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Original article

Role of interleukin-12 in determining differential kinetics of invariant natural killer T cells in response to differential burden of *Listeria monocytogenes*

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Abstract

Invariant (i) natural killer (NK) T cells are unique T lymphocytes expressing NKR-P1B/C (NK1.1), which recognize glycolipids, notably α-galactosylceramide (α-GalCer) presented by CD1d. The characteristic phenotype of these iNKT cells undergoes dramatic changes following *Listeria monocytogenes* infection, and interleukin (IL)-12 is involved in these alterations. Here we show that liver iNKT cells in mice are differentially influenced by the load of infection. Liver α-GalCer/CD1d tetramer-reactive (α-GalCer/CD1d$^+$) T cells expressing NK1.1 became undetectable by day 2 following *L. monocytogenes* infection and concomitantly cells lacking NK1.1 increased regardless of the severity of infection. Whereas α-GalCer/CD1d$^+$NK1.1$^+$ T cells remained virtually undetectable on day 4 following low-dose infection, considerable numbers of these cells were detected in high-dose-infected mice. Whereas numbers of IL-12 producers in the liver on day 4 post infection were comparable in low- and high-dose-infected mice without *in vitro* restimulation with heat-killed *Listeria*, those were more prominent in low-dose-infected mice than in high-dose-infected mice after restimulation despite the fact that higher numbers of macrophages and granulocytes infiltrated the liver in high-dose-infected mice than in low-dose-infected mice. Our results indicate that NK1.1 surface expression on iNKT cells is differentially modulated by the burden of infection, and suggest that a high bacterial load probably causes loss of IL-12 production.

Keywords: *Listeria monocytogenes*; NKT cell; NK1.1; Liver; Interleukin-12

1. Introduction

Listeria monocytogenes, a Gram-positive facultative intracellular bacterium, preferentially propagates in macrophages and liver parenchymal cells [1]. Innate immunity orchestrated by multiple cell types is pivotal for the elimination of *L. monocytogenes*, and granulocytes and macrophages play a central role as a first line of defense [1]. The primary control of *L. monocytogenes* infection depends on the ability of the host to induce efficient type 1-immune responses [1], and interferon (IFN)-γ secreted by natural killer (NK) cells and T helper 1 cells plays a critical role in protection [1–3]. Interleukin (IL)-12 secreted by macrophages [4] and granulocytes [5] is also important for protection against *L. monocytogenes*, with the primary function of inducing IFN-γ secretion from responding cells including NK1.1$^+$ cells [6,7].

NKT cells represent a unique T-cell population, which surface expresses the type II C-type lectin, NKR-P1B and C (NK1.1) [8]. In the mouse, the majority of NKT cells express

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a canonical Vα14/Jα18 T-cell receptor (TCR)α-chain associated with a limited set of TCRβ subfamilies (invariant (i) NKT cells) [8]. These iNKT cells recognize glycolipid antigens including α-galactosylceramide (α-GalCer) in the context of CD1d [8,9]. iNKT cells are abundant in the liver, where they secrete large quantities of both IFN-γ and IL-4 upon activation through their TCR [7,10–12]. iNKT cells become undetectable after *in vivo* administration of anti-CD3 monoclonal antibody (mAb) [13,14], α-GalCer [15–19], IL-12 [7,12,14,20,21], and infection with various microorganisms [7,11,12,20–23]. Although it was originally assumed that apoptosis causes the disappearance of iNKT cells *in vivo* [14,24], accumulating evidence suggests that the failure to detect iNKT cells is caused by a loss of the NK1.1 and/or TCR, previously considered as a reliable marker of this cell population [7,17–19].

We previously reported that iNKT cells in the liver of mice become undetectable immediately after *L. monocytogenes* infection and that endogenous IL-12, but not bacterial components, participates in this mechanism [11,20]. Moreover, we have recently shown that the disappearance of iNKT cells during listeriosis is caused by a loss of surface-expressed NK1.1 [7,25]. In the present study, we compared NK1.1 surface expression on iNKT cells in the liver of mice following high- and low-dose *L. monocytogenes* infection. We found differential kinetics of NK1.1 surface expression on iNKT cells following high- and low-dose infection, which depended on IL-12.

2. Materials and methods

2.1. Mice

C57BL/6 mice were maintained under specific pathogen-free conditions at our animal facilities, and weight-matched female mice were used at 8–12 weeks of age.

2.2. Antibodies

MAbs against TCRβ (H57-597), NK1.1 (PK136), Fcγ receptor (R) (2.4G2), and IL-12 (C15.6.7; C17.8) were purified from hybridoma culture supernatants. MAb against TCRβ was conjugated with fluorescein isothiocyanate (FITC), and mAbs against NK1.1 and IL-12 (C15.6.7) were biotinylated by conventional methods. Unconjugated anti-Ly6G mAb (RB6-8C5), FITC-conjugated anti-Ly6G mAb (RB6-8C5), and biotinylated mAbs against NK1.1 (PK136), CD11b (M1/70) and mouse immunoglobulin (Ig)G2a (R19-15) were purchased from BD PharMingen (Hamburg, Germany). AMCA-conjugated anti-rabbit IgG Ab, Cy5-conjugated anti-rabbit IgG Ab, and Cy2-conjugated anti-rat IgG Ab were obtained from Jackson Immunoresearch (Cambridgeshire, UK). Anti-pan Cadherin mAb (CH-19) was purchased from Sigma–Aldrich (Schnelldorf, Germany). MAb against F4/80 (CI: A3-1) was obtained from Serotec (Dusseldorf, Germany).

2.3. Bacteria and infection

L. monocytogenes (strain EGD) recovered from infected livers of mice were grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37 °C for 18 h and aliquots were frozen at −80 °C until used. The final concentration of viable bacteria was enumerated by plate counts on tryptic soy agar (Difco). Mice were infected intravenously (i.v.) with either 1.5×10^3 (low dose) or 4.5×10^3 (high dose) *L. monocytogenes*. Heat-killed *Listeria* (HKL) were prepared by heating bacteria in a water bath at 80 °C for 3 h. HKL were washed three times with PBS and frozen at −20 °C for later use.

2.4. α-GalCer/CD1d tetramer

Mouse α-GalCer/CD1d tetramers were prepared using the baculovirus expression system as described previously [7,16].

2.5. Cell preparation and flow cytometry

Mice were killed by cervical dislocation and livers were collected. Hepatic leukocytes (HLs) were prepared as described previously [10]. Cells were incubated with anti-FcγR mAb and then stained with conjugated mAbs at 4 °C for 15 min. Biotinylated mAbs were visualized by streptavidin (SA)-conjugated Cy5 (BD PharMingen). Stained cells were washed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Serva, Heidelberg, Germany) and 0.1% sodium azide (Merck, Darmstadt, Germany), fixed with 1% paraformaldehyde (Merck), acquired by FACScan® or FACS Calibur® (BD Biosciences, Mountain View, CA), and leukocytes were analyzed with CellQuest software (BD Biosciences). Cells were stained with phycoerythrine (PE)-labeled α-GalCer/CD1d tetramer for 15 min at room temperature after blocking.

2.6. Determination of colony forming units (CFUs)

Mice were killed by cervical dislocation on day 4 post infection (p.i.). The liver was perfused with 10 ml sterile PBS to wash out bacteria in blood vessels and CFUs in the liver were determined by plating serial dilutions of liver homogenates on tryptic soy agar plates after sonication.

2.7. In vivo depletion of Kupffer cells

Mice were injected i.v. with 200 μl of dichloromethylene diphosphonate encapsulated in multilamellar liposomes (Cl2MBP-L) (containing 1 mg of Cl2MBP) suspended in PBS as described previously [26,27]. As a control, mice were injected i.v. with 200 μl of liposome-encapsulated PBS (PBS-L). The depletion of Kupffer cells on day 3 after Cl2MBP-L treatment was immunohistochemically verified [26,27].

2.8. Enzyme-linked immunospot assay

IL-12 production was measured by the enzyme-linked immunospot (ELISPOT) method as described previously [20].