

Association of Increased Levels of Heavy-Chain Ferritin with Increased CD4⁺ CD25⁺ Regulatory T-Cell Levels in Patients with Melanoma¹

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ABSTRACT

We have shown previously that melanoma cells in culture release heavy-chain ferritin (H-Ferritin) into supernatants and that this is responsible for the suppression of responses of peripheral blood lymphocytes stimulated by anti-CD3. These effects were mediated by activation of regulatory T cells to produce interleukin (IL)-10. In the present study, we examined whether a similar relation might exist between levels of H-Ferritin and activation of regulatory T cells in patients with melanoma. Ferritin levels were evaluated by ELISA and regulatory T-cell numbers were assessed by three-color flow cytometry to identify CD4⁺ CD25⁺ CD69⁻ T cells. CD69 positive cells were excluded to avoid inclusion of normal activated CD4, CD25 expressing T cells. Measurements of H- and light-chain (L)-Ferritin by ELISA revealed that H- but not L-Ferritin was elevated in the circulation of melanoma patients. In addition, these studies revealed a marked increase in the number of CD4⁺ CD25⁺ CD69⁻ T cells in such patients, compared with age-matched controls. The ratio of H-Ferritin:L-Ferritin correlated with the levels of regulatory T cells consistent with a causal relation between unbound H-Ferritin levels and the activation of regulatory T cells. H-Ferritin or regulatory T cells did not, however, correlate with the stage of the melanoma. These results provide evidence for the importance of H-Ferritin in the induction of regulatory T cells in patients with melanoma and provide additional insight into the suppression of immune responses in such patients.

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INTRODUCTION

Ferritin is a major tissue iron-storage protein (1) and, in its native form, has a molecular weight of $M_r \sim 500,000$. It is composed of 24 subunits consisting of acid/H³ and basic/L chains (2, 3). The 24-subunit polymer may form iso-ferritins, which are either more acidic (H-rich) or more basic (L-rich) depending on the relative proportions of H and L chains. Liver and spleen ferritins are basic because they are made up mainly of L chains and very few H chains. In contrast, heart, kidney, and placental ferritins are highly acidic because they are composed of mostly H chains (4). The ferritin found in cancer cells was found to consist mainly of H chains (5, 6). Ferritin is localized predominantly intracellular; however, small amounts are present in plasma (7), urine (8), cerebrospinal fluid (9), ascitic fluid (10), milk (11), and all body fluids thus far tested (12), which suggests that it is actively secreted by cells.

A number of functions have been attributed to the extracellular ferritins: regulation of myelopoiesis (13), regulation of lymphocyte migration (14), and as an immunosuppressive agent (15). The immunological and structural properties of extracellular ferritin vary in different fluids (12), which probably relates to its origin and function. A large body of evidence indicates that the level of plasma ferritin parallels the concentration of storage iron within the body, regardless of the cell type in which it is stored (16). This relationship of plasma ferritin to body iron stores, however, is altered in inflammatory states and liver disease, conditions in which ferritin is disproportionately elevated (17). Elevated levels have also been reported in the plasma of patients with different malignancies; however, the source of the ferritin is unknown (18–20). We have previously shown that there was marked variability found in the ratios of H-Ferritin:L-Ferritin released from melanoma cultures. It was shown that some melanoma cells released predominantly H-Ferritin, whereas others contained more equal proportions of H- and L-Ferritin (21).

In addition, we have previously shown that H-Ferritin from melanoma cells may suppress immune responses (21). This was attributable to changes in antigen presenting cells, which resulted in the preferential activation of regulatory T cells that produce interleukin 10 (22). The regulatory T cell implicated in this suppression appeared to be a particular subpopulation of CD4⁺ T cells, which constitutively express the CD25 component of the interleukin 2 receptor and the CTLA-4 T cell activation receptor for CD80 (B7.1) and CD86 (B7.2; Refs.

³The abbreviations used are: H, heavy (chain); L, light (chain); H-Ferritin, heavy-chain ferritin; L-Ferritin, light-chain ferritin; MAb, monoclonal antibody; AJCC, American Joint Commission of Cancer; PBL, peripheral blood lymphocytes.

Table 1 Description of melanoma patients

Melanoma patient no.	Sex	Age	Stage of disease ^a	Clinical evidences of disease	L-Ferritin (μg/L) ^b	H-Ferritin (μg/L) ^b	Ratio H-Ferritin: L-Ferritin	% of CD4 ⁺ CD25 ⁺ CD69 ⁻ regulatory T cells
1	M	20	III	No	96.5	197.6	2.0	n.a. ^c
2	M	23	IV	Yes	80.4	103.0	1.3	14.9%
3	F	30	IV	Yes	33.6	121.2	3.6	n.a.
4	F	30	II	No	3.0	12.7	4.2	n.a.
5	M	40	IV	Yes	131.5	272.1	2.1	8.9%
6	F	40	III	No	73.9	170.1	2.3	17.3%
7	M	45	III	No	86.3	245.3	2.8	n.a.
8	M	47	III	No	101.3	221.0	2.2	13.8%
9	M	48	II	No	162.2	381.2	2.4	15.9%
10	M	53	IV	Yes	135.9	238.6	1.8	n.a.
11	M	53	II	No	149.7	413.5	2.8	11.4%
12	F	54	II	No	87.6	166.5	1.9	4.4%
13	M	56	II	No	117.1	393.3	3.4	n.a.
14	F	59	IV	Yes	171.2	368.4	2.2	6.1%
15	F	60	IV	Yes	116.6	203.5	1.7	7.5%
16	M	60	III	No	122.3	209.1	1.7	19.1%
17	F	62	III	Yes	29.8	54.5	1.8	13.7%
18	M	63	IV	No	30.7	57.0	1.9	18.3%
19	M	64	II	No	160.1	339.5	2.1	n.a.
20	M	66	IV	No	121.3	283.1	2.3	14.9%
21	M	67	IV	No	102.2	170.0	1.7	n.a.
22	F	68	III	No	73.8	173.6	2.4	n.a.
23	M	68	III	No	165.4	365.7	2.2	15.7%
24	M	69	IV	No	133.0	443.1	3.3	12.9%
25	F	71	IV	No	42.7	65.2	1.5	26.6%
26	M	76	II	No	53.2	132.3	2.5	20.6%
27	M	77	IV	Yes	32.2	57.2	1.8	28.9%
28	M	77	III	No	105.4	346.6	3.3	14.6%
29	M	78	II	Yes	146.8	285.8	1.9	n.a. ^c
30	M	82	IV	No	120.0	284.7	2.4	14.2%

^a Using the AJCC and the International Union against Cancer Classification.

^b Detection using ferritin ELISA.

^c Not available.

23–27). CD4⁺ T cells with this phenotype have been previously shown to suppress autoimmune disease in several animal models (28, 29) and to facilitate the survival of transplants (30–32) and hence their description as CD4⁺CD25⁺ regulatory T cells. Elevated levels of CD4⁺CD25⁺ regulatory T cells have been described in a number of cancers including patients with lung, ovarian (33), breast, and pancreatic carcinoma (34).

In view of these findings, the present study examined whether H-Ferritin levels were elevated in patients with melanoma and whether the relation between H-Ferritin and regulatory T cells shown *in vitro* may also apply in patients with melanoma.

MATERIALS AND METHODS

Patients. Blood was collected from 30 melanoma patients attending either the Sydney or the Newcastle Melanoma Units. Details on the patients are described in Table 1. Clinically evident disease was present in nine of the patients. Blood was also collected from 19 age-matched subjects with no history of melanoma. Included among the control subjects were eight patients attending the Royal Newcastle Dermatology Clinic for non-melanoma-related conditions (Table 2). The median age for melanoma patients and normal subjects was 60 and 53 years, respectively.

Antibodies and Recombinant Proteins. The MAbs RH02 and LF03, specific for human H- and L-Ferritin, respec-

tively, was kindly provided by Dr. Paolo Arosio (Università di Brescia, Brescia, Italy) and are described elsewhere (35, 36). Anti-CD3 was the OKT3 MAb (37). The MAb against CD25 was purchased from Endogen, (Woburn, MA). The MAbs against CD3, CD4, CD8, CD14, CD45RO, CD56, CD69, CD152 (CTLA-4), and IgG1 isotype were purchased from PharMingen, (San Diego, CA). The MAb against PD-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibody against placenta ferritin was purchased from Fitzgerald (Concord, MA). Recombinant H-Ferritin (rH-Ferritin) and L-Ferritin (rL-Ferritin) was kindly provided by Dr. Paolo Arosio and is described elsewhere (38, 39).

Blood Samples. For collection of plasma, blood was collected in 4-ml vacutainers containing 7.2 mg of K₂EDTA (Becton Dickinson, San Jose, CA). The plasma was separated from packed blood cells by centrifugation at 500 × g for 10 min at 20°C. The plasma was then stored in aliquots at –80°C.

For studies on lymphocytes, blood was defibrinated on glass beads and lymphocytes (PBL) separated by centrifugation on Ficoll-Paque gradient (Amersham Pharmacia Biotech, Buckinghamshire, England) as described previously (22).

Preparation of T-Cell Subpopulations. PBLs were resuspended in DMEM, containing 10% FCS (CSL Biosciences, Sydney, New South Wales, Australia), and adherent cells were removed by adherence in 75-cm² plastic flasks (Sarstedt, Newton, NC) for 2 h in a 37°C incubator supplemented with 5%

Table 2 Description of normal control subjects

Normal subject no.	Sex	Age	Dermatology Clinic treatment ^a	L-Ferritin (μg/L) ^b	H-Ferritin (μg/L) ^b	Ratio H-Ferritin: L-Ferritin	% of CD4 ⁺ CD25 ⁺ CD69 ⁻ regulatory T cells
1	F	23		76.7	88.1	1.1	7.9%
2	F	23		18.4	28.3	1.5	n.a. ^c
3	M	27		50.9	42.4	0.8	3.7%
4	M	29		117.0	105.9	0.9	10.1%
5	M	39		127.7	104.6	0.8	4.2%
6	M	39	Psoriasis	96.1	133.7	1.4	10.7%
7	F	40		92.2	109.8	1.2	7.9%
8	F	47		50.8	20.9	0.4	n.a.
9	M	50		126.6	138.3	1.1	8.2%
10	M	53	Psoriasis	178.6	146.6	0.8	10.2%
11	M	55		104.9	129.5	1.2	11.0%
12	F	60		110.6	70.8	0.6	14.4%
13	F	62	Pruritis	54.8	44.3	0.8	n.a.
14	M	62		250.7	157.5	0.6	3.8%
15	F	65	Basal cell carcinoma	47.3	50.5	1.1	15.5%
16	M	68	Dermatitis	88.9	120.2	1.4	n.a.
17	F	70	Basal cell carcinoma	86.6	87.3	1.0	14.9%
18	M	75	Solar keratosis	138.2	109.0	0.8	4.8%
19	M	81	Squamous carcinoma	139.9	119.7	0.9	n.a.

^a Attending the Royal Newcastle Dermatology Clinic.

^b Detection using ferritin ELISA.

^c n.a., not available.

CO₂, CD25⁺ or CD69⁺ cells were enriched or depleted by positive or negative selection, respectively, on midiMACS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany). In brief, nonadherent PBLs were first incubated with anti-CD25 or CD69 MAb for 30 min on ice in PBS-2 mM EDTA (Sigma, St. Louis, MO) supplemented with 1% human AB plasma and then were incubated in the same buffer with antimouse IgG1 MicroBeads (Miltenyi Biotec) for 15 min. The cell suspension was then added to a miniMACS column for 5 min and was washed four times with PBS-2 mM EDTA. The eluted fraction contained the phenotype-depleted cells. The magnetically labeled positive fraction was retained on a midiMACS column. To isolate this positive fraction, the column was removed from the magnetic field, and the cells were eluted with PBS-2 mM EDTA. Isolation of CD69⁻ CD25⁺ cells required a two-step isolation process, involving a CD69-negative selection followed by a CD25-positive isolation. The purified T-cell populations were treated with 25 μg/ml mitomycin-C (Kyowa Hakko Kogyo CO., Ltd., Tokyo, Japan) at 37°C for 1 h, before being added to the proliferation assay.

Proliferation Assay. Proliferation assays were set up as described by Gray *et al.* (21). Briefly, 2 × 10⁵ PBLs were cultured with 1 × 10⁵ or a 2-fold dilution (1 × 10⁵ to 3.125 × 10³) of mitomycin-C (Kyowa Hakko Kogyo CO.)-treated CD25⁺ CD69⁻ cells in 200 μl of DMEM containing 10% FCS (CSL Biosciences) in a 96-well U-bottomed plate (Falcon, Becton Dickinson, Franklin Lakes, NJ). For blocking studies, MAb against CTLA-4 was prebound to the plate with 10 μg/ml anti-CD3 in carbonate buffer [0.1 M NaHCO₃ (pH 8.2)] overnight at 4°C. Cultures were then incubated in a 37°C incubator supplemented with 5% CO₂ for 72 h. Two μCi of [5-¹²⁵I]iodo-2(1)-deoxyuridine ([¹²⁵I]UdR; Amersham, Aylesbury, United Kingdom) was added for the final 4 h before cell harvesting. [¹²⁵I]UdR incorporation was then measured on a Compu-

Gamma CS gamma counter (Wallac, Oy, Finland). The degree of inhibition of anti-CD3 stimulation was calculated as described elsewhere (40).

Ferritin ELISA. The ferritin ELISA was set up similar to methods described by Vaisman *et al.* (41). Optimum concentrations for rabbit polyclonal anti-Ferritin (placenta), RH02, LF03, and biotin-labeled rabbit antimouse immunoglobulin antibodies were determined using a checkerboard ELISA assay. In brief, 100 μl of rabbit polyclonal anti-Ferritin (placenta) at 2 μg/ml in coating buffer [0.1 M NaHCO₃ (pH 8.2)] was added per well in a 96-well plate (Maxisorp Nunc, Copenhagen, Denmark), which was sealed using an acetate plate sealer (ICN Biomedicals, Aurora, OH) and was incubated overnight at 4°C. The plate was washed three times with PBS-Tween (500 μl of Tween 20 to 1 liter of PBS) and then was blocked with 200 μl of PBS-Tween-1% BSA (Sigma, St. Louis, MO)-10% FCS for 2 h (Sealed). After an additional three washes with PBS-Tween, the plasma or standards (100 μl diluted in PBS-Tween-0.5% BSA-10% FCS) was/were added and incubated (sealed) overnight at 4°C. The plate was washed six times with PBS-Tween; and 100 μl of RH02 or LF03 MAb (2 μg/ml) for H- or L-Ferritin detection, respectively, was placed in each well for 90 min (sealed) at room temperature. The plate was subsequently washed six times with PBS-Tween before the addition of 100 μl of biotin-rabbit antimouse immunoglobulin (1:2000) and was incubated (sealed) for 1 h at room temperature. The plate was washed an additional six times with PBS-Tween. A 1-mg/ml solution of avidin-peroxidase (Sigma, Clayton, Victoria, Australia) was diluted 1:400 in PBS-Tween-0.5% BSA-10% FCS and was added to each well (100 μl) and incubated (sealed) for 30 min at room temperature. The plate was then washed eight times with PBS-Tween, and 100 μl of ABTS [300 μg/ml; 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 0.1 M citric acid (pH 4.35), 3% H₂O₂ (added before use)] were placed in

each well; the reaction was allowed to develop for 10–80 min. The plate was read at $A_{405\text{ nm}}$. The concentration of ferritin in each sample was calculated from the log/linear regression curve obtained from the standard. The linear region of the standard curve was between 15 and 500 ng/ml. For samples with greater concentrations, a dilution of the sample was assayed.

IFN- γ ELISA. An anti-CD3 proliferation assay was set as previously described. After 24 h, the supernatants were collected from the proliferation assay. IFN- γ was assayed using a biotinylated ELISA system (PharMingen) according to the manufacturer's instructions.

Flow Cytometry. Analysis was carried out using a Becton and Dickinson (Mountain View, CA) Facscan flow cytometer. Appropriate concentrations of MAbs were added to the cells in 100 μ l of PBS containing 1% human plasma, and the cells were incubated for 30 min at 4°C. Cells were washed twice with PBS and were analyzed. For indirect labeling, cells were incubated with F(ab')₂ fragment affinity-isolated phycoerythrin-conjugated sheep antimouse (Silenus; Amrad Biotech, Boronia, Victoria) or FITC rabbit anti-goat immunoglobulin (Chemicon International, Inc., Temecula, CA) plus 1% human plasma for 30 min at 4°C. Cells were then washed once in PBS before analysis. A minimum of 5000 cells were analyzed by flow cytometry. The percentage of positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (42).

RESULTS

Definition of CD4⁺ CD25⁺ CD69⁻ Regulatory T Cells in Blood of Normal Subjects and Melanoma Patients.

CD25⁺ T cells within an unstimulated PBL population isolated from a normal subject was found to be 84.7% CD4⁺, 5.5% CD8⁺, and 9.7% CD4⁻ CD8⁻ T cells. The CD25⁺ cells also expressed CD3, CTLA-4, CD45RO, and PD-1 (83.5, 87.0, 68.4, and 86.0% of CD25⁺ cells, respectively), but not CD14 or CD56 (1.6 and 2.8% of CD25⁺ cells, respectively).

CD4⁺ CD25⁺ T cells may also represent normal activated CD4⁺ T cells. We were concerned that this population may be present at higher levels in patients with melanoma and may give rise to falsely high levels of regulatory T cells. For this reason CD4⁺ CD25⁺ T cells that also expressed the CD69 activation antigen were excluded. Detection of CD4⁺ CD25⁺ CD69⁻ T cells in a melanoma patient using three-color flow cytometry analysis is shown in Fig. 1a. Sixteen % of the unstimulated PBLs were CD4⁺ CD25⁺ and 1.7% were CD4⁻ CD25⁺. In addition it shows that 31.2% of CD4⁺ T cells were CD25⁺, and of these, 22.6% were CD69⁺. Therefore, the number of CD4⁺ CD25⁺ T cells expressing CD69⁺ was 7%, indicating that the number of CD4⁺ CD25⁺ CD69⁻ regulatory T cells made up 24.2% of the unstimulated CD4⁺ cell population. The different functional properties of CD4⁺ CD69⁺ and CD4⁺ CD69⁻ cells from a normal subject are shown in Fig. 2. CD4⁺ CD69⁺ cells stimulated the anti-CD3 stimulated PBL response, whereas CD4⁺ CD69⁻ cells inhibited this response. Similarly, anti-CD3-stimulated IFN- γ production was increased in the presence of CD4⁺ CD69⁺ cells but was inhibited by CD4⁺ CD69⁻ cells.

The percentage of unstimulated CD4⁺ T cells with a CD4⁺

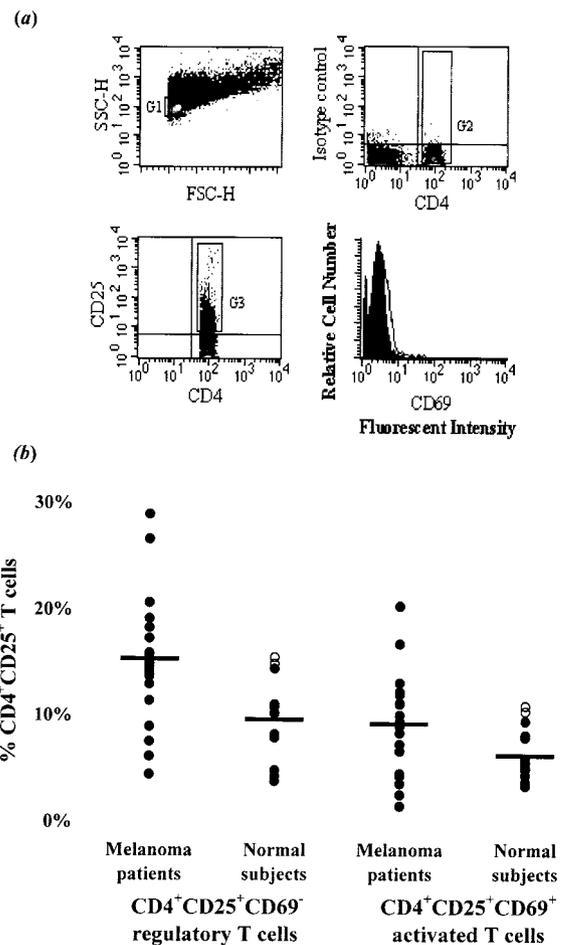


Fig. 1 Examination of CD4⁺CD25⁺CD69⁻ regulatory T cell levels and function in blood from melanoma patients and aged-matched normal subjects. *a*, detection of CD4⁺CD25⁺CD69⁻ regulatory T cells in unstimulated PBLs from a melanoma patient's blood by flow cytometry. *Gate G1*, isolation of lymphocytes; *gate G2*, isolation of CD4⁺ population; *gate G3*, isolation of CD4⁺CD25⁺ population; combined *gates G1 + G2 + G3*, detection by histogram plot of CD69⁺ population in unstimulated CD4⁺CD25⁺ population. *b*, increased levels of CD4⁺CD25⁺CD69⁻ regulatory T cells and CD4⁺CD25⁺CD69⁺ T cells in unstimulated PBLs from melanoma patients detected by three-color flow cytometry. ○, normal subjects undergoing treatment for basal cell carcinoma.

CD25⁺ CD69⁻ or CD69⁺ phenotype in PBLs from 20 melanoma patients and 14 normal subjects is shown in Fig. 1b. The patients and controls used in this study are described in Tables 1 and 2, respectively. There was a significant elevation in the level of CD4⁺ CD25⁺ CD69⁻ regulatory T cells in melanoma patients in comparison with the control subjects tested ($P = 0.007$, by paired t test). The median percentage of CD4⁺ T cells with a CD4⁺ CD25⁺ CD69⁻ phenotype was 14.7% for melanoma patients and 9.1% for normal subjects. Two samples from the control subjects with the above average levels of CD4⁺ CD25⁺ CD69⁻ had been treated for basal cell carcinoma (subjects 15 and 17). There was also a (nonsignificant) trend for CD4⁺ CD69⁺ cells to be increased in patients with melanoma.

The suppressive effects of CD25⁺ CD69⁻ regulatory T

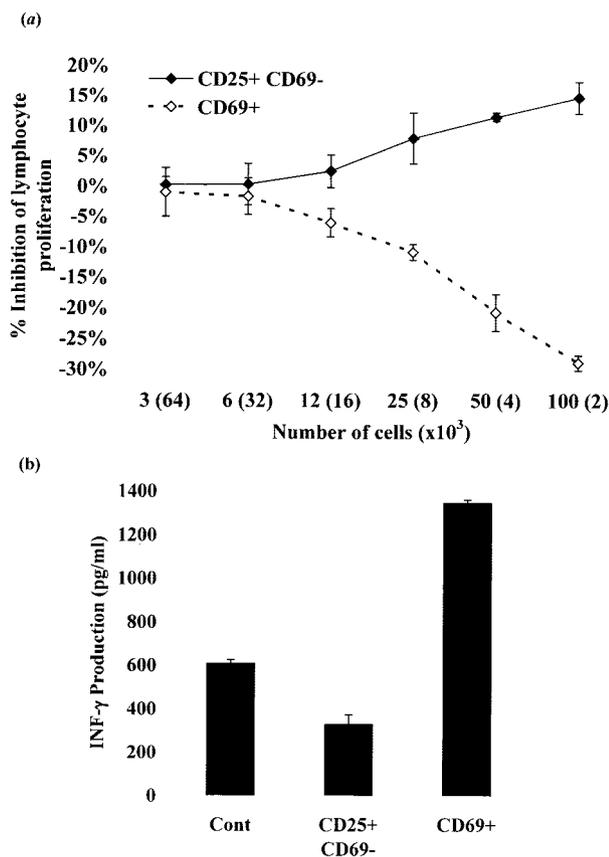


Fig. 2 Effect of CD25⁺CD69⁻ regulatory T cells and CD69⁺ T cells on anti-CD3-stimulated lymphocyte proliferation and IFN-γ production. The purified subpopulations were isolated from unstimulated PBLs from a normal subject and then were pretreated with mitomycin-C before being added to anti-CD3-stimulated PBLs. *a*, CD25⁺CD69⁻ regulatory T cells induced suppression (3210.7 ± 75 cpm at maximum suppression) of lymphocyte proliferation, whereas CD69⁺ T cells induced increased proliferation (4854 ± 240 cpm at maximum proliferation. Control, 3764 ± 98 cpm). The numbers in parentheses, ratio of normal PBLs:CD25⁺CD69⁻ regulatory or CD69⁺ T cells. The proliferative response was measured at 72 h, and percentage inhibition was calculated as stated in "Materials and Methods." *b*, CD25⁺CD69⁻ T cells inhibited IFN-γ production from anti CD3-stimulated-lymphocytes, whereas CD69⁺ T cells induced an increase in IFN-γ production. IFN-γ production was measured by ELISA in triplicate at 24 h.

cells from three melanoma patients were compared with those of similar cells isolated from three normal subjects. As shown in Fig. 3*a*, the addition of CD25⁺CD69⁻ regulatory T cells from the melanoma patients to anti-CD3-stimulated PBLs resulted in a more marked suppression of lymphocyte proliferation on a per-cell basis than did the addition of CD25⁺CD69⁻ regulatory T cells isolated from normal subjects. The suppressive effect was partially inhibited by the MAb against CTLA-4 (Fig. 3*b*).

Expression of H- and L-Ferritin in Plasma of Melanoma Patients. It has been suggested that assay of H-Ferritin may detect abnormal levels of ferritin that may not be evident in assays against L-Ferritin (5). Therefore in the present study, an ELISA was developed using a polyclonal rabbit anti-placenta ferritin capture antibody (which binds both H- and L-Ferritin) combined with MAbs RH02 and LF03, which are specific for H-

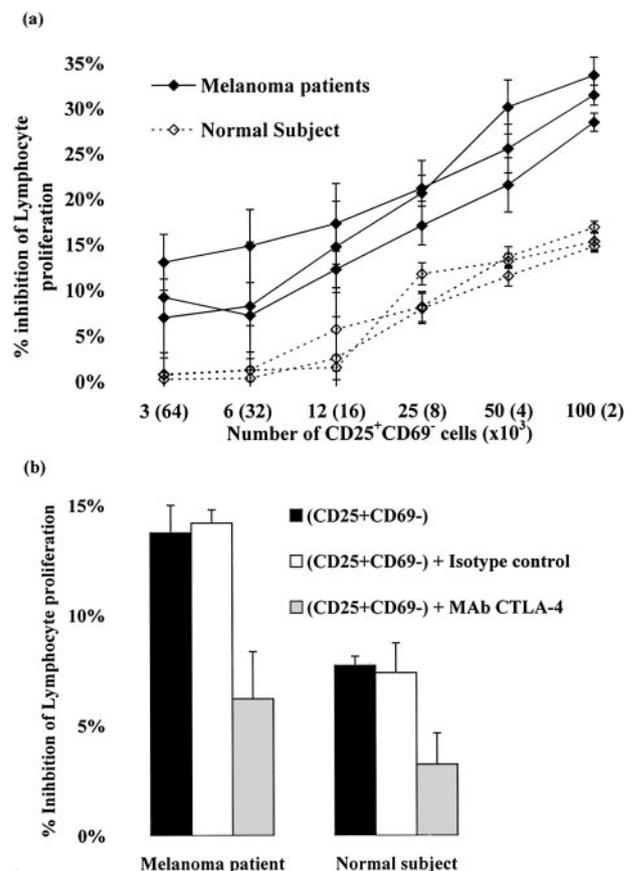


Fig. 3 Comparison of CD4⁺CD25⁺CD69⁻ regulatory T cell function in blood collected from melanoma patients and age-matched normal subjects. *a*, suppression of anti-CD3-stimulated autologous PBL by unstimulated CD25⁺CD69⁻ regulatory T cells isolated from melanoma patients or normal controls. Representative maximum counts in absence of regulatory T cells was 9579 ± 78 cpm for a melanoma patient and 10185 ± 35 for a normal subject. In the presence of the regulatory T cells the counts at maximum suppression were 6453 ± 218 and 8688 ± 42 respectively. The numbers in parentheses indicate ratio of normal PBL:CD25⁺CD69⁻ regulatory T cells. The purified CD25⁺CD69⁻ subpopulations were pretreated with mitomycin-C before being added to anti-CD3-stimulated PBLs. The proliferative response was measured at 72 h, and the percentage inhibition was calculated as stated in "Materials and Methods." *b*, the suppressive effects of CD25⁺CD69⁻ regulatory T cells were partially inhibited by MAb against CTLA-4. MAb against CTLA-4 was prebound to the plate at 10 μg/ml in carbonate buffer overnight at 4°C. Counts per min in presence of CD25⁺CD69⁻ regulatory T cells from melanoma patients was 8317 ± 111 in absence of CTLA-4 and 9195 ± 198 in presence of CTLA-4. Cpm in presence of regulatory T cells from normal subjects in absence and presence of CTLA-4 was 8736 ± 42 and 9006 ± 123 respectively.

and L-Ferritin, respectively. Plasma from blood collected in EDTA was used for the assays to avoid the release of ferritin from red cells in the blood (43).

A representative study on plasma collected from 30 melanoma patients and 19 normal subjects (described in Tables 1 and 2, respectively) is shown in Fig. 4. There was an ~2-fold increase in the expression of H-Ferritin in plasma from melanoma patients compared with the normal subjects ($P = 0.0002$, by paired *t* test; Fig. 4*a*). However there was no significant

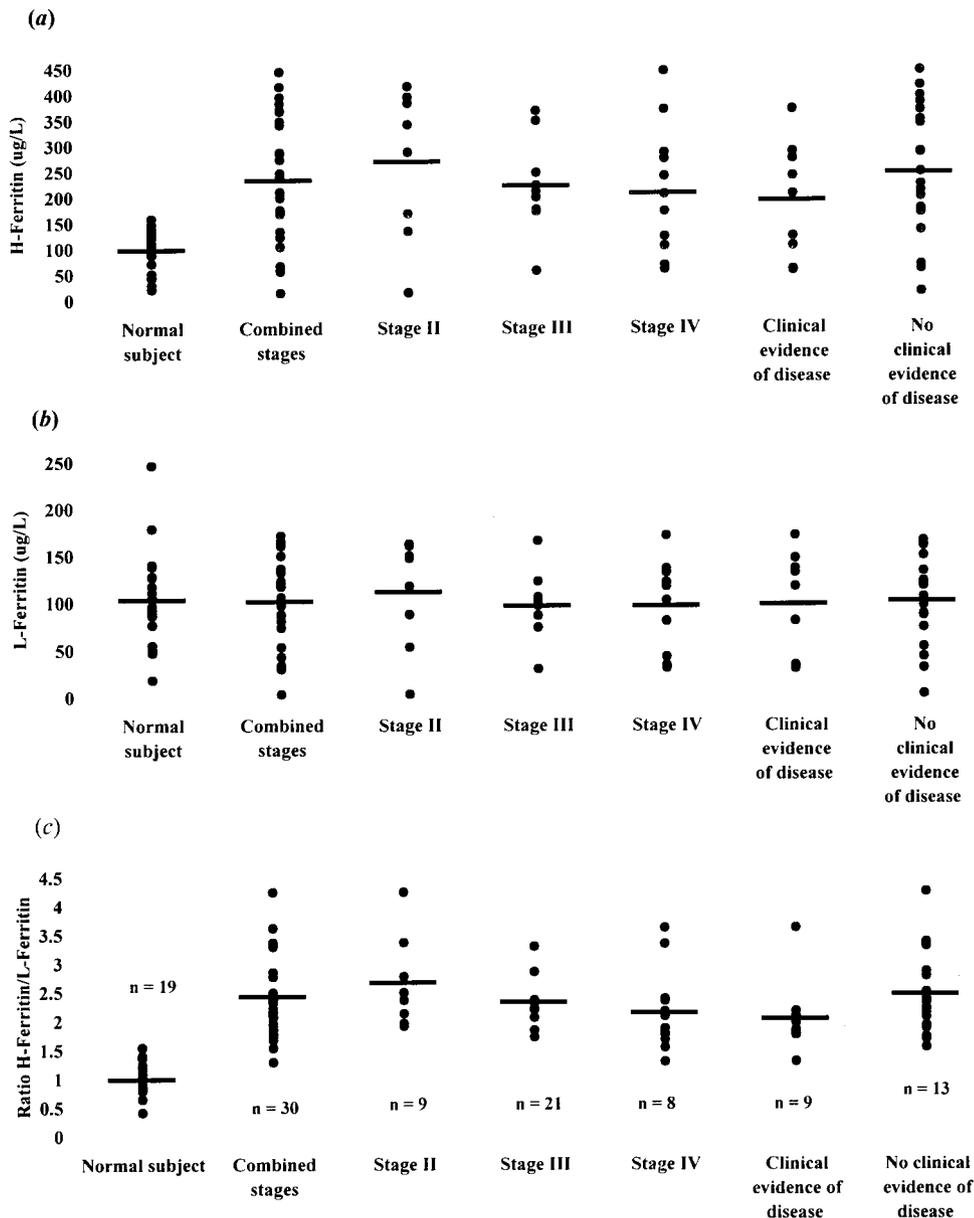


Fig. 4 Correlation between detection of H-Ferritin (a) and L-Ferritin (b) and ratio between H and L-Ferritin (c) in plasma from melanoma patients and stage of disease. *n*, number of patients. The ratio between H-Ferritin and L-Ferritin expressed in the plasma samples was calculated by dividing the concentration of H-Ferritin by the concentration of L-Ferritin.

difference in L-Ferritin expression in the plasma from melanoma patients and normal subjects ($P = 0.7851$; Fig. 4b). The ratio of H-Ferritin:L-Ferritin expressed in the plasma samples was calculated by dividing the total expression of H-Ferritin by the total expression of L-Ferritin. As shown in Fig. 4c, there was a significant difference in the H:L ratio between melanoma patients and normal subjects ($P < 0.0001$).

The expression of H-Ferritin or L-Ferritin or the H:L ratio in relation to clinical evidence or the stage of disease is also shown in Fig. 4. No significant difference was detected between the expressions of H-Ferritin (Fig. 4a), L-Ferritin (Fig. 4b), or the H:L ratio (Fig. 4c) among melanoma patients with and without clinical evidence of disease ($P = 0.339$, $P = 0.951$, and $P = 0.100$ respectively, by paired *t* test).

There was also no significant difference between stage of

disease (using the American Joint Commission on Cancer and the International Union against Cancer Classification) and expression of H-Ferritin (stage II/III, $P = 0.542$; stage II/IV, $P = 0.193$; stage III/IV, $P = 0.836$), L-Ferritin (stage II/III, $P = 0.646$; stage II/IV, $P = 0.512$; stage III/IV, $P = 0.913$) or the H:L ratio (stage II/III, $P = 0.421$; stage II/IV, $P = 0.165$; stage III/IV, $P = 0.470$).

Relation of H-Ferritin in Plasma to Regulatory T Cells in Blood of Patients with Melanoma.

Regression analysis was carried out to determine whether there was a correlation between the numbers of $CD4^+ CD25^+ C69^-$ regulatory T cells and expression of H- and L-Ferritin in melanoma patients or normal subjects (Fig. 5). As shown in Fig. 5, a and b, there was an inverse correlation between the level of $CD4^+ CD25^+ CD69^-$ regulatory T cells and the levels of L-Ferritin, but no correlation with the level of H-Ferritin ($P = 0.416$, $r = 0.025$).

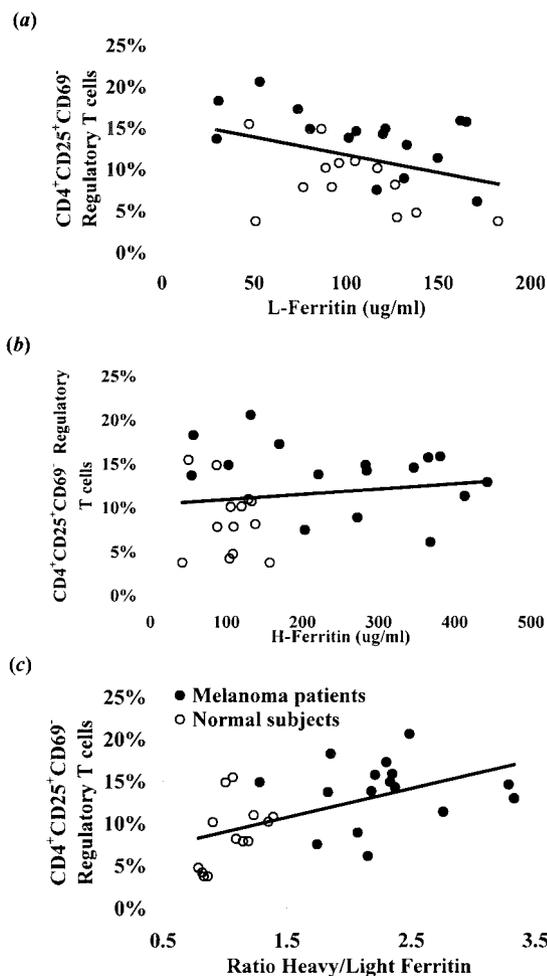


Fig. 5 Regression analysis of the level of $CD4^+CD25^+CD69^-$ regulatory T cells in blood of melanoma patients and normal subjects and in expression in plasma (a) L-Ferritin (melanoma patients, $P = 0.031$, $r = 0.291$; normal subjects, $P = 0.838$, $r = 0.247$; combined $P = 0.040$, $r = 0.146$); b) H-Ferritin (melanoma patients, $P = 0.148$, $r = 0.144$; normal subjects, $P = 0.512$, $r = 0.040$; combined $P = 0.416$, $r = 0.025$) and (c) H-Ferritin:L-Ferritin ratio (melanoma patients, $P = 0.736$, $r = 0.008$; normal subjects, $P = 0.064$, $r = 0.279$; combined, $P = 0.002$, $r = 0.307$).

A positive correlation was also seen between regulatory T cells and the ratio of H-Ferritin:L-Ferritin (Fig. 4c; $P = 0.002$, $r = 0.307$). L-Ferritin binds to H-Ferritin; therefore, the ratio of H-Ferritin:L-Ferritin is a reflection of the amount of free H-Ferritin. The latter results are, thus, consistent with our previous studies *in vitro* showing induction of regulatory T cells by H-Ferritin (22).

Sequential Studies on $CD4^+CD25^+CD69^-$ Regulatory T Cells and H- and L-Ferritin Expression in Melanoma Patients. Additional studies were undertaken to determine whether there was a correlation between the ratio of H-Ferritin:L-Ferritin and regulatory T cells in patients during the course of their disease. H and L-Ferritin expression and numbers of $CD4^+CD25^+CD69^-$ regulatory T cells were assayed in blood samples collected from three AJCC stage-III patients (patients DA,

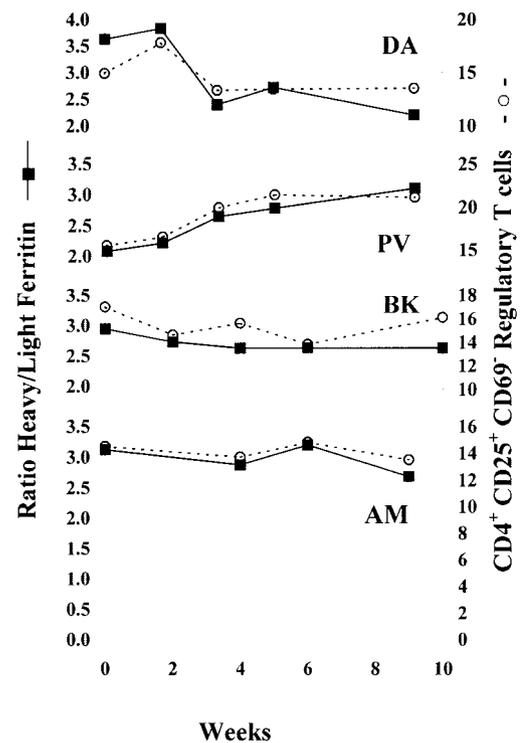


Fig. 6 Sequential expression of $CD4^+CD25^+CD69^-$ regulatory T cells and H- and L-ferritin expression in melanoma patients DA, PV, BK, and AM, showing correlation between the two measures over time *in vivo*.

PV, and AM) and one stage-IIb patient (BK) over a 10–12-week period.

As shown in Fig. 6, the ratio of H-Ferritin:L-Ferritin changed in parallel with the percentage of $CD4^+CD25^+CD69^-$ regulatory T cells in the melanoma patients. There was, however, no consistent change in these parameters during the period of the study. The expression of H-Ferritin/L-Ferritin and the percentage of $CD4^+CD25^+CD69^-$ regulatory T cells decreased in patient DA (Fig. 5a); conversely, in patient PV, there was a small increase in the expression of H-Ferritin/L-Ferritin and the percentage of $CD4^+CD25^+CD69^-$ regulatory T cells (Fig. 5b). In patient BK or AM (Fig. 5, c and d, respectively), no change in expression of H-Ferritin or in the number of $CD4^+CD25^+CD69^-$ regulatory T cells was observed.

DISCUSSION

We have shown previously that H-Ferritin suppresses the response of blood lymphocytes to anti-CD3 *in vitro* by activating $CD4^+$ regulatory T cells in blood (22). The present studies extend these findings by showing that levels of H-Ferritin and regulatory T cells were increased in the circulation of patients with melanoma. Moreover, there was a correlation between the two findings, suggesting that the elevated levels of H-Ferritin may be causally related to the elevated levels of regulatory T cells.

Elevated levels of ferritin have been reported in patients

with renal cell carcinoma (20), neuroblastoma (1, 18), and melanoma (19). In the latter study, elevated levels of ferritin was found in nine patients with disseminated melanoma but not in patients with resected primary or regional lymph node metastases. Most of the assays in these studies were based on antibodies raised against the whole ferritin molecule, most commonly from liver, which is rich in L- rather than H-Ferritin (4). It is, therefore, possible that elevated levels of H-Ferritin may not have been detected in these studies. This would explain why, in the present study, elevated levels of H-Ferritin were detected in melanoma patients in all stages of the disease and not just in patients with advanced progressive disease (19). Another critical factor in such assays is the avoidance of hemolysis during blood collection, which would result in falsely high ferritin levels. This was avoided in the present study by EDTA anticoagulation of the samples. The present studies are consistent with those of Hazard and Drysdale (5) who demonstrated increased levels of H-Ferritin relative to L-Ferritin in plasma samples from patients with breast, ovarian, pancreatic, and stomach carcinomas.

The source of the elevated levels of H-Ferritin remains of much interest. We have previously reported that melanoma supernatants contained elevated levels of H-Ferritin, and we assumed that H-Ferritin in the circulation of patients may be from their melanoma (21). If that were the case, it might be expected that there would be a correlation with the clinical stage of the disease and, particularly, the bulk of the tumor in the patient. This was not the case; and it is, therefore, possible that the source was normal body tissue. Most of the latter, however, is rich in L-Ferritin, which was not elevated in the circulation of melanoma patients. Nonspecific activation of the reticuloendothelial system has been reported to result in abnormal iron metabolism (44). It is not clear whether this is associated with elevated H-Ferritin levels, but, if so, this may explain the present findings. Additional studies are needed on this aspect. A lack of correlation between ferritin levels and disease stage or tumor bulk was reported in studies on patients with lung (45) and renal carcinoma (20).

In view of the association *in vitro* between H-Ferritin and the induction of regulatory T cells (22), it was of much interest to find that a similar correlation existed in patients with melanoma. The association between H-Ferritin and regulatory T cells was also supported by sequential studies on four patients showing parallel changes in both assays over time. The identification of regulatory T cells in patients by MAb against CD4 and CD25 as reported by others (24, 46–48) was considered potentially misleading because normal CD4⁺ T cells may express CD25 when activated by antigen. To avoid this possibility, the activation marker CD69 was used to identify activated CD4 T cells and to exclude them from the analysis using three-color flow cytometry. A small proportion of regulatory T cells may also express this marker; therefore, the results in this study represent a minimal estimate of the number of regulatory T cells in the patients. Even so, it was apparent that up to 30% of CD4⁺ T cells in melanoma patients were regulatory T cells. When tested *ex vivo*, the latter cells appeared to suppress responses of lymphocytes stimulated by anti-CD3 to a greater degree than regulatory T cells from normal subjects, indicating that both the number and their function were elevated in melanoma patients.

The cause of the increased numbers of regulatory T cells in

melanoma patients is not clear. Several studies have indicated that the antigens recognized by CD4⁺ CD25⁺ cells are largely “self” antigens. Interaction of high-affinity thymocytes with self-peptides was shown to induce CD4⁺ CD25⁺ regulatory T cells and appeared distinct from the process of positive selection or deletion (49). Repeated antigen stimulation was also shown to induce regulatory T cells (32, 50). Many of the human melanoma antigens recognized by T cells are self antigens, so that it is possible that repeated exposure to these antigens may account for the high numbers of regulatory T cells in melanoma patients. It is not clear whether H-Ferritin also increases the number of regulatory T cells or whether it is principally involved in activation of the cells as shown *in vitro* (22). Additional studies are needed to examine this question. No consistent change in the H-/L-Ferritin or regulatory T cell levels were seen, but studies on a larger number of patients over longer periods of time are needed to further examine this aspect.

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Clinical Cancer Research

Association of Increased Levels of Heavy-Chain Ferritin with Increased CD4⁺ CD25⁺ Regulatory T-Cell Levels in Patients with Melanoma

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