REGULATORY T-CELL MODULATION BY GREEN TEA IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Regulatory T cells (Tregs) are considered to be key immunomodulatory cells of the immune system and are increased in chronic lymphocytic leukemia (CLL). Rai stage 0 identifies patients with early stage CLL for which there is no effective intervention at the present time and a "wait and see" policy is usually adopted. Some biological and clinical studies have reported that green tea constituents, such as epigallocatechin-gallate (EGCG), have antitumor effects on hematologic malignancies including CLL. We report data on a clinical trial in which green tea extracts were given orally to 12 patients with stage 0 CLL and 12 healthy subjects. Ten patients and 10 controls completed the 6-month scheduled therapy. Two patients and 2 controls stopped therapy within 1 month because of tachycardia and epigastralgia. Eight out 10 evaluable patients (80%) showed a reduction of lymphocytosis and absolute number of circulating Tregs, as well. One patient (10%) had a stabilization of lymphocytosis and a reduction of Tregs, and 1 patient (10%) showed an increase of both lymphocytosis and Tregs. Only the non-responding patient progressed after 5 months from the end of green tea administration and chemotherapy was given. Interestingly, both IL-10 and TGF-β serum levels declined throughout the green tea intake period, in both patients and controls. These data seem to indicate that green tea is able to modulate circulating Tregs in CLL patients with early stage of the disease. This can result in the control of lymphocytosis as well as in the prevention of disease progression.

Green tea is one of the most ancient and popular beverages consumed around the world. Black tea is the most widely used in Western countries, while green tea is the most popular tea in Japan and China. Green tea has a history dating back almost 5,000 years because of its known medicinal effects and it is becoming more and more popular in Western countries for many health benefits (1, 2). Green

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tea contains polyphenols, which have antioxidant properties. The major group of polyphenols are catechins, and the most important of them seems to be epigallocatechin-gallate (EGCG) (3). It is known that green tea has antimicrobial effects, prevents tooth decay, can lower cholesterol levels and blood pressure and reduce the risk of stroke (4). Finally, several researchers have suggested that green tea may be also effective in cancer prevention (5, 6).

Treg cells constitute a small subset of T cells that are considered to play a key role in the regulation of immune responses to cancer (7, 8). Moreover, Treg cell number has been found increased in cancer and in chronic lymphocytic leukemia (CLL), the most frequent form of leukemia in Western countries (9-11). It has been proposed that green tea and its major component EGCG, could have clinical effects in patients with CLL (12-15). However, no data is reported on the mechanism by which green tea and its compounds work on this occasion. Wong and co-workers recently showed that EGCG is able to modulate Tregs *in vitro* in Jurkat T cells, and *in vivo* in mice via an epigenetic mechanism (16).

In this paper we report the effect of green tea given orally to a cohort of patients with low risk CLL, aiming to verify whether a modulation in the Treg-cell number occurs. This may be useful to speculate on the possible immunological role of Tregs in controlling CLL neoplastic clone.

MATERIALS AND METHODS

Cohort of patients and treatment

Twelve previously untreated patients diagnosed with Rai stage 0 CLL (17, 18), (5 male and 7 female; mean age 61 years; range 40–82 years) and 12 healthy subjects (6 male and 6 female; mean age 60 years; range 45–80 years) were enrolled in this trial. The study protocol was approved by the Local Ethics Committee and all subjects enrolled in the study gave written informed consent. Inclusion and exclusion criteria are reported in Table I. The main clinical and biological characteristics of the CLL cohort are reported in Table II. Both patients and controls were screened before starting treatment, at 3 months and at the end of the scheduled treatment. Each participant in the study received 4 capsules per day of a green tea extract (The Verde Monoconcentrato Opercoli, Aboca, Sansepolcro, AR, Italy) for the first month, then 6 capsules per day for the following 5 months. The composition of green tea extract capsules is reported in Table III. We chose to use green tea extract instead of the common infusion of green tea leaves because: i) in the former there is a greater dose of EGCG, the principal catechin of green tea due to the presence of a lyophilized extract; ii) the amount of EGCG is standardized; iii) the constant composition of green tea used and geographical origin (only Gunpowder and Chun Mee types were used). Six capsules of extract green tea contain 4602 mg of green tea leaves and 189 mg of EGCG and 97.5 mg of caffeine, respectively. Patients and controls were asked to report each sign and/or symptom occurring during the treatment period.

Flow cytometry and cytokine assays

Blood samples were taken every 3 months as scheduled and whole blood count, flow cytometry and cytokines assays were performed.

Briefly, EDTA-anticoagulated peripheral blood was used for flow cytometric analysis. One hundred μL of whole blood were incubated at 4°C in the dark for 30 min with 10 μL of each monoclonal antibody (MoAb) combined as follows: CD127-PE, CD4-PerCP, CD25-PE-Cy7, CD45-APC-Cy7. All MoAbs were purchased from Becton Dickinson Biosciences (BDB, San Jose, CA, USA). After lysis of red blood cells and repeated washing, the samples were analyzed by flow cytometry, by acquiring a minimum of 20,000 events for each sample, using a FACSCanto II cytometer equipment (BDB). All subsequent analyses were performed using FACS-Diva software (BDB).

Treg cells were evaluated according to the gating strategy protocol previously described by Baecher-Allan and coworkers (CD4+ cells expressing high levels of CD25) modified by the addition of CD127 monoclonal antibody (19). Treg cells were evaluated as percentage of all (B and T) lymphocytes. Overall, Treg cells were defined by the expression of CD4, CD25 at high density and CD127 low density or undetectable levels (20). This gating strategy was chosen because of being more discriminating between Treg cells and activated T-cells that normally up-regulate CD25 antigen that recognizes the interleukin (IL)-2 receptor (21).

A commercially available enzyme-linked immunosorbent assay (ELISA) was used according to the instructions of the supplier (Instant ELISA, eBioscience, Vienna, Austria) to assay for IL-10 and transforming growth factor (TGF)-β into plasma samples of 8 patients and 8 controls at baseline, at 3 months and at the end of treatment, respectively.

Statistical analysis

Data are expressed as mean ± standard deviation, median and range obtained from independent

determination, each one performed in triplicate. Differences between data sets were evaluated by performing unpaired Student's *t*-test. A p value < 0.05 was considered as statistically significant.

RESULTS

As shown in Table IV, both circulating lymphocytes and Tregs were found increased in CLL patients in respect to controls at baseline ($p < 0.001$).

Two female patients dropped out of the study after just 2 weeks of green tea administration, due to tachycardia and epigastralgia, respectively. The same was observed in 2 control subjects (1 male and 1 female) who exited the study because of epigastralgia after 20 days and 30 days, respectively. Ten patients (4 male and 6 female; mean age 57.8 years; range 40–70 years) and 10 healthy subjects (5 male and 5 female; mean age 57.6 years; range 45–70 years) completed the scheduled 6 months of therapy and were evaluable for the present analysis. The reported side effects are summarized in Table V. No case displayed hepatic, renal or thyroid laboratory abnormalities.

As depicted in Fig. 1a-c, 8 patients (80%) had a reduction in the absolute number of circulating Tregs as well as B-lymphocytes. One patient had a stabilization of lymphocytosis and a reduction of Tregs, while the remaining patient showed an increase of both lymphocytosis and Tregs. Only the non-rèsponding patient progressed after 5 months from the end of green tea administration and chemotherapy was given. Two patients chosen to continue to intake green tea for a further 3 and 6 months, respectively. One showed an increase of lymphocytosis at 9 months while Tregs remained stable after the initial reduction, the other had a stabilization of lymphocytosis and maintained the

Table I. *Criteria to include/exclude CLL patients into the study.*

Inclusion criteria	Exclusion criteria
Diagnosis of CLL	CLL in active phase according to NCI criteria (14)
Rai 0 clinical stage	Uncontrolled arterial hypertension
	Cardiac failure
	Cardiac arrhythmias
	Hyperthyroidism and hypothyroidism
	Gastroduodenal ulcers in active phase
	Sideropenic anemia
	Renal failure
	HBV and/or HCV-related or alcoholic chronic hepatitis
	HIV positivity

Table II. *Clinical biological features of CLL patients at study entry.*

UPN	Sex	Age (years)	Lymphocytes (/μL)	FISH	CD38 (%)	ZAP-70 (%)	IgVH (Mutated/Unmutated)
1	M	51	7000	Normal	2	7	Mutated
2	F	40	6700	Normal	4	24	Unmutated
3	F	62	10000	del13q14	13	30	Mutated
4	F	59	16000	Normal	36	14	Mutated
5	F	58	25300	Normal	42	25	Unmutated
6	M	50	19300	Normal	5	8	Mutated
7	F	70	45900	del13q14	2	10	Mutated
8	M	65	32500	trisomy 12	1	5	Mutated
9	M	60	12900	Normal	10	24	Unmutated
10	M	63	15420	Normal	16	17	Mutated
11	F	74	27000	trisomy 12	23	19	Mutated
12	F	82	6000	Normal	5	2	Mutated

Trisomy 12, del13q14, del17p, del11q were tested by FISH. The last two patients dropped out of the study because of tachycardia and epigastralgia, respectively. Positivity is considered when ≥ 20% B-cells coexpress CD38 or ZAP-70 antigen.

Table III. *Composition of green tea total concentrate capsules (Aboca).*

Composition of 1 capsule	Amount of green tea leaves corresponding to 1 capsule	Amounts of EGCG and caffeine in 1 capsule	Amount of green tea leaves corresponding to 6 capsules	Amounts of EGCG and caffeine in 6 capsules
Total Green Tea Concentrate 404 mg	657 mg	31.5 mg EGCG 16.25 mg caffeine	4602 mg	189 mg EGCG 97.5 mg caffeine

The Total Green Tea Concentrate capsules - Dietary supplement has the following composition:
- *Green tea (Camellia sinensis) leaves powder,*
- *Green Tea (Camellia sinensis) leaves freeze-dried extract,*
- *gelatin.*

Each capsule contains 404 mg of green tea total concentrate (a combination of powder and freeze-dried extract) that, thanks to the particular concentration of freeze-dried extract (D.E.R. 3-5:1), corresponds on average to 767 mg of green tea leaves and contributes 31.5 mg of EGCG. The product can be eaten as it is or can be dissolved in a cup of hot water and taken as a drink. The daily recommended dose of the integrator is 4 capsules (corresponding to 1620 mg of green tea leaves); the dosage may be increased depending on the amount that you want to administer, for example, a dose of 7 capsules corresponds to 5369 mg of green tea leaves (220 mg of EGCG, 108.5 of caffeine). Specifically, the product to be used in the study will be accompanied by a tracing in HPLC fingerprint, which provides the fingerprint of the product itself.

initial reduction of Tregs. Interestingly, serum levels of both IL-10 and TGF-β were found to decrease in controls as well as in patients (Fig. 1e-f).

DISCUSSION

Tregs are a small subset of CD4+ T lymphocytes that is thought to play a key role in modulation of the immune response. These cells are involved in the development and in the control of many autoimmune conditions and in the control of immune responses towards transformed cells. Tregs have been associated to cancer development and progression, in many types of cancer, including CLL in which

Table IV. *Treg cell and B-lymphocyte number (/μL).*

			baseline	*3 months*	*6 months*
T_{reg}	**CLL 0A**	mean ± sd	92.5±27.92	65.48±35.62	59.32±44.65
		median (range)	101 (49 - 128)	63.1 (20 - 120)	49.5 (15 - 151)
	Healthy volunteers	mean ± sd	30.17±12.52	26.24±11.97	27.4±11.09
		median (range)	29.5 (12 - 50)	23.5 (11.4 - 43)	26 (14 - 42)
		p value	**p<0.001**	**p<0.01**	p>0.05
B-Lymphocytes	**CLL 0A**	mean ± sd	18494.4±10991.18	19350±11985.11	18814±13241.05
		median (range)	15710 (6700 - 39800)	15650 (7800 - 45000)	13195 (6150 - 49200)
	Healthy volunteers	mean ± sd	2169±379.45	2118±261.61	2185.6±373.12
		median (range)	2050 (1800 - 2800)	2090 (1800 - 2560)	2100 (2185.6 - 373.12)
		p value	**p<0.001**	**p<0.001**	**p<0.001**

Levels of IL-10 (pg/ml) and TGF-β (ng/ml)

			baseline	*3 months*	*6 months*
IL-10	**CLL 0A**	mean ± sd	4.5±0.57	3.8±0.66	3.04±1.05
		median (range)	4.65 (3.4 - 5.1)	3.95 (2.5 - 4.6)	2.9 (1.8 - 5)
	Healthy volunteers	mean ± sd	2.81±1.74	2.44±1.28	2.1±1.18
		median (range)	2.8 (.7 - 5.1)	2.35 (.7 - 4.2)	1.8 (.7 - 4.2)
		p value	p<0.05	p<0.05	p>0.05
TGF-β	**CLL 0A**	mean ± sd	20724.4±5293.4	18928.1±5507.6	15474.5±7347
		median (range)	19900 (15212 - 29000)	19500 (11425 - 27000)	16700 (581 - 25265)
	Healthy volunteers	mean ± sd	23932.8±4384.6	19851.6±3876.3	14672.2±9939.1
		median (range)	23950 (19200 - 29300)	19579.5 (15000 - 25512)	16750 (748 - 29228)
		p value	p>0.05	p>0.05	p>0.05

their number is found increased in peripheral blood (9-11). Tregs were originally defined on the basis of the expression of the IL-2 receptor (CD25) at high density on CD4+ T-cells (19). Moreover, the nuclear factor foxp3 has been recognized as a master regulation and lineage-specification factor for Tregs (22). However, its routine use is limited by the intracellular localization and consequent difficulties in standardizing protocols and results. More recently, low expression of the IL-7 receptor (CD127) has been recognized as a further Treg marker (20-21). We used a combination of CD4/CD25/CD127 to evaluate, by flow cytometry, the number of circulating Treg cells in 10 evaluable patients with early stage CLL treated with standardized doses of an oral extract green tea, in order to verify whether a modulation of such cells and peripheral blood neoplastic B-cells occurs during beverage intake.

We found that 8 (80%) patients experienced a reduction of lymphocytosis and absolute number of circulating Tregs as well, while one patient had a stabilization of lymphocytosis and a reduction of Tregs, and the remaining patient showed an increase of both lymphocytosis and Tregs. The observed reduction of circulating Tregs suggests a modulation of immune system regulation by a green tea

Table V. *Side effects reported by patients and controls.*

	Side effects	Number of patients
Controls		
	Epigastralgia	2
	Meteorism	2
Patients		
	Epigastralgia	1
	Tachycardia	1
	Meteorism	2

component, such as EGCG. This is further confirmed by the observation that both IL-10 and TGF-β serum concentrations decreased as effect of green tea intake. IL-10 is a pleiotropic cytokine that plays a special role in the regulation of lymphoid and myeloid cell function (23-25). TGF-β is a polypeptide that plays a critical role in growth regulation and development function (23-25). Both these cytokines are thought to be involved in humoral Treg-mediated suppression (26). We chose IL-10 and TGF-β, among others, because they are probably the most relevant Tregs-associated cytokines with suppressive activity in tumor microenvironment.

CLL still remains an incurable disease displaying an extremely variable clinical course. In fact, one-third of patients have an aggressive form of disease requiring treatment immediately after diagnosis and die within 2 to 3 years. On the contrary, another third of patients have a very benign, indolent course and live for 10 to 20 years without needing therapy. The final third of patients have a disease course somewhere in between the two extremes, displaying disease progression at some time and requiring therapy with a reduced half-life. Several efforts have been made to identify the prognosis of patients with CLL at diagnosis (18) and, among others, circulating number of Tregs have been shown to correlate with clinical-biological features and to predict time to first treatment. Moreover, Tregs seem to be implicated in the pathogenesis and disease progression of CLL as well as other cancers (7).

Green tea is a popular beverage in China and Japan and is becoming increasingly popular in Western countries due to its beneficial effects. Several reports have investigated on the mechanism by which green tea components exert their effects on cancer cells (5). A number of mechanisms have been proposed including antiangiogenetic properties, DNA damage, and antiapoptotic mechanism inhibition. More specifically, Lee and co-workers demonstrated that EGCG displays its effect on clonal B cells *in vitro* by means of caspase-dependent death induction (12). A downregulation of antiapoptotic proteins (MCL1 and XIAP), responsible for B-CLL cells resistance to apoptosis, was found along with VEGF receptor phosphorylation decrease. At the same time, it was observed that some patients with low grade lymphomas who used green tea as self-administration experienced objective clinical response on both lymphocytosis and adenopathies (13). On this basis, at the Mayo Clinic, Shanafelt and co-workers (14) started a phase I trial in patients with asymptomatic Rai stage 0-II CLL. They treated 36 patients for 6 months with excalating doses of EGCG (from 400 mg to the maximum tolerated dose of 2000 mg twice per day). The most common adverse side effects were transaminitis, abdominal pain, and nausea. A significant and sustained reduction in absolute lymphocytosis was observed in 33% of patients and 92% of 12 patients presenting adenopathy showed a significant reduction of their volume. This data has been subsequently confirmed by the same Author in a phase II randomized trial in which durable declines in the absolute lymphocyte count and/or lymphadenopathy were observed in the majority of patients with asymptomatic, Rai stage 0 to II CLL (15).

In our study we used doses of EGCG at a lower degree (189 mg/daily) in respect to the North American trial, aiming to evaluate the pattern of

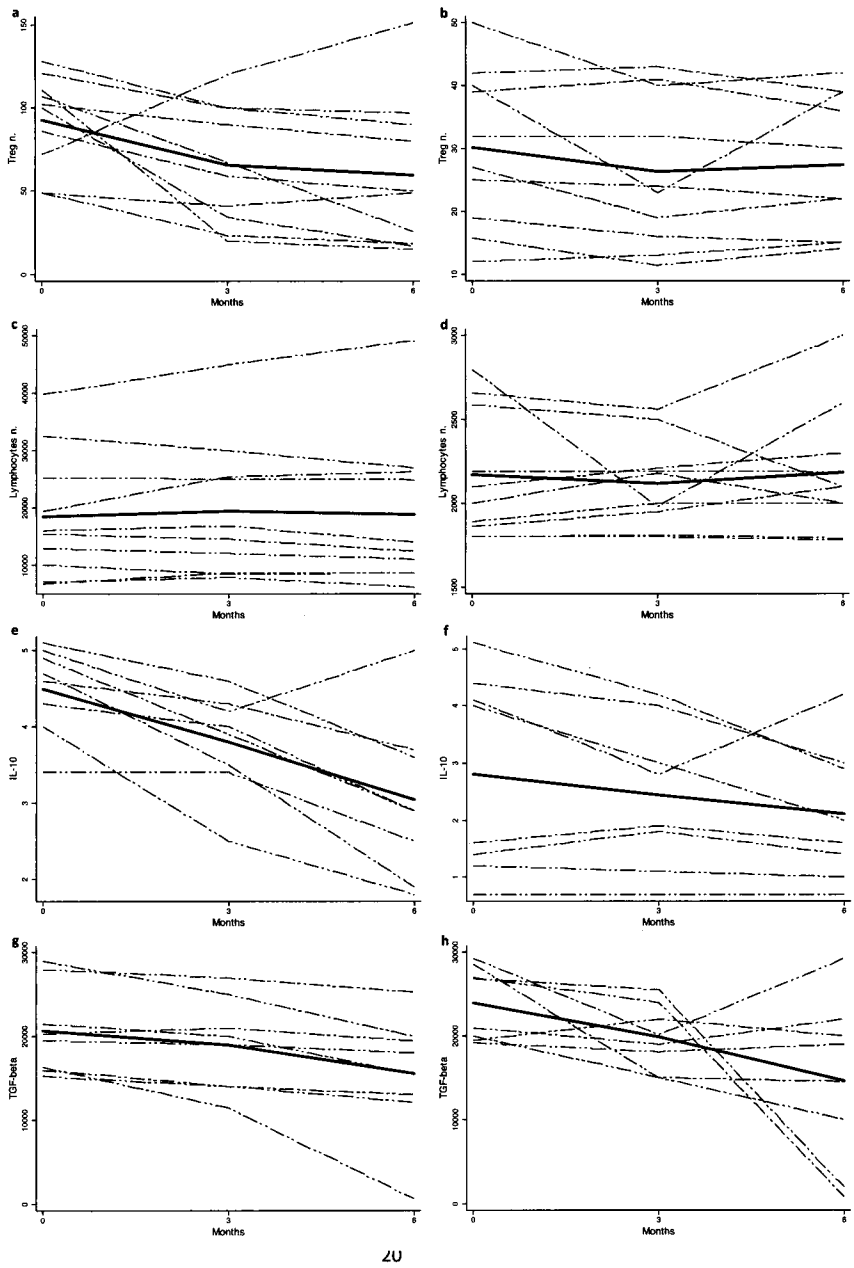

Fig. 1. *Graphical representation of Treg number/μL (**a**, CLL patients; **b**, healthy volunteers), lymphocyte number/μL (**c**, CLL patients; **d**, healthy volunteers), IL-10 serum levels/pg/ml (**e**, CLL patients; **f**, healthy volunteers) and TGF-β serum levels/ng/ml (**g**, CLL patients; **h**, healthy volunteers) at time of treatment with green tea extracts. Longdash-shortdash lines represent values for each individual, solid lines represent the mean value.*

modulation of circulating Tregs. Our assumption was that Tregs reduction could be induced by oral green tea and that this modification could impact on clinical and biological features of the disease. In fact, in patients in whom Tregs have reduced their number, a decrease in lymphocytosis was also observed. If Tregs are actors or just innocent bystanders of immunological phenomena far more complex and not yet fully understood has to be clarified with more and better targeted *in vitro* studies. In the future a

randomized trial between CLL patients treated or not with green tea is scheduled.

Taken together, these data strongly encourage the use of green tea at a given point of clinical course and follow-up of patients with CLL. In fact, oral green tea is a well tolerated and "pleasant" tool that could be useful to prolong the early stage phase of CLL disease and to prolong the remission phase after therapy in progressed patients underwent disease specific treatment (27).

On the other hand, the use of non-conventional medicines, especially herbal remedies, other than green tea, is common in cancer patients, including hematological neoplasias (28-31). Potential side effects and herb-drug interactions must be taken into account. The oncology community must be aware of this unconventional approach, adequately advise their patients, warning them of potential side effects, controindications and risks.

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