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Phenotypical and functional alterations of CD8 regulatory T cells in primary biliary cirrhosis

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Abstract

The mechanisms that lead to loss of tolerance in autoimmune disease have remained both elusive and diverse, including both genetic predisposition and generic dysregulation of critical mononuclear cell subsets. In primary biliary cirrhosis (PBC), patients exhibit a multilineage response to the E2 component of pyruvate dehydrogenase involving antibody as well as autoreactive CD4 and CD8 responses. Recent data from murine models of PBC have suggested that a critical mechanism of biliary destruction is mediated by liver-infiltrating CD8 cells. Further, the number of autoreactive liver-infiltrating CD4 and CD8 cells is significantly higher in liver than blood in patients with PBC. Based on this data, we have studied the frequencies and phenotypic characterization of both CD4 and CD8 regulatory T cell components in both patients with PBC and age–sex matched controls. Our data is striking and indicate that CD8 Treg populations from PBC patients, but not controls, have significant phenotypic alterations, including increased expression of CD127 and reduced CD39. Furthermore, in vitro induction of CD8 Tregs by incubation with IL10 is significantly reduced in PBC patients. Importantly, the frequencies of circulating CD4+CD25+ and CD8+ and CD28– T cell subpopulations are not significantly different between patients and controls. In conclusion, these data identify the CD8 Treg subset as a regulatory T cell subpopulation altered in patients with PBC.

Keywords

Autoimmune cholangitis CD8 T regulatory cells; IL7-R; CD39

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1. Introduction

Primary biliary cirrhosis (PBC) is a chronic inflammatory disease of the liver characterized by progressive destruction of small and medium size intrahepatic bile ducts leading to cirrhosis and may ultimately require liver transplantation for survival [1]. Genetic, environmental and immunological factors have all been shown to contribute to the pathogenesis of PBC [2,3]. In particular, the presence of circulating autoantibodies, mainly directed to mitochondrial and nuclear antigens (AMA and ANA, respectively), is the hallmark of PBC [4]. Furthermore, affected livers from PBC patients are heavily infiltrated by both CD4 and CD8 T lymphocytes [5]. Such liver cell infiltrates are highly enriched for T cells reactive against mitochondrial autoantigens (E2 subunits of the pyruvate dehydrogenase complex) [6,7]. Finally, alterations in a number of other cell lineages have also been documented, including the frequency of NK and Th17 cells and function [8,9]. Despite this extensive and critical evaluation of both autoantibody and autoreactive T cells, the mechanisms that lead to loss of tolerance has proven elusive. From a generic perspective there is considerable discussion that suggests that deficiencies in the regulatory T cell compartments are responsible for antigen specific loss of tolerance [10–12]. However, this has been difficult to prove in humans and further, despite widely observed, quantitative and/or functional impairments of regulatory T lymphocytes in humans and animal models, it has been difficult to link these observations to liver specific autoimmunity.

PBC is overwhelmingly a syndrome of adults, although interestingly, there is a PBC-like disease reported in a child with IL-2 receptor α (CD25) deficiency [13]. This observation is interesting because there is data from murine models of autoimmune cholangitis that support a deficiency of CD25 expression, impairing Treg function that leads to liver pathology [14–16]. In this respect we note that CD8 T cells play a critical role in loss of CD4+ Treg function in mice [17]. We should also note that quantitative and functional analysis of intrahepatic and circulating Tregs in humans with PBC suggest a loss of T regulatory function [18–20], but these studies have focused entirely on CD4+CD25 Treg cells. Based on the murine data, suggesting a role for CD8 Treg populations, we have specifically addressed and analyzed both CD24+CD25+ and CD8+CD28– Tregs. Our data suggest a defect in CD8 Tregs in patients with PBC and we submit that these cells reflect a dysregulatory population that leads to the loss of tolerance in PBC.

2. Materials and methods

2.1. Patients

The studies reported herein included a total of 15 patients with PBC; all patients were female and ranged from 42 to 80 years of age. The first 11 AMA positive patients were consecutively enrolled without any bias. In addition, four well-documented AMA negative patients with PBC were also included. In all cases, the diagnosis was based on internationally accepted criteria which required the fulfilment of at least two of the following features: elevated serum alkaline phosphatase levels for longer than six months, compatible or diagnostic liver histology, and positive serum AMA [1]. All patients underwent informed consent. Of these 15 patients, 12/15 had Ludwig's histologic stages III–IV and 3/15 had stage I/II [21]. At the time of enrolment, all patients were receiving ursodeoxycholic acid (UDCA) at a dose of 12–15 mg per kilogram of body weight per day for at least 6 months as the only form of treatment. To address the possible role of UDCA, 5 patients consented to a 2-month UDCA wash-out period and the same studies were repeated. These 5 patients served as a nested subset that allowed to test both the reproducibility of the data as well as the potential role of UDCA. The demographics and disease characteristics of all patients enrolled in the study are summarized in Table 1. In addition, fifteen age and sex-matched healthy donors were studied as controls. Reproducibility was evaluated by

analysing two different blood samples from 6 out of the 15 PBC patients and 5 of the 15 healthy controls.

2.2. Immunofluorescence analyses

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque gradient for 30 min at 1800 rpm. The cell surface markers expressed by lymphoid cell subsets were determined using standard flow cytometry. Aliquots of the gradient purified lymphocytes (1×10^5 cells in 100 μ l of PBS) were incubated with specific mAbs at 4 °C for 30 min in the dark. To characterize the phenotype of CD4+ Tregs and CD8+CD28– Treg the following mAbs were used: APC conjugated anti-CD3, PE-conjugated anti-CD25, PerCP 5.5 conjugated anti-CD8, FITC-conjugated anti-CD4, phycoerythrin or fluorescein isothiocyanate conjugated anti-CD28, phycoerythrin conjugated anti-CD25, allophycocyanin–cyanin 7 conjugated anti-CD3 FITC-conjugated anti-CD45RA, PE-conjugated anti-CD127, PE-conjugated anti-CCR7 (all from BD Biosciences, Franklin Lakes, NJ USA). Isotype-matched, FITC- or PE-conjugated mAbs, specific for non-relevant antigens, were used as negative controls. After staining procedures the cells were acquired and analyzed by a FACSCan to flow cytometer (BD Biosciences) using the FACSDIVA software. All analysis was performed in a “blinded” fashion with the evaluator unaware of whether the data was obtained from a patient or a control.

2.3. Purification of CD4+CD25+ and CD8+CD28– T lymphocytes

Highly enriched populations of CD4+CD25+ and CD8+CD28– T cell subsets were isolated from PBMC by sequential cycles using commercially available magnetic beads (Milenyi Biotec, Bergisch Gladbach, Germany). While the CD4+CD25+ regulatory T cell isolation kit was used for purification of CD4+CD25+ T cells, the MultiSort CD8 microBeads followed by depletion using microbeads conjugated with the anti-CD28 9.3 monoclonal antibody (mAb) [22] were used for the purification of CD8+CD28– T lymphocytes. The purity of sorted cells resulted $\geq 95\%$ as demonstrated by flow cytometric analysis. The viability of cells was always $>95\%$.

2.4. In vitro generation of CD8 Tregs from PBMC of PBC patients and controls

Purified CD8+CD28– T lymphocytes (1×10^5 cells/well) were re-suspended in culture medium, consisting of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) including 10% fetal calf serum, 2% glutamine and penicillin 100 U/ml-streptomycin 0.1 mg/ml (Sigma, St. Louis, MO, USA). The cells were then incubated with or without 20 U/ml of IL-2 (Chiron, Emeryville, CA, USA) and 40 ng/ml of IL10 (R&D System, McKinley Place NE, MN, USA), in 96 well flat bottomed plates (Corning Costar, Lowell, MA, USA) at 37 °C for 7 days. At the end of the incubation period the cells were collected, washed and used as suppressors in a proliferation suppression assay.

2.5. Proliferation suppression assay

PBMC pulsed with the anti-CD3 UCTH-1 mAb (5 μ g/ml) were cultured for 5 days in a 96 well flat bottomed plate (1×10^5 cells/well) in the presence (or absence) of either CD4+CD25+ Treg or *ex vivo* freshly isolated CD8+CD28– T lymphocytes or *in vitro* generated CD8 Treg (1×10^5 cells/well). Twelve hours before harvesting, 0.5 μ Ci of 3H-thymidine were added to each well. The radioactivity of cells from individual wells was measured by a beta-counter (Hidex Oy, Mustionkatu 2, Finland).

2.6. Statistical analysis

All results are expressed as mean $\pm$ standard deviation. The chi-square or Fisher's exact test was used to derive statistical data. For the analysis of continuous variables, the Student's *t*

and Mann–Whitney *U* tests were used to compare two groups. *P* values less than 0.05 were defined as significant. All of the analyses were two-tailed. The statistical comparisons were made using GraphPad Prism 4.0 Software (GraphPad Software, Inc, La Jolla, CA, USA).

3. Results

3.1. Altered expression of CD127 and CD39 on CD8 Treg from PBC patients

Although no selective cell surface markers have yet to be identified that uniquely identifies Tregs, it is widely accepted that CD4+CD25^{hi} and CD8+CD28[−] phenotypic patterns identify the majority of Tregs belonging to either the CD4 or the CD8 T cell lineages, respectively [23,24]. Hence, in order to achieve information on the frequency of both Treg subpopulations in the peripheral blood of PBC patients, their frequency and absolute numbers were comparatively analyzed in blood samples from PBC patients and healthy subjects. Similar frequencies of circulating CD4+CD25⁺ among CD4 cells were found in PBMC samples from PBC patients and compared with healthy controls (1.08% vs 1.31%, *P* = 0.32), and the same for CD8+CD28[−] among CD8 cells (38.46% vs 47%, *P* = 0.2). It was reasoned that in the absence of numerical defects, qualitative alterations could be at play in determining the functional dysregulation of the Treg compartment. Hence, the phenotypic patterns expressed by both the CD4 and CD8 Treg subsets were evaluated in PBC patients taking into consideration the expression of CD127 (the IL7-R), IL10Ra, CCR7, GITR, CD39, CD49d membrane molecules. The analyses performed on *ex vivo* isolated CD4+CD25⁺ Treg did not show any significant difference as compared with values obtained with cells from healthy subjects (data not shown). Concerning the CD8 T cell compartment, both *in vitro* generated CD8 Treg (Fig. 1a and c) and *ex vivo* freshly isolated CD8+CD28[−] T cells (Fig. 1b and d) were studied. Remarkably, the frequency of CD127⁺ cells was found significantly enhanced in *in vitro* generated CD8 Treg from PBC patients as compared with cells from healthy subjects (Fig. 1a), which is likely due to the down modulation during the generation of Treg function induced by incubation with IL10. Moreover, *in vitro* generated CD8 Tregs from PBC patients showed decreased expression of the CD39 antigen as compared with cells from control (Fig. 1c). These findings demonstrate that in PBC patients CD8 Treg are affected with phenotypic alterations potentially contributing to their function. No significant differences in the expression of CD127 and CD39 were noted in samples from PBC patients prior to (Fig. 1b and d) and following UDCA withdrawal (not shown). In addition, no significant differences were also noted in the frequency and density of cells that expressed IL10Ra, CCR7, GITR and CD49d (not shown) in samples from PBC patients as compared with controls. In 6 patients with PBC and 5 healthy subjects two blood samples were analyzed to test the reproducibility of the data and we obtained similar results (not shown).

3.2. Altered suppressive activity of CD8 Treg from PBC patients

In order to verify whether the phenotypic anomalies observed on CD8 Treg from PBC patients could be related to altered function, the suppressive activity of CD8 Treg from PBC patients was analyzed in comparison with that of control cells. Again, the analysis was performed on both *ex vivo* freshly isolated CD8+CD28[−] T cells and *in vitro* generated CD8 Treg. The suppressive activity of CD4+CD25⁺ T cells was analyzed in parallel with the aim to eventually unveil functional deficits affecting both Treg subpopulation. *Ex vivo* freshly isolated CD8+CD28[−] T cells demonstrated significant suppressive activity (≥25% – threshold for significant suppressive activity) in 7 out of 15 PBC patients, which was not noted in similar cells from control subjects. However, incubation of circulating CD8+CD28[−] T cells with IL10 failed in the *in vitro* generation of CD8 Treg to exert suppressive activity in 10 out of 15 PBC patients. Notably, similar *in vitro* incubation with IL10 led to the generation of CD8 Tregs in 100% of the healthy controls. Indeed, the mean

percent suppressor activities of CD8 Treg from PBC patients was significantly lower than in controls (13.93% vs 67.13%, $P = 0.0001$) (not shown). No significant differences were detected in samples from PBC patients prior to influencing UDCA and treated or not treated with UDCA (not shown). No alteration of the suppressive activity of CD4+CD25+ Treg was detected in our series of PBC patients compared to controls (70.62% vs 64.62%, $P = 0.19$) (not shown). In a nested subset of 6 patients with PBC and 5 healthy subjects we analyzed two blood samples in parallel and we obtained similar results (not shown).

4. Discussion

The results of this study demonstrate that: a) the frequencies and absolute numbers of circulating CD4+CD25+ and CD8+CD28- T cell subpopulations do not significantly differ in PBC patients as compared with healthy controls; b) CD8 Tregs from PBC patients reflect increased expression of CD127 and reduced expression of CD39; c) the *in vitro* generation of CD8 Treg by incubation of cells with IL10 was markedly compromised in cells from PBC patients leading to deficient suppressor activity.

One leading hypothesis on the pathogenesis of autoimmune diseases proposes the existence of an unbalanced ratio between effector and regulatory arms of the immune system [12,25–32]. In particular, numerical and/or functional impairments of Treg are postulated to be at play. Indeed, alterations of circulating Treg have been demonstrated in several autoimmune diseases [10] including rheumatoid arthritis [33,34], systemic lupus erythematosus [35–39], autoimmune thyroiditis [40–42], type 1 diabetes mellitus [43,44] and multiple sclerosis [45]. In particular, the most common finding has been the decreased number of circulating CD4+CD25+ Treg.

In PBC, both autoreactive CD4 and CD8 T cells are clearly involved in the pathogenesis of PBC and liver infiltration of these cells is one of the major features of the disease [3]. However, a growing number of findings point to a predominant role for the CD8 T subpopulation in PBC [17,46]. Also, data support the role of Treg in various murine models of autoimmune cholangiopathies [14–17]. In contrast, scanty and conflicting data are available on the frequency and function of circulating and liver-infiltrating CD4+CD25+ Treg in patients with PBC [18–20]. In the current study, we confirm in an independent series that PBC patients have a normal frequency of CD4+CD25+ Treg in the peripheral blood [18], which indicates that the pathogenic mechanisms in PBC is different from that involved in others autoimmune diseases. Indeed, this assessment is supported by the finding of a normal suppressive function exerted by these cells in PBC patients.

CD8+CD28- T cells constitute the T cell subpopulation which includes the CD8 Treg precursors [47]. In particular, CD28, a major co-stimulatory receptor, is considered responsible for the optimal antigen-mediated T cell activation, proliferation and survival of T cells. Moreover, it has been demonstrated that the accumulation of CD8+CD28- T cells is a key change during T cell homeostasis and function associated with aging in humans [48,49].

In our series of patients with PBC circulating CD8+CD28- T cells, although numerically unaltered, were found unable to mount a regulatory immune response, upon incubation with IL10 in 10 out of 15 (66%) patients. Thus, qualitative defects affecting the CD8 Treg function are likely at play as exemplified by their reduced capacity to generate effective Treg cells. Unfortunately, in the absence of specific markers identifying CD8 Treg precursors, it is not possible at the moment to achieve quantitative data relative to the exact frequency of these cells in the circulation. Concerning qualitative defects, two phenotypic alterations were identified in this study: the increased expression of the CD127 antigen and

the reduced/absent expression of the CD39 molecule. The CD127 antigen, the receptor for IL7, is mainly expressed on effector cells, being physiologically down modulated on Treg [50]. Thus, its elevated expression on CD8 Treg likely indicates the maintenance of effector activities by these cells and their resistance at being committed to Treg function [51]. The CD39 antigen is a nucleosidase present at the cell surface whose activity has been strictly correlated with normal Treg function [52,53]. Interestingly, it was found lowered on *in vitro* generated CD8 Tregs, indicating the impairment of the CD39-dependent mechanism of suppression in these cells.

Collectively, our study demonstrates that both reduced generation and altered function of CD8 Treg coexist in PBC patients, hence suggesting the presence of a profound alteration of the CD8 T cell-dependent regulatory circuit. In this scenario, the finding that *ex vivo* freshly isolated CD8+CD28⁻ T cells from 6 out of 15 (40%) PBC patients spontaneously exert suppressor activity, an unexpected finding since this was not observed in healthy subjects, is relevant. In fact, it may suggest that the immune system of PBC patients tries to respond to the chronic inflammatory process and compensation for the impaired activity of CD8 Treg by targeting their generation at the immune competent tissue sites by inducing their homing to the liver from the circulation. Likely this process progressively decreases the reservoir of CD8 Treg precursors worsening the balance between pathogenic and regulatory immune responses. Altogether, such observations seems to suggest that PBC pathogenesis involves a defect of the CD8 Treg lineage, similarly to other autoimmune diseases [35,54]. Collectively, our data indicates that PBC is an autoimmune disease, which appears to involve a defect of a specific Treg subpopulation, the CD8 Treg, which is not only dysfunctional but its generation for progenitor cells may be impaired. Finally, we join the other contributors to the special volume in congratulating Dr. Harry Moutsopoulos for his contributions and his recognition in this special series on distinguished immunologists in the Journal of Autoimmunity and Autoimmunity Reviews [55–58].

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Abbreviations

PBC	primary biliary cirrhosis
AMA	anti-mitochondrial antibodies
ANA	anti-nuclear antibodies
Treg	regulatory T lymphocytes
UDCA	ursodeoxycholic acid
PBMC	peripheral blood mononuclear cells
IgG	immunoglobulin G
IgM	Immunoglobulin M

References

1. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. N Engl J Med. 2005; 353:1261–73. [PubMed: 16177252]

2. Lleo A, Invernizzi P, Mackay IR, Prince H, Zhong RQ, Gershwin ME. Etiopathogenesis of primary biliary cirrhosis. *World J Gastroenterol.* 2008; 14:3328–37. [PubMed: 18528930]
3. Gershwin ME, Mackay IR. The causes of primary biliary cirrhosis: convenient and inconvenient truths. *Hepatology.* 2008; 47:737–45. [PubMed: 18098322]
4. Invernizzi P, Lleo A, Podda M. Interpreting serological tests in diagnosing autoimmune liver diseases. *Semin Liver Dis.* 2007; 27:161–72. [PubMed: 17520516]
5. Van de Water J, Ansari A, Prindiville T, Coppel RL, Ricalton N, Kotzin BL, et al. Heterogeneity of autoreactive T cell clones specific for the E2 component of the pyruvate dehydrogenase complex in primary biliary cirrhosis. *J Exp Med.* 1995; 181:723–33. [PubMed: 7836925]
6. Shimoda S, Nakamura M, Ishibashi H, Hayashida K, Niho Y. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. *J Exp Med.* 1995; 181:1835–45. [PubMed: 7536796]
7. Shimoda S, Nakamura M, Ishibashi H, Kawano A, Kamihira T, Sakamoto N, et al. Molecular mimicry of mitochondrial and nuclear autoantigens in primary biliary cirrhosis. *Gastroenterology.* 2003; 124:1915–25. [PubMed: 12806624]
8. Chuang YH, Lian ZX, Tsuneyama K, Chiang BL, Ansari AA, Coppel RL, et al. Increased killing activity and decreased cytokine production in NK cells in patients with primary biliary cirrhosis. *J Autoimmun.* 2006; 26:232–40. [PubMed: 16730427]
9. Lan RY, Salunga TL, Tsuneyama K, Lian ZX, Yang GX, Hsu W, et al. Hepatic IL-17 responses in human and murine primary biliary cirrhosis. *J Autoimmun.* 2009; 32:43–51. [PubMed: 19101114]
10. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev.* 2008; 223:371–90. [PubMed: 18613848]
11. Jordan MA, Baxter AG. The genetics of immunoregulatory T cells. *J Autoimmun.* 2008; 31:237–44. [PubMed: 18550334]
12. Abraham M, Karni A, Dembinsky A, Miller A, Gandhi R, Anderson D, et al. In vitro induction of regulatory T cells by anti-CD3 antibody in humans. *J Autoimmun.* 2008; 30:21–8. [PubMed: 18191540]
13. Aoki CA, Roifman CM, Lian ZX, Bowlus CL, Norman GL, Shoenfeld Y, et al. IL-2 receptor alpha deficiency and features of primary biliary cirrhosis. *J Autoimmun.* 2006; 27:50–3. [PubMed: 16904870]
14. Wakabayashi K, Lian ZX, Moritoki Y, Lan RY, Tsuneyama K, Chuang YH, et al. IL-2 receptor alpha(–/–) mice and the development of primary biliary cirrhosis. *Hepatology.* 2006; 44:1240–9. [PubMed: 17058261]
15. Zhang W, Sharma R, Ju ST, He XS, Tao Y, Tsuneyama K, et al. Deficiency in regulatory T cells results in development of antimitochondrial antibodies and autoimmune cholangitis. *Hepatology.* 2009; 49:545–52. [PubMed: 19065675]
16. Oertelt S, Lian ZX, Cheng CM, Chuang YH, Padgett KA, He XS, et al. Antimitochondrial antibodies and primary biliary cirrhosis in TGF-beta receptor II dominant-negative mice. *J Immunol.* 2006; 177:1655–60. [PubMed: 16849474]
17. Yang GX, Lian ZX, Chuang YH, Moritoki Y, Lan RY, Wakabayashi K, et al. Adoptive transfer of CD8(+) T cells from transforming growth factor beta receptor type II (dominant negative form) induces autoimmune cholangitis in mice. *Hepatology.* 2008; 47:1974–82. [PubMed: 18452147]
18. Lan RY, Cheng C, Lian ZX, Tsuneyama K, Yang GX, Moritoki Y, et al. Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis. *Hepatology.* 2006; 43:729–37. [PubMed: 16557534]
19. Sasaki M, Ikeda H, Sawada S, Sato Y, Nakanuma Y. Naturally-occurring regulatory T cells are increased in inflamed portal tracts with cholangiopathy in primary biliary cirrhosis. *J Clin Pathol.* 2007; 60:1102–7. [PubMed: 17158635]
20. Sakaki M, Hiroishi K, Baba T, Ito T, Hirayama Y, Saito K, et al. Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis. *Hepatol Res.* 2008; 38:354–61. [PubMed: 18021223]

21. Ludwig J, Dickson ER, McDonald GS. Staging of chronic nonsuppurative destructive cholangitis (syndrome of primary biliary cirrhosis). *Virchows Arch A Pathol Anat Histol.* 1978; 379:103–12. [PubMed: 150690]
22. Riley JL, Mao M, Kobayashi S, Biery M, Burchard J, Cavet G, et al. Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc Natl Acad Sci U S A.* 2002; 99:11790–5. [PubMed: 12195015]
23. Piccirillo CA. Regulatory T cells in health and disease. *Cytokines.* 2008; 43:395–401.
24. Filaci G, Suci-Foca N. CD8+ T suppressor cells are back to the game: are they players in autoimmunity? *Autoimmun Rev.* 2002; 1:279–83. [PubMed: 12848981]
25. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008; 133:775–87. [PubMed: 18510923]
26. Foustier G, Dave A, Juntti T, von Herrath M. CD103 is dispensable for anti-viral immunity and autoimmunity in a mouse model of virally-induced autoimmune diabetes. *J Autoimmun.* 2009; 32:70–7. [PubMed: 19162441]
27. Venkatesh J, Kawabata D, Kim S, Xu X, Chinnasamy P, Paul E, et al. Selective regulation of autoreactive B cells by FcγRIIB. *J Autoimmun.* 2009; 32:149–57. [PubMed: 19327966]
28. Crispin JC, Tsokos GC. Transcriptional regulation of IL-2 in health and autoimmunity. *Autoimmun Rev.* 2009; 8:190–5. [PubMed: 18723131]
29. Rajaiah R, Moudgil KD. Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev.* 2009; 8:388–93. [PubMed: 19121415]
30. Hondowicz BD, Fields ML, Nish SA, Larkin J, Caton AJ, Erikson J. Autoantibody production in lpr/lpr gld/gld mice reflects accumulation of CD4+ effector cells that are resistant to regulatory T cell activity. *J Autoimmun.* 2008; 31:98–109. [PubMed: 18539433]
31. Mackay IR, Leskovsek NV, Rose NR. Cell damage and autoimmunity: a critical appraisal. *J Autoimmun.* 2008; 30:5–11. [PubMed: 18194728]
32. Lleo A, Selmi C, Invernizzi P, Podda M, Gershwin ME. The consequences of apoptosis in autoimmunity. *J Autoimmun.* 2008; 31:257–62. [PubMed: 18513925]
33. Cao D, van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. *Arthritis Res Ther.* 2004; 6:R335–46. [PubMed: 15225369]
34. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum.* 2004; 50:2775–85. [PubMed: 15457445]
35. Filaci G, Bacilieri S, Fravega M, Monetti M, Contini P, Ghio M, et al. Impairment of CD8+ T suppressor cell function in patients with active systemic lupus erythematosus. *J Immunol.* 2001; 166:6452–7. [PubMed: 11342672]
36. Lyssuk EY, Torgashina AV, Soloviev SK, Nassonov EL, Bykovskaia SN. Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in patients with systemic lupus erythematosus. *Adv Exp Med Biol.* 2007; 601:113–9. [PubMed: 17712998]
37. Liu MF, Wang CR, Fung LL, Wu CR. Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand J Immunol.* 2004; 59:198–202. [PubMed: 14871297]
38. Eisenberg R. Why can't we find a new treatment for SLE? *J Autoimmun.* 2009; 32:223–30. [PubMed: 19329279]
39. Fernandez D, Perl A. Metabolic control of T cell activation and death in SLE. *Autoimmun Rev.* 2009; 8:184–9. [PubMed: 18722557]
40. Kong YC, Morris GP, Brown NK, Yan Y, Flynn JC, David CS. Autoimmune thyroiditis: a model uniquely suited to probe regulatory T cell function. *J Autoimmun.* 2009; 33:239–46. [PubMed: 19822405]
41. Morris GP, Brown NK, Kong YC. Naturally-existing CD4(+)CD25(+)Foxp3(+) regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. *J Autoimmun.* 2009; 33:68–76. [PubMed: 19375891]
42. Jacobson EM, Huber A, Tomer Y. The HLA gene complex in thyroid autoimmunity: from epidemiology to etiology. *J Autoimmun.* 2008; 30:58–62. [PubMed: 18178059]

43. Mikulkova Z, Praksova P, Stourac P, Bednarik J, Strajtova L, Pacasova R, et al. Numerical defects in CD8+CD28- T-suppressor lymphocyte population in patients with type 1 diabetes mellitus and multiple sclerosis. *Cell Immunol.* 2010; 262:75–9. [PubMed: 20219185]
44. Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. Autoreactive human T-cell receptor initiates insulinitis and impaired glucose tolerance in HLA DR4 transgenic mice. *J Autoimmun.* 2008; 30:197–206. [PubMed: 17949947]
45. Moraes-Fontes MF, Rebelo M, Caramalho I, Zelenay S, Bergman ML, Coutinho A, et al. Steroid treatments in mice do not alter the number and function of regulatory T cells, but amplify cyclophosphamide-induced autoimmune disease. *J Autoimmun.* 2009; 33:109–20. [PubMed: 19362805]
46. Hsu W, Zhang W, Tsuneyama K, Moritoki Y, Ridgway WM, Ansari AA, et al. Differential mechanisms in the pathogenesis of autoimmune cholangitis versus inflammatory bowel disease in interleukin-2Ralpha(-/-) mice. *Hepatology.* 2009; 49:133–40. [PubMed: 19065673]
47. Filaci G, Fravega M, Negrini S, Procopio F, Fenoglio D, Rizzi M, et al. Non-antigen specific CD8+ T suppressor lymphocytes originate from CD8+CD28- T cells and inhibit both T-cell proliferation and CTL function. *Hum Immunol.* 2004; 65:142–56. [PubMed: 14969769]
48. Weng NP, Akbar AN, Goronzy J. CD28(-) T cells: their role in the age-associated decline of immune function. *Trends Immunol.* 2009; 30:306–12. [PubMed: 19540809]
49. Konya C, Goronzy JJ, Weyand CM. Treating autoimmune disease by targeting CD8(+) T suppressor cells. *Expert Opin Biol Ther.* 2009; 9:951–65. [PubMed: 19522557]
50. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ Treg cells. *J Exp Med.* 2006; 203:1701–11. [PubMed: 16818678]
51. McKay FC, Swain LI, Schibeci SD, Rubio JP, Kilpatrick TJ, Heard RN, et al. CD127 immunophenotyping suggests altered CD4+ T cell regulation in primary progressive multiple sclerosis. *J Autoimmun.* 2008; 31:52–8. [PubMed: 18406576]
52. Mizumoto N, Kumamoto T, Robson SC, Seigny J, Matsue H, Enjyoji K, et al. CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. *Nat Med.* 2002; 8:358–65. [PubMed: 11927941]
53. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood.* 2007; 110:1225–32. [PubMed: 17449799]
54. Filaci G, Rizzi M, Setti M, Fenoglio D, Fravega M, Basso M, et al. Non-antigen-specific CD8(+) T suppressor lymphocytes in diseases characterized by chronic immune responses and inflammation. *Ann N Y Acad Sci.* 2005; 1050:115–23. [PubMed: 16014526]
55. Ansari AA, Gershwin ME. Navigating the passage between Charybdis and Scylla: recognizing the achievements of Noel Rose. *J Autoimmun.* 2005; 33:165–9. [PubMed: 19682857]
56. Whittingham S, Rowley MJ, Gershwin ME. A tribute to an outstanding immunologist – Ian Reay Mackay. *J Autoimmun.* 2008; 31:197–200. [PubMed: 18502096]
57. Gershwin ME. Bone marrow transplantation, refractory autoimmunity and the contributions of Susumu Ikehara. *J Autoimmun.* 2008; 30:105–7. [PubMed: 18243658]
58. Gershwin ME. The mosaic of autoimmunity. *Autoimmun Rev.* 2008; 7:161–3. [PubMed: 18190871]

Fig. 1.

Analysis of the expression of the CD127 and CD39 antigen on CD8 Tregs from PBC patients and healthy subjects. Panel a: Percentage of CD127 positive cells in *in vitro* generated CD8 Treg from PBC patients and healthy controls and comparison of the mean percentages of CD8+CD28-CD127+ cells before (t0) and after (t7) CD8 Treg *in vitro* generation with cells from PBC patients (black bars) and healthy controls (white bars). A statistically significant decrease of CD127 antigen expression was observed on control cells but not on cells from PBC patients ($P = 0.05$). Panel b: Percentage of CD127 positive cells in *ex vivo* freshly isolated CD8+CD28- T lymphocytes from PBC patients and healthy controls. Panel c: Percentage of CD39 positive cells in *in vitro* generated CD8 Treg from PBC patients and healthy controls and comparison of the mean percentages of CD8+CD28-CD39+ cells before (t0) and after (t7) CD8 Treg *in vitro* generation with cells from PBC patients (black bars) and healthy controls (white bars). A statistically significant difference was observed between the two groups ($P = 0.0032$). Panel d: Percentage of CD39 positive cells in *ex vivo* freshly isolated CD8+CD28- T lymphocytes from PBC patients and healthy controls. All the experiments were conducted in duplicate and data are expressed as percentage means $\pm$ SD of the two results obtained.

Table 1

Clinical, biochemical and serological characteristics of PBC patients.

	AMA+	AMA–	All PBC patients
Number of subjects	11	4	15
Mean age (years)	68 (42–80)	64 (46–75)	67 (42–80)
Duration of disease (years)	14 (4–28)	15 (4–28)	14 (4–28)
Liver cirrhosis (%)	8 (53)	4 (27)	12 (80)
Total bilirubin (mg/dl) (n.v. < 1.0)	0.61 ± 0.11	0.77 ± 0.29	0.66 ± 0.18
Alkaline phosphatase (IU/l) (n.v. < 279)	294 ± 175	736 ± 400	412 ± 311
Alanine aminotransferase (U/l) (n.v. < 50)	30 ± 6	59 ± 27	38 ± 19
Albumin (g/dl) (n.v. > 3,5)	4.41 ± 0.58	4.32 ± 0.18	4.39 ± 0.50
IgG (mg/dl) (n.v. < 1700)	1134 ± 179	1295 ± 134	1177 ± 180
IgM (mg/dl) (n.v. < 280)	289 ± 178	245 ± 109	277 ± 160

Mean values ± standard deviation unless otherwise stated.