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T Cell Immunoglobulin Mucin-3 (TIM-3) Expression on Peripheral Blood Lymphocytes in Chronic Hepatitis Virus C Infection

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Abstract

Introduction: T cell Immunoglobulin Mucin-3 (TIM-3) TIM-3 acts as a negative regulator of (T helper-1)Th1/ (T Cytotoxic-1)Tc1 cell function by triggering cell death upon interaction with its ligand Galectin-9, a feature observed in chronic viral diseases.

Objective: To demonstrate the level of expression of TIM-3 on Peripheral Blood Mononuclear cells (PBCs) in cases of chronic HCV as a number of emerging molecules and pathways have been implicated in mediating the T-cell exhaustion characteristic of chronic viral infection. Patients and Methods: This study included 90 subjects, divided up as follows: Group 1 (35 patients) included HCV antibody positive with normal liver functions (Compensated), Group 2 (35 patients) comprised HCV antibody positive patients with abnormal liver functions (decompensated), and controls (Group 3) involved 20 apparently healthy persons (HCV antibody negative persons). The following laboratory investigations were performed for all participants in the 3 groups: Complete Blood Count (CBC), Blood Chemistry (liver functions), Special investigations (Flowcytometric study, and PCR for HCV RNA).

Results: Comparing the control, compensated and decompensated groups regarding lymphocytic counts, ratios of TIM-3 positive cells within CD4, CD8, CD14 and CD56 cells in the three groups. Ratio of CD4 cells was higher in the compensated and control groups, than in the decompensated group, with non-significant difference. CD8 cells were maximum in the decompensated groups and minimum in the compensated group, with a significant p value. CD14 cells were maximum in the compensated group, followed by decompensated and minimum in the control group, again with a significant difference. CD56 showed non-significant differences between the three groups. A steady increase in the percentage of TIM +ve CD4, CD8, CD14 and CD 56 cells, with maximum percentages among the decompensated liver disease group, and least percentage among the control group was seen. The differences were significant regarding CD8 and CD56 and highly significant regarding CD4 and CD14 cells.

Conclusions: Accumulation of TIM-3+ T cells is associated with functional impairment, and consequently with development of persistent HCV. The present study provides a basis for improving current therapies by simultaneous blockade of multiple inhibitory pathways that could result in additive efficacy without excessive toxicity. These findings have implications for the development of novel immunotherapeutic approaches to this common viral infection.

Key words:

TIM-3; Lymphocytes; Hepatitis C virus

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, affecting approximately 200 million people throughout the world. There is a broad array of functional impairments of virus-specific T cells including decreased antiviral cytokine production and cytotoxicity; with impaired proliferative capacity and arrested stages of differentiation [1-3].

In liver infections, CD81 T cells may show features of cells that did not receive sufficient help. Thus, in chronic lymphocytic choriomeningitis virus in mice, failure to eliminate the virus is associated with "exhausted" T cells that persist, but do not function. [4] These cells express a characteristic surface phenotype, including the markers programmed cell death 1 (PD- 1), T-cell 3 immunoglobulin and mucin domain containing protein 3 (TIM-3), and lymphocyte activation gene 3 (Lag-3)[5,6]which are also expressed on human exhausted T cells [7]. In chronic hepatitis C virus (HCV) infection, the lack of a detectable CD41 T-cell response is one of the clearest correlates of failure to eliminate the virus [8,9]. HCV-infected individuals also harbor exhausted or "stunned" CD81 T cells, defined both functionally as cells that cannot make effector cytokines [10,11], and phenotypically as cells that express PD-1 and TIM-3 [12]. Based on these data, one plausible model for liver tolerance is that, when CD81 T cells are primed in the liver, appropriate CD41 T-cell help may not always be available. The consequence is dysfunctional, exhausted CD81 T cells and thus failure to eliminate the pathogen. However, many other factors complicate this satisfyingly simple. model, in particular, the prevalence of liver antigen presenting cells (APCs) that express coinhibitory ligands, such as programmed death ligand 1 (PDL1), and which stimulate regulatory T (Treg) cells. All of these factors may contribute to immune failure through parallel mechanisms and also The T-cell immunoglobulin mucin-3 (TIM-3) receptor was

recently shown to inhibit cytotoxic and cytokine responses of NK cells upon interaction with galectin-9 (Gal-9) or phosphatidylserine (PtdSer) on target cells [13–16].

TIM-3 which was first identified as a molecule specifically expressed on IFN-gamma-secreting T helper 1 and T cytotoxic 1 cells in both mice and humans, acts as a negative regulator of Th1/Tc1 cell function by triggering cell death upon interaction with its ligand, Galectin-9. This negative regulatory function of TIM-3 has now been expanded to include its involvement in establishing and/or maintaining a state of T cell dysfunction or "exhaustion" observed in chronic viral diseases. Given that an increasing body of data support an important role for TIM-3 in both autoimmune and chronic inflammatory diseases in humans [17-19]. A recent analysis of human immunodeficiency virus (HIV) infection demonstrates that TIM-3 is upregulated on both CD4 and CD8 T cells from patients with chronic infection relative to uninfected individuals and that virus-specific cells expressing high levels of TIM-3 secrete less IFN- than do TIM-3-negative cells [20]. In light of these findings, this study assessed the expression of TIM-3 in chronic HCV infection. We found a higher frequency of TIM-3-expressing CD4 and CD8 T cells in chronic HCV infection. These findings have implications for the development of novel immunotherapeutic approaches to this common disease.

Aim of the study:

To demonstrate the level of expression of TIM-3 on Peripheral Blood Mononuclear cells (PBCs) in cases of chronic HCV during different stages and to clarify its possible role in the pathology of the disease.

Patients and Methods

This study included 90 subjects, divided as follows: Patients group was split into two groups: Group 1 (35 patients) included HCV antibody positive/ HCV RT- PCR positive patients with normal liver functions (Compensated), Group 2 (35 patients) comprised HCV antibody positive/HCV RT-PCR positive patients with abnormal liver functions (decompensated), and controls (Group 3) involved 20 apparently healthy persons (HCV antibody negative individuals).

The following laboratory investigations were performed for all participants in the Three groups:

* Routine laboratory investigations

Complete Blood Count (CBC)

Blood Chemistry:

Alanine Aminotransferase (ALT)

Aspartate Aminotransferase (AST)

Alkaline Phosphatase (ALP)

Total Protein (TP)

Albumin (ALB)

Total Bilirubin (TBIL)

Direct Bilirubin (DBIL)

Prothrombin Time and concentration and INR.

* Special investigations

Using FACSCalibur Flow cytometer (Becton Dickinson- USA) and CellQuaest software, in Sohag university hospitals , Egypt. PBMCs will be stained by monoclonal antibodies for the following antigens:

-Anti-TIM-3-PE (BioLegend,USA , Catalog number: 345006),

-FITC Mouse Anti-Human CD4 (T helper cells) (BD Pharmingen, USA,Catalog number: 555346)

-PerCP Mouse anti-human CD14 (BD Pharmingen, USA, Catalog No. 345786) (Monocytes).

- FITC Mouse Anti-Human CD8 (BD Pharmingen, USA,Catalog number 555366) (Cytotoxic T cells)

-APC – Mouse anti-human CD56 (BD Pharmingen, USA - Catalog No. 341027). (Natural killer cell).

All monoclonals were provided as separate reagents and PBMCs were stained with monoclonal antibodies for the following antigens together as triple markers:

-Anti-TIM-3-PE and anti-CD4-FITC (T helper cells) and anti-CD14-PerCP (Monocytes).

-Anti-TIM-3-PE and anti-CD8-FITC (Cytotoxic T cells) and anti-CD56 -APC (Natural killer cell).

BD Falcon tubes used on the FACS Calibur flow cytometer which is equipped with a 488 nm laser capable of detecting light scatter (forward and side) and 3-color fluorescence with emission detectable in 3 ranges: 515–545, 562–607, and more than 650 nm. Setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument (Figure 1).

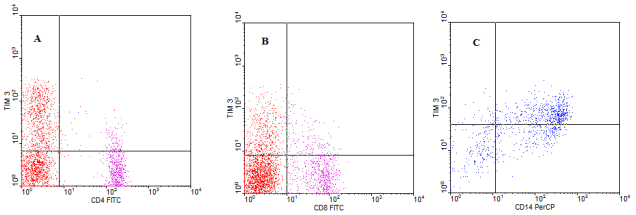

Figure 1: A) shows CD4 vesus TIM-3. Upper right quadrant shows CD4+/TIM-3+ lymphocytes, lower right quadrant shows CD4+/TIM-3- lymphochtes, upper left quadrant shows CD4-/TIM-3+ lymphocytes and lower left quadrant shows CD4-/TIM-3- lymphocytes respectively; B) shows CD8 vesus TIM-3. Upper right quadrant shows CD8+/TIM-3+ lymphocytes, lower right quadrant shows CD8+/TIM-3- lymphochtes, upper left quadrant shows CD8-/TIM-3+ lymphocytes and lower left quadrant shows CD8-/TIM-3- lymphocytes respectively; C) shows CD14 vesus TIM-3. Upper right quadrant shows CD14+/TIM-3+ cells, lower right quadrant shows CD14+/TIM-3- cells, upper left quadrant shows CD14-/TIM-3+ cellss and lower left quadrant shows CD14-/TIM-3- cells respectively.

Statistical analysis

Statistical package for social sciences (IBM-SPSS), version 19 IBM-Chicago, USA was used for statistical data analysis. Mean and standard deviation were used as descriptive value for quantitative data. Pearson

correlation test and 't' test were used to compare two quantitative variables.

Results

Regarding CBC and liver functions, all investigations, with the exception of Hb levels, showed highly significant difference among the three study groups. PCR levels, showed non-significant difference between compensated and decompensated liver disease groups (Table 1). Comparing the control, compensated and decompensated groups regarding lymphocytic counts, ratios of TIM-3 positive cells within CD4, CD8, CD14 and CD56 cells in the three groups. The absolute numbers of lymphocytes and the percentage of lymphocytes was maximum in the control group, followed by the decompensated group and minimum in the compensated group, with a significant p value. CD4 cells was higher in the compensated and control groups, than in the decompensated group, with non-significant difference. CD8 cells were maximum in the decompensated groups and minimum in the compensated group, with a significant p value. CD14 cells were maximum in the compensated group, followed by decompensated and minimum in the control group, again with a significant difference. CD56 showed non-significant differences between the three groups. A steady increase in the percentage of TIM +ve CD4, CD8, CD14 and CD 56 cells, with maximum percentages among the decompensated liver disease group, and least percentage among the control group was seen. The differences were significant regarding CD8 and CD56 and highly significant regarding CD4 and CD14 cells (Table 2).

Test	Control		Decompensated Liver Disease		Compensated Liver Disease		Total		P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
PLT (103/µL)	276	82.4	69.28	17.53	173.9	78.01	169.5	103.8	<0.001
HB (g/dL)	13.33	1.25	8.47	0.96	16.28	5.13	12.97	16.12	0.309
WBCs (103/µL)	8.13	1.72	4.94	1.46	7.02	2.43	6.68	2.32	<0.001
INR	1.02	0.03	1.7	0.27	1.28	0.38	1.32	0.39	<0.001
PC (%)	97.5	5.77	44.28	9.35	79.78	22.95	73.54	26.41	<0.001
PT (Second)	11.86	0.33	19.58	2.97	14.3	4.46	15.28	4.49	<0.001
IBIL (mg/dL)	0.36	0.12	1.68	0.79	0.85	0.97	0.97	0.89	<0.001
DBIL (mg/dL)	0.19	0.08	1.69	0.83	0.7	1.09	0.87	1.02	<0.001
TBIL (mg/dL)	0.54	0.17	3.37	1.54	1.54	1.94	1.84	1.87	<0.001
TP (g/dL)	7.74	0.26	6.56	0.789	7.421	0.915	7.236	0.878	<0.001
ALB (g/dL)	4.86	0.41	2.35	0.504	3.726	0.931	3.61	1.194	<0.001
SGPT (U/L)	21.06	5.62	70.56	38.898	33.087	27.097	41.543	34.292	<0.001
SGOT (U/L)	17.31	7.47	93.5	40.932	33.347	26.721	47.842	42.74	<0.001
PCR (Copy/ml)	-	-	1625915	3087926	1449068	4795099	1537491		0.893

Table 1: CBC, liver functions and PCR of the three groups; PLT: Platelets; HB: Haemoglobin; WBCs: White blood cells; INR: International normalized ratio; PC: Prothrombin concentration; PT: Prothrombin Time; IBIL: Indirect bilirubin; DBIL: Direct bilirubin; TBIL: Total bilirubin; TP: Total protein; ALB: Albumin; SGPT: Serum Glutamic-Pyruvic Transaminase; SGOT: Serum Glutamic Oxaloacetic Transaminase PCR: Polymerase Chain reaction.

By applying Pearson correlation the following was discovered ; the correlation between TIM-3 expression and ALT level is variable, sometimes positive and others negative, and all were non-significant correlations, with the only exception of the negative, moderate, significant correlation between ALT and CD56 TIM-3 among de-compensated population (Table 3); the correlation between TIM-3 and albumin is variable, sometimes positive and sometimes negative, and all were non-significant correlations (Table 4);the correlation between TIM-3 and total bilirubin is variable, sometimes positive and others negative, and all were non-significant correlations (Table 5); the correlation between TIM-3 and INR is variable, whether positive or negative, and all were non-significant correlations. The only exception is the positive moderate, significant correlation between INR and CD56 TIM-3 among compensated group, and the positive, highly significant correlation between INR and CD56 TIM-3 among de-compensated group (Table 6).

The percentage of TIM-3+ve cells (amongst all cell populations) showed increase in the decompensated group as compared to the compensated, although the increase wasnot always statistically significant.(Table 7).

Test	Controls	Decompensated	Compensated	P value

	Mean	SD	Mean	SD	Mean	SD	
Lymphocytes /mm3	2907	1065.2	1918.57	841.43	2672.33	938.74	0.004
Lymphocytes%	31.49 %	9.57%	19.19 %	8.41%	28.87 %	8.95%	<0.001
CD4 Total%	37.17 %	8.21%	38.56 %	12.02 %	32.13 %	14.32 %	0.221
CD4 TIM-3 +ve (%)	3.12%	2.13%	5.78%	5.3%	16.61 %	16.82 %	<0.001
CD4 TIM-3 -ve (%)	96.87 %	2.13%	94.22 %	5.3%	83.39 %	16.82 %	<0.001
CD8 total (%)	20.82 %	5.79%	17.95 %	9.07%	36.67 %	29.12 %	0.004
CD8 TIM-3 +ve (%)	11.18 %	4.18%	17.82 %	10.16 %	27.52 %	30.86 %	0.043
CD8 TIM-3 +ve (%)	88.82 %	4.18%	82.18 %	10.16 %	72.48 %	30.86 %	0.042
CD14 total (%)	5.99%	2.17%	9.51%	3.99%	7.76%	3.27%	0.008
CD14 TIM-3 +ve (%)	42.09 %	26.3%	55.55 %	16.19 %	79.09 %	17.26 %	<0.001
CD14 TIM-3 (%)	57.91 %	26.34 %	44.45 %	16.19 %	20.93 %	17.27 %	<0.001
CD56 total (%)	0.54%	0.33%	0.45%	0.5%	0.5%	0.34%	0.792
CD56 TIM-3 +ve(%)	21.36 %	18.3%	22.18 %	22.94 %	46.26 %	31.92 %	0.007
CD56 TIM-3 -ve (%)	78.7%	18.29 %	77.82 %	22.94 %	53.74 %	31.92 %	0.007

Table 2: Comparison between the three groups regarding lymphocytes; CD: Cluster of differentiation; SD: Standard Deviation ; SE: Standard Error;TIM-3: T-cell immunoglobulin domain and mucin domain 3.

Test		Controls	Compensated	Decompensated
CD4 TIM-3 +ve (%)	r	0.071	-0.175	-0.245
	p	0.794	0.425	0.328
CD8 TIM-3 +ve (%)	r	0.169	-0.065	-0.457
	p	0.532	0.767	0.065
CD14 TIM-3 +ve(%)	r	0.203	0.117	-0.281
	p	0.451	0.594	0.274
CD56 TIM-3 +ve(%)	r	-0.215	-0.113	-0.505
	p	0.442	0.607	0.046

Table 3: Pearson correlation between TIM-3 expression and ALT.

Parameters		Controls	Compensated	Decompensated
CD4 TIM-3 +ve(%)	r	0.223	0.019	-0.075
	p	0.407	0.933	0.769
CD8 TIM-3 +ve(%)	r	0.106	-0.187	-0.186
	p	0.696	0.394	0.475
CD14 TIM-3 +ve(%)	r	0.048	0.205	-0.238
	p	0.860	0.347	0.358
CD56 TIM-3 +ve(%)	r	-0.022	-0.361	-0.364
	p	0.939	0.091	0.166

Table 4: Pearson correlation between TIM-3 expression and albumin.

Test		Controls	Compensated	Decompensated
CD4 TIM-3 +ve(%)	r	0.169	-0.230	0.224
	p	0.530	0.292	0.371
CD8 TIM-3 +ve(%)	r	-0.250	-0.018	-0.281
	p	0.350	0.935	0.275
CD14 TIM-3 +ve(%)	r	0.010	-0.147	-0.132
	p	0.971	0.504	0.614
CD56 TIM-3 +ve(%)	r	-0.072	0.350	0.079
	p	0.799	0.102	0.771

Table 5: Pearson correlation between TIM-3 expression and bilirubin.

Test		Controls	Compensated	Decompensated
CD4 TIM-3 +ve(%)	r	-0.137	-0.124	0.275
	p	0.613	0.572	0.269
CD8 TIM-3 +ve(%)	r	-0.212	0.017	0.323
	p	0.431	0.937	0.207
CD14 TIM-3 +ve(%)	r	-0.357	-0.199	0.433
	p	0.174	0.363	0.083
CD56 TIM-3 +ve(%)	r	-0.064	0.560	0.748
	p	0.820	0.005	0.001

Table 6: Pearson correlation between TIM-3 expression and INR.

Test	Group	Mean	Std. Deviation	P value
Lymphocytes%	Compensated Liver Disease	19.19%	8.41%	0.001

	Decompensated Liver Disease	28.87%	8.95%	
CD4_Total%	Compensated Liver Disease	38.56%	12.02%	0.126
	Decompensated Liver Disease	32.13%	14.32%	
CD4 TIM-3 +ve(%)	Compensated Liver Disease	5.78%	5.30%	0.016
	Decompensated Liver Disease	16.61%	16.82%	
CD4 TIM-3-ve (%)	Compensated Liver Disease	94.22%	5.30%	0.016
	Decompensated Liver Disease	83.39%	16.82%	
CD8 total (%)	Compensated Liver Disease	17.95%	9.07%	0.02
	Decompensated Liver Disease	36.67%	29.12%	
CD8 TIM-3 +ve(%)	Compensated Liver Disease	17.82%	10.16%	0.228
	Decompensated Liver Disease	27.52%	30.86%	
CD8 TIM-3-ve (%)	Compensated Liver Disease	82.18%	10.16%	0.228
	Decompensated Liver Disease	72.48%	30.86%	
CD14 total (%)	Compensated Liver Disease	9.51%	3.99%	0.149
	Decompensated Liver Disease	7.76%	3.27%	
CD14 TIM-3 +ve(%)	Compensated Liver Disease	55.55%	16.19%	<0.001
	Decompensated Liver Disease	79.09%	17.26%	
CD14 tim-ve (%)	Compensated Liver Disease	44.45%	16.19%	<0.001
	Decompensated Liver Disease	20.93%	17.27%	
CD56 total (%)	Compensated Liver Disease	0.45%	0.50%	0.724
	Decompensated Liver Disease	0.50%	0.34%	
CD56 TIM-3 +ve(%)	Compensated Liver Disease	22.18%	22.94%	0.016
	Decompensated Liver Disease	46.26%	31.92%	
CD56 TIM-3-ve (%)	Compensated Liver Disease	77.82%	22.94%	0.016
	Decompensated Liver Disease	53.74%	31.92%	

Table 7: Comparison between compensated and decompensated liver disease groups.

Discussion

The present study included 90 subjects (70 HCV +ve patients and 20 normal controls). HCV infected patients were subdivided into two groups; patients with compensated liver functions and patients with decompensated liver functions, and each subgroup involved 35 patients. The study population groups were age and sex matched. Regarding CBC, there was steady downward degradation from the control to compensated to decompensated groups, with those of the decompensated group showed the worst figures. Similar to CBC, INR showed also best results in the normal group and worst in the decompensated group.

Liver functions were, as expected, all impaired in the decompensated group, and showed highly significant difference compared to the control group and the compensated group. However, comparing control to compensated groups, some of the liver functions showed non-significant differences as both groups fell in the "normal" range for liver functions.

Our results for the control, compensated and decompensated groups as regards lymphocyte counts, ratios of TIM-3 positive cells within CD4, CD8, CD14 and CD56 cells in the three groups showed that the absolute numbers of lymphocytes and the percentage of lymphocytes was maximum in the control group, followed by the decompensated group and minimum in the compensated group, with a significant p value. Also, the ratio of CD4 cells was higher in the compensated and control groups, than in the decompensated group, with non-significant difference. While Mason et al. [21] found that TIM-3 expression may play an important pathogenic role in with chronic HCV patients

In our study, CD8-TIM-3 positive cells were maximum in the decompensated groups and minimum in the compensated group, with a significant p value. Similar results were found by Mason et al. [21],

McMahan et al. (2010) [2], Kaufmann et al. [22] as they found that TIM-3 expression is increased significantly on CD4+ and CD8+ T cells in chronic hepatitis C virus (HCV) infection compared to the control regardless of the liver function. They also demonstrated that early accumulation of PD-1+ TIM-3+ T cells is associated with functional impairment, and consequently with development of persistent HCV.

There was experimental evidence implicating CD8+ T cells as pivotal in host defense against HCV infection [23-26]. McMahan et al [2], found that a significantly higher percentage of total CD4+ and CD8+ T cells and HCV-specific CTLs within the hepatic compartment co-expressed TIM-3 and PD-1, consistent with the hypothesis that the liver is enriched for T cells that are functionally exhausted. However, McMahan et al. [2] found that the kinetics of TIM-3 up regulation in early infection and whether TIM-3 correlates with development of persistence versus spontaneous recovery remains undefined. It is generally accepted that HCV-specific CD4+ T-helper cell responses, critically important for priming and maintaining HCV-specific CTL effector responses and progressively lost as HCV-related disease advances. [27].

In our study CD14 cells were maximum in the compensated group, followed by decompensated and minimum in the control group, again with a significant difference. Henning et al. [28] demonstrate the expansion of CD14+CD16+ monocytes in the circulation and liver of CLD-patients upon disease progression and suggest their functional contribution to the perpetuation of intrahepatic inflammation and profibrogenic HSC activation in liver cirrhosis while Medhat Eman et al. 2015 [29] found that The serum sCD14 level was significantly higher in chronic HCV-infected patients compared to healthy control subjects . The serum sCD14 level was significantly directly correlated with the hepatic fibrosis score, histological activity index, and serum aminotransferases. Peng et al. [30] found no significant differences in the levels of CD14+CD16- and CD16+CD14- monocytes after HCV infection, although there were differences in response to TLR8-ligation or LPS stimulation. Therefore, they suggest that CD16+CD14- monocytes do not appear to have a role in HCV pathogenesis, although they may differentiate into Kupffer or Dendritic cells. However, there is no reason to expect that levels of monocytes would be related to the pathogenesis of HCV, so their conclusions don't seem reasonable.

We also found that CD56 showed non-significant differences between the three groups, with an increase in the percentage of TIM +ve CD4, CD8, CD14 and CD56 cells, with maximum percentages among the decompensated liver disease group, least percentage among the control group. Bjorkstrom et al. [31] stated that immunogenetic association data suggest that NK cells also influence the course of chronic viral infections, such as infections with HIV-1 and hepatitis C virus (HCV). Chronic stages of these infections have a negative impact on NK cell function and promote the appearance of phenotypically and functionally abnormal NK cells.

Conclusion

In conclusion, our findings demonstrated that accumulation of TIM-3+ T cells is associated with decompensated persistant HCV infection. More work needs to be carried out to determine whether this increase is the cause or result of persistant HCV infection. This should provide a basis for improving current therapies by simultaneous blockade of multiple inhibitory pathways that could result in additive efficacy without excessive toxicity.

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