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CD4⁺ T Regulatory Cell Induction and Function in Transplant Recipients after CD154 Blockade Is TLR4 Independent

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Although the role of CD4⁺ T regulatory cells (Treg) in transplantation tolerance has been established, putative mechanisms of Treg induction and function in vivo remain unclear. TLR4 signaling has been implicated in the regulation of CD4⁺CD25⁺ Treg functions recently. In this study, we first examined the role of recipient TLR4 in the acquisition of operational CD4⁺ Treg following CD154 blockade in a murine cardiac transplant model. Then, we determined whether TLR4 activation in allograft tolerant recipients would reverse alloimmune suppression mediated by CD4⁺ Treg. We document that donor-specific immune tolerance was readily induced in TLR4-deficient recipients by a single dose of anti-CD154 mAb, similar to wild-type counterparts. The function and phenotype of CD4⁺ Treg in both wild-type and TLR4 knockout long-term hosts was demonstrated by a series of depletion experiments examining their ability to suppress the rejection of secondary donor-type skin grafts and to inhibit alloreactive CD8⁺ T cell activation in vivo. Furthermore, TLR4 activation in tolerant recipients following exogenous LPS infusion in conjunction with donor-type skin graft challenge, failed to break Treg-mediated immune suppression. In conclusion, our data reveals a distinctive property of CD4⁺ Treg in tolerant allograft recipients, whose induction and function are independent of TLR4 signaling.


By engaging pathogen-associated molecular patterns, TLR represent the host sentinel system that is responsive to infections and important for optimal cellular immune responses against viruses and bacteria (1). It is now well-established that TLR4 activation on APCs contributes to the initiation of adaptive immune responses by inducing the expression of costimulatory molecules on APCs (1). In addition to products derived from infectious agents, endogenous TLR ligands generated during the cellular stress or tissue damage have been recently identified, including heat-shock proteins and fragmented extracellular matrix products (2). Thus, innate immunity may also regulate adaptive immunity in the absence of infection. Obviously, in organ transplantation, surgery procedure itself as well as organ ischemia/reperfusion injury all lead to the generation of multiple types of endogenous TLR ligands. Indeed, the role of TLR system in allograft rejection cascade has been recently confirmed by the inability of minor MHC-mismatched allografts to undergo rejection or to trigger Th1-type response in the absence of MyD88, a key adaptor molecule in multiple TLR-signaling pathways (3). Our own studies have also documented the requirement for TLR4 in the pathophysiology of Ag-independent liver ischemia/reperfusion injury (4). Additionally, the TLR system plays a role in regulating T cell responses by controlling the function of CD4⁺CD25⁺ T regulatory cells (Treg). Indeed, TLR4 activation on APCs was able to overcome CD4⁺CD25⁺ Treg-mediated suppression on other T cells via an IL-6-dependent mechanism (5). Recent studies have shown that several TLRs, in particular TLR4, 5, 7 and 8, may be selectively expressed on CD4⁺CD25⁺ Treg (6). In vitro stimulation of CD4⁺CD25⁺ T cells with LPS elicits their proliferation and enhances the suppressive activity in the absence of APCs.

Alloantigen-specific immune regulation represents a key mechanism in the induction and maintenance of transplantation tolerance. Although multiple types of T lymphocytes express regulatory functions, CD4⁺CD25⁺ T cell is the dominant and best-characterized population in allograft recipients (7). However, despite extensive studies, putative mechanisms of alloreactive Treg induction and their function to suppress effector T cells from rejecting allografts in vivo remain unclear. In this study, we focused on the role of TLR4 signaling in regulating alloreactive Treg, particularly how TLR4 activation affects the induction and maintenance of transplant tolerance mediated by CD4⁺ Treg. As TLR activation associates with a myriad of infections that frequently occur in humans, its impact on tolerance maintenance is of critical clinical significance, particularly in long-term allograft recipients treated with novel immunosuppressive agents, such as T cell costimulatory blockers. We have recently adapted a murine cardiac transplant model (8), in which alloreactive CD8⁺ T cells represent the dominant effectors in rejecting MHC fully mismatched heart grafts (BALB/c to B6) (9). A single dose of anti-CD154 mAb at the time of transplantation prolonged graft survival to >60 days. In this report, we have first determined the induction and functions of operational CD4⁺ Treg in maintaining allograft survival and suppressing activation of alloreactive CD8⁺ T cells in wild-type (WT) long-term recipients. We then used TLR4 knockout (KO) mice to assess the role of TLR4 signaling in the generation and function of alloreactive CD4⁺ Treg. Finally, we tested

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3 Abbreviations used in this paper: Treg, T regulatory cell; WT, wild type; KO, knockout; CTL, CTL effector; HPRT, hypoxanthine phosphoribosyltransferase; s, sense; as, antisense; Tbet, T box expressed on T cells; MST, mean survival time.
whether activation of TLR4 system could overcome CD4⁺ Treg-mediated alloimmune tolerance.

Materials and Methods

Animals and transplantation models

WT BALB/c (B/c; H-2b), C57BL/6 (B6; H-2d), C3H/HeJ (C3H; H-2h) male mice, all at age of 8–12 wk (20–25 g), were obtained from The Jackson Laboratory. TLR4 KO mice of the B6 background were obtained from the laboratory of Dr. G. Cheng (Department of Microbiology, Immunobiology, and Molecular Genetics, University of California Los Angeles, Los Angeles, CA). Animals were housed in the UCLA animal facilities under specific pathogen-free conditions.

B6 mice were used as recipients of intraabdominal B/c heart transplants, as described (6, 10). Graft survival was assessed daily by palpation. The time of rejection was defined as the day of cessation of heartbeat, and was verified by autopsy and pathological examination. Orthotopic full-thickness skin grafts (~0.5 cm in diameter) from B/c or C3H donors were sutured bilaterally onto the flanks of B6 recipients. Graft survival was assessed daily by palpation. The time of rejection was defined as the day of cessation of heartbeat, and was verified by autopsy and pathological examination. Orthotopic full-thickness skin grafts (~0.5 cm in diameter) from B/c or C3H donors were sutured bilaterally onto the flanks of B6 recipients. Graft survival was followed by daily visual inspection, and rejection of skin grafts was defined as the complete necrosis and loss of the viable skin tissue.

Ab therapy

Anti-mouse CD154 mAb (MR1; BioExpress) was administered at the time of cardiac engraftment (0.5 mg/mouse i.v.). Control recipients were treated with relevant doses of hamster Ig. To deplete T cell subsets in long-term B6 recipients of B/c hearts, rat anti-mouse CD8 (2.43) or CD4 (GK1.5, BD Pharmingen) mAb was administered at 0.25 (CD8) and 0.5 (CD4) mg/mouse/day, respectively, i.v. (day −1, 1). Control animals were given relevant doses of rat Ig. To deplete CD25⁺ cells, rat-anti-mouse CD25 mAb (PC61) was administered (day −1, 1) at 1 mg/mouse i.v.

CTL effector (CTLe) differentiation in vivo

One million RBC-free PBLs were used for Ab staining in ice-cold PBSA (PBS with 1% BSA). Cells were processed for staining with antibodies (CD62L and CD44 expression). CTLe were identified as CD8⁺ CD62LlowCD44high (11).

Quantitative RT-PCR

Two and a half micrograms of RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies). Quantitative-PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research). In a final reaction volume of 25 μL, the following were added: 1× SuperMix (Platinum SYBR Green qPCR kit; Invitrogen Life Technologies), CDNA and 0.5 mM of each primer. Amplification conditions were: 50°C (2 min), 95°C (5 min) followed by 50 cycles of 95°C (15 s), 60°C (30 s). Primers used to amplify a specific gene fragment are listed as follows: hypoxanthine phosphoribosyltransferase (HPRT): sense (s), ttgagatccatgccgttg; antisense (as), tgcattgttttaccagtg; TNF-α: s, gtagccacctgtgaa, as, ttgacatctagcttg; FoxP3: s, cactgaacctcactctg; as, ttgacatctagcttg; T box expressed on T cells (Tbet): s, caacagggggcgacagtt, as, ttgacatctagcttg; CD8: s, ctctacatccctctg, as, actgagggctctgct.

Statistical analysis

The results are shown as mean ± SD. Statistical analyses were performed using Student’s t test with p < 0.05 considered as significant.

Results

Induction and function of CD4⁺ Treg after CD154 costimulation blockade

WT B6 recipients of B/c cardiac allografts were treated with a single dose of anti-CD154 mAb (MR1; 0.5 mg) at day 0, the time of transplantation. Unlike untreated mice, which reject cardiac grafts uniformly within 7 days, those treated with MR1 mAb maintained their transplants for >60 days. To test whether donor-specific immune tolerance was induced, test skin grafts from donor (B/c) or third-party (C3H) strains were placed at day 50 postcar-
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The ratio of CD4<sup>+</sup> T cells and maintains graft survival in our model, we used anti-CD25 mAb treatment, which has been shown to efficiently deplete CD4<sup>+</sup>CD25<sup>+</sup> T cells in both autoimmune disease and tumor models (14, 15). Depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells resulted in the rejection of donor-type skin grafts in ~70% of long-term hosts, albeit with somewhat delayed kinetics (MST ± SD = 14 ± 2 days; n = 4, Fig. 1b), as compared with those in naive or CD4-depleted mice. In parallel with skin graft rejection, alloreactive CD8<sup>+</sup> activation was restored (Fig. 3b, CD8<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup> PBLs increased from 3.4 ± 2.0% to 12.3 ± 4.1%, n = 4, p = 0.0008). Thus, CD4<sup>+</sup>CD25<sup>+</sup> Treg controlled alloreactive CD8<sup>+</sup> T cell activation in long-term recipients.

**FIGURE 2.** Suppression of donor-alloreactive CD8<sup>+</sup> T cell activation in tolerant recipients. a, Donor-type (B/c) skin grafts were transplanted onto tolerant B6 recipients at 50 days postcardiac engraftment. PBLs were sampled before the skin and 10 days postskin challenge. The ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells were transplanted onto tolerant B6 recipients 50 days postcardiac engraftment. PBLs were sampled before the skin and 10 days postskin challenge. The ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells were analyzed by FACS. The percentage numbers represent the CD44<sup>high</sup>CD62L<sup>low</sup>CD8<sup>+</sup> T cells in total CD8<sup>+</sup> T cells. Results are representatives of three separate experiments.

**FIGURE 3.** Activation of donor-alloreactive CD8<sup>+</sup> T cells by donor type skin grafts after depletion of: (a) CD4<sup>+</sup> T cells or (b) CD25<sup>+</sup> T cells in tolerant recipients. Depleting anti-CD4 (GK1.5), or depleting anti-CD25 (PC61) mAb was administered at the time of skin grafting in tolerant B6 recipients (day 50 postcardiac engraftment), as described in Materials and Methods. PBLs were sampled before the skin and 10 days postskin challenge. The ratios of CD4<sup>+</sup>/CD8<sup>+</sup> and CD25<sup>+</sup>/CD4<sup>+</sup> T cells (right panels) and the activation of alloreactive CD8<sup>+</sup> T cells were analyzed by FACS. The percentage numbers represent the CD44<sup>high</sup>CD62L<sup>low</sup>CD8<sup>+</sup> T cells in total CD8<sup>+</sup> T cells. Results are representatives of three to six separate experiments.

To further establish the involvement of CD4<sup>+</sup> Treg in maintaining cardiac allografts after CD154 blockade, we examined intragraft expression of FoxP3, a transcriptional factor closely associated with both natural and induced CD4 Treg (16), and of Tbet, another transcriptional factor associated with IFN-γ-producing type I CD4 and CD8 T cells (10). Cardiac allografts were harvested at day 50 from separate groups of MR1 Ab preconditioned recipients that remained otherwise either untreated, or depleted of CD4<sup>+</sup> T cells, as well as from acutely rejecting controls at day 5 posttransplant. Quantitative RT-PCR-assisted Foxp3/HPRT and Tbet/HPRT, as well as Foxp3:Tbet ratios were calculated to represent intragraft accumulation of FoxP3-expressing Treg, Tbet expression of alloaggressive proinflammatory T cells, and their relative ratios, respectively. As shown in Fig. 4a, there was a significant decrease of Tbet expression in tolerant grafts (3.7-fold) as compared with rejecting allografts; FoxP3 levels were similar in both groups. Interestingly, however, there was an ~7-fold increase in Foxp3:Tbet ratio in long-term grafts, as compared with rejecting counterparts. This implies that CD154 blockade inhibited the accumulation of Tbet rather than FoxP3-expressing cells in the tolerant grafts. Although depletion of CD4 T cells in long-term recipients before transplant harvest reduced the expression of both FoxP3 and Tbet, the reduction of FoxP3 was more profound, as evidenced by ~50% decrease of FoxP3:Tbet ratio. This may have resulted from sparing of alloreactive CD8 expressing Tbet by anti-CD4 treatment. CD4 depletion before the test donor-type skin graft challenge led to rejection of otherwise long-term cardiac allografts, implying that alloreactive CD8 T cells in tolerant recipients were capable to reject cardiac allografts in the absence of CD4 Treg. Thus, graft-sequestered Foxp3-expressing CD4<sup>+</sup> T cells might control alloreactive CD8 T cells, and play a key role in maintaining the survival of long-term transplants.

**DISRUPTION OF TLR4 SIGNALING DOES NOT AFFECT THE INDUCTION AND FUNCTION OF OPERATIONAL CD4<sup>+</sup> TREG**

To determine the functional relevance of TLR4 signaling at the T cell site in the induction/function of CD4<sup>+</sup> Treg, we then used TLR4 KO mice as cardiac graft recipients. Indeed, untreated TLR4 KO (B6) mice were capable of rejecting MHC-fully mismatched (B/c) hearts, albeit with a somewhat delayed kinetics (MST ± SD = 10 ± 1 days; n = 4). In parallel, the frequency of activated CD8<sup>+</sup> T effector cells (CD8<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup> PBLs) in TLR4 KO mice at day 10 posttransplant increased from 3.9% to 24 ± 17.5% (vs 44 ± 10.7% in WT; Fig. 5a; n = 4/group, p = NS). CD154 blockade in TLR4 KO hosts uniformly produced long-term (>60 day) cardiac graft acceptance (n = 10). MR1 mAb-treated recipients accepted donor, but promptly rejected third-party test
Skin grafts. Interestingly, alloreactive CD8+ T cells remained inactivated in TLR4 KO hosts despite simultaneous donor-type alloantigen stimulation (4.0 – 4.6% in Fig. 5b). However, CD4 depletion before skin grafting resulted in prompt rejection of both test skin and original cardiac allografts, similar to WT tolerant counterparts. In parallel, CD8 activation was restored in the absence of CD4+ T cells in TLR4 KO tolerant recipients (Fig. 5c, 4.1 ± 1.2% to 21.3 ± 4.6%, n = 3, p = 0.0025). Thus, the absence of TLR4 signaling in T cells did not impair the induction/function of CD4+ Treg, as evidenced by the full protection of allograft survival and suppression of alloreactive CD8+ T cells after CD154 blockade in TLR4 KO recipients.

TLR4 activation fails to overcome CD4+ Treg-mediated immune suppression

One of the proposed mechanisms of Treg function to suppress effector T cells might be mediated by APCs. CD4+ Treg can suppress inflammation-induced APC activation. When Treg and effector T cells are in close proximity and interacting with the same APCs, Treg-suppressed APCs cannot activate effector T cells. In contrast, LPS directly activates APCs via TLR4 by increasing the expression of co-stimulatory molecules and the secretion of proinflammatory cytokines, such as IL-1, TNF-α and IL-6, which will overcome CD4+CD25+ Treg-mediated suppression. Thus, we administered LPS i.p. into long-term recipients in concert with donor-type skin graft to test as to whether or not the direct TLR4 activation could break CD4+ Treg-mediated immune unresponsive state. Both rejection of test skin and original heart grafts, and the activation of alloreactive CD8+ T cells were monitored.

Compared with controls, LPS infused mice did not elicit rejection of either allograft. In parallel, peripheral CD8+ T cells remained suppressed as no increase in the frequency of activated effector CD8+ T cells by donor Ags was recorded (Fig. 6a, 11.5 ± 3.4% to 10.1 ± 2.7%, n = 4).

The in vivo effect of LPS infusion was confirmed by FACS by a significant increase in circulating polymorphonuclear leukocyte numbers in long-term recipients (25.3–63.0%, in Fig. 6b). We also used quantitative RT-PCR to analyze TNF-α induction in both cardiac allografts and spleens harvested 3 h and 3 days after LPS infusion (Fig. 4b). A highly significant 20-fold increase in TNF-α gene expression was detected at 3 h in cardiac allografts. However, this induction was not sustained, as intragraft TNF-α levels at day 3 were even lower than in untreated controls. Similar TNF-α induction pattern was observed in the spleens of LPS-infused mice, but the fold of change was lower. The activation of TLR4 by LPS and its potential effects on Treg was further analyzed by assessing intragraft expression of Foxp3 and Tbet at day 3 post-LPS infusion (Fig. 4c). Although LPS caused significant reduction of intragraft expression of both genes, Foxp3 depression was more profound, as evidenced by decreased Foxp3:Tbet ratio. A similar expression pattern of the two genes was noted in host splenocytes after LPS infusion, indicative of nonselective LPS mediated mobilization of lymphocytes out of the allograft and spleen. This is in sharp contrast to the effect of CD154 blockade, which targeted primarily
Tbet expressing cells. Interestingly, intragraft CD8 levels did not increase despite enhanced TNF-α expression at 3 h (Fig. 4d). Peripheral CD8 T cells were mobilized by LPS, as shown by decreased spleen CD8 levels.

Discussion

Aiming to address the mechanisms of CD4⁺ Treg induction and function with respect to innate TLR4 activation in transplant tolerance, this study first provides direct in vivo evidence that CD4⁺ Treg cells were induced by CD154 blockade, and were responsible for maintaining unresponsiveness in a murine cardiac transplant model. We determined that one of the major subsets of CD4⁺ Treg in our model was indeed CD4⁺CD62LlowCD8 T cell activation in naive TLR4 KO mice. The suppression of allograft survival by CD154-targeted therapy in transplant tolerance model, and that TLR4 activation cannot reverse established immunological tolerance (14, 15, 17). The results of our experiments in in vivo setting of primary tolerant recipients have confirmed several previous findings primarily from adoptive transfer cell systems, that CD4⁺ Treg cells are indeed essential for long-term effects of CD154 blockade in transplant models (18–20). Moreover, this study adds to our appreciation as to how CD4⁺ Treg function and regulate alloreactive CD8 T cell activation. Thus, at both graft and cellular levels, CD4⁺ Treg cells could be monitored functionally, which provides us an excellent platform to accurately determine the cross talk between TLR4 and CD4⁺ Treg.

The role of TLR system in regulating the activation of T cells has been recently revealed (21). TLR-mediated recognition of microbial components by DCs induces their maturation by up-regulating the expression of costimulatory molecules and production of proinflammatory cytokines. This enables the DCs, which also present antigenic peptides in MHC complex, to activate naive T cells, initiating Ag-specific adaptive immune responses (1). Independent of costimulatory molecules, TLR activation has a second mechanism to initiate T cell activation. It was demonstrated that IL-6, a product of TLR activation in DCs, may break CD4⁺CD25⁺-mediated suppression of T cell activation (5). Multiple TLRs, including TLR2, 4, etc., are involved in dendritic cell (DC)-centered immune activation. In the absence of infection, TLRs may also be activated and involved in regulating immune responses. Endogenous ligands produced during disease processes have been demonstrated, such as, heat-shock proteins, heparan sulfate, and hyaluronan (22–25), which are mostly targeted to TLR4. In organ transplantation, these molecules are obviously present and may well become up-regulated during the initial injury or inflammation. Thus, TLRs are relevant in initiating host alloimmune responses. This has been recently confirmed by showing that MyD88 deficiency, which disrupts multiple TLR-signaling pathways prevents minor MHC disparate skin graft rejection and impairs the generation of alloreactive Th1 immune reaction (3). Additionally,
TLRs expressed on T cells also exert immune regulatory functions. Indeed, TLR4 expression was found to be restricted to CD4+CD25+ T cells. TLR4 activation on these natural Tregs promotes their proliferation and enhances immune suppressive function (6, 26). It was also shown that activated mouse CD4+ T cells express TLR3/TLR9, but not TLR2/TLR4 (27).

Our observations that TLR4 KO recipients had near normal rejection kinetics and alloreactive CD8 activation frequencies against MHC-fully mismatched cardiac allografts are consistent with the idea that recipient’s other TLRs or donor TLR4 were sufficient to complement single recipient TLR4 deficiency for allogeneic activation. This has been shown in other transplant models of either major or minor MHC-disparate skin grafts (28). The new finding in this study is that TLR4 was not required for alloreactive CD4 Treg induction and function. There are at least two possible reasons for this negative finding on the TLR4 involvement in CD4+ Treg. Treg subsets in transplant models may include both CD25+ and CD25− cells. Thus, if the impact of TLR4 is limited to CD25+ subset, Treg function might not be so obviously deficient, or becomes complemented by increased CD25− subset. Alternatively, multiple TLRs may be involved in the proliferation and function of CD4+ Treg, similar to DC activation by TLRs.

Interestingly, we have found that exogenous LPS infusion failed to break tolerance mediated by CD4+ Treg. This is opposite to a recent report showing that LPS injection into a tumor tolerant mouse resulted in the activation of tumor reactive CD8+ T cells, and tumor rejection in the presence of CD4−CD25+ Treg (29). In this tumor model, however, DCs were the critical elements in controlling CD8 proliferation, and CD4−CD25+ Treg prevented DCs from activating tumor-reactive CD8+ T cells. Thus, our results indicate that DC activation was not the limiting factor or sufficient in our model to overcome the inhibition of alloreactive CD8 activation by CD4+ Treg. In our experiments, LPS indeed activated inflammatory responses by up-regulating intragraft TNF-α levels, but failed to activate alloreactive CD8+ T cells (no increases in peripheral CTLe or intragraft CD8 level), even in the presence of “fresh” alloantigen on secondary donor-type skin grafts. One observation noticed is that TLR4 activation by LPS did cause a reduction of intragraft Foxp3:Tbet ratio, indicating that there was a flush of proinflammatory cells into the tolerant grafts. Thus, the immune suppression in tolerant recipients was alloantigen-specific and it did not affect host ability to mount inflammatory responses. In contrast, Ag nonspecific inflammation could not overcome allograft “tolerance”, a finding of a major clinical significance.

At least two issues warrant additional consideration in our model. First, what consists of CD4+ Treg population in the presence or absence of TLR4 signaling? Unlike autoimmune disease models in which naturally occurring CD4+CD25+ Treg are dominant, various Treg populations were identified in transplant models, including CD4−CD25− (20), CD4+CD25− (30), double negative T cells (31), CD8−CD28− (32), and NKT cells (33). It is unknown, however, whether these different regulatory subsets can coexist and/or exert complementary functions in vivo. Our observation that depletion of CD25+ cells resulted in variable outcomes in eliciting graft rejection, implies the existence of both CD25+ and CD25− Treg populations. TLR4 deficiency may differentially influence CD4+ Treg subsets. The second issue is how other TLRs, alone or in combination, may affect CD4+ Treg. Although the answer to that question remains beyond the scope of this study, others have recently shown that TLR8 activation can reverse CD4 Treg function in vitro (34). Because DCs are sensitive to the defective TLR function, one possible approach to address the question in vivo is to produce chimeras with WT DCs but deficient T cells.

In summary, CD154 blockade facilitates long-term allograft survival and generates allo-specific CD4+ Treg that suppress CD8+ T effectors. TLR4 deficiency does not alter the rejection kinetics significantly nor the tolerant nature induced by anti-CD154 mAb treatment. Furthermore, TLR4 activation in tolerant recipients could not reverse immune suppression mediated by CD4+ Treg. Thus, TLR4 signaling is not required for the induction and function on CD4+ Treg in vivo in transplant tolerance.

Disclosures
The authors have no financial conflict of interest.

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