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Analiza zależności pomiędzy wyczerpaniem komórek T, zmiennością genetyczną epitopów wirusa zapalenia wątroby typu C (HCV) rozpoznawanych przez komórki T, a leczeniem przeciwwirusowym względem HCV

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

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Key words: HCV, hepatitis C virus, CHC, chronic hepatitis C, genetic heterogeneity, T-cells, immune exhaustion, ant-HCV treatment, DAA, direct-acting antivirals

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Spis treści

Wykaz skrótów

CD	antygen różnicujący
DAA	leki o bezpośrednim działaniu przeciwwirusowym
ELISA	test immunoenzymatyczny
Eomes	eomesodermina
HBV	wirus zapalenia wątroby typu B
HCC	rak wątrobowokomórkowy
HCV	wirus zapalenia wątroby typu C
HIV	wirus nabytego niedoboru odporności
HLA	ludzki antygen leukocytarny
IFN	interferon
IL	interleukina
iRs	receptory hamujące
NK	komórki NK
NS	białko niestrukturalne
PBMC	komórki jednojądrzaste krwi obwodowej
PD-1	receptor programowanej śmierci komórki 1
PD-L	ligand dla receptora programowanej śmierci komórki 1
Pwzw C	przewlekłe zapalenie wątroby typu C
RNA	kwas rybonukleinowy
SVR	trwała odpowiedź wirusologiczna
Tc	komórka T cytotoksyczna
TCR	receptor komórek T
Th	komórka T pomocnicza
TIGIT	Immunoglobulina limfocytów T z domeną G oraz domeną immunoreceptorowego motywu opartego o tyrozynę
Tim-3	transbłonowa immunoglobulina i mucyna 3 limfocytu T
TNF	czynnik martwicy nowotworu
Treg	komórka T regulatorowa
WHO	Światowa Organizacja Zdrowia

Streszczenie

W przewlekłym zapaleniu wątroby typu C (pwzw C), wywoływanym przez zakażenie wirusem zapalenia wątroby typu C (HCV) dochodzi do „wyczerpania" funkcji komórek T, co skutkuje spadkiem uwalniania cytokin efektorowych, upośledzoną eliminacją zakażonych komórek i zmniejszonym potencjałem proliferacyjnym. Zjawisko to jest zależne od ciągłej stymulacji antygenowej, rozwija się wraz z czasem trwania zakażenia i towarzyszy mu ekspresja receptorów „hamujących", m. in.: PD-1 (białka programowanej śmierci komórki 1), jak i Tim-3 (transbłonowej immunoglobuliny i mucyny 3 limfocytu T) na całkowitych i swoistych dla HCV komórkach T. Receptory te hamują aktywację tych komórek po rozpoznaniu antygenu. Dodatkowo, dochodzi do zwiększenia wydzielania cytokin przeciwzapalnych, np. IL-10.

Jak dotychczas, badania nad „wyczerpaniem" komórek T w pwzw C koncentrowały się na opisie tego zjawiska, natomiast nie zostało wyjaśnione, czy „wyczerpanie" komórek T jest uzależnione od wariantu antygenowego HCV. Nie jest również pewne, czy leczenie pwzw C wpływa na to zjawisko.

Celami pracy doktorskiej były:

1) określenie zależności pomiędzy zmiennością epitopów HCV rozpoznawanych przez komórki T $CD8^+$, a „wyczerpaniem" odpowiedzi immunologicznej tych komórek w pwzw C,

2) określenie wpływu skutecznego leczenia pwzw C z udziałem DAA na stopień „wyczerpania" odpowiedzi komórek T krwi obwodowej.

Materiałem do badań była krew 97 pacjentów z pwzw C, zakwalifikowanych do terapii lekami o bezpośrednim działaniu przeciwwirusowym. Próbki pobierano przed rozpoczęciem terapii jak i sześć miesięcy po jej zakończeniu. Materiałem kontrolnym była krew 18 osób zdrowych (anty-HCV^-). Markery „wyczerpania" (poziom ekspresji PD-1 oraz Tim-3 na całkowitych oraz HCV-swoistych komórkach T) określono za pomocą wieloparametrycznej cytometrii przepływowej, a stężenie IL-10 w osoczu za pomocą metody ELISA. Zmienność genetyczną epitopów HCV rozpoznawanych przez komórki T oszacowano za pomocą sekwencjonowania następnej generacji (Illumina).

Realizacja celu 1

Wyniki analizy wykazały zależność pomiędzy sekwencjami analizowanych epitopów a fenotypem „wyczerpania" limfocytów T $CD8^+$. Zakażenie wariantem o sekwencji epitopu $NS3_{1406}$ nie reprezentującym prototypu charakterystycznego dla HCV 1b (KLSGLGLNAV), ani też wariantu reaktywnego krzyżowo (KLSSLGLNAV, KLSGLGINAV lub KLSALGLNAV) było związane z wyższym odsetkiem limfocytów T $CD8^+PD\text{-}1^+Tim\text{-}3^+$ swoistych dla HCV. Zmienność (co najmniej dwa warianty) sekwencji epitopu $NS3_{1406}$ była związana ze zwiększonym odsetkiem obwodowych komórek T o fenotypie $CD8^+PD\text{-}1^+Tim\text{-}3^+$ i niższym odsetkiem komórek T $CD8^+PD\text{-}1^-Tim\text{-}3^-$. Zakażenie dominującym wariantem epitopu $NS3_{1073}$ innym niż prototyp dla HCV 1b (CVNGVCWTV) było związane z niższym odsetkiem obwodowych limfocytów T $CD8^+PD\text{-}1^+Tim\text{-}3^+$. Wyniki te wskazują, że istnieje zależność pomiędzy odsetkiem komórek T z ekspresją receptorów PD-1/Tim-3 a sekwencją epitopów HCV rozpoznawanych przez komórki T oraz ich zmiennością oraz sugerują, że analiza zjawiska „wyczerpania" komórek T wymaga oceny kontekstu sekwencji epitopów wirusa.

Realizacja celu 2

Przed leczeniem, odsetki obwodowych komórek o fenotypie $CD4^+PD\text{-}1^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ oraz $CD8^+PD\text{-}1^+Tim\text{-}3^+$ oraz poziom IL-10 w osoczu były istotnie statystycznie wyższe, a odsetki komórek $CD4^+PD\text{-}1^-Tim\text{-}3^-$ oraz $CD8^+PD\text{-}1^-Tim\text{-}3^-$ niższe w grupie pacjentów, niż w grupie kontrolnej. Leczenie spowodowało znamienne zmniejszenie odsetków komórek T $CD4^+Tim\ 3^+$, $CD8^+Tim\text{-}3^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ oraz $CD8^+PD\text{-}1^+Tim\text{-}3^+$ oraz poziomu IL-10 w osoczu oraz równoczesny wzrost odsetków komórek T fenotypu $CD4^+PD\text{-}1^-Tim\text{-}3^-$ oraz $CD8^+PD\text{-}1^-Tim\text{-}3^-$. Nie było istotnych zmian w odsetku występowania komórek T $CD4^+PD\text{-}1^+$, podczas gdy odsetek komórek T $CD8^+PD\text{-}1^+$ znamiennie wzrósł.

Ważnym dodatkowym odkryciem było wykazanie, że pacjentów z zaawansowanym zwłóknieniem wątroby charakteryzował wyższy poziom ekspresji PD-1 i niższy poziom ekspresji Tim-3 na komórkach T $CD4^+$, a leczenie miało niewielki lub żaden wpływ na ekspresję markerów „wyczerpania" u tych pacjentów.

Częstość występowania obwodowych komórek T $CD8^+$ swoistych dla HCV uległa znamiennemu obniżeniu po leczeniu, ale poziom ekspresji PD-1 i Tim-3 na tych komórkach pozostał bez zmian.

Na podstawie powyższych wyników można stwierdzić, że skuteczne leczenie przewlekłego wirusowego zapalenia wątroby typu C jest związane ze zmniejszeniem poziomu IL-10 w osoczu oraz redukcją poziomu ekspresji markerów „wyczerpania" immunologicznego komórek T, ale efekt ten nie występuje u pacjentów z zaawansowanym zwłóknieniem wątroby. Sugeruje to, że długotrwałe zakażenie przewlekłe HCV powoduje nieodwracalne zmiany fenotypu tych komórek.

Summary

Analysis of relationships between T-cell exhaustion, genetic heterogeneity of hepatitis C virus (HCV) epitopes recognized by T-cells and anti-HCV treatment

During chronic hepatitis C (CHC), caused by hepatitis C virus (HCV) infection, T-cell functions become exhausted, which is manifested in decline in effector cytokines release, impaired elimination of infected cells, and decreased proliferative potential. This phenomenon is mediated by continuous antigenic stimulation, progresses along with time of infection and is accompanied by the expression of "inhibitory" receptors, including: PD-1 (programmed cell death protein 1) and Tim-3 (T-cell immunoglobulin and mucin domain containing protein 3) on total and HCV-specific T-cells. These receptors inhibit T-cell activation of upon antigen recognition. In addition, there is an increase in the secretion of anti-inflammatory cytokines, e.g., IL-10.

To date, studies on T-cell exhaustion in chronic hepatitis C were focused on characterization of this phenomenon, whereas it is still largely unknown whether T-cell exhaustion may be determined by HCV immune epitope variant. It is also uncertain how anti-HCV treatment modifies T-cell exhaustion.

The aims of the PhD thesis were:
1) to analyze of the relationship between T-cell exhaustion and variability of HCV epitopes recognized by $CD8^+$ T-cells;
2) to assess the effect of successful anti-HCV therapy on T-cell exhaustion.

The study material included whole blood of 97 patients with chronic hepatitis C, qualified for therapy with direct antiviral drugs. Samples were collected before the start of therapy and six months after its completion. Controls comprised blood from 18 healthy controls (anti-HCV^-). Exhaustion markers (PD-1 and Tim-3 expression on total and HCV-specific T-cells) were determined by multiparametric flow cytometry, while plasma IL-10 levels were assessed by ELISA. The genetic variability of HCV T-cell epitopes was assessed by next-generation sequencing (Illumina).

Aim 1

The results showed a relationship between the sequences of the analyzed epitopes and the phenotype of $CD8^+$ T lymphocyte exhaustion. Infection with an epitope $NS3_{1406}$ sequence not representing the HCV 1b prototype (KLSGLGLNAV) or a cross-reactive variant (KLSSLGLNAV, KLSGLGINAV or KLSALGLNAV) was associated with a higher percentage of HCV-specific $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells. Variability (at least two variants) of the $NS3_{1406}$ epitope sequence was associated with an increased percentage of $CD8^+PD\text{-}1^+Tim\text{-}3^+$ peripheral T-cells and a lower percentage of $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells. Infection with a dominant variant of the $NS3_{1073}$ epitope other than the prototype for HCV 1b (CVNGVCWTV) was associated with a lower percentage of peripheral $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells. These results indicate that there is a relationship between the percentage of T-cells expressing PD-1/Tim-3 receptors and the HCV epitopes sequence and their variability. They also suggest the importance of evaluating autologous viral epitope sequence in the investigation of $CD8^+$ T-cell exhaustion in HCV infection.

Aim 2

Before treatment the percentages of peripheral $CD4^+PD\text{-}1^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells and plasma IL-10 levels were statistically significantly higher, and the percentages of $CD4^+PD\text{-}1^-Tim\text{-}3^-$ and $CD8^+PD\text{-}1\text{-}Tim\text{-}3^-$ lower in patients than in the control group. Treatment resulted in significant decrease in the percentages of $CD4^+Tim\text{-}3^+$, $CD8^+Tim\text{-}3^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells and plasma IL-10 levels, and concomitant increase in the percentages of the $CD4^+PD\text{-}1^-Tim\text{-}3^-$ and $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells. There was no significant change in the percentage of $CD4^+PD\text{-}1^+$ T cells, while the percentage of $CD8^+PD\text{-}1^+$ T cells significantly increased.

An important additional finding was that patients with advanced liver fibrosis had higher PD-1 as well as lower Tim-3 expression levels on $CD4^+$ T-cells, and treatment had little or no effect on the expression of exhaustion markers in these patients.

The frequency of peripheral HCV-specific $CD8^+$ T-cells has significantly declined after treatment, but the expression level of PD-1 and Tim-3 on these cells remained unchanged.

Based on the above results, it can be concluded that successful treatment of chronic hepatitis C is associated with a reduction in plasma IL-10 levels and a reduction in the immune exhaustion markers expression on T-cells, but this effect was not present in

patients with advanced liver fibrosis. This suggests that long-term chronic HCV infection is related to irreversible changes in the phenotype of these cells.

Wstęp

Zakażenie wirusem zapalenia wątroby typu C (HCV) stanowi przyczynę rozwoju przewlekłego wirusowego zapalenia wątroby typu C (pwzw C), czego konsekwencją może być włóknienie, marskość, a także pierwotny rak wątrobowokomórkowy (HCC). Światowa Organizacja Zdrowia (WHO) szacuje, że na całym świecie zakażonych HCV jest 58 mln osób, a rocznie przybywa około 1.5 mln nowych zakażeń (aktualizacja 24 czerwca 2022r.) [1]. W większości przypadków (ok. 80%), zakażenie przebiega bezobjawowo, przez co jego diagnostyka na wczesnym etapie (tzw. fazie ostrej) jest utrudniona. Ponad połowa (55-80%) pacjentów rozwija zakażenie przewlekłe, a samoistna eliminacja wirusa występuje tylko w fazie ostrej zakażenia [2, 3]. Wprowadzenie nowej terapii względem HCV z zastosowaniem leków o bezpośrednim działaniu przeciwwirusowym (DAA) pozwala na wyleczenie (czyli uzyskanie tzw. trwałej odpowiedzi wirusologicznej SVR) aż 95%-100% przypadków zakażenia, co daje nadzieję na globalną eradykację HCV przy wprowadzeniu odpowiednich strategii diagnostyczno-terapeutycznych [4]. Niestety, ze względu na utrudnioną diagnostykę, wysokie koszty leczenia oraz brak profilaktycznej szczepionki, a także wybuch pandemii COVID-19, osiągnięcie tego celu jest istotnym wyzwaniem. Ponadto, dotychczasowe doświadczenia z użyciem DAA pokazują, że ryzyko rozwoju HCC u pacjentów z zaawansowaną marskością wątroby po skutecznym leczeniu nadal pozostaje wysokie [5].

Swoista odpowiedź immunologiczna gospodarza, w szczególności silna odpowiedź swoistych komórek T (pomocniczych $CD4^+$ oraz cytotoksycznych $CD8^+$) pełni bardzo ważną rolę w kontroli zakażenia HCV [6, 7]. Ich prawidłowa aktywacja, proliferacja, cytotoksyczność oraz zdolność do produkcji cytokin efektorowych jest kluczowa dla redukcji wiremii HCV oraz eliminacji wirusa z organizmu [8, 9]. Niestety, wraz z rozwojem zakażenia dochodzi do spadku funkcji efektorowych komórek T, czyli tzw. „wyczerpania" ich odpowiedzi (ang. T-cell exhaustion). Stopniowo tracą one zdolność do proliferacji, cytotoksyczności oraz produkcji interleukiny 2 (IL-2), czynnika martwicy nowotworu alfa (TNF-α), interferonu gamma (IFN-Ɣ), co przyczynia się do niewydolnej odpowiedzi przeciwwirusowej [10, 11].

„Wyczerpanie" odpowiedzi komórek T opisane zostało również w innych przewlekłych procesach zapalnych, w tym innych zakażeniach, m in. wirusem zapalenia

wątroby typu B (HBV), czy wirusem nabytego niedoboru odporności (HIV) [7, 12-15]. Uważa się, że zjawisko to przyczynia się do rozwoju przewlekłej postaci tych zakażeń [16]. Za główną przyczynę zjawiska „wyczerpania" odpowiedzi komórek T wskazuje się długotrwałą stymulację antygenową, która prowadzi do wzrostu ekspresji receptorów hamujących (iRs) na powierzchni tych komórek [12, 17-19]. Dodatkowo, w rozwoju tej dysfunkcji kluczową rolę odgrywa aktywacja komórek T regulatorowych (Treg) oraz produkcja cytokin przeciwzapalnych, m. in. interleukiny-10 (IL-10) [17, 20]. Wysoka i długotrwała ekspresja receptorów hamujących na powierzchni komórek T negatywnie wpływa na ich funkcję, m. in. poprzez kompetycję z cząsteczkami ko-stymulującymi, zaburzanie sygnałów z receptorów ko-stymulujących bądź receptorów limfocytów T (TCRs) lub aktywację genów odpowiedzialnych za dysfunkcję komórek T [21, 22].

Spośród poznanych receptorów hamujących występujących na komórkach T, najlepiej opisanymi są receptor programowanej śmierci komórki 1 (PD-1) oraz transbłonowa immunoglobulina i mucyna 3 limfocytu T (Tim-3) [12, 23]. Zarówno PD-1, jaki i Tim-3 są obecne na komórkach T $CD4^+$ oraz $CD8^+$, a receptor Tim-3 dodatkowo występuje na komórkach dendrytycznych, komórkach NK oraz na monocytach [24-26]. Interakcja PD-1 z jego ligandami na powierzchni komórek prezentujących antygen obniża wrażliwość komórek T na stymulację antygenową, natomiast analogiczna interakcja z udziałem Tim-3 obniża produkcję IFN-ϒ oraz indukuje apoptozę komórki [24, 27, 28]. Podwyższona ekspresja PD-1 i/lub Tim-3 została zaobserwowana na komórkach T całkowitych, jak i komórkach swoistych dla wirusa w zakażeniach HBV, HCV i HIV [14, 29-31]. Wykazano, że równoczesna ekspresja obu receptorów cechuje komórki w zaawansowanej fazie „wyczerpania" [28]. Jak pokazują badania, wykorzystując przeciwciała monoklonalne względem receptorom hamującym lub ich ligandom, można przywrócić komórkom T utracone funkcje [16, 24, 28, 32, 33]. Podczas gdy blokada PD-1 wpływa na polepszenie właściwości proliferacyjnych komórki oraz poprawia zdolność do produkcji IL-2 czy IFN-ϒ, jej właściwości cytotoksyczne są przywrócone dopiero przy jednoczesnej blokadzie PD-1 oraz Tim-3, wskazując na synergistyczne działanie obu receptorów [11, 29].

W przewlekłych zakażeniach wirusowych (HBV, HCV i HIV), komórki T $CD4^+$ i $CD8^+$ wykazujące ekspresję PD-1 i Tim-3 wytwarzają interleukinę 10 (IL-10) o właściwościach immunosupresyjnych, która ma chronić tkanki przed nadmierną aktywnością układu

immunologicznego, lecz dodatkowo przyczynia się do osłabienia odpowiedzi przeciwwirusowej [28, 34, 35]. Z tego względu ta również jest uznawana za marker „wyczerpania" komórek T.

HCV cechuje się niezwykle wysoką zmiennością genetyczną, która wyraża się nie tylko w postaci istnienia wielu genotypów i subtypów wirusa, ale także zmienności wewnątrzosobniczej (ang. interhost diversity), polegającej na współwystępowaniu dużej liczby wariantów w organizmie zakażonego gospodarza [40]. Zmienność wewnątrzosobnicza HCV jest wynikiem dużego tempa replikacyjnego oraz wysokiego potencjału mutacyjnego wirusowej polimerazy RNA [41]. Występowanie heterogennej genetycznie populacji HCV w jednym organizmie, określane jako zjawisko *quasispecies*, uważane jest za jedno z najważniejszych mechanizmów unikania odpowiedzi immunologicznej przez wirusa, gdyż zwiększa ono prawdopodobieństwo pozytywnej selekcji wariantów w warunkach presji układu immunologicznego, w szczególności wariantów niosących mutacje w sekwencji epitopów, które znoszą możliwość ich rozpoznawania (ang. escape mutations, mutacje warunkujące „ucieczkę") [41-44]. Zmienność HCV uznawana jest zatem za ważny czynnik sprzyjający rozwojowi zakażenia przewlekłego HCV [44].

Wzajemne oddziaływanie pomiędzy poziomem odpowiedzi komórek T, a zmiennością epitopów rozpoznawanych przez te komórki w zakażeniu HCV wydaje się być kluczową kwestią warunkującą rozwój, bądź eliminację zakażenia, gdyż największą dynamikę mutacji warunkujących „ucieczkę" (50% epitopów rozpoznawanych przez komórki T) obserwuje się w pierwszych sześciu miesiącach od zakażenia [45-48]. Zjawisko to obserwuje się niezwykle rzadko w przewlekłej fazie zakażenia, co prawdopodobnie wynika z obniżenia presji immunologicznej komórek T na skutek „wyczerpania" ich odpowiedzi [47]. Jednakże, szczegółowy wgląd we wzajemne oddziaływania pomiędzy zmiennością epitopów wirusa a „wyczerpaniem" komórek T u pacjentów z pwzw C nie był jak dotychczas przedmiotem badań.

Mimo, że są dostępne badania opisujące zjawisko „wyczerpania" odpowiedzi komórek T, funkcję receptorów hamujących oraz ich wpływ na rozwój zakażenia przewlekłego, wciąż aktualne pozostaje pytanie, czy eliminacja przewlekłego zakażenia HCV na drodze leczenia z udziałem DAA powoduje odnowienie odpowiedzi komórek T i obniżenie ekspresji receptorów hamujących oraz stężenia IL-10. Wysoka skuteczność

terapii z zastosowaniem leków DAA dostarcza doskonałego modelu do analizy tego zagadnienia.

Odnowienie przeciwwirusowej aktywności komórek T może być ważne dla powodzenia terapii, gdyż komórki te mogą brać udział w eliminacji resztkowego wirusowego RNA, wspomagając jej efekty [36, 37]. Jest ono również istotne ze względu na ryzyko wtórnego zakażenia HCV u pacjentów leczonych DAA [38, 39]. Ponadto, skuteczne szczepienie względem HCV u pacjentów wyleczonych także będzie wymagało prawidłowego funkcjonowania komórek T.

Założenia i cel pracy

Celem przedstawionej rozprawy było:

1) określenie zależności pomiędzy zmiennością genetyczną epitopów HCV rozpoznawanych przez komórki T $CD8^+$, a „wyczerpaniem" odpowiedzi immunologicznej tych komórek w pwzw C,

2) określenie wpływu skutecznego leczenia pwzw C z udziałem DAA na stopień „wyczerpania" odpowiedzi komórek T krwi obwodowej.

Odpowiedzi na powyższe postawione cele badawcze zawarto w cyklu publikacji, które ukazały się w wysoko punktowanych czasopismach o zasięgu międzynarodowym.

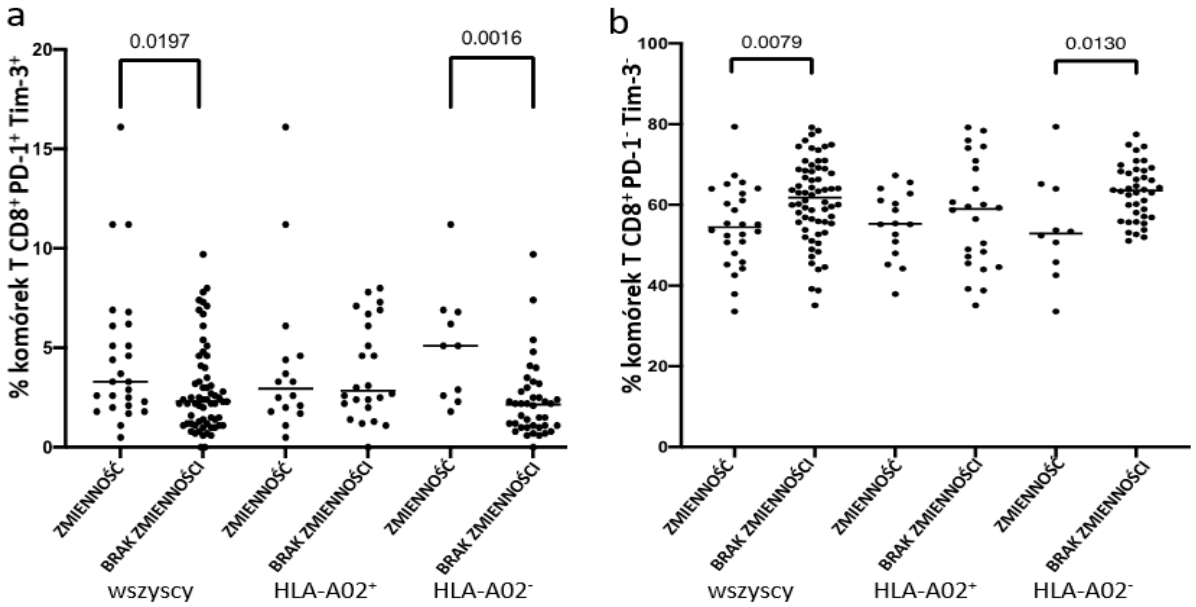
Realizacja celu 1

Analizę zmienności epitopów HCV w kontekście a fenotypu „wyczerpania" komórek T $CD8^+$ przeprowadzono u 97 pacjentów w przewlekłej fazie zakażenia genotypem 1b wirusa, od których prospektywnie pobrano próbki krwi obwodowej. Analizie poddano sekwencje najbardziej immunogennych epitopów rozpoznawanych przez te komórki, zawarte w regionie kodującym białko niestrukturalne NS3/4a: $NS3_{1073}$ oraz $NS3_{1406}$, rozpoznawane w kontekście allelu HLA-A*02, jak i $NS3_{1436}$, rozpoznawany w kontekście HLA-A*01. Sekwencje otrzymano w wyniku sekwencjonowania następnej generacji (NGS) na platformie Illumina. Metoda ta pozwala na uzyskanie milionów odczytów sekwencji, co zapewnia pełne odtworzenie różnorodności genetycznej oraz wykrywanie niszowych wariantów. Dokonano korekty błędów sekwencjonowania i rekonstrukcji wariantów epitopów przy pomocy analizy bioinformatycznej z użyciem programu Quasirecomb.

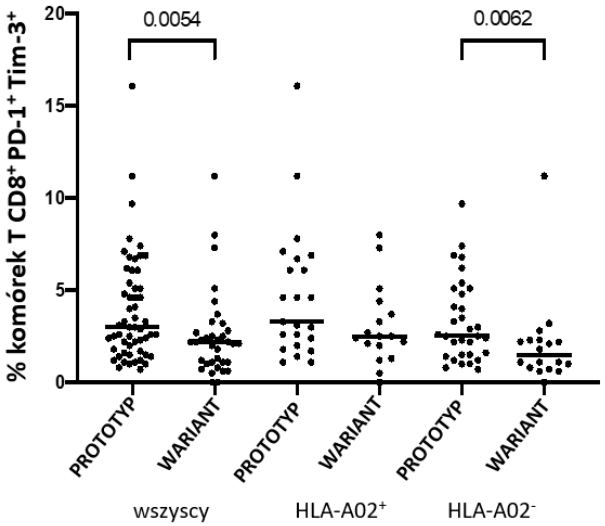
Poziom ekspresji PD-1/Tim-3 na populacji komórek T $CD8^+$ krwi obwodowej oceniono za pomocą wieloparametrycznej cytometrii przepływowej, w tym komórek T $CD8^+$ swoistych względem HCV u pacjentów HLA-$A*02^+$ wykorzystując pentamer HLA-A*02 MHC oraz metodę wzbogacenia magnetycznego.

Wyniki analizy wykazały zależność pomiędzy sekwencjami analizowanych epitopów a fenotypem „wyczerpania" komórek T $CD8^+$. W szczególności, zakażenie dominującym wariantem o sekwencji epitopu $NS3_{1406}$, nie reprezentującym prototypu

charakterystycznego dla HCV 1b (KLSGLGLNAV), ani też wariantu reaktywnego krzyżowo (KLSSLGLNAV, KLSGLGINAV lub KLSALGLNAV) było związane z wyższym odsetkiem limfocytów T $CD8^+PD\text{-}1^+Tim\text{-}3^+$ swoistych dla HCV, P=0,0102. Zmienność (co najmniej dwa warianty) sekwencji epitopu $NS3_{1406}$ była związana ze zwiększonym odsetkiem obwodowych komórek T o fenotypie $CD8^+PD\text{-}1^+Tim\text{-}3^+$ (P=0,0197) i niższym odsetkiem komórek T $CD8^+PD\text{-}1^-Tim\text{-}3^-$ (P=0,0079) (Ryc. 1). Zakażenie dominującym wariantem epitopu $NS3_{1073}$ innym niż prototyp dla HCV 1b (CVNGVCWTV) było związane z niższym odsetkiem obwodowych limfocytów T $CD8^+PD\text{-}1^+Tim\text{-}3^+$ (P=0,0054) (Ryc. 2). Rodzaj obserwowanej zależności wydaje się więc swoisty dla kontekstu danego epitopu.



Rycina 1. Odsetek komórek T $CD8^+$ wykazujących ekspresję PD-1 i Tim-3 (a) oraz brak ekspresji tych markerów (b) u zakażonych pacjentów, w zależności od występowania zmienności epitopu $NS3_{1406}$, gdzie brak zmienności oznacza obecność pojedynczego wariantu, a zmienność wskazuje na ≥ 2 warianty sekwencji. Linie poziome przedstawiają wartości mediany. Liczby nad każdą klamrą wyrażają wartości P.



Rycina 2. Odsetek komórek T $CD8^+$ wykazujących ekspresję PD-1 oraz Tim-3 u pacjentów, u których wykryto zakażenie sekwencją prototypową epitopu $NS3_{1073}$ charakterystyczną dla HCV 1b (CVNGVCWTV) lub wariantem tej sekwencji jako szczepem dominującym. Linie poziome przedstawiają wartości mediany. Liczby nad każdą klamrą wyrażają wartości P.

Wyniki te wskazują, że istnieje zależność pomiędzy odsetkiem komórek T z ekspresją receptorów PD-1/Tim-3 a sekwencją epitopów HCV rozpoznawanych przez komórki T oraz poziomem ich zmienności, co było widoczne nie tylko na poziomie całkowitych komórek T $CD8^+$, ale również swoistych dla wirusa, i sugerują, że analiza zjawiska „wyczerpania" komórek T wymaga oceny kontekstu sekwencji epitopów wirusa.

Wyniki te doceniono za innowacyjność i opublikowano w prestiżowym czasopiśmie immunologicznym (Frontiers in Immunology) w pracy pt. „$CD8^+$ T-Cell Exhaustion Phenotype in Chronic Hepatitis C Virus Is Associated With Epitope Sequence Variation".

Realizacja celu 2

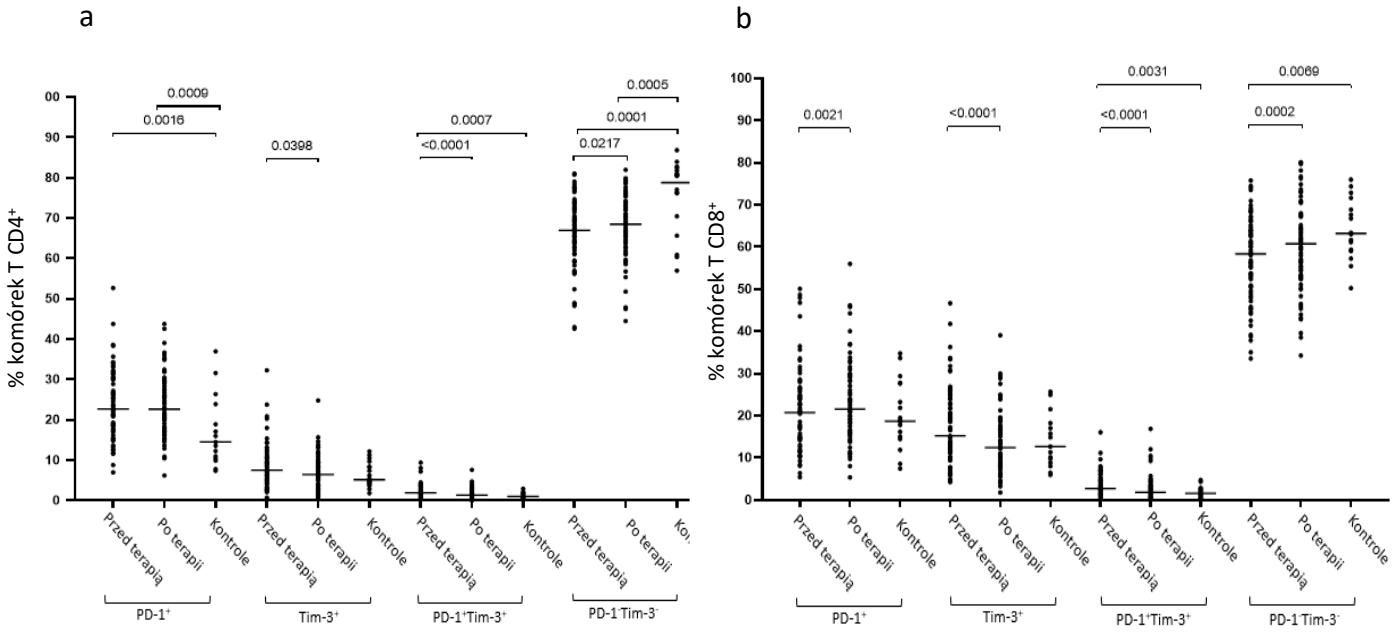
Wykorzystano pobierane prospektywnie próbki pochodzące od siedemdziesięciu sześciu pacjentów z pwzw C, zakwalifikowanych do terapii względem HCV z użyciem leków DAA. Próbki krwi pełnej były pobieranie przed rozpoczęciem terapii oraz sześć miesięcy po jej zakończeniu. Wszyscy pacjenci uzyskali trwałą odpowiedź wirusologiczną (SVR). Jako kontrole wykorzystano próbki pochodzące od osiemnastu pacjentów o potwierdzonym ujemnym statusie przeciwciał anty-HCV.

Zastosowanie nowoczesnych technik laboratoryjnych pozwoliło na wnikliwą ocenę badanych parametrów. Do analizy ekspresji receptorów hamujących na powierzchni komórek T posłużyła wieloparametryczna cytometria przepływowa. Hierarchiczny

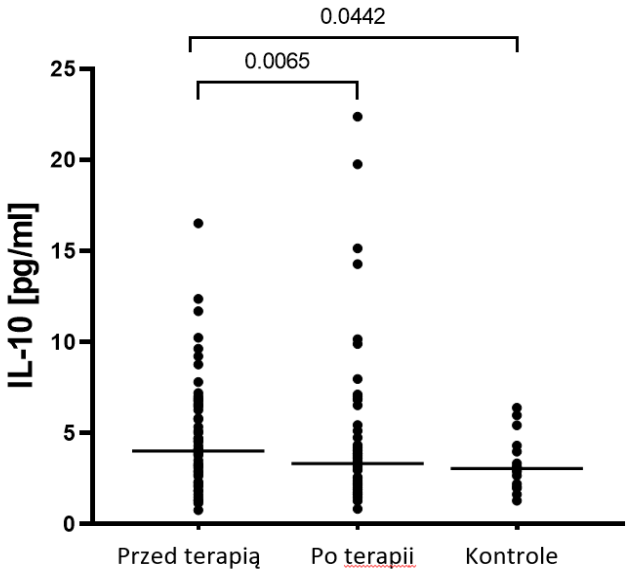
schemat bramkowania komórek pozwolił na wyodrębnienie komórek T $CD4^+$ oraz $CD8^+$, a następnie ocenę ekspresji receptorów PD-1 oraz Tim-3 na ich powierzchni.

O ile fenotyp "wyczerpania" całkowitych komórek T we krwi obwodowej jest stosunkowo łatwo ocenić, komórki HCV-swoiste są niezwykle rzadkie i trudne do wykrycia. Aby umożliwić analizę "wyczerpania" komórek T $CD8^+$ HCV-swoistych, skorzystałam z metody ich wzbogacenia w zawiesinie wg protokołu prof. Roberta Thimme (Uniwersytet we Fryburgu, Niemcy), która opierała się na połączeniu znakowania komórek z użyciem pentameru HLA-A*02 MHC sprzężonego z fluorochromem oraz ich separacji magnetycznej z użyciem mikrosfer magnetycznych wykazujących powinowactwo do tego fluorochromu. To podejście zapewniło wielokrotne zwiększenie odsetka komórek T $CD8^+$ HCV-swoistych w zawiesinie, które następnie poddano analizie cytometrycznej. Dodatkowo, zbadano stężenie IL-10 w osoczu pacjentów przy użyciu testu ELISA.

Przed leczeniem, odsetki obwodowych komórek $CD4^+PD\text{-}1^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$, $CD8^+PD\text{-}1^+Tim\text{-}3^+$ oraz poziom IL-10 w osoczu były istotnie statystycznie wyższe (odpowiednio P=0,0016, P=0,0007, P=0,0031 oraz P=0,0442), a odsetki komórek $CD4^+PD\text{-}1^-Tim\text{-}3^-$ oraz $CD8^+PD\text{-}1^-Tim\text{-}3^-$ niższe (odpowiednio P=0,0001, P=0,0069) w grupie pacjentów, niż w grupie kontrolnej (Ryc. 3 i 4). Leczenie spowodowało znamienne zmniejszenie odsetków komórek T $CD4^+Tim\text{-}3^+$ (P=0,0398), $CD8^+Tim\text{-}3^+$ (P<0,0001), $CD4^+PD\text{-}1^+Tim\text{-}3^+$ (P<0,0001) oraz $CD8^+PD\text{-}1^+Tim\text{-}3^+$ (P<0,0001) oraz poziomu IL-10 (P=0,0065) w osoczu oraz równoczesny wzrost odsetków komórek T fenotypu $CD4^+PD\text{-}1^-Tim\text{-}3^-$ (P=0,0217) oraz $CD8^+PD\text{-}1^-Tim\text{-}3^-$ (P=0,0002) (Ryc. 3 i 4). Nie było istotnych zmian w odsetku komórek T $CD4^+PD\text{-}1^+$, podczas gdy odsetek komórek T $CD8^+PD\text{-}1^+$ znamiennie wzrósł (P=0,0021) (Ryc. 3).



Rycina 3. Ekspresja markerów PD-1 oraz Tim-3 na powierzchni komórek T (a) $CD4^+$ (b) $CD8^+$ u 76 pacjentów przed i po skutecznej terapii pwzw C oraz u 18 niezakażonych kontroli. Linie poziome reprezentują mediany, a liczby powyżej klamer wyrażają wartości P.



Rycina 4. Stężenie IL-10 w osoczu 76 pacjentów przed i po skutecznej terapii pwzw C oraz u 18 niezakażonych kontroli. Linie poziome reprezentują mediany, a liczby powyżej klamer wyrażają wartości P.

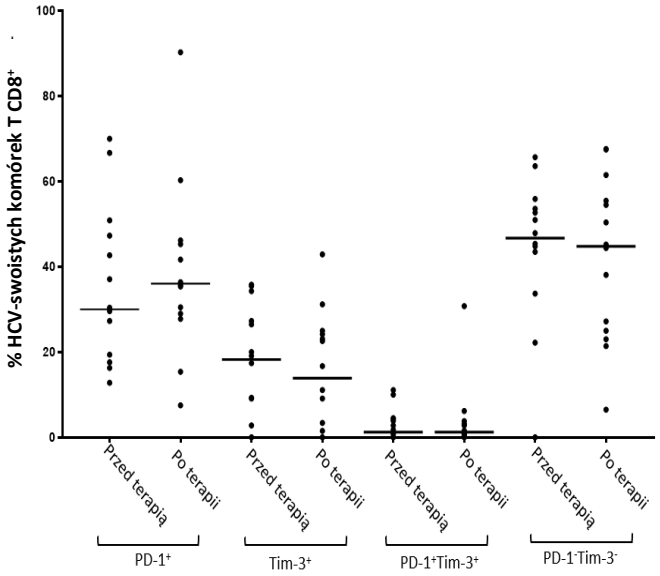
Ważnym dodatkowym odkryciem było wykazanie, że pacjentów z zaawansowanym zwłóknieniem wątroby charakteryzował wyższy poziom ekspresji PD-1 i niższy poziom ekspresji Tim-3 na komórkach T $CD4^+$, a leczenie miało niewielki lub żaden wpływ na ekspresję markerów „wyczerpania" u tych pacjentów (Tabela 1).

Tabela 1. Podsumowanie zmian w odsetkach komórek T $CD4^+$ oraz $CD8^+$ z różnym fenotypem „wyczerpania" (PD-1/Tim-3) z podziałem pacjentów ze względu na stopień zaawansowania zwłóknienia wątroby.

	Brak/łagodne zwłóknienie wątroby (F0/1)* n=38	**Umiarkowane zwłóknienie wątroby (F2)* n=25**	**Zaawansowane zwłóknienie wątroby(F3)* n=13**
SPADEK [%]	$CD4^+Tim\text{-}3^+$ $CD4^+PD\text{-}1^+Tim\text{-}3^+$ $CD8^+Tim\text{-}3^+$ $CD8^+PD\text{-}1^+Tim\text{-}3^+$ IL-10	$CD4^+PD\text{-}1^-Tim\text{-}3^-$ $CD4^+PD\text{-}1^+Tim\text{-}3^+$ $CD8^+Tim\text{-}3^+$ $CD8^+PD\text{-}1^+Tim\text{-}3^+$	$CD4^+PD\text{-}1^+Tim\text{-}3^+$
WZROST [%]	$CD4^+PD\text{-}1^+$ $CD8^+PD\text{-}1^+$ $CD8^+$ $PD\text{-}1^-Tim\text{-}3^-$	$CD8^+$ $PD\text{-}1^-Tim\text{-}3^-$	

*Stopień zwłóknienia wątroby w skali Metavir

Częstość występowania obwodowych komórek T CD8$^+$ swoistych dla HCV uległa znamiennemu obniżeniu po leczeniu (P=0,0003), ale poziom ekspresji PD-1 i Tim-3 na tych komórkach pozostał be zmian (Ryc. 5).



Rycina 5. Ekspresja markerów PD-1 oraz Tim-3 na powierzchni komórek T HCV-swoistych CD8$^+$ u 32 pacjentów przed i po skutecznej terapii pwzw C. Linie poziome reprezentują mediany, a liczby powyżej klamer wyrażają wartości P.

Na podstawie powyższych wyników można stwierdzić, że skuteczne leczenie pwzw C jest związane ze zmniejszeniem poziomu IL-10 w osoczu oraz redukcją poziomu ekspresji markerów „wyczerpania" immunologicznego komórek T, ale efekt ten nie występuje u pacjentów z zaawansowanym zwłóknieniem wątroby. Sugeruje to, że długotrwałe zakażenie przewlekłe HCV powoduje nieodwracalne zmiany fenotypu tych komórek.

Powyższe wyniki opublikowano w pracy oryginalnej pt. *„Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C".*

W trzeciej publikacji pt. *„Reversal of T Cell Exhaustion in Chronic HCV Infection",* mającej charakter pracy poglądowej, dokonano syntezy aktualnego stanu wiedzy na temat odwracalności zjawiska „wyczerpania" u pacjentów z pwzw C. Wyniki badań skupiających się na zmianach w subpopulacjach całkowitych komórek T po leczeniu w oparciu o schematy DAA pokazują, że skuteczne leczenie prowadzi do wzrostu frekwencji

komórek $CD4^+$ oraz $CD8^+$ [49, 50], a także przesunięcia w stronę populacji komórek pamięci, przy jednoczesnym spadku populacji komórek dziewiczych [49, 51]. Ponadto, zwiększa się odsetek komórek produkujących IFN-ϒ, IL-17 oraz IL-22, co wskazuje na odzyskiwanie funkcji efektorowych [52]. Wykazano również spadek ekspresji PD-1 oraz TIGIT, zarówno na komórkach $CD4^+$ jaki i $CD8^+$ [49, 51]. Niektórzy badacze obserwowali także różnice w poziomie aktywacji komórek T po skutecznym leczeniu, czego dowodem był spadek ekspresji HLA-DR oraz $CD38^+$, co również było widoczne w przypadku koinfekcji HIV-1/HCV [50, 51, 53, 54].

Podobnie do wyników uzyskanych w przedstawionej rozprawie, zespół Vranjkovic et al. zaobserwował, że zmiany fenotypu komórek T po skutecznym leczeniu są zależne od stopnia zaawansowania włóknienia wątroby. Podczas gdy u pacjentów z brakiem lub z łagodnym stopniem zwłóknienia wątroby eliminacja zakażenia skutkowała redukcją ekspresji markerów aktywacji oraz spadkiem stężenia cytokin prozapalnych, u pacjentów z zaawansowanym zwłóknieniem wątroby nie zaobserwowano takich zmian [55].

Badania nad HCV-swoistymi komórkami T $CD8^+$ również wykazały zmiany w fenotypie „wyczerpania", jak i poziomie aktywności tych komórek po skutecznej terapii [49, 56-60]. Obejmowały one spadek odsetka komórek z ekspresją PD-1 oraz wzrost funkcjonalności wyrażony polepszeniem potencjału proliferacyjnego, czy zdolności produkcji cytokin [56, 59]. Jednakże, odzyskanie stanu pełnej i prawidłowej aktywności swoistych komórek T może być niemożliwe po wieloletniej ekspozycji na antygeny HCV [58, 60]. Badania wskazują, że po skutecznym leczeniu komórki te, zwłaszcza komórki pamięci, wykazują odmienny fenotyp od tych, które powstają przy samoistnej eliminacji zakażenia (zwiększona ekspresja PD-1 oraz Eomes, lepsze zdolności efektorowe) [58, 59].

Podsumowanie i wnioski

1. Zaobserwowano zależność pomiędzy odsetkiem komórek T z ekspresją receptorów PD-1/Tim-3 a sekwencją epitopów HCV rozpoznawanych przez komórki T oraz poziomem ich zmienności, co było widoczne nie tylko na poziomie całkowitych komórek T $CD8^+$, ale również swoistych dla wirusa. Rodzaj obserwowanej zależności wydaje się swoisty dla kontekstu danego epitopu. W szczególności, zakażenie dominującym wariantem o sekwencji epitopu $NS3_{1406}$, nie reprezentującym prototypu charakterystycznego dla HCV 1b (KLSGLGLNAV), ani też wariantu reaktywnego krzyżowo (KLSSLGLNAV, KLSGLGINAV lub KLSALGLNAV) była związana z wyższym odsetkiem limfocytów T $CD8^+PD\text{-}1^+Tim\text{-}3^+$ swoistych dla HCV. Analiza zjawiska „wyczerpania" komórek T powinna brać pod uwagę kontekst sekwencji epitopów wirusa.

2. Skuteczne leczenie przewlekłego wirusowego zapalenia wątroby typu C z zastosowaniem leków o bezpośrednim działaniu przeciwwirusowym (DAA) jest związane ze zmniejszeniem poziomu IL-10 w osoczu oraz redukcją poziomu ekspresji markerów „wyczerpania" immunologicznego komórek T, ale efekt ten nie występuje u pacjentów z zaawansowanym zwłóknieniem wątroby. Sugeruje to, że długotrwałe zakażenie przewlekłe HCV powoduje nieodwracalne zmiany fenotypu tych komórek.

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CD8$^+$ T-Cell Exhaustion Phenotype in Chronic Hepatitis C Virus Infection Is Associated With Epitope Sequence Variation

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Background and Aims: During chronic hepatitis C virus (HCV) infection, CD8$^+$ T-cells become functionally exhausted, undergoing progressive phenotypic changes, i.e., overexpression of "inhibitory" molecules such as PD-1 (programmed cell death protein 1) and/or Tim-3 (T-cell immunoglobulin and mucin domain-containing molecule-3). The extreme intrahost genetic diversity of HCV is a major mechanism of immune system evasion, facilitating epitope escape. The aim of the present study was to determine whether T-cell exhaustion phenotype in chronic HCV infection is related to the sequence repertoire of NS3 viral immunodominant epitopes.

Methods: The study population was ninety prospective patients with chronic HCV genotype 1b infection. Populations of peripheral blood CD8$^+$ T-cells expressing PD-1/Tim-3 were assessed by multiparametric flow cytometry, including HCV-specific T-cells after magnetic-based enrichment using MHC-pentamer. Autologous epitope sequences were inferred from next-generation sequencing. The correction of sequencing errors and genetic variants reconstruction was performed using Quasirecomb.

Results: There was an interplay between the analyzed epitopes sequences and exhaustion phenotype of CD8$^+$ T-cells. A predominance of $NS3_{1406}$ epitope sequence, representing neither prototype KLSGLGLNAV nor cross-reactive variants (KLSSLGLNAV, KLSGLGINAV or KLSALGLNAV), was associated with higher percentage of HCV-specific CD8$^+$PD-1$^+$Tim-3$^+$ T-cells, P=0.0102. Variability (at least two variants) of $NS3_{1406}$ epitope sequence was associated with increased frequencies of global CD8$^+$PD-1$^+$Tim-3$^+$ T-cells (P=0.0197) and lower frequencies of CD8$^+$PD-1$^-$Tim-3$^-$ T-cells (P=0.0079). In contrast, infection with $NS3_{1073}$ dominant variant epitope (other than prototype CVNGVCWTV) was associated with lower frequency of global CD8$^+$PD-1$^+$Tim-3$^+$ T-cells (P=0.0054).

Conclusions: Our results indicate that PD-1/Tim-3 receptor expression is largely determined by viral epitope sequence and is evident for both HCV-specific and global $CD8^+$ T-cells, pointing to the importance of evaluating autologous viral epitope sequences in the investigation of $CD8^+$ T-cell exhaustion in HCV infection.

Keywords: hepatitis C virus, T-cell exhaustion, PD-1, Tim-3, epitope sequence

INTRODUCTION

Adaptive immune responses play a critical role in the clinical course of infection with hepatitis C virus (HCV) (1). Vigorous and polyfunctional cellular responses are indispensable for rapid viral load reduction and spontaneous recovery from HCV infection, which is observed in about 20%-50% of patients with acute hepatitis C (2–5). Previous studies have shown that $CD8^+$ T-cell responses targeting multiple epitopes, in both structural and nonstructural viral proteins, are associated with viral clearance (5), while only a narrow set of epitopes are targeted in chronic infection (6, 7).

The extreme intrahost genetic diversity of HCV is the result of fast replication and high error rate of viral replicase (RNA-dependent-RNA polymerase) (8). It is manifested by the *quasispecies* phenomenon, which is the concomitant presence of closely related, but not identical genetic variants within an infected host, facilitating adaptive dynamics of the virus (8). This feature is postulated to be a major mechanism of immune system evasion, because of the increased probability of positive selection of escape variants within immune epitopes under immune pressure of the host (9–11). The heterogeneity of HCV epitopes may have direct clinical implications, including the development of chronic infection (11).

Previous studies have shown that there is an interplay between the strength of T-cell response in HCV infection and epitope escape; this may determine the outcome of acute infection, in particular the positive selection of mutations, which induce only a weak and thus potentially insufficient $CD8^+$ T-cell response (12, 13). Evidence for $CD8^+$ T-cell-mediated pressure was found both in the envelope and non-envelope proteins, including non-structural protein 3 (NS3) (14–16). NS3-derived antigens from HCV genotype 1B are presented in the HLA (human leucocyte antigen) context and contain many well defined $CD4^+$ and $CD8^+$ T cell epitopes, which are considered to be the most immunogenic among all HCV antigens (17).

Viral escape mutations typically occur within the first six months of infection in approximately 50% of the $CD8^+$ T-cell-targeted epitopes (18, 19). In contrast, escape mutations during chronic infection are rare, which is likely due to the weak T-cell-mediated selection pressure (18). Escape mutations may be located at various positions within virus-specific $CD8^+$ T-cell epitopes: HLA class I binding anchor, T-cell receptor contact site or the flanking region (20, 21). Amino acid substitutions may lead to altered proteasomal cleavage, including the loss of the original epitope (15, 22), impaired binding to the MHC molecule (19, 23), or compromised TCR recognition of mutated peptide–MHC complex (19).

In contrast to acute resolving infection, the quality of T-cell responses significantly deteriorates during chronic antigen stimulation, including progressive negative changes of their phenotype, function, and both epigenetic and transcriptional profile (24, 25). While both $CD8^+$ and $CD4^+$ HCV-specific T-cells may be present in liver tissue and peripheral blood, they are unable to clear the infection in most patients and do not prevent reinfection with HCV due to their functional exhaustion (1). T-cell exhaustion manifests itself as impairment of antiviral effector functions of antigen-specific T-cells: decline in the effector cytokines production, impaired elimination of infected cells, and decrease in the proliferative potential after antigen exposure *in vitro* (26–28). It is the persistent antigen exposure which is believed to be the major factor promoting T-cell exhaustion, but $CD4^+$ T-cells deletion, activation of regulatory T-cells and increased anti-inflammatory cytokine (e.g. IL-10) production contribute as well (29, 30).

Phenotypic hallmarks of T-cell exhaustion are increased expression of "inhibitory" molecules, among them PD-1 (programmed cell death protein 1) and Tim-3 (T-cell immunoglobulin and mucin domain-containing molecule-3) on global and antigen-specific T-cells, which deliver negative signals precluding cell activation after antigen exposure (31).

PD-1/PD-L1 (programmed cell death protein 1 receptor/programmed cell death protein 1 ligand) inhibitory molecules are part of a regulatory pathway that has been reported to inhibit the virus-specific $CD8^+$ cell function in lymphocytic choriomeningitis virus (LCMV) infection (32, 33). Engagement of the PD-1 receptor and its ligand inhibits cell cycle and synthesis of effector cytokines (32, 33). Previous studies performed both in acute and in chronic HCV infections indicated that PD-1 is expressed by HCV-specific $CD8^+$ and $CD4^+$ T-cells and that the blocking of the PD-1/PD-L1 pathway by anti-PD-L1 antibodies can improve proliferation of HCV-specific $CD8^+$ cells (27, 28).

Exhausted T-cells do not always express PD-1, and the blocking of the PD-1/PD-L1 signaling pathway does not necessarily reconstitute Th1/Tc1 cytolytic function, suggesting that other inhibitory molecules may contribute to the exhaustion associated with chronic viral infections (34–36). One such molecule is Tim-3. Increased frequencies of Tim-3-expressing $CD4^+$ and $CD8^+$ T-cells have been observed in chronic HCV infection and were particularly high on HCV-specific $CD8^+$ T-cells (37). Tim-3 expression correlates with a dysfunctional phenotype and reduced Th1/Tc1 cytokine production, but not with viral load. Blocking the Tim-3/Tim-3L interaction *in vitro* enhanced T-cell proliferation and cytolytic function in response to HCV antigens (36, 37). A single expression of PD-1 or other

co-inhibitory receptors does not necessarily define a state of exhaustion in contrast to co-expression of multiple co-inhibitory receptors (38, 39). Interestingly, these co-expression patterns are functionally related, as a concurrent blocking of these multiple co-inhibitory receptors leads to synergistic reversal of exhaustion (40–42). For example, *in vitro* blocking of PD-1 alone failed to restore the functions of hepatic PD-1$^+$CTLA-4$^+$ HCV-specific CD8$^+$ T-cells, but a concurrent blocking of CTLA-4 and PD-1 reinvigorated these cells in a CD4$^+$ T-cell–independent manner (26).

The quality of T-cell response in the context of HCV viral autologous sequence in chronic infection is poorly understood, especially with respect to immune exhaustion. In particular, the relationship between PD-1/Tim-3 T-cell exhaustion phenotype, especially in the context of the co-expression of these receptors and the in-depth diversity of immune epitopes, is largely unknown. Furthermore, methodological limitations of conventional Sanger sequencing of cloned viral variants allow for the detection of major variants in a genetically diverse viral population. With the advent of next-generation sequencing (NGS), it is now possible to routinely detect variants present at low frequencies, which would remain undetected by standard sequencing methods (43, 44).

The diversity of viral epitopes and immune exhaustion represent major hindrances to spontaneous viral clearance and successful vaccine design (45). Since recent HCV vaccines failed in clinical trials, it is of major importance to elucidate the mechanisms behind successful HCV-specific immunity (46, 47). HCV infection represents a unique immunological experimental model, since it is one among few human viral infections with a dichotomous outcome (viral clearance *vs* chronic infection) and can be cured by highly specific small molecule drugs (45, 48). Thus, the aim of the present study was to determine whether T-cell exhaustion phenotype in chronic HCV infection is related to the sequence repertoire of viral immune epitopes. The analysis was restricted to immunodominant epitopes within NS3 viral gene: $NS3_{1073}$ and $NS3_{1406}$, which are commonly recognized in HLA-A*02-positive patients and $NS3_{1436}$ which are recognized in HLA-A*01-positive patients. The study provides evidence that T-cell exhaustion phenotype in chronic HCV infection is related to the polymorphisms of HCV NS3 immune epitopes, but this is highly dependent on the restricting HLA allele context.

MATERIALS AND METHODS

Patients

The study encompassed 90 prospectively enrolled patients with chronic HCV infection (anti-HCV$^+$, HCV RNA$^+$), presenting for treatment at the Outpatient Clinic of the Warsaw Hospital for Infectious Diseases. The source and timing of infection were unknown in most patients. However, all patients were HCV RNA positive for at least six months prior to therapy. All but one patient achieved sustained virologic response (SVR) (negative PCR test detecting HCV RNA six months post-treatment of sensitivity ≤15 IU/mL). Inclusion criteria were infection with genotype 1b, no evidence of cirrhosis, and no other potential cause of chronic liver disease. Thirty-six mL of EDTA-anti-coagulated whole blood was collected from all patients before treatment and six months post-treatment in the single non-responder. HCV genotype was determined by Inno-LiPA HCV II (Innogenetics N.V., Gent, Belgium) and baseline viral load as well as SVR status were assessed by RealTime HCV Viral Load Assay (Abbott) (sensitivity 12 IU/mL). Some clinical and virological characteristics of analyzed patients are presented in **Table 1**.

Antibodies and Pentamers

Mouse anti-human anti-CD3-peridinin-chlorophyll protein-Cyanine5.5 (PERCP-CY5.5) clone UCHT1, anti-CD4-BD Horizon V500 clone RPA-T4, anti-PD-1-Alexa Fluor 647 clone EH12.1 and anti-Tim-3 (CD366)-Brilliant Violet (BV421) Clone 7D3, antibodies were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Anti-CD8-fluorescein isothiocyanate (FITC) LT8 clone antibody and custom Pro5 Recombinant MHC class I Pentamer containing HLA-A*02-restricted HCV $NS3_{1406}$ immunodominant epitope KLSGLGLNAV (corresponding to genotype 1b), conjugated with phycoerythrin (PE), were purchased from Pro-Immune (Oxford, United Kingdom). The latter epitope is one of the most immunogenic in chronically HCV-infected patients and displays cross-reactivity with other epitope variants (50, 51). BD Horizon Fixable Viability Stain 780 (BD Biosciences), an amine-reactive dye, was used to discriminate viable from non-viable lymphocytes. As isotype controls, IgG1 κ ALEXA 647 and IgG1 κ BV421 (BD Biosciences) were used.

HLA-A Typing

The presence of the HLA-A*02 allele was verified by flow cytometry using anti-HLA-A*02-FITC clone BB7.2 antibody (BD Biosciences) and by quantitative PCR as described elsewhere (52). The presence of the HLA-A*01 allele was

TABLE 1 | Clinical, laboratory and virological characteristics of 90 patients with chronic hepatitis C.

Sex [male/female]	29/61
Age [years]	
median (range)	58.0 (25-88)
mean±SD	56.7±1.6
Serum ALT activity [U/mL]	
median (range)	61 (19-389)
mean±SD	79.1±6.3
normal values: 7–56 U/mL	
Fibrosis score determined by FibroScan[a]	F0/1, n=50 F2, n=26 F3, n=14 F4, n=0
Viral load [U/mL]	
median (range)	8.4×10^5 (6.2×10^3 -1.1×10^7)
mean±SD	$1.4\times10^6\pm1.8\times10^5$

[a]*F0/F1 represents no or minimal fibrosis, F2 moderate fibrosis, F3 severe fibrosis, and F4 represents cirrhosis (49).*

verified by flow cytometry using anti-HLA-A*01-biotin conjugated antibody and streptavidin-PE (both from United States Biological, Salem, USA) and by qualitative PCR as described previously (53).

T-Cell Phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from 36 ml of EDTA-anticoagulated blood by density gradient centrifugation using Lymphoprep, Stemcell Technologies Inc., Vancouver, British Columbia, Canada, according to the manufacturer's protocol. After isolation, cells were passed through a 70 µm cell strainer (BD Biosciences), resuspended in Phosphate Buffered Saline pH 7.2 (Life Technologies, Carlsbad, USA), and counted. Next, 25 million freshly isolated PBMCs were stained with BD Horizon Fixable Viability Stain 780 (BD Biosciences), resuspended in the Pharmingen Stain Buffer with 0.2% (w/v) bovine serum albumin (BD Biosciences) and pre-incubated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells from HLA-A*02-positive patients were subjected to enrichment of HCV-specific $CD8^+$ T-cells by magnetic separation. In brief, pentamer was added to the cells and the mixture was incubated for 10 minutes at room temperature in the dark. The cell suspension was then washed with 4 ml of MACS Buffer (Miltenyi Biotec). Cell pellets were resuspended with Anti-PE Micro Beads (Miltenyi Biotec) and MACS Buffer and incubated for 20 minutes at 4˚C, protected from light. The cell suspension was washed twice with MACS Buffer and passed through a 70 µm cell strainer (BD Biosciences). Magnetic MS Columns (Miltenyi Biotec) were used to perform the separation following the manufacturer's recommendations. Enriched cells were counted and stained with anti-CD3, -CD4, -CD8, -PD-1, -Tim-3 antibodies for 20 minutes at 4°C. Controls consisted of unstained cells and fluorescence minus one (FMO) with Anti-IgG1 Alexa Fluor 647 and Anti-IgG1 BV421 instead of anti-PD-1 and anti-Tim-3, respectively. After washing twice with PBS, cells were resuspended in 300 µl of the Pharmingen Stain Buffer, immediately acquired on FACS Canto II instrument (Becton-Dickinson, Mountain View, USA) and analyzed by BD FACS Diva software (Becton-Dickinson). Typically, one million stained cells per sample were analyzed. Additionally, a separate PBMC sample (both from HLA-A*02-positive and HLA-A*02-negative subjects) was directly stained with antibodies against surface molecules (without pentamer staining step) and analyzed as above.

Next-Generation Sequencing of Immune Epitopes

Diversity analysis of HLA-restricted $NS3_{1073}$, $NS3_{1406}$ and $NS3_{1436}$ immunodominant epitopes contained within NS3/4a viral gene was conducted by next-generation amplicon sequencing. First, RNA was extracted from one mL of plasma using NucleoSpin RNA Virus-Kit (Macherey-Nagel, Düren, Germany), purified from any contaminating DNA using DNA-free DNA Removal Kit (Ambion, Austin, Texas, United States) and then subjected to reverse transcription using PrimeScript Reverse Transcriptase (Takara, Kusatsu, Shiga, Japan). Amplicon of 2223 bp encompassing the NS3/4a and containing $NS3_{1073}$, $NS3_{1406}$ and $NS3_{1436}$ encoding regions (nt 3466-5689 of H77 reference genome, GenBank accession number AF009606) was obtained in two-step PCR using outer primers FW 5'-GGCGTGTGGGGACATCATC-3' (nt 3314-3332), RV 5'-GGCTGTGAATGCCATCAGTGATG-3' (nt 5704-5726) and inner primers FW 5'- GCATCATCACTAGCCTCACAGG-3' (nt 3466-3487), RV 5'-CCAGGCAGAGTGGACAAGC-3' (positions 5671-5689) and Platinum *Taq* DNA High Fidelity Polymerase on GeneAmp 9700 cycler (Applied Biosystems Foster City, California, USA). PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and elongation at 68°C for 2 min 30s. Each PCR product was purified from agarose gel by Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel) and subjected to tagmentation and double indexing using Nextera XT Sample preparation Kit (Illumina, San Diego, California, United States). The run was performed on MiSeq (Illumina) platform using MiSeq Reagent v3 kit 2x300 bp, (Illumina).

Data Analysis

For cytometric analyses, an initial lymphocyte gate was set based on side scatter (SSC)/forward scatter (FSC) and additional gates (single T-cells, live cells, $CD3^+$, $CD4^+$, $CD8^+$, pentamer-positive, $PD\text{-}1^+$, $Tim\text{-}3^+$ cells) were introduced based on the appropriate controls of unstained cells and FMO.

For NGS analyses, amplicon sequence reads were filtered for quality (Phred score >20) yielding a depth of 53483.9 ± 18676 reads per sample covering epitopes $NS3_{1406}$ and $NS3_{1436}$ and 10119.4 ± 4758.2 per sample covering epitope $NS3_{1073}$ (mean ± SD), corrected for sequencing errors and reconstructed into populations of genetic variants using Quasirecomb software (54). The latter uses a jumping hidden Markov model to infer *quasispecies* sequences along with their frequencies from the next-generation sequencing data. Based on the previously estimated sequencing error of the similar analysis (including MMLV-based reverse transcriptase, *Taq* polymerase, and Illumina sequencing), the maximal aggregate error rate would be about 1×10^{-2} per site (55) and thus, accordingly, one percent cutoff of frequency was applied to the reconstructed variants in order to exclude erroneous variants from the analysis. Next, the amino acid composition of epitopes variants ($NS3_{1073}$, $NS3_{1406}$ and $NS3_{1436}$) was assessed using MEGA 6.0 software (56) and represented graphically using WebLogo generator (57). The GenBank EU255962.1 sequence was selected as the prototype 1b HCV strain, as it showed the highest similarity to patients' epitope sequences. Variability of epitope was defined as the presence of at least two variants at a frequency >1%. Minor epitope variant was defined to be the second and subsequent most frequent in a sample and its frequency was between 1% and 30%.

Statistical Analysis

Results were verified for normal distribution by the Kolmogorov–Smirnov test and expressed as mean values ± standard error (SE) or median (range). The Mann-Whitney U test/Fisher Exact Test was used to compare expression of

immune exhaustion markers on $CD8^+$ T-cells and viral epitope diversity parameters. Immune selection was assumed to be present when mutations within the viral epitope were more frequent in patients carrying the relevant HLA allele than in patients without the allele. All P-values were two-tailed and considered significant when ≤0.05.

Ethical Statement

The study protocol followed ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Bioethical Committee of the Medical University of Warsaw (Approval Number KB/77/A/2015). All patients provided written informed consent.

RESULTS

Frequency of HLA-A*01 and HLA-A*02 Alleles

Forty (44.4%) patients displayed the presence of the HLA-A*02 allele, and 30 (33.3%) displayed the HLA-A*01 allele. Of these, five patients (5.6%) manifested the presence of both HLA-A*01 and HLA-A*02 alleles. Twenty-five patients (27.8%) were both HLA-A*01 and HLA-A*02-negative. The diversity analysis of HLA-A*02-restricted $NS3_{1073}$, $NS3_{1406}$ HCV epitopes and HLA-A*01-restricted $NS3_{1436}$ HCV epitope was successful in all 90 patients.

HLA-A*02-Restricted $NS3_{1073}$ Epitope Was Moderately Conserved

$NS3_{1073}$ epitope was moderately conserved, both at interhost (i.e., between patients) and intrahost (i.e., within patient) levels. Only in eight patients (8.9%) a maximum of two variants were present (major and minor variant), while in the remaining 82 (91.1%) a single variant was detected. The prototype $NS3_{1073}$ CVNGVCWTV variant, representing the most common variant found of HCV 1b (e.g., GenBank EU255962.1) was present in 57 (63.3%) patients (in 55 as a single variant and in two as a minor variant). The remaining patients displayed the presence of various epitope sequences, among which: CINGVCWTV [previously described as a 1b escape variant (58)] was present as a major variant in 25 patients and as a minor variant in five; CINGVCWSV was a major variant in two patients; CINGACWTV was a major variant in three patients; CVNGACWTV was a major variant in two patients; CINGVCWTA, CVNGVCWSV, and CLNGVCWTV were major variants in one patient each, respectively; and CVNGVC*TV was a minor variant in one. Distribution of dominant aminoacid sequences in epitope $NS3_{1073}$ among HLA-A*02-positive and HLA-A*02-negative patients are presented on **Supplementary Figure S1**.

There was no difference in the presence of intrahost aminoacid variability at $NS3_{1073}$ epitope in neither HLA-A*02-positive nor HLA-A*02-negative patients (2/38 *vs* 6/44, P=0.2920). Similarly, there was no significant difference between HLA-A*02-positive and HLA-A*02-negative patients in the prevalence of dominant prototype $NS3_{1073}$ CVNGVCWTV *vs* variant epitope sequence (*i.e.*, other than prototype $NS3_{1073}$ CVNGVCWTV as a dominant sequence) (n=23/17 *vs* 32/18), P=0.6638 (**Figure 1**).

Infection With a Variant $NS3_{1073}$ Epitope Was Associated With Lower PD-1/Tim-3 Expression on $CD8^+$ T-Cells

When analyzing the entire cohort (*i.e.*, both HLA-A*02-positive and HLA-A*02-negative patients) there were no differences in the percentages of global $CD8^+$ T-cells expressing either PD-1 and/or Tim-3 or negative for both PD-1 and Tim-3 in patients with presence/absence of intrahost aminoacid variability in the $NS3_{1073}$ epitope. Similarly, no differences were present in either HLA-A*02-positive or HLA-A*02-negative subgroups (**Figure 2**).

When analyzing the entire cohort (i.e., both HLA-A*02-positive and HLA-A*02-negative patients) there were no differences in the percentages of global $CD8^+$ T-cells expressing either PD-1 or Tim-3 or expressing neither PD-1 nor Tim-3 in patients harboring prototype or variant epitope as the dominant strain (**Figure 2**). However, infections with a variant epitope (i.e., other than the prototype $NS3_{1073}$ CVNGVCWTV) as the major sequence were associated with significantly lower percentages of global $CD8^+$ T-cells co-expressing PD-1 and Tim-3 (median 2.2 *vs* 3.0, P= 0.0054 (**Figure 2**). This was also true for both HLA-A*02-positive and HLA-A*02-negative subgroups, but the difference reached statistical significance only in the latter (1.45 *vs* 2.55, P=0.0062).

HLA-A*02-Restricted $NS3_{1406}$ Epitope Displayed High Level of Variability

$NS3_{1406}$ epitope was the most variable, both at interhost and intrahost levels. The most prevalent $NS3_{1406}$ epitope sequence [which was present in 35 (28.23%) of patients] was KLSGLGLNAV, consistent with the prototype 1b sequence (GenBank EU255962.1) and pentamer, followed by KLSSLGLNAV [previously shown to be cross-reactive with prototype sequence (50)] (n=27, 21.77%), KLSSLGINAV (n=13, 10.48%), KLSGLGINAV [cross-reactive with prototype (50)], (n=12, 9.68%), KLSALGINAV (n=5, 4.03%), KLSALGLNAV [cross-reactive with prototype (51)] and KLSSLGVNAV in three patients each (2.42%), KLLSLGINAV, KLSGLGLNAI, and QLSSLGLNAV in two patients each (1.61%), RLLALGINAV, QLSGLGVNAV, KLMGLGVNAV, KLSGLGMNAV, KLSSLSINAV, KLTALGINAV, KLSNLGINAI, KLLSLGLNAV, KLSTLGINAV, KLLALGINAV, KLLGLGINAV, KLVGLGVNAV, KLTALGLNAV, KLSGLGFNAV, KLSSLGVSAV, KLSSLGISAV, QSSSLGLNAV, KLSTLGLNAV, QLSSLGINAV, and KLSSLSIN-A in one patient each (0.8%). Distribution of dominant $NS3_{1406}$ amino acid sequences in HLA-A*02-positive and HLA-A*02-negative patients is presented in **Supplementary Figure S2**.

Sixty-four (71.11%) patients displayed no variability within this epitope. Among the remaining 26 patients, two variants were present in 22 (24.44%), three in two (2.22%) and five variant sequences were present in the other two patients (2.22%). Only four amino acid positions (1407, 1410, 1413, 1414) were conserved among the analyzed variants (**Supplementary Figure S2**). The number of circulating variants was higher in HLA-A*02-positive than in HLA-A*02-negative patients (mean 1.6 *vs* 1.2), P=0.0210.

Prototype 1b $NS3_{1406}$ KLSGLGLNAV was the dominant sequence in 14 out of 40 (37.5%) HLA-A*02-positive and in 15 out of 50 (30%) HLA-A*02-negative patients (P=0.6548;

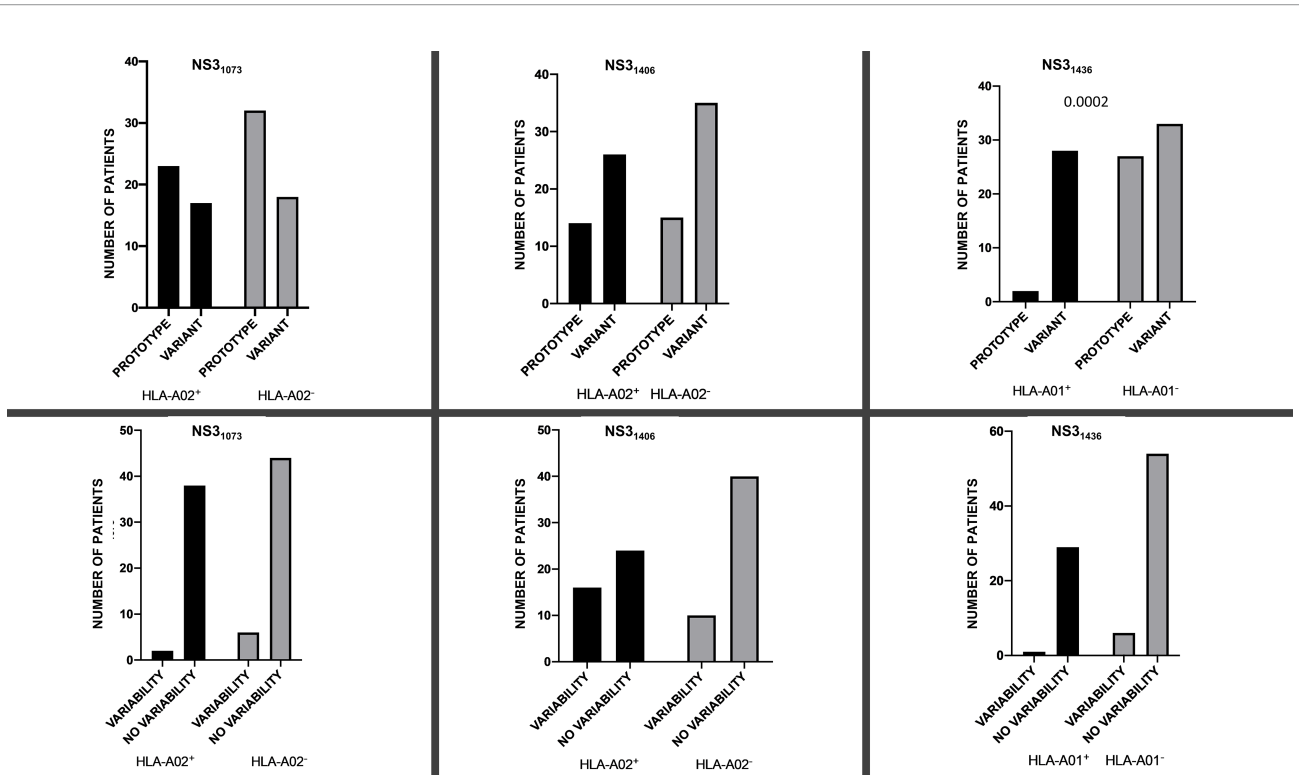

FIGURE 1 | Prevalence of prototype 1b (GenBank EU255962.1) variant (upper panel) dominant sequence and presence/absence of variability (*i.e.*, ≥2 variants) (lower panel) for $NS3_{1073}$, $NS3_{1406}$ and $NS3_{1436}$ epitopes in the context of the restricting HLA-A*01/HLA-A*02 alleles.

Figure 1). Similarly, there was no significant difference in the prevalence of domination of 1b prototype $NS3_{1406}$ KLSGLGLNAV or cross-reactive KLSSLGLNAV, KLSGLGINAV or KLSALGLNAV sequence between HLA-A*02-positive (25 out of 40, 62.5%) and HLA-A*02-negative (35 out of 50, 70%) patients, P=0.5044. While HLA-A*02-positive patients were more likely to display intrahost aminoacid variability in the $NS3_{1406}$ epitope (≥ 2 sequences), this difference did not reach statistical significance (P=0.0601; **Figure 1**).

$NS3_{1406}$ Epitope Sequence Variability Was Associated With Higher PD-1/Tim-3 Expression on CD8$^+$ T-Cells

Variability of $NS3_{1406}$ epitope was not associated with PD-1, Tim-3 and PD-1 and Tim-3 expression on global peripheral CD8$^+$ T-cells in HLA-A*02-positive subjects, but when all enrolled patients were analyzed, variability (≥ 2 variants) was associated with increased percentage of global CD8$^+$ PD-1$^+$Tim-3$^+$ T-cells (P=0.0197) and with lower percentage of CD8$^+$ T-cells negative for both exhaustion markers (P=0.0079) (**Figure 3**). A similar relationship was found in HLA-A*02-negative subjects: $NS3_{1406}$ epitope variability was associated with higher percentage of global CD8$^+$ PD-1$^+$Tim-3$^+$ T-cells (P=0.0016) and lower percentage of CD8$^+$ PD-1$^-$Tim-3$^-$ T-cells (P=0.0130) (**Figure 3**).

When analyzing the entire cohort and either HLA-A*02-positive or HLA-A*02-negative patients, there were no statistically significant differences in the percentages of global CD8$^+$ T-cells expressing either PD-1 and/or Tim-3 or neither PD-1 nor Tim-3 between patients with the prototype $NS3_{1406}$ KLSGLGLNAV or variant epitope as a dominant sequence (**Figure 3**).

$NS3_{1406}$ Epitope Sequence Other Than Prototype or Cross-Reactive Variants Was Associated With Higher PD-1/Tim-3 Expression on HCV-Specific CD8$^+$ T-Cells

In HLA-A*02-positive subjects there was no association between the variability within the $NS3_{1406}$ epitope and the percentage of HCV-specific cells or PD-1/Tim-3 expression on these cells. Furthermore, patients in whom the prototype sequence dominated were not different with respect to the above parameters from the other patients (**Figure 4**). However, predominance of an epitope sequence representing neither prototype $NS3_{1406}$ KLSGLGLNAV nor cross-reactive variants (KLSSLGLNAV, KLSGLGINAV or KLSALGLNAV) was associated with significantly higher percentage of HCV-specific CD8$^+$ T-cells with co-expression of PD-1 and Tim-3, P= 0.0102.

HLA-A*01 -Restricted $NS3_{1436}$ Epitope Was Highly Conserved

$NS3_{1436}$ epitope was the most conserved both at interhost and intrahost levels as only two sequence variants were identified (ATDALMTGY and ATDALMTGF) and only in 7 patients (7.8%) ≥ 2 sequences were present. The prototype ATDALMTGY $NS3_{1436}$ variant (GenBank EU255962.1), representing the most common variant found in HCV 1b, was present in 34 (37.8%)

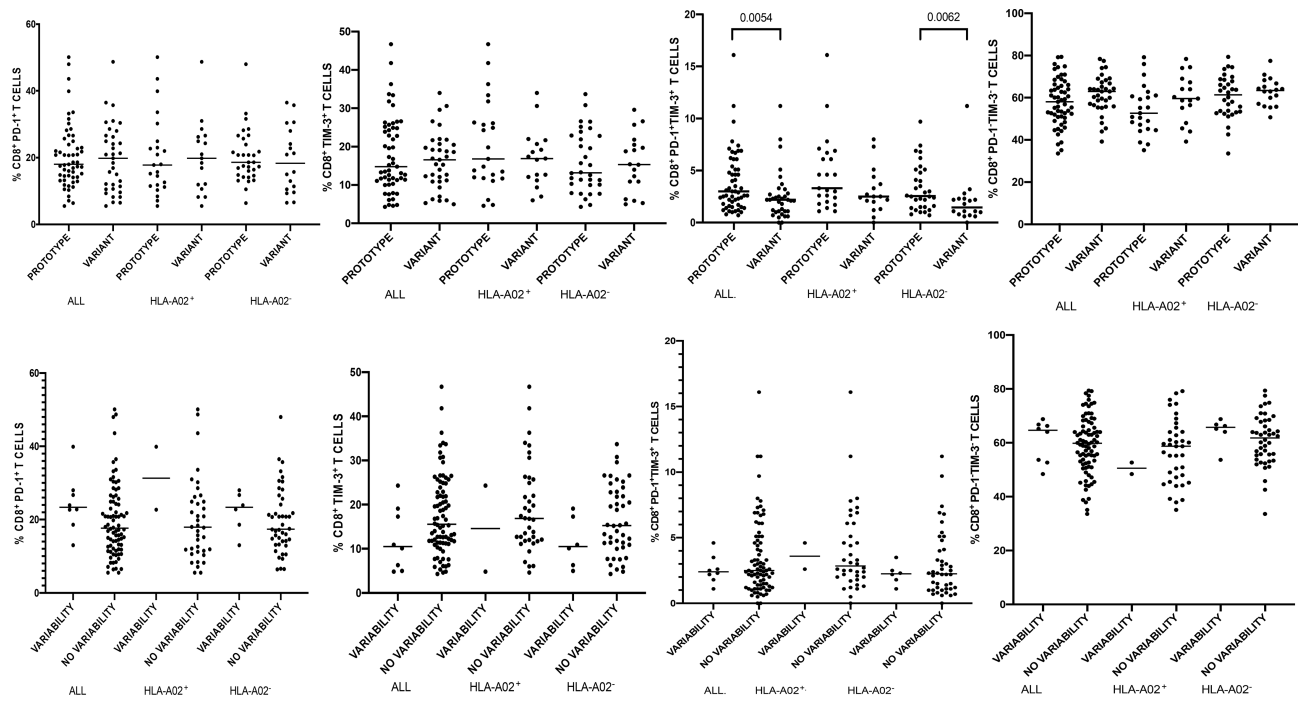

FIGURE 2 | Percentages of peripheral blood CD8$^+$ T-cells expressing PD-1/Tim-3 in patients infected with either $NS3_{1073}$ prototype (GenBank EU255962.1) or variant epitope sequence as the dominant strain (upper panel). Lower panel shows intrahost aminoacid variability of this epitope where no variability denotes the presence of a single variant and variability indicates ≥ 2 variant sequences. Horizontal lines represent median values. Numbers above each bracket express P values.

patients, (in 29 as a single variant and in five as a minor variant). ATDALMTGF, which was previously described in both prospective studies of primary infection and in cross-sectional studies of chronic disease as a viral escape variant in HLA*A1-positive patients (6, 59), was present in 61 patients as a sole variant and in two as a minor variant (70.0% of patients overall).

There was no difference in the presence of intrahost aminoacid variability among either HLA-A*01-positive or HLA-A*01-negative patients (one out of 30 *vs* 6 out of 60, respectively P= 0.4170), **Figure 1**. However, in almost all HLA-A*01-positive patients (28 out of 30, 93.3%), the ATDALMTGF escape sequence predominated, while in patients without this allele this was much less common (33 out of 60, 55%, P= 0.0002 (**Figure 1**). Interestingly, in HLA-A*01-positive patients all minor variants were prototypes while in HLA-A*01-negative patients, two were escape variants and one was a prototype. Distribution of dominant $NS3_{1436}$ sequence in HLA-A*01-positive and in HLA-A*01-negative patients is presented on **Supplementary Figure S3**.

$NS3_{1436}$ Epitope Sequence Variation and CD8$^+$ T-Cell PD-1/Tim-3 Expression Phenotype

When analyzing the entire cohort (i.e., both HLA-A*01-positive and HLA-A*01-negative patients), there were no significant differences in the percentages of global CD8$^+$ T-cells expressing either PD-1 and/or Tim-3 or neither PD-1 nor Tim-3 in patients with the presence and absence of intrahost aminoacid variability within $NS3_{1436}$ epitope (**Supplementary Figure S4**). Similarly, no significant differences were present in the HLA-A*01-negative subgroup, while in HLA-A*01-positive patients there were too few values for a meaningful statistical comparison.

When all patients harboring either prototype ATDALMTGY or variant epitope ATDALMTGF as the dominant sequence were compared, there were no differences in the percentages of global CD8$^+$ T-cells expressing either PD-1 and/or Tim-3 or expressing neither PD-1 nor Tim-3 (**Supplementary Figure S4**). This comparison could not be done for the HLA-A*01-positive subgroup, as only two patients were infected with ATDALMTGY. However, these two patients displayed lower PD-1 and higher Tim-3, as well as PD-1 + Tim-3 expression, on global CD8$^+$ T-cells than patients harboring HCV ATDALMTGF variant (**Supplementary Figure S4**).

Within HLA-A*01-negative patients, there were no differences in the expression of exhaustion markers on global CD8$^+$ T-cells between patients infected with different variants (**Supplementary Figure S4**).

Evolution of Epitopes and Exhaustion Markers in the Patient Not Responding to Treatment

We investigated dynamics of exhaustion markers in the context of viral epitopes variability in the only treatment-naïve non-responder patient treated for 8 weeks with Harvoni. Despite negative viral load at week 4 and 8 of treatment, this patient experienced viral load rebound

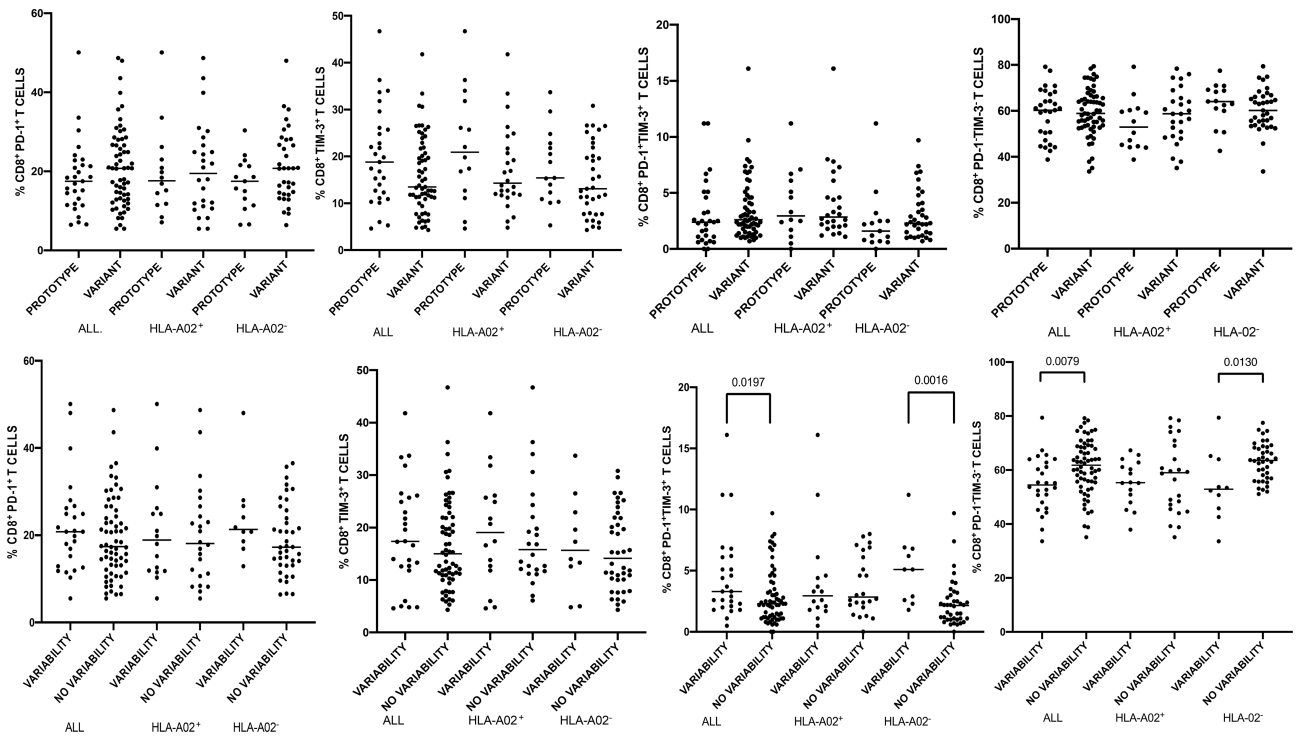

FIGURE 3 | Percentages of peripheral blood CD8$^+$ T-cells expressing PD-1/Tim-3 in patients infected with either $NS3_{1406}$ prototype (GenBank EU255962.1) or variant epitope sequence as the dominant strain (upper panel). Lower panel shows intrahost aminoacid variability of this epitope where no variability denotes the presence of a single variant and variability indicates ≥ 2 variant sequences. Horizontal lines represent median values. Numbers above each bracket express P values.

between the end of treatment and SVR assessment six months post-treatment. His viral load was 821000 before and 643000 IU/ml after treatment. The patient was HLA-A*02-positive, HLA-A*01-negative. Initially (*i.e.*, prior to treatment), this patient displayed the presence of $NS3_{1073}$ escape epitope CINGACWTV which reverted to prototype CVNGVCWTV after unsuccessful treatment. Concomitantly, there was a change in $NS3_{1406}$ sequence from KLSALGLNAV to QLSSLGLNAV. Furthermore, despite the absence of HLA-A*01 allele in this patient, the viral escape $NS3_{1436}$ epitope sequence ATDALMTGF reverted to the prototype ATDALMTGY (**Supplementary Figure S5**). All these post-treatment variants were not detected prior to treatment even below the cutoff value of 1%. Concomitantly, the frequency of global CD8$^+$ T-cells expressing either PD-1 and/or Tim-3 have decreased and the frequency of CD8$^+$ global T-cells expressing neither PD-1 nor Tim-3 increased (**SupplementaryFigure S5**).

DISCUSSION

The aim of the present study was to determine whether T-cell exhaustion phenotype in chronic HCV infection is related to the repertoire of viral immune epitopes. According to our best knowledge, this is the first study of its kind. We studied a large prospective cohort of chronic hepatitis C patients infected with the same viral subgenotype (1b), as it was previously shown that the patterns of viral adaptation to host's immune pressure reflected by HLA-associated viral epitope polymorphisms may vary considerably between the genotypes, despite the presence of the same HLA allele (60). We assessed exhaustion phenotype at a single-cell level on both global CD8$^+$ and HCV-specific T-cells employing multiparametric flow cytometry combined with magnetic-based enrichment of the latter cells using MHC-pentamers.

We found that the PD-1/Tim-3 inhibitory receptors expression pattern is associated with specific autologous viral epitope sequence and its intrahost variability and this was evident for both HCV-specific and global CD8$^+$ T-cell populations. Importantly, the type of association seemed to be epitope-specific, which warrants evaluation of autologous viral epitope sequences when investigating CD8$^+$ T-cell exhaustion.

Although viral pathogens encode numerous potentially immunogenic determinants, CD8$^+$ T-cells recognize and respond only to a very small fraction of these potential epitopes. This phenomenon is called immunodominance and is strongly restricted by particular HLA alleles (61). Immunodominant HCV-specific CD8$^+$ T-cell epitopes located in E2, NS3 or NS5B are targeted in the vast majority of patients expressing the respective HLA type (62–64). Available studies of natural HCV infection and using adenovirus-based vaccine immunogens revealed that NS3 immunodominant CD8$^+$ T-cell responses to HCV 1b include $NS3_{1406}$ KLSGLGINAV, $NS3_{1073}$ CVNGVCWTV restricted by HLA class-I A*02 allele, and $NS3_{1436}$ ATDALMTGY restricted by

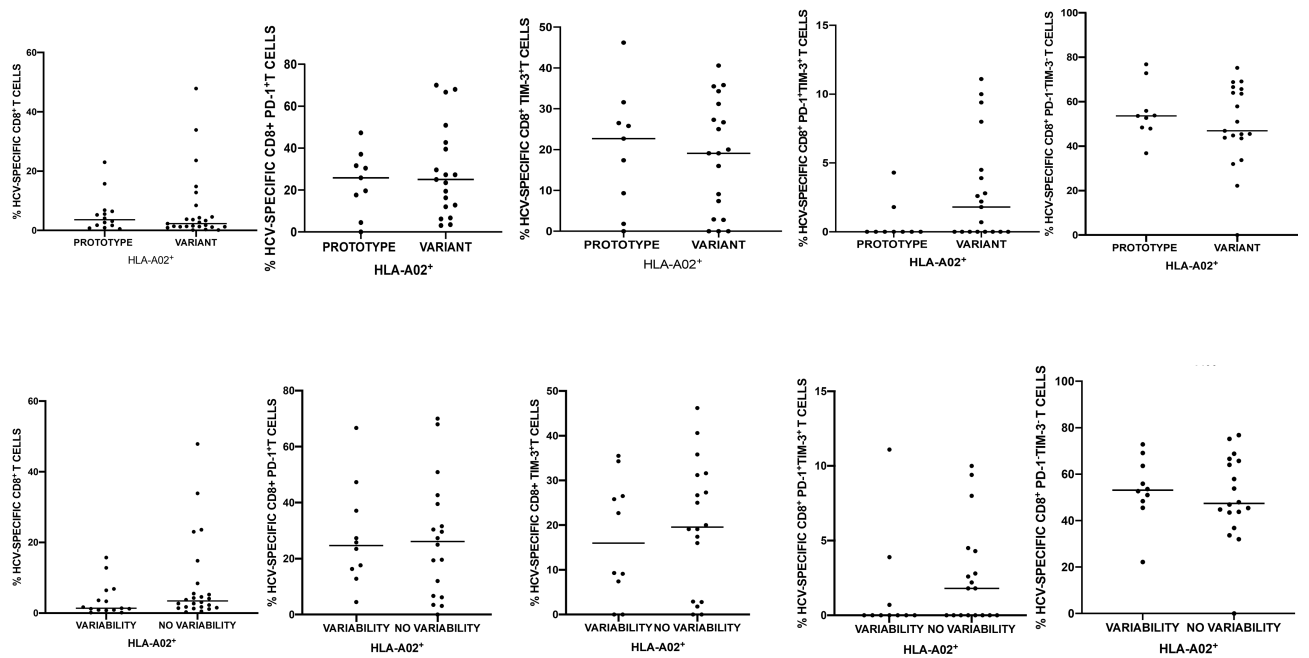

FIGURE 4 | Percentages of peripheral blood HCV-specific $CD8^+$ T-cells expressing PD-1 and Tim-3 in 40 HLA-A*02-positive patients harboring $NS3_{1406}$ prototype (GenBank EU255962.1) or variant epitope sequence as the dominant strain (upper panel). Lower panel shows intrahost aminoacid variability of this epitope where no variability denotes the presence of a single variant and variability indicates ≥ 2 variant sequences. Horizontal lines represent median values.

HLA-A*01 alleles (6, 16, 17, 50, 59, 65). Since these two HLA-A alleles are the most prevalent in the Polish population (44.4% of patients were HLA-A*02-positive, 33.3% patients were HLA-A*01-positive), we narrowed the analysis to immunodominant epitopes $NS3_{1073}$, $NS3_{1406}$ and $NS3_{1436}$.

The analyzed epitopes displayed variable levels of amino acid diversity. The most variable both at interhost and intrahost levels was $NS3_{1406}$ epitope, suggesting the highest tolerance of this region to amino acid substitutions (**Supplementary Figure S2**). This is consistent with the study by Kelly et al. in which epitope $NS3_{1406}$ was highly variable, with single amino acid sequence present in only 25% of genotype 1 infected individuals (50). In contrast, in our study epitope $NS3_{1073}$ was only moderately variable (**Supplementary Figure S1**) and $NS3_{1436}$ displayed almost no variability (**Supplementary Figure S3**), which is consistent with previous observations (22) and implies strong functional constraints and high "fitness cost" related to viral regions encoding these epitopes. Viral escape mutations of high "fitness cost" have been previously observed in some epitopes with a limited sequence variability in the NS3/NS4a region (66). Mutations at positions 1074, 1075, 1076 and 1079 of the $NS3_{1073}$ epitope were reported to negatively affect the replicative fitness of the virus (66) and thus some escape mutations within $CD8^+$ T-cell epitopes may be restricted due to incompatibility with replicative viral capacity (67–69). Consequently, certain viral escape mutations can only evolve together with compensatory mutations that retain viral replication (70, 71).

Importantly, our study showed that immunodominance exerts selective pressure within the analyzed NS3 epitopes, since there were statistically significant differences in epitope sequence variability and/or their number (complexity) in the presence or absence of the restricting HLA-A allele. In particular, in patients harboring HLA-A*02 allele, the number of $NS3_{1406}$ epitope variants was higher than in HLA-A*02-negative patients. Similarly, the HLA-A*01 allele was associated with escape of the $NS3_{1436}$ epitope, since almost all HLA-A*01-positive patients displayed dominant ATDALMTGF escape epitope sequence (Y1444F substitution), while patients without this allele displayed either dominant escape ATDALMTGF or prototype ATDALMTGY (**Supplementary Figure S3**). This suggests that HLA-A*01-positive subjects quite commonly target this epitope. In line with this hypothesis, it was previously found that 39% of HLA-A*01-positive subjects had a detectable *ex vivo* response against this epitope (59). It was reported that the Y1444F substitution impairs binding to the HLA-A*01 molecule and is sufficient to abrogate $CD8^+$ T-cell recognition, which may have an important impact on the ability to prime a functional response upon infection (72). On the other hand, this mutation was shown to have a negative impact on viral fitness, since a helicase activity of the protein containing the Y1444F substitution is reduced when compared to the prototype sequence (72). Interestingly, our study showed that minor epitope variants in HLA-A*01-positive patients were exclusively prototypes while in HLA-A*01-negative patients these were mostly escape variants which, again, may indicate immune-driven selection of this epitope and high fitness cost of the Y1444F substitution.

Previously, the Y1444F substitution has been observed in association with the HLA-A*01 allele in subjects with chronic

infection indicating that mutational escape is common (16, 59). This may confirm the presence of the previously reported HLA class I associated epitope sequence polymorphisms, so called "HLA class I footprints", observed on a population level and manifesting as mutations in autologous viral sequences only in patients positive for the restricting HLA class I allele (10, 14, 16, 59, 60, 73, 74). Moreover, it was revealed that, at least for some CD8 epitopes, identical escape mutations are selected in subjects sharing the same restricting HLA class I alleles (16, 59, 74, 75). The first evidence for selective pressure by $CD8^+$ T-cells comes from studies on chimpanzees sharing the same MHC class I alleles and persistently infected with HCV. These studies demonstrated that mutations within viral epitopes occur shortly after infection and are not random, as the rate of nonsynonymous substitutions was higher in MHC class I restricted epitopes compared to non-restricted epitopes or nearby regions (76). For example, within $NS3_{1073}$, $NS3_{1131}$ and $NS3_{1169}$ epitopes, the mutation rate was 10-fold higher than in conserved regions (77). Similarly, analysis of an Irish outbreak cohort has shown that mutations within the known HLA class I-restricted epitopes were more common than mutations at other sites (74). Furthermore, mutations in these epitopes were more frequent in the presence of particular alleles, pointing to immune selection (74). Studies using a population-based approach have shown that most viral escape mutations revert to wild-type prototype sequence upon transmission to a new host negative for the restricting HLA class I allele because of the high fitness cost incurred by these mutations (15, 78). The fact that these mutations are stable in chronic HCV- infected subjects suggests that selective pressures still operate during the chronic phase of infection (15).

Our study suggests that there was an interplay between the analyzed epitope sequences and the exhaustion phenotype of $CD8^+$ T-cells, reflected by PD-1/Tim-3 expression. In particular, in HLA-A*02-positive patients the predominance of an epitope sequence representing neither prototype $NS3_{1406}$ KLSGLGLNAV nor cross-reactive variant (KLSSLGLNAV, KLSGLGINAV or KLSALGLNAV) was associated with significantly higher percentage of HCV-specific cells $CD8^+$ T-cells with co-expression of PD-1 and Tim-3. Given that it is the co-expression of PD-1 and Tim-3 that defines the state of more profound exhaustion (40, 41), these results indicate that variations from the genotype-specific consensus sequence may be associated with immune exhaustion.

When analyzing the entire cohort, i.e., both HLA-A*02-positive and HLA-A*02-negative patients, $NS3_{1406}$ intrahost variability (i.e., the presence of at least two epitope variants) was associated with increased frequencies of global $CD8^+$ T-cells with PD-1 and Tim-3 co-expression, and with lower frequencies of $CD8^+$ T-cells without these exhaustion markers (**Figure 3**). This suggests that the higher exhaustion is related to the presence of multiple variants, possibly due to limited immune surveillance and natural viral evolution. However, this observation was not confirmed in the subgroup of HLA-A*02-positive patients in whom this epitope should be recognized, as the analyzed group was probably too small.

In the case of $NS3_{1073}$ epitope, infection with a variant epitope (i.e., other than the genotype-specific prototype) was associated with significantly lower percentage of global $CD8^+$ T-cells with co-expression of PD-1 and Tim-3, implying lower exhaustion (**Figure 2**). While this was statistically significant for the entire group (both HLA-A*02-positive and HLA-A*02-negative patients), it was not for the subgroup of HLA-A*02-positive patients in whom this epitope should be recognized, possibly due to too small number of patients analyzed. A similar phenomenon was observed in the case of $NS3_{1436}$ epitope where HLA-A*01-positive patients harboring $NS3_{1436}$ escape sequence displayed higher percentages of PD-1^+ $CD8^+$ T-cells and lower percentages of Tim-3^+ as well as PD-1^+Tim-3^+ $CD8^+$ T-cells than patients with the prototype sequence (**Supplementary Figure S4**). However, these data should be interpreted with caution due to the limited number of observations.

Although there are only a few studies investigating PD-1/Tim-3 expression on $CD8^+$ T-cells in the context of epitope variability in HCV infection, some studies showed that the phenotype of HCV-specific $CD8^+$ T-cells is determined by the level of antigen-specific stimulation. Expression of memory marker CD127 defines $CD8^+$ T-cells that do not recognize cognate antigen because of viral variation (79). Similarly, Bengsch et al. found that co-expression of $CD8^+$ inhibitory receptors such as 2B4, CD160 and KLRG1 in association with PD-1 represented exhausted phenotype and was associated with low and intermediate levels of CD127 expression, an impaired proliferative capacity, an intermediate T-cell differentiation stage, and an absence of sequence variations within the corresponding viral epitopes, indicating ongoing antigen triggering (38). Noteworthy, opposite expression profiles of inhibitory receptors were observed within the same patient depending on the autologous epitope sequence: in the presence of $NS3_{1406}$ epitope escape all $NS3_{1406}$ specific $CD8^+$ T-cells expressed CD127 and low levels of PD-1, 2B4, CD160 and KLRG1, whereas an opposite phenotype was observed in case of $NS5_{2594}$ specific $CD8^+$ T-cells, which were characterized by low CD127 expression, but high PD-1, 2B4, CD160 and KLRG1 (38). Similar studies on HIV-1 and SIV infection revealed high level of PD-1 expression upon recognition of the cognate epitope and subsequent decrease after *in vivo* selection of cytotoxic T lymphocyte escape mutations in the respective epitopes (80–82). These results again imply that the mechanisms responsible for inhibitory receptor expression operate in an epitope-specific manner depending on the autologous virus sequence. Thus, both viral escape and T-cell exhaustion, defined by the expression of multiple inhibitory receptors, seem to contribute to the ineffective viral control present in chronic HCV infection. Currently, it is unclear whether HCV epitopes sequence variation drives the T-cell exhaustion observed in chronic HCV infection or whether it is the consequence of exhausted T-cell phenotype and ensuing loss of viral control.

Administration of anti-HCV treatment may change the repertoire of the viral *quasispecies* because of the new selective drug-related pressure acting on the virus (83). Consequently, unsuccessful therapy may be related to alteration of the composition of the viral variants, which would affect the T-cell exhaustion phenotype. Therefore, by studying the evolution of the viral antigens driven by treatment, we can verify how this affects T-cell exhaustion. Thus, we aimed to investigate the

dynamics of exhaustion markers expression in the context of viral epitopes sequence in one treatment-naïve HLA-A*02-positive, HLA-A*01-negative patient who experienced a viral relapse between the end of 8-week treatment with Harvoni and SVR assessment, and thus represented a unique case because of otherwise highly effective treatment. Based on epidemiological data, this patient was unlikely to be re-infected with a different HCV strain. Interestingly, reversal of escape $NS3_{1073}$ epitope CVNGVCWTV to prototype CVNGVCWTV after treatment was accompanied by change of $NS3_{1406}$ sequence from KLSALGLNAV to QLSSLGLNAV (**Supplementary Figure S5**). Furthermore, despite the absence of HLA-A*01 allele, $NS3_{1436}$ escape epitope sequence ATDALMTGF changed to the prototype ATDALMTGY. Importantly, these post-treatment variants were not detected prior to treatment at any frequency, which suggests the presence of pre-existent minor strain at a very low frequency, or even *de novo* evolution. Concomitantly, the exhaustion markers expression on global $CD8^+$ T-cells has decreased. Although multiple other factors could have contributed to the decrease of exhaustion phenotype after treatment, these results may also indicate that epitope sequence is a factor affecting exhaustion phenotype. In this case, loss of the escape variant (possibly due to treatment-related pressure) and appearance of prototype sequence (possibly a more replication-competent form) could have contributed to the reinvigoration of T-cell response after treatment. This could be because $CD8^+$ T-cells targeting escaped epitopes were not exposed to constant T-cell receptor stimulation anymore and thus acquired a memory-like state rather than an exhaustion phenotype and sustained proliferative potential (38, 79). Similarly, in the study of Wieland et al., viral relapse and thus antigen re-exposure led to phenotypic and qualitative changes including a vigorous expansion of HCV $NS3_{1073}$-specific $CD8^+$ T-cells that was accompanied by re-generation of terminally exhausted effector subsets (84). These results also suggest that $CD8^+$ T-cells are able to re-expand efficiently in response to antigen re-exposure.

Despite being the largest of its kind, our study has several limitations. First of all, with the exception of one patient, the analysis was confined to a single time point and thus the changes of $CD8^+$ T-cell exhaustion markers and HCV NS3 epitope variant sequences over time remain unknown. Furthermore, our study was focused on phenotypical rather than functional markers of $CD8^+$ T-cell exhaustion and the functional status of these cells remains to be determined.

Next, the analysis was confined to patients infected with subgenotype 1b, which, while reducing the confounding effects of varied genotypes, implies that the findings are not necessarily valid for other genotypes due to epitope polymorphism (6, 58, 60, 79).

It is known that various immune cells may support low level HCV replication. While $CD8^+$ T-cells are relatively rarely infected (85, 86) it cannot be ruled out that infection of these cells *per se* and/or the presence of unique, lymphotropic viral variants, could have affected their exhaustion status. Undoubtedly, elucidating the effects of extrahepatic HCV infection on $CD8^+$ T-cells exhaustion status would offer an interesting future direction of research.

Finally, although all patients fulfilled the criteria for chronic infection, having been HCV RNA positive for at least 6 months prior to the study, the exact duration of infection was unknown for the majority of them. However, this is not unusual as symptomatic manifestation of *de novo* infection is rare, subclinical course of the disease is the norm, and potential exposure (*e.g.*, parenteral drug abuse, infected family member, infected sex partner) is often spread over many years. Furthermore, in a substantial proportion of patients the route of transmission remains unknown as no risk factors are identified (87). Transfusion of blood or blood products, once the major source of HCV infection, became extremely rare in the developed countries after the introduction of sensitive tests for blood screening in 1992 (88).

CONCLUSIONS

In summary, our study showed that analyzed NS3 epitopes are exposed to HLA-A allele related selective pressure, which was manifested in the respective restricting epitope sequence polymorphisms (escape of the $NS3_{1436}$ epitope in HLA-A*01-positive patients) or intrahost variability (higher number of $NS3_{1406}$ epitope variants in HLA-A*02-positive patients). Furthermore, our results provide evidence that the PD-1/Tim-3 inhibitory receptors expression pattern is associated with specific autologous viral epitope sequence and/or its intrahost variability and is evident for both HCV-specific and global $CD8^+$ T-cell populations. Importantly, the type of observed association seems to be epitope specific. In particular, infection with predominant $NS3_{1406}$ epitope representing neither prototype nor cross-reactive sequence was associated with higher percentage of $CD8^+$ PD-1^+Tim-3^+ HCV-specific T-cells. Our study points to the importance of evaluating autologous viral epitope sequences in the investigation of $CD8^+$ T-cell exhaustion in HCV infection.

DATA AVAILABILITY STATEMENT

The NGS datasets presented in this study can be found in the NCBI SRA BioProject, accesion no: PRJNA795441.

ETHICS STATEMENT

The study protocol followed ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Bioethical Committee of the Medical University of Warsaw (Approval Number KB/77/A/2015). All patients provided a written informed consent.

AUTHOR CONTRIBUTIONS

Conceptualization: KC, MR, and TL. Data curation: SO, KP, and HB. Data analysis: KC, SO, KP, AP, and MZ. Funding acquisition:

KC. Investigation: SO, KC, and IB-O. Methodology: SO, KC, MR, and HB. Software: KP, KC, and MZ. Supervision: MR, TL, and KC. Manuscript review: SO, TL, KP, HB, IB-O, AP, MZ, MR, and KC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.832206/full#supplementary-material

Supplementary Figure S1 | Distribution of dominant aminoacid sequences in epitope $NS3_{1073}$ among HLA-A*02-positive and HLA-A*02-negative patients. Amino acids are colored according to their chemical properties: polar amino acids **(G, S, T, Y, C, Q, N)** are green, basic **(K, R, H)** blue, acidic **(D, E)** red, and hydrophobic **(A, V, L, I, P, W, F, M)** amino acids are black. Height of letters within the stack indicates the relative frequency of each amino acid at this position. Sequence logos were generated using WebLogo generator (57).

Supplementary Figure S2 | Distribution of dominant aminoacid sequences in epitope $NS3_{1406}$ among HLA-A*02-positive and HLA-A*02-negative patients. Amino acids are colored according to their chemical properties: polar amino acids **(G, S, T, Y, C, Q, N)** are green, basic **(K, R, H)** blue, acidic **(D, E)** red and hydrophobic **(A, V, L, I, P, W, F, M)** amino acids are black. Height of letters within the stack indicates the relative frequency of each amino acid at this position. Sequence logos were generated using WebLogo generator (57).

Supplementary Figure S3 | Distribution of dominant aminoacid sequences in epitope $NS3_{1436}$ among HLA-A*01-positive and HLA-A*01-negative patients. Amino acids are colored according to their chemical properties: polar amino acids **(G, S, T, Y, C, Q, N)** are green, basic **(K, R, H)** blue, acidic **(D, E)** red and hydrophobic **(A, V, L, I, P, W, F, M)** amino acids are black. Height of letters within the stack indicates the relative frequency of each amino acid at this position. Sequence logos were generated using WebLogo generator (57).

Supplementary Figure S4 | Percentages of peripheral blood $CD8^+$ T-cells expressing PD-1/Tim-3 in 90 patients infected with either $NS3_{1436}$ prototype (GenBank EU255962.1) or variant epitope sequence as the dominant strain (upper panel). Lower panel shows intrahost aminoacid variability of this epitope where no variability denotes the presence of a single variant and variability indicates ≥ 2 variant sequences. Horizontal lines represent median values.

Supplementary Figure S5 | Evolution of dominant aminoacid sequences in epitope $NS3_{1073}$, $NS3_{1406}$, $NS3_{1436}$ and percentages of $CD8^+$ T-cells expressing PD-1/Tim-3 in the non-responder to treatment. PRE-TX-before treatment, POST-TX- post-treatment. Amino acids are colored according to their chemical properties: polar amino acids **(G, S, T, Y, C, Q, N)** are green, basic **(K, R, H)** blue, acidic **(D, E)** red and hydrophobic **(A, V, L, I, P, W, F, M)** amino acids are black. Sequence logos were generated using WebLogo generator (57).

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Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C

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During chronic hepatitis C virus (HCV) infection, both $CD4^+$ and $CD8^+$ T-cells become functionally exhausted, which is reflected by increased expression of programmed cell death-1 (PD-1) and T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), and elevated anti-inflammatory interleukin 10 (IL-10) plasma levels. We studied 76 DAA-treated HCV-positive patients and 18 non-infected controls. Flow cytometry measured pretreatment frequencies of $CD4^+PD\text{-}1^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells and IL-10 levels measured by ELISA were significantly higher and $CD4^+PD\text{-}1^-Tim\text{-}3^-$ and $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells were significantly lower in patients than in controls. Treatment resulted in significant decrease of $CD4^+Tim\text{-}3^+$, $CD8^+Tim\text{-}3^+$, $CD4^+PD\text{-}1^+Tim\text{-}3^+$ and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cell frequencies as well as IL-10 levels and increase in $CD4^+PD\text{-}1^-Tim\text{-}3^-$ and $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells. There were no significant changes in the frequencies of $CD4^+PD\text{-}1^+$ T-cells, while $CD8^+PD\text{-}1^+$ T-cells increased. Patients with advanced liver fibrosis had higher PD-1 and lower Tim-3 expression on $CD4^+$T-cells and treatment had little or no effect on the exhaustion markers. HCV-specific $CD8^+$T-cells frequency has declined significantly after treatment, but their PD-1 and Tim-3 expression did not change. Successful treatment of chronic hepatitis C with DAA is associated with reversal of immune exhaustion phenotype, but this effect is absent in patients with advanced liver fibrosis.

Hepatitis C virus (HCV) infection is a common etiologic factor of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). The World Health Organization's Global Hepatitis Report estimates that about 71 million individuals are currently infected with HCV worldwide[1]. The majority of infected subjects (55–80%) develop chronic infection, whereas a minority eliminates the virus spontaneously, almost exclusively in the acute phase[2]. The ultimate outcome of HCV infection is determined by the host immune response, in particular by the strength of specific $CD4^+$ and $CD8^+$ T-cell activity[3,4]. Reduction in viral load and elimination of HCV is related

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to T-cell activation, cytotoxic elimination of infected cells, and effector cytokines production[5]. However, as the HCV infection progresses, there is a gradual decrease of the effector functions of T-cells including diminished proliferative potential and cytotoxicity, and lowered IL-2, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) production. This progressive impairment of the host immune function is referred to as immune exhaustion[6].

T-cell exhaustion has been described in other viral infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) as well as in the murine model infection with lymphocytic choriomeningitis virus (LCMV) and is believed to contribute to the development of chronic infection[3,7–10]. While persistent antigen exposure is thought to be the major factor driving T-cell exhaustion, regulatory T-cell activation, anti-inflammatory cytokines (e.g. IL-10) production and increased expression of inhibitory receptors (iRs) on T-cell surface are all likely to play a contributing role[11,12]. Prolonged up-regulation of multiple iRs negatively affects T-cell function by competing with co-stimulatory molecules, interfering with signals from co-stimulatory molecules or T-cell receptors (TCRs) and by upregulation of genes involved in T-cell dysfunction[13–16]. It has been shown that overexpression of iRs during an early stage of infection facilitates the development of chronic HCV infection[17]. Among known T-cell specific iRs, the best characterized are programmed cell death-1 (PD-1) and T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3)[7,18–22].

PD-1 is expressed on activated $CD4^+$ and $CD8^+$ T-cells and interaction of PD-1 with its ligands results in the suppression of T-cell sensitivity to antigenic stimulation[23,24]. Tim-3 is mainly present on Th1 (helper T-cell 1) and Tc1 (cytotoxic T-cell 1) subsets, but it is also expressed on innate immune cells such as dendritic cells (DCs), natural killer (NK) cells, and monocytes[25]. The effect of its action is downregulation of IFN-γ production and apoptosis induction[26,27]. In chronic HIV, HBV, and HCV infection, elevated PD-1 and/or Tim-3 expression characterize subpopulations of both total and virus-specific exhausted T-cells[9,17,28,29]. Functional exhaustion can be reversed by blocking of interactions of iRs with their ligands[23,27,30–32], but exclusive blockade of the PD-1 pathway with specific monoclonal antibodies does not fully restore cell functionality. McMahan et al.[17] showed that blocking of either the PD-1 or Tim-3 pathway enhances proliferation of HCV-specific $CD8^+$ T-cells in vitro, whereas cytotoxicity against a hepatocyte cell line expressing cognate HCV epitopes was increased exclusively by Tim-3 blocking. Similarly, Urbani et al. showed that while PD-1/PD-L1 blocking enhances IL-2 and IFN-γ production by HCV-specific $CD8^+$ T-cells, there is no improvement of the cytolytic function[33]. However, a combination of antibodies against PD-1 and Tim-3 does restore cell function, including cytotoxity[6]. These findings are congruent with the concept of hierarchical model of functional T-cell exhaustion in which restoration of proliferation capacity is followed by restoration of effector cytokines production and only then by the restoration of cytotoxicity.

It was demonstrated that co-expression of PD-1 and Tim-3 characterizes terminally differentiated, exhausted T-cells[34–36]. In chronic viral infections, $CD4^+$ and $CD8^+$ T-cells with $PD\text{-}1^+Tim\text{-}3^+$ phenotype produce immunosuppressive IL-10 that protects healthy tissues from damage by activated immune cells[27,37,38]. Plasma IL-10 levels are often increased in patients with chronic HCV, HIV, or HBV infections and this could promote transition to chronic disease[39–41]. IL-10 blockade by monoclonal antibodies was found to reduce viral load and PD-1 expression in the murine model of LCMV infection[42].

A number of studies were devoted to the characterization of markers of immune exhaustion in HCV infection and their importance in determining the infection outcome (spontaneous elimination *vs* persistence)[31,43–45]. However, it remains largely unknown whether exhaustion markers normalize after therapy-induced viral clearance. In the present study we analyzed the effect of direct acting antivirals (DAA) treatment-induced elimination of HCV on PD-1 and Tim-3 expression on peripheral $CD4^+$ and $CD8^+$ T-cells, including HCV-specific $CD8^+$ T-cells and on IL-10 plasma levels. We found that an effective antiviral therapy of chronic HCV infection decreases the frequencies of T-cells expressing exhaustion markers and lowers plasma concentrations of IL-10, but these effects are absent in patients with advanced liver fibrosis. HCV-specific $CD8^+$ T-cells frequency significantly declined after treatment, but the PD-1 and Tim-3 expression phenotype of these cells was not affected.

Results

The impact of treatment on PD-1 and Tim-3 expression phenotype of peripheral $CD4^+$ and $CD8^+$ T-cells. *PD-1 and Tim-3 expression phenotype of $CD4^+$ T-cells.* Pretreatment frequencies of $CD4^+$ T-cells expressing PD-1 and PD-1 + Tim-3 were found to be significantly higher in HCV-infected patients than in controls (median 22.7% (range 7.0–52.7%) *vs* 14.5% (7.4–37.0%), P = 0.0016 and 1.9% (0.3–9.4%) *vs* 1.0% (0.3–2.9%), P = 0.0007, respectively); (Fig. 1). The pretreatment frequencies of $CD4^+$ T-cells expressing Tim-3 were also higher in HCV-positive patients, although not statistically significant (7.5% (0.2–32.3%) *vs* 5.1% (1.8–12.2%). In contrast, the pretreatment frequencies of $CD4^+$ expressing neither PD-1 nor Tim-3 were significantly lower in patients than in controls (66.9% (42.6–81.1%) *vs* 78.8% (57.0–86.9%), P = 0.0001).

Treatment resulted a significant decrease of $CD4^+Tim\text{-}3^+$ T-cells frequencies from 7.5% (0.2–32.3%) to 6.4% (0.7–24.8%), P = 0.0398 and $CD4^+$ $PD\text{-}1^+Tim\text{-}3^+$ T-cells from 1.9% (0.3–9.4%) to 1.3% (0.0–7.6%), P < 0.0001; (Fig. 1). The frequency of $CD4^+$ cells expressing PD-1 did not change (22.7% (7.0–52.7%) *vs* 22.6% (6.2–43.8%)). In contrast, $CD4^+PD\text{-}1^-Tim\text{-}3^-$ T-cells increased significantly from 66.9% (42.6–81.1%) to 68.5% (44.5–82.0%), P = 0.0217.

After therapy, with the exception of $CD4^+PD\text{-}1^+$ and $CD4^+PD\text{-}1^-Tim\text{-}3^-$ T-cells (22.6% (6.2–43.8%) *vs* 14.5% (7.4–37.0%), P = 0.0009 and 68.5% (44.5–82.0%) *vs* 78.8% (57.0–86.9%), P = 0.0005, respectively), frequencies of all other analyzed subpopulations did not differ significantly from those in controls. A representative cytometric analysis of treatment-related changes in the expression of exhaustion markers on $CD4^+$ T-cells in two patients and two controls is shown in Fig. 2.

PD-1 and Tim-3 expression phenotype of $CD8^+$ T-cells. Pretreatment frequencies of $CD8^+$ T-cells co-expressing PD-1 and Tim-3 were significantly higher in patients than in controls (median 2.7% range (0.5–16.1%) *vs* 1.6%

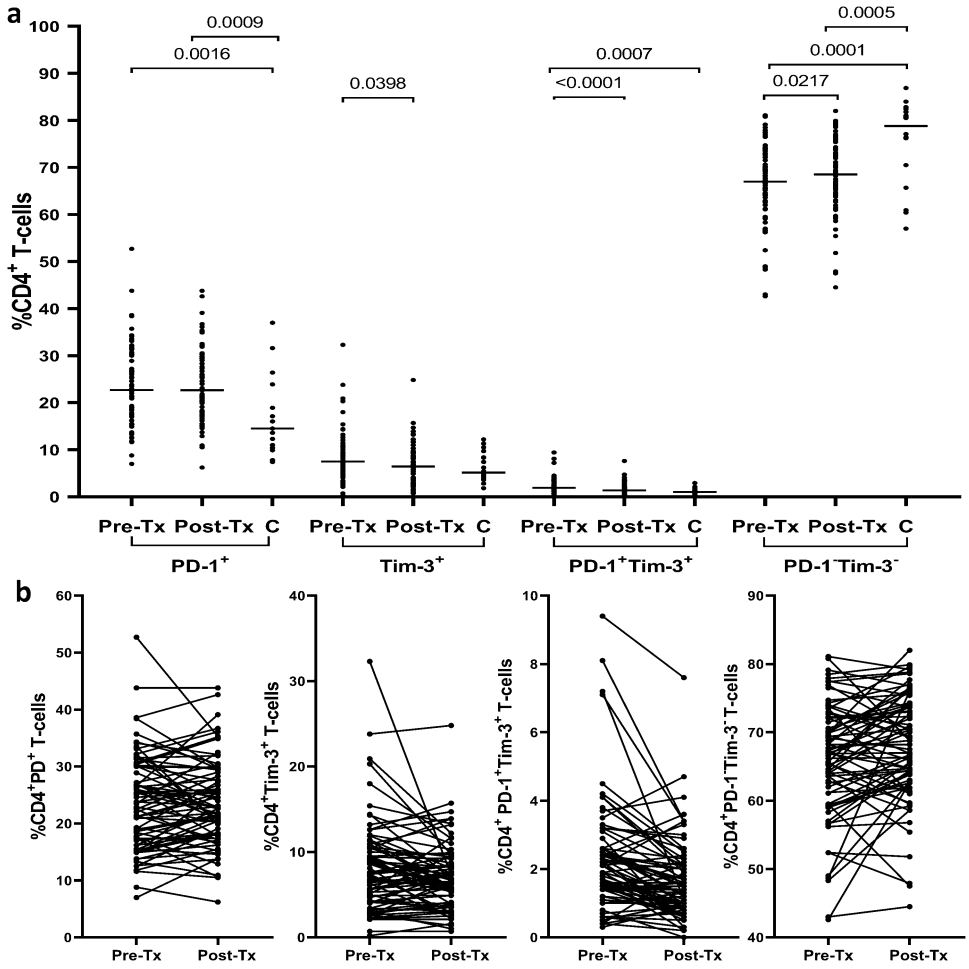

Figure 1. Peripheral blood CD4⁺ T-cells expression of PD-1 and Tim-3 in 76 patients before and after successful therapy of chronic hepatitis C and in 18 non-infected controls (**a**) and individual PD-1 and Tim-3 expression changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P values. *Pre-Tx* before therapy, *Post-Tx* after therapy, C- uninfected controls.

(0.3–4.8%), P = 0.0031; Fig. 3). The pretreatment frequencies of CD8⁺ T-cells expressing PD-1 or Tim-3 were also higher in patients, although not statistically significant (20.8% (5.5–50.1%) *vs* 18.7% (7.5–34.8%) and 15.2% (4.3–46.7%) *vs* 12.7% (6.0–25.7%), respectively). In contrast, the pretreatment frequencies of CD8⁺ expressing neither PD-1 nor Tim-3 were significantly lower in HCV-positive patients than in controls (58.4% (33.6–75.8%) *vs* 63.2% (50.3–76.0%), P = 0.0069).

As shown in Fig. 3, treatment resulted in a marked decrease of CD8⁺ T-cells expressing Tim-3 from 15.2% (4.3–46.7%) to 12.4% (1.8–39.1%), P < 0.0001 and CD8⁺ T-cells expressing PD-1 + Tim-3 from 2.7% (0.5–16.1%) to 1.8% (0.2–16.9%), P < 0.0001. In contrast, the frequency of CD8⁺ T-cells expressing PD-1 increased after treatment from 20.8% (5.5–50.1%) to 21.6% (5.4–56.0%), P = 0.0021 and so did the frequency of CD8⁺ PD-1⁻Tim-3⁻ T-cells (from 58.4% (33.6–75.8%) to 60.8% (34.3–80.1%), P = 0.0002).

After therapy, none of the four CD8⁺ T-cells subpopulations significantly differed from those in controls (21.6% (5.4–56.0%) *vs* 18.7% (7.5–34.8%) for PD-1⁺, 12.4% (1.8–39.1%) *vs* 12.7% (6.0–25.7%) for Tim-3⁺, 1.8% (0.2–16.9%) *vs* 1.6% (0.3–4.8%) for PD-1⁺Tim-3⁺, and 60.8% (34.3–80.1%) *vs* 63.2% (50.3–76.0%) for PD-1⁻Tim-3⁻ T-cells, respectively) (Fig. 3). A representative cytometric analysis of treatment-related changes in CD8⁺ T-cells exhaustion markers in two patients and two controls is shown in Fig. 4.

The effect of clinical and virological parameters on the pretreatment peripheral CD4⁺ and CD8⁺ T-cells PD-1 and Tim-3 expression phenotype and IL-10 plasma levels. Since pretreatment expression of exhaustion markers turned out to be highly variable within the analyzed group of patients, we tried to determine whether they were affected by clinical and/or virological parameters. Multivariate analysis included such factors as age, sex, viral load, ALT activity levels, baseline METAVIR liver fibrosis score, weight, and prior treatment (Table 1). In our analysis male sex was associated with higher IL-10 plasma levels (regression coefficient 1.54, 95% CI 0.03 to 3.04, P = 0.045) and higher percentage of CD8⁺PD-1⁺ T-cells (regression coefficient 7.69, 95% CI 2.39 to 13.00, P = 0.005). Furthermore, older age was associated with higher percentage of CD4⁺PD-1⁺ T-cells (regression coefficient 0.18, 95% CI 0.06 to 0.29, P = 0.003) and CD8⁺PD-1⁺Tim-3⁺ T-cells

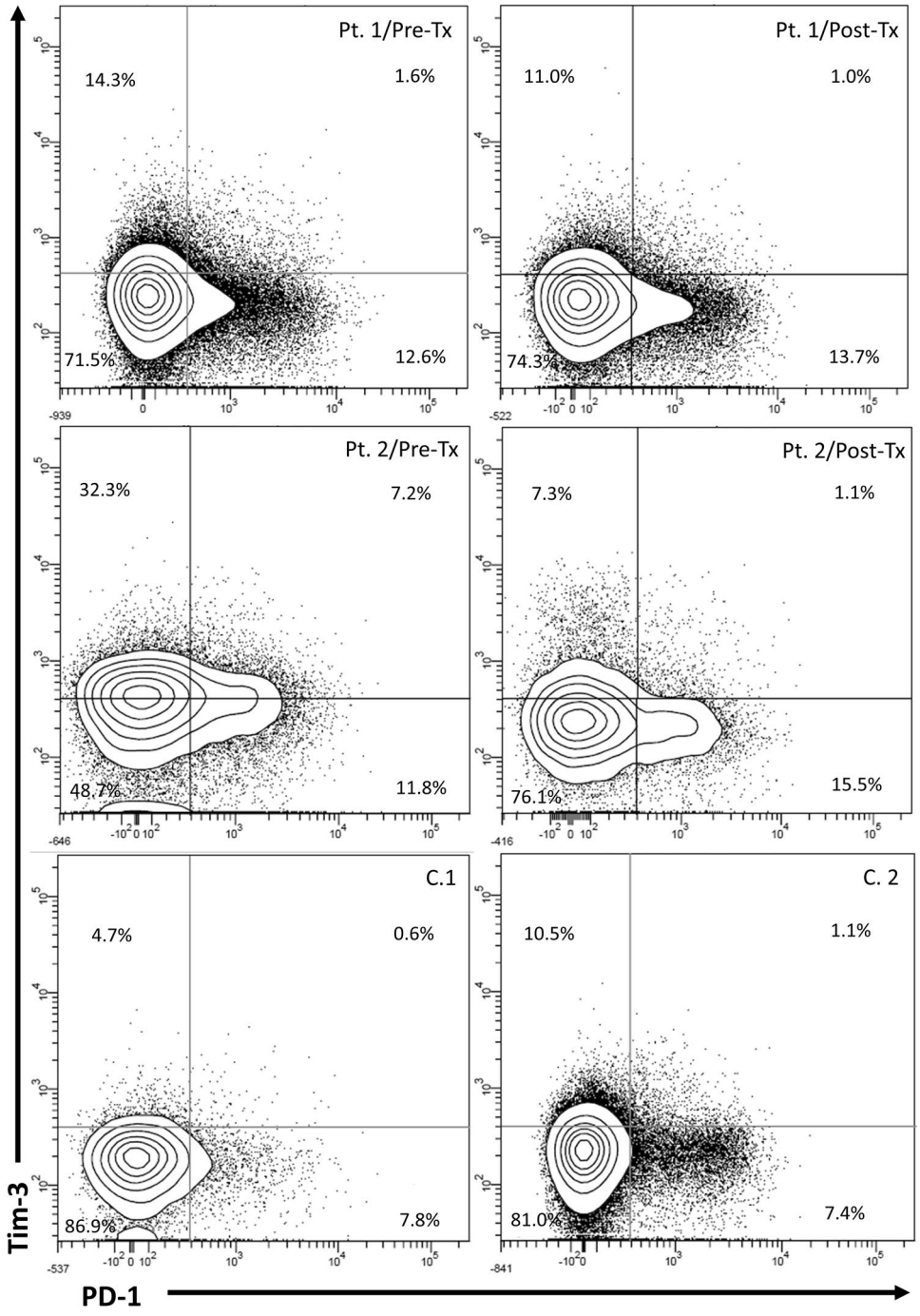

Figure 2. Representative cytometric analysis of peripheral blood $CD4^+$ T-cells expression of PD-1 and Tim-3 before and after successful therapy for chronic HCV infection in Patient 1 (Pt. 1), Patient 2 (Pt. 2) and in two uninfected controls (C.1 and C.2). *Pre-Tx* before therapy, *Post-Tx* after therapy.

(regression coefficient 0.05, 95% CI 0.01 to 0.09, P = 0.025) as well as lower percentage of $CD4^+PD\text{-}1^-Tim\text{-}3^-$ T-cells (regression coefficient − 0.18, 95% CI − 0.30 to − 0.06, P = 0.003). Importantly, when compared to F0/1, F3 liver fibrosis score was associated with higher percentage of $CD4^+PD\text{-}1^+$ T-cells (regression coefficient 5.69, 95% CI 0.13 to 11.26, P = 0.045), but the opposite was true in case of $CD4^+Tim\text{-}3^+$ T-cells (regression coefficient − 3.4, 95% CI − 6.66 to − 0.30, P = 0.032 for F2 and regression coefficient − 5.20, 95% CI − 9.09 to − 1.31, P = 0.010 for F3). Furthermore, F2 stage was associated with higher percentage of $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells (regression coefficient 7.14, 95% CI 1.37 to 12.91, P = 0.016).

Liver fibrosis scores correlation with peripheral T-cell PD-1 and Tim-3 expression phenotype and its treatment-related change. Pretreatment exhaustion markers expression correlated with liver

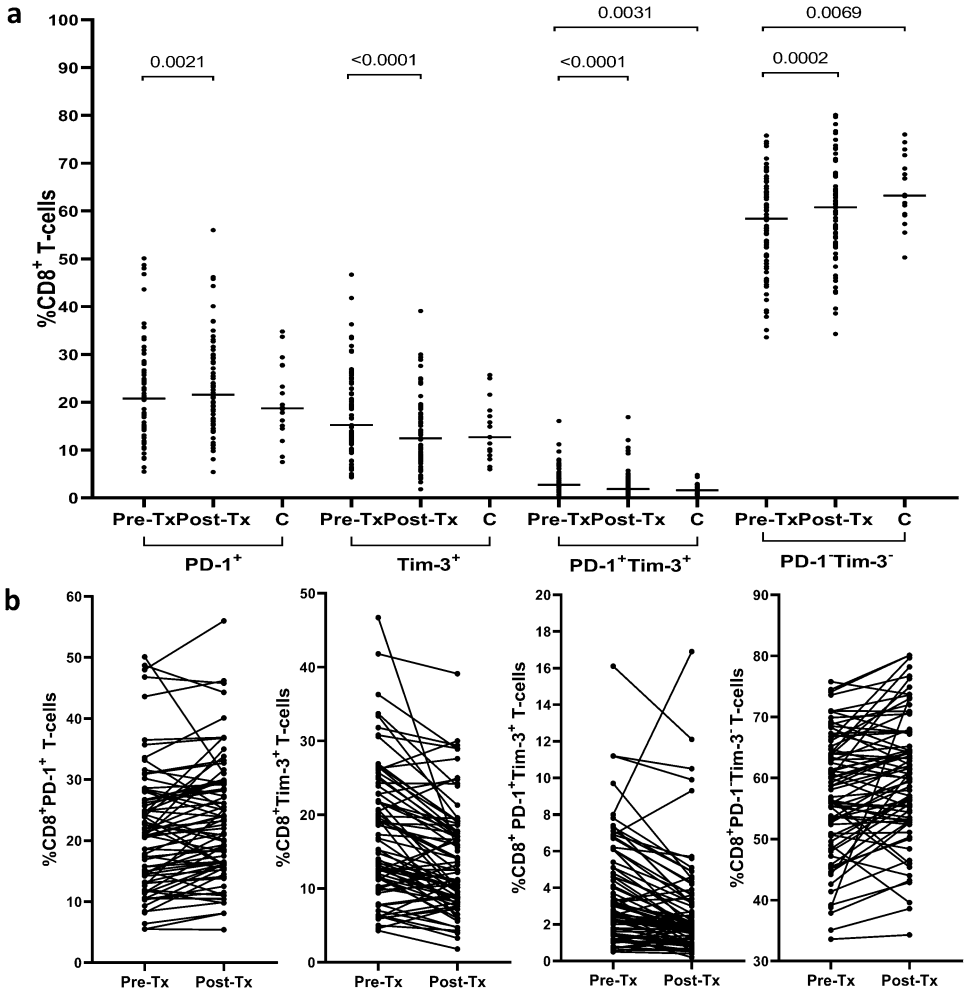

Figure 3. Peripheral blood $CD8^+$ T-cells expression of PD-1 and Tim-3 in 76 patients before and after successful therapy for chronic HCV infection and in 18 non-infected controls (**a**) and individual PD-1 and Tim-3 expression changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy, *C* non-infected controls.

fibrosis score: the more advanced fibrosis, the higher percentage of $CD4^+PD\text{-}1^+$ T-cells (median 20.1% (range 7.0–52.7%) in F0/1 *vs* 25.4% (8.8–43.8%) in F2 *vs* 26.8% (15.0–38.4%) in F3, P = 0.0125). However, in every fibrosis stage the pretreatment percentage of $CD4^+PD\text{-}1^+$ T-cells was higher than in healthy controls (Fig. 5). In contrast, the more advanced fibrosis, the lower the frequency of $CD4^+Tim\text{-}3^+$ T-cells (9.0% (0.7–32.3%) in F0/1 *vs* 5.7% (2.1–23.8%) in F2 *vs* 5.4% (0.2–12.0%) in F3, P = 0.0274). When compared to controls, only F0/1 patients demonstrated higher pretreatment percentage of $CD4^+Tim\text{-}3^+$ T-cells (Fig. 5). While pretreatment percentages of $CD4^+PD\text{-}1^+Tim\text{-}3^+$ T-cells, and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells in all three fibrosis groups were significantly higher than in healthy controls (Figs. 5 and 6), there were no differences between patients with different fibrosis stage. $CD4^+PD\text{-}1^-Tim\text{-}3^-$ and $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells percentages were similar in patients displaying different fibrosis scores but were significantly lower than in healthy controls with the exception of $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells in F2 group which were similar to those in controls (Figs. 5 and 6). For the remaining T-cell subpopulations ($CD8^+PD\text{-}1^+$, $CD8^+Tim\text{-}3^+$) there were no statistically significant differences between different fibrosis stages, and the values were similar to those of healthy controls (Figs. 5 and 6).

Importantly, the more advanced fibrosis, the less likely it was that treatment would change the proportions of cells expressing exhaustion markers. Thus, F0/1 patients experienced increase in $CD4^+PD\text{-}1^+$ T-cells from 20.1% (7.0–52.7%) to 21.6% (10.5–39.1%), P = 0.0026 and $CD8^+PD\text{-}1^+$ T-cells from 20.9% (5.5–46.8%) to 23.7% (8.1–46.2%), P = 0.0002, and increase in $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells from 55.8% (35.1–74.5%) to 59.3 (38.6–80.1%), P = 0.0470 and decrease in $CD4^+Tim\text{-}3^+$ T-cells from 9.0% (0.7–32.3%) to 6.8% (0.7–15.7%), P = 0.0006 and in $CD8^+Tim\text{-}3^+$ T-cells from 19.0% (5.0–46.7%) to 14.1% (3.3–39.1%), P < 0.0001, and decrease in $CD4^+PD\text{-}1^+Tim\text{-}3^+$ T-cells from 2.1% (0.4–7.2%) to 1.3% (0.2–4.7%), P = 0.0032 and in $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T-cells from 2.5% (0.5–16.1%) to 1.9% (0.4–12.1%), P = 0.0014 (Figs. 5 and 6). In contrast, F3 patients displayed no significant changes in frequencies of cells expressing PD-1 and/or Tim-3 with the exception of $CD4^+PD\text{-}1^+Tim\text{-}3^+$ T-cells, which decreased from 1.5% (0.3–4.1%) to 1.4% (0.6–2.9%), P = 0.0215. Patients with stage F2 fibrosis had less pronounced changes than F0/1 patients including decrease in $PD\text{-}1^+Tim\text{-}3^+$ T-cells (both $CD4^+$ (1.6%

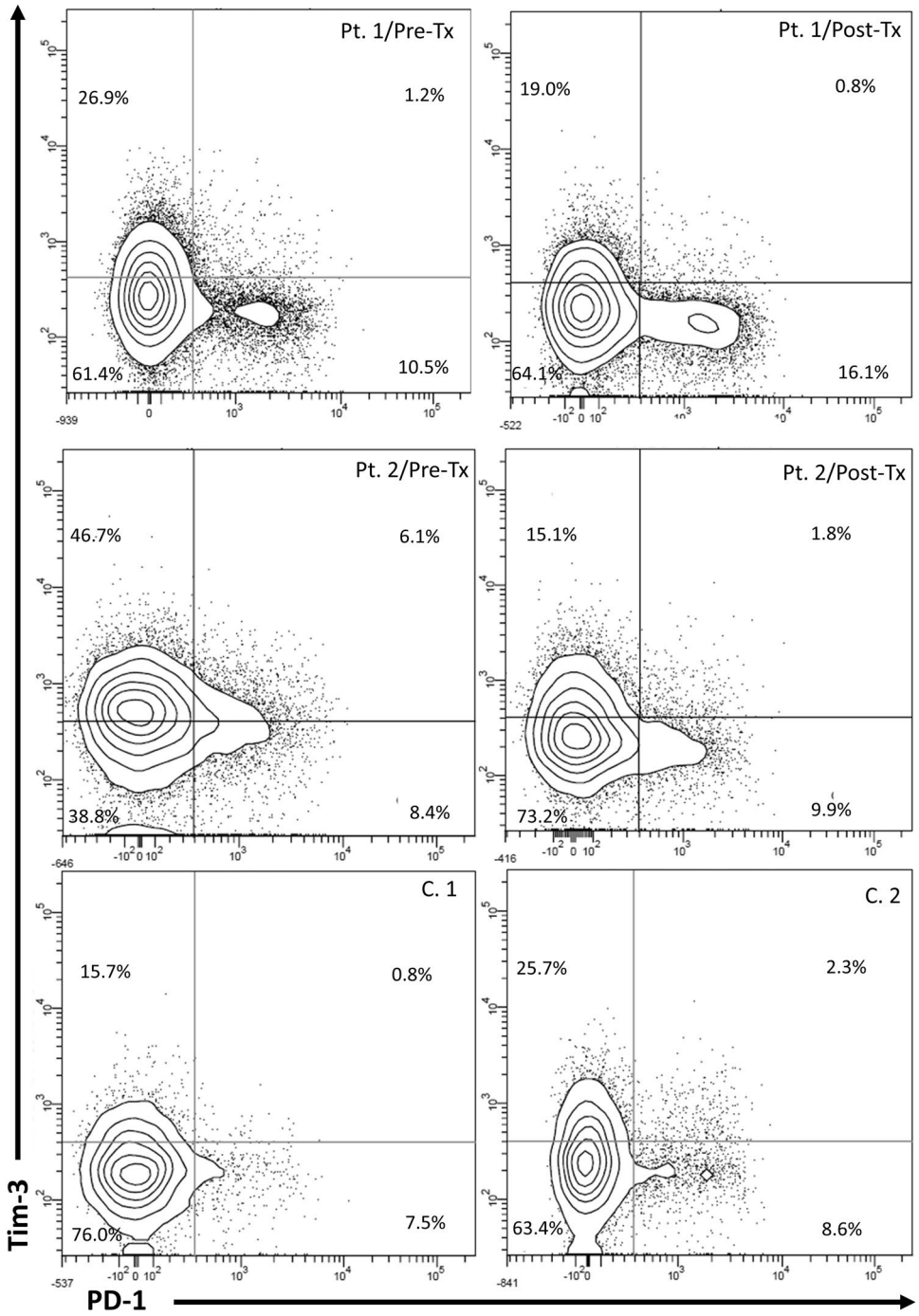

Figure 4. Representative cytometric analysis of peripheral blood $CD8^+$ T-cells expression of PD-1 and Tim-3 before and after successful therapy for chronic HCV infection in Patient 1 (Pt. 1) and Patient 2 (Pt. 2) and in uninfected controls (C.1 and C.2). *Pre-Tx* before therapy, *Post-Tx* after therapy.

(0.4–9.4%) *vs* 1.2% (0.0–7.6%), P = 0.0043) and $CD8^+$ (2.7% (0.6–11.2%) *vs* 1.7% (0.2–10.5%), P = < 0.0001) subpopulations, decrease in $CD8^+Tim\text{-}3^+$ T-cells (12.7% (4.3–36.3%) *vs* 11.1% (1.8–29.4%), P = 0.0018), decrease in $CD4^+PD\text{-}1^-Tim\text{-}3^-$ T-cells (66.9% (43.0–80.8%) *vs* 66.7% (44.5–82.0%), P = 0.0236) and increase in $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells (63.8% (39.2–75.8%) *vs* 64.3% (42.9–80.1%), P = 0.0005) (Figs. 5 and 6).

After treatment, none of the analyzed populations was significantly different between the subgroups of patients with different fibrosis stage.

Despite treatment-induced changes, some of the subpopulations did not reach values seen in healthy controls. These included higher $CD4^+PD\text{-}1^+$ T-cells in F0/1 (P = 0.0022), F2 (P = 0.0125) and F3 (P = 0.0042) groups as well as lower $CD4^+PD\text{-}1^-Tim\text{-}3^-$ T-cells in F0/1 (P = 0.0014), F2 (P = 0.0038) and F3 (P = 0.0131) groups and lower $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells in F0/1 (P = 0.0495) and F3 (P = 0.0203) groups (Figs. 5 and 6).

	Patients n = 76	Controls n = 18
Age [median (range), years]	58.5 (25–88)	49.5 (23–73)
Male/female (%)	29/47 (38.2/61.8)	4/14 (22/78)
Viral load [mean ± SD, IU/mL]	$1.59 \times 10^6 \pm 1.31 \times 10^6$	N/A
ALT activity [mean ± SD, IU/L]; (normal values ≤ 56 IU/mL)	80.1 ± 39.8	27.1 ± 19.5
Treatment scheme Ledipasvir + sofosbuvir/ombitasvir + paritaprevir + ritonavir + dasabuvir (%)	54/22 (71.1/28.9)	N/A
Treatment naïve [Y/N]	53/23	N/A
Weigh [kg]	74.4 ± 13.5	N/A
FibroScan[a]		
F0/1	38	N/A
F2	27	N/A
F3	11	N/A
F4	0	N/A

Table 1. Clinical, laboratory and virological characteristic of patients and controls. *N/A* not available or not applicable. [a]5-point METAVIR scale was used for liver fibrosis grading where F0/F1 represents no or minimal fibrosis, F2 moderate fibrosis, F3 severe fibrosis, and F4 represents cirrhosis[46].

Successful treatment affects HCV-specific CD8⁺ T-cell frequencies but not their PD-1 and Tim-3 expression phenotype. Assessment of HCV-specific $CD8^+$ T-cells frequencies and their exhaustion phenotype was feasible in 32 patients with HLA-A*02 allele. As shown in Fig. 7, treatment resulted in lowering the frequency of these cells from median 2.9% (range 0.1–47.9%) to 0.7% (0–57.2%), P = 0.0003. A representative cytometric analysis of treatment-related changes in HCV-specific $CD8^+$ T-cells frequency in two patients is shown in Fig. 8.

While treatment resulted in some changes in the phenotype of HCV-specific cells, these differences did not reach statistical significance (Fig. 7). Before the therapeutic intervention, HCV-specific T-cells expressed PD-1 less frequently than after the treatment (30.0% (12.8–70.0%) *vs* 36.0% (7.5–90.3%)), were more likely to be $Tim\text{-}3^+$ ((18.2% (0.0–35.8%) *vs* 13.9% (0.0–42.9%)), equally likely to be $Tim\text{-}3^+PD\text{-}1^+$ ((1.2% (0.0–11.1%) *vs* 1.2% (0.0–30.8%)) and more likely to be $PD\text{-}1^-Tim\text{-}3^-$ ((46.7% (0–65.7%) *vs* 44.8% (6.5–67.6%)).

The effect of treatment scheme on the peripheral HCV-specific $CD8^+$ $PD\text{-}1^+$ T-cells frequency. We analyzed the effect of treatment with two different protocols (i.e., ledipasvir + sofosbuvir *vs* ombitasvir + paritaprevir + ritonavir + dasabuvir) correcting for variables differently distributed between the two treatment groups. Patients treated with ledipasvir + sofosbuvir displayed lower liver fibrosis score (59.3% *vs* 27.3% with F0/1, 40.7% *vs* 72.7% with F2/3, P = 0.0216) and higher age (median 61 (range 25–88) *vs* 49.5 (29–78), P = 0.0172). We found that changes in the percentages of cells expressing exhaustion markers were not different for these two protocols, the only exception being HCV-specific $CD8^+PD\text{-}1^+$ T-cells. Thus, while treatment with ledipasvir + sofosbuvir resulted in increase in percentage of HCV-specific $CD8^+PD\text{-}1^+$ T-cells from 28.4% (12.8–70.0%) to 40.8% (15.4–90.3%), treatment with ombitasvir + paritaprevir + ritonavir + dasabuvir resulted in their decrease from 39.9% (17.6–66.7%) to 31.7% (7.5–41.7%) (regression coefficient -39.41% 95% CI -63.71 to -15.11%, P = 0.005). The opposite was found for HCV-specific $CD8^+Tim\text{-}3^+$ T-cells: treatment with ledipasvir + sofosbuvir resulted in decrease in percentage of HCV-specific $CD8^+Tim\text{-}3^+$ T-cells from 18.7% (0–35.80%) to 6.2% (0–31.2%), while treatment with ombitasvir + paritaprevir + ritonavir + dasabuvir resulted in their increase from 14.2% (0–26.5%) to 20.4% (11.1–42.9%). However, these results were not significant in multivariate analysis (P = 0.066). Similarly, treatment with ledipasvir + sofosbuvir resulted in decrease in percentage of HCV-specific $CD8^+PD\text{-}1^-Tim\text{-}3^-$ T-cells from 46.7% (0–65.7%) to 41.6% (6.5–67.6%), while treatment with ombitasvir + paritaprevir + ritonavir + dasabuvir resulted in their increase from 43.6% (22.2–55.9%) to 49.9% (21.4–67.5%). However, these results were also not significant in multivariate analysis (P = 0.063).

Successful treatment diminishes IL-10 levels in plasma. Before therapy, patients displayed significantly higher plasma IL-10 levels than controls (median 4.0 (range 0.8–16.5) pg/mL *vs* 3.0 (1.3–6.4) pg/mL, P = 0.0442) (Fig. 9). Treatment resulted in decrease of IL-10 levels to 3.3 (0.8–22.4) pg/mL, P = 0.0065, which were now similar to the levels observed in controls.

Plasma IL-10 levels were not significantly different between patients displaying different fibrosis scores neither before (4.0 (1.1–16.5) pg/mL in F0/1 *vs* 3.9 (0.8–11.7) pg/mL in F2 *vs* 4.3 (0.8–6.9) pg/mL in F3), nor after treatment (3.5 (0.8–19.8) pg/mL in F0/1 *vs* 3.3 (1.3–22.4) pg/mL in F2 *vs* 3.2 (1.6–8.0) pg/mL in F3). Plasma IL-10 levels were also not significantly different between patients with different fibrosis stage and healthy controls, neither before nor after treatment. However, a significant decrease of IL-10 levels after treatment was observed but was limited to F0/1 patients (P = 0.0395) (Fig. 10).

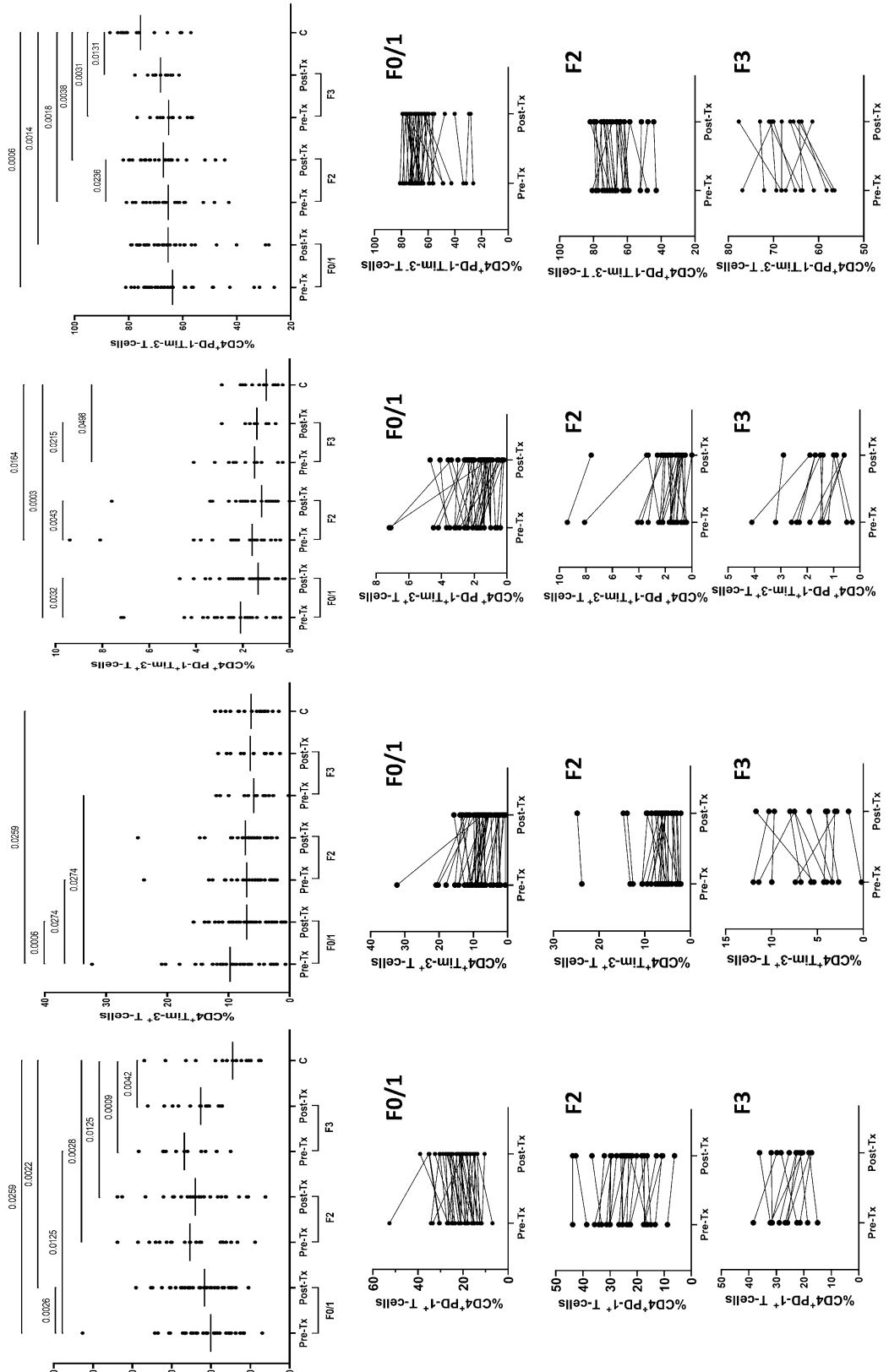

Figure 5. Peripheral blood $CD4^+$ T-cells expression of PD-1 and Tim-3 in patients with different liver fibrosis scores (F0/1, F2, F3) before and six months after successful therapy for chronic HCV infection and in 18 non-infected controls (**a**) and individual PD-1 and Tim-3 expression changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy, *C* non-infected controls.

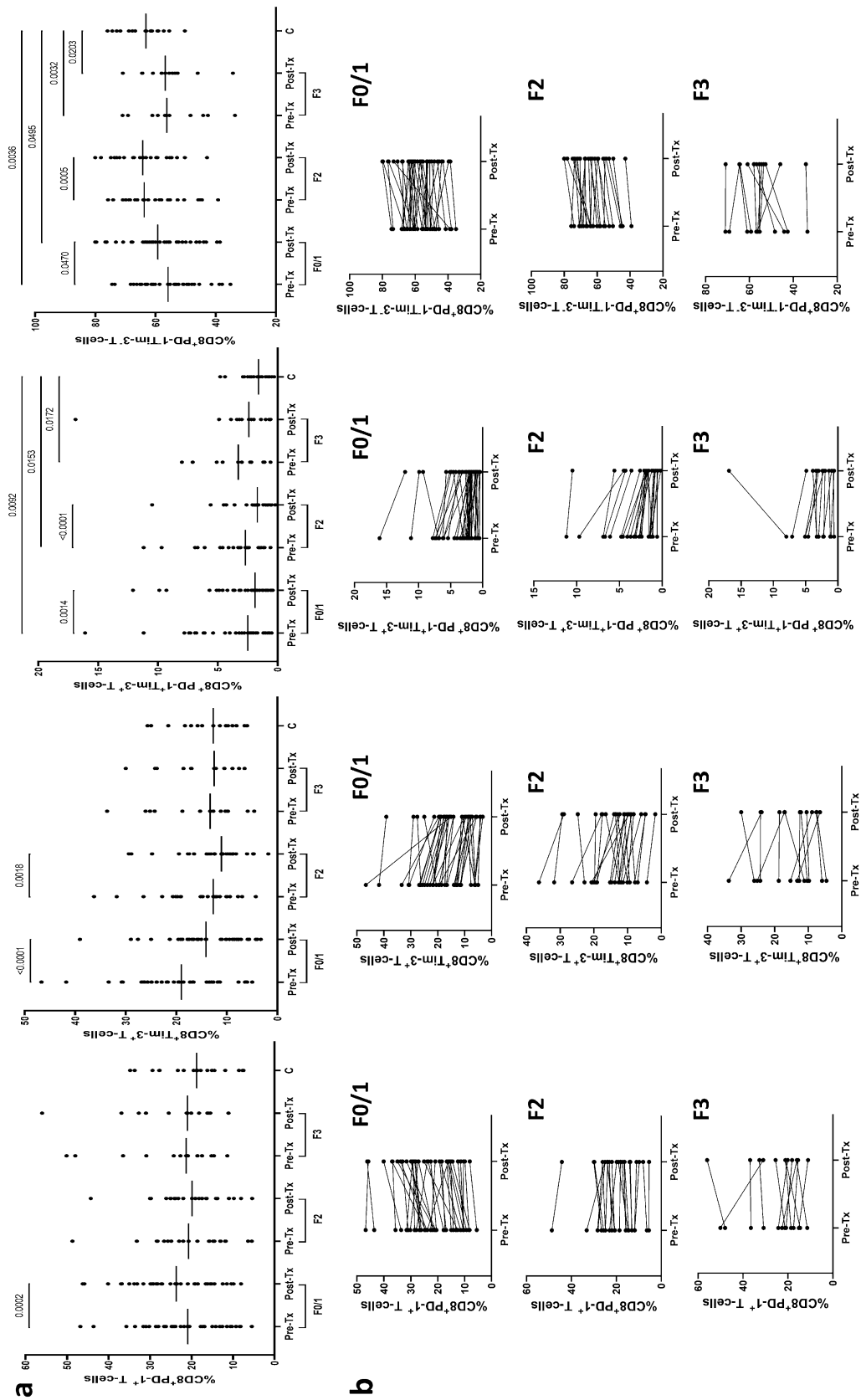

Figure 6. Peripheral blood $CD8^+$ T-cells expression of PD-1 and Tim-3 in patients with different liver fibrosis stage (F0/1, F2, F3) before and six months after successful therapy for chronic HCV infection and in 18 non-infected controls (**a**) and individual PD-1 and Tim-3 expression changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy, *C* non-infected controls.

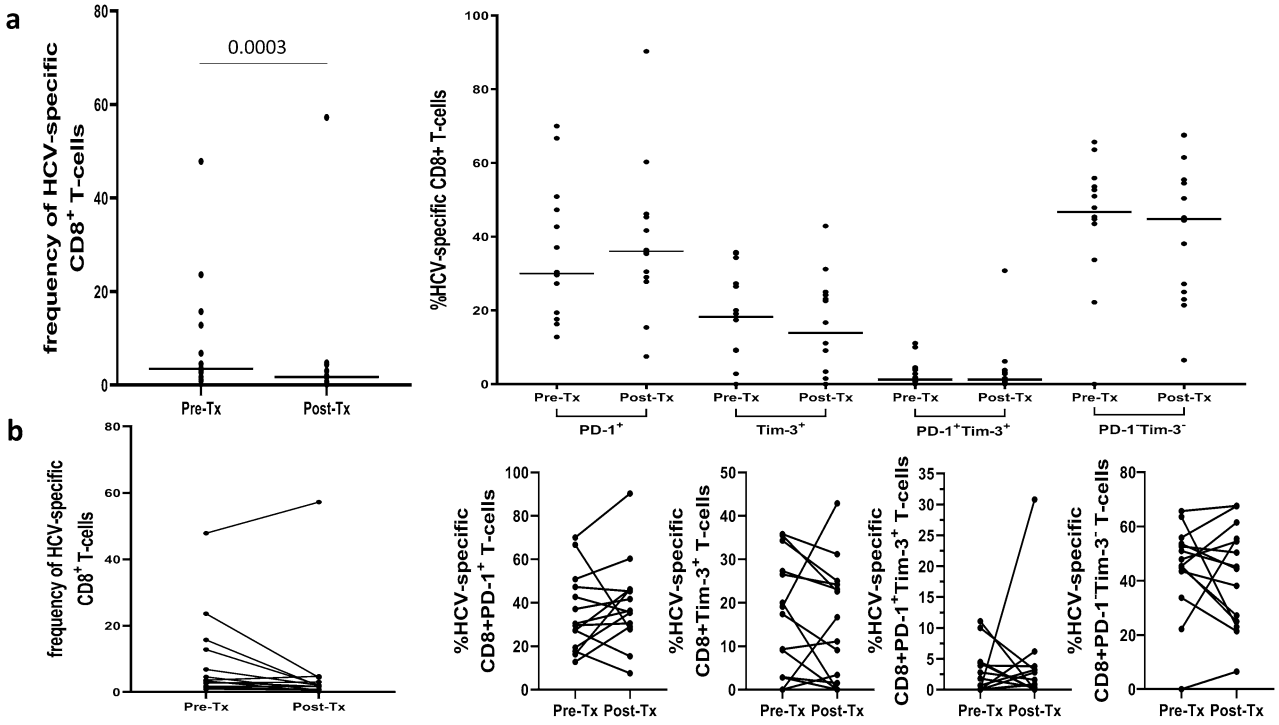

Figure 7. Peripheral blood HCV-specific CD8⁺ T-cells frequency and expression of PD-1 and Tim-3 in 32 patients before and six months after successful therapy of chronic HCV infection (**a**) and individual PD-1 and Tim-3 expression changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy.

Discussion

So far relatively few studies reported on the effects of treatment-induced HCV elimination on the immune exhaustion status of T-cells, and especially on HCV-specific cells. Furthermore, these studies were often inconclusive, carried out on small cohorts of patients infected with various HCV genotypes, assessed different immunological parameters, confined to the analysis of only one inhibitory T-cell receptor, or were conducted on patients treated with IFN or even experimental drugs, which were eventually abandoned[47–54].

The current standard of care of chronic HCV infection advocates the use of DAA, which are successful in over 95% of patients[55]. However, their effect on the immune exhaustion status of T-cells has not been analyzed in detail, especially with respect to CD4⁺ T-cell population. Importantly, it is the restoration of antiviral immunity, manifested by the reversal of the exhausted T-cells phenotype, that may be critical for successful treatment, since the presence of HCV-RNA in serum at the end of DAA-based therapy does not preclude sustained virologic response (SVR)[56,57].

Similar to other authors, we found that chronic HCV infection is associated with increased expression of exhaustion markers on peripheral blood CD4⁺ and CD8⁺ T-cells as compared to non-infected controls[28,58]. Furthermore, in our study CD4⁺ and CD8⁺ T-cells expressing Tim-3 and co-expressing PD-1 and Tim-3 decreased after successful treatment and this was accompanied by increased frequency of PD-1⁻Tim-3⁻ CD4⁺ and CD8⁺ T-cells, suggesting that the immune exhaustion induced by prolonged infection may be at least partially reversed once the infecting pathogen is eliminated. A number of mechanisms could facilitate this reversal of functional T-cells exhaustion such as rapid reduction of viral burden, up-regulation of soluble factors involved in T-cell activation, elimination of viral proteins known to inhibit innate immune responses and reduction in the production of immunosuppressive cytokines including IL-10[59–61]. Indeed, in our study IL-10 plasma values returned to near normal levels after the elimination of HCV. While increase of IL-10 blood levels in chronic HCV infection is likely to be driven by chronic inflammation and immune regulatory mechanisms[40], HCV proteins could also stimulate IL-10 production directly[62].

In contrast to Tim-3 expression and PD-1 + Tim-3 co-expression, the frequencies of CD4⁺ T-cells expressing PD-1 did not change significantly after treatment and the CD8⁺PD-1⁺ population even increased. Similar observation was reported by Zhang et al.[54] who found no significant differences in the population of CD4⁺ T-cells expressing PD-1 before and after DAA-induced SVR. The mechanisms behind this phenomenon are unclear. Since PD-1 expression may vary within particular subsets of CD4⁺ and CD8⁺ T-cells and is increased during early and intermediate cell differentiation stages[63], high post-treatment PD-1 expression among our patients could have been the result of a switch to some earlier differentiation stage of T-cell populations once the infection has been cleared. Alternatively, the relative stability or increase of PD-1⁺ T-cell subpopulations may have been the result of PD-1⁺Tim-3⁺ cells becoming single positive PD-1⁺ cells.

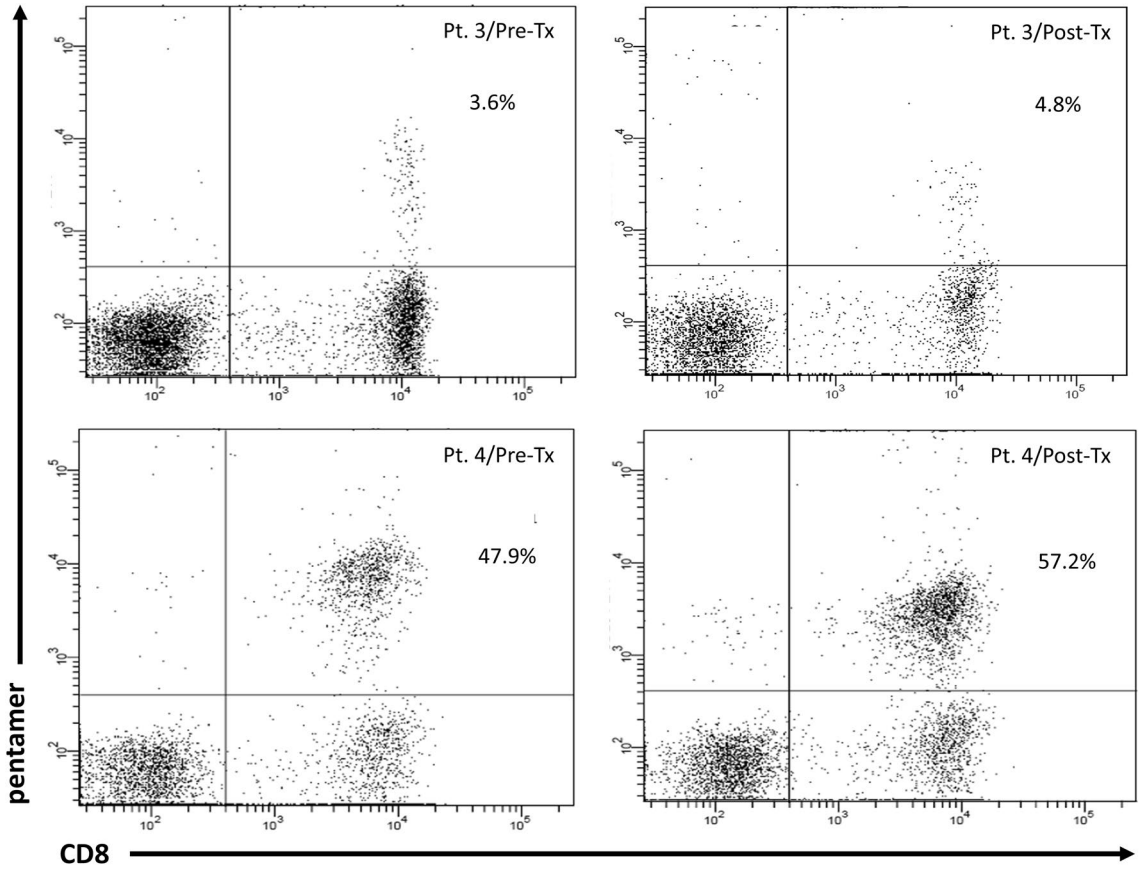

Figure 8. Representative cytometric analysis of peripheral blood HCV-specific CD8⁺ T-cells before and after successful therapy for chronic HCV infection in Patient 3 (Pt. 3) and Patient 4 (Pt. 4). *Pre-Tx* before therapy, *Post-Tx* after therapy.

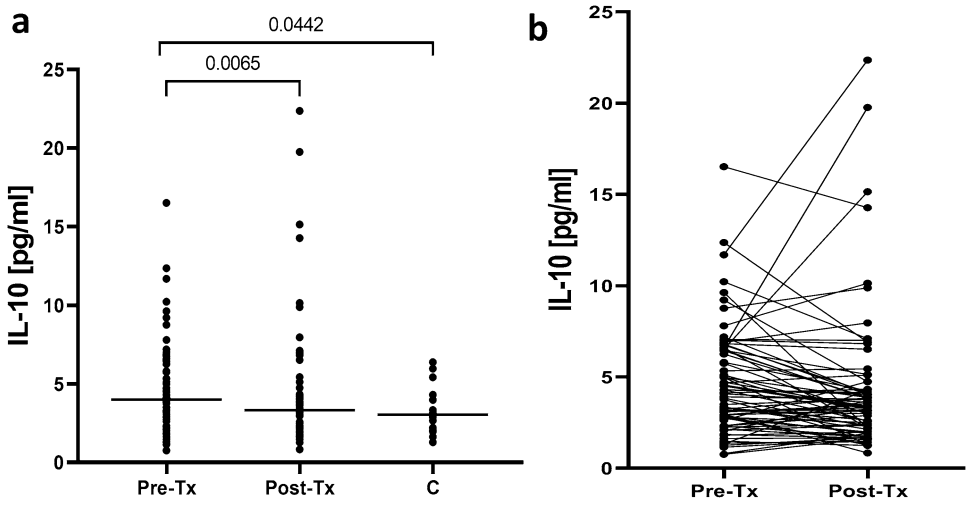

Figure 9. Plasma levels of IL-10 in 76 patients before and six months after therapy for chronic HCV infection and in 18 non-infected controls (**a**) and individual IL-10 changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy, *C* non-infected controls.

Earlier studies conducted on HIV-infected patients suggested that PD-1 is a marker of early T-cell exhaustion representing a stage of impaired proliferation but still relatively well preserved function manifested in the ability of cytokine synthesis[64], whereas Tim-3 is a marker of more advanced T-cell exhaustion associated with

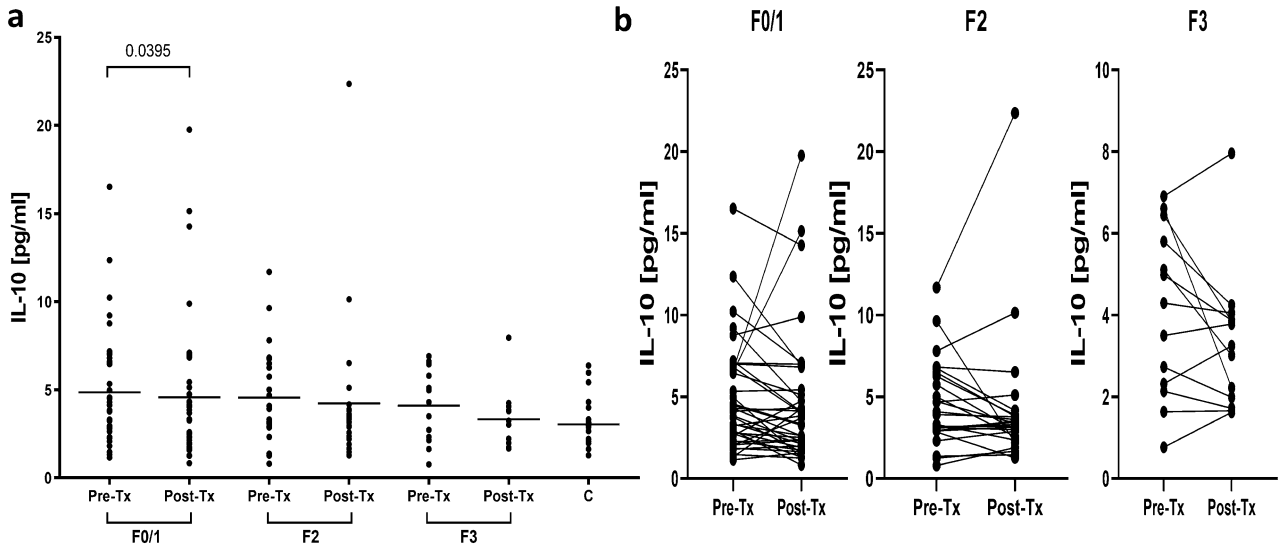

Figure 10. Plasma levels of IL-10 in 76 patients in groups of patients displaying different liver fibrosis scores (F0/1, F2, F3) before and six months after therapy for chronic HCV infection and in 18 non-infected controls (**a**) and individual IL-10 changes before and after treatment (**b**). Horizontal lines represent median values. Numbers above each brace express P-values. *Pre-Tx* before therapy, *Post-Tx* after therapy.

cytokine synthesis impairment and susceptibility to apoptosis[27,65]. Furthermore, it was previously proposed that co-expression of PD-1 and Tim-3 receptors characterizes the most exhausted and dysfunctional T-cell subset[27]. This view is compatible with the observation that the frequency of PD-1$^-$Tim-3$^-$ HCV-specific T-cells is much higher than those of PD-1$^+$Tim-3$^+$ phenotype in patients with acute resolving infection but not in patients in whom acute infection progressed to chronicity[17]. Similarly, in a study employing a murine LCMV infection model, dual expression of these iRs was associated with progression to chronic infection[27]. Our finding of uncommon co-expression of these two markers is also congruent with the existence of a hierarchical model of exhaustion, in which this process is initiated by PD-1 expression, followed by Tim-3 and finally by co-expression of the latter two markers.

In our study we made a novel observation that the pretreatment PD-1 and Tim-3 phenotypes of T-cells correlated with liver fibrosis scores. Thus, the more advanced fibrosis, the higher the frequency of CD4$^+$PD-1$^+$ and the lower the frequency of CD4$^+$Tim-3$^+$ cells. Given the hierarchical model of T-cell exhaustion mentioned earlier, it is likely that in advanced long-lasting infection reflected by pronounced fibrosis, a substantial proportion of Tim-3$^+$ cells may have already been deleted. Furthermore, we found that in patients with advanced fibrosis, successful treatment was not followed by changes in the populations of cells expressing exhaustion markers. Thus, while F0/1 patients experienced an increase in the frequencies of PD-1$^+$ CD4$^+$ and CD8$^+$ and PD-1$^-$Tim-3$^-$ CD8$^+$ T-cells and decrease in the frequencies of Tim-3$^+$ CD4$^+$ and CD8$^+$ T-cells and PD-1$^+$Tim-3$^+$ CD8$^+$ T-cells, this was not the case in F3 patients. Similarly, successful DAA treatment was associated with a significant lowering of IL-10 levels in plasma only in F0/1 patients, having no effect in F2 or F3 patients. Therefore, it seems that advanced liver fibrosis marks patients with advanced and poorly reversible immune-related changes. Vranjkovic et al.[66] found that HCV-infected patients with pronounced liver fibrosis (F4) displayed hyperfunctional activity of peripheral CD8$^+$ T-cell subsets sustained up to a year after treatment, and the impact of successful DAA therapy on T-cell activation depended largely on the stage of liver fibrosis. Furthermore, in F4 individuals DAA therapy had no effect on elevated concentrations of systemic inflammatory cytokines and decreased levels of inhibitory TGF-β in plasma. These data suggest that HCV-infected patients with advanced liver disease have a long-lasting and irreversible immune exhaustion. However, it is unclear whether this effect is due to fibrosis itself or rather to a long-lasting infection of which fibrosis is only a manifestation, as the length of infection in the majority of patients could not be determined.

In our patients HCV-specific CD8$^+$ T-cells frequency was significantly lower after treatment, which was likely due to the elimination of these cells after HCV clearance. Similarly, Han et al.[49] observed that HCV-specific CD8$^+$ T-cells, including antigen-experienced (KLRG1$^+$CCR7$^-$) HCV-specific CD8$^+$ T-cell subset, decreased after SVR. We also found that the expression of PD-1 on HCV-specific CD8$^+$ T-cells was more frequent while that of Tim-3 less frequent after treatment, but these differences did not reach statistical significance. Aregay et al.[67] demonstrated that HCV-specific CD8$^+$ T-cell function was not restored following HCV eradication by means of DAA treatment, since expression of CD5, LAG-3, PD-1 and Tim-3 on HCV-specific CD8$^+$ T-cells, impaired IFN-ϒ and MIP-1β production, metabolic deregulation and mitochondrial dysfunction did not change. These findings imply that in chronically infected patients, HCV-specific CD8$^+$ T-cells exhaustion phenotype may not be restored following DAA-induced viral eradication and suggest that a reversal of this phenotype, similar to that observed in spontaneous viral clearance, is not achievable. Thus, it is likely that chronic stimulation with HCV antigens is related to irreversible changes in the HCV-specific CD8$^+$ T-cell population.

In our study the type of DAA treatment received was found to affect exhaustion markers of HCV-specific $CD8^+$ T-cells differently. In particular, treatment with ledipasvir + sofosbuvir led to an increased expression of PD-1 whereas treatment with ombitasvir + paritaprevir + ritonavir + dasabuvir resulted in decreased expression of PD-1^+. The reason for this phenomenon is unclear and the only difference between the two treatments was different number of drug targets. While ledipasvir + sofosbuvir are NS5A and NS5B inhibitors, respectively, ombitasvir + paritaprevir + ritonavir + dasabuvir are NS5A, NS3/4A/CYP3A4 and NS5B inhibitors, respectively. Shrivastava et al.[68] reported that the most complete restoration of HCV-specific immune response in HIV/HCV coinfected patients was observed in those treated with a regimen that inhibits three distinct stages of the HCV life cycle.

Interestingly, in our study older age was associated with the presence of higher percentage of $CD4^+$PD-1^+ and $CD8^+$PD-1^+Tim-3^+ T-cells. This can be due to the direct effect of age or it could reflect the duration of infection[69]. Similarly, male sex was related to higher IL-10 plasma levels and higher percentage of $CD8^+$PD-1^+ T-cells. The reason probably lays in the fact that immunity in males is characterized by weaker humoral and cellular immune responses and this could be partly due to estrogen and testosterone effects on immunity[70].

Conclusions

In summary, we found that a successful therapy of chronic HCV infection with DAA lowered expression of T-cell exhaustion markers to near normal values and reduced IL-10 levels in plasma, but these changes were largely confined to patients with minimal or no liver fibrosis. DAA treatment was found to lower HCV-specific $CD8^+$ T-cells frequency but had little effect on the expression of exhaustion markers by these cells which suggests that long-term antigenic stimulation results in irreversible changes to the HCV-specific T-cell compartment.

Methods

Patients and controls. Seventy-six patients with chronic HCV infection (47 women and 29 men, median age 58.5 years, range 25–88), who underwent anti-HCV therapy at the Warsaw Hospital for Infectious Diseases Outpatient Clinic in the years 2016–17 were studied prospectively. All were HCV- RNA positive for at least 6 months prior to therapy, and all were infected with HCV genotype 1 (genotype 1b was present in 74 and genotype 1a was present in two patients). Fifty-four patients were treated with ledipasvir and sofosbuvir (Harvoni, Gilead Sciences Inc, Foster City, CA, USA) 90 and 400 mg per day, respectively, and 22 patients received ombitasvir, paritaprevir, ritonavir (Viekirax, AbbVie Inc., Lake Bluff, IL, USA) at doses of 25, 150, and 100 mg per day, respectively, along with dasabuvir (Exviera, AbbVie Inc.) 500 mg per day.

Treatment was administered for 12 weeks in 48 patients, whereas in 19 patients receiving Harvoni and in 9 patients receiving Viekirax and Exviera, the duration of therapy was 8 weeks. Clinical effectiveness of treatment was assessed 6 months post-treatment using a quantitative PCR test (Abbott RealTime HCV Viral Load Assay, Abbott Laboratories, Abbott Park, IL, USA; sensitivity 12 IU/mL). Sustained virologic response (SVR) was achieved in all 76 patients (100%).

Eighteen healthy anti-HCV negative volunteers served as control group. Characteristics of the study and control groups are presented in Table 1.

Thirty-six mL of EDTA-anti-coagulated whole blood was collected from all patients before and 6 months post-treatment while blood from control subjects was collected only once.

The study protocol followed ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Bioethical Committee of the Medical University of Warsaw (Approval Number KB/77/A/2015). All patients and controls provided written informed consent.

PBMC staining and flow cytometric analysis. Peripheral blood mononuclear cells (PBMC) and plasma were separated from whole blood using Lymphoprep reagent (Stemcell Technologies Inc, Vancouver, British Columbia, Canada). Plasma samples were stored at – 80 °C while PBMC were analyzed immediately after isolation.

HLA-A*02 typing. The presence of HLA-A*02 allele was verified by flow cytometry using Mouse Anti-Human HLA-A2 Clone BB7.2 antibody (BD Pharmingen, San Diego, CA, USA) and by quantitative PCR as described elsewhere[71].

T-cell phenotyping. Isolated PBMC were resuspended in PBS pH 7.2 (Life Technologies, Carlsbad, USA), stained with BD Horizon Fixable Viability Stain 780 (BD Biosciences, San Diego, CA, USA) and subsequently mixed with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's protocol. Next, one million cells were resuspended in Stain Buffer with 0.2% (w/v) bovine serum albumin (BD Pharmingen). mixed with 5 µl of BV421 Mouse Anti-Human Tim-3 (CD366) Clone 7D3 (BD Horizon, San Diego, CA, USA), 5 µl of Alexa Fluor 647 Mouse Anti-Human PD-1 (CD279) Clone EH12.1, 5 µl of PerCP-Cy 5.5 Mouse Anti-Human CD3 Clone UCHT1, (both from BD Pharmingen), 5 µl of V500 Mouse Anti-Human CD4 Clone RPA-TY (BD Horizon) and 1 µl of Mouse Anti-Human CD8 FITC Clone LT8 (ProImmune Oxford, United Kingdom). Cells with added antibodies were incubated for 20 min at 4 °C. After incubation, stained cells were washed twice with PBS pH 7.2 (Life Technologies) and resuspended in 300 µL of Stain Buffer.

Controls included unstained cells and isotype controls consisting of cells stained with 5 µl of Mouse Anti-Human IGG1 Alexa Fluor 647 and 2.5 µl of Mouse Anti-Human IGG1 BV421 instead of Alexa Fluor 647 Mouse Anti-Human PD-1 (CD279) and BV421 Mouse Anti-Human Tim-3 (CD366), respectively (both from BD Pharmingen). For data acquisition, one million stained cells were used. The results were acquired immediately

after staining by BD FACS Canto II Flow Cytometer (BD Biosciences), using BD FACS Diva version 6.0 program (BD Biosciences).

HCV-specific CD8⁺ T-cells enrichment and phenotyping. HCV-specific CD8⁺ T-cell populations are difficult to detect directly[72]. We employed a combination of MHC multimer staining, magnetic-bead enrichment, and multiparametric flow cytometry for ex vivo detection and characterization of rare antigen-specific CD8⁺ T-cells[73,74]. In 32 HLA-A*02⁺ patients (genotype 1b HCV infection), 25 million of PBMC were subjected to this procedure. In brief, custom PE-labeled Pro5 Recombinant MHC class I Pentamer containing HLA-A*02-restricted HCV $NS3_{1406}$ immunodominant epitope KLSGLGLNAV (corresponding to genotype 1b) (ProImmune) was added to cells resuspended in Stain Buffer and incubated for 10 min at room temperature in the dark. The cell suspension was then washed with MACS Buffer (Miltenyi Biotec) and cell pellet was resuspended with Anti-PE Micro Beads (Miltenyi Biotec) and MACS Buffer and incubated for 20 min at 4 °C, protected from light. Next, cells were washed twice with MACS Buffer and passed through a 70 µm Cell Strainer (BD Biosciences). Magnetic MS Columns (Miltenyi Biotec) were used for cell separation following manufacturer's instructions. Enriched cells were counted and stained with anti-CD3, -CD4, -CD8, -PD-1, -Tim-3 antibodies for 20 min at 4 °C as described above.

Efficiency of the HCV-specific T-cell enrichment. Without the enrichment, CD8⁺ specific T-cells were rarely detectable at measurable numbers in our patients (detectable in 14 (43.7%) of cases at mean frequency of 0.05% of total CD8⁺ T-cells before treatment and in 18 (56.2%) of cases at mean frequency of 0.07% of total CD8⁺ T-cells after treatment). However, using the enrichment procedure, detectability increased to 32 of cases (100%) and the average frequency increased 136-fold (to 6.8% of total CD8⁺ T-cells) before treatment and to 29 of cases (90.6%) and the average frequency increased 41.4-fold (to 2.9% of total CD8⁺ T-cells) after treatment.

Cytometric data analysis. For data analysis, the initial gate was set on lymphocytes on the forward scatter (FSC) *vs* side scatter (SSC) dot plot. Subsequently, singlet cells gate was set on FSC-H versus FSC-A dot plot. Next, based on SSC *vs* APC-Cy7 dot plot, only live cells were gated. Additionally, the following gates were employed: $CD3^+$, $CD4^+$, $CD8^+$, pentamer⁺, $PD\text{-}1^+$, $Tim\text{-}3^+$, $PD\text{-}1^+Tim\text{-}3^+$ and $PD\text{-}1^-Tim\text{-}3^-$.

IL-10 plasma levels measurement. IL-10 levels were measured in plasma by ELISA (Human IL-10 ELISA Max Kit; BioLegend, San Diego, CA, USA) following manufacturer's protocol. The ELISA Analysis program available from www.elisaanalysis.com was used to calculate IL-10 concentrations in plasma, expressed as pg/mL.

Statistical analysis. Percentages of gated cell populations and IL-10 levels were expressed as median (range). Wilcoxon matched-pairs signed ranks test was used to compare percentages of T-cells expressing exhaustion markers and IL-10 levels before and after treatment while Mann–Whitney U test was used to compare patients with controls. Kruskall-Wallis test was used to compare T-cell exhaustion markers and IL-10 levels between groups with different stage of fibrosis. A general linear model (GLM) was used to test independent pretreatment factors and treatment scheme on percentages of T-cells expressing exhaustion markers and IL-10 levels in plasma. All P values were two-tailed and considered significant when ≤ 0.05.

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Review

Reversal of T Cell Exhaustion in Chronic HCV Infection

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Abstract: The long-term consequences of T cell responses' impairment in chronic HCV infection are not entirely characterized, although they may be essential in the context of the clinical course of infection, re-infection, treatment-mediated viral clearance and vaccine design. Furthermore, it is unclear whether a complete reinvigoration of HCV-specific T cell response may be feasible. In most studies, attempting to reverse the effects of compromised immune response quality by specific blockades of negative immune regulators, a restoration of functional competence of HCV-specific T cells was shown. This implies that HCV-induced immune dysfunction may be reversible. The advent of highly successful, direct-acting antiviral treatment (DAA) for chronic HCV infection instigated investigation whether the treatment-driven elimination of viral antigens restores T cell function. Most of studies demonstrated that DAA treatment may result in at least partial restoration of T cell immune function. They also suggest that a complete restoration comparable to that seen after spontaneous viral clearance may not be attained, pointing out that long-term antigenic stimulation imprints an irreversible change on the T cell compartment. Understanding the mechanisms of HCV-induced immune dysfunction and barriers to immune restoration following viral clearance is of utmost importance to diminish the possible long-term consequences of chronic HCV infection.

Keywords: chronic HCV infection; T cell exhaustion; inhibitory receptors; direct acting antivirals

1. Introduction

WHO's Global Hepatitis Report estimates that about 71 million people are infected with hepatitis C virus (HCV) worldwide [1]. HCV is a blood-borne virus which is mainly transmitted via parenteral exposure as a consequence of intravenous drug use, or through reuse of injection needles or syringes [2]. The risk of parenteral infection is also increased among health care employees with frequent exposure to blood. HCV can also be transmitted sexually through permucosal exposure, especially in individuals with multiple sex partners, or in HIV-infected men who have sex with men (MSM) [2,3]. Furthermore, mother-to-child vertical transmission occurs in 2–8% of HCV-infected mothers [4]. About 80% of HCV-infected individuals do not exhibit any symptoms at the initial stage of infection, while the remaining 20% of acute cases develop mildly symptomatic infection. The virus is cleared spontaneously in 15–45% of individuals with acute infection, whereas the remaining 55–85% of cases develops chronic hepatitis C [5].

Clinical observations show that approximately 20% of chronically-infected patients develop advanced fibrosis and cirrhosis [6], which may lead to hepatocellular carcinoma (HCC), causative of

approximately 399,000 of deaths per year [1,2]. HCV-induced HCC usually develops within 20–40 years of infection and is observed in about 1–7% of infected patients with liver cirrhosis per year [7].

Currently, there is no vaccine available preventing HCV infection, but advances in diagnostic procedures and direct-acting antiviral (DAA) treatment resulted in substantial improvement in clinical care of individuals with hepatitis C, which is crucial in preventing HCV-related morbidity and mortality. Nevertheless, despite highly effective therapeutic options, the risk of HCC development in cirrhotic patients persists even after successful treatment [8].

The extraordinary genetic heterogeneity of HCV is a result of fast replication and high error rate of viral RNA-dependent RNA polymerase and is manifested by the *quasispecies* phenomenon, the concomitant presence of closely related genetic variants within an infected host, largely facilitating the adaptive dynamics of the virus [9]. HCV genetic heterogeneity is a major mechanism of immune system evasion, because of the increased probability of positive selection of escape variants in the immune pressure of the host [10]. The occurrence of mutations within the viral T cell epitopes was associated with diminished recognition by virus-specific T cells [11]. Viral escape occurs early during acute infection, indicating that it contributes to HCV persistence [12], but is also observed in approximately 50% to 70% of viral epitopes targeted by virus-specific $CD8^+$ T cell in chronic infection [12,13].

2. T Cell Exhaustion in HCV Infection

Adaptive immune responses play a critical role in the clinical course of infection with HCV [14,15]. HCV elimination coincides with strong and sustained multi-specific $CD4^+$ and $CD8^+$ T cell immunity which remains detectable after the spontaneous resolution of infection [15]. However, the quality of this response is substantially deteriorated once chronic infection is established [16]. Both $CD4^+$ and $CD8^+$ HCV-specific T cells are commonly present in liver tissue and in peripheral blood, however, in most patients, these cells are unable to clear the infection and do not prevent re-infection with HCV [14,15,17]. The underlying immune impairment phenomenon has been termed T cell exhaustion, defined as weak antigen-specific T cell responses, manifested as the deterioration in antiviral effector functions of antigen-specific T cells, such as a decline in effector cytokines' production, the decreased capability to eliminate infected cells and impaired proliferation after antigen exposure in vitro [18,19]. The consequence of this phenomenon is loss of control over the ongoing infection, and emerging data suggest that exhaustion is a crucial factor determining viral persistence [20–23]. T cell exhaustion is not uniquely observed in HCV infection, but also in other chronic viral infections, particularly with lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV) or hepatitis B virus (HBV), as well as in tumors [20,24–27].

Although most findings are based on the LCMV mouse model, the pathway of T cell exhaustion seems to be universal. The decline in T cell effector functions is sequential and hierarchical, being initiated by the loss of interleukin (IL)-2 expression, followed by the decreased expression of tumor necrosis factor (TNF) and ultimately interferon (IFN)-γ, β-chemokines, as well as impaired cytotoxicity [28,29]. Moreover, exhausted $CD8^+$ T cells downregulate the expression of IL-7 and IL-15 receptors, which physiologically sustain the proliferation and survival of memory T cells [30–32]. Despite substantial functional impairment, exhausted T cells may continue to express proteins associated with effector function [27]. It is believed that T cell exhaustion has evolved as a host-driven mechanism to limit the severity of the immune response and protect from immunopathology [33].

T cell exhaustion is mediated by continuous antigen stimulation, progresses along the time of infection, and is accompanied by transcriptional, translational, metabolic, nucleosomal and epigenetic changes [34–38]. In consequence, exhausted T cells display a characteristic phenotypic and functional pattern distinct from effector and memory T cells, pointing out that exhaustion represents a separate branch of CD8+ T cell differentiation [39–41].

On a phenotypic level, T cell exhaustion during chronic infection is manifested as upregulation of inhibitory receptor (iR) protein molecules, which deliver negative signals precluding cell activation

after antigen recognition and downregulate the functional and proliferative potential of the responding cells [37,40,42]. In acute infection, iRs function to limit immune responses, but are downregulated when the pathogen is cleared. It has been demonstrated that iRs negatively affect T cell function and activation at several levels: (i) through competition with co-stimulatory receptors for shared ligands; (ii) by interfering with signals from co-stimulatory receptors or TCR; (iii) by the upregulation of genes involved in T cell dysfunction [43,44]. IRs, which have been linked to T cell exhaustion, include but are not limited to programmed cell death-1 (PD-1/*CD279*), cytotoxic T cell antigen 4 (CTLA-4/CD152), B- and T-lymphocyte attenuator (BTLA/CD272), CD160, CD200, NK cell type I receptor protein (2B4/CD244), lymphocyte-activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), Ig superfamily-related receptors (GP49/CD85k) and T cell immunoglobulin and mucin-domain containing-3 (Tim-3) (reviewed in [40,43,44]). Furthermore, molecular iR pathways seem to be mechanistically related, and it is the co-expression of multiple iRs defining the state of exhaustion rather than their mono-expression, which was confirmed by the observation that the simultaneous blockade of several iRs results in better effects on the reversal of exhaustion [40].

The underlying changes in transcriptional program which drive the phenotypic and functional signature of exhausted $CD8^+$ T cells are complex, specific and sequential [45]. An additional factor complicating this landscape is the heterogeneity of exhausted $CD8^+$ T cell populations [46–49]. Although a number of transcription factors have been attributed to the pathogenesis of T cell exhaustion (e.g., B lymphocyte-induced maturation protein-1 (Blimp-1), basic leucine zipper ATF-Like transcription factor (Batf), eomesodermin (Eomes), and nuclear factor of activated T cells (NFAT) as well as T-box protein expressed in T cells (T-bet/TBX21) [48,50–53], recent discoveries pointed to the paramount role of T cell factor family member TCF-1 (Tcf7), as well as HMG-box transcription factor TOX [23,54,55]. TCF-1 is a key transcription factor for the "stem-like", progenitor exhausted $PD\text{-}1^+TCF\text{-}1^+$ $CD8^+$ T cell population, which sustains $CD8^+$ T cell responses during chronic viral infection and gives rise to $PD\text{-}1^+Tim3^+$ $TCF\text{-}1^-$ exhausted and terminally differentiated cytotoxic cells [55,56]. Similarly, TOX has been identified as a critical transcriptional and epigenetic coordinator of exhausted $CD8^+$ T cell programming, being robustly expressed in exhausted T cells, but transiently and at low levels during acute viral infection. TOX is necessary and sufficient to induce major features of exhausted T cells, including transcriptional changes, the expression of iRs and decreased function [23,54]. The establishment and maintenance of exhausted T cells depends on TOX, TCF-1, Eomes and T-bet as essential components regulating their development and balance [36,48,55,57]. Beltra et al. delineated a four-stage developmental exhausted $CD8^+$ T cell hierarchy driven by transcription factor cascade conversion from progenitor 1 quiescent and resident $TCF\text{-}1^{hi}TOX^{hi}$ to progenitor 2 proliferative circulating $TCF\text{-}1^{int}TOX^{hi}$ to intermediate circulating mildly cytotoxic $TCF\text{-}1^{neg}T\text{-}bet^{hi}TOX^{int}$ and, finally, to terminally exhausted resident $TCF\text{-}1^{neg}T\text{-}bet^{lo}TOX^{hi}Eomes^{hi}$ cells [36].

In contrast to $CD8^+$ cells, $CD4^+$ T cell exhaustion is still poorly understood, which is mainly due to the lower ex vivo frequency of peripheral blood virus-specific $CD4^+$ T cells during chronic infection as well as technical limitations, particularly in generating MHC class II/antigen complexes by recombinant methods, and lower affinity binding of $CD4^+$ TCR to cognate epitope on MHC class II molecule [58,59]. To date, it has been shown that exhausted $CD4^+$ T cells lose the ability to secrete TNF-α, IFN-γ and IL-2, but increase production of IL-10 and IL-21 [60,61]. Furthermore, some data suggest that virus-specific $CD4^+$ T cells lose effector function sooner than $CD8^+$ T cells [62].

Although phenotypically similar, exhausted $CD4^+$ and $CD8^+$ T cells display certain qualitative differences. In particular, CTLA-4 and CD200 expression, as well as an increase in KAROS family zinc finger 2 (Ikzf2) (Helios) and Kruppel-like factor 4 (Klf4), are more specific to $CD4^+$ T cells [63].

HCV infection represents an extraordinary opportunity to study the pathogenesis of T cell exhaustion in humans, since the spontaneous and complete resolution of infection is uniquely feasible among human chronic viral infections, even after exposure to a strain which previously established chronic infection in other subjects [64]. Furthermore, being the only chronic viral infection in both humans and chimpanzees which can be cured by a highly specific, small molecule-based DAA treatment,

the effect of the removal of constant stimulation with viral antigens, the underlying cause of immune exhaustion, can be genuinely studied [49,65]. The use of magnetic bead enrichment of HCV-specific T cells, as well as recent advances in next-generation sequencing technologies, facilitated studies of these extremely rare populations [16]. Research on the dysregulation of transcriptional, metabolic, nucleosomal, and immune processes in HCV-specific $CD8^+$ T cells preceding the overt establishment of T cell exhaustion in this infection is currently underway. Recently, the transcriptomic profile of HCV-specific $CD4^+$ and $CD8^+$ T cells during acute resolving vs. chronic infection has been delineated. Similar to the LCMV mouse model, it was found that TOX is induced by high antigen stimulation of the T cell receptor during chronic HCV infection in humans and correlates with the exhausted phenotype, in particular with PD-1 expression levels [23]. Furthermore, TOX was detectable in chronic but not in spontaneously resolved HCV infection or influenza-specific memory T cells, pointing out that TOX is crucial for direction of adaptive T cell responses toward exhaustion. Similarly, progression to chronic HCV infection was characterized by higher expression of strategic regulators of T cell immune function, proliferation, and survival, i.e., TBK1, SIRT1, BCOR, and BCL2L11, while acute resolving infection was marked by the higher expression of regulators of T cell differentiation and memory, e.g., TCF7 and its transcriptional target LEF1 [22]. This suggests that $CD8^+$ T cells T cell differentiation might be negatively impacted very early during chronic infection, manifested as the rapid dysregulation of genes that are crucial for orchestration and direction of adaptive T cell responses.

3. Reversibility of T Cell Exhaustion in HCV Infection

Understanding the molecular pathways of T cell exhaustion, in particular the contribution of inhibitory receptors, has helped to identify potential strategies aiming to restore T cell functionality and to improve infection control [66,67]. These included the specific blocking of immunosuppressive cytokines, iRs or their ligands with monoclonal antibodies, antiviral treatment and vaccination (Figure 1). Promising results obtained by blocking of iRs signaling pathways in HIV and LCMV infections have prompted similar studies in in vitro models of HCV infection (Table 1). Because of their better characterization, most studies explored the aspect of $CD8^+$ T cells' exhaustion.

Blockades of PD-1 and/or Tim-3 pathways using monoclonal antibodies could restore functional features of HCV-specific $CD8^+$ T cells, such as proliferation and effector cytokines secretion, whereas improvement in the cytolytic function was observed exclusively in the case of Tim-3 blockade [18,68–71]. These findings are congruent with the concept of hierarchical model of functional T cell exhaustion, with an easier restoration of proliferation capacity, followed by the restoration of effector cytokines production, but with a general lack of effect on cytotoxicity.

Encouraging beneficial effects of in vitro experiments warranted studies employing in vivo immunotherapy of chronic HCV infection using antibodies against iRs (Table 1) [72–74]. Fuller et al. [72] investigated the effect of anti-PD-1 antibodies treatment on T cell responses in three chimpanzees with chronic HCV infection. A significant reduction in HCV viral load without signs of hepatocellular injury was observed in one animal, which rebounded when antibody treatment was discontinued. The viral load drop in this animal was accompanied by the restoration of intrahepatic $CD4^+$ and $CD8^+$ T cell immunity to multiple HCV proteins.

Gardiner et al. [73] presented the findings of a proof-of-concept, placebo-controlled, single-ascending-dose study in 54 patients with chronic HCV infection treated with PD-1-targeting nivolumab (*BMS-936558*). Five patients were reported to respond with reduction in viral load, including two patients with HCV RNA levels below the lower limit of detection. Importantly, treatment was not related to any evidence of immune deficit, since neither clinically relevant changes in immunoglobulin subsets nor changes in mean serum levels of major cytokines were observed during follow-up. Furthermore, substantial quantitative changes in immune cell subsets were not evident and cell counts generally returned to pretreatment levels after one week of treatment.

A similar pilot clinical trial was conducted by Sangro et al. [74] to test the antitumor and antiviral effect of tremelimumab (anti-CTLA-4 monoclonal antibody) in 20 patients with chronic HCV infection

and HCC. Tremelimumab induced a decrease in viral load in most patients followed-up for at least three months. Fifteen percent of patients experienced a transient complete viral response. In parallel, an increased number of virus-specific, IFN-γ-producing lymphocytes were detected, suggesting that antiviral effect was most likely a result of enhanced T-cell-mediated immunosurveillance. *Quasispecies* changes, manifested as new emerging hypervariable region 1 variants, were observed, coinciding with the second cycle of tremelimumab.

The use of immune checkpoint inhibitors in cancer immunotherapy was shown to increase the risk of immune-related adverse events (irAEs) as a result of the elevated activity of immune cells [75]. IrAEs can affect multiple organs, i.e., liver, skin, digestive system, lung, endocrine glands and potentially other tissues [75]. In particular, liver damage manifesting as hepatitis occurs in 1–17% of treated patients [76]. Nevertheless, in the study of Sangro et al., a good safety profile was recorded, including no severe irAEs requiring the administration of steroids.

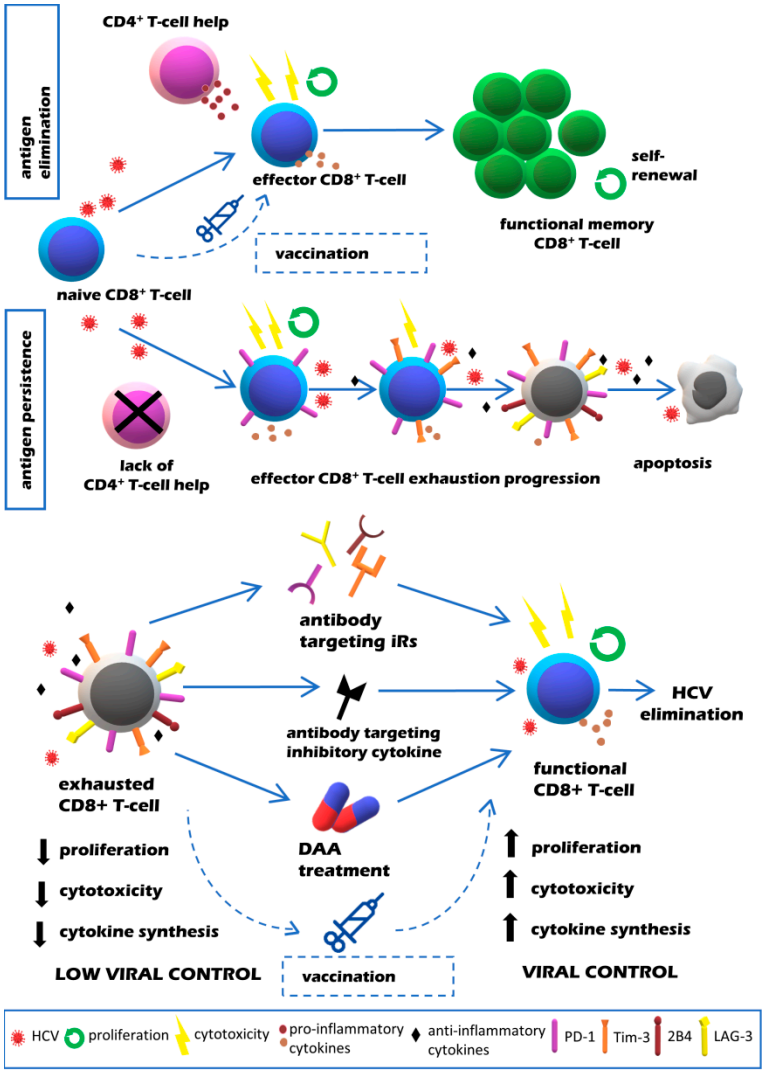

Figure 1. Quality of CD8+ T cell responses in resolving vs. chronic HCV infection (upper panel) and potential strategies aimed at restoration of exhausted T cell function in chronic HCV infection (lower panel).

Table 1. Studies reporting the effect of immune checkpoint inhibitor blockades on CD8+ T cell function in HCV infection.

Reference	Immune Checkpoint Blocked	Stage of HCV Infection	Number of Subjects	Character of the Study	Results
Golden Mason et al. [68]	PD-ligand 1 (PD-L1) PD-ligand 2 (PD-L2)	chronic	7	In vitro	↑ proliferation of HCV-specific $CD8^+$ T cells ↑ IFN-Y and IL-2 secretion by HCV-specific $CD8^+$T cells
Golden Mason et al. [69]	Tim-3	chronic	4	In vitro	↑ proliferation of HCV-specific $CD8^+$ T cells ↑ IFN-Y secretion by HCV-specific $CD8^+$ T cells ↓ IL-10 secretion by HCV-specific $CD8^+$ T cells
Penna et al. [18]	PD-L1	chronic	8	In vitro	↑ expansion of HCV-specific $CD8^+$ T cells ↑ frequency of both IFN-γ– and IL-2–secreting HCV-specific $CD8^+$ T cells
Urbani et al. [70]	PD-L1	acute	8	In vitro	↑ expansion and IFN-γ and IL-2 production but not the cytolytic activity of HCV-specific $CD8^+$ T cells.
McMahan et al. [71]	Tim-3, PD-L1, PD-L2	acute/chronic	6/4	In vitro	↑ proliferation of HCV-specific $CD8^+$ T cells achieved by either PD-1 or Tim-3 blockade ↑ cytotoxicity of HCV-specific $CD8^+$ T cells (increased expression of CD107a, killing of hepatocytes cell line expressing cognate HCV epitopes) achieved exclusively by Tim-3 blockade
Fuller et al. [72]	PD-1	chronic	3 chimpanzees	In vivo	↓ HCV viral load in one of three treated animals ↑ frequencies and IFN-Y production of intrahepatic HCV-specific $CD4^+$ and $CD8^+$ T cells in the same animal
Gardiner et al. [73]	PD-1	chronic	54	In vivo	↓ viral load in five patients (two patients achieved undetectable HCV RNA)
Sangro et al. [74]	CTLA-4	chronic	20	In vivo	↓ viral load sustained in most patients for 3 months follow-up; transient complete viral response in 15% of patients during follow-up ↑ HCV-specific T cell response (IFN-Y production)

↑ —increase; ↓ —decrease.

Cytokines are also regarded as an attractive therapeutic target for the modulation of immune response in chronic viral infections. It was shown that selective blockade of the IL-10 receptor (IL-10R) can result in virus control and the reinvigoration of immune response in chronic LCMV infection in vivo [77]. Translating these findings into HCV infection, Rigopoulou et al. [78] showed that the in vitro monoclonal antibody-induced blockade of IL-10R resulted in a dose-dependent increase in $CD4^+$ T cell proliferative responses to the HCV core, as well as non-structural proteins 3 (NS3) and 4 (NS4). Furthermore, the blockade of IL-10R altered the balance in favor of type 1 antiviral T cell immunity with an increased frequency of HCV-specific, IFN-γ-producing $CD4^+$ T cells.

Taken together, the available findings, although sparse, imply that HCV-induced immune dysfunction may be reversible. They demonstrated that the restoration of proliferation and effector functions of HCV-specific T cells could be observed in vitro and in vivo, the latter being accompanied by durable objective responses and good safety profiles. However, because of the recent advances in and introduction of highly effective therapies based on direct-acting antivirals (DAA), the scope of T cell research has shifted, and the abovementioned immunotherapeutic approaches have been mostly abandoned.

4. Impact of DAA Treatment of Chronic HCV Infection on T Cell Exhaustion

In recent years, substantial progress has been achieved in treatment of chronic HCV infection, which is expected to reduce the extent of virus-related morbidity and mortality (reviewed in [79]). The assessment of therapy effectiveness involves the periodic screening of HCV RNA in patient's serum by polymerase chain reaction (PCR). A sustained virologic response (SVR), defined as the absence of HCV RNA evaluated at 12 weeks (formerly 24 weeks) post-treatment, is regarded as a therapeutic success [80]. Until recently, the standard of care for chronic hepatitis C was a combination of pegylated-IFN-α (PEG-IFN-α) with ribavirin (RBV). IFN-α plays an immunoregulatory, antiviral and anti-proliferative role, while ribavirin inhibits viral RNA polymerase [80]. The newly recommended IFN-free treatment schemes include DAA drugs (NS3/4A, NS5A and NS5B inhibitors) [81]. DAAs exhibit antiviral activity via interference with HCV replication cycle (inhibition of HCV polyprotein maturation and HCV RNA synthesis) [82]. These treatment regimens are short (i.e., last routinely 8–12 weeks), safe, well tolerated, highly effective (SVR rates above 95%), and can be optimized by combining drugs with synergistic or additive effects [83].

The impact of anti-HCV treatment commenced in chronic phase of infection on already-established T cell exhaustion is largely unknown and commonly inconclusive, which may be due to methodological differences (e.g., assessing various immunological effects of treatment, small patient cohorts, different treatment schemes, inclusion of patients infected with different HCV genotypes as well as different follow-up time points) [70,84–87]. Theoretically, the successful therapy of chronic HCV infection might reverse the functional T cell exhaustion by a number of mechanisms, e.g., by a rapid reduction in viral load and immune system stimulation with viral antigens or the elimination of viral proteins known to inhibit immune responses [15,88–90].

Studies of T cell function in IFN-based therapy of chronic HCV infection have shown that it reduces the numbers and impairs the functional potential of antiviral T cells [65,91–93]. While successful IFN-based treatment of acute HCV infection results in highly functional T cell responses comparable to those in spontaneous resolvers, SVR attained by IFN-based treatment of chronic HCV infection does not result in the functional restoration of HCV-specific $CD8^+$ T cells [92,94]. This implies that, in chronic HCV infection, the endogenous T cell population does not contribute to the IFN-based treatment's success [65,91–93]. In contrast, the restoration of antiviral immunity, manifested by the reversal of the exhausted T cells' phenotype and immune-driven elimination of residual viral replication, may be necessary for successful DAA treatment, since the presence of HCV-RNA in serum at the end of treatment (EOT) does not preclude SVR [95,96]. In agreement with that, some findings suggest that DAA treatment may result in certain positive effects on the T cell immune function or phenotype (Tables 2 and 3).

Table 2. Studies reporting the effect of direct-acting antiviral (DAA) treatment on peripheral T cell phenotype or function in chronic HCV infection.

Reference	HCV Genotype	Number of Subjects	Effect of Treatment/Effect of Successful Treatment	Follow-up	Results
Shrivastava et al. [97]	1	22 HIV/HCV co-infected	Effect of successful treatment	12 weeks after the end of treatment (EOT) (sustained virologic response (SVR) 12)	↓ PD1 and TIGIT expression on $CD4^+$ and $CD8^+$ T cells ↓ $Eomes^{hi}$ $T\text{-}bet^{lo}$ $CD4^+$ and $CD8^+$ T cells ↑ $T\text{-}bet^{hi}$ $Eomes^{lo}$ $CD4^+$ and $CD8^+$ T cells ↓ BLIMP-1 expression on $CD4^+$ T cells ↓ CD38 expression on both $CD4^+$ and $CD8^+$ T cells ↑ T_{em} (effector memory) population ↓ naïve T cell subset
Burchill et al. [98]	1a/1b	19	Effect of successful treatment	24 weeks post-EOT (SVR24)	↑ frequency of $CD4^+$ T cells; ↓ expression of TIGIT on $CD4^+$ and $CD8^+$ T cells; ↑ percentage of T_{em} in both $CD4^+$ and $CD8^+$ T cells compartments
Najafi Fard et al. [99]	1–4	HCV mono-infection $n = 18$; HCV/HIV-1 co-infection ($n = 17$)	Effect of successful treatment	12 weeks post-EOT (SVR12)	↑ peripheral $CD4^+$ and $CD8^+$ T cells producing IFN-γ, IL-17, and IL-22 no significant impact on the status of $CD4^+$ and $CD8^+$ T cells activation
Meissner et al. [100]	1	95	Effect of treatment	up to 20 weeks after treatment initiation	↑ peripheral $CD4^+$ and $CD8^+$ T cells early after treatment initiation ↓ $HLA\text{-}DR^+CD38^+$ T-cells during observation ↑ expression CXCR3 on T cells early after treatment initiation
Lattanzi et al. [101]	1–4	45	Effect of treatment	at first month of treatment (T1), at EOT (T2) and 12 weeks post-EOT (T3, SVR12)	stable percentage of $CD4^+$ and $CD8^+$ T cells at T1 when compared to baseline ↓ $HLA\text{-}DR^+$ $CD38^+$ $CD4^+$ and $CD8^+$ T cells at T3 with respect to baseline
Emmanuel et al. [102]	NA	HCV mono-infection ($n = 161$); HIV/HCV co-infection ($n = 59$)	Effect of successful treatment	1 or 2 years post-SVR	↓ $HLA\text{-}DR^+CD38^+$ $CD4^+$ and $CD8^+$ T cells in both HCV infection and HIV/HCV co-infection
Vranjkovic et al. [103]	NA	18	Effect of successful treatment	24 weeks post-SVR12	phenotypic distribution of peripheral $CD8^+$ T cell subsets in patients with advanced liver fibrosis (F4) different from those with minimal fibrosis (F0-1) which remained unchanged after viral elimination sustained hyperfunctional activity (perforin production and cytotoxicity) of $CD8^+$ T cell subsets in patients with liver fibrosis (F4) up to a year post-treatment initiation sustained elevated concentrations of systemic inflammatory cytokines and decreased levels of TGF-β in plasma of patients with liver fibrosis (F4)

↑—increase; ↓—decrease; NA—not available.

Table 3. Studies reporting the effect of DAA-treatment on peripheral HCV-specific T cell phenotype or function in chronic HCV infection.

Reference	HCV Genotype	Number of Subjects	Effect of Treatment/Effect of Successful/Unsuccessful Treatment	Follow-up	Results
Romani et al. [104]	1a/1b	26	Effect of successful/unsuccessful treatment	at the end of treatment (EOT), at week 4 and 12 weeks post-EOT (sustained virologic response (SVR) 12)	higher levels of PD-1$^+$ HCV-specific T cells at baseline and at EOT in patients who achieved SVR ↓ PD-1$^+$ HCV-specific T cell subset at SVR12 in responders
Burchill et al. [98]	1a/1b	7	Effect of successful treatment	24 weeks post -EOT (SVR24)	no significant change in the frequency of HCV-specific CD8$^+$ T cells ↓ PD-1 expression
Martin et al. [105]	1	51	Effect of successful/unsuccessful treatment	treatment week 4, 12 and 24 weeks post-treatment (SVR24)	↑ HCV-specific CD8$^+$ T cells frequency after in vitro expansion in patients with SVR from baseline to 24 weeks after completion of treatment no change in HCV-specific CD8$^+$ T cells frequency in patients with treatment failure
Shrivastava et al. [97]	1	22 HIV-1/HCV co-infected	Effect of successful treatment	12 weeks post-EOT (SVR12)	↑ HCV-specific CD8$^+$ T cells ↑ IL-2 and IFN-γ production ↑ polyfunctionality (co-expression of IFN-γ and TNF-α) ↑ cytolytic capacity (CD107A expression and perforin and granzyme B secretion)
Wieland et al. [106]	1a/1b	21	Effect of successful treatment	at EOT and 12 weeks post-EOT (SVR12)	↓ terminally exhausted HCV-specific CD8$^+$ T cells (TCF-1$^-$CD127$^-$PD1^{hi}) after antigen elimination persistence of memory-like HCV-specific CD8$^+$ T cells (TCF-1$^+$CD127$^+$PD-1$^+$) with ability of self-renewal and proliferation
Han et al. [107]	1b/2a	41	Effect of successful/unsuccessful treatment	treatment week 4, 12, 24 (EOT) and 12 weeks post-treatment (SVR12) or week 4, 12 (EOT), and 12 weeks post-treatment (SVR12)	↑ HCV-specific CD8$^+$ T cell response (IFN-Ɣ production, cytotoxicity) at week 4, which diminished at later weeks ↓ PD-1$^+$Eomes^{hi}T-bet^{low} HCV-specific CD8$^+$ T cells at week 4 ↓ HCV-specific CD8$^+$ T cell frequency at SVR12, including antigen-experienced KLRG1$^+$CCR7$^-$ HCV-specific T cells no change in TCF-1$^+$CD127$^+$PD-1$^+$ HCV-specific CD8$^+$ T cells responsible for recall proliferation over observation time defective restoration of HCV-specific T cell responses in SVR$^-$ group
Aregay et al. [108]	1a/1b	40	Effect of successful treatment	at EOT and 24 weeks post-EOT (SVR24)	unaltered expression of PD-1, Tim-3, LAG-3 and CD5 on HCV-specific CD8$^+$ T cells sustained impaired IFN-Ɣ, MIP-1β production, mitochondrial dysfunction and metabolic deregulation ↓ HLA-DR$^+$CD38$^+$ HCV-specific CD8$^+$ T cells maintenance of memory-like TCF-1$^+$CD127$^+$PD-1$^+$ HCV-specific CD8$^+$ T cells
Hartnell et al. [109]	NA	21	Effect of successful treatment	average 6 weeks post-treatment (range 0–26 weeks)	unchanged proliferative capacity and cytokine production (TNF-α, IFN-Ɣ MIP-1β) of exhausted HCV-specific CD4$^+$ T cells
Smits et al. [110]	1, 2, 3	40	Effect of successful treatment	week 2, either 8, 12, 16 or 24 week of treatment (EOT) and 24 weeks post-treatment (SVR24)	↑ HCV-specific CD4$^+$ T cells within the initial two weeks of treatment unchanged percentages of HCV-specific CD4$^+$ T cells expressing PD-1, BTLA and TIGIT ↑ follicular T helper cells (Tfh) ↓ germinal center activity and HCV-specific neutralizing antibodies

↑—increase; ↓—decrease; NA—not available.

Some studies have demonstrated that DAA treatment of chronic HCV infection leads to the reconstitution of peripheral T cell populations, as evidenced by an increase in the frequency of $CD4^+$ [98,100] and $CD8^+$ T cells [100], and shift toward T_{em} (effector memory) population [97,98], with a concomitant decrease in the naïve T cell subset [97]. Effector function reinvigoration was also observed, manifested by increased frequencies of circulating T helper and cytotoxic T cells, producing IFN-γ, IL-17, and IL-22 [99]. Furthermore, a reduction in the expression of PD-1 [97] as well as TIGIT [97,98] on both $CD4^+$ and $CD8^+$ T cells was reported. Similarly in our study, DAA-treatment resulted in significant decreases in $CD4^+PD\text{-}1^+Tim\text{-}3^+$ and $CD8^+PD\text{-}1^+Tim\text{-}3^+$ T cell frequencies to levels observed in controls, while $CD8^+PD\text{-}1^+$ T cells significantly increased (manuscript submitted). Furthermore, the DAA effect was also observed in decreased IL-10 plasma levels.

Most studies focused on the impact of successful DAA treatment of chronic HCV infection on the status of $CD4^+$ and $CD8^+$ T cell activation. While one study did not show any significant changes after treatment [99], other studies did show a decline in T cell activation status, as evidenced by reduced HLA-DR and/or CD38 expression after treatment [97,100–102]. This was also demonstrated in the case of HIV-1/HCV co-infection [102]. However, Vranjkovic et al. [103] observed that the impact of successful DAA treatment on T cell activation depends mostly on the status of liver fibrosis. The hyperfunctional activity of peripheral $CD8^+$ T cell subsets (naïve, effector, early effector memory, late effector memory and central memory) was sustained in HCV-infected patients with liver fibrosis (F4) up to a year after treatment, particularly manifesting as elevated perforin production and cellular cytotoxicity when compared to patients with minimal fibrosis (F0-1). Furthermore, DAA treatment had no effect on elevated concentrations of systemic inflammatory cytokines and decreased levels of inhibitory TGF-β in plasma of F4 patients, suggesting that HCV infection and advanced liver disease result in a long-lasting immune activating microenvironment. According to the authors, the sustained hyperfunction of $CD8^+$ T cells long after DAA treatment may have significant long-term consequences, including compromised antitumor immunity, often aggressive forms of HCC, increased risk of HCC recurrence [111] and extrahepatic cancers [112]. Other concerns include potential failure to generate effective HCV vaccine responses and vulnerability to HCV re-infection [113].

The abovementioned studies mostly concerned the effect of DAA-based treatment on the total peripheral T cell populations, typically not investigating HCV-specific T cells. Because of limited numbers of such cells in circulation, only a few studies addressed this issue [97,98,104,106,107] (Table 3).

Romani et al. [104] found the SVR-predictive value of $PD\text{-}1^+$ HCV-specific $CD8^+$ T cell subset with cytotoxic capacity (degranulation and cytokine production) in short-duration DAA treatment. Higher levels of $PD\text{-}1^+$ $CD8^+$ HCV-specific T cells were observed in patients who achieved SVR, both at baseline and at EOT, compared to relapsers, which points to the essential role of these cells in DAA-mediated viral clearance. However, 12 weeks after EOT, the frequency of this subset of cells was significantly reduced exclusively in the SVR^+ group, which possibly reflects the elimination of $PD\text{-}1^+$ HCV-specific cells after successful antigen removal. Thus, these results confirm the hypothesis of the active role of immunity in DAA-mediated viral clearance.

Burchill et al. [98] observed that although the frequency of $PD\text{-}1^+$ HCV-specific T cells significantly decreased, the frequency of HCV-specific $CD8^+$ T cells was not significantly altered post-successful DAA treatment.

Martin et al. [105] showed that some functional capabilities, e.g., proliferation, could be renewed. A significant increase in the frequency of HCV-specific $CD8^+$ T cells after in vitro expansion was observed in most of SVR^+ patients from baseline to 24 weeks after completion of treatment, but not in patients with treatment failure.

Shrivastava et al. [97] demonstrated that successful DAA treatment of HCV infection in HIV-1/HCV co-infected patients led to the improvement in HCV-specific T cell function, including cytokine production (IL-2 and IFN-γ), polyfunctionality (measured as an increase in the proportion of cells co-expressing IFN-γ and TNF-α) and cytolytic capacity (increase in CD107A expression and perforin and granzyme B secretion). Comparing therapies with two or three different DAAs, they showed

that the most profound restoration of HCV-specific immune responses was observed in the group of patients treated with a regimen that inhibits three distinct stages of the HCV life cycle. Whether this was due to more potent suppression of HCV in vivo or an independent effect on the immune system remained unknown.

Wieland et al. [106] showed that the restoration of HCV-specific $CD8^+$ T cells' ability to proliferate was associated with changes in their composition. While terminally exhausted HCV-specific $CD8^+$ $TCF\text{-}1^-CD127^-PD1^{hi}$ T cells disappeared after antigen elimination, the memory-like HCV-specific $CD8^+$ T cells ($TCF\text{-}1^+CD127^+PD\text{-}1^+$), with a retained ability of self-renewal and proliferation, persisted, even in the absence of HCV antigen. Moreover, these cells displayed the capacity of robust secondary expansion in a patient with a viral relapse. However, they did not fully resemble memory HCV-specific $CD8^+$ T cells observed after spontaneous viral clearance since they displayed higher PD-1 and Eomes expression, indicative of T cell exhaustion and impaired cytokines production.

Han et al. [107] observed that although successful DAA treatment increased the proliferative capacity of HCV-specific $CD8^+$ T cells, their ex vivo function, which manifested as IFN-ϒ production capabilities, cytotoxicity as well as diminished exhausted marker expression, was only transient (i.e., observed only at week 4 of treatment, which coincided with viral clearance), but attenuated and returned to baseline levels at SVR12. While $TCF\text{-}1^+CD127^+PD\text{-}1^+$ HCV-specific $CD8^+$ T cells responsible for recall proliferation after antigen re-challenge remained unchanged over time, ex vivo HCV-specific $CD8^+$ T cell frequency decreased at SVR12, including antigen-experienced ($KLRG1^+CCR7^-$) HCV-specific $CD8^+$ T cell subset. In contrast, patients experiencing viral breakthrough or relapse exhibited defective restoration of HCV-specific T cell immunity, manifested as significantly lower T cell responses at any timepoint of observation. These findings suggest that the ex vivo function of HCV-specific T cells from chronically infected patients may not be enhanced after successful DAA treatment, despite transient functional restoration during early treatment.

Aregay et al. [108] observed that HCV clearance following DAA therapy was not able to fully restore HCV-specific $CD8^+$ T cells function. The expression of exhaustion markers (PD-1, Tim-3, LAG-3 and CD5) on HCV-specific $CD8^+$ T cells, impaired cytokine production (IFN-ϒ, MIP-1β), mitochondrial dysfunction and metabolic deregulation (reduced mitochondrial polarization, increased mitochondrial mass and increased mitochondrial ROS level), and did not alter after successful DAA treatment. Furthermore, the impaired proliferative potential of HCV-specific $CD8^+$ T cells was only partially restored. In contrast, a significant reduction in HCV-specific $CD8^+$ T cells expressing activation markers (CD38 and HLA-DR), which could be due to the associated loss of terminally differentiated $CD39^+$ HCV-specific $CD8^+$ T cells, was seen following HCV elimination. Similar to Wieland et al.'s and Han et al.'s studies [106,107], memory-like HCV-specific $CD8^+$ T cells ($TCF\text{-}1^+CD127^+PD\text{-}1^+$) remained unaltered after HCV clearance. Interestingly, the proliferative capacity could be increased upon PD-1/PD-L1 pathway blockade exclusively in HCV-specific $CD8^+$ T cells, whose proliferative potential was not restored after HCV clearance by means of DAA therapy.

Although epidemiological data show cases of HCV re-infection after DAA-mediated viral clearance, it is currently unclear how the eventual restoration of $CD8^+$ HCV-specific T cell response would assure protection or influence the clinical course of re-infection [114]. However, a recent study employing a chimpanzee model of chronic HCV infection has demonstrated that the HCV-specific $CD8^+$ T cell population which persisted after DAA-mediated viral clearance did not prevent the development of chronic infection after re-infection [115].

In contrast to $CD8^+$ T cells, there are almost no data on DAA-treatment-induced alterations within the $CD4^+$ HCV-specific T cell subset. In the study by Hartnell et al. [109], it was demonstrated that helper responses in chronic HCV infection was infrequently detected and poorly functional, and did not consistently recover following HCV cure, since DAA treatment did not promote proliferative capacity and TNF-α, IFN-ϒ and MIP-1β production by HCV-specific $CD4^+$ T cells.

In contrast, Smits et al. [110] observed that the very low baseline frequency of HCV-specific $CD4^+$ T cells increased within the initial two weeks of DAA treatment. Although percentages of

HCV-specific $CD4^+$ T cells expressing PD-1, BTLA, and TIGIT were maintained during observation (from baseline to follow-up (SVR24)), analyses of the mean fluorescence intensity (MFI) revealed a significant reduction in the expression levels of PD-1. Importantly, although cells with a Th1 phenotype were the predominant subset at baseline, cells with phenotypic and transcriptional characteristics of follicular T helper cells (Tfh) significantly increased from baseline to follow-up (SVR24), suggesting the antigen-independent survival of this subset. These changes were accompanied by a decline in the germinal center activity and HCV-specific neutralizing antibodies, indicating that these cells may be involved in maintaining HCV-specific humoral immunity during chronic infection.

$CD4^+$ regulatory T cells (Treg, $Foxp3^+$ $CD25^+$ $CD4^+$) limit the in vitro responses of effector $CD8^+$ T cells via suppression of their activation [116]. Although the direct role of Tregs in exhaustion of $CD8^+$ T cells remains unclear, they may play a role, considering that Tregs are a source of immune-suppressive IL-10, TGF-β and IL-35 [66]. Langhans et al. [117] showed that the percentage of Tregs was significantly higher in chronically HCV-infected patients than in healthy controls. After successful DAA therapy, their frequency slightly decreased, although up to 51 ± 14 weeks post-EOT it did not reach levels similar to those observed in healthy controls. The expression of activation/regulation markers on Tregs (i.e., GARP, OX-40, CTLA-4, GITR, Tim-3 and galectin-9) was only slightly reduced after therapy and remained higher than in healthy controls. Wu et al. [118] demonstrated that, in patients treated with DAAs for chronic HCV infection, the frequency of Tregs and their inhibitory function on proliferation of $CD4^+CD25^-$ effector T cells decreased from baseline to EOT, although it subsequently increased from EOT to SVR12 to levels close to baseline. Consequently, these changes coincided with fold changes in IFN-γ and TNF-α secretion, which were the highest at EOT and then decreased at SVR12, whereas an inverse relationship was observed in the case of IL-10, which decreased at EOT and then increased at SVR12. Thus, the concept of decreases in the frequency and activation status of Tregs toward physiological levels following DAA treatment did not find support in the above studies.

5. Effect of Vaccines on T Cell Exhaustion in Chronic HCV Infection

Although the advent of DAAs has revolutionized the management of chronic HCV infection, in many countries the scale of new HCV cases outnumbers those that are cured [119]. In addition, there is still a risk of re-infection in patients after successful anti-HCV treatment [114,120]. Therefore, HCV vaccine development is paramount to limit the spread of the virus. The main goal of HCV vaccination is to increase the antiviral activity of virus-specific $CD4^+$ and $CD8^+$ T cells to protect against HCV infection or re-infection. Hartnell et al. [109] demonstrated that the immunization of healthy volunteers with a novel experimental heterologous recombinant vaccine (chimpanzee adenovirus (ChAd3) combined with modified vaccinia Ankara virus (MVA), encoding the non-structural region of HCV (NSmut)) in a prime/boost regimen, induced the generation of long-lived memory $CD127^+$ HCV-specific $CD4^+$ T cells with increased expression of CD28 co-stimulatory receptor, while the expression of T-bet decreased. Furthermore, vaccination promoted the vigorous production of TNF-α, IFN-γ and MIP-1β, as well as the robust proliferative capacity of HCV-specific $CD4^+$ T cells. Similar qualities of response were demonstrated in patients after spontaneous virus elimination, however, as mentioned above, this was not observed in patients after successful DAA treatment of chronic infection [109].

Multi-specific, high-magnitude responses observed following HCV vaccination in healthy volunteers have prompted an investigation as to whether therapeutic vaccination of patients with chronic HCV infection would result in the generation of vigorous immunity. However, Swadling et al. [121] and Kelly et al. [122] demonstrated no significant induction of HCV-specific T cells and no impact on viral load after ChAd3-NSmut/MVA-NSmut prime/boost vaccination of chronically infected patients treated with PEG-IFNα/rib (with lower baseline viral load) or untreated (with higher baseline viral load). In both studies, vaccine-induced T cells were functionally impaired and did not proliferate after stimulation. Strikingly, the expression of exhaustion markers (PD-1, Tim-3, CTLA-4 and 2B4) on HCV-specific T cells, as well as the transcription factor T-bet/Eomes co-expression pattern, were similar to those seen in healthy volunteers long-term after vaccination [121]. Interestingly,

T cell induction was the lowest in patients in whom vaccine immunogen and circulating virus variants displayed the same sequence, which may have been a result of T cell exhaustion. Conversely, T cells were induced in the context of sequence mismatch between vaccine immunogen and circulating virus, despite a common failure to recognize circulating epitope variants and only partially functional phenotype [121,122].

The effect of HCV vaccination on antiviral $CD8^+$ T-cell function has also been studied in two chronically infected chimpanzees during treatment with DAAs. Callendret et al. showed that although a combination of DAAs and genetic vaccines encoding the HCV NSmut improved peripheral blood $CD8^+$ T-cell function, vaccine-induced antiviral $CD8^+$ T-cells mostly did not recognize circulating persistent virus [123]. These cells did not preclude the replication of DAA-resistant HCV variants which evolved during therapy. Furthermore, exhausted intrahepatic $CD8^+$ T-cells targeting conserved epitopes did not expand after vaccination, and failure to control HCV replication was likely caused by compartmentalized $CD8^+$ T-cell exhaustion in the liver, as manifested by high PD-1 expression. These observations point out that the major challenge in successful therapeutic vaccine design would be to overcome T cell exhaustion in chronic HCV infection.

6. Conclusions and Future Perspectives

The long-term consequences of the impairment of T cell responses in chronic HCV infection are not entirely characterized, though they may be essential in the context of clinical course of infection, re-infection or treatment-mediated viral clearance and vaccine design. Furthermore, it is still uncertain whether a complete restoration of HCV-specific T cell response may be feasible, and whether this restoration would guarantee protection or influence the clinical course of re-infection.

The available studies imply that chronic HCV infection is accompanied by substantial and progressive phenotypic and functional alterations, including upregulation of multiple inhibitory receptors, which downregulate the functional and proliferative potential of the responding cells. Attempts to reverse the effects of compromised immune response quality included specific blockades of inhibitory receptors or immunosuppressive cytokines by means of monoclonal antibodies. In most studies, a restoration of functional competence (proliferation and effector functions) of HCV-specific T cells could be observed in vitro and in vivo, the latter accompanied by viral load decline. This implies that HCV-induced immune dysfunction may be reversible. Another investigated aspect was whether the elimination of viral antigens during chronic infection by means of HCV-targeted direct acting antiviral treatment could restore T cell function. Although the collected data were largely fragmentary and sometimes even contradictory, in most of them there is evidence that DAA treatment may result in at least partial restoration of T cell immune function. Absence of these hallmarks of immune restoration in non-responders is indicative of an active role of immunity in DAA-mediated viral clearance. Despite initial enthusiasm, they also suggest that a complete restoration, comparable to that seen after spontaneous viral clearance, may not be attained, which points out that long-term antigenic stimulation imprints an irreversible change on the T cell compartment. Understanding the mechanisms of HCV-induced immune dysfunction and barriers to immune restoration following viral clearance is of the utmost importance to diminish the possible long-term consequences of chronic HCV infection.

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Opinia Komisji Bioetycznej

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KB/...77.../A/2015

Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym

w dniu 4 listopada 2015 r. po zapoznaniu się z wnioskiem:

prof. dr hab. n. med. Marka Radkowskiego
Zakład Immunopatologii Chorób Zakaźnych i Pasożytniczych
ul. Pawińskiego 3c, 02-106 Warszawa

dotyczącym: akceptacji programu badania „Analiza zależności między „wyczerpaniem" komórek T, zmiennością genetyczną wirusa zapalenia wątroby typu C (HCV), a wpływem leczenia przeciwwirusowego u pacjentów z zakażeniem HCV oraz współzakażeniem HIV-1/HCV", Informacji dla pacjenta oraz Formularza świadomej zgody – wersji 2 z 15.10.2015 r.

wyraża następującą
o p i n i ę

- stwierdza, że są one dopuszczalne i zgodne z zasadami naukowo-etycznymi*.
- ~~stwierdza, że są one niedopuszczalne i niezgodne z zasadami naukowo-etycznymi.*~~

Komisja działa na podstawie art.29 ustawy z dnia 5.12.1996r. o zawodzie lekarza /Dz.U.nr 28/97 poz.152 wraz z późn.zm./, zarządzenia MZiOS z dn.11.05.1999r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych /Dz.U.nr 47 poz.480/, Ustawy prawo farmaceutyczne z dnia 6 września 2001r. (Dz.U.Nr 126, poz. 1381 z późn. zm.) oraz Zarządzenie nr 56/2007 z dnia 15 października 2007r. w sprawie działania Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym /Regulamin Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym/.
Komisja działa zgodnie z zasadami GCP .
W załączeniu: skład komisji oraz lista obecności

Przewodniczący Komisji Bioetycznej

/Prof. dr hab. n. med. Maria ROSZKOWSKA - BLAIM/

*niepotrzebne skreślić

Oświadczenia współautorów publikacji

Warszawa, dn. 28.11.22

Prof. Dr hab. n. med. Tomasz Laskus
Zakład Immunopatologii
Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w określeniu celów i założeń pracy, nadzór nad pracami badawczymi i pisaniem pracy, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....

Podpis

Warszawa, dn. 28.11.22

Dr. n. med. Karol Perlejewski
Zakład Immunopatologii
Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w zbieraniu danych klinicznych, udział w analizie danych, komputerowa obróbka danych, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 2%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Podpis

Warszawa, dn. 29.11.2022

Lek. Med. Hanna Berak
Wojewódzki Szpital Zakaźny
W Warszawie

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w zbieraniu danych klinicznych, udział w opracowaniu metodologii, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch

H Berak

Podpis

Warszawa, dn. 29.11.2022

Dr hab. n. med. i n. o zdr. Iwona Anna Bukowska-Ośko
Zakład Immunopatologii
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OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w prowadzeniu prac badawczych, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Bukowska-Ośko

Podpis

Warszawa, dn. 29.11.22

Dr hab. n. med. Agnieszka Pollak
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OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w analizie danych, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

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Podpis

Warszawa, dn. 01.12.2021

Magdalena Zielenkiewicz

Instytut Matematyki

Uniwersytetu Warszawskiego

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w analizie danych, komputerowa obróbka danych, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....

Podpis

Warszawa, dn. 28.11.22

Prof. Dr hab. n. med. Marek Mirosław Radkowski

Zakład Immunopatologii
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Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w określeniu celów i założeń pracy, opracowanie metodologii, nadzór nad pracami badawczymi i pisaniem pracy, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 76%,

obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....

Podpis

Warszawa, dn. 29.11.2022

Dr hab. n. med. i n. o zdr. Kamila Caraballo Cortés
Zakład Immunopatologii
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OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Perlejewski Karol, Berak Hanna, Bukowska-Ośko Iwona Anna, Pollak Agnieszka, Zielenkiewicz Magdalena, Radkowski Marek Mirosław, Caraballo Cortés Kamila. CD8+ T-cell exhaustion phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation. Frontiers in Immunology. 2022; 13:1-15", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: określenie celów i założeń prac, pozyskiwanie funduszy, opracowanie metodologii, nadzór nad pracami badawczymi i pisaniem pracy, pisanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

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obejmował on: zbieranie danych klinicznych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Kamila Caraballo Cortés

Podpis

Warszawa, dn. 29.11.22

Prof. Dr hab. n. med. Tomasz Laskus
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Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w określeniu celów i założeń pracy, nadzór nad pracami badawczymi i pisaniem pracy, redagowanie manuskryptu.

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obejmował on: zbieranie danych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, przygotowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....
Podpis

Lek. Med. Hanna Berak
Wojewódzki Szpital Zakaźny
W Warszawie

Warszawa, dn. 29.11.2022

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w zbieraniu danych klinicznych, opracowaniu metodologii, zbieraniu próbek badanych, redagowaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 72%,

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Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....
Podpis

Warszawa, dn. 29.11.22

Dr. n. med. Karol Perlejewski
Zakład Immunopatologii
Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w zbieraniu danych, komputerowa obróbka danych, redagowanie manuskryptu.

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Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Perlejewski

Podpis

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Prof. Karin J. Metzner, MD

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Zurich, November 30, 2022

STATEMENT

To whom it concerns

As a co-author of the publication "Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", I declare that my scientific contribution included involvement in conceptualization, supervision as well as manuscript review.
My contribution percentage was 5%.
Scientific contribution of the PhD student to the publication was: 72%.
The PhD student's contribution to the article involved data curation, data analysis, investigation, methodology as well as manuscript review.
I hereby declare that I am aware that the publication of which I am a co-author will be a part of PhD dissertation by the PhD student.

Kind regards,

Karin Metzner

Dr hab. n. med. i n. o zdr. Marcin Paciorek
Wojewódzki Szpital Zakaźny
W Warszawie

Warszawa, dn. 28.11.21

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w zbieraniu danych klinicznych, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 72%,

obejmował on: zbieranie danych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, przygotowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Marcin Perlejewski

Podpis

Warszawa, dn. 28.11.22

Prof. Dr hab. n. med. Marek Mirosław Radkowski
Zakład Immunopatologii
Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Laskus Tomasz Jacek, Berak Hanna, Perlejewski Karol, Metzner Karin J., Paciorek Marcin, Radkowski Marek Mirosław, Caraballo Cortés Kamila. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Scientific Reports. 2020; 10(1): 1- 17", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: udział w określeniu celów i założeń pracy, udział w opracowaniu metodologii, nadzór nad pracami badawczymi i pisaniem pracy, redagowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 72%,

obejmował on: zbieranie danych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, przygotowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

.....
Podpis

Warszawa, dn. 29.11.2022

Dr hab. n. med. i n. o zdr. Kamila Caraballo Cortés
Zakład Immunopatologii
Chorób Zakaźnych i Pasożytniczych;
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

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Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 72%,

obejmował on: zbieranie danych, analizę danych, prowadzenie prac badawczych, opracowanie metodologii, przygotowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Kamila Caraballo Cortés

Podpis

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Zurich, November 30, 2022

STATEMENT

To whom it concerns

As a co-author of the publication ""Osuch Sylwia, Metzner Karin, Caraballo Cortés Kamila. Reversal of T Cell Exhaustion in Chronic HCV Infection. Viruses. 2020; 12(8): 1-20.", I declare that my scientific contribution included involvement in supervision, manuscript review as well as editing.
My contribution percentage was 10%.
Scientific contribution of the PhD student to the publication was: 80%.
The PhD student's contribution to the article involved conceptualization, writing and visualization.
I hereby declare that I am aware that the publication of which I am a co-author will be part of the PhD dissertation by the PhD student.

Kind regards,

Karin Metzner

Warszawa, dn. 29.11.2022

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OŚWIADCZENIE

Jako współautor pracy pt. „Osuch Sylwia, Metzner Karin, Caraballo Cortés Kamila. Reversal of T Cell Exhaustion in Chronic HCV Infection. Viruses. 2020; 12(8): 1-20", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: określenie koncepcji pracy, finansowanie, nadzór nad pisaniem pracy.

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Wkład mgr Sylwii Osuch w powstawanie publikacji określam jako 80%,

obejmował on: określenie koncepcji pracy, przygotowanie manuskryptu, przygotowanie grafiki.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Sylwii Osuch.

Podpis

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Dorobek naukowy

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1. Skrzat-Klapaczyńska Agata, Bieńkowski Carlo, Kowalska Justyna Dominika, Paciorek Marcin, Puła Joanna, Krogulec Dominika, Stengiel Jarosław, Pawełczyk Agnieszka Maria, Perlejewski Karol, **Osuch Sylwia**, Radkowski Marek Mirosław, Horban Andrzej Jerzy. *The Beneficial Effect of the COVID19 Vaccine Booster Dose among Healthcare Workers in an Infectious Diseases Center*. Vaccines. 2022;10(4): 1-7.

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Sylwia Osuch, Kamila Caraballo Cortés, *T-cell exhausted phenotype in chronic hepatitis C virus infection is associated with epitope sequence variation*. The 17th International Symposium on Viral Hepatitis and Liver Disease, Global Hepatitis Summit 2020/2021, Taiwan (June 18-20, 2021), prezentacja plakatu.

Sylwia Osuch, Kamila Caraballo Cortés, *Plasma levels of soluble markers of immune "exhaustion" sPD-1 and sTim-3 in patients with chronic hepatitis C: effect of liver fibrosis and successful treatment.* 32nd European Congress of Clinical Microbiology and Infectious Diseases (23 - 26 April, Lisbon, Portugal), wystąpienie ustne.

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2. Grant Narodowego Centrum Nauki Sonata 10, nr 2015/19/D/NZ6/01303) pt. *Analiza zależności między "wyczerpaniem" komórek T, zmiennością genów kodujących epitopy wirusa zapalenia wątroby typu C (HCV) rozpoznawane przez komórki T, a wpływem leczenia przeciwwirusowego u pacjentów z zakażeniem HCV oraz współzakażeniem HIV-1/HCV*. Wykonawca projektu.

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