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Research paper

Targeting the IL-17/IL-6 axis can alter growth of Chronic Lymphocytic Leukemia *in vivo/in vitro*

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ABSTRACT

The tumor microenvironment (TME) is critical to the longevity of tumor B cells in chronic lymphocytic leukemia (CLL). Bone marrow mesenchymal stem cells (BMMSCs) and the cytokines they produce including IL-6 are important components of the TME in CLL. We found BMMSCs supported the survival of CLL cells *in vitro* through an IL-6 dependent mechanism. IL-17 which induces IL-6 generation in a variety of cells increased production of IL-6 both in CLL cells and BMMSCs *in vitro*. In a xenograft CLL mouse model, BMMSCs and the culture supernatant of BMMSCs increased engraftment of CLL cells through an IL-6 mediated mechanism with human recombinant IL-6 showing similar effects *in vivo*. Human recombinant IL-17 treatment also increased CLL engraftment in mice through an IL-6 mediated mechanism. Plasma of CLL patients showed elevated levels of both IL-6 and IL-17 by ELISA compared with healthy controls, with levels of IL-6 linearly correlated with IL-17 levels. CLL patients requiring fludarabine based chemotherapy expressed higher levels of IL-6 and IL-17, while CLL patients with the lowest levels of IgA/IgM had higher levels of IL-6, but not IL-17. These data imply an important role for the IL-17/IL-6 axis in CLL which could be therapeutic targets.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease characterized by progressive accumulation of $CD5^+$ B cells in peripheral blood, bone marrow and secondary lymphoid organs [1]. CLL cells in peripheral blood stay in the G0/G1 phase and tumor proliferation mainly occurs in proliferation centers in bone marrow and secondary lymphoid organs [2]. The disease is indolent in many patients but it exhibits aggressive behavior and causes death in others [3]. Current standard chemotherapy and novel signal transduction inhibitors are unable to cure the disease.

The tumor microenvironment (TME) has been demonstrated to be critical to the survival and proliferation of CLL cells. It is thought that this reflects the interaction of CLL cells and other nonmalignant cells in the TME, including T cells, nurse like cells, myeloid cells and mesenchymal cells, along with exposure to cytokines and chemokines made by these cells [4–7]. It has been reported that bone marrow mesenchymal stem cells (BMMSCs) are involved in the progression of CLL, but the mechanisms are poorly characterized [8,9]. BMMSCs are derived from bone marrow and have the potential to differentiate into a variety of other cells, including osteoblasts, chondrocytes and adipocytes [10]. BMMSCs can release multiple cytokines and chemokines which are potentially of importance to the regulation of tumor cell growth [11,12]. One of these cytokines, IL-6, has been shown to be secreted at high levels [13,14]. IL-6, also known as B cell stimulating factor 2, is an important mediator of normal B cell differentiation and proliferation [15]. Although some studies have suggested that high IL-6 levels in peripheral blood of CLL patients are correlated with a poor prognosis [16,17], especially in elderly patient who died from progressive CLL [18]. Improved understanding of the pathophysiological role of IL-6 produced by BMMSCs on CLL cells may help pinpoint some therapeutic alternatives for this disease. IL-8, a chemokine, has also been reported to be produced by BMMSCs [19]. There are reports that this molecule too can improve the survival of CLL *in vitro*, and high levels predict a poorer prognosis in CLL patients. Nevertheless, like IL-6, the role of IL-8 in CLL remains a controversial issue and is in need of further study.

IL-17A is an inflammatory cytokine that induces the production of IL-6 and IL-8 in a variety of cells [20–22]. The major source of IL-17A is thought to be a subtype of $CD4^+$ T cells, Th17 [22]. The frequency of

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Th17 in CLL is reported to be increased compared to healthy controls and high levels of IL-17 are correlated with a poor clinical outcome [23]. However, the role of IL-17, and its effect on IL-6 induction in the progression of CLL is unclear. Other groups have claimed that Th17/IL-17 plays a protective role, and that CLL patients with more advanced disease stage have lower levels of Th17/IL-17 [24,25].

In the study below, we have examined the role of IL-6, IL-8 and IL-17 on the survival of CLL cells and their potential roles, alone or in combination, in helping to explain the affect of BMMSCs on CLL growth both *in vitro* and in NOD-SCIDγc^{null} (NSG) mice. We have also analyzed the relationship between IL-17/IL-6 levels in CLL patient serum, and clinical correlates in CLL patients.

2. Methods and materials

2.1. Cells

Fresh CLL cells were isolated from the peripheral blood of consenting patients by negative selection with a RosetteSep kit (Stemcell technologies, Vancouver, Canada). The percentage of $CD5^+CD19^+$ cells after isolation was higher than 95%. Peripheral blood mononuclear cells were isolated with Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, USA). Patients were untreated for at least 3 months before the peripheral blood was collected. The characteristics of the patients whose samples were used in this study are listed in Table 1. Primary human bone marrow mesenchymal stem cells were purchased from Lonza (Lonza, Basel, Switzerland) and used in studies of growth of CLL [26]. Three different cell lines were obtained from 3 different donors.

2.2. Cell culture and transwell assay

CLL cells were cultured in AIM-V medium (Life technologies, Carlsbad, USA). Bone marrow mesenchymal stem cells (BMMSCs) were cultured in mesenchymal stem cell growth medium (MSCGM: Lonza, Basel, Switzerland). Both cell populations were cultured in 10 cm dishes or 24 well plates at 37 °C in a 5% CO_2 incubator. To prepare BMMSC conditioned medium, BMMSCs were cultured in MSCGM to 70% confluence, with the medium then replaced with AIM-V medium for 24 h. This conditioned AIM-V medium was used in both *in vitro/in vivo* experiments as indicated.

In a co-culture assay, 0.5 ml of CLL cell suspension (5×10^6/mL) was incubated on a layer of adherent BMMSCs. For indirect co-culture, the same number of CLL cells was incubated in a transwell insert with 0.4 μm filters (Fisher scientific, Waltham, USA) with mesenchymal cells cultured on the bottom of a 24 well plate. 10 μg/mL tocilizumab (Roche, Basel, Switzerland) or 0.5 μg/mL anti-IL8 antibodies (R&D System Inc., Minneapolis, USA) were added to wells in some experiments. Viable cells were stained with Trypan blue (Fisher scientific, Waltham, USA) and counted under a microscope with a hemocytometer. In other studies 10 ng/mL hrIL-6 or 10 ng/mL hrIL-17A (Biolegend, San Diego, USA) were used to stimulate CLL cells, BMMSCs or co-cultures of these two cells.

2.3. CLL cell viability analysis

Purified CLL cells were kept in culture for 72 h. In some studies 10 ng/mL hrIL-6 (Biolegend, San Diego, USA) and/or 500 ng/mL sIL-6R (R&D System Inc., Minneapolis, USA) were added to the culture. 7-AAD (Biolegend, San Diego, USA) was used to stain CLL cells. 5 μl of 7-AAD was added to a suspension of 1×10^6 cells. Cells were incubated in the dark for 15 min before analysis on a BD LSR flow cytometer.

2.4. Cytokine measurements

A Multi-Analyte ELISArray kit (Qiagen, Venlo, Netherlands) was used to check multiple cytokines in the culture supernatant of BMMSCs or CLL cells alone, or in co-culture. Levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GM-CSF were measured. The array analysis was performed according to the manufacturer's instruction. CLL plasma was collected from consenting CLL patients at routine clinic follow-up. Plasma from healthy donors was obtained from 10 sex and age matched individuals. IL-6 and IL-17A levels in the CLL plasma and culture supernatant were tested with a human IL-6 ELISA

Table 1
Clinical characteristic of patients in plasma IL-17/IL-6 analysis.

Pt ID	Age	Sex	Stage	WBC	β_2M	%CD38	FISH	LDT	Tx
1	48	F	0	16	1.8	< 1	ND	N/A	None
2	61	M	2	63	2.7	2	ND	36	None
3	63	M	4	42	3	20	ND	8	None
4	81	M	0	18	3	1	ND	19	None
5	77	M	4	223	12.8	20	T12	12	None
6	67	F	3	51	2.5	1	13q-	40	None
7	39	F	3	382	2	8	13q-	48	Splen
8	58	M	4	50	2.3	20	ND	29	None
9	80	F	4	92	3.8	4	ND	23	None
10	64	F	4	34	6.5	44	11q-	36	None
11	60	M	4	205	4.9	1	T12	2	C, P, Splen, FC, BR
12	65	M	0	43	2	1	ND	ND	None
13	75	M	4	393	5	NA	T12	14	B
14	87	F	4	120	12	NA	T12	8	C, P
15	66	F	3	113	3.7	18	T12	6	C, FC, BR
16	61	M	4	33	5.4	NA	13q-	14	FR,BR
17	57	F	4	26	4.8	2	13q-,T12	60	None
18	84	F	2	38	6.8	34	17p-	10	HDGC
19	65	M	0	12	1.3	1	ND	48	None
20	89	F	3	236	7.5	NA	11q-	24	S
21	72	M	4	15	2.7	21	ND	90	None
22	48	M	4	84	3.6	5	ND	14	None
23	78	F	4	30	4.5	1	Normal	10	R
24	68	M	2	16	1.5	1	ND	72	None
25	59	F	4	58	2.4	2	13q-	150	None
26	65	M	4	35	2.1	1	Normal	36	Splen
27	66	M	4	239	4.5	1	13q-	26	FCR
28	68	F	0	14	5.6	3	Normal	72	HDGC
29	78	F	2	44	7.3	1	13q-	70	FR
30	68	F	3	826	7.6	1	Normal	14	HDGC, R
31	72	F	4	172	4	1	ND	22	R
32	59	M	4	127	3.1	1	13q-	38	S
33	63	M	4	208	4.4	1	13q-,T12	12	FCR, Splen
34	66	M	4	74	5	14	ND	14	Splen, HDGC, R
35	67	F	4	227	3.8	43	13q-	6	S, FR
36	69	F	3	70	3.9	1	ND	26	S, R
37	51	M	2	22	2.1	NA	13q-	42	FCR
38	77	F	4	180	5.3	1	Normal	18	R
39	52	F	4	169	2.1	1	ND	14	FCR
40	83	F	4	103	15	NA	13q-	4	FC, HDGC, C, RADS
41	78	F	4	30	4.5	1	Normal	10	R
42	68	M	4	65	2.1	1	ND	120	None
43	91	M	4	50	5.8	3	T12	14	S
44	75	F	3	90	5.2	1	13q-	7	C, Splen, HDGC
45	58	M	4	280	2.9	15	Normal	11	C
46	66	M	3	213	2.3	7	ND	29	P
47	67	M	4	209	3.3	11	13q-	13	HDGC, Splen, R
48	66	M	4	150	2.5	1	13q-	60	None
49	66	M	3	18	3.7	12	13q-,11q-	2	FCR, BR
50	80	M	4	36	10.4	2	ND	72	None

Abbreviation: NA, not available; F, fludarabine; C, chlorambucil; P, prednisone; R, rituximab; S, steroids; Splen, splenoectomy; B, bendamustine; FR, fludarabine/rituximab; FCR, fludarabine/cyclophosphamide/rituximab; FC, fludarabine/cyclophosphamide; HDGC, high dose glucocorticoids; BR, bendamustine/rituximab.
Rai stage 0, lymphocytosis; I, with adenopathy; II, with hepatosplenomegaly; III, with anemia; IV, with thrombocytopenia.
WBC, white blood cell count (10^6/ml)in peripheral blood.
β2M, plasma β2microglobulin level, mg/L. T12, trisomy 12.