Accepted Article

Article type : Original

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Characterising HCV specific CD4+ T-cells following viral-vectored vaccination, directly acting anti-virals and spontaneous viral cure

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/hep.31160

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Abstract:

Background: Induction of functional helper CD4+ T cells is the hallmark of a protective immune response against HCV, associated with spontaneous viral clearance. Heterologous prime/boost viral vectored vaccination has demonstrated induction of broad and polyfunctional HCV specific CD8+ T-cells in healthy volunteers, however much less is known about CD4+ T-cell subsets following vaccination.

Methods: We analysed HCV specific CD4+ T-cell populations using MHC Class II tetramers in volunteers undergoing HCV vaccination with novel recombinant HCV adenoviral/MVA viral vectors. Peptide-specific T cell responses were tracked over time and functional (proliferation and cytokine secretion) and phenotypic (cell surface and intranuclear) markers were assessed using flow cytometry. These were compared to CD4+ responses in 10 HLA-matched individuals with HCV spontaneous resolution and 21 chronically infected patients treated with directly acting antiviral therapy (DAA).

Results: Vaccination induced tetramer positive CD4+ T cells that were highest 1-4 weeks after boosting (mean 0.06%). Similar frequencies were obtained for those tracked following spontaneous resolution of disease (mean 0.04%). In addition, the cell surface phenotype (CD28, CD127) memory subset markers and intranuclear transcription factors, as well as functional capacity of peptide specific CD4+ T cell responses characterized after vaccination are comparable to those following spontaneous viral resolution. In contrast, helper responses in chronic infection were infrequently detected and poorly functional, and did not consistently recover following HCV cure.

Conclusions: Helper CD4+ T-cell phenotype and function following HCV viral vectored vaccination resembles “protective memory” that is seen following spontaneous clearance of HCV. DAA cure does not promote resurrection of exhausted CD4+ T cell memory in chronic infection.

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Hepatitis C virus (HCV) is a global pathogen infecting approximately 71 million people with an estimated 1.75 million new cases annually [1]. Following primary infection, most people develop chronic liver disease which may result in decompensated liver cirrhosis and HCC [2]. Despite the introduction of highly efficacious directly acting antiviral agents (DAA), there remains a strong rationale for preventative HCV vaccination. It is estimated that only 20% of people living with HCV have been diagnosed largely due to the clinically silent nature of the virus and many present only once liver fibrosis is established [1]. Of those diagnosed, only 7.4% of patients have had treatment, in part due to the financial barriers of accessing DAAs [1]. Finally, those who are treated remain at risk of reinfection, providing a rationale for a preventative approach.

A T-cell mediated vaccine may be an ideal candidate for a preventative HCV strategy. Both CD4+ and CD8+ T-cells have been shown to play a crucial role in immune control against HCV. This was first demonstrated in chimpanzee challenge studies, where it was shown that following previous successful spontaneous viral resolution (SR), antibody mediated CD4+ [3] or CD8+ [4] T-cell depletion led to prolonged viraemia after HCV reinfection. In particular, early robust Th1 CD4+ T-cell responses are thought to be critical in HCV clearance [5, 6]. It is hypothesised that functional CD4+ T-cells prime an effective CD8+ T-cell response against the virus [7], and the absence of this early priming can lead to an exhausted/dysfunctional immune response seen in chronic HCV infection [6].

We have previously shown that a heterologous prime-boost strategy using chimpanzee adenovirus (ChAd3) and modified vaccinia Ankara virus (MVA) encoding the non-structural (NS) region of HCV induces robust HCV-specific T-cell responses against a broad range of HCV epitopes in healthy volunteers [8, 9]. However, these vaccine studies, as well as other studies assessing antigen specific T-cells in natural HCV infection, have largely focused on the behaviour of HCV specific CD8+ T-cells, and there is a paucity of data assessing the behaviour of HCV specific CD4+ T-cells following both vaccination and infection. The reasons for this include very small population numbers (typically 0.001 to 0.1%) of CD4+ T-cells [10] and limited tools with which to assess these. Proliferation or stimulation assays are often used; however, these do not allow for accurate assessment of ex-vivo phenotype and function. In this study, we have used a panel of HCV specific MHC Class II tetramers on large populations of peripheral blood

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mononuclear cells (PBMCs), in addition to intracellular cytokine staining (ICS) to identify and perform a comprehensive *ex-vivo* phenotypic analysis on HCV specific CD4+ T-cells following viral vectored vaccination, for the first time. We have compared these to the "gold standard" CD4+ responses seen following SR as ideally vaccination would aim to recapitulate these events. Finally, we have interrogated CD4+ T-cell behaviour following DAA treatment since this group is a target group for vaccination and the restoration of CD4+ T cell function following DAA cure may determine the immune response to subsequent vaccination.

Materials and Methods

Recruitment of subjects

DAA patients

Patients with chronic HCV infection (n=29) including 21 receiving DAA therapy were identified at the John Radcliffe Hospital, Oxford and recruited following written informed consent. Inclusion criteria included a negative HCV viral load at the end of treatment with HLA matching the tetramer panel. Additional clinical data was collected (**Supplementary Figure 1A-B**).

Vaccine Trials

Healthy volunteers were recruited at the Churchill Hospital, Oxford into two separate vaccine studies both trialling identical HCV candidate vector vaccines (Endura CT 2009-018260-10 [8] and 2014-000730-30 [11]). All volunteers received intramuscular vaccination with experimental vaccines ChAd3-NSmut1 (ChAd3) and MVA-NSmut (MVA). 10 volunteers were selected on the basis of a positive ELISpot response, a matching HLA type for the tetramer panel and availability of stored vaccine sample (**Supplementary Figure 1C and D**).

Spontaneous HCV resolution

Individuals with SR were identified from the John Radcliffe Hospital, Oxford defined as HCV antibody positive, HCV PCR negative. All were treatment naïve with HLA matching the tetramer panel, and recruited following written informed consent. Where possible, information was gathered about the date of HCV transmission and clearance (**Supplementary Figure. 1E**).

Vaccines

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Development of the ChAd3 and MVA vectors have been described previously [8, 12, 13]. Both vectors encoded the NS3-5b region of HCV genotype 1b BK strain (1985 amino acids [aa]). Development of the HCV immunogen has also been described previously [14].

Peptides, antigens and tetramers

A panel of MHC Class II tetramers were donated from the NIH Core Tetramer Facility (Atlanta, GA, USA) and Proimmune (Oxford, UK). A total of 11 tetramers were used in the study (**Supplementary Figure 2A).** Peptides matching the MHC Class II tetramer sequence were obtained from Mimotopes. HCV genotype-1a (H77) and genotype-1b (J4 strain [structural regions] and BK strains [NS regions]) peptides spanning the entire HCV genome were used for vaccinated volunteers and DAA patients (obtained from BEI resources). Each peptide was between 15-18aa in length (overlapping by 11aa) and arranged in pools representing HCV viral proteins.

Cell lines

Short term cell lines were used for tetramer and ICS analyses. 2-3x10^6 PBMCs were stimulated with peptide and co-stimulatory purified mouse anti-human antibody CD28 (1µg/ml, BD Bioscience) in 1ml hR10 at 37 degrees Celsius. Media was changed and recombinant IL-2 (50U/mL, Roche) was added at days 3, 7 and 10. Cells were harvested on day 13 and left to rest overnight in RH10 prior to ICS and tetramer staining assays.

MHC class II Tetramer, cell surface marker and intranuclear staining

MHC class II tetramer staining was performed on cultured and *ex-vivo* PBMCs. Tetramers were based on immunodominant HCV epitopes previously described within the NS region [15-18]. Cultured cells were rested overnight prior to staining, and approximately 2x10^5 PBMCs were used per tetramer. For the ex-vivo tetramer staining, 6-8x10^6 cells were thawed using RH10 medium with DNase (0.01mg/ml) prior counting (Guava Personal Cell Analysis system). Tetramers were centrifuged for 5 minutes at 14000g at 4°C prior to staining. The cells were stained with fixable live/dead dye for 5 minutes followed by tetramer staining (PE-labelled) for 60 minutes at 37°C (1µg/100µL) in 50 µl of RH10. Following these cells were stained with the surface marker panel

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for 30 minutes. Additionally, for intranuclear staining, cells were then fixed (1% paraformaldehyde), permeabilised (eBioscience 10x perm buffer) and then stained with internal antibody cocktail for 60 minutes (see **Supplementary Figure 3** for full list of antibodies and fluorochromes).

A positive tetramer response was defined as a discrete cluster of cells and >0.004% tetramer+/CD4+ T cells. This cut off was determined after an analysis of tetramer+ CD4+ cells in healthy individuals. In addition, following vaccination a positive tetramer+ CD4+ cloud was required to be 3x baseline (pre-vaccination).

Furthermore, each tetramer was trialed with HLA matched and mismatched individuals that had not been exposed to either HCV infection or HCV peptides. All tetramers demonstrated clean staining with low background (**Supplementary Figure 2B-G**).

Intracellular cytokine stains (ICS)

ICS was performed following in-vitro expansion using short term cell lines. Prior to staining the cells were left to rest overnight in 1ml RH10 and 37°C. Cells were then plated at 1-5 $x10^5$ PBMC/well in 96 well U bottom plates. PBMCs were stimulated using individual peptides matching the tetramer sequence or pooled peptides F+G+H (matching NS3-4) plus unstimulated (controlled for DMSO) and PMA/Ionomycin (50 and 500ng/mL respectively). Brefeldin A (10µg/mL) was added after one hour and the cells were stimulated overnight at 37°C. Cells were then stained with fixable live/dead dye, fixed (1% paraformaldehyde), permeabilised (eBiosciences 10x perm buffer) and stained with the antibody panel (**Supplementary Figure 3).**

All FACS data were analysed by a LSRII flow-cytometer (BD, Bioscience, Franklin Lakes, NJ, USA). Data were collected with BD FACS DIVA software (San Jose, CA, USA) and analysed with Treestar Flow Jo software (Flowjo, LLC, Ashland, OR, USA).

Statistical analysis

GraphPad prism version7 was used for all statistical analysis. Non-parametric or parametric two tails tests were used, based on the distribution of the population: paired T tests were used for comparisons for matched samples and unpaired T tests for unrelated sample comparisons. *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001; ****p ≤ 0,0001. Only statistically significant results were reported

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in the figures. Unless stated otherwise, all values will be shown as mean population with 95% CI range.

RESULTS

HCV heterologous prime-boost vaccination with ChAd3 and MVA-NS induces HCV specific MHC class II tetramer + CD4+ T-cells

All vaccinated volunteers chosen for tetramer analysis received priming vaccination with AdCh3NSmut1 (dose 2 x 10^{10} pfu), and 8 out of 10 volunteers received boosting vaccination with MVA-NSmut at week 8 (dose 2 x 10^{8} vp). The remaining two volunteers (HCV003347 and HCV003374) received boosting with MVA-NSmut at week 8 at doses of 2 x 10^{7} and 2 x 10^{6} vp respectively. Some volunteers also received either an additional round of ChAd3/MVA vaccination or single additional MVA vaccination as part of their trial protocol. Characteristics of vaccine volunteers can be found in **Supplementary Figure 1C**.

In these volunteers, MHC class II tetramer positive populations were assessed over time; at baseline before vaccination, after prime vaccination, after boost vaccination and at the end of study. Examples plots are shown in **Figure 1A** for two vaccinated volunteers at different time points.

The highest frequency of HCV specific T-cells was observed following boosting vaccination with MVA (mean 0.062% [0.023%-0.10%] of CD4+ T-cells). The population was significantly larger compared with other trial time points. The control population, healthy HLA mismatched PBMCs not exposed to HCV virus or vaccination, was comparable to baseline staining (**Figure 1B**).

Robust and durable ex vivo tetramer positive CD4+ T-cell populations are detectable also in spontaneous resolved but not in chronic HCV patients following DAA therapy.

We then sought to compare the HCV tetramer specific CD4+ T-cell population induced following vaccination to a natural model of HCV clearance (SR) as well as to cured HCV infection with DAA therapy. Given the widely published data suggesting negligible or absent HCV specific CD4+ T-cells in chronic infection [16, 19], we were interested if these populations recover following DAA-mediated viral clearance.

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10 samples from volunteers with SR and 21 patients receiving DAA therapy were selected (where HLA matched available tetramers). Samples were taken between 0-52 weeks prior to commencing treatment (pre-DAA) and on average 6 weeks following treatment completion (range 0-26 weeks) (post-DAA).

MHC Class II tetramer+ CD4+ T-cells were detectable following SR (mean 0.036% [0.0-0.08%] of total CD4+ T-cells) (**Figure 2B-C)**. This population was comparable in magnitude to the vaccine groups, in particular at the final trial time point (mean 0.027% [0.017-0.038%). There were few detectable CD4+ tetramer positive T-cells following ex vivo staining in the DAA-treated patient groups at either time-point (**Figure 2A and C).**

HCV vaccination induces high levels of long-lived memory (CD127) and co-stimulatory (CD28) markers in ex vivo HCV specific CD4+ T-cells analogous to spontaneous resolution

We assessed the phenotypic profile of ex vivo HCV specific tetramer+ CD4+ T-cells in chronic HCV patients, vaccinated volunteers and SR individuals. Following staining with MHC class II tetramers, PBMCs were stained with a surface panel containing co-stimulatory and memory markers (gating strategy showed in **Supplementary Figure 4**).

CD28 (a critical costimulatory molecule for T-cell activation) was highly expressedat all time points after vaccination, with peak expression post boost (mean 96.6% [94.3-98.9%]). CD28 expression was significantly lower in patients with chronic HCV (mean 81.4% [71.1-91.8%]) (**Figure 3A**).

CD127 (IL7R) expression of tetramer+ T-cells increased during the course of the trial and reached peak expression at the EOT time point (mean 74.3% [64.8-83.8%]). CD127 expression at the EOT was comparable to that seen in SR (mean 71.05% [60.02-82.1%]), and both these groups demonstrated significantly higher expression compared with both prime and boost vaccination and chronic HCV patients. **(Figure 3B).**

Memory subsets were further assessed as follows: stem memory T-cells (Tscm: CCR7+/CD45RA+), effector memory T-cells (Tem: CCR7-/CD45RA-); central memory T-cells (Tcm: CCR7+/CD45RA-) and effector memory RA positive cells (Temra: CCR7-/CD45RA+).

Central and effector memory subsets were the most predominant at all vaccine time points as well as in SR (**Figure 3C**). The percentage of Tcm cells were similar between these groups,

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ranging from 29.9% [20.3-39.1%] to 38% [28.15-47.7%] of tetramer+ CD4+ T-cells (prime and SR respectively). Chronic HCV patients comparatively had lower expression (mean 23.8% [12.5-35%]), significantly so when compared with SR (p=0.04).

Similarly, robust Tem populations were observed in all groups and was highest following boosting vaccination (49.6% [33.8-65.6%]), reducing significantly by EOT (mean 31.6% [20.9-42.4%] (p=0.0075). The populations of Tscm's were observed to contract between prime and boost vaccination (mean 22.8% [12.4-33.3%] to 13.2% [4.5-21.8%], p=0.01%), and then re-expand at the end of the trial (mean 25.3% [17.2-33.5%], p=0.005%).

Temra's were the smallest population observed out of the memory marker subsets. However, the numbers increased significantly at the end of the trial (mean 9.8% [2.1-17.5%]) compared with prime (mean 6.1% [0.75-11.5%]) (p=0.01) and boost vaccination (mean 4.6 [0-11.2%]) (p=0.007).

(Figure 3C).

T-bet and Eomes expression following vaccination mirrors spontaneous viral resolution

Intranuclear phenotypic analysis was performed on two transcription factors (TF) important in T-cell activation and differentiation into effector and memory cells, T-box TF TBX21 (T-bet) and Eomesodermin (Eomes). Robust T-bet expression was demonstrated following prime and boost vaccination, however was reduced at the EOT (mean 41.4% [15.9-55%] of tetramer+ CD4+ T-cells), which reached significance when compared with T-bet staining following boosting vaccination (mean 61.5% [29.3-84.6%]) (p=0.03). T-bet expression in SR was lower than all other groups, reaching significance when compared with prime (p=0.04) and boost vaccination (p=0.009) **(Figure 4A).** There are fewer published studies assessing Eomes behavior in CD4+ T-cells [20, 21]. Expression of Eomes was low at all vaccine time points as well as in chronic HCV patients and in SR individuals **(Figure 4B)**.

Further there is a growing body of evidence that changing levels of T-bet and Eomes expression occur on the pathway to T cell exhaustion resulting in a final T-bet^{lo}/Eomes^{hi} population [22]. This exhausted population was very low in chronic HCV patients, and in each time point after the study, although it was significantly higher at the EOT compared to SR (p=0.0087) **(Figure 4C)**.

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In summary, the expression of T-bet significantly decreased over the course of the trial, and was lowest in SR.

Proliferative capacity of HCV tetramer specific CD4+ T-cells following HCV vaccination is robust, but limited following DAA mediated cure

We sought to assess the proliferative capacity of HCV specific CD4+ T-cells following DAA mediated cure (n=21), and compare with HCV vaccination (n=5) and SR (n=10). The proliferative capacity of HCV specific CD4+ T-cells in all groups was assessed using MHC class II tetramers following *in vitro* culture with peptide corresponding to the tetramer and IL-2 for two weeks (**Figure 5A-D)**. Between 1 and 6 tetramers were used with each sample (depending on HLA specificity) with the highest magnitude tetramer for each individual sample chosen for analysis (**Supplementary Figure 1D**)**.**

There was a robust population of HCV tetramer+ CD4+ T-cells induced following viral vectored vaccination (mean 10.94% [0-26.6%] at boost and 6.7% [0-14.6%] at EOT). Likewise, there was a comparable, though smaller population in SR (mean 3.6% [0.6-6.6%] (**Figure 5B-D**). In comparison, there was a poor proliferative response in the DAA patient cohorts (mean 1.3% [0-3.1%] and 0.8% [0.02-1.7% for pre-DAA and post-DAA respectively) (**Figure 5A and D**). There was no clear pattern or trend between the pre- and post-DAA treatment group, with subgroup analyses comparing patients which had both a two-fold increase and two-fold decrease in tetramer+ populations showing no significant differences in phenotypic characteristics (**Supplementary Figure 5**).

Single epitope sequences that were contained within the tetramers were compared following *in vitro* culture to assess the immunogenicity of each. The peptide sequence contained in tetramer 14 ($NS3_{1806-1818}$) restricted to HLA DRB1*0101 was by far the most immunogenic across all four groups. In comparison, T-cell populations binding tetramers 22 ($NS2_{794-810}$) and 29 ($NS5A_{1957-1975}$) were rarely detectable (**Supplementary Figure 6**).

Functionality of CD4+ T-cells in pre- and post-DAA treatment, vaccinated and SR groups.

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To evaluate the capacity of cytokine secretion following HCV vaccination, SR and following DAA treatment, ICS assays were performed following *in vitro* expansion using peptide pools F-H (corresponding to NS3-4), CD28 and periodic addition of IL-2. These peptides pools were chosen based on their immunogenicity demonstrated in HCV vaccine trials to date [8, 9] as well as published data identifying a number of immunogenic CD4+ epitopes in NS3-NS4 proteins [23, 24]. Representative ICS plot are shown in **Figure 6A.**

Overall there was poor cytokine production in the DAA patient cohort, with no recovery of cytokine production observed following viral cure and no significant differences between the two groups (**Figure 6B**). In comparison, vigorous cytokine production was observed for three out of the four cytokines tested in both SR and following vaccination. Production of TNF-α, IFN-γ and MIP-1β was significantly greater in these groups compared to both pre- and post-DAA treatment, peaking at 21.2% [10.18-32.2%]) for TNF-α production in SR. No significant difference was observed between SR and vaccination in any measured cytokine (**Figure 6B**). We observed a strong correlation of cytokine production between CD4+ and CD8+ T-cells measured at the same time points after vaccination (r=0.73, p=<0.0001) **(Supplementary Figure 7).**

DISCUSSION

This research addresses two important areas of HCV research; the behaviour of viral specific CD4+ T-cells following HCV vaccination and the extent of immune recovery following DAA mediated HCV cure. CD4+ T-cells are widely believed to play a vital role in the early immune response to HCV infection. However, HCV specific CD4+ T-cells have been challenging to study, largely due to limited/absent populations and limited tools for assessing them *ex-vivo*. The use of MHC class II tetramers to predictably assess CD4+ responses has been limited by the promiscuous epitope binding to MHC class II alleles [18] and a variable length of amino acids optimal for binding to the MHC [25]. Here we employed a panel MHC class II tetramers, ICS and peptide-stimulated cell lines to perform a detailed analysis of the functional and phenotypic behaviour of CD4+ T-cells following HCV viral vectored vaccination, in chronic HCV infection before and following DAA-mediated HCV cure and in SR.

Using large input cell numbers (6-8x10^6 cells), we were able to detect HCV specific tetramer+ CD4+ T-cells following ChAd3/MVA prime-boost viral vectored vaccination in *ex-vivo* analysis.

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Tetramer populations were identified at every time point after vaccination and peaked following boosting vaccination, comparable to the kinetics of CD8+ T-cells following identical vaccination schedules [8]. Within our panel of 10 tetramers, we observed variable peptide sequence immunogenicity, however epitopes restricted to NS3, notably DRB1*01 restricted sequence $NS3_{1806\text{-}1818}$ were the most immunogenic, supporting previous publications [16]. Importantly the size of the tetramer+ cell population at the final trial time point and thus the best predictor of the residual memory population following vaccination was comparable to that seen in SR.

Although the number of HCV specific CD4+ T-cells induced by vaccination is undoubtedly important, the phenotypic and functional properties of the CD4+ T-cells are likely to be significant determinants of a protective response. We hypothesise that vaccine induced CD4+ T-cells that function in an analogous way to CD4+ T-cells following SR may be predictive of a protective response. We analysed a number of cell surface markers associated with T-cell activation (CD28) and memory differentiation (CD127, CCR7, CD45RA) in chronic HCV patients, vaccinated volunteers and SR individuals, using tetramer+ populations in *ex vivo* CD4+ T-cells.

A critical component of a successful vaccine is one that induces long lived memory cells capable of homeostatic proliferation [26], with appropriate co-stimulatory molecules. CD28 is a critical co-stimulatory molecule in T-cell activation [27], and CD4+CD28- T-cells are widely observed to be present in chronic viral infections such as CMV [28], HIV [29] and HBV [30]. CD28 was highly expressed at all time points after vaccination. Significantly lower levels were expressed in chronic HCV patients compared vaccinated and SR volunteers. CD127 (IL-7rα) is strongly associated with T memory cell development. Using adoptive transfer models in mice, Kaech et al showed that the expression of CD127 was required as a precursor for functional memory cell development [31]. IL7 (the ligand for CD127) has been shown to play a critical role in the maintenance of a polyclonal and functionally diverse repertoire of human CD4(+) memory T cells in the absence of ongoing antigen stimulation [32]. We have shown CD127 expression to be progressively upregulated in ex vivo HCV tetramer+ CD4+ T-cells following HCV vaccination, suggesting this marker is associated with memory cell development. When compared to expression in natural HCV clearance, expression was similar to the final trial time point (mean 74.3% at EOT and 71% for SR group) and significant lower in chronic HCV patients (mean 49%).

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Much work has been directed at elucidating and characterising T-cell memory subsets with a focus on CD8+ T-cells and a paucity of data assessing CD4+ T memory subsets. Here we provide a valuable insight into the behaviour of these cell populations in tetramer+ CD4+ T cells in chronic HCV patients, vaccinated volunteers and SR individuals. In all settings Tcm's and Tem's were the predominant memory subset induced, in keeping with published data in CD8+ populations. These two populations are perhaps the most widely studied, and both contribute critical functions to a successful memory response – rapid differentiation on antigen re-exposure (Tem's) and long half-life and proliferative capacity (Tcm's). Tscm's have been recently described as long-lived multipotent memory cells [33, 34]. We show a robust population of Tscm's in all study groups. Notably, Tscm expression expanded significantly between boosting and EOT (mean 13.2% to 25.3%), similar to previous observations in CD8+ T cells following ChAd3/MVA prime-boost [8]. CD45RA re-expression in long lived memory cells is important for the ability to self-renew, and to differentiate into other memory subsets, whilst CCR7 re-expression is important for exposure to circulating antigen – both critical components of memory cells. They have been suggested to play a role in the persistence of latent HIV reservoirs in CD4+ T-cells of infected hosts [35], but their role in CD4+ T-cells in maintaining protection following both viral clearance and vaccination – although presumed – has been challenging to identify. We observed small populations of Temra's in all groups consistent with previous observations in CD4+ T cells [36] and, similarly to Tscm, the expansion of this subset between boosting and EOT may reflect and evolving CD4+ memory phenotype with time. The role of Temra's in CD4+ T-cells has not been fully elucidated, although have been associated with CX3CR1 expression, suggesting a potential cytotoxic phenotype [37, 38].

Transcription factor (TF) analysis was performed on tetramer gated *ex vivo* CD4+ T-cells specific for HCV. T-bet, a central regular in CD4+ Th1 differentiation and widely studied in both CD4+ T and CD8+ subsets, was observed to decrease in expression over time following vaccination, reflecting previous observations that T-bet expression declines as CD4+ T-cells gain a more memory-like phenotype [21]. The reduced T-bet expression in SR (on average sampled many years post HCV exposure) further supports this. Unlike its CD8+ counterpart, there is a lack of literature assessing Eomes expression in CD4+ T-cells following antigen exposure. Knowledge to date is largely confined to assessing Eomes in bulk CD4+ phenotyping, with low expression

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observed, particularly in Tcm's compared with Tem's and effector cells [20, 21]. We observed low levels of Eomes expression throughout the vaccine trial and in SR, in keeping with published data. Importantly, the comparable expression of both TF's following HCV vaccination – particularly at the final trial time point – with SR indicates vaccination may induce a memory CD4+ T-cell with a protective transcriptional signature.

In the chronic HCV group, we observed the memory phenotype and TF expression of HCV tetramer+ CD4+ T-cells to be similar to all vaccine time points and SR, with the exception of a significant decrease in Tcm in chronic HCV compared to SR. A possible explanation for this similarity is selection bias, in that those CD4+ T-cells detectable in chronic infection are relatively functionally preserved, whilst the most functionally exhausted are deleted.

The ability of antigen specific memory T-cells to rapidly expand and produce cytokine upon re-exposure to antigen is a critical component of a functional and protective cell mediated response [39-41]. Following peptide-stimulated short-term culture, we observed robust proliferation as well as effector cytokine production after ChAd3/MVA vaccination, comparable to SR. In particular antiviral cytokines IFNγ and TNFα were readily produced following ICS stimulation, which are well defined correlates of highly effective T-cells. IL-2 production was attenuated; progressively diminishing IL-2 production has previously been observed following T-cell activation via a negative feed lack loop [42, 43]. We hypothesise poor IL-2 secretion was due to IL-2 downregulation following activation and proliferation in the cell lines. Overall, these results mirror the behaviour of HCV specific CD8+ T-cells following vaccination [8, 9] and there was a high correlation of cytokine production between CD4+ and CD8+ T-cells suggesting a coordinated response to vaccination. Similar phenotypic and functional correlations between peptide specific CD4+ and CD8+-T cells have been observed in highly effective viral vaccines such as yellow fever vaccine [44]. The fact that CD4+ vaccine induced responses are phenotypically and functionally analogous to that seen in natural HCV clearance is suggestive of a protective immune response. Importantly, our observations in SR, made on average many years or decades following exposure, suggests that the phenotype observed is long-lived, as suggested by previous studies [45].

The other critical question addressed here is the state of the host immune system after DAA mediated HCV cure. T-cell exhaustion, or the hierarchical loss of effector functions and eventual anergy and deletion [46], is a well-described process in chronic infection, including HCV [47, 48].

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The new era of DAA-treatment gives a unique opportunity to interrogate immunological recovery following cure of chronic viral infection. Using tetramer assays and ICS following peptide stimulation, we were unable to detect an increase in proliferative capacity or production of antiviral cytokines following DAA-mediated HCV cure. There are a number of hypotheses to explain this. Firstly, more time may be needed for the host immune system to 're-set' following cure and functional ability to be restored. Secondly, antigen re-priming with naïve thymic emigrants may be required following viral cure to induce a functional immune response. Thirdly, all the patients in this cohort had liver cirrhosis, well described to induce an immunosuppressive state in the host [49]. A final hypothesis however, is that the host HCV specific immune response in chronic HCV infection is terminally exhausted and unable to be reversed following cure. Our observations contrast with a previous study demonstrating T cell recovery following interferon-free therapy [50]; the study by Martin et al assessed CD8 T cells in treatment naïve non-cirrhotic patients, suggesting the limited recovery observed in our population may be due to previous or current interferon treatment or the presence of cirrhosis. However, HCV re-infection is known to occur following DAA treatment in those with ongoing HCV exposure, including patients without cirrhosis. These clinical data show that DAAs do not consistently restore HCV specific immune responses to protect against re-infection. An important remaining question is whether HCV -specific immune responses can be restored following DAA cure to a level that will enable effective HCV specific vaccination strategies. Clinical trials in progress (NCT03688061) will address this question directly.

In summary, we provide a detailed analysis of phenotypic and functional characteristics of HCV specific CD4+ T-cells. Following vector-based HCV vaccination, HCV specific CD4+ T-cells are analogous to those in the gold-standard setting of SR in both phenotypic and functional parameters, suggesting the induction of effective and protective CD4+ T-cells following vaccination. Furthermore, we show minimal functional recovery of the same population of cells following DAA-mediated cure. This work has wider implications to both T-cell vaccine development and chronic viral infection. Further studies assessing both *in vivo* responses to HCV vaccines and behaviour of CD4+ T-cells following vaccination in DAA-mediated cure are required.

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Acknowledgements:

The vaccine trials were supported by funding from the EU Sixth (HEPACIVAC) and Seventh (FP7/2007-2013) Framework Programmes. This research was supported by the Wellcome Trust (WT109965MA to PK), NIHR Senior Fellowships (PK and EJB), NIH U19 I082360 (PK), and MRC (EB). The authors wish to acknowledge the BRC Oxford GI Biobank and BRC Oxford IBD Cohort in collecting and making available the samples/data used in the generation of this publication. The research was also funded/supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. The authors would also like to acknowledge the hepatology nurses at the John Radcliffe Hospital, Oxford and the staff at the CCVTM, Oxford.

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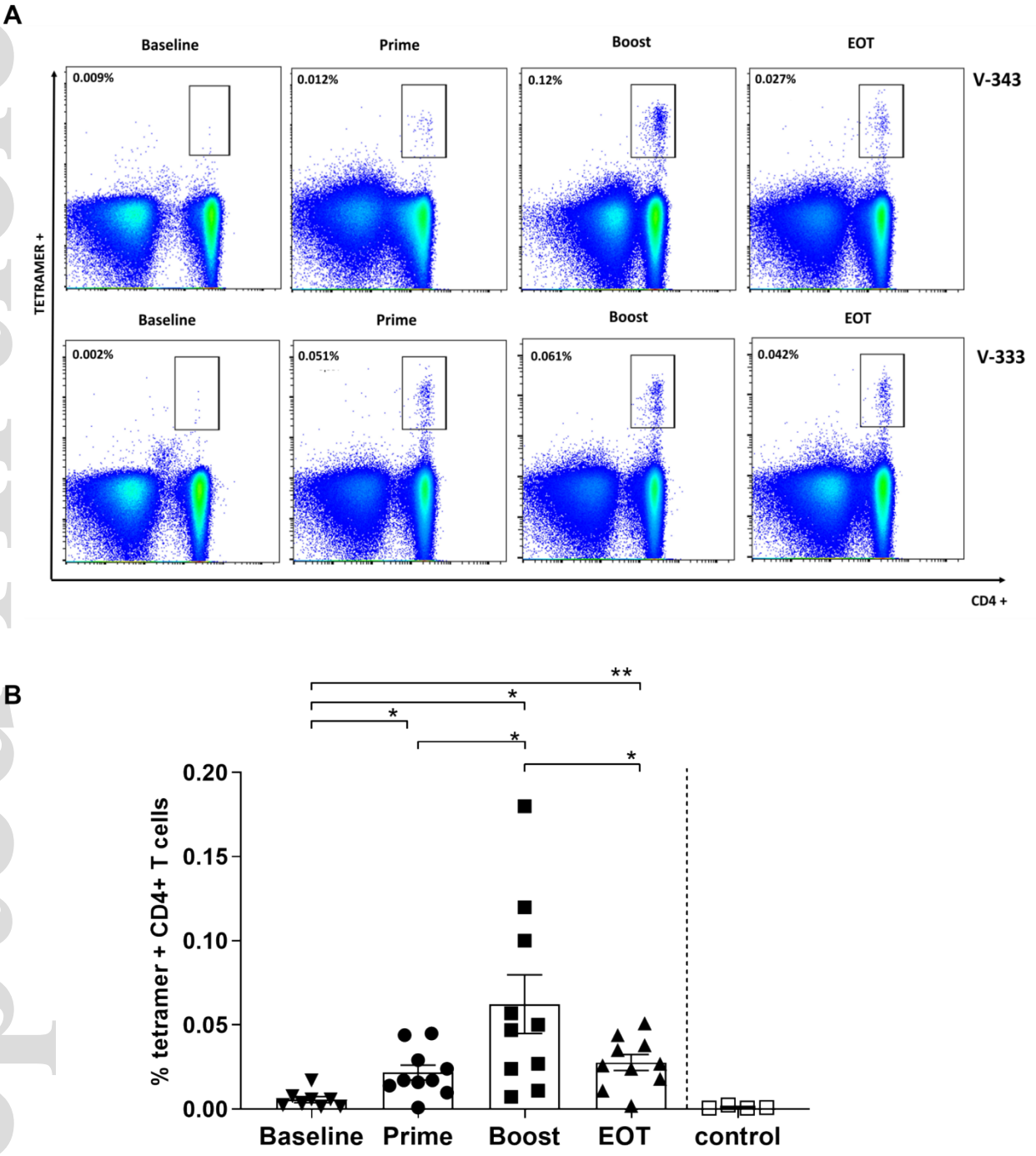

Figure 1: HCV specific MHC class II tetramer+ CD4+ T cells following viral vectored vaccination *A) Example FACS plot of staining with tetramers 14 HCV-NS4B$_{1806\text{-}1818}$ in two vaccinated healthy volunteers over the study course. Gating is on live CD3+ cells. Values indicate the percentage of CD4+ T cells binding the tetramer. B) Percentage of tetramer+ CD4+ cells after* ex vivo *staining in vaccinated healthy volunteers at different time points of the*

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vaccination regimen. Controls are represented by healthy volunteers stained with mismatched MHC class II tetramer. Error bars represent the standard error of mean (SEM).

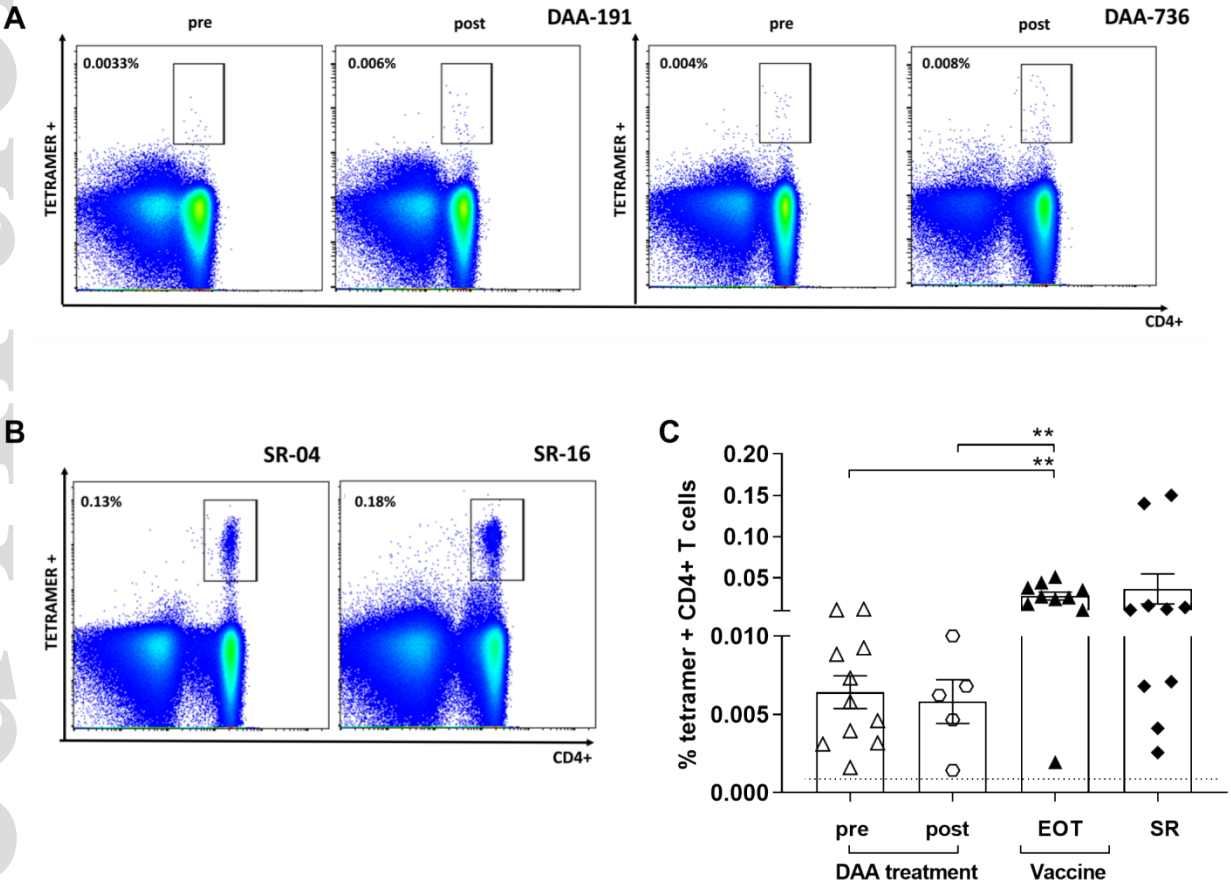

Figure 2: Assessment of ex- vivo tetramer+ CD4+ T cells pre- and post-DAA treatment, following vaccination and in SR volunteers.

A-B) FACS example plot of staining with tetramers 14 HCV-NS4B$_{1806-1818}$, 24 HCV-NS3$_{1535-1551}$ or pool of tetramers 17 HCV-NS3$_{1582-1597}$, tetramer 18 HCV-NS3$_{1411-1425}$ and tetramer 19 HCV-NS3$_{1535-1551}$ in two DAA patients pre and post therapy and in two SR. The gating is on live CD3+ cells. Values indicate the percentage of CD4+ T-cells binding the tetramer. C)Percentage of tetramer+ CD4+ cells after ex vivo staining at the end of study of vaccinated healthy volunteers, in DAA pre- and post- therapy and in SR individuals. Error bars represent the standard error of mean (SEM). Only statistical differences shown.

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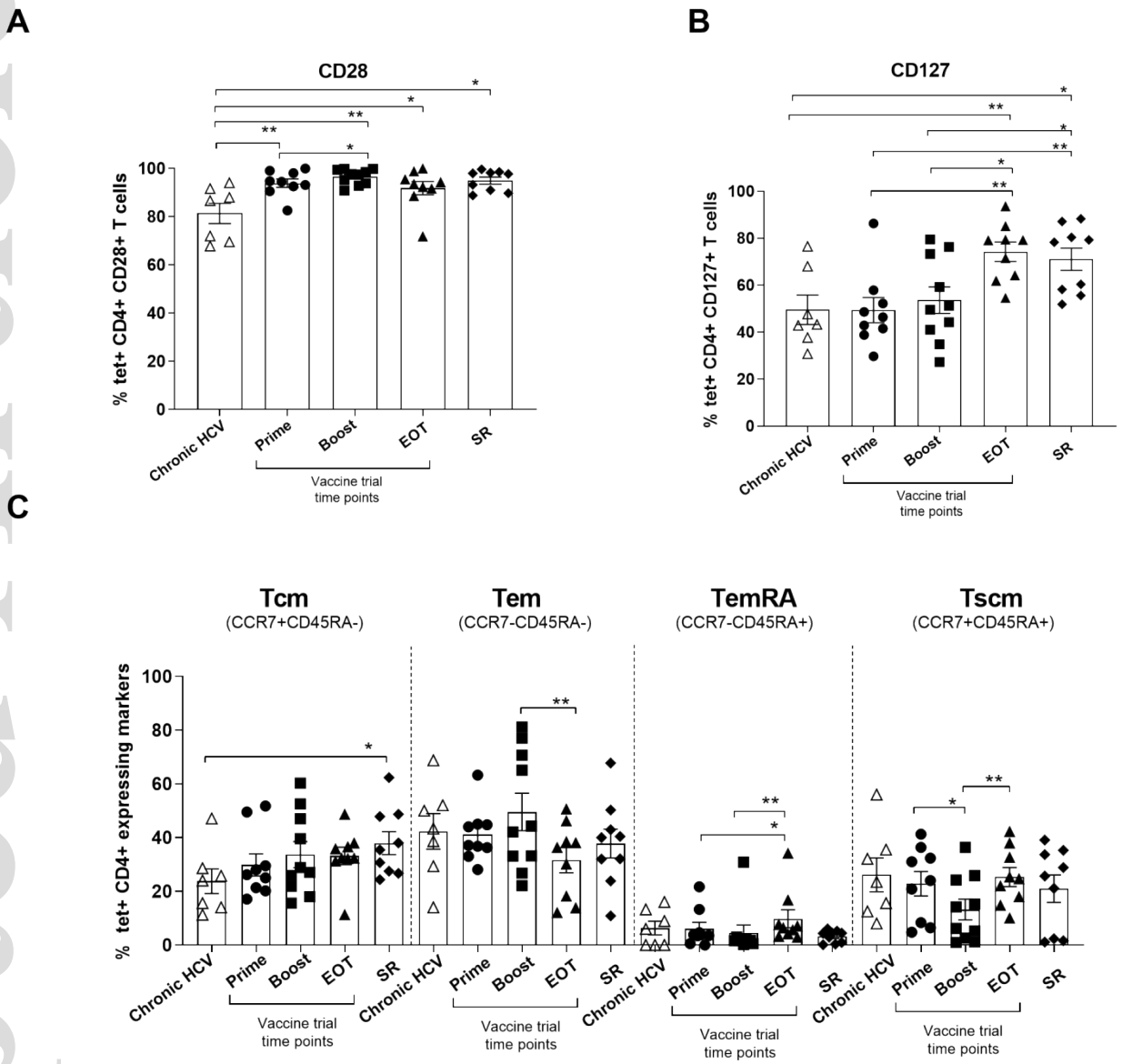

Figure 3: Analysis of co-stimulatory and memory cell-surface markers in chronic HCV, vaccinated and SR volunteers.

A-B) Thawed PBMCs were co-stained with MHC class II tetramers and anti-CD127 and CD28 antibodies in chronic HCV patients, vaccinated volunteers at different trial time points and in SR individuals. C) Proportion of tetramer+ cells expressing Tcm, Tem, Temra and Tscm phenotype in the same cohorts. All tetramer staining and phenotyping were performed ex vivo. *Error bars represent the standard error of mean (SEM). Only statistical differences are shown.*

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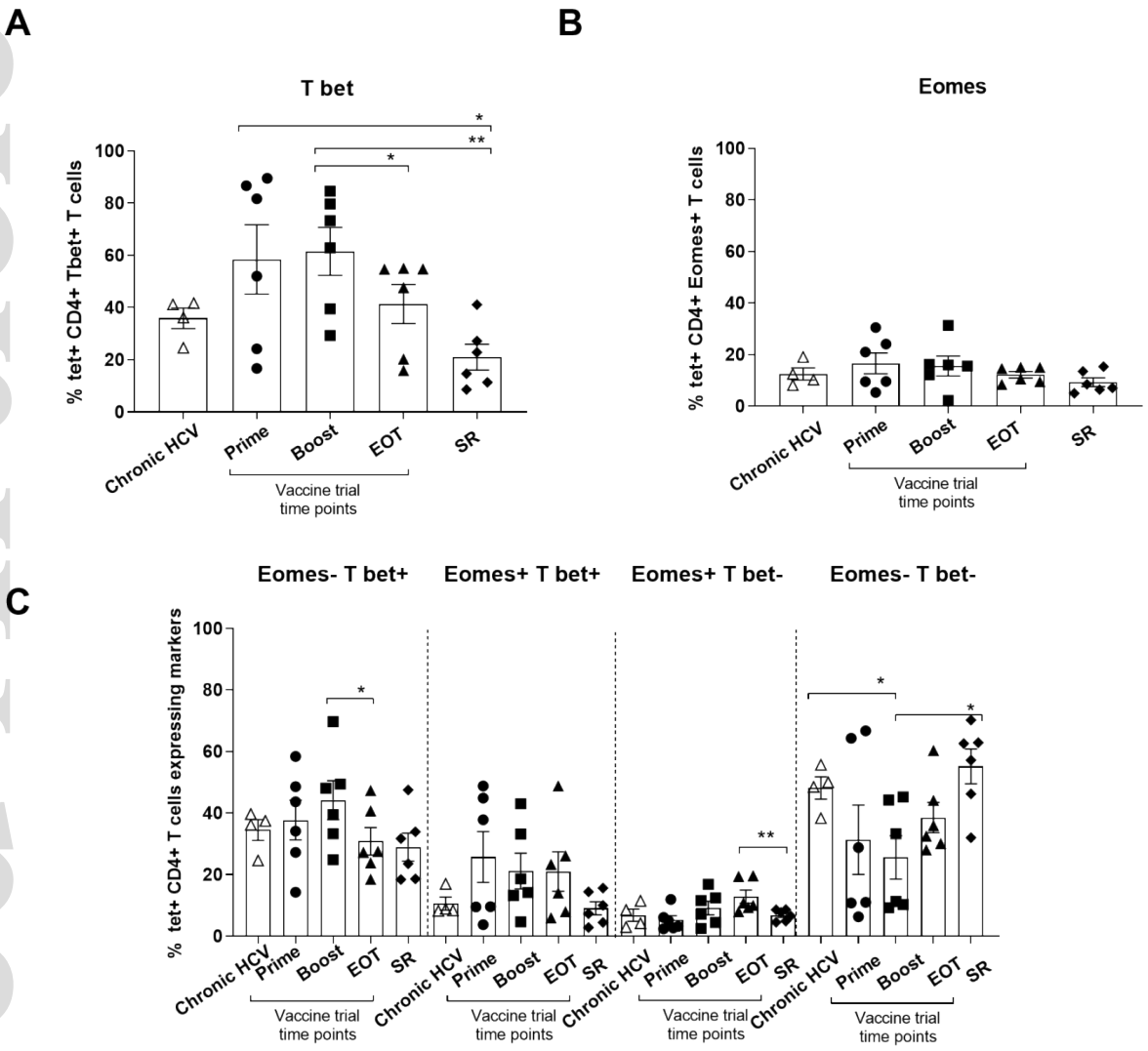

Figure 4: Transcription factor analysis in chronic HCV, vaccinated and SR volunteers.

A-B) Percentage of tetramer+ CD4+ T cells expressing T bet and Eomes in chronic HCV patients, vaccinated volunteers and SR individuals. C) Co-staining with class II tetramers and anti-human T bet and Eomes antibodies in the same cohorts. Error bars represent the standard error of mean (SEM). Only statistical differences shown.

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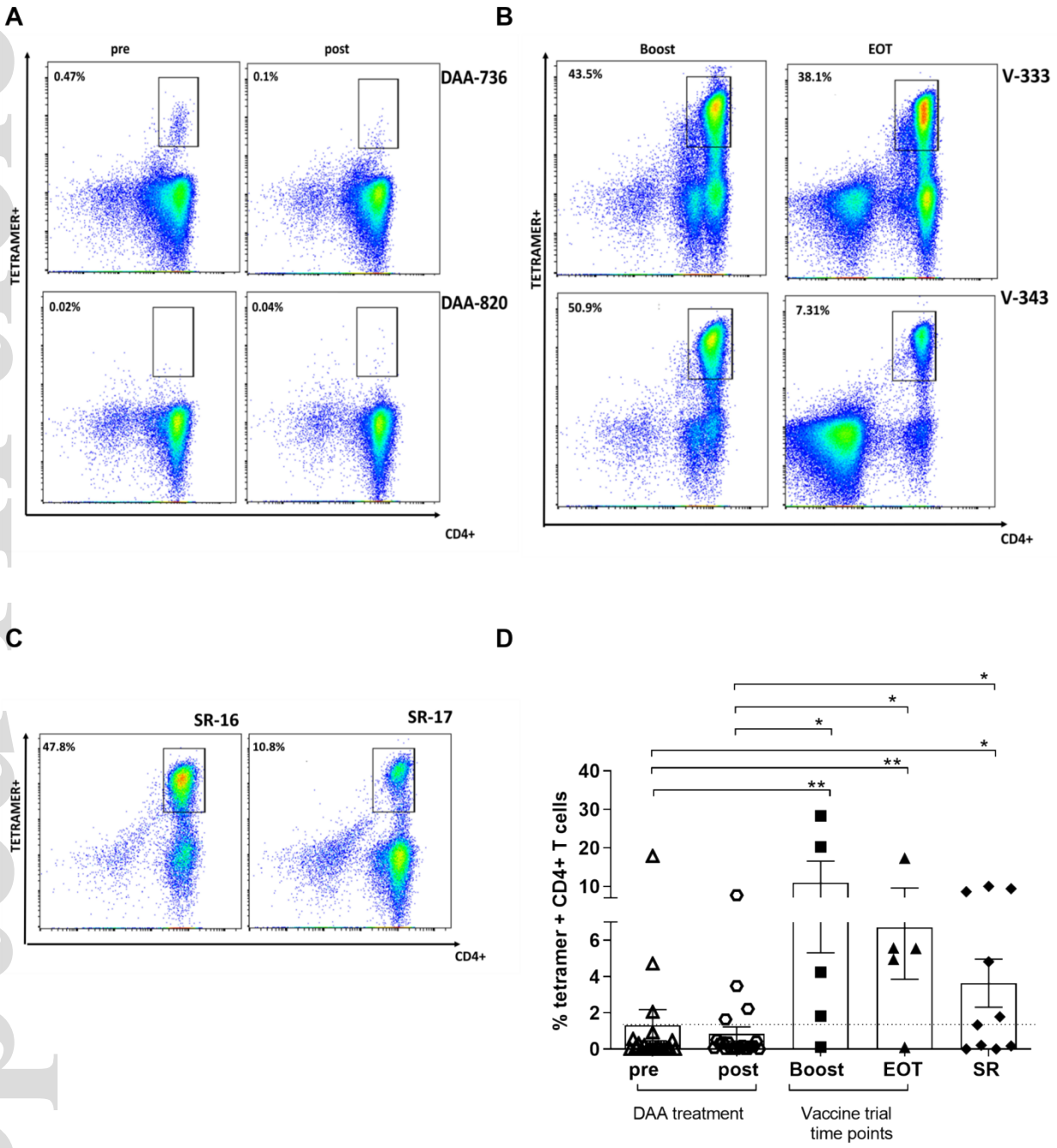

Figure 5: Proliferative capacity of CD4+T cells in pre- and post-DAA treated patients, vaccinated and SR volunteers. *A-C) Example FACS plots of tetramer+ CD4+ T-cells after 14 days of culture with peptide matching tetramer sequence in two DAA patients pre- and post-treatment (A), two vaccinated volunteers at boost and EOT (B), and two SR individuals (C). Values indicate the percentage of CD4+ T-cells binding tetramer. D) Scatter plot with bar showing the percentage of tetramer+ CD4+ T-cells after culture in pre- and post-DAA,*

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vaccination and SR. Error bars represent the standard error of mean (SEM). Only statistical differences are shown.

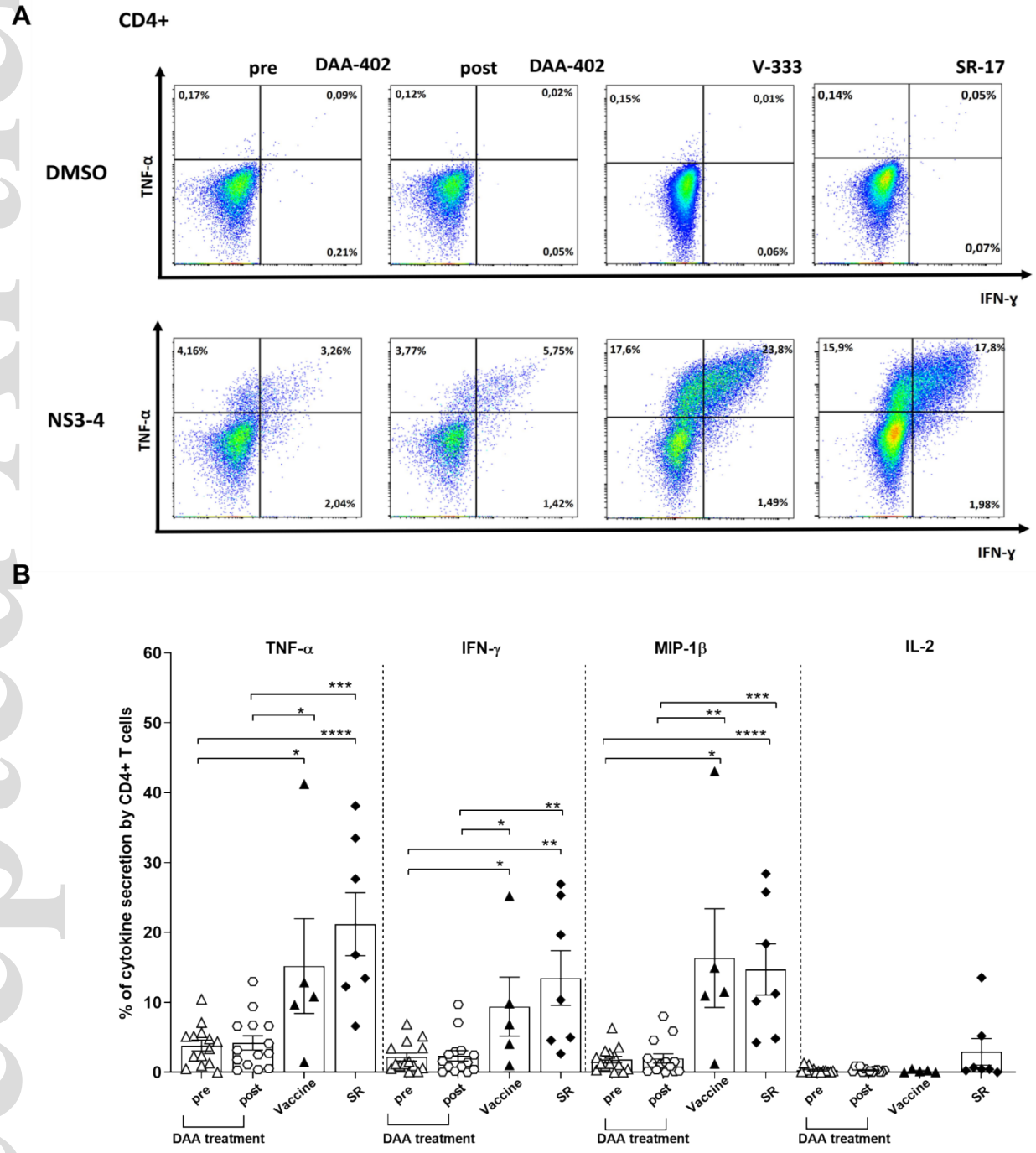

Figure 6: Functional capacity of CD4+T cells in pre- and post-DAA treated patients, vaccinated and SR volunteers.

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A) Example FACS plots showing TNFα/IFN-γ after intracellular cytokine staining are shown for CD4+ T-cells stimulated with NS3-4 or DMSO control in DAA patient pre- and post-treatment, vaccine volunteer (after boost vaccination) and in SR. B) Comparison of cytokine production by CD4+ T cell pre- (n=14) and post-DAA treatment (n=14), after ChAd3/MVA vaccination (n= 5) and in SR (n= 7). PBMC were cultured for 14 days with peptide matching NS3-4 (pools F+G+H), rested and re-stimulated with the same peptides overnight. Staining in DMSO wells was subtracted. Error bars represent the standard error of mean (SEM). Only statistical differences shown.

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