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MAJOR ARTICLE

Markers of NK cell exhaustion in HIV/HCV coinfection and their dynamics after DAA mediated HCV clearance

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Background. Liver fibrosis is a leading cause of morbi-mortality in people withHIV/HCV. NK cells are linked with amelioration of liver fibrosis, however, NK cells from HIV/HCV-coinfected individuals with cirrhosis displayed impaired functionality and high PD-1 expression. Here, we aim to study PD-1, TIGIT and Tim3 as potential exhaustion markers in NK cells from HIV/HCV-coinfected individuals with mild and advanced liver fibrosis. The role of PD-1 expression on NK-cells after HCV clearance by direct-acting antivirals (DAA) was evaluated as well.

Methods. PBMC were isolated from HIV/HCV-coinfected individuals (n=54; 27 METAVIR F0/F1; 27 F4, evaluated by transient elastography). In 26, samples were collectedbefore, at the end, and 12 months after successful DAA treatment. Frequency, immunophenotype (PD-1, TIGIT and Tim3 expression) and degranulation capacity (CD107a assay) of NK cells were determined by flow cytometry.

Results. Unlike PD-1, Tim3 and TIGIT were comparably expressed between individuals with mild and advanced fibrosis. Degranulation capacity was diminished in NK/TIGIT$^+$ cells in both fibrosis stages, while NK/PD-1$^+$ cells showed a lower CD107aexpression in cirrhotic individuals.

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After 12 months post DAA treatment, subjects with advanced fibrosis showed an improved NK-cell and reduced NK/PD-1^{+}-cell frequency, but nochanges in CD107a expression. In individuals with mild fibrosis, neither PD-1 nor NK-cell frequency was modified, although percentage of NK/CD107a^{+} cells was improved at 12 months post-treatment.

Conclusions. Although DAA improved exhaustion and frequency of NK cells in cirrhotic individuals, functionality was only reverted in mild liver fibrosis, remarking theimportance of an early DAA treatment.

KEY WORDS: HIV, HCV, HIV/HCV coinfection, liver fibrosis, NK cell exhaustion, immunology, direct acting antivirals.

KEY POINTS

- PD-1 and TIGIT are associated with impaired NK cell functionality in HIV/HCV^{+}-individuals.
- NK cell degranulation capacity improved in individuals with mild fibrosis after DAA-treatment.
- In individuals with cirrhosis, although frequency of NK cells was restored, loss offunctionality was not.

INTRODUCTION

Nearly 71 million people worldwide are chronically infected with hepatitis C virus (HCV) [1]. Estimates indicate that 25%-30% of those individuals will develop cirrhosis within 20 years of infection [2]. Among other factors, HIV-coinfection has been demonstrated to significantly accelerate fibrosis progression and development of end-stage liver disease [3, 4], even in the presence of antiretroviral therapy (ART) [3]. With a global assessment of 2.3 million HIV/HCV-coinfected individuals [5], monitoring hepatic disease and preventing liver damage constitute essential recommendations for the clinical management of this population [6].

Direct acting antivirals (DAA) represent a major advancement in the treatment of HCV infection, since HCV clearance can be achieved in more than 90% of the cases, with minimal side effects [7]. However, the impact of DAA treatment on hepatic fibrosis still needs to be evaluated in larger populations and broader follow-ups [8]. Currently, studies show that DAA treatment is associated with the resolution of hepatic inflammation and improvement of fibrosis, especially among individuals with mild to moderate levels of liver damage. Nevertheless, a great proportion of subjects with baseline advanced liver disease stay within cirrhotic scores [8] Unraveling the mechanisms that regulate liver fibrosis is vital since nearly half a million people die annually from decompensated cirrhosis related to chronic HCV infection [1]. Moreover, end-stage liver disease constitutes a major cause of death among HIV/HCV-coinfected individuals [9].

Natural Killer (NK) cells play an important role in inhibiting hepatic fibrosis by killing early activated or senescence-activated Hepatic Stellate Cells [10]. It has been shown that peripheral NK cell frequency is significantly decreased in both HCV monoinfection and HIV/HCV coinfection [11, 12]. Functionality of these cells has also been reported to be severely compromised in both groups of individuals [11, 13, 14]. Previously, we demonstratedthat HIV/HCV-coinfected individuals with advanced fibrosis are characterized by having low peripheral NK cell frequencies compared to healthy volunteers and subjects with minimal fibrosis. Furthermore, NK cell degranulation and cytokine secretion were significantly diminished in samples from individuals with higher levels of fibrosis. When PD-1 expression was assessed on the NK cell compartment, PD-1 expression was significantly upregulated in cirrhotic subjects, despite presenting similar times of known HIV and HCV infection, time of ART, HCV viral load and HCV genotype of those observed in individuals with mild hepatic disease [12, 15].

PD-1 molecule has been extensively studied in T, B and dendritic cells [16], however, less is known about PD-1 expression and NK cell functionality. Several studies have shown that PD-1 is highly expressed on peripheral and tumor-infiltrating NK cells from individuals suffering from different malignancies, and that PD-1 axis blocking significantly enhances cytokine production, degranulation and viability of these cells [17]. PD-1 expression on NK cells was also linked to chronic HCV infection [18], although the relationship between progression of liver fibrosis and the PD-1 axis in NK cells has not been completely addressed. Additional surface proteins have been evaluated as potential NK cell immune checkpoints, including CD96, LAG3, T cell immunoglobulin and mucin-domain containing-3 (Tim3) and T cellimmunoreceptor with Ig and ITIM domains (TIGIT) [19, 20, 21]. Of those, Tim-3 and TIGIT have been studied in viral hepatitis, and it has been suggested that expression of these markers is linked to exhausted phenotypic characteristics [22].

The aim of this work was to characterize PD-1, TIGIT and Tim3 as potential biomarkers for NK-cell exhaustion among HIV/HCV-coinfected individuals with different degrees of liver fibrosis. In this regard, we evaluated the association between NK-cell exhaustion and progression of liver damage. Additionally, we explored how baseline hepatic fibrosis levels impact PD-1 expression and NK-cell dynamics following HCV eradication by DAA. By studyingPBMC from HIV/HCV-coinfected individuals with mild and advanced liver fibrosis, we showedthat PD-1 is a selective marker of NK-cell exhaustion, and that is significantly linked to advanced fibrosis stages. Lastly, although NK-cell frequency is mildly improved, HCV- clearance does not completely restore NK-cell functionality in HIV/HCV-coinfected individualswith advanced liver fibrosis.

METHODS

Study cohort

HIV/HCV-coinfected (n=54, 27 with METAVIR F0/F1 and 27 with METAVIR F4), HIV-seropositive (HIV^+, n=6), HCV-seropositive (HCV^+, n=9) and HIV/HCV-seronegative individuals (HC: healthy controls, n=5) were included in this study. Written informed consent was obtained, and 60 mL of peripheral blood was drawn. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Bioethics Committee of *Fundación Huésped*. HIV/HCV-coinfected individuals were allocated in two groups based on their level of fibrosis according to transient hepatic elastography (FibroScan® and SuperSonic Imagine's Aixplorer®). Subjects with a result of ≤7.1 Kpa were classified as compatible with a METAVIR score of F0/F1: absent or minimal fibrosis; and those with ≥12.5 Kpa as compatible with METAVIR F4: cirrhosis [23]. Individuals enrolled in this study were not acutely or chronically HBV-infected (determined by serology); and denied current use of recreational drugs and more than 14 units/week of alcohol intake on a regular basis. From 26 out of 54 coinfected individuals (14 with METAVIR F0/F1 and 12 with METAVIR F4), blood samples were collected at three different times: Baseline (prior to DAA treatment), End of Treatment (EOT) and 12 months post-DAA treatment (12MPT).

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC): PBMC were obtained from whole blood by Ficoll-Hypaque centrifugation (GE Healthcare, UK) and cryopreserved at -80°C for up to 4 months. Cells were cultured in complete RPMI-1640 medium (cRPMI) containing 10% fetal bovine serum, 2 mM l-glutamine, 100 IU/mL penicillin and 100 µg/mL of streptomycin (all reagents, Gibco SRL, USA). *K562 cell line*: Three days before the experiments, the chronic myelogenous leukemia K562 cell line was thawed and grown at 37°C and 5% CO_2 in cRPMI.

CD107a assay

For degranulation assays, the K562 cell line was used as both a stimulus and a sensible target for NK cells. PBMC were thawed and cultured for 2 hours in cRPMI, at 37°C with 5% CO_2. Next, one million viable PBMC were co-incubated for five hours with 10^5 K562 cells, in the presence of anti-CD107a-FITC mAb, brefeldin and monensin (4 µL, 10 µg/mL and 0.7 µg/mL respectively, BD Biosciences, San Diego, CA, USA). To assess basal levels of degranulation, PBMC were incubated in the absence of K562 cells.

Multicolor flow cytometry

Cells were immunophenotyped by flow cytometry on a FACS Canto Flow Cytometer (BD Biosciences). For antibodies and gating strategies, see Supplementary Table 1 and Supplementary Figure 1. Flow cytometry data was analyzed with FlowJo software v.10 (BD Biosciences). Phenotype and functionality assays were performed according to cell availability.

Data analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego,CA. EEUU). Data normality was assessed using the Shapiro-Wilk test and subsequently analyzed by non-parametric methods (see each Figure legend for specific details). All tests were considered significant when $p < 0.05$.

RESULTS

To evaluate biomarkers for NK-cell exhaustion and its relationship with liver fibrosis, blood samples were obtained from HIV/HCV-coinfected individuals with mild and advanced hepatic fibrosis, as assessed by transient elastography and METAVIR staging. Only participants with extreme F0/F1 and F4 levels were included in this study. This strategy was used in order to minimize METAVIR misclassification by transient hepatic elastography which is less accurate than liver biopsies to determine liver fibrosis. PBMC were isolated as described previously and summarized in Material and Methods. The characteristics of study participants are summarized in Table 1. In accordance with fibrosis METAVIR stage, individuals with advanced fibrosis presented higher indicators of liver stiffness, APRI score, AST and total bilirubin than individuals with mild fibrosis, and a lower platelet count.

PD-1 expression in NK cells associates with cell activation and exhaustion in HIV/HCV-coinfected individuals.

In comparison with HIV/HCV-coinfected individuals with mild fibrosis, NK cells from individuals with advanced fibrosis displayed a higher median fluorescence intensity (MFI) of PD-1 (Figure 1A). Also, the frequency of NK/PD-1^+ cells peaked in advanced liver fibrosis as previously demonstrated [15]. When PD-1 expression was further analyzed in peripheral NK cells from HIV/HCV-coinfected individuals, PD-1 was found to be mainly subscribed to the $CD56^{dim}$ NK cell subset (Figure 1A), as was described by others [24] [25, 26]. Evaluation of the activation markers CD25 and CD69 showed that PD-1 expression was also significantly associated with an activated NK cell phenotype in individuals with mild and advanced hepatic fibrosis. In addition to this result, a reduction in Nkp46 expression was registered in NK/PD-1^+ cells from subjects with mild fibrosis. No differences were found in NKG2D expression between NK/PD-1^+ and NK/PD-1^- cells from individuals with mild or advanced fibrosis (Figure 1B). In order to evaluate whether or not PD-1 expression was linked to an impaired NK cell functionality, degranulation capacity (externalization of CD107a) of both NK/PD-1^+ and NK/PD-1^- cells was studied as previously described [15]. Briefly, following stimulation with K562 cells, externalization of CD107a was monitored. Compared to the PD-1^- NK cell subset, expression of CD107a was significantly affected in PD-1^+ NK cells (Figure 1C). Stratified analysis of F0/F1 and F4 groups suggests that impaired NK cell degranulation is more frequently observed in subjects suffering from advanced liver fibrosis (Figure 1C). In line with these latter results, when serum biochemical variables were

analyzed, frequency of peripheral NK/PD-1$^+$ cells wasnegatively associated to albumin levels and prothrombin time, and directly correlated with liver stiffness, APRI score, and AST levels of HIV/HCV-coinfected individuals (Figure 1D). In sum, these results suggest that PD-1 is not only a marker for mature and activated NK cells, but also is linked to an exhausted phenotype and to more altered liver function tests.

PD-1 is a selective marker of NK cell exhaustion in HIV/HCV-coinfected individuals with liver fibrosis.

Next, we evaluated if PD-1$^+$ exhausted NK cells also expressed additional markers ofdysfunction. Tim3 and TIGIT have been reported as NK cell exhaustion markers in hepatic diseases [22, 27-29]. To characterize Tim3 and TIGIT expression in NK cells from HIV/HCV- coinfected individuals, resting PBMC were analyzed via flow cytometry. When comparing frequencies of Tim3$^+$ or TIGIT$^+$ NK cells, no differences were found between groups with mildand advanced fibrosis (Figure 2A). Similar results were obtained when MFI was analyzed (results not shown). To additionally identify alterations in cell functionality due to Tim3 or TIGITexpression, a CD107a degranulation assay was performed as described above. While similar frequencies of CD107a$^+$ NK cells were registered based on Tim3 expression (Figure 2B), in individuals with both mild and advanced liver fibrosis the proportion of degranulating NK cells was significantly reduced when TIGIT was expressed (Figure 2B). Finally, the distribution of NK cell populations defined by the expression of PD-1, Tim3 and TIGIT in HIV/HCV-coinfectedindividuals with mild and advanced liver fibrosis was studied. Although there was a visible expansion of NK cell populations expressing PD-1 and either other marker among individuals with advanced fibrosis, global distribution between F0/F1 and F4 groups did not differstatistically (Figure 2C). Nonetheless, the coexpression of PD-1, Tim3 and TIGIT is rarely observed in NK cells in both groups studied. In sum, results show that PD-1 and TIGIT are associated with impaired NK cell functionality in HIV/HCV-coinfected individuals, but only PD-1 is differentially expressed throughout the liver fibrosis stages.

Exhaustion and dysfunctionality of NK cells from HIV/HCV-coinfected individuals with advanced liver fibrosis is not restored by DAA treatment.

Liver fibrosis in HIV/HCV-coinfected individuals is associated with functional exhaustion of the NK compartment, and a reduction in the frequency of such cells. To evaluate whether HCV clearance with DAA impacts exhaustion and functionality of NK cells, and further explore a possible role of hepatic fibrosis on this phenomenon, frequency, PD-1 expression, and degranulation capacity of NK cells were evaluated in HIV/HCV-coinfected individuals with mild and advanced fibrosis treated with DAA. Immunophenotype was studied at baseline, at the end of treatment (EOT), and 12 months post treatment (12MPT) with anti-HCV therapy. Clinical characteristics of the studied individuals are depicted in Table 2. Briefly, at 12MPT, individuals with advanced fibrosis presented lower counts of CD4-cells than individuals with mild fibrosis, which is also reflected in a lower CD4/CD8 ratio. As expected, in individuals with advanced

fibrosis, ALT and AST levels diminished at EOT and after 12MPT. Consequently, APRI score diminishes too.

First, we evaluated PD-1 expression dynamics over time, in both F0/F1 and F4 groups. As observed in Figure 3A, baseline levels of PD-1 expression in HIV/HCV-subjects are significantly higher than displayed by HCV and HIV-monoinfected individuals or healthy controls. Although all individuals achieved a sustained virological response after DAA treatment, globally, exhaustion of NK cells was not modified. Interestingly, when subjects withadvanced fibrosis were evaluated, HCV clearance was associated with decreased cell exhaustion in individuals with NK cells showing high PD-1 expression at baseline. On the contrary, on those few individuals who had low frequency of PD-1^+ cells, the expression of this marker increased along time, although not significantly (Figure 3B). NK-cell percentage and degranulation capacity were not directly associated in either of the liver fibrosis stageevaluated. While the frequency of NK cells did not change after DAA treatment in individuals with mild fibrosis, their degranulation capacity was significantly improved (Figure 3C and 3D, left panel). In individuals with advanced fibrosis, although the percentage of those cells was restored, loss of functionality was not (Figure 3C and 3D).

DISCUSSION

The arrival of DAA treatment in the last decade has represented a great advance in HCV treatment, since viral clearance is possible in more than 90% of cases, with minimal adverse effects and short treatment schedules (2-3 months) [7]. However, the capacity of DAA therapyto improve liver fibrosis, especially in individuals with advanced liver disease, is yet less clear [8, 30]. Liver fibrosis is directly correlated with increased morbidity and mortality due to liver failure and hepatocellular carcinoma [31-33]. As advanced fibrosis persists, HCV elimination is not enough to restore health, and options to decrease the level of liver damage are needed.NK cells play a crucial role in the modulation of hepatic stellate cells, key cells in the generation of liver fibrosis. In the present study, we were able to add further evidence regarding NK cells and their association with liver fibrosis in HIV/HCV-coinfected individuals. We have shown that NK cells from HIV/HCV-coinfected subjects with advanced hepatic fibrosis express higher levels of PD-1 than those individuals with mild liver disease (Figure 1A). Also, we demonstrated that PD-1 is significantly associated with a lower capacity of NK cells to degranulate (Figure 1C). The possibility of increasing NK cell functionality could potentially benefit individuals and have an impact on liver tissue remodeling. Since the improvement of NK cell degranulation capacity was only observed in samples from individuals with lower fibrosis, this underscore the importance of designing strategies that could apply as possible treatments (e.g.: anti PD-1 that has already been approved as adjunctive therapy to chemotherapy) in order to revert exhaustion and modify *in vivo* the function of NK cells.

Regarding additional markers of NK cell exhaustion that have been previously reported, we observed that the expression of TIGIT was associated with a decreased impaired degranulation, in

individuals with both mild and advanced fibrosis (Figure 2B). Nevertheless, TIGIT was not differentially expressed on NK cells from individuals with different degrees of liver fibrosis (Figure 2A). Anti-TIGIT therapies are being studied for cancer treatment [34], so it is plausible to consider them as a therapy to improve NK functionality and secondary liver fibrosis. Tim3 was not associated with a lower capacity of degranulation in NK cells (Figure 2B). However, it is observed that there is a higher percentage of NK/Tim3$^+$ cells, both in individuals with mild and advanced fibrosis when comparing with the control group (data not shown). Future analysis should aim to completely understand the role that this marker plays, both in the context of monoinfection and in HIV/HCV coinfection.

HCV elimination by DAA treatment may exert a differential effect on different immune parameters, depending on the level of liver fibrosis displayed by an individual. Here, multiple NK cell parameters were longitudinally monitored after HCV eradication with DAA treatment, by analyzing three sampling times: before DAA treatment (Baseline), the end of treatment (EOT) and 12 months after completion of DAA treatment (12MPT). In individuals with mild fibrosis, an increase in the NK cell degranulation capacity was observed at 12MPT (Figure 3D). On the other hand, in individuals with advanced fibrosis, this improvement in degranulation capacity was not found. Nevertheless, an increase in the frequency of NK cells was observed at 12MPT (Figure 3C) and a decrease in the percentage of NK/PD-1$^+$ cells at the EOT and 12MPT was detected (Figure 3B). This incomplete recovery of the NK cell compartment may suggest a persistent exhaustion of NK cells even after HCV has been eliminated by DAA treatment, particularly in individuals with advanced fibrosis, who are unable to improve their NK cell degranulation capacity. This is consistent with data reported by other groups, describing a limited capacity of DAA treatments to revert the METAVIR score in those individuals with F3 and F4 scores [35-37]. This data reinforces the possibility to use the above-mentioned immune checkpoint inhibitors to improve the functionality of NK cells and, as a consequence, modify their impact on liver tissue fibrosis.

When modulation of PD-1 expression in NK cells was evaluated following DAA therapy, no significant overall changes were observed in samples from subjects with either mild or advanced fibrosis. Nevertheless, while NK/PD-1$^+$ cell frequency is significantly higher at baseline than in control groups, this significance is lost after DAA treatment, suggesting a trend that could be better elucidated with a larger sample size and at longer follow-ups. Additionally, when analyzing samples of individuals with advanced fibrosis we observed that PD-1 expression at baseline was very heterogenous and we could also observe different outcomes over time. In F4 participants with high baseline expression of PD-1, a decrease of NK/PD-1$^+$ cell frequency was observed both at EOT and 12MPT, compared to baseline (Figure 3B, left panel). This result was as expected as it has been observed in the cohorts of individuals with advanced fibrosis [12, 15] and could indicate an improvement in NK cell homeostasis, which, however, does not seem to be enough to restore its NK cell degranulation capacity since no significant changes in this parameter were detected over time. With respect to individuals with a low baseline percentage of NK/PD-1$^+$ cells, a paradoxical behavior is observed, since this frequency increases after HCV clearance (Figure 3B). In these

participants with low PD-1 baseline expression in their NK cells, high cellular death was observed in the viability control in the flow cytometry analysis. Therefore, one possible explanation for this observation is that in those samples, NK cells presented a very high level of exhaustion before DAA and died preferentially during the in vitro experimental conditions. If this is the case, the real proportion of PD-1+ NK cells would be underestimated in these samples and could explain the paradoxical outcome observed in this group. As for individuals with mild fibrosis, although there is no significant decrease in the percentage of NK PD-1^+ cells as a function of time (Figure 3A), there is a significant increase in the degranulation capacity of NK cells at 12MPT (Figure 3D). This would suggest that at least in people with mild fibrosis, it is possible to recover the functionality of NK cells once HCV is cleared with DAA.

From the results reported in the present manuscript, it could be inferred that the presence of advanced liver damage exerts a direct effect on NK cells, regardless of the presence of HCV. From our results, PD-1 and TIGIT emerge as markers of exhaustion in NK cells and of compromised degranulation capacity. However, only PD-1 is differentially expressed, with a higher percentage of PD-1^+ cells in individuals with advanced fibrosis. This observation could reflect a more pronounced immune system exhaustion in people with advanced fibrosis. In line with this, it has been previously reported that those NK cells that coexpress Tim3 and TIGIT are increased in individuals with advanced fibrosis [28].

One of the limitations that faced this study included the great heterogeneity of the data obtained from samples of HIV/HCV-coinfected individuals, with both mild and advanced fibrosis. A larger sample size could help to elucidate the role of each of the studied markers more clearly. Another limitation is that the functionality of NK cells was evaluated *ex vivo*, under conditions that do not allow taking into account the multiple interactions that occur within the organism, both at the level of direct cellular interactions and soluble molecules, such as cytokines.

CONCLUSIONS

The results obtained suggest that DAA therapy is not sufficient per se to reverse the state of general exhaustion of the NK cells and their loss of functionality in the short term, particularly in people with advanced fibrosis. Nevertheless, they shed light on possible modifiable immune parameters that could be involved in NK cell alterations. These results highlight the importance of the active search of new therapies, such as checkpoint inhibitors, that allow to revert the described exhausted immune phenotype that, consequently, may improve the level of liver fibrosis and reduce the risk of mortality associated with it.

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Ethics: The study was conducted in accordance with the Declaration of Helsinki and was reviewed and approved by the Bioethics Committee of *Fundación Huésped* (Buenos Aires, Argentina).

Patient consent statement: Individuals were invited to participate at INBIRS Institute. Study participants provided written informed consent prior to enrollment and for use of their data and samples for future studies.

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Table 1. Subject characteristics at baseline.

Characteristics	F0/F1 n=27	F4 n=27	p value
Age (years)[1]	48.0 (47.5-52.0)	48 (43.8-53.2)	0.766
Male sex (n, %)[2]	12 (44.4)	20 (74.1)	0.027
CD4 count (cells/μL)[1]	698 (473-900)	596 (352-789)	0.341
CD8 count (cells/μL)[1]	903 (618-1253)	912 (659-1438)	0.850
NK cells (%)[1]	8.57 (4.92-14.08)	6.09 (2.97-14.30)	0.171
CD4/CD8 ratio[1]	0.70 (0.55-1.05)	0.61 (0.32-1.03)	0.410
Time of HCV infection (years)[1]	14 (8-22)	17 (12-22)	0.339
Time of HIV infection (years)[1]	20 (13-23)	21 (19-22)	0.685
HCV viral load (log_{10} copies)[1]	6.30 (5.93-6.68)	6.28 (5.94-6.80)	0.941
Time on ART (years)[1]	13 (9-17)	13 (9-18)	0.919
Routes of transmission (n, %)[2]			--
IDU	12 (44.4)	12 (44.4)	
Heterosexual	2 (7.4)	5 (18.5)	
MSM	--	1 (3.7)	
MTC	1 (3.7)	--	
Unknown	12 (44.4)	9 (33.3)	
HCV genotype (n, %)[2]			--
1a	17 (63.0)	13 (48.1)	
1b	3 (11.1)	2 (7.4)	

1	2 (7.4)	1 (3.7)	
2	--	1 (3.7)	
3	--	3 (11.1)	
4	--	1 (3.7)	
Unknown	5 (18.5)	6 (22.2)	
Liver stiffness (Kpa)[1]	5.80 (4.95-7.05)	20.6 (15.0-25.4)	<0.0001
APRI score[1]	0.45 (0.31-0.86)	1.59 (0.56-2.11)	0.0009
ALT (IU/L)[1]	65.0 (47.0-85.5)	79.5 (49.5-114.5)	0.291
AST (IU/L)[1]	41.0 (33.5-74.0)	79.0 (46.8-115.8)	0.009
Albumin (g/dL)[1]	4.3 (4.0-4.5)	4.3 (3.7-4.5)	0.623
Platelets (x10^3/mm^3)[1]	188 (172-239)	144 (106-198)	0.014
Total bilirubin (µg/dL)[1]	0.5 (0.3-0.6)	0.9 (0.7-1.0)	0.0003

Abbreviations: ART: antiretroviral therapy, ALT: alanine aminotransferase, AST: aspartate transaminase, IDU: Injecting drug user, MSM: men who have sex with men, MTC: mother to child transmission. 1- Median (IQR). P value determined by Mann-Whitney test. 2- Number of cases (number/total in %). P value was determined by unpaired Chi-square test.

Table 2. Subject characteristics for the longitudinal analysis (n = 26).

Characteristics	F0/F1n = 14			F4 n = 12			p value
	BSL	**EOT**	**12MPT**	**BSL**	**EOT**	**12MPT**	
Age (years)[1]	49.5 (48.0-55.0)	--	--	46 (43-52)	--	--	0.117
Male sex (n,%)[2]	5 (35.71)	--	--	8 (66.67)	--	--	0.116
CD4 count (cells/µL)[1]	698 (570-750)	974 (314-1549)	773 (640-1061)	384 (291-776)	623 (360-856)	334 (108-499)	F0/F1 vs F4, 12MPT = 0.029
CD8 count (cells/µL)[1]	869 (780-1041)	729 (450-1054)	924 (809-979)	946 (783-1374)	1089 (1006-1307)	965 (350-1569)	F0/F1 BSL vs 12MPT = 0.710/ F4 BSL vs 12MPT = 0.715
NK cells (%)[1]	11.75 (6.27-22.28)	11.15 (9.30-17.15)	18.0 (9.6-22.0)	5.61 (3.52-10.73)	9.07 (4.97-19.03)	17.40 (9.65-23.35)	F4 BSL vs 12MPT = 0.0393/ F0/F1 vs F4, BSL = 0.069
CD4/CD8 ratio [1]	0.78 (0.60-1.12)	0.72 (0.50-1.21)	0.80 (0.65-1.20)	0.50 (0.31-0.94)	0.64 (0.43-0.82)	0.34 (0.23-0.55)	F0/F1 vs F4, 12MPT = 0.006
Time of HCV infection (years)[1]	15.0 (9.5-19.8)	--	--	14 (12-22)	--	--	0.805
Time of HIV infection (years)[1]	21.0 (14.3-25.5)	--	--	19.0 (13.5-22.0)	--	--	0.453
HCV viral load (log_{10} copies)[1]	6.27 (5.98-6.80)	All < 1.3	All < 1.3	6.04 (5.90-6.60)	All < 1.3	All < 1.3	F0/F1 vs F4, BSL = 0.345
Time on ART (years)[1]	14.0 (10.3-19.3)	--	--	10.5 (6.3-17.5)	--	--	0.349
Routes of transmission (n,%)[2]							--
IDU	7 (50)	--	--	6 (50)	--	--	
Heterosexual	1 (7.1)	--	--	2 (16.7)	--	--	
ND	6 (42.9)	--	--	4 (33.3)	--	--	
HCV genotype							--

(n,%)[2]							
1a	8 (57.1)	--	--	7 (58.33)	--	--	
1b	2 (14.3)	--	--	2 (16.7)	--	--	
1	2 (14.3)	--	--	1 (8.33)	--	--	
3	--	--	--	1 (8.33)	--	--	
ND	2 (14.3)	--	--	1 (8.33)	--	--	
Liver stiffness (Kpa)[1]	5.5 (4.8-6.2)	ND	ND	15.7 (12.4-19.0)	ND	ND	<0.0001
APRI score[1]	0.52 (0.30-0.86)	0.36 (0.17-0.38)	0.30 (0.05-0.46)	1.70 (0.79-2.30)	0.60 (0.24-0.95)	0.50 (0.30-0.86)	F0/F1 BSL vs 12MPT = 0.090/ F0/F1 vs F4, 12MPT = 0.031
ALT (IU/L)[1]	82 (53-89)	22 (21-26)	28 (23-41)	87 (72-115)	ND	25 (18-40)	F4 BSL vs 12MPT = 0.016/ F0/F1 vs F4, BSL=0.414
AST (IU/L)[1]	47 (33-68)	18 (15-25)	24 (22-38)	90 (70-127)	ND	33 (22-38)	F4 BSL vs 12MPT = 0.016/ F0/F1 vs F4, BSL = 0.026
Albumin (g/dl)[1]	4.30 (3.95-4.45)	ND	4.7 (4.4-4.9)	4.30 (3.60-4.53)	4.4 (4.1-4.8)	4.4 (4.1-5.0)	F0/F1 BSL vs 12MPT = 0.313
Platelets (x10^3/mm3)[1]	176 (163-208)	166 (126-219)	195 (155-235)	142 (106-236)	143 (105-245)	151 (125-193)	F0/F1 BSL vs 12MPT = 0.219/ F0/F1 vs F4, BSL = 0.366
Total bilirubin (µg/dl)[1]	0.5 (0.3-0.7)	0.3 (0.2-0.5)	0.4 (0.4-0.9)	0.85 (0.56-0.98)	0.7 (0.5-1.0)	0.70 (0.60-0.88)	F0/F1 vs F4, BSL = 0.051

Abbreviations: BSL: baseline. EOT: end of treatment. 12MPT: 12 months post-treatment. ART: antiretroviral therapy, ALT: alanine aminotransferase, AST: aspartate transaminase, ND: not determined. IDU: Injecting drug user. MSM: men who have sex with men. 1- Median (IQR). 2-Number of cases (number/total in %). Wilcoxon and Mann-Whitney test were used to determine p values as appropriate.

Figure 1: Functionality of PD-1$^+$ NK cells from HIV/HCV-coinfected individuals with mild and advanced liver fibrosis at baseline, before DAA treatment. A: Expression of PD-1 was evaluated by flow cytometry in resting PBMC fromhealthy subjects, and HIV/HCV-coinfected individuals with METAVIR F0/F1 or F4 scores. NK cells wereidentified as CD3$^-$/CD56$^+$ lymphocytes; MFI of PD-1 expression on NK cells from F0/F1 and F4 groups, together with frequency of PD-1$^+$ cells in both CD56^{dim} and CD56^{bright} subsets are shown. B: Expression of CD25, CD69, Nkp46, NKG2D and PD-1 were evaluated in resting PBMC by flow cytometry. Frequency of NK cells positive for each activation marker is shown according to PD-1 status in F0/F1 (upper panel) or F4 scores (bottom). C: CD107a externalization in PBMC from HIV/HCV-coinfected individuals incubated with K562 cells. Representative cytometry plots for CD107a expression, followingstimulation, are shown for PD-1$^-$ and PD-1$^+$ NK cells (left panel). Frequency of CD107a$^+$ NK cells according to PD-1 expression in the whole population (middle panel) and both F0/F1 and F4 groups (right). D: Correlation between frequency of PD-1$^+$ NK cells and several clinicopathological characteristics. In each graph, the correlation coefficient and p values are shown. Blue and red points represent mild and advanced fibrosis individuals, respectively. Statistical comparisons were performedusing the Wilcoxon matched-pairs signed rank test (A-C) and Spearman correlation (D). Each set of points represents a different individual.

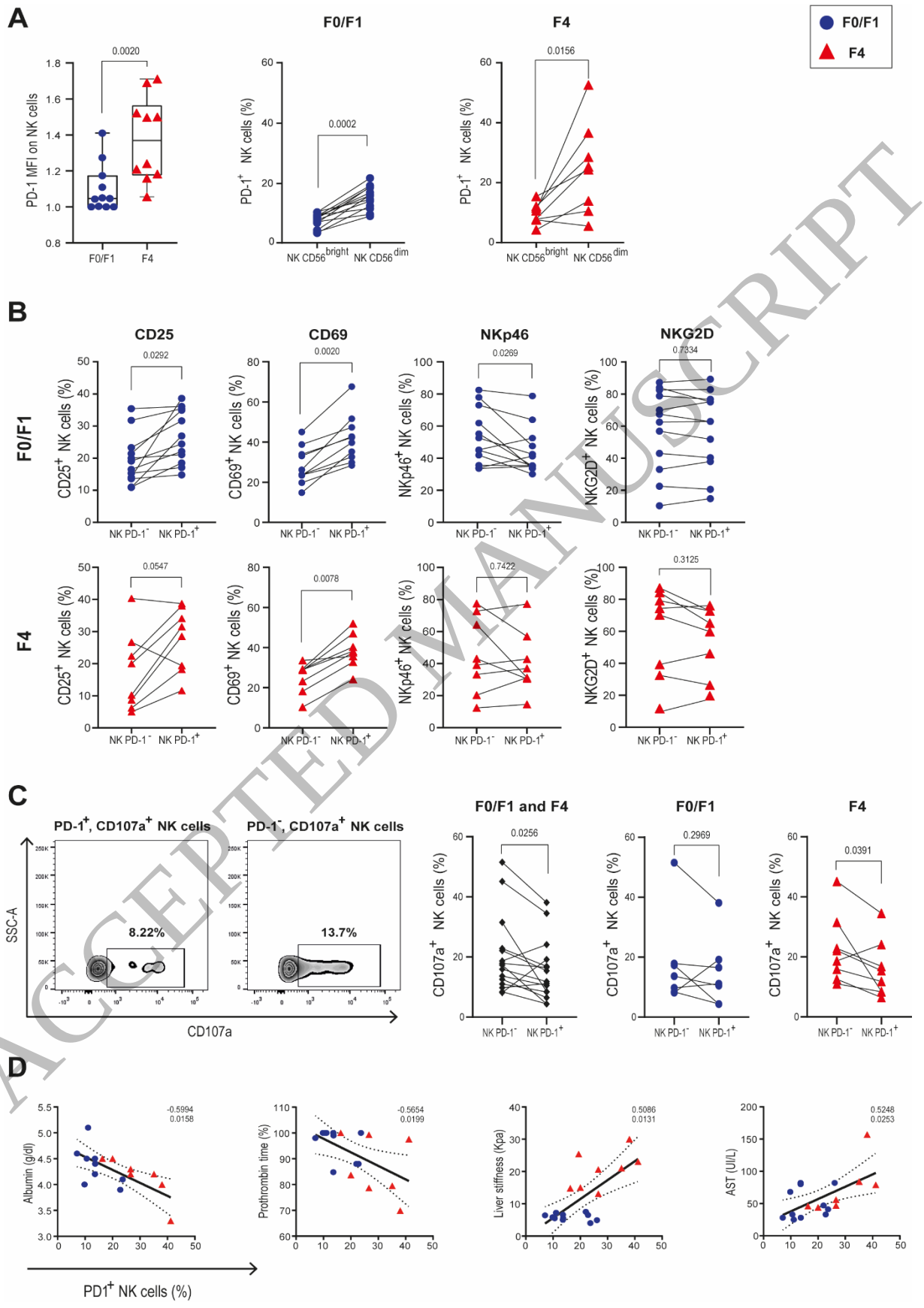

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Figure 2: Coexpression of PD-1, Tim3 and TIGIT in NK cells from HIV/HCV-coinfected individualswith mild or advanced liver fibrosis. A: Expression of Tim3 (upper panel) and TIGIT (bottom) were evaluated by flow cytometry in resting PBMC from HIV/HCV-coinfected individuals with METAVIR F0/F1 or F4 scores. Representative cytometry plots are shown in the left. Individual NK cell frequencies, median and 25th and 75th percentiles are indicated. B: CD107a externalization in PBMC from HIV/HCV-coinfected individuals incubated with K562 cells. Representative cytometry plots for CD107aexpression following stimulation are shown for both Tim3 or TIGIT positive and negative NK cell populations (left panel). Frequency of $CD107a^+$ NK cells according to Tim3 or TIGIT expression in F0/F1 and F4 groups (right panel). Each set of points represents a different individual. C: PD-1, Tim3 and TIGIT coexpression analysis in individuals with METAVIR F0/F1 or F4 scores, using the SPICE 6.0 software. Individual NK cell frequencies are indicated. Statistical comparisons were performed using the Mann Whitney (A), Wilcoxon matched-pairs signed rank (B), Permutation analysis (C, left panel) and Wilcoxon Rank Sum test (C, right panel).

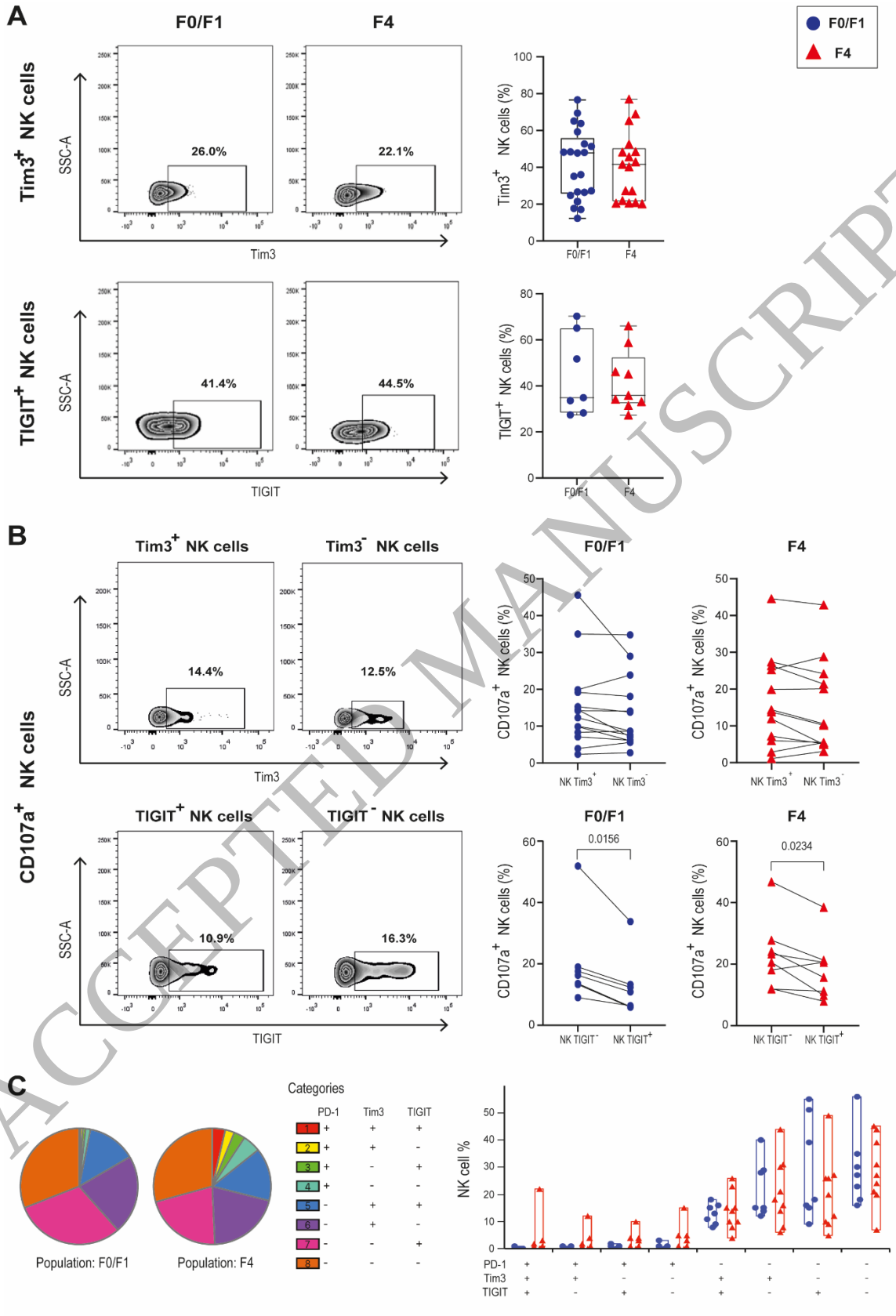

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Figure 3. Evolution of the NK cell compartment after DAA treatment in HIV/HCV-coinfected individuals with minimal or advanced liver fibrosis. A: Frequency of PD-1$^+$ NK cells in individuals with METAVIR F0/F1 or F4 scores before DAA treatment (Baseline), at the end of treatment (EOT) and 12 months post DAA-treatment (12MPT). PD-1$^+$ NK cell percentage is also shown for HIV (HIV$^+$) and HCV-monoinfected individuals (HCV$^+$) as well as healthy controls. B: Frequency of NK/PD-1$^+$ cells in individuals with METAVIR F4 with high (>5%, left panel) or low (<5%, right panel) baseline PD-1 expression. C: NK cell percentage in individuals with METAVIR F0/F1 or F4 scores at Baseline, EOT and 12MPT, HIV$^+$ or HCV$^+$ individuals, and healthy donors. D: CD107a externalization assessed in NK cells from individuals with METAVIR F0/F1 or F4 scores at Baseline, EOT and 12MPT, following incubation with K562 cells. Frequency of NK/CD107a$^+$ cells is also shown for HIV$^+$ or HCV$^+$ subjects and healthy controls. Statistical comparisons were

performed using the Wilcoxon and Mann-Whitney test.s

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