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CORRESPONDENCE

Iron Overload and Anemia in Transferrin Immune Complex Disease, an Overlooked Monoclonal Gammopathy of Clinical Significance

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To the Editor,

Transferrin-Immune Complex Disease (TICD) is an acquired monoclonal gammopathy characterized by the presence of anti-transferrin (TF) autoantibodies, leading to remarkable hypertransferrinemia and hypersideremia [1–8]. The condition is not benign since TICD patients present a high risk of developing hemochromatosis and multiple myeloma. Anemia might contemporaneously occur. So far, few cases of TICD have been reported (Table 1), but the disease's frequency is likely underestimated. Here, we identified two novel TICD cases (patients 10 and 11, Table 1) and reported mechanistic studies aimed at characterizing the pathophysiology of the disease.

Patient 10 is a 65-year-old man presenting with hypersideremia (706 μg/mL), hypertransferrinemia (652 mg/dL), high TF saturation (76%), and a monoclonal IgG-κ peak (2740 mg/dL). Liver Iron Concentration (LIC) quantification by Magnetic Resonance T2* (MR-T2*) confirmed iron overload (8 mg Fe/g liver dry weight, normal range < 3.2 mg Fe/g liver dry weight). Due to mild anemia, the patient underwent moderate phlebotomies (200 mL each time). In spite of the hypersideremia, hepcidin was extremely low (< 0.5 ng/100 mL). Erythroferrone (ERFE) was remarkably high (7.4 ng/mL).

Patient 11 is a 72-year-old woman with hypersideremia (580 μg/mL), hypertransferrinemia (445 mg/dL), high TF saturation (70%), and a monoclonal IgG-κ peak (2232 mg/dL). She was identified as having MGUS since the age of 55. MR-T2* LIC quantification showed mild iron overload (6.4 mg Fe/g liver dry weight). After diagnosis, the patient developed progressive anemia, and phlebotomy therapy became poorly tolerated. No data on hepcidin and ERFE levels are available.

Patient 10 IgGs were purified by protein A/G and analyzed by SDS/PAGE, showing a light chain with an abnormally higher molecular weight and the presence of a large amount of TF. Through these data and direct laboratory analyses (Supporting Information: 1, SM1), we established that about 70% of TF was IgG-bound and determined the stoichiometry of the immunocomplex, containing two IgG molecules for each TF molecule (Table S1). Iron co-purified with the TF-immunocomplexes. Using the same procedure, patient 11 IgGs were only partially

Gian Luca Forni and Emanuela Stampone contributed equally as co-first authors.

TABLE 1 | Clinical and laboratory data of identified TICD patients.

Patient (ref.)	Gender (age)	Serum iron μg/dL	Serum TF mg/dL	% TF Satura-Tion	Serum ferritin ng/mL	Serum Igs (mg/dL)	Hb g/dL at diagnosis	ERFE ng/mL	Hepcidin nmol/L	Liver Iron overload[a]	Evolution
1 [1]	F (73)	735	390	132	—	IgG1-κ (NA)	—	—	—	Mild	—
1 [2]	F (71)	780	—	—	—	IgG1-κ (NA)	12.5	—	—	Mild	Anemia
2 [3]	M (49)	—	—	—	—	IgG-λ (1640)	—	—	—	—	—
3 [4]	M (79)	781	579	94	947	IgA1-κ (NA)	11.8	—	—	Increased density	MM
4 [5]	F (55)	669	540	89	—	IgG2-κ (1460)	14.1	—	—	—	Spontaneous remission
5 [5]	M (53)	809	638	91	200	IgG-κ (840)	15.5	—	—	—	Spontaneous remission
6 [6]	M (62)	710	570	100	800	IgG-λ (2258)	14.9	1.64	0.15 MIU/mM creatinine	Mild	MM/Anemia
7 [7]	F (46)	400	500	61	77	IgG-κ (500)	13	<0.16	3.6	No (MRI)	—
8 [7]	M (73)	385	600	48	109	IgM-κ (1460)	14.9	—	3.3	No (MRI)	—
9 [8]	— (82)	541	500	77.5	608	IgG-κ (NA)	—	—	Hepcidin/ferritin ratio decreased < 0.1[b]	No	—
10	M (65)	706	652	76	2132	IgG-κ (2740)	12.1	7.04	<0.5	Moderate	MM/Anemia
11	F (72)	580	445	91	100	IgG-κ (2232)	13.7	—	—	Mild	—

Note: F, Female. M, Male. Normal range values: serum iron, F 37–145 μg/dL, M 59–158 μg/dL; serum transferrin, 200–360 mg/dL; transferrin saturation, 18%–60%; serum ferritin, F 13–150 ng/mL, M 30–400 ng/mL; Hb, F 12.3–15.3 g/dL, M 14–17.5 g/dL; Serum hepcidin, F 39–45 years, 2.6–7.15 nmol/L, M 73–79 years, 7.02–10.7 nmol/L [9]. Urinary hepcidin, 0.49 MIU/mM creatinine, 95% CIs 0.31–0.66 [10]. Serum erythroferrone (ERFE), F 0.01–0.76 ng/mL, M 0.32–1.80 ng/mL [11]. MM, multiple myeloma; –, not available.

[a]LIC values: normal, < 3.2 mg/g liver dry weight; mild, 3.2–7 mg/g liver dry weight; moderate, 7–15 mg/g liver dry weight; severe, > 15 mg/g liver dry weight.

[b]Normal range hepcidin/ferritin, 4–20 as reported in Reference [8].

purified, and the fraction of IgG-bound TF could not be precisely estimated. No molecular weight alteration of the κ-light chain was evident.

We investigated in vitro whether the IgG/TF occurrence affects iron supply to cells. 10% control, or patient 10 sera were added to Hep-G2, a human hepatocellular carcinoma-derived cell line. An immunofluorescence punctate pattern (Supporting Information: SM2) demonstrated TF incorporation. Thus, the TF-immunocomplex does not hamper TF entry. Intriguingly, we evidenced TF localization at the mid-body (Supporting Information: SM2), suggesting TF's still unknown roles. Hep-G2 cells were then incubated with patient 11 serum as well, and cytosolic/nuclear fractions were analyzed (experimental details in Figure 1A). While TF incorporation was clearly demonstrated, no signal for IgG (detected as a light chain) was evidenced, allowing the conclusion that the immunocomplexes do not enter cells. The finding was confirmed with the other two TICD sera available (Figure 1B) and in two other liver cell lines, Lx-2 and Hep-3b (Supporting Information: SM3). Surprisingly, when K562 cells (an erythroleukemic cell line) were incubated with sera from patients 10, 11, and all other patients available (Figure 1C–E), we detected IgG (light chain with abnormal molecular weight) signals in the cytosolic fractions, clearly demonstrating that the TF/IgG complex enters these cells. Additionally, IgGs from two MGUS (non-TICD) patient sera were not internalized (Supporting Information: SM4), confirming the specificity of the immunocomplex entry. Thus, in hepatic cells, only free TF can be internalized, whereas IgG-bound TF can enter K562 cells. To unravel the discrepancy between the two cell lines, we investigated the cellular levels of the transferrin receptors TFR1 and TFR2. TFR1 was highly expressed in both cell lines, while TFR2 was faintly present in Hep-G2 compared to the high K562 expression (Supporting Information: SM5). The finding suggested that the IgG/TF complex might be internalized in K562 through TFR2. The hypothesis was confirmed by the IgG/TF inability to enter a different TFR2-negative cell line (Lan5 cells) (Supporting Information: SM5). To further validate the TFR2 role in immunocomplex entry, we followed two different approaches: (i) selectively hamper TFR2 function by preincubating K562 cells with anti-TFR2 antibodies, and (ii) rescue TFR2 expression in Hep-G2 cells by transient transfection. In line with our hypothesis, while TFR2 inactivation reduces anti-TF IgG entry, the TFR2 overexpression enables Hep-G2 to incorporate TF-immunocomplexes (Figure 2A–C). Subsequently, we investigated whether the TF/IgG complex might hamper TFR1-mediated endocytosis of free TF. Hep-G2 cells, expressing high TFR1 and very low TFR2 levels, were grown in the presence of a healthy-human-serum-containing medium supplemented (or not) with the protein-A/G-purified IgGs from patient 10. The coincubation with the TF/IgG complex had no significant effect on TF endocytosis by TFR1 (Supporting Information: SM5), demonstrating the absence of competition between IgG-bound and free TF for TFR1-mediated uptake. From these data, we concluded that the TF-immunocomplexes interact only with TFR2 and not with TFR1. Due to the TFR2 role in hepcidin production and/or EPO responsiveness, we assayed serum hepcidin in patient 10 and found levels under the detectable value (Table 1). Moreover, normal/low serum hepcidin levels or urinary content were detected in all the patients analyzed for this hormone (our patients 6, 7, and 8 and patient 9 reported by others) [6–8] (Table 1). The finding indicated an altered hepcidin response to hypersideremia occurring in TICD patients, probably due to altered TFR2 activity. To note, we evidenced that in TICD patients, a large part of serum TF is bound to IgGs, and the quantification of the iron content in the purified material along with the capture assay results (Supporting Information: SM6) suggested that the interaction between the monoclonal antibody and TF does not affect TF's ability to bind iron. Similar findings have been previously found for patient 8 [7]. On the other hand, iron bound to immunocomplex is not available for cells and apparently, although TICD patients are hypersideremic, they might show mild anemia due to iron deficiency. This is suggested by ERFE quantification that in patient 10 serum is unusually high (Table 1). In accord with this mechanism, Westerhausen and Meuret reported the occurrence of iron-restricted erythropoiesis in their TICD patient, which was rescued by the immunosuppressive treatment [2]. Although anemia was not common in our case history, we also observed it in patient 6 [6] (Table 1). Bone marrow aspiration showed a normal plasma cell count and signs of iron-restricted erythropoiesis with Perl's staining (absence of stainable iron in red cell precursors), while some hemosiderin was present in macrophages [6]. Moreover, patient 10 exhibited hemoglobin values at diagnosis of 12.1 g/dL (Table 1). This led to the decision to limit phlebotomies (required to reduce iron overload risk) to max 200 mL each time. Physiologically, to sustain erythropoiesis, erythroblasts increase the synthesis and release of ERFE to suppress the hepatic production of hepcidin and thus increase serum iron availability. However, in TICD patients, as we observed, anti-TF autoantibodies sequester most of the iron in the immunocomplexes, reducing iron bound-TF endocytosis through TFR1, and might also alter the TFR-2-assisted erythropoietin (EPO) response via EPO receptor [12]. This scenario might explain the occurrence of mild anemia instead of erythrocytosis, which is usually observed with such levels of sideremia and ERFE. Probably, in TICD patients, increased (or abnormally high in comparison to sideremia) ERFE contributes to the further suppression of hepcidin synthesis in the attempt to enhance erythropoiesis.

In summary, the complexity of TICD symptoms, that is, hypersideremia, hypertransferrinemia, iron-restricted anemia, and high risk of hemochromatosis, might be explained, at least in part, by alteration of the ERFE/hepcidin axis due to interaction of IgG/TF with TFR2. Similar clinical signs are also observed in the most recognized type 3 hemochromatosis due to *TFR2* mutations [15]. It should be underlined that the in vivo effect of the TF/IgG complexes might not be easily anticipated due to the variability of the mAbs' structure, although we have demonstrated the immunocomplexes' internalization via TFR2 for all the TICD sera investigated. It is also possible that other cases of MGUS involve production of anti-TF antibodies, but the immunocomplexes can still enter the cells through TFR1, thereby not causing hypersideremia or hypertransferrinemia and escaping TICD diagnosis. We suggest that the disease is strongly underestimated since only our team identified five TICD patients (approximately 40% of all reported cases) by selecting subjects with hypersideremia, hypertransferrinemia, and monoclonal gammopathy. We propose that TICD could be classified as a novel type of MGCS (monoclonal gammopathy of clinical significance)

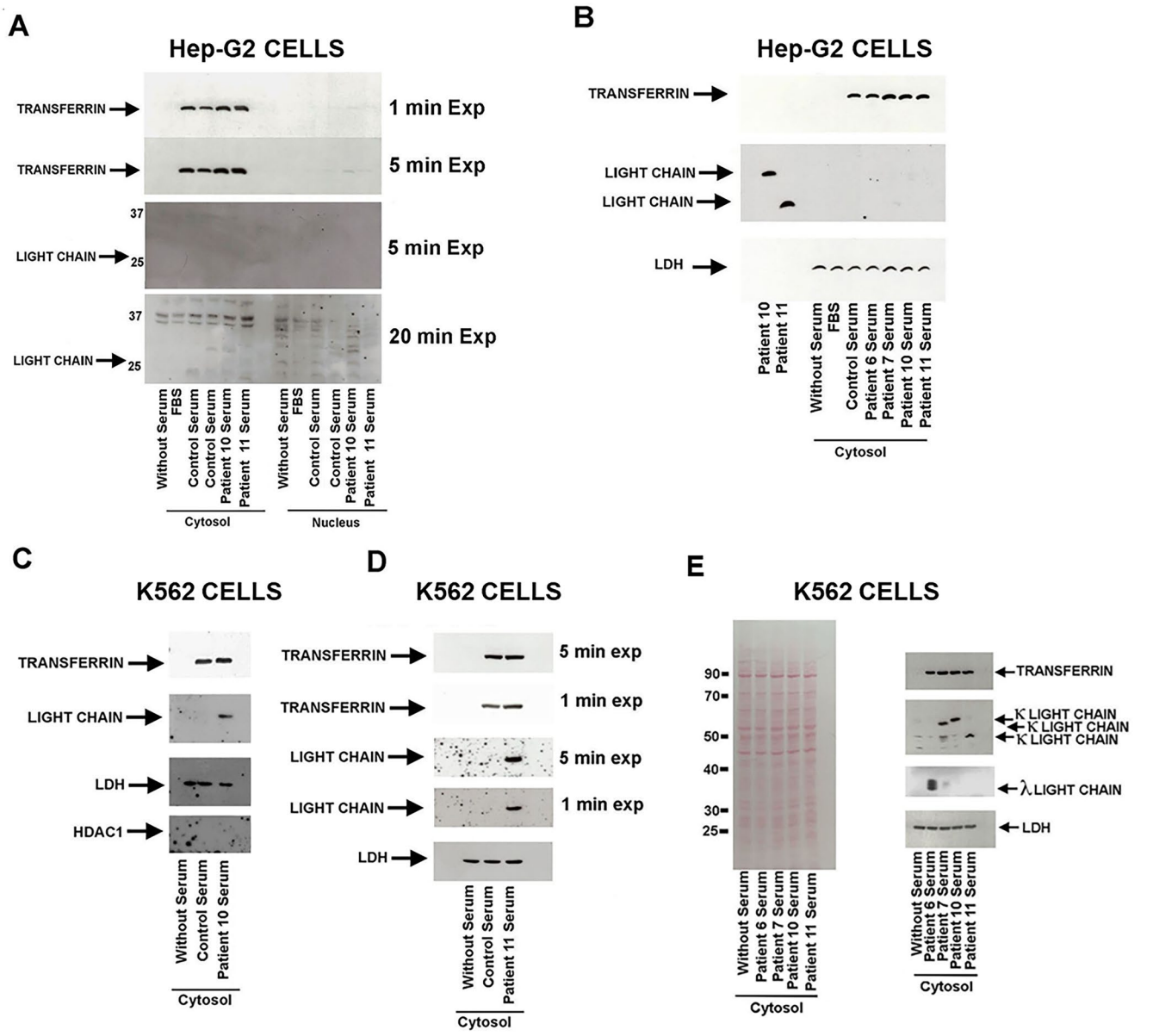

FIGURE 1 | Endocytosis of TF-immunocomplexes occurs in K562 cells, but not in Hep-G2 cells. Panel A. TF entry in Hep-G2 cells was investigated employing control and patients 10 and 11 sera. Hep-G2 cells were incubated for 4 h with growth medium without serum, or containing 10% FBS, 10% human control serum, 10% patient 10 and 11 sera. Subsequently, cells were recovered and washed. Then, cytosolic and nuclear fractions were prepared using the Nuclear Extraction kit (Merck KGaA, Darmstadt, Germany), following the manufacturer's instructions and separated by SDS-polyacrylamide gel electrophoresis. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis as in Reference. The filter was analyzed by immunoblotting by using antibodies against transferrin and κ light chain as in Reference [7]. Films at different exposure times are shown. Panel B. The same experiment as in panel A was performed employing sera from patients 6, 7, 10, and 11. The first two lines represent patient 10 and 11 sera and are loaded as markers for light chains molecular weight. The filter was analyzed by antibodies against transferrin and light chains. Note that the antibodies employed for detecting light chains were a mixture of anti-κ (A80-115P, Bethyl Laboratories) and anti-λ isotypes (A80-116P, Bethyl Laboratories). LDH (Lactic Dehydrogenase) was evaluated as a marker of the cytosolic fraction (anti-LDH Ab sc-137 243). Panels C and D. K562 cells were incubated for 4 h with a growth medium without serum, or alternatively containing 10% human control serum, or 10% patient 10 serum (Panel C), or 10% patient 11 serum (Panel D). Then, cells were processed as for Hep-G2 cells. After blotting, the filter was analyzed for the content of the TF and κ light chains. The κ light chain presence was evaluated since it is indicative of the monoclonal anti-TF antibodies. LDH and HDAC1 (histone deacetylase 1, anti-HDAC1 Ab sc-8410) were employed as markers of cytosolic and nuclear fractions, respectively. Different exposure times of the filter were employed to appreciate the relative signal intensity. Panel E. As in panels C and D, except that the sera from patients 6, 7, 10, and 11 were contemporaneously employed for investigating the complex TF/IgG entry in K562 cells. On the left, the Red Ponceau S staining of the filter is shown. On the right, western blotting analysis of transferrin and human antibody light chain contents in the cytosol of exposed cells. LDH was employed as marker of the cytosolic fraction. Note that the light chain from patient 6 was of λ-type and, accordingly, a goat antiserum specific for human λ chains was used to evidentiate its occurrence.

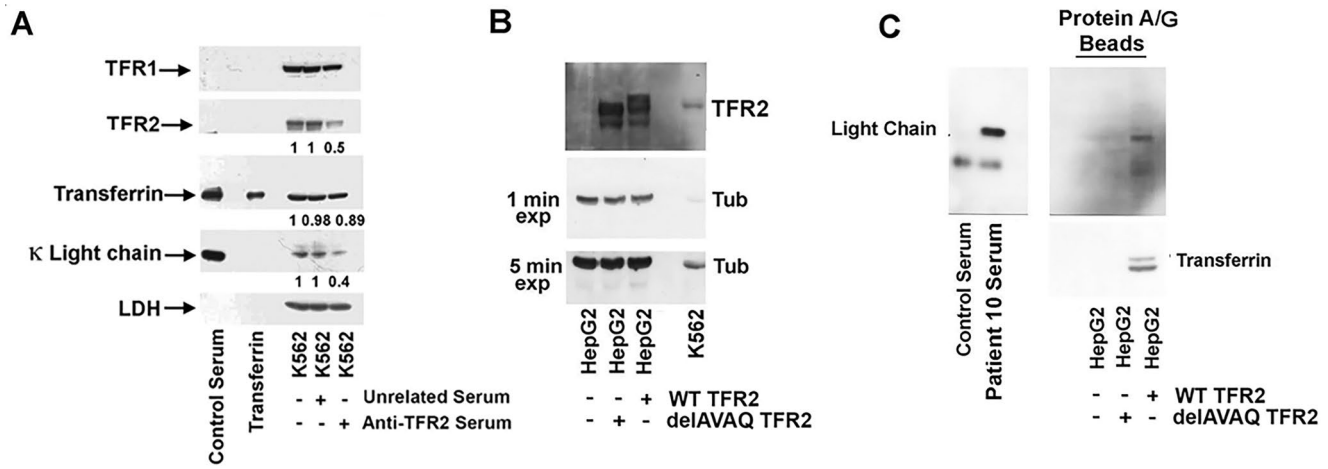

FIGURE 2 | Assessment of TFR2 involvement in TF-immunocomplex endocytosis. Panel A. K562 cells were preincubated with anti-TFR2 antiserum (sc-32271) for 2 h, then washed and incubated with patient 7 antiserum. Finally, levels of the cytosolic TFR2, κ light chain, and TF were evaluated in the cytosol. The experiment employed two different controls, namely untreated K562 and K562 preincubated with an unrelated commercial antiserum (normal mouse IgG sc-2025). Panel B. Hep-G2 cells were transfected for 48 h using an expression vector encoding for TFR2 and a vector for a TFR2 bearing deletion that hampers its membrane localization (delAVAQ-TFR2) [13]. An aliquot of Hep-G2 transfected cells was lysed in loading sample buffer and analyzed for TFR2 content to verify the efficiency of transfection [14]. Panel C. After 48 h of transfection as in Panel B, Hep-G2 cells were incubated with the serum of patient 10 (10% final concentration). Antibodies from the cytosolic fraction were then purified with protein A/G beads as in References [7]. After three washes with PBS, the immunoprecipitated materials were eluted in loading sample buffer and analyzed by SDS-PAGE and immunoblotting for their content in κ light chains and TF.

within the category in which tissue injury is caused by monoclonal immunoglobulins targeting a clearly identified specific antigen. TICD might also be considered a novel acquired condition of hemochromatosis due to anti-TF autoantibodies. Further studies and longer follow-up are needed to better characterize the prognosis of TICD, the impact of iron overload risk, and the potential anemia.

In conclusion, cases with monoclonal peaks associated with hypersideremia, hypertransferrinemia need to be examined for the occurrence of anti-TF antibodies, given the detrimental clinical consequences of TICD. The simultaneous presence of iron overload and anemia makes treatment with phlebotomy difficult, and given the risk of progression to myeloma, we recommend considering an early therapeutic approach for MGCS. To this end, antibody characterization is important. Furthermore, hepcidin and ERFE dosage might assist in identifying TICD patients with the highest risk of hemochromatosis, anemia, and progression of the disease.

Ethics Statement

Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. All cases included in the study were fully anonymized.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supporting Information.