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Hepatitis C virus-induced myeloid-derived suppressor cells regulate T-cell differentiation and function via the signal transducer and activator of transcription 3 pathway

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Summary

T cells play a pivotal role in controlling viral infection; however, the precise mechanisms responsible for regulating T-cell differentiation and function during infections are incompletely understood. In this study, we demonstrated an expansion of myeloid-derived suppressor cells (MDSCs), in particular the monocytic MDSCs (M-MDSCs; $CD14^+$ $CD33^+$ $CD11b^+$ $HLA-DR^{-/low}$), in patients with chronic hepatitis C virus (HCV) infection. Notably, HCV-induced M-MDSCs express high levels of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and interleukin-10 (IL-10) compared with healthy subjects. Blocking STAT3 signalling reduced HCV-mediated M-MDSC expansion and decreased IL-10 expression. Importantly, we observed a significant increase in the numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells following incubation of healthy peripheral blood mononuclear cells (PBMCs) with MDSCs derived from HCV-infected patients or treated with HCV core protein. In addition, depletion of MDSCs from PBMCs led to a significant reduction of $Foxp3^+$ Treg cells developed during chronic HCV infection. Moreover, depletion of MDSCs from PBMCs significantly increased interferon-γ production by $CD4^+$ T effector (Teff) cells derived from HCV patients. These results suggest that HCV-induced MDSCs promote Treg cell development and inhibit Teff cell function, suggesting a novel mechanism for T-cell regulation and a new strategy for immunotherapy against human viral diseases.

Keywords: hepatitis C virus; interleukin-10; myeloid-derived suppressor cells; regulatory T cells; signal transducer and activator of transcription 3.

Introduction

Hepatitis C virus (HCV) is a blood-borne virus characterized by a high rate (over 80%) of chronic infection. HCV has evolved multiple strategies to evade host immunity, becoming an excellent model to study the mechanisms of persistent infections in humans.[1,2] Although the use of direct antiviral agents has resulted in a significant improvement in the outcome of HCV treatment, this therapeutic cocktail is costly and already facing new issues such as viral mutation, relapse and re-infection following therapy.[3,4] Additionally, the lack of a vaccine against HCV is a major hurdle to control this global infection. The failure to successfully manage HCV chronic infection in specific populations, and to develop an effective vaccine, stems from our incomplete understanding of the host immune response that permits HCV persistence.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that are generated due to aberrant myelopoiesis under

pathological conditions, such as cancer, inflammatory and infectious diseases.[5–7] These cells have gained special attention recently due to their potential to suppress immune responses by multiple mechanisms, including over-expression of nitric oxide synthase, arginase 1, reactive oxygen species, interleukin-4 receptor-α (IL-4Rα), programmed death-1 ligand, IL-10, tumour growth factor β (TGF-β), and phosphorylated signal transducer and activator of transcription 3 (pSTAT3), all of which are important mediators of innate immune and inflammatory responses against pathogenic infections.[8–14] In mice, MDSCs are defined by the co-expression of the Gr-1 and CD11b surface antigens. Human MDSCs are less well-characterized because of the lack of uniform phenotypic markers.[13–15] However, they usually express the common myeloid markers CD33 and CD11b, but lack the maturation marker HLA-DR. Human MDSCs are primarily dissected into two subsets: monocytic MDSCs (M-MDSCs; $CD14^+ CD11b^+ CD33^+ HLA-DR^{-/low}$) and granulocytic MDSCs (G-MDSCs; $CD14^- CD11b^+ CD33^+ HLA-DR^{-/low}$). These two subsets of MDSCs may have different biological functions and use different mechanisms for immune suppression.[6,7]

Although MDSCs may contribute to immune homeostasis after infection by limiting excessive inflammatory processes, their expansion may be at the expense of pathogen elimination and so may lead to infection persistence. Despite many studies reporting expansion of MDSCs in various disease models including viral infections,[16–27] the phenotypic and functional features of MDSCs remain controversial and their role in regulating T-cell responses and viral persistence are far from clear. For example, although some studies have shown that M-MDSC frequencies are remarkably elevated and play a role in the pathogenesis of HIV or HCV infection,[20–25] Bowers *et al.*[26] reported an immunosuppression by neutrophils during HIV-1 infection, whereas Nonnenmann *et al.*[28] found no significant increases in MDSCs, which lack suppressive functions in peripheral blood of chronically HCV-infected individuals. These discrepancies in MDSC frequency and suppressive capability might partially result from measuring different MDSC subsets, using different methodologies, investigating different patient populations, and/or focusing on different disease stages with specific cytokine milieus that may induce distinct MDSC phenotypes. Therefore, further phenotypic and functional characterization of these particular types of cells will delineate the mechanisms by which MDSCs promote immune suppression under specific disease conditions.

In this study, we characterized the phenotypes and functions of MDSCs in patients with chronic HCV infection, with the primary focus on the role of MDSCs in regulating T-cell differentiation and function, a mechanism by which HCV might modulate T-cell responses to

facilitate the establishment and maintenance of chronic infection. We provide evidence that HCV-induced MDSCs promote regulatory T (Treg) cell development and inhibit effector T (Teff) cell function via the STAT3 pathway, representing a novel mechanism for T-cell regulation during viral infection.

Materials and methods

Subjects

The study protocol was approved by the joint institutional review board at East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN). The study subjects were composed of two populations: 129 chronically HCV-infected individuals and 59 healthy subjects (HS). HCV genotype (70% type 1, 30% type 2 or 3) and viral load (ranging from 12 300 to 50 000 000 IU/ml) were performed by Lexington VAMC, and all subjects were virologically and serologically positive for HCV, before antiviral treatment. Healthy subjects were serologically negative for hepatitis B virus, HCV, and HIV infection and blood buffy coats were obtained from Key Biologics, LLC, Memphis, TN. Written informed consent was obtained from all participants.

PBMC isolation and flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque gradient centrifugation (GE Heathcare, Piscataway, NJ). For phenotypic analysis of MDSCs, PBMCs were stained with CD14-Peridinin chlorophyll protein 710 (PerCP710), CD11b-phycoerythrin (PE), CD33-allophycocyanin (APC) (eBioscience, San Diego, CA), and HLA-DR-FITC (BD Biosciences, San Jose, CA) antibodies or isotype controls. Cytometric data were collected on an Accuri C6™ flow cytometer (BD, Franklin Lakes, NJ) and analysed using FLOWJO software (Tree Star, Inc., Ashland, OR). For intracellular cytokine staining, PBMCs were stimulated with 1 µg/ml lipopolysaccharide (Santa Cruz Biotechnology, Santa Cruz, CA) plus 2·5 µg/ml R848 (Santa Cruz) for 6 hr, and 1 µg/ml Brefeldin A (BioLegend, San Diego, CA) was added 5 hr before harvest. The cells were fixed and permeabilized using Inside Stain Kit (Miltenyi Biotec, Auburn, CA), and then stained with IL-10-PE, TGF-β-PE, programmed death-1 ligand (CD274)-PE (BD Bioscience), IL-4Rα-PE (R&D, Minneapolis, MN), p47[phox] and rat anti-mouse IgG1-PE. For pSTAT3-PE (pY705, BD Bioscience) staining, PBMCs were fixed with Fix Buffer I and permeabilized with Perm Buffer III (BD) following the BD Phosflow™ protocol. The cells were also stained with surface antigens CD14-PerCP710, CD33-APC (clone p67.6, eBioscience) and HLA-DR-FITC, then analysed by flow cytometry. The fluorescence minus one strategy and isotype controls were used to adjust multicolour compensation for cell gating and to determine background levels.

MDSC induction by HCV core protein

The PBMCs from HS were incubated with recombinant HCV core or a control protein β-gal (1 μg/ml, ViroGen, Watertoen, MA) for 72 hr *in vitro*, followed by analysis of MDSC induction and their IL-10 or TGF-β expressions by flow cytometry.

Treg cell induction

(i) CD33$^+$ myeloid cells isolated from HCV PBMCs, using CD33 microbeads (Miltenyi Biotec., cell purity > 95%), were co-cultured with healthy PBMCs in a ratio of 1 : 5 in the presence of 20 U/ml IL-2 (eBioscience) for 5 days. (ii) The CD33$^+$ myeloid cells or CD33$^-$ non-myeloid cells, purified from healthy PBMCs treated with HCV core or β-gal control protein (1 μg/ml, ViroGen) *in vitro* for 5 days, were also co-cultured with autologous naive CD4$^+$ T cells in a ratio of 1 : 3 in the presence of 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Bioscience) for 3 days. (iii) The CD33$^+$ myeloid cells isolated from HCV PBMCs were also co-cultured with autologous naive CD4$^+$ T cells in a ratio of 1 : 3 in the presence of 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Bioscience) for 3 days. (iv) For depletion experiments, HCV PBMCs and CD33$^+$-depleted PBMCs were incubated *ex vivo* for 5 days in the presence of 20 U/ml IL-2 (eBioscience), respectively. (v) To determine whether the conversion is a cytokine-dependent event, we also incubated the CD33$^{+/-}$ cells with CD4$^+$ T cells in the presence of 10 μg/ml anti-IL-10 blocking antibody or IgG control (Biolegend, San Diego, CA) for 3 days. (vi) To determine whether cell–cell direct contact is required for Treg cell induction, a transwell plate containing 0·4-μm pores (Corning, Corning, NY) was employed to culture the isolated cells in the same condition. To analyse *in vitro*-generated Treg cells, the cells were stained with CD4-PE, CD25-Alexa488, Foxp3-PE-Cy5 (eBioscience) or isotype controls using Foxp3 Staining Buffer Kit (Miltenyi Biotec). The stained cells were collected by flow cytometry and analysed using FLOWJO software as described above.

Teff cell interferon-γ assays

The PBMCs derived from HCV-infected subjects, with or without depletion of CD33$^+$ myeloid cells, were stimulated with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Bioscience) for 3 days. CD4$^+$ Teff intracellular cytokine expression was assessed by interferon-γ (IFN-γ) -PE (BD Bioscience) or isotype control staining, followed by flow cytometric analysis as described above.

STAT3 inhibition

The PBMCs from patients with chronic HCV infection were cultured in the presence of the specific STAT3 inhibitor STA-21 (Santa Cruz, 20 μM) or DMSO control for 48 hr. The cells were stimulated by lipopolysaccharide/R848 for 6 hr and Brefeldin A was added for 5 hr before harvest, then subjected to flow analysis for the MDSC phenotype as well as IL-10 and TGF-β expression in M-MDSCs as described above.

Statistical analysis

The data were summarized as mean ± standard error of the mean (SEM) or median with interquartile, depending on the characteristics of the data distribution. An independent *t*-test or paired *t*-test was used to compare the difference of mean between two groups. A Mann–Whitney test or Wilcoxon signed rank test was used to compare the difference of median between two groups. All the data were analysed using GRAPHPAD PRISM 5·0 (GraphPad Prism, San Diego, CA). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ were considered significant or very significant.

Results

Expansion of MDSCs in patients with chronic HCV infection

As an initial approach to characterize the role of MDSCs in the chronicity of HCV infection, we first compared the phenotypic frequencies of MDSCs in the peripheral blood of 56 chronically HCV-infected individuals and 22 HS. As shown in Fig. 1, the representative dot plots and summary data of flow cytometry, PBMCs were first gated on CD11b$^+$ and CD33$^+$ myeloid cells, and then analysed for the immature HLA-DR$^{-/low}$ populations within the monocytic (CD14$^+$) and granulocytic (CD14$^-$) subsets (Fig. 1a). Although the overall numbers of myeloid cells (CD33$^+$ CD11b$^+$) in chronic HCV patients were significantly lower than those in HS (Fig. 1b), the numbers of MDSCs (CD33$^+$ CD11b$^+$ HLA-DR$^{-/low}$, Fig. 1c), in particular M-MDSCs (CD14$^+$ CD33$^+$ CD11b$^+$ HLA-DR$^{-/low}$, Fig. 1d), but not G-MDSCs (CD14$^-$ CD33$^+$ CD11b$^+$ HLA-DR$^{-/low}$, Fig. 1e), were significantly higher in patients with chronic HCV infection. Of note, we did not find any direct relationship between MDSC frequencies and HCV genotype, viral load, and alanine aminotransferase or aspartate aminotransferase levels in this study.

Elevation of regulatory proteins in M-MDSCs from patients with chronic HCV infection

MDSCs are more accurately identified by their immunosuppressive functions rather than phenotypic surface markers. MDSCs exert their suppressive activities by producing copious amounts of immunosuppressive
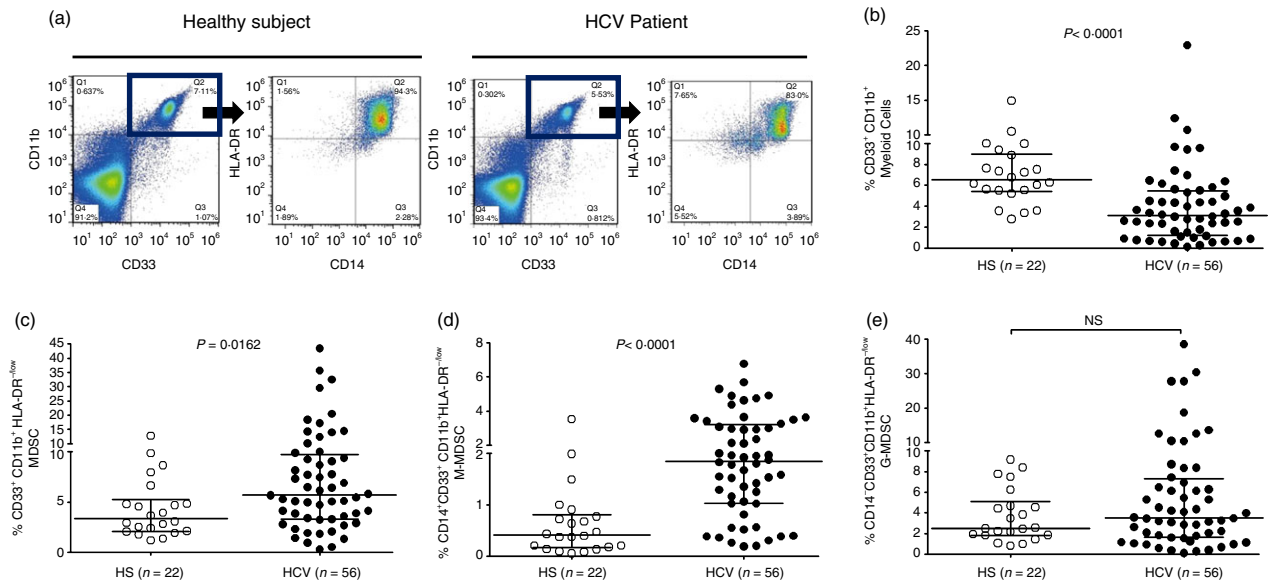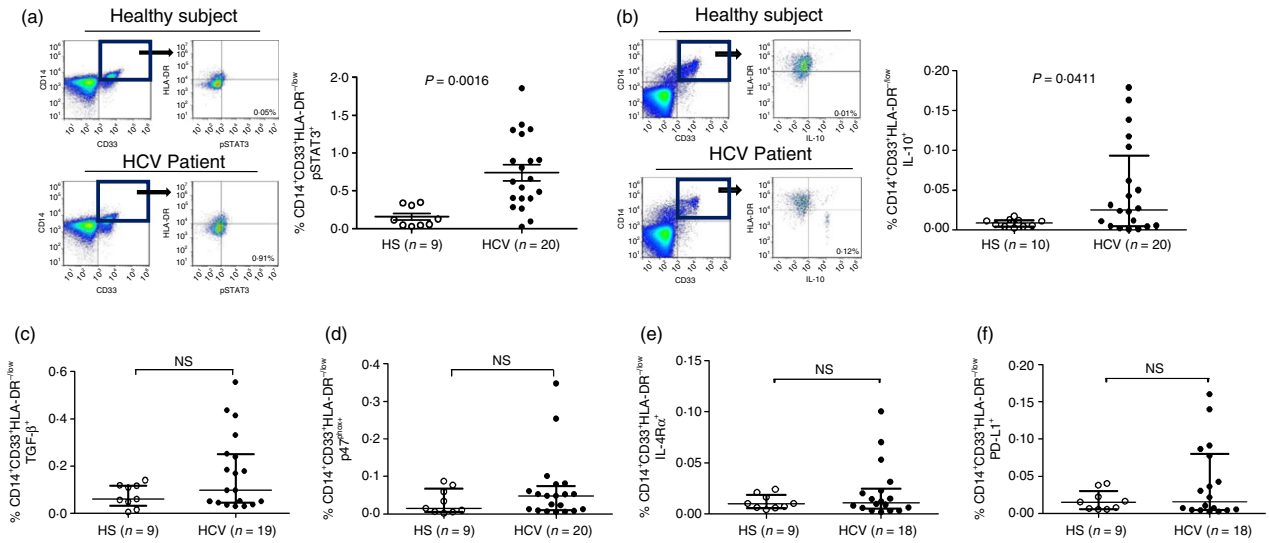
Figure 1. Phenotypic analysis of myeloid cell frequencies in chronic hepatitis C virus (HCV) patients versus healthy subjects (HS). (a) Representative dot plots analysis of myeloid-derived suppressor cells (MDSCs) by flow cytometry. $CD33^+$ $CD11b^+$ myeloid cells were first gated in peripheral blood mononuclear cells (PBMCs) of HS and HCV patients; monocytic MDSCs (M-MDSC, $CD33^+$ $CD11b^+$ $CD14^+$ HLA-DR$^{-/low}$) and granulocytic MDSCs (G-MDSC, $CD33^+$ $CD11b^+$ $CD14^-$ HLA-DR$^{-/low}$) were further characterized by the expression levels of CD14 and HLA-DR, respectively. The values in the quadrants indicate the percentages of the related subsets of cells. (b) Summary data of myeloid cell ($CD33^+$ $CD11b^+$) frequencies in HS (hollow circle, $n = 22$) versus chronically HCV-infected patients (filled circle, $n = 56$). (c) Summary data of immature myeloid cell ($CD33^+$ $CD11b^+$ HLA-DR$^{-/low}$) frequencies in HS versus HCV. The frequencies of circulating MDSCs in the pooled data were calculated by the frequencies of $CD33^+$ $CD11b^+$ cells in PBMCs multiplied by the frequencies of $CD14^{+/-}$ HLA-DR$^{-/low}$ cells. (d) Summary data of M-MDSC ($CD14^+$ $CD33^+$ $CD11b^+$ HLA-DR$^{-/low}$) frequencies in HS versus HCV. (e) Summary data of G-MDSC ($CD14^-$ $CD33^+$ $CD11b^+$ HLA-DR$^{-/low}$) frequencies in HS versus HCV. Each symbol represents an individual subject, and the horizontal bars represent median with interquartile. P-value with significant difference is shown in each panel; NS, no significance.

mediators.[5–14] To functionally characterize MDSCs in patients with chronic HCV infection, we measured the most common inhibitory cytokines or proteins, including pSTAT3, IL-10, TGF-β, p47^{phox}, IL-4Rα and programmed death-1 ligand, which have been suggested to play a role in MDSC development and/or suppressive functions.[6,7] As shown in Fig. 2, the representative dot plots and summary data of flow cytometry, we first gated the monocytic myeloid cell populations ($CD14^+$ $CD33^+$) in PBMCs, and then measured the expressions of these regulatory proteins in the immature (HLA-DR$^{-/low}$) myeloid cells. Although M-MDSCs express these regulator proteins, only pSTAT3$^+$ and IL-10$^+$ M-MDSCs (Fig. 2a,b) were found to be significantly elevated in PBMCs in patients with chronic HCV infection.

Induction of MDSCs and suppressive proteins by HCV (core) via the STAT3 pathway

Despite several reports showing increases in the numbers of MDSCs in HCV-infected patients, the factors responsible for MDSC expansion during HCV infection are unclear. As patients with chronic HCV infection often suffer from confounding factors that may drive MDSC expansion, we determined whether the increases of M-MDSCs in patients with HCV infection were directly caused by HCV or other factors. To this end, purified PBMCs from HS were incubated with recombinant HCV core protein, an important HCV structural protein that has been found to circulate in the peripheral blood of HCV patients and exerts immunosuppressive activity,[29,30] or a control protein β-gal (1 µg/ml, ViroGen) for 3 days, followed by the analysis of immature myeloid cell differentiation and maturation by flow cytometry. As shown in Fig. 3(a), healthy PBMCs treated with HCV core protein *in vitro* generated an increased number of M-MDSCs ($CD14^+$ $CD33^+$ HLA-DR$^{-/low}$ cells) when compared with those exposed to the control protein. In addition, healthy PBMCs treated with HCV core protein generated higher numbers of pSTAT3$^+$ M-MDSCs (Fig. 3b), IL-10$^+$ M-MDSCs (Fig. 3c), and TGF-β^+ M-MDSCs (no significance, Fig. 3d) compared with those exposed to the β-gal control protein. Moreover, healthy PBMCs co-cultured with Huh-7R hepatocytes expressing HCV also resulted in an increase in MDSC frequency, though the increase is not statistically significant compared with those co-cultured with Huh-7 cells without HCV expression (data not shown).

Figure 2. Analysis of regulatory protein expression in monocytic myeloid-derived suppressor cells (M-MDSCs) from hepatitis C virus (HCV) patients versus healthy subjects (HS). Peripheral blood mononuclear cells (PBMCs) isolated from HS and HCV patients were stimulated with Toll-like receptor (TLR) ligand lipopolysaccharide (LPS)/R848 for 6 hr, immune stained for phosphorylated signal transducer and activator of transcription 3 (pSTAT3), interleukin-10 (IL-10), transforming growth factor-β (TGF-β), p47^{phox}, IL-4Rα, and programmed death-1 ligand (PD-L1) in M-MDSC, followed by flow cytometric analysis. (a), and (b) left panel: representative dot plots for pSTAT3 and IL-10 expression in M-MDSCs from HS and HCV patients; right panel: summary data for the percentage of pSTAT3$^+$ or IL-10$^+$ M-MDSCs in PBMCs from HS (hollow circle) and HCV patients (filled circle). The percentages of pSTAT3$^+$ (or IL-10$^+$) M-MDSCs in the pooled data were calculated by the frequencies of CD14$^+$ CD33$^+$ cells in PBMCs multiplied by the frequencies of HLA-DR$^{-/low}$ pSTAT3$^+$ (or IL-10$^+$) cells. (c), (d), (e), and (f) Summary data of TGF-β^+, p47$^{phox\ +}$, IL-4Rα^+ and PD-L1$^+$ M-MDSCs in PBMCs from HS and HCV patients. Each symbol represents an individual subject, n = the number of subjects to be studied, and the horizontal bars represent mean $\pm$ SEM (pSTAT3) or median with interquartile. P-value with significant difference is shown in each panel; NS, no significance.
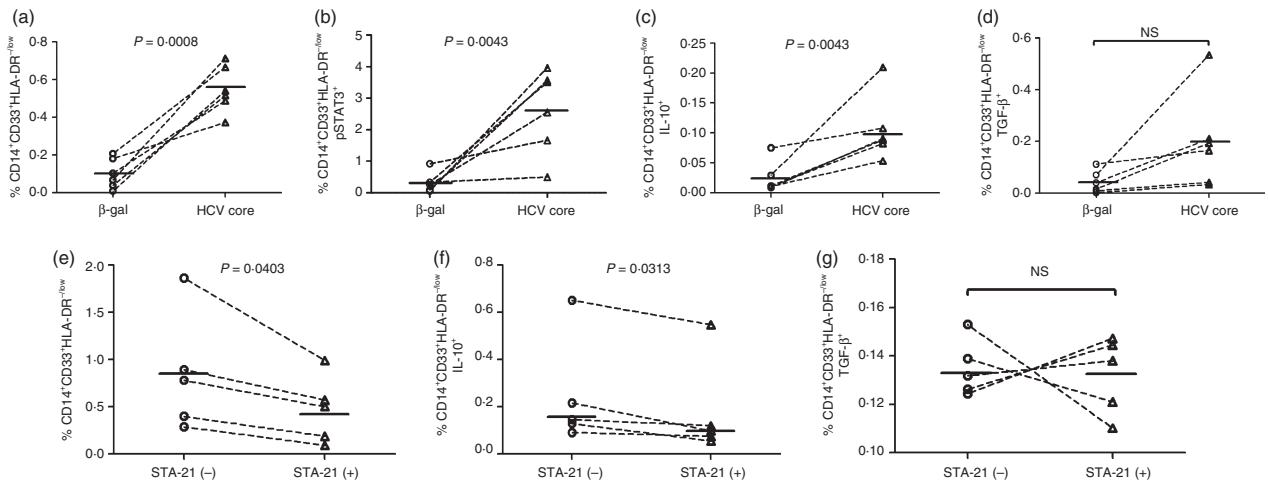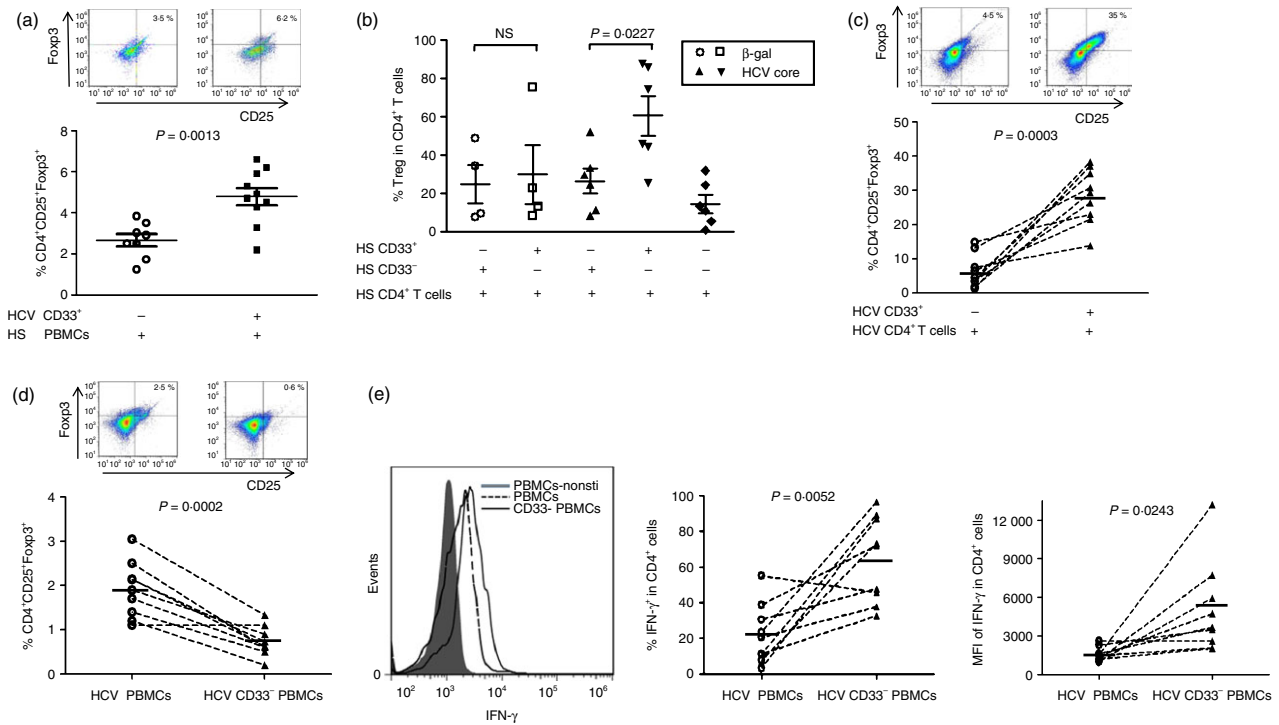

Figure 3. Hepatitis C virus (HCV) induces myeloid-derived suppressor cell (MDSC) differentiation and regulatory cytokine expression via the signal transducer and activator of transcription 3 (STAT3) pathway. (a), (b), (c), and (d) HCV core protein induces monocytic MDSCs (M-MDSCs) and their expression of pSTAT3, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). Healthy peripheral blood mononuclear cells (PBMCs) (n = 6) were incubated with HCV core or β-gal control protein (1 μg/ml) *in vitro* for 3 days, stimulated with lipopolysaccharide (LPS)/R848 for 6 hr before harvesting, followed by flow cytometric analysis for the development of M-MDSCs (a: CD14$^+$ CD33$^+$ HLA-DR$^{-/low}$ cells) and the frequencies of pSTAT3$^+$ M-MDSCs (b), IL-10$^+$ M-MDSCs (c), and TGF-β^+ M-MDSCs (d) in PBMCs. (e), (f) and (g) Involvement of STAT3 signalling in the development of MDSCs and expression of regulatory cytokines. PBMCs isolated from HCV patients (n = 5) were cultured *ex vivo* in the presence of the specific STAT3 inhibitor STA-21 (20 μM) for 48 hr, stimulated by LPS/R848 for 6 hr, and Brefeldin A for 5 hr before harvest, then subjected to flow cytometric analysis for M-MDSC frequency (e) as well as IL-10$^+$ M-MDSCs (f) and TGF-β^+ M-MDSCs (g) in PBMCs. P values are shown in each panel from a paired t-test or by a Wilcoxon signed rank test (f); NS, no significance.

Although MDSCs were increased in chronic HCV patients, the precise mechanisms involved in their differentiation and suppressive function remain elusive. Because the HCV-induced transcription factor STAT3 activation is accompanied by the increases in IL-10 and TGF-β expression (Fig. 2a–c and Fig. 3b–d), we further investigated whether pSTAT3 promotes MDSC expansion and their production of IL-10 or TGF-β. To this end, we blocked STAT3 signalling *ex vivo* using the STAT3-specific inhibitor STA-21[31] in PBMCs from HS and HCV-infected patients, then analysed the frequencies of M-MDSCs as well as IL-10 and TGF-β expression in these cells. As shown in Fig. 3(e), blocking STAT3 signalling significantly reduced the numbers of CD14$^+$ CD33$^+$ HLA-DR$^{-/low}$ M-MDSCs, which

were increased during chronic HCV infection, whereas the percentage of M-MDSCs in PBMCs from HS was not altered (data not shown). In addition, the expression levels of the suppressive cytokine IL-10 (Fig. 3f), but not TGF-β (Fig. 3g), in HCV M-MDSCs were inhibited after blocking STAT3 signalling.

MDSCs regulate T-cell differentiation and function in chronic HCV infection

In addition to producing inhibitory proteins, it has been suggested that MDSCs exert their immunosuppressive functions by inducing CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cell development in cancer or transplant patients.[12,15,32]

Figure 4. Myeloid-derived suppressor cells (MDSCs) from hepatitis C virus (HCV) patients promote Foxp3$^+$ regulatory T (Treg) cell differentiation and suppress interferon-γ (IFN-γ) expression by effector T (Teff) cells. (a) CD33$^+$ myeloid cells from HCV patients induce Treg cell generation in healthy peripheral blood mononuclear cells (PBMCs). Healthy PBMCs were co-cultured with or without CD33$^+$ myeloid cells derived from HCV patients for 5 days, followed by immunostaining for CD4, CD25, Foxp3 and flow cytometric analysis for Treg cell development. Representative dot plots and cumulative results from 10 independent experiments are shown. (b) CD33$^+$ myeloid cells from healthy PBMCs treated with HCV core protein promote Treg cell generation. Healthy CD4$^+$ T cells were co-cultured with CD33$^+$ or CD33$^-$ cells isolated from PBMCs exposed to HCV core or β-gal control protein for 3 days, followed by flow cytometric anlysis for Foxp3$^+$ Treg cell frequencies. Cumulative results from four independent experiments are shown. (c) Induction of Treg cell development by co-culture of CD4$^+$ T cells with CD33$^+$ myeloid cells derived from HCV patients. CD4$^+$ T cells isolated from HCV patients were co-cultured with or without autologous CD33$^+$ myeloid cells for 3 days, respectively, followed by immune staining and flow analysis for CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells. Representative dot plot and cumulative results from nine HCV patients are shown. (d) Depletion of CD33$^+$ myeloid cells from PBMCs of HCV patients reduces Treg cells accumulated during HCV infection. HCV PBMCs, with or without CD33$^+$ myeloid cell depletion, were cultured *ex vivo* for 5 days, followed by immune staining and flow analysis for CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells. Representative dot plot and cumulative results from nine HCV patients are shown. (e) Depletion of CD33$^+$ myeloid cells from PBMCs of HCV patients boosts the IFN-γ production by CD4$^+$ Teff cells. PBMCs isolated from chronic HCV patients, with or without depletion of CD33$^+$ myeloid cells, were cultured *ex vivo* in the presence of anti-CD3/CD28 stimulation, followed by flow cytometric analysis for IFN-γ expression in CD4$^+$ T cells. Representative overlaid histogram (left panel) and cumulative results for the percentage (middle panel) as well as the mean fluorescence intensity (MFI) (right panel) of IFN-γ expression in CD4$^+$ T cells from nine HCV patients are shown. *P*-values are shown in each panel.

Therefore, we next sought to determine whether HCV-associated MDSCs can induce Foxp3$^+$ Treg cell generation. To this end, we first co-cultured healthy PBMCs with or without CD33$^+$ myeloid cells derived from HCV patients for 5 days, and then analysed Foxp3$^+$ Treg cell development by flow cytometry. As shown in Fig. 4(a), the representative dot plots of flow cytometry and summary data of co-culture experiments, healthy PBMCs co-cultured with HCV CD33$^+$ myeloid cells (enriched in MDSCs) induced a significant increase in CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells when compared with those cultured without HCV myeloid cells. To address the concern of allo-stimulation in the co-culture system, we also observed Treg induction using healthy CD4$^+$ T cells incubated with autologous myeloid cells isolated from the same PBMCs treated with HCV-core protein for 5 days in vitro. As shown in Fig. 4(b), CD33$^+$ myeloid cells (but not CD33$^-$ non-myeloid cells) derived from healthy PBMCs treated with HCV core protein, but not from PBMCs exposed to β-gal protein, can significantly induce Foxp3$^+$ Treg cell development. In addition, purified HCV CD4$^+$ T cells incubated with autologous HCV CD33$^+$ myeloid cells led to a high number of Foxp3$^+$ Treg cells; whereas HCV CD4$^+$ T cells (enriched in Treg cells, but lacking MDSCs) incubated alone without the presence of HCV CD33$^+$ myeloid cells resulted in a significant decrease in Foxp3$^+$ Treg cells (Fig. 4c). We also tested whether depletion of CD33$^+$ myeloid cells from HCV PBMCs might lead to a decrease in Foxp3$^+$ Treg cells that were expanded during chronic HCV infection. As shown in Fig. 4(d), the representative dot plots of flow cytometry and summary data of depletion experiments, depletion of CD33$^+$ myeloid cells from HCV PBMCs resulted in a significant decrease in the numbers of CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells when compared with HCV PBMCs with MDSCs in culture, suggesting that generation and maintenance of Treg cells need the presence of MDSCs.

As HCV-induced MDSCs were able to produce high levels of IL-10 (Fig. 2) that are capable of triggering Treg cell induction, we tested whether Treg cell induction by MDSC is merely a cytokine-dependent event. To this end, we incubated HCV CD33$^+$ myeloid cells or CD33$^-$ non-myeloid cells with autologous CD4$^+$ T cells in the presence of anti-IL-10 blocking antibody or IgG control (Biolegend, San Diego, CA) for 3 days, followed by flow cytometric analysis for Treg cell induction. Not surprisingly, simply blocking cytokine IL-10 alone could not abrogate the HCV MDSC-induced Treg cell induction (data not shown). To determine if direct contact of MDSCs with naive T cells is required for differentiation into Treg cells, we isolated CD33$^+$ myeloid cells (CD33$^-$ non-myeloid cells as control) and naive CD4$^+$ T cells from HCV patients and co-cultured in the same conditions (1 : 3 cell ratio, stimulated by anti-CD3/CD28 for 3 days) using a transwell plate (Corning, Corning, NY). Interestingly, the induction of Treg cells by

HCV CD33$^+$ myeloid cells was not observed in this transwell system when compared with CD33$^-$ non-myeloid cells (data not shown), suggesting that direct contact of myeloid cells with naive T cells is required for differentiation into Treg cells in HCV patients.

In addition to inducing or maintaining the differentiation of Treg cells, we also studied whether MDSCs can inhibit the function of Teff cells that are usually suppressed or exhausted during chronic viral infections.[33,34] To this end, PBMCs derived from chronically HCV-infected patients, with or without depletion of CD33$^+$ myeloid cells, were stimulated with anti-CD3 and anti-CD28 for 72 hr, followed by intracellular staining for the IFN-γ expression in CD4$^+$ T cells. As shown in Fig. 4(e), the representative overlaid histogram (left panel) and summary data (middle and right panel) from the flow cytometric analysis, depletion of CD33$^+$ myeloid cells from PBMCs of chronic HCV patients significantly increased the IFN-γ production by CD4$^+$ T cells. This result holds true by analysing both the percentage of positive cell frequency and mean fluorescence intensity of IFN-γ production in CD4$^+$ T cells.

Discussion

Compelling studies clearly implicate an important role for MDSCs in tumour progression and the antitumour immune response; however, the significance of MDSCs in viral infections is far from clear. In this study, we demonstrate a significant increase in the monocytic subset of myeloid cells (CD14$^+$ CD33$^+$ CD11b$^+$) that are immature (HLA-DR$^{-/low}$) and immunosuppressive in patients with chronic HCV infection. These HCV-induced M-MDSCs express high levels of pSTAT3-mediated IL-10, since blocking STAT3 signalling reduces the expression of IL-10 and decreases the numbers of M-MDSCs developed in chronic HCV infection. In addition, we demonstrated that HCV-induced MDSCs promote Foxp3$^+$ Treg cell development and inhibit CD4$^+$ Teff cells producing IFN-γ. Of note, both Treg cell expansion and Teff cell inhibition are associated with viral persistence.[1,2]

The mechanism underlying MDSC expansion in virus-infected individuals remains to be determined. It has been suggested that MDSCs can be induced or expanded by the virus itself or its coding proteins, including HIV, HCV and lymphocytic choriomeningitis viruses.[21,23,27] We and others have demonstrated that HCV core, the first protein to be secreted from HCV-infected hepatocytes and circulating in the blood of virus-infected patients, can activate STAT3 phosphorylation, prevent myeloid cell maturation and promote M-MDSC development, an effect that can be abrogated by adding STAT3 inhibitor in vitro[35] (Fig. 3). These results indicate that HCV can regulate MDSC differentiation and suppressive functions via the STAT3 signalling pathway. Indeed,
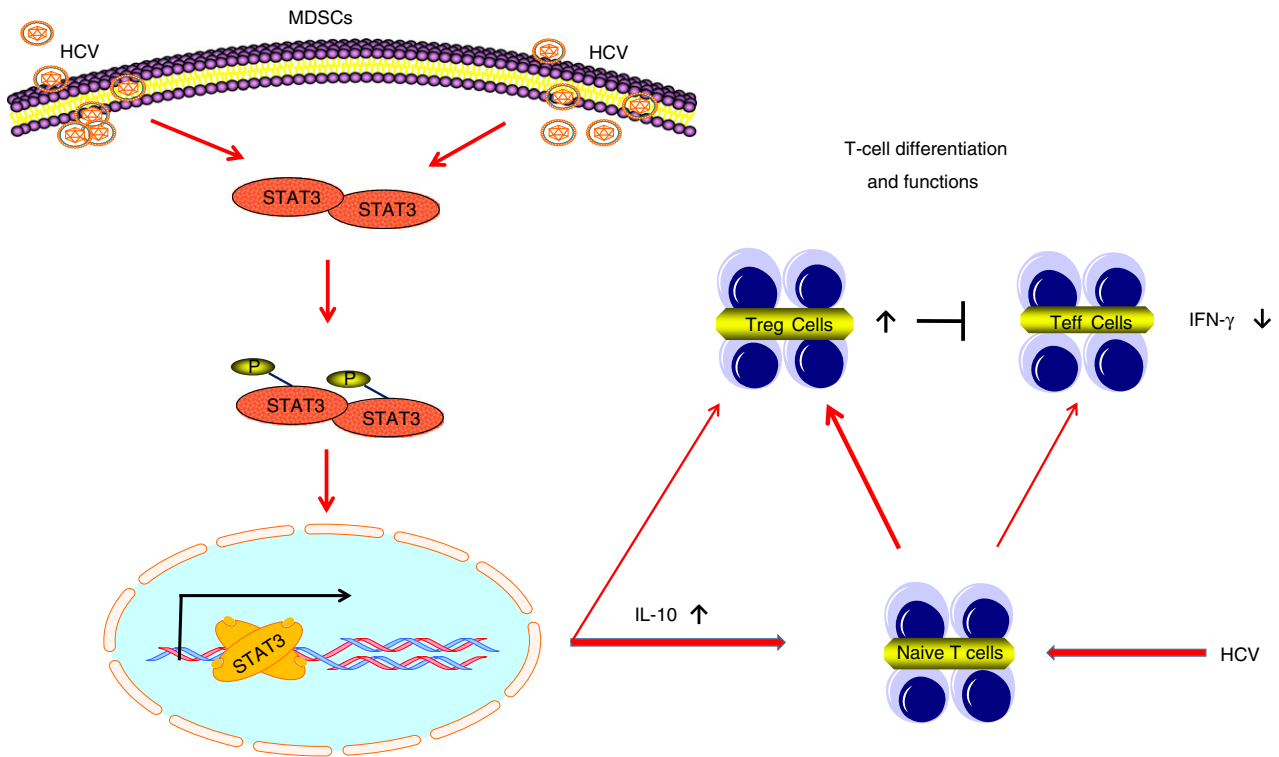
Figure 5. A schematic model for hepatitis C virus (HCV) -induced myeloid-derived suppressor cells (MDSCs) regulate T-cell differentiation and function via signal transducer and activator of transcription 3 (STAT3) signalling. HCV infection activates STAT3 phosphorylation and interleukin-10 (IL-10) expression and drives MDSC expansion, which in turn, promotes CD4^{+} CD25^{+} Foxp3^{+} regulatory T (Treg) cell differentiation and inhibits CD4^{+} effector T (Teff) cell interferon-γ (IFN-γ) production. Therefore, inhibition of the STAT3/IL-10 pathway and MDSC development may provide a novel approach for HCV immunotherapy. Of note, we and others have previously shown that HCV can directly induce Treg cell differentiation and suppress Teff cell functions.[29,30,33,34,47,48] Hence, direct antiviral therapy is the key to controlling infection and correcting HCV-induced immune dysregulation.

ablation of STAT3 using conditional knockout mice reduces the expansion of MDSCs and improves T-cell responses in tumour-bearing mice.[36] Additionally, myeloid cells expressing toll-like receptor 3 that can interact with double-stranded RNA released from non-viable virus in the blood may serve as a plausible mechanism for MDSC differentiation by HCV. Another factor that might contribute to the increases in numbers of MDSCs during HCV infection is an inflamed liver, a microenvironment where active HCV replication and persistent inflammation occurs and so may modify myeloid cell differentiation, resulting in expansion of MDSCs.

Although dysregulated myelopoiesis by inflammatory cytokines, such as IL-6, IL-10, TGF-β and prostaglandin E_2 that are up-regulated during chronic HCV infection,[37–43] may partially explain the expansion of MDSCs, the molecular nature of this process is unclear. Previous studies have shown that increased expression of arginase 1, nitric oxide synthase or reactive oxygen species is the primary mechanism by which MDSCs promote immunosuppression during chronic HCV infection.[23–25] HCV core-treated CD33^{+} cells exhibit a CD14^{+} CD11b^{+} HLA-DR^{−/low} phenotype with up-regulated expression of p47[phox], a component of the NOX2 complex that is critical for reactive oxygen species production,[23] and STAT3 has been shown to directly regulate the production of immunosuppressive mediators, such as IL-6, IL-10, IL-4Rα and p47[phox].[31,35,36] Our results showed that the MDSCs that have accumulated during chronic HCV infection expressed higher levels of pSTAT3 and IL-10 regulatory molecules that are essential and not redundant to their suppressive functions, a feature not seen in MDSCs from healthy controls.[44,45] The detailed mechanism of how STAT3-mediated signalling pathways are activated and regulate the MDSC functions during chronic HCV infection requires further investigation. Recently, Condamine and Gabrilovich[46] proposed two overlapping STAT3-mediated pathways that regulate the distinct features of MDSCs: one signalling pathway of STAT3, along with survival factors, such as c-Myc, Cyclin D and Survivin, promotes proliferation but prevents differentiation and maturation of immature myeloid progenitors, which may contribute to MDSC expansion in mouse. A second signalling pathway uses STAT-3,

phosphatidyl inositol 3-kinase and nuclear factor-κB transcription factors to induce production of the immuno-suppressive mediators arginase 1, reactive oxygen species, IL-10 and TGF-β in MDSCs. These factors produced at inflammation sites or in a tumour microenvironment may expand MDSCs and enhance their immunosuppressive functions.[7]

We and others have previously shown that HCV (core protein) has direct effects on T-cell differentiation and responses.[29,30,33,34,47,48] Based on our new findings of an HCV-induced, STAT3/IL-10-mediated MDSC expansion and regulation of T-cell differentiation and function, we propose a model, as depicted in Fig. 5, that represents a novel mechanism by which HCV suppresses immune responses, in particular T-cell differentiation and function, during chronic viral infection. HCV-induced MDSCs may trigger naive CD4 T-cell differentiation into Foxp3$^+$ Treg cells, and they may also promote natural occurring and adaptive Treg cell expansion. The mechanisms regarding how HCV induces pSTAT3 expression in MDSCs to dampen immune responses to viral infection and antiviral activities are far from clear, and we continue to investigate these potential mechanisms in our laboratory, focusing on microRNA-based regulatory mechanisms. Nevertheless, this study demonstrates that HCV-induced MDSCs control T-cell activities through STAT3 signalling, shedding new light on the features of MDSCs and providing a novel mechanism for T-cell immune suppression during chronic viral infection. Hence, targeting STAT3 signalling and/or MDSC expansion may represent a promising strategy for immunotherapy to treat human viral diseases.

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Disclosure

The authors declare no competing interests.

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