Circulating classical CD14^{++}CD16^{-} monocytes predict shorter time to initial treatment in chronic lymphocytic leukemia patients: Differential effects of immune chemotherapy on monocyte-related membrane and soluble forms of CD163

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Received March 19, 2015; Accepted May 29, 2015

DOI: 10.3892/or.2015.4088

Abstract. Three main monocyte subsets: classical CD14^{++}CD16^{-}, intermediate CD14^{++}CD16^{+} and non-classical CD14^{+}CD16^{++}, differentially regulate tumor growth and survival. Thereby, in the present study we aimed to determine the role of distinct monocyte subsets in the prognostication of chronic lymphocytic leukemia (CLL). Moreover, we set out to analyze the effects of standard immune chemotherapy on different monocyte subsets and levels of membrane-associated and soluble forms of CD163, a monocyte/macrophage-related immunomodulatory protein. We demonstrated that the number of peripheral blood classical CD14^{++}CD16^{-} monocytes assessed at the time of diagnosis was negatively correlated with lymphocytosis and was decreased in the CLL patients who required immediate treatment as opposed to patients who qualified to 'watch and wait' strategy. Notably, lower baseline levels of classical CD14^{++}CD16^{-} monocytes in CLL patients who were qualified for 'watch and wait' therapy were associated with shorter time to initial treatment. Notably, therapy with rituximab, cyclophosphamide and fludarabine resulted in a significant reduction in the number of non-classical CD14^{+}CD16^{++} monocytes and soluble form of CD163 but upregulation of membrane-associated monocyte CD163. Our data indicate that distinct monocyte subsets and two forms of CD163 are differentially modulated by both CLL and immune chemotherapy. Moreover, we proposed that quantification of classical monocytes at the time of diagnosis contributes to better prognostication of CLL patients.

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Key words: monocytes, chronic lymphocytic leukemia, prognostication, CD163, sCD163

Introduction

The past decade has brought significant advances in our understanding of the pathogenesis of chronic lymphocytic leukemia (CLL), accompanied by a significant increase in the number and range of treatment options. However, despite these opportunities, the cure for CLL is still unavailable (1). Both intrinsic defects affecting the regulation of programmed cell death (apoptosis) and an altered, survival-stimulating microenvironment are considered to be the major pathogenic factors for CLL (1-3). Thus, it is now clear that the expansion of the malignant clone depends not only on its intrinsic characteristics (such as the expression of anti-apoptotic molecules), yet also on delivery of stimulating signals from stromal cells infiltrating neoplastic cells. This tumor microenvironment is constituted mostly by mononuclear phagocytes, namely monocytes and monocyte-derived macrophages. Monocytes are a heterogeneous population that comprises cells at different maturation levels and with different immunomodulatory potential (4,5). As demonstrated by numerous studies including ours, monocytes can be divided into 3 distinct subsets defined by differential expression of CD14 and CD16 molecules (6-8). In healthy conditions, the majority of monocytes are referred to as classical monocytes that can be delineated by the CD14^{++}CD16^{-} phenotype. These classical monocytes play largely phagocytic and antitumor roles. Importantly, this monocyte subset gives rise to so called M1 macrophages that, in some contrast to M2 macrophages, exert numerous potent antitumor effects (9,10). The other two subsets of monocytes are named intermediate CD14^{++}CD16^{+} and non-classical CD14^{+}CD16^{++} monocytes (6). Notably, we and others demonstrated that these two monocyte subsets

with CD16 expression were significantly expanded in patients with numerous inflammatory and/or malignant disorders such as (but not only) asthma, HIV, atherosclerosis and breast cancer (7,8,11-13). Moreover, we recently proved that non-classical CD14^{+}CD16^{++} monocytes are capable of secreting significantly higher TNF-α levels than classical CD14^{++}CD16^{-} and intermediate CD14^{++}CD16^{+} monocytes (8). Notably, TNF-α promotes the proliferation of leukemic B cells and plays an important role in the progression of B-CLL (14). Indeed, circulating monocytes derived from CLL patients have been recently shown to play an important role in leukemic cell survival (15). In addition, monocytes from CLL patients were shown to differentiate *in vitro* into large, adherent cells capable of protecting leukemic cells from spontaneous and drug-induced apoptosis (16,17). Notably, higher numbers of non-classical CD14^{+}CD16^{++} have been recently detected in CLL patients. This anomaly was observed as more prominent in CLL cases with adverse genomic aberrations (18). However, to date, it is still unknown whether enhanced numbers of non-classical or classical monocytes in CLL patients could be related to an unfavorable prognosis. To date, it also remains unclear whether immune chemotherapy is capable of affecting the numbers of these pro-inflammatory monocyte subsets.

Monocytes/macrophages are important for tumor cell migration, invasion and metastasis (19). Fusion between tumor-associated macrophages (TAMs), particularly M2 type, which express CD163 and cancer cells causes hybrids with an increased metastatic potential. Indeed, recent studies have proven the significance of CD163-expressing M2 TAMs in the growth of tumor cells (20). Enhanced levels of M2 TAMs were reportedly associated with a worse prognosis in patients with numerous malignant tumors, including lymphomas (20). To date, however, the role of CD163 was not investigated in the context of CLL and no evaluation of CD163 expression was performed in patients subjected to standard immune chemotherapy. Knowing that CD163 can be shed from monocytes/macrophages into the bloodstream as a soluble form (referred to as sCD163), we wished to investigate here whether soluble CD163 can be associated with CLL stage or response to therapy. CD163 is a monocyte/macrophage-restricted receptor involved in the clearance of hemoglobin-haptoglobin complexes and regulation of inflammatory processes (21-23). CD163 is widely considered as a marker of low-grade inflammation that is enhanced in such disorders as sepsis, coronary atherosclerosis and myeloid leukemia (24,25). Elevation of sCD163 can reflect the status of inappropriate activation of macrophages. In the present study, we performed both cross-sectional and time-course analysis of different monocyte subsets in newly diagnosed CLL patients who were further subjected to either 'watch and wait' strategy or immune chemotherapy. We demonstrated that newly diagnosed CLL patients who qualified for the 'wait and watch' strategy that presented with higher absolute numbers of classical monocytes had a longer time to treatment. Notably, we found that CLL patients who had progressive disease at diagnosis and thus required immediate treatment had a lower baseline expression of CD163 as compared to these CLL patients who were qualified for the 'wait and watch' strategy. Notably, we showed that immune chemotherapy resulted in a significant enhancement of membrane-associated CD163 expression and a significant decrease in the pro-inflammatory non-classical CD14^{+}CD16^{++} monocytes and soluble CD163 levels.

Patients and methods

Patients. A total of 56 patients with newly diagnosed B lineage CLL were enrolled in the present study (Table I). Their median age at the time of sample collection was 64 years and the range was 55-69. There were 23 male and 35 female subjects. Patients with an acute or chronic infection, inflammatory processes and liver or kidney diseases (creatinine >2.0 mg/dl or creatinine clearance rate CrCl <60 ml/min), or those who received corticosteroids before the beginning of the treatment course, or whose co-morbid conditions possibly required systemic corticosteroids were excluded from the study.

The diagnosis of CLL was based on clinical observation, morphological composition of the peripheral blood (flow cytometry to identify immunophenotype of leukemic cells), bone marrow aspiration, trephine biopsy and computer tomography from neck to pelvis, according to the Hallek recommendation (26). Patient characteristics at the time of CLL diagnosis are summarized in Table I.

At the time of diagnosis, patients were staged according to the Rai staging system (27) as follows: stage 0 (7 cases), stage I (11 cases), stage II (24 cases), stage III (8 cases) and stage IV (6 cases). Thirty patients with stable disease did not receive chemotherapy and 24 patients with progressive disease (including 10 cases at stage 2, with massive lymphadenopathy confirmed by CT scan) were treated at the Department of Hematology of the Medical University of Bialystok from 2010 to 2014.

Patients who were qualified for treatment received FCR therapy: intravenous (iv) fludarabine 25 mg/m^2/day and cyclophosphamide 250 mg/m^2/day for 3 days, repeated every 28 days for a total of 6 cycles and rituximab 375 mg/m^2 by iv infusion on day 1 of the first cycle and 500 mg/m^2 iv on day 1 of the subsequent cycles with premedication (oral acetaminophen and an antihistamine). Prophylaxis for tumor lysis syndrome (including allopurinol) and prophylactic antimicrobials (sulfametoksazol + trimetoprim and acyclovir) were required for all our patients.

Disease status after the treatment was assessed by regular blood counts, clinical examination and computed tomography (CT) scans in all cases according to The International Workshop on Chronic Lymphocytic Leukemia (IWCLL) 2008. Cases of complete remission (CR) were confirmed by bone marrow biopsy and trephine biopsy.

For the patients who qualified for the 'wait and watch' strategy, the follow-up relied on the assessment of the evaluation of each patient every 3 months until progression.

Additionally, 21 age-matched healthy blood donors were enrolled in the study. There were 13 male and 8 female subjects (median age 66.6 years; range 60-79).

All samples were collected following informed consent and upon approval of the Ethics Committee of the Medical University of Bialystok, who approved the research protocol.

Methods

Flow cytometry. Freshly obtained EDTA-anti-coagulated whole blood samples were stained by means of mouse anti-

Table I. Clinical and molecular characteristics of the studied patients.

Characteristics	Data
Patients, n	56
Age, mean (range) in years	64 (55-69)
Rai stage, n (%)	
0	7 (12.5)
I	11 (19.64)
II	24 (41.85)
III	8 (14.28)
IV	6 (10.71)
WBC (x10^3), median (range)	80.9 (9.87-360.1)
Lymphocytes (x10^3), median (range)	58.930 (5.580-229.000)
Hemoglobin (mg/dl), median (range)	13 (6.6-16)
Platelets (x10^3), median (range)	160 (32-312)
β2m (g/l), median (range)	3.94 (2.094-5.357)
LDH (IU/l), median (range)	221 (7.3-477)
Creatinine level (mg/dl), median (range)	0.91 (0.55-239)
Hierarchical cytogenetic subgroup (%)	
Sole 13q deletion	30.1
Normal	34.0
Trisomy 12 (no 17p13 or 11q22 deletion)	10.7
11q22 deletion (no 17p13 deletion)	14.3
17p13 deletion	10.7
ZAP70 >30%	30.4
Patients at 'wait and watch' strategy, n	30
Patients qualified to immune chemotherapy, n	26
Response rate after treatment, n	
Patients with CR response	7
Patients with PR response	11
Patients with SD response	5

HGB, hemoglobin; β2m, β-2-microglobulin; LDH, lactate dehydrogenase; TB, trephine biopsy; BM, bone marrow; PLT, platelets count; WBC, white blood cells; CR, complete remission; PR, partial remission; SD, stable disease.

human monoclonal antibodies, according to stain and then lyse and wash protocol. Briefly, 100 μl of whole blood was stained with 5 μl of the following murine anti-human monoclonal antibodies: anti-CD16 FITC (clone, 3G8), anti-CD14 PE (clone, M5E2) and anti-HLA-DR APC (clone, TU36) (all from BD Biosciences) and incubated for 30 min at room temperature in the dark. Thereafter, erythrocytes were lysed by adding 2 ml of FACS lysing solution (BD), followed by incubation for 15 min in the dark. Cells were washed twice with cold phosphate-buffered saline (PBS) and fixed with Cell Fix (BD Biosciences). Fluorescence minus one (FMO) controls were used for setting compensation and to assure correct gating. Specimen acquisition was performed using a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences). The obtained data were analyzed with FlowJo version 7.6.5 software (Tree Star).

Cytokine assay. sCD163 levels were quantified by means of commercially available enzyme-linked immunosorbent assays (ELISA). Initially, all samples were diluted 1,000-fold with reagent diluent [1% BSA (Sigma-Aldrich) in PBS]. Next, the specimens were assayed using sCD163 DuoSet ELISA kit (R&D Systems), according to the manufacturer's instruction. Finally, the protein levels in the diluted specimens were calculated from a reference curve generated using reference standards (range 156-10,000 pg/ml), and the final results were obtained by an appropriate multiplication. The samples were analyzed with automated light absorbance reader (LEDETEC 96 system) at 450 nm wavelength, and the results were calculated by MicroWin 2000 software.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 6 (GraphPad software). Categorical variables were analyzed with the Fisher's exact test while continuous variables were analyzed with the Mann-Whitney U test. Survival curves were created by the application of the Kaplan-Meyer method, and the log-rank test was used to determine differences between survival proportions. Spearman correlation coefficient was used to determine correlations between variables. The differences were considered statistically significant at $p<0.05$. The results are presented as medians with interquartile range (IQR).

Results

First, we demonstrated that the quantitative distribution of different monocyte subsets (classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{++}$) in newly diagnosed CLL patients was comparable at different stages of the disease (for all $p>0.05$) (Fig. 1).

Notably, in the group of CLL patients under the 'wait and watch' strategy, the study established a significantly longer time to initial treatment in the patients with lower than median absolute counts of $CD14^{++}CD16^{-}$ compared to those with initially higher $CD14^{++}CD16^{-}$ amounts (Fig. 2A). There were no differences in the time to initial treatment in patients with higher amounts of non-classical and intermediate monocytes values compared to patients with lower counts of the above mentioned subsets (Fig. 2B and C).

Next, we correlated the numbers of each monocyte subset with a number of widely acknowledged disease progression parameters of prognosis and tumor load in CLL. Our analysis demonstrated that numbers of classical, intermediate and non-classical monocytes were positively correlated with the total absolute numbers of neutrophils and monocytes (Table II). In some contrast, we did not find significant correlations between C-protein levels and any subsets of monocytes (for all, $p>0.05$).

Furthermore, we demonstrated decreased levels of the CD163 membrane-associated monocyte expression in newly diagnosed CLL patients in advanced disease stages according to Rai classification (Fig. 3A). However, we found that these CLL patients who had progressive disease at the time of diag-

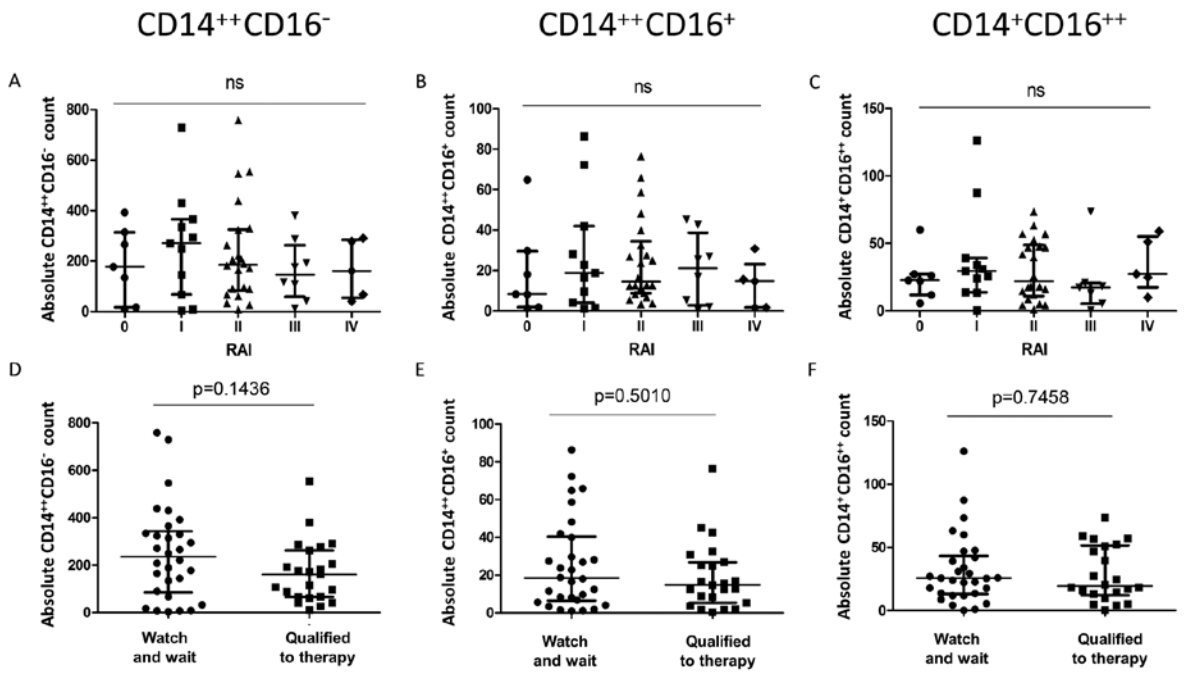

Figure 1. The summary of analyses of baseline absolute numbers of different monocyte subsets in (A-C) CLL patients at different stages of the disease and (D-F) patients with stable ('wait and watch' strategy) and progressive (classified to treatment) CLL. CLL, chronic lymphocytic leukemia.

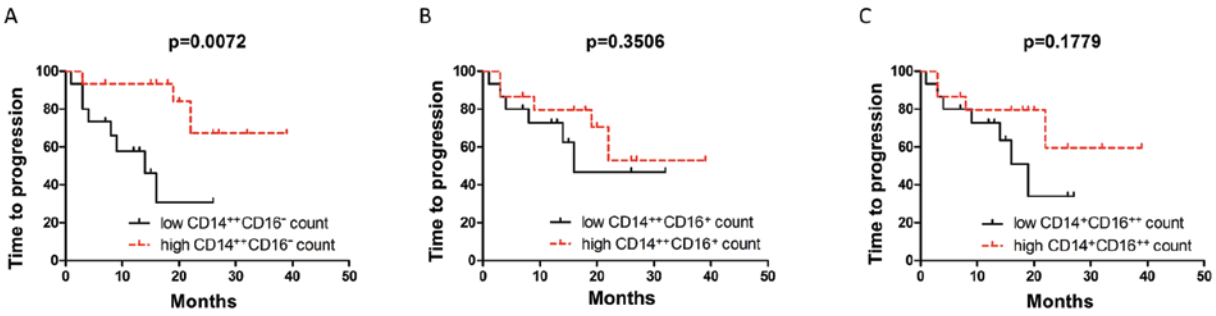

Figure 2. Kaplan-Meier estimates of CLL time to progression (time to initial treatment) for (A) classical monocyte absolute numbers (CD14^{++}CD16^{-}), (B) intermediate monocyte absolute numbers (CD14^{++}CD16^{+}) and (C) non-classical monocyte absolute count (CD14^{+}CD16^{++}). CLL, chronic lymphocytic leukemia.

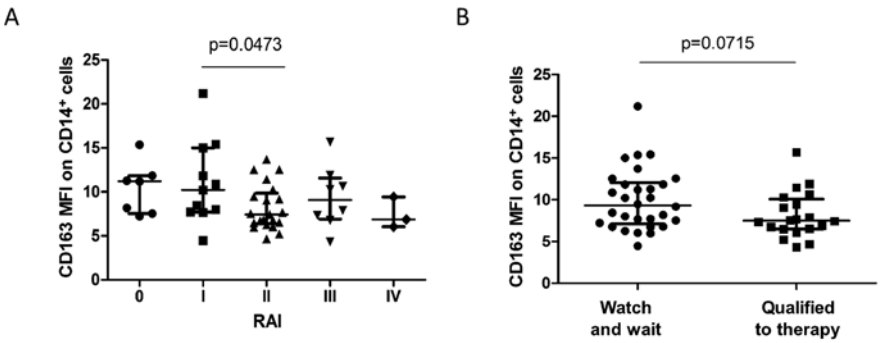

Figure 3. The summary of analyses of baseline CD163 mean fluorescence intensity on monocytes in (A) CLL patients at different stages of the disease and (B) patients with stable ('wait and watch' strategy) and progressive (classified to treatment) disease. CLL, chronic lymphocytic leukemia.

nosis and were qualified for immediate treatment tended to have lower CD163 expression as compared to those patients who, due to the stable character of their disease, were qualified for the 'wait and watch' strategy (Fig. 3B).

In some contrast to membrane-associated CD163, levels of the soluble form of CD163 (sCD163) were significantly increased in the CLL patients (622.3 μg/ml) (428.3-832.2) as compared to the healthy controls (386.6 μg/ml) (322.3-

Table II. Correlations between pre-treatment counts of monocyte subsets and selected hematological and biochemical parameters in 'wait and watch' patients, and patients qualified for immediate treatment.

	CLL patients subjected to 'watch and wait' strategy n=30						CLL patients qualified for immediate treatment n=22					
	Absolute $CD14^{++}CD16^{-}$ count		Absolute $CD14^{++}CD16^{+}$ count		Absolute $CD14^{+}CD16^{++}$ count		Absolute $CD14^{++}CD16^{-}$ count		Absolute $CD14^{++}CD16^{+}$ count		Absolute $CD14^{+}CD16^{++}$ count	
Parameters	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
WBC ($10^3/\mu l$)	0.06251	0.7428	0.1390	0.4637	0.1889	0.3175	-0.2006	0.3588	0.08696	0.6932	0.2388	0.2844
HGB (mg/dl)	-0.3072	0.0986	-0.2140	0.2562	-0.09707	0.6098	-0.01236	0.9554	0.1706	0.4365	0.08701	0.7002
PLT ($10^3/\mu l$)	0.2935	0.1223	0.1072	0.5799	0.1759	0.3613	0.1749	0.4246	0.2753	0.2036	0.07851	0.7284
Frequencies of lymphocytes (%)	-0.1454	0.4434	-0.1767	0.3502	-0.2544	0.1749	-0.2525	0.2451	0.04208	0.8488	-0.002264	0.9920
Absolute lymphocyte count ($10^3/\mu l$)	0.03270	0.8638	0.1261	0.5066	0.1591	0.4011	-0.2332	0.2842	0.09980	0.6505	0.2196	0.3260
Frequencies of neutrophils (%)	-0.003	0.9879	-0.1595	0.4174	-0.07237	0.7144	**0.4635**	**0.0259**	0.1817	0.4066	0.08095	0.7203
Absolute neutrophil count ($10^3/\mu l$)	**0.5610**	**0.0029**	**0.4776**	**0.0136**	**0.4605**	**0.0179**	0.4794	0.0602	0.3765	0.1506	**0.6714**	**0.0061**
Frequencies of monocytes (%)	**0.5034**	**0.0088**	0.3264	0.1036	**0.4188**	**0.0332**	**0.6773**	**0.0007**	0.3689	0.0998	0.3979	0.0740
Absolute monocyte count ($10^3/\mu l$)	**0.8549**	**<0.0001**	**0.7621**	**<0.0001**	**0.7474**	**<0.0001**	**0.4998**	**0.0152**	**0.5818**	**0.0036**	**0.8831**	**<0.0001**
IgG (mg/dl)	-0.2802	0.3538	-0.3297	0.2713	0.07143	0.8166	-0.3941	0.1309	**-0.5618**	**0.0235**	-0.1536	0.5848
LDH (IU/l)	0.009032	0.9636	0.1642	0.4037	0.2472	0.2048	0.03313	0.8866	0.1351	0.5593	0.07898	0.7407
β2m (g/l)	**0.6029**	**0.0134**	**0.6824**	**0.0036**	**0.6471**	**0.0067**	0.2527	0.4048	-0.07143	0.8166	-0.2363	0.4371
CRP (mg/l)	0.2119	0.2698	0.04218	0.8280	0.05649	0.7710	0.1261	0.5859	-0.08583	0.7115	0.003251	0.9888
TP (g/dl)	-0.08893	0.6939	-0.2118	0.3439	0.02322	0.9183	0.04425	0.8707	**-0.5133**	**0.0420**	-0.1209	0.6555
Creatinine (mg/dl)	-0.1010	0.6021	-0.1927	0.3166	0.09708	0.6164	-0.2429	0.2641	-0.04353	0.8437	-0.1787	0.4263
% of lymphocytic cells in smear BM	0.2484	0.3919	0.1473	0.6154	0.01099	0.9703	-0.4037	0.0966	**-0.6402**	**0.0042**	-0.1925	0.4591
% of lymphocytic cells in TB	0.5593	0.8113	0.3647	0.4483	0.3040	0.2475	-0.01879	0.9673	-0.3147	0.3415	-0.05592	0.3869

WBC, white blood cells; HGB, hemoglobin; PLT, platelet counts; IgG, immunoglobulin G; LDH, lactate dehydrogenase; β2m, β-2-microglobulin; CRP, C-protein; TP, total protein; BM, bone marrow; TB, trephine biopsy.

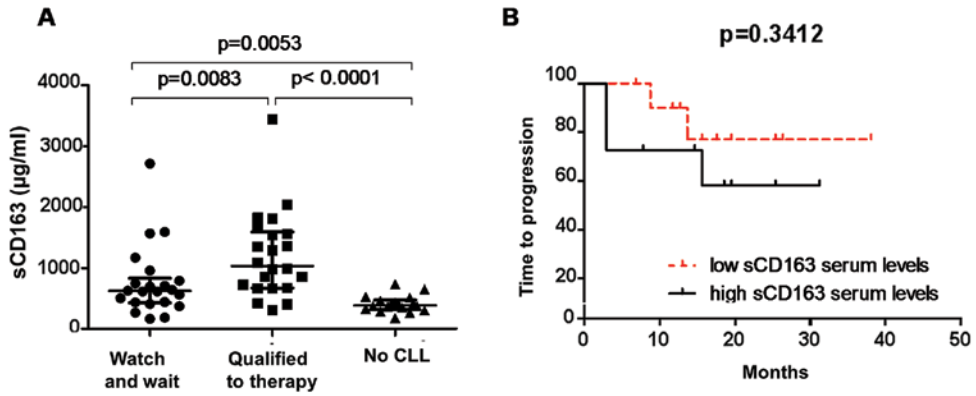

Figure 4. (A) Baseline sCD163 serum levels in CLL patients with stable ('wait and watch' strategy) and progressive (qualified to treatment) disease and normal donors. (B) Kaplan-Meier estimates for CLL time to progression (time initial treatment) for serum sCD163 levels. CLL, chronic lymphocytic leukemia.

Table III. Correlations between pre-treatment serum sCD163 levels and selected hematological and biochemical parameters in 'wait and watch' patients, and patients qualified for immediate treatment.

	sCD163					
	All patients n=44		Untreated 'watch and wait' n=22		Qualified for treatment n=22	
Parameters	r	p-value	r	p-value	r	p-value
WBC ($10^3/\mu$l)	**0.2895**	**0.0597**	0.05391	0.8165	0.3362	0.1261
HGB (mg/dl)	**-0.3907**	**0.0096**	-0.110^3	0.6342	**-0.4820**	**0.0231**
PLT ($10^3/\mu$l)	**-0.2763**	**0.0766**	-0.1667	0.4823	-0.3051	0.1674
Frequencies of lymphocytes (%)	-0.04357	0.7815	0.1504	0.5152	-0.1939	0.3873
Absolute lymphocyte count ($10^3/\mu$l)	0.2430	0.1164	0.01104	0.9621	0.3288	0.1351
Frequencies of neutrophils (%)	**-0.4101**	**0.0077**	-0.2855	0.2360	**-0.4822**	**0.0231**
Absolute neutrophils count ($10^3/\mu$l)	-0.2577	0.1350	-0.2405	0.3214	-0.2104	0.4340
Frequencies of monocytes (%)	-0.1567	0.3474	-0.1019	0.6874	-0.1558	0.5118
Absolute monocyte count ($10^3/\mu$l)	-0.01534	0.9222	-0.03769	0.8712	0.2326	0.2975
IgG (mg/dl)	0.1853	0.3972	-0.4048	0.3268	0.1769	0.5281
LDH (IU/l)	**0.3500**	**0.0290**	0.3872	0.1015	0.1686	0.4773
β2m (g/l)	**0.4884**	**0.0211**	-0.05455	0.8916	0.3217	0.3079
CRP (mg/l)	0.1187	0.4658	0.1974	0.4043	0.02260	0.9247
TP (g/dl)	0.1188	0.5471	-0.06630	0.8296	-0.01166	0.9671
Creatinine (mg/dl)	0.07183	0.6512	0.05156	0.8291	-0.1758	0.4339
% of lymphocytic cells in smear BM	**0.5096**	**0.0093**	0.3571	0.3894	**0.5267**	**0.0298**
% of lymphocytic cells in TB	**0.4498**	**0.0804**	-0.2571	0.6583	**0.9119**	**0.0002**
Absolute CD14^{++}CD16^{-} count ($10^3/\mu$l)	-0.2537	0.0966	-0.1457	0.5176	-0.07175	0.7510
Absolute CD14^{++}CD16^{+} count ($10^3/\mu$l)	-0.1649	0.2847	-0.1265	0.5748	-0.1480	0.5109
Absolute CD14^{+}CD16^{++} count ($10^3/\mu$l)	-0.05452	0.7284	-0.03954	0.8613	0.1462	0.5273
CD163 MFI on monocytes	-0.206	0.1962	-0.2129	0.3414	-0.1659	0.4972

WBC, white blood cells; HGB, haemoglobin; PLT, platelets counts; IgG, immunoglobulin G; LDH, lactate dehydrogenase; β2m, β-2-microglobulin; CRP, C-protein; TP, total protein; BM, bone marrow; TB, trephine biopsy.

473.8) (p=0.0053, Fig. 4A). Moreover, patients that were qualified to immediate treatment due to advanced stage of disease were found with significantly higher sCD163 levels as compared to patients subjected to 'watch and wait' therapy [1,036 µg/ml (672.6-1,592) vs. 622.3 µg/ml (428.3-832.2), p=0.0083, respectively, Fig. 4A]. However, analysis of baseline levels of sCD163 in 'watch and wait' patients did not reveal differences between patients with different times

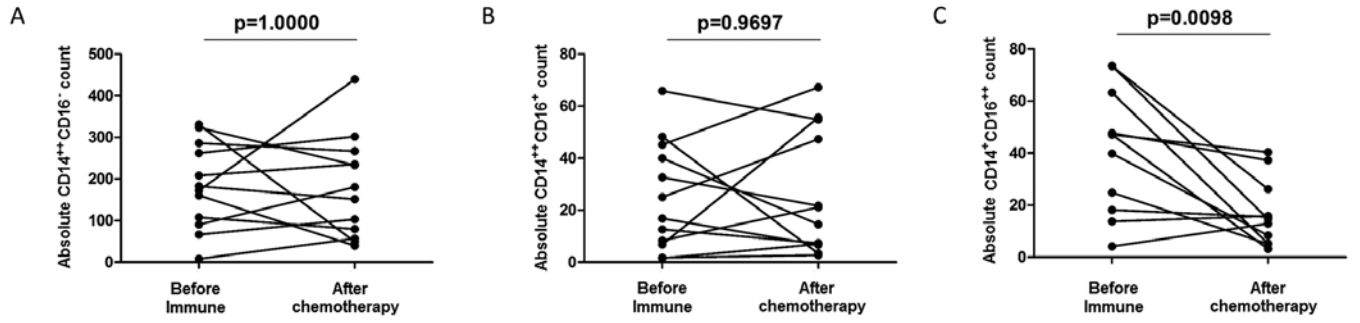

Figure 5. Effects of immune chemotherapy on the absolute numbers of (A) classical ($CD14^{++}CD16^{-}$), (B) intermediate ($CD14^{++}CD16^{+}$) and (C) non-classical ($CD14^{+}CD16^{++}$) monocytes. CLL, chronic lymphocytic leukemia.

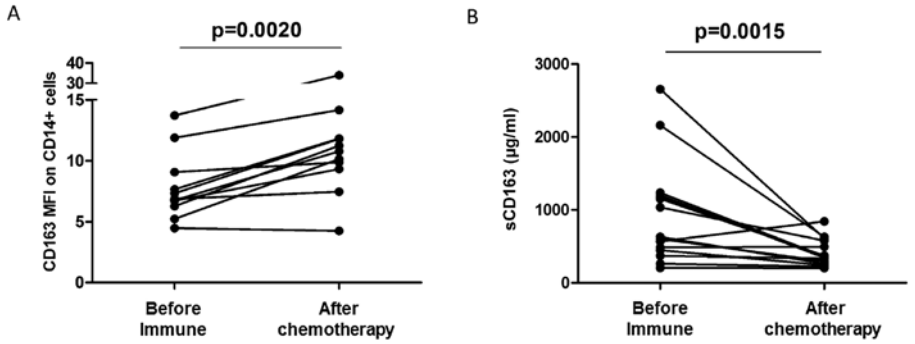

Figure 6. Effects of immune chemotherapy on the (A) CD163 mean fluorescence intensity on monocytes and (B) sCD163 serum levels.

to initial treatment (p>0.05, Fig. 4B). Quite surprisingly, we did not find an expected negative correlation between levels of soluble and membrane-associated CD163 (p>0.05, Table III).

Next, we set out to analyze the effects of immune chemotherapy on monocyte subsets and surface and soluble CD163. We demonstrated that applied chemotherapy (FCR) resulted in a statistically significant reduction in the absolute numbers of non-classical $CD14^{+}CD16^{++}$ monocytes in a group of patients who achieved at least partial remission (Fig. 5C). There were no significant changes in the numbers of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocyte subsets (Fig. 5A and B). Importantly, the study established that initial numbers of classical monocytes predicted better response to immune chemotherapy as the patients who achieved CR had significantly (p=0.0297) higher baseline numbers of $CD14^{++}CD16^{-}$ monocytes as compared to patients who achieved only PR [182.7 (115.5-262.4) vs. 66.65 (40.89-160.7), respectively].

Notably, we found that monocytes of CLL patients who underwent immune chemotherapy and achieved at least partial response had significantly upregulated expression of the anti-inflammatory surface protein CD163 (Fig. 6A). In clear contrast to membrane-associated CD163, standard immune chemotherapy resulted in a significant decrease in soluble CD163 levels (Fig. 6B).

Discussion

During tumorigenesis, monocytes are destined to provide the antitumor response of the host and act both as cells presenting tumor-associated antigens to tumor-infiltrating lymphocytes and as cytotoxic effector cells (28). However, cancer cells have developed mechanisms that inhibit immune surveillance (27). Indeed, it was shown that circulating monocytes are actively recruited to tumor beds, where they are 'conditioned' to promote the survival of malignant cells both directly and indirectly via the suppression of host immunity (29,30). Practically, extensive DNA microarray and cytokine antibody array data, on the basis of survival-inducing CLL cultures, identified a variety of inflammatory cytokines and signaling pathways which upregulated the expression and increased the secretion or activation of B-CLL (31,32). Moreover, experiments with normal B cells isolated from peripheral blood samples of healthy donors further indicate that monocytes harbor the survival-inducing activity for B cells in general (31).

In the present study, we demonstrated that quantification of different monocyte subsets served as a novel predictive marker of time that elapses before the treatment of CLL patients was needed. We found that detection of higher baseline numbers of classical $CD14^{++}CD16^{-}$ monocytes (but not non-classical and intermediate monocytes) in patients subjected to 'watch and wait' strategy was associated with significantly longer time to treatment. This finding was supported by identification of a negative correlation between the levels of lymphocytosis and absolute counts of classical monocytes. As above mentioned, classical $CD14^{++}CD16^{-}$ monocytes exhibit high antitumor and phagocytic capacities confirmed by both functional assays (9,33,34) and genomic analyses (35). Moreover, classical monocytes represent the first line of immune defense against microbial pathogens (36). Thus, our data suggest that monocyte

subsets with high antitumor and phagocytic potential can play an important role in a series of processes that can either protect from or delay CLL progression. It remains unclear however whether the putative beneficial effects of classical monocytes in CLL are indeed directly associated with their phagocytic potential or rather some immunomodulatory actions directed at either neoplastic cells or other cell types. Nevertheless, our findings warrant further functional studies on the role of classical monocytes in the pathogenesis of CLL as these cells may appear as potential novel targets of immune therapy of CLL.

In concert with putative protective roles of classical monocytes, we showed in the present study that CLL patients who qualified for immediate treatment due to progressive disease that presented with higher baseline levels of classical and lower baseline levels of non-classical monocytes, demonstrated better clinical response to applied therapy. It is difficult now to conclude whether these differential relationships of classical and non-classical monocytes were dependent directly on these monocyte subsets or rather their subsequent developmental fates. In fact, classical monocytes mainly differentiate into M1 macrophages that are known to play pro-inflammatory and tumor-suppressive roles (9,37). In contrast, non-classical monocytes differentiate into M2 macrophages that exert potent immunosuppressive and tumor-promoting effects (25). Moreover, it remains unknown whether the positive correlation of non-classical monocytes with lymphocytosis reflects direct B-CLL driven activation of monocytes or rather their involvement in the regulation of inflammatory reaction induced by the growth of neoplastic cells. Regardless of the exact mechanism, our findings point to the differential roles of classical and non-classical monocytes not only in the course of CLL but also in modulation of response to immune chemotherapy.

To the best of our knowledge, the present study is the first to investigate changes in monocyte subsets in CLL in the context of applied immune chemotherapy. Our standard therapy regimen included both chemotherapeutic agents (fludarabine-cyclophosphamide) and rituximab. For ethical reasons, we could not introduce treatment with either component administered alone. To date, it is known that rituximab treatment of CLL induced *in vitro* a substantial loss of CD20 on B cells that are stripped out of B cells by monocytes/macrophages in a reaction mediated by FcγR (38-40). Such antigenic modulation mediated by monocytes/macrophages, if occurring *in vivo*, severely compromised the therapeutic efficacy of rituximab treatment. In the present study, we found that higher numbers of classical and lower numbers of non-classical monocytes allow for prediction of the type of clinical response to immune chemotherapy. Our data suggest indirectly that classical monocytes could play certain roles in promoting the beneficial effects of anti-CLL therapy whereas non-classical monocytes can be linked to actions compromising efficacy of immune chemotherapy. On the other hand, however, the opposite actions, namely the effects of rituximab on monocyte subsets were not sufficiently studied. Here, we showed a significant treatment-related reduction in the absolute numbers of pro-inflammatory non-classical CD14^{+}CD16^{++} monocytes. This suggests that this cell subset can be identified as a novel target of CLL-specific immune chemotherapy. Thus, our data indicate that chemotherapy, beyond its anti-neoplastic actions, also exerts anti-inflammatory effects via elimination of the CD14^{+}CD16^{++} pro-inflammatory cell subset, known to constitute an effective source of TNF-α (8). In support of this notion, the present study, in line with other studies, confirmed the anti-inflammatory effects of CLL-specific therapy (significant reduction in CRP levels following chemotherapy).

In order to further the explore putative anti-inflammatory effects of immune chemotherapy, we investigated changes in the expression of monocyte protein CD163 whose anti-inflammatory functions have been previously identified by numerous research group including ours (4,7,22,41-44). In addition, CD163-expressing macrophages are involved in the resolution of inflammation by limiting free-hemoglobin associated damage (45), secreting anti-inflammatory cytokines in response to inflammation (46,47) and inhibiting T cell-mediated responses. The role of CD163 in the pathogenesis of solid tumors seems quite complex. In breast cancer, monocyte/macrophage CD163 expression was found to be significantly decreased as compared to healthy controls (48-50). In contrast, in a recent study, Tiainen *et al* (51) showed that detection of CD163-positive macrophages in breast cancer patients was related to poor prognosis. Similarly, accumulation of CD163-postive macrophages was associated with poor outcome of a few types of solid tumors (52-54). To date, the role of the differential patterns of monocyte CD163 expression in the pathogenesis of CLL were not studied. In the present study, we found that these CLL patients who had progressive disease at diagnosis and were therefore qualified for immediate treatment presented with lower levels of surface CD163 expression yet higher levels of soluble CD163 as compared to the patients who were qualified for the 'wait and watch' strategy due to the stable character of their disease. In concordance with this notion, the application of immune chemotherapy resulted in significant upregulation of surface CD163 expression and downregulation of soluble CD163 in a group of patients with at least partial remission. This pattern of CD163-related alterations reflects the dynamic monocyte response to intensity and extent of inflammatory processes driven by expanding B-CLL cells. In that case, a decrease in inflammation initiated by immune chemotherapy could have resulted in decreasing the intensity of CD163-related anti-inflammatory compensatory mechanisms. On the other hand, one cannot exclude that in CLL, in contrast to breast cancer, CD163 exerts some protective antitumor effects limiting the growth and expansion of neoplastic cells. Similarly, the literature on soluble CD163 in the pathogenesis of neoplastic disorders is very scarce and its role in malignancy is not yet elucidated (55,56). The present study is the first to our knowledge to investigate the role of soluble CD163 in CLL. It remains unclear whether our notion of elevated levels of sCD163 in CLL patients (particularly in those with more advanced disease) reflects yet unknown mechanisms fueling the progression of CLL or it rather represents the compensatory counter-action against malignant process and on-going inflammation. Regardless of the exact mechanism, our data indicate that the role of CD163 in the regulation of the development and progression of CLL warrants further studies.

In summary, we presented in the present study a number of significant relationships between classical monocytes (but not the other subsets) and markers of more favorable CLL prognosis or positive response to anti-CLL treatment. Our

data warrant further studies that could explore in more detail the beneficial potential of this cell subset in CLL treatment. Moreover, the present study provides initial evidence proving that diminishing the CLL-related inflammation by immune chemotherapy can be explained, at least to some degree, by targeting specific subsets and molecules of circulating monocytes. These monocyte-directed anti-inflammatory effects of immune chemotherapy could represent underappreciated benefits in the treatment of CLL. What still remains unclear, however, is whether the reduced levels of non-classical monocytes and the upregulated expression of CD163 also accounts for the general beneficial effects of chemotherapy. This issue deserves further investigation as it can help to establish novel monocyte-directed strategies, potentially enhancing the effectiveness of the current therapeutic regimens.

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