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

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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.

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

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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). αGalCer (KRN7000) was provided by S. Porcelli (Albert Einstein College of Medicine, Bronx, NY). Murine IL-12 and rIL-18 were purchased from R&D Systems. CD1d/αGalCer tetramers were made as described previously (12).

Animals

CXCR6<sup>+/EGFP</sup> 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 (×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 animals before Ag challenge, quantitative changes in means of NKT cell activation. First, flow cytometric analysis of Cxcr6<sup>+/EGFP</sup>CD1d-tetramer<sup>+</sup> liver and splenic mononuclear cells showed increased cell surface expression of the activation marker CD69 on liver NKT cells in animals treated with 50 μg of GalA-GSL 2 h posttreatment (Fig. 1B). NKT cell activation with 50 μg of GalA-GSL demonstrated an acute rise in serum arrest is presented here due to its significance. The arrest coefficient is the fraction of time that a cell is stopped (velocity ≤ 2 μm/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 μg of GalA-GSL administered i.v. did not affect NKT cell arrest (Fig. 1A and supplemental video 1).<sup>4</sup> Conversely, 50 μg 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<sup>+/EGFP</sup>CD1d-tetramer<sup>+</sup> liver and splenic mononuclear cells showed increased cell surface expression of the activation marker CD69 on liver NKT cells in animals treated with 50 μg of GalA-GSL 2 h posttreatment (Fig. 1B). NKT cell activation with 50 μg of GalA-GSL demonstrated an acute rise in serum

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<sup>+/EGFP</sup>CD1d-tetramer<sup>+</sup>-gated splenic and liver mononuclear cells were analyzed for CD69 by flow cytometry, n = 3 (saline, gray line; 50 μg 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 μg of GalA-GSL (saline, gray squares; 50 μg of GalA-GSL, filled squares).

4 The online version of this article contains supplemental material.
IL-4 (Fig. 1C) but no IFN-γ (not shown). No effect on cytokine or CD69 was seen with 20 μg 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**

αGalCer is defined as a strong agonist because only nanogram quantities are required to activate NKT cells in vivo. As with GalA-GSL, αGalCer was titrated while quantitatively examining NKT cell patrolling in liver sinusoids. Although 50 ng of αGalCer 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 αGalCer at this lower dose may further decrease our ability to detect statistically significant alterations in migration. Conversely, 250 ng of αGalCer induced NKT cell arrest (Fig. 2A and supplemental video 4). This extends earlier results showing that injection of 5 μg of αGalCer induces NKT arrest in the liver (11).

As with GalA-GSL, we examined biologic readouts of NKT cell activation after challenge with αGalCer. 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 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 αGalCer can modulate NKT patrolling by inducing acute changes in arrest, corresponding with NKT cell activation in the sinusoid.

**FIGURE 2.** NKT arrest and activation in response to αGalCer. 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 αGalCer. A, left panel, Fifty nanograms of αGalCer (closed circles) had a variable effect on the arrest coefficient vs saline (open circle). Right panel, Two hundred-fifty nanograms of αGalCer (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 αGalCer, dashed line; 250 ng of αGalCer, solid line). C, Serum IL-4 was detected at 1 h in both 50 ng (hatched bar; p = 0.05) and 250 ng of αGalCer (solid bar; p = 0.05).

**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 affect and IL-18 had minimal affects, 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.

**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 TCRβ and intracellular IFN-γ.
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 after the presentation of 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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