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**IN AUTOIMMUNE LIVER DISEASE DEFECTIVE REGULATORY T-CELL
RESPONSIVENESS TO IL-2 RESULTS IN LOW IL-10 PRODUCTION AND
IMPAIRED SUPPRESSION**

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Abbreviations: AIH, autoimmune hepatitis; AILD, autoimmune liver disease; AISC autoimmune sclerosing cholangitis; ANA, anti-nuclear antibody; AST, aspartate aminotransferase; SMA, smooth muscle antibody; T-regs, regulatory T-cells

Keywords: autoimmune hepatitis, autoimmune sclerosing cholangitis, disease activity, immune-regulation, effector cells

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ABSTRACT

Defective immune regulation plays a permissive role enabling effector cells to initiate and perpetuate tissue damage, eventually resulting in autoimmune disease. Numerical and functional regulatory T-cell (T-reg) impairment has been previously reported in autoimmune liver disease (AILD, including autoimmune hepatitis and autoimmune sclerosing cholangitis). In these early reports, however, T-regs were phenotypically defined as CD4⁺CD25⁺ or CD4⁺CD25^{high} cells. In the current study we re-examined phenotypic and functional properties of T-regs by adopting a more refined definition of these cells that also includes negativity or low level of expression of CD127. We studied 43 AILD patients and 22 healthy subjects (HS) and found that CD4⁺CD25⁺CD127⁻ T-regs were decreased in the former. This decrease was more marked in patients with active disease than in those in remission. In AILD T-reg frequencies correlated inversely with parameters of disease activity and were not affected by the immunosuppressive treatment. We also document for the first time that in AILD *bona fide* T-regs produce less IL-10 and are impaired in their ability to suppress CD4⁺CD25⁻ target cell proliferation, a feature that in HS but not in AILD is dependent, at least in part, on IL-10 secretion. Decreased IL-10 production by T-regs in AILD is linked to poor responsiveness to IL-2 and p-STAT5 up-regulation.

Conclusion: T-regs are numerically impaired in AILD, this impairment being more prominent during active disease. Notably, defective IL-10 production, resulting from low T-reg responsiveness to IL-2, contributes to T-reg functional impairment.

INTRODUCTION

Imbalance between effector and regulatory mechanisms results in the breakdown of immune-tolerance and consequent development of autoimmune disease. Numerical and functional defects of CD4⁺CD25^{high}FOXP3⁺ regulatory T-cells (T-regs) - a subset central to the maintenance of immune homeostasis - play a permissive role enabling autoimmune responses to occur and persist.

Despite T-regs are unanimously regarded as possessing distinct functional properties both *in vitro* and *in vivo*, their phenotypic identification remains challenging as the majority of the T-reg defining markers, i.e. CD25, CTLA-4 and FOXP3 - the T-reg transcription factor - are also up-regulated by effector T-cells during their activation.

Bona fide T-regs have been defined by absence or low level expression of CD127 - the IL-7 receptor α chain - that is normally present on activated effector T-cells (1). Human CD4⁺CD25⁺CD127^{low} T-regs were found to express high levels of intracellular FOXP3 and CTLA-4, to exert suppression and to respond poorly to TCR signalling (2). Moreover, expression of CD127 negatively correlates with that of FOXP3, as result of FOXP3 binding to the CD127 promoter, which has a repressor function (3). Collectively these reports indicate that lack or low expression of CD127 differentiate *bona fide* T-regs from activated T-cells.

Numerical and functional impairment of T-regs has been reported in autoimmune hepatitis (AIH), a progressive inflammatory liver disorder characterised by hypergammaglobulinaemia, circulating autoantibodies and a florid mononuclear cell infiltration on histology, referred to as interface hepatitis (4, 5). In AIH the liver damage is mainly perpetrated by CD4 effector T lymphocytes, the extent to which they proliferate

and produce pro-inflammatory cytokines being correlated with the activity and severity of the disease (6). We have previously shown that the magnitude of these autoreactive CD4 T-cell immune responses is associated with the degree of T-reg impairment. In these earlier studies, however, the definition of T-regs primarily relied on their functional suppressive properties and, phenotypically, on their CD25 and FOXP3 expression (7, 8). At variance to our findings, a recent paper from Peiseler and colleagues reports that in AIH T-regs - identified as CD4⁺CD25^{high}CD127⁻FOXP3⁺ cells - are not functionally and numerically defective (9). In the present work we re-examined phenotypic and functional properties of *bona fide* T-regs in our AIH patients, and went on to investigate mechanisms that might affect T-reg ability to suppress.

SUBJECTS AND METHODS

Patients and controls

Forty-three patients with anti-nuclear (ANA) and/or anti-smooth muscle (SMA) positive AIH were studied. Twenty-two patients were female. A liver biopsy performed at the time of or close to diagnosis showed histological features of interface hepatitis in all patients. Sixteen of the 43 patients had bile duct changes characteristic of sclerosing cholangitis on retrograde cholangiography and were diagnosed as having autoimmune hepatitis/sclerosing cholangitis (AISC) overlap syndrome (10); 3 of the 27 AIH patients had inflammatory bowel disease (IBD), including 2 with ulcerative colitis (UC) and 1 with Crohn's disease; 9 of the 16 AISC patients had concomitant UC. When considered together, AIH and AISC are henceforth indicated as autoimmune liver disease (AILD).

Twenty-five patients were studied during drug-induced remission (i.e. normal transaminase levels; [R] patients) while 18 patients had active disease ([A] patients) at the time of study. From 6 of the 18 [A] patients, blood was obtained at diagnosis before treatment was initiated; the remaining 12 [A] patients were studied at relapse during immunosuppression tapering. Patients demographic, clinical and laboratory data are summarised in Table 1. Patients were treated with prednisolone (2.5-5 mg daily at remission and 1-2 mg/kg/day at relapse) either alone or in combination with azathioprine (1-2 mg/kg/day). One [R] and 3 [A] patients were on prednisolone and mycophenolate mofetil (MMF 40 mg/kg/day). In AISC patients, ursodeoxycholic acid (UDCA) at a dose of 15-20 mg/kg/day was added to the immunosuppressive regimen.

Twenty-two healthy subjects (HS) (median age: 27.5 years, range 22.8-35.7, 70% female) served as normal controls. The age difference between AILD patients and HS is due to ethical constraints in obtaining blood from healthy children. Informed consent was obtained from all patients - or their guardians if they were younger than 16 years of age - and from controls. The study was approved by the Ethics Committee of King's College Hospital, London, United Kingdom.

Cell separation

Peripheral blood mononuclear cells (PBMCs) were obtained as previously described (8). Mononuclear cell viability, determined by Trypan blue exclusion, exceeded 98%.

Flow cytometry

The frequency and phenotypic properties of T-regs, identified as CD4⁺CD25⁺CD127^{-low} cells, were determined by flow cytometry. PBMCs were stained with allophycocyanin (APC)-cychrome (Cy)-7-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-

conjugated or phycoerythrin (PE)-Cy7-conjugated anti-CD25, and FITC or PE-conjugated anti-CD127 (all from BD Bioscience Discovery Labware, Oxford, UK). Cells were incubated at 4°C in the dark for 30 minutes, washed with phosphate buffered saline (PBS)/1% foetal calf serum (FCS), resuspended and analysed by flow cytometry on a Becton Dickinson fluorescent activated cell sorter (FACSCanto II, Becton Dickinson Immunocytometry Systems, San José, CA); FACSDiva or FlowJo (TreeStar Inc) software were used for analysis. A minimum of 2×10^4 gated events was acquired for each sample.

Intracellular staining: the frequency of cells positive for FOXP3, T-bet, GATA-3 and ROR- γ t, transcription factors of T-regs, Th1, Th2 and Th17 cells respectively, and CTLA-4 was determined by intracellular staining after cell fixation and permeabilization with Cytofix/Cytoperm (BD Bioscience) and counterstaining with FITC or APC-conjugated anti-FOXP3 (clone PCH101), peridinin chlorophyll protein (PerCP)-Cy5-conjugated anti-T-bet, Pe-Cy7-conjugated anti-GATA3, PE-conjugated anti-ROR- γ t (all from eBioscience, Hatfield, UK) or APC-conjugated anti-CTLA-4 monoclonal antibodies (BD Bioscience). The frequency of IFN γ , IL-17, IL-10, TGF- β and IL-2 producing cells was assessed after exposure to phorbol 12-myristate 13-acetate (PMA) (10 ng/ml)/Ionomycin (500 ng/ml) (both from Sigma Aldrich Company Ltd., Gillingham, UK), incubation with Brefeldin A (10 μ g/ml, Sigma Aldrich) for 5 hours and counterstaining with AlexaFluor 488, PE or APC-conjugated anti-IL-17 (eBioscience), anti-IFN γ (IQ Products, Groningen, The Netherlands), anti-IL-10, anti-IL-2 (BD Bioscience) and PerCP-conjugated anti-TGF- β monoclonal antibodies (R&D Systems, Abingdon, UK). To assess the expression of the phospho signal transducer and activator

of transcription 5 (p-STAT5), cells were initially treated with intracellular IC fixation buffer (eBioscience), incubated at room temperature for 30 minutes, and centrifuged at 600xg for 5 minutes. After addition of ice-cold 100% methanol, cells were incubated at 4°C for 30 minutes and then stained with PE-conjugated p-STAT5 (Y694) monoclonal antibodies (eBioscience). Flow cytometry was performed as indicated above.

Cell purification

CD4⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻ cells to be used in cell polarization experiments (CD4⁺) and suppression assays (CD4⁺CD25⁺ and CD4⁺CD25⁻) were purified from PBMCs using immunomagnetic beads (DynaL Invitrogen, Oslo, Norway) as previously described (7, 8). CD4⁺CD25⁺ cells were further purified according to the expression of CD127. Briefly, CD4⁺CD25⁺ cells were incubated with PE-conjugated anti-CD127 for 30 minutes, then with microbeads conjugated with monoclonal anti-PE antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 15 minutes at 4°C. The CD4⁺CD25⁺CD127⁻ cell population was purified by negative selection using MS columns (Miltenyi Biotec) according to the manufacturer's instructions. The purity of CD4⁺ and CD4⁺CD25⁻ cells exceeded 95%; that of CD4⁺CD25⁺CD127⁻ cells was consistently higher than 92%.

Cell polarization

The transcription factor and cytokine profile of CD4⁺CD25⁺CD127⁻ cells was analysed after exposing purified CD4⁺ cells to different polarizing conditions, respectively favouring the lymphocyte polarization into T-regs, Th1, Th2 or Th17 cells. Culture conditions promoting the development of T-regs consisted of recombinant human (rh) TGF-β at 10 ng/ml, IL-2 at 100U/ml and anti-CD3/anti-CD28 T-cell expander (ratio bead/cell 1:2; Dynal Invitrogen). Th1 cell culture conditions comprised rhIL-12 (20

ng/ml) and anti-IL-4 (10 µg/ml). Conditions driving CD4⁺ lymphocytes into Th2 cells consisted of rhIL-4 (10 ng/ml) and anti-IFN γ (10 µg/ml), while those promoting Th17 cell development comprised rhIL-23 (20 ng/ml), IL-6 (50 ng/ml), IL-1 β (10 ng/ml), TGF- β (3 ng/ml; all recombinant cytokines and neutralizing antibodies were from R&D Systems) and anti-CD3/anti-CD28 T-cell expander (ratio bead/cell 1:50). CD4⁺CD25⁺CD127⁻ cell transcription factor and cytokine profile was analysed at baseline and following 4 days in culture in the presence of T-reg, Th1, Th2 and Th17 polarizing conditions and assessed by flow cytometry as described above.

Suppression assay

CD4⁺CD25⁺CD127⁻ cells were added at 1:8 ratio to CD4⁺CD25⁻ target cells (7, 11). Parallel cultures of CD4⁺CD25⁻ cells on their own were set up under identical conditions. Cells were co-cultured at 37°C and 5% CO₂ for 5 days in the presence of anti-CD3/anti-CD28 T-cell expander (DynaL Invitrogen) (ratio bead/cell 1:2) and IL-2 (30 U/ml). All experiments were performed in duplicate. For the last 18 hours cells were pulsed with 0.25 µCi/well ³H-thymidine and harvested using a multi-channel harvester. The percentage inhibition was calculated using the formula: [1-count per minute (cpm) in the presence of CD4⁺CD25⁺CD127⁻ cells/cpm in the absence of CD4⁺CD25⁺CD127⁻ cells]. As we have previously shown that the extent of inhibition of cell proliferation measured by either ³H-thymidine incorporation or carboxy fluorescein succinimidyl ester (CFSE) staining is comparable (12), the ³H-thymidine incorporation method was selected as it requires fewer lymphocytes and it is therefore suitable when dealing with low yield cell samples.

Neutralization assay

To assess whether the suppressive function of CD4⁺CD25⁺CD127⁻ cells is related to and/or influenced by the release of cytokines, purified mouse monoclonal anti-human IL-10 (clone 23278), TGF- β (clone 9016), IFN γ (clone 25723) and IL-17 (clone 41809) neutralizing antibodies (all from R&D Systems) were added at a final concentration of 10 μ g/ml to purified CD4⁺CD25⁺CD127⁻ cells. After 12 hours, these populations were added to autologous CD4⁺CD25⁻ cells used as targets. Following a 5-day co-culture, cells were tested for their proliferative response.

IL-2 stimulation

Responsiveness of CD4⁺CD25⁺CD127⁻ cells to IL-2 was measured by p-STAT5 expression after 20 minutes stimulation in the presence of 10 ng/ml IL-2. An aliquot of CD4⁺CD25⁺CD127⁻ cells from the same subjects was tested for the frequency of IL-10 producing cells after 4-day stimulation in the presence of the same IL-2 concentration.

Statistical analysis

The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness-of-fit-test; once the hypothesis of normality was accepted ($P > 0.05$), comparisons were performed by paired or unpaired Student t test as appropriate when variables were compared. A one-way analysis of variance, followed by Tukey's multiple comparisons test, was used to compare means of multiple samples. Results are expressed as mean \pm standard error of the mean (SEM), unless otherwise stated, and P values < 0.05 were considered significant. Data were analysed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) and SPSS software (IBM; Hampshire, UK).

RESULTS

The frequency of CD4⁺CD25⁺CD127⁻ T-regs is decreased in AILD and inversely correlated with markers of disease activity

T-regs were identified by flow cytometry as CD4⁺CD25⁺CD127⁻ cells. The gating strategy adopted for their identification is depicted in Figure 1A. T-reg frequency was determined in 32 AILD patients (14 [A] and 18 [R]) and 14 HS. By deploying the gating strategy shown in Figure 1A, we observed decreased proportions of T-regs in AILD patients compared to HS (Figure 1B), also when [A] and [R] patients were considered separately, [R] having higher percentages than [A] (Figure 1C,D). The difference in T-reg frequencies between AILD and HS and within AILD between [A] and [R] patients was also evident when the CD25^{high} subset was analysed (Figure 1 B-D). Within [A] patients, no difference in T-reg frequencies was found between those studied at diagnosis before immunosuppression was started and those studied during an episode of relapse, also when the CD25^{high} T-reg subset was considered (Figure 1E). Within AILD patients, no differences were found between AIH and AISC, or between those with or without concomitant IBD. T-reg frequency was inversely correlated with the levels of AST, γ GT, bilirubin, IgG and with ANA titre (Figure 1F).

T-regs from AILD patients contain markedly lower percentages of IL-10⁺ lymphocytes

The transcription factor and cytokine profile of T-regs was then investigated in 10 of the same AILD patients and in 5 HS (Figure 2A). Compared to HS, T-regs from AILD patients contain lower frequencies of CTLA-4⁺ and FOXP3⁺ cells - in agreement with our

previous findings (8) -, similar frequencies of T-bet⁺, GATA-3⁺, ROR- γ t⁺, IFN γ ⁺, IL-17⁺, IL-2⁺ and TGF- β ⁺ cells, but a markedly lower frequency of IL-10⁺ lymphocytes (Figure 2A). Of note, only a minority of IL-10⁺ T-regs were also FOXP3⁺. The frequency of IL-10⁺FOXP3⁺ cells tended to be lower in AILD than HS (Figure 2B).

As the assays described in the following results sections require high number of cells, they could only be performed in those subjects for whom a sufficient number of lymphocytes could be isolated from the peripheral blood.

In AILD T-regs derived from CD4 cells upon exposure to T-reg polarizing conditions are impaired in their ability to produce IL-10

The transcription factor and cytokine profile of T-regs within CD4⁺ cells exposed to Th1, Th2, Th17 and T-reg polarizing conditions was assessed in 4 AILD patients (1 [A] and 3 [R]) and 4 HS. Upon exposure of CD4⁺ cells to Th1, Th2, Th17 and T-reg polarizing conditions, the proportion of T-bet⁺, GATA-3⁺, ROR- γ t⁺ and FOXP3⁺ cells within T-regs purified at the end of the cultures did not differ between AILD and HS (data not shown). While the frequencies of IFN γ ⁺, IL-17⁺, IL-2⁺ and TGF- β ⁺ cells within T-regs purified at the end of the cultures were similar in AILD and HS (Figure 3), the percentage of IL-10⁺ T-regs isolated from CD4 cells upon exposure to T-reg polarizing conditions was markedly lower in AILD than HS (Figure 3), suggesting also a defect in the differentiation of IL-10 producing T-regs.

T-reg ability to suppress is impaired in AILD and is partially dependent on IL-10 in HS but not in AILD

The suppressive function of T-regs, as assessed by their ability to inhibit the proliferation of autologous CD4⁺CD25⁻ responder cells, was examined in 8 patients (5 [A] and 3 [R]) and 6 HS. Addition of T-regs reduced the mean cpm count of CD4⁺CD25⁻ target cells less effectively in AILD than in HS (Figure 4A), the percentage of inhibition being lower in the former (42%) than in the latter (67%, P<0.001). Within AILD the percentage of inhibition of responder cell proliferation was lower in [A] than [R], though the difference did not reach statistical significance (Figure 4B).

To investigate whether T-reg suppressive function was related to or influenced by cytokine secretion, neutralizing antibodies to IL-10, TGF- β , IFN γ and IL-17 were added to T-regs 12 hours prior to co-culture with responder cells. Exposure to anti-TGF- β , anti-IFN γ and anti-IL-17 neutralizing antibodies did not change T-reg ability to suppress in both AILD and HS, while anti-IL-10 exposure decreased the T-reg ability to suppress in HS but not in AILD patients (Figure 4C).

Following IL-2 stimulation T-regs from AILD patients do not up-regulate p-STAT5 and do not increase IL-10 production

To explore possible causes for impaired IL-10 production by T-regs from AILD patients, we studied the relation between IL-2 stimulation, expression of p-STAT5 - a molecule with a key role in the IL-2/IL-2R signalling and in the development of naturally occurring T-regs (13-15) - and induction of IL-10 (16) in isolated T-regs stimulated with IL-2. The expression of pSTAT5 was tested after 20 min stimulation with IL-2; the expression of IL-10 (determined as proportion of IL-10 producing cells) was tested after a 4-day culture in the absence or presence of IL-2. These experiments were performed in 10 AILD

patients (2 [A] and 8 [R]) and 8 HS. Short-term stimulation with IL-2 resulted in up-regulation of p-STAT5 expression in T-regs from HS but not AILD patients (Figure 5A,B). The proportion of IL-10⁺ cells within T-regs after a 4-day culture was lower in AILD than HS both in the absence and presence of IL-2 stimulation (Figure 5C,D). T-reg culture in the presence of IL-2 resulted in increase in the frequency of IL-10 producing T-regs in HS but not in AILD patients (Figure 5C,D).

DISCUSSION

In the present study we show that in AILD *bona fide* T-regs - defined as CD4⁺CD25^{+/high}CD127⁻ cells - are numerically and functionally defective, and that their impairment is likely to result from decreased IL-10 production.

CD4 T-cells exerting suppressive function were originally defined as those expressing high levels of the IL-2 receptor (CD25) and of the transcription factor FOXP3 (17, 18). Using this definition, we have previously reported low T-reg numbers in AILD (7, 8, 11). The group of Bluestone has subsequently shown that absence or low level expression of the IL-7 receptor (CD127) acts as a more reliable marker of regulatory T-cells (3).

In the present study, where we have included negativity/low expression of CD127 for the phenotypic identification of T-regs, we confirm our previous observations and show reduced *bona fide* T-reg counts, associated with decreased suppressor function, in patients with AILD compared to healthy subjects, both when patients were studied during active disease or during remission, with markedly lower numbers of T-regs characterising the former compared to the latter.

At variance to our findings, Peiseler et al (9) failed to demonstrate a numerical and functional impairment of T-regs, defined as CD4⁺CD25^{high}CD127⁻FOXP3⁺, in adult patients with AIH. This difference in results may be due to the fact that our patients have the more aggressive juvenile form of the disease, though reduced number, but importantly also function of T-regs, defined as CD4⁺CD25^{high}FOXP3⁺, have been reported in adult patients by others (19). A critical evaluation of the techniques used in purifying *bona fide* T-regs and in assessing their function is crucial to allow comparison between different laboratories. In the paper by Peiseler et al the T-reg frequency was reported to be higher in AIH patients with active disease than in those in remission, a finding interpreted as an effect on T-reg number by immunosuppressive treatment (9). Our data do not support this possibility, as no significant differences in the proportions of T-regs were noted between patients studied at diagnosis before starting immunosuppressive treatment and patients at relapse while on immunosuppression.

That a defective T-reg number may be involved in promoting liver damage is supported by the strong inverse correlation with biochemical (AST, γ GT and bilirubin levels) and serological (IgG and ANA levels) indices of disease activity.

Of note, we observed that T-regs from AILD patients, in addition to expressing lower levels of FOXP3 - a finding confirming our previous observations (8) - are also impaired in their ability to produce IL-10. This impairment is noted as well when CD4 cells are exposed to T-reg polarizing conditions, suggesting an intrinsic defect of the CD4 subset to differentiate into IL-10 producing regulatory cells. Previous investigations from our group had already suggested that IL-10 production is impaired in T-regs from AILD patients and indicated that defects in this immune-modulatory cytokine contribute, at

least in part, to T-reg functional impairment (20). The present work extends this finding and shows that neutralization of IL-10 significantly decreases T-reg suppressor ability to control CD4 target cell proliferation in HS, but not in AILD patients, whose T-regs, being defective in their ability to produce IL-10, are not responsive to IL-10 blockade.

In multiple sclerosis it has been reported that removal of T-reg-antagonistic CD127⁺ cells ameliorates T-reg function (21); in contrast, we find that removal of CD127⁺ cells does not restore the suppressive function of *bona fide* T-regs, suggesting that CD127⁻ T-regs in AILD are intrinsically impaired in their ability to suppress.

Tsuji-Takayama et al have shown that IL-2 stimulation of T-regs results in the production of IL-10 through activation of STAT5, the intron 4 of IL-10 locus containing a STAT-responsive element (16). In order to explore the mechanisms leading to impaired IL-10 production by T-regs in AILD, we studied the relationship between IL-2 stimulation, expression of p-STAT5 and IL-10 production. Exposure of *bona fide* T-regs to IL-2 resulted in p-STAT5 up-regulation and increase in IL-10 production in HS, but not in AILD, suggesting that decreased frequencies of IL-10 producing lymphocytes within T-regs from patients derive from poor responsiveness to IL-2 stimulation. A crucial influence of IL-2 and STAT5 on FOXP3 expression and T-reg function has been shown by Garg and colleagues in type 1 diabetes, where the presence of an autoimmune disease-related *IL-2RA* haplotype was associated with defective responsiveness of antigen-specific CD4 T-cells to IL-2 - measured by p-STAT5a expression - and correlated with lower T-reg expression of FOXP3 and impaired suppressor ability (22).

A recent study has shown accumulation of intrahepatic CD4⁺FOXP3⁺ cells, deemed to be T-regs, in patients with AIH, though no functional phenotype or suppressor function were

investigated (23). It would be of interest to characterise in greater depth these liver infiltrating CD4⁺FOXP3⁺ cells in order to determine their expression of IL-7 receptor, their ability to produce IL-10 and to suppress.

In conclusion, our findings suggest that T-reg impairment in AILD results from poor responsiveness to IL-2 leading to defective p-STAT5 expression and IL-10 production. The reduced number of T-regs and their impaired ability to secrete IL-10 are likely to underpin tolerance breakdown in autoimmune liver disease. Future studies should concentrate on manoeuvres capable of restoring T-reg IL-10 secretion by boosting their responsiveness to IL-2.

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FIGURE LEGENDS

Figure 1. Frequencies of CD4⁺CD25⁺CD127⁻ T-regs in AILD are lower than in HS and inversely correlated with markers of disease activity. (A) Gating strategy used to define CD4⁺CD25⁺CD127⁻ T-regs in the circulation. Lymphocytes were initially gated based on their forward (FSC) and side scatter (SSC) patterns. CD4⁺ lymphocytes and CD25⁺CD127⁻ cells within them were subsequently gated. CD25^{high} cells within CD25⁺CD127⁻ lymphocytes are also shown. Representative plots from one HS. (B) Cumulative data showing the mean (\pm SEM) frequency of CD4⁺CD25⁺CD127⁻ and CD4⁺CD25^{high}CD127⁻ cells in HS (n=14) and AILD patients (n=32). (C) Flow cytometry

plots showing CD4⁺CD25⁺CD127⁻ cells in one representative HS, in one patient in remission [R] and in one patient with active disease [A]. (D) Cumulative data showing the mean (\pm SEM) frequency of CD4⁺CD25⁺CD127⁻ and CD4⁺CD25^{high}CD127⁻ cells in HS (n=14), [R] patients (n=18) and [A] patients (n=14). (E) Mean (\pm SEM) frequency of CD4⁺CD25⁺CD127⁻ and CD4⁺CD25^{high}CD127⁻ cells in [A] patients studied at diagnosis (n=6) or during relapse (n=8). *: P \leq 0.05, **: P \leq 0.01, ***: P \leq 0.001. (F) Correlation between frequencies of CD4⁺CD25⁺CD127⁻ cells and levels of AST, γ GT, bilirubin, IgG and ANA titres.

Figure 2. T-regs from AILD patients contain lower frequencies of cells positive for CTLA-4, FOXP3 and IL-10. (A) Mean (\pm SEM) frequency of cells positive for CTLA-4, FOXP3, T-bet, GATA-3, ROR- γ t, IFN γ , IL-17, IL-2, TGF- β and IL-10 in 5 HS and 10 AILD (1 [A] and 9 [R]) within T-regs. (B) (A) Mean (\pm SEM) frequency of cells positive for FOXP3 and IL-10 in HS and AILD patients.

Figure 3. In AILD CD4 cell exposure to T-reg polarizing conditions results in T-regs impaired in their ability to produce IL-10. CD4 cells isolated from 4 HS and 4 AILD patients (3 [R] and 1 [A]) were exposed to Th1, Th17 and T-reg polarizing conditions for 4 days and their cytokine profile was assessed thereafter. Mean (\pm SEM) frequency of IFN γ ⁺, IL-17⁺, IL-2⁺, TGF- β ⁺ and IL-10⁺ cells in HS and AILD patients. **: P<0.01.

Figure 4. T-regs from AILD patients are impaired in their ability to suppress. The ability of T-regs to suppress was evaluated as decrease in CD4⁺CD25⁻ cell cpm count using a ³H-thymidine incorporation assay. (A) Mean (\pm SEM) CD4⁺CD25⁻ cpm count in the absence and presence of T-regs in HS (n=6) and AILD patients (n=8) including 5 [A] and 3 [R]. (B) Mean (\pm SEM) percentage (%) inhibition of responder cell proliferation by

T-regs in 3 [R] and 5 [A] AILD patients. (C) Ability of T-regs to suppress was also tested in the absence or presence of anti-IL-10, anti-TGF- β , anti-IFN γ or anti-IL-17 neutralizing antibodies. Bars represent mean (\pm SEM) % inhibition of responder cell proliferation by untreated or neutralizing antibody treated T-regs. While T-regs from HS decrease their ability to suppress following treatment with anti-IL-10 neutralizing antibodies, T-regs from AILD patients do not.

Figure 5. Following stimulation with IL-10 T-regs from AILD patients do not up-regulate p-STAT5 and IL-10

T-reg p-STAT5 expression and IL-10 production after exposure to IL-2. (A) p-STAT5 mean fluorescence intensity in the absence and presence of IL-2 stimulation. Representative histograms from one HS and one patient. (B) Mean (\pm SEM) of p-STAT5 mean fluorescence intensity (MFI) in the absence and presence of IL-2 stimulation in 8 HS and 10 AILD patients (2 [A] and 8 [R]). (C) Frequency of IL-10 producing T-regs in the absence and presence of IL-2 stimulation. Representative flow cytometry plots of CD4 (x axis) and IL-10 (y axis) fluorescence in one HS and one AILD patient. (D) Mean (\pm SEM) frequency of IL-10 producing T-regs in the absence and presence of IL-2 stimulation in 5 HS and 7 AILD patients (2 [A] and 5 [R]).

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