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Agonist Ligands Expressed by Thymic Epithelium Enhance Positive Selection of Regulatory T Lymphocytes from Precursors with a Normally Diverse TCR Repertoire

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CD4\(^+\)CD25\(^+\) regulatory T lymphocytes play a crucial role in inhibition of autoimmune pathology. In accordance with this physiological role, it is now well established that the repertoire of these lymphocytes is strongly enriched in autospecific cells. However, despite extensive investigation, the thymic mechanisms involved in development of regulatory T cells remain incompletely defined. To address the issue of selection of regulatory T cell precursors in mice with a naturally diverse TCR repertoire, we have analyzed development of superantigen-specific regulatory T cells in hemopoietic chimeras in which endogenous superantigens are exclusively presented by thymic epithelial cells. Our results demonstrate that recognition of agonist ligands expressed by thymic epithelium does not lead to deletion but substantially enhances development of mature regulatory T cells. Interestingly, also development of a small subpopulation of CD25-expressing T cells lacking expression of the transcription factor Foxp3, by thymic epithelium substantially enhances their positive selection.

The precise role of positive selection in development of Treg remains unclear. It has been proposed that interaction with agonist ligands directs developing thymocytes to the Treg lineage. However, studies with mice doubly transgenic for TCR and agonist ligand have led to conflicting results. In some cases, evidence of increased selection of CD4\(^+\)CD25\(^+\) Treg by agonist ligand was observed (21–25). In other TCR/ligand doubly transgenic mice, the relatively increased percentage of CD4\(^+\)CD25\(^{shhi}\) Treg has been attributed to deletion of CD25\(^-\) cells rather than increased development of CD4\(^+\)CD25\(^+\) cells (19). The potential differences reported in the distinct doubly transgenic mice may be due to the particular avidity of the chosen TCR/ligand pair and/or to variations in the expression pattern of the ligand. Therefore, it remains unclear 1) whether TCR-mediated signals are involved in the Treg lineage choice and 2) whether differences exist between positive selection of conventional and regulatory T lymphocytes.

To gain insight into the mechanisms of thymic Treg differentiation, we analyzed the role of agonist ligands expressed by TE in the development of Treg precursors with a naturally diverse TCR repertoire. We generated bone marrow chimeras in which superantigen (sAg) presentation is limited to TE and analyzed the development of peripheral Treg repertoire highly enriched in self-reactive cells (14–16). These observations raise important questions concerning the selection of these cells in the thymus. In this organ, CD4\(^+\)CD25\(^+\) Treg are positively selected by cortical epithelial cells, as are conventional CD4\(^+\) lymphocytes (17). Surprisingly, Treg precursors have been shown to be susceptible to thymic negative selection (14, 17, 18). However, although autospecific Treg precursors are negatively selected by APC of bone marrow origin (14), they appear relatively resistant to negative selection induced by thymic epithelium (TE) (13, 19). Therefore, TE plays an important role in development of dominant tolerance, as previously postulated based on the observation that TE can induce dominant transplantation tolerance (20).

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sAg-specific Treg. Our results indicate that agonist ligands considerably enhance positive selection of Treg.

Materials and Methods

Mice

All mice were used at 6–10 wk of age. C57BL/6 and DBA/2 mice were purchased from Janvier. B10.D2 mice were originally obtained from Harlan France and maintained in our specific-pathogen-free animal facilities. C57BL/6 mice deficient in MHC expression (MHC°) because of targeted deletions in the β2-microglobulin (26) and IAPβ genes (27) were obtained from the Centre de Développement des Techniques Avancées-National de la Recherche Scientifique and were maintained in our specific-pathogen-free animal facility. All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines (Institut National de la Santé et de la Recherche Médicale; approval no. 51-13) and have been approved by the local ethics committee (Midipyrénées, France; Reference MP/01/51/10/03).

Antibodies

The following Abs were used for phenotypic analysis: FITC-labeled anti-TCR Vβ3, 4, 5, 6, 14, 17*, PE-labeled anti-CD25, PE-Cy7-labeled anti-CD4 (BD Pharmingen), PE-labeled anti-Foxp3, and allophycocyanin-labeled anti-CD8 and anti-CD25 Abs (eBioscience).

Flow cytometry

Thymi were homogenized, washed in medium, and resuspended in 2.4G2 (anti-FcγR mAb) (28) hybridoma supernatant. After incubation of 30 min on ice, saturating concentrations of Ab were added. Twenty minutes later, cells were washed in PBS, 2.5% FCS, and 0.02% NaN3. Labeled cells were analyzed using a FACS Calibur and CellQuest software (BD Biosciences). Dead cells were excluded using appropriate forward scatter/side scatter gates.

For TCR Vβ analysis, thymi of three mice were pooled, depleted of CD8+ cells by treatment with anti-CD8 mAb 31.1.2 (29) and complement (Saxon Europe), followed by Lympholyte-M gradient (Cederlane Laboratories). The efficiency of CD8 depletion was verified in every experiment and was routinely >99%.

Bone marrow chimeras

Irradiation bone marrow chimeras were generated by lethally irradiating (8.5 Gy gamma) C57BL/6 hosts using a 140 Cs source (7 Gy/min). Next day, irradiated mice were reconstituted by i.v. injection of 107 bone marrow cells. Chimeras were kept on antibiotics containing water (0.2% Bactrim; Roche) for the complete duration of the experiment.

Statistical analysis

Statistical significance of differences between subpopulations was assessed using Student’s t test and is indicated as not significant (p > 0.05); *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Results

Superantigens presented by Treg enhance differentiation of CD4+ CD8+ CD25high Treg

To study the role of naturally expressed agonist ligands in thymic selection of Treg precursors with a normally diverse TCR repertoire, we generated irradiation chimeras in which sAg are exclusively presented by Treg. As hosts, we used (lethally irradiated) DBA/2 mice which present endogenous sAg encoded by mouse mammary tumor viruses 1, 6, 7, 8, 11, and 13. These sAg are high-affinity ligands for Vβ3, Vβ5, and Vβ6 but they do not interact with Vβ4 and Vβ14 (30). MHC-deficient (MHC°) bone marrow was used to prevent thymic deletion of Treg precursors induced by APC of hemopoietic origin (14). These MHC°→DBA/2 chimeras were compared with MHC°→C57BL/6 (B6) mice, in which no sAg-mediated thymic deletion is known to occur (30). Controls consisted of B6→B6 and DBA/2→DBA/2 chimeras.

Chimeras were analyzed by flow cytometry 6 wk after reconstitution. The percentage of thymocytes of donor origin was always superior to 99%. In the spleen, >99% of T and B lymphocytes were of donor origin. Less than 7% of host CD11c+CD11b− thymic DC remained in the chimeras. Moreover, the ratio of CD8low:CD8high cells among remaining host DC was identical to that found in unmanipulated animals (data not shown). In the thymus of DBA/2→DBA/2 chimeras, significantly higher percentages of CD25high Treg among CD4+CD8+ (CD4SP) thymocytes were observed than in B6→B6 chimeras (Fig. 1, A and B). This result confirms genetically determined quantitative differences in Treg development, due to thymocyte-intrinsic factors, that we have reported previously (31). Significantly reduced percentages of sAg-specific Vβ3-, Vβ5-, and Vβ6-expressing CD4SP CD25+ and CD25high cells were found in DBA/2→DBA/2 as compared with B6→B6 chimeras (Fig. 1, C and D). No difference in percentages of Vβ4 and Vβ14 T cells (which do not react with sAg presented in DBA/2 mice) was observed between the two types of chimeras. These data confirm that Treg precursors are sensitive to thymic deletion, as we and others have previously reported (14, 18).

We next analyzed irradiation chimeras in which bone marrow derived cells did not express MHC molecules and therefore could not present sAg. As compared with B6→B6 and DBA/2→DBA/2 chimeras, in MHC°→B6 and MHC°→DBA/2 chimeras significantly increased percentages of CD4SP thymocytes were found (Fig. 1B). These results are due to substantially reduced induction of apoptosis of autospecific cells in these chimeras, as previously reported (2).

Interestingly, a 2-fold higher percentage of CD25− (but not CD25+) CD4SP Vβ5+ (but not Vβ3, Vβ6, Vβ4, or Vβ14) cells was found in MHC°→B6 than in B6→B6 chimeras (Fig. 1D). These results show that Vβ5-specific deletion of CD25− but not CD25+ thymocytes by bone marrow-derived APC occurs in B6→B6 chimeras.

In contrast to chimeras in which TE and APC express sAg, in MHC°→DBA/2 mice sAg-specific Vβ3+ cells were not deleted and only partial deletion of sAg-specific Vβ5 and Vβ6 CD4SP CD25− cells was observed. These results are consistent with previous work documenting the limited role of thymic (medullary) epithelium in deletion of autoreactive precursors (32–34). Control Vβ4+ and Vβ14+ thymocytes were not deleted.

As compared with MHC°→B6 chimeras, in MHC°→DBA/2 mice a substantial increase in the percentage of CD4SP CD25high regulatory thymocytes expressing sAg-specific Vβ3, Vβ5, and Vβ6 was observed (Fig. 1, C and D). Since in the MHC°→B6 and MHC°→DBA/2 chimeras the total number of thymocytes and the percentage of CD4SP cells were similar, and in MHC°→DBA/2 chimeras the percentage of CD25high Treg among CD4SP cells is increased (Fig. 1B), the increase in percentage corresponded to an increase in absolute cell numbers of sAg-specific Treg in MHC°→DBA/2 chimeras. Comparable percentages of Vβ4 and Vβ14 CD4SP CD25high Treg developed in both types of chimeras. These results indicate that natural agonist ligands presented by Treg substantially enhance the generation of CD4SP CD25high Treg.

sAg enhance generation of CD4+Foxp3+ Treg and a subpopulation of CD4+ Foxp3+ thymocytes

CD25 is widely used as marker for Treg (35). However, it is also expressed on activated conventional CD4+ and CD8+ lymphocytes and does not identify all Treg. A better marker for Treg is Foxp3 (7, 8, 36), a forkhead/winged helix transcription factor. We therefore assessed whether sAg presented by TE also enhanced differentiation of CD4+Foxp3+ regulatory T cells. As compared with MHC°→B6 chimeras, in MHC°→DBA/2 chimeras substantially increased percentages and numbers of Foxp3+ CD4+ thymocytes expressing sAg-specific Vβ3, Vβ5, and Vβ6 (but not control
Vβ4 and Vβ14) were observed (Fig. 2, A–C). In contrast, sAg-specific Foxp3+ CD4SP thymocytes were either partially deleted (Vβ5 and 6) or not affected (Vβ3) by sAg presented by TE (Fig. 2B).

The MHC° mice we used as bone marrow donors were on the C57BL/6 background, in which very few mouse mammary tumor virus genomes are present (30). Since hemopoietic cells are known to produce sAg, sAg presentation in MHC° DBA/2 and DBA/2 chimeras is not only qualitatively but also quantitatively different. One could therefore argue that enhanced positive selection of sAg-specific Treg in MHC° DBA/2 chimeras might be due to the lower overall avidity of the interaction of thymocytes with stromal cells, independently of the nature (DC or TE) of the presenting cell. To assess this possibility we generated MHC°→B6 and MHC°→DBA/2 chimeras in which the expression levels of sAg were the same but the nature of the cells presenting them differed. As shown in Fig. 2C, in contrast to MHC°→DBA/2 chimeras, in B10.D2→DBA/2 chimeras sAg-specific Vβ3+, Vβ5+, and Vβ6+Foxp3+ CD4SP thymocytes were deleted. Importantly, also substantially less sAg-specific Foxp3+ CD4SP thymocytes were observed in B10.D2→DBA/2 than in MHC°→DBA/2 chimeras. These data unequivocally show that the nature of the stromal cell type presenting the sAg determines the outcome of selection in our experimental model: DC induce deletion and TE induces positive selection of Treg precursors.

Foxp3+ CD4SP thymocytes express varying levels of CD25 (Fig. 3A) as previously reported (37). We therefore next assessed whether sAg differentially enhanced development of CD25−, CD25int, or CD25high Foxp3+ thymocytes (Fig. 3B). sAg presented by TE substantially enhanced differentiation of all three Foxp3+ subpopulations (Fig. 3B, cf MHC°→B6 with MHC°→B6 chimeras).

We also analyzed selection of a small population of CD4SP CD25intFoxp3+ thymocytes (Fig. 3, A and C). These cells may be thymic precursors for recently described peripheral T lymphocytes with the same phenotype (8, 38). Interestingly, as compared with MHC°→B6 chimeras, in MHC°→DBA/2 chimeras a statistically significant increase in the percentage of CD4SP CD25highFoxp3+ thymocytes expressing sAg-reactive Vβ3, Vβ5, and Vβ6 (but not control Vβ4 and Vβ14) was observed (Fig. 3C). The CD4SP CD25highFoxp3+ population therefore appears to be enriched in self-Ag-specific T lymphocytes as previously suggested (8, 38).

**FIGURE 1.** sAg presented by TE enhance generation of CD4SP CD25high Treg. A, Analysis of CD25 expression by electronically gated CD4SP thymocytes. B, Total numbers of thymocytes (left panel), percentage of CD4SP cells among total thymocytes (middle panel), and percentage of CD25high cells among CD4SP thymocytes (right panel) in indicated chimeras. C, Flow cytometry analysis of TCR Vβ expression by electronically gated CD4SP CD25− and CD25high thymocytes in indicated chimeras. D, Percentages of CD4SP CD25− and CD25high thymocytes expressing indicated Vβ in the distinct chimeras. ***p < 0.001; **p < 0.01; ns, not significant; Student’s t test. Error bars indicate SD (B6→B6 and DBA/2→DBA/2: n = 3, MHC°→B6 and MHC°→DBA/2: n = 6).
sAg do not enhance positive selection of precursors for conventional T cells

The presented data indicate that generation of Treg is substantially enhanced by interaction with agonist ligand. We next investigated whether thymic-positive selection of conventional (i.e., nonregulatory) T cells can also be increased by interaction with agonist ligand. To this end, we analyzed $V_{\beta}$ expression by CD69-expressing CD4$^{+}$CD8$^{-}$ (DP) thymocytes. Whereas it is impossible at this very early stage of development to distinguish between precursors for conventional and Treg, since the vast majority of mature thymocytes are conventional cells, analysis of the whole population in essence reflects analysis of conventional T cell precursors. Analysis of $V_\beta$ expression by CD69$^+$ DP revealed two levels of $V_\beta$ expression: low and high (Fig. 4A). $V_\beta^{low}$ thymocytes are recently activated (positively selected) cells that are precursors for mature CD4sp and CD4$^+$CD8$^+$ (CD8sp) thymocytes (39). Using a TCR-transgenic mouse system, it has previously been shown that these cells have not yet been submitted to thymic deletion (34). $V_\beta^{high}$ cells are more mature, have been submitted to thymic deletion, and are precursors for CD8sp thymocytes (34, 39, 40). We therefore analyzed the percentage of recently positively (but not yet negatively) selected $V_\beta^{low}$ thymocytes in MHC$^+$→B6 and MHC$^+$→DBA/2 chimeras (Fig. 4B). No difference between the percentages of sAg-specific $V_\beta^{low}$ and $V_\beta^{low}$ and control $V_\beta^{low}$ and $V_\beta^{low}$ thymocytes in sAg-presenting vs nonpresenting chimeras was observed. We therefore conclude that positive selection of precursors for conventional T cells is not enhanced by agonist ligands.

Discussion

This is the first study on the role of natural agonist ligands on Treg development in mice with a normally diverse TCR repertoire. We found that sAg-specific Treg precursors were deleted in mice in which both TE and APC of bone marrow origin presented sAg, as we have previously reported (14). In contrast, in bone marrow chimeras in which sAg are exclusively expressed by TE, we found that development of sAg-specific Treg (but not conventional T cells) was substantially enhanced. Therefore, our data unambiguously show that agonist ligands substantially enhance development of Treg.
Specificity for sAg is determined by the Vβ region expressed by T lymphocytes. In mice containing endogenous mouse mammary tumor viruses, sAg-specific thymocytes are deleted during T cell development in the thymus (reviewed in Ref. 30). We have previously reported that precursors for Treg are not an exception and are efficiently deleted during development (14). When sAg are only presented by TE, deletion of precursors for conventional T cells is much less efficient and tolerance is mediated by clonal anergy (41). In our MHC° DBA/2 chimeras, in which sAg are exclusively presented by TE, we indeed found that deletion of sAg-specific Foxp3⁺ thymocytes was much less efficient than in control DBA/2→DBA/2 chimeras in which sAg can also be presented by APC. However, depending on the Vβ region expressed, partial deletion clearly took place, confirming earlier reports showing that TE can directly induce deletion of precursors for CD4SP thymocytes (32–34).

Importantly, agonist ligand expressed by TE did not induce any deletion of Treg precursors. In contrast, it substantially enhanced development of Treg. By studying MHC class II transfer from thymic stroma to developing thymocytes, we have previously observed that precursors for CD25⁺, but not CD25⁻, CD4SP thymocytes are sensitive to negative selection induced by TE (13). In TCR/ligand doubly transgenic mice in which Ag is expressed by TE, significant deletion of CD25⁻ but not of CD25⁺ CD4SP precursors has previously been observed (19, 21, 22, 25). However, in these studies it was unclear whether deletion was due to presentation of Ag directly by TE or indirectly by APC. In these and other transgenic mouse models, the agonist ligand induced development of increased percentages of CD25⁺ Treg among CD4SP thymocytes (19, 21, 22, 24, 25, 42). Whether or not expression of the agonist ligand led to development of more Treg, in terms of absolute cell numbers, was unclear in most (21, 22, 24, 42) but not all (25) of these reports, and this was formally dismissed in another (19). However, even if numerically more Treg developed in TCR/Ag doubly transgenic mice, this would not necessarily be due to agonist ligand-mediated recruitment into the Treg lineage or selection of these cells. In this light, we have previously reported that development of CD8SP thymocytes is limited by homeostatic mechanisms (43). When development of CD4SP thymocytes is strongly inhibited by negative selection, as in the TCR/Ag doubly transgenic mice, more Treg might develop due to homeostatic mechanisms. Such complications are related to the fact that in
TCR-transgenic mice practically all precursors express the same TCR. In contrast, in the experimental model we presented here a substantially smaller fraction of precursors was involved. Indeed, the total thymocyte numbers and percentages of CD4SP were similar in the two types of irradiation chimeras we compared. As compared with chimeras in which no sAg was presented, in chimeras in which sAg were presented only by TE substantially higher numbers of sAg-specific Treg developed. Since in our experimental model it is difficult to imagine how results could be due to homeostatic mechanisms, we conclude that recognition of agonist ligand substantially enhanced development of Treg.

An unexpected observation merits particular attention. It has previously been reported that in B6 thymi Vβ5 expression is skewed to the CD8SP population (44). This result was attributed to a less efficient positive selection of Vβ5+ cells into the CD4SP than the CD8SP population. Also, peripheral Vβ5 expression in I-EΔ (C57BL/10) mice is skewed to the CD8+ population (45). With age the CD4:CD8 ratio of Vβ5-expressing T cells gradually decreases, and this is known to be due to Mtv-8- and Mtv-9-mediated peripheral deletion of CD4+, but not CD8+ Vβ5+ T cells (46). We observed that 2-fold more CD4SP Vβ5+ thymocytes developed in absence than in presence of thymic deletion by bone marrow-derived APC. Vβ5+ thymocytes are therefore partially deleted, probably by Mtv-8 and 9. Interestingly, also 2-fold more Vβ5 CD8SP thymocytes developed in MHC~→B6 than in B6→B6 chimeras (data not shown). Therefore, thymic skewing of Vβ5 expression toward the CD8 lineage cannot be explained by differences in thymic deletion mediated by APC of bone marrow origin. In MHC~→B6 chimeras, the percentage of Vβ5+ cells among freshly positively selected CD69+ DP thymocytes is similar to that among CD4SP cells but significantly lower than that among CD8SP cells (data not shown). Together, these data indicate that the thymic skewing of Vβ5 expression toward the CD8 lineage is indeed most probably explained by more efficient positive selection into the CD8 lineage. Moreover, since Mtv-8 and 9 presentation by I-Aa leads to only partial deletion of Vβ5+ thymocytes, clearly low-avidity interactions are involved.

Whereas the TCR repertoires of CD25+ Treg and CD25− conventional T cells show only very limited overlap (16), their TCR Vβ repertoires are very similar (14, 18, 47). An exception to this rule is Vβ5, which, in B6 mice is ~2-fold more represented in the CD25+ than in the CD25− population. In absence of negative selection by APC (in MHC~→B6 chimeras), the percentage of Vβ5+ cells among CD25−, but not CD25+ CD4SP thymocytes was increased. As compared with conventional T cell precursors, precursors for Treg appear therefore less susceptible to APC-mediated deletion by low-avidity ligands. This conclusion is consistent with our earlier observation that the magnitude of deletion of Treg precursors by APC seemed slightly lower than that of conventional T cell precursors (14).

Interestingly, no more Vβ5+ Treg developed in MHC~→B6 than in B6→B6 chimeras. Therefore, in contrast to high-avidity I-Eα/sAg ligands, low-avidity I-Aα/sAg ligands did not induce enhanced positive selection of Treg. This result is consistent with earlier data showing that high avidity, but not low-avidity interactions allow for Treg development in TCR/ligand doubly transgenic mice (24).

The precise mechanism involved in sAg-mediated enhanced development of Treg is uncertain. However, it appears unlikely that recognition of the agonist ligand on TE by uncommitted precursors recruits them to the Treg lineage. If this were the case, one would expect an even higher increase in development of sAg-specific Treg in MHC~→DBA/2 (as compared with MHC~→B6) chimeras. For example, TE expression of sAg led to deletion of ~5 × 105 CD25−Vβ5+ CD4SP, while only 2 × 104 more CD25−high/Vβ5+ cells developed. Our data are more compatible with the hypothesis that recognition of the agonist ligand allows for positive selection of precursors already committed to the Treg lineage. Alternatively, recognition of the agonist ligand expressed in a thymic niche specialized in Treg development may allow for simultaneous positive selection and Treg lineage commitment. This view is consistent with the observation that thymocytes expressing Treg-derived TCR preferentially, but not exclusively, develop into Treg (16). The second scenario is also consistent with the observation that expression of Foxp3, a master switch in Treg development, requires TCR-ligand interaction (8).

To evaluate the effect of agonist ligand expression by TE on positive selection of conventional T cells, we analyzed Vβ expression by TCRVβ+/CD69+ DP thymocytes in MHC~→B6 and MHC~→DBA/2 chimeras. These cells have initiated positive selection, but have not yet been submitted to negative selection (34). Comparable percentages of sAg-specific cells were found in the two types of chimeras. We conclude therefore that positive selection of conventional T cells is not enhanced by recognition of agonist ligand. Differentiation of conventional T cells has been shown to require continued TCR-mediated signaling occurring after the initial phase of positive selection (48). Therefore, enhanced positive selection of Treg by interaction with agonist ligand may very well occur at later stages of development (e.g., in the medulla). In contrast, continued interaction of conventional T cell precursors with agonist ligands does not enhance positive selection but leads to negative selection.

Under physiological conditions, peripheral T lymphocytes of CD4+CD25−Foxp3− phenotype produce IL-2, required for in vivo maintenance of Treg (38). It has previously been proposed that this population is enriched in self-reactive T cells (8, 38). Intriguingly, we found that recognition of the agonist ligand expressed by TE enhanced development of CD4SP CD25−Foxp3− thymocytes. Precursors for these cells therefore appear resistant to negative selection induced by ligands expressed by TE and their positive selection is enhanced by recognition of agonist ligand. Our results strongly suggest that the autologous CD4+CD25−Foxp3+ T cell repertoire is, at least in part, generated in the thymus. These data therefore indicate that at least two apparently distinct T cell lineages, Treg and CD4+CD25−Foxp3− T cells, use identical modes of thymic selection.

In conclusion, natural agonist ligands expressed by TE do not induce deletion of Treg precursors but substantially enhance their positive selection. Importantly, medullary thymic epithelium (but not bone marrow-derived DC) expresses a large variety of “tissue-specific” Ags (49). Therefore, TE will enhance development of Treg specific for ubiquitously expressed as well as for tissue-specific Ags, but DC will induce deletion of Treg precursors specific for ubiquitously expressed Ags. Thus, a repertoire of Treg exquisitely appropriate for protection against autoimmune aggression appears to develop.

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Disclosures
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