Chemotherapy-induced augmentation of T cells expressing inhibitory receptors is reversed by treatment with lenalidomide in chronic lymphocytic leukemia

While T-cell dysfunction occurring alongside chronic lymphocytic leukemia (CLL) is well documented (reviewed by Pleyer *et al.*[1] and Hamblin *et al.*[2]), it was recently reported that also T-cell exhaustion is associated with CLL, reflected in the presence of $PD\text{-}1^+$ CLL T cells and higher levels of PD-L1 on the surface of CLL cells.[3-5] It was suggested that T-cell exhaustion might impede proper synapse formation of CLL-specific T cells and, hence, impair a cytolytic attack.[6] Therefore, therapeutic interference with T-cell exhaustion in combination with chemotherapy has been proposed as a promising treatment option, aimed at restoring a functional cytotoxic T-cell pool to eradicate the malignant cells.

In this study, we assessed the level of T cells expressing the inhibitory surface markers PD-1 (programmed death receptor-1, CD279), 2B4 (natural killer cell receptor 2B4, CD244) and CD57 (human natural killer-1), which were previously associated with T-cell exhaustion and senescence as they counteract intracellular activation signals triggered upon recognition of target cells.[7,8] CD57 expression is thereby associated to impaired proliferation of $CD8^+$ but also $CD4^+$ T cells,[9] and PD-1 engagement counteracts T-cell receptor (TCR)/CD3-induced T-cell proliferation and cytokine production, while expression of 2B4 on $CD8^+$ T cells correlates with PD-1 expression.[10] We evaluated the expression of PD-1, 2B4 and CD57 in peripheral blood T cells from 131 unselected CLL patients and compared it to the expression levels of 14 age-matched healthy donors (median age 68.8 or 66.1 years, respectively) by flow cytometric analysis of whole blood samples. From the analyzed CLL patients, 82 were chemonaive (no prior CLL-related treatment) and 49 had received therapy, 10 of whom had been treated with a regimen including the immunomodulatory drug lenalidomide.[11] *Online Supplementary Tables S1* and *S2* list detailed patients' characteristics and treatment regimes. As summarized in Table 1, we observed that within the $CD4^+$ T-cell subset of chemonaive patients, the percentage of cells expressing the analyzed individual markers (i.e. PD-1, 2B4, CD57) or expressing two or all three inhibitory receptors was comparable to age-matched healthy controls. While all analyzed markers were slightly increased within the $CD8^+$ T-cell population, only the expansion of $CD8^+$ T cells expressing CD57 alone or in combination reached statistical significance. Interestingly, we noted a significant increase in $CD8^+$ T cells expressing PD-1 either alone or in combination with 2B4 and/or CD57 in chemonaive patients with advanced RAI stage and high leukocyte counts (*Online Supplementary Tables S3*). While RAI stage also had a significant impact on the frequency of $CD4^+$ $PD\text{-}1^+$ T cells, none of the other analyzed prognostic markers, i.e. CD38, Zap-70, IgVH mutations or cytogenetic aberrations in the malignant B cells was associated with PD-1, 2B4 or CD57 expression on $CD4^+$ or $CD8^+$ T cells. We found a significantly higher expression of 2B4 and CD57, but not PD-1 in $CD4^+$ and $CD8^+$ T cells of patients seropositive for human cytomegalovirus (HCMV) (*data not shown*). While data on the HCMV serostatus of the healthy donors in this study were not available, HCMV seroprevalence in healthy elderly has been suggested to be 50-80%,[12] which is comparable to or even higher than the rate of seropositivity in our CLL cohort (53%). We, therefore, suppose that the impact of HCMV infection is comparable between healthy controls and CLL patients.

Furthermore, we observed that all of the T-cell subsets except $CD8^+$ T cells expressing PD-1 alone or in combination were increased in samples from chemotherapy-treated patients (Table 1). Conversely, these T-cell subsets were significantly reduced in samples from patients receiving lenalidomide combinational treatment, leading to a reversion of T-cell numbers to levels found in-between healthy controls and chemonaive CLL patients. To further elucidate the functional exhaustion of T-cell subsets expressing the respective inhibitory receptors, we tested their proliferative capacity to T-cell receptor-dependent stimulation *in vitro*. Thereby, we found that $CD57^+$ as well as $2B4^+$ T cells exhibited the most distinct reduction of proliferative potential upon CD3/CD28 stimulation (Figure 1). Interestingly, PD-1 expression has a different impact on proliferating $CD4^+$ or $CD8^+$ T cells. While PD-

Table 1. Percentage of $CD4^+$ and $CD8^+$ T cells of CLL patients or healthy controls expressing single or multiple exhaustion markers.

		1. Healthy controls (n=14)	2. Chemonaive CLL (n=82)	*P* (1 *vs.* 2)	3. Non-lenalidomide treated CLL (n=39)	*P* (2 *vs.* 3)	4. Lenalidomide treated CLL (n=10)	*P* (2 *vs.* 4)	*P* (3 *vs.* 4)
$CD4^+$	$PD\text{-}1^+$	40.5 %±10.6	42.1 %±15.7	n.s.	64.0 %±14.7	<0.001	55.5 %±14.9	0.028	0.013
	$2B4^+$	14.5 %±22.1	12.9 %±14.4	n.s.	20.4 %±19.9	0.017	13.4 %±17.0	n.s.	0.035
	$CD57^+$	13.7 %±19.4	14.5 %±12.9	n.s.	25.0 %±16.1	<0.001	14.9 %±17.1	n.s.	0.002
	$PD\text{-}1^+$ $2B4^+$	8.6 %±10.7	9.2 %±10.8	n.s.	13.9 %±13.4	0.014	9.7 %±14.5	n.s.	0.042
	$PD\text{-}1^+$ $CD57^+$	7.1 %±8.7	11.8 %±10.9	0.015	18.0 %±12.2	0.001	10.5 %±14.2	n.s.	<0.001
	$2B4^+$ $CD57^+$	11.3 %±19.5	9.4 %±12.7	n.s.	13.9 %±15.6	0.052	10.1 %±15.7	n.s.	0.047
	$PD\text{-}1^+$ $2B4^+$ $CD57^+$	5.9 %±9.6	6.4 %±9.2	n.s.	8.8 %±9.4	0.036	6.6 %±12.8	n.s.	0.009
$CD8^+$	$PD\text{-}1^+$	47.3 %±15.9	52.0 %±16.6	n.s.	52.4 %±19.9	n.s.	49.5 %±18.1	n.s.	n.s.
	$2B4^+$	65.6 %±22.3	76.7 %±15.8	0.055	82.3 %±16.2	0.021	66.9 %±28.7	n.s.	0.039
	$CD57^+$	36.6 %±18.1	52.3 %±19.2	0.008	64.4 %±17.0	0.001	47.5 %±23.4	n.s.	0.005
	$PD\text{-}1^+$ $2B4^+$	39.3 %±16.8	46.2 %±15.3	n.s.	46.4 %±18.0	n.s.	36.9 %±16.2	n.s.	n.s.
	$PD\text{-}1^+$ $CD57^+$	19.6 %±11.0	31.7 %±14.2	0.003	36.1 %±16.5	n.s.	24.4 %±11.5	n.s.	0.040
	$2B4^+$ $CD57^+$	36.9 %±18.6	53.1 %±19.2	0.004	64.0 %±17.8	0.003	46.9 %±25.3	n.s.	0.008
	$PD\text{-}1^+$ $2B4^+$ $CD57^+$	18.8 %±10.9	29.7 %±14.0	0.006	33.8 %±15.8	n.s.	21.5 %±12.1	n.s.	0.023

Percentage of positive cells ± standard deviation; n.s. not significant.

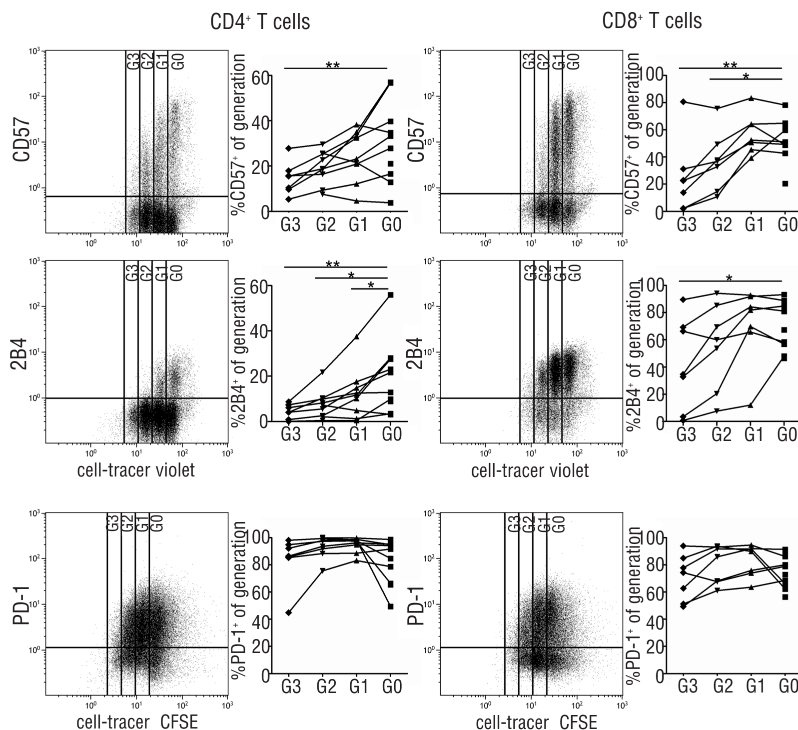

Figure 1. Proliferation of T-cell subsets expressing inhibitory receptors. PBMCs from CLL patients were stained with CellTracer CFSE or violet (Life Technologies, CA, USA) and incubated for three days in the presence of activating CD3/CD28 antibodies *in vitro*. Proliferation was determined by measuring dilution of CellTracer using flow cytometry. Representative plots are shown for CD4+ (left) and CD8+ (right) subset. The graphs display the results of independent experiments performed on samples from 7 different patients (4 chemonaive, 3 previously treated), showing the percentage of T cells expressing the respective inhibitory receptor within each cell division. On the x-axis, the number of cell divisions according to CellTracer dilution is indicated (G0: undivided; G1-3: 1-3 cell divisions; significance levels: **P*<0.05, ***P*<0.01).

1+ CD4+ T cells showed no impaired proliferation, PD-1+ CD8+ T cells tended to be reduced in their proliferative potential, albeit not significantly.

Our results are consistent with recent studies which reported the association of the PD-1/PD-L1 axis and T-cell exhaustion with T-cell dysfunction and progressive disease in CLL.[3-5] In our study, we confirm the phenotypic marker profile of exhausted T cells in CLL and beyond that show that an expanded subset of low-proliferative, CD57+ CD8+ T cells is observed in CLL patients, which was further increased upon treatment of patients with chemotherapeutic regimen.

We showed that T-cell dysfunction is not solely reflected by increased PD-1 levels, which were comparable in CLL patients and healthy donors in our study samples, but rather by expression of other inhibitory receptors such as 2B4 and CD57, which seem to be more closely related to functional inhibition of CLL T cells. This is particularly important, as we show that CD57+ as well as 2B4+, but not PD-1+ single positive T cells are significantly impaired in their proliferative response to TCR-dependent stimulation in vitro and might contribute to paralyzed immune surveillance towards the CLL clone. While patients receiving chemotherapy showed an increase in T-cell subsets expressing inhibitory receptors, combined treatment with lenalidomide induced a reduction of these T-cell compartments and, thus, most likely counteracts therapy-induced accumulation of silenced T cells. We have already shown a substantial shift in T-cell subset composition towards an antigen-experienced Th1 memory phenotype after fludarabine treatment.[13] Reducing expression of inhibitory signaling molecules by immunomodulatory drugs may, therefore, represent an important factor for enduring tumor surveillance and contribute to maintaining long-term remissions. Our results corroborate recent findings on the improvement of T-cell immune synapse formation upon lenalidomide treatment *in vitro* and *in vivo*[16,14] which also suggests lenalidomide-induced reversion of T-cell functionality.

In summary, our results show that chemotherapy may hamper the patients' T-cell-mediated immune surveillance while a combined treatment with lenalidomide may contribute to establishment of a more robust tumor control. Future work will be necessary to assess CLL specificity of T cells expressing one or more of the inhibitory receptors analyzed in our study and to test whether lenalidomide treatment can improve cytotoxicity of CLL-specific T cells, in particular in combination with immune therapeutic antibodies specific for PD-1 or CTLA4, which also counteracts TCR/CD3 mediated signaling pathways.[15]

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