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Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis

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Follicular regulatory T cells (TFR) have been extensively characterized in mice and participate in germinal center responses by regulating the maturation of B cells and production of (auto)antibodies. We report that circulating TFR are phenotypically distinct from tonsil-derived TFR in humans. They have a lower expression of follicular markers, and display a memory phenotype and lack of high expression of B cell lymphoma 6 and ICOS. However, the suppressive function, expression of regulatory markers, and FOXP3 methylation status of blood TFR is comparable with tonsil-derived TFR. Moreover, we show that circulating TFR frequencies increase after influenza vaccination and correlate with anti-flu Ab responses, indicating a fully functional population. Multiple sclerosis (MS) was used as a model for autoimmune disease to investigate alterations in circulating TFR. MS patients had a significantly lower frequency of circulating TFR compared with healthy control subjects. Furthermore, the circulating TFR compartment of MS patients displayed an increased proportion of Th17-like TFR. Finally, TFR of MS patients had a strongly reduced suppressive function compared with healthy control subjects. We conclude that circulating TFR are a circulating memory population derived from lymphoid resident TFR, making them a valid alternative to investigate alterations in germinal center responses in the context of autoimmune diseases, and TFR impairment is prominent in MS. The Journal of Immunology, 2015, 195: 832–840.

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most accessible source to analyze immune responses in healthy control subjects (HCs) and patients with AID. Therefore, we first determined whether blood TFR are a good representation to investigate TFR responses ongoing in secondary lymph nodes. To do so, a pairwise comparison was made between TFR derived from blood and tonsils of HCs. Next, circulating TFR were monitored after influenza vaccination to define GC-induced changes in this subset. Finally, circulating TFR alterations were investigated in MS patients and compared with HCs to evaluate their involvement in AID.

**Materials and Methods**

Ethics approvals were obtained from each institute’s human ethics committee. Tonsils and blood were obtained from adult patients without AIDs who were undergoing routine tonsillectomies at Hospital East-Limburg (Genk, Belgium; for detailed information, see Supplemental Table I). Adult healthy volunteers were recruited for the vaccination of inactivated influenza vaccine Influvac S 2013/2014 (ABBOTT BIOLOGICALS B.V., Brussels, Belgium; for detailed information, see Supplemental Table I). MS patients were recruited from the Rehabilitation and MS-Center (Overpelt, Belgium). Detailed clinical characteristics are listed in Supplemental Table I.

**PBMC purification and flow-cytometric analysis**

PB was collected in heparin-coated tubes (Venesafe plastic tubes; Terumo Europe N.V., Leuven, Belgium). Tonsils were cut into small pieces and single cells were obtained using a cell strainer (EASYstrainer 70 μm; Greiner Bio-One BVBA/SPRL, Wemmel, Belgium). After collection of the plasma, density centrifugation was used to isolate the PBMCs (Lymphocyte; Cedara Laboratories, SanBio B.V., Uden, the Netherlands). In line with recent publications, specific flow-cytometric markers were used to identify circulating TFR and TFH in human blood (13, 14). For flow-cytometric analysis using FACSDiva software (BD Biosciences, Erembodegem, Belgium; and CD25 allophycocyanin-Cy7 and CD27 allophycocyanin (from Biolegend, ImTec Diagnostics N.V., Antwerp, Belgium). B cell analysis in the plasma, density centrifugation was used to isolate the PBMCs (Lym-CA); and CD25 allophycocyanin-Cy7, CD31 allophycocyanin-H7, CXCR3 PE-CF594, CCR6-PerCP-Cy5.5, BCL-6 PE-CF594, Foxp3 PE-CF594 (all from BD Biosciences, Erembodegem, Belgium). CD25 PerCP-Cy5.5, CD127 PE, PD-1 PE-Cy7, CD45RA allophycocyanin-Cy7, CD31 allophycocyanin-H7, and CXCR5 PE-CF594, CD25 allophycocyanin-Cy7, SAP-P, Helios allophycocyanin (all from eBioscience, San Diego, CA); and CD25 allophycocyanin-Cy7 and CD27 allophycocyanin (from Biolegend, ImTec Diagnostics N.V., Antwerp, Belgium). B cell analysis was performed with CD19 PerCP-Cy5.5, IgD allophycocyanin-Cy7, and CD27 PE-Cy7 (all from BD Biosciences). Appropriate isotype controls were used to establish the proper gating strategies (all from BD Biosciences). All flow-cytometric analyses were performed on a FACSAriaII flow cytometer and analyzed with FACS DIVA software (BD Biosciences). For Foxp3 intranuclear staining, the eBioscience kit was used; for other intracellular stains, the BD Cytofix/Cytoperm kit (BD) was used according to the manufacturers’ guidelines.

**Purification of CD4 T cell subsets**

CD4 T cells were purified using CD4 negative selection (STEMCELL Technologies SARL, Grenoble, France). CD25 positive selection (STEMCELL Technologies SARL) was used to obtain a CD25+ enriched population and a CD25− population. CD4+CD25+CXCR5+PD-1− TFR were sorted from the CD25− population, CD4+CD25+CXCR5+PD-1 TFR were sorted from the CD25+ population using the following Abs: CD4 FITC (BD), CD25 PerCP-Cy5.5 (eBioscience), CD127 PE (eBioscience), CXCR5 Alexa Fluor 647 (BD), and PD-1 PE-Cy7 (eBioscience) using a FACSAria II (BD). Purity of the isolated cells was confirmed. Flow cytometric analysis was performed using FACS Diva software (BD Biosciences) and FlowJo V10.

**Suppression assays**

A 96-well round-bottom plate (Nunc, Roskilde, Denmark) was coated for 2 h on 37°C with 0.01 μg/ml anti-CD3 (HIT3; BD) and washed with PBS. CD4+CD25+CXCR5+PD-1 Treg (labeled with 4 μM CFSE; Invitrogen) were cultured at 1 × 10^3 cells/well with 1 × 10^2 irradiated autologous PBMCs (feeder cells) in the presence or absence of the same number of Tregs or TFR in duplicate. The isolated Treg population thus also includes the CXCR5−PD-1− TFR population. Cell cultures were also stimulated with soluble anti-CD28 (BD) for 4 d. The following controls were used: 1) a non-labeled stimulated control to serve as reference for setting the Treg gate; 2) a labeled non-stimulated control to serve as reference for setting the nonproliferated gate; and 3) a labeled stimulated control with double amount of responder cells to exclude possible nutrient deprivation effects. Cocultures were analyzed on a FACS Aria II on day 4. The suppressive capacity (percentage) of Tregs toward Tresp in coculture was calculated relative to the maximal proliferation of the Tresp alone: [100 − (% proliferation Tresp alone% proliferation Tresp + Treg)]×100.

**Foxp3 methylation assay**

CD4+CD25+ cells (purified as described earlier) were sorted to obtain CD4+CD25+CXCR5−PD-1− TFR, and CD4+CD25− cells were sorted to obtain CD4+CD25+CXCR5+PD-1− TFR cells using FACS Aria II (BD). Purity of the obtained cells was confirmed. Purified cells were pelleted and frozen at −80°C. Next, the proportions of cells with a demethylated FoxP3 intron 1 allele were quantified by quantitative PCR on bisulfite-treated genomic DNA, as described previously (15).

**Glutination inhibition (HAI) assay**

Statistical analyses were done using SAS 9.3, SAS Jump, and GraphPad Prism 6. Graphics were made using GraphPad Prism 6. Data sets were checked for normality, and for effect of age and sex. Analyses of the vaccination study was done using a mixed model (multiple measurements SAS 9.3). Analysis of multiple groups was done using ANOVA, non-parametric testing (Kruskal–Wallis), or linear correlations using SAS JUMP. A Mann–Whitney U test was used for nonparametric unpaired data. Wilcoxon matched-pair test was used for nonparametric paired data. Tests were considered significant when p < 0.05 (two-sided tests).

**Study approval**

All human blood samples and tonsils were obtained with ethical approval of each institute’s human ethics committee, the Medical Ethical Commission of University Hospital Leuven and Hospital East-Limburg, respectively. Written, informed consent was obtained from all study subjects.

**Results**

**Human circulating TFR comprise a phenotypically distinct population**

The presence and origin of genuine follicular subsets in the circulation remains controversial (2, 16). This led us to question whether circulating TFR are phenotypically bona-fide TFR or rather represent a distinct population. First, circulating TFR were phenotypically characterized in detail comparing blood and tonsils of HC (for detailed information, see Supplemental Table I). Distinct subpopulations of Treg (CD4+CD25+CXCR5−) and conventional T cells (CD4+CD25+CXCR5+) were found to express the follicular markers CXCR5 and PD-1 in blood and in tonsils (Fig. 1A). Based on that, CD4+CD25+CXCR5+PD-1− and CD4+CD25+CXCR5+PD-1+ were defined as (circulating) counterparts of, respectively, TFR and TFH. In tonsils, TFR and TFH comprise a much larger population compared with the blood (Fig. 1A, 1B).
FIGURE 1. Circulating T_{FR} comprise a distinct population compared with tonsil-derived T_{FR}. (A) Gating strategy to identify circulating T_{FR} (CD4+CD25−CD127−CXCR5+PD-1+) and T_{FH} (CD4+CD25−CD127+CXCR5+PD-1+) within the Tregs and conventional T cells (Tconv) gate, respectively, in tonsils and blood. (B) Percentage of T_{FR} and T_{FH} in blood and in tonsils. (C) Percentage of follicular markers (ICOS, SAP, and BCL-6) on both T_{FR} and T_{FH} from blood and tonsils. The MFI of BCL-6 is shown. (D) Expression levels of follicular markers CXCR5, PD-1, BCL-6, and (Figure legend continues)
We next analyzed whether circulating follicular cells are phenotypically similar to those derived from tonsils using both follicular (ICOS, SAP, and BCL-6) and regulatory (Foxp3, Helios, and CD31) markers (Fig. 1C–E). Circulating TFR and TFH did not express ICOS, whereas tonsil-derived cell subsets did. SAP, essential for T–B cell interaction, was significantly less expressed on circulating compared with tonsil-derived TFH, whereas no significant differences were found for TFR. A significantly higher proportion of TFR from tonsil express BCL-6 compared with circulating TFR, whereas TFH did not significantly differ in percentage of BCL-6+ cells. In contrast, the mean fluorescent intensity (MFI) of BCL-6 is significantly decreased on both blood TFR and TFH compared with their tonsil-derived counterparts. In addition, the expression levels of essential follicular markers (CXCR5, PD-1, BCL-6, and ICOS) are shown in Fig. 1D, again highlighting the phenotypical difference in follicular expression on blood TFH and TFR. The regulatory markers Foxp3 and Helios were equally expressed by TFR in tonsils and blood. No expression of regulatory markers was seen in TFH from any source. CD31 is a key molecule for the regulation of T cell homeostasis, effector function, and trafficking (17–19). We found an increased expression of CD31 on the surface of circulating follicular cells, whereas the tonsil-derived counterparts did not express this marker. Taken together, these data show that human circulating TFR are not bona fide TFR because they lack high expression of CXCR5, PD-1, BCL-6, and ICOS, all essential follicular marker proteins.

Lastly, we determined the differentiation stage and effector phenotype of human circulating TFR. As a negative and positive control, we compared these cells with naive T cells and memory T cells, respectively, based on their CD45RO expression. Tonsil-derived TFR and TFH cells have a CD45RO+ and CD45RO2 cell population unlike the circulating follicular cell subsets, which are all CD45RO+ indicating a memory phenotype (Fig. 2A). To further characterize this memory phenotype, we used CCR7 and CD62L to distinguish effector memory T cells (TEM) (Fig. 2B) from central memory T cells (TCM) (Fig. 2C). Although all tonsil-derived follicular T cells have a TEM phenotype (CCR72CD62L2), only half of the circulating TFR have a TEM and a minority is TCM. We next examined the effector phenotype based on the expression of chemokine receptors CXCR3 and CCR6. On TFH (CD4+CXCR5+), CXCR3 expression is reported to represent a Th1 phenotype, whereas CCR6 indicates a Th17 phenotype (20). Combining both markers gives a more elaborate view on the effector phenotype (Th2; CXCR32CCR62, Th17; CXCR32CCR6+; and Th1; CXCR3•CCR6•). We found that circulating TFR have a significantly higher percentage of CXCR3•CCR6• cells (Th1-like phenotype) compared with tonsil-derived TFR (p = 0.03; Fig. 2D). A trend toward an increase in the percentage of CXCR3•CCR6• cells (Th17-like phenotype) was found (p = 0.079; Fig. 2F). An overview of the gating strategy of the flow-cytometric markers can be found in Supplemental Fig. 1.

Together, these data suggest that human circulating TFR are phenotypically distinct from their counterparts in the secondary lymphoid organs (tonsils were used as a model) because they
express lower levels of follicular markers, are a memory population, and have a proinflammatory effector phenotype.

Influenza vaccination boosts the number of circulating TFR

To investigate whether follicular cell activity is measurable in the blood, we next assessed the effect of vaccination on circulating follicular and B cell subsets. PB from HCs (n = 24; for detailed information, see Supplemental Table I) was taken before (D0), and 1 d (D1), 1 wk (D7), and 3 wk (D21) after influenza vaccination. The percentage of circulating TFR cells significantly increased after 24 h (p < 0.001) and 7 d (p < 0.001) after vaccination, and returned back to baseline after 3 wk (Fig. 3A). Memory TFR (CD45RO+CD45RA2 TFR) significantly increased (D1 and D7, p < 0.0001) after vaccination (Fig. 3B), whereas the percentage of naive TFR (CD45RO−CD45RA+) decreased significantly after 1 and 7 d (p = 0.01, data not shown). In line with other groups (21, 22), we confirmed a significant increase in circulating TFH after vaccination (all time points, p < 0.001; Fig. 3C). Similar to memory TFR, memory TFH increased significantly after influenza vaccination (D1 and D7; p < 0.001, D21: p < 0.05; Fig. 3D). CXCR5 and PD-1 levels of circulating TFR and TFH decreased after vaccination (D1 and D7, p < 0.001) and returned to baseline after 3 wk (Fig. 3E–H).

Next to circulating T cells, we also evaluated various B cell subtypes after vaccination. Similar to a previous report (21), we show that percentage of plasmablasts (CD19+CD27+CD138+) increased after vaccination, although it did not reach statistical significance (D7, p = 0.07; Fig. 3I). Although no difference in the percentage of class-switched B cells (CD19+IgD−CD27+) was found (data not shown), the ratio of non–class-switched B cells (NCS B cells, CD19+IgD+CD27+) over class-switched B cells (CS B cells, CD19+IgD−CD27+) was significantly increased after 21 d compared with D1 (p < 0.001) and D7 (p = 0.03) (Fig. 3J).

To investigate whether the changes in the circulating TFR compartment are linked with the production of protective Ab responses, we performed HAI assays on plasma samples collected at baseline and D21 using two vaccine virus strains (A/California/7/2009 [H1N1pdm] and A/Texas/50/2012 [H3N2]). For both strains,
the percentage of circulating TFR at D7 was positively correlated with postvaccination (D21) geometric mean titers (GMTs; \( p = 0.05 \) and \( p = 0.03 \); Fig. 3K, 3L). Moreover, the percentage of plasmablasts significantly correlated with postvaccination H1N1pdm GMTs titers (\( p = 0.02 \); Fig. 3M), but not with postvaccination H3N2 GMTs (Fig. 3N).

In summary, we show that seasonal influenza vaccination in HCs leads to a significant increase in circulating (memory) TFR and TFH, which significantly correlate with titers of anti-vaccine Abs. We therefore conclude that circulating TFR are a relevant source to measure TFR activity and regulation of Ab responses in response to GC reactions.

**Human circulating and tonsil-derived TFR are equally suppressive in vitro**

To assess the functionality of circulating TFR, we made a pairwise comparison of the suppressive capacity of sorted blood- and tonsil-derived TFR from the same donor using a CFSE-based coculture assay in vitro. The shown percentages are proliferation of Tresp with or without TFR. (B) TFR from blood and tonsil of HCs (\( n = 7 \)) were sorted and a CFSE-based coculture was used to determine the suppressive capacity in vitro. Tregs were incorporated as a positive control for suppressive capacity. (C) Demethylation status of FOXP3 of sorted TFR, Tregs, and TFH from HCs (\( n = 4 \) for tonsil, \( n = 9 \) for blood). Tregs and TFH were incorporated as a positive control and negative control, respectively. Data are median with range. Differences were assessed using a Wilcoxon matched-pair test.

Circulating TFR frequencies are decreased in patients with MS

Various groups, including ours, have reported an impairment in the Treg compartment of MS patients (10, 26–28). Moreover, elevated levels of autoantibodies have been reported in MS (29, 30). In light of these findings, we investigated whether the TFR com-
partment known to regulate humoral immunity is disturbed in MS. We show a decreased frequency of circulating TFR in MS patients \((n=172;\) for more information, see Supplemental Table I) compared with HCs \((n=107;\) for more information, see Supplemental Table I; \(p<0.0001;\) Fig. 5A). Moreover, the TFR/TFH ratio was significantly increased in blood of MS patients \((p<0.0001;\) Fig. 5B), indicating a relative imbalance between these interacting T cell populations. Age and sex had no effect on the frequency of circulating TFR in MS patients. We also looked into the effect of treatment but did not observe any effect (Supplemental Fig. 2A).

Moreover, in a 1-y follow-up study of patients treated with fingolimod, we did not detect an effect of treatment on percentage of blood TFR (Supplemental Fig. 2B). In addition, no significant association could be found between TFR frequencies and Expanded Disability Status Scale, MS disease type, and disease duration (Fig. 5C–E).

Next, we assessed the TFR effector phenotypes in MS. TFR with a Th17-like phenotype are increased in MS patients \((n=14\) for MS patients and \(n=16\) for HC, \(p=0.0033;\) Fig. 5F; for more information, see Supplemental Table I), whereas other effector phenotypes did not differ. In contrast with TFR, no alterations were found in the TFH compartment of MS (Supplemental Fig. 3).

Taken together, our results show that the frequency of circulating TFR is significantly decreased in patients with MS, and that the Th17 effector subpopulation of MS-derived TFR is increased.

Circulating TFR are functionally impaired in patients with MS

The functionality of MS-derived circulating TFR was tested using the in vitro coculture suppression assay described earlier (Fig. 6A). MS-derived TFR displayed a strongly impaired suppression compared with HCs (HC: \(n=15,\) MS: \(n=12,\) \(p<0.0001;\) for more information, see Supplemental Table I; Fig. 6B). We further confirmed that conventional Tregs from these MS patients were also significantly impaired \((p<0.0001;\) Fig. 6B).

When correlating the suppressive function of both Tregs and TFR from the same donor, a significant positive association was found for both MS patients and HCs (Fig. 6C). For one MS patient (relapsing-remitting MS, no treatment), the suppressive capacity of Tregs and TFR (at a 1:1 ratio) at both time points was in the same range (data not shown), confirming the reproducibility and stability of the assay, as well as the constant nature of Treg/TFR function.

In conclusion, we found that both Tregs and TFR isolated from MS patients had a reduced capacity to suppress the proliferation of Tresp showing a functional impairment of circulating TFR in MS patients.

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**FIGURE 5.** Frequency of circulating TFR in MS patients and HCs. (A) The percentage of circulating TFR in HCs and MS patients compared with HCs \((p<0.0001, n=172 \text{ for MS patients and } n=107 \text{ for HCs});\) (B) The TFR/TFH ratio MS patients and HCs \((p<0.0001, n=172 \text{ for MS patients and } n=107 \text{ for HC});\) (C) Correlation of clinical scores (Expanded Disability Status Scale) and percentage TFR. (D) The percentage of TFR in different MS disease types (one-way ANOVA). (E) Correlation between the percentage of circulating TFR and the disease duration in patients with MS. (F) Within the circulating TFR, the percentage of effector cells was investigated using CXCR3 and CCR6: Th2-like; CXCR3\(^+\)CCR6\(^-\), Th17-like; CXCR3\(^+\)CCR6\(^+\), and Th1-like; CXCR3\(^+\)CCR6\(^-\) in MS patients and HCs \((n=14 \text{ for MS patients and } n=16 \text{ for HC}, p=0.0033, \text{Mann–Whitney test});\) Data are mean ± SEM. Correlations were made using a standard linear regression model. **\(p<0.01, ****p<0.0001.\)**
Discussion

In this study, we provide an insight into the phenotype and function of circulating TFR in humans. Although circulating TFR represent a population that is phenotypically distinct from their tonsil-derived counterparts, they are an eligible source to measure GC responses ongoing in secondary lymphoid organs. We showed a decreased frequency and impaired functionality of circulating TFR in MS patients, indicating their involvement in breakdown of self-tolerance in human AIDs. These conclusions led us to two main questions: What is the origin and fate of these cells? And what explains the impairment of the circulating TFR population in MS?

Circulating TFR are not considered bona-fide TFR because they lack high expression of essential follicular markers, such as BCL-6 and ICOS. These results are consistent with findings in circulating TFH and likely indicate that follicular markers upregulate only after homing to the GC (16). This notion is further supported by the decreased expression of CXCR5 and PD-1 on circulating TFR after influenza vaccination, which could reflect homing of CXCR5hiPD-1hi TFR to the GC. In mice, TFR were shown to originate from thymic-derived Tregs (4). In line with these findings, we showed that human blood TFR express Helios and demethylated FOXP3 to a similar extent as tonsil-derived TFR and are fully functional. Furthermore, we characterized these cells as central memory with a higher expression of both CCR7 and CD62L compared with tonsil-derived TFR, allowing recirculation to the lymph nodes. He et al. (16) showed that circulating TFH also have a higher expression of these markers. In addition, we found a significant increase in the percentage of memory TFR after seasonal influenza vaccination. It is therefore possible that they originate from GC TFR, migrate to the circulation after a GC response, and become a central effector memory population that is long-lived and has the capacity to recirculate. Another theory proposed by He et al. (16) suggested that circulating TFH cells are a population of cells that leave the GC response in its early phase, before developing in mature TFH cells, governing a “precursor memory” population. We showed that although the expression of Foxp3 and Helios was the same in TFR, blood-derived follicular cells are CD31+ whereas the tonsil-derived follicular cells are negative. It has been reported that CD31 expression regulates T cell activation and can thus prevent hyperactivation (17, 19). In addition, recent evidence showed that the loss of CD31 after activation leads to a stable interaction of T cells with B cells (18). This could indicate that circulating TFR are indeed a central memory population that is quiescent by nature and will lose CD31 expression upon activation. In line with this, a recent mouse study (31) reported that circulating TFR are a long-lived memory population that homes to GC after reactivation. A human study showed that follicular T cell populations do not need an ongoing GC response for their maintenance because treatment with rituximab, known to eliminate GC B cells, had no effect on the follicular T cell compartment (32).

Together all these data indicate that circulating TFR are a distinct effector memory population that persists for a long time and is able to recirculate to the lymph nodes when needed.

MS, an AID of the CNS, was used as a model to investigate the role of circulating TFR in autoimmunity. A functional impairment of Tregs in MS was shown by various groups (26–28, 33, 34). We...
confirmed an impairment of Tregs in MS patients and found that TFR from the same patients are also defective in their capacity to suppress Tresp. Also, a decreased percentage of blood TFR in MS patients was found compared with HCs. Based on the chemokine markers suggested by Morita et al. (20), we show that the amount of Th17-like TFR is increased in MS patients. A more proinflammatory phenotype could explain the decreased suppressive function of circulating TFR. Furthermore, this impairment could originate from a defect in CTLA-4 signaling because CTLA-4 is essential in TFR function (35). An alternative explanation for the reduced frequency and suppressive activity in the circulating TFR compartment could be that the most potent TFR homed to the lymph nodes to suppress the ongoing GC response. Sage et al. (31) showed that circulating TFR in mice require dendritic cells for their development and cytokine production. Circulating dendritic cells in MS were shown to have a decreased regulatory character and their development and cytokine production. Circulating dendritic cells in MS were shown to have a decreased regulatory character and could in this way contribute to an impairment of functional circulating TFR (36).

In conclusion, we believe that follicular populations in the blood form a source of responsive memory cells that quickly react when encountering an Ag again. Impairment of circulating TFr and TFr could contribute to the pathogenesis of various AIDs, including MS, highlighting their importance in conserving normal immunity.

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Disclosures
The authors have no financial conflicts of interest.

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