

# Role of IL-10 for Induction of Anemia During Inflammation<sup>1</sup>

Herbert Tilg,\* Hanno Ulmer,<sup>‡</sup> Arthur Kaser,\* and Günter Weiss<sup>2†</sup>

Anemia is frequently observed in patients suffering from chronic inflammatory disorders. Recent *in vitro* data suggest that Th2 cytokines, such as IL-10, could be involved in its pathogenesis. We analyzed 1) changes in hemoglobin values in 329 patients with chronic active Crohn's disease receiving the anti-inflammatory cytokine IL-10 as part of a randomized, double-blind, placebo-controlled study, 2) serum iron parameters in a subgroup of these patients ( $n = 54$ ), and 3) the *in vitro* effects of IL-10 on ferritin transcription and translation in human monocytic cells (THP-1) by means of Northern blot and immunoprecipitation after metabolic labeling. Patients receiving higher doses of IL-10 developed anemia and presented with a dose-dependent increase of ferritin and soluble transferrin receptor levels, an indicator of iron restriction to erythroid progenitor cells. According to our *in vitro* data, hyperferritinemia may result from direct stimulation of ferritin translation by IL-10 in activated monocytic cells, most likely by cytokine-mediated reduction of the binding affinity of translational repressors, iron-regulatory proteins, to the 5'-untranslated region of ferritin mRNA. In patients, all observed changes were most pronounced at the end of therapy (day +29), and thereafter hemoglobin levels and serum iron parameters returned to baseline levels within 4 wk of follow-up. Our data demonstrate that IL-10 causes anemia in patients with inflammatory bowel disease which may be referred to the induction of imbalances in iron homeostasis by the cytokine, leading to hyperferritinemia and limited iron availability to erythroid progenitor cells, a condition typically seen in the anemia of chronic inflammation. *The Journal of Immunology*, 2002, 169: 2204–2209.

Anemia is frequently observed in patients suffering from chronic inflammatory, infectious, or tumor diseases. This moderate anemia, termed as anemia of chronic disease (ACD),<sup>3</sup> is characterized by typical disturbances of iron homeostasis. Normally, iron is mainly transported in the circulation after binding to the protein transferrin, with one molecule transferrin accepting up to two molecules of iron. This iron-transferrin complex binds to specific cell surface receptors, known as transferrin receptors (TfR), and the resulting TfR-transferrin-iron complex is then taken up by cells via endocytosis. Iron is then released from transferrin and transported by an as yet not fully understood mechanism into the cytoplasm. Either iron is then used for heme biosynthesis or generation of heme/iron enzymes or the metal is incorporated into the protein ferritin, which forms a core structure consisting of 24 subunits of H and L chain ferritin, where it can be stored and from where it can be mobilized in case of an iron demand.

In ACD, the imbalances of iron homeostasis lead to reduced serum iron concentrations and transferrin saturation, whereas ferritin levels, reflecting body iron stores, are often elevated (1–4). In addition, transferrin concentrations are usually normal, and serum TfR levels are slightly increased (5). One central mechanism underlying ACD is a diversion of iron traffic which leads to with-

drawal of iron from the sites of erythropoiesis and the circulation to the storage compartment in the reticuloendothelial system, thus causing at the same time hypoferrinemia and hyperferritinemia. Other mechanisms involved in the pathophysiology of ACD may include marrow progenitor inhibition and a poor response to erythropoietin (EPO). Several cytokines have been demonstrated to affect iron homeostasis by various mechanisms (6, 7). Proinflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , induce hypoferrinemia by modulating macrophage iron metabolism via induction of ferritin biosynthesis (8–11). The Th1 cytokine IFN- $\gamma$  may contribute to ACD mainly by blocking erythropoiesis directly (3); however, its role in iron homeostasis is controversial (6, 7, 12–15). Interestingly, recent *in vitro* evidence has suggested that the anti-inflammatory cytokines IL-4 and IL-13 may contribute to the diversion of iron traffic in ACD by increasing iron uptake and storage in activated macrophages (16). Thus pro- and anti-inflammatory cytokines may collaborate in the development of ACD.

IL-10 suppresses inflammation by various pathways including diminished production of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-8, and toxic radicals such as NO (17). This cytokine has been recently introduced for the treatment of chronic inflammatory conditions such as Crohn's disease (18). On the basis of *in vitro* evidence showing profound effects of anti-inflammatory cytokines on iron homeostasis (16), we assessed in the present study possible effects of rHuIL-10 on iron metabolism *in vivo* and subsequently toward an involvement of the cytokine in the anemia of inflammation.

Divisions of \*Gastroenterology and Hepatology and <sup>†</sup>General Internal Medicine, Department of Medicine, University Hospital Innsbruck, and <sup>‡</sup>Department of Biostatistics, University of Innsbruck, Innsbruck, Austria

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<sup>2</sup> Address correspondence and reprint requests to Dr. Günter Weiss, Department of Internal Medicine, University Hospital, Anichstrasse 35, A-6020 Innsbruck, Austria. E-mail address: guenter.weiss@uibk.ac.at

<sup>3</sup> Abbreviations used in this paper: ACD, anemia of chronic disease; CACD, chronic active Crohn's disease; EPO, erythropoietin; TfR, transferrin receptor; IRP, iron-regulatory protein; sTfR, soluble TfR; rHuIL-10, recombinant human IL-10; LDH, lactate dehydrogenase.

## Materials and Methods

### Patients

A multicenter, randomized, double-blind, placebo-controlled study was conducted in patients with chronic active Crohn's disease (CACD). Patients had to be between 18 and 65 years of age and to have active steroid-resistant Crohn's disease involving the colon or both the ileum and colon, with or without external fistula. Active disease was defined as having a Crohn's disease activity index of between 200 and 400 despite treatment with prednisone (10–40 mg/day for at least 3 mo) before study given alone or in combination with 6-mercaptopurine or azathioprine. These therapies

had to be stable during the study. Patients were allowed to take aminosalicylates and/or antibiotics, providing that the used dosages were kept stable during the study. The protocols and consent procedure were approved by the local medical ethics committees.

### Study design

One of four doses (1, 4, 8, and 20  $\mu\text{g}/\text{kg}$  body weight) of IL-10 (Schering Plough, Kenilworth, NJ) was administered once daily s.c. during 28 consecutive days. A total of 329 patients were included in the study. Patients were screened for eligibility during a period of 14 days. Therapy was initiated at day +1. The study medication was administered at the same time of the day during the study period. After the 28-day treatment period, patients were followed up for 4 wk.

### Measurement of hemoglobin and hematocrit

A total of 329 patients were analyzed for changes in hemoglobin and hematocrit concentrations during the study. The study population included 66 patients in the placebo group, 67 patients in the 1- $\mu\text{g}/\text{kg}$  group, 64 patients in the 4- $\mu\text{g}/\text{kg}$  group, 65 patients in the 8- $\mu\text{g}/\text{kg}$  and 67 patients in the 20- $\mu\text{g}/\text{kg}$  body weight IL-10 group. Analyses were performed twice in the pretreatment period, on days +8, +15, and +29 during therapy, and at wk 2 and 4 of follow-up.

### Measurement of iron, transferrin, transferrin saturation, soluble TfR (sTfR), ferritin, and hematological laboratory variables

Serum levels of iron, transferrin, sTfR, and ferritin were analyzed in a subgroup of the patients described above consisting of 10 patients treated with placebo, 12 patients treated with 1  $\mu\text{g}/\text{kg}$ , 12 patients treated with 4  $\mu\text{g}/\text{kg}$ , 10 patients treated with 8  $\mu\text{g}/\text{kg}$ , and 10 patients treated with 20  $\mu\text{g}/\text{kg}$  body weight IL-10 s.c. for 28 days. Serum levels of iron, transferrin, sTfR, and ferritin were assessed on days -1, +15, and +29 of therapy and after 4 wk of follow-up. Serum levels of transferrin and ferritin were determined using an immunoturbidimetric assay (both from Roche Diagnostics, Mannheim, Germany) and sTfR was assessed using a specific ELISA (Orion Diagnostics, Helsinki, Finland). Serum EPO concentrations were determined by ELISA (DRG Diagnostics, Marburg, Germany), lactate dehydrogenase (LDH) as well as reticulocyte, erythrocyte, and platelet counts were assessed by automated laboratory test systems.

### In vitro studies

THP-1 cells, a human myelomonocytic cell line, were investigated in this study. Cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (Life Technologies, Vienna, Austria) at 37°C in humidified air containing 5%  $\text{CO}_2$ . Cells were treated with 100–500 U/ml of human rIFN- $\gamma$  (Life Technologies) for 24 h, with no further supplements or after preincubation with rHuIL-10 (Schering-Plough) for 1 h. Treatment with the indicated concentrations of IFN- $\gamma$  and/or IL-10 did not result in reduced viability of cells in comparison with untreated controls as checked by trypan blue exclusion (data not shown).

### Northern blotting

After 24 h, cells were harvested and washed, and RNA was extracted by a guanidinium thiocyanate method and further subjected to Northern hybridization exactly as described elsewhere (16).

### Metabolic labeling and immunoprecipitation

THP-1 cells ( $1 \times 10^7$ ) were stimulated with cytokines as described above for 24 h. After this, cells were washed twice with methionine-free medium and labeled with [ $^{35}\text{S}$ ]methionine (50  $\mu\text{Ci}$ ) for 2 h. Quantitative immunoprecipitation of equal amounts of trichloroacetic acid-insoluble radioactivity was conducted with polyclonal ferritin Abs (Roche Diagnostics, Indianapolis, IN) and analyzed by 12.5% SDS-PAGE with subsequent autoradiography as described (16).

For determination of intracellular ferritin content, cell extracts were prepared by repeated freezing and thawing, and ferritin concentrations were then determined in the supernatants using a commercially available ELISA (DRG Diagnostics).

### Statistical analysis

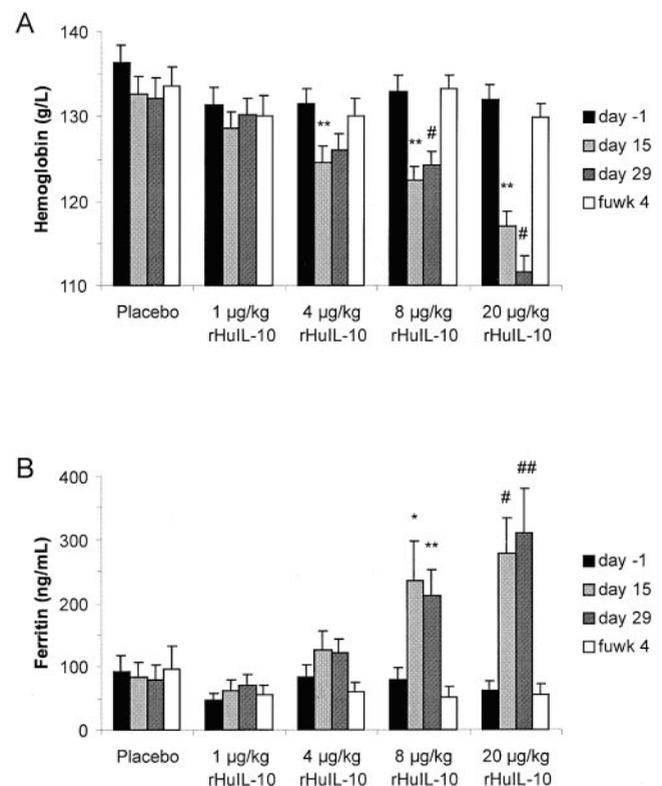
ANOVA for repeated measurements was used to analyze differences in means between and within treatment groups. Hypotheses were tested two-sided on a global significance level of 0.05. Post hoc multiple comparisons between IL-10 groups and placebo were performed with Dunnett's *t* test. Descriptive statistics are presented using means  $\pm$  SEM. Comparisons

within groups were assessed by ANOVA. Calculation of correlations was assessed by Spearman rank correlation technique using Bonferroni correction for multiple tests.

## Results

### Dose-dependent decrease of hemoglobin and hematocrit values during therapy with IL-10

In a total of 329 patients, hemoglobin and hematocrit values were evaluated before IL-10 treatment, on days +15 and +29 of therapy and at 4 wk of follow-up. As hematocrit values paralleled hemoglobin values, only hemoglobin values are reported. Pretreatment hemoglobin values did not differ significantly between the various treatment groups (Fig. 1A). Although no changes in hemoglobin levels during treatment were observed in the placebo group and in patients receiving the lowest dose of IL-10 (1  $\mu\text{g}/\text{kg}$ ), treatment with higher dosages of IL-10 ( $\geq 4$   $\mu\text{g}/\text{kg}$ ) resulted in a highly significant decrease of hemoglobin concentration (Fig. 1A). This effect was dose dependent and most pronounced in patients receiving 20  $\mu\text{g}/\text{kg}$  body weight IL-10. In these patients, a significant



**FIGURE 1.** A, Hemoglobin values in patients with CACD treated with placebo ( $n = 66$ ) or 1  $\mu\text{g}/\text{kg}$  ( $n = 67$ ), 4  $\mu\text{g}/\text{kg}$  ( $n = 64$ ), 8  $\mu\text{g}/\text{kg}$  ( $n = 65$ ), or 20  $\mu\text{g}/\text{kg}$  body weight rHuIL-10 (IL-10) ( $n = 67$ ) s.c. during 28 days. Data obtained before (day -1), after 2 wk (day 15), and at the end of treatment (day 29) and 4 wk after end of treatment (follow-up, fuwk 4) are presented as means  $\pm$  SEM. Multiple comparisons between the different dose levels and placebo were performed with a two-sided Dunnett test (day 15: \*\*,  $p < 0.005$  compared with placebo and  $p < 0.001$  comparing the 20- $\mu\text{g}/\text{kg}$  dose level with all other groups; day 29: #,  $p < 0.05$  comparing the 8- $\mu\text{g}/\text{kg}$  dose level with placebo and  $p < 0.001$  comparing the 20- $\mu\text{g}/\text{kg}$  dose level with all other groups). B, Ferritin values (nanograms per milliliter) in a subgroup of patients with CACD treated with placebo ( $n = 10$ ) or 1  $\mu\text{g}/\text{kg}$  ( $n = 12$ ), 4  $\mu\text{g}/\text{kg}$  ( $n = 12$ ), 8  $\mu\text{g}/\text{kg}$  ( $n = 10$ ), or 20  $\mu\text{g}/\text{kg}$  body weight rHuIL-10 (IL-10) ( $n = 10$ ) s.c. during 28 days. \*,  $p < 0.05$  compared with the placebo and 1  $\mu\text{g}/\text{kg}$  rHuIL-10 dose group; \*\*,  $p < 0.05$  compared with the placebo and 1  $\mu\text{g}/\text{kg}$  rHuIL-10 dose group; #,  $p = 0.009$  compared with placebo and 1 and 4  $\mu\text{g}/\text{kg}$  rHuIL-10; ##,  $p = 0.006$  compared with placebo and 1 and 4  $\mu\text{g}/\text{kg}$  IL-10.

Table I. Serum levels of ferritin and sTfR in patients with CACD treated with placebo (n = 10), 1 µg/kg (n = 12), 4 µg/kg (n = 12), 8 µg/kg (n = 10), or 20 µg/kg body weight IL-10 (n = 10) s.c. over 28 days<sup>a</sup>

	Pretreatment	Day +15	Day +29	p	Follow-up wk 4
<b>Ferritin (ng/ml)</b>					
Placebo	91.3 ± 25.7	84.3 ± 23.5	79.2 ± 23.1	NS	97.3 ± 34.5
1 µg/kg	47.1 ± 10.0	61.7 ± 18.1	69.7 ± 18.1	NS	55.3 ± 16.3
4 µg/kg	83.3 ± 20.4	125.2 ± 30.1	122.9 ± 20.6	0.03	60.2 ± 14.6
8 µg/kg	80.0 ± 17.6	235.6 ± 61.5	212.2 ± 40.5	0.002	51.2 ± 16.5
20 µg/kg	62.0 ± 14.6	279.1 ± 55.4	310.6 ± 71.1	0.003	55.3 ± 16.8
<b>sTfR (mg/L)</b>					
Placebo	4.3 ± 0.5	4.8 ± 0.5	4.2 ± 0.4	NS	4.1 ± 0.5
1 µg/kg	5.4 ± 0.9	5.6 ± 0.9	5.9 ± 0.8	NS	5.6 ± 0.9
4 µg/kg	4.5 ± 0.6	5.4 ± 0.8	5.2 ± 0.6	NS	4.9 ± 0.6
8 µg/kg	4.3 ± 0.5	5.1 ± 0.7	5.9 ± 0.6	NS	5.0 ± 0.8
20 µg/kg	4.4 ± 0.4	5.2 ± 0.5	7.2 ± 0.8	0.004	5.1 ± 0.7

<sup>a</sup> Data are presented as means ± SEM, and statistical analysis of trends during IL-10 treatment were assessed by ANOVA.

decrease in hemoglobin developed even within 1 wk of cytokine treatment, and hemoglobin levels further decreased progressively, reaching the lowest values at the end of therapy (day +29) (Fig. 1A). One patient randomized to receive 20 µg/kg IL-10 developed anemia (hemoglobin, 6.5 mg/dl) at day +15 of therapy (pretreatment value 12.1 mg/dl) and received a blood transfusion. Because this patient achieved clinical remission, she was kept on IL-10 and her anemia recovered at follow-up wk 2. Hemoglobin values recovered 4 weeks after discharge of therapy in all IL-10 treatment groups, and hemoglobin values were comparable with those before initiation of therapy (Fig. 1A).

#### Changes in iron metabolism during therapy with IL-10

To see whether the observed decrease in hemoglobin levels and the development of anemia in patients receiving IL-10 are paralleled by disturbances of iron homeostasis, we investigated serum parameters of iron metabolism. In patients receiving 4 µg/kg IL-10 or more, serum ferritin levels increased within 2 wk (Fig. 1B and Table I), and the maximum differences were observed at the end of therapy (day +29) as compared with patients receiving placebo (Fig. 1B and Table I). Although there was a significant increase in ferritin levels during therapy in patients receiving 4 µg/kg IL-10 as estimated by ANOVA (Table I), differences in ferritin concentrations at day +15 and day +29 as compared with patients treated with placebo at the same time points became significant only in the 8-µg/kg and 20-µg/kg IL-10 group as estimated by Dunnett's test (Fig. 1B). After the end of therapy, ferritin levels returned to normal or to pretreatment levels within 4 wk (Fig. 1B and Table I).

In patients receiving the highest IL-10 dosage, the observed elevation of ferritin concentrations was succeeded by a constant in-

crease of sTfR levels which is an indicator for depletion of metabolically available tissue iron (5, 19) (Table I). However, sTfR increased with a much slower kinetics than ferritin levels showing significant differences as compared with pretreatment sTfR levels only at the end of therapy (day +29;  $p < 0.05$ ). Thereafter, sTfR levels returned to pretreatment levels (Table I). No significant changes or trends were observed for serum iron, serum transferrin levels, total iron-binding capacity, and transferrin saturation in either treatment group (details not shown).

#### Chancing associations between hemoglobin and iron parameters under IL-10 therapy

To determine whether the changes in ferritin, sTfR, and hemoglobin levels may be interconnected, we separately calculated Spearman rank correlations in patients receiving IL-10 at dosages  $\geq 8$  mg/ml and in patients receiving placebo or the lowest dose of IL-10 (1 µg/kg). In both groups, sTfR levels were inversely related to hemoglobin concentrations, which supports the role of sTfR in indicating iron availability to erythroid progenitor cells, with sTfR being high when iron supply to progenitor cells is limited (5, 20). When investigating the interactions of ferritin with hemoglobin and sTfR, contrasting differences become evident between the two groups. Whereas ferritin is positively related to hemoglobin levels in placebo patients (Table II) a significant inverse relationship is found in patients receiving IL-10 at higher dosages (Table II). Moreover, whereas sTfR was negatively correlated to ferritin levels in the placebo group, just the opposite was true in patients receiving IL-10 (8 µg/dl) (Table II).

Table II. Spearman rank correlations between hemoglobin and indicators of iron homeostasis during IL-10 therapy

	Placebo or Low IL-10 Dose Level (IL-10 1 µg/kg Body Wt) Group <sup>a</sup>					Patients Receiving IL-10 $\geq 8$ µg/kg Body Wt <sup>b</sup>				
	Hb 2	Hb 3	Ferritin 2	Ferritin 3	sTfR2	Hb 2	Hb 3	Ferritin 2	Ferritin 3	sTfR 2
Ferritin 2 <sup>c</sup>	0.23	0.41				-0.69 <sup>#</sup>	-0.87 <sup>§</sup>			
Ferritin 3	0.21	0.43	0.87 <sup>§</sup>			-0.56 <sup>#</sup>	-0.86 <sup>§</sup>	0.90 <sup>§</sup>		
sTfR 2	-0.47	-0.55 <sup>#d</sup>	-0.54 <sup>#</sup>	-0.59 <sup>#</sup>		-0.87 <sup>§</sup>	-0.68 <sup>#</sup>	0.79 <sup>#</sup>	0.47	
sTfR 3	-0.62 <sup>#</sup>	-0.57 <sup>#</sup>	-0.42	-0.49	0.82 <sup>§</sup>	-0.80 <sup>§</sup>	-0.70 <sup>#</sup>	0.86 <sup>§</sup>	0.63 <sup>#</sup>	0.83 <sup>§</sup>

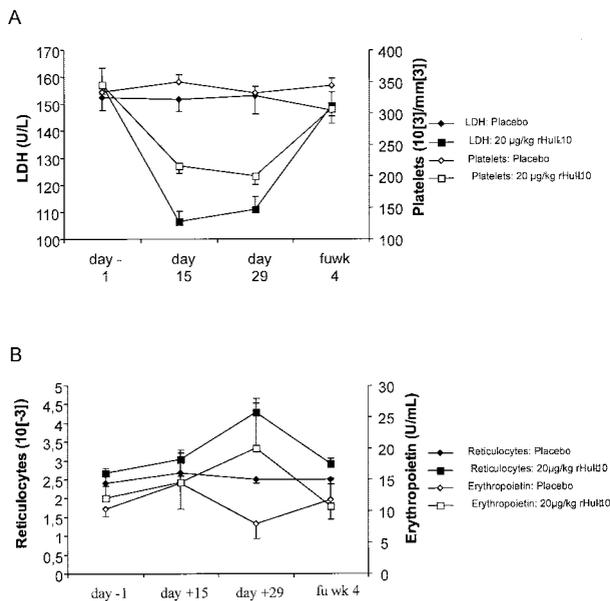
<sup>a</sup> Because patients receiving the lowest dose level of IL-10 and controls were not different from each other with respect to changes of hemoglobin levels and parameters of iron metabolism, they were analyzed together to increase the statistical power of the calculations and to avoid biasing of results by a few outliers ( $n = 22$ ). Trends were almost the same when analyzing only patients receiving placebo (details not shown).

<sup>b</sup> This group consisted of patients receiving the two highest dose levels of IL-10 ( $n = 20$ ).

<sup>c</sup> Ferritin 2, ferritin levels at day 15 of therapy; ferritin 3, ferritin levels at end of therapy (day +29). The same applies to hemoglobin (Hb) and sTfR levels.

<sup>d</sup> Spearman rank correlation coefficients are shown.

#,  $p < 0.05$ ; §,  $p < 0.01$ .



**FIGURE 2.** Effects of IL-10 treatment on various laboratory and hematological parameters. Patients with CACD were treated with placebo ( $n = 66$ ) or 1  $\mu\text{g}/\text{kg}$  ( $n = 67$ ), 4  $\mu\text{g}/\text{kg}$  ( $n = 64$ ), 8  $\mu\text{g}/\text{kg}$  ( $n = 65$ ) or 20  $\mu\text{g}/\text{kg}$  body weight rHuIL-10 ( $n = 67$ ) s.c. during 28 days. Values from patients treated with placebo ( $\blacklozenge$  and  $\diamond$ ) or 20  $\mu\text{g}/\text{kg}$  ( $\blacksquare$  and  $\square$ ) are shown before (day -1), after 2 wk (day 15), and at the end of treatment (day 29) and 4 wk after end of treatment (follow-up, fu wk4) are presented as means  $\pm$  SEM. *A*, LDH serum concentrations ( $\blacklozenge$  and  $\blacksquare$ ) and platelet counts ( $\diamond$  and  $\square$ ). *B*, Reticulocyte counts ( $\blacklozenge$  and  $\blacksquare$ ) and EPO levels ( $\diamond$  and  $\square$ ).

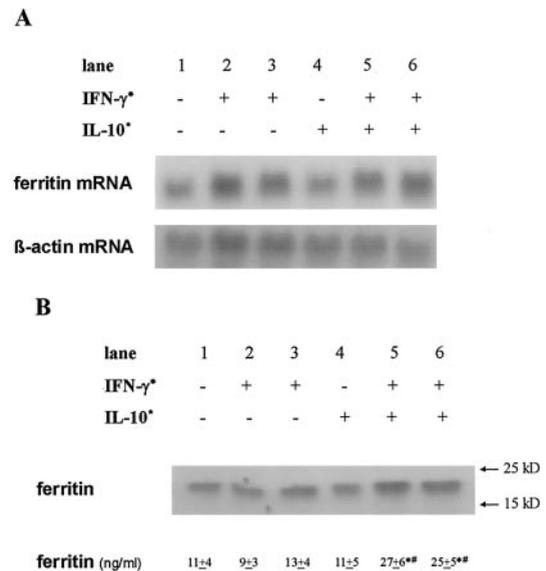
#### Effects of IL-10 on hematological laboratory variables

To study whether or not other mechanisms may also be involved in the development of anemia in patients treated with IL-10, we retrospectively analyzed additional hematological parameters. Neither mean corpuscular hematocrit nor mean corpuscular volume changed in patients receiving placebo or IL-10 at the dosages used (details not shown). To examine a possible hemolysis-inducing effect of IL-10, we analyzed serum levels of LDH. As demonstrated in Fig. 2*A*, LDH concentrations did not change significantly over time in control patients, whereas a significant reduction during therapy was observed in patients receiving the highest dosage of IL-10 (20  $\mu\text{g}/\text{kg}$ ).

The reduction in LDH levels during IL-10 therapy is paralleled by a decrease in platelet counts. With the highest dose level of IL-10, we observed a significant drop of platelet counts immediately after onset of therapy ( $p < 0.001$ ). Platelet concentrations then remained low during therapy and returned to normal after the end of IL-10 treatment (Fig. 2*A*). No changes in platelet counts were observed in patients receiving placebo.

We then analyzed a possible effect of IL-10 on erythropoiesis. When investigating reticulocyte counts, no significant changes over time were observed in patients receiving placebo (Fig. 2*B*). In patients receiving the highest dose level of IL-10, reticulocyte numbers progressively increased during IL-10 treatment ( $p < 0.001$  by ANOVA) and were almost 2-fold higher at the end of therapy (day +29,  $0.45 \pm 0.03\%$ ) than at baseline (Fig. 2*B*). After the end of therapy, reticulocyte counts returned to pretreatment values during 4 wk of follow-up (Fig. 2*B*).

We then investigated the possibility that IL-10 may affect the endogenous production of EPO. EPO levels increased significantly over time in patients receiving the highest IL-10 dosages ( $p < 0.01$  by ANOVA) and paralleled the increase in reticulocyte counts (Fig. 2*B*). Moreover, EPO concentrations in the IL-10 treatment



**FIGURE 3.** Effects of IL-10 on ferritin expression in activated monocytic cells. THP-1 cells were stimulated with IFN- $\gamma$  (100 U/ml, lanes 2 and 5; or 500 U/ml, lanes 3 and 6) for 24 h after no pretreatment or preincubation with IL-10 (120 U/ml = 10 ng/ml, lanes 5 and 6). As controls, cells were left untreated (lane 1) or were treated with IL-10 alone (lane 4). *A*, RNA was extracted and probed for ferritin mRNA (upper panel). As an internal control, cohybridization with the housekeeping gene  $\beta$ -actin was performed (lower panel). One of three independent experiments is shown. *B*, THP-1 cells were treated as described above, and metabolic labeling and immunoprecipitation for ferritin were conducted as described in *Materials and Methods*. One of four independent experiments is shown. Intracellular content of ferritin was determined in cell extracts by ELISA. Results are shown as means  $\pm$  SD for five independent experiments performed in duplicates. \*,  $p < 0.05$  as compared with control; #,  $p < 0.05$  as compared with the respective IFN- $\gamma$  treatment without IL-10.

group were inversely correlated to hemoglobin levels at these time points ( $p < 0.001$  at day +29 as estimated by Spearman rank correlation). EPO levels returned to normal after the end of IL-10 therapy and the subsequent normalization of hemoglobin levels.

#### In vitro effects of IL-10 on ferritin production in human monocytic cells

To see whether or not changes in ferritin levels observed in vivo may be caused directly by IL-10, we studied the effects of IL-10 on ferritin synthesis in activated THP-1 cells. Treatment of THP-1 cells with IFN- $\gamma$  slightly increased ferritin mRNA levels (Fig. 3*A*), whereas IL-10 added alone had no effect on ferritin mRNA levels compared with controls. Moreover, IL-10 did not counteract the slightly increased ferritin mRNA expression observed after IFN- $\gamma$  treatment when THP-1 cells were incubated with IL-10 before stimulation with IFN- $\gamma$  (Fig. 3*A*).

When investigating ferritin de novo synthesis by means of metabolic labeling and immunoprecipitation, we found that although IFN- $\gamma$  increased ferritin mRNA levels, ferritin synthesis was not significantly changed as compared with untreated control cells (Fig. 3*B*). IL-10 alone did not affect ferritin translation as compared with the control (lane 4). However, preincubation of IFN- $\gamma$ -stimulated cells with IL-10 led to an increase of ferritin de novo synthesis (Fig. 3, lanes 5 and 6) as compared with cells treated with IFN- $\gamma$  alone (Fig. 3, lanes 2 and 3).

These results were confirmed on determination of intracellular ferritin concentrations by an ELISA-based assay. The increased de novo synthesis of ferritin in cells treated with IL-10 before IFN- $\gamma$

stimulation (Fig. 3, lanes 5 and 6) was paralleled by significantly higher amounts of ferritin in cytoplasmic cell extracts than in cells treated with IFN- $\gamma$  alone. These results go along with a posttranscriptional regulation of ferritin expression by IL-10 via modulation of iron-regulatory protein (IRP)-controlled ferritin mRNA translation.

## Discussion

In patients with chronic inflammatory disorders, clinicians frequently observe anemia, which was thus called ACD. Although this type of anemia is the most frequent one observed in hospitalized patients, the underlying mechanisms remain still elusive. Several factors are believed to play a role in its pathophysiology. The most important are 1) a diversion of iron traffic, thus causing withdrawal of iron from the sites of erythropoiesis and storage of the metal in the reticuloendothelial system, 2) a suppressed erythropoiesis which is mainly referred to a direct effect of IFN- $\gamma$  on the proliferation of erythroid progenitor cells, 3) erythrophagocytosis which may also contribute to these imbalances in iron distribution, and 4) a blunted response to EPO (for review, see Refs. 1–4).

Our data now suggest that the anti-inflammatory cytokine IL-10, which is up-regulated in most inflammatory disorders of the body, alters iron metabolism *in vivo*, induces anemia, and may thus be involved in the pathogenesis of ACD.

Previous studies have suggested that proinflammatory cytokines, such as IL-1, IL-6, or TNF- $\alpha$  cause disturbances of iron homeostasis. These cytokines induce hypoferrremia by increasing ferritin synthesis via both transcriptional and translational mechanisms in various cell types (8–11). However, IL-1 $\beta$  and TNF- $\alpha$  are at the same time potent inducers of IL-10 formation *in vitro* and *in vivo* (17). Thus, it would appear also reasonable that the observed effects of IL-1 $\beta$  and TNF- $\alpha$  on ferritin synthesis *in vitro* and *in vivo* can be in part referred to induction of IL-10 synthesis by these proinflammatory cytokines.

Recent observations demonstrated that IL-4 and IL-13, both cytokines with an anti-inflammatory profile, modulate iron metabolism in activated macrophages by different pathways (16): 1) by opposing activation of IRP thus increasing ferritin translation; and 2) by an IRP-independent augmentation of TfR mRNA expression. Thus, Th2-derived cytokines are able to increase iron uptake and storage in activated macrophages. These *in vitro* data are in accordance with our *in vivo* findings in patients with CACD treated with IL-10, another Th2-derived anti-inflammatory cytokine, and are further confirmed by our *in vitro* data demonstrating that IL-10 increases ferritin expression in activated/IFN- $\gamma$ -treated monocytic cells although mRNA levels are unchanged (Fig. 3). This suggests that, as in murine macrophages, IL-10 increases ferritin translation in human monocytes by decreasing the binding affinity of IRPs to the ferritin iron-responsive element.

This leads to the question of whether the induction of hyperferritinemia by IL-10 and the development of anemia are cause effective. Such a notion is supported by the results obtained by calculation of Spearman rank correlations (Table II). In patients receiving placebo, hemoglobin concentrations were associated positively with ferritin and negatively with sTfR, indicating that under these conditions sufficient erythropoiesis depends on a normal supply of iron to erythroid progenitor cells. This is also reflected by the negative association between sTfR and ferritin, given that high sTfR indicated low iron availability to erythroid cells as does low ferritin under normal conditions (Table II).

In contrast, in patients receiving IL-10 therapy, we found ferritin to be associated negatively with hemoglobin levels and positively with sTfR concentrations. Moreover, we demonstrated that ferritin levels at day 15 were more closely associated to hemoglobin and

sTfR levels at day 29 than the opposite, namely, correlation of hemoglobin or sTfR levels at day 15 with ferritin levels at day 29 (Table II). This provides strong evidence that an IL-10-induced increase in serum ferritin concentrations leads to a subsequent decrease of iron availability to erythroid cells as indicated by a rise in sTfR levels and the development of anemia according to a cause and effect relationship.

Anemia may result from stimulation of ferritin translation by IL-10 in cells of the reticuloendothelial system and consecutive incorporation of metabolically available iron into the protein (16). This decreases the amount of metabolically available iron to erythroid progenitor cells, thus resulting in impaired heme biosynthesis and subsequent development of anemia. However, one might claim that we did not observe significant changes in serum iron levels or transferrin saturation, although ferritin and sTfR levels progressively increased during IL-10 therapy. This could be because: 1) sTfR is a more sensitive parameter for reflecting the need of iron in tissues and/or the bone marrow than serum iron levels and transferrin saturation (5); or 2) the increase of sTfR levels observed in patients treated with the highest IL-10 dosage may also be influenced by a regulatory effect of IL-10 on TfR mRNA expression as indicated from *in vitro* data (16).

Nevertheless, other mechanisms by which IL-10 could induce anemia have also been taken into account.

First, there is the possibility that like IL-6 the application of IL-10 may cause a dilution anemia even if ferritin levels increase (20). However, because neither mean corpuscular hematocrit nor mean corpuscular volume changed significantly during therapy, nor did patients under IL-10 therapy had a significant gain in weight (details not shown), this is a rather unlikely explanation.

Second, IL-10 could cause growth-depressing effects toward erythroid progenitor cells. Such a possibility is supported by *in vitro* data showing direct negative effects of IL-10 toward erythroid progenitor cell proliferation (21) and by the observations that ferritin may also inhibit cell proliferation (22).

However, reticulocyte levels did not change over the first 2 wk of treatment and then progressively increased under IL-10 therapy. This increase in reticulocyte counts was paralleled by an increase of endogenous EPO levels, the latter being negatively associated with hemoglobin concentrations at these time points (Fig. 2B). This suggests that the increase of EPO may result from the physiological response to anemia/hypoxia which then leads to stimulation of reticulocyte proliferation.

These observations argue against a strong inhibitory effect of IL-10 toward erythroid cell proliferation and against an inhibition of EPO production by IL-10. This is supported by a recent finding showing that the administration of IL-10 to healthy volunteers in a dose of 8  $\mu$ g/kg did not result in a change of granulocyte-macrophage CFUs, mixed lineage CFUs (granulocyte-macrophage-monocyte-CFUs); or erythroid burst-forming units when comparing the IL-10- vs placebo-treated groups (23).

However, a mild antiproliferative effect of IL-10 toward erythroid progenitor cells cannot be fully excluded by our investigation (see below). Such an effect could be exerted directly by IL-10 or indirectly via induction of ferritin or cytokine synthesis, a notion which is supported by the finding that in high doses IL-10 induces the formation of IFN- $\gamma$  (24), a potent inhibitor of erythroid progenitor cell development (3).

Third, we also investigated whether or not IL-10 may induce hemolysis by monitoring LDH levels. In contrast to what would be expected on hemolysis, IL-10 significantly reduced LDH levels. At the same time, a substantial decline in platelet numbers was observed (Fig. 2A), whereas white blood cell counts were not significantly affected by IL-10 therapy (not shown). This suggests that

IL-10 may not cause hemolysis but rather negatively affects the possibility of megakaryopoiesis via modulation of the activity or formation of proteins needed for stimulation of platelet formation, such as thrombopoietin, or GM-CSF or by influencing platelet pooling in the spleen (23, 25).

Fourth, recent data provide evidence that IL-10 induces the expression of heme oxygenase 1 (26). This may lead to excessive heme degradation, an increase in the amount of iron within monocytes, and a subsequent incorporation of the metal into ferritin which may thus contribute to iron retention within the reticuloendothelial system and the development of anemia.

In summary, our data provide direct evidence for an involvement of IL-10 in the pathogenesis of anemia of inflammation. This can be mainly referred to a direct effect of IL-10 on ferritin translation and presumably subsequently storage of iron within activated monocytes/macrophages which may limit the availability of iron to erythroid progenitor cells. The cause and effect relationship of IL-10 for inducing hyperferritinemia and anemia is sustained by the finding that after the end of cytokine therapy all abnormalities in iron homeostasis and anemia returned to normal without any further intervention.

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