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Increased killer B cells in chronic HCV infection may lead to autoimmunity and increased viral load

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Summary

Regulatory B (B_{reg}) cells are characterized by various membrane markers and the secretion of different inhibitory cytokines. A new subset of B_{reg} cells was identified as $CD5^{hi}$ Fas-ligand $(FasL)^{hi}$. Their main reported role is to suppress anti-viral and anti-tumour immune responses, and, hence they have been dubbed 'killer' B cells. In this study, we aim to assess the role of these cells in chronic hepatitis C virus (HCV) infection, and determine if they contribute to the increased viral load and persistence of HCV and its related autoimmunity. (i) FasL expression on $CD5^{hi}$ B cells is increased significantly in HCV-infected patients compared to healthy individuals [28·06 ± 6·71 mean fluorescence intensity (MFI) ± standard error of the mean (s.e.m.), median = 27·9 *versus* 10·87 ± 3·97 MFI ± s.e.m., median = 10·3, respectively, $P < 0·0001$]. (ii) Killer B cells from HCV patients increased autologous $CD4^+$ T cell apoptosis compared to the apoptosis in healthy individuals [39·17% ± 7·18% mean ± standard deviation (s.d.), median = 39·6 *versus* 25·92 ± 8·65%, mean ± s.d., median = 24·1%, $P < 0·0001$, respectively]. A similar increase was observed in $CD8^+$ T cell apoptosis (54·67 ± 15·49% mean ± s.d., median = 57·3 *versus* 21·07% ± 7·4%, mean ± s.d., median = 20%, $P = 0·0006$, respectively). (iii) By neutralizing FasL with monoclonal anti-FasL antibodies, we have shown that the induction of apoptosis by killer B cells is FasL-dependent. (iv) Increased expression of FasL on $CD5^{hi}$ B cells is correlated positively with an increased viral load and the presence of anti-nuclear antibodies and rheumatoid factor in HCV. This is the first study in which killer B cells have been suggested to play a pathogenic role in HCV. They seem to be involved in HCV's ability to escape efficient immune responses.

Keywords: autoimmunity, B cell, viral load

Introduction

Hepatitis C (HC) is the second most common infectious disease worldwide, with 130–150 million cases, and 500 000 deaths annually due to cirrhosis or liver cancer. The efficient host immune reaction against the various hepatitis C virus viral proteins determines viral persistence, the extent of infected hepatocytes and the severity of liver inflammation. It has been shown that the persistence of the hepatitis C virus (HCV) is associated with the increased presence of autoantibodies. Anti-nuclear antibodies (ANA), rheumatoid factor (RF) and anti-cardiolipin antibodies (aCL) are the most reported autoantibodies in HC patients with a prevalence of positivity in more than 20% among them [1]. Once primary immunization has occurred, the repeated generation of apoptotic material (during persistent viral infection) might efficiently rechallenge the primed immune system. This capacity for immune-derived autoamplification is possibly a critical principle underlying systemic autoimmune disorder [2]. The first line of defence is always the efficient peripheral immune responses against HCV antigens, mainly cytotoxic T cell response and natural killer (NK) cell activity. Both T

and NK cells were shown to eliminate HCV-infected cells by over-expressing Fas ligand (FasL) and/or producing granzyme B (GranB). In the early phase of infection, B cells produce neutralizing antibodies against different virus epitopes, mainly against envelope glycoproteins E1 and E2 [3]. Most of these antibodies fail to block the entry of the virus into host cells, and therefore the role of humoral anti-viral responses remains limited. Chronic HCV infection and its persistence were reported to be the result of impaired NK cell function and insufficient interferon (IFN)-γ production, in addition to increased secretion of interleukin (IL)-10, leading to the failure of HCV cleaning. We reported previously that enhanced peripheral T cell apoptosis in chronic HCV infection was associated with an increased viral load, autoimmunity and liver disease severity [4,5]. Of the many possible mechanisms by which HCV infection increases the tendency of T cells to undergo apoptosis, worth mentioning are: (1) down-regulation of major histocompatibility complex (MHC) class II and B7 molecules on HCV-infected dendritic cells, which attenuates the delivery of co-stimulation; (2) inhibition of the production of IFN-γ and IL-12, leading to an enhanced responsiveness to IL-10 modulating effects and down-regulation of T helper type 1 (Th1) responses; (3) induction of autoimmune responses due to cross-reactions between HCV core proteins and cryptic epitopes on activated T cells; and (4) enhancement of T cell apoptosis by causing G1/S arrest and c-myc up-regulation [5]. In order to facilitate an efficient process of anti-viral cytotoxicity, a balance between regulatory T cell (T_{reg}) function and efficient effector T cell function is required. In this respect, T_{reg} expansion and their increased suppressive function (induced by HCV signalling) was found to be correlated with altered abilities of effector T cell responses and the persistence of HCV infection followed by its escape to the liver [6]. In this regard, $CD4^+CD25^{hi}$ cells from chronic HCV patients produce higher amounts of GranB, consequently suppressing autologous $CD4^+CD25^{low}$ effector T cells and reducing $CD4^+$ T cell responses against HCV [7]. In parallel with T_{regs}, regulatory B cells (B_{regs}) are involved in suppressing autoimmunity and inflammation. These are characterized by different membrane markers and producing inhibitory cytokines, of which IL-10 is the most dominant [8]. Some studies describe B_{regs} as $CD24^{hi}CD38^{hi}CD1d^{hi}$ with an IL-10-dependent suppressive ability, and is altered in patients suffering from autoimmune diseases such as systemic lupus erythematosus [9]. Other researchers have identified B_{regs} as $CD25^{hi}CD86^{hi}CD1d^{hi}$, IL-10 and transforming growth factor (TGF)-β-producing and showed an increase in T_{reg} function through a cell-to-cell mechanism [10]. In recent years, many studies have reported that $CD5^{hi}$ B cells are a main source of IL-10 production, resulting in them being considered to be another member of the B_{reg} cell family [11,12]. $CD5^{hi}$ B_{reg} cells were also presented as expressing FasL, thereby showing that they play a role in regulating different immune responses by inducing effector T cell apoptosis [13]. In one study, FasL expressing B lymphocytes were purified from the spleen of MRL/lpr mice, and were shown to be potent cytotoxic effectors against Fas-positive targets. The level of FasL expression increased with the extent of the cell-surface activation marker CD69, indicating that expression of FasL is up-regulated in parallel with the activation state of B cells [14]. Later, in a T cell-receptor transgenic mouse model of collagen-induced arthritis, altered T cell death and enhanced severity of arthritis correlated with reduced splenic $CD5^+FasL^+$ B cells. Appropriately dubbed 'killer B' cells, it was suggested that this subset of B_{regs} may provide a novel mechanism for inducing T cell death as a treatment for arthritis [15]. With this in mind, $CD5^{hi}FasL^{hi}$ B cells were reported to play a crucial role in the persistence of some pathogens and in their escape from efficient T cell immune responses. In a very early study, in a murine model of *Schistosoma mansoni* infection, a subset of splenic $CD19^+$ B cells were shown to be FasL-expressing and mediators of $CD4^+$ T cell apoptosis, thus inhibiting anti-pathogen immune responses [16]. Several viral infections, including the human immunodeficiency virus and Epstein–Barr virus (EBV), have been reported to increase Fas (CD95) on effector $CD4^+$ T cells and FasL expression on B cells, leading to increased T cell apoptosis and the evasion of viruses from cellular cytotoxic immune responses. The mechanisms by which viruses and parasites induce FasL expression are still not clear enough [17]. Although it has been mentioned in the literature throughout the last decade, the issue of killer B cells and their contribution to viral persistence remains enigmatic [18]. In this study we aim to analyse, for the first time, the status of this unique subset of B_{regs} in patients suffering from chronic HCV infection. We assume that increased numbers and expression of FasL on $CD5^{hi}$ B cells is followed by these cells' increased killing function, and are thus possibly associated with HCV persistence, increased viral load and disease severity.

Patients and methods

Patients

Our study included 41 patients (15 females and 26 males, mean age = 53·4 years, range = 34–66 years) in whom chronic HCV infection was established by their having increased liver enzymes, positive serum anti-HCV antibodies [assessed by enzyme-linked immunosorbent assay (ELISA) II; Abbot Laboratories, North Chicago, IL, USA] and detectable HCV RNA (polymerase chain reaction). All patients were studied before any specific anti-viral therapy was given. We excluded HCV patients in whom other immune-mediated diseases were diagnosed and to whom any immune-modulated treatments were given. HCV-infected patients are followed at the Liver Disease Clinic,

Bnai-Zion Medical Center and at the Liver Disease Center of Carmel Medical Center, Haifa, Israel. All patients were assessed for the presence of liver enzymes, serum HCV antibodies, a panel of autoantibodies including ANA, anti-smooth muscle antibodies (anti-sm), RF and aCL. A viral load assessment was performed on all patients and was tracked periodically as part of the follow-up. Forty-five healthy age- and sex-matched individuals served as a control group. Informed consent was obtained from all the participating individuals and the study was approved by the local ethics committee for clinical studies.

Methods

HCV quantification. HCV RNA concentration in serum was measured using an Amplicor Monitor Test Kit (Roche Diagnostic Systems, Basel, Switzerland). This test includes an RNA quantification standard of a known copy number that is co-amplified with a target and is used to calculate the copy level of the sample by colorimetric assay following hybridization to a specific probe. All antibodies were detected using commercial ELISA kits as part of our routine evaluation of all HCV patients.

T and B cell isolation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque density gradient. The $CD4^+$ T cells or $CD8^+$ T cells were positively isolated from PBMCs using anti-human CD4 or CD8 microbeads, respectively [magnetic-activated cell sorting (MACS) technology; Milteyni Biotec, Bergisch Gladbach, Germany], according to the manufacturer's instructions. Achieved purity was assessed by fluorescence activated cell sorter (FACS) analysis and was always found to be $> 96\%$. The $CD19^+$ B cells were positively isolated in order to characterize $CD5^{low}$ *versus* $CD5^{hi}$ B cells. However, B cells were also negatively isolated from PBMCs (to be used later for B cell sorting) using the EasySep™ human B cell enrichment kit (StemCell Technologies Inc., Vancouver, BC, Canada), according to the manufacturer's instructions. The desired unlabelled fraction was poured off into a final collection tube, thereby achieving a purity of $> 96\%$.

Assessment of $CD5^{hi}FasL^{hi}$ B cells by flow cytometry

Isolated $CD19^+$ B cells (both naive and activated) were divided into three tubes, and stained as follows: (1) phycoerythrin/cyanin 5 (PE/Cy5) anti-$CD19^+$PE anti-$CD5^+$ Alexa Fluor 488 anti-FasL; (2) fluorescein isothiocyanate (FITC) anti-$CD19^+$PE anti-CD5, PE/Cy7 anti IL-10; and (3) FITC anti-$CD19^+$PE/Cy5 anti-$CD5^+$PE anti-perforin or +Alexa Fluor 647 anti-granzyme B. The staining of intracellular markers was performed using the Fix and Perm Cell Permeabilization Kit™ (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions. The above markers were assessed on naive and activated purified B cells. Cells were cultured overnight with cytosine–phosphate–guanosine-oligodeoxynucleotide (CpG-ODN) and anti-CD40L, as reported previously [10]. Purified B cells were stained and gated on $CD19^+CD5^{hi}$ and $CD19^+CD5^{low}$ and then evaluated for the expression of FasL, GranB, perforin and IL-10.

$CD5hiFasL^{hi}$ B cell sorting. Peripheral B cells were stained with anti-human CD19 PE/Cy5, anti-human CD5 PE and anti-human CD3 peridinin chlorophyll (PerCP)-Cy 5.5. Stained B cells were sorted by BD FACS ARIA III into two subpopulations: (1) cells defined as $CD19^+CD5^{hi}$ but not CD3; and (2) cells defined as $CD19^+CD5^{low}$ but not CD3. Following this sorting process, cells were washed twice in PBS and incubated overnight in a fresh sorting medium at 37°C. Sorted B cells ($CD19^+CD5^{low}$ and $CD19^+CD5^{hi)}$ were then stained with PE/Cy5 anti-$CD19^+$PE anti-$CD5^+$ Alexa Fluor 488 anti-FasL, in order to check $CD5^{hi}$ purity and to double-check FasL expression on each subpopulation. Both $CD5^{hi}$ purity and FasL expression were evaluated by flow cytometer (FC500 and CXP software; Beckman Coulter Life Sciences, Indianapolis, IN, USA).

Assessment of $CD4^+$ and $CD8^+$ T cell apoptosis

Purified $CD4^+$ and $CD8^+$ T cells were immune-stained directly (one step) with propidium iodide (PI) and FITC-labelled recombinant human annexin V (annexin V kit; MedSystems Diagnostics GmbH, Vienna, Austria). Flow cytometry was carried out with a FACS operating with CellQuest software (Becton Dickinson, Mountain View, CA, USA). The total population of viable cells was gated according to their typical forward- and right-angle light-scatter. The percentage of cells stained by annexin V alone or PI/annexin V was determined, taking into account only positive-stained cells. The data were displayed on a dot-plot where each was generated from at least 10^4 events.

The induction of T cell apoptosis following co-culture with killer B cells. Autologous $CD4^+$ and $CD8^+$ T cells were activated overnight with anti-CD3 and anti-CD28 in a medium containing 5 units/ml of IL-2. T cells were then cultured alone for the detection of spontaneous apoptosis, or co-cultured with sorted $CD5^{hi}FasL^{hi}$ or with $CD5^{low}$-$FasL^{low}$ (non-killer B cells) at a 1 : 1 ratio in a 96-well plate for 48 h at a final concentration of 10^5 cells/well. Cells undergoing early apoptosis were those stained with annexin V only, whereas those co-stained with annexin/PI were considered cells in late apoptosis or necrosis.

The induction of apoptosis by killer B cells is FasL-dependent. Aiming to establish that the induction of T cell apoptosis by killer B cells is FasL-dependent, we added anti-FasL neutralizing antibody [FasL monoclonal antibody (mAb) (3C82); Alexis Biochemicals, Nottingham, UK] or isotype control antibody (Alexis Biochemicals) to the co-culture of the cells. When comparing the efficacy of three doses in neutralizing FasL (1, 2·5 and 5 μg ml^{-1}), we found the dosage of 2·5 μg ml^{-1} to be the ideal choice.

Table 1. Clinical and laboratory characteristics of hepatitis C virus (HCV)-infected patients and healthy individuals

	HCV patients $n = 41$	Healthy individuals $n = 45$
Age range	34–66 years	29–65 years
Sex F/M	15/26	21/24
ALT (U/l)	123 ± 95	35 ± 12
HCV RNA titre ($\times 10^5$ copies/ml)	Mean: 14 ± 11 (range: 5–40)	0
RF	16 (39%)	3 (7%)
ANA	17 (41%)	4 (9%)
Anti-cardiolipin	3 (7%)	0

F/M = female/male; RF = rheumatoid factor; ANA = antinuclear antibodies; ALT = alanine transaminase.

Statistical analysis

The assessment of data normality was performed using the D'Agostino–Pearson test, and a comparison of the differences between two groups and assessment of the median values was performed using the Mann–Whitney nonparametric test. A comparison among three groups was performed using Kruskal–Wallis one-way analysis of variance (ANOVA), followed by Dunn's *post-hoc* test. In order to determine the difference between the different autoantibody levels (negative $\leq$ low $\leq$ high), ANOVA and *post-hoc* tests using Tukey's procedure were performed. A two-tailed *P*-value of 0·05 or less was considered to be statistically significant.

The correlation coefficient (r_s) of the correlation between the percentage of killer B cells and viral load was determined using Spearman's correlation test. In order to determine the difference between the different autoantibody levels (negative $\leq$ low $\leq$ high), ANOVA and *post-hoc* tests using Tukey's procedure were performed. A two-tailed *P*-value of 0·05 or less was considered to be statistically significant.

Results

Hepatitis C patients

All patients were studied after HCV infection was confirmed by detecting HCV RNA genomes, and by recording increased levels of antibodies to the hepatitis C virus (anti-HCV). All patients had elevated liver enzymes to at least one of the following: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT). In all patients, serology was performed for the detection of HCV-related autoantibodies. Positive RF was found in 16 of 41 patients (39%), but only in three of 45 healthy controls (7%). ANA positivity was found in 17 of 41 patients (41%), but only in four of 45 healthy controls (9%). For detailed results, see Table 1.

Characterization of killer B cells

In order to define the phenotypical markers of killer B cells, purified B cells from 12 healthy controls were positively selected from PBMCs using CD22 microbeads (purity > 98%) and assessed for 48 h as either non-stimulated or stimulated cells, as described above. In normal controls, the amount of $CD19^+CD5^{low}$ cells was comparable to that of $CD19^+CD5^{hi}$ cells. $CD5^{low}$ and $CD5^{hi}$ B cells were compared in terms of their expression of FasL, GranB, perforin and IL-10. These were found to be expressed significantly more highly on $CD5^{hi}$ B cells. Specifically, the expression of FasL on $CD19^+CD5^{hi}$ cells was significantly higher than that on $CD19^+CD5^{low}$ cells [12·18 ± 2·77 mean fluorescence intensity (MFI) ± standard error of the mean (s.e.m.), median = 12·3 *versus* 4·14 ± 2·0 MFI ± s.e.m., median = 3·2, $P < 0·0001$, respectively]. (Fig. 1). The expression of IL-10 and the death molecules GranB and perforin by $CD5^{low}$ and $CD5^{hi}$ B cells were also significantly higher on $CD5^{hi}$ B cells (2·37 ± 1·04 MFI ± s.e.m., median = 2, *versus* 5·41 ± 1·82 MFI ± s.e.m., median = 5·9, $P = 0·0003$; 2·07 ± 0·80 MFI ± s.e.m., median = 1·9 *versus* 11·59 ± 8·94 MFI ± s.e.m., median = 8·6, $P < 0·0001$; 1·82 ± 1·02 MFI ± s.e.m., median = 1·8 *versus* 4·15 ± 2·25 MFI ± s.e.m., median = 3·8, $P = 0·0069$, respectively). This expression was increased significantly when B cells were stimulated. Again, the above-mentioned markers, namely FasL, IL-10, GranB and perforin, were increased further on both stimulated $CD5^{low}$ and $CD5^{hi}$ when compared to that on naive B cells. This expression was significantly higher on $CD5^{hi}$ compared to $CD5^{low}$ B cells; see detailed data in Table 2.

Killer B cells in healthy controls *versus* HCV patients

Aiming to establish the difference in the amount and level of Fas-ligand expression of killer B cells in HCV patients compared to healthy controls, B cells were isolated from the peripheral blood of 41 patients suffering from chronic HCV infection and from 45 healthy controls and then sorted to $CD5^{high}/CD5^{low}$ B cells (see Methods section). Using flow cytometry analysis, we could not detect any difference in FasL expression on the surface of $CD19^+CD5^{low}$ B cells in HCV patients compared to healthy controls (6·91 ± 1·33 MFI ± s.e.m., median = 6·3 *versus* 4·62 ± 1·98 MFI ± s.e.m., median = 4·5 respectively, $P = 0·053$; data not shown). The expression of FasL on the surface of sorted $CD19^+CD5^{hi}$ cells, however, was found to be significantly higher in HCV patients when compared to the healthy controls (28·06 ± 6·71 MFI ± s.e.m., median = 27·9 *versus* 10·87 ± 3·97 MFI ± s.e.m., median = 10·3, respectively, $P < 0·0001$); see Fig. 2a. Furthermore, killer B cells ($CD19^+CD5^{hi}FasL^{hi}$), were found to be increased significantly in HCV patients when compared to the amount

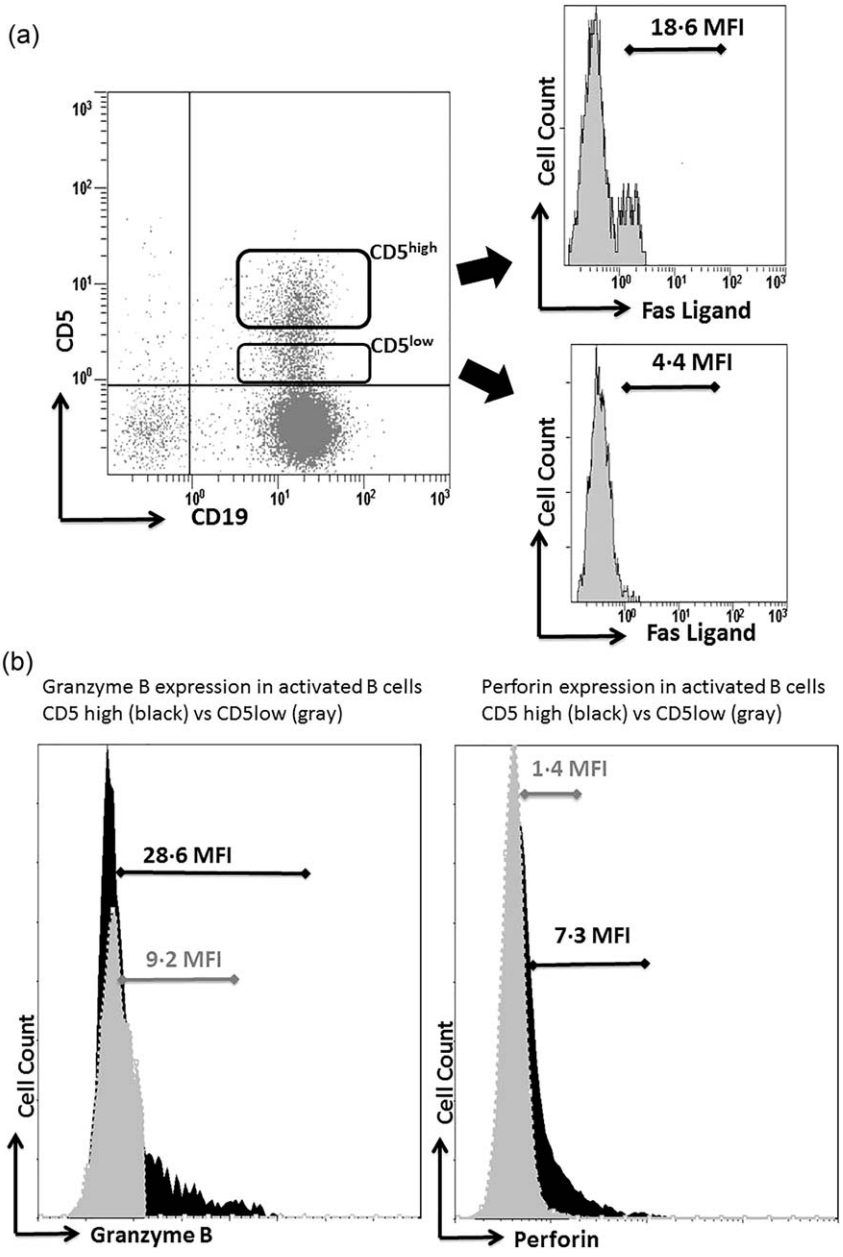

Fig. 1. (a) Fas-ligand (FasL) expression on $CD5^{low}$ and $CD5^{hi}$ B cells in normal individuals. A representative dot-plot experiment presenting $CD5^{low}$ and $CD5^{hi}$ B cells in normal individuals. As can be seen, FasL expression is increased significantly on $CD5^{hi}$ B cells [18·6 mean fluorescence intensity (MFI)] when compared to that on $CD5^{low}$ (4·4 MFI) (on histogram). (b) Left panel: a representative fluorescence activated cell sorter (FACS) histogram showing increased expression of granzyme B in $CD5^{high}$ activated B cells (black) *versus* $CD5^{low}$ activated B cells (grey). Right panel: a representative FACS histogram showing increased expression of perforin in $CD5^{high}$ activated B cells (black) *versus* $CD5^{low}$ activated B cells (grey).

present in healthy controls [44 ± 15·95% mean ± standard deviation (s.d.), median = 41·7 *versus* 29·46 ± 12·58%, mean ± s.d., median = 26·4%, respectively, median = 41·7 *versus* 26·4, respectively, $P = 0·0038$]; see Fig. 2b.

The effect of killer B cells on autologous T cells

Killer B cells were assessed for their ability to induce increased $CD4^+$ and $CD8^+$ T cell apoptosis. To evaluate this, we co-cultured sorted $CD19^+CD5^{hi}FasL^{hi}$ or

Table 2. The expression of regulatory cytokines and death-signal markers (MFI) in primary and activated CD5low vs. CD5hi B cells.

	Primary			Activated		
	$CD19^+CD5^{low}$	$CD19^+CD5^{hi}$	*P*-value	$CD19^+CD5^{low}$	$CD19^+CD5^{hi}$	*P*-value
FasL	4·14 ± 2·0	12·18 ± 2·77	$P < 0·0001$	11·58 ± 3·59	32·03 ± 8·65	$P < 0·0001$
Interleukin-10	2·37 ± 1·04	5·41 ± 1·82	$P = 0·0003$	18·36 ± 4·15	36·44 ± 5·45	$P < 0·0001$
Granzyme B	2·07 ± 0·80	11·59 ± 8·94	$P < 0·0001$	8·48 ± 3·77	27·68 ± 12·98	$P < 0·0001$
Perforin	1·82 ± 1·02	4·15 ± 2·25	$P = 0·0069$	1·33 ± 0·48	6·98 ± 2·34	$P < 0·0001$

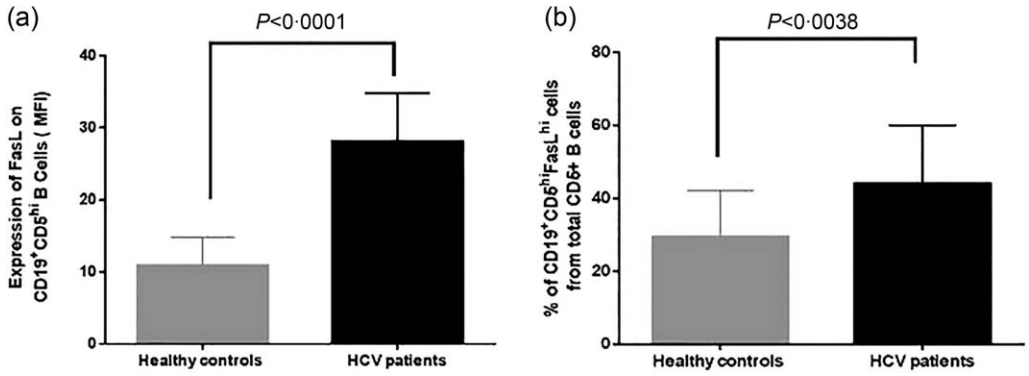

Fig. 2. Characterization of killer B cells in hepatitis C (HC) patients *versus* healthy controls. Flow cytometry analysis of B cells from healthy controls ($n = 41$) and hepatitis C virus (HCV) patients ($n = 45$) showed that (a) Fas-ligand (FasL) expression on $CD19^+CD5^{hi}$ B is significantly higher in HCV patients compared to healthy controls [28·06 ± 6·71 *versus* 10·87 ± 3·97 mean fluorescence intensity (MFI) ± standard error of the mean (s.e.m.), respectively, $P < 0·0001$). (b) The percentage of killer B cells subpopulation is higher in HCV patients compared to healthy controls [44 ± 15·95 *versus* 29·46 ± 12·58%, mean ± standard deviation (s.d.), respectively, $P = 0·0038$].

$CD19^+CD5^{low}FasL^{low}$ cells with purified and activated $CD4^+$ and $CD8^+$ T cells, as described above in the Methods section. We analysed 17 experiments from both HCV patients and healthy controls and assessed the apoptosis of T cells using fluorescent antibodies to CD4/$CD8^+$ T cells and annexin V and PI, as described above in the Methods section. In healthy controls, as can be seen in the representative Fig. 3a, early spontaneous apoptosis of activated $CD4^+$ T cells was 1·5% and late apoptosis/necrosis was 7·4%. Induced early apoptosis in co-cultured $CD4^+$ T cells with $CD19^+CD5^{low}FasL^{low}$ B cells was 4·5%, while the late apoptosis/necrosis rate was 11·7%. When $CD4^+$ T cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$, however, the rate of early apoptosis rose to 17%, and the late apoptosis/necrosis rate was 20·7%. Figure 3b summarizes the apoptosis rate of co-cultured $CD4^+$ T cells with $CD5^{low}$ *versus* $CD5^{hi}$ in normal individuals. In HCV patients, Fig. 3c represents FACS results of the $CD4^+$ T cell apoptosis rate following their co-culture with $CD5^{low}$ *versus* $CD5^{hi}$ in HCV patients. Here as well, a significant increase of T cell apoptosis was noticed following the co-culture of $CD4^+$ T cells with $CD19^+CD5^{hi}FasL^{hi}$.

As can be seen in Fig. 4, $CD19^+CD5^{hi}FasL^{hi}$ B cells from HCV patients caused significantly more $CD4^+$ T cell apoptosis compared to healthy controls (39·17 ± 7·18%, mean ± s.d., median = 39·6 *versus* 25·92 ± 8·65%, mean ± s.d., median = 24·1%, $P < 0·0001$, respectively). Spontaneous $CD4^+$ T cell apoptosis and the rate of apoptosis when T cells were cultured with $CD5^{low}FasL^{low}$ B cells were comparable in HCV patients and healthy individuals.

The same situation was found when we analysed the apoptotic rate in autologous $CD8^+$ T cells: killer B cells induced more apoptosis of autologous $CD8^+$ T cells in HCV patients compared to healthy controls. To determine this, we analysed seven experiments. In healthy controls, spontaneous apoptosis of $CD8^+$ T was 8·6 ± 4·52%, mean ± s.d., median = 7·3%, but when cultured with $CD19^+CD5^{low}FasL^{low}$ B cells, the degree of apoptosis increased to 12·94 ± 5·82%, mean ± s.d., median = 11%, $P = 0·68$, and when $CD8^+$ T cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$ the apoptosis rate rose to 21·07 ± 7·4%, mean ± s.d., median = 20%, $P = 0·0067$ (ANOVA significance = 0·0087). In HCV patients, spontaneous apoptosis of $CD8^+$ T cells was 16·91 ± 7·48%, mean ± s.d., median = 17·9%, but when co-cultured with $CD19^+CD5^{low}FasL^{low}$ B cells, the apoptosis rate increased to 30·64 ± 14·93%, mean ± s.d., median = 28·7%, $P = 0·43$. When these cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$, apoptosis rose to 54·67 ± 15·49%, mean ± s.d., median = 57·3%, $P = 0·0017$ (ANOVA significance = 0·0005). Here as well, $CD19^+CD5^{hi}FasL^{hi}$ B cells induced more $CD8^+$ T cell apoptosis in HCV patients compared to healthy controls (54·67 ± 15·49%, mean ± s.d., median = 57·3 *versus* 21·07 ± 7·4%, mean ± s.d., median = 20%, $P = 0·0006$, respectively).

The induction of T cell apoptosis by killer B cells is prevented by the anti- FasL neutralizing antibody

Aiming to prove the concept of apoptosis being dependent upon FasL expression on killer B cells, we used anti-FasL neutralizing monoclonal antibody to block biological activity of FasL. As described above, we co-cultured T cells with killer B cells, but this time added anti-FasL neutralizing monoclonal mAbs or isotype control mAbs, as described in the Methods section. In healthy controls, the analysis of the results obtained from 10 experiments demonstrated that the spontaneous apoptosis of $CD4^+$ T was comparable in the presence of the isotype control or in the presence of the anti-FasL neutralizing antibody, $P = 0·78$. A similar degree of apoptosis was also recorded when $CD4^+$ T cells were co-cultured with $CD19^+CD5^{low}FasL^{low}$ B cells, either in the presence of the isotype control or anti-FasL neutralizing antibody; $P = 0·72$. However, when $CD4^+$ T cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$, the apoptosis rate rose

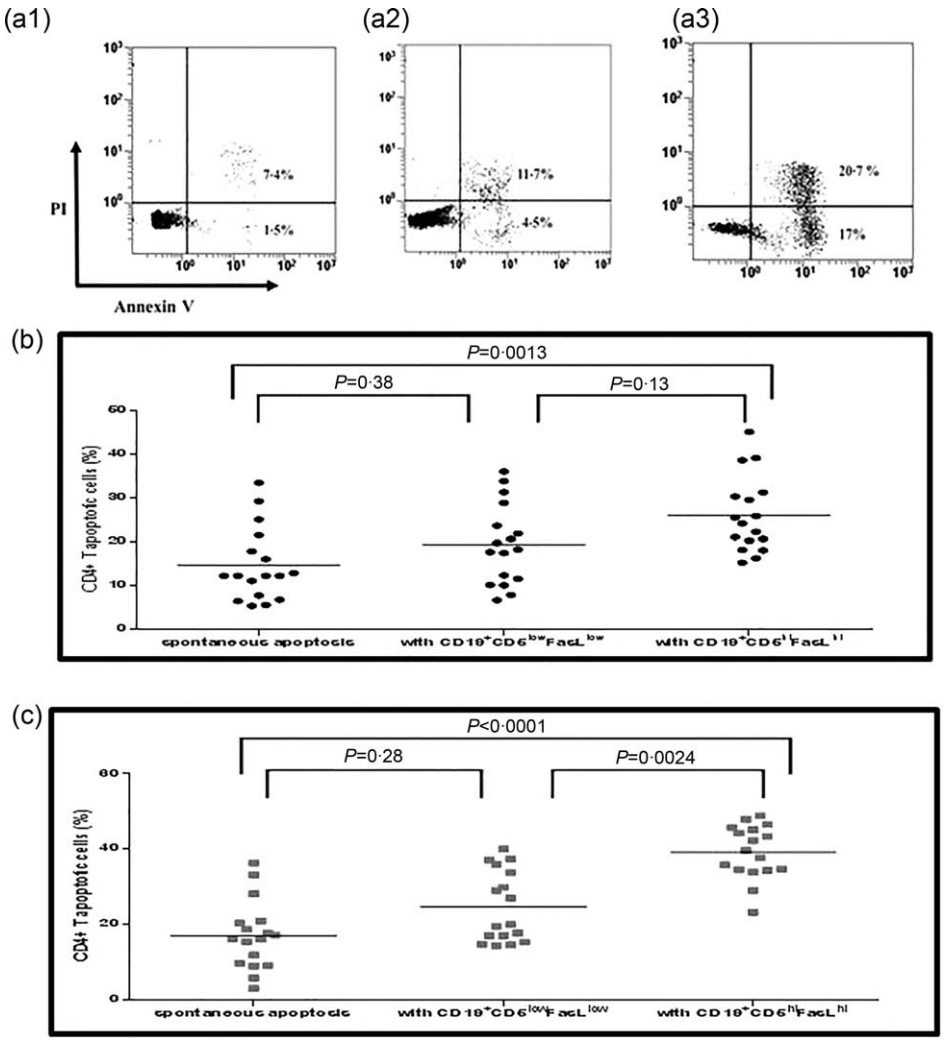

Fig. 3. The effect of killer B cells on autologous $CD4^+$ T cell apoptosis in healthy controls and hepatitis C virus (HCV) patients. (a) Representative fluorescence activated cell sorter (FACS) results of $CD4^+$ T cells apoptosis: I. Spontaneous apoptosis II. Apoptosis induced by $CD19^+CD5^{low}$ B cells. III. Apoptosis induced by $CD19^+CD5^{hi}$Fas-ligand $(FasL)^{hi}$ B cells. (b) Summary of 17 experiments, results of healthy controls [analysis of variance (ANOVA) significance = 0·0019]: the spontaneous apoptosis of $CD4^+$ T cells was 14·53 ± 8·36% mean ± standard deviation (s.d.), median = 12·2%. When $CD4^+$ T cells were co-cultured with $CD19^+CD5^{low}$ the rate of apoptosis increased to 19·24 ± 9·13% mean ± s.d., median = 18·2%, $P = 0·38$. However, when $CD4^+$ T cells were co-cultured with $CD19^+$ $CD5^{hi}FasL^{hi}$, the rate of apoptosis rose to 25·92 ± 8·65% mean ± s.d., median = 24.1%, $P = 0·0013$. (c) Summary of 17 experiments results of HCV patients (ANOVA significance < 0·0001): the spontaneous apoptosis of $CD4^+$ T cells was 16·9 ± 9·1% mean ± s.d., median = 16·1. When $CD4^+$ T cells were co-cultured with $CD19^+CD5^{low}$ the rate of apoptosis increased to 24·66 ± 9·44% mean ± s.d., median = 19·9, $P = 0·28$. However, when $CD4^+$ T cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$, the rate of apoptosis rose to 39·17 ± 7·18% mean ± s.d., median = 39·6, $P < 0·0001$.

to 33·7 ± 9·76%, mean ± s.d., median = 32·9% in the presence of the isotype control, and decreased significantly to 21·17 ± 5·72%, mean ± s.d., median = 21·25% in the presence of anti-FasL neutralizing antibody; $P = 0·0039$; see Fig. 5a. In HCV patients, the analysis of results obtained from seven experiments demonstrated that the same situation as was found when $CD4^+$ T cells were cultured with the isotype control or with neutralizing monoclonal antibody in healthy controls. When $CD4^+$ T cells were co-cultured with $CD19^+CD5^{hi}FasL^{hi}$, however, the apoptosis rate rose to 44·63 ± 10·13%, mean ± s.d., median = 45·9% in the presence of the isotype control, and decreased significantly to 22·33 ± 11·83%, mean ± s.d., median = 20·05% in the presence of the anti-FasL neutralizing antibody; $P = 0·0041$; see Fig. 5b.

An increase in the number of killer B cells is associated with an increased viral load and autoimmunity

Aiming to prove the concept that an increased viral load and the presence of autoimmunity in HCV patients are

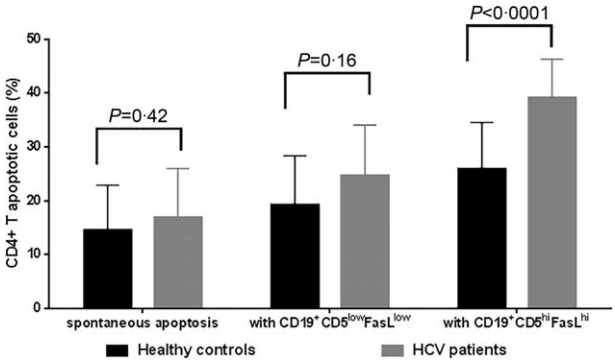

Fig. 4. The effect of killer B cells on autologous $CD4^+$ T cells, comparison between HCV patients and healthy controls. The results demonstrated that killer B cells induce more apoptosis of autologous $CD4^+$ T cells compared to non-killer B cells ($CD19^+CD5^{low}$) in both hepatitis C virus (HCV) patients and healthy controls. However, the rate of $CD4^+$ T cell apoptosis induced by killer B cells was significantly greater in HCV patients than in healthy controls [39·17 ± 7·18% mean ± standard deviation (s.d.), median = 39·6 *versus* 25·92 ± 8·65% mean ± s.d., median = 24·1, $P < 0·0001$, respectively].

associated strongly with increased killer B cells, the percentage of $CD19^+CD5^{hi}FasL^{hi}$ B cells was assessed in correlation with the viral load and with different autoantibodies in HCV patients. We were able to demonstrate that the viral load in HCV patients is correlated strongly and positively with the increase of killer B cells, $r_s = 0·55$, $P < 0·01$; see Fig. 6a. One-way ANOVA was used to show how the presence of RF and ANA effects the percentage of killer B cells (overall ANOVA significance: $P = 0.0264$ and $P = 0·0424$, respectively). *Post-hoc* tests using Tukey's procedure show that the order of the levels are negative ≤ low ≤ high and that the difference between high and negative is significant ($P = 0·0198$, $P = 0·0357$, respectively). See Fig. 6b,c.

Discussion

Historically, most studies have focused upon understanding how HCV infection is cleared by efficient immune responses; namely, natural killer cells and cytotoxic T cells. These responses, however, sometimes fail, and when they do HCV infection becomes chronic, thereby damaging the liver by inducing hepatocyte apoptosis. Seeking to understand that HCV chronicity and its escape from efficient immune responses is frequent, researchers have looked for the mechanisms by which this evasion occurs. The exhaustion of HCV-specific T cells and NK cells is usually balanced by an increase in immune regulatory function; namely, an increased level of anti-inflammatory T_{regs}. Many researchers have linked the failure of HCV clearance and progression to chronic infection with significantly higher IL-10 production and a relative absence of IFN-γ and IL-2 production. In contrast, deficient $CD4^+CD25^+$ cells increased HCV-specific $CD4^+$ and $CD8^+$ T cell proliferation. The failure of $CD4^+CD25^+$ T_{reg} suppressive function was shown to be TGF-β-dependent in a cell-to-cell manner. These data support the idea that high T_{reg} cell frequency is correlated positively with HCV RNA titre [19]. Both $CD4^+$ and $CD8^+$ effector T cells and $CD4^+CD25^{hi}$ T_{regs} are HCV antigen-specific. Subjects who cleared the virus had efficient HCV-specific $CD4^+$ T cell responses dominated by IFN-γ-producing cells and down-regulation of IL-10-producing cells [20]. In chronic HCV infection, IFN-α-based therapy gradually enhances $CD4^+$ T cell responses, whereas IL-10 and TGF-β serum levels are decreased [21]. In one of our early studies, we demonstrated that increased spontaneous $CD4^+$ T cell apoptosis in peripheral blood may be an important mechanism by which HCV escapes efficient immune responses. In the last few years, the role of B cells in fighting HCV infection has been gaining more attention. Being the source of neutralizing anti-HCV antibodies, they are believed to prevent envelope glycoproteins

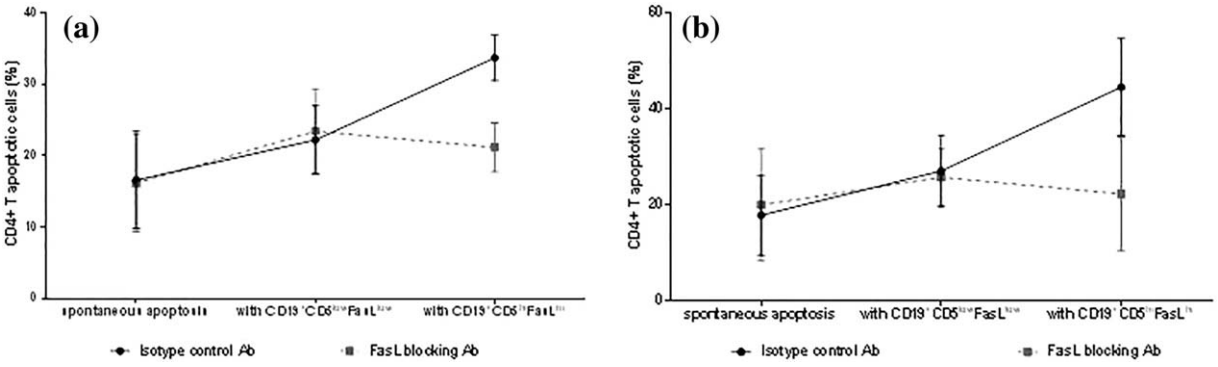

Fig. 5. Killer B cells induce apoptosis of $CD4^+$ T cells in a Fas-ligand (FasL)-mediated mechanism. The results showed that in the presence of isotype control antibody, killer B cells but not non-killer B cells induce increase $CD4^+$ T cells apoptosis. However, when anti-FasL blocking antibody was added, the level of $CD4^+$ T cell apoptosis induced by killer B cells was reduced significantly, with no significant change in the rate of apoptosis induced by non-killer ones. These results are valid for both (a) healthy controls ($n = 10$) and (b) HCV patients ($n = 7$).

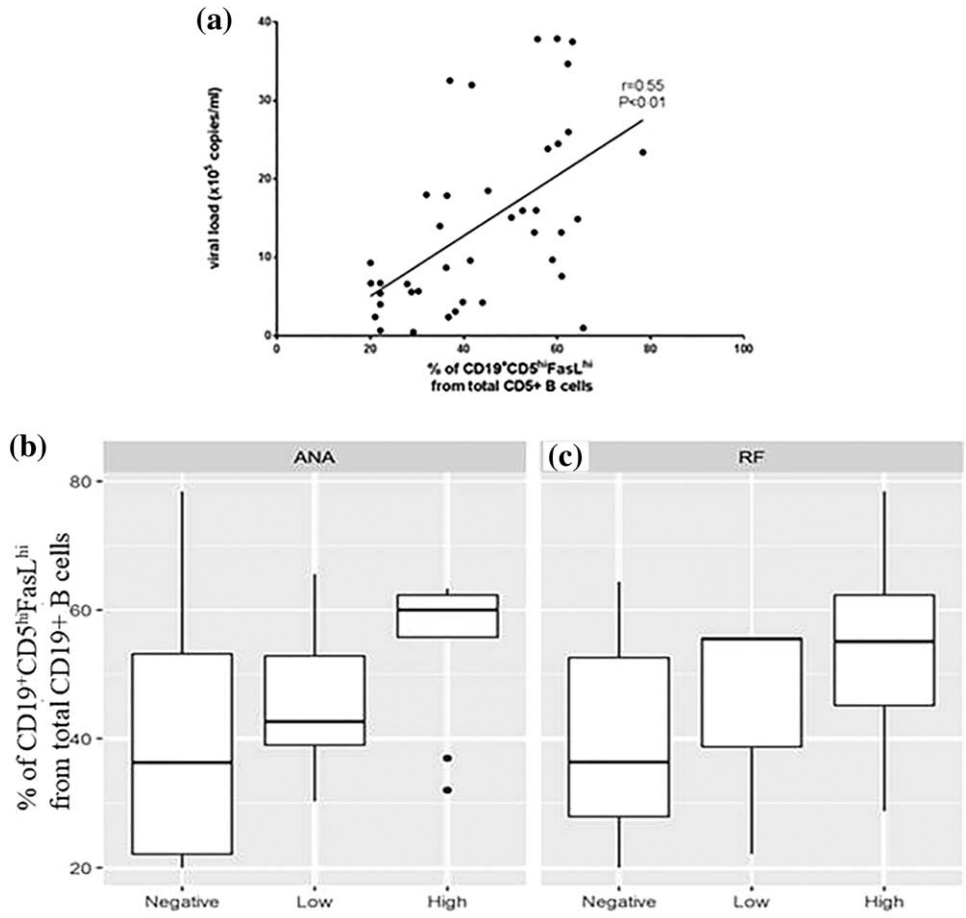

Fig. 6. Killer B cells – association with increased viral load and autoantibodies. (a) Expansion of killer B cells is correlated highly with viral load ($r_s = 0{\cdot}55$, $P < 0{\cdot}01$). Strong correlation was found between killer B cels percentage and viral load. Box-plot analysis between the percentage of killer B cells and the level of (b) anti-nuclear antibodies and (c) rheumatoid factor.

E1 and E2 from entering host cells and modifying the course of acute HCV infection. Chronic HCV infection, by itself, is not sufficient to activate mature memory B cells. The presence of RF in the serum of these patients with chronic HCV infection, however, was found to be associated with a more pronounced state of over-representation of these memory B cells, and that by persisting in the blood, the latter probably play a role in the attempt to clean HCV from the host [22]. So far, little is known about the role of B_{reg} cells in the persistence of HCV. However, in chronic HBV, $CD19^{+}CD24^{hi}CD38^{hi}$-producing IL-10 B_{reg} cells were reported to increase when compared to healthy controls and were able to suppress the response of HBV-specific $CD8^{+}$ T cells [23]. Another subset of B_{reg} cells, $CD19^{+}CD5^{+}CD1d^{hi}IL10^{hi}$, was also found to be increased in both HCV and HBV patients, and to be correlated with HCV RNA in monocytes of patients' sera, as well as with poor virus elimination [24]. Recently, studies have found that $CD5^{hi}$ B_{reg} cells are important in producing IL-10 when specifically activated [25]. Similar to the above-mentioned subsets of B_{reg} cells, these were also reported to play a role in immune-mediated disorders. Of great interest are $CD5^{hi}$ B cells highly expressing FasL, and are therefore called killer B cells. Their role in the spread of malignancy and the persistence of viral infections such as EBV and HIV has been emphasized in the literature [17,26]. Many studies have discussed the issue of HCV entry into immune cells, alluding to its importance for its spread and maintenance of infection. CD81, CLDN1 and other receptors were assessed for their ability to attach HCV. Their over-expression on NK or T cells (although not fully proved) were suggested to be entry receptors for HCV. All this may explain why both FasR and FasL are over-expressed on the T cells of chronic HCV patients, leading to their increased apoptosis and the escape of HCV from clearance. It is important to mention that increased T cell apoptosis is a result of antigen-specific mediated killing, and therefore is not associated with any global T cell immune suppression. Our main aim in this study was to demonstrate the expansion of killer B cells in HCV-infected patients and how they induce effector T cell apoptosis in a FasL-dependent mechanism. It should be mentioned, however, that the well-reported increase of FasR expression in virally infected T cells is an independent factor contributing to the increased effector T cell apoptosis. In addition, we demonstrate the important fact that increased FasL expression on $CD5^{hi}$ B cells and the expansion of this subpopulation of B cells in HCV patients is correlated positively with an increased

viral load and with related HCV autoimmunity. Unfortunately, we were unable to show that this increase is correlated positively with HCV disease severity. This limitation in our study was due to the fact that the severity of HCV was evaluated using different assays in the two centres where HCV patients are followed. Future studies should evaluate this issue. In this respect, it is of importance to assess the level of killer B cell expansion *vis-à-vis* HCV severity and its possible down-regulation following successful treatments and HCV elimination. The over-expression of death molecules such as programmed cell death 1 (PD-1), perforin, GranB and FasL on immune cells, mainly those infected with HCV, suggests that targeting them may become a successful therapeutic strategy in the battle against HCV. In this regard, HCV-specific T cell function was assessed. Anti-viral T cell responses were restored following their incubation with anti-PD-1 antibody. This suggests that PD-1/programmed cell death ligand 1 (PD-L1) blockade should be considered a beneficial therapeutic option in long-lasting chronic HCV infection [27]. Anti-PD-1 antibody was also given to chimpanzees with persistent HCV infection. Here, the control of HCV replication was associated with restoration of intra-hepatic $CD4^+$ and $CD8^+$ T cell immunity against multiple HCV proteins [28]. The beneficial effect of anti-Fas ligand in animal models of chronic hepatitis was reported more than a decade ago. In an animal model of chronic hepatitis, the development of hepatocellular carcinogenesis, hepatocyte apoptosis, proliferation and liver inflammation were all prevented by the neutralization of FasL [29]. Anti-perforin neutralizing antibody was assessed for its ability in reducing myocardial damage in BALB/c mice (used as a model for viral myocarditis). The injection of anti-perforin antibody into these mice reduced myocardial viral titres, the extent of cardiomyocyte apoptosis and down-regulation of messenger ribonucleic acid and caspase-3 expression [30]. Our finding of FasL being over-expressed on killer B cells strengthens the theory regarding their important role in the persistence of HCV and, hence, the therapeutic direction of combining anti-FasL antibodies in the arsenal of anti-viral therapy. Future studies should focus upon evaluating killer B cells following anti-viral therapy and to determine whether this could restore the over-activity of this B cell subset. It is possible that by adding anti-FasL and anti-PD-1 antibodies to other anti-viral therapies we could achieve better and, perhaps, longer-lasting clearance of HCV. In conclusion, killer B cells should be taken into consideration when HCV persistence is studied, and their role in HCV chronicity and disease severity should be defined more clearly.

Disclosure

The authors declare that they have no conflicts of interest.

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