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Postpartum innate responses and control of viral replication

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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Postpartum innate responses and control of viral replication

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Magna cum laude

Advisor: Arash Grakoui, Ph.D.

An abstract of

A dissertation submitted to the faculty of the James T. Laney School of Graduate Studies
of Emory University in partial fulfillment of the requirements for the degree of
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Microbiology and Molecular Genetics

2016

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Abstract

The hepatitis C virus (HCV) persists in 75% of infected individuals, predisposing to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Following childbirth, however, women persistently infected with HCV may experience a significant decrease in plasma viral load or even clear infection. This work explores factors that may contribute to the postpartum decrease in viral load. In addition, we report the use of the Cas9 RNA-guided endonuclease platform derived from prokaryotes to restrict HCV infection. Together these studies highlight the complementary forces of the immune system and antivirals in the control of HCV.

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Chapter 1

An introduction to hepatitis C virus, pregnancy, and viral control

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Chapter 1

An introduction to hepatitis C virus, pregnancy, and viral control

Hepatitis C virus epidemiology and global impact

The hepatitis C virus (HCV) has infected over 185 million people worldwide (1). The virus was identified and cloned in 1989 and has been detected in regions all across the world (1-3). Seven HCV genotypes have been categorized, with sequence variance as high as 30-35% between genotypes (4). Genotypes are geographically distributed, with genotype 1 most commonly found within the United States (3). Though global prevalence is not fully calculated, it has been estimated that 1.3% of Americans are infected with HCV, while up to 2 percent of individuals in Europe are afflicted, depending on the country (3). More disconcerting, however, is the percentage of individuals infected in countries such as Egypt, where the prevalence rate is as high as 14.7% of the total population (3). Nevertheless, asymptomatic infection likely convolutes these prevalence estimates, as does the rapidly evolving field of HCV therapeutics. Incredibly, the treatment of HCV infection has been revolutionized by the introduction of new direct acting antiviral regimens, which can cure nearly all chronic infections (5).

Spontaneous clearance of HCV following the acute phase of infection is associated with robust antiviral immune responses. However, HCV persists in about 75% of infected individuals, predisposing to complications such as liver inflammation, cirrhosis, and hepatocellular carcinoma (6). In fact, HCV is the leading cause of liver transplantation in the U.S., and poses a significant burden on our healthcare system, as each transplant is

estimated to total greater than $250,000 (7). Further, the demand for livers for transplantation vastly exceeds the available supply.

HCV may be transmitted either sexually or percutaneously (8). Prior to screening of blood products for HCV, blood transfusions and organ transplants were the leading modes of transmission. Screening protocols have been established, and presently injection drug use is the primary cause of new infections in industrialized countries, especially among young adults and adolescents (8, 9). An alarming statistic is that approximately a quarter of individuals infected with human immunodeficiency virus (HIV) are co-infected with HCV (10). This number increases dramatically to approximately 90% among those that inject drugs (10). Given the rate of transmission, global prevalence, and economic burden, HCV will likely continue to negatively impact our world until a vaccine is developed. The development of a pan-genotypic vaccine will require extensive understanding of the viral life cycle, as well as immune responses to HCV infection.

Viral life cycle

HCV is a member of the family *Flaviviridae* and genus *Hepacivirus* (11). It is an enveloped, positive sense, single stranded RNA virus composed of approximately 9.6 kilobases that encode ten proteins (**Figure 1**) (12). The sequence diversity of the seven HCV genotypes has been attributed to the high mutation rate by the error-prone RNA polymerase, which serves as a major obstacle to vaccine development. It is estimated that the replication rate of HCV is upwards of 10^{12} virions per day, and as the mutation

frequency of the HCV polymerase is one in every 10^4 bases (one mutation per HCV genome), this equates to 10^{12} mutations per day (13).

The HCV genome is translated by cellular machinery into a single polyprotein that is subsequently cleaved by both viral and cellular proteases. Three of these ten proteins are structural, whereas the remaining proteins are non-structural (14). Core proteins form the capsid of the HCV particle, while the envelope glycoproteins E1 and E2 are involved in receptor binding, fusion, and cellular entry. The remaining proteins, p7-NS5B, are nonstructural and are not incorporated into the viral particle (14).

The HCV life cycle can be broken down into several general steps (11). First, the hepatitis C virion must bind cellular surface receptors for entry. Multiple entry factors are necessary for HCV particle entry into permissive cells, primarily hepatocytes. These include the human tetraspanin CD81, scavenger receptor BI (SRB-1), claudins, and occludin, among others (15-19). Second, the virion membrane must fuse with the cellular membrane. The low pH within the endosome may trigger this fusion process so that the HCV genome can be released into the cytoplasm (11, 20). Third, the genome is translated to generate viral proteins necessary for both the formation of new virions and replication along membranous webs. An internal ribosome entry site located within the 5' untranslated region (UTR) mediates genome translation. As shown in **Figure 1**, both the 5' and 3' UTRs contain extensive RNA secondary structure required for both viral translation and replication (21, 22). Finally, HCV genomes are packaged into fully formed virions and are released from the cell via the secretory pathway (11).

Interestingly, HCV is one of the only viruses known to utilize a cellular microRNA for its life cycle. miR-122 binds within the 5' UTR to enhance genome replication (23). Another unique feature of the HCV is that it does not circulate within peripheral blood as a single particle. Rather, the virus exists as a virolipoparticle, composed of the HCV particle in conjunction with low-density lipoproteins (LDL) or very low-density lipoproteins (VLDL) (11). Association with LDL or VLDL is advantageous for the virus because certain apolipoproteins have been shown to enhance binding of the HCV E2 glycoprotein to the cell surface receptors (24), resulting in enhanced infectious capacity. In addition, it is thought that apolipoproteins shield the HCV glycoproteins from neutralizing antibodies (25). However, these unique aspects of HCV replication and infection also serve as challenges because they must be recapitulated in effective *in vivo* animal models.

HCV model systems

In vitro. It wasn't until ten years after the discovery of HCV that an *in vitro* model system was established. This genotype 1b replicon system allowed for the study of replication in the hepatocellular carcinoma cell line Huh7 (26). However, fully infectious virus particles are not produced by HCV replicons, preventing the study of the full HCV life cycle. This obstacle was partially overcome in 2003, when HCV pseudoparticle systems were first generated (27). In pseudoparticle systems, the HCV E1 and E2 surface glycoproteins are incorporated into lentivirus particles, allowing for HCV entry and neutralizing antibody studies (27). A fully infectious cell culture virus production system was established in 2005 following the isolation and sequencing of the genotype 2a isolate

Japanese Fulminant Hepatitis Virus-1 (JFH) (28, 29). This particular virus strain did not require mutations for replication and is consistently utilized for viral fitness studies and analysis of antiviral therapeutics. The work of chapter 4 utilizes an HCV cell culture system composed of sequences of the genotype 2a J6 and JFH viruses that produce high titers of infectious virions (30).

In vivo. The only truly effective animal model system for studying HCV is the chimpanzee. However, government regulations have restricted the use of chimpanzees for HCV research in the United States, thus leading investigators to pursue other animal models. HCV is incapable of naturally infecting small rodents, although mice engrafted with human liver tissue (31), mouse-adapted HCV strains (32), and humanized mice (33) have been used to overcome this obstacle. Alternatively, infection of tamarins with GB virus-B, a virus closely related to HCV, has been used as a surrogate model of HCV infection (34). The development of these *in vitro* and *in vivo* models for the study of HCV has been imperative for the discovery of new drugs that effectively inhibit the virus.

HCV therapeutics: past, present, and future

Even before HCV was officially identified and isolated, interferon-α was used as an antiviral agent to treat non-A non-B hepatitis (35), and continued to be administered for decades. IFN-α therapy was not without its drawbacks, however, as the sustained virologic response (SVR) rate only ranged from 7-11%, and individuals undergoing treatment were riddled with severe side effects (36). Years later individuals infected with HCV were treated with a combination therapy composed of pegylated (slow-release)

interferon-α and Ribavirin, a broad nucleoside inhibitor, which increased the SVR rate to nearly 50% in patients with genotype 1 infection (37-39). More importantly, a new class of drugs, direct acting antivirals (DAAs), has revolutionized the field of HCV treatment, as certain drug regimens can drastically reduce side effects and achieve much higher SVR rates than previous IFN-based therapies.

DAAs can be placed into three broad categories, based upon the particular HCV protein that is inhibited (40). These categories include NS3 protease inhibitors, NS5A phosphoprotein inhibitors, and NS5B RNA-dependent RNA polymerase inhibitors. Overall, multiple treatment options are currently available for individuals with HCV infection, and factors that contribute to the choice of DAA regimen include HCV genotype, subtype, level of hepatic damage, treatment history, viral load, and HIV co-infection status (41).

The U.S. Food and Drug Administration approved the first NS3 protease inhibitors (Telaprevir and Boceprevir) for HCV genotype 1 infections in 2011, though they required concurrent administration of Peg-IFN and Ribavirin (42, 43). Although these first-generation NS3 inhibitors were quite potent, they had several disadvantages including high resistance rates due to viral amino acid mutation, and the inability to broadly inhibit the NS3 protease of multiple HCV genotypes. NS5B polymerase inhibitors, on the other hand, are generally pan-genotypic and treatment results in sustained virologic response (SVR) rates as high as 100% (40). The first polymerase inhibitor to receive FDA approval was Sofosbuvir, a once daily pill for individuals infected with genotypes 1, 2, 3,

and 4. Sofosbuvir was also the first drug for HCV infection approved for individuals co-infected with human immunodeficiency virus (HIV). Since the approval of Sofosbuvir in 2013, several treatment options are now available for individuals with genotype 1 infection, which is the most prevalent HCV genotype within the U.S.A, as mentioned previously. These options include the brand name drugs Harvoni, Olysio, and Viekira Pak, each of which is a combination drug taken in pill form. New drugs for the treatment of individuals with genotype 3 or 4 infections have now been approved, as well. Incredibly, all oral IFN-free DAA regimens have now achieved SVR rates ranging from 93-100% (44).

An emerging technology being investigated to inhibit both DNA and RNA virus infections, including HCV, is based on the prokaryotic adaptive immune system. The majority of prokaryotes possess antiviral defense mechanisms called clustered, regularly interspaced, short palindromic repeats - CRISPR associated (CRISPR-Cas) systems. These systems confer protection to invasion by phages or foreign nucleic acids upon challenge and have been harnessed for antiviral use in eukaryotic cell types (45). Advances in harnessing the prokaryotic adaptive immune system as a eukaryotic antiviral defense are described in detail in chapter 3, and the efficacy of the Cas9 platform to inhibit HCV in cell culture is demonstrated in chapter 4. However, this study utilizing Cas9 to bind and inhibit HCV RNA is currently a proof of principle. It is currently unknown whether Cas9 can be used as an antiviral prophylactic or therapeutic in human infections.

Though incredible progress has been made in treating HCV infection with DAAs, a primary drawback to the use of these regimens is the extremely high cost. In 2015, the NS5B polymerase inhibitor Sofosbuvir was released onto the U.S. market at a cost of $1,000 per pill, totaling roughly $80,000 for a single pill per day 12-week regimen (46). This high cost renders treatment inaccessible to many patients afflicted by HCV. Further, HCV diagnosis often occurs at late a stage of infection when liver disease and hepatocellular carcinoma may be advanced, so viral clearance does not always negate the need for liver transplantation. In addition, DAAs do not provide lasting immunity. As the current primary mode of transmission is injection drug use, reinfection remains a major concern. Given these obstacles, global eradication of HCV seems unlikely without the development of a vaccine.

Two HCV vaccine candidates have been tested in human clinical trials (ClinicalTrials.gov NCT01436357 and NCT01436357) (47). The first, developed by Chiron/Novartis, is composed of subunits of the HCV E1 and E2 glycoproteins delivered in an oil:water vehicle to induce neutralizing antibodies and $CD4^+$ T cells (48, 49). Unfortunately, plans for future studies on this vaccine are currently unknown. The second candidate, developed by Okairos/GlaxoSmithKline, utilizes recombinant viral vectors to deliver HCV non-structural proteins to induce $CD8^+$ T cell immunity (50), and a Phase I/II clinical trial is currently underway. In the following sections we will discuss immune responses to HCV infection, and the importance of neutralizing antibodies and T cell responses for effective immunization and viral clearance.

The immune response to HCV

Innate immunity

Interferons. Innate immune responses are the first line of defense against infection with HCV. HCV infection of hepatocytes results in the induction of a general interferon (IFN)-induced antiviral state that is not specific to HCV. Both hepatocytes and cells of the innate immune system produce type I (IFN-α and IFN-β) and type III IFNs (IFN-λ1, λ2, and λ3, also called IL29, IL28A, and IL28B, respectively) (51). An additional type III IFN has recently been discovered and is termed IFNL4 (52).

Virus infections are sensed by toll-like receptors (TLRs), RNA helicase retinoic acid inducible gene-I (RIG-I), and melanoma differentiation antigen 5 (MDA5). These pathways converge and result in the activation of the transcription factors NFKB, IRF3, and IRF7. These transcription factors are then able to bind type I and type III promoter sequences, thereby inducing transcription of IFNs (53).

Type I IFNs bind to the IFN receptor, composed of IFNAR1 and IFNAR2, and induce a signaling cascade resulting in the transcriptional activation of hundreds to thousands of interferon-stimulated genes (ISGs), each of which possesses antiviral effector function. Upon binding of type I IFN to the receptor, Janus-kinase (JAK) 1 and Tyk2 kinases phosphorylate signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2), which then form a trimer with interferon response factor 9 (IRF9). This trimer, known as the interferon-stimulated gene factor 3 (ISGF3), then translocates to the nucleus and binds to interferon sensitive response elements (ISREs) in the promoter

regions of ISGs, inducing ISG transcription (53).

Though type III IFN signaling results in the induction of similar gene sets as type I IFN signaling, receptor usage is distinct. Type III IFNs bind to a receptor composed of the IFNLR1 and IL10R2 subunits, though downstream signaling also includes activation of the ISGF3 complex that binds to ISREs (53). IFNLR1 expression is more restricted than IFNAR1 and IFNAR2. Receptor expression is typically found along epithelial and mucosal barriers, where virus exposure is common (51). Type III IFNs are particularly important for HCV infection as hepatocytes are a major cell type capable of producing these IFNs. In addition, polymorphisms within type III IFN genes have been associated with the outcome of infection with HCV (54-57).

Impact of genetic polymorphisms on the outcome of infection. Certain genetic polymorphisms are associated with the spontaneous and treatment-induced clearance of HCV by both IFN and IFN-free treatment regimens. Genome-wide association studies identified a single nucleotide polymorphism (rs12979860) near the IFNL3 gene that is strongly associated with the level of ISGs found within HCV-infected livers. TT or CT genotypes at this SNP are indicative of high ISG levels, whereas patients with a CC genotype generally express low levels of ISGs (58). Further, the CC genotype is associated with spontaneous resolution of HCV infection, while TT or CT genotypes are associated with failure to clear persistent HCV and a poor response to IFN-based therapy (54-57).

The mechanisms by which TT or CT alleles impact interferon responses have not been fully elucidated, although a dinucleotide variant ss469415590 (TT or ΔG) is in linkage disequilibrium with rs12979860. A ΔG genotype results in expression of a novel transcript encoding the IFN-λ4 protein, which is associated with impaired clearance of HCV and induction of ISGs, possibly prolonging IFN signaling in individuals with this genotype (52). Further work is needed to elucidate the mechanisms contributing to prolonged ISG expression. It also remains to be determined if cells of the innate immune system contribute, as they are also potent producers of IFNs (53).

Innate immune cells. Certain innate immune cells, specifically myeloid and plasmacytoid dendritic cells (DCs), are key directors for the induction of adaptive immune responses. DC impairment may contribute to the establishment of chronic infection, though studies examining the functional capacity of DCs from HCV-infected individuals describe varying levels of functional impairment (59-61). One consistent observation is the decrease in frequency of circulating DC populations in HCV-infected individuals. Our lab has corroborated these findings, and has also demonstrated that DC populations are enriched in the liver relative to blood. In this study, six populations of professional antigen-presenting cells (APCs) were identified within the liver (62). Frequencies of myeloid DCs were enriched in the livers of HCV-infected individuals relative to uninfected livers. Further, myeloid DCs expressed high levels of activation and maturation molecules (62).

In general, there is evidence for extensive innate immune cell infiltration within livers

persistently infected with HCV. Many of these cells, including dendritic cells (DCs), monocytes, and macrophages, are characterized by an increase in activation marker expression and proinflammatory cytokine production that may contribute to liver damage and fibrosis (63). Though phenotypically activated, innate immune cells may be unable to efficiently present viral antigens to $CD4^+$ T cells, and by extension, impairment of innate immune cells may then lead to impairment of adaptive immune cells.

Adaptive Immunity

Humoral immunity. While antibodies targeted to HCV non-structural proteins have been detected in HCV-infected individuals, most HCV-neutralizing antibodies target the E2 surface glycoprotein. E2 contains three hypervariable regions, and mutation within these regions has been demonstrated to mediate neutralizing antibody escape (64). However, the overall role of antibody-mediated clearance of acute HCV infection remains controversial.

Antibody-mediated neutralization of HCV has been demonstrated in chimpanzees (65, 66) and humanized mouse models of HCV infection (67, 68). In humans, broadly neutralizing antibodies develop faster and to higher titers than in acutely infected individuals (69, 70). Further, cross-reactive antibodies have been demonstrated to confer protection in injection drug users upon re-infection with HCV (71). However, other studies suggest that infection can be resolved in the absence of detectable HCV glycoprotein-specific antibody responses (72, 73). In these studies the resolution of infection was attributed to robust virus-specific T cell mediated responses. It is therefore

likely that an HCV vaccine will need to induce both neutralizing antibodies and anti-HCV T cell responses.

T-cell mediated immunity. T cell immunity is vital for control of HCV infection but fails in most individuals. When acute HCV infection is successfully controlled, the effective immune response predictably includes expansion of highly functional HCV-specific CD4+ and $CD8^+$ T cells targeted to multiple viral epitopes. $CD8^+$ T cell populations secrete antiviral cytokines such as IFN-γ, TNF-α, and IL-2 and exert cytotoxic activity that is sustained until viremia resolves. Similarly, $CD4^+$ T cell populations are responsible for antiviral cytokine secretion and $CD8^+$ T cell and B cell help. Depletion of either $CD8^+$ or $CD4^+$ T cells in chimpanzees challenged with HCV results in failure to clear infection, demonstrating the importance of T cells in the control of HCV (74, 75).

In the more common scenario of progression to chronic infection, HCV-specific T-cell responses are variable in the acute phase of infection, but inevitably develop functional exhaustion or select viral variants that escape T cell recognition (76, 77). Exhausted HCV-specific $CD8^+$ T cells exhibit poor cytotoxicity, cytokine secretion, and proliferative capacity, and express high levels of inhibitory molecules such as programmed death 1 (PD-1) and low levels of self-renewal markers such as the IL-7 receptor alpha chain CD127 (78). In addition, elevated numbers of T_{regs} have been identified in the livers of chronically infected individuals that may further attenuate T cell effector function (76).

In the chronic phase of infection HCV-specific $CD8^+$ T cells are often difficult to detect in the peripheral blood but are maintained in the liver. Whether exhausted cells exert any antiviral activity *in vivo*, particularly within the liver, and their ability to be rescued from an exhausted state, are questions of intense interest. Reversing T cells from the exhausted state may present a novel way of restricting persistent viruses, and the postpartum period is a unique opportunity to study the restoration of virus-specific T cell immunity.

Pregnancy and the postpartum period in healthy women

Successful pregnancy requires that maternal immune cells tolerate paternal alloantigens expressed by the fetus. Failure to do so has been linked to early onset of labor and fetal death (79). The placental maternal-fetal interface is thus replete with mechanisms to prevent or subdue untoward allogeneic immune responses. To understand these tolerance mechanisms, I will first briefly review the anatomy and physiological roles of the placenta.

Placental anatomy and physiology. Upon embryonic implantation into the wall of the maternal uterus, a structure, known as the placenta, forms around the developing fetus. The placenta is derived from fetal tissue and thus must be tolerated by the maternal immune system. Placental cells are termed trophoblasts, and can be categorized as syncytiotrophoblasts or cytotrophoblasts, as they are oriented toward maternal tissue or the fetus, respectively (80).

The human placenta plays three primary physiological roles during pregnancy. First, the placenta is responsible for the synthesis of hormones involved in the regulation of pregnancy, such as estrogen and progesterone. Second, the placenta serves as a physiological structure to house the fetus, and finally, serves to transport oxygen and nutrients to the developing fetus. Due to the anatomy of the placenta, maternal blood comes in direct contact with fetal tissue (80), thus the maternal immune system must adapt to prevent rejection of the semi-allogeneic fetus.

Tolerance of the semi-allogeneic fetus. Numerous mechanisms exist to allow the maternal immune system to tolerate the presence of the semi-allogeneic fetus. These include antigen presentation by tolerogenic non-classical class I molecules such as HLA-G, tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO), inhibition via programmed cell death 1 ligand 1 (PDL1), and expansion of immunosuppressive $FOXP3^+$ $CD4^+$ T regulatory cells (T_{regs}), many of which are specific for paternal antigens (80, 81).

Immunomodulatory mechanisms are concentrated in the placenta. However, some of these mechanisms also exert influence on systemic cellular immunity during pregnancy. For instance, T_{regs} with robust immunosuppressive activity expand systemically during pregnancy and may inhibit $CD8^+$ T cell activation, proliferation, and cytokine production in the periphery by secreting IL-10 and TGF-β, depleting available IL-2, and secreting extracellular adenosine (82, 83). Further, pregnancy hormones such as progesterone and estrogen have been shown to affect $CD8^+$ T cell activity (84, 85).

This dampening of adaptive immunity during pregnancy may render mothers susceptible to more severe infections during gestation (86-91). Therefore it has been proposed that innate immune activation may partially compensate for the altered adaptive system (92, 93). These compensatory changes include increased frequencies of monocytes and granulocytes, as well as the upregulation of activation markers such as CD11b and CD64 on these cell types. Further, granulocytes are thought to possess enhanced phagocytic capability during pregnancy, as well as produce high levels of oxygen radicals (94). However, the restoration of the innate immune system following delivery is not fully understood and will be examined further in the work of chapter 2.

Parturition and the postpartum period. The induction of labor is controlled by many factors, including levels of estrogen, progesterone, and oxytocin, among other hormones. It has also been demonstrated that a proinflammatory milleu within the cervix and placenta may further enhance labor (95). Specifically, placental $CD14^+$ cells are phenotypically activated and secrete extremely high levels of TNF-α, IL1-β, and IL-6, suggesting a role for these cells in parturition (96).

Together the processes of labor and delivery place tremendous stress on a mother's body. In fact, Mayo Clinic obstetricians recommend waiting a minimum of 18 months following birth before becoming pregnant again to allow the mother's body to fully recuperate from the stress of delivery. Multiple factors contribute to the time required for the body to heal from birth trauma, but during this time, many new mothers may

experience pain, swelling, and fatigue, nutritional deficiencies, as well as mental disorders such as postpartum depression (97).

In contrast to the aforementioned difficulties posed by childbirth, the postpartum period is also associated with enhanced control of persistent viruses such as HCV and hepatitis B virus (HBV). Some women may experience a drastic decrease in viral load following delivery. The plasma viral load obtained during pregnancy and following delivery from a woman chronically infected with HCV is depicted in **Figure 2**. Decreases in HCV load have been attributed, at least in part, to restoration of virus-specific $CD8^+$ T cell responses from an "exhausted" state. The following section discusses hepatitis C viral load dynamics during pregnancy and following delivery, and the cellular immune components that contribute to the postpartum decrease in viral load. These observations form the basis for the work of chapter 2.

HCV infection during pregnancy and following parturition

Robust, poly-functional $CD8^+$ T cell responses targeted to multiple viral epitopes have been associated with successful resolution of acute HCV infection. In most individuals however, immunity fails and chronic viremia ensues as $CD8^+$ T cells become functionally exhausted or select viral variants with escape mutations in class I epitopes. During pregnancy, maternal-fetal tolerance processes such as the expansion of regulatory T cell populations or hormonal changes may further impair dysfunctional HCV-specific $CD8^+$ T cell responses.

Viral load dynamics. Recognition that HCV viral loads tend to climb during pregnancy and frequently fall sharply after delivery (98, 99), supports the hypothesis that HCV-specific T-cell function may be further suppressed during pregnancy and potentially rebound after delivery. Recent evidence suggests that HCV adapts to relaxed maternal $CD8^+$ T cell pressure in pregnancy by shedding unfit escape mutations in certain class I epitopes, resulting in the selection of viruses with more efficient replication. Emergence of HCV variants with enhanced replicative capacity during pregnancy has ramifications for mother to child transmission, the primary route of HCV infection in children, and could potentially be relevant to other important vertically transmissible persistent viruses such as hepatitis B virus (HBV) and human immunodeficiency virus (HIV).

Since $CD8^+$ T-cells are major drivers of the evolution of HCV genomes, we recently attempted to gain insight into the effects of pregnancy on HCV-specific cellular immunity by examining evolution of HCV genomes in serial blood samples of two persistently infected women followed through consecutive pregnancies. Viremia dropped markedly after the first delivery in both women and viruses with amino acid substitutions in one or more HLA class I epitopes emerged. These substitutions prevented recognition by $CD8^+$ T cell lines derived from the women, confirming that they functioned as immune escape mutations (98). Surprisingly, several of these effective escape mutations were lost in the second pregnancy, only to appear again after the second delivery, coincident with another sharp fall in viremia. This unusual pattern of amino acid substitution suggested that HCV-specific $CD8^+$ T cell selection pressure was transiently reduced during pregnancy. Using cell culture adapted HCV we demonstrated that the

escape mutations lost in pregnancy significantly impaired *in vitro* production of infectious viruses relative to viruses bearing the "wild-type" epitope sequences (98). These findings confirmed for the first time that maternal-fetal tolerance mechanisms of pregnancy do indeed impair HCV-specific $CD8^+$ T cell responses, which has important implications for vertical transmission.

Vertical transmission. More than one million children are born to HCV infected mothers each year, and 3-5% acquire the infection *in utero* or at delivery, making vertical transmission the leading route of pediatric HCV infection in the developed world (100). Improved viral replication through loss of unfit escape mutations in pregnancy may contribute to higher viral loads that have been linked to increased risk of vertical transmission. One subject in our study had viral loads in excess of 10^7 IU/ml in both pregnancies and vertical transmission occurred with each. Sequencing of virus from the 2^{nd} child confirmed that he had received the fit "wild-type" sequence of the epitopes that had lost escape mutations in the mother during pregnancy, suggesting that relaxed $CD8^+$ T cell immune pressure during pregnancy may permit viruses with the highest replicative and infectious capacity to be passed to offspring (98).

As discussed above, the postpartum period is associated with enhanced control of persistent HCV infection. Longitudinal sequencing of viral genomes during pregnancy and following delivery indicated that $CD8^+$ T cells exert pressure on the virus to escape immune recognition (98). We hypothesized that additional cell types, including those of the innate immune system, could contribute to the postpartum control of HCV. As less

than one percent of pregnant women are estimated to be persistently infected with HCV (100), we chose to study the transcriptional profiles of innate immune cells from healthy, uninfected women. Chapter 2 demonstrates the potential role for innate immune cells and IFN signaling in the postpartum control of persistent viruses, while unpublished data examining antiviral components of postpartum plasma and future directions for this work are highlighted in chapter 5.

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Figures and Figure Legends

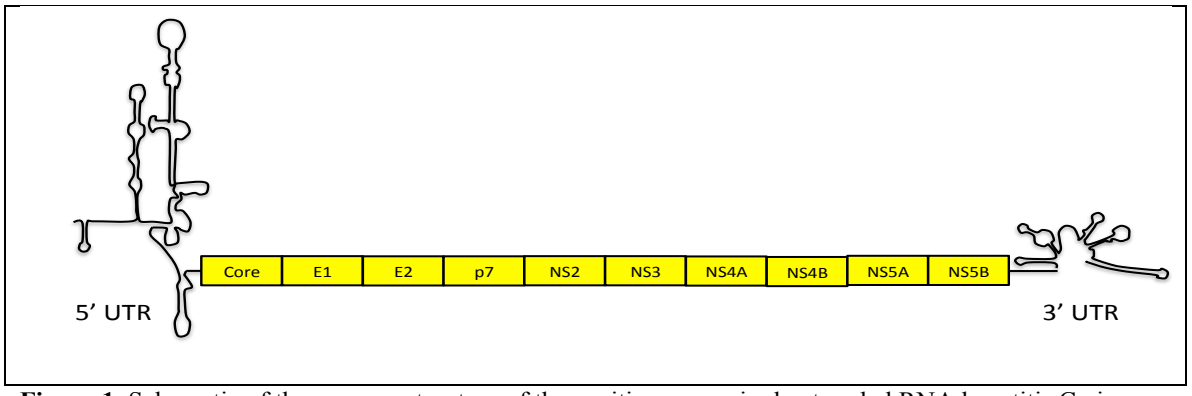

Figure 1. Schematic of the genome structure of the positive sense single-stranded RNA hepatitis C virus.

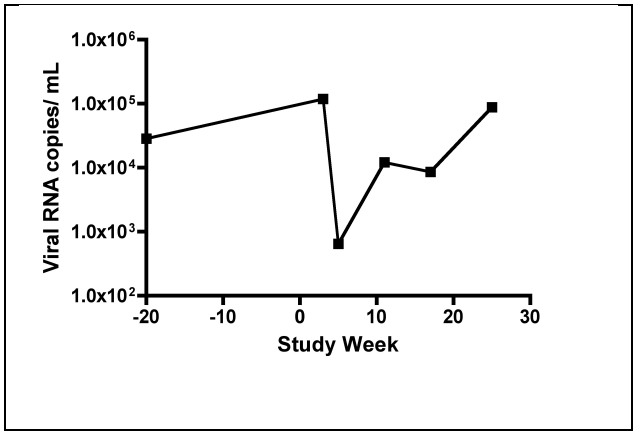

Figure 2. Plasma viral load is decreased following delivery in a woman chronically infected with HCV. Study week is relative to delivery (week 0).

Chapter 2

Prolonged activation of innate antiviral gene signature after childbirth is determined by IFNL3 genotype

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Prolonged activation of innate antiviral gene signature after childbirth is determined by *IFNL3* genotype

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Keywords: postpartum, parturition, interferon, CD14, innate immunity, antiviral genes

Abstract

Maternal innate and adaptive immune responses are modulated during pregnancy to concurrently defend against infection and tolerate the semi-allogeneic fetus. The restoration of these systems after childbirth is poorly understood. We reasoned that enhanced innate immune activation may extend beyond gestation while adaptive immunity recovers. To test this hypothesis, the transcriptional profiles of total PBMCs following delivery in healthy women were compared to those of non-pregnant control subjects. Interestingly, interferon stimulated genes (ISGs) encoding proteins such as IFIT1, IFIT2, and IFIT3, as well as signaling proteins such as STAT1, STAT2, and MAVS, were enriched postpartum. Antiviral genes were primarily expressed in $CD14^+$ cells and could be stratified according to genetic variation at the interferon-λ3 gene (*IFNL3*, also named IL28B) single nucleotide polymorphism (SNP) rs12979860. Antiviral gene expression was sustained six months following delivery in mothers with a CT or TT genotype but quickly returned to basal levels following delivery in mothers with a CC genotype. CT and TT IFNL3 genotypes have been associated with persistent elevated ISG expression and failure to clear infection in individuals chronically infected with hepatitis C virus. Together these data suggest that postpartum, the normalization of the physiological rheostat controlling interferon signaling is dependent on *IFNL3* genotype, which may influence the outcome of certain chronic viral infections.

Significance

In this study we examined the possibility that the maternal innate immune system is modulated following delivery. We identified an interferon stimulated gene signature that was primarily expressed in $CD14^+$ cells circulating in the peripheral blood. Postpartum antiviral gene expression was dependent on the interferon-λ3 (*IFNL3*) single nucleotide polymorphism rs12979860, which suggests that *IFNL3* genotype may influence a mother's ability to combat pathogens following delivery.

Introduction

Maternal immuno-regulatory adaptations such as the expansion of regulatory T cells (T_{regs}) with potent immunosuppressive capacity serve to maintain fetal tolerance during pregnancy (1, 2). Although tolerance mechanisms are necessary for fetal survival, suppressed cellular immunity renders pregnant women susceptible to severe infection with certain pathogens (3-8). Augmented innate immunity may partially offset the reduced adaptive immune protection in pregnancy (9, 10). Following delivery, however, the maternal immune system is released from the constraints of pregnancy.

The postpartum period is associated with enhanced control of several chronic viruses. Women persistently infected with hepatitis C virus (HCV) or hepatitis B virus (HBV) may experience a precipitous decrease in viral load in the months following delivery (11-15). Factors that contribute to the enhanced postpartum viral control are not fully understood, though in the context of HCV infection the decrease in viral load was linked in 2 women to the emergence of viral variants with escape mutations in class I epitopes, suggesting a restoration of virus-specific $CD8^+$ T cell selection pressure (11).

There is evidence, however, that a brief lag phase in T cell function may occur following delivery. Though absolute numbers of T cells normalize by one month postpartum (16, 17), these T cells may possess functional effector deficits such as decreased IFN-γ production (17). We hypothesized that maternal innate immune activation extends beyond gestation, thus compensating for the lag phase in postpartum T cell immunity and possibly contributing to the postpartum control of persistent viruses. We tested this

hypothesis by comparing the transcriptional profiles of total peripheral blood mononuclear cells (PBMCs) from healthy mothers following delivery in relation to non-pregnant control subjects (NPCs). We identified an interferon-stimulated gene (ISG) signature that was primarily expressed in $CD14^+$ cells and dependent upon the interferon-λ3 (*IFNL3*, also known as IL28B) single nucleotide polymorphism (SNP) rs12979860. This SNP has been associated with the spontaneous and treatment-induced clearance of viruses such as HCV (18, 19) suggesting that *IFNL3* genotype may influence the outcome of persistent viral infections following delivery.

Results

We utilized a systems biology approach to identify gene expression patterns and pathways that may contribute to postpartum control of viral infections. Peripheral blood mononuclear cells (PBMCs) were collected two weeks following delivery from two healthy mothers, UM07 and UM08, and two non-pregnant control subjects (NPCs), NPC354 and NPC357. All NPC subjects were women in their childbearing years who had never been pregnant. Postpartum genes enriched at least two-fold over NPCs were included in the analysis. 982 genes were differentially expressed in both UM07 and UM08. Further analysis of these genes by DAVID bioinformatics indicated that the top five enriched biological processes were related to immune response (GO:0006955), positive regulation of defense response to virus by host (GO:0002230), response to virus (GO:0009615), defense response to virus by host (GO:0050691), and regulation of immune effector process (GO:0002697) (**Figure 1a**). Remarkably, all five biological processes were associated with immunity, and three of these were specifically related to

antiviral immunity.

Due to the number of antiviral genes within the 'defense response to virus' biological process, this category was investigated further. The enriched antiviral defense genes encoded proteins with antiviral effector function (IFIT1, IFIT2, IFIT3, and OAS1) as well as signaling function (STAT1, STAT2, MAVS, and TLR7). Interestingly, the IFN-α/β signaling pathway was enriched postpartum (data not shown). Network analysis identified STAT1 and STAT2 to be the regulatory nodes within the network (**Figure 1b**). STAT1 and STAT2 are key modulators of type I and type III interferon signaling, and together these data are consistent with upregulated antiviral interferon signaling in peripheral blood cells following delivery in UM07 and UM08.

We then examined the duration of antiviral gene enrichment following delivery. Gene expression analysis was performed on PBMCs from UM07 and UM08 obtained 2, 4, 12, and 24 weeks following delivery and compared to the gene signatures from NPCs (**Figure 1c**). Interestingly, many antiviral genes were enriched as late as 24 weeks following delivery. Expression patterns varied from transient expression for 2 to 4 weeks postpartum, as in the case of *MAVS* and *STAT2*, to prolonged expression from 2 to 24 weeks following delivery, as exemplified by *IFIT1*, *IFIT2*, and *IFIT3* (**Figure 1c**).

IFIT1, *IFIT2*, *IFIT3*, and *OAS1* were chosen for further analysis due to their relative fold increases in transcript levels within total PBMCs. These genes are canonical ISGs that when translated possess antiviral function against numerous viruses. The interferon-induced protein with tetratricopeptide repeats (IFIT) family proteins function by

inhibiting distinct steps of the viral life cycle (20), while oligoadenylate synthetase 1 (OAS1) activates RNAse L to cleave viral RNA (21, 22). RT-PCR confirmed the elevated ISG levels obtained by gene array. *IFIT1* levels were similar between UM07 and UM08, with both mothers displaying approximate six-fold increases relative to NPCs. *IFIT2* levels were enriched by nearly six-fold in UM07 but were not significantly enriched in UM08, whereas *IFIT3* levels were enriched by approximately four-fold in UM08 but not UM07 (**Figure 2a**). Similarly, levels of *IFIT1*, *IFIT2*, *IFIT3*, and *OAS1* were enriched in total PBMCs obtained from UM332 following delivery.

We next set out to determine if antiviral genes were expressed in all circulating cell types. $CD8^+$ T lymphocytes, $CD4^+$ T lymphocytes, $CD19^+$ B lymphocytes, and $CD14^+$ monocytes were separated based on surface marker expression and analyzed for antiviral gene expression. Surprisingly, the highest postpartum transcript levels of genes encoding IFIT1, IFIT2, IFIT3, and OAS1 were detected within the isolated $CD14^+$ population (**Figure 2a**). Transcript levels in $CD14^+$ cells between NPCs did not vary by more than approximately 2 fold, whereas *IFIT1, IFIT2, IFIT3,* and *OAS1* levels were elevated postpartum in UM07, UM08, and UM332. In contrast, transcript levels of these genes in cells expressing CD4, CD8, CD19, or in the remaining cell subsets did not appear to exceed the range of expression seen in NPCs (**Figure 2a**). To ensure that the postpartum ISG enrichment observed in total PBMCs was not solely due to elevated numbers of $CD14^+$ cells, flow cytometry was utilized for analysis of cell frequency. The frequencies of $CD14^+$ cells within total PBMCs used for transcript analysis were not significantly different between NPCs and postpartum subjects (**Figure 2b**).

To extend the findings from these two mothers, we analyzed antiviral gene expression in $CD14^+$ cells from an additional thirteen mothers and eleven NPC subjects. Characteristics of the mothers and NPCs enrolled in this study are shown in Supplementary Tables 1 and 2. $CD14^+$ cells were isolated, and the levels of *IFIT1*, *IFIT2*, *IFIT3*, and *OAS1* were examined by RT-PCR. NPC transcript levels were tightly clustered in each of the genes tested (**Figure 3a**), whereas highly variable antiviral transcript levels were observed postpartum. Multiple factors could influence postpartum gene expression, so we examined a potential role for genetic factors.

The *IFNL3* SNP rs12979860 is strongly associated with the level of ISGs found within HCV-infected livers. TT or CT genotypes at this SNP are linked to high ISG levels, whereas patients with a CC genotype generally express low levels of ISGs (23). Further, the CC genotype is associated with spontaneous resolution of HCV infection, while TT or CT genotypes are associated with failure to clear persistent HCV and a poor response to interferon-based therapy (18, 19, 24, 25). Given the relationship between this SNP and antiviral gene expression, we hypothesized that the antiviral transcript levels from postpartum $CD14^+$ cells could also be stratified based on *IFNL3* genotype at rs12979860. Donor *IFNL3* genotype was determined using a PCR-based allelic discrimination assay (**Figure 3a**). Donors with TT or CT genotypes were grouped together due to their similar patterns of antiviral gene expression. Purified $CD14^+$ cells from NPCs displayed consistent baseline levels of ISG transcripts, regardless of *IFNL3* genotype. Interestingly, transcript levels of *IFIT1*, *IFIT2*, *IFIT3*, and *OAS1* within isolated $CD14^+$ cells from

mothers homozygous for the CC alleles were not significantly different from NPCs (**Figure 3a**).

In comparison, $CD14^+$ cells isolated from women with either TT or CT genotypes at rs12979860 displayed significantly enriched transcript levels of *IFIT1*, *IFIT2*, *IFIT3*, and *OAS1*, both relative to NPC and homozygous CC genotype postpartum samples. Although a similar trend in *IFIT5* and *ISG15* expression was observed relative to NPCs and CC mothers, the levels did not reach statistical significance (data not shown). It is interesting to note that within the postpartum TT/ CT cohort, the relative enrichment of the highest data point within each analyzed gene was not recurrently obtained from the same donor. The donor displaying the highest postpartum *IFIT1*, *IFIT2*, *IFIT3*, and *OAS1* transcript level was UM07, UM1001, UM332, and UM06, respectively (**Figure 3a**). These results indicate that postpartum antiviral transcript levels in $CD14^+$ cells from uninfected mothers may be stratified according to *IFNL3* genotype at SNP rs12979860, which to our knowledge is the first observation of the association between postpartum antiviral gene expression and *IFNL3* genotype in healthy mothers.

Since the initial microarray was performed on PBMCs isolated following delivery from two mothers with CT alleles at SNP rs12979860 (**Figure 1a, b, c**), we next investigated whether ISG enrichment in postpartum PBMCs from individuals expressing homozygous CC alleles would more closely resemble NPCs. Total PBMCs were isolated two weeks postpartum from two CC donors, UM10 and UM12, and compared to the same two NPC donors (NPC354 and NPC357) that were utilized for the gene expression analysis in

Figure 1. Analysis of antiviral genes indicated little to no enrichment from postpartum CC donors (**Figure 3b**), a finding in agreement with our analysis of purified $CD14^+$ cells (**Figure 3a**). This lack of enrichment in CC donors was in stark contrast to the high level of antiviral transcripts observed two weeks postpartum in CT donors UMO7 and UM08 (**Figure 3b**). Taken together, postpartum PBMC ISG expression is prolonged at least six months postpartum in mothers with a CT genotype (**Figure 1c**), but quickly diminishes in mothers with a CC genotype (**Figure 3b**).

Discussion

We initially hypothesized that innate antiviral immunity may be augmented in the weeks after childbirth to protect women from infections while adaptive immune function recovers. Here we found that some women indeed exhibited prolonged up-regulation of interferon-stimulated antiviral genes in $CD14^+$ cells after delivery. Strikingly, this signature was limited to women with a particular *IFNL3* rs12979860 genotype. To our knowledge these findings are novel and raise important questions about the mechanisms and potential benefits or harms of protracted interferon signaling in $CD14^+$ cells after childbirth.

Interestingly, the observed effects of this SNP on ISG expression in circulating $CD14^+$ cells after delivery (**Figure 1c, 3a**) parallel findings in HCV-infected livers (23). The *IFNL3* SNP at position rs12979860 was originally identified by genome wide association studies to be predictive of spontaneous and treatment-induced resolution of infection with HCV (18, 19, 24, 25). HCV-infected individuals with the favorable homozygous CC

genotype exhibit low intrahepatic ISG levels, while those with the unfavorable CT or TT genotypes exhibit higher intrahepatic antiviral gene expression (18, 19, 23-25). Though the association between impaired clearance of HCV and high intrahepatic ISG levels is counterintuitive, it has been suggested that interferon exerts its protective effect early following infection, whereas prolonged interferon signaling may lead to "interferon exhaustion." Indeed, in a mouse model of chronic infection, blockade of type I interferon prior to infection with lymphocytic choriomeningitis virus clone 13 enhanced viral load during the acute phase of infection, but led to viral clearance at later time points (26, 27).

Some evidence suggests that protracted interferon signaling may also impair $CD14^+$ function. Tilton and colleagues described an association between ISG enrichment in $CD14^+$ cells and decreased pro-inflammatory cytokine production in patients infected with human immunodeficiency virus (HIV) (28). This study hints that in certain settings, the postpartum antiviral transcript enrichment in $CD14^+$ cells could actually be less favorable due to decreased cytokine production.

Our findings indicate that maternal *IFLN3* genotype may affect postpartum control of viral infections. In support of this, recent work determined that the sharp drop in HCV viremia that sometimes follows pregnancy (11-13) occurs more commonly in women with the *IFNL3* CC genotype (Honegger *et al.* personal communication). This suggests that rapid down-regulation of innate antiviral genes in mothers is likely favorable for the control of HCV infection following delivery. It is possible that a similar effect will be seen for other chronic viral infections that exhibit unique postpartum dynamics such as

the hepatitis B virus (14, 15). However, we cannot rule out the possibility that enrichment of antiviral genes after pregnancy associated with the *IFNL3* CT or TT genotype could be advantageous for other infections. It will be exciting to learn which viral infections are impacted by *IFNL3* genotype and the variable $CD14^+$ ISG expression following delivery.

It is also tempting to speculate that the *IFNL3* genotype effects on interferon signaling could influence other postpartum complications including bacterial infections, autoimmune diseases, or even depression. Puerperal sepsis, a bacterial infection originating in the uterus following delivery, is the leading infectious cause of maternal death worldwide (29). Type I IFN signaling has been associated with increased severity of infection with a number of relevant pathogens, including staphylococcal and pseudomonas species (30, 31). Likewise, elevated levels of type I interferon are associated with pathogenesis of certain autoimmune diseases such as systemic lupus erythematosus (SLE) (32). As SLE flares are common during and following pregnancy (33), perhaps the rapid control of IFN signaling in mothers with a CC genotype could influence flare severity. In contrast, patients with multiple sclerosis are often treated with recombinant type I IFN (32). Some women are prone to postpartum relapses (34), suggesting that prolonged IFN signaling could be protective following delivery. Further, depression is a side effect of therapeutic treatment with exogenous IFN-α (35), and has been associated with aberrant pro-inflammatory cytokine production (36). A CC genotype could potentially be favorable for women prone to postpartum depression. Though we have not studied these potential connections, they are intriguing possibilities

worth examining in the future.

The mechanisms underlying the prolonged and selective ISG up-regulation in $CD14^+$ cells of *IFNL3* CT/TT women after childbirth remain elusive. First, the mechanism by which the *IFNL3* rs12979860 SNP exerts an effect on ISG signaling in any cell type is unsettled, with uncertainty about whether the SNP is actually functional or whether its effect is mediated by other *IFNL* SNPs in high linkage disequilibrium (37). Second, an effect of *IFNL3* genotype on ISG expression has not been previously described for $CD14^+$ cells to our knowledge. Monocytes are known to increase in both frequency and activation marker expression during pregnancy and labor (10, 17, 38), with roles in placental vascularization, initiation of labor and potentially immune protection during a period of reduced adaptive immunity. Nonetheless, the stimulus for continued interferon signaling in $CD14^+$ cells after delivery is unknown. Regardless of the source of $CD14^+$ cell stimulation, postpartum $CD14^+$ cells from CC donors may have improved ability relative to their TT and CT counterparts to physiologically down-regulate interferon signaling following delivery.

Overall, we identified a prolonged ISG signature that was primarily expressed in $CD14^+$ cells following delivery in healthy women. ISG enrichment was dependent upon *IFNL3* genotype, which to our knowledge is the first report of *IFNL3* SNP-dependent gene expression in $CD14^+$ cells. Together these data may have important ramifications for the maternal response to viral infections and other inflammatory diseases following delivery.

Methods

Study approval

Healthy non-pregnant control subjects and mothers were recruited from the Yerkes National Primate Research Center Healthy Donor Protocol under the approval of the institutional review board at Emory University. Donors provided written consent for blood donation. Healthy mothers (negative for hepatitis B surface antigen, hepatitis C antibody, and HIV antibody) were also recruited from the Ohio State University Substance Treatment, Education and Prevention in Pregnancy (STEPP) Program. Following informed consent, blood collections were performed at Nationwide Children's Hospital in Ohio under the approval of the institutional review boards at Ohio State University and Nationwide Children's Hospital. Donor characteristics are highlighted in Supplementary Tables 1 and 2.

Blood collection and processing

Blood samples were collected in Vacutainer tubes (BD, Franklin Lakes, NJ, USA) and PBMCs were isolated using Ficoll gradient centrifugation. Cells were suspended at a concentration of 5×10^6 cells/ml in fetal bovine serum (FBS, Hyclone, Logan, UT, USA) containing 10% dimethyl sulfoxide (DMSO) and stored at -150 degrees C.

RNA purification for microarray analysis

PBMCs were stored in 350 µl of RLT buffer (Qiagen, Hilden, Germany) containing 1% beta-mercaptoethanol. Total RNA extraction was performed using the RNeasy mini kit according to the manufacturer's specifications; on-column DNAse digestion was also

performed to remove genomic DNA. RNA integrity of the extracted RNA was assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) capillary electrophoresis on an RNA 6000 NanoChip; all samples had an RNA Integrity Number (RIN) score of 8.5 or higher. Quantitative analysis was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA).

Microarray hybridization

For each individual sample, cDNA synthesis and amplification was performed using the NuGEN Ovation Pico WTA V2 system (NuGEN, San Carlos, CA, USA). Briefly, 25 ng of total RNA were used for cDNA synthesis followed by whole transcriptome amplification by NuGEN's Ribo-SPIA® technology. The Ribo-SPIA® technology uses DNA/RNA chimeric primers to amplify cDNA isothermally maintaining the stoichiometry of the input RNA. The amplified single stranded DNA was purified using the AMpure XP beads (Beckman, Indianapolis, IN, USA). Qualitative and quantitative analyses were performed on the Bioanalyzer and NanoDrop respectively to assess the size distribution of the amplified DNA and quantity. 5 μg of the amplified DNA were used for biotinylation and fragmentation using the NuGEN Ovation Encore Biotin Module (NuGEN, San Carlos, CA, USA).

Microarray analysis and bioinformatics

Data were analyzed using TIBCO Spotfire with OmicsOffice for Microarrays (Integromics Biomarker Discovery). Primary microarray data has been submitted to Gene Expression Omnibus (GEO) in accordance with proposed Minimum Information About a

Microarray Experiment (MIAME) standards. DAVID Bioinformatics Resources and Ingenuity Pathways Analysis were employed to identify biological pathways and processes that were enriched in postpartum samples over non-pregnant controls. These programs analyze and categorize gene sets within known biological pathways or networks.

Cell purification

Total PBMCs were thawed and washed twice with complete RPMI containing 10% FCS and 100-μg/ml penicillin and streptomycin and once with cell isolation buffer (PBS containing 1% FCS). $CD14^+$ cells were isolated using human CD14 MicroBeads and MACS columns according to the manufacturer's instructions (Miltenyi Biotech, Paris, France). Other subpopulations were purified using CD8, CD4, and CD19 Pan B cell Dynabeads for human cell isolation, also according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Isolated cells were immediately washed and placed in 350 μL Buffer RLT containing 1% beta-mercaptoethanol and stored at -80 degrees C.

RT-PCR

Total RNA was extracted using an RNeasy Mini Kit and treated with an RNAse Free DNAse Set (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription reactions were performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). To determine gene expression levels, 20 ng of cDNA per reaction were analyzed using the *Power* SYBR

Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7500 apparatus. Transcript levels were normalized to GAPDH and fold changes were calculated using the formula $2^{-\Delta\Delta C}{}_{T.}$

Allelic discrimination assay

Genotypes of NPC and postpartum subjects at SNP rs12979860 were determined as previously described (23). Briefly, genomic DNA was isolated from patient samples using the PureLink Genomic DNA Mini Kit (Qiagen, Valencia, CA, USA) per the manufacturer's protocol. Allelic discrimination was carried out using Taqman Genotyping Master Mix and the following primer/probe combination: Forward Primer 5'-GCCTGTCGTGTACTGAACCA-3', Reverse Primer 5' -GCGCGGAGTGCAATTCAAC-3'; 5'-VIC-TGGTTCGCGCCTTC-3' and 5'- FAM-CTGGTTCACGCCTTC-3' (Life Technologies) Samples were run on an ABI 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA), and donor genotype was assigned by automated algorithm included in the SDS v1.3.1 software suite (Applied Biosystems).

Flow cytometric analysis

PBMCs were stained with antibodies to CD3 (FITC, clone UCHT1, Beckman Coulter, Brea, CA, USA), CD8 (PE, clone SK1, Biolegend, San Diego, CA, USA), HLA-DR (PerCP – Cy5.5, clone L243, Biolegend), CD19 (violet fluor 450, clone HIB19, Tonbo Biosciences, San Diego, CA, USA), and CD14 (PE-Cy7, clone M5E2, BD Biosciences, (Franklin Lakes, NJ, USA) for analysis of cell frequencies and isolation purity.

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Figures and Figure Legends

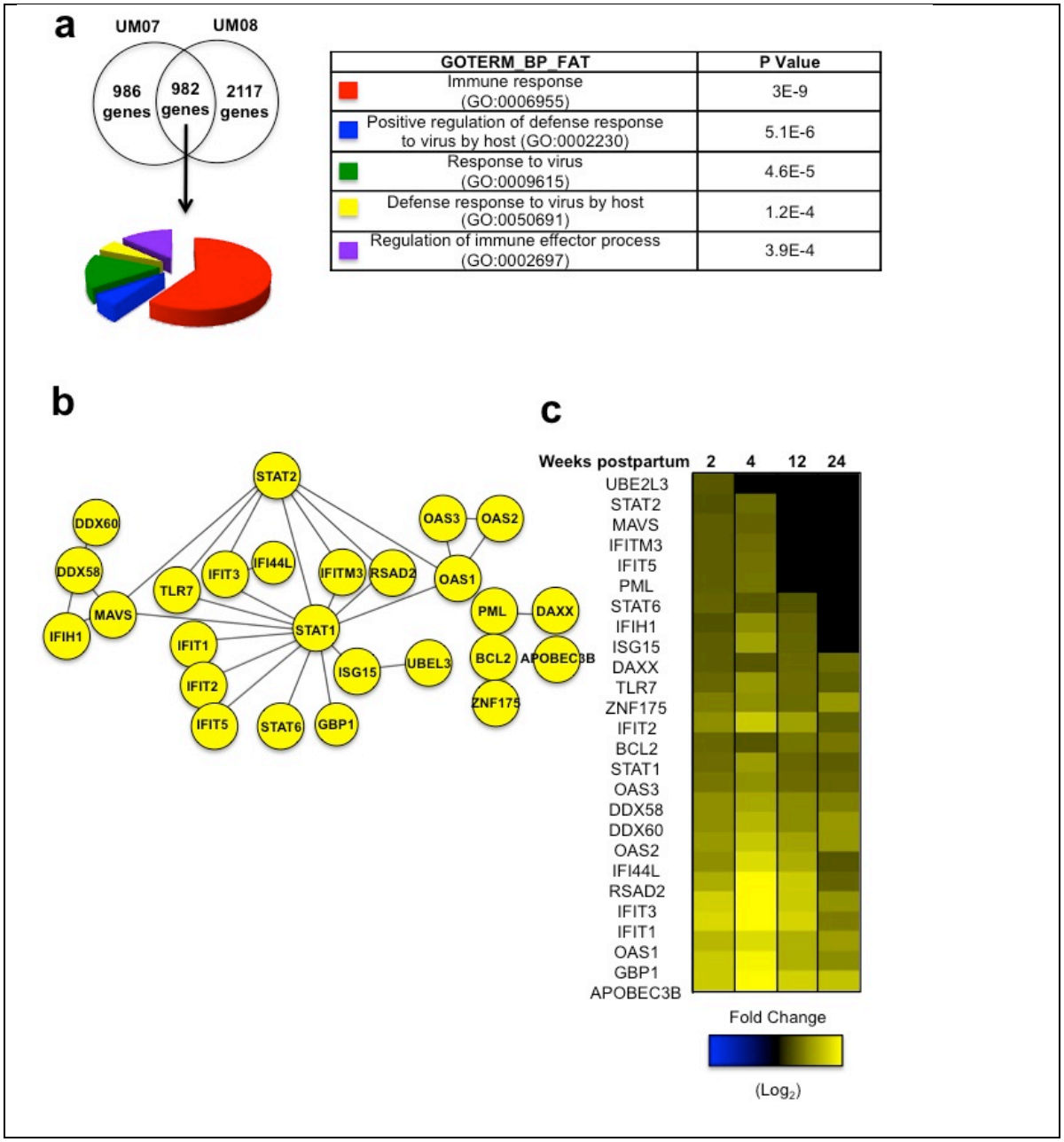

Figure 1. Antiviral defense genes are sustained six months postpartum in PBMCs from healthy mothers. (A) Top five biological processes increased two weeks postpartum over NPCs. Fold changes were calculated relative to the average of NPC subjects. DAVID Bioinformatics Resource was used for functional annotation, while gene accession numbers for biological processes are listed. (B) Network analysis of antiviral genes enriched two weeks postpartum. Each node represents one gene within the biological process. (C) Heat map of antiviral gene expression in total PBMCs obtained from UM07 and UM08 between two and 24 weeks postpartum. Fold changes were calculated relative to the average transcript levels from NPC354 and NPC357.

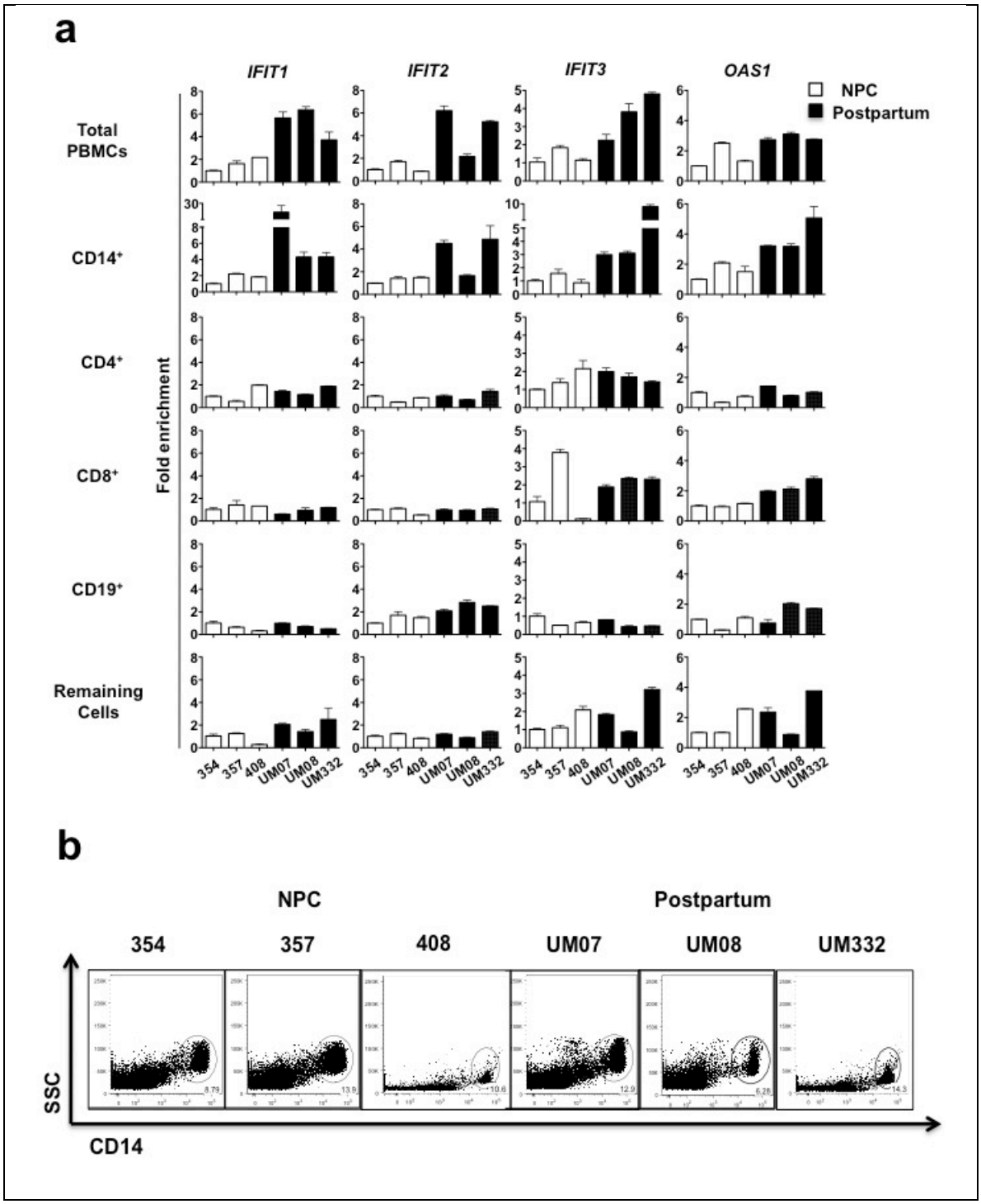

Figure 2. Antiviral defense genes are enriched postpartum in $CD14^+$ cells. (A) Antiviral gene expression levels of *IFIT1, IFIT2, IFIT3, and OAS1* from isolated $CD14^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cell populations were analyzed by RT-PCR and shown as fold change relative to NPC354 (bars represent SEM). (B) Frequency of $CD14^+$ cells in total PBMCs used for transcript analysis in (A).

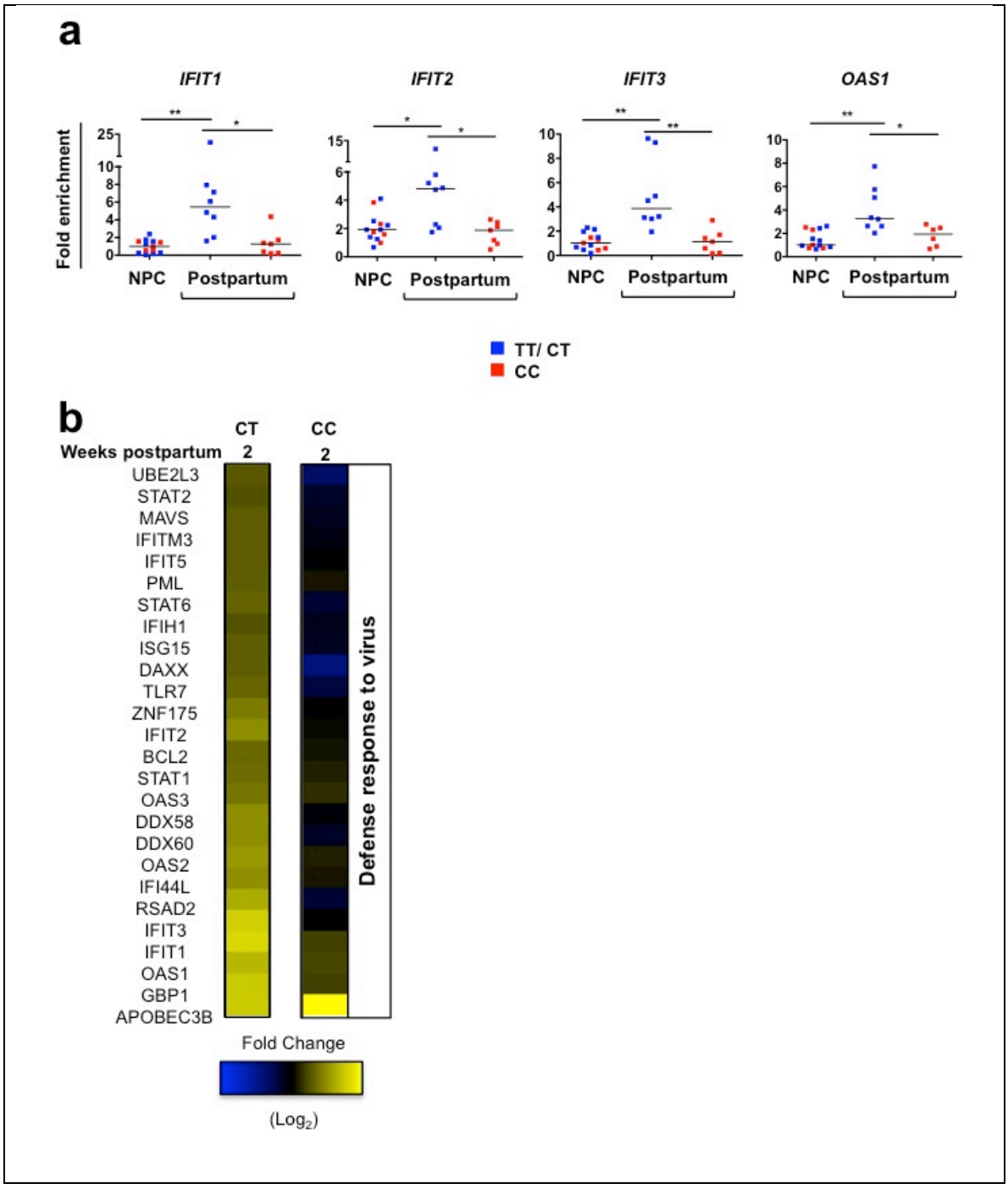

Figure 3. Postpartum antiviral defense transcript enrichment can be stratified based upon allele expression at interferon-λ3 SNP rs12979860. (A) RT-PCR analysis of antiviral genes. Gene expression levels from isolated $CD14^+$ cells are expressed as fold change relative to healthy NPC354. Each dot represents one individual donor, and the median of each group is shown. (Kruskal-Wallis test with subsequent Dunn's test for pairwise comparison, (*) $p<.05$, (**) $p<.001$). (B) Gene expression analysis from total PBMCs obtained postpartum from two mothers with CT genotypes (UM07 and UM08) and two mothers with CC genotypes (UM10 and UM12). Fold changes were calculated relative to the average transcript levels from NPC354 and NPC357.

Supplemental Table 1. IFNL3 genotype and demographics of uninfected mothers.

Donor	Age at Delivery	Race	Ethnicity	Total Pregnancies	Total Deliveries	Delivery Mode	IFNL3 rs12979860
UM01	24	Caucasian	Non-Hispanic	2	1	Vaginal	TT
UM02	22	Caucasian	Non-Hispanic	1	1	Vaginal	TT
UM06	33	Caucasian	Non-Hispanic	4	2	Vaginal	CT
UM07	25	Caucasian	Non-Hispanic	3	2	Vaginal	CT
UM08	21	Caucasian	Non-Hispanic	3	1	Vaginal	CT
UM10	33	Caucasian	Non-Hispanic	5	4	Vaginal	CC
UM12	22	Caucasian	Non-Hispanic	1	1	Vaginal	CC
UM14	22	Caucasian	Non-Hispanic	5	3	Caesarian	CT
UM332	38	Caucasian/ African American	Hispanic	5	4	Vaginal	CT
UM1001	U*	Caucasian	Non-Hispanic	2	2	Caesarian	CT
UM1003	U*	Asian	Non-Hispanic	1	1	Vaginal	CC
UM1004	U*	Caucasian	Non-Hispanic	1	1	Vaginal	CC
UM1005	33	Caucasian	Non-Hispanic	1	1	Vaginal	CC
UM1006	34	Caucasian	Non-Hispanic	2	2	Vaginal	CC
UM1007	36	Caucasian	Non-Hispanic	2	2	Vaginal	CC

U* unknown

Supplemental Table 2. IFNL3 genotype and demographics of non-pregnant control subjects.

NPC Donor	Age	Race	Ethnicity	IFNL3 rs12979860
125	39	Caucasian	Non-Hispanic	CC
354	28	Caucasian	Non-Hispanic	CC
357	25	Caucasian	Non-Hispanic	CC
380	37	Caucasian	Non-Hispanic	CT
393	27	Caucasian	Non-Hispanic	CT
408	24	Caucasian	Non-Hispanic	CT
426	37	African American	Non-Hispanic	TT
427	25	Caucasian	Non-Hispanic	CT
428	33	Caucasian	Non-Hispanic	CT
435	26	Asian	Non-Hispanic	CC
447	25	Asian	Non-Hispanic	CC
457	28	Caucasian/ Asian	Non-Hispanic	TT
461	31	African American	Non-Hispanic	CT

Chapter 3

Harnessing the prokaryotic adaptive immune system as a eukaryotic antiviral defense

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Harnessing the Prokaryotic Adaptive Immune System as a Eukaryotic Antiviral Defense

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Abstract

Clustered, regularly interspaced, short palindromic repeats - CRISPR associated (CRISPR-Cas) systems are sequence specific RNA-directed endonuclease complexes that bind and cleave nucleic acids. These systems evolved within prokaryotes as adaptive immune defenses to target and degrade nucleic acids derived from bacteriophages and other foreign genetic elements. The antiviral function of these systems has now been exploited to combat eukaryotic viruses throughout the viral life cycle. Here we discuss current advances in CRISPR-Cas9 technology as a eukaryotic antiviral defense.

The Prokaryotic Immune System

Prokaryotic cells possess innate and adaptive immune systems that conceptually parallel those found in eukaryotic organisms (1). Prokaryotic restriction endonucleases function as an 'innate' immune defense mechanism, recognizing conserved nucleotide sequences and subsequently cleaving foreign nucleic acids. These endonucleases have formed the platform for the generation of powerful tools such as recombinant DNA technology and genome sequencing. Alternatively, CRISPR-Cas systems serve as the 'adaptive' immune system of bacteria and archaea. By incorporating short sequences of bacteriophage-derived or other foreign nucleic acids into their own genome, prokaryotes use CRISPR-Cas systems to recognize new targets and degrade these invaders upon secondary encounter, analogous to a memory response in eukaryotic organisms (2).

Trends

- Cas9 technology has been utilized to inhibit pathogenic DNA and RNA viruses in cell culture. This has been accomplished by modulating expression of host factors required for viral entry, directly targeting virus genomes, transcriptionally activating antiviral genes, and cleaving the DNA stage of viruses that integrate into the host cell chromosome.
- The Cas9 machinery has been shown to be efficacious in restricting pathogenic viruses in small rodent models.
- Transgenic plants have recently been developed that inhibit the replication stage of geminiviruses, a major cause of crop losses worldwide.
- Cas9 technology is a promising tool for the generation of virus-resistant transgenic plants and animals, as well as antiviral therapeutics.

Cas9-mediated Targeting of Bacteriophages

The clustered, repetitive sequences (CRISPR array) that form the central feature of CRISPR-Cas systems were first discovered in 1987 in *Escherichia coli* (3), although it was not until 2007 that the function of CRISPR sequences and their conserved, adjacent

cas genes (CRISPR-associated genes) was first described. Barrangou and colleagues demonstrated that upon bacteriophage infection, *Streptococcus thermophilus* integrated phage genomic sequences into the CRISPR array, and that these sequences, in conjunction with the *cas* genes, provided protection from subsequent viral challenge (4). CRISPR-Cas systems have now been identified in over 90% of sequenced archaea, as well as roughly 50% of bacterial species (5, 6). The class II CRISPR-Cas9 system has been the most extensively studied and is discussed in detail below.

There are two distinct stages of Cas9-mediated immunity (**Figure 1**). During the acquisition phase, a bacterium encounters foreign nucleic acid, such as that of a phage genome. A portion of the phage genome is incorporated into the CRISPR array, and is termed a spacer (4, 7, 8). The subsequent immunity phase occurs when the bacterium again encounters an identical foreign nucleic acid and proceeds to catalyze its cleavage. In this phase, Cas9 is guided by two small RNAs, the trans-activating RNA (tracrRNA) and the CRISPR RNA (crRNA) (9, 10). When the associated crRNA, transcribed from the CRISPR array, has complementarity to the invading phage sequence, the two Cas9 endonuclease domains (RuvC and HNH) mediate cleavage of the targeted sequence. Endonuclease activity also requires that a short sequence on the foreign DNA adjacent to that bound by the crRNA, known as the protospacer adjacent motif (PAM), is recognized by Cas9 (10) (**Figure 1**).

In addition to its natural role within bacteria, the Cas9 system has been harnessed for diverse applications in eukaryotic cells. Cas9 can be programmed to target and cleave

double-stranded DNA (dsDNA) sequences of interest by engineering single chimeric guide RNAs (gRNAs) composed of portions of the tracrRNA and crRNA (10) (**Figure 2**). Target specificity is achieved by simply modifying the short spacer region of the gRNA to a sequence complementary to the target. Co-expression of the gRNA with Cas9 in the cell of interest leads to target cleavage. Genomic alterations have already been performed in diverse cell types, including those from zebrafish (11, 12), mice (13-15), humans (10, 14, 16, 17), and an abundance of other organisms. Further, Cas9-based technologies have now been successfully exploited against eukaryotic viruses at different stages of their life cycle.

Cas9-mediated Inhibition of Eukaryotic Viruses

While directly targeting viral nucleic acids is an obvious strategy to inhibit viral replication, Cas9 has also been targeted to disrupt host factors critical for the viral life cycle. Additional strategies include using Cas9-transcriptional fusion proteins to reactivate viruses and render them susceptible to killing by the immune system, or to induce transcription of antiviral genes. Together, these avenues may provide effective means to inhibit, or even clear, viral infections. In this review, we discuss examples of pathogenic eukaryotic viruses that have been targeted by Cas9, as well as current obstacles, future implications, and questions regarding this technology.

Human immunodeficiency virus

More than 35 million people worldwide are infected with human immunodeficiency virus (HIV) (18). HIV infects cells using the primary receptor CD4 (19) and chemokine co-

receptors CCR5 (20) or CXCR4 (21). If left untreated, infection can lead to progressive impairment and depletion of immune cells, particularly $CD4^+$ T cells, and acquired immunodeficiency syndrome (AIDS) (18). Despite significant advances in antiretroviral regimens, treatment toxicity and viral resistance are limitations of current therapies. A vaccine remains elusive despite intense research effort (22-24).

Certain individuals possess a naturally occurring 32-nucleotide deletion within CCR5 that confers resistance to CCR5-tropic HIV-1 infection (25). In the highly publicized case of the Berlin patient, Timothy Brown received a bone marrow transplant for myeloid leukemia from a donor who was homozygous for this 32-nucleotide deletion in the *ccr5* gene. As a consequence, viremia was undetectable following the transplant, even after cessation of antiviral therapy (26, 27). This fortuitous occurrence highlighted the potential for this CCR5 deletion to be harnessed therapeutically.

Since then, several gene editing strategies have been utilized to generate the CCR5 deletion, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and Cas9 (28-31). Briefly, ZFNs are recombinant proteins comprised of sequence-specific zinc finger DNA binding domains fused to the endonuclease domain of the bacterial FokI restriction enzyme. ZFNs are designed in pairs, and following DNA binding, FokI endonuclease domains dimerize to induce site-specific cleavage (32). Like ZFNs, TALENs are proteins consisting of novel DNA binding domains fused to the endonuclease domain of FokI. Each TALE repeat confers specificity for a single nucleotide, so virtually any DNA sequence can be targeted. ZFNs

and TALENs have been reviewed extensively elsewhere (33-35), but importantly, one of the major drawbacks of these technologies is that a new protein must be engineered for each target sequence, so protein design and synthesis remain significant obstacles. Consequently, the emerging Cas9 platform has quickly become the preferred approach for genome engineering due to its ease of use. Only an RNA needs to be altered to program the system for each new target.

Ye *et al.* recently demonstrated that deletion of the 32 bp region of the *ccr5* gene with Cas9:gRNA complexes conferred resistance to CCR5 tropic HIV-1 in monocytes and macrophages differentiated from induced pluripotent stem cells (28). Other groups have also reported CCR5 disruption in HIV-1 permissive cell lines (29), primary $CD4^+$ T cells (30, 31), as well as hematopoietic stem and effector cells (31). Together, these findings reveal the utility of Cas9 in generating CCR5 deletion mutations to inhibit HIV entry and infection.

As HIV-1 may also use CXCR4 as a co-receptor for cellular entry (21), Cas9 has been utilized to disrupt the *cxcr4* gene in GHOST (a human osteosarcoma cell line expressing CXCR4) and Jurkat cell lines, as well as *Rhesus macaque* primary $CD4^+$ T cells *in vitro* (36). p24 capsid antigen levels were reduced by approximately four-fold, seven days post infection in the supernatants of primary $CD4^+$ cells transfected with Cas9 and CXCR4-specific gRNA (36). This concept of host gene modulation by Cas9 could potentially be applied to countless other viruses with life cycles that are dependent upon specific host proteins.

An alternative strategy to combat HIV is to directly target the HIV provirus by Cas9-mediated cleavage. The provirus is generated when virally encoded polymerase (Pol) protein reverse transcribes the RNA genome of the virion into a DNA form. RNase H, also virally encoded, degrades the RNA strand, thus allowing the DNA polymerase activity of Pol protein to synthesize the complementary DNA strand (37, 38). This dsDNA form, when integrated into the host chromosome is termed the provirus, which serves as a fundamental hurdle to curing HIV infection (39-42). An HIV provirus can be transcriptionally silent, though in the more common scenario, the provirus serves as a key transcriptional template for HIV RNAs (42). Antiviral therapies target transcriptionally active proviruses, while transcriptionally inactive proviruses escape both therapeutics and immune recognition (42). Cells containing the provirus maintain a persistent reservoir that poses a long-lasting health risk to infected individuals.

Several studies have demonstrated that the HIV provirus can be disrupted or excised from latently infected cell lines using Cas9 (43-45). Ebina *et al.* utilized an HIV vector expressing GFP under the control of a long terminal repeat (LTR) promoter to mimic latent HIV infection. Transfections of plasmids encoding Cas9 or gRNA targeted to the LTR in Jurkat cells reduced GFP expression by more than half relative to cells transfected with a control vector (43). Expression of Cas9 and gRNA targeted to the LTR region of an HIV-1 reporter virus in microglial cells also reduced the frequency of GFP expressing cells from 76% to 4% (44). Others have shown that Cas9 treatment was efficacious in latently infected U1 monocytic cells, and that when HeLa cells stably

expressing Cas9 and an LTR-specific gRNA were infected with a GFP reporter virus, the percentage of GFP^+ cells was reduced to less than 10%, as compared to roughly 60% in cells expressing a control gRNA (44). Further, Zhu and colleagues designed a panel of 10 gRNAs targeted to different regions of the HIV genome. Expression of three multiplexed gRNAs and Cas9 in Jurkat cells expressing a full length HIV-1 reporter virus reduced viral particle production by more than 20-fold relative to vector controls (45). Liao *et al.* expanded on these studies to demonstrate that the viral cDNA may be disrupted prior to chromosomal integration (46). More importantly, Cas9 and HIV-specific gRNA expression within primary $CD4^+$ T cells and induced monocyte/macrophage cells reduced virus production by approximately three-fold and ten-fold, respectively (46).

An additional approach to inhibition of HIV has been based on Cas9-transcriptional activator proteins (fusion proteins of catalytically inactive Cas9 to a transcriptional activation domain from another protein), which were originally engineered to induce eukaryotic host gene expression (47, 48). This approach has been applied to activate transcription of the antiviral restriction factor *APOBEC3B*, which can inhibit HIV-1 in cell culture (49). Another strategy, termed 'shock and kill', relies on Cas9 to reactivate transcriptionally inactive virus from cellular reservoirs, which will then be cleared by host immune defenses and/or with conventional antiretroviral therapy. Several groups have engineered Cas9-transcriptional activator proteins that specifically target the HIV-1 LTR promoter sequence. These fusion proteins may provide enhanced specificity relative to traditional agents used for provirus reactivation (50-52). Taken together, the aforementioned studies highlight the diverse ways in which Cas9 technology is being

used to directly target the HIV provirus and/or the receptors important for its entry, reactivate latent virus, or stimulate antiviral gene induction. A critical question is how these technologies will translate to potential use in humans.

Although the aforementioned studies are promising, the efficient delivery of Cas9 and gRNAs *in vivo* poses a major challenge, not only due to the sheer number of intended recipient $CD4^+$ T cells within the body, but also to the anatomic locations of viral reservoirs. Common viral vectors such as adeno-associated viruses (AAV) and lentiviruses are currently unsuitable for *in vivo* delivery for any of the approaches described above. For example, AAV does not readily transduce T cells (53), and lentiviruses are generally produced at titers of no greater than 10^9 transducing units/mL, even after extensive concentration, (54) which does not meet the necessary requirement for the *in vivo* transduction of each $CD4^+$ T cell within the body, estimated to total $>10^{11}$ cells (55). Until these obstacles can be overcome, targeted modification of *ccr5* or *cxcr4* by Cas9 may be the most immediate approach. However, this approach has three obvious disadvantages. First, this technique requires *ex vivo* genomic alteration and cellular expansion, which will likely hinder its widespread use. Second, although lentiviruses are currently being utilized as *ex vivo* Cas9 delivery systems, transduction itself is a mutagenic event, which poses an inherent risk should the virus integrate at a site that could cause harm to the host cell. Finally, latent cellular reservoirs would still persist in spite of HIV co-receptor modification. Given the great potential of Cas9 in combating HIV, it will be interesting to see if and how these obstacles are overcome in the future.

Hepatitis B virus

Hepatitis B virus (HBV) is an enveloped partially dsDNA virus that primarily infects hepatocytes (56). Despite the development of a highly effective HBV vaccine (57) as well as nucleoside and nucleotide analog therapeutics used in combination with interferon (56), HBV still affects approximately 250 million people worldwide (58), predisposing these individuals to complications such as liver fibrosis, cirrhosis, and hepatocellular carcinoma.

Following cellular entry, the relaxed circular, partially dsDNA HBV genome (rcDNA) is converted to covalently closed circular DNA (cccDNA), which serves as the transcription template for the generation of viral RNAs (56, 59). Though antiviral therapies inhibit HBV replication, they do not eliminate cccDNAs, which stably persist in the nuclei of infected hepatocytes (60).

Several groups have demonstrated that both HBV rcDNA and cccDNA are susceptible to Cas9-mediated cleavage in cell culture (61-69) as well as *in vivo* (63-66, 69). Numerous cell types and Cas9:gRNA delivery methods were used for the cell culture studies, but one feature common among several of the reports was a two to three fold reduction in HBV surface antigen (HBsAg) level in the lysates or supernatants of cells expressing HBV-specific gRNAs relative to cells expressing control gRNA (61, 63-66). *In vivo*, NRG mice hydrodynamically injected with two different plasmids encoding HBV or Cas9 with a gRNA specific for the HBV polymerase-coding region displayed a four-fold decrease in viremia relative to mice injected with HBV and Cas9/control gRNA

plasmids. The viral titer correlated with a mild reduction in serum HBV surface antigen (HBsAg) level (63). Another group utilized the hydrodynamic injection system in which plasmids encoding HBV and plasmids encoding Cas9 with either control gRNA or gRNAs targeting the HBV surface (S) and X proteins, were injected into Balb/c nude mice (64). Three days following injection, a 90% reduction in serum HBsAg level was observed (64). Similar inhibition was retained up to ten days following injection, and Cas9-induced mutations were detected in up to 75% of total HBV genomes (64). An additional study demonstrated at least a five-fold decrease in serum HBV DNA level and HBsAg four days post Cas9:gRNA treatment in Balb/c mice (69).

Perhaps the least efficacious report of the ability of Cas9 to inhibit HBV *in vivo* was performed in immunocompetent mice. C57BL/6 mice were hydrodynamically injected with two different plasmids encoding HBV and Cas9 with either control gRNA or gRNAs specific for the HBV P1 or X coding sequences (66). Serum HBsAg levels were only slightly reduced in Cas9 expressing mice relative to those expressing the vector control, 24 and 72 hours post treatment, and Cas9-mediated insertions and deletions within the HBV sequence were only detected in four to five percent of total HBV genomes (66). However, discrepancies in the level of HBV inhibition between this study and others could be due to multiple reasons other than the intact immune system, such as gRNA target region, injection time course, and/or ratio of injected HBV plasmid to Cas9 gRNA plasmid.

One caveat to the use of hydrodynamic injection of plasmids encoding the HBV genome

is that cccDNAs are not efficiently generated, and therefore this system does not closely resemble the hepatocytes of chronically infected HBV patients which may harbor up to 40 copies of cccDNA per cell (70). To overcome this obstacle, Dong and colleagues utilized an alternative *in vivo* model in which Balb/c mice were injected with plasmids that allowed for high expression of cccDNAs. Subsequently, hydrodynamic injection with Cas9/gRNA plasmids resulted in a two to threefold decrease in liver cccDNA, and a corresponding approximate fivefold decrease in HBeAg six days post injection (65). Regardless of the mouse model and treatment length, Cas9/gRNA complexes did not facilitate 100% viral clearance in any of the aforementioned studies, highlighting the importance of effective delivery systems in which each infected cell receives Cas9 and gRNA.

In vivo delivery of Cas9 for therapeutic targeting of HBV and other hepatotropic viruses may be more readily achievable than for other eukaryotic viruses, as recombinant adeno-associated virus 8 (AAV8) is capable of efficiently transducing hepatocytes (71). Recombinant AAV has gained popularity as a potential Cas9 delivery vector, and offers the advantages of high-titer production and continuous transgene expression (72). However, recent observations that chromosomal insertions of AAV2 may activate proto-oncogenes in human hepatocellular carcinomas suggest a pathogenic role for this vector (73). Further studies will need to address the potential oncogenic role for AAV8, a serious consideration given that viruses such as HBV are associated with hepatocellular carcinomas. An additional point to consider is that size constraints in many of these viral vectors may not allow for the full expression of *S. pyogenes* Cas9, gRNA, and their

respective promoters. To overcome packaging restraints, split-enzyme Cas9 proteins have been generated. Each half of the enzyme can be packaged within a separate vector, and then the protein domains dimerize and form a functional endonuclease upon chemical induction or recruitment by gRNA (74, 75). Further, smaller proteins, such as Cas9 from *Staphylococcus aureus,* are being investigated as alternatives (76, 77). Despite the obstacles, Cas9 may have intriguing potential to combat hepatotropic viruses, specifically in the case of HBV and the ablation of cccDNA.

Papillomavirus

Human papillomavirus (HPV) encodes a circular dsDNA genome that may integrate into the host cell chromosome. HPV DNA is detected in greater than 99% of cervical cancers, and aberrant expression of HPV proteins promotes cervical cell malignant transformation and tumor expansion (78). The HPV proteins E6 and E7 disrupt normal expression of the host cell cycle proteins p53 and retinoblastoma (Rb), respectively, enhancing cellular immortalization (79, 80). Although the prophylactic vaccines, Gardasil ® (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm111283.htm) and Cervarix ® (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm186959.htm), have been developed, there is still a need for therapeutics that effectively control established HPV infection and associated tumors. Presumably, excision of these HPV oncogenes could restore expression and normal functioning of p53 and Rb tumor suppressors, leading to cell cycle arrest and inhibition of tumor/cancer progression. Cas9 was used with cognate gRNAs to disrupt the HPV E6 and E7 coding sequences

within the siHa cervical tumor cell line that retains integrated HPV-16, a subtype of HPV that has been associated with greater than 50% of cervical tumors (81). Forty-eight hours following transfection of plasmids encoding Cas9 and gRNAs, viral transcript levels were reduced over 90% relative to cells transfected with control vectors (82). The reduction in E6 and E7 transcript levels correlated with an increase in host p53 and p21 (a cyclin-dependent kinase inhibitor used as a readout for Rb) production, as well as a significant reduction in cell viability (82). Kennedy *et al.* demonstrated similar findings in both siHa and HeLa cells, which retain integrated coding sequences from HPV-subtype 18 (83). Further, expression of Cas9 and HPV targeting gRNAs impeded tumorigenesis of siHa cells in a xenograft mouse model (82). No mice were completely free of tumors 45 days following transplantation, but tumor weights were reduced by three-fold relative to those from control treated mice (82). However, in this model, siHa cells were transiently transfected with plasmids encoding Cas9 and HPV-targeting gRNAs, and subsequently implanted into immunodeficient Balb/c nude mice. Other models such as naturally occurring rabbit papillomavirus perhaps more closely resemble the course of infection and tumorigenesis of HPV, and it would be interesting to test the Cas9 system in these models.

It is plausible that AAV may be a potential delivery vehicle for Cas9:gRNAs that target HPV, since certain AAV serotypes can readily transduce HeLa cells (53), a cervical cancer cell line, and AAV sequences have been detected in primary cervical samples (84). However, AAV tissue tropism is not limited to cervical cells (53). Localized introduction of recombinant AAV in the female cervix could potentially minimize

systemic cellular transduction. Similarly, the generation of mosaic capsid proteins to express peptide moieties or antibody light chains for a specific receptor may provide the specificity required for transducing a particular cell type (85). Nonetheless, an underlying concern is again the potential oncogenic role of AAV. However, if delivery concerns can be overcome, Cas9 may hold considerable promise for the targeting HPV.

Epstein-Barr virus

Epstein-Barr virus (EBV) is a linear dsDNA herpes virus and causative agent of infectious mononucleosis. EBV infection can lead to cancers such as Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma, among others, when maintained as an episome (86). The first report of Cas9 in the study of EBV was for promoter studies and the generation of an EBV reporter virus (87). Later, Wang and colleagues utilized Raji cells, a B cell line derived from a patient with Burkitt's lymphoma that harbors episomal copies of EBV (88), to demonstrate that Cas9 with gRNAs specific for EBV could excise the coding sequences of EBNA1 and EBNA3C, viral proteins involved in genome replication and host cell transformation, respectively (89). Further, expression of Cas9 with seven multiplexed EBV-targeting gRNAs could arrest cellular proliferation and induce apoptosis (89). Because it has been described that EBV proteins inhibit apoptotic processes in host cells, the induction of apoptosis may be due to the loss of these viral proteins (90). Single cell quantification of EBV episomal copy number within Raji cells expressing both Cas9 and these multiplexed gRNAs indicated that the copy number was widely divergent from cell to cell. Of the 71-sorted cells expressing both Cas9 and gRNA, 19 had undetectable EBV genome levels, whereas

22 cells retained copy numbers identical to untreated cells. The remaining 30 retained varying EBV copy numbers (89). Although this study demonstrates that Cas9 and targeting gRNAs can induce EBV genome disruption in Raji cells, the reasons why Cas9 and EBV gRNAs were only effective in reducing the EBV genome copy number in a portion of cells remain to be elucidated. To our knowledge, a delivery vector that specifically targets only B cells has yet to be developed, which would serve as a limitation in implementing Cas9 technology to combat EBV.

Geminiviruses

Geminiviruses are one of the largest families of plant viruses and are the causative agents of major crop losses in both the U.S. and around the world. These small, single stranded DNA viruses disrupt host machineries, often leading to plant developmental defects (91). However, recent progress has been made in engineering plants that inhibit geminivirus replication. Ji and colleagues generated transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* plants expressing Cas9 and gRNA specific for beet severe curly top virus. Plants expressing high levels of Cas9 were protected from virus challenge, and no leaf curling was observed (92). Similarly, Baltes *et al.* demonstrated that DNA loads of bean yellow dwarf viruses were reduced by approximately four-fold in transgenic *N. benthamiana* plants expressing Cas9 and gRNA (93), while others have demonstrated a reduction in tomato yellow leaf curl virus DNA accumulation in Cas9-transgenic plants (94). These studies are extremely encouraging and may pave the way for the generation of other transgenic plants and animals that are resistant to viral pathogens through Cas9-mediated targeting.

Cas9 Targeting of RNA

In addition to their role in bacterial adaptive immunity, it has been demonstrated that type II CRISPR-Cas systems can regulate endogenous gene expression. In conjunction with a small, CRISPR-Cas-associated RNA (scaRNA) and tracrRNA, *Francisella novicida* Cas9 (FnCas9) can target an endogenous mRNA encoding a bacterial lipoprotein (BLP) (95). Recognition of BLP by the host innate immune receptor Toll-like receptor 2 (TLR2) triggers an important pro-inflammatory cascade that contributes to host defense. By targeting BLP mRNA, Cas9 suppresses BLP expression and promotes evasion of TLR2-mediated host immune activation and *F. novicida* virulence (95, 96).

Further, O'Connell *et al.* described the ability of purified Cas9 from *Streptococcus pyogenes* (SpCas9) along with gRNA to bind and cleave single stranded RNA in the presence of an exogenous PAM oligonucleotide, termed the PAMmer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA could also be isolated and precipitated from HeLa cell lysate using SpCas9:gRNA in the presence of a PAMmer (97). Taken together, these studies highlight the ability of Cas9 to target RNA.

Given the success of Cas9 in targeting DNA for genome engineering in eukaryotic cells, it was theorized that the ability of Cas9 to target RNA might also be harnessed for use in eukaryotic cells. Such ability could be useful in targeting viruses that contain no DNA stage in their life cycle. Eukaryotic cells possess a mechanism of RNA interference (RNAi) in which small RNAs direct cellular machinery to mRNAs to dampen gene expression (98, 99). RNAi has been harnessed to inhibit the RNA stage of numerous

eukaryotic viruses, and does not require the presence of an exogenously expressed protein, as does the Cas9 platform. However, certain viruses have developed methods of circumventing RNAi (100, 101). Eukaryotic viruses have not evolved in the presence of Cas9 and likely cannot inhibit it, thus raising a potential advantage of Cas9-based systems.

FnCas9 was recently used to target the genome of the positive sense single-stranded RNA (+ssRNA) virus, hepatitis C virus (HCV), as a proof of principle (102). HCV is an important human pathogen that can cause liver fibrosis, cirrhosis, and may lead to hepatocellular carcinoma (103). Transient expression of Cas9 and RNA-targeting guide RNA (rgRNA) complexes targeted to either the 5' or 3' untranslated regions of the genome prior to infection of a hepatoma cell line with HCV resulted in a 50-60% reduction in viral protein expression relative to cells transfected with vector controls. In a model of established infection, FnCas9-mediated inhibition was approximately 40%, once again highlighting the need for efficient delivery systems in which each cell receives the Cas9:rgRNA complexes. The endonuclease domains of FnCas9 were dispensable for FnCas9-mediated inhibition of HCV, as was the presence of a canonical PAM (102). Collectively, these data suggest that inhibition was most likely due to a blockade of cellular translation and viral replication machineries that associate with the viral RNA, rather than HCV genome cleavage. This application of Cas9 technology more closely resembles CRISPR interference (CRISPRi), in which catalytically inactive Cas9 proteins are programmed to dampen gene expression, (104, 105) rather than canonical genomic editing. One potential obstacle to the use of Cas9:rgRNA complexes to directly

target RNA viral genomes *in vivo* would likely be the requirement for continuous high-level Cas9:rgRNA expression during the course of viral infection. Nonetheless, these studies highlight the potential for using Cas9 to target RNA in diverse applications.

Obstacles to the Use of Cas9 as an Antiviral in Humans

Despite the advances demonstrating Cas9-mediated inhibition of eukaryotic viruses, significant obstacles must be overcome before Cas9 can be used for human therapeutics. One primary obstacle is the current lack of data addressing how the mammalian immune system responds to Cas9. It is currently unknown whether Cas9 elicits cross-reactive antibodies or T cell responses. This concern may be alleviated if delivery systems are developed that allow for transient expression of Cas9:gRNAs, as would likely be required for DNA cleavage. The introduction of a Cas9-destabilization domain or inducible promoter could also allow for transient Cas9 expression.

Outstanding questions

- Can multiplexing gRNAs in transgenic plants and animals simultaneously facilitate resistance to multiple viruses, as occurs naturally with crRNAs in CRISPR arrays in prokaryotes? In addition, can multiplexed gRNAs targeting the same virus prevent viral escape?
- Can current antiviral therapies synergize with Cas9:gRNA to promote viral clearance?
- Are there off target effects on the host when Cas9 technology is used *in vivo*? How can such off-target effects be limited? Tissue-restricted expression of Cas9 and gRNA, as well as continued engineering to generate increased Cas9:gRNA specificity are possibilities.
- How does the mammalian immune system respond to expression of the foreign Cas9 protein? The large Cas9 proteins from *Streptococcus pyogenes, Francisella novicida*, and other organisms likely contain numerous B and T cell epitopes. Cas9 proteins may be immunogenic and result in clearance of the protein:RNA complex. Additional studies are also needed to address whether these epitopes are cross-reactive to host epitopes and induce aberrant autoimmune responses.

In contrast to using Cas9 to cleave DNA targets, prolonged Cas9 expression would likely be required for the transcriptional activation of antiviral effector proteins and latent viruses, as well as direct targeting of the RNA stage of virus infections. Sustained expression may not be feasible if the immune system is activated to kill cells expressing Cas9. It is plausible that truncated Cas9 proteins may be utilized to target the RNA stage of viral infections, which could potentially offer the advantages of a smaller packaging size for delivery systems such as AAV, as well as a decreased number of epitopes to be presented to the immune system, assuming a direct correlation between protein size and number of recognizable epitopes. As RNA virus inhibition may not be reliant on Cas9 endonuclease activity, shorter proteins could potentially protect the gRNA from nuclease digestion while still inhibiting translation and/or replication of viral nucleic acid. Further, it is possible that complete Cas9-mediated viral clearance may not be required for viruses that do not establish latency, as a Cas9-mediated decrease in viral load may synergize with other antiviral therapeutics and/or the immune system to fight infection, thus lessening the length of time Cas9 would need to be expressed.

A second major obstacle, as discussed in several sections above, is the development of a highly effective Cas9 delivery vehicle. Ensuring that the Cas9:gRNA machinery is expressed in the correct anatomic locations and proper cell types is absolutely necessary to translate this technology from the bench to the bedside. While transgenic plants and livestock may be readily produced to bypass delivery issues, the potential for human therapeutics is limited at this time. Nevertheless, numerous viral vectors such as

adenoviruses, adeno-associated viruses (AAV), and lentiviruses or retroviruses are potential approaches for the delivery of the antiviral Cas9 machinery. Other approaches include non-viral delivery methods such as lipopeptide nanoparticles, cell-penetrating peptides conjugated to Cas9:gRNA complexes, and DNA nanoclews. Each of these approaches offers both advantages and drawbacks that have been discussed in detail elsewhere (72, 85, 106-109), but underlying concerns include cell target specificity, proper Cas9 expression levels, and host safety. An additional concern is the immunogenicity of the delivery vehicle itself. For viral vectors, untoward immune responses, such as the production of neutralizing antibodies, could prevent effective vector entry and transgene expression in the cell type of interest, which is especially concerning if multiple rounds of treatment are warranted. Even if delivery methods are established so that every infected cell receives Cas9:gRNAs, the necessary ratio of the number of Cas9:gRNA molecules per virus genome or genomic cleavage site will still need to be established.

Finally, Cas9 is a powerful tool that could wreak havoc on the host should cleavage occur at genomic off-target sites. Generating Cas9 proteins with altered PAM specificities or enhanced target recognition are approaches being pursued as methods of reducing off-target effects (110, 111). Other methods include utilizing truncated gRNAs, paired Cas9 nickases, and high-fidelity Cas9 nucleases in order to alleviate this hurdle (15, 112, 113).

Concluding Remarks

Overall, significant advances have been made in employing the Cas9:gRNA machinery to

inhibit pathogenic eukaryotic viruses, though questions still remain (see Outstanding Questions). gRNA multiplexing could facilitate resistance to a panoply of different viruses in transgenic plants and animals, and may also circumvent viral escape. Targeting host factors necessary for viral infection and replication represents yet another route of viral inhibition. However, the possibility of off target effects on the host remains a major concern. Further research will need to address the feasibility of co-administration of currently available antiviral therapeutics and Cas9:gRNA, as well as potentially damaging immunological responses generated toward the foreign Cas9 protein. Despite its limitations, the use of the prokaryotic adaptive immune system as a eukaryotic antiviral defense holds considerable promise. With continued progress and engineering feats, Cas9-based technologies may represent the next generation of antiviral prophylactics and therapeutics.

Presently, however, the majority of studies have been performed in cell culture, with a small number of *in vivo* experiments. Surmounting the existing challenges will likely require intensive collaborative efforts from experts in the fields of immunology, molecular biology, bacteriology, and biomedical engineering. Undoubtedly huge strides will be made in the near future, but the use of Cas9 in humans should be weighed very carefully given the numerous potential risks.

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Figures and Figure Legends

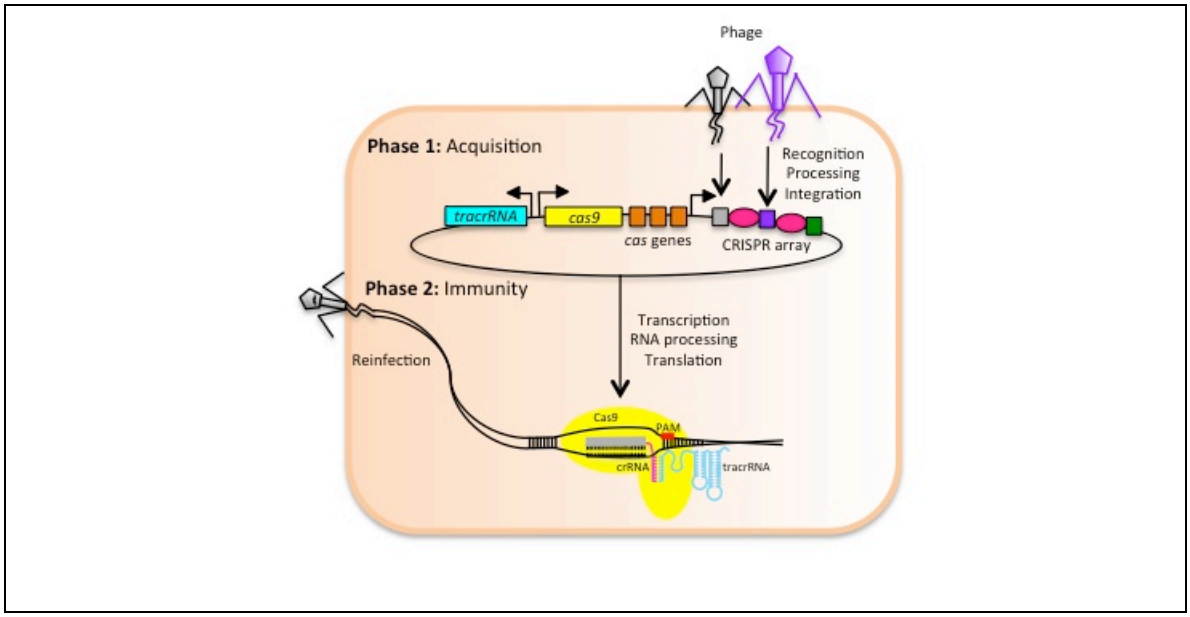

Figure 1. Cas9-mediated immunity in type II CRISPR-Cas systems. Phase 1: Acquisition. Upon encounter with foreign nucleic acid, such as that of a bacteriophage genome, a short sequence of the phage DNA is recognized, processed into small portions, and integrated into the CRISPR array by Cas proteins. Each sequence, termed a spacer, (gray, purple, and green boxes) is incorporated between identical CRISPR repeats (pink ovals). Phase 2: Immunity. In this stage, the bacterium encounters an identical foreign nucleic acid. Two small RNAs, the crRNA and the tracrRNA, guide the Cas9 effector protein to the phage genome. If there is sufficient sequence complementarity between the crRNA and the phage genome and a protospacer adjacent motif (PAM) is present, Cas9 then cleaves both strands of the target.

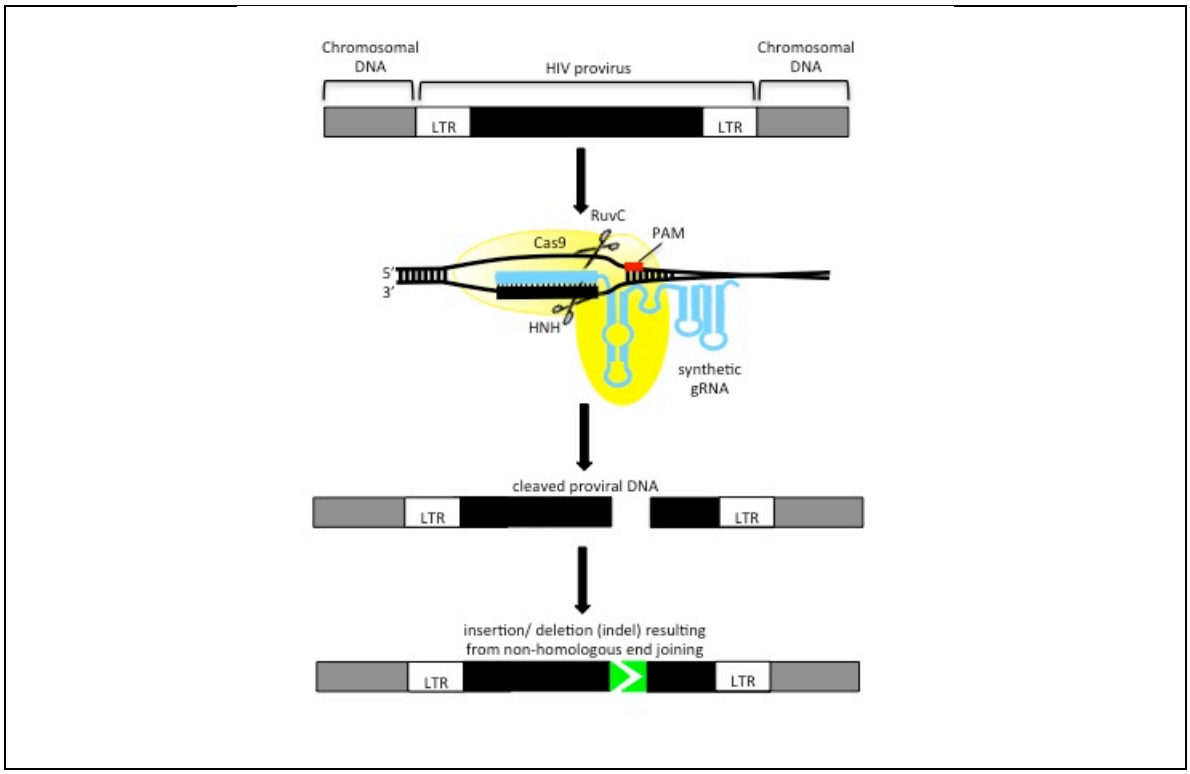

Figure 2. Cas9-mediated cleavage of the HIV provirus. A synthetic guide RNA (gRNA), shown in blue, is composed of a duplex of the crRNA and tracrRNA and may be engineered to target virtually any sequence of interest. Cas9 targeting is dependent on sequence complementarity with the target, in this case the HIV provirus, and a protospacer adjacent motif (PAM), shown in red. Following DNA recognition and binding, two Cas9 endonuclease domains, the HNH and RuvC domains, cleave the complementary and non-complementary DNA strands, respectively.

Chapter 4

Cas9-mediated targeting of viral RNA in eukaryotic cells

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Cas9-mediated targeting of viral RNA in eukaryotic cells

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Short Title : Cas9-mediated RNA virus targeting in eukaryotic cells.

Abstract:

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) - Cas (CRISPR-associated) systems are prokaryotic RNA-directed endonuclease machineries that act as an adaptive immune system against foreign genetic elements (1, 2). Utilizing small CRISPR RNAs (crRNAs) that provide specificity, Cas proteins recognize and degrade nucleic acids (3). We previously demonstrated that the Cas9 endonuclease from *Francisella novicida* (FnCas9) is capable of targeting RNA (4). Here, we show that FnCas9 can be directed by an engineered RNA-targeting guide RNA (rgRNA) to target and inhibit a human +ssRNA virus, hepatitis C virus, within eukaryotic cells. This work reveals a versatile and portable RNA targeting system that can effectively function in eukaryotic cells and be programmed as an antiviral defense.

Significance:

The Cas9 endonuclease has quickly become a revolutionary tool in genome engineering. Utilizing small guiding RNAs, Cas9 can be targeted to specific DNA sequences of interest, where it catalyzes DNA cleavage. We now demonstrate that Cas9 from the Gram-negative bacterium *Francisella novicida* (FnCas9) can be reprogrammed to target a specific RNA substrate in eukaryotic cells, the genome of the +ssRNA virus, hepatitis C virus (HCV). Further, this targeting results in inhibition of viral protein production. Overall, programmable Cas9-mediated viral RNA targeting likely represents one of myriad potential applications of FnCas9 in RNA targeting in eukaryotic cells.

Introduction

Our recent work revealed a unique form of prokaryotic gene regulation, whereby Cas9 from *Francisella novicida* (FnCas9) targets a bacterial mRNA, leading to decreased mRNA stability and ultimately gene repression (4). Given the ability of specific Cas9 proteins to be reprogrammed to target and cleave DNA in numerous biological systems (5-7), we hypothesized that FnCas9 could be retargeted to a distinct RNA in eukaryotic cells and lead to its inhibition. To eliminate any confounding interactions of FnCas9 with DNA we targeted FnCas9 to the +ssRNA virus, hepatitis C virus (HCV), which has no DNA stage in its lifecycle. HCV is an important human pathogen associated with liver fibrosis, cirrhosis, hepatocellular carcinoma, and is the leading cause of liver transplantation (8, 9).

To target the RNA of HCV, we engineered a small RNA, which we term an RNA-targeting guide RNA (rgRNA). The rgRNA is similar in structure to that naturally created by the *F. novicida* tracrRNA and scaRNA, which are utilized for endogenous mRNA targeting (4). It consists of a dsRNA region thought to be important for interaction with Cas9, and a ssRNA targeting sequence complementary to a portion of the highly conserved HCV 5' untranslated region (UTR), involved in both translation of the viral polyprotein and replication of the viral RNA (Fig. 1A, and Fig. S1). Vectors encoding either this rgRNA or FnCas9 (Fig. S2 and Supplementary Sequence File) were transfected into human hepatocellular carcinoma cells (Huh-7.5) and subsequently infected with a previously described HCVcc genotype 2a recombinant virus encoding *Renilla* luciferase (10). Expression of both the 5' UTR-targeting rgRNA and FnCas9

together reduced the levels of viral proteins, as measured by immunostaining for the E2 glycoprotein (Figs. 1B, C) or quantification of luciferase production (Fig. 1D). Conversely, expression of either the rgRNA or FnCas9 alone had no effect (Figs. 1B-D), nor did expression of a non-specific rgRNA and FnCas9 (Figs. 1B-D), demonstrating the specificity of this system. Additionally, an rgRNA complementary to a portion of the 3' UTR, necessary for replication of viral RNA, decreased viral protein levels similarly (Figs. 1A-D), demonstrating that the effect was not specific to a single site in the HCV genome. Therefore, FnCas9 can be programmed by a single rgRNA to target the RNA of a human virus in eukaryotic cells, leading to viral inhibition.

In order to determine if FnCas9 was directly associated with HCV RNA, we performed co-immunoprecipitation experiments. We transfected cells with an HA epitope-tagged version of the protein (which maintained its ability to inhibit HCV (Figs. S3A, B)) as well as the 5' UTR-targeting rgRNA and subsequently infected the cells with HCV. FnCas9 was immunoprecipitated from cell lysates, associated RNA was purified, and quantitative real-time PCR was performed for the rgRNA and HCV RNA. The rgRNA was present in association with FnCas9 (Fig. 2A), as was HCV RNA (Fig. 2B), suggesting that HCV RNA was directly targeted by the FnCas9:rgRNA complex. A non-specific rgRNA did not facilitate this interaction (Fig. 2B), but it did associate with FnCas9 (Fig. 2C). Thus, FnCas9 can be targeted to directly associate with viral RNA within eukaryotic cells.

We next sought to determine how FnCas9 was inhibiting HCV. We first found that

addition of a nuclear localization signal (NLS) to FnCas9 abrogated its repression of viral protein production (Fig. 3A and Fig. S4), in line with its targeting of cytosolic HCV RNA. Since Cas9 proteins including FnCas9 are known to cleave DNA through two conserved structural domains, the RuvC and HNH endonuclease domains (7), it is possible that these regions are important for inhibiting HCV. We therefore generated alanine point mutations in the conserved RuvC and HNH active sites of FnCas9 (D11A and H969A, respectively). Despite mutation in one or both of these domains, FnCas9 maintained its ability to inhibit HCV (Fig. 3B). However, mutation in the RNA-binding arginine-rich motif (ARM; R59A), which is necessary for interaction with nucleic acids both within *F. novicida* and in orthologous Cas9 (4, 11, 12), resulted in diminished HCV inhibition (Fig. 3B). It is therefore likely that FnCas9 inhibits HCV not through target cleavage, but instead by associating with the target RNA and preventing the function of the translational and/or replication machineries.

We subsequently tested whether FnCas9 could inhibit translation of HCV. We performed an *in vitro* translation reaction using immunoprecipitated FnCas9 from transfected Huh-7.5 cells, purified RNA from cells transfected with either the 5' UTR-targeting rgRNA or the non-targeting RNA, as well as HCV genomic RNA. Addition of both FnCas9 and the 5' UTR-targeting rgRNA resulted in decreased translation of the HCV genome, as measured by viral luciferase production (Fig. 3C). Furthermore, a catalytically inactive FnCas9 (D11A/H969A) maintained its ability to inhibit translation of HCV, while the ARM (R59A) mutant displayed less translational inhibition (Fig. 3C). Taken together, these data suggest that FnCas9 is capable of directly inhibiting translation of target RNA.

In order to determine whether FnCas9 also inhibits replication of HCV RNA, we targeted the negative-sense strand (generated during replication and which is untranslated) of the 5' UTR. Such targeting resulted in inhibition of HCV (Fig. S5), suggesting that FnCas9 is capable of inhibiting viral replication as well. Overall, these data strongly support a model whereby FnCas9 binds HCV RNA, does not cleave the target, but inhibits the function of both translational and replication machineries.

We subsequently tested the sequence requirements for RNA targeting. Cas9 proteins require a short sequence adjacent to the targeted region, called a proto-spacer adjacent motif (PAM), to cleave DNA (13). We sought to determine if a similar conserved adjacent region was necessary for HCV inhibition. A 5' UTR targeting rgRNA shifted to lack similar adjacent sequences still inhibited HCV (Fig. 4A). In fact, no common features are observed in the sequences adjacent to the targets of rgRNAs utilized in this study (Fig. S6A). In contrast, DNA targeting by FnCas9 endogenously within *F. novicida* absolutely requires a PAM (Figs. S6B, C). Together, these data demonstrate that FnCas9-mediated inhibition of HCV is PAM-independent.

DNA targeting RNAs utilized by Cas9 require a 3' seed sequence within the targeting region, and even a single mismatch in this region can abrogate function (14, 15). To test if there was a similar requirement for RNA targeting, we generated a panel of rgRNA mutants containing mismatches within the targeting sequence. We found that mutation of up to 6 bases within the 3' targeting region of the rgRNA were tolerated, without loss of HCV inhibition (Fig. 4B). Longer regions of mismatched bases at the 3' end resulted in a

loss of activity (Fig. 4B). Specific mismatches in the 5' region of rgRNA were also non-functional (Fig. 4C). A single mismatch in either the first or second base was sufficient to abrogate viral inhibition (Fig. 4C). However, a mismatch in the third base alone did not lead to a loss of activity (Fig. 4C). These data strongly suggest that unlike DNA targeting by other Cas9 proteins, FnCas9 inhibition of HCV requires a critical sequence in the 5' end rather than the 3' end of the targeting region of the guiding RNA (14, 15).

The previous experiments demonstrated that FnCas9 could target an RNA and facilitate resistance to HCV infection. We next tested whether FnCas9 could be introduced following an established viral infection and inhibit the virus (Fig. 5A). Transfection of HCV-infected Huh-7.5 cells with FnCas9 and 5' or 3' UTR targeting rgRNAs resulted in decreased viral protein production (Fig. 5B), while expression of either FnCas9 or rgRNAs alone was not sufficient to inhibit HCV infection (Fig. 5B). Thus, the FnCas9:rgRNA machinery can combat both new and established viral infections.

These data demonstrate the successful adaptation of the CRISPR-Cas prokaryotic immune system as an intracellular eukaryotic antiviral defense. While other CRISPR-Cas systems can target RNA in archaea (16-18) and bacteria (19), and recently SpCas9 has been shown to cleave RNAs *in vitro* (20), this work represents the reprogramming of a Cas protein (FnCas9) to target RNA within a eukaryotic cell. Intriguingly, we find that orthologous Cas9 proteins from diverse Type II CRISPR-Cas families, including *Streptococcus pyogenes, S. thermophilus,* and *Neisseria meningitidis*, are also capable of inhibiting HCV during cellular infection (Figs. S7,8). This suggests a broader capability

of diverse Cas9 proteins to target and associate with RNA targets. Our results further demonstrate that FnCas9 inhibition of HCV is PAM-independent, unlike the *in vitro* RNA targeting ability of *Streptococcus pyogenes* Cas9 which requires exogenous PAM encoding oligomers (20). Thus, FnCas9-mediated RNA inhibition may be more flexible in its targeting. Importantly, this targeting is highly specific. Compared to cells with a vector control, significant changes in host cell gene expression were not observed in cells expressing Cas9 in conjunction with either the HCV targeting or non-specific rgRNA, demonstrating the specificity of these complexes (Fig. S9 and Supplementary Table 1). Furthermore, the presence of FnCas9 in the cytosol is necessary to inhibit HCV, in contrast to DNA targeting by Cas9 that relies on nuclear localization (21). Cytosolic RNA targeting would potentially limit FnCas9 off-target effects on host DNA. Since the lifecycles of viruses with both RNA and DNA genomes require an RNA stage (generated during transcription, replication, or both), and FnCas9 can target both negative- and positive-sense strands of RNA and inhibit by blocking both viral translation and replication machineries (Fig. S10), it is likely that the FnCas9:rgRNA machinery can be utilized to target diverse viruses, including both +ssRNA viruses (such as flavivirus, poliovirus, and rhinovirus) and –ssRNA virus (such as filovirus, paramyxovirus and orthomyxovirus). Furthermore, some eukaryotic viruses have mechanisms to circumvent eukaryotic RNA-targeting antiviral defenses, such as classical RNAi systems (22-24); however, these viruses have not evolved in the presence of Cas9, so it is unlikely that they have Cas9 evasion strategies. Thus, the FnCas9:rgRNA machinery could facilitate the targeting of viruses as soon as their genome sequences are available, without knowledge of the virus lifecycle or host receptors.

Given the vast success of Cas9 as a mediator of genome engineering in a multitude of species (5-7, 14, 25-31) our data suggest that FnCas9 could be used in a broad range of systems, representing a new paradigm in Cas9-mediated genetic engineering. This work demonstrates a portable, inter-domain machinery capable of viral inhibition, likely just one of myriad potential biotechnological and medical applications of Cas9-mediated RNA targeting.

Materials and Methods

Plasmid Construction. FnCas9 was amplified using the primers found in Supplementary Table 2 and cloned into the *XbaI* and *PmeI* sites of pcDNA3.3 (Invitrogen, Grand Island, NY). FnCas9 point mutants were amplified from strains published previously(4). StCas9, SpCas9, and NmCas9 were amplified from Addgene vectors #48669, 41815, 47872, respectively. To create rgRNA vectors, *F. novicida* CRISPR repeat sequences and the indicated targeting sequence were cloned into the gRNA-encoding plasmid from the Church Lab (Addgene #41824) (5), within the pCR-Blunt-II (Invitrogen) backbone, using overlapping PCR and the primers indicated in Supplementary Table S1.

Cell lines and culture conditions. Huh-7.5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 100 ug/ mL of penicillin/ streptomycin (Cellgro, Herndon, VA) at 37 degrees in 5% CO_2.

Plasmid Transfection. Huh-7.5 cells were seeded to 80% confluence in 24 well plates in DMEM without antibiotics the day prior to transfection. Eight hundred ng of total plasmid DNA were transfected using Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Six hours following transfection, cell supernatants were aspirated and replaced with DMEM supplemented with FBS and antibiotics.

Virus transcription and transfection. Rluc virus utilized for luciferase assays encodes

the *Renilla* luciferase gene between the p7 and NS2 coding sequences of the previously described J6/ JFH genotype 2a infectious clone Cp7(10). Rluc and Cp7 viral RNA were prepared as previously described(10). Purified plasmid encoding the cDNA copy of the full-length viral genome was linearized and 5' overhangs were digested with Mung Bean Nuclease (New England Biolabs, Ipswich, MA). Linearized DNA was purified with phenol-chloroform extraction and ethanol precipitation. Transcription of the linearized DNA template was performed using a MEGAscript^{TM} T7 kit and the linear template was treated with DNAse I (Ambion, Austin, TX). RNA was purified with phenol-chloroform extraction and isopropanol precipitation, and concentration and purity were determined using a Nanodrop spectrophotometer and standard agarose gel electrophoresis.

Huh-7.5 cells were grown to 70% confluence, trypsinized, and washed twice in PBS. Ten μg purified RNA were mixed with .4 mL Huh-7.5 cells suspended at a concentration of 2 x 10^7 cells/ mL. Samples were electroporated in BTX 2 mm gap cuvettes (Harvard Apparatus, Inc., Holliston, MA) using an ECM 830 apparatus (BTX Genetronics, San Diego, CA) with five pulses of 99 usec at 820 V over 1.1 sec. Cells were suspended in 25 mL complete DMEM and virus was harvested and stored at –80 degrees C three days following transfection.

Immunoprecipitation. Anti-HA IP was performed according to the manufacturer's instructions from lysates of Huh-7.5 cells infected with HCVcc and transfected with FnCas9 and rgRNA expression vectors as indicated (Sigma-Aldrich, St. Louis, MO). Total RNA was extracted from the precipitated fraction using an RNeasy Mini Kit

(Qiagen, Valencia, CA) according to the manufacturer's instructions.

In vitro translation assay. Immunoprecipitated FnCas9 was incubated with 1μg HCV RNA, and 500ng RNA from Huh-7.5 cells transfected with either the 5' UTR – targeting rgRNA or the Control rgRNA, in conjunction with the rabbit reticulocyte lysate *in vitro* translation kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Translated viral luciferase was measured as described below.

Quantitative RT-PCR. Quantitative reverse transcription reactions were performed using Taqman One Step RT-PCR Master Mix Reagent (Applied Biosystems, Hammonton, NJ) and primers specific for the 5' untranslated region of HCV (Supplementary Table 1). Sample analysis was performed on an Applied Biosystems 7500 apparatus. rgRNAs were quantified via Syber Green One Step RT-PCR, with primers found in Supplementary Table 1.

Luciferase Assays. Huh-7.5 cells in a 96 well plate format were lysed and analyzed for relative light activity using the Renilla Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 50 ul of Renilla substrate in assay buffer was added to 50 ul of cell lysate and relative light units were quantitated on a Clarity 4.0 microplate luminometer (Biotek, Winooski, VT).

Immunohistochemistry. Six thousand Huh-7.5 cells per well were plated in collagen coated 96 well plates. The following day, cells were infected with the J6/JFH genotype 2a

virus Cp7. Following three days of incubation, cells were fixed with methanol, washed twice with PBS, and permeabilized with PBS containing .1% Tween-20 (PBS-T). Fixed cells were incubated in blocking buffer containing 1% bovine serum albumin and .2% skim milk for thirty minutes. Endogenous peroxidase was blocked using 3% H_2O_2, then cells were washed twice with PBS and once with PBS-T. Cells were then incubated with the 2C1 monoclonal antibody to HCV E2 glycoprotein for one hour at room temperature. After two washes with PBS and one with PBS-T, cells were incubated with ImmPress anti-mouse HRP (Vector Laboratories, Burlingame, CA), washed, and developed using DAB substrate (Vector Laboratories).

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Figure 1

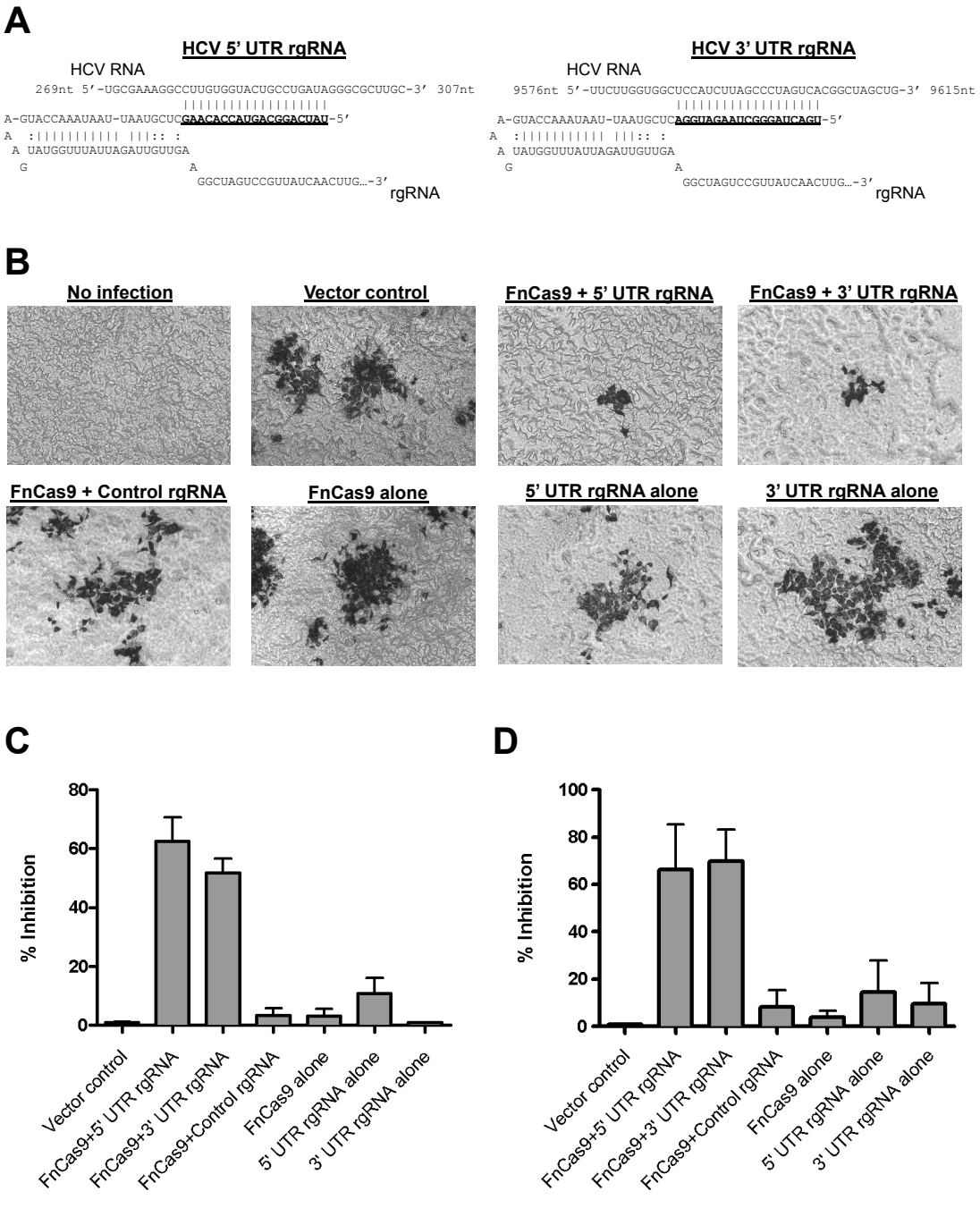

Figure 1. FnCas9 can be reprogrammed to inhibit viral protein production in eukaryotic cells. (A) rgRNA schematic with targeting sequences (gray highlight) against the 5′ or 3′ UTR of HCV genomic RNA. (B) Huh-7.5 cells were transfected with the indicated combinations of FnCas9 and rgRNA and infected 48 h later with HCV encoding Renilla luciferase. At 72 h, cells were fixed and stained with anti-E2 antibody and imaged. (C) E2-positive foci from B were quantified and plotted as percent inhibition compared with the vector control. (D) Quantification of viral luciferase production displayed as percent inhibition compared with the vector control (n = 3; bars represent the SEM; data are representative of at least six experiments).

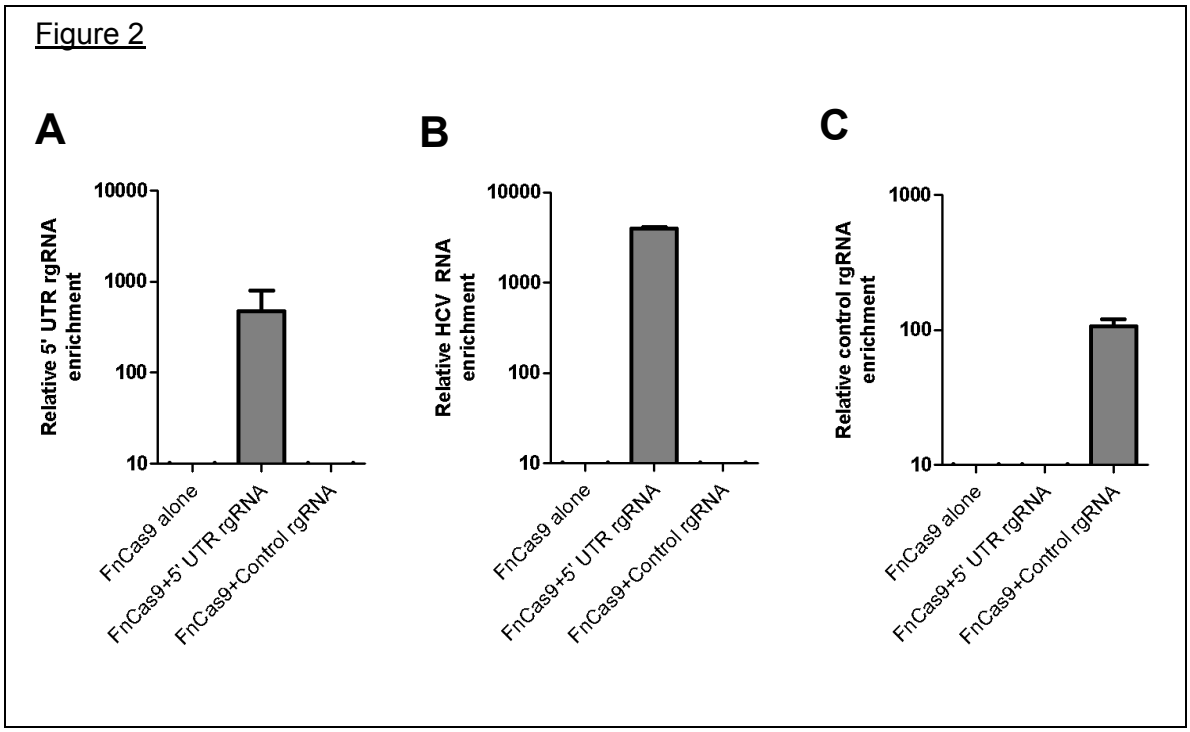

Figure 2. FnCas9 targets and associates with HCV RNA. Huh-7.5 cells producing an HA epitope-tagged FnCas9 alone, or with either the 5′ UTR targeting rgRNA or the control rgRNA, were infected with HCV. At 72 h postinfection, lysates were immunoprecipitated with anti-HA. Coprecipitating RNA was purified and analyzed by quantitative real-time PCR to detect the relative enrichment of the (A) 5′ UTR rgRNA, (B) HCV RNA, or (C) control rgRNA, normalizing to gapdh mRNA levels (n = 4; bars represent the SEM; data are representative of three experiments).

Figure 3

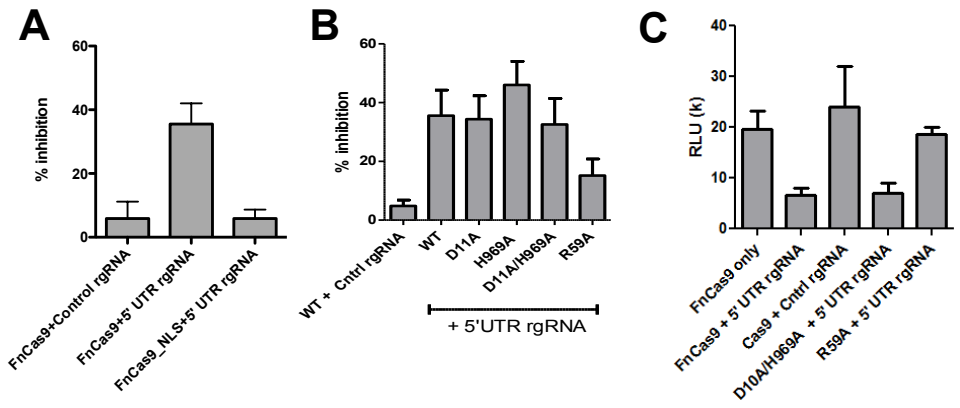

Figure 3. Molecular requirements for FnCas9-mediated HCV inhibition. (A) Huh-7.5 cells were transfected with FnCas9 ± NLS, the 5′ UTR-targeting rgRNA, and HCV. At 72 h, viral luciferase was quantified and the percent inhibition compared with the nontargeting rgRNA is displayed (n = 8; data compiled from three in- dependent experiments). (B) Experiments were performed as above, using alanine point mutants in the RuvC domain (D11A), HNH domain (H969A), the double mutant (D11A/H969A), or the ARM (R59A) (n = 8; data compiled from three independent experiments). (C) Rabbit reticulocyte lysate in vitro translation assays of HCV luciferase were performed using the indicated Cas9 and RNAs and viral luciferase measured (n = 4; data are rep resentative of four experiments).

Figure 4

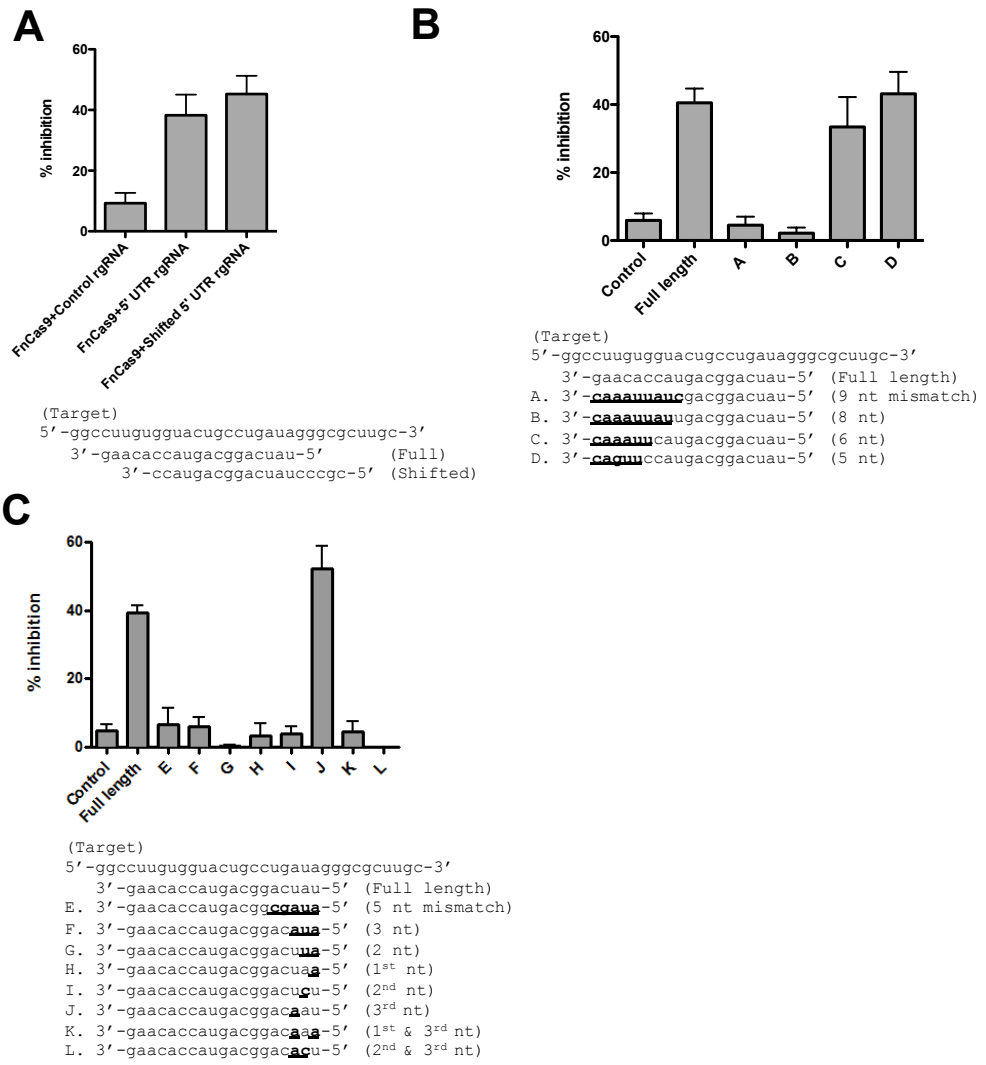

Figure 4. RNA sequence requirements for FnCas9 inhibition of HCV. (A) Huh-7.5 cells were transfected with FnCas9 using the rgRNA mutants in the indicated shifted alignments. At 72 h, viral luciferase was quantified and the percent inhibition compared with the nontargeting rgRNA is displayed (n = 12; data are compiled from three independent experiments). (B) Experiments were performed as above, using the mutants in the 3′ region indicated in the alignment below the figure (n = 12; bars represent the SEM; data are compiled from three independent experiments). (C) Experiments were performed as above, using the mutants in the 5′ region indicated in the alignment below the figure (n = 12; bars represent the SEM; data are compiled from three independent experiments).

Figure 5

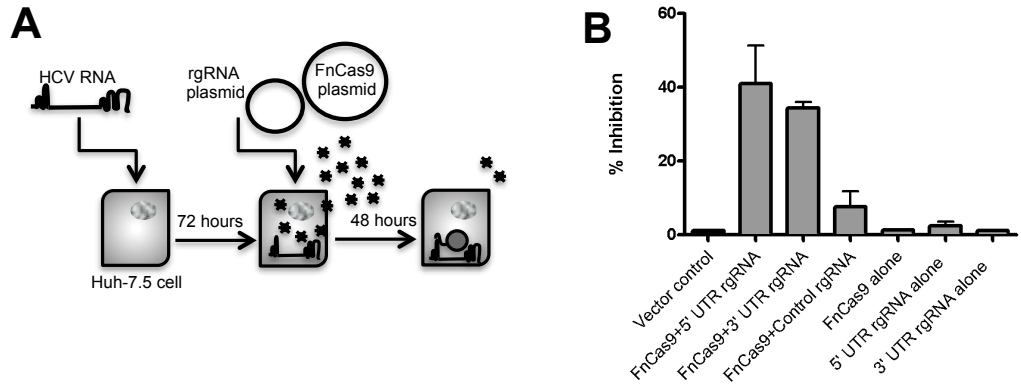

Figure 5. FnCas9 can inhibit an established viral infection. (A) Experimental outline. HCV-transfected Huh-7.5 cells were transfected with FnCas9 and the in- dicated targeting RNAs, after 72 h postinfection. (B) Quantification of viral luciferase production, displayed as percent inhibition compared with the vector control (n = 3; bars represent the SEM; data are representative of at least 12 experiments).

Supplementary Figure 1

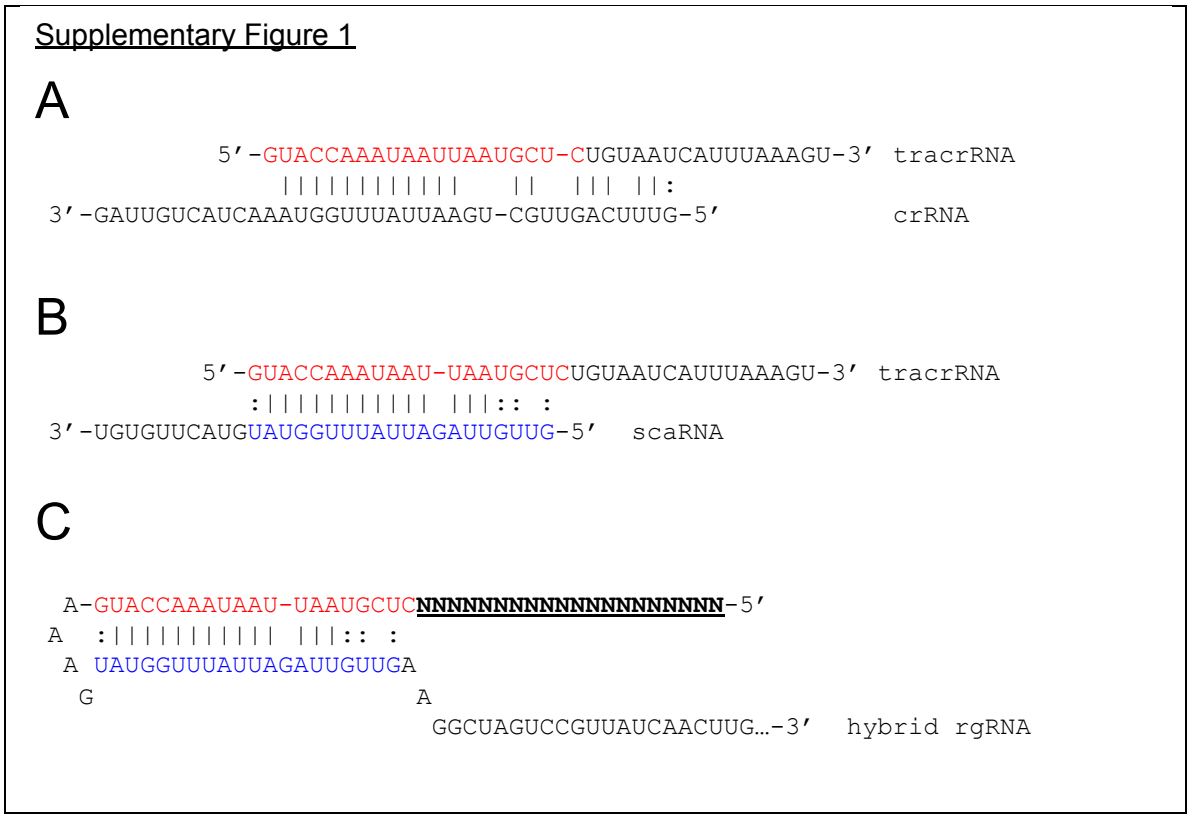

Fig. S1. Design of the rgRNA. (A and B) Schematic of the interaction between the (A) F. novicida U112 CRISPR repeat sequence and the tracrRNA (red) or (B)the scaRNA (blue) and the tracrRNA. (C) Design of the rgRNA based on the interaction between the tracrRNA (red) and the scaRNA (blue) with the RNA targeting region (black).

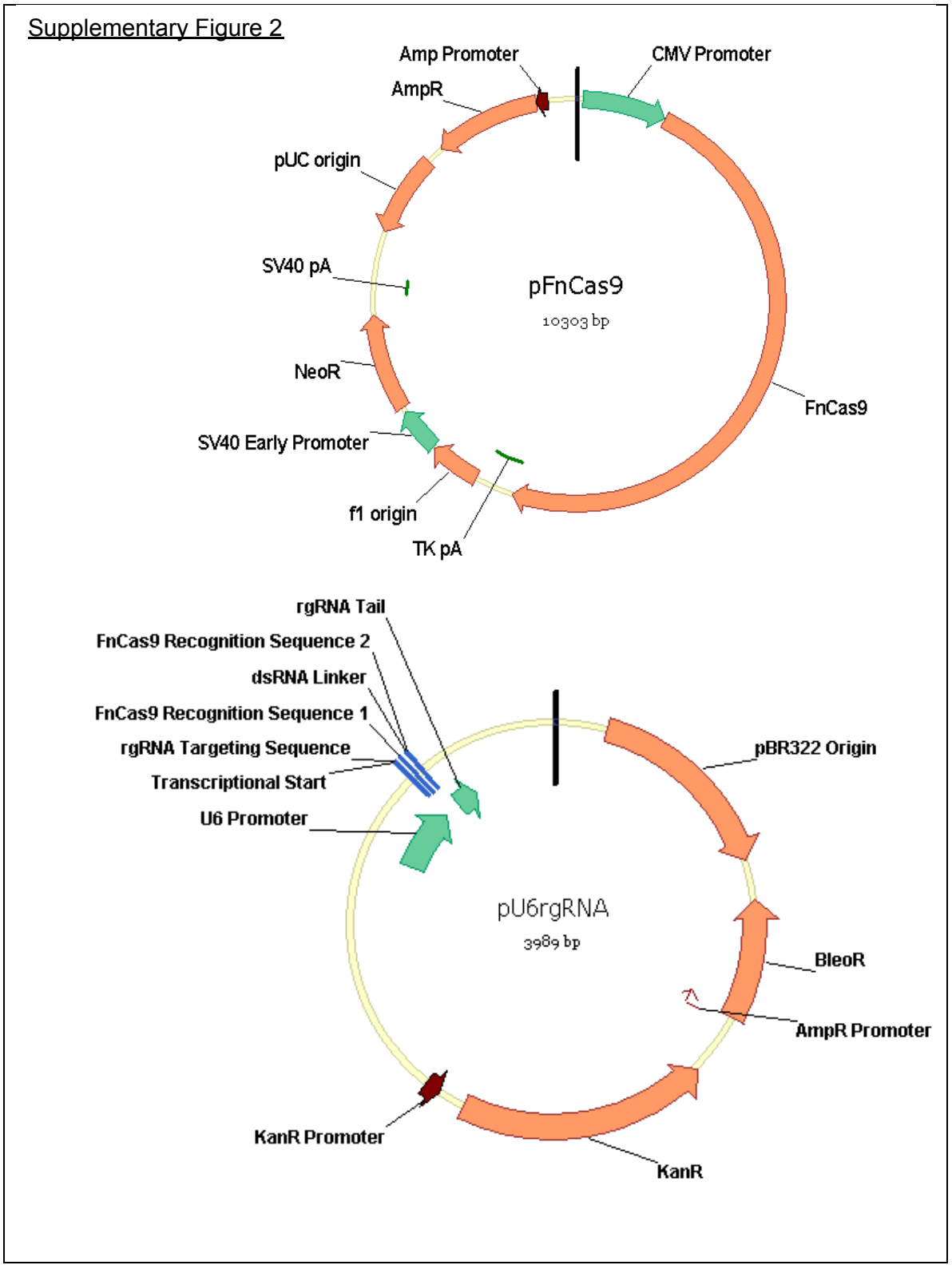

Fig. S2. Plasmid maps of the FnCas9 and rgRNA encoding vectors.

Supplementary Figure 3

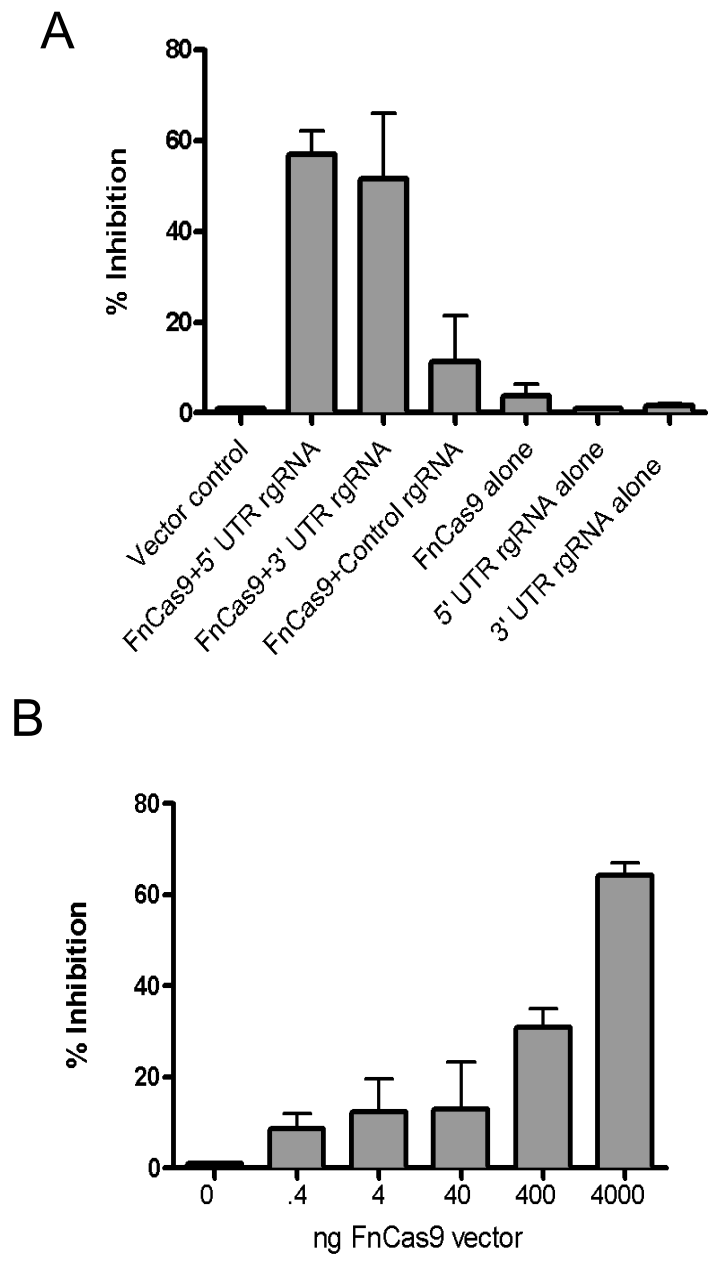

Fig. S3. HA epitope-tagged FnCas9 retains the ability to inhibit HCV luciferase production in a dose-dependent manner. (**A**) Huh-7.5 cells were transfected with the indicated combinations of HA epitope-tagged FnCas9 and rgRNA as well as HCV RNA. At 72 h postinfection, viral luciferase production was measured and displayed as percent inhibition compared with vector controls (**n** = 3; bars represent the SEM; data are representative of at least five experiments). (**B**) Experiments were performed as above, varying the concentration of FnCas9 (**n** = 3; bars represent the SEM; data are representative of at least two experiments).

Supplementary Figure 4

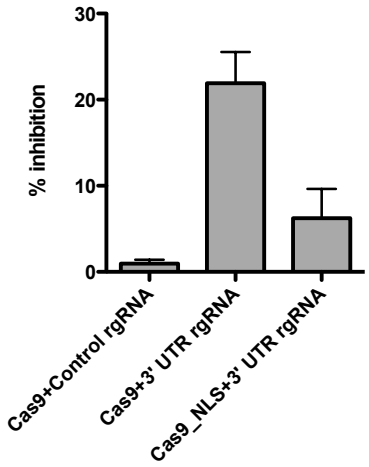

Fig. S4. NLS abrogates FnCas9 inhibition of HCV luciferase production. Huh-7.5 cells were transfected with the indicated combinations of FnCas9 (with or without an NLS) and rgRNA targeting the 3′ UTR as well as HCV RNA encoding Renilla luciferase. At 72 h postinfection, viral luciferase production was measured and displayed as percent inhibition compared with vector controls (n = 3; bars represent the SEM; data are representative of at least five experiments).

Supplementary Figure 5

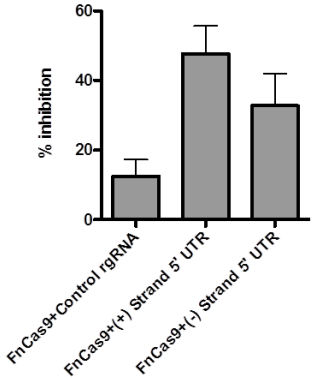

Fig. S5. FnCas9 can inhibit HCV through positive or negative sense strand targeting. Huh-7.5 cells were transfected with the indicated combinations of FnCas9 and an rgRNA targeting either the positive or negative sense strand of the 5′ UTR as well as HCV RNA encoding Renilla luciferase. Viral luciferase production was measured and displayed as percent inhibition compared with FnCas9 and control targeting rgRNA (n = 3; bars represent the SEM; data are representative of at least two experiments).

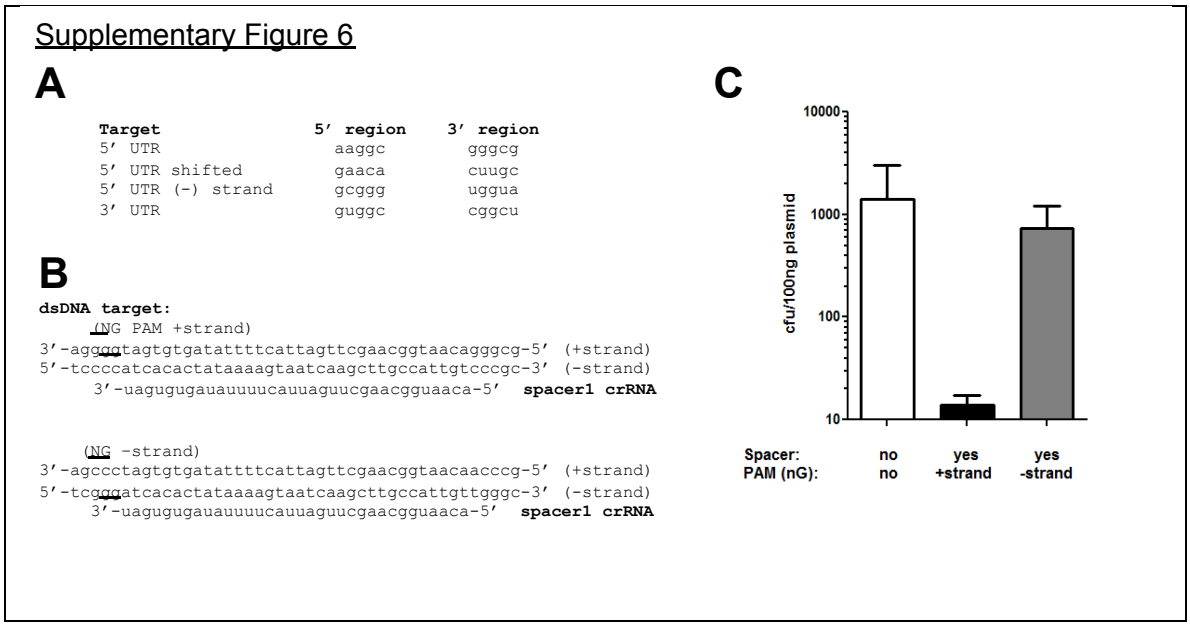

Fig. S6. FnCas9 targeting of DNA is PAM-dependent, whereas RNA targeting is PAM-independent. (**A**) Alignment of the adjacent sequences to both the 5′ and 3′ end of the regions targeted by the rgRNAs in this manuscript. (**B**) Schematic outline of the natural crRNA 1 and proto-spacer interaction in the context of the PAM sequence (–NG) (1). (**C**) Transformation efficiency (plotted as cfu per 100 ng of plasmid) of wild-type **F. novicida** U112 with either empty vector, a vector containing spacer 1 of the **F. novicida** crRNA array, and the predicted PAM on the nontargeted strand (which would be recognized by Cas9) or a predicted PAM on the targeted strand (which is not recognized by Cas9) (**n** = 3; bars represent the SEM; data are representative of at least two experiments).

Supplementary Figure 7

Fig. S7. rgRNAs for orthologous Cas9 proteins. Schematic of the rgRNAs generated to target the 5' UTR of HCV RNA by orthologous Cas9 proteins from (A) *S.thermophilus*, (B) *S.pyogenes*, and (C) *N.meningitidis*.

Supplementary Figure 8

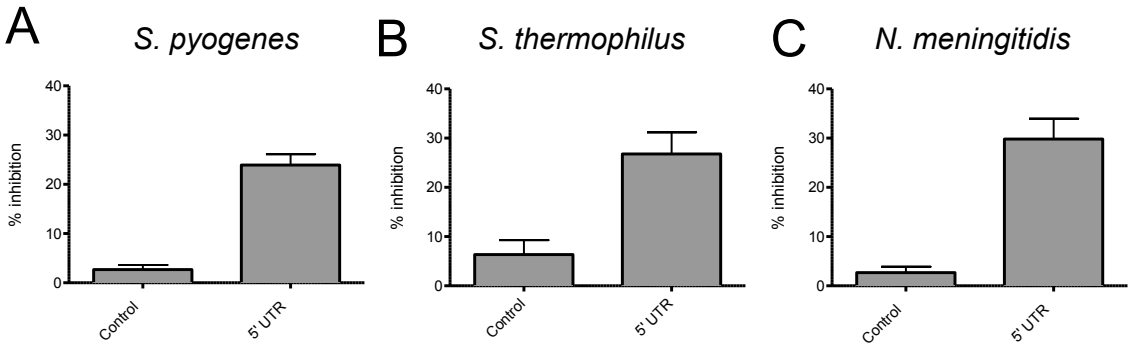

Fig. S8. Orthologous Cas9 proteins are capable of HCV inhibition. Huh-7.5 cells were transfected with Cas9 proteins from (**A**) **S. pyogenes**, (**B**) **S. thermophilus**, or (**C**) **N. meningitidis**, as well as a cognate control or 5′ UTR-targeting rgRNA (Fig. S7), and infected with HCV. At 72 h, viral luciferase was quantified. The percent inhibition compared with the control rgRNA is displayed (**n** = 8; data were compiled from three independent experiments).

Supplementary Figure 9

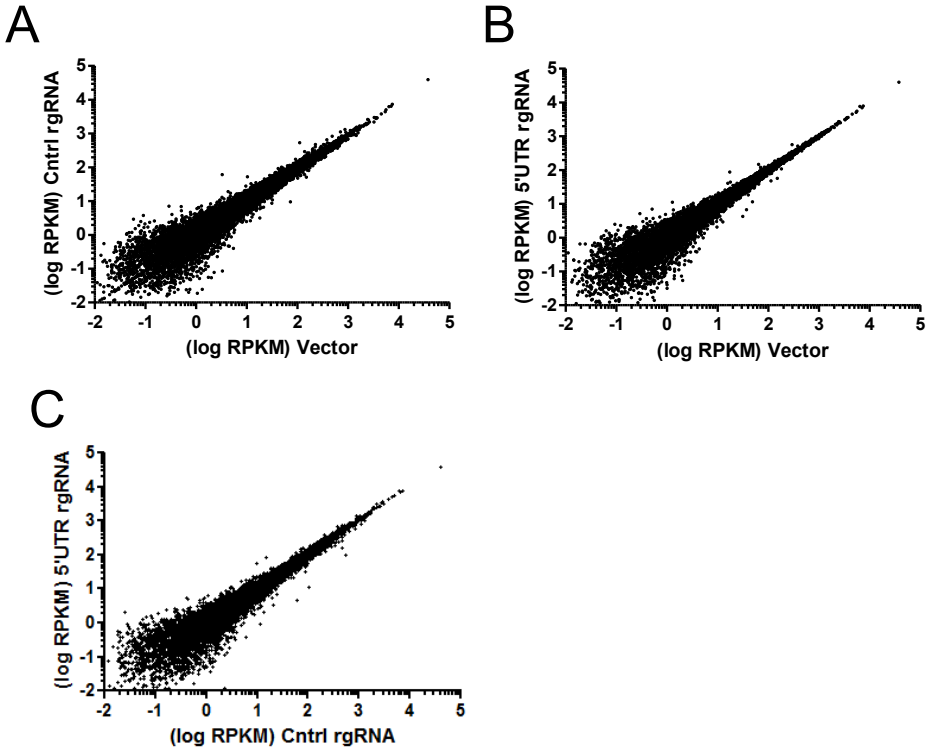

Fig. S9. Global gene expression of Huh-7.5 cells transfected with the FnCas9 machinery. Huh-7.5 cells were transfected with FnCas9 and either the nontargeting control rgRNA, 5′ UTR-targeting rgRNA, or an empty vector alone. RNA was extracted and sequenced to analyze gene expression. Data are plotted to compare the reads per kilobase per million mapped reads for each individual gene. (**A**) FnCas9 + control rgRNA compared with vector alone. (**B**) FnCas9 + 5′ UTR-targeting rgRNA compared with vector alone. (**C**) FnCas9 + control rgRNA compared with FnCas9 + 5′ UTR-targeting rgRNA.

Chapter 5

Conclusions and Future Directions

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HCV adaptations to altered $CD8^+$ T-cell immunity during pregnancy

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Chapter 5

Conclusions and Future Directions

The studies presented herein examine two very different immune systems, one maternal, and the other from the bacterium *Franciscella novicida*. The intersection of these drastically different immune systems is their ability to restrict HCV infection. In the second chapter we explored the possibility that maternal innate immune activation extends beyond gestation until adaptive immune function is fully restored. We found that prolonged ISG expression in circulating PBMCs following delivery is dependent upon maternal genotype at IFNL3 SNP rs12979860. Further, this antiviral gene signature was primarily expressed in $CD14^+$ cells and not all circulating cell types and may have important implications for women persistently infected with HCV. In chapter four we demonstrated the use of Cas9 to restrict HCV infection in cell culture. The remainder of this work will address outstanding questions, future directions, and implications for these studies.

Potential sources of $CD14^+$ cell stimulation. One caveat to the work of chapter two is that it did not address the source of $CD14^+$ cell stimulation. $CD14^+$ cells may be stimulated within a localized environment in which other circulating cell types are not privy, or have a more robust response to interferon stimulation than other cell subsets. Hepatocytes are potent producers of type I and III IFNs (1), and both HCV and HBV are susceptible to IFN-mediated signaling. Further, HBV viremia tends to decrease following delivery (2, 3). Though it has not been demonstrated that IFN is produced by hepatocytes during pregnancy and/or following delivery, this may be a possibility worth examining

since the liver undergoes significant physiological changes during gestation. For example, mouse studies have demonstrated that during pregnancy, each hepatocyte nearly doubles in size to accommodate the increase in circulating hormones such as estrogen and progesterone (4, 5). As the liver is the primary site of steroid hormone metabolism, hormonal changes could potentially impact cellular hepatocyte function and cytokine secretion. However, the study of this correlation poses a significant technical challenge, as liver biopsies would have to be obtained from healthy women both during pregnancy and following delivery.

Alternatively, $CD14^+$ cells may be exposed to IFN during pregnancy and the maintenance of the ISG signature is dependent upon IFNL3 genotype. Placental recruitment of monocytes and macrophages is required for the vascular remodeling that occurs during pregnancy (6). Humans undergo haemochorial transplantation, in which placental syncytiotrophoblasts invade maternal blood vessels, which allows maternal blood to directly contact semi-allogeneic syncytiotrophoblasts (7). One hypothesis is that $CD14^+$ cells circulating within maternal blood are exposed to IFN produced by these placental syncytiotrophoblasts. Physical interactions between syncytiotrophoblasts and monocytes involving ICAM-1, LFA-1, and fractalkine have been described, so it is plausible that $CD14^+$ interferon stimulation *in vivo* requires cell-to-cell contact (8-10). Continuous exposure to interferon during the gestational period could account for the enriched $CD14^+$ ISGs following delivery. Regardless of the location of $CD14^+$ stimulation, we speculate that postpartum $CD14^+$ cells from CC donors display an advantage over their TT and CT counterparts in their ability to dampen interferon signaling.

We also hypothesize that the prolonged IFN signature identified following delivery in uninfected mothers could have potential implications for mothers persistently infected with HCV. Given the parallel between postpartum ISG expression in mothers with a CC genotype and intrahepatic ISG expression in individuals infected with HCV, we predict that a CC genotype may correlate with the observed postpartum decrease in viral load observed in certain women chronically infected with HCV. In contrast, we predict the mothers with TT or CT genotypes are more likely to retain steady viral loads throughout pregnancy and following delivery. In the next section we will discuss the viral load dynamics during pregnancy and following delivery of other persistent viruses such as HBV and HIV. We focus on the role of IFN signaling in control of these viruses, as well as certain acute virus infections. Further, we will examine other potential factors that may contribute to the viral load differences among HCV, HBV, and HIV.

Relevance to other viral infections.

Persistent infections. Pregnancy has been associated with increased disease severity and/or shedding of a variety of RNA and DNA viruses, but for many viral pathogens there is no unique phenotype in pregnancy (11). HBV and HIV, two other vertically transmissible viruses with persistent viremia, seem to offer instructive illustrations of differing natural histories during pregnancy. HBV viral loads in pregnant women appear to parallel those of women infected with HCV (2, 3). An increase in HBV antigen titers, as well as DNA replication, is observed during pregnancy, followed by a precipitous decrease in viral load following delivery. In addition, alterations in serum alanine

aminotransferase (ALT) levels indicate liver disease activity is increased postpartum in women chronically infected with HBV (3). Like HCV, HBV viral loads in pregnancy are clinically relevant as they correlate with risk of vertical transmission.

Natural history studies of HIV in pregnancy performed prior to the era of standard anti-retroviral therapy did not identify significant changes in HIV viral load during or after pregnancy (12), suggesting that HIV may not be affected by the immunoregulatory changes of pregnancy. Multiple factors could account for the discrepancies in viral load dynamics of HIV versus HCV and HBV. HCV and HBV are both hepatotropic viruses, suggesting liver specific immunity might be particularly susceptible to regulation in pregnancy and following delivery. Another similarity is that HCV and HBV are both sensitive to IFN signaling, whereas the sensitivity of HIV to IFN and its role in pathogenesis remains controversial. In fact, during the chronic phase of infection, type I IFN may further contribute to $CD4^+$ cell depletion and immune cell exhaustion (13).

Alternatively, it could be hypothesized that $CD8^+$ T cells specific for HCV and HBV may be more sensitive to the expansion and contraction of T_{regs} during and after pregnancy than those targeting HIV. Further, uncontrolled HIV infection leads to the depletion of $CD4^+$ immune cells, resulting in progressive immune system failure and susceptibility to opportunistic infections. Even if HIV-specific $CD8^+$ T cells are activated postpartum, insufficient $CD4^+$ T cell help may hinder $CD8^+$ T cell effector function. Certain $CD4^+$ cells also express the surface marker CD14. Depletion of $CD4^+$ $CD14^+$ antigen-presenting cells could also potentially impact antiviral immune responses. Nonetheless,

the parallel dynamics of viruses as dissimilar as HCV, a +ssRNA virus, and HBV, a dsDNA virus, highlight the potentially broad and potent effects of pregnancy and parturition on cellular immunity, with conceivable implication for vaccines, cancers, and acute viral infections.

Acute infections. It is also possible that IFNL3 genotype may have implications for viruses that do not establish persistent infection, such as the Zika virus. More than 80% of infections with Zika virus are asymptomatic, though in rare cases symptoms include fever, headache, and rash (14). Viremia has not been reported to last more than seven days (15), but new studies suggest that there is a connection between maternal infection during pregnancy and fetal microcephaly (14, 16). Given that HCV and Zika virus are both positive sense single stranded RNA viruses and members of the family *Flaviviridae*, it is possible that Zika may be particularly susceptible to IFNL3 genotype-dependent IFN signaling. Whether IFNL3 genotype plays a role in maternal susceptibility to the virus or likelihood of vertical transmission remains to be elucidated. However, given the rapid transmission and recent concern over the virus, continued studies are warranted.

Future directions

Overall, our studies have raised an abundance of questions that remain to be addressed. First, what is the mechanism causing the prolonged IFN signaling in the $CD14^+$ cells from women with CT or TT genotypes? It has yet to be determined if the prolonged ISG expression is due to an IFNL3 genotype-dependent signaling process within the $CD14^+$ cells themselves. Alternatively, it is possible that an external inflammatory process

continues unabated in the months after delivery that particularly influences ISG expression in women with an IFNL3 TT or CT genotype. If a continuous external inflammatory process is responsible, then an additional question is raised. Is this continuous exogenous stimulus present in women with CT, TT, and CC genotypes, or only in women with CT and TT genotypes?

One of the greatest obstacles we have faced with this project is finding new mothers willing to donate blood following childbirth. For this reason we are currently examining the ISG signatures of NPC $CD14^+$ cells both during and following the removal of IFN-α, IFN-λ, and other proinflammatory cytokines. We hope to generate a surrogate system that recapitulates postpartum genotype-dependent ISG signatures and will supplement our studies on postpartum blood samples. Should this system utilizing the stimulation of NPC $CD14^+$ cells be successful, we will then attempt to generate immortalized cell lines (THP-1, etc.) possessing the IFNL3 rs12979860 SNPs using the CRISPR-Cas9 platform. We foresee the use of these cell lines to perform chromatin immunoprecipitation studies on the promoter region of IFNL3 to determine if a difference in the binding of transcription factors could be responsible for the prolonged ISG signatures observed individuals with TT or CT genotypes.

Further, we would like to examine the possibility that IFN-λ4 could be contributing to the prolonged ISG signatures in women with CT and TT genotypes. Deletion of the G at this position results in expression of the IFN-λ4 protein that has been shown to retain IFN signaling capability. All of the NPC subjects and mothers from our study were genotyped

at ss469415590, and in each case there was a direct linkage between genotype at IFNL3 SNP rs12979860 and IFNL4 ss469415590. We will examine whether $CD14^+$ cells from individuals with CT and TT genotypes produce IFN-λ4, and whether it functions in an autocrine fashion to prolong IFN signaling.

An additional question that remains is what are the downstream consequences of prolonged IFN signaling in postpartum $CD14^+$ cells from mothers with TT or CT genotypes, particularly on adaptive immune responses? Since monocytes and myeloid dendritic cell subsets express CD14, we would like to examine the possibility that $CD14^+$ cells from CC donors are functionally capable of antigen presentation and cytokine secretion following delivery, thus activating downstream adaptive immune responses. Though we hypothesize that the increase in postpartum $CD14^+$ cell ISG transcripts from CT and TT genotypes renders them less functional than their CC counterparts, as they may be refractory to proinflammatory stimulation, this has not yet been determined experimentally. We hope to expand our cohort of both healthy and HCV-infected mothers and perform extensive phenotypic and functional analyses of postpartum $CD14^+$ cells. Further analysis will be required to determine if postpartum $CD14^+$ ISG expression can be stratified according to genotype in women persistently infected with HCV.

We have previously reported that postpartum $CD8^+$ T cell escape mutations are lost during pregnancy, then regained following a second delivery in women who experience a precipitous decrease in plasma hepatitis C viral load, which suggests that $CD8^+$ T cells are activated postpartum and exerting pressure on the virus (17). Blockade of type I

interferon signaling in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (a strain that persists in mice with features resembling human chronic infections such as HCV) reduced dendritic cell PDL1 expression, reinstated $CD4^+$ T cell IFN-γ production and promoted viral clearance (18, 19). In addition, Zheng and colleagues described a decrease in PDL1 frequency and MFI on $CD14^+$ cells from HCV treatment responders versus non-responders (20), although the impact on adaptive immunity was not assessed. If women with homozygous CC alleles possess an inherent advantage in their ability to dampen interferon responses following delivery, perhaps this has downstream consequences similar to the effect of IFNR1 blockade observed in LCMV-infected mice. $CD14^+$ cells from CC donors could potentially be functional, secrete pro-inflammatory cytokines and present viral antigen to $CD4^+$ T cells, which then provide help for virus specific $CD8^+$ T cells. We hope to pursue this hypothesis for how $CD14^+$ cells and IFNL3 signaling could determine the outcome of chronic HCV infection.

Understanding methods of modulating viral control during pregnancy and following parturition may provide new insight into the intricacies of maternal immune regulation. Thus far we have examined the possibility that IFNL3 genotype, innate IFN signaling, and $CD8^+$ T cells may contribute to the postpartum decrease in hepatitis C viral load. In the next section we briefly discuss unpublished data regarding a potential role for a circulating antiviral factor in postpartum plasma.

Other factors that may contribute to the postpartum decrease in HCV load. We first tested the ability of postpartum plasma to inhibit HCV in cell culture. Plasma samples obtained from HCV-uninfected women were utilized for these studies to prevent convoluting results from neutralizing antibodies or virus already present in the plasma. Briefly, plasma samples were heated to inactivate complement, and Huh-7.5 cells were treated with plasma and subsequently infected with HCV cell culture virus. Three days following infection, cells were permeabilized and stained for the E2 glycoprotein. Focus-forming units were quantitated and the percent inhibition was calculated relative to untreated cells. These *in vitro* inhibition assays suggest that plasma obtained from uninfected women between one and two weeks post delivery displays a low level of anti-viral activity when compared to plasma obtained from NPC subjects (**Figure 1**).

Given the ability of IFN to inhibit HCV replication in cell culture, we first sought to determine if IFN was present in the postpartum plasma. A549 cells (a human alveolar adenocarcinoma cell line that is extremely sensitive to type I, II, and III interferon), NPC PBMCs, and Huh-7.5 cells were incubated with NPC, pregnant, or postpartum plasma from UM07 and UM08 and analyzed for ISG induction. Regardless of cell type and length of incubation, treatment with postpartum plasma did not induce higher ISG transcript expression than treatment with plasma from NPC subjects (**Figure 2**), suggesting that IFN is not responsible for the ability of postpartum plasma to inhibit HCV.

An additional approach utilized to determine the plasma factor responsible for HCV

inhibition was 2-D gel analysis and mass spectrometry. Plasma samples isolated during pregnancy or following delivery were sent to Kendrick Labs at the University of Wisconsin for 2-D gel analysis, which allowed for comparison of protein composition and concentration in the plasma samples. 2-D gel analysis revealed significant differences in protein composition of plasma samples drawn pre and post delivery (**Figure 3**). Individual proteins were chosen for further analysis based upon fold difference in concentration and significance testing. For protein identification, mass spectrometry was performed on the chosen 2-D gel spots by the proteomics core at Columbia University. One of the proteins identified by mass spec was apolipoprotein C (**Figure 3**). As mentioned in chapter 1, lipids are imperative for the HCV life cycle and certain apolipoproteins enhance the infectious capacity of HCV particles.

We first quantified apolipoprotein C levels within NPC, pregnant, and postpartum plasma. Plasma levels of apolipoprotein C I and C II remained steady throughout pregnancy and postpartum, while apolipoprotein C III levels were significantly decreased following delivery (**Figure 4**). However, apolipoproteins do not circulate *in vivo* as distinct proteins. Rather, they form HDL, LDL, and VLDL particles that transport cholesterol throughout the body. We analyzed levels of single apolipoproteins during pregnancy and following delivery. Further study is warranted to determine if elevated levels of HDL and VLDL during pregnancy could contribute to the increased HCV replication observed during pregnancy. Following delivery, if HDL and VLDL levels are drastically decreased, this could potentially reduce the infectivity of HCV virions and contribute to the decrease in postpartum plasma viral load. However, these data are both

preliminary and purely correlative. Proof of causation is unlikely without an *in vivo* animal model of HCV and pregnancy.

The future of Cas9-mediated inhibition of HCV and other RNA viruses. To our knowledge the inhibition of RNA viruses, other than HCV, by Cas9 has not been demonstrated. Numerous DAAs are currently available to cure persistent HCV infection with limited side effects. Therefore, it is unlikely that the use of Cas9 will be extensively pursued for HCV infection in the near future. However, I would like to end this dissertation with a consideration for an additional intersection of the maternal and bacterial immune systems. Maternal susceptibility to the infectious Zika and Ebola viruses raises important considerations for the use of the Cas9 platform to restrict these infections. The 2014-2015 Ebola outbreak in Africa claimed 11,315 lives (21, 22), and mortality rates greater than 95 percent have been reported for pregnant women. In a study of fifteen pregnant women infected by Ebola virus in the Democratic Republic of the Congo, only one mother and no offspring survived (23). Equally unsettling is the possibility of a Zika virus epidemic. Infection during pregnancy has been associated with microcephaly in newborns, and in Brazil alone over 4,000 new cases of microcephaly have been reported (14).

The postpartum period is associated with enhanced control of persistent viruses such as HCV, yet during gestation the maternal immune system is unable to control infection and prevent vertical transmission of other infectious RNA viruses such as Ebola and Zika. It is undeniable that antiviral therapeutics and vaccines are desperately needed for these

infections, but it remains to be seen if Cas9 will serve as an effective defense against these viruses.

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Figures and Figure Legends

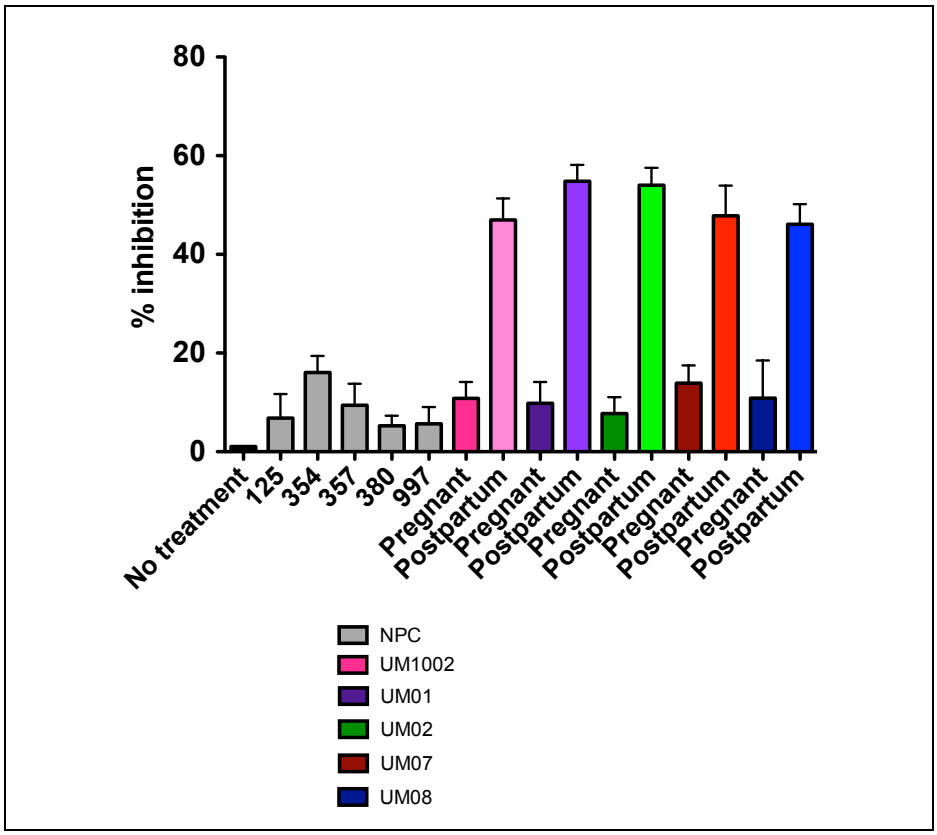

Figure 1. Postpartum plasma obtained from healthy women inhibits hepatitis C virus in cell culture.

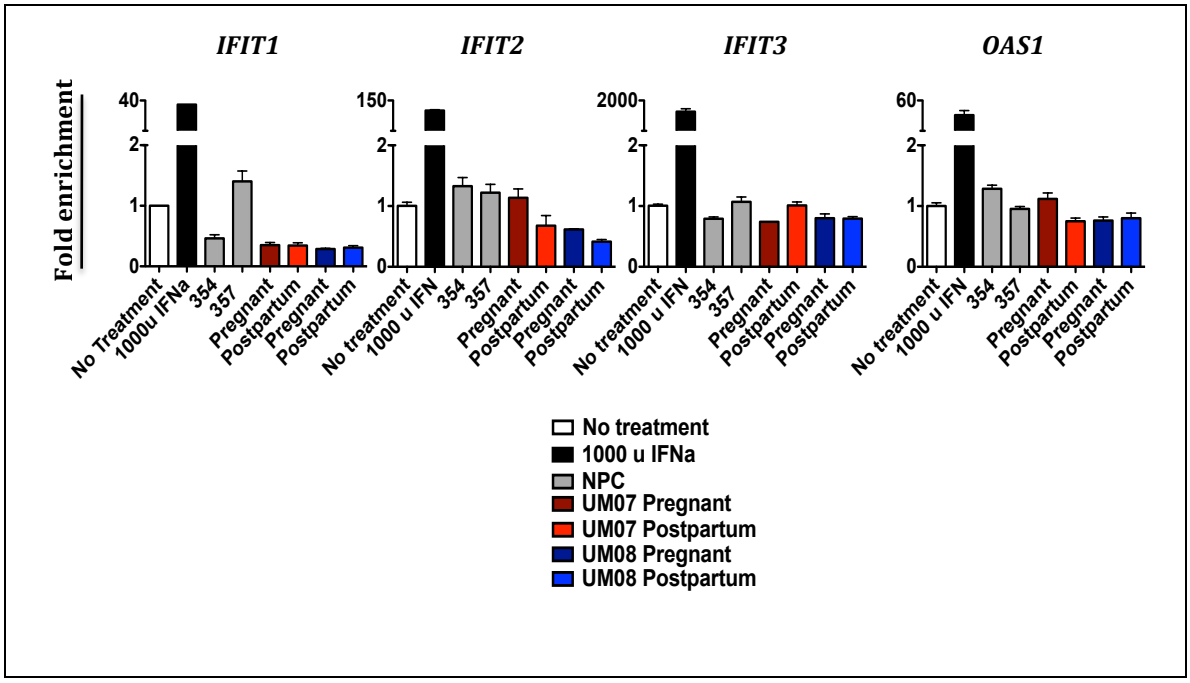

Figure 2. Treatment of A549 cells with postpartum plasma does not induce *IFIT1*, *IFIT2*, *IFIT3*, or *OAS1* transcript expression.

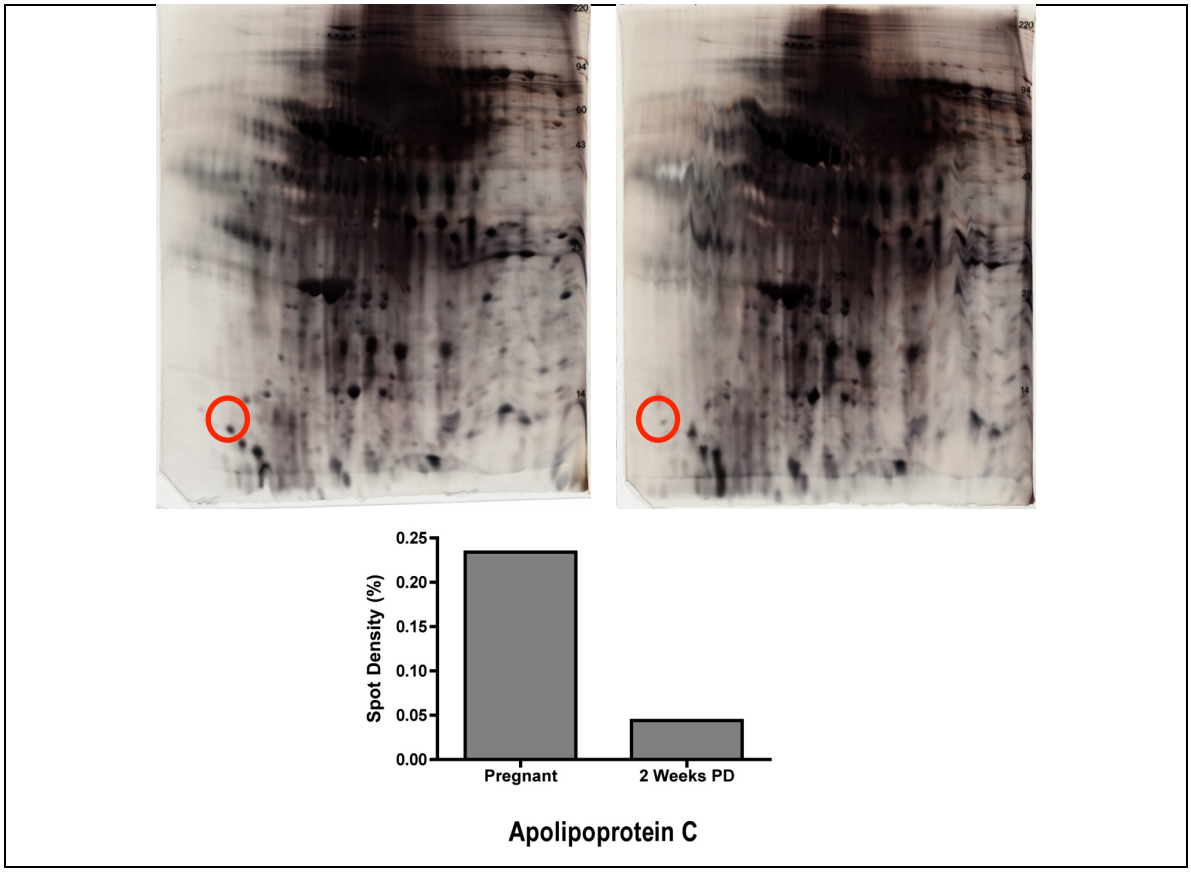

Figure 3. 2-D gel analysis and mass spectrometry indicate that apolipoprotein C is decreased following delivery in a healthy mother.

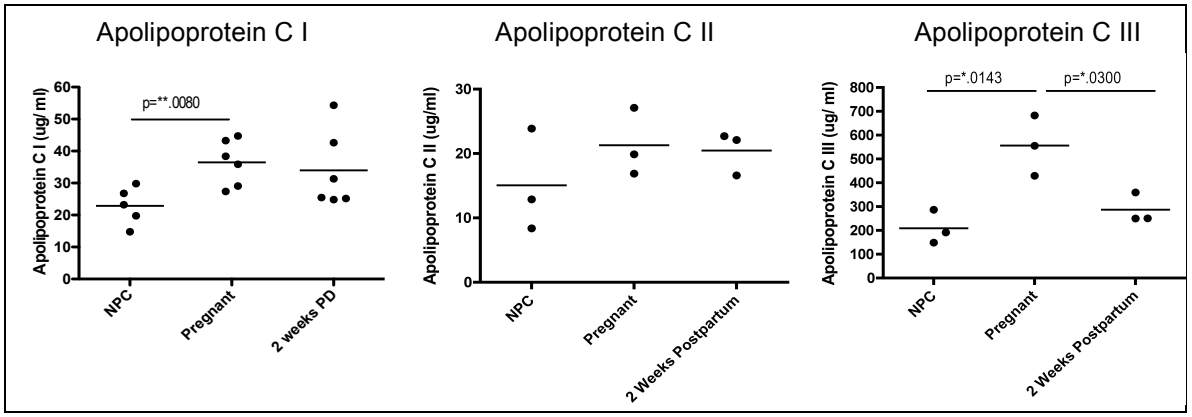

Figure 4. Plasma apolipoprotein C III levels are decreased following delivery in uninfected women.