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Chronic Lymphocytic Leukemia and Regulatory B Cells Share IL-10-Competence and Immunosuppressive Function

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Abstract

Chronic lymphocytic leukemia (CLL) can be immunosuppressive in humans and mice, and CLL cells share multiple phenotypic markers with regulatory B cells that are competent to produce IL-10 (B10 cells). To identify functional links between CLL cells and regulatory B10 cells, the phenotypes and abilities of leukemia cells from 93 patients with overt CLL to express IL-10 were assessed. $CD5^+$ CLL cells purified from 90% of the patients were IL-10-competent and secreted IL-10 following appropriate *ex vivo* stimulation. Serum IL-10 levels were also significantly elevated in CLL patients. IL-10-competent cell frequencies were higher among CLLs with IgV_H mutations, and correlated positively with TCL1 expression. In the TCL1-transgenic (TCL1-Tg) mouse model of CLL, IL-10-competent B cells with the cell-surface phenotype of B10 cells expanded significantly with age, preceding the development of overt, CLL-like leukemia. Malignant CLL cells in TCL1-Tg mice also shared immunoregulatory functions with mouse and human B10 cells. Serum IL-10 levels varied in TCL1-Tg mice, but *in vivo* low-dose lipopolysaccharide treatment induced IL-10 expression in CLL cells and high levels of serum IL-10. Thus, malignant IL-10-competent CLL cells exhibit regulatory functions comparable to normal B10 cells that may contribute to the immunosuppression observed in patients and TCL1-Tg mice.

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Disclosure of Conflict of Interest

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Keywords

B10 cells; regulatory B cell; CLL; leukemia; IL-10; immunosuppression

Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in North America and Europe. It is characterized by the monoclonal expansion of small, mature, $CD5^+CD23^+CD19^+$ B lymphocytes.[1] CLL cells share phenotypic markers (e.g., cell surface expression of CD5 and other activation markers) with several cell types, including activated mature B cells, marginal zone B cells, memory B cells, and B1 cells.[2–4] CLL cells generally have restricted patterns of immunoglobulin V (IgV) gene utilization, although some patients express unmutated IgV sequences, while others have mutated IgV receptors. These two categories (unmutated and mutated) define patient groups with distinct clinical courses.[1] Generalized immunosuppression is also frequently observed in CLL patients.[5] A better understanding of the etiology and functional capacity of CLL cells may reveal new management strategies for this incurable leukemia.

The Eμ-TCL1 transgenic (TCL1-Tg) mouse with B cells that overexpress TCL1 is a well-characterized model for CLL development and progression.[6–8] A clonal preleukemic population of $CD5^+B220^{int}$ B cells develops in the blood and lymphoid tissues of TCL1-Tg mice by 4–6 mo of age; this clonal population gives rise to a CLL-like leukemia. CLL in TCL1-Tg mice is characterized by splenomegaly, lymphadenopathy, and a lethal malignant lymphocyte infiltration into vital organs.[6,8–10] The transfer of leukemic CLL cells from TCL1-Tg mice also transfers disease.[11] TCL1-Tg mice also represent a model for CLL-associated immunosuppression, with decreased T cell activation, proliferation, and cytokine production.[12] Thus, TCL1-Tg mice share multiple characteristics with CLL patients, and TCL1 expression is proposed to correlate with different molecular subtypes of human CLL.[13,14]

A subset of regulatory B cells has been functionally defined in humans and mice by their ability to express IL-10.[15–18] Regulatory B cells that are competent to express IL-10 following 5 h of *ex vivo* stimulation have been functionally labeled as B10 cells to distinguish them from other regulatory B cell subsets that modulate immune responses through IL-10-independent mechanisms.[18,19] B10 cells are found in the tissues of young naïve mice at low frequencies (1–5%), but expand with age or autoimmunity.[20] In the spleen, *ex vivo* B10 cells predominantly represent a subset of the $CD1d^{hi}CD5^+CD19^{hi}$ B cell subpopulation[16,17,21] that shares overlapping cell surface markers with multiple phenotypically defined B cell subsets, including marginal zone B cells and B1 cells.[22–24] Additional $CD1d^{hi}CD5^+$ B10 progenitor (B10pro) cells have also been functionally identified that are induced to become IL-10-competent during 48 h *in vitro* cultures with agonistic CD40 monoclonal antibody (mAb).[18,20] Human B10 and B10pro cells that are functionally comparable to their mouse counterparts have also been identified.[15] B10 and B10pro cells are generally rare in healthy individuals, represent a subset within the circulating $CD24^{hi}CD27^+$ "memory" B cell subpopulation, and can expand significantly in patients with autoimmune disease.[15] The capacity of human and mouse B10pro and B10 cells to express IL-10 is central to their ability to negatively regulate inflammation, autoimmunity, and adaptive and innate immune responses.[15–18,20,21,25–27]

Since CLL cells and B10 cells share several phenotypic markers and can be immunosuppressive, detailed studies were carried out to assess the ability of CLL cells to express IL-10 in humans and mice. Unexpectedly, CLL cells from 90% of patients and from

100% of TCL1-Tg mice were competent to express IL-10 and thereby shared regulatory functions with B10/B10pro cells. These results suggest that the regulatory functions of IL-10-competent CLL cells may contribute to the immunosuppression observed in patients.

Materials and methods

Patients

Patients with CLL and age-matched healthy controls were recruited from the Duke University and Durham Veterans Affairs (VA) Medical Centers. CLL diagnosis and staging were according to NCI Working Group criteria.[28] All 93 patients had not received therapy for ≥4 weeks, did not exhibit other pathologic complications before blood isolation, and gave informed consent according to protocols approved by the VA and Duke University Institutional Review Boards. Blood CLL cells were purified using ficoll-hypaque density gradients, with B cell enrichment using the RosetteSep Human B Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC).[29] CLL preparations were generally >98% pure, and were always >90% $CD5^+CD19^+$ B cells. Normal B cells in CLL patient's blood samples were generally <1% of $CD19^+$ cells as identified by their normal cell surface distributions of κ and λ light chains and absence of CD5 expression.

Mice

TCL1-Tg mice on a B6/C3 background[6] and $TCL1^{-/-}$ mice on a C57BL/6 background were as described.[30] Other mice were from the Jackson Laboratories (Bar Harbor, ME). All mice were housed in a conventional environment facility. In some experiments, mice were injected i.p. with LPS (10 μg; Sigma Aldrich, St. Louis, MO). The Animal Care and Use Committees of the Duke University and Durham VA Medical Centers approved all studies.

Antibodies and immunofluorescence analysis

Single-cell leukocyte suspensions from mouse tissues were prepared as described.[31] Mouse and human blood mononuclear cells were isolated by centrifugation over a discontinuous Lymphoprep (Axis-Shield PoC As, Oslo, Norway) gradient. For multi-color immunofluorescence analysis, single cell suspensions of mononuclear cells (10^6 cells) were stained as described.[31] Intracellular IL-10 analysis, and B10 cell and B10+B10pro cell enumeration were as described.[15,32] Fc receptors were blocked using FcγR-Binding inhibitor (Biolegend, San Diego, CA). Cells with the forward and side light scatter properties of single viable lymphocytes were analyzed using Live/Dead Viability/Vitality reagent (Invitrogen) and a Becton Dickinson (BD; San Jose, CA) FACSCanto flow cytometer. Background staining was assessed using non-reactive, isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA).

Mouse CD20 (MB20-11)[33] and CD22 (MB22-8)[34] mAbs were conjugated to Alexa488 or biotin (Invitrogen; Carlsbad, CA). FITC-, PE-, PE-Cy5-, APC, PE-Cy7, or biotin-conjugated mouse B220 (RA3–6B2), F4/80 (BM8), CD1d (1B1), CD4 (H129.19), CD5 (57–7.3), CD8 (53–6.7), CD11b (M1/70), CD19 (6D5), CD21 (7G6), CD23 (B3B4), CD24 (M1/69), CD93 (AA4.1), CD138 (281–2), IL-10 (JES5–16E3), and TNFα (MP6-XT22) were from Biolegend (San Diego, CA). Fluorophore- or biotin-conjugated human IgM (MHM-88), IgD (IA6-2), CD1d (51.1), CD5 (UCHT2), CD11b (ICRF44), CD19 (HIB19), CD20 (2H7), CD21 (LT21), CD22 (HIB22), CD23 (EBVCS-5), CD24 (ML5), CD27 (O323), CD38 (HIT2), CD43 (CD43-10G7), TCL1 (1–21), and IL-10 (JES3–19F1) mAbs and unconjugated low endotoxin, azide-free IL-10 mAb were from Biolegend. PE-conjugated anti-mouse IgM (II/41) mAb was from eBioscience (San Diego, CA). Anti-mouse IgD (11–26) mAb was from Southern Biotech (Birmingham, AL). PE- or PE-Cy7-conjugated streptavidin was from BD.

IL-10 ELISA and in vitro suppression assays

Serum, plasma, or secreted IL-10 levels were quantified using IL-10 OptEIA ELISA kits (BD). Magnetic bead (Miltenyi Biotech; Auburn, CA) purified B cells (4×10^5) were cultured in 0.2 ml of medium.[15,16]

Purified spleen CLL and B cells from 12–14-mo-old TCL1-Tg mice were cultured with LPS +CD40 mAb for 24 h, as described.[32] Purified bone marrow $CD11b^+$ cells from wild type mice were cultured in replicate wells either alone (10^6/ml), with washed LPS+CD40 mAb-stimulated B cells (10^6/ml), or with unstimulated B cells (10^6/ml) for 20 h. Neutralizing anti-IL-10 mAb (10 μg/ml) was added as indicated. LPS (1 μg/ml) and Brefeldin A were added during the final 5 h of culture, and the cells were subsequently stained for cell surface CD11b, F4/80, and cytoplasmic TNFα expression before flow cytometry analysis.

TCL1 quantification

Purified patient's CLL cells were frozen and lysed in Mammalian Protein Extraction Reagent® (Pierce, Rockford, IL) containing a protease inhibitor. A standard lysate of Ramos cells was used as a quantitation control in each individual gel. Cell lysates (50 μg total protein per lane) were electrophoresed and blotted onto PVDF membranes. The membranes were then rinsed in distilled water and incubated in 0.1% Ponceau S Solution (Sigma) as described.[35] Blocked membranes were incubated with anti-TCL1 antibody followed by anti-goat IgG antibody-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). Quantified band densities were expressed as a ratio of TCL1 band density/Ponceau band density (total proteins) and expressed relative to TCL1 in Ramos cell lysates (arbitrarily established as 100).

Statistical analysis

Data are shown as means (±SEM). Significant differences between sample means were determined using the Student's *t* test. Correlations between IL-10^+ cell frequencies and clinical parameters (Table 1) were assessed using the Mann-Whitney test. IL-10 expression was considered significant if IL-10^+ cell frequencies were mean+2 SD greater than Brefeldin A culture results.

Results

CLL cells from most patients are IL-10-competent

B10 cells normally represent 0.62±0.05% of blood B cells in healthy individuals, while B10+B10pro cells represent 3.8±0.3% of normal B cells (Fig. 1A).[15] While measuring blood B10 and B10pro cell frequencies among 72 normal individuals and 92 patients with autoimmunity,[15] a pemphigus foliaceus patient with 29% blood B10pro cells, but low B10 cell frequencies was identified (Fig. 1B). On detailed assessment, this patient was diagnosed with early stage CLL one month later, with the vast majority of B cells representing $CD5^+CD20^{int}$ clonal CLL cells (10,000 lymphocytes/μl). Among healthy individuals, one control subject had few if any B10 cells, while 30% of their blood $CD19^+$ B cells resembled B10pro cells and acquired IL-10-competence during 48 h cultures (Fig. 1C). The 30% value is 6- and 4-fold above the mean for healthy individuals and patients with autoimmune disease, respectively. A further evaluation revealed that >90% of the B cells in this individual were clonal $CD5^+CD20^{int}$ cells, representing classical monoclonal B cell lymphocytosis (MBL; a precursor condition to CLL) with a normal lymphocyte count (3,000 cells/μl). The extraordinarily high frequency of IL-10-competent cells in these individuals suggested that patients with overt CLL might share this functional property.

Malignant $CD5^+$ B cells purified from the blood of 93 patients with overt CLL were therefore assessed for IL-10-competence. $CD5^+$ CLL cells from 25 of 91 patients (27%) were competent to express IL-10 (range 0.4–6.1%) after 5 h stimulation with CpG plus PMA, ionomycin, and brefeldin A (PIB) relative to background IL-10 staining in Brefeldin A cultures (Fig. 1D). Moreover, mean blood $IL\text{-}10^+$ CLL cell numbers were 18- to 30-fold ($p<0.0001$) greater than mean absolute blood B10 cell numbers in healthy age-matched individuals. By contrast, $CD5^+$ CLL cells from 78 of 91 patients (86%) expressed IL-10 after 48 h of *in vitro* stimulation (range 2.1–27%; Fig. 1E). On average, $CD5^+$ CLL cells expressed IL-10 at significantly higher frequencies (6.7±0.8%, $p<0.0001$) after 48 h stimulation than blood B10+B10pro cell frequencies (3.2±0.3%) among age-matched healthy individuals. Moreover, mean numbers of blood CLL cells capable of being induced to express IL-10 after 48 h cultures (B10+B10pro cells, CD40L+CpG cultures) were 98-fold ($p<0.0001$) higher than blood B10+B10pro cell numbers in healthy controls. Thus, despite heterogeneity among CLL cases, $CD5^+$ CLL cells from only 10% of patients were unable to express measurable IL-10 after *ex vivo* stimulation.

Human CLL and B10 cells share a common phenotype

Blood B10pro and B10 cells from healthy donors express a $CD24^{hi}CD27^+$ phenotype.[15] Blood $CD5^+$ CLL cells also shared this $CD24^{hi}CD27^+$ phenotype, although decreased and variable IgM, IgD, CD20, and CD38 expression were also noted (Fig. 2A,B), as reviewed by others.[1] Importantly, IL-10 induction by PIB stimulation did not affect *ex vivo* cell surface molecule expression due to the presence of Brefeldin A and the short 5 h stimulation period.[15,16] Thus, human CLL cells and B10 cells share similar phenotypes.

Human CLL cells can secrete IL-10

Whether purified CLL cells secrete IL-10 when stimulated *in vitro* was assessed as described for human B10 cells.[15] Following CpG or CD40L/CpG stimulation, CLL cells secreted IL-10, and the quantity of IL-10 correlated directly with cytoplasmic $IL\text{-}10^+$ CLL cell frequencies in cell samples (Fig. 3A). Plasma IL-10 levels varied among CLL patients, but mean IL-10 levels were 1.5-fold ($n=42$, $p=0.008$) higher than those from age-matched normal donors (Fig. 3B). However, plasma IL-10 levels did not correlate with absolute numbers of circulating IL-10-competent CLL cells, suggesting that specific triggers may induce IL-10 secretion by CLL cells *in vivo*. Thus, human CLL cells had the functional capacity to both express and secrete IL-10.

IL-10 competence correlates with CLL cell mutation status, cytogenetics and TCL1 expression

The clinical characteristics of the CLL patients are shown in Table 1. The frequency of IL-10-competent CLL cells among cases did not segregate with CD38 or Zap70 expression, Rai stage, length of follow-up, treatment, or death in the current subset of patients. However, the mean frequency of IL-10-competent cells after 5 h of *ex vivo* stimulation was significantly higher among CLL cases with mutated IgV_H than for CLL cases with unmutated IgV_H (Fig. 3C). $IL\text{-}10^+$ CLL cell frequencies after 48 h of *in vitro* stimulation were not significantly different between CLL cases with mutated or unmutated IgV_H, suggesting the possibility that IL-10 competence could be driven by antigen stimulation among CLL cases with mutated IgV_H. Furthermore, none of the cells from six CLL cases with 11q chromosomal deletions expressed significant levels of IL-10 after 5 h stimulation with PIB relative to background IL-10 staining in Brefeldin A cultures (Fig. 3D). Nonetheless, the CLL cases with 11q chromosomal deletions were able to express IL-10 following 48 h *in vitro* cultures. IL-10-competent CLL cell frequencies did not correlate with any other cytogenetic alterations.

Since TCL1 expression correlates with different molecular subtypes of human CLL,[13,14] overall TCL1 protein expression was quantified in CLL cell samples from 52 patients. Remarkably, TCL1 protein levels correlated positively with IL-10-competent CLL cell frequencies (Fig. 3E) and their absolute numbers ($p=0.015$). TCL1 expression by IL-10$^+$ CLL cells was therefore examined by immunofluorescence staining in patients with diverse frequencies of IL-10-competent CLL cells. TCL1 expression was significantly higher ($17\pm3.8\%$, $p=0.003$, $n=11$) in IL-10$^+$ CLL cells than in IL-10$^-$ CLL cells from the same patients (Fig. 3F). Thus, TCL1 overexpression by CLL cells may contribute to their IL-10-competent phenotype.

B10-like cells expand with age in TCL1-Tg mice

TCL1 overexpression in IL-10-competent human CLL cells, prompted studies examining IL-10 production in TCL1-Tg mice overexpressing human TCL1 (Fig. 3F).[6–8] Compared to humans with CLL, these mice are amenable to mechanistic *in vivo* studies. As TCL1-Tg mice have a mixed B6 x C3H/HeJ (C3) genetic background, spleen B10 cells were quantified in 2-mo-old B6, C3, and B6/C3 F_1 mice. B10 cells were identified by cytoplasmic IL-10 expression following 5 h of *ex vivo* stimulation with PMA and ionomycin, with monensin (PIM) included to block IL-10 secretion.[16–18] Monensin-treated cells served as controls for background IL-10 staining. B10 cell frequencies were ~2.0-fold greater in young C3 mice when compared with B6 mice ($p<0.01$), while B6/C3 F_1 mice had intermediate B10 cell numbers (Fig. 4A). Likewise, 2-mo-old TCL1-Tg and B6/C3 F_1 mice had intermediate IL-10$^+$ B cell numbers ($p\leq0.01$) relative to B10+B10pro cell frequencies in C3 and B6 mice following *in vitro* CD40 mAb stimulation for 48 h.[16–18] In contrast to young mice, the vast majority (50–90%) of B cells from ≥12-mo-old TCL1-Tg mice were IL-10-competent ($p<0.0001$, Fig. 4B,C). B10 cell and B10+B10pro cell frequencies were not significantly increased in old B6 or C3 mice. Similar results were obtained under all culture conditions used to induce B cell IL-10 expression. Thus, B10 cells develop normally in young TCL1-Tg mice, while B cells in aged TCL1-Tg mice were IL-10-competent.

TCL1-Tg CLL cells are IL-10-competent

An age-associated expansion of blood and spleen IL-10-competent CD5$^+$B220^{int} B cells in TCL1-Tg mice was frequently apparent by 6-mo of age (Fig. 5A). In all cases, the ability of B cells to express IL-10 correlated linearly with the acquisition of a CD5$^+$B220^{int} B cell phenotype and with circulating B cell numbers. By 12 mo of age, significant expansion of the IL-10-competent CD5$^+$B220^{int} B cell subset occurred at the expense of other B cells. In fact, most aged TCL1-Tg mice (12–14 mo-old) had dramatically (>4 fold) increased numbers of clonally expanded, IL-10-competent CD5$^+$B220^{int} B cells within the blood and spleen. In contrast to B cells, blood CD4$^+$ and CD8$^+$ T cell numbers changed minimally with age (Supplementary Fig. 1A). IL-10-competent CD5$^+$B220^{int} B cell frequencies also increased with age in the bone marrow, lymph nodes, and peritoneal cavity (Fig. 5B, and Supplementary Fig. 1B–D). Thus, a >4-fold expansion in blood or spleen IL-10-competent CD5$^+$B220^{int} B cell numbers was interpreted to indicate progression to CLL.

Some TCL1-Tg mice retained normal frequencies and absolute numbers of blood and spleen IL-10-competent B cells through 16 mo of age (Fig. 5A–B), demonstrating that neither the TCL1 transgene nor age alone drove expansion of this IL-10-competent B cell subset. There was also heterogeneity among mice. In one example, some mice exhibited an expansion of blood CD5$^+$B220^{int} B cells but only a fraction of these cells expressed IL-10 during 5 h assays (Fig. 5C). While overall blood and spleen B cell numbers remained normal, the majority of spleen CD5$^+$B220^{int} B cells were IL-10-competent. Thus, differences in IL-10-competence can occur between cells from different tissues. By contrast, blood and spleen CD5$^+$B220^{int} B cells from mice with overt CLL were more uniformly IL-10-competent (Fig.

5D). Regardless, expansion of the IL-10-competent $CD5^+B220^{int}$ B cell subset always preceded the development of overt, malignant CLL in TCL1-Tg mice.

TCL1-Tg CLL cells and regulatory B10 cells share a common phenotype

The IL-10-competent $CD5^+B220^{int}$ B10 cells from aged TCL1-Tg mice with >4-fold increases in spleen B cell numbers exhibited the characteristic $IgM^+CD11b^{hi}CD23^{low}CD43^{hi}CD19^+$ phenotype of mouse CLL cells (Fig. 6A) as described previously.[6,8] These CLL cells also shared similar phenotypes with $CD5^+$ B cells from 2-mo-old TCL1-Tg mice (Fig. 6A) and IL-10-competent B10 cells from 2 mo-old B6/C3 and TCL1-Tg mice (Fig. 6B). Importantly, IL-10 induction by LPS+PIM stimulation does not affect *ex vivo* cell surface molecule expression due to the presence of monensin and the short 5 h stimulation period.[16] Thus, IL-10-competent regulatory B10 cells and CLL cells share multiple phenotypic characteristics.

TCL1-Tg CLL cells are immunoregulatory

Assays for human and mouse B10 cell regulatory function[15,27] were used to determine whether purified TCL1-Tg CLL cells were also able to suppress monocyte/macrophage function. Activated CLL cells significantly inhibited macrophage TNFα production in co-culture assays (63%, $p<0.01$), while resting CLL cells were not able to significantly inhibit macrophage TNFα production (Fig. 7A). CLL cell inhibition of macrophage TNFα production was reversed by the addition of a neutralizing anti-IL-10 mAb to the co-cultures (1.8-fold, $p<0.0001$). Thus, CLL cells from TCL1-Tg mice functionally parallel human and mouse B10 cells and suppress macrophage activation in a stimulation- and IL-10-dependent manner.

TCL1-Tg CLL cells produce IL-10 in vivo

The relationship between CLL cell numbers and serum IL-10 levels was assessed using aged TCL1-Tg mice with CLL expansion (as determined by >4-fold increases in spleen B cell numbers). *Ex vivo* IL-10 secretion was highest for CLL cases with higher frequencies of cytoplasmic $IL\text{-}10^+$ cells after 5 h of *in vitro* LPS+PIM stimulation (Fig. 7B). Spleen TCL1-Tg CLL cells secreted significantly more IL-10 when compared with cultured B cells from 2-mo-old wild type B6/C3 mice ($p<0.0001$). After stimulation, CLL cells also secreted significantly more IL-10 than activated wild type B cells, with mean differences of 7.8- to 11-fold depending on the stimulus ($p\leq0.04$). Thus, CLL cells actively secreted IL-10 *ex vivo* and after *in vitro* stimulation.

Serum IL-10 increased with age in most TCL1-Tg mice, but was not detectable in B6/C3 or TCL1-Tg mice younger than 8 mo of age (data not shown, and Fig. 7C). As in humans, there was no close relationship between serum IL-10 levels and blood $CD5^+B220^{int}$ B cell frequencies or numbers in individual mice (data not shown). Therefore, the ability of inflammation to induce serum IL-10 production was measured in aged TCL1-Tg mice with overt CLL but with low serum IL-10 levels. Remarkably, low-dose LPS treatment *in vivo* induced serum IL-10 levels in TCL1-Tg mice with CLL. One hour after LPS administration, IL-10 levels were 159-fold ($p=0.003$) higher in TCL1-Tg mice than in wild type mice (Fig. 7D). Thus, low-level *in vivo* inflammatory signals induced serum IL-10 in mice with CLL. This indicates that inflammatory signals are likely to induce CLL cell IL-10 secretion *in vivo*, and this likely explains the observed variability in serum IL-10 levels among aged TCL1-Tg mice and CLL patients.

Whether CLL cells themselves could be induced to produce IL-10 *in vivo* was assessed by staining CLL and clonal $CD5^+B220^{int}$ preleukemic B cells for cytoplasmic IL-10 following *in vivo* low-dose LPS treatment. In mice with overt CLL, $IL\text{-}10^+$ CLL cell frequencies

increased significantly at 24, 48 and 72 h after LPS treatment in parallel with significantly increased serum IL-10 levels (Fig. 7E). In fact, *ex vivo* examination showed that up to 15% of CLL cells produced IL-10 after *in vivo* low-dose LPS treatment. Spleen B cells from TCL1-Tg mice with $CD5^{+}B220^{int}$ B cell expansions but normal total blood B cell numbers also expressed significantly higher frequencies of $IL\text{-}10^{+}$ $CD5^{+}B220^{int}$ B cells. By contrast, spleen B cells from young B6 or aged C3 mice did not express detectable IL-10 following *in vivo* low-dose LPS treatment relative to isotype-matched control mAb staining. Thus, inflammatory stimuli can induce significant CLL cell IL-10 production *in vivo*.

Discussion

These collective studies demonstrate that CLL cells in both humans and mice share a molecular mechanism for immunosuppression that is also utilized by regulatory B10 cells. CLL cells expressed the immunoinhibitory cytokine IL-10 both *in vitro* (Figs. 1–5) and *in vivo* (Fig. 7). Moreover, mouse CLL cells suppressed monocyte/macrophage activation through IL-10-dependent pathways (Fig. 7A), a feature previously noted in human and mouse B10 cells.[15,27] The $CD24^{hi}CD27^{+}$ phenotype of human CLL cells also matched the phenotype of human blood B10pro and B10 cells (Fig. 3A–B).[15] Human CLL and B10 cells also shared a common $CD5^{+}IgM^{hi}IgD^{low}CD23^{low}$ phenotype with cells from TCL1-Tg mice (Fig. 6). Despite their similar phenotypes, IL-10-competent CLL cells were clearly separable from normal B10pro and B10 cells in both patients and in TCL1-Tg mice, and cannot be explained as normal B10 cell contamination of the CLL cell populations. In fact, as many as 30% of some human CLL cell samples were induced to express IL-10 (Figs. 1–2), and >90% of CLL cells in TCL1-Tg mice were IL-10-competent (Figs. 4–5). Thus, shared CD5 expression, a shared capacity to secrete IL-10, and the similar phenotypes of CLL, B10pro, and B10 cells in humans and mice suggests shared regulatory functions for these cells.

Plasma IL-10 levels were significantly elevated in some patients with high frequencies of circulating IL-10-competent CLL cells (Fig. 3E). Previous studies have also identified some CLL patients with increased plasma IL-10.[36,37] Blood IL-10 levels were also increased with age in most TCL1-Tg mice (Fig. 7C). However, detectable CLL cell IL-10 expression *in vivo* was primarily induced following stimulation. For example, *in vivo* low-dose LPS treatment induced significant serum IL-10 levels in TCL1-Tg mice with CLL in parallel with significantly increased numbers of IL-10-expressing CLL cells. By contrast, *in vivo* low-dose LPS treatment did not significantly affect serum IL-10 levels in disease-free or wild type mice, thereby demonstrating that CLL cells were a significant source of serum IL-10 *in vivo*. Inflammatory signals are likely to also induce serum IL-10 in CLL patients; this may explain differences in IL-10 levels among patients in the current study (Fig. 3B) and the heterogeneous levels of IL-10 reported in CLL patients from other studies.[37–39] Based on these findings, infections or host inflammatory responses to CLL may induce CLL cell IL-10 secretion and contribute directly to the systemic immunosuppression observed in CLL patients.[5]

CLL-derived IL-10 may also reduce responsiveness to CD20 (Rituximab) immunotherapy. Even small numbers of endogenous B10 cells can inhibit CD20 mAb-induced lymphoma depletion in a mouse model.[27] In the present study, CLL cell IL-10 production significantly inhibited monocyte activation (Fig. 7A), which is required for CD20 mAb-induced tumor clearance *in vivo*.[27,40] Monocytes also express high levels of IL-10 receptor,[41] and B10 cell-derived IL-10 inhibits monocyte activation *in vivo* and *in vitro*.[15,27] CLL cell-derived IL-10 may also inhibit normal and anti-tumor immune responses[42] by altering T cell frequencies[43], T cell activation[44,45], regulatory T cell numbers[46], CTLA-4 expression,[47] and T cell gene expression and function.[5] Thus, IL-10 production by CLL cells may reduce the effectiveness of CD20 immunotherapy, as well as other immune cell-based treatments.

CLL and B10 cells may develop IL-10-competence and a shared phenotype as a consequence of (a) similar molecular events or activation signals, (b) shared self-reactive antigen receptors, (c) common cellular origins, or (d) the malignant transformation of CLL cells. The finding that IL-10^{+} human CLL cells expressed higher levels of TCL1 than IL-10^{-} CLL cells (Fig. 3F) along with the observation that essentially all CLL cells in TCL1-Tg mice express IL-10 (Fig. 5) suggests that shared molecular events or activation signals contribute to CLL cell IL-10 production. However, TCL1 expression was not essential for B10 cell development (Supplementary Fig. 2A). Constitutive TCL1 overexpression was also insufficient to drive CLL development since some TCL1-Tg mice retained normal blood B cell numbers and subset distributions until 16 months of age (Fig. 5A). However, TCL1-Tg mice developed IL-10-competent pre-leukemic CD5^{+}B220^{int} B cells that progressively increased in frequency and displaced other B cell subsets (Fig. 5A–B), but otherwise remained stable until CLL conversion and subsequent expansion.[6,8,9,48] By contrast, human CLL cells may lose IL-10-competence with disease progression as genetic alterations accumulate or various clones outgrow their IL-10-competent parental cells. This may explain why IL-10-competence was more common in less aggressive CLL cells with mutated IgV_H as compared to the more aggressive CLLs with unmutated IgV_H (Fig. 3C). The observation that CLL cells with 11q deletions had low numbers of CLL cells with a capacity to rapidly express IL-10 (Fig. 3D) suggests that a genetic element on chromosome 11 may contribute to signals that drive IL-10-competence *in vivo*. Alternatively, the strong correlation between IL-10 competence and mutated IgV_H expression suggests that IL-10 production could be induced by antigen stimulation *in vivo* rather than genetic mutations. Thereby, the correlations between IL-10 competence, mutated IgV_H genes, and TCL1 expression in patients may not be discordant. Regardless of the differences in IL-10-competent cell frequencies among CLL patients, CLL cells from most patients exhibited a functional program that is shared with the functionally defined regulatory B10 cell subset.

There are valid arguments that CLL cells represent malignant CD5^{+} B cells, memory B cells, marginal zone B cells, or antigen-stimulated follicular cells, or that they possess a heterogeneous etiology as reflected in different patients.[1,3,4,49] Similar arguments have been made for IL-10 producing regulatory B cells.[16,17,22–24] Nonetheless, human CLL cells frequently express self-reactive antigen receptors,[50] and B10 and B10pro cell numbers are frequently increased in patients and mice with autoimmune disease.[15,20] Consistent with antigen encounter *in vivo*, human B10pro and B10 cells also predominantly express a "memory" CD24^{hi}CD27^{+} phenotype,[15] and B10 cells respond rapidly to mitogenic stimuli *in vivo* and *in vitro* following TLR stimulation.[15,16,18,20] Antigen receptor signals are also critical for the generation of mouse B10 cells that additionally quickly mature to secrete antibodies following mitogenic activation.[20] B10 cells in wild type mice also predominantly express cell surface IgM that is unmutated, autoreactive, and poly-reactive.[51] Similarly, CD5^{+}B220^{int} B cells in TCL1-Tg mice predominantly express unmutated IgM, and these B cells can express poly-reactive antibodies.[10] Additional studies will therefore be required to establish the molecular basis for shared properties between CLL and B10 cells, but the current findings clearly demonstrate their shared functional capacity to express IL-10.

These collective data support the hypothesis that the capacity of CLL cells to produce IL-10 in response to external stimuli may contribute to immunodeficiency and thereby influence disease progression or outcome. Consequently, the further identification, characterization and enumeration of IL-10-competent CLL cells may help explain some of the heterogeneous features of CLL disease, prognosis, and outcome. Larger patient populations and longitudinal studies will also determine whether IL-10-competence correlates with known CLL subtypes and clinical course (Table 1). These studies should also reveal whether IL-10-competent CLL cell frequencies vary in patients in response to *in vivo* triggers such as

during infections or inflammation as was observed in mice (Fig. 7E). Nonetheless, current and future therapies should consider that CLL cells have the potential for IL-10-competence and production, and that increased plasma IL-10 levels may affect therapy outcome. Future studies may also identify further links between CLL and B10 cells and help determine whether they develop IL-10-competence as a consequence of shared molecular events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005; 352:804–815. [PubMed: 15728813]
2. Damle RN, Ghiotto F, Valetto A, Albesiano E, Fais F, Yan XJ, et al. B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. Blood. 2002; 99:4087–4093. [PubMed: 12010811]
3. Nordgren TM, Joshi SS. The etiology of chronic lymphocytic leukemia: another look at the relationship between B1 cells and CLL. Open Leuk J. 2010; 3:69–73.
4. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. Blood. 2011; 117:1781–1791. [PubMed: 21148333]
5. Gorgun G, Holderried TA, Zahrieh D, Neuberg D, Gribben JG. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. J Clin Invest. 2005; 115:1797–1805. [PubMed: 15965501]
6. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci, USA. 2002; 99:6955–6960. [PubMed: 12011454]
7. Hamblin TJ. The TCL1 mouse as a model for chronic lymphocytic leukemia. Leuk Res. 2010; 34:135–136. [PubMed: 19726084]
8. Efanov A, Zanesi N, Nazaryan N, Santanam U, Palamarchuk A, Croce CM, et al. $CD5^{+}CD23^{+}$ leukemic cell populations in TCL1 transgenic mice show significantly increased proliferation and Akt phosphorylation. Leukemia. 2010; 24:970–975. [PubMed: 20357824]
9. Johnson AJ, Lucas DM, Muthusamy N, Smith LL, Edwards RB, De Lay MD, et al. Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. Blood. 2006; 108:1334–1338. [PubMed: 16670263]
10. Yan XJ, Albesiano E, Zanesi N, Yancopoulos S, Sawyer A, Romano E, et al. B cell receptors in TCL1 transgenic mice resemble those of aggressive, treatment-resistant human chronic lymphocytic leukemia. Proc Natl Acad Sci, USA. 2006; 103:11713–11718. [PubMed: 16864779]
11. Enzler T, Kater AP, Zhang W, Widhopf GF 2nd, Chuang HY, Lee J, et al. Chronic lymphocytic leukemia of Eμ-TCL1 transgenic mice undergoes rapid cell turnover that can be offset by extrinsic CD257 to accelerate disease progression. Blood. 2009; 114:4469–4476. [PubMed: 19755673]
12. Gorgun G, Ramsay AG, Holderried TA, Zahrieh D, Le Dieu R, Liu F, et al. Eμ-TCL1 mice represent a model for immunotherapeutic reversal of chronic lymphocytic leukemia-induced T-cell dysfunction. Proc Natl Acad Sci, USA. 2009; 106:6250–6255. [PubMed: 19332800]

13. Herling M, Patel KA, Khalili J, Schlette E, Kobayashi R, Medeiros LJ, et al. TCL1 shows a regulated expression pattern in chronic lymphocytic leukemia that correlates with molecular subtypes and proliferative state. Leukemia. 2006; 20:280–285. [PubMed: 16341048]

14. Browning RL, Geyer SM, Johnson AJ, Jelinek DF, Tschumper RC, Call TG, et al. Expression of TCL-1 as a potential prognostic factor for treatment outcome in B-cell chronic lymphocytic leukemia. Leuk Res. 2007; 31:1737–1740. [PubMed: 17659340]

15. Iwata Y, Matsushita T, Horikawa M, DiLillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011; 117:530–541. [PubMed: 20962324]

16. Yanaba K, Bouaziz J-D, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique $CD1d^{hi}$ $CD5^{+}$ phenotype controls T cell-dependent inflammatory responses. Immunity. 2008; 28:639–650. [PubMed: 18482568]

17. Matsushita T, Yanaba K, Bouaziz J-D, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008; 118:3420–3430. [PubMed: 18802481]

18. DiLillo DJ, Matsushita T, Tedder TF. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann N Y Acad Sci. 2010; 1183:38–57. [PubMed: 20146707]

19. Ray A, Basu S, Williams CB, Salzman NH, Dittel BN. A novel IL-10-independent regulatory role for B cells in suppressing autoimmunity by maintenance of regulatory T cells via GITR ligand. J Immunol. 2012 in press.

20. Yanaba K, Bouaziz J-D, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J Immunol. 2009; 182:7459–7472. [PubMed: 19494269]

21. Haas KM, Watanabe R, Matsushita T, Nakashima H, Ishiura N, Okochi H, et al. Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/W F_1 mice. J Immunol. 2010; 184:4789–4800. [PubMed: 20368280]

22. Spencer NF, Daynes RA. IL-12 directly stimulates expression of IL-10 by $CD5^{+}$ B cells and IL-6 by both $CD5^{+}$ and $CD5^{-}$ B cells: possible involvement in age-associated cytokine dysregulation. Int Immunol. 1997; 9:745–754. [PubMed: 9184920]

23. Brummel R, Lenert P. Activation of marginal zone B cells from lupus mice with type A(D) CpG-oligodeoxynucleotides. J Immunol. 2005; 174:2429–2434. [PubMed: 15699180]

24. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR, et al. Novel suppressive function of transitional 2 B cells in experimental arthritis. J Immunol. 2007; 178:7868–7878. [PubMed: 17548625]

25. Bouaziz J-D, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunol Rev. 2008; 224:201–214. [PubMed: 18759928]

26. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling EAE initiation and late-phase immunopathogenesis. J Immunol. 2010; 185:2240–2252. [PubMed: 20624940]

27. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J Clin Invest. 2011; 121:4268–4280. [PubMed: 22019587]

28. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008; 111:5446–5456. [PubMed: 18216293]

29. Volkheimer AD, Weinberg JB, Beasley BE, Whitesides JF, Gockerman JP, Moore JO, et al. Progressive immunoglobulin gene mutations in chronic lymphocytic leukemia: evidence for antigen-driven intraclonal diversification. Blood. 2007; 109:1559–1567. [PubMed: 17082314]

30. Kang SM, Narducci MG, Lazzeri C, Mongiovi AM, Caprini E, Bresin A, et al. Impaired T-and B-cell development in Tcl1-deficient mice. Blood. 2005; 105:1288–1294. [PubMed: 15479728]

31. Zhou L-J, Smith HM, Waldschmidt TJ, Schwarting R, Daley J, Tedder TF. Tissue-specific expression of the human CD19 gene in transgenic mice inhibits antigen-independent B lymphocyte development. Mol Cell Biol. 1994; 14:3884–3894. [PubMed: 7515149]
32. Matsushita T, Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10. Methods Mol Biol. 2011; 677:99–111. [PubMed: 20941605]
33. Uchida J, Lee Y, Hasegawa M, Liang Y, Bradney A, Oliver JA, et al. Mouse CD20 expression and function. Int Immunol. 2004; 16:119–129. [PubMed: 14688067]
34. Haas KM, Sen S, Sanford IG, Miller AS, Poe JC, Tedder TF. CD22 ligand binding regulates normal and malignant B lymphocyte survival *in vivo* . J Immunol. 2006; 177:3063–3073. [PubMed: 16920943]
35. Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. Anal Biochem. 2010; 401:318–320. [PubMed: 20206115]
36. Kitabayashi A, Hirokawa M, Miura AB. The role of interleukin-10 (IL-10) in chronic B-lymphocytic leukemia: IL-10 prevents leukemic cells from apoptotic cell death. Int J Hematol. 1995; 62:99–106. [PubMed: 8590779]
37. Fayad L, Keating MJ, Reuben JM, O'Brien S, Lee BN, Lerner S, et al. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. Blood. 2001; 97:256–263. [PubMed: 11133769]
38. Knauf WU, Ehlers B, Bisson S, Thiel E. Serum levels of interleukin-10 in B-cell chronic lymphocytic leukemia. Blood. 1995; 86:4382–4383. [PubMed: 7492801]
39. Ramachandra S, Metcalf RA, Fredrickson T, Marti GE, Raveche E. Requirement for increased IL-10 in the development of B-1 lymphoproliferative disease in a murine model of CLL. J Clin Invest. 1996; 98:1788–1793. [PubMed: 8878429]
40. Minard-Colin V, Xiu Y, Poe JC, Horikawa M, Hamaguchi Y, Haas KM, et al. Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage Fc γ RI, Fc γ RIII, and Fc γ RIV. Blood. 2008; 112:1205–1213. [PubMed: 18495955]
41. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001; 19:683–765. [PubMed: 11244051]
42. de Waal, Malefyt R.; Haanen, J.; Spits, H.; Roncarolo, MG.; te Velde, A.; Figdor, C., et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med. 1991; 174:915–924. [PubMed: 1655948]
43. Matutes E, Wechsler A, Gomez R, Cherchi M, Catovsky D. Unusual T-cell phenotype in advanced B-chronic lymphocytic leukaemia. Br J Haematol. 1981; 49:635–642. [PubMed: 6459116]
44. Chiorazzi N, Fu SM, Montazeri G, Kunkel HG, Rai K, Gee T. T cell helper defect in patients with chronic lymphocytic leukemia. J Immunol. 1979; 122:1087–1090. [PubMed: 376702]
45. Lauria F, Foa R, Mantovani V, Fierro MT, Catovsky D, Tura S. T-cell functional abnormality in B-chronic lymphocytic leukaemia: evidence of a defect of the T-helper subset. Br J Haematol. 1983; 54:277–283. [PubMed: 6221752]
46. Beyer M, Kochanek M, Darabi K, Popov A, Jensen M, Endl E, et al. Reduced frequencies and suppressive function of $CD4^{+}CD25^{hi}$ regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood. 2005; 106:2018–2025. [PubMed: 15914560]
47. Motta M, Rassenti L, Shelvin BJ, Lerner S, Kipps TJ, Keating MJ, et al. Increased expression of CD152 (CTLA-4) by normal T lymphocytes in untreated patients with B-cell chronic lymphocytic leukemia. Leukemia. 2005; 19:1788–1793. [PubMed: 16094420]
48. Pekarsky Y, Zanesi N, Aqeilan RI, Croce CM. Animal models for chronic lymphocytic leukemia. J Cell Biochem. 2007; 100:1109–1118. [PubMed: 17131382]
49. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001; 194:1625–1638. [PubMed: 11733577]
50. Hodgson K, Ferrer G, Montserrat E, Moreno C. Chronic lymphocytic leukemia and autoimmunity: a systematic review. Haematologica. 2011; 96:752–761. [PubMed: 21242190]

51. Maseda D, Smith SH, DiLillo DJ, Bryant JM, Candando KM, Weaver CT, et al. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. J Immunol. 2012; 188:1836–1048.

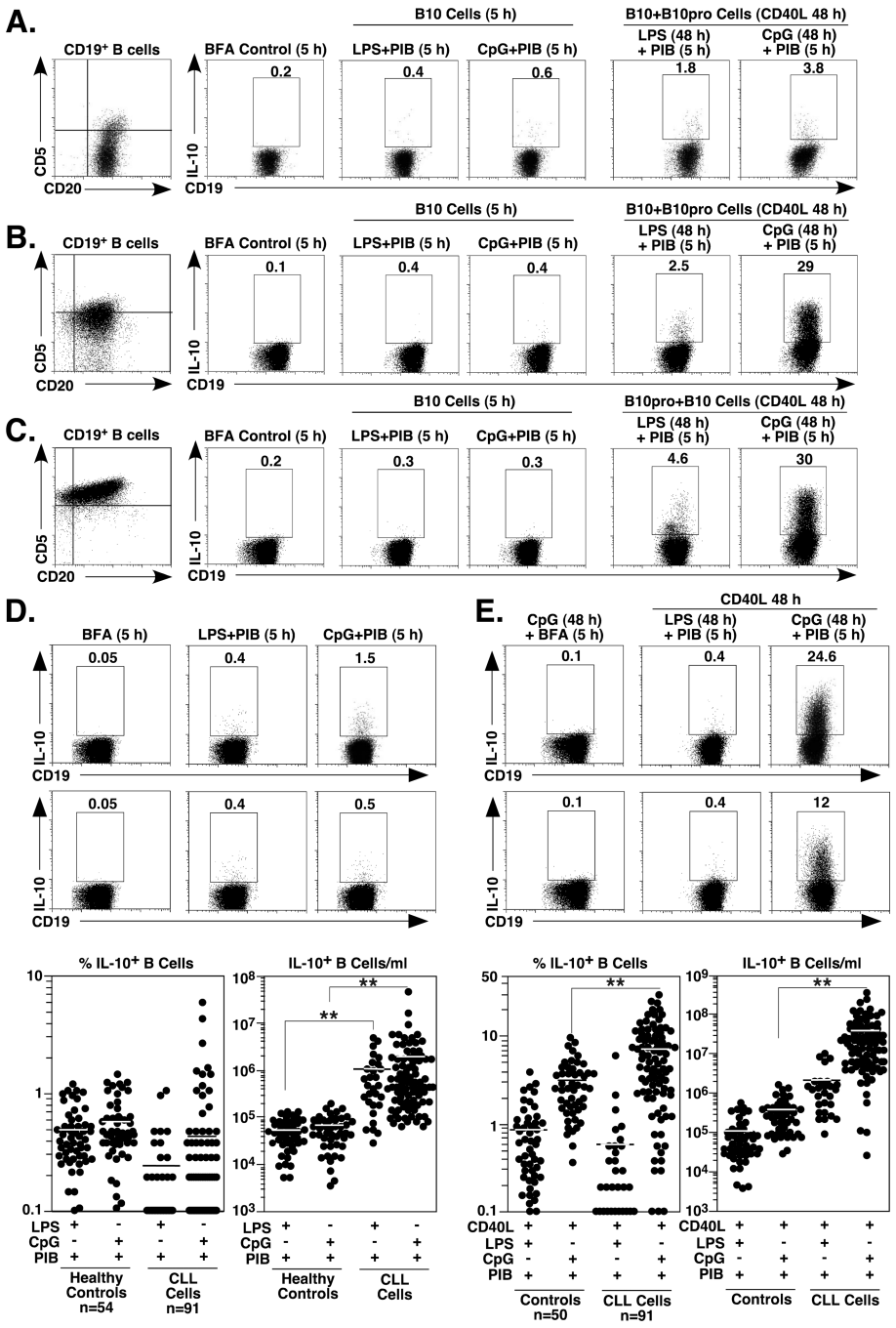

Figure 1. Human CLL cells are IL-10-competent

(**A**) *Representative cytoplasmic IL-10 expression by blood B10 cells and B10+10pro cells from a healthy control.* Representative CD5 vs. CD20 surface staining and/or cytoplasmic IL-10 staining by viable, single $CD19^+$ B cells is shown in the flow cytometry dot-plots, with percentages of $IL\text{-}10^+$ cells within the gates indicated. Blood B cells were immunophenotyped or cultured with Brefeldin A (BFA Control), or LPS, PMA, ionomycin, and Brefeldin A (PIB), or CpG+PIB for 5 h before cell surface CD19 and cytoplasmic $IL\text{-}10^+$ staining to enumerate B10 cell frequencies. To enumerate B10+B10pro cell frequencies, the cells were also cultured with CD40L+LPS or CD40L+CpG for 48 h, with

PIB added during the final 5 h of culture. (**B**) *Blood B cell cytoplasmic IL-10 expression by a patient with pemphigus 1 mo before early CLL diagnosis*. Cell analysis was as in (A). (**C**) *Blood B cell cytoplasmic IL-10 expression by an individual with MBL*. Cell analysis was as in (A). (**D–E**) $CD5^+$ CLL cells are IL-10-competent. Representative cytoplasmic IL-10 expression by purified CLL cells from 2 patients (>98% CLL cells, <1% normal B cells) that were stimulated for 5 (D) or 48 (E) h as in (A). Graphs in the lower frames of D and E show the frequencies (left panel) and absolute numbers (right panel) of $IL\text{-}10^+$ CLL cells after treatment with the indicated stimuli in comparison with normal B cells from age-matched healthy donors. Horizontal bars indicate group means. Dashed lines indicate 95% confidence intervals (mean + 2 SD) for background IL-10 staining in unstimulated samples. Significant differences between means are indicated: ** $p<0.01$.

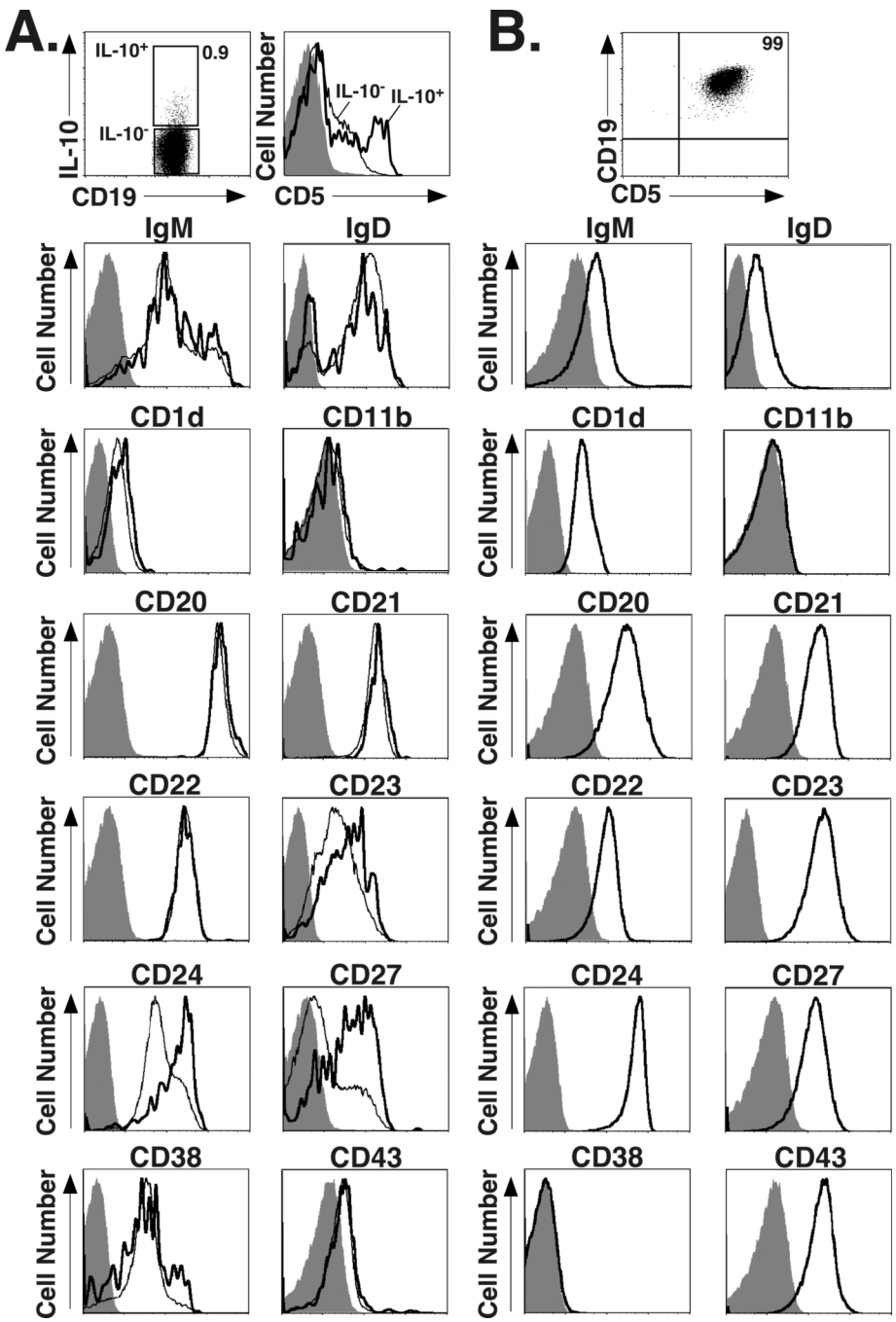

Figure 2. Human B10 cells and CLL cells share a common phenotype

(**A**) *Representative B10 cell phenotype*. Blood B cells from a normal donor were stimulated with LPS, PMA, ionomycin, and Brefeldin A (PIB) for 5 h before cell surface and intracellular immunoflourescence staining with flow cytometry analysis. Representative IL-10 vs. CD19 and CD5 flow cytometry dot plots are shown gated on viable, single $CD19^+$ B cells (top panels). Percentages indicate cell frequencies within the indicated gates and quadrants. Representative surface molecule expression by $IL\text{-}10^+$ (heavy lines) and IL-10 (thin lines) $CD19^+$ cells from 5 normal donors is shown (lower panels). (**B**) *Representative CLL cell surface molecule phenotype*. Purified blood CLL cells were assessed by

immunofluorescence staining with flow cytometry analysis as in (A). Representative cell surface molecule expression levels by $CD19^{+}CD5^{+}$ CLL cells (heavy lines) from 5 CLL patients are shown (lower panels).

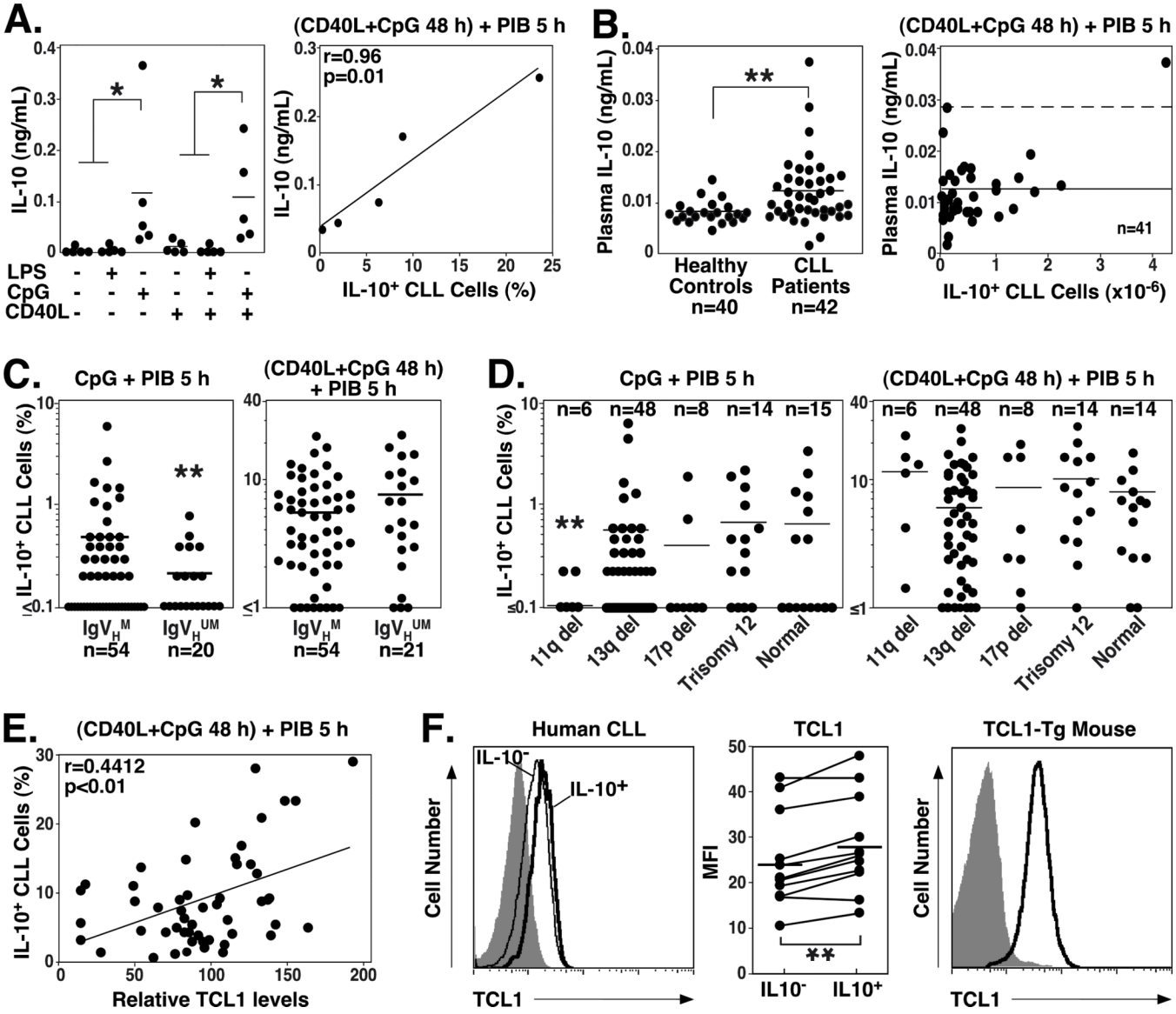

Figure 3. Human CLL cell IL-10 competence correlates with disease markers

(**A**) *CLL cells secrete IL-10 in vitro*. Purified blood CLL cells from five individual patients (dots) were cultured with the indicated stimuli for 72 h. Horizontal bars indicate mean values. The right panel shows supernatant fluid IL-10 concentrations vs. the frequency of IL-10$^+$ cells detected by intracellular cytokine staining (after 48 h culture with CD40L and CpG, with PIB stimulation during the final 5 h of culture) from the corresponding sample. A regression line is shown for reference. (**B**) *Plasma IL-10 concentrations in CLL patients and age-matched healthy donors*. The graph (right panel) shows plasma IL-10 concentration vs. absolute numbers of blood IL-10$^+$ CLL cells after 48 h cultures with CD40L and CpG, with PIB stimulation during the final 5 h of culture. The horizontal bars and line designate means. The dashed line indicates the mean + 2 SD. (**C**) *CLL cell IL-10 competence correlates with IgV_H mutation status*. B10 and B10+B10pro cell frequencies were determined as in figure 1C–D. Mutated (M) or unmutated (UM) status was assessed as in table 1. **D**) *CLL cell IL-10 competence correlates with cytogenetic abnormalities*. B10 and B10+B10pro cell frequencies were determined as in figure 1C–D, with cytogenetic abnormalities assessed as

in table 1. (**E**) *CLL cell IL-10 competence positively correlates with TCL1 protein expression*. Relative TCL1 protein levels in CLL cells from 52 patients were determined by quantitative Western blot analysis. IL-10-competent CLL cell frequencies were determined after 48 h stimulation with CD40L/CpG stimulation. (**F**) *Human TCL1 expression by human CLL cells and TCL1-Tg mouse B cells*. Representative intracellular TCL1 staining of human $CD19^+$ CLL cells (n=27; left panel) and $CD19^+$ cells from 12 to 14-mo-old TCL1-Tg mice (n=4; right panel). Human CLL cells were stimulated with CD40L and CpG for 48 h before the addition of PMA, ionomycin, and brefeldin A for 5 h. Mouse TCL1-Tg B cells were stained directly *ex vivo*. TCL1 expression levels by $IL\text{-}10^+$ cells (heavy lines) and IL-10 cells (thin lines) are shown. Shaded lines in histograms indicate background staining using isotype-matched control mAb. Representative differences in TCL1 expression densities (MFI, mean fluorescence intensities) between $IL\text{-}10^+$ and IL-10 CLL cells are shown (n=11, middle panel). (A–D, F) Significant differences between means are indicated: * $p<0.05$; ** $p<0.01$.

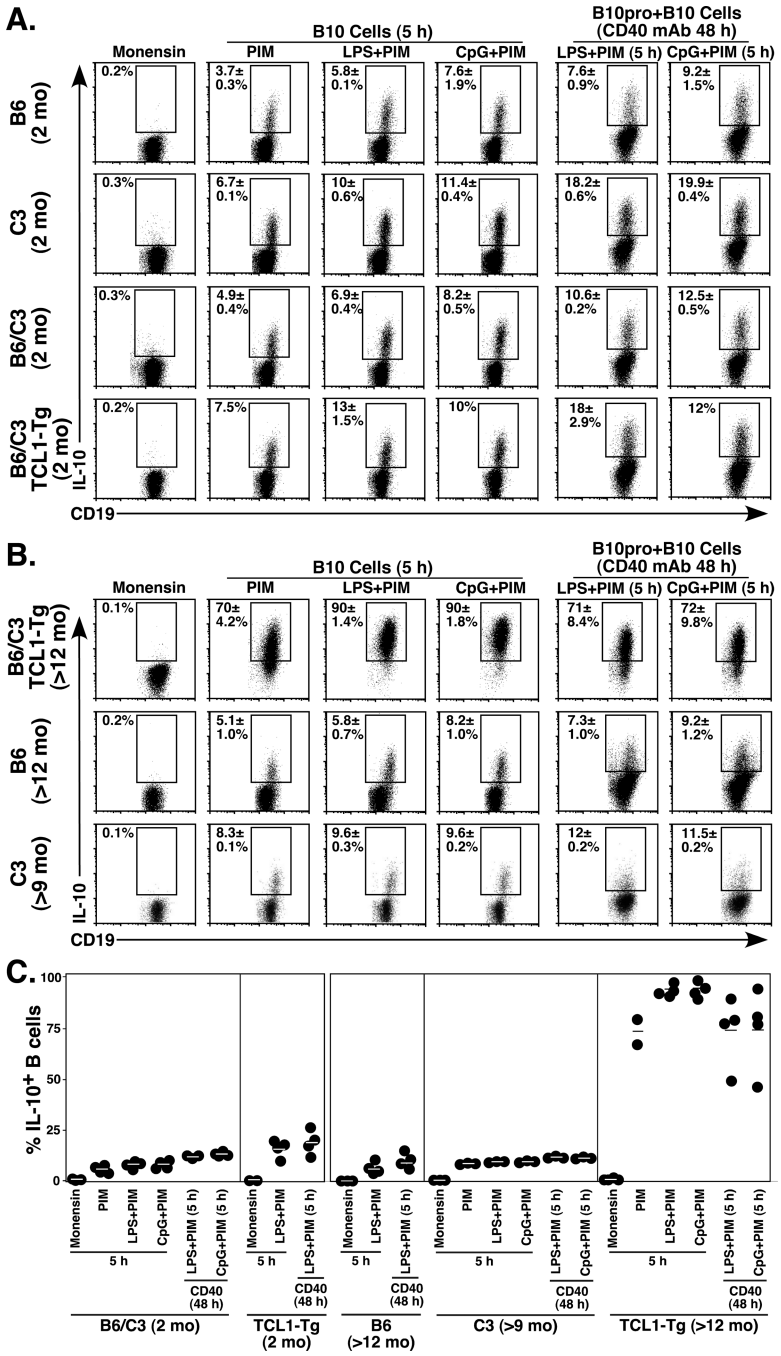

Figure 4. IL-10-competent cells dominate in aged TCL1-Tg mice

(**A**) *Representative B cell IL-10 production in young (2-mo) wild type B6, C3, B6/C3, and B6/C3 TCL1-Tg mice*. Splenocytes were cultured with monensin alone, PMA, ionomycin, plus monensin (PIM), LPS+PIM, or CpG+PIM for 5 h and stained for cell viability, cell surface CD19, and cytoplasmic IL-10. Alternatively, B10+B10pro cell frequencies were determined after *ex vivo* culture with CD40 mAb for 48 h, with LPS+PIM or CpG+PIM added during the final 5 h of culture. Representative cytoplasmic IL-10 staining by viable, single $CD19^+$ B cells is shown in the flow cytometry dot-plots. Numbers indicate mean ± SEM cytoplasmic $IL\text{-}10^+$ B cell frequencies within the indicated gates from 4 mice.

Splenocytes cultured with monensin alone before immunofluorescence staining served as negative controls; background staining in these negative controls was similar to that obtained using isotype-matched control mAbs. (**B**) *Representative B cell IL-10 production in old (>9 mo) wild type B6, C3, and B6/C3 TCL1-Tg mice*. (**C**) IL-10^{+} B cell frequencies among CD19^{+} cells from mice after culture under the indicated conditions as in (A) and (B). Horizontal bars indicate means.

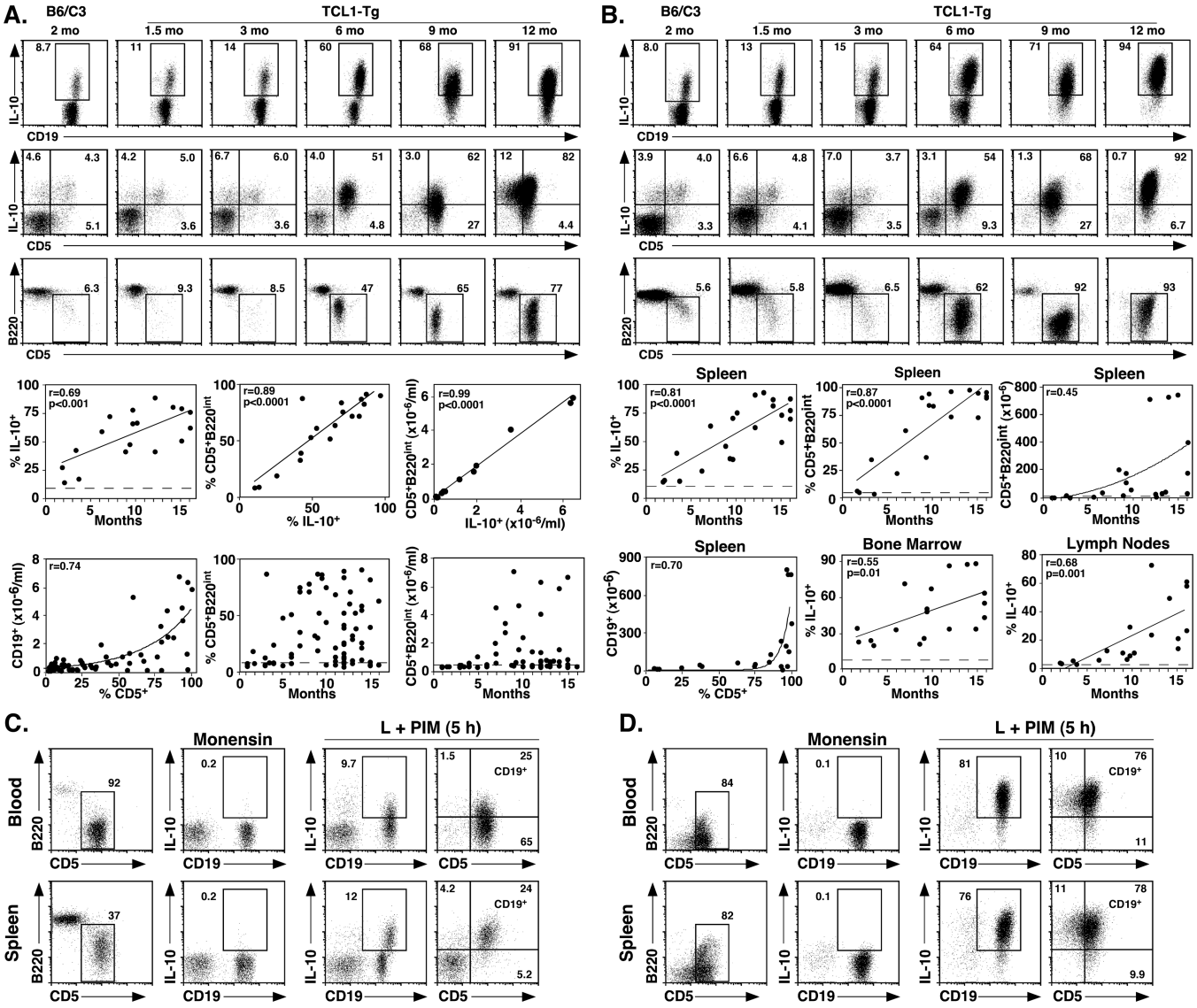

Figure 5. IL-10-competent CLL cells expand with age in TCL1-Tg mice

(**A**) *Blood and* (**B**) *spleen CD5*$^{+}$*B220*^{int} *B10 cells in TCL1-Tg mice*. Cells were isolated from B6/C3 and TCL1-Tg mice of the indicated ages and assessed by immunofluorescence staining with flow cytometry analysis. Splenocytes were cultured with LPS, PMA, ionomycin, and monensin for 5 h and stained for cell viability, cell surface CD5, B220, and CD19 expression, and cytoplasmic IL-10. Representative dot plots (top panels) show IL-10 vs. CD19, IL-10 vs. CD5, and B220 vs. CD5 staining by viable, single CD19^{+} cells. Cell frequencies within the gates are indicated. Each point within the graphs represents an individual mouse. Horizontal dashed lines indicate mean values from 2-mo-old wild type B6/C3 mice (n≥4). Regression lines are shown for reference. Axillary, brachial, and inguinal lymph nodes were pooled. (**C**) Representative B cell IL-10 expression in a 3-mo-old TCL1-Tg mouse with high frequencies of blood and spleen CD5^{+}B220^{int} B cells, but normal blood and spleen CD19^{+} B cell frequencies (43 and 48%, respectively), and CD19^{+} B cell numbers (blood, 0.17×10^{6}/ml; and spleen, 46×10^{6}). (**D**) Representative B cell IL-10 expression in a 12-mo-old TCL1-Tg mouse with high frequencies of blood and spleen CD5^{+}B220^{int} B cells, and high blood and spleen CD19^{+} B cell numbers (blood, 56×10^{6}/ml; and spleen, 774×10^{6}).

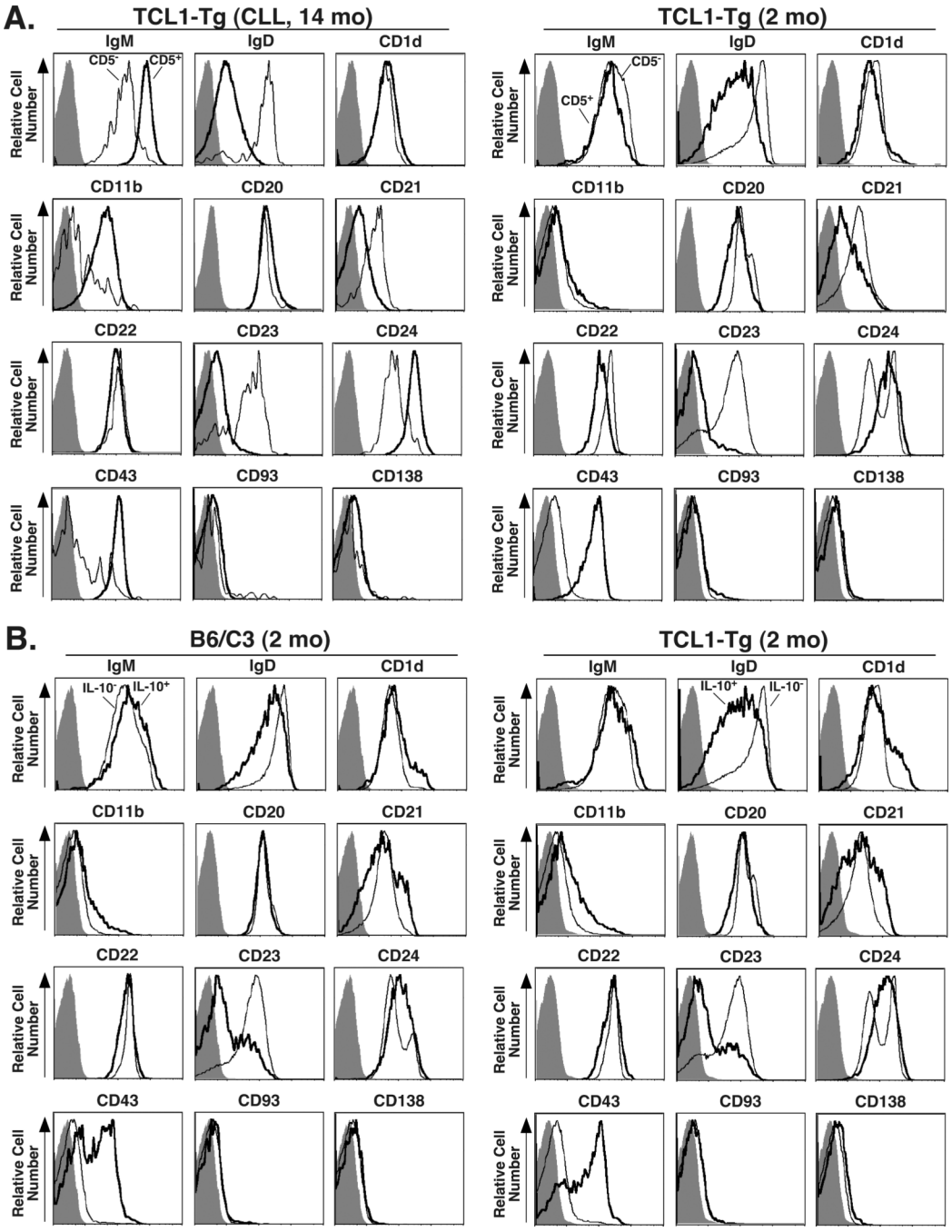

Figure 6. CD5^{+}B220^{int} CLL cells and B10 cells share cell-surface phenotypes in TCL1-Tg and wild type mice

(**A**) *Representative cell surface molecule expression by CD5^{+}B220^{int} cells (heavy lines) and CD5B220^{+} B cells (thin lines) from 14- and 2-mo-old TCL1-Tg mice*. (**B**) *Representative cell surface molecule expression by IL-10^{+} (heavy lines) and IL-10 cells (thin lines) from 2-mo-old wild type B6/C3 and 2-mo-old TCL1-Tg mice after 5 h stimulation with LPS, PMA, ionomycin, and monensin*. (A–B) Flow cytometry histograms are representative of ≥3 mice. Shaded lines in all histograms indicate background isotype-matched control mAb staining.

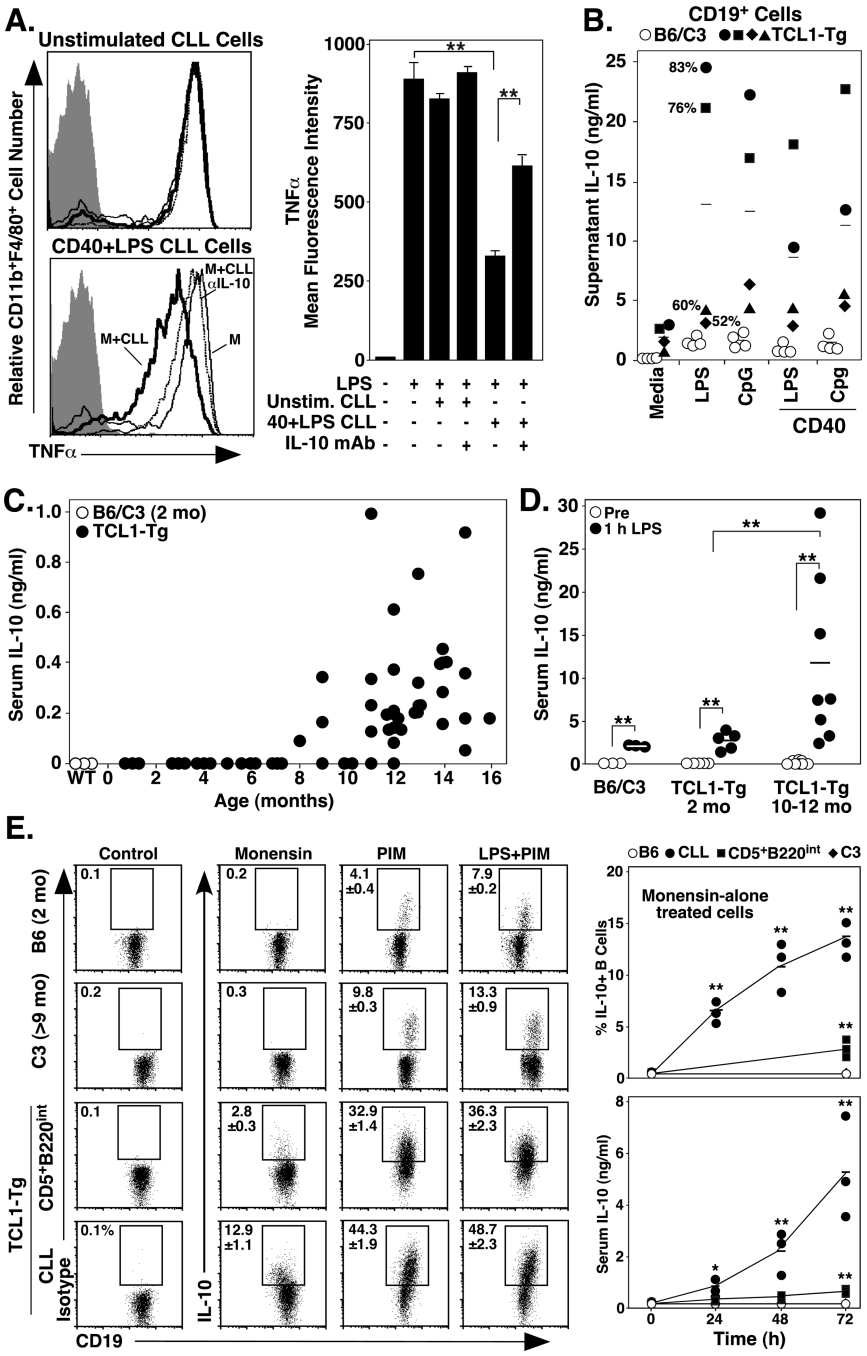

Figure 7. CLL cells from TCL1-Tg mice are regulatory

(**A**) *CLL cells from TCL1-Tg mice suppress monocyte activation in vitro*. Histograms show TNFα expression by $CD11b^{+}F4/80^{+}$ cells cultured alone (M, thin line), cultured with unstimulated (top histogram) or CD40+LPS-stimulated (bottom histogram) CLL cells (M +CLL, heavy line), or with CLL cells plus neutralizing anti-IL-10 mAb (M+CLL αIL-10, dotted line). Monocytes cultured with Brefeldin A alone served as background staining controls (shaded lines). Graph values indicate mean ± SEM TNFα fluorescence staining intensities from six independent experiments using CLL cells from 12–14-mo-old TCL1-Tg mice with >4-fold increases in total spleen B cell numbers. Significant differences between

means are indicated: **, p<0.01. (**B**) *CLL cells from TCL1-Tg mice secrete IL-10 in vitro*. Purified spleen $CD19^+$ B cells from wild type mice (open symbols) or spleen CLL cells from 12–14-mo-old TCL1-Tg mice (filled symbols) were cultured with the indicated stimuli for 72 h. Culture supernatant fluid IL-10 was quantified by ELISA. In separate assays, 5–6% of wild type B cells and 83% (circle), 76% (square), 60% (triangle), and 52% (diamond) of CLL cells expressed intracellular $IL\text{-}10^+$ after 5 h of LPS+PIM stimulation. (**C**) *Serum IL-10 levels increase with age in TCL1-Tg mice*. Each point represents serum IL-10 concentrations from individual mice at the indicated ages. (**D**) *LPS elevates serum IL-10 in TCL1-Tg mice*. Serum samples from wild type (2-mo-old) and TCL1-Tg (2- or 12-mo-old) mice were obtained before and 1 h after low-dose (10 mg) LPS treatment. (**e**) CLL cells express IL-10 in vivo. Young B6, aged C3, and TCL1-Tg mice with clonal preleukemic populations of $CD5^+B220^{int}$ B cells or CLL cells (n=3 mice per group) were given low-dose (10 μg) LPS. Representative histograms show frequencies of spleen B cells, $CD5^+B220^{int}$ B cells, or CLL cells expressing IL-10 as assessed by immunofluorescence staining at 72 h. Control cells were cultured with monensin alone (5 h) before staining with an isotype control mAb, while other cells were cultured with monensin, PIM or LPS plus PIM for 5 h before IL-10 staining. The top graph shows mean $IL\text{-}10^+$ cell frequencies for monensin-only treated $CD19^+$ cells, with serum IL-10 levels shown below. Serum samples were collected before and after LPS treatment as indicated, with IL-10 levels measured by ELISA. The values for B6 and C3 mice are superimposed. (A–E) Significant differences between means are indicated: *, p<0.05; **, p<0.01. Individual symbols represent individual mice. Horizontal bars indicate mean values.

Table 1

CLL patient clinical features

Age at diagnosis [median (IQR [a])]	61.0 (range 53.5 – 67.0) years		n = 93
Men & women	65 (70%) men		n = 93
	28 (30%) women		
VA & Duke patients	15 (16.1%) VA		n = 93
	78 (83.9%) Duke		
Race	82 Caucasian (88.2%)		n = 93
	8 African American (8.6%)		
	2 Asian (2.2%)		
	1 Native American (1.1%)		
Rai stage: 0	63 (67.7%)		n = 93
1	18 (19.4%)		
2	4 (4.3%)		
3	2 (2.2%)		
4	6 (6.5%)		
CD38 expression	84 negative (90.3%)		n = 93
	9 positive (9.7%)		
Zap70 expression	40 negative (43%)		n = 93
	53 positive (57%)		
IgV_H mutation status	21 unmutated (27.6%)		n = 76 [b]
	55 mutated (72.4%)		
Cytogenetics	Normal	15 (18.1%)	n = 83 [b]
	13q del alone	43 (55.4%)	
	13q del ± other abn.	49 (59.0%) [c]	
	Trisomy 12 alone	10 (12.7%)	
	Trisomy 12 ± other abn.	14 (17.7%)	
	17p del	4 (4.8%)	
	17p del ± other abn.	8 (9.6%)	
	11q del alone	3 (3.8%)	
	11q del ± other abn.	6 (7.6%)	
Length of follow-up (median, IQR)	4.3 (1.9–8.2) years		n = 93
Treated during the course of disease	33 (35.5%)		n = 93
Died	3 (3.2%)		n = 93

[a] IQR signifies interquartile range (25^{th} to 75^{th} percentile).

[b] Not all of these analyses were performed on samples from each patient.

[c] "±" signifies that the patient had the designated cytogenetic abnormality alone or in combination with additional cytogenetic abnormalities.