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IL-10–Producing Regulatory B Cells in the Pathogenesis of Chronic Hepatitis B Virus Infection

Abhishek Das,* Gidon Ellis,* Celeste Pallant,* A. Ross Lopes,* Pooja Khanna,*[†] Dimitra Peppas,* Antony Chen,* Paul Blair,[‡] Geoffrey Dusheiko,[†] Upkar Gill,[§] Patrick T. Kennedy,[§] Maurizia Brunetto,[¶] Pietro Lampertico,^{||} Claudia Mauri,[†] and Mala K. Maini*

A regulatory subset of B cells has been found to modulate immune responses in autoimmunity, infection, and cancer, but it has not been investigated in the setting of human persistent viral infection. IL-10 is elevated in patients with chronic hepatitis B virus infection (CHB), but its cellular sources and impact on antiviral T cells have not been addressed. We investigated the role of IL-10 and regulatory B cells in the pathogenesis of CHB. Serum IL-10 levels were studied longitudinally in patients with CHB undergoing spontaneous disease flares. There was a close temporal correlation between IL-10 levels and fluctuations in viral load or liver inflammation. Blockade of IL-10 in vitro rescued polyfunctional virus-specific CD8 T cell responses. To investigate the potential contribution of regulatory B cells, their frequency was measured directly ex vivo and after exposure to stimuli relevant to hepatitis B virus (HBV) (CpG or HBV Ags). IL-10–producing B cells were enriched in patients, and their frequency correlated temporally with hepatic flares, both after stimulation and directly ex vivo. Phenotypically, these cells were predominantly immature (CD19⁺CD24^{hi}CD38^{hi}) ex vivo; sorted CD19⁺CD24^{hi}CD38^{hi} cells suppressed HBV-specific CD8 T cell responses in an IL-10–dependent manner. In summary, these data reveal a novel IL-10–producing subset of B cells able to regulate T cell immunity in CHB. *The Journal of Immunology*, 2012, 189: 3925–3935.

B cells have been identified as potent regulators of T cell immune responses in studies of autoimmunity, infection, and cancer (1–7). IL-10 is the primary mechanism by which B cells modulate other immune cells; however, additional contributory mechanisms include engagement of costimulatory molecules (CD80/CD86), Ag presentation, and induction of regulatory T cell populations (8). These regulatory B cells (Bregs) are pathogenic in parasitic infections, subverting CD4 T cells toward a Th2 phenotype favorable to pathogen survival, and they can reduce

Ag-specific CD8 T cell responses through production of IL-10 in murine viral infection (9, 10). In contrast, IL-10–producing B cells are protective in the setting of autoimmune disease, chronic intestinal inflammation, and allograft survival (2, 3, 11). For instance, adoptive transfer of these cells can ameliorate established disease in murine models of collagen-induced arthritis and experimental autoimmune encephalomyelitis; absence of these cells is associated with increased Th1 and Th17 responses, reduced numbers of Foxp3⁺ regulatory T cells, and exacerbated disease (12). Therefore, B cell function is pleiotropic and not confined to Ab production.

In humans, it was recently demonstrated that analogous Breg subsets can suppress CD4 T cell proliferation and IFN- γ and TNF- α production by CD4⁺ T cells, as well as regulate TNF- α release by monocytes (13–15). The suppressive effects are mediated by IL-10 and are TGF- β independent. Phenotypically, B cells producing IL-10 were found to be enriched within CD19⁺CD24^{hi}CD38^{hi} transitional B cells (13), CD19⁺CD24^{hi}CD27⁺ B10 cells (15), or both CD27⁺ memory and CD38^{hi} transitional B cell subsets together (14); it remains to be determined whether these phenotypic distinctions are partially dependent on the mode of stimulation. Dysfunction within these cells has also been described. B cells from patients with systemic lupus erythematosus or multiple sclerosis have impaired capacity to produce IL-10; these cells are refractory to CD40 stimulation in the former disease and recover their IL-10 production upon treatment in multiple sclerosis (13, 16).

A role for Bregs in human viral infection has not been reported. To investigate whether IL-10 and Bregs contribute to the pathogenesis of chronic hepatitis B virus infection (CHB), we studied a cohort of patients undergoing spontaneous flares of liver disease. These rapid fluctuations in disease activity provide a unique opportunity to study the relationship between immune and clinical parameters of viral control and liver damage. Longitudinal analysis in these patients revealed that serum IL-10 levels and the frequency of IL-10–producing B cells were enriched in temporal

*Division of Infection and Immunity, University College London, London WC1E 6JF, United Kingdom; [†]Centre for Hepatology, Royal Free Campus, University College London, London NW3 2QG, United Kingdom; [‡]Division of Medicine, University College London, London WC1E 6JF, United Kingdom; [§]Centre for Digestive Diseases, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London E1 4AT, United Kingdom; [¶]Gastroenterology and Hepatology Unit, United Hospitals of Santa Chiara, I-56124 Pisa, Italy; and ^{||}First Gastroenterology Unit, University of Milan, Milan 20122, Italy

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A.D., P.B., C.M., and M.K.M. conceived and designed the study; A.D., G.E., C.P., A.R.L., P.K., D.P., and A.C. collected the data; A.D., G.E., C.P., A.R.L., P.K., D.P., A.C., C.M., and M.K.M. analyzed and interpreted the data; A.D., C.M., and M.K.M. wrote the manuscript; A.D., G.D., U.G., P.T.K., M.B., P.L., C.M., and M.K.M. critically reviewed the manuscript; and P.K., G.D., U.G., P.T.K., M.B., and P.L. provided technical or material support.

Address correspondence and reprint requests to Prof. Mala K. Maini, Division of Infection and Immunity, Rayne Institute, 5 University Street, University College London, London WC1E 6JF, U.K. E-mail address: m.maini@ucl.ac.uk

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Abbreviations used in this article: ALT, alanine aminotransferase; Breg, regulatory B cell; CHB, chronic hepatitis B virus infection; HBcAg, HBV core Ag; HBsAg, HBV e Ag; HBsAg, HBV surface Ag; HBV, hepatitis B virus.

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correlation with the peak of viral load or liver inflammation. Ex vivo phenotypic characterization of IL-10-producing B cells revealed that these cells were predominantly contained within the immature B cell subset. Depletion of this B cell subset resulted in an expansion of functional hepatitis B virus (HBV)-specific CD8⁺ T cells in vitro. To our knowledge, these data provide the first demonstration of the capacity of Bregs to regulate Ag-specific CD8⁺ T cells in humans and implicate these cells in HBV pathogenesis.

Materials and Methods

Patients and controls

Written informed consent was obtained from all patients. The study was approved by the local ethical committees (Royal Free Hospital, Royal London Hospital, Camden Primary Care Ethics Review Board). All patients recruited were negative for HIV and hepatitis C virus Abs. A total of 57 patients and 22 healthy donors took part in the study. All patients were untreated. Patient demographics are shown in Table I. Serum samples were collected at multiple time points over the course of spontaneous flares of liver disease from 10 patients with HBV e Ag (HBeAg)⁻ CHB and five patients with HBeAg⁺ CHB. Viral load was measured either by an in-house real-time PCR-based assay or by the Bayer Versant HBV DNA 1.0 Assay. Liver biopsies (surplus to diagnostic requirements) were obtained from eight patients with CHB; paired peripheral blood samples were available from five of these patients (Table II).

Quantification of IL-10 in serum and supernatant

Serum IL-10 was quantified using the Cytometric Bead Array inflammation kit (BD Biosciences), according to the manufacturer's instructions.

To detect IL-10 in supernatant, PBMCs from patients and controls were stimulated with PMA (3 ng/ml) and ionomycin (100 ng/ml) (Sigma) for 5 h, after which supernatant was collected and stored at -80°C. IL-10 in batch-collected supernatant samples was quantified with the Human IL-10 Elisa Kit (Diacclone), per the manufacturer's instructions. The range of detection for this kit was between 1.56 and 50 pg/ml. Alternatively, PBMCs from healthy donors were stimulated with HBV core Ag (HBeAg; Rhein Biotech), HBV surface Ag (HBsAg; Rhein Biotech) or HBeAg (Siemens Healthcare Diagnostics Products) for 96 h, after which IL-10 was measured in supernatant.

Isolation of PBMCs and intrahepatic lymphocytes

PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and used immediately or frozen. Intrahepatic lymphocytes were isolated as described previously (17).

IL-10 production by intracellular cytokine staining

PBMCs were incubated with 1 μM CpG-B ODN2006 (InvivoGen) for 96 h at 37°C. PMA (3 ng/ml) and ionomycin (100 ng/ml) were added during the last 4 h in the presence of 10 μg/ml brefeldin A (Sigma). Alternatively, PBMCs were stimulated with HBeAg (10 μg/ml; kindly provided by Rhein Biotech, Düsseldorf, Germany) for 16 h. Cells were then surface stained for the markers CD19 PerCP (BD), CD24 PE, and CD38 FITC, fixed, permeabilized, and stained intracellularly with anti-IL-10 allophycocyanin (BD Pharmingen). PBMCs were acquired on a FACSCalibur flow cytometer (Becton Dickinson). FlowJo software (TreeStar) was used for analysis. For ex vivo analysis of IL-10 production, unstimulated PBMCs were stained by the same protocol as above.

FACS of immature B cells

PBMCs were isolated as described above and surface stained with anti-CD38 FITC (BD Pharmingen), anti-CD19 PE-Cy7 (eBioscience), and anti-CD24 PE (BD Pharmingen). Immature B cells were depleted from PBMCs on the basis of high expression of CD24 and CD38 by FACSARIA (Becton Dickinson). Additionally, PBMCs that had been stained with the same Abs were passed through the machine untouched as a control.

Detection of HBV-specific CD8 T cell responses with IL-10/IL-10R blockade

PBMCs were seeded in duplicate into a 96-well plate (0.25 × 10⁶/well) in the presence of 1 μM viral peptide and 50 U/ml IL-2, with or without anti-IL-10 (5 μg/ml; eBioscience) and anti-IL-10R (10 μg/ml; BD Pharmingen). HLA-A2⁺ patients were stimulated with a panel of peptides representing immunodominant HLA-A2-restricted epitopes from HBV

(envelope: FLLTRILTI, WLSLLVPFV, LLVPFVQWFV, GLSPTVWLSV; core: FLPSDFPFSV; and polymerase: GLSRYVARL, KLHLYSHPI) or CMV (pp65: NLVPMVATV) (Proimmune). HLA-A2⁻ patients were stimulated with overlapping peptides (pool of 15mer peptides overlapping by 10 residues) spanning the core of HBV genotype D or the pp65 protein of CMV. Medium was refreshed on day 4, with further addition of exogenous IL-2 (50 U/ml), anti IL-10 (2.5 μg/ml), and anti IL-10R (5 μg/ml). On day 10, PBMCs were pulsed for 5 h with 1 μM peptide in the presence of brefeldin A (10 μg/ml) and then stained with anti-CD8 allophycocyanin, anti-CD3 PerCPy5.5 (BD Biosciences) and intracellularly stained with anti-IFN-γ FITC (R&D Systems). The frequency of IFN-γ⁺CD3⁺CD8⁺ T cells represented the virus-specific CD8 T cell population.

Determination of CD8 T cell polyfunctionality

In six individuals with chronic disease, polyfunctionality of virus-specific CD8⁺ T cell responses was analyzed. PBMCs were stimulated with viral peptides, and production of IFN-γ, IL-2, and TNF-α, proliferation (CFSE), and degranulation (CD107a) were determined after 10 d of in vitro culture. The following panel of Abs was used: anti-CD3 ECD (IOTest), anti-CD107a PE (BD Pharmingen), anti-IFN-γ V450 (BD), anti-TNF-α allophycocyanin (BD Biosciences), CFSE FITC (Serotec), and LIVE/DEAD Near-IR Fluorescent Reactive Dye (Invitrogen). The above-described protocol for detection of virus-specific CD8 T cell responses was used, with the exception that PBMCs were additionally stained with CFSE dye on day 0, and anti-CD107a Ab and monensin were added in addition to brefeldin A upon restimulation with peptide on day 10. After gating on live CD3⁺CD8⁺ T cells, the percentages of the 16 different combinations of IFN-γ, TNF-α, CFSE, and CD107 responses were determined. Boolean gate arrays created in FlowJo were exported to PESTLE (version 1.7) for background subtraction (from medium-alone samples), and graphical representations of polyfunctional CD8 T cell responses were generated using Simplified Presentation of Incredibly Complex Evaluations software, version 5.1 (M. Roederer, National Institutes of Health, Bethesda, MD) (18).

Detection of HBV-specific CD8 T cell responses after depletion of immature B cells

PBMCs or PBMCs depleted of CD19⁺CD24^{hi}CD38^{hi} B cells isolated by FACSARIA were stimulated with HBV peptides in the presence of IL-2, as described above. The frequency of virus-specific CD3⁺CD8⁺IFN-γ⁺ T cells was determined on day 10.

Functional B cell assays

Sorted immature (CD19⁺CD24^{hi}CD38^{hi}) B cells (0.6 × 10⁵) were stimulated with PMA/ionomycin for 2 h, washed in RPMI 1640, and cocultured at a 1:4 ratio with PBMCs (2.5 × 10⁵) derived from the same HLA-A2⁺ patients in the presence of viral peptide and IL-2, as described above. The frequency of virus-specific CD8 T cells was determined at day 10 and compared with PBMCs that had been stimulated with HBV or CMV peptides in the absence of immature B cells.

Statistical analysis

Statistical significance was calculated by the nonparametric Mann-Whitney U test. A *p* value < 0.05 was deemed significant. All paired samples were analyzed by the Wilcoxon matched-pairs test.

Results

Temporal correlation between serum IL-10 levels and flares of liver disease

To determine the role of IL-10 in CHB infection, serum levels were measured longitudinally in 15 patients sampled repeatedly during disease fluctuations. Ten patients had HBeAg⁻ CHB characterized by recurrent spontaneous hepatic flares; for comparison, we studied five patients with HBeAg⁺ CHB (Table I). In all HBeAg⁻ patients undergoing hepatic flares, IL-10 levels showed dynamic changes, with a close temporal correlation with disease activity. IL-10 peaked in tandem with the increase in viral load or liver inflammation or both, and it decreased at the same time or shortly after the resolution of liver inflammation (Fig. 1). For comparison, we examined five patients with HBeAg⁺ disease in whom there was persistently elevated HBV DNA (>10⁶ log at all time points). In four of five patients with HBeAg⁺ CHB, IL-10 levels were

Table I. Patient characteristics

Patient Characteristics	Overall CHB	Healthy Control
<i>n</i>	57	22
Median age (y; range)	37 (19–65)	31.5 (24–48)
Gender (% male)	58	48
Median serum HBV DNA (IU/ml [range])	1.5×10^6 (blq– 5.27×10^8)	NA
Median ALT (IU/l [range])	62 (18–900)	NA
HBeAg status (% positive)	30	NA

blq, DNA positive but below limit of quantification (i.e., <50 IU/ml); NA, not applicable.

greater than the low levels seen in healthy donors and in HBeAg⁻ CHB between flares (Fig. 1) (19). However, unlike the close temporal correlation between IL-10 and recurrent hepatic flares in

HBeAg⁻ disease, it was not possible to detect any correlation between IL-10 levels and disease activity in these HBeAg⁺ patients.

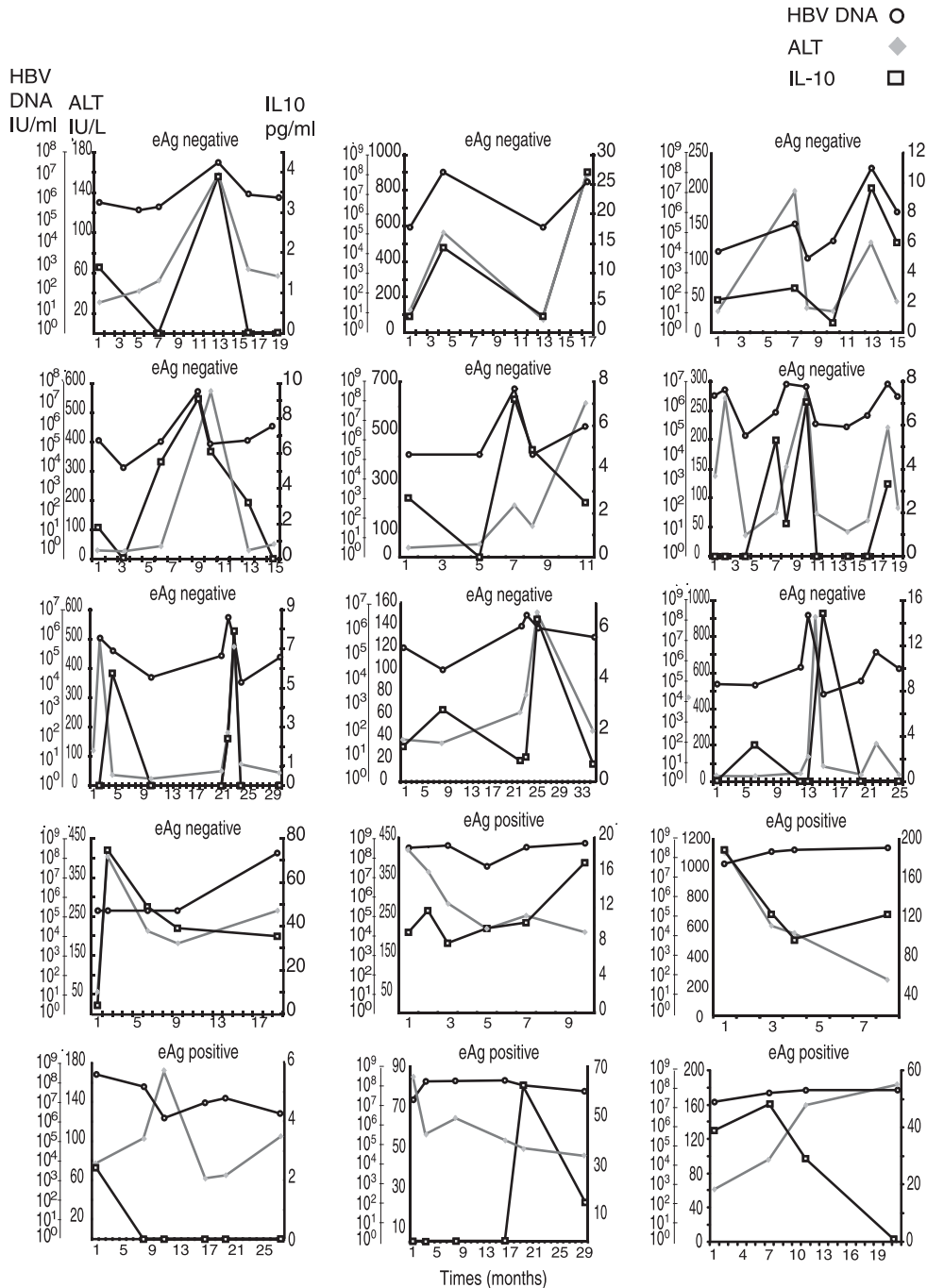


FIGURE 1. Temporal correlation between serum IL-10 and chronic flares of liver disease. IL-10 levels were measured in serum samples collected before, during, and after chronic flares of liver disease in 10 HBeAg⁻ and 5 HBeAg⁺ patients with CHB. A graph for each patient shows the temporal longitudinal fluctuations in IL-10 (pg/ml) in association with viral load (IU/ml) and ALT (IU/l).

Role of IL-10 in suppression of HBV-specific CD8 T cell responses

We next investigated a potential role for IL-10 in modulation of the antiviral immune response. HBV-specific CD8 T cell responses were quantified following *in vitro* stimulation with a pool of HBV peptides with or without blockade of IL-10 and its receptor (Fig. 2A). There was little response above background in patients with high viral load; this is in keeping with previous data that characterized profound qualitative and quantitative defects in the HBV-specific CD8 T cell response in high-level carriers (20, 21). Eight of fifteen CHB-infected patients showed recovery of HBV-specific responses upon IL-10 blockade (Fig. 2B). In five individuals, blockade of IL-10 revealed new HBV-specific CD8 T cell responses that were previously undetectable. There was no relationship between response to blockade and viral load, liver inflammation, or HBeAg status in this small group of individuals studied. To determine whether IL-10 blockade could recover additional T cell effector functions, we investigated the polyfunctionality of CD8 T cell responses (defined as ability to produce IFN- γ , IL-2, or TNF- α ; proliferate [CFSE]; or degranulate [CD107]) after stimulation with HBV or CMV peptides in six patients. Contour plots depict representative effector responses of HBV-specific CD8 T cells after IL-10 blockade (Fig. 2C). No IL-2 production above background was detected in any of the patients studied. As shown in Fig. 2D, we observed recovery of cytolytic potential in three of six patients with CHB. Analysis of CD8 T cell polyfunctionality was performed using Simplified Presentation of Incredibly Complex Evaluations software, grouping responding CD8 T cells by their ability to produce one, two, or three or more effector functions. By this method, the contributions of each individual response profile to the total virus-specific CD8 T cell response before and after IL-10 blockade were analyzed (Fig. 2E). From this analysis, we observed that, in five of six patients, IL-10 blockade uncovered novel polyfunctionality, with an increased proportion of the total CD8 T cell response represented by dual and polyfunctional cells (Fig. 2F). One patient, who already had an unusually polyfunctional response to HBV, did not show any enhancement of this upon IL-10 blockade (Fig. 2F). Polyfunctionality of CMV-specific CD8 T cell responses from patients with CHB was also enhanced upon IL-10 blockade (Supplemental Fig. 1), suggesting that IL-10 may have a global suppressive effect. This is in line with our previous finding that CD8 T cells are functionally impaired in CHB, regardless of their Ag specificity (22). These data imply that different effector functions of virus-specific CD8 T cells are susceptible to suppression by IL-10. IL-10 blockade was not effective in all patients studied, in line with findings showing that multiple mechanisms are known to contribute to the exhaustion of the T cell response in CHB (17, 20, 23).

IL-10-producing B cells are enriched in CHB and correlate temporally with disease flares

IL-10-producing B cells were recently identified in humans as potent regulators of T cell proliferation and cytokine production in healthy donors (14) and in autoimmune disease (13, 15). However, their role in human infection is unknown. To first assess the role of circulating, rather than intrahepatic, mononuclear cells in IL-10 release in CHB, we quantified levels in supernatant after stimulation with mitogens (PMA and ionomycin). As shown in Fig. 3A, PBMCs from patients with CHB produced more IL-10 than did those from healthy donors ($p < 0.05$). We next investigated the contribution of IL-10-producing B cells to this enhanced capacity for IL-10 production within circulating leukocytes. PBMCs were stimulated with CpG, a TLR-9 agonist, relevant in the context of the DNA virus HBV, which was shown to potently induce B cell IL-10 release (14, 24, 25).

In response to this stimulus, we observed a mean 5-fold increase in the frequency of B cells producing IL-10 in patients ($n = 25$) compared with controls ($n = 14$; $p < 0.0001$) (Fig. 3B, *upper panel*). Additionally, the frequency of IL-10-producing B cells was enriched during flares of disease activity (Fig. 3B, *lower panel*). As shown in Fig. 3C, there was no difference in the frequency of total CD19⁺ B cells between healthy donors and patients, suggesting that a subset of IL-10-producing B cells may arise from the existing pool during active flares of disease.

We next investigated whether B cells could produce IL-10 in response to HBV-derived Ags. HBV core Ag potently induced IL-10 production from healthy donor PBMCs (Fig. 4A). Stimulation with HBsAg and HBeAg did not induce IL-10, suggesting that the effect of HBcAg was not mediated by a nonspecific contaminant in the Ag preparations. The effect of HBcAg could be due to its unique ability to activate B cells, regardless of their Ag specificity, by cross-linking surface receptors (26). Similarly, there was an enrichment of B cells producing IL-10 in response to HBV core Ag stimulation that mirrored the spontaneous flares of disease observed in two patients (Fig. 4B).

Immature CD19⁺CD24^{hi}CD38^{hi} B cells producing IL-10 are enriched during the peak of disease flares

To investigate the phenotype of IL-10-producing B cells, peripheral blood leukocytes were surface stained for the markers CD19, CD24, and CD38 poststimulation, which differentiate B cells into mature (CD24^{int}CD38^{int}), memory (CD24^{hi}CD38⁻), and immature (CD24^{hi}CD38^{hi}) subsets. Upon CpG stimulation, all B cell subsets could produce IL-10 (Fig. 5A, representative plot). We next studied whether the contribution of each B cell subset to total IL-10 production varied according to disease activity. During the peak of liver inflammation, immature B cells showed a 5–7-fold increase in their capacity to produce IL-10 (Fig. 5B, *upper panels*). In an HBeAg⁻ patient undergoing a flare, almost a third of total IL-10 production (28%) was produced by immature B cells during this peak, despite this subset representing the minority of total B cells. IL-10 production by immature cells did not show analogous large fluctuations in a patient with consistently high levels of viremia and liver inflammation (HBeAg⁺, high DNA, high alanine aminotransferase [ALT], Fig. 5B, *upper panel*). Mature B cells produced IL-10 at constitutively higher levels than did immature or memory subsets in all individuals. However, the proportion of mature or memory subsets able to produce IL-10 did not fluctuate over the course of a flare (Fig. 5B, *lower panels*).

Immature B cells produce IL-10 directly ex vivo in patients with active HBV infection

In vitro stimulation with CpG may alter the phenotype of Bregs. To identify the putative Breg subset without the influence of *in vitro* stimulation, we analyzed the phenotype of IL-10⁺ B cells directly *ex vivo* before, during, and after a hepatic flare of disease. As shown in Fig. 6A, IL-10⁺ B cells were detectable at low frequencies in the absence of any stimulation. IL-10-producing B cells were enriched and clustered together, predominantly within the CD19⁺CD24^{hi}CD38^{hi} immature cells at the peak of viral load. There was no comparable clustering of IL-10-producing CD24^{hi}CD38^{hi} B cells on examination of four healthy donors *ex vivo* (Fig. 6A, *lower panel*). Total immature B cell frequency correlated temporally with peak viral load or liver inflammation in two patients undergoing flares but not in an HBeAg⁺ CHB patient with a persistently elevated viral load and liver inflammation consistently four times greater than the upper limit of normal (>200 IU/l) (Fig. 6B, *upper panels*). In this case, immature B cells were persistently elevated at twice the mean

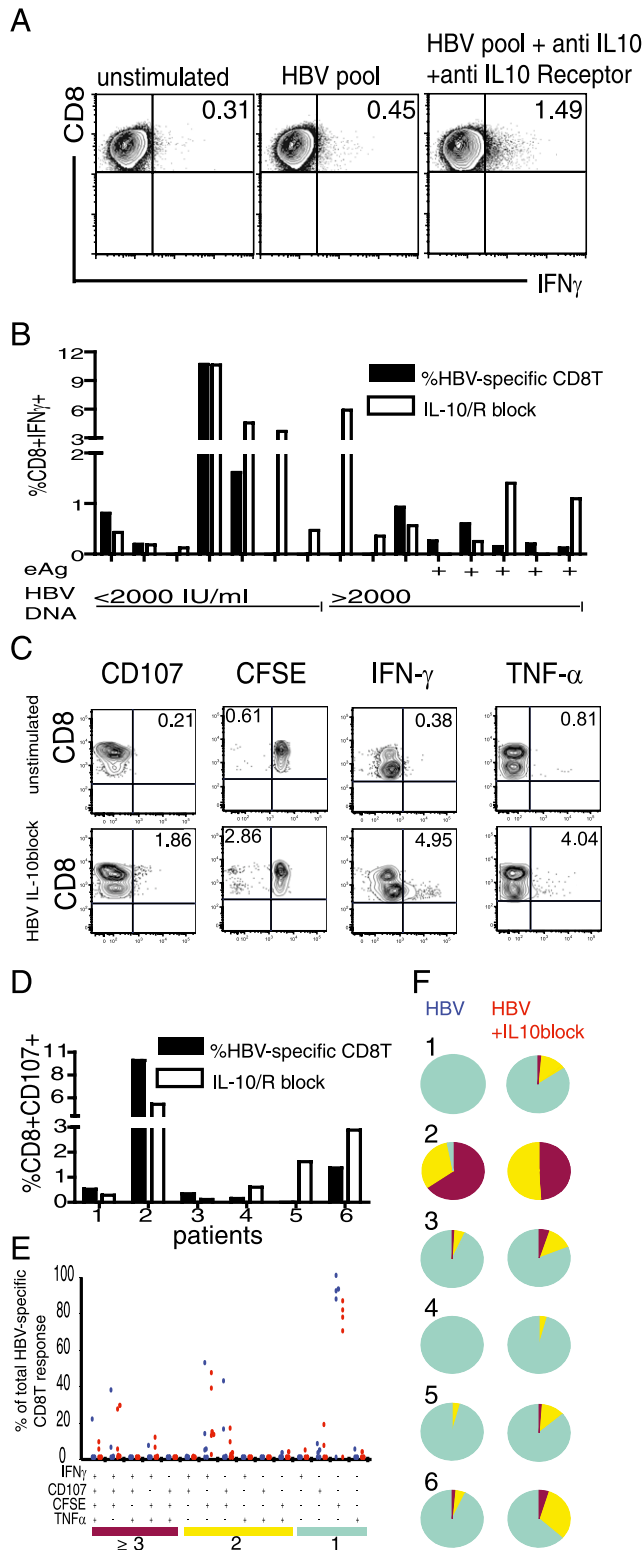


FIGURE 2. Blockade of IL-10 rescues polyfunctionality of virus-specific CD8 T cell responses. PBMCs were stimulated with a pool of HBV peptides, with or without blockade of IL-10 and its receptor. **(A)** Representative contour plots show the effect of IL-10/R blockade on the HBV-specific CD8 T cell response (percentage of CD8⁺IFN- γ ⁺ cells) in a high-level carrier. **(B)** Summary data for 15 patients after subtraction of background from an unstimulated well. **(C)** The polyfunctionality of HBV-specific responses was assessed by their capacity to produce IFN- γ and TNF α , to proliferate (CFSE), and to degranulate (CD107a). Representative contour plots show the gating of each CD8 T cell effector response. **(D)** Summary data from six individuals show the effect of IL-10 blockade on

frequency observed in patients with high viral load (A. Das and M.K. Maini, unpublished observations). Temporal fluctuations in mature, memory, and total B cell frequencies did not correlate with either viral load or liver inflammation (Fig. 6B, *lower panels*). Thus, not only do immature cells have an increased capacity for IL-10 production during the peak of liver inflammation relative to other B cell subsets, they also are enriched in number in patients with CHB *ex vivo*.

Little is known about the presence of different B cells within the human liver, the site of HBV replication. By extracting lymphocytes from surplus tissue available from liver biopsies from patients with CHB (Table II), we determined that the percentage of total B cells and their subset composition was not significantly different from that seen in the periphery of the same patients. Immature B cells (CD19⁺CD24^{hi}CD38^{hi}), which had not previously been identified in the human liver, were present at a frequency of 3–7.3%, suggesting that they may also participate in IL-10-mediated regulation in this setting (Fig. 6C). In support of this, a small population of intrahepatic B cells from patients with CHB showed the capacity to produce IL-10 in response to CpG stimulation (Supplemental Fig. 2).

Functional suppression of HBV-specific CD8 T cell responses by immature B cells

To investigate whether Bregs play a role in the immune tolerance characteristic of this disease, CD19⁺CD24^{hi}CD38^{hi} B cells were depleted from total peripheral blood by FACS, and HBV-specific CD8 T cell responses were determined after 10 d of *in vitro* stimulation with pooled HBV peptides. We observed a 2–4-fold increase in the HBV-specific responses in six of nine patients following selective depletion of immature B cells only, despite this subset accounting for <1% of total peripheral blood leukocytes (Fig. 7A). We observed a trend toward a greater fold change in HBV-specific responses compared with CMV-specific responses from individuals in the same cohort (Fig. 7B).

To further probe the potential of immature B cells to actively suppress antiviral responses, sorted highly purified CD19⁺CD24^{hi}CD38^{hi} B cells were added back to *in vitro* culture in a 1:4 ratio with peripheral blood leukocytes. In all three cases there was suppression of HBV-specific CD8 T cell responses compared with PBMCs depleted of immature B cells (Supplemental Fig. 3; representative example Fig. 7C).

Human immature B cells were recently shown to mediate their suppressive activity in healthy controls and those with autoimmune disease through IL-10. To define the role for IL-10 in the suppressive activity of immature B cells in the setting of a persistent viral infection, we added them into a short-term expansion of HBV-specific CD8 T cell responses with or without IL-10 blockade. The coadministration of Abs to block IL-10 and IL-10R almost completely abrogated the suppression of HBV-specific CD8 T cell responses mediated by CD19⁺CD24^{hi}CD38^{hi} B cells (Fig. 7C). Therefore, immature B cells show regulatory potential and can

degranulate (CD107a) in virus-specific CD8 T cells. **(E)** Graph shows the contribution of each individual response pattern (*x*-axis) as a percentage of the total virus-specific CD8 T cell response (*y*-axis). The response patterns are grouped by the number of positive functions (turquoise, 1 function; yellow, 2 functions; purple, ≥ 3 functions). Summary data are shown for six patients with CHB: blue dots, HBV responses; red dots, HBV responses with IL-10 blockade. **(F)** Pie charts compare the proportion of the total virus-specific CD8 T cell response represented by any combination of one, two, or three or more functions in six individuals before and after IL-10 blockade.

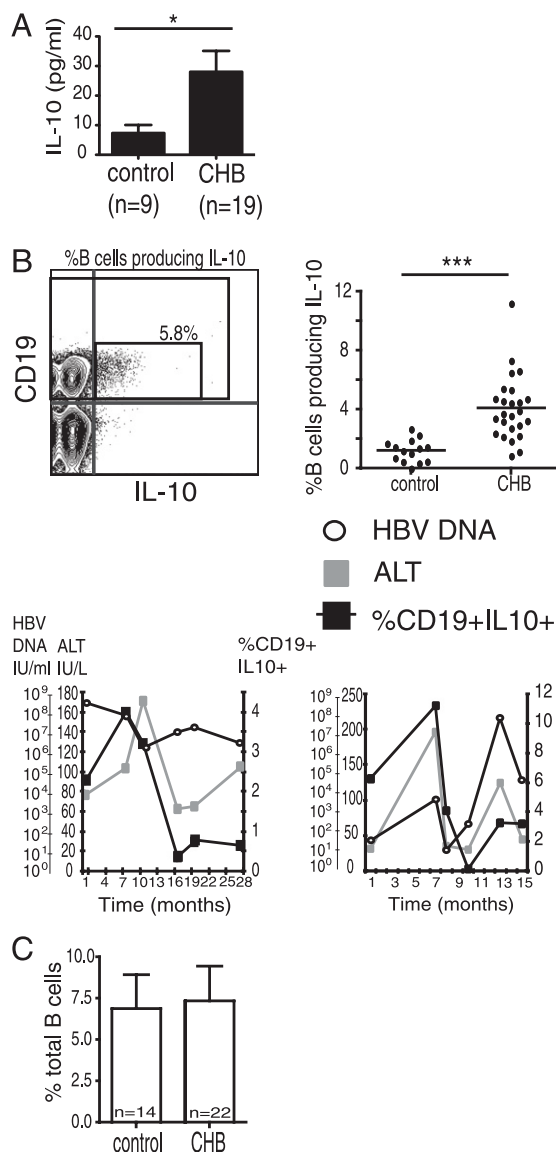


FIGURE 3. IL-10-producing B cells are enriched during active flares of disease. **(A)** Cross-sectional comparison of IL-10 levels in supernatant between patients with CHB ($n = 19$) and controls ($n = 9$) following a 5-h stimulation of PBMCs with mitogens (PMA and ionomycin). **(B)** Representative dot plot depicts the percentage of B cells producing IL-10 following stimulation of PBMCs with CpG for 4 d (PMA/ionomycin added for last 4 h) (*upper left panel*). Cross-sectional data are shown in the *upper right panel*. Longitudinal fluctuations of CD19⁺IL-10⁺ B cells over the course of a flare of liver inflammation in two patients (*lower panels*). **(C)** Bar graph comparing the percentage of total B cells following CpG stimulation in 14 healthy donors and 22 controls; bars represent mean \pm SEM. * $p < 0.05$, *** $p < 0.001$.

suppress HBV-specific CD8 T cell responses, in part through production of IL-10.

Discussion

In this study we showed that serum IL-10 levels correlated with spontaneous flares of liver disease, and IL-10 blockade in vitro could restore HBV-specific CD8 T cell polyfunctionality. Bregs contributed to IL-10 release and were enriched in CHB, in particular during flares. Unstimulated IL-10-producing B cells were of immature phenotype *ex vivo*; depletion of this small subset restored HBV-specific CD8 T cell responses. All B cell subsets were also found in the liver, suggesting that they may be able to

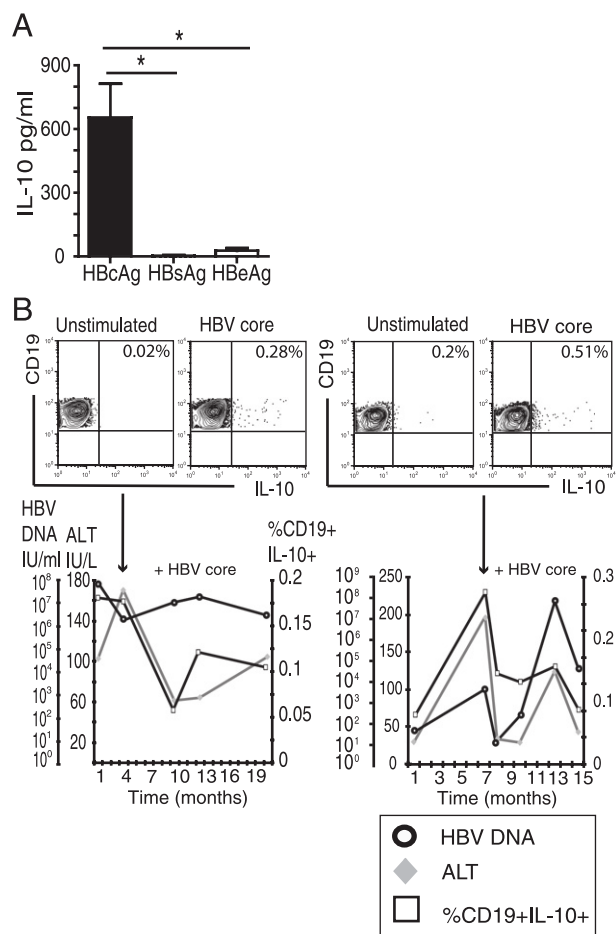


FIGURE 4. B cells produce IL-10 in response to HBcAg. **(A)** IL-10 production in supernatant in response to stimulation of healthy donor PBMCs ($n = 4$) with HBcAg, HBsAg, or HBeAg (10 μ g/ml). **(B)** Longitudinal analysis shows the frequency of IL-10-producing B cells over the course of a flare of liver inflammation following 16 h of stimulation with HBcAg (10 μ g/ml) in two patients (background subtracted) (*lower panels*). Representative dot plots show the frequency of IL-10-producing B cells in medium alone or in response to HBcAg at the peak of liver inflammation (*upper panels*). * $p < 0.05$.

exert regulatory effects on HBV-specific CD8 T cell responses both in the circulation and the liver. These data point toward a pathogenic role for B cells and IL-10 in CHB.

We had access to a unique cohort of patients undergoing spontaneous flares of liver disease in whom we could correlate serum IL-10 levels with disease activity over time. In all individuals with HBcAg⁻ CHB, IL-10 was increased at the time of the peak in viral load or liver inflammation. It was not possible to infer whether IL-10 was more closely linked to viral load or liver inflammation because of their close association during the peak of flares, unlike in acute HBV infection in which IL-10 can start to increase in tandem with viral load, sometimes weeks before the onset of liver inflammation (27). However, previous cross-sectional data showed elevated serum IL-10 in patients with liver inflammation and increased IL-10 during flares induced by treatment withdrawal (19, 28). Thus, it is likely that, in the setting of spontaneous flares of CHB, IL-10 constitutes a physiological mechanism to dampen liver inflammation.

We next investigated whether IL-10 impairs HBV-specific CD8 T cell responses in this setting. As shown previously, IL-10 is known to have a dual role during infection (29). It can both protect the host against Th1-mediated immunopathology during malaria

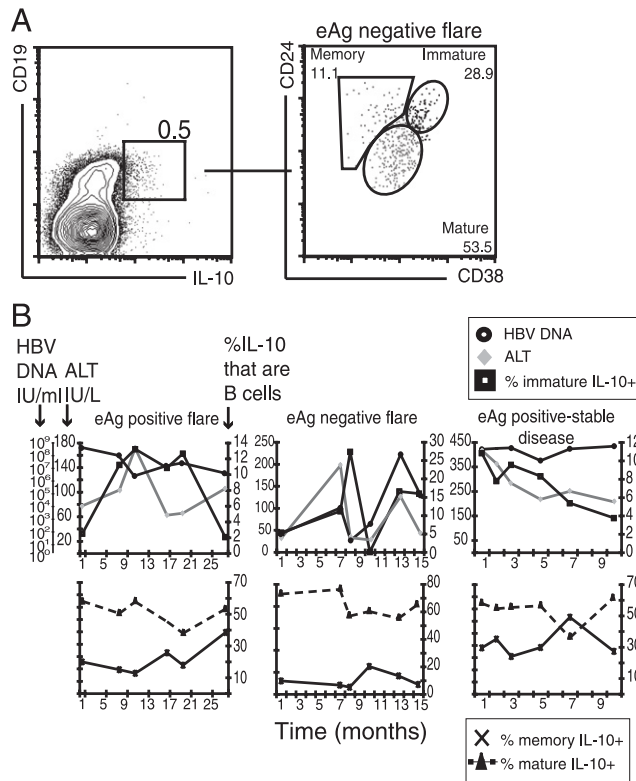


FIGURE 5. Immature B cells are enriched in CHB and have increased capacity for IL-10 production at the peak of liver disease. PBMCs were stimulated with the TLR ligand CpG (96 h) and PMA/ionomycin (4 h), following which the phenotype of IL-10-producing B cells was determined. **(A)** Representative dot plot illustrates that IL-10-producing B cells express markers for mature (CD19⁺CD24^{int}CD38^{int}), memory (CD19⁺CD24^{hi}CD38^{int}), and immature B cells (CD19⁺CD24^{hi}CD38^{hi}). **(B)** The proportion of IL-10 produced by each B cell subset for two patients undergoing flares of liver disease and an HBeAg⁺ patient with nonfluctuating high-level viremia and liver inflammation.

and *Toxoplasma gondii* infections, yet conversely tolerize local and systemic antiviral responses and favor viral persistence in murine chronic infection (19, 30–32). Blockade of IL-10 in these studies improved vaccine efficacy and disease resolution (33–35). In this study, we showed that IL-10 blockade in vitro could recover polyfunctional virus-specific CD8 T cell responses. Based on this, we hypothesize that increased IL-10 during hepatic flares may serve a physiological role in attenuating inflammation; however, a bystander effect may result in an inadvertent dampening of adaptive immune responses. This is supported by studies of *Leishmania major*, in which IL-10-producing T cells suppressed immunopathology in subjects with high Ag load; however, it additionally rendered Th1 responses ineffective, resulting in prevention of lesion healing (36).

The source of IL-10 in CHB is likely to be multicellular, as noted in HIV infection (37). Within the liver, TLR-activated Kupffer cells (38), liver sinusoidal endothelial cell-primed CD4 T cells (39), CD4⁺CD25^{hi}Foxp3⁺ (40), and Ag-induced CD8 T cells are known to produce IL-10 (41), which can suppress T cell-proliferative responses and NK cell function (19, 42). In this study, we showed that mitogen-activated PBMCs were additionally capable of IL-10 production ex vivo, and, upon expansion with CpG, B cells were potent IL-10 producers. Recent findings suggest that IL-10-producing B cells may play a primary role in the induction and maintenance of other regulatory populations, such as regulatory T cells (8); it will be interesting to investigate whether

their suppressive effects can be amplified by other cell types in CHB.

B cells have classically been associated with Ab production and humoral immunity; anti-envelope Abs have been associated temporally with clearance of HBV, and persisting memory B cells maintain the ability to mount a rapid humoral response upon Ag re-encounter, even in patients who have clinically subtherapeutic Ab responses (43, 44). B cells are more activated in CHB compared with healthy donors (45), and they can uniquely interact with viral proteins; the core Ag of HBV can cross-link surface BCRs, irrespective of their Ag specificity, and it is postulated to be presented mainly by B cells not dendritic cells (45, 46). The unique capacity of HBV core Ag to cross-link the BCR in a non-Ag-specific manner suggests that it may be an alternative stimulus in addition to activation through HBV-specific BCRs. This is supported by our observation that B cell IL-10 responsiveness to HBcAg correlated with disease activity.

The potential importance of B cells in ongoing control of HBV was highlighted recently by cases of HBV reactivation triggered by the B cell-depleting drug rituximab (47). An immunoregulatory role is attributed to B cells in another liver disease, primary biliary cirrhosis. Rituximab treatment in this setting exacerbates cholangitis, associated with a large T cell inflammatory infiltrate into the liver and proinflammatory cytokine production (48). In concordance with these observations, recent studies showed an emerging role for non-Ab-dependent B cell functions: primarily modulation of T cell responses through production of soluble cytokines, Ag presentation, and interaction with costimulatory molecules (8). Bregs suppress inflammation and autoimmunity in mice (2, 3), and they can suppress T cell proinflammatory cytokine production in healthy human donors (13); this is mediated primarily by IL-10 production. Importantly, the absence of endogenous B cell-derived IL-10 alone was shown to favor a proinflammatory environment in murine arthritis, with preferential generation of Th1 and Th17 cells over regulatory T cells. B cell-derived IL-10 was similarly shown to suppress pathogenic T cell responses in experimental autoimmune encephalomyelitis (7) and *Salmonella* infection (49). These data highlight that a relatively small fraction of total B cells have the capacity to potently modulate T cell responses in vivo (12).

Naive B cells express TLR9 and TLR10, which are upregulated upon activation, in contrast to TLR4, which lacks significant expression on human B cells (50, 51). Stimulation via TLRs was shown to induce regulatory function and IL-10 production in B cells by a two-step model: TLR stimulation first primes B cells toward IL-10 production and they are then further potentiated toward a regulatory phenotype upon BCR and CD40 ligation (52). In this study, we observed that stimulation with CpG, an agonist of TLR-9, could induce IL-10 production by B cells and that IL-10 B cells were more enriched in CHB than in healthy donors.

IL-10 B cells were previously assigned distinct phenotypic profiles, including CD19⁺CD24^{hi}CD38^{hi} transitional or CD27⁺CD24^{hi} B10 cells (13, 15). However, we observed that all B cell subsets had the capacity for IL-10 production, similar to that reported by Bouaziz et al. (14), who showed a heterogenous spread of IL-10 B cells among immature and memory B cell compartments. Discrepancies in phenotype between studies is due, in part, to an imperfect panel of markers to characterize this subset at this time, as well as different methods of stimulating these cells, including TLR ligands, CD40, and stimulation through the BCR, all of which could impact on their phenotype. To remove any potential bias of stimulation, we phenotyped IL-10⁺ B cells directly ex vivo, in the absence of stimulation. In this setting, we observed

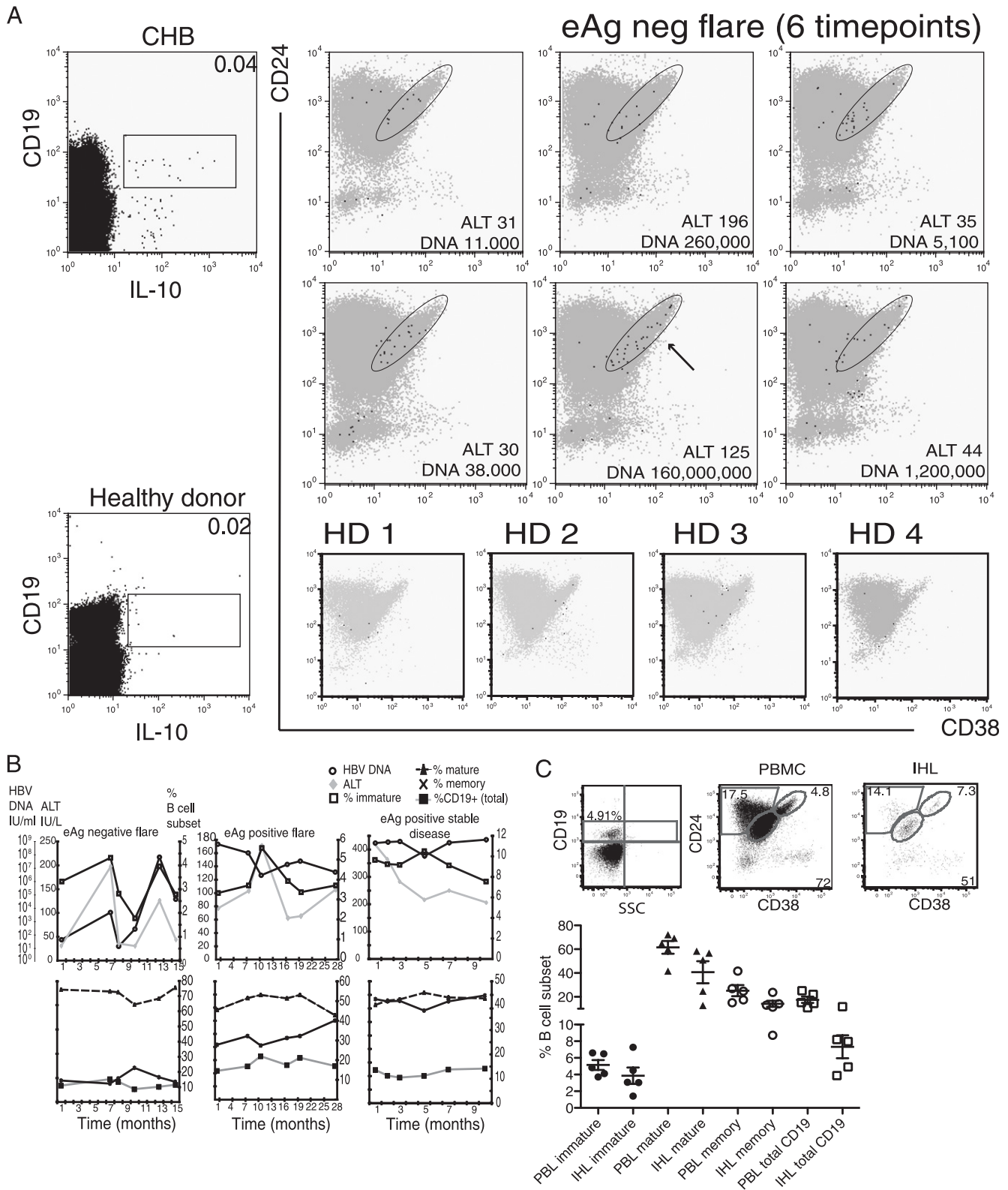


FIGURE 6. Ex vivo characterization of IL-10–producing B cells. **(A)** Dot plots show the phenotypic characteristics of IL-10–producing B cells (unstimulated, ex vivo) over multiple time points in an HBeAg[−] patient undergoing a flare (*upper and middle panels*) or in four healthy donors (HD1-4; *lower panels*). Black dots represent CD19⁺IL-10⁺ B cells overlaid on top of gray dots (CD19⁺IL-10[−] B cells). **(B)** CD19⁺CD24^{hi}CD38^{hi} (□), CD19⁺CD24^{int}CD38^{int} (▲), CD19⁺CD24^{hi}CD38[−] (×), and total B cell (■) frequencies are shown in temporal correlation with HBV DNA (○; IU/ml) and ALT (◆, IU/l) for two patients undergoing flares of liver inflammation and one HBeAg⁺ high-level carrier with stable disease. **(C)** Representative dot plots comparing the frequency of B cell subsets between intrahepatic lymphocytes obtained from biopsy specimens and paired peripheral blood in a patient with CHB (*upper panels*). Summary data comparing the frequency of intrahepatic versus peripheral B cells subsets in five patients with CHB (*lower panel*).

Table II. Patient characteristics for liver samples

Subject	Age (y)	Gender	HBeAg	ALT (IU/l)	HBV DNA (IU/ml)
IHL 1	46	Male	Positive	80	20
IHL 2	40	Female	Negative	7	135
IHL 3	39	Female	Negative	51	1.4×10^6
IHL 4	38	Female	Negative	23	1,027
IHL 5	28	Female	Positive	32	39×10^6
IHL 6	28	Female	Negative	35	23,442
IHL 7	55	Female	Positive	43	151×10^6
IHL 8	21	Female	Positive	56	218×10^6

IHL, Intrahepatic lymphocyte.

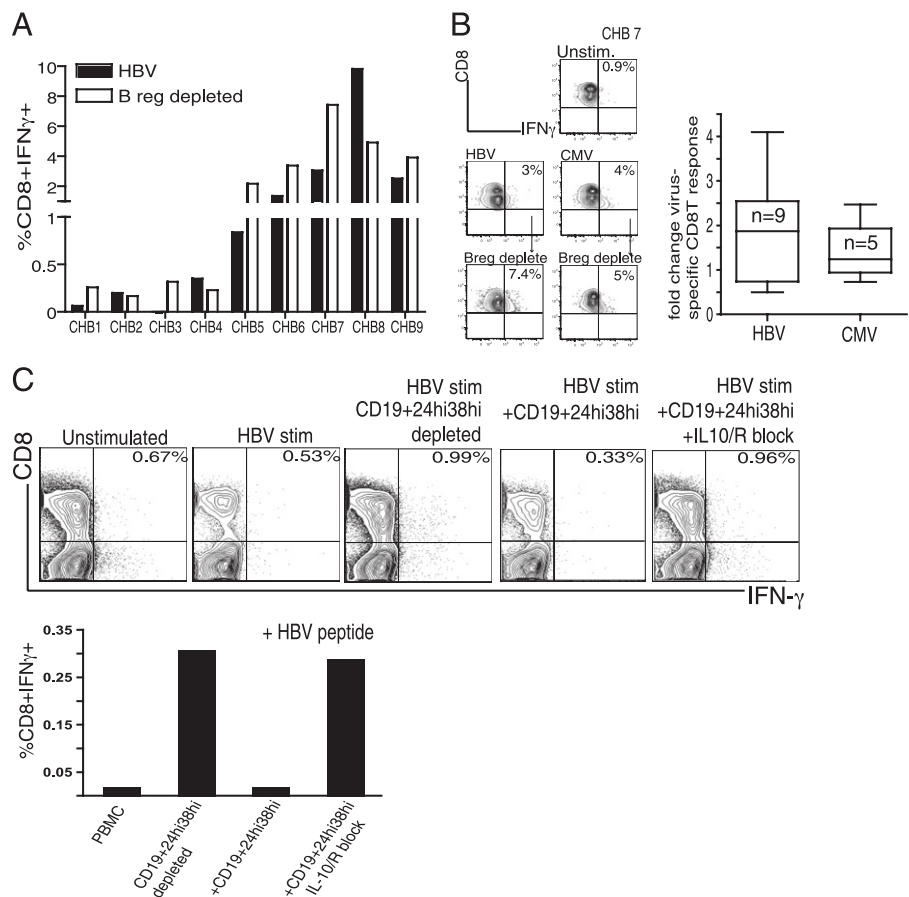
that IL-10-producing B cells were predominantly of the immature phenotype, and immature B cells were the only B cell subset to correlate with hepatic fares *ex vivo*. This is consistent with a phenotypic subset of human transitional Bregs described by Blair et al. (13) and is in line with studies of HIV and hepatitis C virus infection, in which there is a peripheral enrichment of this immature subset (53, 54).

On this pretext, we chose to study immature B cells as the putative regulatory subset in CHB. Depletion of this small subset by cell sorting recovered virus-specific CD8 T cell responses *in vitro*. These observations are similar to those seen in murine arthritis, in which an analogous transitional two marginal zone precursor B cell subset suppressed Ag-specific T cell responses and ameliorated established disease (1). Immature B cells are generated in the bone marrow and traffic to the spleen, where they can mature after encounter with Ag and CD4 T cell help. Recirculation to non-splenic secondary lymphoid organs and sites of inflammation has been reported; however, immature cells actively downregulate

integrin-mediated homing to lymph nodes (55). In this study, we observed that all B cell subsets, including immature B cells, were present in the liver at a similar frequency to that observed in paired peripheral blood. This was despite the absence of significant liver inflammation at the time when liver biopsies were taken from patients. To our knowledge, this is the first demonstration of the presence of immature B cells in the human liver, and it sheds new light on the unexpected trafficking potential of this subset. Although the intrahepatic localization of this subset remains to be defined, a recent study showed that B cells have the potential to infiltrate hepatic parenchyma and form lymphoid aggregates (56). These data allude to an additional site where B cells may encounter and modulate T cells apart from the peripheral blood and spleen, the principal sites where human immature B cells have been identified (57).

Thus, B cells have the capacity to suppress CD8 T cell responses, which could further constrain antiviral responses already dysregulated by coinhibitory signals and proapoptotic pathways (17, 20, 23). The capacity of Bregs to suppress virus-specific CD8 T cell responses implicates a pathogenic role for Bregs and IL-10 in CHB. An *in vivo* role for Bregs is supported by the mirroring between their expansion/contraction and disease activity. We hypothesize that IL-10 may be triggered in this setting as a feedback mechanism to dampen liver inflammation, but it results in suppression of HBV-specific CD8 T cell responses as an inadvertent bystander effect. Our findings highlight the capacity of Bregs to curtail proinflammatory adaptive responses, as well as to downregulate Ag-specific T cell responses. Taken together, to our knowledge, our data provide the first evidence for a role of Bregs in human infection, revealing that they can potently modulate the immune response to HBV.

FIGURE 7. Suppression of HBV-specific CD8 T cell responses by immature B cells. PBMCs from HLA-A2⁺ donors were depleted of CD19⁺CD24^{hi}CD38^{hi} immature B cells by cell sorting. **(A)** Summary data show the effect of CD19⁺CD24^{hi}CD38^{hi} B cell depletion on the HBV-specific CD8 T cell responses in nine patients with CHB. **(B)** Representative contour plots comparing the effect of immature B cell depletion on the HBV- and CMV-specific CD8 T cell response in one CHB patient (*left panel*). Box and whisker plots depict the mean fold change in HBV- and CMV-specific CD8 T cell responses after depletion of immature B cells (*right panel*). **(C)** Sorted CD19⁺CD24^{hi}CD38^{hi} B cells were added back to PBMCs at a 1:4 ratio with or without additional IL-10 blockade; dot plots show the effect of this on the HBV response in a patient with high viral load (*upper panels*). A summary is shown in the bar graph (*lower panel*).



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Disclosures

The authors have no financial conflicts of interest.

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