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CORE-SPECIFIC ADAPTIVE REGULATORY T CELLS IN DIFFERENT OUTCOMES OF HEPATITIS C

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Abstract

Background: CD4⁺ regulatory T cells (Tregs) probably contribute to the impaired virus-specific T cell responses in chronic hepatitis C virus (HCV) infection. However, their antigen-specificity has remained elusive.

Methods: We analyzed peripheral blood CD4⁺ Tregs in patients with chronic and subjects with self-limited HCV infection and characterized individual Treg clones obtained from both patient groups at the phenotypic and functional level.

Results: Foxp3⁺CD25⁺CD4⁺ Tregs were detected more frequently in patients with chronic than self-limited HCV infection, which responded to HCV core stimulation and inhibited proliferation of reporter cells. Cloning under limiting dilution conditions resulted in fourteen and six hypoproliferative Foxp3⁺CD25⁺CD127⁻CD4⁺ T cell clones from patients with chronic and self-limited HCV infection, respectively. All clones expressed Treg markers and produced IL-10 upon mitogen stimulation. However, exclusively Treg clones from chronic hepatitis C produced IL-10 in response to HCV core and inhibited proliferation of reporter T cells. These core-specific Treg clones recognized epitopes in two regions of HCV core (aa1-44 and aa79-113). Co-culture inhibition assays demonstrated Tregs to inhibit reporter T cells via secretion of IL-10 and IL-35 rather than cell-contact-dependent mechanisms. Finally, the HCV-specific Treg clones lost their functional capacity along with Foxp3 expression, if kept in culture without HCV core exposure.

Conclusions: We identified functionally active HCV core-specific Tregs in patients with chronic hepatitis C, which share their epitopes with conventional T cells and require the continued presence of antigen to maintain their functional differentiation. Thus, HCV core-specific Tregs may contribute to the immunoregulatory balance in chronic hepatitis C.

Introduction

Hepatitis C virus (HCV) efficiently establishes chronic persistency in the majority of individuals who encounter the infection, and thus, it is one of the leading causes of chronic liver disease in the world [1]. While strong and multi-specific lymphocyte responses including CD4⁺ and CD8⁺ T cells, NK cells, and dendritic cells contribute to viral elimination, chronic hepatitis C is associated with inefficient and delayed T cell responses [2]. Although the mechanisms of impaired antiviral immunity in patients with chronic hepatitis C are only incompletely understood it has been proposed that CD4⁺ regulatory T cells (Tregs) might contribute to T cell dysfunctions during ongoing HCV infection.

Several studies demonstrated an overall increased frequency of CD25^{high}CD4⁺ Tregs in chronic hepatitis C which produce IL-10 [3] and transforming growth factor (TGF)-beta [4], express Foxp3 (forkhead transcription factor box3) [5], can suppress interferon (IFN)-gamma production [6] and proliferation of virus-specific CD8⁺ T cells [7, 8]. Thus far, analyzed Tregs carry phenotypic and functional characteristics of so-called natural CD4⁺ Tregs [9, 10], because they are Foxp3⁺ [5], express high levels of CD25 [3-5] and predominantly inhibit reporter cells via contact-dependent mechanisms [3, 4, 6, 8]. Since thus far expansion of Tregs in chronic hepatitis C is largely considered to reflect HCV non-specific Tregs, the role of HCV-specific adaptive Tregs has still remained a matter of debate.

Antigen-induced adaptive Tregs are generated in the periphery upon antigen exposure inhibiting effector T cells via release of the immunosuppressive cytokines IL-10 and TGF-beta [11-13]. In general, adaptive Tregs exhibit heterogeneous phenotypes with variable expression of CD25 and Foxp3 and can be further subdivided according to their individual phenotypes and cytokine profiles [13, 14].

The HCV core protein is highly conserved among different HCV isolates [15] and is frequently recognized by the host's cellular antiviral immune response [16]. Thus, HCV core is an exquisite model antigen to search for HCV-induced adaptive Tregs in chronic hepatitis C. Studying subjects with different outcomes of HCV infection offers the opportunity to understand the relationship of Tregs to continued antigen exposure. With this approach we studied HCV core-specific Tregs in the peripheral blood of patients with chronic hepatitis C and compared them to corresponding cells in anti-HCV positive subjects who had resolved their infection spontaneously.

Methods

Study groups

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque gradient centrifugation (PAA Laboratories GmbH, Linz, Austria) from heparinized blood in 31 treatment-naïve, non-cirrhotic patients with chronic hepatitis C (15 males and 16 females; age: 46 (30-68) years, median (range)), and 12 subjects with self-limited hepatitis C (all males; age: 43 (27-65) years). Patients with chronic hepatitis C had detectable HCV RNA genotype 1 by PCR for more than 6 months. Stages of fibrosis were obtained either by histology or Fibroscan and are reported as Metavir scores. Subjects with self-limited hepatitis C consisted of carefully selected individuals who had spontaneously recovered from a well-documented episode of acute hepatitis infection 27 months to 30 years prior to the study, which in retrospect had unequivocally been identified as hepatitis C. All control subjects were anti-HCV positive (AXSYM System, HCV version 3.0, Abbott, Wiesbaden, Germany) but had consistently normal aminotransferases and a negative HCV PCR on repeated assessment. Clinical data of both study groups are summarised in Table 1.

The study has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and has been approved by the local ethics committee. Informed consent had been obtained prior to inclusion into the study.

Reagents

Recombinant HCV core protein (genotype 1, amino acids (aa) 1-115; <4.0 pg/μg LPS) was purchased from Mikrogen GmbH (Martinsried, Germany). For epitope mapping a 25-mer HCV peptide panel (P1 to P12) covering region aa 1-133 of the HCV core protein with 10 aa overlap was synthesised as described previously [17, 18].

Human recombinant IL-2 (rIL-2) was obtained from R&D Systems (Wiesbaden, Germany), anti-CD28 from BD Biosciences (Heidelberg, Germany), OKT-3 (human anti-CD3) from Janssen-Cilag GmbH (Neuss, Germany), and cytomegalovirus (CMV) glycoprotein p65 from AUSTRAL Biologicals, San Ramon, California, USA.

RPMI 1640 medium, supplemented with 10% human AB serum, glutamine (200 mg/ml), gentamycin (50 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all PAA Laboratories GmbH) was used for cell culture.

Analysis of CD4⁺ Treg subsets prior to in vitro expansion

To identify the spectrum of CD4⁺ Tregs prior to in vitro expansion, subsets of CD4⁺ T cells carrying Treg markers were quantitatively determined by flow cytometry in freshly isolated PBMC of patients with chronic hepatitis C and subjects with self-limited HCV infection. Next, PBMC were studied after a single round of in vitro stimulation with HCV core to search for HCV-responsive cells. In brief, PBMC (5x10⁵ cells/well) were stimulated with HCV core protein (10 μg/ml) in culture medium for 10 days. PBMC were stained with anti-CD25 (PE-labeled), anti-CD4 (APC-labeled), anti-Foxp3, anti-GITR, anti-CTLA-4, anti-PD-1, anti-CD134 (all FITC-labeled).

In co-culture experiments, we analyzed the inhibitory capacity of freshly isolated CD25^{high}CD4⁺ and CD25^{int}CD4⁺ T cells on CD25⁻CD4⁺ reporter T cells. Briefly, CD25^{high}CD4⁺ and CD25^{int}CD4⁺ T cells were purified from unexpanded PBMC by stepwise immunomagnetic separation using the CD4⁺CD25⁺ Treg isolation kit and high affinity CD25 Microbeads (Miltenyi

Biotech, Gladbach, Germany). Resulting Tregs were routinely greater 95% pure and comprised less than 5% non-vital cells (7-aminoactinomycin D staining). The remaining CD25⁺CD4⁺ T cells were used as reporter cells in co-culture stimulation experiments. CD25^{high}CD4⁺ or CD25^{int}CD4⁺ Tregs were added to CD25⁺CD4⁺ reporter T cells at 1:1 ratio and stimulated with HCV core or anti-CD3/anti-CD28 presented via 10% autologous monocytes. Proliferation was assessed via [³H] thymidine uptake after 5 days.

Generation and characterization of HCV-specific Treg clones

Generation of Treg clones was achieved by adapting previously established protocols [19, 20]. In detail, freshly isolated PBMC (5×10^5 cells/ml) were stimulated with core protein (10 µg/ml) and cultured in medium with rIL-2 (100 U/ml). After 10 days, cells were re-stimulated with HCV core protein and supplemented with irradiated autologous feeder cells (5×10^5 PBMC/ml). After 20 days, cells were re-stimulated for 72 h with phytohemagglutinin (PHA, 1 µg/ml) together with irradiated feeder cells. Next, cells were seeded in Terasaki plates (Greiner bio-one, Frickenhausen, Germany) under conditions of limiting dilution (0.1 and 0.3 cells/ well). After 10 days, clones were picked from plates with less than 10% positive wells and re-stimulated at 10-14 day intervals in the presence of rIL-2.

Clones were classified as Tregs by analyzing proliferation and cytokine secretion (IL-4, IL-10, IFN-gamma, TGF-beta1) in response to either HCV core protein or anti-CD3/anti-CD28. Recombinant CMV p65 (10 µg/ml) served as a control antigen to check HCV-specificity. Functional and phenotypic studies were always performed in the resting phase 10 days after re-stimulation. In the presence of antigen T cell clones maintained their original functionality for culture periods up to 100 days.

Flow cytometric analysis of Treg clones

Treg clones (5×10^5 cells) were stained with APC-labeled anti-CD4 and PE-conjugated anti-CD25 (clone M-A251), anti-CD45R0 (clone UCHL1), anti-CD62L (clone Dreg-56), and anti-CD127 (clone SB/199), respectively. FITC-labeled antibodies were used for detection of Treg markers GITR (clone 110416), CTLA-4 (clone 48815), PD-1 (clone J116), CD134 (clone ACT35) and CD30 (clone Ki-1) (all BD Biosciences). Moreover, we analyzed expression of GARP (LRRC21, clone Plato-1; ALEXIS) and nuclear expression of Foxp3 using the Foxp3 staining kit (clone PCH101, IgG2a isotype control (clone eBR2a)) according to the manufacturer's instructions (NatuTec, Frankfurt, Germany). T cells were examined on a FACSCalibur flow cytometer and analyzed by Flow Jo software (Tree Star). CD25⁺ T cells were segregated into CD25-intermediate (mean fluorescence intensity (MFI): 10-300) and CD25-high cells (MFI: >300) with reference to KARPAS-299 cells, which carry the CD25^{high}CD4⁺ phenotype of natural Tregs (Supplementary Figure 1) [21].

Proliferative responses of HCV core-specific Treg clones

5×10^4 cells from each prospective HCV-specific Treg clone were stimulated with either HCV core protein (10 µg/ml) or anti-CD3/anti-CD28 (1 and 2.5 µg/ml) together with 1×10^5 irradiated autologous APC. After 4 days, cultures were pulsed with 1 µCi [³H] thymidine for 18 h. Incorporated radioactivity was measured by liquid scintillation spectrometry (TopCount@NXT, Perkin Elmer™ life sciences, Boston, USA). Stimulation indices (SI) were calculated as the ratio of [³H] thymidine uptake relative to the medium controls. SIs >5 were considered positive.

Cytokine profiles of HCV core-specific Treg clones

1×10^5 cells of each candidate Treg clone were stimulated separately either with HCV core protein or anti-CD3/anti-CD28. 72 h after stimulation supernatants were harvested to measure IL-4, IFN-gamma, IL-10, and TGF-beta1 by ELISA. TGF-beta1 was determined by the OptEIA human TGF-beta1-Set from BD Biosciences. IL-4, IL-10 and IFN-gamma were captured with primary antibodies 8D4-8, JES3-9D7, and NIB42 and bound cytokines detected with corresponding antibodies MP4-25D2, JES3-268, S.B3 (all BD Biosciences), respectively. Plates were measured in an ELISA-Reader (Orgentec, Mainz, Germany) at 450 nm. Cytokine values >50 pg/ml were considered positive and defined as high if >250 pg/ml. Clones were classified as TH1, TH2 and Tregs based on cytokine and proliferation profiles in response to anti-CD3/anti-CD28 stimulation (Supplementary Table 1).

HLA-restriction of HCV core-specific Treg clones

HLA-restriction was determined by blocking HCV core-specific IL-10 production with antibodies to L243 (HLA-DR), Tü22 (HLA-DQ; both American Type Culture Collection) or HI43 (HLA-DP; Biozol, Eching, Germany), respectively. Briefly, 1×10^5 cells of HCV-specific Treg clone were stimulated with HCV core protein (10 μ g/ml) in the presence or absence of HLA-DR, -DQ or -DP antibodies. After 72 h, supernatants were harvested to measure IL-10 by ELISA.

Identification of Treg epitopes on HCV core

Individual Treg clones (1×10^5) were stimulated with single peptides P1-P12 (1 μ g/ml) in the presence of autologous APC (1×10^5). After 72 h, supernatants were harvested to measure IL-10 by ELISA.

Analysis of in vitro suppressor function of HCV core-specific Treg clones

The suppressive function of individual Treg clone was studied using co-culture assays with stimulated autologous TH1 and TH2 reporter clones. Reporter cells (5×10^4 cells/well) were stimulated either with anti-CD3/anti-CD28 or recombinant HCV core protein in the presence of irradiated autologous APC (5×10^4 cells/well). Putative Treg clones were added at 1:4, 1:2 and 1:1 ratio. After 5 days, proliferation was measured in both TH1 and TH2 reporter clones via [3 H] thymidine incorporation.

To decipher the predominant mechanism of inhibition we compared the inhibitory capacity of anti-CD3/anti-CD28-stimulated Treg clones in direct co-culture with TH1 and TH2 reporter clones (1:1 ratio) versus transwell culture, which disrupts cell-cell contacts between Tregs and reporter cells (tissue culture inserts, 0.2 μ m Anopore® Membrane, Nunc). In addition, we performed blocking experiments using either a neutralising antibody to IL-10 (clone 23738; R&D Systems; 10 μ g/ml) or to the shared β -chain of IL-35 (EBI3 - ATI136, Axxora; 10 μ g/ml).

Statistics

All calculations were performed using Prism software package 4.0 (Graph Pad Software, San Diego California, USA). Differences between experiments were compared by Fisher's exact test, Kruskal Wallis test, Wilcoxon signed rank test, and Student's t test as appropriate. $P < 0.05$ was considered as statistically significant.

Results

HCV-reactivity of freshly isolated Treg subsets in peripheral blood

In line with previous reports, we observed significantly higher baseline numbers of $\text{Foxp3}^+\text{CD25}^+\text{CD4}^+$ T cells in freshly isolated PBMC from patients with chronic than from subjects with self-limited hepatitis C. Using KARPAS-299 cells which carry the $\text{Foxp3}^+\text{CD25}^{\text{high}}\text{CD4}^+$ phenotype of natural Tregs as a reference [21] we found that both $\text{Foxp3}^+\text{CD25}^{\text{high}}\text{CD4}^+$ and $\text{Foxp3}^+\text{CD25}^{\text{int}}\text{CD4}^+$ T cell subsets were increased in chronic hepatitis C (Table 2).

To study the functional contribution of both $\text{CD25}^+\text{CD4}^+$ T cell subsets, we purified $\text{CD25}^{\text{high}}\text{CD4}^+$ and $\text{CD25}^{\text{int}}\text{CD4}^+$ T cells from PBMC of patients with chronic hepatitis C (Figure 1A) and analyzed their suppressive activity. As shown in Figures 1B and C, addition of $\text{CD25}^{\text{high}}\text{CD4}^+$ and $\text{CD25}^{\text{int}}\text{CD4}^+$ T cell subsets significantly decreased proliferation of $\text{CD25}^+\text{CD4}^+$ reporter T cells both after stimulation with mitogen and HCV core. We also noted that $\text{Foxp3}^+\text{CD25}^{\text{high}}\text{CD4}^+$ and $\text{Foxp3}^+\text{CD25}^{\text{int}}\text{CD4}^+$ T cells from patients with chronic hepatitis C increased after in vitro stimulation with HCV core protein (Table 2). Of note, expansion of $\text{Foxp3}^+\text{CD25}^+\text{CD4}^+$ T cells after core stimulation corresponded to increased numbers of $\text{CTLA-4}^+\text{CD25}^{\text{high}}$ and $\text{CTLA-4}^+\text{CD25}^{\text{int}}\text{CD4}^+$ T cells (Table 2), while expression of the other Treg markers GITR, PD-1 and CD134 remained unaffected (data not shown). In contrast to chronic hepatitis C, CD4^+ Treg subsets from subjects with self-limited HCV infection remained unaltered after in vitro stimulation with HCV core. Thus, patients with chronic hepatitis C but not subjects with previous self-limited HCV infection harbor $\text{Foxp3}^+\text{CD25}^+\text{CD4}^+$ Tregs, which specifically respond to stimulation with HCV core.

When numbers of $\text{Foxp3}^+\text{CD25}^{\text{high}}$, $\text{Foxp3}^+\text{CD25}^{\text{int}}$, $\text{CTLA-4}^+\text{CD25}^{\text{high}}$ and $\text{CTLA-4}^+\text{CD25}^{\text{int}}$ T cells in patients with chronic hepatitis C were correlated to age, viral loads, liver enzymes and Metavir scores, significant trends towards increased numbers of $\text{Foxp3}^+\text{CD25}^{\text{int}}$ Tregs in advanced stages of fibrosis were detected both at baseline ($r=0.46$; $p=0.02$) and after HCV core stimulation ($r=0.41$; $p=0.04$). In contrast, none of the other T cell subpopulations revealed any correlation to clinical parameters (data not shown).

To characterize HCV-specific Tregs in detail, we studied T cell clones generated in the presence of HCV core antigen and abundant IL-2.

Identification of antigen-specific Treg clones in chronic hepatitis C

Overall, we succeeded in generating 242 CD4^+ T cell clones from seven patients with chronic hepatitis C and 65 clones from six subjects with self-limited HCV infection. Based on their proliferation characteristics and cytokine profiles in response to anti-CD3/anti-CD28 stimulation, we identified 23 and 14 TH1 clones, 34 and 21 TH2 clones as well as 14 and 6 Treg clones from patients with chronic and subjects with self-limited hepatitis C, respectively (Supplementary Tables 1 and 2).

All putative Treg clones both from chronic hepatitis C (#1 to #14) and self-limited HCV infection (#15 to #20) were Foxp3^+ and CD25^{int} (range of MFI: 49-297) (Figure 2A). In addition they expressed variable levels of the other Treg markers CTLA-4, GITR, PD-1, CD30, CD134, and GARP but were consistently CD127^- (Supplementary Figure 2). Thus, all clones matched the expected phenotype of Tregs. However, Treg clones from patients with chronic hepatitis C expressed significantly higher levels of CD25 and CTLA-4 than Treg clones from subjects with

self-limited HCV infection (Figure 2B). Concerning their cytokine profiles in response to anti-CD3/anti-CD28 all Treg clones produced IL-10 (Supplementary Table 2); and two clones from chronic hepatitis C also produced TGF-beta1 (clone #5: 187.0 pg/ml and clone #6: 963.1 pg/ml). Of note, there was a fundamental difference in antigen-specificity between Treg clones from chronic versus self-limited hepatitis C: twelve of the fourteen Treg clones from chronic hepatitis C produced IL-10 after stimulation with HCV core antigen, whereas none of the Treg clones from self-limited HCV infection recognized the core antigen (Figure 3A). HCV core-specificity of IL-10 responses in Treg clones from chronic hepatitis C was confirmed by the absence of reactivity to a recombinant CMV antigen (Figure 3A). HCV core-specific IL-10 responses were restricted by HLA-DR (Supplementary Figure 3). Using overlapping peptides we identified single epitopes, which were clustered in regions aa 1-44 and aa 79-113 on the HCV core protein (Figure 3B).

Inhibitory capacity of Treg clones in chronic hepatitis C

When Treg clones were analyzed in suppressor assays against autologous TH1 and TH2 reporter clones, all Treg clones from chronic hepatitis C but none of the clones from self-limited HCV infection inhibited proliferation of reporter cells in a dose-dependent manner upon mitogenic stimulation (Figures 4A, B and Table 3). In two Treg clones from chronic hepatitis C (#4 and #5) sufficient numbers of cells were available to also study inhibitory activity against autologous reporter cells after activation with HCV core. Of note, both Treg clones inhibited proliferation of reporter cells also upon antigen-specific activation (Figure 4C).

Since adaptive Tregs are considered to require ongoing stimulation to maintain their regulatory activity, we next studied the *in vitro* stability of Treg clones from chronic hepatitis C if they were further expanded with Treg expander beads (Dyna, Germany) instead of HCV antigen. As illustrated in Figure 5 Treg clones gradually lost their suppressor function after multiple rounds of non-specific re-stimulation. This loss of function was closely correlated to reduced Foxp3 expression.

Mechanism of suppression mediated by HCV-specific Treg clones

Treg cells can act by contact-dependent and contact-independent mechanisms. Therefore, we performed suppressor assays in a transwell system and in the presence of neutralizing antibodies. Figure 6 illustrates that suppression of reporter cells remained unaffected if Tregs were directly co-cultured with reporter cells or kept in transwells. Thus, disrupting contact between Tregs and reporter T cells did not abrogate their inhibitory capacity suggesting that inhibition was mediated via soluble factors. In line with this concept, suppression of reporter cells by the Treg clones was markedly reduced when co-culture experiments were done in the presence of neutralising IL-10 antibody (Figure 6). We suspected additional soluble factors other than IL-10, possibly explaining the functional difference between Treg clones from chronic and self-limited hepatitis C. Since IL-35 has recently been described as a novel inhibitory cytokine of Tregs [22, 23], we also co-cultured Tregs and reporter cells in the presence of a neutralizing IL-35 antibody and found that addition of anti-IL-35 also significantly blocked Treg-mediated inhibitory effects (Figure 6).

Conclusion

Accumulating evidence indicates that CD4⁺ Tregs are expanded in man during acute HCV infection, maintained at increased levels during the chronic stage and lowered to normal levels upon spontaneous or treatment-induced recovery [24], suggesting that a certain balance exists between Tregs and effector T cells. Our data support this concept and provide several levels of evidence that HCV core-specific adaptive Tregs are present in chronic hepatitis C. First, we demonstrated that freshly isolated CD25^{high} and CD25^{int}CD4⁺ T cells in chronic hepatitis C express Foxp3 as well as other markers of Tregs and can inhibit CD25⁻CD4⁺ reporter T cells not only after stimulation with mitogen but notably also after activation with HCV core. In line with previous reports [3-5, 16, 25, 26] our data also confirmed increased numbers of Foxp3⁺CD25⁺CD4⁺ T cells in patients with chronic hepatitis C. In addition in vitro stimulation with HCV core further increased the Foxp3⁺CD25^{high}CD4⁺ and Foxp3⁺CD25^{int}CD4⁺ Treg subsets in patients with chronic hepatitis C but not in subjects with previous self-limited HCV infection; despite the fact that HCV-specific CD4⁺ T cells usually persist long-term after elimination of the virus [27]. Finally, numbers of Foxp3⁺CD25^{int}CD4⁺ Tregs appeared to increase in patients with more advanced fibrosis. A rather similar relationship between Foxp3⁺CD25⁺CD4⁺ Tregs and hepatic fibrosis in chronic hepatitis C has also been reported by Marché and coworkers in the liver [28]. However, their study had not addressed antigen specificity.

To further study HCV core-specific Tregs we established T cell clones in the presence of HCV core including abundant IL-2 and succeeded in generating fourteen and six Treg clones from patients with chronic hepatitis C and subjects with self-limited HCV infection, respectively. At a first glance Treg clones from either patient group exhibited rather similar features: all clones were hypoproliferative, expressed Foxp3 as well as other Treg markers, and produced substantial amounts of IL-10. However, Tregs clones were CD25^{int} and expressed rather moderate levels of Foxp3, which distinguished them from natural Tregs [9-10].

Nevertheless we found marked differences between Treg clones from chronic and self-limited HCV infection: Treg clones from self-limited hepatitis C had lower CD25 and CTLA-4 expression levels, were not active in autologous suppressor assays and did not respond to HCV core. In contrast, Treg clones from chronic hepatitis C inhibited proliferation of autologous TH1 and TH2 reporter clones and were specifically activated by recombinant HCV core in HLA-DR-restricted fashion. Although our experiments were somewhat limited by the low number of available cells, we also found that activation with HCV core lead to inhibition of reporter T cells by the two Treg clones, which we could study in antigen-specific suppressor assays. Taken together, these findings indicated that we had cloned HCV core-specific adaptive Tregs from chronic hepatitis C, whereas T cells with a rather similar phenotype from self-limited HCV infection did neither exhibit HCV-specificity nor functional activity.

Epitope mapping with 25-mer peptides confirmed that Tregs from chronic hepatitis C were activated by single epitopes which clustered to two regions on HCV core (aa 1-44 and aa 79-113). It is noteworthy that the same regions have previously been shown to harbor major epitopes of HCV core-specific effector T cells [17, 18]. Thus, shared HCV core epitopes recognized by both conventional effector T cells and Tregs may be a hint, that adaptive Tregs in chronic hepatitis C are activated in parallel to maintain a certain balance in the regulation of the immune response [29].

HCV core protein is abundant during chronic infection but absent after viral elimination. Importantly, HCV core-specific adaptive Tregs appear to be closely linked to continued antigen exposure. This idea is supported by our *in vitro* studies, which indicated that Treg clones gradually lost their Foxp3 expression and inhibitory activity if cultured without HCV core protein. Thus, in analogy to our experiments in Treg clones, freshly isolated Foxp3⁺CD25⁺CD4⁺ T cells from subjects with previous self-limited hepatitis C might have not responded to HCV core stimulation, because HCV core-specific Tregs had disappeared after antigen-elimination. This mechanism can also explain why we were unable to establish HCV core-specific Treg clones from subjects with self-limited HCV infection.

Our HCV core-specific Treg clones differ from Tregs in HCV infection described by other groups with respect to several features [3-8]: For instance, they differ from the HCV-specific Tregs described by Ebinuma *et al.*, which had been studied after stimulation with non-structural proteins NS3 and NS4 [30], because Ebinuma's Tregs were CD25^{high} and did not produce IL-10 or TGF-beta1. Thus, it remains an interesting but unproven idea, that several types of distinct adaptive CD4⁺ Treg subsets exist, the phenotypic and functional differentiation of which may reflect activation by distinct HCV antigens (core in our study versus NS3/4 in Ebinuma's study).

A further difference concerns the mechanisms underlying the inhibitory activity in our Treg clones, because most Tregs in hepatitis C seem to involve contact-dependent mechanisms of inhibition [3, 4, 6, 8]. Despite some heterogeneity in the individual expression of Treg markers, which is a characteristic feature of adaptive Tregs [11-13], all Treg clones from chronic hepatitis C expressed CTLA-4 or PD-1, which are pivotally involved in contact-mediated T cell inhibition. Moreover, CTLA-4 expression was substantially higher on Treg clones from chronic hepatitis C than from self-limited HCV infection. Nevertheless, our transwell co-culture experiments clearly indicated that Treg clones from chronic hepatitis C suppressed reporter T cells via the inhibitory cytokines IL-10 and IL-35. These findings are in line with recent observations in mice suggesting that activation of Tregs might require some cell-contact, while in activated cells immunosuppressive functions were mediated via release of IL-10 and IL-35 [31]. Foxp3 expression appears to be an important factor in this process, since Foxp3 can enhance IL-10 secretion, because *in vitro* experiments transfecting Foxp3 into CD25⁺CD4⁺ T-cells had resulted in increased IL-10 mRNA levels [32, 33]. This relationship between Foxp3 and cytokine enhancement possibly explains our observation that Treg clones lost their immunosuppressive capacity when Foxp3 expression disappeared.

Several studies have already provided some hints that Tregs may be antigen-specific during HCV infection [4, 5, 34]. Li *et al.* reported that unlike controls *in vitro* culture of PBMC from HCV-infected patients with HCV-derived peptides resulted in rapid induction of Foxp3⁺CD25⁺CD4⁺ Tregs [5]. Furthermore, *in vivo* expansion of Foxp3⁺CD25⁺CD4⁺ Tregs has been noted during acute hepatitis C [34-36]. Nevertheless, the functional role of Tregs has remained unclear. While Perella *et al.* identified Treg expansion as a risk factor for chronic infection [34], other groups reported that increases in Treg numbers were independent of the clinical outcome of disease [34, 36]. Finally the finding of relationship between numbers of Tregs and fibrosis in the report by Marché [28] and our study suggests that Tregs may be linked in some way to tissue damage in hepatitis C. To this discussion our data also add the novel finding that functionally active Tregs with specificity for HCV core exist in chronic hepatitis C. These cells require continued antigen exposure to maintain their Foxp3 expression and inhibitory capacity. Since we could not detect such adaptive Tregs in subjects after spontaneous resolution

of their HCV infection, this type of Tregs is either not generated during acute HCV infection or lost after spontaneous resolution in the subjects with self-limited HCV infection. Thus, evolution of Tregs should be further studied during the various phases of infection as well as in the liver in order to better understand their contribution to the altered balance of immunoregulation and tissue damage in hepatitis C.

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Figure legends

Figure 1: Inhibition of CD25⁻CD4⁺ reporter T cells by CD25⁺CD4⁺ Treg subpopulations in chronic hepatitis C

Figure 1A shows separation of freshly isolated PBMC into CD25⁻CD4⁺, CD25^{int}CD4⁺ and CD25^{high}CD4⁺ T cells subsets by stepwise serial immunomagnetic separation. **Figure B** illustrates inhibition of proliferation in CD25⁻CD4⁺ T effector cells induced by CD25^{high} and CD25^{int} T cell subsets after stimulation with anti-CD3/anti-CD28. **Figure C** illustrates inhibition of proliferation by CD25^{high} and CD25^{int} T cell subsets after antigen-specific stimulation with HCV core.

Figures 1B and 1C illustrate the inhibitory capacity of CD25^{high}CD4⁺ and CD25^{int}CD4⁺ Treg subsets (stimulation index) in five independent experiments of patients with chronic hepatitis C. CD25⁻CD4⁺ effector T cells (1x10⁵ cells/well) had been stimulated with anti-CD3/anti-CD28 (1 µg/ml and 2.5 µg/ml; **Figure 1B**) and HCV core protein (10 µg/ml; **Figure 1C**), respectively. Proliferative responses were studied in the presence or absence of purified CD25^{high}CD4⁺ T cells or CD25^{int}CD4⁺ T cells at a 1:1 ratio. Cell proliferation was measured by [³H] thymidine incorporation after 5 days. Statistical comparisons were done by paired t test.

Figure 2: Phenotypic characteristics of Treg clones from subjects with different outcomes of HCV infection

Figure 2A shows representative examples of Foxp3, CD25, and CTLA-4 expression in Treg clone #2 from chronic hepatitis C and clone #15 from self-limited HCV infection.

Figure 2B summarizes quantitative expression levels of the Treg markers (MFI) in all fourteen Treg clones from chronic hepatitis C (shaded columns) and the six Treg clones from self-limited HCV infection (unshaded columns). Results are given as box plots indicating 10, 25, 50, 75 and 90 percentiles. Treg clones had been studied in the resting phase 10 after re-stimulation. Statistical comparisons were done by unpaired t test.

Figure 3: Identification of HCV core-specific Treg clones in chronic hepatitis C

Figure 3A illustrates that HCV core-specific secretion of IL-10 was selectively found in Treg clones from patients with chronic hepatitis C (**left**), but not in Treg clones from subjects with self-limited HCV infection (**right**). Antigen-specificity was confirmed by the absence of reactivity upon stimulation with CMV control antigen, respectively. Both Treg clones from chronic and from self-limited HCV infection secreted IL-10 upon stimulation with anti-CD3/anti-CD28. Statistical comparisons were done by unpaired t test. "Ø" designates unstimulated cells.

Figure 3B demonstrates that HCV core-specific Treg clones from chronic hepatitis C responded to distinct peptides of the HCV core protein. Tregs were stimulated with single peptides (P1 to P12; 1 µg/ml) from our HCV core 25-mer peptide panel in the presence of autologous antigen presenting cells. After 72 h, IL-10 secretion was measured in the supernatants by ELISA. This figure illustrates representative stimulation experiments in two Treg clones (clone #2 upper left and clone #6 upper right) selectively responding to peptide P2 (aa 10-34) and P10 (aa 89-113), respectively. The table at the bottom summarizes peptide epitope specificities demonstrating that recognized peptides were clustered in regions aa 1-44 and aa 79-113 on the HCV core protein.

Figure 4: Functional characterization of Treg clones from subjects with different outcomes of HCV infection

Figure 4A illustrates that Treg clones raised from chronic hepatitis C can inhibit proliferation of autologous reporter clones in a dose-dependent manner. The example illustrates that co-culture of anti-CD3/anti-CD28-stimulated Tregs (Treg clone #1) in increasing ratios (1:4, 1:2 and 1:1) and a TH1 reporter clone (clone T5_4; Trep; 50,000 cells/well) significantly reduced proliferation of reporter cells. The inhibition of reporter clone T5_4 by Treg clone #1 was confirmed in 4 independent experiments. Statistical comparison was done by the Kruskal Wallis test. A summary of all co-culture suppression assays is given in Table 2.

Figure 4B shows that hypoproliferative, IL-10-producing CD4⁺ Treg clones from subjects with self-limited HCV infection fail to inhibit proliferation of reporter clones. The figure illustrates a representative co-culture experiment using anti-CD3/anti-CD28-stimulated (1µg/ml; 2.5µg/ml) autologous TH2 reporter clone DK5_6 (Trep; 50,000 cells/well) with Treg clones #15, #16, and #17 (at a 1:1 ratio) generated from subjects with self-limited HCV infection. Proliferative responses were analyzed via [³H] thymidine incorporation (counts per minute = cpm) after 5 days. Statistical comparison was done by the Kruskal Wallis test.

Figure 4C exemplifies that Treg clones from chronic hepatitis C suppress proliferation of autologous reporter clones upon stimulation with HCV core antigen. This figure demonstrates the immunosuppressive capacity of the two available HCV-specific Treg clones generated from patients with chronic hepatitis C (clone #4 and #5) against their autologous reporter clones after stimulation with HCV core protein. After 5 days, proliferative responses were analyzed via [³H] thymidine incorporation.

Figure 5: Maintenance of suppressor function in HCV-specific Treg clones in the absence of antigen

Figure 5 illustrates that in the absence of antigen-specific activation HCV core-specific Treg clones from chronic hepatitis C gradually lost Foxp3 expression, which closely correlated to reduced immunosuppressive function in co-culture assays. In brief, following multiple rounds of non-specific antigen stimulation (each 10 days apart) levels of detectable Foxp3 within Tregs decreased. Histograms of Foxp3 staining (thick lines) and isotype controls (thin lines) are shown on the right side. In correlation, Treg clones also lost their ability to suppress autologous reporter T cells. The right panel shows reduction of [³H] thymidine uptake as percent of specific inhibition after 5 days of co-culture.

Figure 6: Mechanisms of inhibition mediated by HCV-specific Treg clones

This figure illustrates that inhibition of reporter cells was not affected when direct cell-contact was prevented during co-culture suppression assays of reporter clones and Treg clones in a transwell system. However, blocking experiments with neutralising IL-10 and IL-35 antibodies revealed consistent abrogation of Treg-mediated suppression of reporter cells indicating that Treg clones mainly acted via secretion of both IL-10 and IL-35 rather than contact-dependent mechanisms.

Table 1: Demographic and clinical data of the study groups

A) Chronic hepatitis C

Patient	Sex	Age (years)	ALT (U/l)	Viral load (IU/ml)	Genotype	Metavir Fibrosis Score ^{b)}
#1 ^{a)}	m	55	92	53 821	1b	n/a
#2 ^{a)}	m	46	67	5 306 449	1a	n/a
#3 ^{a)}	f	46	38	163 250	1b	n/a
#4 ^{a)}	m	45	45	605	1a	F0
#5	f	49	103	755 026	1a	F2
#6	m	57	279	243 942	1b	n/a
#7	m	68	172	9 692	1a	F3
#8 ^{a)}	f	64	111	671 893	1b	F3
#9	m	42	148	43 782	1a	F3
#10	m	60	160	1 172 054	1	F2
#11	f	49	34	144 000	1a	F1
#12 ^{a)}	m	30	70	3 269 055	1a	F1
#13	f	52	67	1 065	1b	n/a
#14	m	35	134	152 454	1a	F2
#15	f	32	12	621	1a	F1
#16	m	35	357	9 330	1b	F0
#17 ^{a)}	m	31	71	2 000 000	1	F2
#18	f	33	34	3 355 733	1b	F0
#19	f	55	65	323 206	1b	F0
#20	f	54	132	5 009 277	1a/1b	F1
#21	f	47	34	2 494 991	1ab	F1
#22	m	46	102	382 644	1	F3
#23	m	47	40	536 579	1b	F3
#24	f	62	70	974 862	1b	F2
#25	f	56	69	664	1b	F0
#26	m	30	163	4 091 895	1	F0
#27	f	51	42	1 562 602	1	F1
#28	f	50	48	707 266	1a	F0
#29	f	30	12	56 940	1b	F0
#30	m	30	103	67 657	1a	F1
#31	f	30	33	1 429	1b	F1

B) Self-limited hepatitis C

Subject	Sex	Age (years)	ALT (U/l)
SL#1 ^{a)}	m	34	17,1
SL#2 ^{a)}	m	32	27,5
SL#3 ^{a)}	m	36	22,1
SL#4	m	40	24,2
SL#5 ^{a)}	m	65	23,6
SL#6	m	35	23,7
SL#7	m	27	26,2
SL#8	m	60	22,9
SL#9	m	48	14,0
SL#10	m	30	30,2
SL#11 ^{a)}	m	55	18,7
SL#12 ^{a)}	m	49	16,6

a) These individuals had been randomized to T cell cloning.

b) Metavir scores had been obtained in 9 and 17 patients by liver biopsy and fibroscan, respectively.

n/a = data not available

Table 2:

CD4⁺ Treg subsets in unexpanded PBMC at baseline and after short-term *in vitro* stimulation with HCV core (Mean ± SEM)

	chronic hepatitis C (n=31)		self-limited hepatitis C (n=12)	
	baseline	HCV core stimulation ^{a)}	baseline	HCV core stimulation ^{a)}
Foxp3⁺ CD25^{int}	p<0.001			
	2.79 ± 0.35%	3.62 ± 0.41%	0.86 ± 0.20%	0.85 ± 0.17%
Foxp3⁺ CD25^{high}	p<0.001			
	1.27 ± 0.20%	1.73 ± 0.25%	0.23 ± 0.08%	0.16 ± 0.05%
CTLA-4⁺ CD25^{int}	p<0.001			
	0.84 ± 0.24%	1.04 ± 0.24%	0.62 ± 0.18%	0.88 ± 0.36%
CTLA-4⁺ CD25^{high}	p=0.001			
	0.57 ± 0.11%	0.83 ± 0.15%	0.11 ± 0.03%	0.08 ± 0.02%

^{a)} T cell subset composition was measured in freshly isolated PBMC 10 days after stimulation with HCV core (10 µg/ml).
Of note, results in patients with self-limited hepatitis were similar to a group of healthy controls (data not shown).

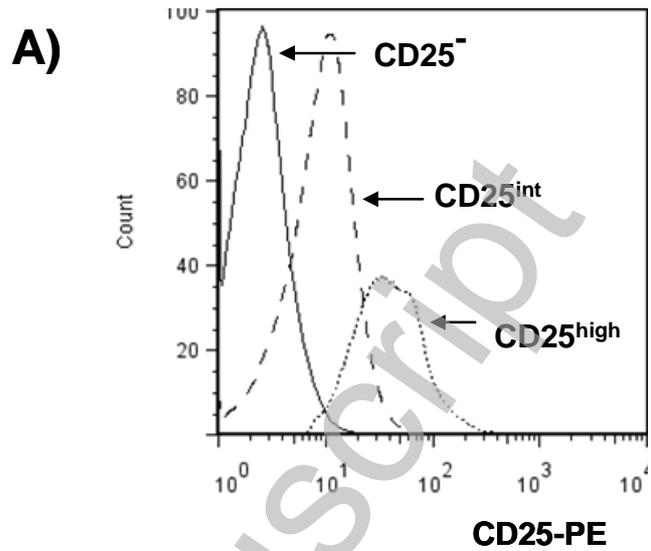
Table 3: Inhibitory capacity of Foxp3⁺CD25⁺CD4⁺ Treg clones from chronic and self-limited HCV infection^{a)}

chronic hepatitis C				self-limited hepatitis C			
clones		Proliferation inhibition of		clones		Proliferation inhibition of	
		autologous TH1 clones ^{b)}	autologous TH2 clones ^{b)}			autologous TH1 clones ^{b)}	autologous TH2 clones ^{b)}
#1	T1_7	50% (T5_4)	36% (T3_6)	#15	DK1_2	n.d.	0% (DK5_6)
#2	T6_2	20% (T5_4)	27% (T3_6)	#16	DK3_2	n.d.	0% (DK5_6)
#3	T7_2	50% (T5_4)	n.d.	#17	HG15_3	0% (HG4_7)	0% (HG9_1)
#4	M5_11	n.d.	13% (M7_7)	#18	HG5_1	0% (HG4_7)	0% (HG9_1)
#5	H7_3	46% (H4_11)	63% (H3_14)	#19	HG5_6	0% (HG4_7)	0% (HG9_1)
#6	H7_16	36% (H4_11)	n.d.	#20	HG7_4	0% (HG4_7)	0% (HG9_1)
#7	R13_3	n.d.	50% (R10_3)				
#8	R8_3	n.d.	59% (R10_3)				
#9	R19_8	n.d.	53% (R11_8)				
#10	Z6_3	13% (Z13_4)	57% (Z6_4)				
#11	F15_1	n.d.	33% (F10_6)				
#12	F12_3	14% (F8_6)	45% (F4_6)				
#13	G5_6	n.d.	60% (G22_4)				
#14	G3_1	n.d.	34% (G22_4)				

^{a)} The data refer to Treg : Trep cell ratios of 1 : 1.

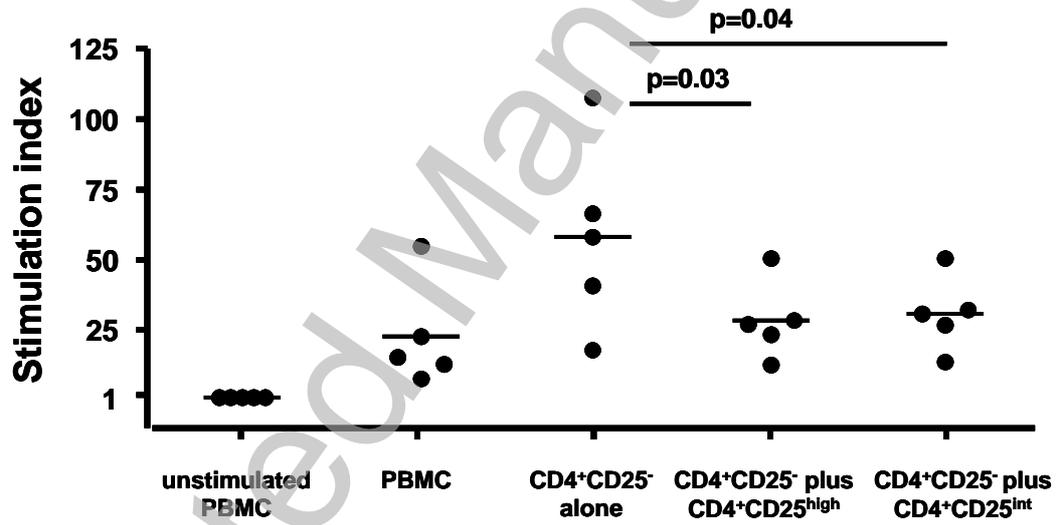
^{b)} Labels in brackets refer to the tested reporter clones (Trep).

Figure 1



B)

Stimulation with anti-CD3/CD28



C)

Stimulation with HCV core

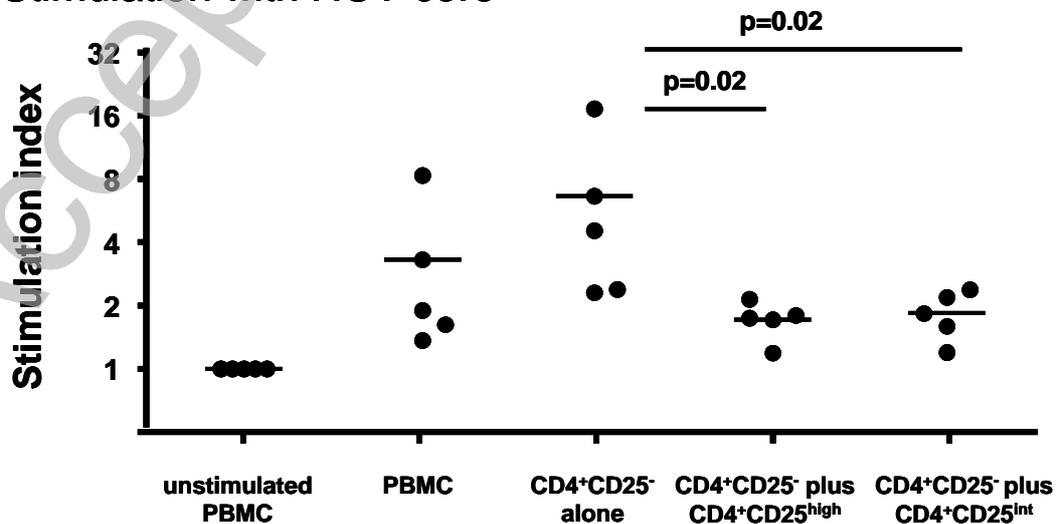
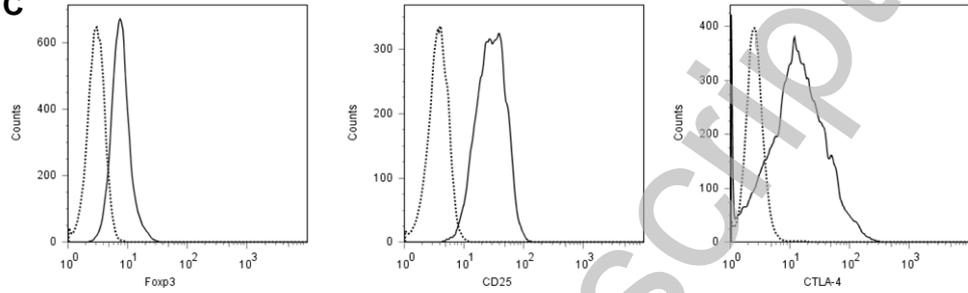


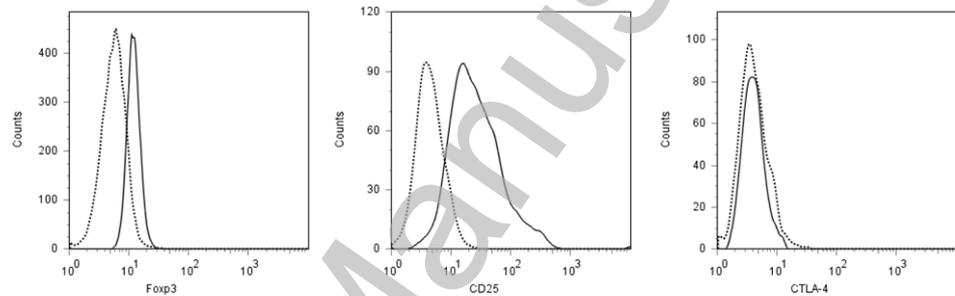
Figure 2

A)

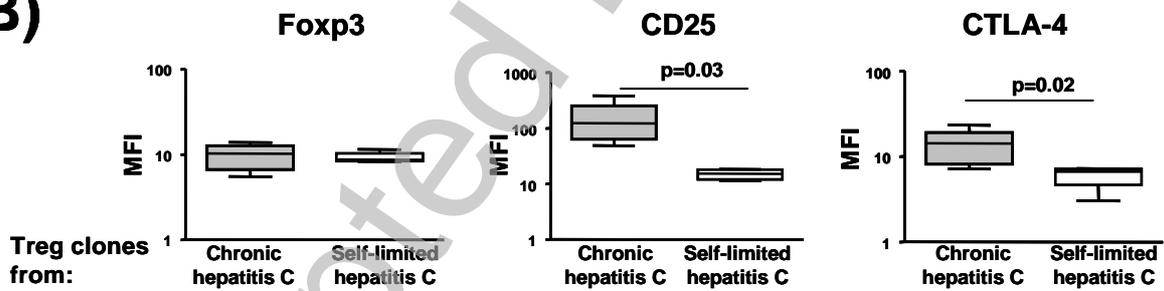
Chronic hepatitis C



Self-limited hepatitis C

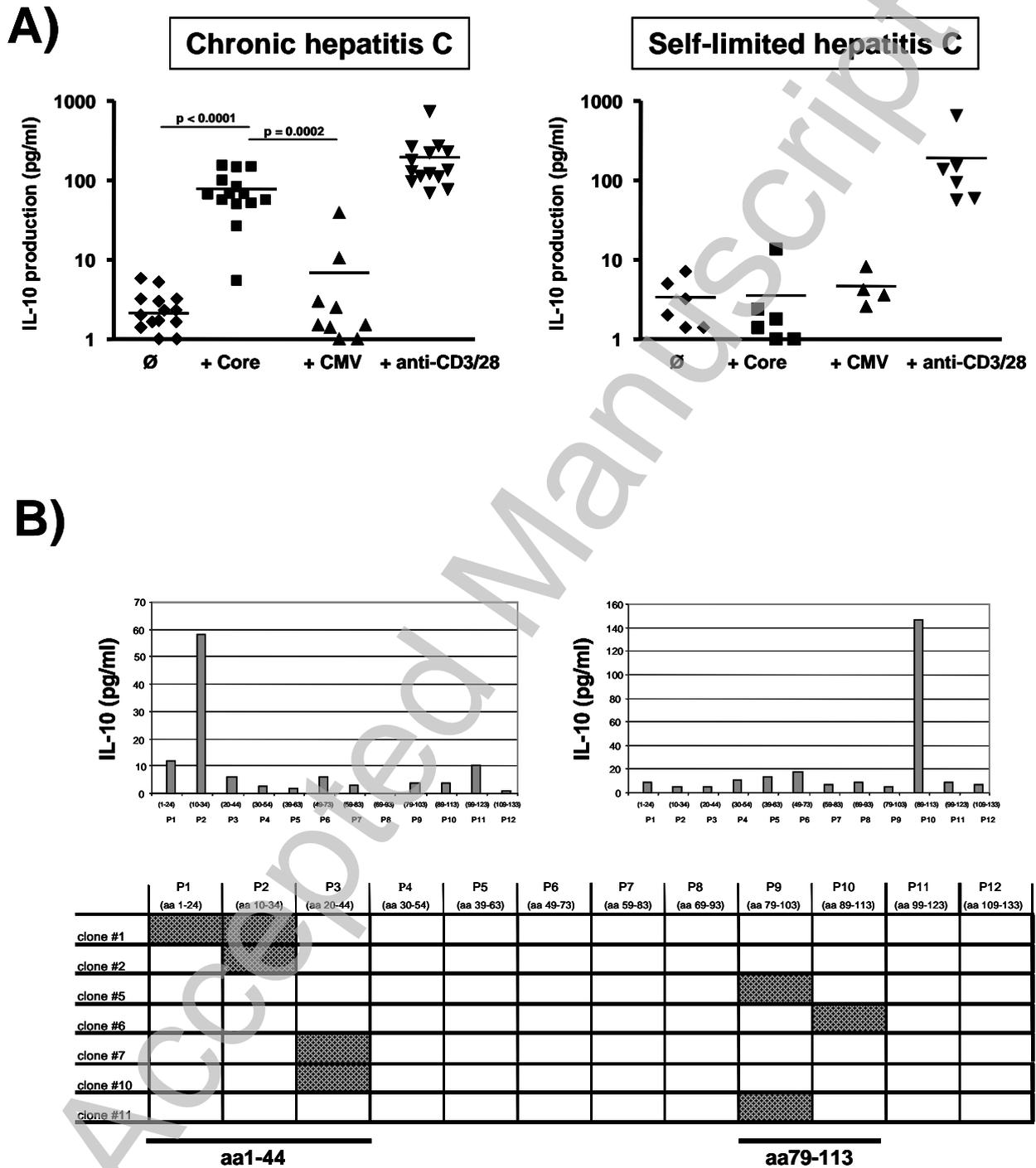


B)



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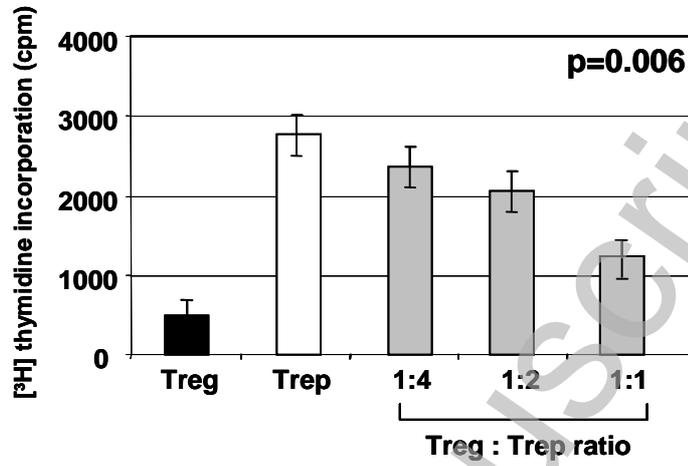
Figure 3



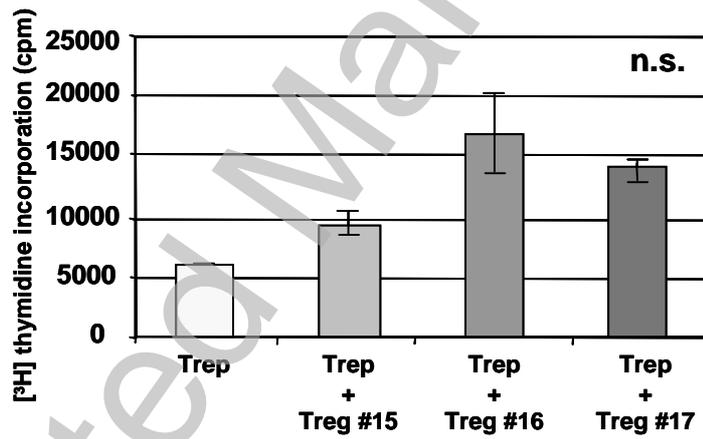
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Figure 4

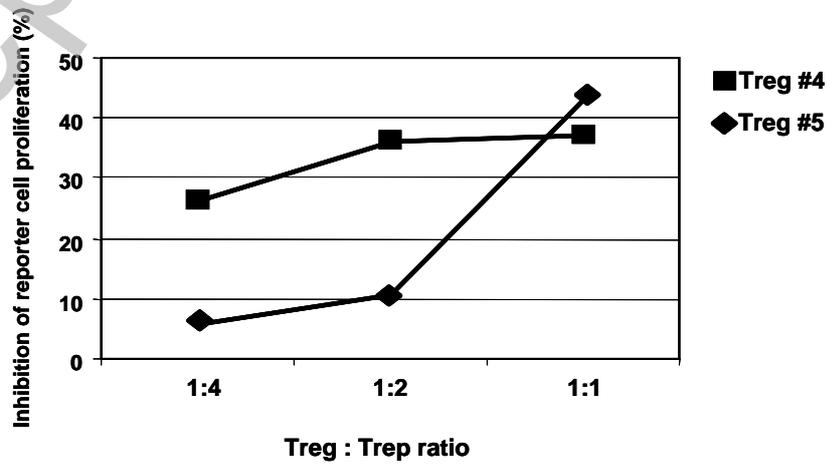
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B)



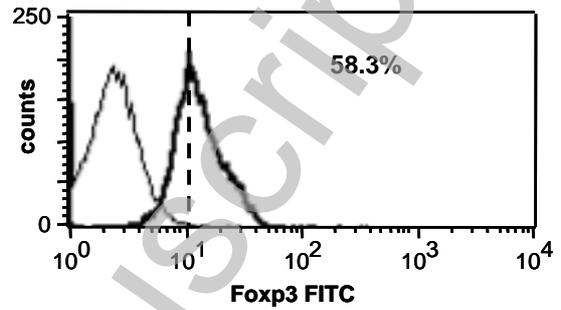
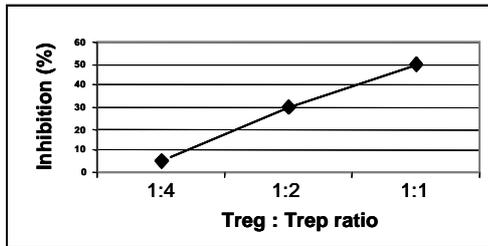
C)



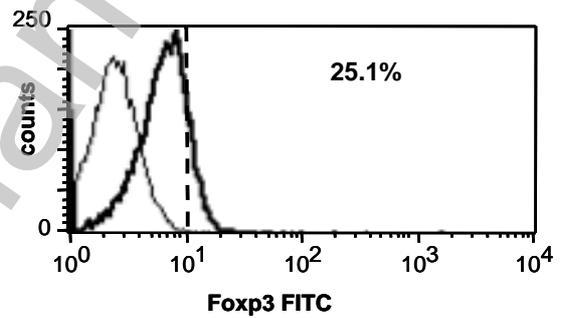
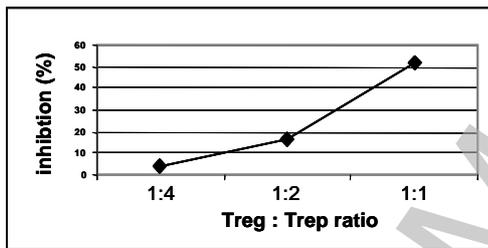
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Figure 5

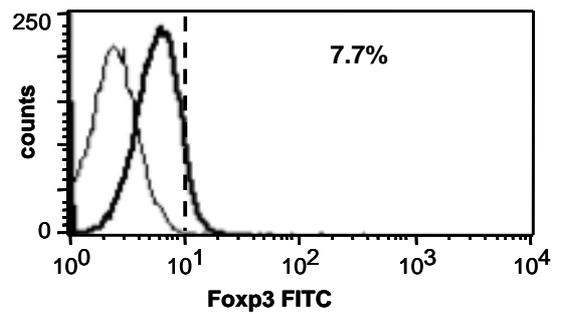
Round 1 of re-stimulation



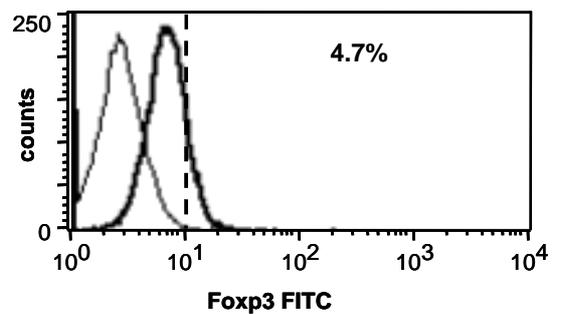
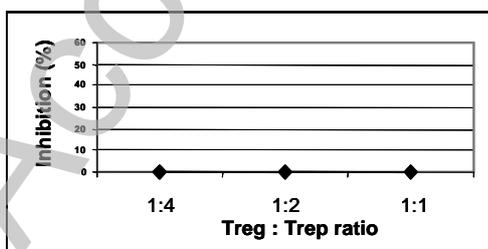
Round 2 of re-stimulation



Round 3 of re-stimulation

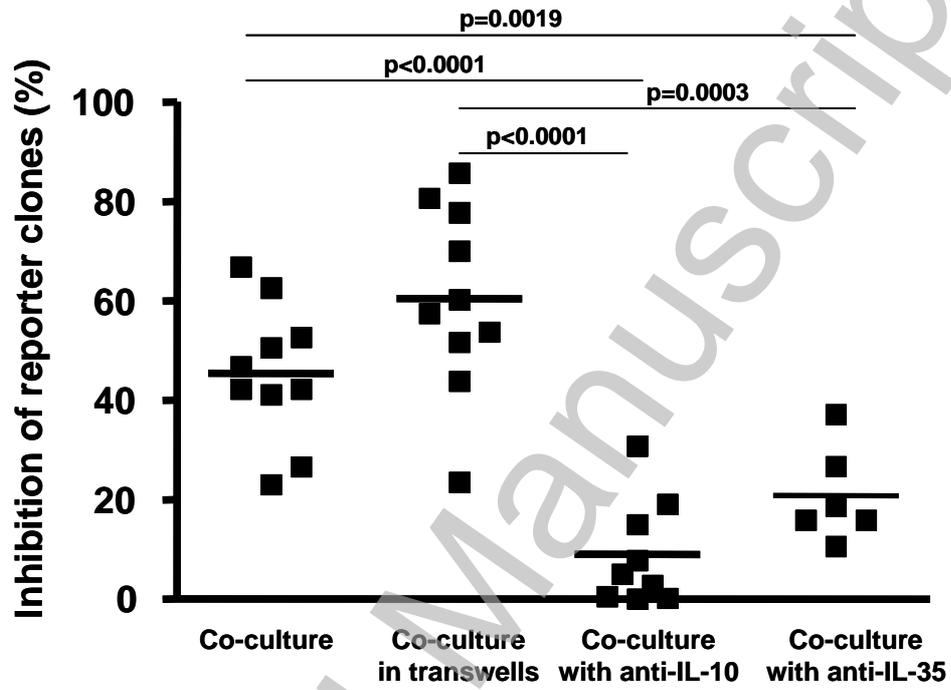


Round 4 of re-stimulation



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Figure 6



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