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Diminished Expression and Function of TLR in Lymphatic Filariasis: A Novel Mechanism of Immune Dysregulation

Subash Babu,1* Carla P. Blauvelt,* V. Kumaraswami, † and Thomas B. Nutman* 

Lymphatic filariasis is a disease characterized by immune dysregulation involving APC and T cell populations. To assess the contribution of TLR in mediating this dysregulation, we examined the expression of TLR1, TLR2, TLR4, and TLR9 on B cells and monocytes of filaria-infected and uninfected individuals. Baseline expression of TLR was significantly lower in B cells but not in monocytes of the filaria-infected group compared with the uninfected group. Upon stimulation with filarial Ag, a diminished up-regulation of TLR was observed in both B cells and monocytes of infected individuals. Finally, stimulation of B cells and monocytes with TLR ligands resulted in decreased B cell and monocyte activation/cytokine production, indicating a state of immune tolerance. This dysregulation is associated with diminished CD4+ T cell production of IFN-γ and IL-5. The diminished expression and function of TLR is thus a likely consequence of chronic Ag stimulation and could serve as a novel mechanism underlying the dysfunctional immune response in filariasis. The Journal of Immunology, 2005, 175: 1170–1176.

Parasitic helminths have evolved mechanisms to overcome and evade host immune responses to thrive in immune-exposed locations such as lymphatics, bloodstream, and gastrointestinal tract. Most helminth infections induce relatively little disease despite extraordinarily high loads of infection. This subversion of the host immune response is achieved through the induction of multiple layers of immunoregulation. For example, lymphatic filariasis and schistosomiasis are associated with diminished Ag-specific immune responsiveness and a significant attenuation of responses to bystander Ag and vaccinations (1). Immunomodulation induced by filarial parasites has been shown to act on different arms of the immune system including lymphocytes and APC (2). T cells from filarial patients exhibit an impaired production of IL-2, IFN-γ, and IL-5, as well as decreased Ag-specific proliferation (3), whereas B cells exhibit diminished precursor frequencies and in vitro unresponsiveness to the parasite (4). In addition, filarial parasites have been shown to induce a profound dysfunction in multiple types of APC including dendritic cells, macrophages and Langerhans cells (5), a dysfunction that occurs at the level of gene transcription (6), and in the induction of cytokines and chemokines (7). However, more importantly, filaria Ag-primed APC exhibit a diminished capacity to stimulate CD4+ T cells (6–10).

The interactions of helminth parasites with APC are likely to involve TLR (2). TLR are pattern-recognition receptors that form a key component of microbial detection and host defense and are important in the initiation of host immune responses (11). TLR are involved in the recognition of a wide spectrum of pathogens by binding to pathogen-associated molecular patterns. In addition, TLR control multiple APC functions and activate signals critically involved in the initiation of adaptive immune responses (11).

The potential role of TLR in mediating interactions between helminth parasites and the host immune system has recently been described. For example, filarial parasites harbor a Wolbachia endosymbiont that can interact with the innate immune system through TLR2 and TLR4 (12). Similarly, schistosomes have been shown to induce immune responses through TLR2 (13). Finally, the pathogenesis underlying lymphatic and ocular disease in filarial infections, at least in murine models, seems to depend on TLR4 signaling (14).

Down-regulation of TLR-mediated immune responses through dampening TLR-mediated cell signaling or through diminished TLR expression seems to be an important immune evasion mechanism in some bacterial pathogens (15), a process that may be applicable to helminth parasites. Thus, children with schistosomiasis have diminished responses to TLR ligands compared with uninfected children in the same endemic area (16). Because filarial activation of the immune system via TLR causes disease, and as most filarial infections are chronic and asymptomatic, it is conceivable that filarial parasites have evolved mechanisms to limit immunopathology by modulating TLR expression or function.

To elucidate the role of TLR in filarial infections, we examined the expression of TLR1, TLR2, TLR4, and TLR9 on B cells and monocytes in a group of infected and uninfected individuals. We demonstrate that filaria-infected individuals (compared with uninfected individuals) exhibit diminished expression of TLR on B cells and diminished parasite-induced up-regulation of TLR in both B cells and monocytes. Moreover, B cell and monocyte responses to TLR ligands are significantly impaired in infected individuals. This study suggests an important role for TLR expression and function in chronic filaria infection-induced immune dysregulation.

Materials and Methods

Study population

We studied a group of 20 individuals in an area endemic for lymphatic filariasis in Tamil Nadu, South India (Table I). This included ten clinically asymptomatic, infected individuals (INF)2 and ten uninfected individuals

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2 Abbreviations used in this paper: INF, infected individual; BmA, Brugia malayi Ag; GM, geometric mean; MFI, mean fluorescence intensity; PPD, mycobacterial protein derivative; Treg, regulatory T cell; UN, uninfected individual; CT, threshold cycle.
The infected individuals were positive by both the ICT filarial Ag test (Binax) and the Trop Bio Og4C3 ELISA (Trop Bio Pty) test for circulating filarial Ag, and they were microfilaria positive by night blood examination. Each UN was circulating filarial Ag negative and had no history or signs/symptoms of filarial infection.

Brugia malayi Ag (BmA)-specific IgG4 levels in INF ranged from 1389 to 7830, with a geometric mean (GM) of 4176.7 pg/ml, whereas no BmA-specific IgG4 was detected in UN. BmA-specific total IgG in INF ranged from 97.4 to 643.8 ng/ml (GM 280.2) and from 4.3 to 350.5 ng/ml (GM 40.1) in UN, indicating likely exposure. BmA-specific IgG4 and IgG ELISA were performed exactly as described previously (17). A group of uninfected and unexposed North American blood donors (n = 5) were included as controls. All individuals were examined as part of a clinical protocol approved by Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center, and informed consent was obtained from all participants.

Isolation of PBMC

Heparinized blood was collected and peripheral blood lymphocytes isolated by Ficoll diatrizoate gradient centrifugation (LSM; ICN Biomedicals). Erythrocytes were lysed using ACK lysis buffer (BioSource International).

Parasite and control Ag

Saline extracts of B. malayi adult worms were used as the parasite Ag (BmA), and mycobacterial purified protein derivative (PPD) (Serum Statens Institute) was used as the control Ag. Final concentrations were 5 μg/ml for BmA and 10 μg/ml for PPD. Endotoxin level of final soluble BmA was <0.1 U/ml using the QCL-1000 chromogenic LAL test kit (BioWhittaker). In addition, we have excluded any functional role for endotoxin in our Ag preparations by the use of endotoxin inhibitors polymyxin B, recombinant bactericidal/permeability increasing protein, and anti-TLR4 Ab (18).

TLR ligands

The TLR ligands (Invitrogen Life Technologies) used were TLR2 ligand Pam3CysSerLys4 (hereafter Pam3Cys), TLR5 ligand flagellin from Salmonella typhimurium, TLR4 ligand ultra-pure LPS, and TLR9 ligand CpG ODN M362 (hereafter CpG).

Table I. Study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Infected (n = 10)</th>
<th>Uninfected (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (Range)</td>
<td>36 (27–55)</td>
<td>28 (25–50)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>7/3</td>
<td>8/2</td>
</tr>
<tr>
<td>Pathology</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>W. bancrofti circulating Ag levels U/ml a</td>
<td>254–49,069 (&lt;32)</td>
<td>32 (Median) (6,429)</td>
</tr>
<tr>
<td>BmA-specific IgG (μg/ml)</td>
<td>97.4–643.8</td>
<td>4.3–350.5 (280.2)</td>
</tr>
<tr>
<td>BmA-specific IgG4 (ng/ml)</td>
<td>1,389–7,830</td>
<td>0 (GM 4176.7)</td>
</tr>
</tbody>
</table>

a The lower limit of the assay detection was 32 U/ml.

FIGURE 1. Diminished expression of TLR in B cells but not monocytes in patent lymphatic filariasis. A and B, Histograms depicting baseline expression of surface TLR1, 2, 4, and intracellular TLR9 on B cells (A) and monocytes (B) in a representative filaria INF and UN. C and D, TLR1, 2, 4, and 9 expression on B cells (C) and monocytes (D) of INF (n = 10) and UN (n = 10) are shown as geometric (MFI) + 95% CI. NS, not significant.
In vitro culture and cell purification
PBMC were cultured with BmA or PPD in 24-well tissue culture plates (Corning) at concentrations of 5 x 10^5/well. After 24 h of culture, B cells (positive selection) and monocytes (negative selection) were isolated by magnetic cell purification using the respective selection kits (Miltenyi Biotec). The cells were >90% pure in all our experiments as estimated by flow cytometry. For TLR ligand stimulation, PBMC were cultured with Pam3Cys (10 μg/ml), ultra-pure LPS (10 μg/ml), Cpg DNA (5 μM), or flagellin (1 μg/ml) for 24 h.

Reagents for flow cytometry
Ab used for phenotype analysis were obtained from BD Pharmingen, and Ab used for TLR analysis were purchased from eBiosciences. The Ab used in the study were as follows: FITC-labeled anti-human CD14 and CD4; PE-labeled anti-human TLR1, 2, 4, 9, CD80, CD86, HLA-DR, IFN-γ, TNF-α, IL-4, -5, -6, -12, and -10; PerCP-labeled anti-human CD19; and allophycocyanin-labeled anti-human CD3.

Flow cytometry
PBMC were washed in PBS/0.1% BSA buffer and stained for TLR expression. Staining for TLR9 was performed using a permeabilization buffer (PBS/0.1% BSA/0.1% saponin) for detection of intracellular expression. Fluorescence was measured on a FACSCalibur (BD Biosciences) using 50,000 gated lymphocytes.

RNA preparation
B cells and monocytes were lysed using the reagents of a commercial kit (QiAmp Shredder; Qiagen). Total RNA was extracted according to the manufacturer’s protocol (RNeasy mini kit; Qiagen), and RNA was dissolved in 50 μl of RNase-free water.

cDNA synthesis
RNA (1 μg) was used to generate cDNA using TaqMan reverse transcription reagents according to the manufacturer’s protocol (Applied Biosystems). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe reverse transcriptase.

Real-time RT-PCR
Real-time quantitative RT-PCR was performed in an ABI 7700 sequence detection system (Applied Biosystems) using TaqMan Assays-on-Demand reagents for TLR1, 2, 3, 4, and 9 and an endogenous 18S ribosomal RNA control. The threshold cycle (CT) is the PCR cycle at which a statistically significant increase in the reaction concentration is first detected. The CT is calculated for the different TLR and 18S control and used to determine the relative transcript levels. Relative transcripts were determined by the formula: 1/2(CTtarget - CTcontrol), where CT is the threshold cycle during the exponential phase of amplification.

Statistical analysis
Comparisons between groups were done using the nonparametric Mann-Whitney U test. Spearman rank test followed by Bonferroni correction for multiple testing was used for correlation statistics. All statistics were performed with StatView 5 (SAS Institute).

Results
Decreased expression of TLR1, 2, 4, and 9 in B cells but not monocytes of INF
We compared the baseline expression of TLR on B cells and monocytes of INF (n = 10) and UN (n = 10) by flow cytometry. As shown in a representative histogram of B cells (Fig. 1A) and monocytes (Fig. 1B), surface expression of TLR1, 2, and 4 and intracellular expression of TLR9 was lower in INF compared with UN in B cells but not in monocytes. The geometric mean fluorescence intensity (MFI) of TLR1 (75 in UN vs 35 in INF), TLR2 (76 vs 34), TLR4 (90 vs 30), and TLR9 (450 vs 250) were significantly lower in B cells (Fig. 1C), whereas the geometric MFI of TLR1 (57 vs 59), TLR2 (66 vs 63), TLR4 (75 vs 80), and TLR9 (450 vs 470) was not significantly different in monocytes (Fig. 1D). We also examined baseline expression of TLR1, 2, 4, 5, and 9 in a subset (n = 5) of individuals in both groups by real-time RT-PCR.

The mRNA expression levels of TLR1, 2, and 9 were significantly lower in B cells (Fig. 2A) but not monocytes (Fig. 2B) of INF.

Increased expression of TLR1, 2, 4, and 9 in response to filarial Ag in UN but not INF
To determine the regulation of TLR expression in response to parasite or nonparasite Ag, we stimulated PBMC with BmA or PPD, respectively, and purified B cells and monocytes for real-time RT-PCR. B cells exhibit a significantly differential regulation of TLR1 (0.76 GM -fold change in INF vs 3.93 in UN), TLR2 (1.35 vs 3.92), TLR4 (0.72 vs 1.79), and TLR9 (0.68 vs 1.69) but not TLR5 (1.64 vs 1.63) in INF compared with UN in response to BmA but not PPD (Fig. 3A). Similarly, a significantly differential regulation of TLR1 (0.69 GM -fold change in INF vs 1.87 in UN), TLR2 (0.86 vs 4.47), TLR4 (0.58 vs 2.6), and TLR9 (0.64 vs 8.27) but not TLR5 (0.91 vs 0.94) was observed in monocytes in response to BmA but not PPD (Fig. 3B). Thus, although B cells and monocytes of UN up-regulate TLR expression in response to BmA, B cells and monocytes of INF show a complete absence of up-regulation or active down-regulation of TLR expression. The expression of TLR was not altered by exposure to BmA in B cells and monocytes, obtained from five unexposed North American blood donors (data not shown).

B cell responses to TLR ligands are diminished in INF
To determine whether the altered TLR expression in B cells of INF translated to functional diminution in responses to TLR ligands, we stimulated PBMC with the ligands for TLR2 (Pam3Cys), TLR4

FIGURE 2. Expression of TLR1, 2, 4, 5, and 9 at the mRNA level. The expression of TLR1, 2, 4, 5, and 9 on B cells (A) and monocytes (B) of UN (n = 5) and INF (n = 5) was determined by RT-PCR. The expression levels are shown as the GM relative transcript level (see Materials and Methods).
FIGURE 3. Parasite and nonparasite Ag-induced regulation of TLR in filarial infections. The expression of TLR1, 2, 4, 5, and 9 in B cells (A) and monocytes (B) following 24-h stimulation with BmA or PPD is depicted as fold change over media control in UN (n = 5) and INF (n = 5).

FIGURE 4. Stimulation of B cells by TLR ligands. A. The up-regulation of CD80, CD86, and HLA-DR in B cells of UN (n = 5) and INF (n = 5) in response to ligands for TLR2 (Pam3Cys), TLR4 (LPS), TLR5 (flagellin), and TLR9 (CpG) is shown as the geometric MFI + 95% CI. B. The percentage of CD19+ B cells staining positive for intracellular cytokines TNF-α, IL-6, and IL-10 is shown as the GM percentage + 95% CI in response to TLR ligands in UN (n = 5) and INF (n = 5).
(LPS), TLR9 (CpG), and, as a control, TLR5 (flagellin) and examined the B cell response by flow cytometry in INF (n = 5) and UN (n = 5) (Fig. 4A). Although baseline expression of all markers and cytokines examined was not different between the two groups (data not shown), TLR2, 4, and 9 ligands induced a significant up-regulation of CD80, CD86 (except Pam3Cys), and HLA-DR in UN compared with INF. When B cell cytokine production was examined by intracellular flow cytometry, we found that Pam3Cys, LPS, and CpG DNA each increased the percentage of B cells expressing TNF-α, IL-6, and IL-10 in UN but not in INF (Fig. 4B). The TLR5 ligand flagellin did not alter the expression of any of the activation markers or nature of cytokine secretion, suggesting that the functional responses measured correlated with the expression patterns of TLR in INF and UN.

Monocyte costimulatory induction and IL-6 and IL-12 responses to TLR ligands are impaired in INF

To determine whether the diminished TLR expression in monocytes of INF resulted in a functional dysregulation, we stimulated PBMC with the ligands for TLR2 (Pam3Cys), TLR4 (LPS), TLR5 (flagellin), and TLR9 (CpG) and, as a control, TLR5 (flagellin) and examined the monocyte response by flow cytometry in INF (n = 5) and UN (n = 5). Whereas baseline expression of all markers and cytokines examined was not different between the two groups (data not shown), Pam3Cys, LPS, and CpG DNA each induced significant up-regulation of CD80 and CD86 but not HLA-DR (except for LPS) in UN compared with INF (Fig. 5A). Notably, unlike B cells, monocytes of INF were significantly impaired only in their ability to produce IL-6 and IL-12 but not TNF-α in response to TLR ligands (Fig. 5B). As expected, and similar to that seen in B cells, flagellin had no differential effect on monocyte responses.

Decreased expression of IFN-γ and IL-5 in CD4+ T cells of INF

To examine whether the diminished APC expression/up-regulation of TLR could have an impact on the cytokine response of CD4+ T cells to parasite Ag, we stimulated PBMC with BmA for 24 h and examined the production of IFN-γ, IL-4, and IL-5 in CD4+ T cells by flow cytometry. As shown in Fig. 6, the percentage of CD4+ T cells expressing IFN-γ and IL-5 was significantly lower in INF in comparison with UN. Of interest, BmA-induced TLR2 expression levels on monocytes positively correlated with BmA-induced IFN-γ (p = 0.034, p = 0.852) and IL-5 (p = 0.043, p = 0.806) expression on CD4+ T cells (data not shown).

Discussion

One hallmark of helminth infections is their chronicity, which is felt to reflect the varied host evasion mechanisms used by the parasites. Thus, in filarial infections, multiple mechanisms have been postulated to play a role in down-regulating host immune responses. These include regulatory T cells (Treg) and cytokines, altered function of APC, T cell and APC apoptosis, conditioned Th2 cells, alternatively activated macrophages, and inducible NO synthase (2). The alteration of APC function and regulation as a
FIGURE 6. Cytokine response of CD4+ T cells to parasite Ag. The percentage of CD4+ T cells expressing IFN-γ, IL-4, and IL-5 is shown as the GM percentage ± 95% CI in UN (n = 5) and INF (n = 5).

major mechanism of altering immune responses has recently begun to emerge (5). In mouse models of filarial infection, parasites elicit a population of alternatively activated macrophages, which have been shown to down-modulate T cell responses (8). Similarly, in human studies, it has been shown that different stages of filarial parasites can modulate the function of dendritic cells and macrophages (7, 19), Langerhans cells (6), and monocytes. These filaria-conditioned APC are poor inducers of T cell responses.

The TLR family, the best characterized class of pattern-recognition receptors in humans, currently includes ~11 members. TLR detect multiple pathogen-associated molecular patterns including LPS (detected by TLR4), bacterial lipoproteins (detected by TLR2), flagellin (detected by TLR5), the unmethylated CpG DNA of bacteria and viruses (detected by TLR9), dsRNA (detected by TLR3), and viral ssRNA (detected by TLR7) (11). Because activation of APC is primarily through TLR recognition and signaling, we examined TLR expression and function on the APC present in peripheral blood monocytes and B cells. Both B cells and monocytes are known to express a wide variety of TLR including TLR1, 2, 4, 5, and 9 (20–22). Although monocyte expression of TLR9 (23) and B cell expression of TLR4 (24, 25) is not common, it is clear that such expression is present only under certain conditions. We consistently observed expression of TLR on both cell types by flow cytometry as well as by real-time RT-PCR. Our first major observation was the diminished expression of TLR1, 2, 4, 5, and 9 on B cells both at the protein and mRNA levels in INF compared with UN. Notably, no difference in TLR expression patterns was noted on monocytes obtained from blood of the two distinct clinical groups; however, upon examination of TLR regulation in response to BmA or PPD, we found that both monocytes and B cells of INF exhibit severe impairment in their ability to up-regulate TLR1, 2, 4, and 9 but not TLR5 selectively in response to BmA. Although it is well known that a variety of bacterial pathogens can cause an increase in TLR expression (15), our data provide evidence that parasites also induce regulation of TLR expression on monocytes and B cells. On B cells, both baseline and Ag-induced TLR expression patterns are altered in chronically infected individuals, whereas on monocytes, Ag-induced but not baseline expression patterns are altered. Interestingly, B cells and monocytes of uninfected and unexposed controls did not exhibit any alteration of TLR expression and function. This suggests that chronic stimulation by filarial parasites in infected individuals is the major determinant in the down-regulated TLR responses.

Down-regulation of TLR expression is an important immune evasion strategy used by bacterial pathogens. TLR4 down-regulation and tolerance by LPS and TLR2 down-regulation by bacterial lipoprotein is a common mechanism of immune suppression. Similarly, parasites such as *Entamoeba histolytica* and *Trypanosoma* spp. inhibit immune responses by down-regulating TLR2 expression (26) and TLR signaling (27, 28). In our studies, down-regulation of TLR1, 2, 4, and 9 appears to be an important mechanism of immune evasion in filarial infections. This down-regulation on monocytes is specific to filarial Ag, which would prevent the establishment of a much broader state of immunodeficiency in filarial patients. The down-regulation of TLR expression could be a direct effect of filarial Ag on B cells and monocytes, or an indirect effect resulting from diminished cytokine/chemokine production by APC. Also, the TLR down-regulation was not a generalized phenomenon in that TLR5, for example, was not found to be altered in expression following stimulation with filarial Ag. Human memory B cells do respond to TLR ligand stimulation by producing Ab independently of Ag-specific cognate T cell help (29); therefore, down-regulation of baseline TLR expression would be a mechanism to prevent exaggerated activation of B cells in filarial infections.

TLR expression on APC can modulate adaptive immune responses by two different mechanisms. The first mechanism involves induction of costimulatory molecules CD80 and CD86 on APC (11, 30). TLR-induced expression of costimulatory molecules on APC enables the Ag-specific T cells to receive the necessary second signal resulting in T cell activation. In addition, TLR-mediated production of cytokines such as TNF-α, IL-6, -10, and -12 contribute to the class and type of Ag-specific T cell responses (30). The other mechanism by which TLR modulate adaptive responses is by blocking the suppression maintained by Treg (31). This mechanism is independent of costimulation but mediated by IL-6, which causes the Ag-specific T cells to become refractory to Treg (31). When we examined the induction of costimulatory molecules (CD80 and CD86) as well as HLA-DR by various TLR ligands, we found significant impairment of CD80 and CD86 up-regulation in both B cells and monocytes of INF. As mentioned above, the diminished expression of costimulatory molecules can directly contribute to diminished adaptive immune responses. Moreover, upon examination of cytokine production, we found a diminished percentage of B cells expressing TNF-α, IL-6, and IL-10 and a lower percentage of monocytes expressing IL-6 and IL-12. These patterns were evident in response to TLR2, 4, and 9 ligands but not the control TLR5 ligand, suggesting that functional responses correspond specifically to expression patterns. Impaired production of IL-6 could directly contribute to a failure to overcome Treg-induced suppression in filariasis and the impaired production of IL-12 to diminished Th1 responses. These findings indicate that TLR expression patterns on B cells and monocytes correlate with functional responses to TLR ligands. CD4+ T cell responses to BmA is significantly impaired in terms of IFN-γ and IL-5 production (but not IL-4) in a subset of INF, indicating that down-regulation of TLR in these individuals may mediate, in part, this impairment in CD4+ T cell responses. Indeed, a significant correlation exists between TLR2 expression on monocytes and CD4+ T cell cytokine production, indicating that TLR2 plays a major role in the diminished cytokine response. Thus, compromised TLR expression and function underlie the diminished Ag-specific proliferation and down-regulated cytokine responses characteristic of filarial infections (3) and, in addition, possibly explain the diminished immunity observed for bystander Ag and routine vaccinations (32–34). Because polyclonal T cell stimuli such as anti-CD3 do not depend on TLR-mediated activation, no inhibition in such T cell activation is seen in filarial infections (3). The diminished expression of TLR on B cells could...
presumably result in a different profile of Ab responses, and that is precisely what we observe in INF and UN. Thus, INF mount an Ab profile characterized by the inhibitory isotype IgG4, which is characteristically absent in UN. Hence, the altered TLR expression phenotype could potentially impact the humoral immune responses in filarial infections. It would be interesting to study the effect of differential TLR expression on the class of Ab responses in more amenable animal models of filarial infection.

Our studies suggest a central role for TLR dysregulation as a mechanism for Ag-specific anergy in filariasis. The chronicity of parasite Ag exposure in filarial infection likely suppresses TLR-dependent activation of the innate and adaptive immune system, and the TLR system provides a novel target for the parasite to down-modulate the host immune system.

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Disclosures

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References


