Published in final edited form as:

J Viral Hepat. 2013 July ; 20(7): 453–462. doi:10.1111/jvh.12050.

Impact of oral silymarin on virus- and non-virus-specific T-cells responses in chronic hepatitis C infection

Oluwasayo Adeyemo^{2,¶}, Hiroyoshi Doi^{2,¶}, K. Rajender Reddy², and David E. Kaplan^{1,2} for the SyNCH Trial Investigators

¹Medicine and Research Services, Philadelphia VA Medical Center, Philadelphia PA

²Division of Gastroenterology, Department of Medicine, University of Pennsylvania

Abstract

Silymarin displays anti-inflammatory effects on T-lymphocytes *in vitro*. The immunomodulatory properties of oral silymarin *in vivo* in humans with chronic hepatitis C have not previously been characterized. We hypothesized that silymarin would suppress T-cell proliferation and pro-inflammatory cytokine production of virus- and non-virus-specific T-cells while increasing anti-inflammatory IL-10 production *in vivo*. Patients from one site of the SyNCH-HCV double-masked, placebo-controlled study of oral silymarin in prior interferon non-responders with chronic hepatitis C provided blood samples at baseline and treatment week 20. Mononuclear cells were stimulated with recombinant HCV proteins and controls in ³H-thymidine proliferation assays, IFN γ Elispot and IL-10 Elispot. The frequency of CD4⁺CD25^{hi} and CD4⁺foxp3⁺ regulatory T-cells, serum cytokine levels, serum IP-10 and lymphocyte interferon-stimulated gene expression were also quantified at baseline and week 20. Thirty-two patients were recruited (10; placebo, 11; 420mg three times a day, 11; 700mg three times a day). Serum ALT and HCV RNA titers did not change in any group. HCV-specific CD4⁺ T-cell proliferation and the frequency of IFN γ - and IL-10-producing T-cells were not significantly changed in silymarin-treated subjects. However, C. albicans-induced T-cell IFN γ and phytohemagglutinin-induced T-cell proliferation were suppressed by silymarin therapy. A trend towards augmentation of interferon-induced ISG15 expression was present in the high-dose silymarin group. While no effect on HCV-specific T-cells was identified, these data confirm that high-dose oral silymarin exerts modest non-specific immunomodulatory effects *in vivo*. The impact of this anti-inflammatory effect on long-term liver health in chronic hepatitis C merits future clinical investigation.

Keywords

silymarin; T-cell; lymphocyte; hepatitis C; interferon-gamma; interferon-stimulated gene

INTRODUCTION

T-cells play a critical role in the resolution of hepatitis C virus (HCV) infection. In early infection, HCV-specific T-cell responses are characterized by robust IFN γ production and proliferation in patients who resolve their infections compared to weak and dysfunctional responses in those who progress to chronic infection (1–4). In chronic infection, some but not all studies suggest that treatment-related resolution of chronic hepatitis C is also in part

Corresponding Author: David E. Kaplan, MD, MSc, Philadelphia VA Medical Center, Research Bldg 21, A402A, 3900 Woodland Avenue, Philadelphia, PA 19104, dakaplan@mail.med.upenn.edu.

[¶]Authors contributed equally to manuscript

mediated by antiviral T-cells (5–9). Furthermore, various regulatory T-cell populations have been associated with protection from chronic liver injury in chronic infection (10–12).

Silymarin, an extract of milk thistle (*Silybum marianum*; plant extract of *Asteraceae* family), has been used for two millennia to treat various liver disorders. In the modern era, salutary effects in alcoholic liver disease and chronic viral hepatitis have been ascribed to orally administered silymarin in the absence of rigorous data supporting this practice (13). Modulation of T-cells has been one postulated explanation for the putative anti-inflammatory properties of silymarin. In murine models, silymarin has been variably demonstrated to have both pro- (14) and anti-inflammatory effects on T-lymphocytes (15) (16). Recent human clinical trials somewhat unexpectedly demonstrated that high dose intravenous silibinin exhibits direct antiviral properties in chronic hepatitis C *in vivo* (17, 18), suppressing HCV replication primarily by inhibiting viral entry and transmission (19). *In vitro*, the silymarin preparation MK001 also appeared to suppress virus- and non-virus-specific CD4⁺ T-cell proliferation, as well as both pro- (TNF α , IFN γ) and anti-inflammatory (IL-10) cytokine production (20) likely due to blockade of STAT1-induced NF κ B activation (20, 21). However, the effects of silymarin on human antiviral T-cells *in vivo* have not previously been investigated.

In the NIH-sponsored Phase II multicenter, randomized, placebo-controlled, double-masked, prospective clinical trial completed by the Silymarin in Non-alcoholic Steatohepatitis and C Hepatitis (SynCH) Study Group, two doses of Legalon® 140, a standardized preparation of milk thistle extract, or identical sugar-containing placebo were administered to patients with chronic hepatitis C with previous non-response to interferon-based antiviral therapy. In the clinical trial, there was no statistically significant difference in the proportion of patients in placebo or silymarin arms that achieved serum ALT level of ≤ 45 IU/L or 50% reduction of ALT from baseline level to < 65 IU/L at the end of the 24-week treatment period (22). We present results of a prospective, masked immunological substudy designed to determine the impact, if any, of orally-administered silymarin on hepatitis C-specific CD4⁺ T-cell proliferation and pro-inflammatory cytokine responses *in vivo*.

MATERIALS AND METHODS

Subjects and Samples

Patients from the clinical trial at one site who gave informed consent under a separate IRB-approved protocol provided 40ml of peripheral blood once prior to initiation of study medication or placebo and once at 20 ± 2 weeks during active treatment or placebo.

Peripheral blood mononuclear cells (PBMC)

PBMC were isolated from blood using Histopaque-1077 density centrifugation (Sigma-Aldrich, St. Louis MO) (23, 24). PBMC were suspended in RPMI 1640 containing L-glutamine, 10% human AB serum, 25mM HEPES (Gibco/Invitrogen), and penicillin/streptomycin (Gibco/Fisher scientific).

Antigens and reagents

Recombinant genotype 1a-derived HCV core (SOD-c22, aa 2-120), NS3/4 (SOD-c200, aa 1192-1931) NS5 (SOD-NS5, aa 2054-2995), and control superoxide dismutase (SOD) proteins were generously provided by Novartis Diagnostics (Emeryville, CA) (1, 25–27). Tetanus toxoid 0.5 μ g/ μ l (Sigma) and *Candida albicans* 20 μ g/ μ l (Sigma) were utilized as antigen positive controls.

T-cell proliferative response

CD4⁺ T-cell proliferation assay was performed as previously described (1, 23, 24). Briefly, PBMC (2x10⁵ cells/well) were stimulated for 7 days with SOD-c22, SOD-c200, and SOD-NS5 and controls and harvested after 16 hours of ³H-thymidine incorporation (1μCi/well) (Dupont NEN, Boston, MA). As a positive control, PBMC were stimulated with a T-cell mitogen phytohemagglutinin (PHA) at 2 μg/mL and harvested on day 4. The results were expressed as a stimulation index (SI) with mean counts per minute (cpm) in stimulated wells divided by the mean cpm in control wells. SI greater than 3 was considered positive (24). Testing of *in vitro* T-cell suppression was performed using PBMC (1–2x10⁵ cells/well) with and without 2 μg/mL PHA in presence of silymarin (Sigma) at concentrations ranging from 0.1 to 100 μg/ml.

T-cell IFN γ and IL-10 secretion

The frequency of IFN γ and IL-10 producing T-cells were quantified by Elispot assay, performed using 2 x 10⁵ PBMC/well in triplicates with and without HCV and control antigens as described previously (9, 11, 24). HCV-specific CD4⁺T cell IFN γ (Th1) or IL-10 (Tr1) responses in whole PBMC was examined using recombinant HCV and control proteins (10μg/ml). Spot forming units (SFU) were counted using IFL04 Elispot reader (AID, Hamburg, Germany). HCV-specific IFN γ or IL-10 T-cell frequency was calculated by subtracting the mean SFU in negative control wells from mean SFU in antigen-stimulated wells and expressed as HCV-specific SFU/10⁶ PBMC.

Quantification of regulatory T-cells

2 x 10⁵ PBMC were stained with 7-actinomycin-D/Viaprobe, CD69 FITC, CD25 PE, CD45RO APC, CD4 PE-Cy7, CD4 APC (all BD Bioscience), CD4 APC-Alexa Fluor 750 (eBioscience), foxp3 FITC (eBioscience) as previously described (11). All samples were acquired using FACS Diva software on a FACSCanto, and analyzed using FlowJo (Treestar Inc., Ashland OR).

Serum cytokine analysis

Freshly isolated plasma from whole blood were aliquoted and stored at –80c for cytokine analysis of IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, TNF α , TNF β , and IFN γ using Milliplex MAP Kit (Millipore, Billerica, MA) on a Luminex 200 system (Luminex Corporation, Austin, TX) using Masterplex QT software (Hitachi/MiraiBio, South San Francisco, CA) and IP-10 by ELISA (R&D Systems).

Interferon-stimulated gene expression

Cryopreserved PBMC from pre- and on-treatment samples from 6 patients from each treatment assignment were thawed and cultured overnight in RPMI 10%ABS media with or without stimulation using rhIFN α (0.1ng/ml) or PHA (2 μg/mL). Using Taqman Gene expression kits and Cells-to-CT kit (Applied Bioscience), mRNA levels for 18S RNA, GAPDH, IRF7, Mx1, PP2A, ISG15, USP18 and STAT1 were measured on ABI 7000 rtPCR system (Applied Bioscience) and analyzed by $\Delta\Delta$ Ct methodology.

Statistical analysis

The median values for clinical and immunologic parameters at individual time points were compared using the nonparametric Kruskal-Wallis ANOVA, Wilcoxon Rank Sum or Mann-Whitney U test. MANOVA for repeat measures was utilized to determine the significance of the difference of pre-and on-treatment immunological results across treatment assignment.

Statistical analysis was performed using JMP 9 (SAS Institute Inc, Cary NC). A p-value < 0.05 was considered significant.

RESULTS

Baseline characteristics and clinical outcomes

Thirty-two patients consented to participate in the immunological substudy. The placebo and two treatment arms were similar in terms of age and gender, but there was a modest over-representation of black patients in the silymarin 700mg arm (Table 1). Similar to overall trial results, substudy patients demonstrated no significant on-treatment change in ALT or HCV RNA regardless of treatment assignment.

Oral administration of silymarin has no significant impact on HCV-specific CD4⁺T-cell proliferation in chronic hepatitis C

As shown in Figure 1A–C, neither silymarin treatment arm nor the placebo group demonstrated any significant change in CD4⁺ T-cell proliferation in response to HCV Core, NS3/4 or NS5 proteins from baseline to on-treatment (summed HCV-specific SI 3.7 to 3.2 in placebo arms, 2.6 to 3.0 in 420mg, and 3.8 to 4.0 in the 700mg arm, $p=0.54$). *C. albicans*-specific T-cell proliferation responses were at baseline approximately 1-log greater than HCV-specific responses (median 70 SI units, Figure 1D) but similarly were unchanged between baseline and week 20. By contrast, proliferation responses induced by the T-cell mitogen PHA decreased significantly in silymarin-treated patients (mean 205 to 77 SI units, $p=0.014$ combining 420mg and 700mg arms, Figure 1F) but not in placebo-treated subjects (mean 122 to 144 SI units, $p=0.51$; $p=0.035$ for difference of differences).

HCV-specific T-cell cytokine responses were unchanged by silymarin therapy

To examine the impact of oral silymarin administration on T-cell cytokine production, we performed IFN γ and IL-10 Elispot with freshly isolated PBMC. As shown in Figure 2 and Supplemental Figure 1, there were no significant changes in the frequency of HCV-specific CD4⁺T-cells producing IFN γ or IL-10 between baseline and week 20 of treatment. Interestingly, the frequency of *C. albicans*-specific IFN γ -producing T-cells decreased in both silymarin treatment arms (Figure 2F) without any consistent effect observed on *C. albicans*-specific IL-10 T-cell frequency. Combining the silymarin treatment arms, *Candida*-specific T-cell IFN γ decreased from a mean 256 to 114 SFU/10⁶ PBMC ($p=0.0066$) compared to no change (116 to 107 SFU/10⁶ PBMC, $p=0.85$) in the placebo arm with a strong trend towards a difference in these differences by MANOVA ($p=0.076$). Anecdotally, the single individual in the silymarin 700mg cohort with detectable HCV-specific cytokine and proliferation responses at baseline manifested parallel reductions in HCV- and *Candida*-specific T-cell proliferation and IFN γ production with increased IL-10 responses. While overall HCV-specific T-cell proliferation and cytokine production was not altered by silymarin therapy, we observed evidence that silymarin suppresses mitogen- and recall-antigen-induced proliferation and cytokine responses, respectively, *in vivo*.

Silymarin did not alter regulatory T-cell frequency or global cytokine levels

We then asked if other non-specific signs of reduced inflammatory markers were present in silymarin-exposed subjects. As shown in Table 2, we did not observe any significant reduction in serum pro-inflammatory (IL-6, IL-12p40, IL-17, TNF α , IFN γ) or anti-inflammatory (IL-8, IL-10). Serum IP-10 chemokine levels, a marker of interferon-responsiveness (when low)(28), were similarly unchanged by silymarin 700mg thrice daily. No change in the frequency of CD4⁺foxp3⁺ or CD4⁺CD25^{hi} regulatory T-cells nor activated

CD4⁺CD69⁺ or CD8⁺CD69⁺ activated T-cells were identified between baseline and week 20 in either treatment or placebo group (Figure 3C–F).

Subtle alterations of lymphocyte interferon-stimulated gene expression were observed related to silymarin therapy

To determine if silymarin might alter interferon-associated or interferon-stimulated gene expression in a manner that might suggest a restoration of interferon-sensitivity, we measured baseline and week 20 lymphocyte expression of STAT1, Mx1, ISG15, IRF7, USP18 and PP2A in lymphocytes from six subjects in each group. USP18 and PP2A did not amplify in the majority of samples from all three groups of patients, and therefore these data were excluded. As shown in Table 3, there was no change in expression of STAT1, Mx1, ISG15 and IRF7 in *ex vivo* unstimulated lymphocytes in any arm. There was a trend for a greater increase in interferon-induced ISG15 mRNA expression in the 700mg group (median 4.7 fold versus 2.1 fold, $p=0.12$; 4/6 patients in the 700mg group exhibited a 3-fold increase in interferon-induced ISG15 compared to 0/6 in the placebo arm $p=0.03$, Supplemental Figure 2A). PHA-stimulation led to greater than 5-fold increases in STAT1 and Mx1 in 3/6 (50%) of placebo patients but 0/10 (0%) silymarin-exposed patients ($p=0.035$, Supplemental Figure 2B). Thus, while the highest dose silymarin group exhibited improvement in IFN α -sensitivity, silymarin suppressed PHA-induced lymphocyte activation.

Dose responsiveness of silymarin-induced T-cell proliferation

To address whether or not silymarin at concentrations obtainable by oral administration could potentially suppress *in vivo* lymphocyte responses, we tested PHA-induced lymphocyte proliferation over a broad concentration range. As shown in Figure 4A, no significant suppression of T-cell proliferation developed at silymarin concentrations 1 $\mu\text{g}/\text{dl}$. The IC₅₀ for silymarin appeared variable among subjects within the range of 10–50 $\mu\text{g}/\text{dl}$ (Figure 4B–D).

DISCUSSION

Silymarin, a mixture of 6 major flavonolignans and multiple flavonoids (29), remains the most widely used herbal supplement utilized for chronic liver diseases, including chronic hepatitis C. In addition to pleiomorphic effects on hepatocytes (29), silymarin has been demonstrated to have anti-inflammatory immunomodulatory effects both *in vitro* (20, 21, 30) and in multiple rodent hepatitis models (14, 16). In both normal humans and mice, silymarin at concentrations 50–100 μM *in vitro* has been shown to suppress mitogen and anti-CD3-mediated T-cell proliferation, IFN γ , IL-2 and TNF α production, and NF κ B nuclear translocation (21). Similarly in the concanavalin A T-cell mediated hepatitis model, intraperitoneal administration of silibinin suppressed T-cell pro-inflammatory cytokine production (IFN γ , TNF α , IL-2), increased T-cell production of anti-inflammatory IL-10, and reduced intrahepatic expression of iNOS (16). More recently a direct antiviral effect of silymarin at concentrations of 1–20 μM has been appreciated *in vitro* (19, 31), an effect clinically observed after administration of intravenous silibinin to patients with chronic hepatitis C infection (17, 18). While short term courses of oral silymarin has never consistently shown salutary effects in alcoholic or viral liver disease (13), long-term silymarin use in the HALT-C trial was associated with slower fibrosis progression (32) suggesting that oral silymarin might indeed have anti-inflammatory effects *in vivo* in humans.

The present study reflects the first systematic, prospective assessment of the immunological effects of a well-characterized orally-administered silymarin preparation on human T-cell

responses in chronic hepatitis C *in vivo*. The parent clinical trial identified no significant effect of silymarin on alanine aminotransferase or HCV RNA titers. Overall, we did not identify any significant impact of silymarin on HCV-specific T-cell proliferation, IFN γ production, or IL-10 secretion. Similarly, there was no global alteration of serum cytokines, liver-related chemokines (IP-10), or circulating regulatory or activated T-cell frequencies. The lack of suppression of HCV-specific responses in our study is discrepant with the *in vitro* findings of Morishima et al. (20) who clearly demonstrated that a distinct preparation of silymarin, MK001, at 20 μ g/ml suppressed HCV-specific CD4⁺ and CD8⁺ T-cell proliferative responses from lymphocytes isolated from HCV-infected patients *in vitro*. In that study, similar to the present study, the suppressive effect of silymarin was more convincingly demonstrated with PHA, anti-CD3, *C. albicans* or tetanus toxoid stimulation, due to low frequency and weak HCV-specific responses typically found in chronic hepatitis C patients (9). Indeed, in the single patient in the 700mg silymarin group with detectable HCV-specific responses, suppression of virus-specific proliferation and IFN γ production with increased IL-10 was observed. Combined with the rarity of detectable HCV-specific immune responses in all cohorts, that the plasma concentrations of flavonolignans such as silybin A and silybin B achieved from oral administration were approximately 1–2 log lower than concentrations utilized *in vitro* (33) likely contributed to difficulties in detecting changes in virus-specific T-cell responses. Our *in vitro* PHA proliferation results suggest an IC₅₀ of silymarin in the 10–50 μ g/ml, a concentration not currently attainable with oral silymarin. We cannot discount the possibility that a type II error explains our negative findings, as the sample size of 10–11 per group was below our initial targets of 20 patients per group. Our ability to sample patients only at one timepoint during the study could also have impaired our ability to detect dynamic changes in T-cells responses particularly early after medication initiation. Furthermore, the initial starting population of prior interferon non-responders could also potentially bias against detection of changes in T-cell responses.

Despite these potential limitations, consistent with *in vitro* findings of Morishima et al., we did observe significant suppression *in vivo* of Candida-specific T-cell IFN γ production as well as PHA-induced T-cell proliferation. Notably, suppression of mitogenic or anti-CD3-mediated T-cell activation and proliferation has been consistently demonstrated with various preparations of silymarin *in vitro* (21, 34, 35). One may speculate that this non-specific suppression of T-cell pro-inflammatory effects might explain the association of silymarin exposure and reduced hepatic fibrosis progression observed in the large, long-term HALT-C cohort study (32).

The possibility that silymarin might restore interferon-responsiveness via its oxidant properties was the initial rationale for the human clinical trials that ultimately demonstrated a direct antiviral effect of intravenous silibinin (17). In our laboratory's previous work examining the role of T-cells in the outcome of IFN α /ribavirin therapy for chronic HCV infection, early virological response was paradoxically associated with baseline suppressed PHA-induced proliferation responses (9). Similarly, both suppressed serum levels of the ISG IP-10 (28) and suppressed pre-treatment intrahepatic ISG mRNA (36) have been associated with improved virological responses suggesting that basal ISG hyperactivation is associated with reduced interferon sensitivity. Similar to previous *in vitro* studies (20, 34, 35), we observed significant reductions of mitogen-induced T-cell proliferation *in vivo* and *in vitro* that might be a signature of the restoration of interferon-sensitivity. Focusing on IRF7, ISG15, Mx1 and STAT1 whose intrahepatic expression strongly correlates with interferon-responsiveness (37), we did not observe any significant alteration in basal lymphocyte ISG mRNA expression from silymarin. However, a strong trend toward increased interferon-induced ISG15 expression was observed in patients in the high-dose silymarin group with the caveat that the extent to which lymphocyte ISG induction correlates with intrahepatic ISG induction is unknown. Furthermore, these data suffer from a significant type II error

due to limitations on paired samples available to perform these analyses *post hoc*. Our data and a small clinical trial by Rutter et al. (38) that raised possibility that intravenous silibinin improved interferon sensitivity *in vivo*, provide rationale for a formal prospective evaluation of silymarin's impact on intrahepatic ISG expression and a potential interferon-sensitizing role.

In conclusion, oral silymarin administered at higher than usual dosages had no measurable impact on hepatitis C-specific T-cell proliferation, IFN γ or IL-10 responses *in vivo*. Silymarin also had no effect on global serum cytokines, IP-10, lymphocyte ISG expression or regulatory T-cell frequency. However, silymarin did significantly reduce the pro-inflammatory cytokine response to *Candida albicans* and PHA lectin-induced T-cell proliferation, similar to previous *in vitro* findings, confirming that silymarin does exert an immunomodulatory effect *in vivo*. The impact of this anti-inflammatory effect on long-term liver health in chronic hepatitis C should be examined in future clinical investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported with cooperative agreements to the SyNCH Study Group from the National Institutes of Health (NIH), National Center for Complementary and Alternative Medicine: AT003573, AT003571, AT003566, AT003560, and AT003574 with co-funding from the NIDDK, and with support from the NIH CTSA Division of Research Resources (University of Pennsylvania UL1 RR024134). In addition, Rottapharm/Madaus (Monza, Italy and Cologne, Germany) provided both the silymarin and placebo for the trial. Other support was received from a Research Career Development Award from the Veterans Health Administration (DEK), academic development funds from the University of Pennsylvania (DEK), and Center for Molecular Studies in Digestive and Liver Disease (NIH/NIDDK P30-DK050306, DEK, KRR). The authors would like to thank Amina Wirjosemito for sample acquisition. The authors would also like to thank the patients who contributed samples. The content of this article does not reflect the views of the VA or of the US Government.

Abbreviations

HCV	hepatitis C virus
IFNγ	interferon-gamma
IL	interleukin
IP-10	Interferon gamma-induced protein 10
IRF7	interferon-response factor 7
ISG	interferon-stimulated gene
Mx1	Myxovirus resistance 1
NFκB	nuclear factor kappa B
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
SFU	spot-forming unit
SI	stimulation index
SOD	superoxide dismutase
STAT1	signal transducer and activator of transcription 1

TNFα	tumor necrosis factor-alpha
TNFβ	tumor necrosis factor-beta

References

1. Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, et al. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology*. 2001 Jan; 33(1):267–76. [PubMed: 11124845]
2. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol*. 2002 Sep 15; 169(6):3447–58. [PubMed: 12218168]
3. Ulsenheimer A, Gerlach JT, Gruener NH, Jung MC, Schirren CA, Schraut W, et al. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology*. 2003 May; 37(5):1189–98. [PubMed: 12717401]
4. Kaplan DE, Sugimoto K, Newton K, Valiga ME, Ikeda F, Aytaman A, et al. Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology*. 2007 Feb; 132(2):654–66. [PubMed: 17258733]
5. Barnes E, Harcourt G, Brown D, Lucas M, Phillips R, Dusheiko G, et al. The dynamics of T-lymphocyte responses during combination therapy for chronic hepatitis C virus infection. *Hepatology*. 2002 Sep; 36(3):743–54. [PubMed: 12198669]
6. Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology*. 2000 Feb; 118(2):346–55. [PubMed: 10648463]
7. Rahman F, Heller T, Sobao Y, Mizukoshi E, Nascimbeni M, Alter H, et al. Effects of antiviral therapy on the cellular immune response in acute hepatitis C. *Hepatology*. 2004 Jul; 40(1):87–97. [PubMed: 15239090]
8. Wiegand J, Jackel E, Cornberg M, Hinrichsen H, Dietrich M, Kroeger J, et al. Long-term follow-up after successful interferon therapy of acute hepatitis C. *Hepatology*. 2004 Jul; 40(1):98–107. [PubMed: 15239091]
9. Kaplan DE, Sugimoto K, Ikeda F, Stadanlick J, Valiga M, Shetty K, et al. T-cell response relative to genotype and ethnicity during antiviral therapy for chronic hepatitis C. *Hepatology*. 2005 Jun; 41(6):1365–75. [PubMed: 15915458]
10. Claassen MA, de Knecht RJ, Tilanus HW, Janssen HL, Boonstra A. Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. *Journal of hepatology*. 2010 Mar; 52(3):315–21. [PubMed: 20129690]
11. Kaplan DE, Ikeda F, Li Y, Nakamoto N, Ganesan S, Valiga ME, et al. Peripheral virus-specific T-cell interleukin-10 responses develop early in acute hepatitis C infection and become dominant in chronic hepatitis. *J Hepatol*. 2008 Jun; 48(6):903–13. [PubMed: 18384906]
12. Accapezzato D, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest*. 2004 Apr; 113(7):963–72. [PubMed: 15057302]
13. Rambaldi A, Jacobs BP, Iaquinto G, Glud C. Milk thistle for alcoholic and/or hepatitis B or C liver diseases--a systematic cochrane hepato-biliary group review with meta-analyses of randomized clinical trials. *Am J Gastroenterol*. 2005 Nov; 100(11):2583–91. [PubMed: 16279916]
14. Johnson VJ, He Q, Osuchowski MF, Sharma RP. Physiological responses of a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: III. Silymarin inhibits T-lymphocyte function at low doses but stimulates inflammatory processes at high doses. *Planta Med*. 2003 Jan; 69(1):44–9. [PubMed: 12567278]
15. Johnson VJ, Osuchowski MF, He Q, Sharma RP. Physiological responses to a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: II. alterations in thymic differentiation correlate with changes in c-myc gene expression. *Planta Med*. 2002 Nov; 68(11):961–5. [PubMed: 12451483]

16. Schumann J, Prockl J, Kierner AK, Vollmar AM, Bang R, Tiegs G. Silibinin protects mice from T cell-dependent liver injury. *J Hepatol.* 2003 Sep; 39(3):333–40. [PubMed: 12927918]
17. Ferenci P, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, et al. Silibinin Is a Potent Antiviral Agent in Patients With Chronic Hepatitis C Not Responding to Pegylated Interferon/ Ribavirin Therapy. *Gastroenterology.* 2008 Aug 3.
18. Biermer M, Berg T. Rapid suppression of hepatitis C viremia induced by intravenous silibinin plus ribavirin. *Gastroenterology.* 2009 Jul; 137(1):390–1. [PubMed: 19486953]
19. Wagoner J, Negash A, Kane OJ, Martinez LE, Nahmias Y, Bourne N, et al. Multiple effects of silymarin on the hepatitis C virus lifecycle. *Hepatology.* 2010 Jun; 51(6):1912–21. [PubMed: 20512985]
20. Morishima C, Shuhart MC, Wang CC, Paschal DM, Apodaca MC, Liu Y, et al. Silymarin inhibits in vitro T-cell proliferation and cytokine production in hepatitis C virus infection. *Gastroenterology.* 2010 Feb; 138(2):671–81. 81, e1–2. [PubMed: 19782083]
21. Polyak SJ, Morishima C, Shuhart MC, Wang CC, Liu Y, Lee DY. Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized Silymarin. *Gastroenterology.* 2007 May; 132(5):1925–36. [PubMed: 17484885]
22. Fried MW, Navarro VJ, Afdhal N, Belle SH, Wahed AS, Hawke RL, et al. Effect of silymarin (milk thistle) on liver disease in patients with chronic hepatitis C unsuccessfully treated with interferon therapy: a randomized controlled trial. *JAMA: the journal of the American Medical Association.* 2012 Jul 18; 308(3):274–82. [PubMed: 22797645]
23. Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology.* 2003 Dec; 38(6):1437–48. [PubMed: 14647055]
24. Sugimoto K, Stadanlick J, Ikeda F, Brensinger C, Furth EE, Alter HJ, et al. Influence of ethnicity in the outcome of hepatitis C virus infection and cellular immune response. *Hepatology.* 2003 Mar; 37(3):590–9. [PubMed: 12601357]
25. Diepolder HM, Zachoval R, Hoffmann RM, Jung MC, Gerlach T, Pape GR. The role of hepatitis C virus specific CD4+ T lymphocytes in acute and chronic hepatitis C. *J Mol Med.* 1996 Oct; 74(10):583–8. [PubMed: 8912179]
26. Missale G, Cariani E, Lamonaca V, Ravaggi A, Rossini A, Bertoni R, et al. Effects of interferon treatment on the antiviral T-cell response in hepatitis C virus genotype 1b- and genotype 2c-infected patients. *Hepatology.* 1997 Sep; 26(3):792–7. [PubMed: 9303515]
27. Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, et al. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology.* 1999 Oct; 117(4):933–41. [PubMed: 10500077]
28. Romero AI, Lagging M, Westin J, Dhillon AP, Dustin LB, Pawlotsky JM, et al. Interferon (IFN)-gamma -Inducible Protein-10: Association with Histological Results, Viral Kinetics, and Outcome during Treatment with Pegylated IFN- alpha 2a and Ribavirin for Chronic Hepatitis C Virus Infection. *J Infect Dis.* 2006 Oct 1; 194(7):895–903. [PubMed: 16960776]
29. Flora K, Hahn M, Rosen H, Benner K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol.* 1998 Feb; 93(2):139–43. [PubMed: 9468229]
30. Gharagozloo M, Amirghofran Z. Effects of silymarin on the spontaneous proliferation and cell cycle of human peripheral blood leukemia T cells. *J Cancer Res Clin Oncol.* 2007 Aug; 133(8): 525–32. [PubMed: 17436018]
31. Polyak SJ, Morishima C, Hawke R. Antiviral effects of silymarin against hepatitis C: the jury is still out. *Hepatology.* 2008 Jul; 48(1):345–6. [PubMed: 18509876]
32. Freedman ND, Curto TM, Morishima C, Seeff LB, Goodman ZD, Wright EC, et al. Silymarin use and liver disease progression in the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis trial. *Alimentary pharmacology & therapeutics.* 2011 Jan; 33(1):127–37. [PubMed: 21083592]
33. Hawke RL, Schrieber SJ, Soule TA, Wen Z, Smith PC, Reddy KR, et al. Silymarin ascending multiple oral dosing phase I study in noncirrhotic patients with chronic hepatitis C. *J Clin Pharmacol.* 2010 Apr; 50(4):434–49. [PubMed: 19841158]

34. Deak G, Muzes G, Lang I, Nekam K, Gonzalez-Cabello R, Gergely P, et al. Effects of two bioflavonoids on certain cellular immune reactions in vitro. *Acta Physiol Hung.* 1990; 76(2):113–21. [PubMed: 2082690]
35. Meroni PL, Barcellini W, Borghi MO, Vismara A, Ferraro G, Ciani D, et al. Silybin inhibition of human T-lymphocyte activation. *Int J Tissue React.* 1988; 10(3):177–81. [PubMed: 3265704]
36. Chen L, Borozaan I, Sun J, Guindi M, Fischer S, Feld J, et al. Cell-type specific gene expression signature in liver underlies response to interferon therapy in chronic hepatitis C infection. *Gastroenterology.* 2009 Nov 6.
37. Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response. *Hepatology.* 2007 Nov; 46(5):1548–63. [PubMed: 17929300]
38. Rutter K, Scherzer TM, Beinhardt S, Kerschner H, Stattemayer AF, Hofer H, et al. Intravenous silybinin as ‘rescue treatment’ for on-treatment non-responders to pegylated interferon/ribavirin combination therapy. *Antiviral therapy.* 2011; 16(8):1327–33. [PubMed: 22155914]

Figure 1. CD4⁺T-cell proliferation responses to HCV- and non-HCV-specific stimulation at baseline and during silymarin therapy

Stimulation index for PBMC stimulated with A. c22 (Core), B. c200 (NS3/4), C. NS5 (aa2504-2995), D. *C. albicans*, E. sum of HCV, and F. phytohemagglutinin. Pie charts show fraction of patients with 50% increase to SI greater than 3 (black), 50% decrease from SI greater than 3 to below 3 (white), or no significant change (grey). Active treatment patients demonstrated a statistically significant decrease in PHA-induced T-cell proliferation (mean -128.1 SI units, $p=0.0135$ when 420mg and 700mg groups combined) and the decrease was statistically different from the difference in placebo patients (+22.5 SI units, $p=0.035$).

Figure 2. Frequency of HCV- and non-HCV-specific IFN γ -producing T-cells at baseline and during silymarin therapy

IFN γ spot-forming units per 10⁶ PBMC are shown for PBMC stimulated with A. c22 (Core), B. c200 (NS3/4), C. NS5 (aa2504-2995), D. summed HCV-specific responses, E. Tetanus toxoid, and F. C. albicans. Pie charts show fraction of patients with ≥ 50 SFU/10⁶ increase to SFU/10⁶ greater than 50 (black), ≥ 50 SFU/10⁶ decrease from SFU/10⁶ greater than 50 to below 50 (white), or no significant change (grey). Active treatment (420mg plus 700mg) patients demonstrated a statistically significant decrease in frequency of C. albicans-induced IFN γ production (mean -142 SFU/10⁶, $p=0.007$). There was a trend for the difference in C. albicans response in active treatment patients to be statistically different from the difference in placebo patients (-9 SFU/10⁶, $p=0.076$). G. Composite of changes of ALT, HCV RNA, HCV- and control-specific SI, IFN γ and IL-10 responses for patient in 700mg TID group with detectable HCV-specific T-cell responses at baseline.

Figure 3. Frequency of activated and regulatory T-cells at baseline and during silymarin therapy

A. Gating strategy for identifying CD4⁺CD25^{hi} regulatory T-cells. B. Examples of pre- and post-treatment determination of CD4⁺foxp3⁺ frequency. C. Pre- and on-treatment CD4⁺CD25^{hi} T-cells. D. Pre- and on-treatment CD4⁺foxp3⁺ T-cells. E. Pre- and on-treatment %CD69⁺/CD4⁺ T-cells. F. Pre- and on-treatment %CD69⁺/CD8⁺ T-cells. None of specified populations significantly changed frequency in any group from baseline to on-treatment.

Figure 4. In vitro sensitivity of T-cells to proliferation suppression by silymarin

A. Percentage of maximal proliferation after PHA-stimulation of PBMC from 3 baseline samples in the presence of silymarin 0.1–100 µg/ml. B–D. Percentage of maximal proliferation after PHA stimulation in PBMC from 3 individual subjects in the presence of silymarin 1–100 µg/ml.

* $p < 0.05$ ** $p < 0.001$ compared to maximal proliferation condition

Table 1

Baseline patient characteristics and clinical outcome

	Treatment Assignment			p
	Placebo TID	420mg TID	700mg TID	
At Randomization				
N	10	11	11	
Median Age (IQR)	57 (52–59)	56 (50–60)	55 (48–57)	0.42
Gender (M/F)	9/1	9/2	6/5	0.14
Race (W/B)	10/0	10/1	6/5	0.01
Median ALT (IQR)	128 (106–187)	109 (90–181)	110 (81–165)	0.58
Median log HCV RNA (IQR)	6.1 (5.8–6.6)	6.4 (5.9–6.7)	6.3 (5.9–6.5)	0.69
During Treatment				
Median ALT at week 20 (IQR)	125 (99–189)	104 (73–161)	118 (100–157)	0.35*
Median log HCV RNA at week 12 (IQR)	6.3 (5.7–6.4)	6.4 (5.9–6.7)	6.2 (5.7–6.4)	0.46*
Median log HCV RNA at week 24 (IQR)	6.3 (5.8–6.6)	6.4 (6.1–6.5)	6.2 (6.0–6.4)	0.83*

* comparison across groups for difference from values at randomization

Table 2

Serum cytokine levels at baseline and during silymarin administration

	Placebo TID			420mg TID			700mg TID		
	Pre	Post	N=6	Pre	Post	N=6	Pre	Post	p *
IFN- γ (pg/ml)	7.3 $\pm$ 12.5	23.7 $\pm$ 37.5		< LLD	< LLD		2.8 $\pm$ 2.7	3.0 $\pm$ 3.5	0.11
IL-4 (pg/ml)	< LLD	4.5 $\pm$ 6.9		< LLD	< LLD		< LLD	< LLD	0.36
IL-6 (pg/ml)	4.4 $\pm$ 4.0	8.9 $\pm$ 10.6		5.5 $\pm$ 7.3	< LLD		4.4 $\pm$ 3.3	4.2 $\pm$ 3.5	0.17
IL-8 (pg/ml)	26.5 $\pm$ 32.5	40.6 $\pm$ 48.7		8.3 $\pm$ 5.0	8.8 $\pm$ 6.1		30.1 $\pm$ 58.7	5.0 $\pm$ 6.6	0.20
IL-10 (pg/ml)	5.7 $\pm$ 5.8	10.7 $\pm$ 17.6		7.2 $\pm$ 11.1	3.6 $\pm$ 14.2		4.1 $\pm$ 2.9	4.8 $\pm$ 4.7	0.69
IL-12p40 (pg/ml)	27.5 $\pm$ 44.8	102.2 $\pm$ 135.6		39.7 $\pm$ 86.4	44.8 $\pm$ 97.1		10.9 $\pm$ 11.4	5.4 $\pm$ 6.3	0.04
IL-17 (pg/ml)	51.5 $\pm$ 111.6	54.1 $\pm$ 104.9		< LLD	< LLD		37.7 $\pm$ 79.8	< LLD	0.42
TNF- α (pg/ml)	10.4 $\pm$ 3.2	12.9 $\pm$ 5.9		6.3 $\pm$ 2.4	8.0 $\pm$ 4.2		4.8 $\pm$ 1.1	7.2 $\pm$ 2.1	0.59
TNF- β (pg/ml)	9.5 $\pm$ 18.0	21.5 $\pm$ 446.3		5.0 $\pm$ 7.1	4.1 $\pm$ 10.0		< LLD	< LLD	0.34
IP-10 (pg/ml)	500.7 $\pm$ 173.5	456.8 $\pm$ 205.0		ND	ND		500.7 $\pm$ 187.9	533.0 $\pm$ 220.9	0.06

* p-value for differences in mean change across groups

Table 3

Fold-change of interferon-stimulated gene expression on-treatment relative to pre-treatment

Condition/Gene		Group		
		Placebo	420mg	700mg
Media-stimulated				
IRF7	N	6	4	6
	Median (Min-Max)	0.8 (0.1–83.9)	1.1 (0.1–3.7)	1.8 (0.4–17.8)
Mx1	N	6	4	6
	Median (Min-Max)	1.5 (0.1–109.1)	0.5 (0.0–3.4)	1.6 (0.6–10.2)
ISG15	N	1	2	5
	Median (Min-Max)	25.1 (25.1–25.1)	143.2 (48.3–238.0)	13.1 (3.6–156.0)
STAT1	N	6	4	6
	Median (Min-Max)	0.6 (0.1–94.7)	0.7 (0.1–4.6)	1.0 (0.2–7.5)
IFN α -stimulated				
IRF7	N	6	4	6
	Median (Min-Max)	0.9 (0.1–34.3)	0.5 (0.3–9.9)	1.7 (1.2–11.0)
Mx1	N	6	4	6
	Median (Min-Max)	1.6 (0.1–168.9)	0.5 (0.3–6.9)	2.0 (0.9–6.0)
ISG15	N	5	4	6
	Median (Min-Max)	2.1 (0.1–2.6)	0.3 (0.0–8.8)	4.7 (0.5–13.5) [†]
STAT1	N	6	4	6
	Median (Min-Max)	1.4 (0.2–119.4)	0.6 (0.3–5.4)	2.0 (0.9–6.5)
PHA-stimulated				
IRF7	N	6	4	6
	Median (Min-Max)	4.2 (0.4–112.2)	0.7 (0.2–2.3)	1.3 (0.5–2.6)
Mx1	N	6	4	6
	Median (Min-Max)	4.8 (0.5–117.4)	1.0 (0.1–2.1)	1.6 (0.4–2.7)
ISG15	N	2	3	5
	Median (Min-Max)	19.1 (9.1–29.1)	0.4 (0.1–3.1)	1.0 (0.5–1.7)
STAT1	N	6	4	6
	Median (Min-Max)	3.7 (0.3–147.5)	1.3 (0.5–4.5)	1.3 (0.2–2.1)

[†] p=0.12 for difference between placebo and 700mg group