Role of Molecular Mimickry of Hepatitis C-Virus (HCV) Protein with Platelet GPIIIa in Hepatitis C-related Immunologic Thrombocytopenia

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Running Title: Anti-platelet GPIIIa49-66 Ab in Hepatitis C
Abstract
HIV-1-ITP patients have a unique Ab against platelet GPIIIa49-66 capable of inducing oxidative platelet fragmentation in the absence of complement. HIV-1-seropositive drug abusers are more prone to develop immune thrombocytopenia (HIV-1-ITP) than non-drug abusers and have a higher coinfection with Hepatitis C virus than non-drug abusers (90% vs 30%). Molecular mimickry with a Hepatitis C protein was sought by screening a phage peptide library with anti-GPIIIa49-66 antibody as bait for peptides sharing homology sequences with HCV protein. Several phage peptide clones had 70% homology with HCV protein. Sera from dually infected thrombocytopenic patients with HCV and HIV-ITP reacted strongly with 4 non-conserved peptides from HCV core envelope 1. Reactivity correlated inversely with platelet count, r²=0.7, p<0.01. Ab raised against peptide PHC09 in GPIIIa-/- mice induced severe thrombocytopenia in wild-type mice. Affinity-purified IgG against PHC09 induced oxidative platelet fragmentation in vitro. Drug abusers dually infected with HCV and HIV-1 had a greater incidence and severity of thrombocytopenia as well as greater incidence and titer of anti-GPIIIa49-66/PHC09 Ab. NZB/W F1 mice injected with recombinant core envelope 1 developed Ab vs PHC09 and significantly decreased their platelet count, p<0.001. Thus, HCV core envelope 1 can induce thrombocytopenia by molecular mimicry with GPIIIa49-66.
Introduction
Thrombocytopenic patients with early HIV-1 infection (HIV-1-ITP) have a shortened platelet survival due to an autoantibody against an epitope on platelet surface integrin GPIIIa, GPIIIa49-66 (CAPESIEFPVSEARVLED)\(^1\)\(^-\)\(^3\). Their sera have increased immune complexes which contain platelet fragments as well as anti-GPIIIa49-66 Ab. The presence of anti-GPIIIa49-66 Ab correlates inversely with platelet count (\(r=-0.71\)) and induces severe thrombocytopenia when injected into mice. This antibody is unique in that it induces complement independent platelet fragmentation by oxidative platelet fragmentation due to the release of reactive oxygen species through activation of 12-lipoxygenase and NADPH oxidase \(^4\)\(^-\)\(^6\).

HIV-1 immune-related thrombocytopenia (HIV-1-ITP) is more frequent in drug abusers compared to non-drug abusers (37 vs 16% incidence, respectively); and more severe in HIV-1-seropositive drug abusers than non-drug abusers (platelet count <10,000/ul in 52% vs 9%, respectively) \(^7\)\(^-\)\(^8\). A striking feature of HIV-1 infection in drug abusers is the frequent coinfection with Hepatitis C virus (HCV) \(^9\)\(^-\)\(^{13}\). The overall prevalence of HCV infection among HIV-1-infected individuals is 30-50% \(^9\) in non-drug abusers, with rates of co-infection as high as 90% in intravenous drug abusers \(^9\)\(^-\)\(^{13}\). We asked whether coinfection with HCV facilitates ITP and if so, what would be the mechanism. The presence of a relatively high-affinity immunodominant Ab against GPIIIa49-66 in HIV-1-ITP patients suggested antigen driven B-cell clonal expansion. We therefore investigated whether coinfection of HCV in HIV-1-ITP patients enhances the likelihood of inducing anti-GPIIIa49-66 Ab due to molecular mimicry of Hepatitis C, with GPIIIa49-66 as we have shown for nef with HIV-1-ITP\(^14\).

Patients with HCV commonly develop immunologic thrombocytopenia (HCV-ITP) which correlates with severity of disease (e.g. chronic active hepatitis, cirrhosis)\(^15\)\(^-\)\(^{17}\). The incidence of HCV-ITP in a series of 368 HCV Japanese patients with chronic persistent or chronic active hepatitis was 41%. The incidence of endemic HCV-ITP in 294 chronic patients was 10% which
increased to 32% with advanced liver disease\textsuperscript{15}. The frequency of B cell production of anti-GPIIb-IIIa Ab was 27 fold greater than with control cells in 37 HCV-ITP patients with cirrhosis\textsuperscript{17}; and an inverse correlation was found between platelet count and B cell anti-GPIIb-IIIa Ab production in 51 patients with liver cirrhosis (73% with Hepatitis C). This would suggest some degree of specificity. Like HIV-1-ITP, patients with HCV-ITP have increased serum immune complexes\textsuperscript{16}. We therefore reasoned that a second autoimmune disease with serum immune complex associated immunologic thrombocytopenia could also contain an anti-GPIIIa\textsuperscript{49-66} Ab capable of inducing oxidative platelet fragmentation – induced by molecular mimickry with an HCV peptide in addition to HIV nef peptide in HIV-1-ITP\textsuperscript{15}.

In the present report, we demonstrate that: 1) Four HCV core-envelope peptides from a non-conservative region, display molecular mimickry with GPIIIa\textsuperscript{49-66} by reactivity with anti-GPIIIa\textsuperscript{49-66} Ab; 2) The strongly reactive SAIHIRNASG Peptide (PHC09) was examined more extensively. PHC09 injected into GPIIIa\textsuperscript{-/-} mice induced an Ab capable of inducing oxidative platelet fragmentation in vitro and thrombocytopenia in vivo in wild type mice; 3) Platelet counts of HIV-1 Hepatitis C Drug Abusers correlate inversely with serum titer vs PHC09 ($r^2=0.7$, n=15, $p<0.01$); 4) Injection of rHCV-core-envelope 1 protein into NZB/W F1 mice induces thrombocytopenia which correlates with murine anti-PCH09 Ab level; 5) Thrombocytopenic drug abusers dually infected with HIV-1 and Hepatitis C have a greater incidence and titer of anti-GPIIIa\textsuperscript{49-66} Ab as well as greater incidence and severity of thrombocytopenia.

**Materials and Methods**

**Human Population**

Coded stored frozen sera (sent to the clinical laboratory for platelet-Ab testing) were randomly obtained from thrombocytopenic intravenous drug abusers with both HCV and HIV-infection,
non-drug abuser Hepatitis C patients, non-drug abuser HIV-ITP patients and healthy control subjects. Liver chemistries (albumin, alkaline, phosphatase) were comparable in all 3 groups. These studies were approved by the New York University Medical Center Institutional Review Board.

**Mice**

Female BALB/c and C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). Integrin GPIIIa/⁻ knock out mice and NZB/W F1 mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). Animal work was approved by the New York University School of Medicine Animal Review Board.

**Reagents**

All reagents were obtained from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise designated. 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR).

**Peptides**

Peptide GPIIIa49-66 (CAPESIEFPVSEARVLED), PHC09 (SAIHIRNASG), PHC07 (IFDPGAL), PHC01 (VDWQAPPGARS), PHC09 preceded by cysteine (CSAIHIRNASG), PHC09-H1 (SAYQVRNSSG), PHC09-H2 (SAYQVRNSTG), PHC09-H3 (SAEVRNVSG) and irrelevant 10-mer peptide (GIGALFLGFL) were synthesized by Bio-Synthesis (Lewisville, TX).

**Antibodies**

Human anti-GPIIIa49-66 Ab and rabbit anti-GPIIIa49-66 Ab were obtained or produced in our laboratory as described previously¹,². Mouse Ab against HCV peptides (PHC09) or Mouse Ab against rHCV-core-envelope¹ were purified with protein A affinity chromatography as
recommended by the manufacturer (Sigma Chemical Co.).

**Affinity Purification of Anti-PHC09 IgG**

Serum IgG was isolated from HIV-1 drug abusers by protein A affinity chromatography. PHC09 preceded by cysteine (CSAIHIRNASG) was coupled to an affinity column with the heterobifunctional cross-linker sulfo-succinimidyl 4-\((N\text{-maleimidomethyl})\text{ cyclohexane-1-carboxylate}\) as recommended by the manufacturer (Pierce Chemical Co.). This again cross-links the resin with NH\(_2\)-terminal cysteine of the peptide.

**Screening of the Phage Display Peptide Library**

Anti-GPIIIa49-66 Ab was incubated with \(10^{12}\) to \(10^{13}\) PhD-7 phage from a 7 mer linear peptide library obtained from New England Biolabs (Beverly, MA) in 2% non-fat milk in PBS at room temperature for 3 hrs. Protein A and alternating Protein G-conjugated agarose beads were added overnight at 4°C. The beads were then centrifuged and washed 20 times with 0.1% Tween-20-PBS. Positive phages were eluted with trypsin-PBS by shaking for 10 min at room temperature and eluted phage titered and amplified as previously described\(^{14}\). After the third round of panning, 30 clones were randomly selected for ELISA and the positive clones sequenced. Phage peptide sequences were analyzed for sequence similarity to other proteins using the BLAST algorithm of the blast program and the database of the National Center for Biotechnology Information (NCBI).

**ELISA**

Twenty \(\mu\text{g/ml}\) of peptide in 0.1M sodium bicarbonate buffer (pH 9.6) was adsorbed to a 96-well ELISA plate at 4°C overnight, and blocked with blocking buffer [3% BSA in PBS-Tween (0.1%)] at room temperature for 2h. Primary antibody was added at room temperature for 1h, followed by horse raddish peroxide (HRP)-conjugated secondary IgG for 1h at room temperature. Plates were washed six times with Tris-buffered saline 0.1% Tween 20 at each
step in the procedure. The ABTS reagent used to develop the HRP color reaction at 405 nm was obtained from Pierce.

Production of Anti-PHC09 Ab in GPIIIa-/- Mice
GPIIIa-/- mice aged 6-8 weeks were immunized by intraperitoneal injection of 50ug KLH conjugated PHC09 with equal volume of mineral oil adjuvant for primary immunization. Booster injections were performed three times every two weeks. Immune sera were titered by ELISA on the PHC09 peptide. Positive sera were purified as described.

Assay of Platelet Particle Formation
Gel-filtered murine or human platelets were isolated from EDTA-anticoagulated blood and labeled with anti-CD61-fluorescein isothiocyanate (FITC, human) or anti-CD41- FITC (murine) as previously described. Fluorescent-labeled platelets/particles were measured by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Gates were adjusted for platelets by exclusion of other blood cells. Fluorescent-labeled intact platelets were monitored in the right upper quadrant with the y-axis measuring forward scatter and the x-axis measuring fluorescence. A shift in fluorescence from right upper quadrant to left upper quadrant and left lower quadrant reflected the percentage of platelet particle induction in 10,000 enumerated events.

Assay of Platelet Oxidation
Gel-filtered platelets were loaded with 10 μM DCFH-DA for 30 minutes at 37° C as described and challenged with platelet Ab. Intracellular DCFH is converted to a fluorescent form by hydrogen peroxide generated in this reaction. Oxidation was quantified by measuring the increase in mean fluorescence by flow cytometry.
Induction of Passive Thrombocytopenia in Mice

Six-week-old female BALB/c mice were randomly divided into 4 groups (n=4 per group). Fifty ug of purified control IgG, patient IgG or mouse IgG against peptide PHC09 were injected intraperitoneally into Balb/c mice, and platelet counts followed for 24 hours.

Determination of Mouse Platelet Counts

Platelet counts were determined from 20 ul of blood draw into Unopettes (No.365855, Becton Dickinson), containing optimal anti-coagulant concentration and diluent for quantitating platelet counts by phase-contrast microscopy.

Expression of Recombinant HCV-Core-Envelope 1 in \textit{E.coli}

HCV core envelope 1 C-histidine tag cDNA encoding 24 amino acids (167-191) of the core and 160 amino acids of the envelope (192-350) and containing the PHC09 sequence was cloned into expression vector PET29a (Novagen). PHC09 is composed of amino acids 190-199 which contain the 2 C-terminal amino acids of the core and the 8 N-terminal amino acids of the envelope. \textit{E.coli} BL21 (DE3) PlyS cells were transformed with the pET29a-HCV-core-envelope1 plasmid cultured in LB medium [Bacto-tryptone (1g %), Bacto-yeast extract (0.5%), NaCl (1%), pH7.0] containing carbenicillin (50μg/ml) and chloramphenicol (34μg/ml) with shaking at 37°C until the OD (600nm) was 0.4-0.6. Expression was induced by the addition of 1 mM IPTG at 37°C for 4 hrs with shaking, and purified by Ni-NTA agarose resin. The protein was dialysed in PBS, and the purity verified with 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunodeficiency Animal Model

Two animal models were employed. \textit{NZB/W F1 mouse model}: NZB/W F1 mice were directly challenged with rHCV-core-envelope1 protein containing the corresponding PHC09 sequence, or scrambled peptide (25 ug/mouse, every 10 days for 9 injections). Platelet counts and
antibody titer vs PHC09 were monitored after every injection. BALB/c mouse model: BALB/c mice were immunosuppressed, as described 26 with an initial 6 mg of cyclophosphamide/mouse (Mead Johnson, Princeton, N.J.) and maintained with additional doses of 2 mg/mouse every week. BALB/c mice were challenged with control or rHCV-core-envelope1 protein (25 ug/mouse) every 10 days for 9 injections after the total leukocyte count reached <2,500 cells/ul. Platelet counts and antibody titers vs PHC09 were monitored as above.

Results
Identification and characterization of selected phage clones
To test the hypothesis that an HCV epitope contributes to the generation of anti-GPIIIa49-66 Ab, we screened a filamentous phage display 7-mer peptide library using anti-GPIIIa49-66 antibody as bait, and focused on peptides sharing homology sequences with HCV protein. Thirty phage clones that reacted with anti-GPIIIa49-66 Ab by ELISA were randomly selected. Clones with non-specific cross-reactivity to BSA were also screened by ELISA. Twenty clones reacted positively with anti-GPIIIa49-66 Ab with high OD value and low cross-reactivity with BSA (ratio>3) (Figure 1A). Three positive clones [PHC09 (SIHIRNG), PHC07 (SFDPGLF), and PHC01 (VDWPRSE)] showed sequence homology with HCV protein. Clone PHC09 was in a non-conserved core envelope 1 region of Hepatitis C virus. We also searched the data base for other non-conserved clones with homology for PCH09. Six such clones were found, PHC09-H1-H6. PHC09-H5 and H6 had the highest homology for PCH09 (Table1). These clones were synthesized as 10-mers and were compared for reactivity with GPIIIa49-66, PHC09, conserved PHC01 and PHC07 and nef. Figure 1B demonstrates the strongest Ab reactivity with PHC09 and PHC09-H5. Table1 also records the HIV genotype. Note that PHC09 is a relatively rare genotype (HCV6) in the U.S. (1.5%), whereas PHC09-H1-6 are more common HCV1a or HCV1b genotypes (38 and 36% respectively).

Reactivity of Ab with a modified epitope
Since the core envelope 1 protein is partially cleaved during virus secretion from the infected cell with removal of 2 amino acids from the core, we tested the remaining 8 mer peptide from 3 different HCV isolates. All 3 had similar or greater activity than the PHC09 10 mer peptide, Figure 1C.

Cross-Reactivity of HCV-ITP, HIV-ITP and HCV-HIV-ITP sera with GPIIIa49-66, PHC09, PHC09-H5, PHC07 and nef

Since HIV-1/HCV-ITP drug abusers have a higher incidence and severity of ITP, we reasoned that they might have a greater incidence of anti-GPIIIa49-66 Ab secondary to molecular mimickry with HCV peptides as well as recently reported HIV-nef. Figure 2A, B and C demonstrate considerably increased Ab reactivity vs GPIIIa49-66 in 12/15 HIV/HCV-ITP drug abusers (Figure 2C) compared to 2 of 15 HCV-ITP patients (Figure 2A) (p<0.001) and 6/15 HIV-ITP patients (Figure 2B) (p<0.05), Chi Square analysis. Similar differences were noted for the other 4 molecular mimickry HCV and nef Ag’s in the 3 groups of patients (Table 2). Of further interest was the presence of Ab to all 4 Ag’s in 7 of 15 HIV/HCV-ITP patients compared to 1 HCV-ITP patient and 1 HIV-ITP patient (p=0.019). A greater severity of thrombocytopenia, <30,000/ul (Figure 2D) was also noted in 8 of 15 HIV/HCV-ITP drug abusers compared to 2 HCV-ITP (p<0.025), and 3 HIV-ITP patients, (p=0.06). Thus the greater incidence and severity of thrombocytopenia in HIV-1/HCV-ITP drug abusers could be explained on the basis of molecular mimickry of GPIIIa49-66 with the 4 non-conserved HCV peptides and HIV-1 nef.

In this regard it is of interest to cite the work of Yong and Wang who employed an anti-human HCV core polyclonal Ab to pan a phage peptide library for Hepatitis C epitope mapping. An analysis of their data reveals the presence of a dominant linear epitope at residues 19-25 of the N-terminal end. This peptide, PQXV(I)XFP has 86% homology with platelet GPIIIa51-57 (PESIEFP) which resides within the GPIIIa49-66 peptide, confirming our observations.
Clinical association of HCV peptide specific antibodies and thrombocytopenia

To determine the clinical association of HCV peptide and thrombocytopenia in patients, we obtained 15 sera from thrombocytopenic HIV-1/HCV-infected Drug Abusers and looked for a correlation between their platelet count and titer vs the 3 HCV peptides. Figure 3A, B demonstrates an inverse correlation between patient platelet count and Ab titer vs the PHC09 peptide ($r^2=0.7$, $p<0.01$) and the PHC09-H5 peptide ($r^2=0.53$, $p<0.05$). Since Ab vs PHC09 provided the best correlation (Figure 3A) and highest titers (Figure 2A, B and C), we focused on anti-PHC09 Ab located in HCV core-envelope 1.

Effect of the HCV peptide specific antibodies on Ab-induced oxidative platelet fragmentation

To assess the potential effect of HCV peptide on Ab-induced platelet fragmentation, peptide PHC09 (SAIHIRNASG) was then tested for its ability to inhibit affinity-purified patient GPIIIa49-66 Ab-induced platelet fragmentation in vitro. PHC09 significantly inhibited Ab-induced human platelet fragmentation at an (IC$_{50}$) molar Ab/peptide ratio of 1:5, (similar to GPIIIa49-66 peptide CAPESIEFPVSEARVLED), whereas irrelevant peptide had no effect at 1:100 (Figure 4A).

Induction of oxidative platelet fragmentation with Ab

We next affinity-purified IgG with a PHC09 affinity column and confirmed that (similar to anti-GPIIIa49-66) anti-PHC09 IgG induces oxidative platelet fragmentation in vitro (Figure 4 B, C). We therefore looked for platelet fragments in the immune complexes of 10 HCV-ITP patients by immunoblot. They were found in patient #10 and #13 (Figure 4D). We next isolated IgG from the serum immune complexes of patients #10 and #13 as well as patients #2 and #6 who had undetectable platelet fragments. Both positive patients tested (#10 and #13) had immune complex IgG capable of oxidatively fragmenting platelets, whereas, the 2 control patients (#2 and #6) were unreactive (Figure 4E and F).
Effect of mouse Ab against HCV peptide on induction of thrombocytopenia in mice

The in vivo relevance of this peptide was further tested by raising an Ab against the KLH-conjugated peptide in GPIIIa−/− KO mice, which do not recognize the peptide as self, avoiding the problem of mouse tolerance. The mouse serum Ab against PHC09 was shown to bind to GPIIIa49-66 (data not shown). Affinity-purified mouse anti-PHC09 IgG, mouse control IgG, and rabbit anti-GPIIIa49-66 IgG (50 ug/mouse, n=4/group) were then injected intraperitoneally into Balb/c mice, and platelet counts followed for 24 hours. Figure 5 demonstrates that whereas mouse control IgG had no effect, rabbit anti-GPIIIa49-66 IgG or mouse anti-PHC09 IgG induced thrombocytopenia.

Pathological effect of rHCV-Core-Envelope 1 on mouse platelet counts

Since PHC09-SAIHIRNASG is a molecular mimick of GPIIIa49-66 and located in HCV-Core-Envelope 1, we investigated its in vivo pathological effect on the mouse platelet count. Autoimmune SLE NZB/W F1 mice and immunosuppressed BALB/c mice were challenged with multiple rHCV-core envelope 1 injections. Platelet counts decreased in 8 of 8 NZB/W F1 treated mice, p=0.001, mean platelet count of 1,003 ± 29 vs 870 ± 7.4 x 10^6/ul, (Figure 6A, B) whereas no decrease in platelet count was noted in the comparison groups immunized with control protein or rHCV-core-envelope 1 peptide in BALB/c immuno- suppressed mice (data not shown). In addition, the serum titer to peptide PHC09 was elevated in NZB/W F1 mice immunized with rHCV-core-envelope 1 whereas the control group had no significant effect (Figure 6C). The platelet decrease correlated inversely with serum titer vs PHC09 (r²=0.63, n=16, p<0.05) (Figure 6D).

Since platelet counts represent an equilibrium between platelet production and destruction, we reasoned that the modest decrease in platelet count could have been dampened by a compensatory increase in platelet production by the challenged bone marrow. We therefore affinity-purified the serum IgG with PHC09, to increase its relative concentration. Figure 6E
demonstrates a more pronounced thrombocytopenia (60% decrease in platelet count compared to control IgG).

**Discussion**

Our current data clearly demonstrate that the HCV core envelope 1 can induce functionally active anti-GPIIIa49-66 Ab by mimicking platelet GPIIIa49-66 as antigen and inducing immune thrombocytopenia in NZB/W F1 mice. Anti-HCV core envelope 1 Ab correlates inversely with platelet counts in HIV-1 drug abusers with HCV infection. To our knowledge, this is the first report that an HCV epitope can induce anti-GPIIIa49-66 Ab by molecular mimickry.

We now describe a second immune complex associated platelet immunologic disorder (HCV-ITP) in which a unique Ab is developed against GPIIIa49-66 capable of inducing oxidative platelet fragmentation. It is now apparent that this new pathologic mechanism of platelet destruction is not unique for HIV-1-ITP and could possibly be associated with other immune complex associated disorders in which serum complexes may contain platelet fragments and anti-GPIIIa49-66 Ab.

Attempts to induce thrombocytopenia by injection of HCV peptide PHC09 into immunosuppressed wild type mice were unsuccessful – either due to immune tolerance [mouse GPIIIa has 83% homology with human GPIIIa19] or cyclophosphamide-induced inhibition of anti-GPIIIa49-66 Ab production. However, we were able to induce anti-GPIIIa49-66 Ab and resultant thrombocytopenia in 8 of 8 autoimmune NZB/W F1 mice which correlated inversely with their platelet count. This is likely due to impairment of immune surveillance in these mice with polyclonal B lymphocyte hyperactivity to self antigen which parallels the B cell hyperactivity in early HIV-1 infection20.

Studies by others have demonstrated that the HCV core protein 1 is cleaved by signal
peptidases between residues 191 and 192 to generate the retained E1 envelope $^{21,22}$. PHC09 contains 10 amino acids (SAIHIRNASG). The major (8 amino acids) molecular mimickry sequence (IHIRNASG) is located in the retained HCV 1 envelope region, which is likely to retain the epitope specificity. Indeed, a human monoclonal Ab (H111) derived from a Hepatitis C donor infected with HCV genotype 1b reacts with the N-terminal 192-202 amino acids of E1 from 12 different E1 proteins obtained from virus isolates of genotypes 1a, 1b, 2b and 3a $^{23}$. PHC09-H5, the epitope analyzed in our study has homology with 20 different E1 isolates, with the candidate linear epitope containing the critical amino acids ---RN-SG-Y-. Thus PHC09-H5 appears to be a common Hepatitis C virus epitope.

Of interest were our observations on the greater incidence and severity of ITP in drug abusers coinfected with HIV-1 and HCV compared to HCV-ITP or HIV-ITP. The increased anti-GPIIIa49-66 Ab incidence, titer and cross-reactivity with molecular mimotopes from HCV (core envelope protein 1) as well as HIV-1 (nef) provide an explanation for the clinically observed greater incidence and severity of dually infected drug abusers. This is likely due to HIV-induced dysregulation of immune function in which spontaneous activation of B cells and immunoglobulin production has been reported $^{24,25}$. It is suggested that molecular mimickry of HCV or HIV-1 peptide with other epitopes on the platelet or megakaryocyte surface might also play a role in the induction of HCV/HIV-ITP thrombocytopenia.

Ab against PHC09-H5 was found in 6 of 15 HCV-ITP and 8/15 HIV-HCV-ITP patients. Ab was not detected in the other ~7 patients (~50%), suggesting that molecular mimickry could be dependent upon the fine specificity of the specific mimotope in the non-conserved region, or that other mechanisms can be invoked in these patients. This could vary and is dependent upon the ability of the Hepatitis C virus envelope to avoid host immune surveillance. Never-the-less, it is now apparent that at least some patients with HCV-ITP may develop their ITP due to molecular mimickry. This is supported by our ability to induce immunologic thrombocytopenia
in NZB/WF1 mice with a core-envelope construct. It is well known that thrombocytopenia increases with severity of disease – and has been claimed that this may be due to liver disease rather than Ab. We consider this debatable because of patients whom we have studied with severe liver cirrhosis who had abundant IgG on their platelets as well as in their immune complexes 16.

It is of interest that both HCV and HIV-1 each contain different structural elements that mimic peptide sequences in GPIIIa49-66 and that both viruses are capable of inducing GPIIIa49-66 specific antibodies capable of causing thrombocytopenia. A possible explanation is that both the HCV and HIV-1 mimickry epitopes are in non-conserved highly mutated regions of the 2 viruses, and that mimickry of host proteins serves to help viruses escape host immune surveillance. Therefore the mutations of these mimickry regions are likely to contribute to the autoimmunity in general, and in our study, thrombocytopenia more specifically. It is also likely that coinfection of HCV and HIV-1 increases the chances of molecular mimickry.

In summary, these findings demonstrates the potential of HCV or other pathogens to induce anti-platelet Ab by molecular mimickry – and suggests that platelet membrane GPIIIa49-66 may be a common immunodominant target for these pathogens, particularly HIV-1/HCV for the induction of immunologic thrombocytopenia.
Acknowledgements

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Authorship

Contribution: W.Z. performed and analyzed most of the experiments; M.N. performed the platelet fragmentation experiment; Z.L. designed the phage display experiments; W.B. suggested the HCV genotype experiments; S.K. guided the project and wrote the paper.

Conflict-of-interest disclosure: The authors declare no financial interests.

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REFERENCES


Candidate clone PHC09 is located in a Hepatitis C virus non-conserved region. H1-H6 represent homologous PHC09 isolates. The underlined regions denote an identical amino acid sequence between screened 7-mer peptide and PHC09. Column 1 denotes the % homology of the isolates. Superscript numbers refer to amino acid position in HCV core-envelope 1.

Table 2
Ab Reactivity Against Various Peptides in HCV-ITP, HIV-ITP and HCV-HIV-ITP Patients

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*Chi square analysis of positive Ab reactivity in the various groups. Upper P row is difference between HCV-HIV-ITP and HIV-ITP. Lower P row is difference between HCV-HIV-ITP and HCV-ITP.

FIGURE LEGENDS
Figure 1

**Panning of PhD 7-phage peptide library.** A. Positive clonal analysis for phages following the 3rd round of panning. Clones were subjected to ELISA for binding to anti-GPIIIa49-66 Ab. BSA is used as control antigen. Clones that gave an anti-GPIIIa49-66 Ab/BSA, OD ratio >3 were designated positive. Sequence analysis is given for positive peptides were analyzed by BLAST algorithm of the Blast program and database of the National Center for Biotechnology Information (NCBI). Three clones are molecular mimics with close sequence similarity to HCV proteins. PHC09 is a non-conserved peptide in the core envelope 1 region. B. Anti-GPIIIa49-66 reactivity with various 10 mer peptides: GPIIIa49-66, PHC09, homologous peptides (H1-H6) of PHC09, HIV-nef and irrelevant peptide (IR). C. Anti-GPIIIa49-66 reactivity with various 8 mer peptides. HCV genome map is provided.

Figure 2

**Serum reactivity to GPIIIa49-66 mimicry peptide and thrombocytopenia in HCV, HIV and HIV/HCV-ITP patients.** Serum samples were collected from immunologic thrombocytopenia patients [15 HCV-ITP (A), 15 HIV-ITP (B) and 15 HIV/HCV-ITP intravenous drug abusers (C)]. Serum reactivity to GPIIIa49-66 and its mimicry peptides HCV (PHC09), (PHC09-H5) and (PHC07) as well as HIV-1 peptide nef and irrelevant peptide (IR) was measured by ELISA with patient sera. SEM is given. Horizontal lines refer to 2SD’s. The severity of thrombocytopenia was evaluated by platelet count, (D). Horizontal bar is an arbitrary 30,000/ul cut-off.

Figure 3

**Inverse correlation between anti-HCV peptide Ab titer and thrombocytopenia.** A. Anti-HCV peptide (PHC09). (B) Anti-HCV peptide (PHC09-H5). Serum titer to HCV peptides was measured by ELISA with serial dilutions of sera.

Figure 4
**Inhibition of anti-GPIIIa49-66 Ab-induced oxidative platelet fragmentation by HCV peptides.**

**A.** Effect of HCV peptides on anti-GPIIIa49-66 platelet reactivity. Patient IgG was preincubated with different peptides prior to incubation with gel-filtered platelets. CAPESIEF refers to positive control (CAPESIEFPVSEARVLED). Irrel refers to irrelevant peptide. Dark gray, light gray, white, and black bars refer to molar Ab/peptide ratios of 1:100, 1:10, 1:5, and 1:2, respectively. **B.** Effect of IgG against HCV peptide (PHC09) on platelet fragmentation and **C.** oxidation. Affinity purified IgG from 5 HIV-1/HCV-ITP patients with high reciprocal Ab titer to PHC09 induced platelet fragmentation (B) and oxidation (C) in vitro. C (white bar) indicates control human IgG; P (black bar) indicates HIV-1-ITP patient IgG; IVDA horizontal line refers to affinity purified anti-HCV peptide PHC09 IgG from dually-infected Drug Abusers. The black, grey and white bars refer to doubling dilutions of isolated IVDA IgG (25, 12.5 and 6.25ug/ml, respectively). **D.** Presence of platelet fragments in patients with HCV-ITP. Immunoblots of serum immune complexes run on SDS-PAGE and immunoblotted with anti-GPIIIa49-66. **E.** Effect of HCV IgG isolated from HCV immune complexes on (E) platelet fragmentation and (F) platelet DCFH oxidation. Pt refers to patient. Black, dark gray and light gray bars refer to serial 1:2 dilutions of IgG, starting at 50 ug/ml. SEM is given.

**Figure 5**

**Effect of Ab against HCV peptide on induction of thrombocytopenia in Balb/C mice.** Purified control mouse IgG (Ctl), rabbit GPIIIa49-66 IgG, and mouse IgG against PHC09 (50 ug) were injected intraperitoneally into Balb/C mice, and platelet counts followed for 24 hours. n=4. SEM is given.

**Figure 6**

**Effect of recombinant HCV core-envelope 1 injection on mouse platelet counts.** NZB/W F1 mice were challenged with multiple control (A) or rHCV-core-envelope 1 (B) injections. At 90 days, the platelet count had decreased in 8 of 8 NZB/W F1 mice (X axis small b and a refer
to before and after immunization), n=8, p<0.001 (B). No decrease in platelet count was found in the comparison group immunized with control protein (A). The serum titer to peptide PHC09 was elevated in NZB/W F1 mice immunized with rHCV-core-envelope 1 (HCV) (C), whereas the control protein (control) had no effect. D. Correlation between % platelet count decrease and serum titer vs PHC09 in NZB/W F1 mice. Percent platelet decrease correlated inversely with serum titer vs PHC09 (r²=0.63, n=16, p<0.05). Black and white dots refer to NZB/W F1 mice immunized with rHCV-core-envelope 1 or control protein, respectively. E. In vivo induction of thrombocytopenia in mice injected with affinity-purified anti-PHC09 IgG derived from NZB/W F1 mice immunized with rHCV-core-envelope 1. Purified control mouse IgG (Ctl IgG), and mouse IgG against PHC09 (HCV IgG) (25 ug/mouse) were injected intraperitoneally into Balb/c mice (n=4 per group).
Figure 1

A

Ab Ratio vs Clones

B

OD (405 nm)

HCV Genome

C

OD (405 nm)
Figure 2

A

B

C

D

HCV-ITP Patients

HIV-ITP Patients

HCV-HIV-ITP Patients

Platelet count (x 10^3)

HCV-ITP Patients  HIV-ITP Patients  HCV-HIV-ITP Patients
Figure 3

A

B

\[ R^2 = 0.7 \]
\[ n=15 \]
\[ P<0.01 \]

\[ R^2 = 0.526 \]
\[ n=15 \]
\[ P<0.05 \]
Figure 4

A

B

C

D

E

F

INDUCTION OF PLATELET FRAGMENTATION WITH IgG EXTRACTED FROM IMMUNE COMPLEXES OF HEPATITS C PATIENTS

INDUCTION OF PLATELET OXIDATION WITH IgG EXTRACTED FROM IMMUNE COMPLEXES OF HEPATITS C PATIENTS
Figure 5
Figure 6
Role of molecular mimickry of hepatitis C-virus (HCV) protein with platelet GPIIia in hepatitis C-related immunologic thrombocytopenia

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