Rh AUTOANTIGEN PRESENTATION TO HELPER T CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA BY MALIGNANT B-CELLS

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Running Title: Helper T cell activation in CLL

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Abstract

Chronic lymphocytic leukemia (CLL) is frequently associated with autoimmune diseases directed against constituents of the blood, including hemolytic anemia (AIHA). We hypothesized that CLL cells predispose to hematological autoimmunity by acting as aberrant antigen presenting cells (APC). Initially, it was confirmed that all studied patients with AIHA secondary to CLL harbored activated Th cells specific for epitopes on the dominant red blood cell (RBC) autoantigens in primary AIHA, the Rh proteins. Rh specific Th cells were also detected in a number of CLL patients who, although they did not suffer AIHA, had low levels of anti-RBC antibody in their serum. Fractionation of putative APC populations from the peripheral blood of patients by negative selection showed that CD5+ CLL cells are the most effective cell type in processing and presenting purified Rh protein to autoreactive Th cells. This ability was confirmed using positively selected CD5+ CLL cells. Thus, our study provides the first evidence for malignant cells driving an autoimmune response by acting as aberrant APC.
Introduction

Chronic lymphocytic leukemia (CLL) is a common, low grade malignancy of CD5 expressing B cells that may be either pre- or post-germinal centre. The condition is unusual among leukemias since it is frequently associated with immunologically mediated complications, which contribute substantially to morbidity and mortality. In particular, autoantibodies specific for red blood cells (RBC) are detectable by direct antiglobulin test (DAT) in up to 20% of advanced CLL cases and can cause autoimmune hemolytic anemia (AIHA). Other common immunological complications include pure red cell aplasia and immune thrombocytopenic purpura. In addition, rare autoantibodies can develop against factor VIII, von Willebrand factor, and C1 esterase inhibitor. A striking feature of autoimmunity in CLL is that it appears to be directed against constituents of the blood with no documented propensity to other targets. However, despite the importance of this immune dysregulation in CLL, the causes of autoantibody production remain unclear.

Studies of animal models and human AIHA demonstrate that the activation of autoreactive helper T (Th) cells by antigen presenting cells (APC) is a key event in the induction of disease. The generation of most IgG antibodies is T-cell dependent, and responses to RBC autoantigens appear to be no exception. Thus, in mice, T-cell depletion blocks the autoantibody response induced by cross-reactive rat RBC. Furthermore, spontaneous anti-RBC autoantibody production in NZB mice can be retarded by anti-CD4 monoclonal antibody or CD4 gene deletion, and anemia can be modulated by peptides containing the dominant Th cell epitope. We have previously demonstrated that patients with primary warm type AIHA mediated by IgG autoantibodies harbor activated Th cells specific for the Rh proteins, the dominant human RBC autoantigens, but there have been no studies of RBC-reactive Th cells in AIHA secondary to CLL. If AIHA associated with CLL is Th dependent, the question arises as to why the autoreactive Th cells are activated.
In autoimmune disease, the B cell is largely studied in the context of pathogenic autoantibody production, whilst other functions, including antigen presentation, have often been ignored\textsuperscript{21}. The RBC- and platelet-reactive autoantibodies identified in CLL patients are polyclonal and differ in specificity and isotype from the immunoglobulins secreted directly by CLL cells\textsuperscript{22}. It is, therefore, generally accepted that residual, non-malignant B cells must produce the pathogenic autoantibodies in most cases\textsuperscript{22-24}. We wished to test the hypothesis\textsuperscript{24} that the malignant B cell may, instead, play an important role in presentation of autoantigen to activate pathogenic Th cells.

In a conventional immune response, B cells can act as "professional" APC, with the ability both to process and present antigen, and to express the co-stimulatory molecules necessary to stimulate Th cells\textsuperscript{25,26}. In murine models of autoimmune diseases, such as diabetes\textsuperscript{27} and multiple sclerosis\textsuperscript{28}, B cells have been shown to be important in presenting autoantigen. A role for CD5\textsuperscript{+} CLL B cells in autoantigen presentation has seemed unlikely, since the malignant cells appear to be inefficient APC \textit{in vitro}\textsuperscript{29}. However, this possibility should not be discounted in the light of further reports showing that co-stimulatory molecules such as CD80 are up-regulated, and antigen presentation is enhanced, after encounter with T cells expressing CD40 ligand\textsuperscript{26,30,31}. Self-tolerance is maintained in part by the elimination or silencing of autoreactive lymphocytes\textsuperscript{32,33}, but Th cells specific for many autoantigens, including Rh proteins\textsuperscript{34}, can escape these censoring mechanisms in healthy individuals. There has been considerable interest in the hypothesis that such surviving autoreactive T cells can be activated and cause autoimmune disease following changes in APC function or type that enhance presentation of the epitopes they recognize\textsuperscript{16,17,34,35}.

As already mentioned, it is striking that autoimmunity in CLL predominantly targets blood constituents. We hypothesize that the large numbers of malignant B cells present in CLL can act as an aberrant APC population: when exposed to high concentrations of RBC and platelet breakdown products, there may be sufficient presentation of blood cell autoantigens.
by CLL cells to overcome self-tolerance. Here, we test this proposal by first establishing whether the peripheral blood of CLL patients with AIHA contains Th cells specific for a dominant RBC autoantigen, and then determining the ability of malignant CLL B cells to present this autoantigen.

**Methods**

**Patients**

AIHA was diagnosed in patients with CLL attending the Aberdeen Royal Infirmary on the basis of clinical and hematological investigation, and a positive DAT. The protocol for investigation was approved by the Grampian Health Board and the University of Aberdeen Joint Ethical Committee, and all patients gave informed consent. Details of the cases are summarized in Table 1A. Most blood samples were obtained when patients were receiving low to moderate doses of steroid treatment. A group of patients with CLL, but no evidence of clinical hemolysis, was also included (Table 1B).

**Determination of Autoantibody Specificity**

Autoantibody eluted by ether treatment from the RBC of AIHA patients, or serum antibody, was screened in hemagglutination assays for the ability to bind a panel of RBC consisting of M^K^M^K, U-, Rhnull, D-, LW(a-b-), Oh, Fy(a-b-), Lu(a-b-), Ko, R_1^, R_1, R_2, r, r^r and rr cells. Autoantibody samples were considered to include Rh specificities if they failed to react, or reacted very weakly, with Rhnull cells, but strongly agglutinated other RBC phenotypes tested. Autoantibodies with Rh specificities were detected in all AIHA patients tested (Table 1).
Detection of human anti-red blood cell antibodies by indirect enzyme linked antiglobulin test

The indirect enzyme linked antiglobulin test (IELAT) is a sensitive method of detecting RBC reactive antibodies. Based on a modification of our published method, this test was used to measure the levels of serum anti-human red blood cell IgG in patients with primary and CLL-associated AIHA, CLL patients with no evidence of AIHA and healthy donors. Round bottomed microtiter plates (Nunc, Roskilde, Denmark) were blocked in phosphate buffered saline pH 7.4 (PBS) containing 0.2% bovine serum albumin (BSA), before addition of 50µl 2% v/v washed human D-positive RBC. Test sera were incubated at 50µl per well with triplicate RBC samples for 1 hour at 37°C and washed three times in PBS-BSA before fixing for 30 minutes in 0.15% glutaraldehyde (Sigma, Dorset, UK) to prevent lysis of the cells in the alkaline conditions required later in the test. The fixed RBC were transferred to fresh, pre-blocked, 96-well plates and washed before incubation with 50µl per well 1µg/ml goat anti-human IgG γ-chain specific antibody (Sigma) for 1 hour at 37°C. After washing, the plates were incubated with 50µl per well 1µg/ml rabbit anti-goat IgG alkaline phosphatase (Sigma) for 1 hour at 37°C, washed, and 100µl of phosphatase substrate solution was then incubated in each well for 1 hour at 37°C. After pelleting of the RBC by centrifugation, 50µl of each supernatant was transferred into the wells of fresh, flat-bottomed microtiter plates (Nunc) and the absorbance measured at 405nm, with 492nm as a reference, using a multiscan plate reader (Labsystems, Basingstoke, UK). Each result is expressed as the mean of triplicate wells. Inter-assay variation was controlled for by including previously tested serum samples on each plate.

Antigens and mitogens

A complete panel of 68 15-mer peptides, with five amino acid overlaps, was synthesized (Department of Biochemistry, University of Bristol, UK), corresponding to the sequences of the 30kDa Rh protein associated with expression of the D blood group antigen. For testing in patients who were D negative, a second panel of 15-mer peptides corresponding to the
ce Rh protein was made. In order to ensure purity, peptides was synthesized by fluorenylmethoxycarbonyl chemistry on resin using a base-labile linker, rather than by pin technology, and screened by HPLC and amino acid analysis. As previously optimized\textsuperscript{16,17,42}, the peptides were used to stimulate cultures at 20µg/ml.

As previously described\textsuperscript{17}, RhD protein was purified from D-positive RBC by immunoprecipitation using monoclonal antibody T19 (Scottish National Blood Transfusion Service) bound to magnetic beads (Biomag, PerSeptive Biosystems, MA, USA), and added to cultures at an estimated concentration of 5µg/ml. The control antigen Mycobacterium tuberculosis purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark) was added to cultures at 20µg/ml. PPD readily provokes recall T cell responses \textit{in vitro},\textsuperscript{16,41} since most UK citizens have been immunized with Bacillus Calmette-Guérin (BCG).

\textit{Isolation peripheral blood mononuclear cells and preparation of T cells}

As described elsewhere\textsuperscript{16,17,42}, peripheral blood mononuclear cells (PBMC) were separated from fresh blood samples by density gradient centrifugation. T-cells were positively selected from PBMC using kits of magnetic beads coated with anti-CD2 monoclonal antibody, released and washed according to the manufacturer’s instructions (CELLection, Dynal Biotech, Wirral, UK). Unbound PBMC were removed following magnetic separation and used as a source of APC.

\textit{Preparation of APC fractions by negative selection}

Three APC fractions were prepared from T-depleted PBMC by successive negative selection steps using antibody-coated magnetic beads (Dynabeads, Dynal). The cells were sequentially depleted of monocytes with anti-CD14 coated beads; dendritic cells with a mixture of mouse anti-human CD1a and CD11c (both Pharmingen) immobilized on anti-mouse IgG coated beads; and CLL B cells with mouse anti-human CD5 (Pharmingen) on anti-mouse IgG coated beads.
Positive selection of CLL B cells

In some experiments CLL B cells were isolated from the other potential APC types in T-depleted PBMC by a single positive isolation step. Kits of magnetic beads (Dynal) coated with mouse anti-human CD5 (Pharmingen) were used, and the CD5^+ cells released, according to the manufacturer's instructions (Dynal).

Flow cytometry

The purity of negatively and positively selected PBMC populations was screened by flow cytometry using an EPICS XL cytometer (Beckman Coulter) and Expo v2 analysis software (Applied Cytometry Systems, Yorks, UK). Each negatively depleted population contained fewer than 10% cells expressing the respective marker, and more than 95% of the positively selected CLL cells stained CD5^+.

Cell culture

Cell culture conditions have previously been described^{16,17,42}. Briefly, PBMC were cultured at a concentration of 1.25 x 10^6 cells/ml in the alpha modification of Eagles medium (Gibco, Paisley, UK) supplemented with 5% autologous serum, 4mM L-glutamine (Gibco), 100U/ml sodium benzylpenicillin G (Sigma), 100µg/ml streptomycin sulfate (Sigma), and 20mM HEPES pH7.2 (Sigma), in a humidified atmosphere of 5% CO_2/95% air. Similar conditions were used for T cells cultures, with APC supplied by adding an equal concentration of one of the different APC fractions.

T cell stimulation assays

As described elsewhere^{16,17,42}, PBMC or T cells plus APC were cultured with antigen, and T cell proliferation was estimated from the incorporation of ^3H-thymidine in triplicate microtitre wells five days after stimulation. Proliferation results are presented either as the mean CPM +/- SD of the triplicate samples, or as a stimulation index (SI), expressing the ratio of mean
CPM in stimulated versus unstimulated control cultures. An SI >3 with CPM >1000 is interpreted as representing a significant positive response\textsuperscript{43}.

Inhibition of MHC class II restricted responses

Blocking mAbs specific for HLA -DP, -DQ, or -DR supplied by Pharmingen were dialyzed thoroughly against PBS before addition to PBMC cultures at the previously determined optimum concentration of 2.5 µg/ml\textsuperscript{16,42}.

RESULTS

T cell responses to the RhD protein and peptide panel

We have previously demonstrated that the blood of patients with primary AIHA contains activated Th cells specific for epitopes on the RhD and RhCcEe proteins\textsuperscript{16,17}. Experiments were set up to determine whether such Th cells are also present in AIHA secondary to CLL. PBMC were obtained from twelve CLL patients with concurrent AIHA (details in Table 1) and tested for the ability to proliferate in response to Rh protein and/or the corresponding panel of peptides. Given the ethical constraints on the size of the blood samples, and thus the number of Th cells available, we were only able comprehensively to study responses against either the RhD or RhCcEe sequences in each case. It was important to ensure that the responses observed were autoimmune and therefore that the Rh sequences tested corresponded to "self". Most of the patients were D-positive, and, for these individuals, we focused on the RhD rather than the RhCcEe polypeptide. This enabled us to take advantage of our validated method for immunopurifying RhD protein from RBC\textsuperscript{17}, allowing a comparison with previous data of Th cell responses in primary AIHA patients\textsuperscript{16,17}, and obviated the need to match the type of the polymorphic RhCcEe protein sequence with each patient. Representative results from three of the ten RhD-positive patients, who were screened with purified RhD protein and the RhD peptide panel, are illustrated in Figure 1 and data from all
patients are summarized in Table 2A. In all the RhD-positive patients, purified RhD protein induced proliferative responses by PBMC. Furthermore, it can be seen that one or more peptides from the RhD protein elicited significant proliferation by PBMC from all patients tested, and, typically, multiple peptides were stimulatory. Comparable results were obtained in the two RhD-negative patients, whose PBMC proliferated in response to multiple peptides from the sequence of the Rhce protein autoantigen. As in our previous studies of primary AIHA\textsuperscript{16,17}, proliferation was assessed on day five after stimulation, and cell culture was performed in microtiter plates without enrichment of Th cells, conditions which strongly favor recall, rather than primary, responses\textsuperscript{34,41,42}. It was confirmed that the PBMC proliferating against Rh protein epitopes were helper T cells, since removal of T cells from cultures, or the addition of blocking antibody specific for MHC class II molecules, significantly inhibited responses (results not shown).

A number of publications\textsuperscript{16,17,34,35,42} have demonstrated that, in contrast to AIHA patients, Th cells capable of recall responses \textit{in vitro} to epitopes on the Rh proteins are rarely detected in healthy donors or patients with non-immunological causes of anemia, providing support for the view that such cells provide help for the production of anti-RBC autoantibodies. Examples of this lack of responsiveness in healthy donors are illustrated in Figure 2. We now wished to ascertain whether there may be sub-clinical anti-RBC autoimmune responses in CLL patients with no overt signs of AIHA. It was determined whether the PBMC from ten such CLL patients were able to proliferate in response to Rh protein and/or Rh peptides, and the results are summarized in Table 2B, with representative data from three individuals illustrated in Figure 3. PBMC from eight of ten patients proliferated when stimulated with purified RhD protein, and six of these eight individuals were also responsive to multiple peptides from the Rh sequence.

\textit{Antibody levels}
One explanation for the responsiveness of PBMC from patients with CLL, but no overt AIHA, is that Th cells in these cases are providing help for anti-RBC autoantibodies that are undetectable by DAT and do not cause clinical hemolysis. Accordingly, a sensitive IELAT was used to compare the levels of RBC-reactive antibodies in the sera of healthy control donors, primary AIHA patients, the patients with AIHA secondary to CLL, and the patients with CLL but no clinical hemolysis. Figure 4 demonstrates that, as expected, high levels of antibody were present in the sera of most of the patients with either primary or secondary AIHA, but antibody levels were also elevated in six of the ten CLL patients without overt AIHA. Importantly, all of these antibody-positive, but AIHA-negative, CLL patients showed proliferative responses to the RhD protein.

Comparison of responses to Rh protein epitopes in primary and CLL associated AIHA

The question arises as to how the fine specificities of the Rh-specific Th cells in cases of CLL with secondary AIHA compare with those previously identified in primary AIHA patients. Figure 5 demonstrates a significant correlation ($R_s=0.55$, $p<0.0001$) between the Rh peptides that most commonly induce proliferative responses in primary AIHA patients, and those that are dominant in CLL patients with AIHA.

Capacity of different APC types to stimulate Th cells responsive to Rh protein

Having shown that CLL is associated with responsiveness of Rh-reactive Th cells, we next tested our primary hypothesis that CLL B cells are effective in presenting Rh autoantigen to activate Th cells. T cells were purified from the PBMC of CLL patients with AIHA, and stimulated with RhD protein presented by different putative APC populations from the remaining PBMC. The first approach to obtaining APC fractions was negative selection, by sequentially depleting PBMC. The PBMC of three AIHA positive CLL patients were fractionated in this way, and experiments illustrating the antigen presenting capacity of the fractions are depicted in Figure 6. The proliferative responses against purified RhD protein by T cells from all patients were maintained when CD14$^+$ and then CD1a$^+$/CD11c$^+$ cells were
removed from cultures, but responses decreased significantly after CD5⁺ cells were depleted.

Neither purified T cells nor any T depleted APC fractions proliferated when stimulated with Rh protein or peptides. These results suggest that CLL B cells, rather than monocytes, dendritic cells or B2 cells, play an important role in processing and presenting the RhD protein in vitro.

To confirm that CLL B cells were able to present Rh protein effectively, T cells were stimulated with purified autoantigen in the presence of freshly isolated CD5⁺ cells that had been positively selected from T-depleted PBMC. The results are summarized in Table 3 and representative data are shown in Figure 7A-D. In a total of 13 experiments from nine different patients, positively selected CD5⁺ cells presented RhD protein to stimulate Th cell proliferation, and, on 12 out of 13 occasions, these responses were higher than those supported by APC from unfractionated PBMC. This ability to stimulate RhD protein reactive Th cells contrasts with the lack of responses elicited by the control recall antigen PPD when APC were provided by the CD5⁺ fraction (Figure 7E and F). Thus, although, as previously reported, CLL cells are inefficient APC for conventional antigens, they are very effective in presenting the RhD autoantigen to T cells.

Capacity of different APC types to stimulate Th cells responsive to Rh peptides

There are at least two possible explanations for the ability of CLL B cells to present RhD autoantigen: the tumor cells may either take up and process the antigen more effectively than other APC types, or preferentially present to autoreactive T cells. To address this question, we determined whether the different APC fractions from four CLL patients could stimulate T cells specific for the respective dominant peptides that had been identified earlier. The results are summarized in Table 4 and show that CD5⁺ cells can present effectively to particular peptide specific T cells. However, in comparison with the responses to the purified RhD protein, there is a less clear bias towards peptide presentation by this fraction, and
other APC populations that include dendritic cells and/or B2 B cells can be equally or more effective.

Discussion

We here describe a novel role for malignant B cells in the autoimmune pathology associated with CLL. The results demonstrate that AIHA secondary to CLL is associated with the activation of Th cells specific for epitopes on the dominant RBC autoantigens, the Rh proteins. Thus, as in primary AIHA, the production of pathogenic anti-RBC autoantibodies in CLL appears to be helper dependent. Cell fractionation experiments showed that the CLL B cells were the major APC type that process and present the Rh proteins to stimulate the autoreactive Th cells. This is the first example of malignant cells driving an autoimmune response by acting as aberrant APC.

Murine studies\textsuperscript{12,14-19} have demonstrated that IgG autoantibodies against RBC proteins are dependent on the activation of autoreactive Th cells specific for these antigens, and an Rh-reactive helper response has previously been identified in human patients with primary AIHA\textsuperscript{16,17}. The characterization of patients with AIHA secondary to CLL reveals similar Th responsiveness to the Rh proteins. Thus, Th cells from the peripheral blood of all such patients proliferated in response to purified Rh protein, and, typically, also responded to multiple synthetic peptides derived from the sequence. There are further parallels between the Th responses in primary and CLL-associated AIHA. The patterns of Rh peptides that elicit Th cell proliferation vary between the CLL patients, but particular sequences are commonly stimulatory, and these correspond to the peptides that are dominant in primary AIHA\textsuperscript{17}. Together, these data lead us to conclude that AIHA secondary to CLL is Th driven, and suggest that, as in primary AIHA, the breakdown of self-tolerance may be due to changes in autoantigen presentation\textsuperscript{35}. 
The results also demonstrate that anti-RBC autoimmune responses are more common in CLL patients than the incidence of overt AIHA would suggest. The view that latent autoimmunity is underestimated in CLL is supported by previous studies using a mitogen-stimulated DAT\textsuperscript{44}. Th cells from a number of our CLL patients with no clinical evidence of hemolysis exhibited reactivity to Rh epitopes, and a sensitive enzyme-linked assay revealed that this responsiveness is often associated with elevated levels of anti-RBC antibodies compared to healthy individuals. Although titers are typically lower than in patients diagnosed with AIHA, either primary or secondary to CLL, it is probable that the Th reactivity reflects help provided for production of antibodies that do not result in overt hemolysis. It remains to be determined whether these antibodies appear to be non-pathogenic solely because of their low titer, since many other factors, including autoantibody subclass, galactosylation, and the activation state of phagocytes within the reticulo-endothelial system also influence the rate of hemolysis\textsuperscript{36-38,45,46}. In the remaining CLL patients with Rh-specific Th cells in the absence of hemolysis, but no detectable anti-RBC antibodies, it could be argued that Th activation precedes the antibody response, or may represent the occasional cross-reactivity to environmental antigens seen in healthy control individuals\textsuperscript{16, 17, 34, 35, 42}.

Although CLL B cells can secrete polyreactive autoantibodies\textsuperscript{47}, these are not the cause of hemolysis in AIHA associated with the tumor\textsuperscript{22}. Autoreactive B and T cells form part of the normal immune repertoire, remaining quiescent in healthy individuals, but capable of driving autoimmune disease if activated\textsuperscript{34,35}. It has previously been proposed\textsuperscript{34,35} that autoimmune diseases, including AIHA, are initiated by changes in autoantigen presentation such as the recruitment of new APC types, which then leads to the activation of autoreactive Th cells. We therefore sought to determine whether malignant CLL cells, which could represent a large, abnormal APC population, were capable of presenting the Rh autoantigens to Th cells in patients with secondary AIHA. Although it is well-documented that CLL cells are poor APC for conventional antigens\textsuperscript{29}, and this is supported in the current work, we demonstrated that
they are very effective in stimulating the proliferation of Th cells specific for Rh protein. This ability was shown by experiments to fractionate different APC types by negative selection, and confirmed by positively isolated CD5⁺ B cells from CLL patients stimulating significantly higher responses than the other APC populations in PBMC.

There are precedents for B cells acting as APC to drive autoimmune pathology. For example, myelin oligodendrocyte protein is immunogenic and can induce EAE in wild type, but not B cell deficient, mice⁴⁸. Furthermore, B cells can effectively process and present protein antigens to prime naïve T cells⁴⁹, and are particularly efficient as APC for conformational immunogens⁵⁰. In the context of AIHA, the ability of B-cells to form a synapse and internalize membrane bound or integral antigens⁵¹ provides a mechanism for the uptake and presentation of the Rh proteins from RBC.

There are a number of possible explanations, which are not mutually exclusive, for the preferential presentation of Rh autoantigen by the malignant cells. First, CLL cells may be particularly effective in taking up and/or processing the Rh protein, or, secondly, they may stimulate autoreactive Th cells more efficiently than conventional responses. Both these factors may be relevant, since CLL cells were able to present many synthetic Rh-derived peptides to Th cells, but not so strikingly as the purified Rh protein. An alternative explanation, which we favor, is that CLL cells process Rh protein in such a way as to skew presentation away from epitopes to which the Th cell repertoire is normally tolerant. It is widely believed that such presentation of previously cryptic epitopes can initiate and drive autoimmune diseases⁵¹⁻⁵⁷, including AIHA¹⁶,¹⁷,³⁴,³⁵,⁵⁸. Whatever the reasons for the efficacy of CLL cells as APC for Rh protein, the uncontrolled expansion of these malignant cells in vivo, combined with their localization with blood breakdown products, provides the opportunity to activate autoreactive Th cells specific for RBC, platelet and other blood-derived antigens. It is yet to be determined where the Th cell activity occurs, but the spleen is likely to be important, given its role as a secondary lymphoid organ and a major site where blood cell
breakdown products are available. Indeed, five of our 12 patients with CLL and overt hemolysis exhibited palpable splenomegaly. The observation that the presence of CLL cells does not inevitably lead to AIHA reflects the complex, multifactorial etiology of autoimmune conditions, and that other, as yet identified, genetic/environmental factors contribution to development of the disease.

The recognition that AIHA secondary to CLL is helper dependent, and that the malignant B cells may drive activation of the autoreactive Th cells by acting as APC, has implications for the development of future treatments. For example, specific peptide immunotherapy, based on the induction of tolerance to dominant Th cell epitopes is under development for primary AIHA, and to prevent allo-responses to the RhD blood group antigen, and this approach can now be extended to CLL patients. Such therapy would remove one important complication of this leukemia.
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Figure Legends

Figure 1

Responses to RhD autoantigen by PBMC from CLL patients with secondary AIHA.
Representative experiments showing proliferation by PBMC from patients CLL+AIHA1 (A), CLL+AIHA2 (B) and CLL+AIHA3 (C) after stimulation with a panel of overlapping 15-mer peptides spanning the sequence of the RhD protein (filled bars) or purified RhD protein (hatched bars). Control unstimulated cultures were also included (empty bars). Peptides are numbered 1-68 from the N-terminus. The line ----- indicates the level of response taken as representing a positive response (SI>3).

Figure 2

Responses to Rh protein and peptides by PBMC from healthy control donors
Representative experiments showing absent, or weak proliferation by PBMC from healthy D-positive (A&B) and D-negative (C) control donors after stimulation with panels of overlapping 15-mer peptides spanning the sequences of the RhD (A and B) or Rhce (C) proteins (filled bars) or purified RhD protein (hatched bars). Control unstimulated cultures were also included (empty bars). Peptides are numbered 1-68 from the N-terminus: the ce panel lacks numbers 7,9,13,15,17,19,22,24,26,28,30,32,34,36,38,40,43,45,49,51,53,55,57,59,64, and 66. The line ----- indicates the level of response taken as representing a positive response (SI>3).

Figure 3

Responses to RhD autoantigen by PBMC from CLL patients with no clinical AIHA.
Representative experiments showing proliferation by PBMC from patients CLL1 (A), CLL2 (B) and CLL3 (C) after stimulation with a panel of overlapping 15-mer peptides spanning the sequence of the RhD protein (filled bars) or purified RhD protein (hatched bars). Control unstimulated cultures were also included (empty bars). Peptides are numbered 1-68 from the N-terminus. The line ----- indicates the level of response taken as representing a positive response (SI>3).

Figure 4

Anti-red blood cell antibodies in CLL patients.
IELAT measurements of serum antibodies specific for human red blood cells in control healthy donors (n=10), primary AIHA patients (n=10), CLL patients with AIHA (n=7) and CLL patients with no clinical AIHA (n=10). CLL patients with no clinical AIHA whose PBMC respond to purified RhD protein and/or RhD peptides (n=7) are represented by filled squares, whilst patients in this group with no such responses (n=2) are represented by empty squares.
The lines indicate the mean antibody level in each group. Significant differences are marked (Mann-Whitney U test).

**Figure 5**

**Summary of proliferative responses to RhD autoantigen peptides by PBMC from CLL patients with AIHA.**

Shown here is the proportion of D-positive CLL patients with secondary AIHA (n=8) (filled bars) whose PBMC proliferate in response to peptides spanning the RhD protein. Also shown (empty bars) are the results of parallel studies to map RhD peptides that stimulate PBMC from patients with primary AIHA patients (n=11). The peptide panel is reduced to the 42 sequences with less overlaps used in the previous work and numbered accordingly. There is a significant correlation (R=0.55, p<0.0001) between the abilities of each RhD peptide to elicit proliferative responses in both the primary and secondary AIHA patients.

**Figure 6**

**Responses to RhD protein by T cells from CLL patients with AIHA when different PBMC populations are used as APC.**

T cells were purified from PBMC and the remaining cells fractionated by negative selection into putative APC types. Sequential depletion of CD14+, CD1a+/11c+ and CD5+ cells removes monocytes, dendritic cells and CLL cells respectively. The APC fractions were tested for the ability to stimulate T cell proliferation in response to RhD protein. Proliferative responses in patients CLL+AIHA1 (A), CLL+AIHA4 (B&C) and CLL+AIHA5 (D) is inhibited by the depletion of CLL B cells, but remains unaffected by removal of other APC fractions. Significant differences between cultures stimulated using unfractionated and fractionated PBMC as APC are indicated (Student’s t-test *p<0.05, **p<0.01).

**Figure 7**

**RhD protein, but not the control recall antigen PPD, is presented to T cells by CLL B cells.**

T cells were purified from PBMC and CD5+ CLL B cells positively selected from the remaining cells. The ability of the T-depleted PBMC and the CD5+ CLL B cell fraction to stimulate T cell proliferation in response to RhD protein was compared. CLL B cells are more effective than PBMC in presenting RhD protein to T cells from patients CLL+AIHA2 (A), CLL+AIHA5 (B), CLL+AIHA6 (C) and CLL+AIHA10 (D). In contrast, CLL B cells fail to stimulate proliferative responses against PPD by T-cells from patients CLL+AIHA1 (E) and CLL+AIHA7 (F). Significant differences between cultures stimulated using unfractionated PBMC and CD5+ CLL B cells as APC are indicated (Student’s t-test *p<0.05, **p<0.01).
### Table 1A

**Details of CLL patients with secondary AIHA (CLL+AIHA)**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age</th>
<th>RhD</th>
<th>Coombs</th>
<th>Coombs</th>
<th>Autoantibody Specificity</th>
<th>Stage (Rai)</th>
<th>CLL/AIHA Duration (M)</th>
<th>Blood Count at diagnosis of AIHA at sampling</th>
<th>Previous therapy</th>
<th>Current therapy (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb  g/L</td>
<td>WBC 10^9/L</td>
<td>PLT 10^9/L</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>M</td>
<td>66</td>
<td>+</td>
<td>++++</td>
<td>-</td>
<td>Y N Tp</td>
<td>II</td>
<td>24/24</td>
<td>50</td>
<td>113</td>
<td>6.7</td>
</tr>
<tr>
<td>CLL+AIHA2</td>
<td>M</td>
<td>73</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>Y N III</td>
<td>III</td>
<td>18/8</td>
<td>62</td>
<td>142</td>
<td>5.9</td>
</tr>
<tr>
<td>CLL+AIHA3</td>
<td>M</td>
<td>75</td>
<td>+</td>
<td>++++</td>
<td>++</td>
<td>Y N I</td>
<td>I</td>
<td>3/3</td>
<td>83</td>
<td>114</td>
<td>63</td>
</tr>
<tr>
<td>CLL+AIHA4</td>
<td>F</td>
<td>83</td>
<td>+</td>
<td>++++</td>
<td>-</td>
<td>Y N 0</td>
<td>2 cm</td>
<td>18/8</td>
<td>96</td>
<td>142</td>
<td>12.8</td>
</tr>
<tr>
<td>CLL+AIHA5</td>
<td>M</td>
<td>87</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>Y N 2 cm</td>
<td>2 cm</td>
<td>24/18</td>
<td>48/48</td>
<td>114</td>
<td>159</td>
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<tr>
<td>CLL+AIHA6</td>
<td>M</td>
<td>68</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>Y N IV</td>
<td>6 cm</td>
<td>24/18</td>
<td>68</td>
<td>166</td>
<td>111</td>
</tr>
<tr>
<td>CLL+AIHA7</td>
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<td>69</td>
<td>-</td>
<td>++++</td>
<td>++</td>
<td>Y Y I</td>
<td>IV</td>
<td>25/18</td>
<td>79</td>
<td>339</td>
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</tr>
<tr>
<td>CLL+AIHA8</td>
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<td>82</td>
<td>-</td>
<td>++++</td>
<td>++</td>
<td>Y Y I</td>
<td>I</td>
<td>98/11</td>
<td>76</td>
<td>119</td>
<td>137</td>
</tr>
<tr>
<td>CLL+AIHA9</td>
<td>M</td>
<td>68</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>UA UA</td>
<td>II</td>
<td>42/36</td>
<td>111</td>
<td>134</td>
<td>98</td>
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<tr>
<td>CLL+AIHA10</td>
<td>M</td>
<td>74</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>UA UA</td>
<td>I</td>
<td>43/1</td>
<td>72</td>
<td>111</td>
<td>72.3</td>
</tr>
<tr>
<td>CLL+AIHA11</td>
<td>M</td>
<td>70</td>
<td>+</td>
<td>++++</td>
<td>++</td>
<td>UA UA</td>
<td>IV</td>
<td>4 cm</td>
<td>74/9</td>
<td>42</td>
<td>208</td>
</tr>
<tr>
<td>CLL+AIHA12</td>
<td>F</td>
<td>78</td>
<td>+</td>
<td>++++</td>
<td>-</td>
<td>UA UA</td>
<td>II</td>
<td>5 cm</td>
<td>234/140</td>
<td>67</td>
<td>30.7</td>
</tr>
</tbody>
</table>

**UA Unavailable**

### Table 1B

**Details of CLL patients (CLL)**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age</th>
<th>RhD</th>
<th>Coombs</th>
<th>Coombs</th>
<th>Autoantibody Specificity</th>
<th>Stage (Rai)</th>
<th>CLL Duration (M)</th>
<th>Blood Count at sampling</th>
<th>Previous therapy</th>
<th>Current therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb  g/L</td>
<td>WBC 10^9/L</td>
<td>PLT 10^9/L</td>
</tr>
<tr>
<td>CLL1</td>
<td>M</td>
<td>13</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>85</td>
<td>108</td>
<td>297</td>
<td>86</td>
</tr>
<tr>
<td>CLL2</td>
<td>F</td>
<td>26</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>I</td>
<td>26</td>
<td>143</td>
<td>11.3</td>
<td>121</td>
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<tr>
<td>CLL3</td>
<td>M</td>
<td>58</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>21</td>
<td>140</td>
<td>71.4</td>
<td>97</td>
</tr>
<tr>
<td>CLL4</td>
<td>M</td>
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<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>II</td>
<td>41b</td>
<td>59</td>
<td>163</td>
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<tr>
<td>CLL5</td>
<td>M</td>
<td>31</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>42</td>
<td>149</td>
<td>22</td>
<td>136</td>
</tr>
<tr>
<td>CLL6</td>
<td>F</td>
<td>67</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>120</td>
<td>128</td>
<td>29.8</td>
<td>197</td>
</tr>
<tr>
<td>CLL7</td>
<td>F</td>
<td>30</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>30</td>
<td>132</td>
<td>9.7</td>
<td>288</td>
</tr>
<tr>
<td>CLL8</td>
<td>M</td>
<td>62</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>82</td>
<td>17.6</td>
<td>235</td>
<td>None</td>
</tr>
<tr>
<td>CLL9</td>
<td>F</td>
<td>85</td>
<td>+</td>
<td>-</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>22</td>
<td>134</td>
<td>63.2</td>
<td>338</td>
</tr>
<tr>
<td>CLL10</td>
<td>F</td>
<td>66</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>NA NA</td>
<td>0</td>
<td>84</td>
<td>143</td>
<td>17.3</td>
<td>284</td>
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</tbody>
</table>

**UA Unavailable**

**NA Not Applicable**
Table 2A

Summary of proliferative responses to RhD protein and RhD peptides by CLL patients with secondary AIHA (CLL+AIHA)

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Purified RhD protein induced proliferation</th>
<th>Rh specific peptides stimulating proliferation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL+AIHA1</td>
<td>Y</td>
<td>2, 3, 8, 11, 18, 20, 27, 33, 34, 35, 44, 46, 48, 50, 52, 55, 62, 67, 68</td>
</tr>
<tr>
<td>CLL+AIHA2</td>
<td>Y</td>
<td>2, 5, 26, 34, 57</td>
</tr>
<tr>
<td>CLL+AIHA3</td>
<td>Y</td>
<td>3, 9, 10, 22, 28, 37</td>
</tr>
<tr>
<td>CLL+AIHA4</td>
<td>Y</td>
<td>1, 23</td>
</tr>
<tr>
<td>CLL+AIHA5</td>
<td>Y</td>
<td>19, 68</td>
</tr>
<tr>
<td>CLL+AIHA6</td>
<td>Y</td>
<td>NT</td>
</tr>
<tr>
<td>CLL+AIHA7</td>
<td>NA</td>
<td>12, 20, 50, 58, 63</td>
</tr>
<tr>
<td>CLL+AIHA8</td>
<td>NA</td>
<td>2, 3, 4, 20, 21, 23, 44, 52, 54, 63</td>
</tr>
<tr>
<td>CLL+AIHA9</td>
<td>Y</td>
<td>NT</td>
</tr>
<tr>
<td>CLL+AIHA10</td>
<td>Y</td>
<td>5, 6, 9, 17, 18, 21, 30</td>
</tr>
<tr>
<td>CLL+AIHA11</td>
<td>Y</td>
<td>5, 57</td>
</tr>
<tr>
<td>CLL+AIHA12</td>
<td>Y</td>
<td>21, 46</td>
</tr>
</tbody>
</table>

NA  Not applicable (RhD negative patient)
NT  Not tested
* PBMC from patient tested with the appropriate panel of synthetic RhD or Rhce peptides to match phenotype (Rhce panel tested in patients CLL+AIHA 7 & 8)

Table 2B

Summary of proliferative responses to RhD protein and RhD peptides by CLL patients without overt AIHA (CLL)

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Purified RhD protein induced proliferation</th>
<th>Rh specific peptides stimulating proliferation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL1</td>
<td>N</td>
<td>None</td>
</tr>
<tr>
<td>CLL2</td>
<td>Y</td>
<td>15, 16, 31, 34, 47</td>
</tr>
<tr>
<td>CLL3</td>
<td>Y</td>
<td>17, 40</td>
</tr>
<tr>
<td>CLL4</td>
<td>N</td>
<td>None</td>
</tr>
<tr>
<td>CLL5</td>
<td>Y</td>
<td>2, 3, 17</td>
</tr>
<tr>
<td>CLL6</td>
<td>Y</td>
<td>None</td>
</tr>
<tr>
<td>CLL7</td>
<td>Y</td>
<td>2, 9, 41</td>
</tr>
<tr>
<td>CLL8</td>
<td>Y</td>
<td>None</td>
</tr>
<tr>
<td>CLL9</td>
<td>Y</td>
<td>14, 38</td>
</tr>
<tr>
<td>CLL10</td>
<td>Y</td>
<td>27, 28, 29, 30, 41, 42, 53, 54, 63, 68</td>
</tr>
</tbody>
</table>

* PBMC from patient tested with the panel of synthetic RhD peptides to match phenotype
Table 3
Responses to purified RhD protein by T cells from CLL patients with secondary AIHA when CD5+ CLL cells are used as APC. Responses are shown relative to proliferation stimulated when unfractionated PBMC act as APC.

<table>
<thead>
<tr>
<th>CLL+AIHA PATIENT TESTED</th>
<th>2</th>
<th>2</th>
<th>3</th>
<th>3</th>
<th>4</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CHANGE IN RESPONSE</td>
<td>245</td>
<td>818</td>
<td>201</td>
<td>160</td>
<td>151</td>
<td>85</td>
<td>333</td>
<td>356</td>
<td>173</td>
<td>140</td>
<td>167</td>
<td>239</td>
<td>119</td>
</tr>
</tbody>
</table>
Table 4

Responses to Rh peptides by T cells from CLL patients with secondary AIHA when different PBMC populations are used as APC. PBMC remaining after the T cell purification were fractionated by negative or positive selection into putative APC types, and all results are expressed as % of the response when the unfractionated PBMC act as APC. Sequential depletion of CD14⁺, CD1a⁺/I1c⁺ and CD5⁺cells removes monocytes, dendritic cells and CLL cells respectively.

<table>
<thead>
<tr>
<th>CLL patient</th>
<th>Rh peptide tested</th>
<th>% of response when PBMC are used as APC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Populations depleted from PBMC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD14⁺</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>D33</td>
<td>NT</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>D46</td>
<td>NT</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>D62</td>
<td>65</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>D20</td>
<td>117</td>
</tr>
<tr>
<td>CLL+AIHA1</td>
<td>D27</td>
<td>116</td>
</tr>
<tr>
<td>CLL+AIHA4</td>
<td>D23</td>
<td>25</td>
</tr>
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<td>CLL+AIHA7</td>
<td>ce20</td>
<td>NT</td>
</tr>
<tr>
<td>CLL+AIHA8</td>
<td>ce2</td>
<td>12</td>
</tr>
<tr>
<td>CLL+AIHA8</td>
<td>ce21</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT Not tested
Fig. 1

A

3H-THYMIDINE INCORPORATION (MEAN CPM x10^-3 +/-SD)

B

C

STIMULUS

For personal use only.
Fig. 2

A

B

C

$^{3}H$-THYMIDINE INCORPORATION (MEAN CPM x10$^{-3}$ +/- SD)

STIMULUS
Fig. 3

A

B

C

3^H-THYMIDINE INCORPORATION (MEAN CPM x 10^-3 +/- SD)

STIMULUS
Fig. 3

p = 0.017
p = 0.003
p = 0.017

Fig. 4
Fig. 5

% INDIVIDUALS WITH T CELLS RESPONDING TO RhD PEPTIDE
Fig. 6

**APC POPULATIONS DEPLETED FROM PBMC**
Fig. 7

APC POPULATION ADDED TO T-CELLS
Rh Autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B-cells

Andrew M Hall, Mark A Vickers, Ewan McLeod and Robert N Barker