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Blocking of monocyte-associated B7-H1 (CD274) enhances HCV-specific T cell immunity in chronic hepatitis C infection

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Abstract: The establishment of a chronic hepatitis C (CHC) infection is associated with defective HCV-specific T cell responses. Recent studies suggest that negative T cell regulators such as programmed death 1 (PD-1) contribute to the impairment of virus-specific T cell functions in chronic viral infections. However, the implication of peripheral monocytes from CHC patients in the inhibition of HCV-specific T cell responses is only partially defined. In this study, we found that B7-H1, a ligand of PD-1, was significantly up-regulated on monocytes of CHC patients. Proliferation of T cells in response to anti-CD3 antibody was directly suppressed by B7-H1$^+$CD14$^+$ monocytes, and this suppression was reversed by addition of antagonistic B7-H1 mAb. Furthermore, blocking of monocyte-associated B7-H1 (moB7-H1) significantly enhanced the frequency of IFN-γ-producing, HCV-specific CD4$^+$ and CD8$^+$ effector T cells and the production of Th1 cytokines, such as IL-2 but not Th2 cytokines, including IL-4 and IL-10. Upon B7-H1 blockade, production of perforin was also increased in CD8$^+$ T cells stimulated with HCV peptides. Our findings suggest that moB7-H1 inhibits HCV-specific CD4$^+$ and CD8$^+$ T lymphocyte proliferation and suppresses Th1 cytokine production and perforin secretion. Blockade of the B7-H1 pathway thus represents an attractive approach in the treatment of chronic HCV infection. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: *cosignaling molecule · programmed death 1*

INTRODUCTION

HCV is a parenterally transmitted, hepatotropic RNA virus that is estimated to infect 200 million people worldwide [1]. Failure to eradicate HCV during the acute phase of infection often leads to chronic hepatitis C (CHC), which may cause serious complications, such as liver cirrhosis and hepatocellular carcinoma [2, 3]. Viral clearance during the acute hepatitis C largely depends on vigorous and multispecific IFN-γ$^+$, HCV-specific CD8$^+$ T cell responses, and strong Th1 CD4$^+$ T cell responses [4, 5]. In contrast, in chronically infected patients, HCV-specific T cell responses appear weak, oligospecific, and impaired [6, 7]. An increasing body of evidence indicates that HCV-specific CD8$^+$ T cells from CHC patients are functionally deficient, as demonstrated in vitro by an impaired production of type 1 (Th1) cytokines, including IFN-γ, and limited expansion of T cells in response to HCV peptides [4, 7]. A number of studies suggest that these T cell function impairments may be attributed by multiple mechanisms, including a direct modulation of immune function by viral proteins such as core and NS4 proteins [8, 9], T cell exhaustion as a result of chronic stimulation with HCV [10], and inhibition by CD4$^+$ or CD8$^+$ regulatory T cells (Tregs) [11–13], all of which might contribute to the establishment of persistent HCV infection [12, 14].

Members of the B7 family of cosignaling molecules act as positive or negative regulators of T cell activation and tolerance [15]. Among the B7 family, B7-H1 [also known as programmed death 1 (PD-1) ligand or CD274] is believed to stimulate or inhibit T cell immunity by engaging different receptors on T cells or via reverse signaling through B7- H1 itself toward the inside of T cells and dendritic cells [16, 17]. Several in vivo animal studies suggest that endogenous B7-H1 plays suppressive roles in tumor immunity [18], autoimmune diabetes [19], and contact hypersensitivity [20] by multiple mechanisms, including inducing tolerance or apoptosis of effector T cells via its receptor PD-1 and rendering resistance to CTL lysis. In another study, however, ectopic expression of B7-H1 in β-islet cells promoted graft rejection, suggesting that B7-H1 may also stimulate T cell immune responses through an as-yet-unidentified costimulatory receptor(s) [21].

With regard to the involvement of PD-1 in persistent viral infection in chronic viral diseases such as AIDS and CHB, several studies showed that exhausted CD8$^+$ T cells specific for HIV and HBV, respectively, up-regulated PD-1 expression on the surface and restored functional competence in prolifer-

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ation and IFN-γ and IL-2 production upon in vitro blocking of PD-1 engagement to its ligand. These findings indicate that the PD-1:B7-H1 pathway is largely responsible for the reversible immune dysfunction [22, 23]. Recent ex vivo studies using intrahepatic, HCV-specific $CD8^+$ T cells revealed that PD-1 expression was up-regulated in the T cell population, and in vitro blockade of PD-1 by antagonistic B7-H1 mAb leads to an enhanced proliferation and cytokine production from HCV-specific $CD8^+$ T cells [24, 25]. Although these evidences implicate PD-1 in an impaired antiviral function of $CD8^+$ T cells from CHC patients, the cell population responsible for the defective HCV-specific T cell function is only partially defined.

In the present study, we report that B7-H1 is up-regulated on immune cells, including monocytes, B cells, and T cells, in CHC patients. Furthermore, monocyte-associated B7-H1 (moB7-H1) significantly suppresses the proliferation of HCV-specific $CD4^+$ and $CD8^+$ T cells, the production of Th1 cytokines, and the expression of intracellular perforin. Our findings suggest that moB7-H1 may be responsible for the persistent, functional suppression of HCV-specific T cells during chronic HCV infection.

MATERIALS AND METHODS

Study subjects

The study subjects comprised 24 CHC patients (**Table 1**) enrolled at Busan Paik Hospital of Inje University (Busan, Korea) and 21 age-matched, healthy volunteers (data not shown). Written, informed consent was obtained from all participants, and the Institutional Review Board of Busan Paik Hospital of Inje University approved the study. All healthy donors were serologically negative for HCV, HBV, and HIV. All patients were virologically and serologically positive for HCV and had not been treated with IFN-α or ribavirin at the time of the study.

TABLE 1. Patient Characteristics

ID	Age	Sex	ALT (U/L)	HCV genotype	Viral load ($\times 10^6$)
C01	45	F	102	1b	4.5
C02	55	M	177	1b	4.4
C03	35	M	65	1b	1.4
C04	43	M	294	3a	0.9
C05	48	M	92	2a/2c	0.2
C06	60	M	191	2a/2c	2.1
C07	39	M	24	2a/2c	4.3
C08	35	M	334	1b	4.4
C09	34	M	92	2a/2c	0.2
C10	46	M	97	1b	2.1
C11	62	M	122	1b	1.9
C12	35	M	123	1b	0.1
C13	20	M	193	2a/2c	1.7
C14	43	M	57	1b	0.1
C15	36	M	99	1b	4.2
C16	44	M	63	2a/2c	0.3
C35	48	F	33	2a/2c	4.4
C18	40	M	499	1b	3
C19	47	M	108	2a/2c	0.1
C20	32	F	50	1b	0.2
C21	50	M	26	1b	0.4
C22	45	M	24	2a/2c	4.3
C23	56	M	140	1b	4.4
C24	48	F	114	1b	4.5

The table presents the characteristics of 24 CHC patients who have not been treated with IFN-α or ribavirin. Normal subjects show similar age and sex (data not shown). Viral load in copies/ml. ALT, Alanine aminotransferase.

Antibodies and reagents

The following mAb specific for human surface proteins were purchased from eBioscience (San Diego, CA, USA): FITC-conjugated anti-CD4, -CD8, -CD14, and -CD19; PE-conjugated anti-CD8; FITC-conjugated antiperforin, anti-TRAIL, and anti-Fas ligand (anti-FasL); and PE-Cy5-conjugated anti-CD14. PE-conjugated streptavidin was also purchased from eBioscience. Purified anti-CD3 mAb (OKT3) was purchased from BioLegend (San Diego, CA, USA), and recombinant human (rh)IL-2 was from Roche Laboratories (Nutley, NJ, USA). rNS4 protein fused to β-galactosidase was obtained from ViroGen (Watertown, MA, USA). Chicken OVA peptide $OVA_{257–264}$, HLA-A2-restricted NS3 peptide $NS3_{1073–1081}$ (CINGVCWTV), and NS4 peptide $NS4_{1789–1797}$ (SLMAFTAAV) were acquired from Peptron (Daejeon, Korea). We generated an antagonistic hB7-H1 mAb (clone 5H1) that blocks B7-H1 binding to its cognate receptor. The B7-H1-blocking effect of this clone was described previously [17, 26].

Preparation of monocytes and $CD4^+$ and $CD8^+$ T cells

PBMCs were isolated as described previously [27]. To purify monocytes ($CD14^+$) and T cells ($CD4^+$ and $CD8^+$), FITC-conjugated anti-CD14, -CD4, and -CD8 antibodies were used with anti-FITC microbeads (Miltenyi Biotech, Auburn, CA, USA), according to the manufacturer's instructions. Each isolated population of monocytes, $CD4^+$ and $CD8^+$ T cells, was more than 95% pure. In some experiments, B7-H1-expressing $CD14^+$ cells were isolated by sequential selections using anti-FITC microbeads as follows: First, $CD14^+$ cells were negatively purified using FITC-conjugated anti-CD3, -CD19, -CD56, and -CD11c, and then, $B7\text{-}H1^+CD14^+$ cells were positively selected using biotin-conjugated anti-B7-H1 and streptavidin-coated microbeads (eBioscience). Cells from a flow-through of positive selection were used as $B7\text{-}H1^-CD14^+$ cells. Unless otherwise indicated, freshly isolated PBMCs were cultured in complete RPMI media containing 10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone.

Flow cytometry

PBMCs were stained for surface and intracellular markers with fluorescent antibodies, acquired via FACSort (BD Bioscience, Franklin Lakes, NJ, USA), and analyzed using CellQuest software. The cutoff for each marker was based on a relevant isotype control antibody. For surface staining, FITC-conjugated mAb specific for CD4, CD8, CD14, or CD19 (BD Bioscience) were used. Biotin-labeled anti-B7-H1 mAb (5H1) and PE-conjugated streptavidin were used for B7-H1 staining. For intracellular perforin staining, cells were stained with PE-conjugated anti-CD8 or isotype control antibody (BD Bioscience) and subsequently fixed, permeabilized with the Cytofix/Cytoperm kit (BD Bioscience), according to the manufacturer's instructions, and incubated with FITC-conjugated antiperforin mAb.

Expression analysis by quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from PBMCs with TRIzol reagent (Life Technologies, Frederick, MD, USA) and digested with RNase-free DNase (RQ1 DNase, Promega, Madison, WI, USA) to remove a contaminating genomic DNA. The first-strand cDNA was synthesized using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed using a SYBR Supermix kit and an iCycler system (both from Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Primers for hGAPDH and hB7-H1 were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA), according to the software guidelines. Following are the primer sequences used in the study for qRT-PCR: GAPDH, forward 5′-AAC GAC CCC TTC ATT GAC-3′, reverse 5′-TCC ACG ACA TAC TCA GCA C-3′; B7-H1, forward 5′-ATG GTG GTG CCG ACT ACA AG-3′, reverse 5′-GAA TTG GTG GTG GTG GTC TTA C-3′.

Proliferation assay

For stimulation of T cells by anti-CD3 antibody, 2×10^5 purified T cells were cocultured with 2×10^4 homologous monocytes that had been treated with 50 μg mitomycin C/ml for 20 min. They were then activated with soluble anti-CD3 antibody (1 μg/ml) in the presence of 10 μg/ml control Ig or anti-B7-H1 mAb for 3 days. In some experiments, 50 U/ml rhIL-2 was included in the culture with the anti-CD3 antibody. The cells were pulsed with 1 μCi [^{3}H]-thymidine for the final 16 h of culture, and T cell proliferation was determined by incorporation of [^{3}H]-thymidine. In some experiments, negatively isolated 4×10^5 $CD8^+$ T cells and monocytes were cocultured and stimulated with 10 μM NS3 peptide $NS3_{1073–1081}$ (CINGVCWTV) in the presence of control Ig or anti-B7-H1 mAb for 10 days. NS3 peptide-specific $CD8^+$ T cells were enumerated by PE-labeled pentameric-NS3 peptide-HLA-$A2^+$ complexes (Proimmune Ltd., Oxford, UK). Polymyxin B (10 μg/ml) was also included in the cell proliferation and cytokine assays to neutralize potential endotoxin contamination.

ELISA

The concentrations of IL-2, IL-4, IL-10, and IFN-γ in the culture supernatant fractions were measured with ELISA kits (eBioscience) according to the manufacturer's instructions.

ELISPOT assay

To measure IFN-γ production by HCV-specific T cells in response to HCV protein or peptides, we used an ELISPOT assay as described previously [28]. Briefly, 96-well nitrocellulose-backed plates (Millipore, Bedford, MA, USA) were coated with anti-hIFN-γ mAb (clone 4S.B3) overnight at 4°C, washed in sterile PBS, and blocked for 2 h with 10% FBS. Then, 2×10^5 freshly isolated $CD4^+$ or $CD8^+$ T cells were cocultured with 2×10^4 homologous $CD14^+$ monocytes (HLA-$A2^+$) in complete media containing: 5 μg/ml rNS4 protein or β-galactosidase (for $CD4^+$ T cells), a peptide mix containing 10 μM HLA-A2-restricted NS3 and NS4 peptides, or irrelevant chicken OVA peptide (for $CD8^+$ T cells). Unless otherwise stated, cells were cultured in the presence of 10 μg/ml control Ig or anti-B7-H1 mAb for 30 h. The plates were washed in PBS/0.05% Tween 20 and incubated for 2 h with biotin-conjugated secondary antibody against hIFN-γ (clone myeloid differentiation protein-1). After washing the plates and incubating them for 1 h with HRP-conjugated streptavidin, the assay was developed with Adenylate energy charge substrate (BD Biosciences). The colorimetric reaction was stopped by washing in distilled water. The plates were air-dried, and spots were quantified using an ELISPOT reader system (AID, Strasbourg, Germany).

Statistical analysis

Mean values were compared between groups of normal subjects and patients with CHC using the Mann-Whitney U-test. In some experiments, the Wilcoxon rank-signed test was used to compare the mean values. Statistical analysis was performed using the SPSS-PC (Version 10.0 for Windows). P values <0.05 were considered statistically significant.

RESULTS

B7-H1 is up-regulated in PBMCs of CHC patients

B7-H1 expression was examined initially by qRT-PCR analysis of total RNAs purified from PBMCs from normal subjects and CHC patients. As shown in **Figure 1A**, the level of B7-H1 mRNA expression was significantly higher in patient PBMCs than in normal subject PBMCs ($P=0.001$). Flow cytometric analysis also revealed that B7-H1 was up-regulated on patient immune cells including $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes compared with their normal counterparts (Fig. 1, B and C; $P=0.001$ for $CD4^+$ and $CD8^+$ T cells; $P=0.002$ for $CD14^+$ monocytes; $P=0.040$ for $CD19^+$ B cells). We also compared the expression of PD-1 on the T cells from normal and patient subjects. We found that patient $CD4^+$ and $CD8^+$ T cells significantly up-regulated a median PD-1 expression (i.e., the MFI) on the surface compared with normal subject T cells (Fig. 1D, left panel; $P=0.001$ for $CD4^+$ T cells; $P=0.003$ for $CD8^+$ cells). However, the frequency of PD-1-positive T cells was increased significantly only in patient $CD4^+$ T cells, not $CD8^+$ T cells (Fig. 1D, right panel; $P=0.027$ for $CD4^+$ T cells).

Monocytes from CHC patients inhibit anti-CD3 antibody-mediated T cell proliferation

As it has been suggested that B7-H1-expressing $CD14^+$ monocytes may be involved in AIDS progression [29], we analyzed whether patient monocytes could affect the ability of T cells to proliferate. As shown in **Figure 2A**, although patient $CD4^+$ T cells could expand in response to anti-CD3 antibody, the proliferation was significantly lower than that of normal $CD4^+$ T cells ($P=0.015$). However, patient $CD8^+$ T cell expansion in response to anti-CD3 antibody was not significantly reduced compared with its normal counterpart (Fig. 2B). The expansion of patient $CD4^+$ and $CD8^+$ T cells was suppressed further when homologous $CD14^+$ monocytes were added to the T cell culture ($P=0.028$ for $CD4^+$ T cells; $P=0.045$ for $CD8^+$ T cells). The suppression, however, could be reversed by adding IL-2 to the culture ($P=0.002$ for $CD4^+$ T cells; $P=0.004$ for $CD8^+$ T cells), although the enhancement of proliferation was much less prominent than that observed for normal subject T cells ($P=0.005$ for $CD4^+$ T cells; $P=0.024$ for $CD8^+$ T cells). The proliferation of normal $CD4^+$ and $CD8^+$ T cells in response to anti-CD3 antibody was not affected by the addition of homologous $CD14^+$ monocytes. The results suggest that CHC patient $CD14^+$ monocytes play a role in impaired T cell proliferation, which could be partially reversed by IL-2.

moB7-H1 is involved in suppression of T cell responses

We further investigated whether up-regulation of B7-H1 in patient monocytes is responsible for the inhibition of T cell responses by adding antagonistic B7-H1 mAb (clone 5H1) to cocultures of T cells and mitomycin C-treated monocytes. Blocking of moB7-H1 with anti-B7-H1 mAb dramatically restored the ability of patient T cells, not normal subject T cells, to expand in response to anti-CD3 antibody compared with the group treated with control Ig (**Fig. 3A,** left panel; $P=0.001$ for $CD4^+$ and $CD8^+$ T cells). moB7-H1 blockade also significantly increased the Th1 cytokine production from patient CD4+ T cells, including IFN-γ and IL-2, compared with the control group treated with control Ig (Fig. 3B, upper left panel: $P=0.014$ for $CD4^+$ IFN-γ, $P=0.001$ for $CD8^+$ IFN-γ; lower left panel: $P=0.016$ for $CD4^+$ IL-2, $P=0.046$ for $CD8^+$ IL-2). When monocytes were absent from the culture, there were no significant differences in the proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells, even in the presence of anti-B7-H1 mAb (Fig. 3, A and B, right panels). Interestingly, we observed no significant increase of Th2 cytokine production, such as IL-4 and IL-10, by patient $CD4^+$ or $CD8^+$ T cells

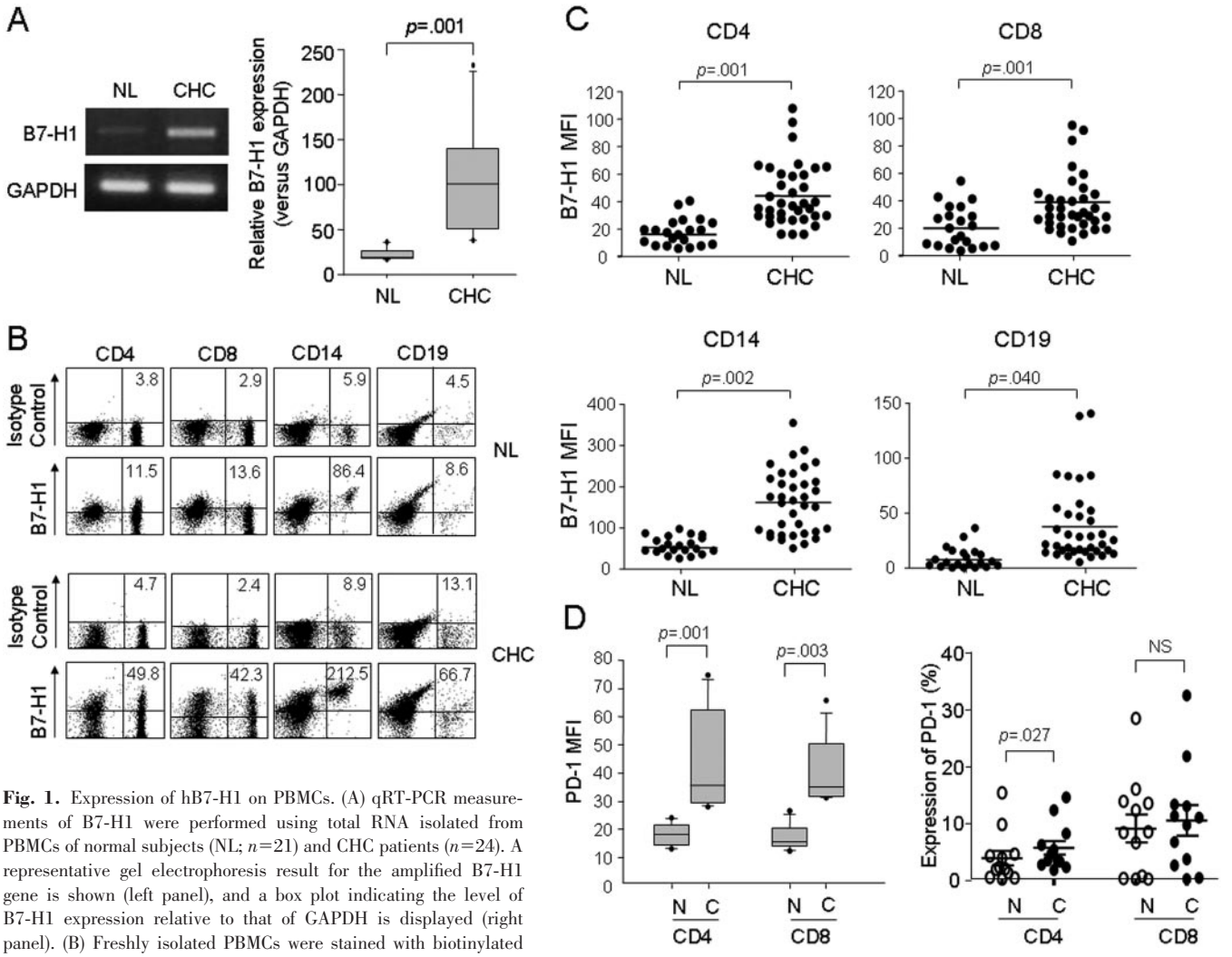

Fig. 1. Expression of hB7-H1 on PBMCs. (A) qRT-PCR measurements of B7-H1 were performed using total RNA isolated from PBMCs of normal subjects (NL; $n=21$) and CHC patients ($n=24$). A representative gel electrophoresis result for the amplified B7-H1 gene is shown (left panel), and a box plot indicating the level of B7-H1 expression relative to that of GAPDH is displayed (right panel). (B) Freshly isolated PBMCs were stained with biotinylated anti-B7-H1 mAb, PE-conjugated streptavidin, and FITC-conjugated mAb specific for CD4, CD8, CD14, or CD19. Cells were analyzed by flow cytometry. The numbers represent mean fluorescence intensity (MFI) of B7-H1 expression on each cell population. (C) MFI of B7-H1 expression on $CD4^+$, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD19^+$ B cells in normal subjects ($n=21$) and CHC patients ($n=24$). (D). MFI and percent of PD-1 expression on $CD4^+$ or $CD8^+$ T cells from healthy individuals (N; $n=10$) and CHC patients (C; $n=10$). Horizontal bars represent median values, the box indicates the interquartile range, error bars denote the range, and circles indicate values outside these percentiles. The P values were calculated using the Mann-Whitney U-test. NS, Not significant.

in response to anti-CD3 antibody with moB7-H1 blockade (data not shown). There was also no significant increase in the proliferation and Th1 and Th2 cytokine production of $CD4^+$ and $CD8^+$ T cells treated with anti-B7-H1 mAb alone without anti-CD3.

To demonstrate a direct involvement of moB7-H1 in a suppressed T cell expansion, we performed a proliferation assay in which T cells were cocultured with B7-$H1^+CD14^+$ or B7-$H1^-$ $CD14^+$ monocytes (each monocyte population, >95% in purity). As shown in Figure 3C, B7-$H1^+CD14^+$ monocytes but not B7-$H1^-CD14^+$ monocytes largely inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells mediated by anti-CD3 antibody ($P<0.01$ for $CD4^+$ and $CD8^+$ T cells). The inhibitory effect of B7-$H1^+CD14^+$ monocytes was reversed by addition of anti-B7-H1 mAb to coculture ($P=0.003$ for $CD4^+$ T cells; $P=0.002$ for $CD8^+$ T cells). Moreover, the suppressive effect of B7-$H1^+CD14^+$ monocytes on the T cell proliferation unlikely resulted from apoptosis-inducing molecules, such as TRAIL and FasL, as there were no detectable differences in TRAIL and FasL expression between the monocyte populations (Fig. 3D). Taken together, these results demonstrate that moB7-H1 substantially suppresses T cell proliferation and Th1 cytokine production in response to a TCR signaling mimicry.

Blockade of moB7-H1 enhances the proliferation of HCV-specific T cells

We also investigated the effect of moB7-H1 blockade on HCV-specific $CD8^+$ T cell proliferation by pentamer staining detecting $CD8^+$ T cells specific for the HLA-A2-restricted HCV $NS3_{1073–1081}$ epitope. As shown in **Figure 4**, although pentamer-positive $CD8^+$ T cells were detectable in the coculture of $CD8^+$ T cells and monocytes stimulated with NS3 peptide, the frequency of pentamer-positive $CD8^+$ T cells was increased greatly when treated with antagonistic B7-H1 mAb rather than with control Ig ($P=0.006$). This result indicates

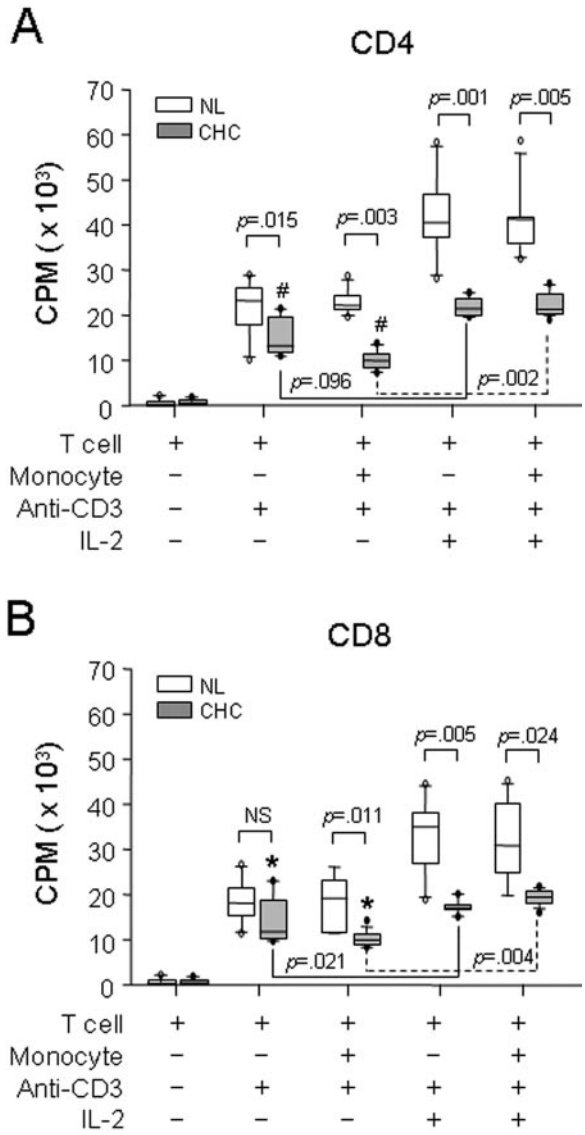

Fig. 2. Patient monocytes inhibit the T cell proliferation mediated by anti-CD3 antibody. Freshly isolated $CD4^+$ (A) or $CD8^+$ (B) T cells (2×10^5) from normal subjects ($n=15$) or CHC patients ($n=18$) were cocultured with or without mitomycin C-treated homologous $CD14^+$ monocytes (2×10^4). They were then stimulated with anti-CD3 antibody (1 μg/ml) in the presence or absence of IL-2 (50 U/ml) for 3 days. Proliferation was determined by [^{3}H]-thymidine incorporation, and the data are displayed as box plots. Horizontal bars represent median values, the box indicates the interquartile range, error bars denote the range, and circles indicate values outside these percentiles. The P values were calculated using the Mann-Whitney U-test and Wilcoxon rank-signed test for paired samples (#, $P=0.028$; *, $P=0.045$).

that moB7-H1 suppresses the proliferation of virus-specific $CD8^+$ T cells.

Neutralization of moB7-H1 restores HCV-specific effector T cell responses

To demonstrate the effect of moB7-H1 on the generation of IFN-γ^+ HCV-specific effector T cells, we stimulated patient $CD4^+$ T cells with HCV NS4 protein and $CD8^+$ T cells with a peptide mix containing HLA-A2-restricted HCV peptide $NS3_{1073-1081}$ and $NS4_{1789-1797}$ in the presence of homologous $CD14^+$ monocytes. We then assessed the frequency of IFN-γ-producing T cells by ELISPOT. Those peptides are known to induce a high cytotoxic T lymphocyte response in CHC patients [30]. In coculture assays, blocking of moB7-H1 resulted in an increased frequency of IFN-γ-producing, NS4-specific $CD4^+$ T cells compared with the control Ig-treated group [**Fig. 5A**; 49±16 and 141±23 spot-forming unit (SFU) for control Ig- and anti-B7-H1 mAb-treated groups, respectively; $P=0.001$]. moB7-H1 blockade had the similar effect on $CD8^+$ T cell response to NS3 and NS4 peptides (Fig. 5B; 69±13 and 159±35 SFU for control Ig- and anti-B7-H1 mAb-treated groups, respectively; $P=0.008$). However, a patient T cell culture without monocytes, otherwise the same as the coculture assays, did not increase the frequency of IFN-γ-producing, NS3-specific $CD8^+$ T cells, even in the presence of anti-B7-H1 mAb (Fig. 5C). In case of normal subjects, there was no significant increase of the frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in response to HCV protein or peptides, even in the presence of anti-B7-H1 mAb. We next sought to determine the effect of moB7-H1 on the cytokine production by HCV-specific T cells. Using a cytokine ELISA assay with culture supernatant fractions from the ELISPOT assays as described above, we found that the production of IL-2, but not IL-4 or IL-10, by $CD4^+$ and $CD8^+$ T cells in response to HCV NS4 protein or NS3 and NS4 peptides increased in the presence of anti-B7-H1 mAb (Fig. 5, D and E; $P=0.041$ for $CD4^+$ IL-2, $P=0.007$ for $CD8^+$ IL-2). Taken together, these findings indicate that moB7-H1 suppresses the generation of IFN-γ-producing, HCV-specific effector $CD4^+$ and $CD8^+$ T cells and inhibits the production of Th1-associated cytokines such as IL-2.

Blocking of moB7-H1 improves the production of perforin

Because of the low frequency of HCV-specific $CD8^+$ T cells to perform ^{51}Cr-release cytotoxicity assay, we analyzed the $CD8^+$ T cell cytotoxic potential by assessing perforin production in $CD8^+$ T cells stimulated with HCV peptides. We cocultured purified $CD8^+$ T cells and homologous patient $CD14^+$ monocytes in the presence of HLA-2-restricted NS3 and NS4 peptides with 10 μg/ml control Ig or anti-B7-H1 mAb. Consistent with our other results described above, intracellular staining of perforin revealed that blocking of moB7-H1 greatly increased the frequency of perforin$^+$ $CD8^+$ T cells from CHC patients compared with the group treated with control Ig (**Fig. 6, A** and **B**; 0.5±0.2 for control Ig-treated group, 5.8±0.4 for anti-B7-H1 mAb-treated group). The result suggests that moB7-H1 is involved in suppression of the cytolytic activity by $CD8^+$ T cells.

DISCUSSION

A hallmark of HCV infection is a lack of functionally competent and phenotypically mature, HCV-specific $CD4^+$ and $CD8^+$ T cells, despite the presence of virus-specific T cells in the peripheral blood and liver [1]. In this study, we demonstrated the contribution of cosignaling molecule B7-H1 (CD274) up-regulated on patient monocytes to the defective

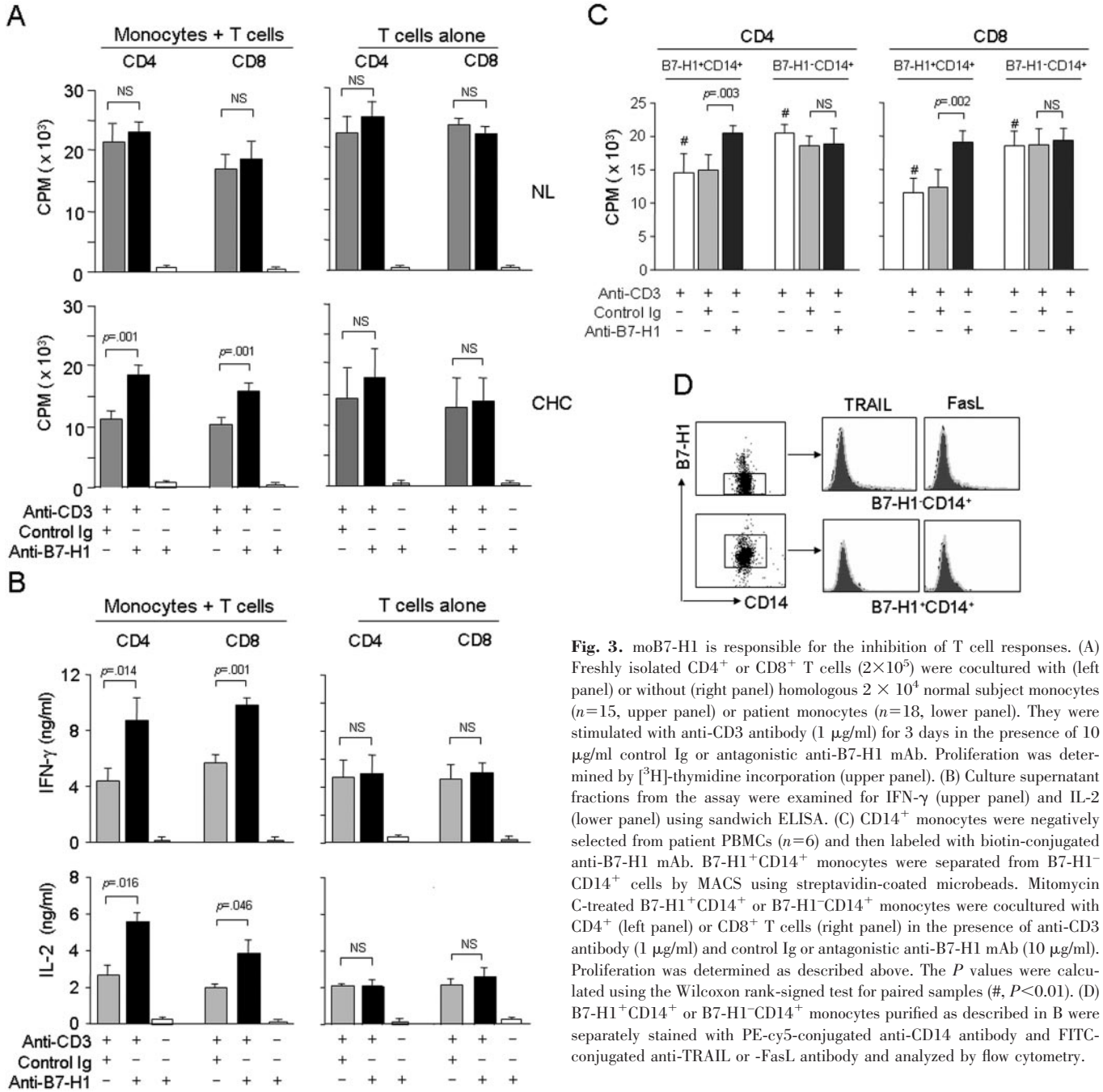

Fig. 3. moB7-H1 is responsible for the inhibition of T cell responses. (A) Freshly isolated $CD4^+$ or $CD8^+$ T cells (2×10^5) were cocultured with (left panel) or without (right panel) homologous 2×10^4 normal subject monocytes ($n=15$, upper panel) or patient monocytes ($n=18$, lower panel). They were stimulated with anti-CD3 antibody (1 μg/ml) for 3 days in the presence of 10 μg/ml control Ig or antagonistic anti-B7-H1 mAb. Proliferation was determined by [^{3}H]-thymidine incorporation (upper panel). (B) Culture supernatant fractions from the assay were examined for IFN-γ (upper panel) and IL-2 (lower panel) using sandwich ELISA. (C) $CD14^+$ monocytes were negatively selected from patient PBMCs ($n=6$) and then labeled with biotin-conjugated anti-B7-H1 mAb. $B7\text{-}H1^+CD14^+$ monocytes were separated from $B7\text{-}H1^-CD14^+$ cells by MACS using streptavidin-coated microbeads. Mitomycin C-treated $B7\text{-}H1^+CD14^+$ or $B7\text{-}H1^-CD14^+$ monocytes were cocultured with $CD4^+$ (left panel) or $CD8^+$ T cells (right panel) in the presence of anti-CD3 antibody (1 μg/ml) and control Ig or antagonistic anti-B7-H1 mAb (10 μg/ml). Proliferation was determined as described above. The P values were calculated using the Wilcoxon rank-signed test for paired samples (#, $P<0.01$). (D) $B7\text{-}H1^+CD14^+$ or $B7\text{-}H1^-CD14^+$ monocytes purified as described in B were separately stained with PE-cy5-conjugated anti-CD14 antibody and FITC-conjugated anti-TRAIL or -FasL antibody and analyzed by flow cytometry.

functions of HCV-specific T cells. A high proportion of patient peripheral APCs, including monocytes and B cells, exhibited up-regulated B7-H1 mRNA and surface expression; this up-regulation was not limited to APCs but was also evident in $CD4^+$ and $CD8^+$ T cells. Interestingly, B7-H1 expression was also increased much more in HCV-specific $CD8^+$ T cells than in non-HCV-specific counterparts (Supplemental Fig. 1). Other studies also demonstrated the up-regulation of B7-H1 on monocytes from patients with chronic infections such as AIDS and CHB [29, 31]. As for factors affecting the B7-H1 up-regulation, there are many in vitro findings that B7-H1 was strongly up-regulated by proinflammatory cytokines, including IFN-γ and TNF-α [32, 33], suggesting that an inflammatory microenvironment may induce B7-H1 expression. However, a recent histological study in autoimmune hepatitis revealed conflicting data that there is no significant correlation between clinical markers of inflammatory activity such as ALT and the histological activity index score and level of B7-H1 expression in liver cells [34]. Furthermore, in an analysis of the relationship between inflammatory activity and B7-H1 up-regulation, we found that there was an inverse correlation between serum the ALT level and MFI of B7-H1 expression on $CD4^+$ and $CD8^+$ T cells and monocytes, not on $CD19^+$ B cells (Supplemental Fig. 2; $B7\text{-}H1^+/CD4^+$: $r^2=0.049$, $P=0.049$; $B7\text{-}H1^+/CD8^+$: $r^2=0.09$, $P=0.039$; $B7\text{-}H1^+/CD14^+$: $r^2=0.132$, $P=0.018$), which was in agreement with another report indicating a significant negative correlation between percentage of $B7\text{-}H1^+CD14^+$ monocytes and serum ALT level

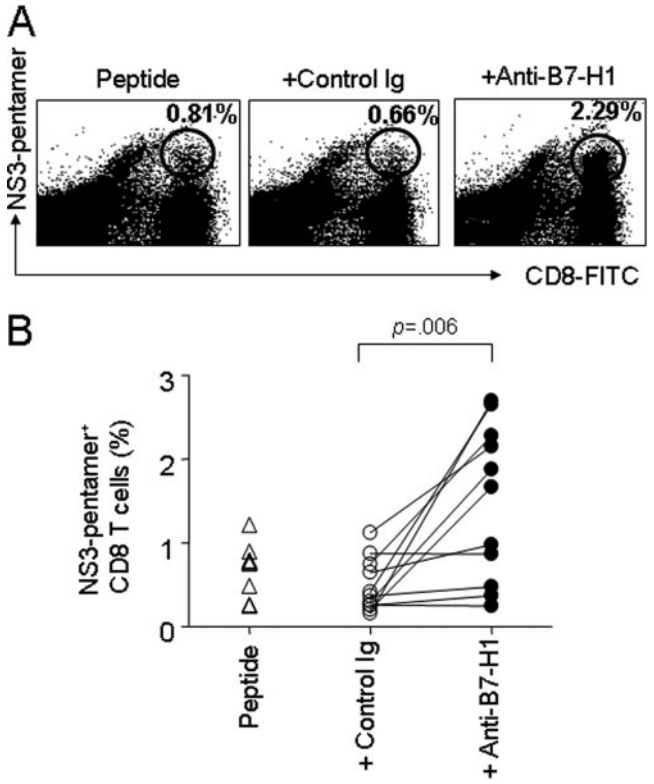

Fig. 4. Blockade of moB7-H1 enhances the proliferation of HCV-specific $CD8^+$ T cells. FITC-conjugated anti-CD19, and -CD56 antibody were used to negatively purify $CD4^+$, $CD8^+$ T cells and monocytes using anti-FITC microbeads, according to the manufacturer's instructions. Negatively isolated 4×10^5 cells were stimulated with 10 μM $NS3_{1073-1081}$ peptide for 10 days (n=10). Cells were stained with PE-conjugated, pentameric-NS3 peptide-HLA-$A2^+$ complexes and analyzed by flow cytometry. Representative dot plot (A) and summary data (B) of $pentamer^+$ $CD8^+$ T cells in coculture in the presence of control Ig versus antagonistic B7-H1 mAb.

in CHB patients [28]. We cannot exclude the possibility, however, that B7-H1 up-regulation on patient immune cells is related to a direct effect of the virus, as replicative forms of positive-stranded HCV RNA are detectable in patient peripheral immune cells [35], which may increase B7-H1 expression through undefined mechanisms, such as TLR signaling.

As a monocyte/macrophage population plays a major role in antigen presentation and interacts with effector T cells, we sought to demonstrate the role of moB7-H1 up-regulation in impaired HCV-specific T cell responses. In coculture assays, we found that through the B7-H1 pathway, patient monocytes directly inhibited T cell proliferation in response to TCR-mediated signaling, as revealed by the finding that B7-H1-positive $CD14^+$ patient monocytes greatly reduced T cell proliferation compared with their B7-H1-negative counterpart, and blocking of moB7-H1 with antagonistic B7-H1 mAb reversed T cell proliferation and increased Th1 cytokine production. Interestingly, although $CD4^+$ T cells also exhibited B7-H1 up-regulation, blocking of T cell-associated B7-H1 in T cell culture without monocytes did not enhance the proliferation of T cells in our experiment settings, suggesting that T cell-B7-H1 is less likely to participate in the suppression of T cell expansion. In line with the results from experiments using anti-CD3 antibody, pentamer staining also revealed that blocking of moB7-H1 augmented the proliferation of HCV NS3 peptide-specific $CD8^+$ T cells. Furthermore, blocking of moB7-H1 increased the frequency of IFN-γ-producing, HCV-specific $CD4^+$ T cells and the production of Th1 cytokines in response to in vitro HCV protein stimulation. Our results are well in agreement with those of previous reports about the involvement of the B7-H1/PD-1 pathway in impaired function of exhausted, HCV-specific $CD8^+$ T cells in CHC patients [24, 25, 36].

Interestingly, we found that the moB7-H1-mediated suppression of virus-specific T cell expansion was not limited to HCV-specific T cells in CHC patients, as shown by the findings that the blockade of moB7-H1 in CHC patients enhanced the proliferation of IFN-γ-producing $CD8^+$ T cells specific for CMV and EBV (Supplemental Fig. 3A). However, blocking of moB7-H1 of normal subjects was not as effective at increasing the frequency of the IFN-γ-producing $CD8^+$ T cells specific for those virus as blockade of patient moB7-H1 (Supplemental Fig. 3B). These findings suggest that moB7-H1 in CHC patients likely globally affects the effector function of virus-specific T cells. This idea is supported in part by the findings by others and us that the total patient $CD8^+$ T cell population exhibited higher PD-1 expression compared with normal $CD8^+$ T cells, and specifically, CMV-specific $CD8^+$ T cells up-regulated PD-1 comparable with that of HCV-specific $CD8^+$ T cells [25], which provides a plausible explanation for the observation that CHC patients have significantly higher prevalence of chronic viral infections such as HIV, HBV, CMV, and HSV infections [37]. Furthermore, the finding that depletion of $CD4^+CD25^+$ Tregs in CHC patients enhanced expansion of EBV- and CMV-specific $CD8^+$ T cells in response to respective viral peptides also partially supports the idea of global impairment of virus-specific $CD8^+$ T cell functions in CHC patients [38]. In contrast to these results, there were conflicting reports showing that PD-1 expression is lower in non-HCV (influenza or CMV)-specific T cells than in HCV-specific T cells, and B7-H1/PD-1 blockade led to an increase of influenza- or CMV-specific T cells in only a small fraction of CHC patients [24, 36]. The discrepancy with the results of these reports can be related to the mode of induction of virus-specific T cell responses. Whereas we used purified patient $CD14^+$ monocytes and $CD8^+$ T cells for the assay, they used PBMC to stimulate virus-specific T cells, wherein B7-H1-expressing immune cells, including $CD19^+$ B cells and $CD16^+$ NK cells other than $CD14^+$ monocytes, likely affect virus-specific $CD8^+$ T cell functions through PD-1 engagement.

In our coculture system, HCV-specific T cells stimulated by HCV NS4 protein did not produce detectable levels of Th2 cytokines, such as IL-4 and IL-10, even in the presence of anti-B7-H1 mAb. This result appears to conflict with a previous result from Brady et al. [9], in which patient PBMCs promoted IL-10 secretion in response to HCV NS4 protein. This discrepancy may be a result of differences in experimental settings and in the population of cells ultimately responsible for IL-10 secretion. Overall, our findings suggest that moB7-H1 likely suppresses the generation of Th1 $CD4^+$ cells rather than

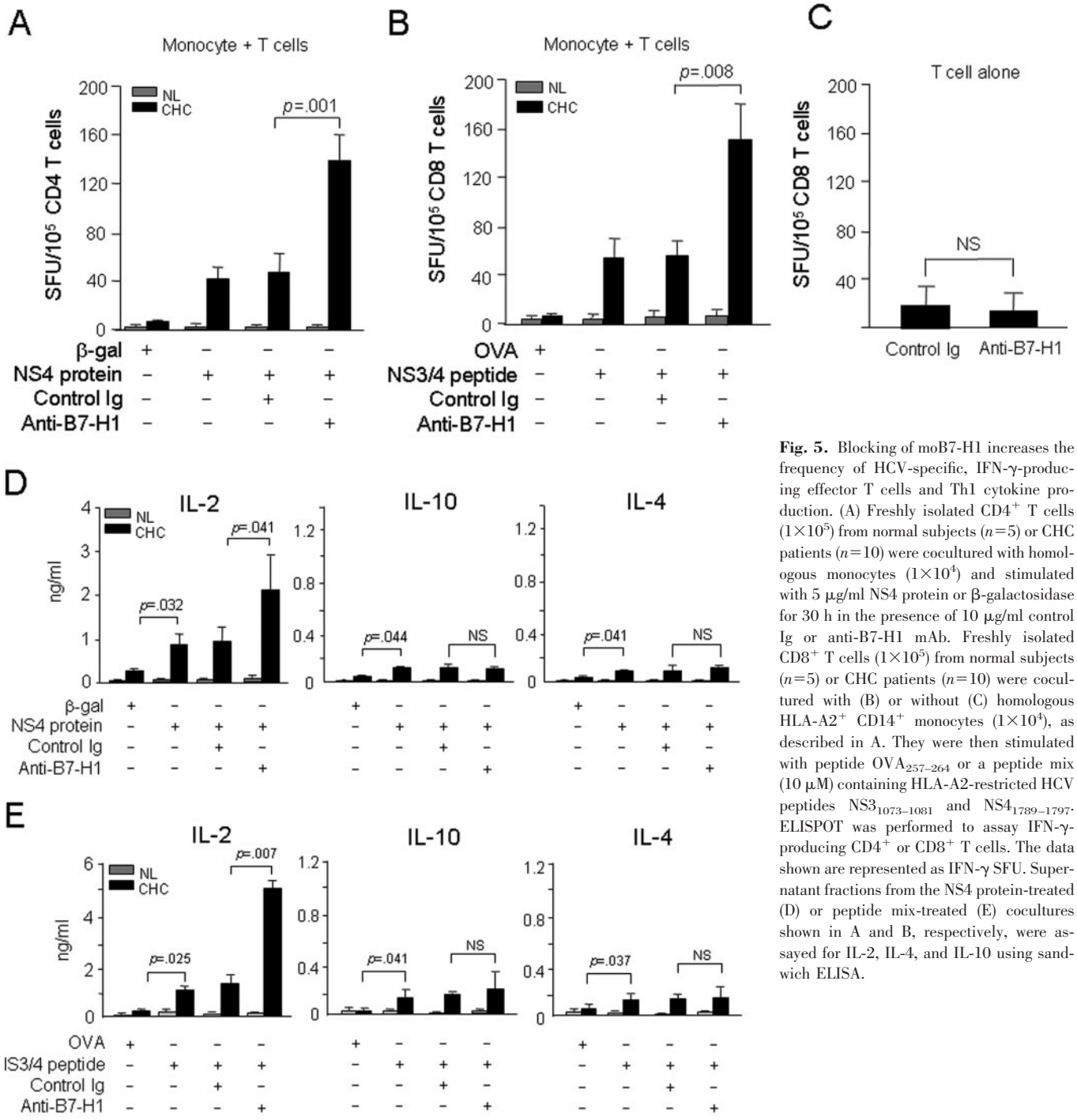

Fig. 5. Blocking of moB7-H1 increases the frequency of HCV-specific, IFN-γ-producing effector T cells and Th1 cytokine production. (A) Freshly isolated $CD4^+$ T cells (1×10^5) from normal subjects ($n=5$) or CHC patients ($n=10$) were cocultured with homologous monocytes (1×10^4) and stimulated with 5 μg/ml NS4 protein or β-galactosidase for 30 h in the presence of 10 μg/ml control Ig or anti-B7-H1 mAb. Freshly isolated $CD8^+$ T cells (1×10^5) from normal subjects ($n=5$) or CHC patients ($n=10$) were cocultured with (B) or without (C) homologous HLA-$A2^+$ $CD14^+$ monocytes (1×10^4), as described in A. They were then stimulated with peptide $OVA_{257-264}$ or a peptide mix (10 μM) containing HLA-A2-restricted HCV peptides $NS3_{1073-1081}$ and $NS4_{1789-1797}$. ELISPOT was performed to assay IFN-γ-producing $CD4^+$ or $CD8^+$ T cells. The data shown are represented as IFN-γ SFU. Supernatant fractions from the NS4 protein-treated (D) or peptide mix-treated (E) cocultures shown in A and B, respectively, were assayed for IL-2, IL-4, and IL-10 using sandwich ELISA.

Th2 $CD4^+$ cells. Furthermore, the observation that the moB7-H1 blockade enhances HCV-specific $CD8^+$ T cell functions without $CD4^+$ T cell help indicates that blockade of the B7-H1 inhibitory pathway has a beneficial effect on the helpless $CD8^+$ T cells, restoring their ability to expand and produce effector cytokines.

Recently, IFN-γ locally produced by HCV-specific T cells has been proposed to purge HCV via a noncytolytic mechanism involving inhibition of HCV protein synthesis and viral replication independently of IFN-α [39]. Based on this observation, we speculate that a moB7-H1-mediated decrease in IFN-γ production by HCV-specific T cells may be responsible for the failure of the T cells to eradicate HCV. Previous in vivo studies using knockout mice have demonstrated that viral clearance is solely dependent on perforin, although virus-induced liver damage develops when the Fas/FasL and perforin/granzyme pathways are involved [40]. In our experiment, perforin production by patient $CD8^+$ T cells in response to NS3 and NS4 peptides increased significantly when moB7-H1 was blocked. Although we cannot rule out the possibility that an increased perforin expression comes from the HCV-nonspecific T cells, at least our data suggest that moB7-H1 may negatively regulate the ability of HCV-specific $CD8^+$ T cells to clear the virus.

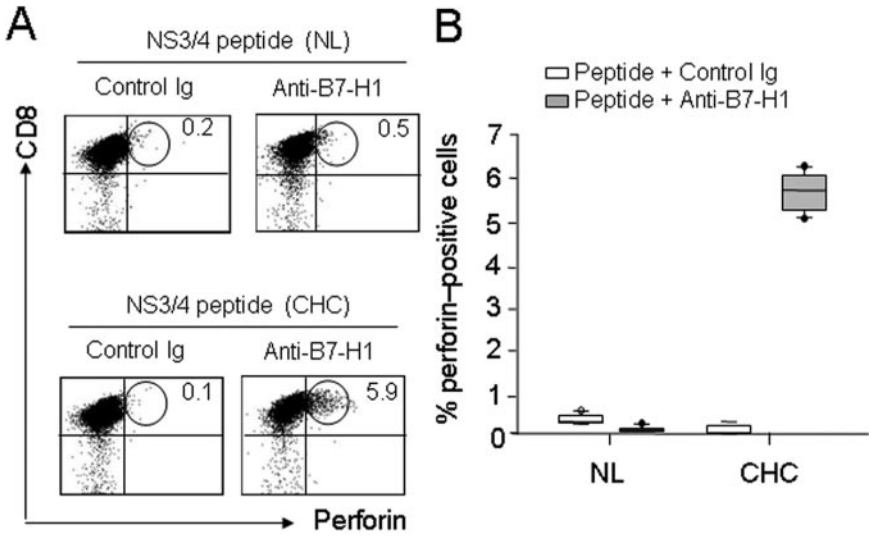

Fig. 6. moB7-H1 blockade improves the perforin production by $CD8^+$ T cells. Freshly isolated $CD8^+$ T cells (1×10^5) from normal subjects ($n=5$; upper panel) or CHC patients ($n=5$; lower panel) were cocultured with homologous HLA-$A2^+$ $CD14^+$ monocytes (1×10^4) in the presence of a peptide mix containing HLA-A2-restricted HCV peptides $NS3_{1073-1081}$ and $NS4_{1789-1797}$. Cells were grown for 30 h in the presence of 10 μg/ml control Ig or anti-B7-H1 mAb. Cells were treated with brefeldin for the final 4 h of culture and then stained for intracellular perforin using FITC-conjugated antiperforin and PE-conjugated anti-CD8 antibodies. Cells were analyzed by flow cytometry. (A) Representative dot plot is shown. (B) The data from A are shown as a box plot. Horizontal bars represent median values, the box indicates the interquartile range, error bars denote the range, and circles indicate values outside these percentiles. The *P* values were calculated using the Mann-Whitney U-test.

Although the crucial role of PD-1 in defective functions of virus-specific $CD8^+$ T cells from patients with chronic viral diseases was largely acceptable [23, 28, 36], our findings—that blocking the B7-H1/PD-1 pathway by soluble PD-1 Ig greatly increased the frequencies of IFN-γ-producing, HCV-specific $CD4^+$ and $CD8^+$ T cells, but the extent of enhancement was significantly lower than those by blocking with antagonistic B7-H1 mAb (Supplemental Fig. 4)—suggest that another receptor(s) other than PD-1 may be engaged in the impairment of $CD8^+$ T cell functions. This notion of the presence of second receptor(s) for B7-H1 was indicated by previous reports [17, 18], which remain to be elucidated using an appropriate experimental setting.

It also remains to be determined whether moB7-H1-mediated suppression of HCV-specific T cell responses in vitro indeed mirrors the pathophysiological events in the liver. Further investigations about the role of moB7-H1 in the impaired response of HCV-specific T cells infiltrated into the inflamed liver tissue of chronically infected patients are needed. Collectively, our results demonstrate that B7-H1 up-regulated on patient monocytes suppresses the ability of HCV-specific $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T lymphocytes to undergo proliferation and generate efficient T cell effector responses. These results provide a plausible explanation for the failure of HCV-specific T cells to eradicate the virus efficiently, leading to viral persistence, and suggest that blockade of the B7-H1 pathway may be one of the effective immunotherapeutic strategies for CHC.

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