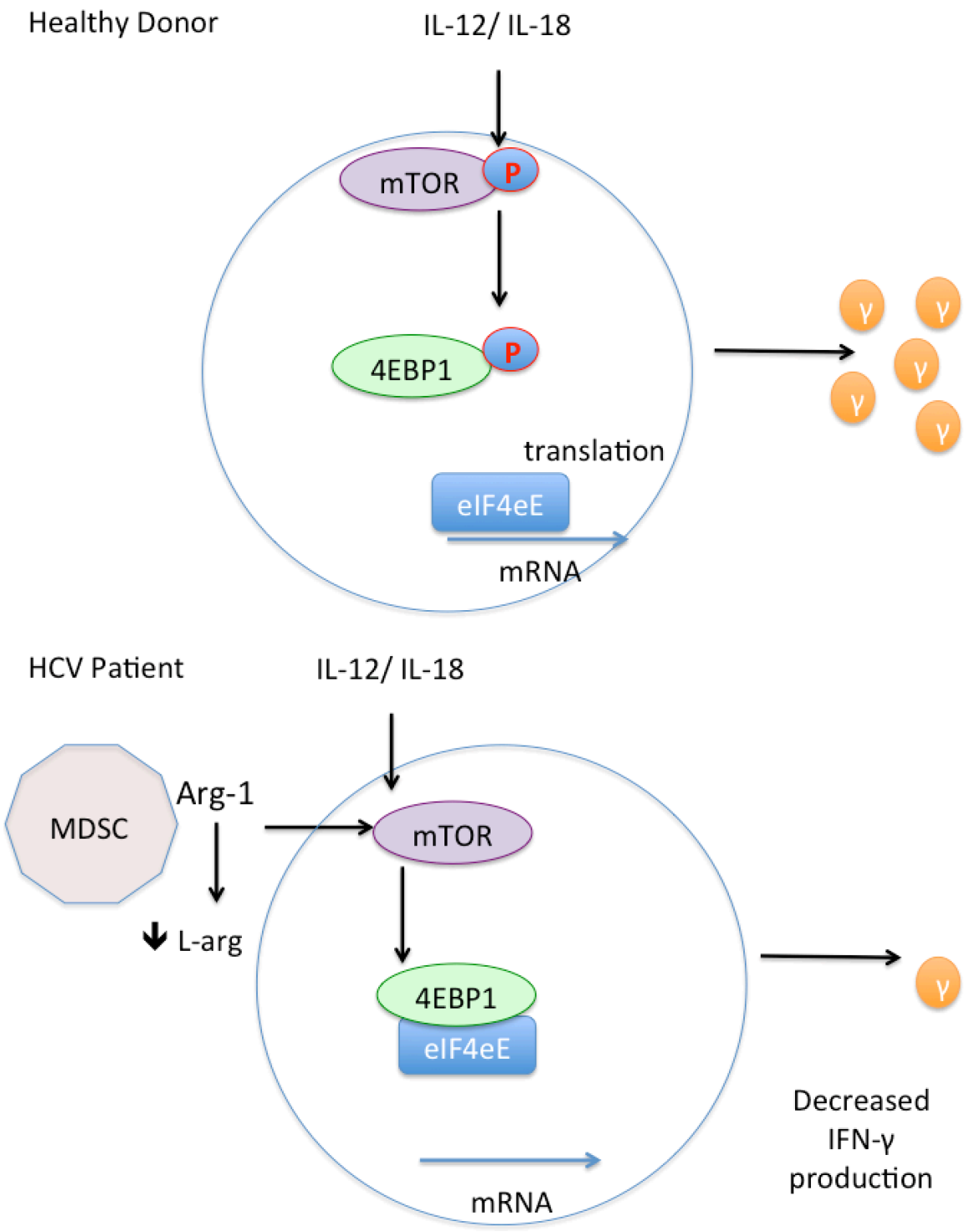


Figure 3.16. Mechanism of HCV-induced MDSCs on NK cell IFN-γ production. When healthy donor NK cells are stimulated by IL-12/IL-18, mTOR is phosphorylated and active. The active mTOR complex phosphorylates and activates 4EBP1. When 4EBP1 is phosphorylated, it frees eIF4e, which binds to 5' cap structure of the mRNA, allowing the assembly of the translation initiation complex. As such, IFN-γ protein can be produced. When HCV-induced MDSCs are present, the elevated levels of arginase activity result in decreased availability of L-arginine to the NK cells. This causes reduced mTOR and thus 4EBP1 activation, allowing 4EBP1 to sequester eIF4e, preventing protein translation and IFN-γ from being made.

Figure 3.16


Healthy Donor
IL-12/ IL-18
mTOR
P
4EBP1
P
translation
eIF4eE
mRNA
γ
γ
γ
γ
γ
HCV Patient
IL-12/ IL-18
MDSC
Arg-1
L-arg
mTOR
4EBP1
eIF4eE
mRNA
γ
Decreased
IFN-γ
production

Chapter 4: Conclusions and Future Directions

I. General Conclusions

HCV infection is highly efficient in the establishment of persistent infection due to the virus' many immune evasion mechanisms (229-231), one of which is the generation of MDSCs (13). As NK cell-produced IFN-γ is important for launching a good immune response (232), this dissertation shows the effects of HCV-generated MDSCs on NK cell functions, and further discusses the mechanisms leading to impaired IFN-γ production. In this dissertation, I demonstrated that the presence of HCV-infected hepatocytes causes an accumulation of MDSCs that are $CD33^{+}CD11b^{lo}HLA\text{-}DR^{lo/-}$ and have enhanced arginase-1 expression and activity. I further confirmed that *in vitro,* a higher viral dose of HCV generated MDSCs at higher frequencies. Also, I showed that these MDSCs exist in chronic HCV patient blood, with a trend that patients with high virus titers expressed MDSCs at higher frequencies than patients with low virus titers.

Importantly, these HCV-induced MDSCs suppress NK IFN-γ production, but not Granzyme B production or cell viability, suggesting that the effects of HCV-induced MDSCs were specific to IFN-γ production rather than a global shutdown of NK cell function.

Next, I explored the interactions between the MDSCs and the NK cells and established that the effect on IFN-γ production is cell-contact independent but is not due to innate cytokines secreted by the MDSCs. In addition, this suppression was mediated by neither

ROS production nor iNOS activity. Instead, the suppression was a result of arginase-1 activity by blocking arginase-1 with Nor-NOHA. Arginase-1 depletes the cell culture medium of L-arginine, and thus decreasing its availability to the NK cells. Thus, I confirmed my findings by replenishing the culture media with L-arginine, restoring NK IFN-γ production, showing that suppression indeed results from the NK cells being deprived of L-arginine.

Developing this research further, I explored the intracellular events within the NK cells that caused the decreased IFN-γ production. I found that the defect is post-transcriptional but not mediated by miR-29 microRNA. Looking into the mTOR pathway, I found that phosphorylation and activation of mTOR and its downstream target, 4EBP1 were both decreased. Hence, arginine starvation decreases IFN-γ production by impairing mTOR signaling, an important pathway in protein translation.

Finally, I found that chronic HCV patients NK cells produce less IFN-γ. Even though I did not obtain statistically significant results, I have noted a trend that chronic HCV patients' NK cells also suffer from defective mTOR signaling, supporting that HCV causes immune dysfunction of NK cells.

In summary, HCV affects the cellular metabolism of NK cells leading to a specific effector function being reduced.

II. Future directions

Other NK cell functions. In addition to producing cytokines, NK cells play other important roles such as direct cytotoxicity of infected hepatocytes via the degranulation and the induction of apoptosis (39, 233, 234) and studies have reported impaired NK cell degranulation and cytotoxicity in chronic HCV patients (233), examining whether MDSCs affect these NK cell functions could be performed. I showed that the amount of granzyme B released into the cell culture media when stimulated with IL-12 and IL-18 was not altered in the presence of HCV-induced MDSCs. As different stimuli activate different NK cell effector functions (235), in order to investigate whether granzyme B production is impaired by HCV-induced MDSCs, other forms of stimulation, such as IL-15, need to be used. In addition, if we find that cytotoxicity and/or degranulation is affected by HCV-conditioned $CD33^+$ cells, the mechanisms of action could be elucidated.

Other pathways affecting IFN-γ production. It is noteworthy that both the percentages of IFN-γ producing cells and phospho-mTOR positive cells are similar in NK cells that experienced MDSCs compared to those that did not, while, on the other hand, both the MFIs of IFN-γ and phospho-mTOR differed between the two groups. These consistent findings are reassuring because it shows that the decrease in IFN-γ production does not result from fewer cells producing the cytokine, but rather, there is a functional change within the NK cells, such that the mTOR pathway is impaired in a way to affect IFN-γ production at the translational level. On the other hand, over 20% of NK cells produced IFN-γ and are affected by the MDSCs, but only 3-7% of NK cells experienced a change

in p-mTOR and p-4EBP1. This suggests that the defect in NK IFN-γ production is caused by more factors than just a change in mTOR signaling and arginine bioavailability. Even though the defective mTOR pathway plays a major role in reducing NK IFN-γ production, further studies showing other pathways that were implicated due to arginine deprivation will allow a fuller restoration of NK IFN-γ production.

Crosstalk between NK cells and MDSCs. In my research, an interesting finding arose: infected-conditioned CD33 produce greater amounts of arginase-1 as well as express higher arginase activity compared to uninfected-conditioned CD33 only after they had been cultured for 2 days with NK cells (Fig. 3.9 C-F) When $CD33^{+}$ cells were obtained from a co-culture of PBMCs and Huh7.5.1 and directly analyzed for arginase activity, the HCV-conditioned CD33+ cells surprisingly expressed lower arginase activity than their uninfected-conditioned counterparts (Fig. 4.1A). In addition, there was no difference in *ARG1* mRNA (4.1B) in these cells. This finding suggests that culturing these $CD33^{+}$ cells for 2 days (likely in the presence of NK cells or their soluble factors) was needed to activate the increased arginase-1 activity.

In vivo, myeloid cells would experience NK cells, and thus it is very probable that in a HCV setting, crosstalk between NK cells and MDSCs exist. This is because there is probable cause to believe that NK cells would trigger a pathway alerting MDSCs to suppress inflammation. This could be as simple as IFN-γ acting as a feedback loop, or some complicated mechanism to turn on MDSC activity. Hence, it would be extremely

interesting to elucidate the effects of NK cells on these myeloid cells leading to the upregulation of their arginase expression and activity.

To test this crosstalk between NK cells and MDSCs, I would propose to investigate if the presence of NK cell bodies is needed. I have shown that cell-cell contact between the two cell types is not needed as there was elevated arg-1 expression and activity even when the cells were separated by a 0.45μm transwell insert (Fig. 3.9C-F). To this end, I propose growing NK cells in complete media or arginine-free media and stimulating them with the IL-12/IL-18 cocktail. After 2 days, I would recover the cell culture media and centrifuge it to remove cell bodies and cell debris, and then culture the $CD33^+$ cells recovered from the co-culture of PBMCs and uninfected or HCV-infected Huh7.5.1 cells for another 2 days. I would then test the $CD33^+$ cells for arginase activity or stain them for CD33, CD11b, HLA-DR, and intracellular Arg-1. As controls, I would use uninfected- or HCV-conditioned $CD33^+$ cells that were grown in fresh PBMC media or $CD33^+$ cells that were not further cultured after being obtained from the previous co-culture (see Fig 4.2). There could be several outcomes of this experiment, but my hypothesis is that $CD33^+$ cells grown in complete (or even arginine-free) NK cell media would express higher arginase-1 activity compared to those cultured in fresh media. I hypothesize that the NK cells secrete some soluble factor, perhaps even IFN-γ, to turn up arginase-1 activity in MDSCs.

IFN-γ has been reported to activate MDSCs (55) and to play a role in the activation of arginase in macrophages (67). This supports my hypothesis IFN-γ or some other soluble factor played a role in the activation of arginase-1 in our HCV-generated MDSCs.

If arginase activity is shown to be increased in $CD33^+$ cells grown in NK cell supernatant, the next step would be to see if the addition of IFN-γ at various concentrations to $CD33^+$ cells grown in fresh media would also achieve this upregulation of arginase-1. If IFN-γ does not change arginase-1 expression, I would run a luminex panel on the cell culture media from NK cells to see if a different cytokine or chemokine was highly elevated. I would then add that cytokine or chemokine exogenously to fresh culture media when culturing the $CD33^+$ cells and evaluate the arginase activity. Preventing the activation of MDSCs could provide an alternative way to combat HCV.

Induction of MDSC accumulation. I showed in my dissertation that HCV-infected hepatocytes caused an accumulation of MDSCs. Two interesting questions arose from this finding— 1) Does the virus directly cause the accumulation of MDSCs? Is viral replication needed to induce MDSC accumulation? Are hepatocytes and non-myeloid cells needed for this accumulation? 2) How are the MDSCs proliferating? How are they accumulating?

To answer the first question, I would conduct the series of experiments in Figure 4.3 to determine MDSC accumulation by staining cells of MDSC markers and analyzing them by flow cytometry. First, I would examine the effect of UV-irradiated JFH1 on the generation of MDSCs. I would add UV-irradiated sucrose gradient-purified JFH1 or competent JFH1 to Huh7.5.1 for 5 days before co-culturing the cells with PBMCs. After 7 days, I would recover the immune cells and stain them for MDSC markers. If MDSCs are not accumulated in the UV-irradiated-conditioned sample, I would know that intact

virus is needed for MDSC accumulation. However, it is also possible that a protein from the virus is need but present in too low a concentration. To address this issue, increasing doses of virus should be used. If MDSCs are accumulated, I would measure the virus titer in recovered supernatant to ensure that the irradiation had rendered the virus unable to replicate. I would also attempt to infect Huh cells with some irradiated virus and visualize intracellular core by fluorescence microscopy to confirm that the irradiation achieved its intended effects. If the irradiation was successful and non-replicating virus was still able to generate MDSCs, I would determine if presence of the hepatocytes is needed. To achieve this, I would use sucrose-purified JFH directly on PBMCs without Huh cells. If MDSCs are not accumulated, I can conclude that the presence of the hepatocytes or even replication of the virus is needed. To confirm that replication is required, negative strand PCR should be performed. Once again, it is possible that a protein from the virus is need but present in too low a concentration. To address this issue, increasing doses of virus should be used.

Next, to determine if a hepatocyte-derived factor is responsible for the accumulation o MDSCs, I would perform a Luminex assay on the cell culture media obtained from HCV-infected Huhs and see if any of the known factors that cause MDSC accumulation are present at elevated amounts. If a factor(s) is identified, this factor(s) can be added exogenously to PBMCs to see if MDSCs are accumulated. One caveat is that a cocktail of factors might be needed and a series of experiments would need to be run to determine the combinations and concentrations.

If the virus is found to accumulate MDSCs in the absence of hepatocytes, either the virus acts directly on the myeloid cells or it acts on other PBMCs, which in act on the myeloid cells to cause MDSC accumulation. To test if the effects of the virus act directly on myeloid cells, I would first magnetically select $CD33^+$ cells and culture them in the presence of purified virus. If I do not get an accumulation of MDSCs, I would try higher doses of virus. Should I still not get an accumulation, I would conclude that the effects of the virus act indirectly on $CD33^+$ cells. Moving forward, I would culture total PBMCs in the presence of JFH1, collect the cell culture supernatant and perform a Luminex assay to see if there are elevated amounts of known factors that cause accumulation. If I do, I would apply that factor(s) directly onto $CD33^+$ cells to see if I get an accumulation of MDSCs. I might need to determine the cocktail of factors other PBMCs produce as a result of HCV to induce MDSC accumulation. One caveat would be that $CD33^-$ PBMCs require cell-cell contact with the $CD33^+$ cells to induce MDSC accumulation. If so, systematically depleting one cell type at a time from the co-culture will tell me which cell type is needed for the accumulation of MDSCs. If I get an accumulation of MDSCs in the absence of $CD33^-$ PBMCs, I can conclude that the virus acts directly on $CD33^+$ cells.

Finally, if purified JFH1 induces and accumulation of MDSCs on $CD33^+$ cell in the absence of other PBMCs, I would add the various viral proteins, such as core, alone or in combination, to determine which viral proteins are directly responsible for inducing the accumulation of MDSCs. If MDSCs are not accumulated, I conclude that intact virus acts on $CD33^+$ cells to induce MDSC accumulation.

To address my second question of whether MDSCs are accumulating, I would measure BrdU incorporation by flow cytometry. Another explanation for the increase in MDSC population would be that the MDSCs failed to mature in monocytes and other mature myeloid cells. Using the data from flow cytometry, I can look at the change in the different myeloid populations over the course of 7 days. This would tell me the time course of MDSC accumulating, and if pre-MDSC progenitors are becoming MDSCs and failing to differentiate into more mature cells. Understanding the crosstalk between NK cells and MDSCs and how HCV induces the accumulation of MDSCs will give a more complete picture of Immunoregulation by HCV-generated MDSCs on NK cells (Fig. 4.4).

NK cells from chronic HCV patients. Finally, it would be interesting to study if NK cells that have impaired IFN-γ production as a result of being cultured with HCV-induced MDSCs or from chronic patients eventually recover their IFN-γ production after being arginine sufficient *in vitro*. Considering that this inhibition to the mTOR pathway is reversible, it is possible that these NK cells will be able to recover their full IFN-γ producing capabilities. This will shed light on therapeutic implications discussed below.

III. Therapeutic Implications

Improving NK cell functions could prevent the onset of a chronic infection. In order to limit or reverse the effects of MDSCs, arginine supplementation may prove therapeutic. Given that robust NK cell IFN-γ production requires arginine, increasing the bioavailability of arginine to the NK cells during the acute stage of infection might boost NK cell responses, especially their IFN-γ production. This would result in a better immune response by both the innate and adaptive branches of the immune system, as well as induce an antiviral state in the hepatocytes, possibly preventing the onset of chronic HCV infection. Arginine has been reported to reduce liver damage and improve liver function in rats and humans (236-238). As such we know that increasing the bioavailability of arginine does not cause widespread inflammation and increased general immune cell responses. If a slow release of arginine benefits patients, perhaps administering a stent in the hepatic portal vein that allows slow release of arginine might prove beneficial.

Also, if NK cells defective in IFN-γ production due to defective mTOR signaling are able to recover their IFN-γ production when cultured with sufficient arginine *in vitro*, removing NK cells from HCV patients and rendering their NK cells functional could be a therapeutic strategy. Chronically infected HCV patients might benefit if NK cells recover their functions after *ex vivo* culture. However, a lot more research has to be done before taking it to the bedside.

Figure 4.1. Arginase activity in CD33$^+$ cells in the absence of NK cells. PBMCs were cultured with Huh7.5.1 cells uninfected or infected with HCV for 7 days after which CD33$^+$ cells were positively selected by magnetic separation. (A) Arginase activity in the CD33$^+$ cells was determined using an arginase assay kit. (B) *ARG1* expression was measured by qRT-PCR and normalized to *GAPDH* expression. Both arginase activity and mRNA expression were normalized to those of the uninfected-conditioned samples from the same donor. Results are the mean of 3-4 independent experiments. *p<0.05, ns denotes not significant two-tailed paired t test

Figure 4.1

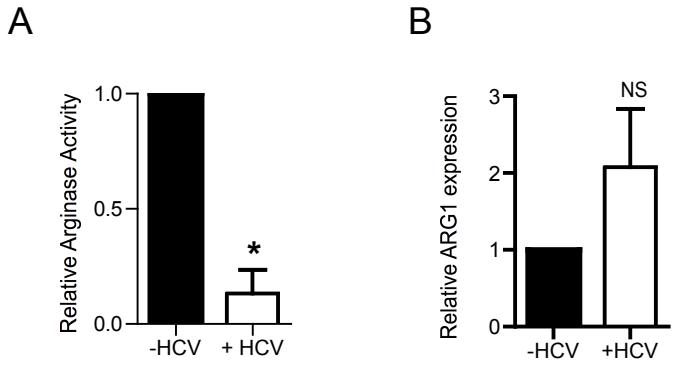
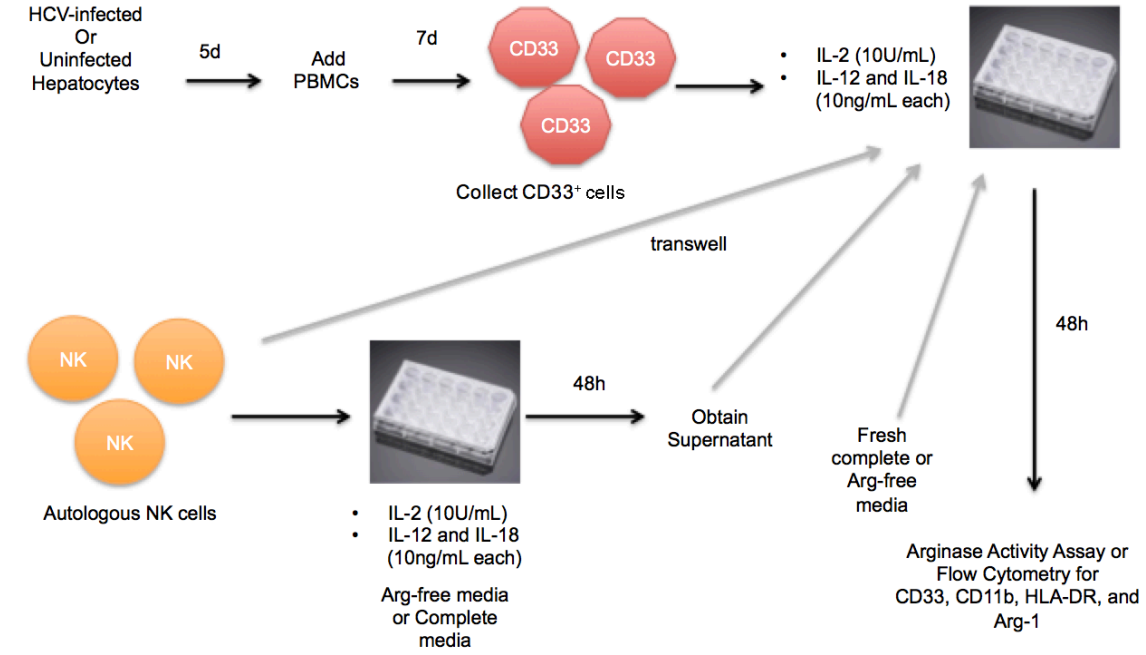

A
B
Relative Arginase Activity
1.0
0.5
0.0
*
-HCV
+ HCV
Relative ARG1 expression
3
2
1
0
NS
-HCV
+HCV

Fig. 4.2. Experimental Set-up to investigate if NK cell presence or their soluble factors are involved in crosstalk with MDSCs. Recover $CD33^+$ cells from a 7-day co-culture of PBMCs with uninfected or HCV-infected Huh7.5.1. Culture the $CD33^+$ cells with autologous NK cells (separated by a 0.45μm transwell insert) or with cell culture supernatant from NK cells that were grown in either complete or arginine-free media, or culture them with fresh complete media or arginine-free media. $CD33^+$ cells that do not undergo further culture should be used as a control. After recovering $CD33^+$ cells, an arginase activity assay should be performed on them. In a separate experiment, the recovered $CD33^+$ cells should be stained for MDSC surface markers and intracellular arginase-1 by flow cytometry.

Fig. 4.2


HCV-infected
Or
Uninfected
Hepatocytes
5d
Add
PBMCs
7d
CD33
CD33
CD33
Collect CD33+ cells
IL-2 (10U/mL)
IL-12 and IL-18
(10ng/mL each)
transwell
NK
NK
NK
Autologous NK cells
IL-2 (10U/mL)
IL-12 and IL-18
(10ng/mL each)
Arg-free media
or Complete
media
48h
Obtain
Supernatant
Fresh
complete or
Arg-free
media
48h
Arginase Activity Assay or
Flow Cytometry for
CD33, CD11b, HLA-DR, and
Arg-1

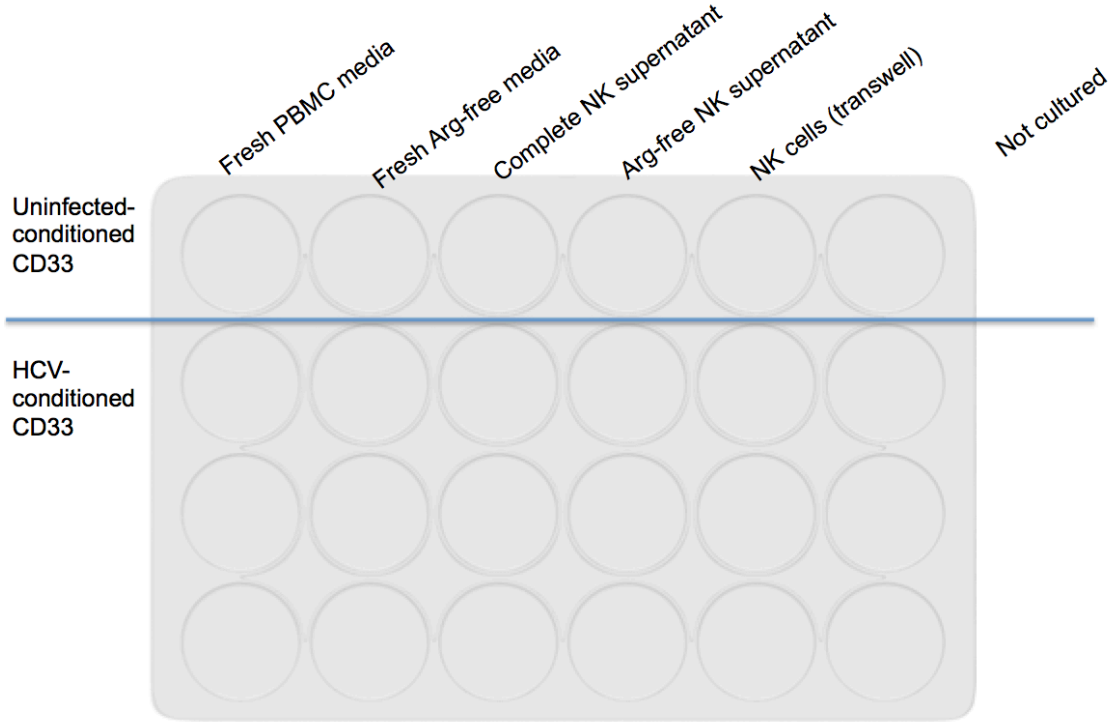

Fresh PBMC media
Fresh Arg-free media
Complete NK supernatant
Arg-free NK supernatant
NK cells (transwell)
Not cultured
Uninfected-
conditioned
CD33
HCV-
conditioned
CD33

Figure 4.3. Flow chart showing experiments to determine if (and what) soluble factors or viral proteins cause the accumulation of MDSCs. The following flow chart shows a series of experimental conditions to identify if viral replication, viral factors, or cell-derived factors are responsible for the accumulation of MDSCs as a result of HCV presence. After culturing the PBMCs or $CD33^+$ cells, stain the cells for MDSC markers and analyze them by flow cytometry.

Start by determining if replication-competent virus is needed to induce MDSC accumulation. If replication-competent virus is needed, determine the presence of hepatocytes is needed. If hepatocytes are required, determine by Luminex if they secrete any known MDSC-generating/accumulating factors. Test to determine if the factor(s) generates MDSCs when directly added to PBMCs in the absence of the virus. Also, determine if viral protein(s) generates MDSCs.

Next determine if the virus acts directly on $CD33^+$ cells. If it does, determine if the viral proteins, alone or in combination, cause an accumulation in MDSCs.

Figure 4.3

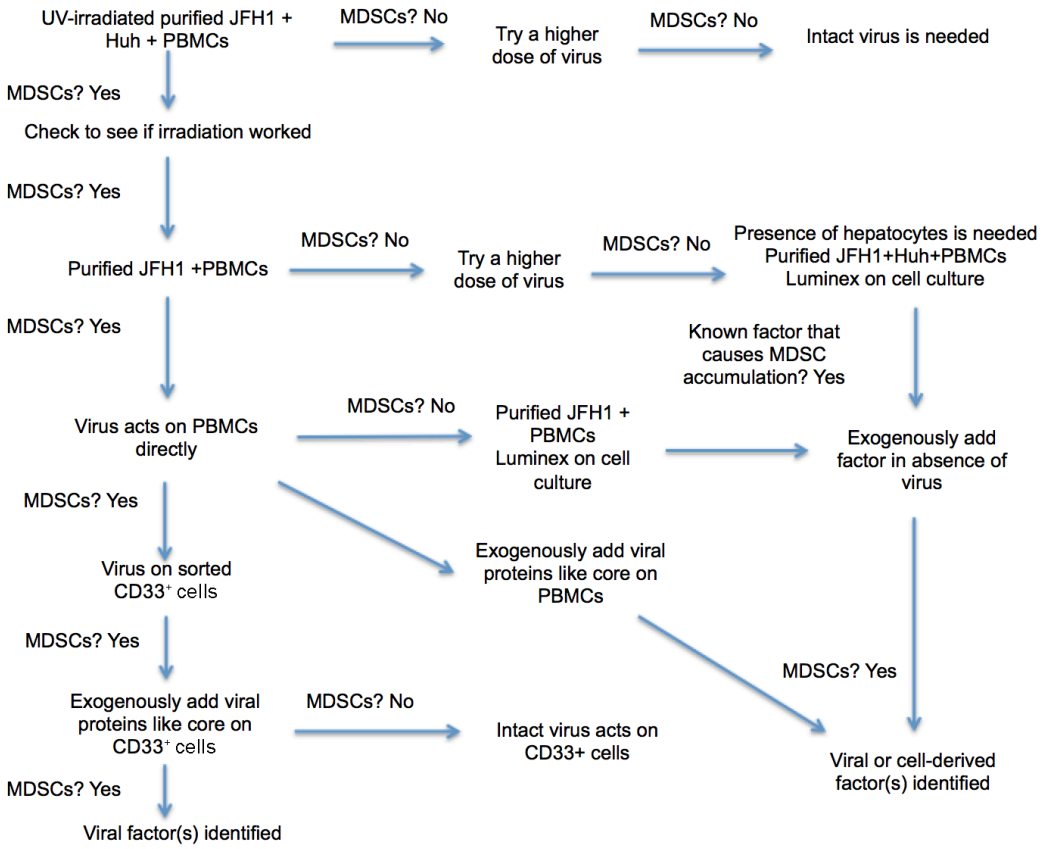
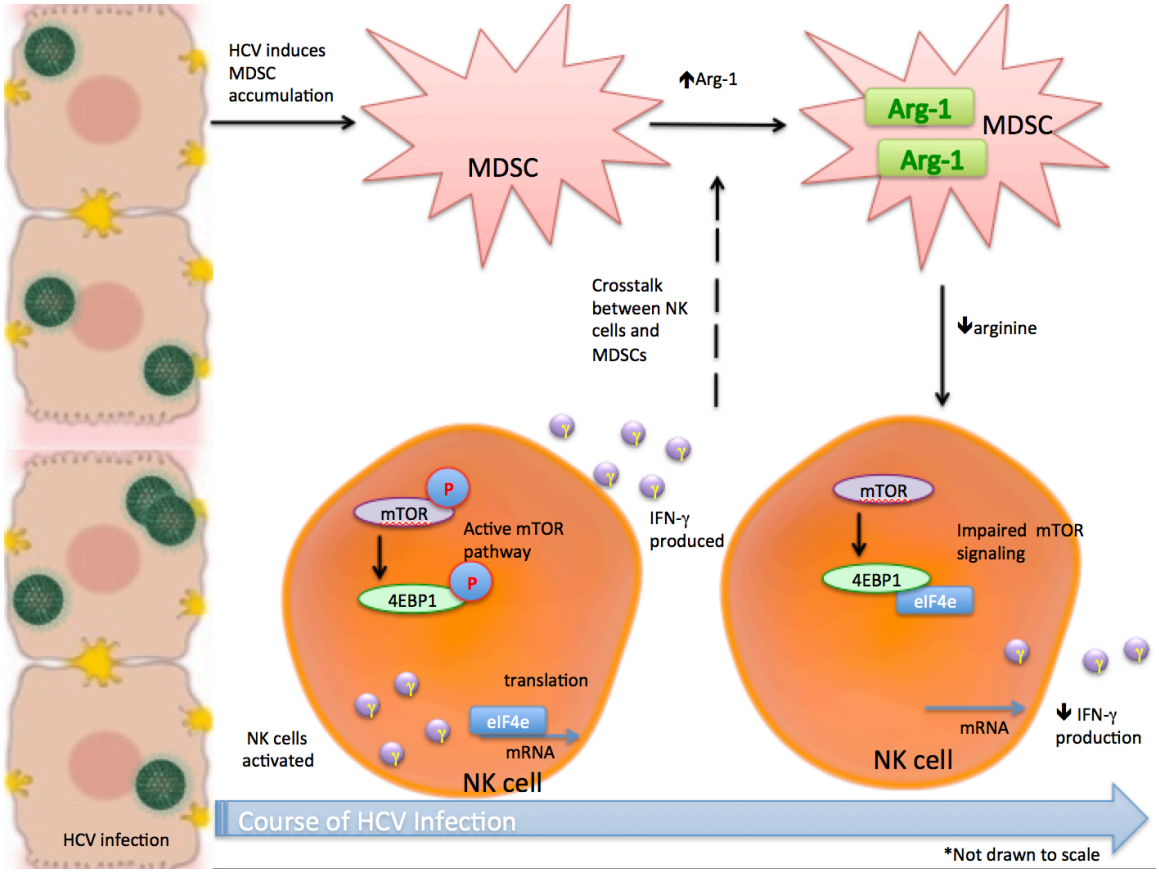

UV-irradiated purified JFH1 + Huh + PBMCs
MDSCs? No
Try a higher dose of virus
MDSCs? No
Intact virus is needed
MDSCs? Yes
Check to see if irradiation worked
MDSCs? Yes
Purified JFH1 +PBMCs
MDSCs? No
Try a higher dose of virus
MDSCs? No
Presence of hepatocytes is needed
Purified JFH1+Huh+PBMCs
Luminex on cell culture
MDSCs? Yes
Known factor that causes MDSC accumulation? Yes
Virus acts on PBMCs directly
MDSCs? No
Purified JFH1 + PBMCs Luminex on cell culture
Exogenously add factor in absence of virus
MDSCs? Yes
Virus on sorted CD33+ cells
Exogenously add viral proteins like core on PBMCs
MDSCs? Yes
MDSCs? Yes
Exogenously add viral proteins like core on CD33+ cells
MDSCs? No
Intact virus acts on CD33+ cells
MDSCs? Yes
Viral factor(s) identified
Viral or cell-derived factor(s) identified

Figure 4.4. Model of interaction between MDSCs and NK cells in HCV infection. HCV-infected hepatocytes induce accumulation of MDSCs with increased arginase-1 activity. This results in a decrease of arginine availability to the NK cells causing a defect in mTOR signaling, which results in decreased translation of *IFNG* mRNA, which in turn results in decreased IFN-γ protein production. There is reason to believe that crosstalk exists between NK cells and MDSCs. This could potentially be mediated via IFN-γ itself or some other mediator and would be interesting to explore.

Figure 4.4


HCV induces MDSC accumulation
MDSC
Arg-1
Arg-1
Arg-1
MDSC
Crosstalk between NK cells and MDSCs
arginine
IFN-γ produced
mTOR
P
Active mTOR pathway
4EBP1
P
translation
eIF4e
mRNA
NK cells activated
NK cell
mTOR
Impaired mTOR signaling
4EBP1
eIF4e
mRNA
IFN-γ production
NK cell
HCV infection
Course of HCV Infection
*Not drawn to scale

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