Targets for the treatment of drug resistant chronic lymphocytic leukemia

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Expansion of effector T cells associated with decreased PD-1 expression in patients with indolent B cell lymphomas and chronic lymphocytic leukemia

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Abstract

In patients with chronic lymphocytic leukemia (CLL), numbers of CD8^+CD45RA^+/−CD27^− effector T cells are expanded. We investigated whether this expansion is also present in other B cell malignancies and the possible mechanism underlying these changes.

Whereas an increase in total CD4^+ and CD8^+ T cell numbers was found only in CLL, CD4^+ and CD8^+ effector T cells were significantly expanded both in CLL and indolent lymphoma, but not aggressive lymphoma and myeloma. Interestingly, PD-1 expression was decreased on effector T cells and inversely correlated with effector T cell numbers, suggesting a functional role for PD-1 in regulating T cell homeostasis. In vitro experiments revealed impaired upregulation of PD-1 upon T cell activation in the presence of malignant but also healthy B cells.

Our data suggest that in CLL and indolent lymphoma, the malignant B cells affect PD-1 expression on effector T cells, resulting in an expansion of these subsets.
Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and mainly affects the elderly. The clinical course is often characterized by considerable morbidity due to an increasing susceptibility to (opportunistic) infections and the occurrence of autoimmune phenomena. A cardinal feature of this immuno-incompetence is progressive hypogammaglobulinemia, but also defects in T cell function play an important role (reviewed in 1;2).

The interaction between malignant B cells and the host immune system is thought to be a dynamic and reciprocal process in which the exact role of T cells is ill-defined. T cells may have an important role in cancer immunosurveillance, but on the other hand, these cells can contribute to clonal selection and tumour growth and survival 3.

Several studies have described an expansion of both CD4+ cells 4;5 and CD8+ cells 4;6;7 in CLL. These T cells were found to have an antigen experienced, activated phenotype 4;6;8. Furthermore, clonal and oligoclonal expansions were demonstrated within both the CD4+ and the CD8+ T cell compartment 4;7;9;10. Hence these expansions have been thought to reflect a tumour-derived antigen directed response. However, in vitro data do not support this hypothesis, as cytotoxic responses against freshly isolated, or even CD40-activated, autologous CLL cells have rarely been observed 6;11;12. In addition numbers of regulatory T (Treg) cells were found to be increased in CLL 13, but also in this case, neither analysis of the T-cell receptor repertoire nor CD45 isoform expression on Treg cells provided evidence for chronic (tumour) antigenic stimulation as a cause for the expansion 14.

Functionally, CD8+ T cells can be divided into three subsets; naïve cells (expressing both CD45RA and CD27), memory cells (expressing CD27 but not CD45RA), and effector cells (which may express CD45RA, but not CD27) 15. We have found earlier that the expansion of CD8+ T cells in CLL is largely confined to CD8+ T cells exhibiting the cytotoxic CD45RA+/-CD27- phenotype 16 and is exclusively seen in CMV-seropositive patients. In CMV infected, but otherwise healthy individuals, CD8+ T cells displaying the cytotoxic CD45RA+CD27- or the non-cytotoxic CD45RA-CD27+ phenotype persist in the latency phase after clearance of the acute infection 17. Indeed, using tetrameric cytomegalovirus (CMV)-peptide complexes, a considerable proportion of the expanded CD8+ CD45RA+/-CD27+ T cell pool in CLL patients was found to be CMV-specific 16. A similar division can be made in CD4+ T cells, based on expression of CD28 and CD45RA. Low frequencies of CD4+ T cells which have lost expression of the co-stimulatory receptor CD28 and have direct cytotoxic capacity (through granzyme B and perforin containing granules) can be found in the peripheral blood of individuals with chronic viral infections among which CMV 18;19. In a very recent study, also the numbers of CMV-specific CD4+ T cells exhibiting the late differentiated CD45RO+CD27-CD28-CCR7- phenotype were found to be increased in CLL 20. It is currently not known whether these changes are due to intrinsic changes in T cells in patients with CLL or whether they
reflect an increased antigenic pressure due to failure of other components of the immune system, possibly induced by the malignancy. The fact that reactivations of CMV-infection are rare in untreated CLL patients does not support the latter hypothesis.

Immuno-incompetence is also a frequent feature of the clinical course of other B cell malignancies, like B cell non Hodgkin lymphoma (B-NHL) and multiple myeloma (MM). Although also in these diseases, changes in the T cell compartment and shifts in T cell-receptor repertoire have been described, much less is known about the dynamics of specific T cell subsets and its relation to CMV-serostatus.

In the present study we address two questions: (1) Are the changes in the T cell compartment specific for CLL or do they also occur in patients with other B cell malignancies, and (2) what are possible mechanisms underlying the alterations in T cell homeostasis.

Materials and methods

Patients

Peripheral blood (PB) mononuclear cells (PBMCs) and plasma samples from 67 previously untreated patients with B cell malignancies were collected during routine follow-up visits at the departments of Hematology of the Academic Medical Centre, the Antoni van Leeuwenhoek hospital and the Onze Lieve Vrouwe Gasthuis in Amsterdam. The numbers per disease entity were: CLL, n=29 (including; small lymphocytic leukemia n=2); indolent lymphoma, n=15 (follicular lymphoma grade I-II n=8; hairy cell leukemia n=2 marginal zone lymphoma n=3, low-grade lymphoma not otherwise specified n=2); aggressive lymphoma, n=13 (follicular lymphoma grade III with an aggressive clinical course n=3, diffuse large B cell lymphoma n=10) and multiple myeloma, n=10.

As a control, blood was drawn from 12 healthy age-matched individuals; all control subjects had a normal lymphocyte count. Approval for these studies was acquired from the Medical Ethical Committee in all participating institutions. Written informed consent was obtained in accordance with the Declaration of Helsinki.

Isolation of peripheral blood mononuclear cells, cell culture and T cell activation

PBMCs were isolated by Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands) and cryopreserved at -180 °C until the day of analysis. Plasma was obtained by centrifugation of blood samples at 1750 rpm for 10 min and subsequently stored at –20 °C.

For T cell activation studies, 96-well flat bottom plates were coated with anti-CD3 (clone 16A9T, generated in our laboratory) overnight at 4°C. Then, PBMCs (0.15x10^6) derived from healthy donors were cultured in these wells in the presence or absence of 0.15x10^6 CLL cells, healthy donor derived CD19^+ B cells (CD2/14/16/36/43/235a...
depleted PBMCs) or CD19 depleted PBMCs (‘second party cells’) using Dynabeads according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Second party cells were labelled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen) in PBS for 15 min at 37 °C and washed in IMDM containing 10%. After 48 hours of culture, cells were washed, stained and analyzed as described below.

**Immunofluorescence staining and flow cytometric analysis**

Mononuclear cells were washed twice with ice-cold PBS containing 1% BSA and 0.01% sodium acetate and stained for 30 min on ice, using saturating amounts of CD3-PE, CD3-APC, CD3-pacific blue, CD3 Pe-Cy7, CD4-FITC, CD4-Percp-Cy5.5, CD8-Percp-Cy5.5, CD8-APC, CD8 Pe-Alexa610 (Invitrogen), CD19-Percp-Cy5.5, CD25-FITC, CD28-PE, CD38-PE, CD127-PE, HLA-DR-FITC, CD45RA-APC, PD-1-PE (all Becton Dickinson (BD), San Jose, Ca, USA) and CD27-FITC (Pelicluster, Amsterdam, the Netherlands). Cells were washed and were acquired on a FACSCanto using FACSDiva Software (BD Biosciences). Results were analyzed using FlowJo MacV8.6.3 (Tree Star, Ashland, OR, USA).

**CMV polymerase chain reaction (PCR) and CMV antibody levels**

CMV copy number in whole blood (EDTA) was analyzed by a quantitative polymerase chain reaction (PCR) as described previously \(^{21}\). To determine CMV serostatus, anti-CMV IgG antibody levels were measured in plasma samples with use of the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, II, USA), according to the manufacturer’s instructions. Measurements were calibrated relative to a standard serum.

**Statistical analysis**

The d’Agostino and Pearson omnibus normality test was performed to assess normal distribution of data sets. In case of Gaussian distribution of data, an unpaired two-sided t test was used to analyze differences between data sets. If there was no Gaussian distribution, a two-tailed Mann-Whitney-U test was used to analyze differences between the groups. Correlations were analyzed by Spearman’s rank correlation test. A p-value <.05 was considered statistically significant.

**Results**

**Expansion of CD4\(^+\) and CD8\(^+\) T cells is restricted to CLL**

The size of the total T cell compartment (CD3\(^+\) cells) and CD4\(^+\) and CD8\(^+\) (gated on CD3\(^+\)) subsets was assessed by flowcytometry in patients with CLL (n=22), indolent lymphoma (n=15), aggressive lymphoma (n=13), multiple myeloma (n=10) and age-
matched healthy controls (n=12). Patient characteristics of these patients are summarized in supplemental Table I. As shown in Table I, total T cell numbers as well as CD4+ as CD8+ T cell numbers were increased in CLL patients, in accordance with our previous findings. In contrast, in patients with indolent non Hodgkin lymphoma and multiple myeloma no significant changes in T cell subsets were found, whereas in aggressive lymphoma, both total T cell numbers and CD4+ T cell numbers were significantly decreased when compared to controls.

Effector T cells are expanded both in CLL and in indolent lymphoma

Next we investigated the distribution of subsets within the CD4+ and CD8+ T cell compartment (Figure 1A). Analysis of subsets within the CD4+ T cell compartment in CLL patients revealed expansion of both memory cells and, more strikingly, effector cells, whereas numbers of naïve cells were not increased as compared to healthy controls (Figure 1B left graph). Regarding CD8+ T cells, an expansion was found only of effector T cells, whereas numbers of naïve and memory cells were unchanged as compared to healthy controls, in line with our previous findings 16.

Subset analysis of the CD4+ and CD8+ T cell compartment in patients with indolent lymphoma revealed a similar increase in effector cells, CD4+ as well as CD8+ (Figure 1C), whereas numbers of naïve cells were decreased (data not shown). Numbers of memory CD4+ and CD8+ T cells were equal. No significant increase in effector T cells was found in patients with aggressive lymphoma or multiple myeloma (Figure 1C). Thus, a common expansion of effector T cells, CD4+ as well as CD8+, was found in patients with CLL and indolent B cell lymphoma; however, the increase was more pronounced in CLL.

Effector T cell expansion is related to latent CMV infection

In line with our previous findings 16, in CLL, the increase in effector T cell numbers (CD4+ as well as CD8+) as compared to healthy controls was predominantly found in CMV-seropositive patients (Figure 2A). The significant increase in effector T cells in
Figure 1. Expansion of the T cell compartment mainly affects effector T cells in CLL but also in indolent lymphoma. (A) Representative dot plots of 6-color immunostaining of T cells in a healthy control. Cells were gated on the ‘lymphogate’ using forward and sideward scatter (FSC/SSC), CD3, and subsequently on CD4 (left panel) and CD8 (right panel). Within the CD4⁺-gate the following subsets were discerned CD45RA⁺CD28⁺ (naïve cells); CD45RA⁻CD28⁺ (memory cells) and CD45RA⁻/CD28⁻ (effector cells). Within the CD8⁺-gate the following subsets were discerned CD45RA⁺CD27⁺ (naïve cells); CD45RA⁻CD27⁺ (memory cells) and CD45RA⁻/CD27⁻ (effector cells). (B) Distribution of T cell subsets (as described in A) within the CD4⁺-gate (left graph) and CD8⁺-gate (right graph) in CLL patients. Bars represent mean ± standard error of mean (SEM). ** p < .005, *** p < .0005. (C) Absolute numbers of effector (CD45RA⁻/CD28⁻) CD4⁺ cells and effector (CD45RA⁻/CD27⁻) CD8⁺ cells in patients with B cell malignancies as indicated. Bars represent mean ± SEM. * p < .05, ** p < .005, *** p < .0005.
indolent lymphoma was also restricted to CMV-seropositive patients, whereas there was no significant difference between CMV-seropositive patients with aggressive lymphoma or multiple myeloma and healthy controls. The mechanism which drives this effector T cell expansion is not known. One hypothesis is that failure of components of the immune system due to the malignant disease increases the antigenic pressure of the latent infection. However, in our study cohort, none of the subjects had a positive CMV-PCR in the peripheral blood (data not shown). We also tested whether the number of effector T cells was related to CMV-serology. No significant relation was found between the height of CMV-antibody titers and effector T cell numbers (Figure 2B).

We found an increased number of Tregs (CD4+CD25hiCD127low) in CLL patients. However, there were no differences in Treg numbers between patients with aggressive and indolent lymphoma or multiple myeloma and healthy controls. Furthermore the expansion of Tregs in CLL patients was not related to CMV-serostatus (Supplemental Figure 1).

**Figure 2. Expansion of effector T cells is related to CMV-infection** (A) Absolute numbers of effector (CD45RA+/−CD28) CD4+ cells and effector (CD45RA+/−CD27) CD8+ cells in CMV-seronegative (CMV−) and CMV-seropositive (CMV+) patients with B cell malignancies as indicated. In CMV-seronegative healthy controls, patients with indolent and aggressive lymphoma and multiple myeloma frequencies of effector (CD45RA+/−CD28) CD4+ cells were extremely low. Bars represent mean ± SEM. * p < .05, ** p < .005. (B) Absolute numbers of effector (CD45RA+/−CD28) CD4+ cells and effector (CD45RA+/−CD27) CD8+ cells in CMV-seropositive patients and healthy controls (pooled), divided according to height of anti-CMV IgG titre (low: < 250 U/l; high: > 250 U/l).
Effector T cells in CLL and indolent lymphoma display decreased PD-1 expression

Chronic viral infections, including CMV, are often characterized by varying degrees of functional impairment of virus-directed T cell responses, which may explain the inability of the host to eliminate the persisting pathogen. A (unique) feature of these exhausted T cells is the expression of the inhibitory activation-associated B7-CD28-family member programmed death-1 (PD-1). Upon interaction with its ligands PD-L1 (inducibly expressed on hematopoietic and parenchymal cells) and PD-L2 (predominantly expressed on macrophages and dendritic cells), signaling via the T cell receptor (TCR) is inhibited (reviewed in 25,26). Blocking interaction of PD-1 with its ligands reverses T cell anergy and results in viral clearance in the experimental setting 24. In contrast to aggressive lymphomas, PD-1 is expressed on low-grade lymphomas including subtypes of follicular lymphoma, SLL and CLL cells and is increased upon CD40-stimulation 27. We hypothesized that the presence of high numbers of CLL cells expressing PD-1 may lead to competition for

Figure 3. Decreased expression level of PD-1 on effector T cells in patients with CLL and indolent B cell lymphoma/ low-grade follicular lymphoma (A) Percentage of cells expressing PD-1 within subsets of CD4+ T cells and CD8+ T cells as described in figure 1A in controls (n=12), patients with CLL (n=14) and patients with follicular lymphoma grade I-II (FL, n=8). Bars represent mean ± SEM. * p < .05, ** p < .005. (B) Percentage of PD-1 positive cells within CD3+CD4+CD45RA+/-CD28- subset (left graph) or within CD3+CD8+CD45RA+/-CD27- subset (right graph) set out against absolute numbers of cells (x 10^9/L) within these gates. Correlation analyzed by assessment of Spearman’s rank correlation coefficient.

Effector T cells in CLL and indolent lymphoma display decreased PD-1 expression

Chronic viral infections, including CMV, are often characterized by varying degrees of functional impairment of virus-directed T cell responses, which may explain the inability of the host to eliminate the persisting pathogen 22,23. A (unique) feature of these exhausted T cells is the expression of the inhibitory activation-associated B7-CD28-family member programmed death-1 (PD-1) 24. Upon interaction with its ligands PD-L1 (inducibly expressed on hematopoietic and parenchymal cells) and PD-L2 (predominantly expressed on macrophages and dendritic cells), signaling via the T cell receptor (TCR) is inhibited (reviewed in 25,26). Blocking interaction of PD-1 with its ligands reverses T cell anergy and results in viral clearance in the experimental setting 24. In contrast to aggressive lymphomas, PD-1 is expressed on low-grade lymphomas including subtypes of follicular lymphoma, SLL and CLL cells and is increased upon CD40-stimulation 27. We hypothesized that the presence of high numbers of CLL cells expressing PD-1 may lead to competition for
PD-1-ligands and subsequent accumulation of exhausted PD-1 expressing effector T cells. Surprisingly, we found a significant decrease in percentage of effector T cells expressing PD-1 in patients with CLL and follicular lymphoma when compared to healthy controls (Figure 3A). The difference was found irrespective of CMV-serostatus, but assessment of PD-1 expression was hampered by the very low frequency especially of CD4<sup>+</sup> effector T cells in CMV-seronegative subjects (data not shown). Interestingly, the level of PD-1 expression inversely correlated with the size of the effector CD4<sup>+</sup> as well as CD8<sup>+</sup> T cell compartment, supporting in vivo relevance of the in vitro data (Figure 3B).

**Extent of PD-1 expression on effector T cells is related to the number of circulating clonal B cells in patients with CLL**

Although PD-1 expression on effector T cells was not related to RAI stage (data not shown), the expression was inversely correlated to numbers of CD19<sup>+</sup> cells in CMV-

![Figure 4](image_url)

**Figure 4. Level of PD-1 expression is related to number of circulating CLL cells** (A) Percentage of PD-1 positive cells within CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>+</sup>-CD28<sup>-</sup> subset or within CD3<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup>-CD27<sup>-</sup> subset set out against absolute numbers of CD19<sup>+</sup> cells (x 10<sup>9</sup>/ L) in CMV-seropositive CLL patients (n=15). (B) Number of circulating CLL cells (left panel), percentage PD-1 expressing effector (CD45RA<sup>+</sup>-CD28<sup>-</sup>) CD4<sup>+</sup> cells and effector (CD45RA<sup>+</sup>-CD27<sup>-</sup>) CD8<sup>+</sup> cells (middle panel), and number of effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells (right panel) in a 62-year old male patient at diagnosis, after chlorambucil treatment (chl), at relapse, immediately and 1 year after completion of fludarabine/ cyclophosphamide (FC) treatment.
seropositive CLL patients (Figure 4A). This may indicate a causal relation between the abundance of malignant B cells and PD-1 expression on effector T cells. Support for this hypothesis comes from data on numbers of circulating CLL cells, effector T cells and PD-1 expression on these cells, in serial blood samples derived from a 62-year old CLL patient (Rai stage III, del13q, mutated IgVH, bulky disease) at diagnosis, after chlorambucil treatment, at disease relapse and immediately after and 1 year after completion of fludarabine / cyclophosphamide treatment respectively. Upon treatment of the disease, numbers of CLL cells decrease, PD-1 expression on CD8+ T cells increases and effector CD8+ T cell numbers decrease, whereas at disease relapse (with an increasing number of circulating CLL cells), PD-1 expression decreases and effector CD8+ T cell numbers increase (Figure 4B). As CD4+ effector T cells were present at a very low frequency, PD-1 expression on these cells could not be measured reliably.

**Upregulation of PD-1 expression upon T cell activation is impaired in the presence of malignant as well as normal B Cells**

Next, we addressed the possible mechanism behind impaired PD-1 expression on effector T cells in patients with CLL and indolent lymphoma. First, we assessed the proportion of activated T cells in all disease categories. Activation was defined by co-expression of CD38 and HLA-DR on CD4+ and CD8+ T cells. An increased proportion of T cells (CD4+ as well as CD8+) was activated in patients with CLL and B cell lymphomas (both indolent and aggressive lymphoma; supplemental Figure 2B).

Next we investigated whether impaired PD-1 expression results from interaction with malignant B cells. In a time course experiment, robust upregulation of PD-1 on CD4+ and CD8+ T cells was seen after 48 hours of stimulation with anti-CD3 antibodies (Figure 5A and data not shown). As shown in Figure 5B, PD-1 expression on CD4+ T cells after 48 hours of activation by anti-CD3 stimulation was hampered when activation occurred in the presence of CLL cells, whereas PD-1 expression was not affected by the presence of PBMCs depleted of CD19+ cells. PD-1 expression on CD8+ cells was similarly hampered in the presence of CLL cells (Figure 5C). To test whether impaired expression of PD-1 in the presence of CLL cells is attributable to features related to malignancy, or rather to a common B cell feature, PD-1 upregulation was studied in the presence of healthy B cells. Interestingly, PD-1 expression upon activation of T cells was also hampered in the presence of healthy B cells (Figure 5B-C). These data suggest that in patients with indolent B cell lymphoma, including CLL, the abundance of B cells interferes with normal T cell activation.

**Discussion**

In this study we found that, although an increase of total CD4+ and CD8+ T cells is confined to patients with CLL, an expansion of effector T cells (CD4+ as well as CD8+),
exists in all patients with indolent B cell lymphoproliferative diseases. On these cells we found a decreased expression of PD-1 compared to the expression on effector T cells derived from healthy controls. Furthermore, the extent of PD-1 expression on effector T cells correlated inversely with absolute numbers of these cells, which may relate to the mechanism behind the expansion.
T cell numbers, and specifically effector T cell numbers, were not significantly different from controls in patients with aggressive lymphoma. An explanation could be that at time of diagnosis (or at the start of treatment), the disease usually has been present for a relatively longer time period in case of indolent lymphoma or CLL when compared to more aggressive diseases, allowing for more extensive interaction of the malignant cells with the immune system. An alternative explanation could be a difference in the quantity and / or quality of interactions between malignant B cells and T cells.

The relevance of the interaction of T cells with malignant B cells is supported by recent studies in T cells of untreated CLL patients, in which altered expression was found of genes, mainly involved in cell differentiation in CD4+ cells, and in cytoskeleton formation, vesicle trafficking, and cytotoxicity in CD8+ cells. Direct contact of both patient-derived and healthy control T cells with CLL cells resulted in defective immunological synapse formation. Interestingly, similar findings were reported in studies on T cells of patients with follicular lymphoma and DLBCL. These changes in peripheral T cells were only found in patients in leukemic phase of the disease. In addition, Christopoulos et al recently showed reduced expression of numerous genes involved in the T cell receptor (TCR) signalling cascade upon TCR and CD28 co-stimulation of CD4+ T cells in very early stage CLL (Rai stage 0). A similar pattern was seen upon co-culture with follicular lymphoma cells in healthy donor derived CD4+ T cells, suggesting that the consequences of the pathologic interaction between malignant B cells and T cells in lymph nodes, persists after re-entry into the circulation.

We now describe that PD-1 upregulation on effector T cells is hampered upon interaction with malignant CLL cells, but notably also in the presence of (large numbers of) normal B cells. As B cell depleted PBMCs did not affect PD-1 expression upon T cell activation, we conclude that a B cell feature, which is preserved in malignancy, influences PD-1 regulation.

Very little is known about the regulation of PD-1. In a recent study, PD-1 gene regulation was found to occur in part via the recruitment of NFATc1 to a novel regulatory element of the PD-1 gene. In a mouse model, the addition of TLR-9 receptor ligand CpG oligonucleotide (ODN) to an intra-tumoral peptide vaccine resulted in decreased PD-1 expression on the ensuing antigen-specific CD8+ T cell clone. This clone was expanded and exhibited enhanced ex vivo function. Also, antigen-independent upregulation of PD-1 on T cells by the common γ chain cytokines IL-2, IL-7, IL-15 and IL-21 has been reported. However, as long as the mechanism of PD-1 regulation upon T cell activation is not known, it is difficult to reason how B cells could influence this process. An appropriate next step to elucidate the role of B cells would be to analyze whether direct cell contact or rather a B cell derived soluble factor is necessary for this effect.

The important role of PD-1/PD-1-ligand interactions in maintaining peripheral tolerance is increasingly appreciated. First, although these interactions may prohibit viral clearance in chronic viral infections and blockade of the interactions greatly enhanced viral control in a LCMV infected mouse model, PD-L1 deficient mice died of...
to the viral infection\textsuperscript{24}. Our finding of decreased PD-1 expression on effector T cells would imply intact cytotoxic functionality. Indeed, we have previously found that CMV-specific effector CD8\textsuperscript{+} T cells derived from CLL patients displayed robust cytotoxic activity, even without ex vivo restimulation\textsuperscript{35}. Furthermore, CMV reactivation in CLL patients is not frequently encountered, with the exception of patients treated with T cell depleting agents like alemtuzumab or fludarabine. Secondly, functional PD-1 deficient mice exhibit clinical signs of autoimmunity\textsuperscript{36} and in humans, polymorphisms within the PD-1 gene have been linked to adverse outcome in multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus\textsuperscript{37-39}. Autoimmune phenomena are frequently encountered in CLL, occurring in 10\% to 25\% of patients at some time during the disease course. Autoimmunity in CLL predominantly targets blood constituents, and autoimmune hemolytic anemia (AIHA) is most commonly seen followed in incidence by auto-immune thrombocytopenia. These phenomena are antibody mediated, but probably also involve auto reactive T cells and regulatory T cells (reviewed by\textsuperscript{40} and\textsuperscript{2}). Furthermore, also non-hematological autoimmunity occurs, although to a much lesser extent. The role of PD-1 expression on auto reactive T cells in the pathogenesis of autoimmunity related to CLL or lymphoma has not been investigated.

Although we found the expansion of effector T cells was largely confined to CMV-seropositive patients, we expect our findings may apply to antigen-primed effector T cells more in general. The immunophenotyping strategy we used was predetermined to identify T cell subsets with specificity for CMV; especially CD4\textsuperscript{+}CD45RA\textsuperscript{+/-}CD28\textsuperscript{-} T cells are highly specific for latent phase CMV infection\textsuperscript{19}. Moreover, CMV is a highly immunodominant virus and CMV-specific T cells comprise an increasingly large proportion of the T cell compartment with increasing age, even in healthy individuals\textsuperscript{41}.

In conclusion, we have identified impaired PD-1 expression as a possible mediator in the expansion of CD4\textsuperscript{+} and CD8\textsuperscript{+} effector T cells in CLL and other indolent lymphomas. As both immuno-incompetence and auto-immunity cause considerable morbidity in patients with CLL and indolent B cell malignancies, our data provide an incentive for further research into the role of PD-1 in the interaction between malignant B cells and the immune system.

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Reference List


Supplemental Figure 1. Increased number of Tregs in CLL (A) Absolute number of Tregs (CD3+CD4+CD25hiCD127low) in patients with CLL, indolent lymphoma, aggressive lymphoma and multiple myeloma compared to healthy controls. Bars represent mean ± standard error of mean (SEM), *** p < .0005. (B) Absolute number of Tregs (CD3+CD4+CD25hiCD127low) in CMV-seronegative (CMV-) versus CMV-seropositive (CMV+) patients with CLL, indolent lymphoma, aggressive lymphoma and multiple myeloma compared to healthy controls. Bars represent mean ± SEM.

Supplemental Figure 2. Percentage of activated T cells in patients with B cell malignancies (A) Representative plots of CD3+CD4+ gated samples characterized by an increasing level of activation as assessed by co expression of CD38 and HLA-DR. (B) Percentage of CD4+ and CD8+ T cells co expressing CD38 and HLA-DR in patients with CLL, indolent lymphoma, aggressive lymphoma and multiple myeloma compared to healthy controls. Bars represent mean ± SEM, * p < .05, ** p < .005, *** p < .0005.
Supplemental Table 1. Patient characteristics.

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Indolent lymphoma: follicular lymphoma grade I-II (8), hairy cell leukemia (2), marginal zone lymphoma (3), low-grade lymphoma n.o.s. (2); aggressive lymphoma: follicular lymphoma grade III (3), diffuse large B cell lymphoma (10). Age in years; mean ± standard deviation. There were no significant differences in age between different disease categories, for total groups as well as upon subdivision in CMV-seropositive and -negative patients.