

CUTTING EDGE

Cutting Edge: Activation by Innate Cytokines or Microbial Antigens Can Cause Arrest of Natural Killer T Cell Patrolling of Liver Sinusoids¹

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Natural killer T (NKT) cells are innate-like lymphocytes that rapidly secrete large amounts of effector cytokines upon activation. Recognition of α -linked glycolipids presented by CD1d leads to the production of IL-4, IFN- γ , or both, while direct activation by the synergistic action of IL-12 and IL-18 leads to IFN- γ production only. We previously reported that in vitro cultured dendritic cells can modulate NKT cell activation and, using intravital fluorescence laser scanning microscopy, we reported that the potent stimulation of NKT cells results in arrest within hepatic sinusoids. In this study, we examine the relationship between murine NKT cell patrolling and activation. We report that NKT cell arrest results from activation driven by limiting doses of a bacteria-derived weak agonist, galacturonic acid-containing glycosphingolipid, or a synthetic agonist, α -galactosyl ceramide. Interestingly, NKT cell arrest also results from IL-12 and IL-18 synergistic activation. Thus, innate cytokines and natural microbial TCR agonists trigger sinusoidal NKT cell arrest and an effector response. The Journal of Immunology, 2008, 180: 2024–2028.

Natural killer T (NKT)³ cells are innate lymphocytes with diverse influences and multiple pathways leading to their activation. NKT cells are activated directly by cytokines such as IL-12 and IL-18 in synergy, independently of exogenous Ag (1, 2), a property that distinguishes them from most conventional, naive lymphocytes (3). Furthermore, NKT cells can be activated when the semi-invariant TCR they express, containing a V α 14J α 18 rearrangement, recognizes glycolipid Ag in the context of CD1d (4).

Activation via the TCR with strong agonists does not require costimulation, demonstrating an effector state of these lympho-

cytes, and it can result in a robust production of IL-4 or IFN- γ (5, 6). IFN- γ but not IL-4 is induced in NKT cells via the synergistic activation by IL-12 and IL-18 produced by LPS-stimulated macrophages and dendritic cells in vivo (1, 7).

Although several NKT cell TCR agonists have been identified, the best studied is the glycolipid α -galactosyl ceramide (α GalCer), originally purified from a marine sponge and optimized by medicinal chemistry. Recent studies have identified bacterial glycolipids derived from sphingomonads that are natural NKT cell TCR agonists (2, 8, 9). In particular, galacturonic acid-containing glycosphingolipid (GalA-GSL) is a ceramide-based glycolipid agonist produced by *Sphingomonas yanoikuyae*. GalA-GSL is at least 100-fold less potent than α GalCer in vitro. Like α GalCer, activation by GalA-GSL is characterized, in part, by an early rapid and robust production of IL-4 by NKT cells (8).

Anatomically, NKT cells are most abundant in the liver and spleen of mice (10). Therefore, understanding the basis of NKT cell activation at these sites in vivo is of major importance. Unlike in the spleen, in the liver NKT cells comprise a major percentage of the lymphocyte population (~10–40%) (10). Also, ~90% of the liver's blood supply comes from the portal vein, which drains the gut mucosa, a major site of Ag entry. Using intravital laser scanning microscopy we have previously shown that resident liver NKT cells patrol liver sinusoids both with and against the blood flow and arrest their migration in response to the presentation of α GalCer by CD1d (11).

The aim of the current study was to gain insight into the relationship between NKT cell patrolling and the different modes of activation known for these cells. Using intravital laser scanning microscopy we examined NKT cell patrolling in response to Ag receptor agonists and innate cytokines. We tested a range of doses of a natural Ag, GalA-GSL, compared with the more potent synthetic strong agonist, α GalCer. Activation in the absence of foreign Ag also was studied using innate cytokines,

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Received for publication June 29, 2007. Accepted for publication December 26, 2007.

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¹ This work was supported by National Institute of Allergy and Infectious Diseases, National Institutes of Health Grants R01 AI055037-03 (to M.L.D.) and R37 AI 17922 (to M.K.), grants from the Irene Diamond Fund (to M.L.D.) and the Cancer Research Insti-

tute (to Y.K.), and National Institute of Diabetes and Digestive and Kidney Disease Grant F32-DK078153 (to P.V.).

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³ Abbreviations used in this paper: NKT, natural killer T; GalA-GSL, galacturonic acid-containing glycosphingolipid; α GalCer, α -galactosyl ceramide; EGFP, enhanced green fluorescent protein.

IL-12 and IL-18. We report the surprising finding that all modes of activation can result in acute NKT cell arrest in liver sinusoids. Although arrest is an important indicator of activation by distinct biologic stimuli, GalA-GSL vs IL-12 and IL-18 synergy, a differential effector response is elicited, IL-4 vs IFN- γ , respectively.

Materials and Methods

Reagents

GalA-GSL was synthesized and provided by Drs. M. Fujio and C.-H. Wong (Scripps Research Institute, La Jolla, CA). α GalCer (KRN7000) was provided by S. Porcelli (Albert Einstein College of Medicine, Bronx, NY). Murine rIL-12 and rIL-18 were purchased from R&D Systems. CD1d/ α GalCer tetramers were made as described previously (12).

Animals

CXCR6^{+/EGFP} animals (where EGFP is enhanced green fluorescent protein) were provided by D. R. Littman of the New York University School of Medicine, Department of Laboratory Animal Research (New York, NY), where they were maintained under specific pathogen-free conditions and studied with the approval of the Institutional Animal Care and Use Committee (IACUC).

Intravital imaging

All surgical procedures were approved by New York University School of Medicine IACUC and performed as previously described (11); images were acquired with Zeiss Plan-Apochromat microscope ($\times 20$ magnification/0.75 objective).

Lymphocyte migration dynamic analysis

Dynamics were quantified using Velocity (Improvision); each cell was tracked semiautomatically and confirmed visually during each frame collected. The number of cells varied between experiments, with ~ 6 –20 cells per viewing field (typically 10–15 cells). Each cell was examined during each acquisition frame (1/30 s) for 5–10 frames. The mean from each animal was then calculated and plotted as a single data point. Therefore, each data point represents a single animal with 30–200 observations (typically 100–150).

Statistics

Statistical analysis was conducted using GraphPad Prism software. All data sets were examined for Gaussian distribution via a D'Agostino and Pearson normality test. For determination of the significance of differences between two groups, a two-tailed nonparametric Mann-Whitney *U* test (non-Gaussian) with a 95% confidence interval was conducted. For multiple groups, a Kruskal-Wallis (non-Gaussian) ANOVA with Dunn's multiple comparison posttest was conducted. Significance was defined as $p \leq 0.05$.

Liver mononuclear cell isolation

Liver homogenate was digested with collagenase/DNase (Roche) at 37°C for 45 min. This suspension was filtered with a 70- μ m cell strainer and separated with 40% Percoll.

Flow cytometry

Cells were stained with CD1d/ α GalCer, CD69-PE-Cy7, or isotype control-PE-Cy7 (BD Biosciences). Data acquisition was performed at Skirball Institute Flow Cytometry Core Facility (New York, NY) using BD FACSCalibur (BD Biosciences). Analysis was conducted on FlowJo software (Tree Star).

Results

Activation via natural bacterial Ag

To understand the relationship between NKT cell patrolling and activation driven by a biologic weak agonist *in vivo*, we titrated GalA-GSL while quantitatively examining NKT liver patrolling via intravital microscopy. Animals with a targeted replacement by the EGFP in a single allele of the CXCR6 locus (CXCR6^{+/EGFP}) were used to visualize liver sinusoidal NKT cells. Expression of this chemokine receptor in liver is confined almost exclusively to NKT cells (11).

Multiple parameters of migration were analyzed to understand the effects of GalA-GSL on NKT cell migration. Only

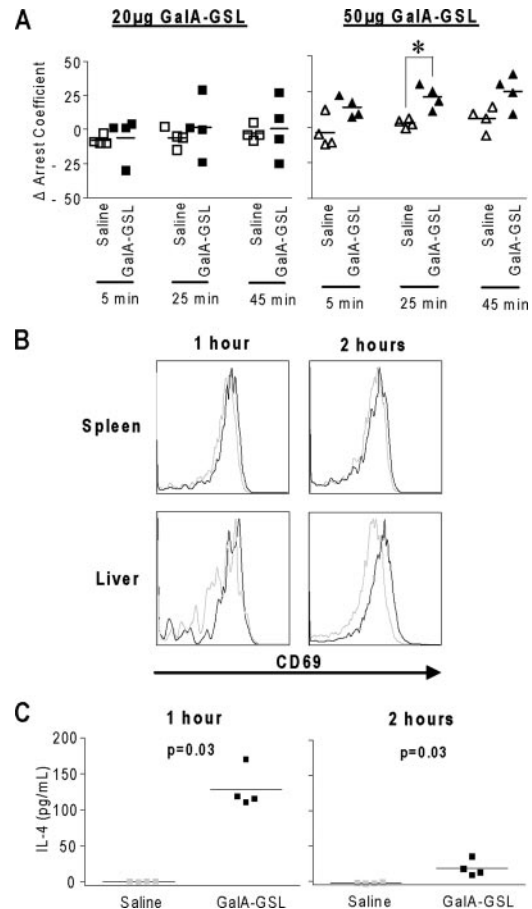


FIGURE 1. NKT arrest and activation in response to GalA-GSL. Changes in the arrest coefficient of liver NKT cells in response to titrating doses of GalA-GSL (A), CD69 (B), and serum cytokine (C). A, Twenty micrograms of GalA-GSL (closed squares) had no effect on the arrest coefficient as compared with saline (open squares). Fifty micrograms of GalA-GSL (closed triangles) induced acute arrest, $p = 0.03$, as compared with saline (open triangle). B, CXCR6^{+/EGFP}CD1d-tetramer⁺ splenic and liver mononuclear cells were analyzed for CD69 by flow cytometry, $n = 3$ (saline, gray line; 50 μ g of GalA-GSL, solid line). C, IL-4 response was detected at 1 h ($p = 0.03$) and was decreased at 2 h, ($p = 0.03$) after treatment with 50 μ g of GalA-GSL (saline, gray squares; 50 μ g of GalA-GSL, filled squares).

arrest is presented here due to its significance. The arrest coefficient is the fraction of time that a cell is stopped (velocity ≤ 2 μ m/min). Because of variations in homeostatic NKT patrolling between animals before Ag challenge, quantitative changes in NKT cell arrest were calculated by subtracting the mean before treatment from the mean at each time point after treatment.

Doses as high as 20 μ g of GalA-GSL administered *i.v.* did not affect NKT cell arrest (Fig. 1A and supplemental video 1).⁴ Conversely, 50 μ g of GalA-GSL resulted in increased NKT cell arrest (Fig. 1A and supplemental video 2).

To understand the biologic importance of these variations in NKT cell patrolling, we examined two well-established parameters of NKT cell activation. First, flow cytometric analysis of CXCR6^{+/EGFP}CD1d-tetramer⁺ liver and splenic mononuclear cells showed increased cell surface expression of the activation marker CD69 on liver NKT cells in animals treated with 50 μ g of GalA-GSL 2 h posttreatment (Fig. 1B). NKT cell activation with 50 μ g of GalA-GSL demonstrated an acute rise in serum

⁴ The online version of this article contains supplemental material.

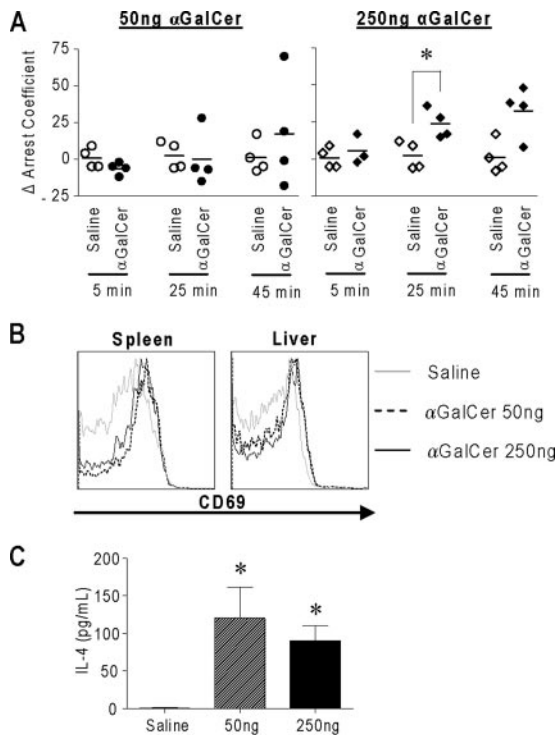


FIGURE 2. NKT arrest and activation in response to α GalCer. Analysis of changes in arrest coefficient (A), CD69 up-regulation (B), and serum cytokine (C) of liver NKT cells in response to titrating doses of the α GalCer. A, *left panel*, Fifty nanograms of α GalCer (closed circles) had a variable effect on the arrest coefficient vs saline (open circle). *Right panel*, Two hundred-fifty nanograms of α GalCer (closed diamonds) resulted in an increased arrest coefficient, $p = 0.03$, vs saline (open diamonds). B, CD69 expression on CXCR6⁺/EGFP/CD1d-tetramer⁺ mononuclear cells, $n = 3$, (saline, gray line; 50 ng of α GalCer, dashed line; 250 ng of α GalCer, solid line). C, Serum IL-4 was detected at 1 h in both 50 ng (hatched bar; $p = 0.03$) and 250 ng of α GalCer (solid bar; $p = 0.03$).

IL-4 (Fig. 1C) but no IFN- γ (not shown). No effect on cytokine or CD69 was seen with 20 μ g of GalA-GSL (not shown). Thus, NKT cell activation driven by the bacterial Ag GalA-GSL was characterized by CD69 up-regulation, acute IL-4 production, and acute NKT cell arrest.

Activation via strong TCR agonist

α GalCer is defined as a strong agonist because only nanogram quantities are required to activate NKT cells in vivo. As with GalA-GSL, α GalCer was titrated while quantitatively examining NKT cell patrolling in liver sinusoids. Although 50 ng of α GalCer did not yield a statistically significant effect on the NKT cell arrest coefficient (Fig. 2A and supplemental video 3), there was considerable variability in the migratory response of NKT cells at this dose, particularly at later time points. This is likely due to a subset of NKT cells arresting below our limit of detection in this system. Slow kinetics of the loading of α GalCer at this lower dose may further decrease our ability to detect statistically significant alterations in migration. Conversely, 250 ng of α GalCer induced NKT cell arrest (Fig. 2A and supplemental video 4). This extends earlier results showing that injection of 5 μ g of α GalCer induces NKT arrest in the liver (11).

As with GalA-GSL, we examined biologic readouts of NKT cell activation after challenge with α GalCer. Detectable CD69 up-regulation was seen at both the 50- and 250-ng doses in liver and spleen NKT cells by 1 h (Fig. 2B), although the effect in

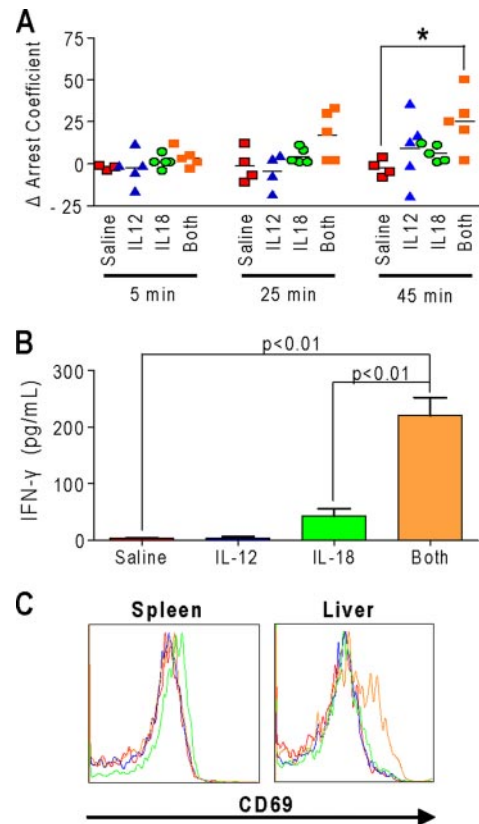


FIGURE 3. Synergistic effects of innate cytokines on NKT arrest and activation. A, Animals were imaged and treated with saline (red), 2 ng of IL-12 only (blue), 200 ng of IL-18 (green), or 2 ng IL-12 plus 200 ng IL-18 (orange). IL-12 or IL-18 alone had no effect on arrest, while the combination of both IL-12 and IL-18 induced acute NKT cell arrest, $p < 0.05$. B, IL-12 and IL-18 induced serum IFN- γ , $p < 0.01$, while IL-12 only had no effect and IL-18 had minimal effects, $p < 0.01$. C, Liver and splenic CXCR6⁺/EGFP/CD1d-tetramer⁺ mononuclear cells were analyzed for CD69. Only the combination of IL-12 and IL-18 induced CD69 up-regulation specifically in liver NKT cells, $n = 3$.

spleen was variable. Serum IL-4 was comparably induced in animals treated at both doses (Fig. 2C) but no IFN- γ was detected (not shown). Thus, low doses of α GalCer can modulate NKT patrolling by inducing acute changes in arrest, corresponding with NKT cell activation in the sinusoid.

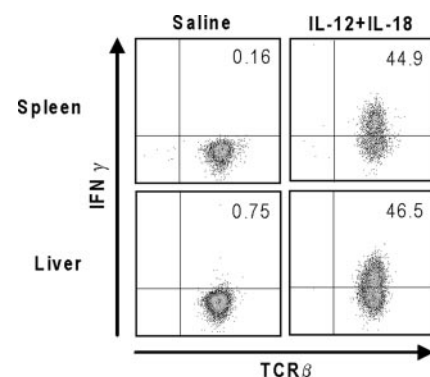


FIGURE 4. Liver NKT cells produce IFN- γ in response to exogenous IL-12 and IL-18. Animals were treated with saline (*left*) or 2 ng of IL-12 plus 200 ng IL-18 (*right*). After 1 h, spleen and liver mononuclear were harvested, stained, and analyzed via flow cytometry. Cells gated on B220⁻CD1d tetramer⁺ were analyzed for TCR β and intracellular IFN- γ .

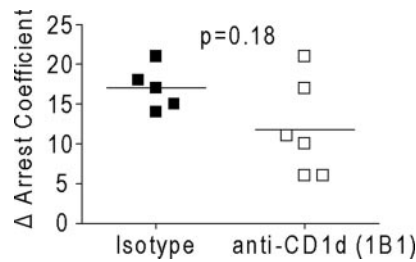


FIGURE 5. Requirement of CD1d for cytokine-induced arrest of liver NKT cells. Animals were treated with IL-12 and IL-18 plus isotype control (closed squares) or anti-CD1d (clone 1B1) Ab (open squares), and change in arrest coefficient was quantified.

NKT activation via innate cytokines

IL-12 and IL-18 synergize to directly activate NKT cells, characterized by acute IFN- γ production and CD69 up-regulation, without IL-4 production (7). It has been reported that the presentation of self-Ag by CD1d participates in this type of activation and, although the putative self-Ags have not been defined, this possibility cannot be excluded (2).

To better understand NKT cell patrolling and activation independent of an exogenous Ag, we examined the effects of stimulation via IL-12 and IL-18 administration in vivo. Animals were treated with saline, IL-12 only, IL-18 only, or the combination of IL-12 and IL-18 while imaging NKT cell migration. The amounts of cytokine injected were similar to those detected in the sera of mice following exposure to LPS (7). In the same animals, serum IFN- γ , IL-4, and CD69 expression by NKT cells was examined. IL-12 alone was insufficient to induce the arrest of sinusoidal NKT cells (Fig. 3*A* and supplemental video 5) or serum IFN- γ (Fig. 3*B*) or CD69 up-regulation in liver or splenic NKT cells (Fig. 3*C*). Similarly, IL-18 alone did not induce NKT arrest (Fig. 3*A* and supplemental video 6) and only weakly induced serum IFN- γ (Fig. 3*B*). Surprisingly, the combination of IL-12 and IL-18 induced NKT arrest (Fig. 3*A* and supplemental video 7). CD69 expression was up-regulated in liver but not in splenic NKT cells (Fig. 3*B*). As previously reported, this cytokine combination also induced robust IFN- γ secretion (Fig. 3*C*) but not IL-4 secretion (not shown). Intracellular cytokine staining confirmed that liver NKT cells are activated to produce IFN- γ (Fig. 4), although splenic NKT cells and conventional NK cells contribute to the serum IFN- γ pool (Fig. 4 and not shown, respectively). To better understand the molecular basis of NKT cell arrest in the liver sinusoids, animals were challenged with a combination of IL-12 and IL-18 with anti-CD1d (clone 1B1) or isotype control. The data are consistent with the hypothesis that CD1d expression and Ag presentation are not absolutely necessary for IL-12 and IL-18-induced stopping, although the trend of the data suggests that CD1d plays a partial role (Fig. 5). Therefore, IL-12 and IL-18 synergized to induce liver sinusoidal NKT arrest and activation characterized by robust IFN- γ production that was not highly dependent on CD1d.

Discussion

This study was aimed at achieving a better understanding of the relationship between NKT cell patrolling in the liver sinusoids and the activation of these cells driven via different pathways. We studied activation through the TCR by using both natural

microbial and synthetic glycosphingolipid agonists of differing potencies. In addition, we examined NKT cell activation driven by the synergistic action of inflammatory cytokines. We report that NKT cell arrest in the liver sinusoid can be mediated by both natural weak agonists and synthetic strong agonists. Surprisingly, signals mediated via the inflammatory cytokines IL-12 and IL-18 can also induce arrest without a strict requirement for CD1d Ag presentation. Importantly, arrest is acute, occurring in less than 1 h, and precedes NKT cell activation. Thus, NKT arrest results from distinct stimuli leading to different effector responses: early IL-4 via TCR stimulation and IFN- γ via the combination of IL-12 and IL-18.

Previous studies have shown that potent stimulation of the NKT cell TCR leads to arrest in the hepatic sinusoid (11). Additionally, we have demonstrated that in vitro cultured dendritic cells loaded with a synthetic strong agonist can activate liver NKT cells (8). We initially predicted that a weak agonist, such as GalA-GSL, would also lead to NKT cell arrest and activation. However, Skokos et al. recently demonstrated the activation of conventional CD4⁺ T cells by weak agonist without any acute arrest (13). Therefore, the current study demonstrates unique dynamics of NKT cell and conventional T cell activation in vivo.

The physiology of liver sinusoids is unique compared with that encountered in the secondary lymphoid organs or tissue parenchyma and may provide a partial explanation of the difference between NKT cell and conventional T cell dynamics in response to a weak agonist. The rapid blood flow in the liver sinusoids results in the rapid removal of proteins there, including Ags, chemokines, and effector proteins. We hypothesize that a stable NKT cell-APC interaction in the sinusoids facilitates an optimal biologic response via at least two possible mechanisms. First, arrest may stabilize the NKT cell-APC interaction in the area of Ag encounter, possibly via the formation of an immunological synapse as has been shown for NKT cells in vitro (14). Second, stable NKT cell-APC interactions may also facilitate the directed secretion of IL-4 and IFN- γ , maximizing their local effects. In conventional T cells, IFN- γ secretion is directed to the synapse while IL-4 secretion is not (15). Although we injected IL-12 and IL-18 i.v., the local production of IL-12 and IL-18 in the tissue during infection combined with the local up-regulation of other cues such as ICAM-1 expression may lead to the appropriate selection of arrest sites and the targeting of IFN- γ secretion by cytokine-activated NKT cells.

In vitro studies have shown that CD1d Ag presentation is not required for the functional activation of NKT cells by the combination of IL-12 and IL-18 (7), although CD1d-mediated presentation of self Ags can play a role in modulating such a response to inflammatory cytokines from innate cells. We show here that CD1d function is not absolutely necessary for IL-12 and IL-18-driven arrest. However, the trend of blocking arrest by anti-CD1d Ab indicates that CD1d may play a partial role in sinusoidal NKT cell arrest. Additionally, we have not ruled out the possibility that other molecules, such as integrins, may also participate in this process in vivo.

Together, these data point to the surprising regulation of sinusoidal NKT cells by distinct receptor pathways and give some insight into how NKT cells perform their effector functions in vivo. Future studies should be aimed at understanding the molecular and biochemical basis of NKT cell

arrest and activation as they apply to infectious disease and at determining whether NKT cell patrolling can be modulated for therapeutic intervention.

Disclosures

The authors have no financial conflict of interest.

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