Hepatitis C Virus-Specific Th17 Cells Are Suppressed by Virus-Induced TGF-β[1]

Aileen G. Rowan,[2]* Jean M. Fletcher,* Elizabeth J. Ryan,* Barry Moran,* John E. Hegarty,[†] Cliona O'Farrelly,* and Kingston H. G. Mills[3]*

IL-17-secreting T (Th17) cells play a protective role in certain bacterial infections, but they are major mediators of inflammation and are pathogenic in organ-specific autoimmune diseases. However, human Th17 cells appear to be resistant to suppression by CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells, suggesting that they may be regulated by alternative mechanisms. Herein we show that IL-10 and TGF-β suppressed IL-17 production by anti-CD3-stimulated PBMC from normal individuals. TGF-β also suppressed IL-17 production by purified CD4$^+$ T cells, whereas the inhibitory effect of IL-10 on IL-17 production appears to be mediated predominantly by its effect on APC. An examination of patients infected with hepatitis C virus (HCV) demonstrated that Ag-specific Th17 cells are induced during infection and that these cells are regulated by IL-10 and TGF-β. PBMC from HCV Ab-positive donors secreted IL-17, IFN-γ, IL-10, and TGF-β in response to stimulation with the HCV nonstructural protein 4 (NS4). Furthermore, NS4 induced innate TGF-β and IL-10 expression by monocytes from normal donors and at higher levels from HCV-infected patients. Neutralization of TGF-β, and to a lesser extent IL-10, significantly enhanced NS4-specific IL-17 and IFN-γ production by T cells from HCV-infected donors. Our findings suggest that both HCV-specific Th1 and Th17 cells are suppressed by NS4-induced production of the innate anti-inflammatory cytokines IL-10 and TGF-β. This may represent a novel immune subversion mechanism by the virus to evade host-protective immune responses. Our findings also suggest that TGF-β and IL-10 play important roles in constraining the function of Th17 cells in general. *The Journal of Immunology,* 2008, 181: 4485–4494.

R ecently, a novel lineage of CD4$^+$ T cells that secrete IL-17, termed Th17 cells,[4] have been described (1). Th17 cells are characterized by the ability to secrete IL-17A, IL-17F, IL-21, IL-22, and IL-6 (2, 3). The ubiquitous expression of the IL-17 receptor means that it can act on a broad range of cellular targets. IL-17A and IL-17F function primarily as proinflammatory mediators, inducing local IL-6, PGE$_2$, and NO production, and specifically recruiting neutrophils (4). IL-22 acts on tissues close to the body surface, for example, the skin and digestive system, and promotes acute-phase responses (5), but it has also been shown to play a protective role against liver inflammation in mice (6). Studies in murine models have highlighted a pathological role for IL-17 in organ-specific auto-immune diseases (7–10). Th17 cells have been detected in T cell-mediated inflammatory lesions in patients with psoriasis and uveitis (2, 11). These cells have also been shown to have protective function in certain bacterial infections (4, 12, 13). To date, there are few reports on Th17 cells in viral infections.

Studies in mice have demonstrated that the induction and expansion of Th17 cells is promoted by either TGF-β and IL-6 or IL-21, or by IL-1 and IL-23 (3, 14–16). In humans, IL-1β in combination with IL-6 or IL-23 is thought to drive the generation of Th17 cells (2, 17), while IL-1, IL-23 (18), and IL-21 (3) promote the production of IL-17 by memory T cells. Interestingly, while CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (Treg) cells have been shown to efficiently suppress Th1 responses, they do not suppress human Th17 cells (19), suggesting that Th17 cells may be regulated by other mechanism, such as by IL-10 and TGF-β.

Hepatitis C virus (HCV) is an RNA virus that primarily infects hepatocytes, and it may lead to the development of fibrosis or cirrhosis of the liver and is a significant risk factor for the development of hepatocellular carcinoma. It is estimated that 80% of individuals exposed to HCV develop chronic infection, and currently an estimated 210 million people are chronically infected. Antiviral immune responses are induced but they fail to eradicate the pathogen in these patients. This has been attributed to a combination of both host and viral factors, which include the immunosuppressive environment of the liver and the high mutation rate of the virus. Additionally, a number of viral proteins can suppress HCV-specific immune responses (20). There is evidence that both IL-10 and TGF-β are up-regulated in chronic HCV infection, and this may be associated with persistence and progression of the disease (21, 22). However IL-10 and TGF-β are both induced as part of the normal immune response to infection where they may play a crucial role in regulating immune responses and in preventing pathogen-induced immunopathology.

HCV-specific CD4$^+$ T cell responses are most readily detected in patients acutely infected with HCV. In particular, the induction of HCV-specific Th1 responses are associated with at least transient control of the infection (23). In contrast, induction of HCV-specific Th2 cells has been associated with viral persistence (24).

We have also reported that IL-10-secreting Treg cells, as well as Th1 cells, are induced against the core protein in HCV infection (21). Furthermore, we have demonstrated defective IFN-γ production by PBMC from HCV-infected patients in response to non-structural protein 4 (NS4), which is reversed by neutralization of IL-10 (25). To date, there have been no reports on the induction of Th17 cells during HCV infection. Like IFN-γ, IL-17 may play a protective role, promoting viral clearance. Alternatively, the potent proinflammatory properties of Th17 cells may contribute to liver damage. IL-17 expression has been detected in the context of inflammatory liver injury in humans and mice (26–28).

In this study we report for the first time the induction of Ag-specific Th17 cells in HCV-infected patients. Furthermore, we demonstrate that TGF-β and IL-10, which are induced by the HCV NS4 protein, suppress Th1 and Th17 responses in HCV-infected patients.

Materials and Methods

Patients and PBMC samples

Patients attending a HCV out-patient clinic at St. Vincent's University Hospital, Dublin, were recruited for this study. Written informed consent was obtained from each patient, and the study received ethical approval from the local Research and Ethics Committee at St. Vincent's University Hospital in accordance with the guidelines of the 1975 Declaration of Helsinki. All patients tested positive for Abs to HCV using a third-generation enzyme immunoassay (Abbott Diagnostics), which was confirmed with a third-generation recombinant immunoblot assay (RIBA-3) (Chiron). A total of 29 patients were recruited: 8 patients had received HCV-contaminated anti-D Ig, 5 patients had received HCV-contaminated blood transfusions, and 6 patients were i.v. drug users. Seventeen patients remained chronically infected with the virus as determined by testing consistently positive for HCV RNA by a qualitative RT-PCR (Amplicor; Roche Diagnostic Systems). The remaining 12 patients tested negative for HCV RNA by RT-PCR. A single experienced histopathologist scored liver biopsy specimens from 14 of the chronically infected patients according to the modified histological activity index (HAI) system (29). There was evidence of mild inflammation in 12 patients, 1 patient had no inflammation, while 1 patient had moderate inflammation. The biopsies were also scored according to the Ishak scoring system for fibrosis (30); 3 of the chronically infected patients scored 2, 2 had a score of 1, and 10 scored 0. Therefore, this cohort of HCV patients had mild liver disease, with no evidence of cirrhosis. All patients who tested HCV PCR⁻ showed no signs of liver disease (either biopsy or normal alanine aminotransferase/aspartate aminotransferase levels). Leukocyte-enriched buffy coats from anonymous normal donors were obtained with permission from the Irish Blood Transfusion Board, St James's Hospital, Dublin, and ethical approval was granted by the Trinity College ethics committee. PBMC were isolated by density gradient centrifugation.

Effect of IL-10 and TGF-β on monocyte cytokine production

CD14⁺ monocytes were isolated from PBMC using positive selection with MACS microbeads (Miltenyi Biotec) and an autoMACS cell sorting instrument. The purity of CD14⁺ monocytes after autoMACS separation was routinely 85–92% as estimated by flow cytometry analysis using FITC-conjugated CD14 (BD Biosciences). Monocytes were stimulated with *Escherichi coli* LPS (Alexis; 100 ng/ml) and IFN-γ (BD Biosciences; 20 ng/ml) in the presence of medium only or increasing concentrations of recombinant human IL-10 or TGF-β (BD Biosciences; 0.001–10 ng/ml). After 48 h, supernatants were removed and the concentrations of IL-1β, IL-6, IL-12p40, IL-12p70, and IL-23 determined by ELISA (eBioscience and BD Biosciences).

Stimulation of innate IL-10 and TGF-β by HCV NS4

Recombinant NS4, corresponding to amino acids 1616–1862 of the HCV polyprotein, was purchased from Mikrogen and was shown to be free of LPS by analysis with a *Limulus* amoebocyte lysate assay (BioWhittaker). LPS (Alexis) was used as a positive control for stimulation of innate cells. CD14⁺ monocytes and CD11C⁺ dendritic cells were purified from PBMC using positive selection with MACS microbeads and an autoMACS cell sorting instrument. PBMC, CD14-depleted PBMC, or purified CD14⁺ or CD11c⁺ cells from healthy controls or HCV-infected donors were cultured at a concentration of 1 × 10⁶/ml with medium only, 0.4 or 2 μg/ml NS4, or 100 ng/ml LPS. After 24 h, cell culture supernatants were harvested and

the concentrations of latent or bioactive TGF-β and IL-10 were assayed by ELISA. Bioactive TGF-β was directly assayed using an ELISA specific for active TGF-β. Latent TGF-β production was determined following acid treatment of culture supernatants.

Detection of TGF-β by flow cytometry

Intracellular IL-10 and TGF-β expression in monocytes was detected after stimulation of PBMC with 2 μg/ml NS4 for 5 h, with 0.4 μM monensin added after the first hour, followed by surface staining with CD14-FITC, fixation and permeabilization (Caltag Laboratories), and intracellular staining with anti-TGF-β (clone TB21, IQ products). Cell-surface expression of TGF-β and latency-associated peptide (LAP), the amino-terminal domain of the TGF-β precursor peptide, was detected by indirect staining after stimulation with 2 μg/ml NS4 for 20 h. Cells were stained for 15 min with 0.05 μg/ml biotinylated anti-LAP Ab (goat polyclonal, BAF246, R&D Systems) in FACS buffer, washed twice, and incubated for a further 15 min with 0.5 μl/test streptavidin PerCP (BD Biosciences), anti-TGF-β, and anti-CD14. The stained cells were washed three times before acquisition.

T cell stimulation

For measurement of Ag-specific cytokine production by PBMC from HCV-infected patients, PBMC were incubated in medium alone or stimulated with 2 μg/ml NS4 for 72 h, after which cell culture supernatants were harvested and cytokine concentration was quantified by ELISA. Neutralizing Abs to IL-10 (clone JES-9D7, BD Biosciences) and TGF-β1,2,3 (clone 1D11, R&D Systems) were added at a concentration of 5 μg/ml 30 min before the addition of NS4. Recombinant IL-10 and TGF-β (both eBioscience; 1 pg/ml to 10 ng/ml) were added to PBMC 2 h before stimulation with anti-CD3 (0.5 μg/ml; BD Biosciences) or to purified CD4⁺ T cells (purified by MACS sorting) stimulated with anti-CD3 and anti-CD28 (1.0 μg/ml; BD Biosciences). After 3 days of culture, IL-17 and IFN-γ concentrations were quantified in the supernatants by ELISA. Cell proliferation was assessed by [³H]thymidine incorporation, with results expressed as cpm.

CFSE amplification assay and intracellular cytokine staining

To amplify Ag-specific cells and monitor T cell proliferation by flow cytometry, cells were labeled with CFSE before in vitro stimulation. Briefly, cells were washed thoroughly with PBS to remove any protein and incubated with 0.5 μM CFSE (Molecular Probes) in PBS for 10 min at 37°C. Complete RPMI 1640 was added to stop the reaction, and the cells were washed twice before being stimulated with 2 μg/ml NS4. After 8 days, cells were harvested and restimulated with PMA (Sigma-Aldrich; 10 ng/ml) and ionomycin (Sigma-Aldrich; 500 ng/ml) for 6 h in the presence of 5 μg/ml of brefeldin A. Cells were stained with CD3-allophycocyanin-Cy7 and CD8-PECy7 (eBioscience), fixed and permeabilized using Fix and Perm reagents (Caltag Laboratories), and stained for intracellular IL-17 (anti-IL-17-PE, eBioscience) and IFN-γ (anti-IFN-γ-allophycocyanin, BD Biosciences) and analyzed by flow cytometry. CD4⁺ T cells were identified as CD3⁺CD8⁻ cells. Ag-specific proliferation was estimated by CFSE dilution. Cells were acquired using a DakoCytomation CyAn flow cytometer and anlayzed using FlowJo software.

Quantification of cytokines by ELISA

IL-17, IFN-γ, TGF-β (all R&D Systems), and IL-10 (BD Biosciences) were quantified in cell culture supernatants by sandwich ELISA. Latent TGF-β was detected after acid treatment of the samples. Due to the cross-reactivity between human and bovine TGF-β present in FCS, the amount of latent TGF-β present in the culture medium was assayed and subtracted from the total latent TGF-β concentration detected for each experimental condition tested.

Results

Exogenous IL-10 and TGF-β inhibit Th1 and IL-17 responses either directly or through their effect on APCs

The immunosuppressive effects of IL-10 and TGF-β on Th1 cells are well documented. However, the role of these anti-inflammatory cytokines in regulating Th17 cells is less clear. In fact, it has been proposed that TGF-β has a positive role in the development of murine Th17 cells (14, 15). To test the ability of IL-10 and TGF-β to suppress IL-17 secretion, PBMC from a healthy donor were stimulated with anti-CD3 in the presence of a range of concentrations of recombinant IL-10 (rIL-10) or recombinant TGF-β (rTGF-β). After 3 days, culture supernatants were removed and IL-17 and
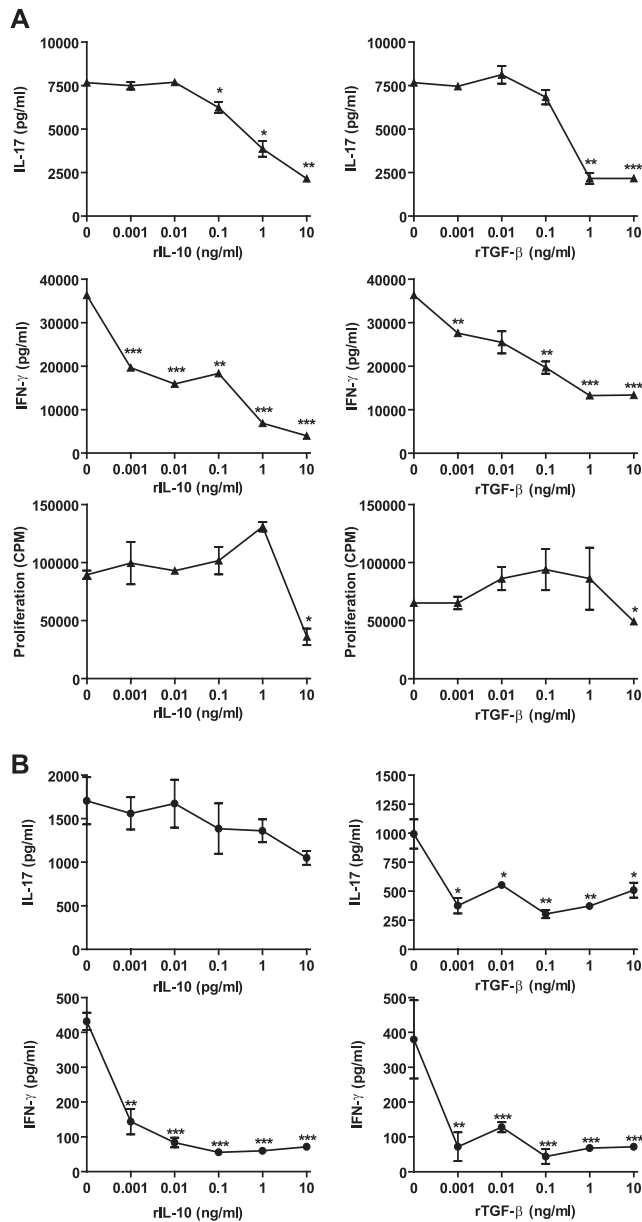
A

B

FIGURE 1. IL-10 and TGF-β inhibit IL-17 and IFN-γ production by anti-CD3-activated T cells. PBMC isolated from a normal individual were stimulated with 0.5 μg/ml anti-CD3 (A), or CD4$^+$ T cells purified from normal PBMC were stimulated with 0.5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 (B) in the presence of increasing concentrations of recombinant IL-10 or TGF-β. After 72 h, culture supernatants were harvested and the concentrations of IL-17 and IFN-γ were determined by ELISA. Cell proliferation was assessed by [^{3}H]thymidine incorporation. PBMC or CD4$^+$ T cells stimulated with medium only did not produce any detectable IL-17 or IFN-γ. The 0 value on the x-axes represents the positive controls, which are cells stimulated with anti-CD3 and anti-CD28 in the absence of added rIL-10 or rTGF-β. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$: with vs without IL-10 or TGF-β by unpaired t test. Data are representative of four experiments.

IFN-γ production was quantified by ELISA. Both IL-10 and TGF-β significantly inhibited anti-CD3-induced IL-17 production by PBMC in a dose-dependent manner (Fig. 1A). Similarly, IFN-γ production was inhibited by IL-10 and TGF-β (Fig. 1A). IL-17 and IFN-γ production was inhibited at concentrations of IL-10 and TGF-β that did not inhibit proliferation of T cells (Fig. 1A), suggesting that production of IL-17 and IFN-γ was more sensitive to

suppression by IL-10 and TGF-β than were proliferative responses.

To determine whether IL-10 and TGF-β inhibited IL-17 and IFN-γ production by acting directly on the T cells or indirectly through the APC, we examined the effect of IL-10 and TGF-β on purified CD4$^+$ T cells stimulated with anti-CD3 and anti-CD28 in the absence of APC. TGF-β significantly suppressed IL-17 and IFN-γ production by CD4$^+$ T cells stimulated with anti-CD3 and anti-CD28 (Fig. 1B). Furthermore, IL-10 suppressed IFN-γ production, but it had little effect on IL-17 production by purified CD4$^+$ T cells (Fig. 1B). We also examined the effect of TGF-β on IL-17 and IFN-γ production by purified naive T cells. Although the concentration of IL-17 induced with anti-CD3 and anti-CD28 (in absence of APC or T cell-activating cytokine) was very low, TGF-β also suppressed IL-17 and IFN-γ production by CD45RA$^+$CD4$^+$ naive T cells stimulated with anti-CD3 and anti-CD28 (without and with TGF-β: IFN-γ, 12,956 reduced to 1,992 pg/ml; IL-17, 26 reduced to 0 pg/ml).

Since IL-10 and TGF-β may target APC as well as T cells directly, we examined their influence on the production of T cell-activating cytokines by purified monocytes. It is well established that innate IL-12 production helps to direct the induction of Th1 cells (31), whereas TGF-β with IL-6 or IL-21 (3) (3, 14, 15) can enhance the development of murine Th17 cells, while IL-1 and IL-23 (18, 16) have also been implicated in promoting differentiation and/or expansion of murine and human Th17 cells. Therefore, we examined the influence of IL-10 and TGF-β on IL-1β, IL-6, IL-12p40, IL-12p70, and IL-23 production by CD14$^+$ monocytes in response to LPS and IFN-γ. IL-10 had a profound suppressive effect on IL-1β, IL-6, IL-12p40, IL-12p70, and IL-23 production by monocytes, with significant inhibition across a range of concentrations (Fig. 2A). In contrast, TGF-β had little effect on IL-1β or IL-6 production, but significantly inhibited IL-12p40 and IL-23 production (Fig. 2B). The effect on IL-12p70 production was more variable and difficult to interpret because of the low concentrations of this cytokine produced by the LPS- and IFN-γ-stimulated monocytes. These findings demonstrate that the anti-inflammatory cytokines IL-10 and TGF-β exert regulatory control on Th17 and Th1 cells, both directly and indirectly by inhibiting innate cytokines that promote the activation or expansion of these CD4$^+$ T cell subtypes. In general, IL-10 had a more profound effect than did TGF-β on monocyte production of T cell-activating cytokines, whereas TGF-β had a more significant direct effect on CD4$^+$ T cell IL-17 production.

NS4 induces innate TGF-β and IL-10 production by monocytes from normal individuals and HCV-infected patients

Pathogen-derived molecules can elicit immunoregulatory cytokines to suppress Ag-specific responses. We have previously demonstrated that HCV infection is associated with the generation of HCV-specific IL-10-secreting T cells and have also demonstrated that the HCV NS4 protein induces innate IL-10 production by monocytes from normal donors (21, 25). Here, we investigated whether NS4 could stimulate production of innate TGF-β and IL-10 by cells from normal donors and HCV-infected patients. PBMC from normal donors were stimulated for 24 h with NS4, medium alone, or with LPS as a positive control. PBMC constitutively secreted high concentrations of latent TGF-β into culture supernatants. Treatment with either NS4 or LPS significantly ($p < 0.01$) enhanced latent TGF-β production by PBMC from normal donors (Fig. 3A). This was accompanied by an increase in concentrations of active TGF-β; however, the concentrations produced in response to both NS4 and LPS were not significantly greater than that produced by cells treated with medium only (Fig.
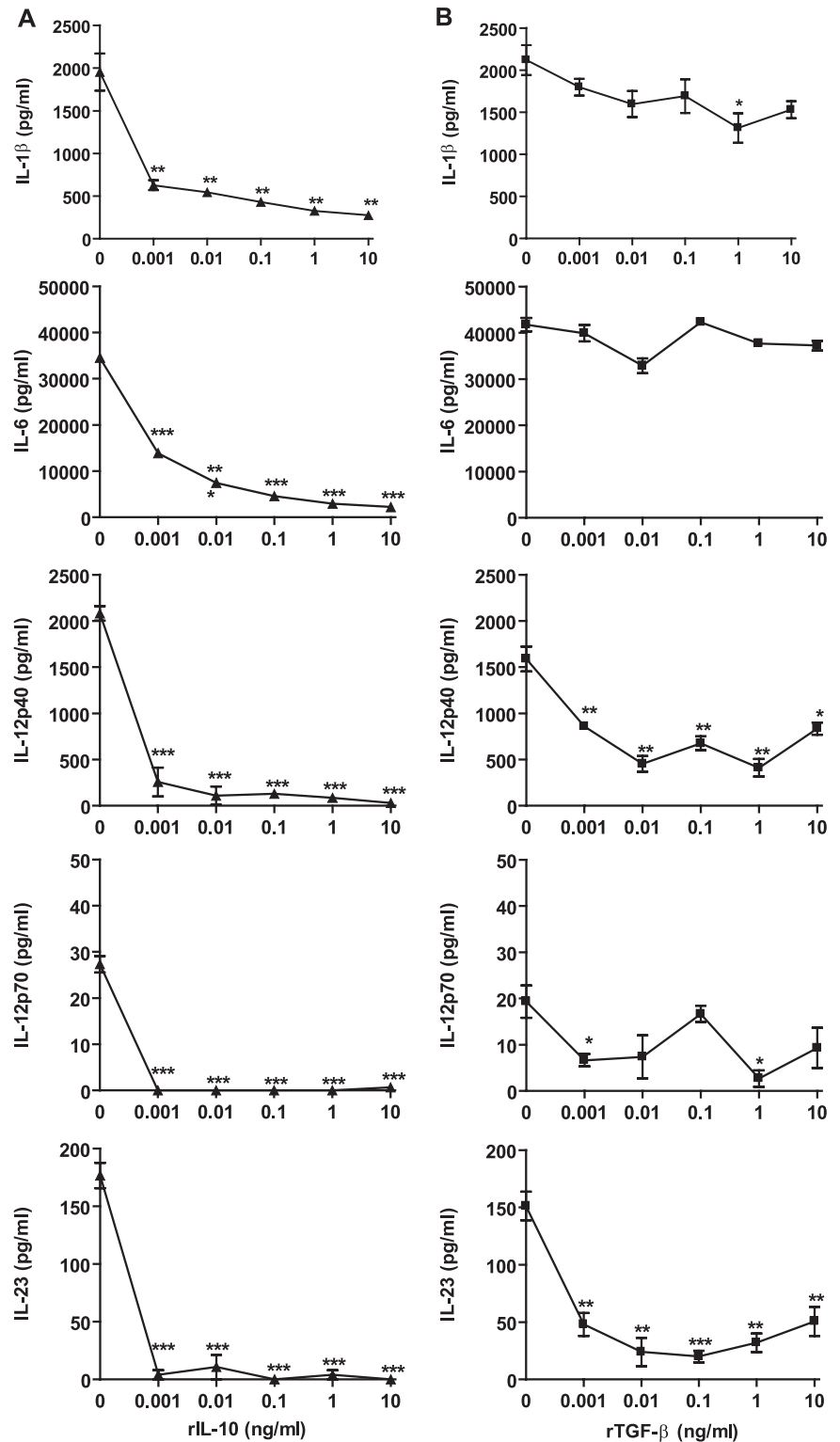
FIGURE 2. Inhibitory effects of IL-10 and TGF-β on monocyte production of T cell-activating cytokines. CD14⁺ monocytes isolated from normal PBMC were stimulated with LPS (100 ng/ml) and IFN-γ (20 ng/ml) in the presence of medium only or increasing concentrations (0.001–10 ng/ml) of recombinant human IL-10 (*A*) or TGF-β (*B*). After 48 h, supernatants were removed and the concentrations of IL-1β, IL-6, IL-12p40, IL-12p70, and IL-23 were determined by ELISA. Monocytes stimulated with medium only did not produce any detectable cytokines. The 0 value on the *x*-axes represents the positive controls, which are cells stimulated with LPS and IFN-γ in the absence of added rIL-10 or rTGF-β. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$: with vs without IL-10 or TGF-β. Results are means ± SD values for triplicate cultures and data are representative of three experiments.

3*A*). NS4 also induced significant IL-10 production by PBMC from normal individuals (Fig. 3*A*; $p < 0.001$). PBMC from HCV-infected patients secreted significant concentrations ($p < 0.001$) of latent TGF-β, which surpassed that generated with LPS (Fig. 3*B*), and also produced substantial concentrations of IL-10 (Fig. 3*B*), which were greater than that detected in NS4-stimulated cells from normal donors (Fig. 3*A*).

Since monocytes and dendritic cells are an important source of TGF-β in vivo, we examined the possibility that CD14⁺ or CD11C⁺ cells were the source of the latent TGF-β in NS4-treated

PBMC. While PBMC from a normal donor secreted IL-10 and latent TGF-β in response to NS4, PBMC depleted of CD14⁺ cells failed to secrete these cytokines in vitro (Fig. 3*C*). CD14⁺ monocytes produced IL-10 in response to NS4, but it did not secrete detectable TGF-β. Furthermore, we could not detect TGF-β or IL-10 production by CD11c⁺ cells (Fig. 3*C*).

We next investigated whether we could detect NS4-induced TGF-β production by monocytes from normal donors and HCV-infected patients using intracellular or surface staining and FACS analysis. PBMC from normal donors were stimulated with NS4 for
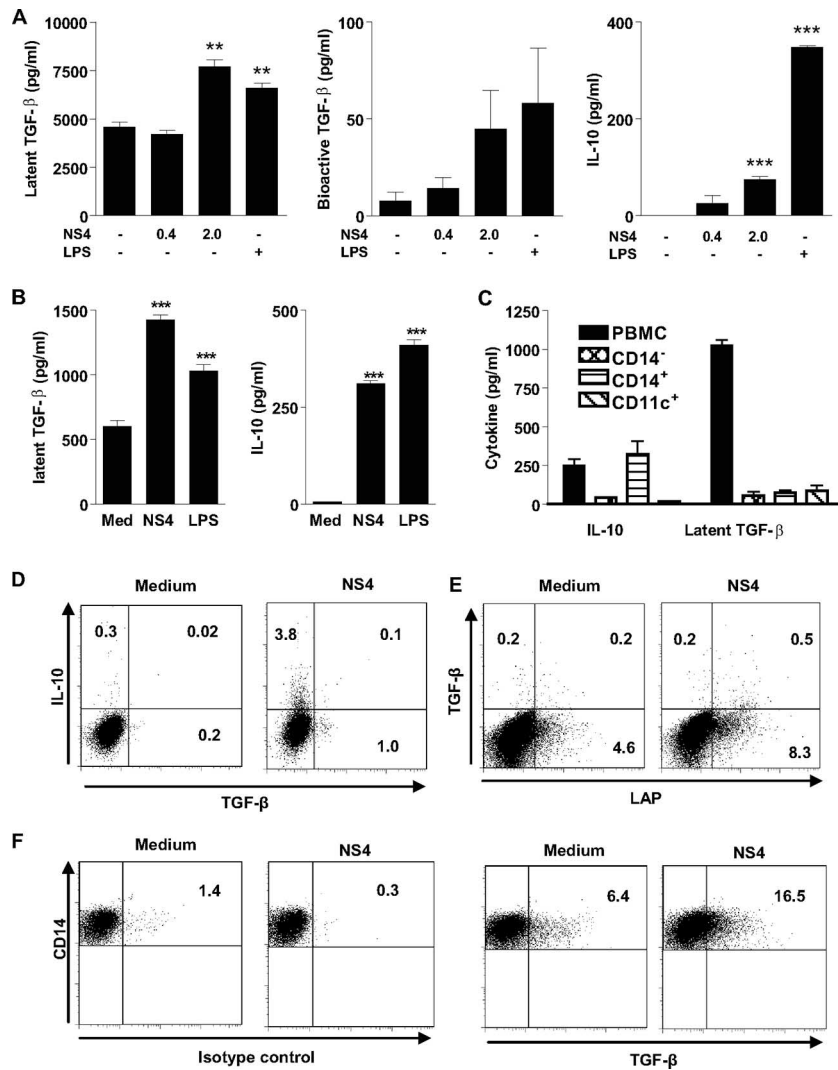
FIGURE 3. NS4 induces synthesis of TGF-β by PBMC and monocytes. *A*, PBMC from a normal donor were cultured with NS4, LPS, or medium only. After 24 h, culture supernatants were harvested and the concentrations of latent TGF-β, bioactive TGF-β, and IL-10 were measured by ELISA. **, $p <$ 0.01 and ***, $p <$ 0.001 for NS4 or LPS vs medium control, unpaired t test. *B*, PBMC from a chronically HCV-infected donor were cultured with NS4 (2 μg/ml), LPS, or medium (Med) only. After 24 h, culture supernatants were harvested and the concentrations of latent TGF-β and IL-10 were measured by ELISA. ***, $p <$ 0.001 for NS4 or LPS vs medium control, by unpaired t test. *C*, PBMC, CD14-depleted PBMC, purified CD14$^+$, or CD11c$^+$ cells from a normal donor were stimulated with medium only or 2 μg/ml NS4, and after 24 h the concentrations of latent TGF-β and IL-10 in supernatants were quantified by ELISA. Results for TGF-β are expressed as net cytokine after subtraction of medium only controls, which were 608 $\pm$ 135, 99 $\pm$ 5.2, 37 $\pm$ 2, and 114 $\pm$ 34 pg/ml, respectively, for PBMC, CD14-depleted PBMC, purified CD14$^+$, or CD11c$^+$ cells. IL-10 was below the limit of detection for cells stimulated with medium only. *D*, PBMC from a normal donor were stimulated with 2 μg/ml NS4 or medium only for 5 h in the presence of monensin for the last 4 h. Cells were stained for surface CD14 and for intracellular expression of IL-10 and TGF-β and analyzed by flow cytometry. Plots represent expression of IL-10 vs TGF-β on CD14$^+$-gated monocytes. *E*, PBMC from a normal donor were stimulated with 2 μg/ml NS4 or medium only for 20 h, followed by cell-surface staining for CD14, TGF-β, and LAP and analysis by flow cytometry. The dot plots show cell-surface TGF-β or LAP expression on CD14-gated monocytes. *F*, PBMC from a HCV-infected patient were stimulated with 2 μg/ml NS4 or medium only for 20 h, followed by cell-surface staining with anti-CD14 and anti-TGF-β or an isotype control Ab and analysis by flow cytometry. The results are representative of four experiments with cells from healthy donors and three from HCV-infected patients.

5 h, after which they were stained for cell-surface expression of CD14 and intracellular IL-10 and TGF-β. Both TGF-β and IL-10 accumulated over time in NS4-treated CD14$^+$ monocytes, but not in medium-treated monocytes (Fig. 3*D*). A higher proportion of monocytes from a normal donor produced IL-10 (3.8%) than TGF-β (1.0%). Interestingly, most monocytes that produced cytokines in response to NS4 produced IL-10 or TGF-β, whereas only 0.1% produced both cytokines.

TGF-β is secreted as a latent complex of a 25-kDa dimeric protein bound to LAP. Biologically active TGF-β is released by cleavage and dissociation from LAP, which is retained on the cell surface. Here, we examined cell-surface expression of both TGF-β

and LAP on monocytes after incubation with NS4 or medium only. LAP was constitutively expressed on ~4.5% of medium only-treated monocytes and this increased to almost 9% following stimulation with NS4 (Fig. 3*E*). Furthermore, a higher percentage of TGF-β^+LAP$^+$ monocytes was detected in PBMC stimulated with NS4 than with medium only. We also examined the ability of NS4 to promote TGF-β production by monocytes from HCV-infected donors. FACS analysis with an anti-TGF-β Ab revealed higher constitutive cell-surface expression of TGF-β in monocytes from HCV-infected compared with normal donors, and the percentage of cells staining positive for TGF-β was substantially enhanced in monocytes from HCV-infected donors following in
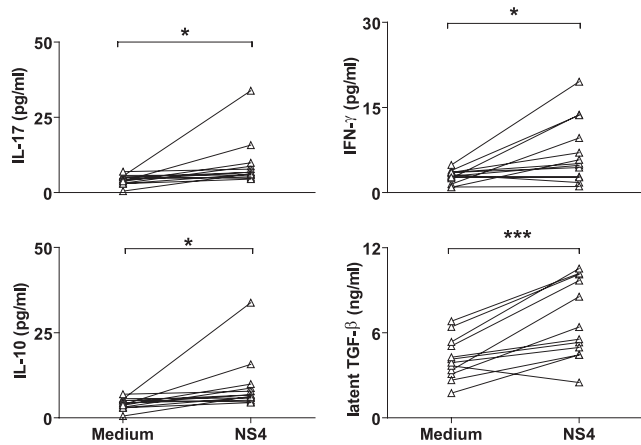
FIGURE 4. NS4 induces IL-17, IFN-γ, TGF-β, and IL-10 production by PBMC from HCV-infected patients. PBMC isolated from HCV-infected patients were cultured with 2 μg/ml NS4 or medium alone. After 72 h, culture supernatants were harvested and the concentrations of IL-17, IFN-γ, IL-10, and TGF-β were determined by ELISA. Results were compared using a Wilcoxon matched pairs test. *, $p < 0.05$ and ***, $p < 0.005$ for NS4 treated vs medium control.

vitro stimulation with NS4 (Fig. 3F). Collectively, these findings demonstrate that HCV NS4 stimulates IL-10 and TGF-β production by human monocytes, which is enhanced in cells from HCV-infected patients.

Induction of pro- and anti-inflammatory cytokines by NS4 in HCV-infected patients

We have previously reported that HCV infection is associated with the induction of Ag-specific IL-10 and IFN-γ production (21).

However, the potential role of IL-17 and TGF-β has not yet been examined. Here, we investigated whether Ag-specific Th17 cells were detectable in a cohort of patients who had all previously mounted an anti-HCV immune response (HCV Ab[+]). We stimulated PBMC with NS4, using an optimal concentration of Ag determined from preliminary experiments (data not shown). After 72 h of culture the concentrations of IL-17, IFN-γ, IL-10, and latent TGF-β were quantified in the cell culture supernatants by ELISA. NS4-stimulated PBMC produced significantly more IL-17 and IFN-γ ($p < 0.05$) than did PBMC cultured with medium only. NS4 treatment also resulted in a significant induction of IL-10 ($p < 0.05$) and latent TGF-β ($p < 0.001$) (Fig. 4).

To confirm that T cells were the source of IL-17 produced in response to NS4, we assessed intracellular cytokine expression by flow cytometry. Consistent with previous studies, the frequency of Ag-specific IFN-γ- or IL-17-producing T cells was too low to detect ex vivo without amplification, and therefore we stimulated CFSE-labeled PBMC with NS4 or medium alone for 8 days and then restimulated the cells with PMA and ionomycin followed by intracellular cytokine staining. The CFSE^{low} cells were those that had proliferated in response to Ag and were therefore assumed to be Ag-specific. We assessed the frequency of CD4^{+} and CD8^{+} T cells that proliferated and secreted IL-17 and/or IFN-γ. Since PMA and ionomycin stimulation down-regulates CD4 expression, CD4^{+} T cells were identified as CD3^{+}CD8^{−} T cells. In the representative example given in Fig. 5A, there was <0.4% nonspecific proliferation in the absence of stimulation, while ~12% of CD4^{+} T cells had proliferated in response to NS4. Both IL-17 and IFN-γ were detectable within the NS4-specific CD4^{+} T cell population. While most cytokine-producing NS4-specific cells were IL-17^{−}IFN-γ^{+}CD4^{+} or IL-17^{+}IFN-γ^{−}CD4^{+} T cells, there was also a small proportion of IL-17^{+}IFN-γ^{+}CD4^{+} T cells (Fig. 5B).

FIGURE 5. Induction of NS4-specific Th17 and Th1 cells in HCV patients. PBMC from HCV-infected patients were labeled with CFSE and cultured with 2 μg/ml NS4 or medium alone for 8 days and then restimulated with PMA and ionomycin in the presence of brefeldin A. After 6 h, the cells were stained for surface CD3 and CD8 and intracellularly for IL-17 and IFN-γ. *A*, Dot plots show CFSE dilution and cytokine production of CD3^{+}CD8^{−} (CD4^{+}) T cells from a representative HCV patient, which were stimulated with NS4 or medium only. *B*, IFN-γ vs IL-17 staining for NS4-specific (CFSE^{low}) CD4^{+} T cells. *C*, The percentage of Ag-specific (CFSE^{low}) cells that produced IL-17, IFN-γ, or IL-17 and IFN-γ in response to NS4 are shown for 10 normal and 10 HCV-infected donors. Results were compared using a Wilcoxon matched pairs test. *, $p < 0.05$ and **, $p < 0.01$ for NS4 treated vs medium control.
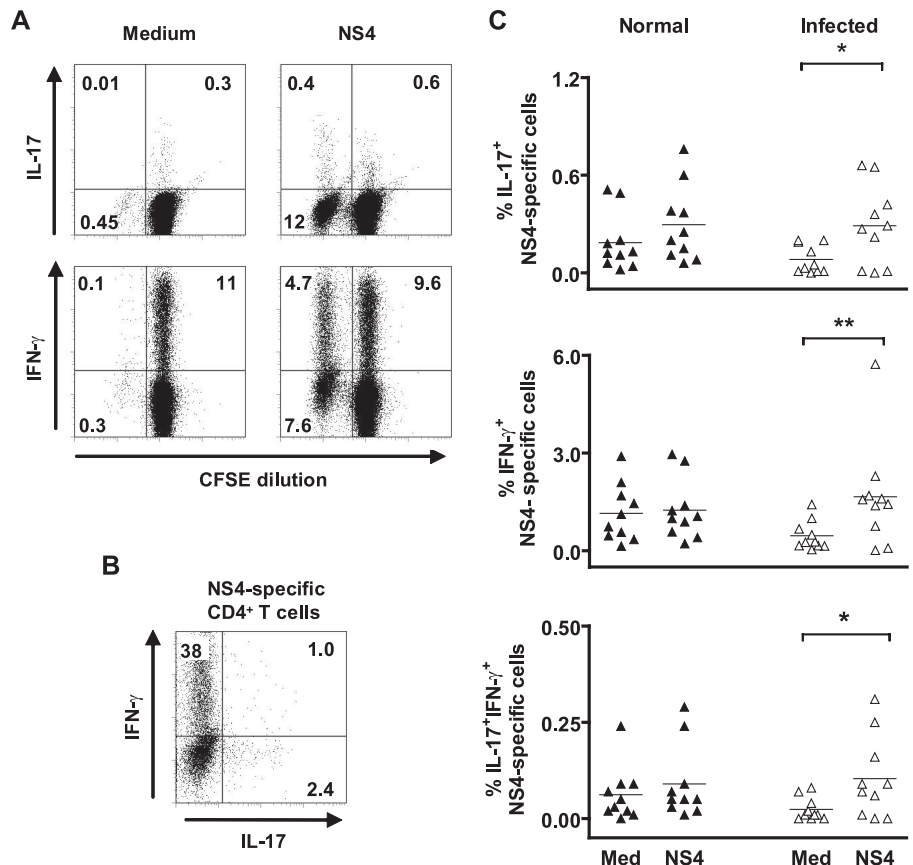
We next compared IL-17 and IFN-γ production by CD4$^+$ T cells from a cohort of HCV-exposed patients ($n = 10$) with that from normal individuals, who had no known history of HCV infection ($n = 11$). For each individual, the number of IL-17$^+$IFN-γ$^-$CD4$^+$, IL-17$^-$IFN-γ$^+$CD4$^+$, or IL-17$^+$IFN-γ$^+$CD4$^+$ T cells that proliferated in response to stimulation with NS4 or medium was calculated and expressed as a percentage of total CD4$^+$ T cells. A small but significant proportion of CD4$^+$ T cells from HCV-infected individuals proliferated and produced IL-17 in response to NS4 (Fig. 5C). Similarly, a small but significant proportion of CD4$^+$ T cells from HCV-infected individuals proliferated and produced IFN-γ in response to NS4. NS4-specific proliferation of Th17 and Th1 cells was not detected in PBMC from normal individuals. NS4-specific IL-17$^+$IFN-γ$^+$CD4$^+$ T cells were also detected in PBMC from a significant number of HCV-infected patients, but not normal individuals (Fig. 5C). In general, approximately one-third of NS4-specific Th17 cells co-produced IFN-γ. In contrast with the findings for CD4$^+$ T cells, only $1.6 \pm 1.0\%$ of CD8$^+$ T cells from HCV-infected patients proliferated in response to NS4, and a very low frequency of these secreted IL-17 ($0.07 \pm 0.07\%$) or IFN-γ ($0.5 \pm 0.5\%$). Furthermore, there was no significant difference in the frequency of NS4-induced proliferation of CD8$^+$ T cells from HCV-infected and normal individuals (data not shown). However, since the T cells were stimulated in vitro with NS4 protein, which would not be processed by the MHC class I pathway, these findings do not rule out the possibility that IL-17-producing CD8$^+$ T cells are induced during HCV infection.

The effect of neutralizing IL-10 and TGF-β on NS4-specific Th1 and Th17 responses

Having shown that NS4 induced IL-10 and TGF-β production by monocytes, we next tested whether these cytokines inhibited HCV-specific Th1 and Th17 responses. PBMC from HCV-infected patients were stimulated with NS4 in the presence of neutralizing anti-IL-10 or anti-TGF-β Ab, and IL-17 and IFN-γ production was quantified by ELISA. Stimulation of PBMC from HCV-infected patients with NS4 induced low concentrations of IL-17 and IFN-γ, but relatively high concentrations of IL-10 and latent TGF-β (Fig. 6). The efficacy of the anti-IL-10 was confirmed by the abrogation of NS4-induced IL-10. Neutralizing IL-10 significantly enhanced the secretion of NS4-specific IL-17 and IFN-γ (Fig. 6A, $p < 0.05$). Although TGF-β was not completely neutralized by the addition of anti-TGF-β, it was sufficient to significantly enhance production of NS4-specific IL-17 ($p < 0.01$) and IFN-γ ($p < 0.001$) by PBMC from HCV-infected patients (Fig. 6B).

We also compared HCV Ab$^+$PCR$^+$ with HCV Ab$^+$PCR$^-$ patients for HCV-specific IL-17 and IFN-γ production and the effect of anti-IL-10 and anti-TGF-β on these responses. NS4-specific IL-17 and IFN-γ production was detectable in both PCR$^+$ and PCR$^-$ patients, and anti-IL-10 and anti-TGF-β enhanced these responses in both cohorts of patients. IFN-γ production was higher in the PCR$^+$ patients and IL-17 higher in the PCR$^-$ patients, but the differences were not statistically significant (IL-17 production (pg/ml) in presence of anti-IL-10, 20.5 ± 11.0 for PCR$^-$ and 12.2 ± 3.4 for PCR$^+$; IL-17 (pg/ml) in the presence of anti-TGF-β, 27.5 ± 16.1 for PCR$^-$ and 24.1 ± 7.4 for PCR$^+$; IFN-γ production (pg/ml) in presence of anti-IL-10, 27.6 ± 18.5 for PCR$^-$ and 44.2 ± 19.9 for PCR$^+$; IFN-γ production (pg/ml) in the presence of anti-TGF-β, 43.4 ± 27.1 for PCR$^-$ and 92.8 ± 39.9 for PCR$^+$).

To verify that IL-10 or TGF-β constrain IL-17 and IFN-γ production by NS4-specific CD4$^+$ T cells, we used CFSE and intracellular cytokine staining. PBMC from HCV-infected or normal individuals were stimulated with NS4 in the presence and absence
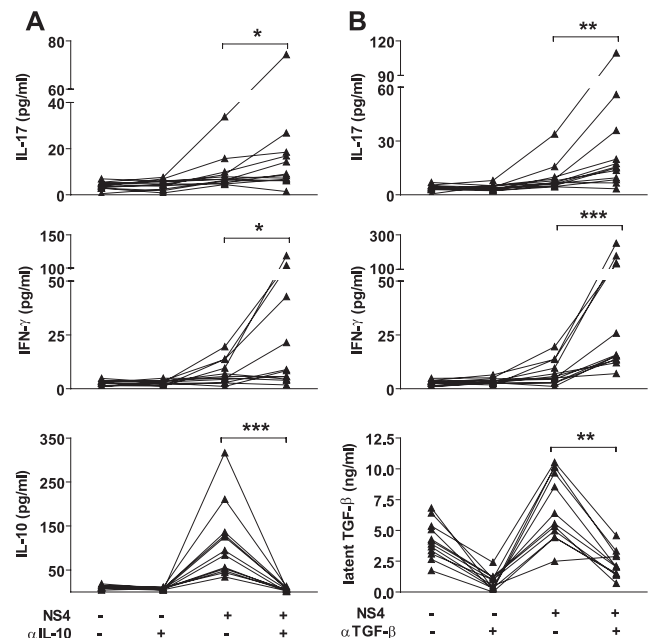
FIGURE 6. Neutralization of IL-10 and TGF-β enhances NS4-specific IL-17 and IFN-γ production by PBMC from HCV-infected patients. PBMC from HCV-infected patients were stimulated with NS4 or medium alone, either in the presence or absence of neutralizing anti-IL-10 (A) or anti-TGF-β Ab (B). After 72 h, cell culture supernatants were harvested and IL-17, IFN-γ, IL-10, and TGF-β concentrations were determined by ELISA. Results were compared using a Wilcoxon matched pairs test. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.005$: NS4 + anti-TGF-β or IL-10 vs NS4 alone.

of neutralizing anti-IL-10 or anti-TGF-β. Although blocking IL-10 significantly increased the secretion of IL-17 and IFN-γ as measured by ELISA (Fig. 7A), we did not find an increase in proliferation of NS4-specific Th1, Th17, or total CD4$^+$ T cells in the presence of anti-IL-10 (IL-17-secreting: NS4, $0.21 \pm 0.05\%$, NS4 + anti-IL-10, $0.24 \pm 0.05\%$; IFN-γ-secreting: NS4, $2.0 \pm 0.2\%$, NS4 + anti-IL-10, $2.1 \pm 0.2\%$; total CD4$^+$ cells: NS4, $5.2 \pm 1.6\%$, NS4 + anti-IL-10, $4.6 \pm 0.7\%$). On the other hand, neutralization of TGF-β significantly enhanced the frequency of proliferating NS4-specific IL-17-secreting CD4$^+$ T cells when compared with cells stimulated with NS4 alone (Fig. 7A, $p < 0.05$). We also examined the combined effects of anti-TGF-β and anti-IL-10 and found that there was no enhancement of IL-17 and IFN-γ production over that observed when cells were cultured with Ag in the presence of either Ab alone (data not shown). The enhancement of IL-17-producing T cells by anti-TGF-β was observed in PBMC from HCV-exposed patients, but not from normal individuals. Anti-TGF-β also significantly enhanced proliferation of Th17 ($p < 0.05$) and Th1 ($p < 0.01$) cells cultured in medium only (Fig. 7, A and B). This was observed using PBMC from HCV-infected patients but not PBMC from normal individuals, suggesting that constitutive expression of TGF-β by PBMC from HCV-exposed patients has an inhibitory effect on steady-state T cells. Neutralization of TGF-β also significantly enhanced the frequency of NS4-specific IL-17$^+$IFN-γ$^+$CD4$^+$ ($p < 0.01$) and IL-17$^+$IFN-γ$^-$CD4$^+$ T cells ($p < 0.05$) in HCV-infected, but not normal, individuals (Fig. 7, C and D). Similarly, anti-TGF-β significantly enhanced the frequency of both IL-17$^+$IFN-γ$^-$CD4$^+$ and IL-17$^+$IFN-γ$^+$CD4$^+$ T cells in medium only-treated PBMC from HCV-infected patients (Fig. 7, C and D; $p < 0.05$).

FIGURE 7. Neutralization of TGF-β enhances the frequency of NS4-specific IL-17 and IFN-γ CD4+ T cells detected in PBMC from HCV-infected patients. PBMC from HCV-infected patients were labeled with CFSE and stimulated for 8 days with NS4 or medium only in the presence or absence of neutralizing anti-TGF-β Ab. The cells were then restimulated with PMA and ionomycin in the presence of brefeldin A and stained for CD3, CD8, and intracellular IL-17 and IFN-γ. The frequency of NS4-specific (CFSE^{low}), IL-17$^+$ (A), IFN-γ$^+$ (B), IL-17$^+$IFN-γ$^+$ (C), and IL-17$^+$IFN-γ$^-$ (D) CD4$^+$ T cells was plotted as a percentage of total CD4$^+$ T cells. Results were compared using a Wilcoxon matched pairs test. *, $p < 0.05$ and **, $p < 0.01$.
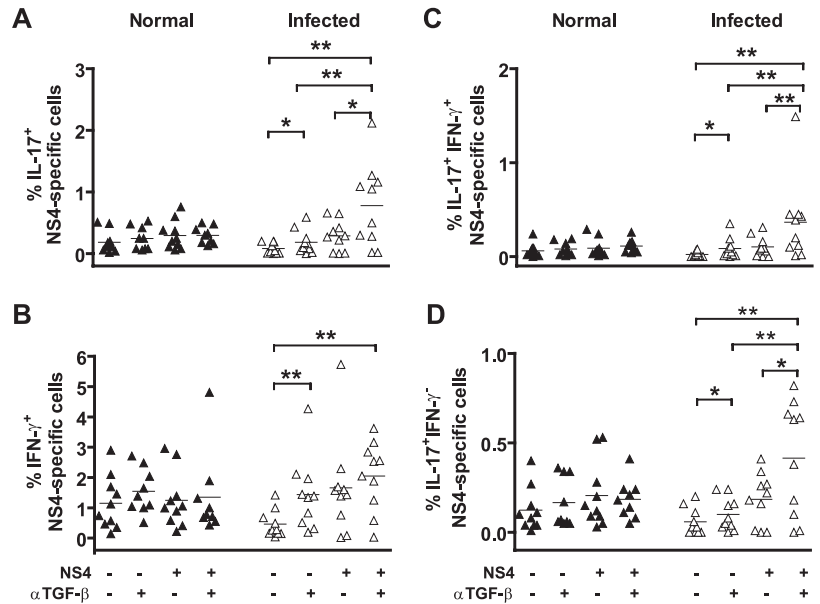
Discussion

In this study, we have demonstrated that human Ag-specific Th17 cells are regulated by TGF-β and IL-10. We report for the first time that infection with HCV is associated with the induction of Ag-specific Th17 cells and that these cells are suppressed by TGF-β, which was expressed by monocytes in response to the HCV NS4 protein. PBMC from HCV-infected donors secreted IL-17, IFN-γ, TGF-β, and IL-10 following in vitro stimulation with NS4. Furthermore, the NS4 protein induced secretion or surface expression of TGF-β, as well as IL-10 by PBMC and monocytes from normal donors. Neutralization of TGF-β, and to a lesser extent IL-10, enhanced NS4-specific IL-17 and IFN-γ production by PBMC from HCV Ab$^+$ donors. Analysis of the frequency of cytokine-secreting NS4-specific CD4$^+$ T cells using intracellular cytokine staining and CFSE labeling revealed a low frequency of Ag-specific Th17 cells in a cohort of HCV-infected donors, which was significantly enhanced following in vitro neutralization of TGF-β. Conversely, direct addition of recombinant TGF-β or IL-10 inhibited IL-17 and IFN-γ production by anti-CD3-activated PBMC, confirming that both Th1 and Th17 cells are regulated by the anti-inflammatory cytokines IL-10 and TGF-β.

It has recently been demonstrated that Th17 cells are the primary pathogenic T cells in organ-specific autoimmune diseases (7–10). Th17 cells have also been shown to have a protective role in certain bacterial and fungal infections, where they promote chemokine and proinflammatory cytokine production and consequent recruitment and activation of neutrophils and macrophages (4, 12, 13, 32). While the inflammatory functions of Th17 cells are important for clearance of bacterial infections, if these cells are uncontrolled they can result in tissue damage, such as that seen in autoimmune diseases. Conversely, studies in a mouse model of liver injury have suggested that IL-22 (which is coexpressed with IL-17 by Th17 cells), but not IL-17, had an important protective role in virus-induced inflammation and hepatocellular carcinoma (6). However, consistent with a recent report (33), we found low concentrations of this cytokine in serum of HCV-infected patients, and there was no significant difference between patients and normal controls (our unpublished observations). We do not rule out a role for IL-22, but a full understanding of its function in HCV infection will require further investigation.

Although the induction and regulation of Th17 in viral infections have received little attention, it is possible that IL-17 is involved in viral clearance or in virus-induced immunopathology. Elevated IL-17 production has been demonstrated in the cornea of mice infected with HSV-1, and mice lacking IL-17R had a transient decrease in neutrophil infiltration, but corneal pathology and viral clearance were not significantly different than in wild-type mice (36). IL-17 production has been demonstrated in CD4$^+$ and CD4$^-$ T cells from HIV-infected individuals following in vitro stimulation with PMA and ionomycin (35). However, the Ag specificity or function of these cells was not addressed. In the present study, we demonstrated that Ag-specific Th17 cells can be detected in the peripheral blood of HCV-infected individuals. Although, limitations in access to, and cell yields from, liver biopsy precluded analysis of these cells in the liver, we did demonstrate that circulating HCV-specific Th17 cells are suppressed by virus-induced TGF-β. Therefore, it is reasonable to speculate that Th17 cells may have a role in immunity to HCV. Interestingly, IL-17-producing T cells were detected in chronic HCV Ab$^+$ PCR$^+$ patients, but also in HCV Ab$^+$ PCR$^-$ patients who had cleared the infection, and although the numbers were too small to reach statistical significance, there was a trend toward higher IL-17 production by NS4-specific CD4$^+$ T cells from patients who had cleared the infection.

Th17 and Th1 cells both play a role in protective immunity to bacterial infections. However, there is evidence to suggest that IFN-γ may exert regulatory control on Th17 cells, especially in autoimmune diseases. In the present study, we found that the frequency of Ag-specific Th17 cells was ~5-fold less than the frequency of Th1 cells in the peripheral blood of HCV-infected donors. Intracellular staining analysis of CFSE-labeled NS4-activated PBMC revealed no obvious difference in the number of cell divisions undergone by Th1 and Th17 cells in vitro, so this difference in frequency may reflect a preferential priming of Th1 responses in vivo. However, we also found that a significant proportion of Ag-specific IL-17-secreting cells coexpressed IFN-γ. IL-17$^+$IFN-γ$^+$CD4$^+$ T cells are gaining recognition as a genuine population of cells primed in vivo, particularly at mucosal sites (36). IL-17$^+$IFN-γ$^+$CD4$^+$ and IL-17$^+$IFN-γ$^-$CD4$^+$ T cells express receptors for both IL-12 and IL-23, and stimulation with

IL-12 enhances IFN-γ production and T-bet expression (37). Interestingly, the present study revealed that neutralization of IL-10 specifically enhanced proliferation of IFN-γ$^+$IL-17$^+$CD4$^+$ T cells, whereas neutralization of TGF-β enhanced proliferation of both IFN-γ$^+$IL-17$^+$CD4$^+$ and IFN-γ$^-$IL-17$^+$CD4$^+$ T cells.

The differentiation and expansion of Th17 cells appear to be generated by products of TLR and NOD-like receptor ligand activation of innate immune cells, including the inflammatory cytokines IL-1, IL-6, IL-21, and IL-23 (13, 18). However, the anti-inflammatory cytokine TGF-β, which is produced by Ag-specific Treg cells and promotes peripheral conversion of natural Foxp3$^+$ Treg cells (37), has also been implicated in promoting differentiation of murine Th17 cells, especially in combination with IL-6 or IL-21 (3, 14, 15). Interestingly, we found that the HCV NS4 protein induced production of IL-1 and IL-6 (our unpublished observations) as well as TGF-β. However, we also demonstrated that recombinant TGF-β suppressed anti-CD3-induced IL-17 production by PBMC and purified CD4$^+$ T cells, whereas addition of neutralizing Abs to TGF-β enhanced NS4-induced IL-17 and IFN-γ production by T cells from HCV-infected donors. Neutralization of TGF-β also significantly enhanced proliferation, IFN-γ and IL-17 production, by anti-CD3-stimulated CD4$^+$ and CD8$^+$ T cells (data not shown). TGF-β present in serum is known to constitutively inhibit resting CD4$^+$ T cells under steady-state conditions (38). Constitutive signaling through the TGF-β receptor can be detected in resting human CD4$^+$ T cells and, to a lesser extent, CD8$^+$ T cells, and removal of TGF-β by serum starvation enhanced CD4$^+$ T cell proliferative responses, particularly in the context of low levels of Ag and/or costimulation (39). Furthermore, it has recently been shown that HCV-specific CD8$^+$ T cells produce TGF-β, which suppress IFN-γ production by HCV-specific T cells (39).

HCV-infected patients have elevated levels of circulating IL-10 and TGF-β (21, 22). In this study we have demonstrated that NS4 can induce TGF-β and IL-10 synthesis by monocytes from normal donors and to a greater extent by monocytes from HCV-infected patients. Although bioactive TGF-β production was not detectable by ELISA in supernatants from NS4-activated purified monocytes, it was detected from NS4-stimuated PBMC, and this response was lost following depletion of CD14$^+$ cells. Furthermore, we found that stimulation of PBMC with NS4 did induce intracellular and surface expression of TGF-β on CD14$^+$ monocytes. It is possible that monocytes require a second cell population to mediate or activate production of TGF-β. Alternatively the intracellular or surface labeling by FACS may be more sensitive than the ELISA employed, which only quantified secreted TGF-β. The active form of TGF-β can also be surface bound, and the latent form of TGF-β is associated with LAP (40). We found that NS4 significantly enhanced surface expression of LAP on CD14$^+$ monocytes. It has been reported that expression of surface-bound TGF-β in association with LAP has important immunomodulatory functions in dendritic cells (41). Taken together, these findings suggest that NS4 may induce immunomodulatory function in monocytes by enhancing expression of TGF-β.

Our finding suggest that TGF-β functions to attenuate Th17 and Th1 responses in HCV infection. These observations are at variance with reports on murine T cells, which have demonstrated that TGF-β, together with IL-6 or IL-21, can promote the differentiation of Th17 cells (3, 14, 15). However, it has recently been reported that low concentrations TGF-β can synergize with IL-6 and IL-21 to enhance differentiation of murine Th17 cells, but that high concentrations of TGF-β favored Foxp3 expression, which inhibited the function of RORγt and Th17 differentiation (42). It has also been reported that TGF-β alone or in the presence of IL-6

does not promote IL-17 production by naive human T cells (2, 18, 43). Additionally, it has been demonstrated that TGF-β can inhibit IL-17 production by naive human T cells stimulated with IL-1β and IL-6 in the presence of anti-CD3 and anti-CD28 (17). Furthermore, IL-22, produced by human Th17 cells, in response to in vitro stimulation with IL-6 or IL-6 and IL-2 is inhibited by TGF-β (2, 44). However, our findings in mice (16) and a study using human cells (18) have shown that IL-1 in combination with IL-23 can also promote the activation or expansion of Th17 cells. Interestingly, we found that TGF-β not only exerted direct inhibitory effects on IL-17 production by T cells, but also suppressed IL-23 and IL-12p40 production by monocytes. In contrast, and consistent with the established effect of IL-10 on APC function, we found that IL-10 had a profound immunosuppressive effect on IL-1, IL-23, IL-12, and IL-6 production by monocytes, but had a less dramatic effect on T cell IL-17 production. Thus, it appears that both TGF-β and IL-10 can suppress Th17 responses, with the former acting more directly on the T cell and the latter acting primarily on the APC. Since human Th17 cells have been shown to be resistant to suppression by CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (19), the induction of TGF-β may be an important mechanism of constraining proinflammatory Th17 responses. While the role of IL-17 in HCV disease remains to be determined, our demonstration that Th17 responses are inhibited by TGF-β and IL-10 may represent another example of an evasion strategy by the virus to subvert protective immune responses of the host.

Acknowledgments

Disclosures

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