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High Accumulation of T Regulatory Cells Prevents the Activation of Immune Responses in Aged Animals

Sanjay Sharma, Ana Lucia Dominguez, and Joseph Lustgarten

In our previous in vivo study we demonstrated that young BALB/c mice effectively rejected the BM-185 tumor cells expressing enhanced GFP (EGFP) as a surrogate tumor Ag. In contrast, old BALB/c mice succumbed to the BM-185-EGFP tumors, indicating that there is a deficiency in old animals preventing the rejection of immunogenic tumors. There is cumulative evidence indicating that regulatory T (T_{reg}) cells control the activation of primary and memory T cell responses. However, very little is known about whether there is a relation between T_{reg} and the lack of immune responses in the aged. We evaluated young and aged animals, and our results demonstrated that there are significantly more CD4^+ CD25^+ FoxP3^+ and CD8^+ CD25^+ FoxP3^+ T_{reg} in the spleen and lymph nodes of old animals when compared with the young. Depletion of CD25^+ cells with anti-CD25 mAb induces the rejection of BM-185-EGFP cells, restores antitumor T cell cytotoxic activity, and results in the generation of a protective memory response against the BM-185 wild-type tumors in old mice. Furthermore, vaccination with CpG-oligodeoxynucleotide decreases the number of T_{reg} cells in old animals to the same levels as young mice, restoring the primary and memory antitumor immune responses against BM-185-EGFP tumors. Taken together, these results indicate that there is a direct correlation between the expansion of T_{reg} cells and immune deficiency in the old, and that depletion of these cells might be critical for restoring immune responses in aged animals. The Journal of Immunology, 2006, 177: 8348–8355.

The mechanism of suppression still remains controversial. Although some studies have shown that T-T cell contact is required for suppression (9, 10), other studies have revealed that suppression is mediated by cytokines IL-10 and TGF-β (7, 8). Furthermore, depletion of CD4^+ CD25^+ T cells by the administration of...
anti-CD25 mAb has been shown to suppress the growth of a variety of different syngeneic tumors in mice (15–17). The observation that the removal of immunoregulatory CD4+CD25+ T cells can abrogate unresponsiveness to syngeneic tumors in vivo, leading to the spontaneous development of tumor-specific responses, indicates that Treg cells negatively regulate tumor immunity and that depletion of these cells might be critical for controlling tumor growth, also indicating that the maintenance of self-tolerance against tumor-self Ags could potentially be lifted.

Several studies have shown that there is an increase in the number of CD4+CD25high T cells in the elderly (18–21). Some of these studies have demonstrated that there is a correlation between a higher number of CD4+CD25high T cells and Treg suppression activity (18, 19), whereas other studies have shown no correlation (20, 21). Although some of these studies analyzed the expression of Foxp3 in CD4+CD25high T cells by PCR, they did not analyze whether these CD4+CD25high T cells were Foxp3 positive by functional Foxp3 protein intracellular flow cytometry staining (22). Currently, there are no studies demonstrating the expression of Foxp3 in CD4+CD25+ T cells in the elderly or old animals or analyzing whether these populations influence the activation an immune response in the aged. We evaluated whether there are differences in the number of Treg cells between young and old animals and whether Treg cells influence the activation of antitumor immune responses in the aged. Our results indicated that old mice contained the double amount of CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+ populations in spleen and lymph nodes when compared with spleens and lymph nodes from young mice. Furthermore, depletion of CD25+ cells with anti-CD25 mAb in old mice resulted in the rejection of BM-185-EGFP tumor cells and the generation of a protective memory response against BM-185 wild-type (w.t.) cells, and restores antitumor T cell cytotoxic activity. We have demonstrated previously that the addition of costimulatory molecules could restore the antitumor response against BM-185-EGFP tumors in old animals (3). TLR ligands such as LPS, flagellin, CpG-oligodeoxynucleotide (ODN), imiquimod, poly(I:C), and others are strong coactivator molecules capable of activating antitumor responses. We tested whether these ligands were capable of restoring an antitumor immune response in old mice. Our results indicate that only CpG-ODN vaccination induced the rejection of BM-185-EGFP tumors in old mice. We observed that after CpG-ODN treatment, the numbers of Treg cells in old animals were reduced to the same levels as young mice. These results indicate that the accumulated Treg cells in the old inhibit or prevent the activation of antitumor responses, and the depletion or reduction of Treg cells might be critical to optimally activate an immune response in the aged.

Materials and Methods

Mice and cell lines

Young (2- to 3-month-old) and old (18- to 22-month-old) BALB/c mice were purchased from the National Institute of Aging and housed under specific pathogen-free conditions. The tumor cell lines, BM-185 w.t., and BM-185 expressing EGFP (BM-185-EGFP) and BM-185-EGFP-CD80 were provided by D. Kohn (University of Southern California, Los Angeles, CA). P815 cells were purchased from the American Type Culture Collection. P815 wild-type (w.t.) cells, and restores antitumor T cell cytotoxic activity.

Flow cytometry

The prevalence of Treg populations in young and old animals was determined by analysis of CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+ Treg cells in the spleen and lymph nodes. Spleen and lymph nodes from young and old animals were first surface stained with anti-CD4 (FITC) or anti-CD8 (PE) and mice were examined twice per week for tumor development and survival. To evaluate whether old mice developed a memory response after treatment with the anti-CD25 mAb, animals were challenged 60 days later with an s.c. injection of 10⁶ BM-185-w.t. cells. Survival analysis used the Breslow modification of the Kaplan-Meier test.

Generation of CTL cultures and cytotoxic activity

Young and old BALB/c mice were immunized with an s.c. injection of 10⁵ BM-185-EGFP cells. Two weeks later, spleens from primed animals were removed and spleen cells were restimulated in vitro with BM-185-EGFP-CD80 cells. After 5 days, CTLs were assayed for lytic activity. The BM-185-w.t., BM-185-EGFP, P815, and P815 pulsed with the H2-Kd-HYLSTQSLAL peptide (a Kd-EGFP-derived immunodominant epitope) were incubated with 150 μCi of ⁴⁶Cr sodium chromate for 1 h at 37°C. Cells were washed three times and resuspended in complete RPMI 1640 medium. For the cytotoxic assay, ¹¹¹I-Cr-labeled target cells (10⁴) were incubated with varying concentrations of effector cells in a final volume of 200 μl in U-bottom 96-well microtiter plates. Supernatants were recovered after 5 h of incubation at 37°C, and the percentage of lysis was determined by the following formula: percentage of specific lysis = 100 × (experimental release – spontaneous release)/ (maximum release – spontaneous release).

Treatment with TLR ligands

TLR ligands, CpG-ODN (1826) and control-ODN, were purchased from Oligos Etc.; imiquimod (a soluble form of this compound was obtained directly from 3M Pharmaceuticals), poly(I:C), and LPS were purchased from Sigma-Aldrich. Flagellin was purified, as previously reported (23). Old animals were injected s.c. with 10⁵ BM-185-EGFP cells on day 0.
Day 5 after tumor inoculation, animals were randomly divided into groups of five mice/group. Animals received s.c. injections of poly(I:C), LPS, flagellin, imiquimod, CpG-ODN, and control-ODN (as a control) in the opposite flank from where the tumor was injected three times per week (30 µg/injection) for 3 wk. Evaluation of memory responses and survival was performed, as described above.

**IL-6 secretion by splenocytes after treatment with TLR ligands**

Spleen cells (10^6) from old mice were plated in 24-well plates and not treated or treated with CpG-ODN, LPS, poly(I:C), imiquimod, and flagellin at 1 µg/ml. After 24 h, supernatants were collected and levels of IL-6 were determined by the Luminex-100 flow cytometry assay.

**Statistical analyses**

Statistical significance of data was determined by Student’s t test to evaluate the p value.

**Results**

*Treg* cells accumulate in high numbers in spleens and lymph nodes of old mice

Spleen and lymph nodes from naïve young and old BALB/c mice were evaluated for the presence of CD4^+ CD25^+ FoxP3^- T cells. As shown in Fig. 1, old mice contained a significantly larger population of CD4^+ FoxP3^- T cell in their spleen (ranging from 5 to 9%) (p < 0.04) and lymph nodes (ranging from 10 to 14%) (p < 0.02) when compared with spleens (ranging from 4 to 6%) and lymph nodes (ranging from 6 to 8%) from young mice. Further analysis of CD4^+ populations in terms of comparative expression of Foxp3 and CD25 demonstrates that a significantly larger population of CD4^+ cells also coexpresses Foxp3 and CD25 in spleens (21%) and lymph nodes (27%).
It has been demonstrated recently that Treg cells are not exclusively confined to CD4+ T cells; CD8+ T cells also express Foxp3 and can act as immunoregulatory cells inhibiting T cell responses (13, 14). We next evaluated whether there were differences in the number of CD8+ CD25+FoxP3+ cells accumulated in spleens and lymph nodes in young and old mice. Our results indicated that spleens (4.9%) and lymph nodes (5.3%) from old mice contained a higher accumulation \((p < 0.05)\) of CD8+ CD25+FoxP3+ cells in comparison with young spleens (2.6%) and lymph nodes (2.5%) (Fig. 3).

**Depletion of CD25+ cells induces antitumor responses in old mice**

Compared with young animals, the aged have a higher prevalence of CD25+Foxp3+ regulatory cells (Figs. 2 and 3). Based on these results, we hypothesize that elimination of CD25+ cells could improve the antitumor immune response of old mice against BM-185-EGFP. Old mice were treated with anti-CD25 mAb on days \(-7, -3,\) and \(-1\), and animals were inoculated with BM-185-EGFP cells on day 0. We included young mice inoculated with BM-185-EGFP cells as a positive control. Our results indicated that 100% of old mice treated with anti-CD25 mAb developed an antitumor response capable of rejecting the BM-185-EGFP cells in the same manner as young mice (Fig. 4). Old mice without anti-CD25 mAb treatment formed tumors (Fig. 4). We next evaluated whether old mice depleted of CD25+ cells developed a protective memory response against the BM-185 w.t. cells. As shown in Fig. 4, challenging the animals on day 60 posttumor implantation, 100% of old animals originally injected with the BM-185-EGFP cells plus anti-CD25 mAb rejected the BM-185-w.t. cells. As expected, young mice inoculated with BM-185-EGFP also developed a protective memory response after the challenge. Our results show that aged animals accumulate Treg cells and inhibit specific immunity, and their depletion might be a simple means to supplement antitumor immunity in the aged hosts.

**Depletion of CD25+ cells restores cytotoxic T cell responses in old mice**

We have demonstrated previously that injection of BM-185-EGFP cells induces T cell responses against EGFP+ tumors and specific CD8 T cell responses against the EGFP-H2-K1-restricted peptide (HYLSTQSAL) in young animals, but not in old animals (3, 24). We evaluated whether depletion of CD25+ cells could restore the activation of EGFP-specific responses in old mice. Young and old mice were treated or not treated with anti-CD25 mAb and immunized with BM-185-EGFP cells. Two weeks later, animals were sacrificed and T cell cytotoxic activity was evaluated. As shown in Fig. 5, young mice (Fig. 5A) demonstrated a robust cytotoxic response against BM-185-EGFP and P815 cells pulsed with EGFP-H2-K1 peptide; in contrast, old mice could not activate a cytotoxic T cell response (Fig. 5B). However, old mice treated with anti-CD25 mAb generate a cytotoxic T cell response against BM-185-EGFP and P815 cells pulsed with EGFP-H2-K1 peptide similar to those observed in young mice (Fig. 5C). Taken together, these results suggested that CD25+ Treg cells negatively modulate the immune response in the old mice, and depletion of CD25+ cells could be used as strategy to restore the immune response in old animals.
Treatment of old animals with CpG-ODN induces rejection of BM-185-EGFP tumors

We have demonstrated previously that costimulatory molecules, such as B7.1, anti-OX40, or anti-4-1BB mAb, could restore the immune responses in old mice. It has been demonstrated that TLR ligands are potent activators of immune responses and significantly up-regulate the costimulatory molecules on APCs. It is not clear which of these adjuvants would be the most effective to induce an immune response in old mice. To this end, we compared the antitumor immune responses in old tumor-bearing mice after injecting poly(I:C) (TLR-3), LPS (TLR-4), flagellin (TLR-5), imiquimod (TLR-7), and CpG-ODN (TLR-9). Old mice were implanted s.c. with 10^5 BM-185-EGFP cells on day 0. On day 5, animals started treatment with s.c. injections of poly(I:C), LPS, flagellin, imiquimod, CpG-ODN, and control-ODN (as a control) in the opposite flank from where the tumor was injected three times per week (30 μg/injection) for 3 wk. Our results demonstrate that only injections of CpG-ODN (p < 0.001) restored the immune responses in old mice, resulting in the complete rejection of tumors (Fig. 6). Treatment with poly(I:C), imiquimod, LPS, or flagellin did not have any effect in controlling the tumor growth. No antitumor effect was observed in old mice injected with control-ODN, indicating that the immune response stimulated by the CpG-ODN was specific against the tumor.

Treatment of old animals with CpG-ODN suppresses the T_{reg} accumulation and restores CTL responses

We evaluated whether vaccination with CpG-ODN influenced the number of CD4^+Foxp3^+ T_{reg} cells in old mice. As shown in Fig. 7A, treatment with CpG-ODN resulted in a significant reduction (~40%) of CD4^+Foxp3^+ T_{reg} cells in the spleens of old animals as compared with aged controls (p < 0.05). Furthermore, the levels of T_{reg} cells in old controls after CpG-ODN treatment were comparable with the level of the young controls. We also evaluated whether CpG-ODN restored the cytotoxic activity against EGFP Ags in old mice. As expected, old mice immunized with BM-185-EGFP cell did not induce an immune response (Fig. 7B), whereas immunization with BM-185-EGFP plus CpG-ODN restored the CTL responses against BM-185-EGFP and P815 cells pulsed with EGFP-H2-K^d peptide (Fig. 7C). These results indicate that CpG-ODN vaccination influences the levels of circulating CD4^+Foxp3^+ T_{reg} cells in old mice by reducing their...
ligands, correlating it with the reduced Treg population in CpG-compared with controls or other groups treated with other TLR splenocytes following incubation with CpG-ODN, LPS, poly(I:C). It has been demonstrated that Treg cells do not express TLR-9. It has been demonstrated that IL-6 is capable of reversing the suppressive activity of these cells (data not shown). These results are in agreement with the data of Caramalho et al. (25), which demonstrated that IL-6 secretion by splenocytes following incubation with CpG-ODN, LPS, poly(I:C), imiquimod, and flagellin. Treatment with CpG-ODN significantly increased the levels of IL-6 with regard to inducing antitumor immunity in old animals is not completely understood. This is the first study that shows quantitative data of Treg cells in old hosts in terms of Foxp3 in context to host antitumor responses.

Our results demonstrate that there is accumulation of CD25\(^{\text{high}}\) Foxp3\(^{+}\) Treg cells in the spleen and lymph nodes of aged animals when compared with young animals. Our results show that depletion of CD25\(^{+}\) cells could restore the immune responses, resulting in the rejection of BM-185-EGFP tumor cells in old mice. Furthermore, depletion of CD25\(^{+}\) cells also induces the generation of a protective memory response against BM-185 w.t. cells in the same manner as young mice. Additionally, we observed that old mice did not have the ability to prime a cytotoxic immune response for optimal stimulation of an immune response in the old mice, and depletion of CD25\(^{+}\) cells could contribute to the immune suppression induced by Treg cells in old animals as compared with young mice. Therefore, both CD4\(^{+}\) and CD8\(^{+}\) Treg cells could contribute to the immune suppression induced by Treg cells in old animals, and for the optimal stimulation of an immune response, it might be necessary to make sure to eliminate both CD4\(^{+}\) and CD8\(^{+}\) Treg cells in the elderly. Taken together, these results suggested that CD25\(^{+}\)-positive Treg cells negatively modulate the immune response in the old mice, and depletion of CD25\(^{+}\) cells could be used as a strategy to restore the immune response in old animals.

Aging is accompanied by numerous functional and phenotypic changes in the immune system; moreover, increases in autoimmunity, infections, and occurrence of cancer have been reported in aged people (1, 2). We have demonstrated previously that transfecting the BM185-EGFP tumors with the CD80 molecule allowed the old mice to reject the tumors (3). However, under these conditions, old animals do not develop memory responses. But when EGFP-CD80 tumors were given in combination with anti-OX40 and anti-4-1BB mAb, old mice developed long-term memory responses capable of rejecting a challenge against w.t. tumors (3, 30). These data indicate that the aged immune repertoire can be exploited for the induction of antitumor responses.

Discussion

The capacity of a host to mount an effective immune response can be limited by the pre-existence of counterregulatory elements. Thus, controlling regulatory mechanisms may represent a powerful strategy for controlling chronic infection or enhancing the efficiency of vaccines. This is of particular significance to the aging population, which has a diminished immune system. Recent evidence has demonstrated that Treg cell-mediated immunosuppression is one of the crucial tumor immune evasion mechanisms, contributes to the failure of tumor immunotherapy, and controls the activation of primary and memory T cell responses. One subset is represented by naturally occurring CD4\(^{+}\) Treg cells, generated in the thymus, which constitutively express CD25 (6, 7). Other CD4\(^{+}\) Treg populations include cells secreting high levels of TGF-\(\beta\), which can be induced by oral Ag administration or stimulation of CD4\(^{+}\)CD25\(^{-}\) T cells in the presence of TGF-\(\beta\). Additionally, Treg class 1 cells secreting IFN-\(\gamma\) and IL-10 can be induced following in vitro stimulation in the presence of exogenous IL-10 (8). These Treg subsets are most likely generated in the periphery, which enables the development of peripheral tolerance to self Ags (27).

**FIGURE 8.** Treatment with CpG-ODN, but not other TLR ligands, induces the secretion of IL-6. We wanted to evaluate the effect of CpG-ODN on Treg cells. We treated in vitro CD4\(^{+}\) CD25\(^{-}\) T cells with CpG-ODN, and our initial results indicate that CpG-ODN does disrupt the suppressive activity of these cells (data not shown). These results are in agreement with the data of Caramalho et al. (25), which demonstrated that Treg cells do not express TLR-9. It has been demonstrated that IL-6 is capable of reversing the suppressive activity of CD4\(^{+}\) Treg cells (26). We evaluated IL-6 secretion by splenocytes following incubation with CpG-ODN, LPS, poly(I:C), imiquimod, and flagellin. Treatment with CpG-ODN significantly increased the levels of IL-6 (p < 0.001) (Fig. 8) as compared with controls or other groups treated with other TLR ligands, correlating it with the reduced Treg population in CpG-ODN-treated old mice.

**Foxp3, also called Scurfen, belongs to the forkhead family of winged-helix transcription factors, and its gene, FOXP3, is located on the X chromosome**

Foxp3 expression is confined only to CD25\(^{\text{high}}\) cells in humans, whereas expression of Foxp3 is distributed throughout the CD25-staining population in mice (10). There is cumulative evidence indicating that CD4\(^{+}\)CD25\(^{-}\)Foxp3\(^{+}\) T cells are capable of suppressing the activation and expansion of other T cells and NK cells (9, 10). Furthermore, the ectopic expression of Foxp3 conferred a suppressive function on peripheral CD4\(^{+}\)CD25\(^{-}\) T cells (9). This demonstrates that Foxp3 is a key player in CD4\(^{+}\)CD25\(^{-}\) suppressor/Treg cells. The prevalence of higher CD4\(^{+}\)CD25\(^{\text{high}}\) T cells with immunosuppressive activity has been described in the elderly (18–20). However, these studies did not evaluate the expression of Foxp3; therefore, it is difficult to predict how many of those cells are truly Treg cells. Additionally, the function of CD4\(^{+}\)CD25\(^{\text{high}}\) T cells does not always correlate with the function of CD4\(^{+}\)Foxp3\(^{+}\) Treg cells (24, 27). Recently, it was shown that CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) T cells of aged mice are comparable with those of young animals in showing potent immunosuppressive activities (28). However, the relation of CD25\(^{+}\)Foxp3\(^{+}\) Treg cells with immunosuppressive activity with regard to inducing antitumor immunity in old animals is not completely understood. This is the first study that shows quantitative data of Treg cells in old hosts in terms of Foxp3 in context to host antitumor responses.

**Treatment with CpG-ODN, but not other TLR ligands, induces the secretion of IL-6**

We wanted to evaluate the effect of CpG-ODN on Treg cells. We treated in vitro CD4\(^{+}\) CD25\(^{-}\) T cells with CpG-ODN, and our initial results indicate that CpG-ODN does disrupt the suppressive activity of these cells (data not shown). These results are in agreement with the data of Caramalho et al. (25), which demonstrated that Treg cells do not express TLR-9. It has been demonstrated that IL-6 is capable of reversing the suppressive activity of CD4\(^{+}\) Treg cells (26). We evaluated IL-6 secretion by splenocytes following incubation with CpG-ODN, LPS, poly(I:C), imiquimod, and flagellin. Treatment with CpG-ODN significantly increased the levels of IL-6 (p < 0.001) (Fig. 8) as compared with controls or other groups treated with other TLR ligands, correlating it with the reduced Treg population in CpG-ODN-treated old mice.
of tumor immunity, and that it is possible to convert aged animals from nonresponder to responder status with the inclusion of additional costimulation (3, 30). Activation of the innate immune system through TLR ligand had been demonstrated to be a good strategy to activate antitumor immune responses. Our result demonstrated that only immunizations with CpG-ODN restored the immune responses in old mice, resulting in the complete rejection of tumors and generation of memory responses. Treatment with LPS, imiquimod, flagellin, or poly(I:C) did not have any effect on controlling the tumor growth. These results have clinical implications, indicating that signaling via different TLR ligands could induce qualitatively or quantitatively different types of responses in old APCs, which ultimately could trigger an antitumor response (S. Sharma and J. Lustgarten, manuscript in preparation). Our responses with CpG-ODN were also comparable with observations of Maletto et al. (31) and Alignani et al. (32), who have shown that CpG-ODN can function as efficient adjuvants in both young and old in a peptide vaccination setting. Analysis of Treg cells in CpG-ODN-immunized old animals revealed that numbers and levels of Treg cells are significantly reduced to the level found in the young animals. Furthermore, we observed that cytotoxic T cell responses were restored after CpG-ODN immunization in old mice. Therefore, there is a relationship between CpG-ODN treatment, reduction in the level of Treg cells, and the induction of T cell responses. Vaccinations with CpG-ODN induce potent antitumor responses by stimulating a proinflammatory response (33, 34). It was demonstrated recently that IL-6 and perhaps other unidentified soluble factors secreted by DCs following stimulation with CpG-ODN reversed the suppressive activity of CD4⁺ Treg cells (26). We evaluated IL-6 secretion by spleen cells following incubation with CpG-ODN and other TLR ligands. Our results indicate that CpG-ODN is the only TLR ligand that significantly increased the levels of IL-6 (p < 0.001), correlating it with down-regulation of Treg cells and the activation of immune responses. We have observed that after CpG-ODN treatment, the levels of IL-6 within the tumor microenvironment are also increased by >20-fold (S. Sharma and J. Lustgarten, manuscript in preparation). This report shows for the first time that one of the effects of immunizing with CpG-ODN results in the inhibition of Treg cells.

In summary, this study has shown for the first time that there are higher pools of CD4⁺ CD25⁺ FoxP3⁺ and CD8⁺ CD25⁺ FoxP3⁺ cells accumulating in spleens and lymph nodes from old mice when compared with young animals. Furthermore, the existence of these suppressor populations leads to an inhibition of an immune activation because depletion of CD25⁺ with anti-CD25 mAb induced an antitumor response in old animals, inducing the rejection of BM-185-EGFP tumors, restoring the cytotoxic T cell response, and generating a protective memory response against w.t. tumors. Immunization of old animals with CpG-ODN inhibits the accumulation of Treg cells and restores the antitumor immune responses against BM185-EGFP tumors. During aging, thymic output may change the pattern of T cells, and Treg homeostasis may also be affected. An imbalance of Treg homeostasis would then predispose the aged to immune dysfunction, resulting in a higher risk of immune-mediated diseases, cancer, or infections. The reason for the accumulation of Treg cells in old mice is not yet understood. It has been demonstrated that there is shift from a Th1 to a Th2 response in aged mice (35), with an increased production of IL-10 (36), which may account in part for the high accumulation of Treg cells in these animals. Further studies are needed to completely understand the reason that Treg cells accumulate or expand in the old and how these populations relate to other immune-regulatory effects, such as cytokines, homeostasis, and APCs, and determine their specificity.

Disclosures

The authors have no financial conflict of interest.

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