Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells

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Abstract

Asthma is an inflammatory lung disease, in which conventional CD4+ T cells producing IL-4/IL-13 appear to play an obligatory pathogenic role. Here we show, in a mouse model of asthma, that activation of pulmonary IL-4/IL-13 producing invariant TCR + CD1d-restricted natural killer T (NKT) cells is sufficient for the development of airway hyperreactivity (AHR), a cardinal feature of asthma, in the absence of conventional CD4+ T cells and adaptive immunity. Respiratory administration of glycolipid antigens that specifically activate NKT cells (α-GalactosylCeramide and a Sphingomonas bacterial glycolipid) rapidly induced AHR and inflammation typically associated with protein allergen administration. Naive MHC class II-deficient mice, which lack conventional CD4+ T but have NKT cells, showed exaggerated baseline AHR and, when challenged with α-GalactosylCeramide, demonstrated even greater AHR. These studies demonstrate an expanded role for NKT cells, in which NKT cells not only produce cytokines that influence adaptive immunity but also function as critical effector cells that can induce AHR. These results suggest that NKT cells responding to glycolipid antigens, as well as conventional CD4+ T cells responding to peptide antigens, may be synergistic in the induction of AHR, although in some cases, each may independently induce AHR.

Conflict of interest statement: No conflicts declared.

Abbreviations: AHR, airway hyperreactivity; NKT cell, natural killer T cell; TCR, T cell receptor; iNKT, invariant TCR+ NKT; α-GalCer, α-GalactosylCeramide; i.n., intranasal; j.l., subcutaneous; GalCer, galactosylceramide; BALB/c, BALB/c mouse; OVA, ovalbumin; MHC, major histocompatibility complex; LPS, lipopolysaccharide.

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Bronchial asthma is an immunological disease resulting from Th2-driven inflammation in the airways. It is characterized by inflammation in the peribronchial space, with increased production of airway mucus, and by airway hyperreactivity (AHR), a cardinal feature of asthma. Over the past decade, investigators focused on the role of conventional CD4+ Th2 cells in orchestrating inflammation in asthma. CD4+ Th2 cells, which are thought to be present in the airways of all patients with asthma (1), secrete key cytokines, such as IL-4 and IL-13 (2, 3), as well as IL-5 and IL-9 (4). Conventional CD4+ T cells recognize exogenous antigens and initiate allergic inflammation in the lungs and, in mouse models of asthma, elimination of CD4+ T cells abrogates the development of AHR (5). Therefore, conventional CD4+ T cells are thought to play an obligatory role in the pathogenesis of asthma (6).

CD4+ is expressed not only by conventional CD4+ T cells but also by natural killer T (NKT) cells, which comprise a unique and relatively rare subset of lymphocytes that have features of both T cells and NK cells. Many NKT cells express a highly restricted T cell receptor (TCR) repertoire consisting of Vα14-Jα18 (in mice) or Vα24-Jα18 (in humans) and are called invariant TCR+ NKT (iNKT) cells (7). Through this invariant TCR, iNKT cells recognize bacterial and endogenous glycolipid antigens presented by the nonpolymorphic MHC class I-like protein, CD1d1, and rapidly produce large quantities of cytokines, including IL-4 and IFN-γ, which enhance the function of dendritic cells, NK cells, and B cells, as well as conventional CD4+ and CD8+ T cells (8–11). Rapid production of cytokines by iNKT cells is a manifestation of innate-like immunity and endows iNKT cells with the capacity to amplify and regulate adaptive immune responses and thus link innate and adaptive immunity. Moreover, iNKT cells have been shown to regulate the development of autoimmune, antimicrobial, antitumor, and antitransplant immune responses (12–14).

Recently, we and others showed in standard mouse models of allergen-induced AHR that iNKT cells producing IL-4 and IL-13 are required for the development of allergen-induced AHR (15, 16). In the absence of iNKT cells, AHR failed to develop after sensitization and challenge with allergen, although Th2 responses and eosinophilia developed in these iNKT cell-deficient mice (15, 16). However, because conventional CD4+ T cells are thought to play an essential role in asthma, and because iNKT cells cannot recognize the protein allergens that drive allergic asthma and AHR, the precise relationship between iNKT cells and conventional CD4+ T cells in the induction of airway inflammation and AHR was not clear.

To define a specific role for iNKT cells in the induction of AHR, and in particular to define the relationship between iNKT cells and conventional CD4+ T cells, we studied the direct activation of iNKT cells by the respiratory administration of iNKT cell-activating glycolipid antigens, α-GalactosylCeramide (α-GalCer) and the glycolipid antigen PS-30, derived from Sphingomonas, which are gram− LPS− opportunistic bacterial pathogens (17).
does not appear to be due to differences in onset kinetics, because i.v. administration of α-GalCer also did not induce AHR at 6 or 40 h (data not shown). i.n. but not i.v. administration of α-GalCer induced pulmonary eosinophilia (Fig. 1e) and increased serum IL-4 at 24 h (Fig. 1f) and peribronchiolar infiltrates (Fig. 1g). These results suggest that direct activation of pulmonary but not systemic iNKT cells was important for AHR, and that activation of iNKT cells by α-GalCer was sufficient to induce AHR and airway inflammation typical of allergen-induced AHR.

**Induction of iNKT Cell Unresponsiveness Prevents Subsequent AHR.**

Treatment with two doses of α-GalCer resulted in a reduced level of AHR (Fig. 2a), suggesting that, although α-GalCer can directly induce AHR, activation with α-GalCer may also cause iNKT cell unresponsiveness, which reduces the subsequent capacity of the iNKT cells to induce AHR. α-GalCer is known to induce iNKT cell anergy in a manner similar to T cells treated with superantigens (18), although α-GalCer may also alter other factors such as iNKT cell trafficking or cytokine production to prevent subsequent AHR. The attenuated AHR observed with two doses of α-GalCer may be similar to recent reports showing that administration of α-GalCer to mice already sensitized to ovalbumin (OVA) prevented the subsequent development of AHR (19–21). However, in a similar OVA-induced AHR system, we found that administration of α-GalCer just before airway challenge with OVA resulted first in exacerbation of AHR, measured 48 h after the α-GalCer challenge, although AHR measured 96 h after the α-GalCer administration was reduced (Fig. 2b). Administration of α-GalCer also reduced airway eosinophilia, increased airway neutrophilia (data not shown) and increased IFN-γ but reduced IL-4 production (Fig. 8, which is published as supporting information on the PNAS web site). It appears that the activation state of iNKT cells plays a critical role in the development of AHR, and that α-GalCer can induce iNKT cell unresponsiveness, which can limit the subsequent development of AHR.

**Activation of iNKT Cells Increases Serum IgE.** Direct activation of iNKT cells by α-GalCer also unexpectedly and rapidly increased serum IgM and IgE, but not IgG1 or IgG2a (Fig. 3). The induction of IgE with α-GalCer has been observed before 1 week after several injections of the glycolipid (22), but a significant rise within 24 h has not been previously reported and was observed in all mouse strains tested (BALB/c B6, C57BL/6, NUB/BNJ, and CBA/J; data not shown). The increase in IgE occurred within 12 h of challenge for BALB/c mice but declined over 2 days after α-GalCer challenge (Fig. 3c); similar kinetics were also observed when α-GalCer was administered i.v. (see also Fig. 9, which is published as supporting information on the PNAS web site), indicating that IgE production may be the product of iNKT cells located in the spleen or other nonpulmonary compartments, but that AHR requires activation of pulmonary iNKT cells. Because i.v. administration of α-GalCer induced IgE but not AHR, an increase in serum IgE is not sufficient for the development of AHR.

**Sphingomonas Glycolipid Antigen Induces AHR.** Specific glycolipid antigens recognized by the invariant TCR of iNKT cells are just now being defined, including endogenous glycolipids (11), glycolipids found in pollens (23), and bacterial glycosphingolipids found in the *Sphingomonadaceae* and *Rickettsiaceae* families (9, 10, 24, 25). Many of these species are gram- LPS- bacteria and include human bacterial pathogens such as *Sphingomonas paucimobilis* and *Ehrlichia chaffeensis*. We tested one antigen found in some *Sphingomonas* species, PS-30 [structure of PS-30 and α-GalCer (Fig. 4a)] and found that the synthetic version of this glycolipid induced AHR within 24 h after i.n. administration to wild-type BALB/c mice (Fig. 4b). The induction of AHR by PS-30 depended on iNKT cells, because it did not occur in CD1d<sup>−/−</sup> mice (Fig. 4b). The development...
Penh, representative of three experiments (tration of anti-mouse IL-5 mAb (TRFK5) administered before i.n. adminis-
IgE with before 3 consecutive days of i.n. challenge with OVA. AHR was assessed on day 8
n
ments (ground), which lack conventional CD4
cells. MHC class II-deficient mice (MHC
level of baseline AHR in naı ¨ve MHC
to B cell-deficient JHD mice demonstrated significant AHR within 24 h (Fig. 5c).
Given the potentially controversial role of eosinophils in the development of asthma, we asked whether IL-5 and pulmonary eosinophilia were important for the induction of AHR by α-GalCer. We challenged BALB/c mice in the presence or absence of anti-mouse IL-5 mAb (TRFK5) administered before i.n. adminis-
tration of α-GalCer. Treatment with anti-IL-5 mAb but not isotype control reduced eosinophil levels in the bronchial lavage fluid by >85% (Fig. 6a) but had no effect upon AHR (Fig. 6b). These results suggest that IL-5 and eosinophils are not critical for AHR induced with α-GalCer.

Conventional CD4+ T Cells Are Not Required for AHR. We examined MHC class II-deficient mice (MHCΔΔ mice, C57BL/6 background), which lack conventional CD4+ T cells but contain iNKT cells. MHCΔΔ mice have an increase in the number of pulmonary iNKT cells versus wild-type mice, and the majority of these iNKT cells are CD4+ (Fig. 7a). These mice have very few CD1d-restricted pulmonary γδ T cells (data not shown). Surprisingly, challenge of the MHCΔΔ mice with α-GalCer induced severe airway inflammation, eosinophilia, and severe AHR that was much greater than that observed in α-GalCer-challenged wild-type mice (Fig. 7b; see also Fig. 10, which is published as supporting information on the PNAS web site). Moreover, the saline-challenged MHCΔΔ mice showed greatly increased baseline responsiveness to methacholine, well beyond that of saline-challenged wild-type mice, although to a much lower degree than α-GalCer-challenged MHCΔΔ mice. Although the AHR response in the MHCΔΔ mice to α-GalCer was not accompanied by a detectable serum IgE response (data not shown), the development of severe AHR response in MHCΔΔ mice demonstrates that AHR can indeed occur in the total absence of conventional CD4+ T cells.

We confirmed that the exaggerated AHR response in the MHCΔΔ mice was due to CD1d-restricted NKT cells and not some other confounding factor due to the loss of conventional CD4+ T cells, because treatment of these mice with an anti-CD1d mAb completely blocked the response to α-GalCer in terms of AHR (Fig. 7c) and airway inflammation (Fig. 10). Importantly, the high level of baseline AHR in naive MHCΔΔ mice was also due to a CD1d-restricted iNKT cell response (presumably in response to endogenous glycolipids), because treatment of the naive MHCΔΔ mice with anti-CD1d mAb completely reversed the elevated baseline airway responsiveness (Fig. 7d).

Discussion
In this paper, we demonstrate that the specific and direct activation of iNKT cells by glycolipid antigens results in the rapid induction of AHR, airway inflammation, and IgE production, which typifies the Th2-driven response normally associated with allergen administration. The induction of AHR with direct iNKT cell activation was independent of eosinophils and B cells and did not occur in iNKT cell-deficient J18Δsi or CD1dΔΔ mice. Surprisingly, the induction of AHR was also completely independent of conventional MHC class II-restricted CD4+ helper T cells and hence of adaptive immunity. In fact, airway responsiveness to methacholine was enhanced in MHC class II knockout mice, which lack conventional CD4+ T cells, but which have increased lung iNKT cells. The AHR response in MHC class II knockout mice was eliminated by the administration of anti-CD1d mAb, establishing that CD1d-restricted iNKT cells play an essential and critical role in the development of AHR independent of conventional CD4+ T cells. Because conventional CD4+ T cells have been thought to perform an obligatory role in the pathogenesis of asthma, our studies demonstrating that AHR can occur in the complete absence of conventional CD4+ T cells suggest that iNKT cells may mediate a previously unrecognized effector pathway contributing to the pathogenesis of AHR.
The possibility that NKT cells function as effector cells is supported by our result that NKT cells induced AHR independent of conventional CD4+ T cells, eosinophils, and B cells, although other cell types could serve as effector cells. In contrast, the conventional wisdom is that NKT cells trigger the immune system by rapidly releasing cytokines on activation, which then influences the function of dendritic cells, macrophages, NK cells, B cells, and conventional CD4+ Th2 cells (26, 27), thereby amplifying the subsequent development of adaptive autoimmune, antimicrobial, antitumor, and transplant immune responses (28–30). For example, IFN-γ release by NKT cells enhances the differentiation of effector CD4+ Th1 cells that mediate autoimmune disease or protection against specific infectious organisms (31–33). Similarly, we previously proposed that NKT cells might “license” conventional Th2 effectors, which would orchestrate the development of AHR and asthma (15).

We now believe, however, that direct activation of NKT cells by antigen allows NKT cells to mediate AHR and Th2 inflammation, suggesting that NKT cells function directly as effector cells in inducing AHR, a role that is distinct from their role in autoimmunity or infection. That NKT cells function as effector cells is consistent with our observation that a large fraction of CD4+ cells in the lungs of human patients with asthma are NKT cells and not conventional CD4+ T cells (17). Moreover, because pulmonary NKT cells are phenotypically similar to conventional CD4+ Th2 effector cells [pulmonary NKT cells are CD4+ and produce Th2 cytokines when activated with α-GalCer or Sphingomonas glycolipids], it is possible that pulmonary NKT “effector” cells may have been mistakenly identified in the past as Th2 cells.

Both α-GalCer and Sphingomonas glycolipid induced a very rapid NKT cell-mediated rise in serum IgE. Increased serum IgE levels of 6-fold 9 days after a single dose or multiple doses of α-GalCer were reported (34), but the rapid rise we observed in serum IgE over 24 h is reminiscent of an innate immune pathway and is distinct from the induction of antigen-specific IgE, which can occur in the absence of NKT cells (35). The rapid rise in IgE may be related to the observation that NKT cells activate B cells to produce IgM (14, 36), and the observation that in vivo treatment with IL-18 results in the NKT cell-dependent production of nonspecific IgE antibody (37). This unique innate-like NKT cell-mediated IgE production may represent a reflex response that might be important for host defense against parasites and bacteria that express NKT cell activating glycolipids (9, 10). Although the development of AHR in our glycolipid-induced AHR model was independent of the rise in IgE, NKT cell-mediated IgE responses might contribute to the severity of atopic diseases and asthma.

Although we found that administration of α-GalCer directly induces AHR, several groups recently found that administration of α-GalCer 24 h before challenge with OVA inhibited allergen-induced AHR (19–21). In contrast, other investigators have found that coadministration of α-GalCer with antigen sensitizes mice to these antigens, thereby enhancing AHR (38, 39). These seemingly contradictory results confirm that NKT cells are critically involved in the regulation of AHR and indicate that the timing of activation of the NKT cells dictates their role in AHR. Thus, activation of NKT cells can function as an adjuvant when NKT cells are activated during the administration of protein antigen (38), can directly induce AHR when they are activated in the absence of other signals (Fig. 1), or can function to “prevent” the development of AHR, when they are rendered unresponsive (possibly anergically) at a critical time point by strong NKT cell activating agents (18). It is possible that the development of human asthma could depend upon the activation state of NKT cells.

In our studies, glycolipid antigens could activate NKT cells and induce AHR independent of conventional CD4+ T cells, suggesting that NKT cells may autonomously induce AHR, at least in some forms of asthma. However, we believe that conventional CD4+ T cells are still likely to play a significant role in asthma. Conventional CD4+ T cells respond to allergens important in asthma and produce IL-4 and IL-13, which are known to amplify and prolong allergic...
with anti-IL-5 mAb blocking antibody (described in Materials and Methods) and challenged with 1.5 μg of α-GalCer i.n. showed >85% reduction in BAL eosinophils at 24–27 h versus isotype control-treated mice (P < 0.05; Student’s t test). Results represent three experiments, shown as in Fig. 1c. (b) Mice depleted of lung eosinophils in a show normal AHR at 24 h, assessed as in Fig. 1a. Data are the mean ± SEM Penh, representative of three experiments (n = 4).

Fig. 6. IL-5 and eosinophils are not required for AHR. (a) BALB/c mice treated with anti-IL-5 mAb blocking antibody (described in Materials and Methods) and challenged with 1.5 μg of α-GalCer i.n. show >85% reduction in BAL eosinophils at 24–27 h versus isotype control-treated mice (P < 0.05; Student’s t test). Results represent three experiments, shown as in Fig. 1c. (b) Mice depleted of lung eosinophils in a show normal AHR at 24 h, assessed as in Fig. 1a. Data are the mean ± SEM Penh, representative of three experiments (n = 4).

The specific glycolipid antigens that might activate iNKT cells in vivo to induce asthma are not yet identified. However, glycolipids that are recognized by the invariant TCR of NKT cells appear to be highly conserved and can include pollens (11, 23). As we show here, a glycolipid constituent of *Sphingomonas* cell membranes, synthesized as PS-30, activates iNKT cells to induce a rapid AHR response after respiratory administration in wild-type but not NKT cell-deficient mice (9, 10). This pulmonary response to a *Sphingomonas* glycolipid indicates that the induction of AHR by glycolipids is not limited to the marine sponge glycolipid, α-GalCer, and suggests a pathway through which microorganisms or pollen antigens may be presented by CD1d+ APC to activate iNKT cells and induce AHR. This might play a role in some forms of asthma in humans.

In summary, we showed that NKT cells activated by glycolipids can directly effect the development of AHR and airway inflammation, independent of conventional CD4+ T cells, eosinophils, and B cells, and hence of adaptive immunity. In view of previous reports showing that the induction of allergen-induced AHR absolutely requires NKT cells, which may have been mistakenly thought in the past to be Th2 effector cells, are critical effector cells that drive the development of asthma, in concert with or instead of conventional CD4+ T cells in some forms of asthma. Therefore, therapies that target NKT cells may be clinically effective in limiting AHR and asthma.

Materials and Methods

Mice. Wild-type BALB/c ByJ and C57BL/6 mice were purchased from The Jackson Laboratory. IL-4–/–/IL-13–/– double knockout and absent in IL-4–/– IL-13–/– double knockout BALB/c mice. Data are the mean ± SEM Penh, representative of four experiments (n = 3–5). (b) Total serum IgE, collected from mice in a 1–2 h after AHR measurement, was reduced in IL-4–/– and IL-13–/– versus wild-type mice (measured by ELISA). Data are the mean ± SEM, representative of four experiments (n = 4–5), two of which examined only IL-4–/– and IL-4–/– IL-13–/– mice. (c) AHR does not require B cells. AHR was assessed as in Fig. 1a for B cell-deficient JHD–/– versus wild-type BALB/c mice at 24 h after 1.5 μg of α-GalCer i.n. challenge. Data are the mean ± SEM Penh, representative of five experiments (n = 4).

Fig. 5. IL-4 and IL-13 but not B cells or IgE are necessary for the full development of AHR. (a) AHR depends upon IL-4 and IL-13. After i.n. challenge with 1.5 μg of α-GalCer, AHR was assessed as in Fig. 1a and was reduced in IL-4–/– and IL-13–/– single knockout and absent in IL-4–/– IL-13–/– double knockout BALB/c mice. Data are the mean ± SEM Penh, representative of four experiments (n = 3–5). (b) Total serum IgE, collected from mice in a 1–2 h after AHR measurement, was reduced in IL-4–/– and IL-13–/– and IL-4–/– IL-13–/– versus wild-type mice (measured by ELISA). Data are the mean ± SEM, representative of four experiments (n = 4–5), two of which examined only IL-4–/– and IL-4–/– IL-13–/– mice. (c) AHR does not require B cells. AHR was assessed as in Fig. 1a for B cell-deficient JHD–/– versus wild-type BALB/c mice at 24 h after 1.5 μg of α-GalCer i.n. challenge. Data are the mean ± SEM Penh, representative of five experiments (n = 4).

Antibodies and Reagents. Neutralizing rat anti-mouse CD1.1 mAb (hybridoma HB323, American Type Culture Collection) and rat anti-mouse IL-5 neutralizing antibody (hybridoma TRFK5, a gift of T. Mossman, DNAX) were purified by ammonium sulfate precipitation and ion-exchange chromatography. The CD1.1 blocking antibody was used at a dose of 500 μg per mouse administered i.p. 1 day before antigen challenge. The IL-5 neutralizing antibody was used at a dose of 500 μg per mouse administered i.p. at 5 days and again 1 day before antigen challenge. Isotype control rat mAb were administered in the same fashion (R & D Systems). α-GalCer (KRN7000) and its corresponding proprietary vehicle control were a gift of Kirin Brewery, Takasaki, Japan. *Sphingomonas* glycolipid antigen, PS-30 (also known as PBS-30), was synthesized as described (9). Both α-GalCer and PS-30 (0.2 mg each per aliquot) were provided as a lyophilized powder that included a vehicle (40). The vehicle included sucrose (56 mg/ml), L-histidine (7.5 mg/ml), cysteine (7.5 mg/ml in PS-30), and polysorbate 20 (5 mg/ml). Glycolipids were brought up in water (to a concentration of 0.2 mg/ml), and this initial stock was further diluted in PBS just before i.n. or i.v. challenge.
μl) to mice anesthetized with ketamine and xylazine. Glycolipid vehicles were administered as controls. Allergen-induced AHR was induced as described (15). AHR was measured described (15). In some experiments, AHR was assessed by invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated modified version of a described method (Buxo Electronics) (15). For additional materials and methods, see Supporting Text, which is published as supporting information on the PNAS web site.

Lymph Node Cell Restimulation and Collection of BAL Fluid and Lung Histology. Lymph node cells were isolated from the OVA-challenged mice by mechanical disruption and were restimulated in vitro (5.0 × 10^6 cells per well in a 96-well plate) with 33 μg/ml OVA. Supernatant were collected after 4 days of culture and assayed for cytokines. BAL and lung histology was collected as described (15).

Flow Cytometry and FACS. Analysis of nK cT cells was performed by preincubation of cells (2 × 10^6) with mAb against the Fcy receptor (2.4G4). After washing, cells were incubated with α-GalCer-loaded mouse CD1d tetramer (16) or mouse CD1d:Ir 

Cytokine and Serum Antibody Measurement. Cytokine ELISAs were completed as described (15). Serum IgE, IgG1, IgG2a, and OVA-specific IgE antibodies were obtained by ELISA as described (15).

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Supplemental Figure 2

**a.**

- TCC
- MO
- EOS
- LYM
- NEU

**b.**

- IL-4 (ng/ml)
- IFN-γ (ng/ml)

- OVA i.n. (saline i.n. day 6)
- OVA i.n. (α-GalCer i.n. day 6)

p = 0.051
p = 0.034
Supplemental Figure 1.
Supplemental Figure 3.
Table 1. Airway response to intranasal (i.n.) αGalCer in different mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Final dose of methacholine, mg/ml</th>
<th>Peak Penh after α-GalCer i.n. challenge, +/- SEM</th>
<th>Peak Penh after vehicle i.n. challenge, +/- SEM</th>
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<tr>
<td></td>
<td></td>
<td>6 hours</td>
<td>24 hours</td>
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<tr>
<td>BALB/c BYJ</td>
<td>40</td>
<td>5.56 +/- 0.34</td>
<td>11.28 +/- 1.35</td>
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<tr>
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<tr>
<td>NZB/BINJ</td>
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<td>9.75 +/- 0.93</td>
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