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ICOS costimulates invariant NKT cell activation☆

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Abstract

It has been reported that costimulatory molecules, CD80/86–CD28 and CD154–CD40, critically contribute to activation of CD1d-restricted invariant NKT (iNKT) cells. Here we have demonstrated that ICOS, a new member of the CD28 family, plays a substantial role in iNKT cell activation. iNKT cells constitutively expressed ICOS as well as CD28 independently, and ICOS expression was further up-regulated 2–3 days after α-galactosylceramide (α-GalCer) treatment. Blockade of ICOS-mediated costimulation by administration of anti-ICOS ligand (B7RP-1) mAb or by ICOS gene knockout substantially inhibited α-GalCer-induced IFN-γ and IL-4 production, cytotoxic activity, and anti-metastatic effect. Moreover, blockade of both B7RP-1–ICOS and CD80/86–CD28 interactions mostly abolished the α-GalCer-induced immune responses. These findings indicate that iNKT cell activation is regulated by CD28 and IOCS independently.

Keywords: NKT cells; α-Galactosylceramide; ICOS; CD28; IFN-γ; IL-4; Cytotoxicity; Anti-tumor effect

The typical invariant NKT (iNKT) cells coexpress a semi-invariant T cell receptor (TCR), consisting of an invariant TCR Vα14Jα18 chain (Vα24Jα15 in humans) and a TCR β-chain that is largely biased towards Vβ8.2 (Vβ11 in humans), Vβ2, and Vβ7, as well as NK cell receptors such as NK1.1 [1,2]. Although the physiological antigens (Ags) for iNKT cells still remain unclear, this semi-invariant TCR recognizes glycolipid antigens, such as α-galactosylceramide (α-GalCer) and its analogs, presented on an MHC class I-like molecule, CD1d [1,2]. iNKT cells secrete various cytokines, including both IFN-γ and IL-4, promptly after TCR ligation [1,2]. Accordingly, iNKT cells are thought to be potent immune-regulatory cells and their activation by ligands has been shown to be a powerful means to modulate various immune responses [1,2].

In addition to the regulation by specific ligands through TCR, activation of iNKT cells is critically regulated by costimulatory signals provided by antigen-presenting cells (APCs). We and others have previously demonstrated that a costimulatory signal mediated through the CD80/86–CD28 interaction is required for optimal activation of iNKT cell functions in response to α-GalCer in vitro and in vivo [3,4]. Moreover, we have also demonstrated that the CD154–CD40 interaction is critically involved in IFN-γ production and induction of Th1-type responses by α-GalCer-activated

☆ *Abbreviations:* iNKT, invariant NKT; TCR, T cell receptor; α-GalCer, α-galactosylceramide; APCs, antigen-presenting cells; B7RP-1, B7-related protein-1.

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iNKT cells [3,5]. Recently, ICOS, a new member of the CD28 family of costimulatory receptors, was identified to be inducibly expressed on conventional T cell upon activation [6]. It has been reported that the interaction of ICOS with its ligand, B7-related protein-1 (B7RP-1), on APCs is important for activation, proliferation, and cytokine production of Ag-primed conventional T cells, and preferentially leads to the generation of Th2 cells and controls Th1 responses [6–11]. Here, we demonstrated a critical contribution of ICOS to α-GalCer-induced iNKT cell activation and functions.

Materials and methods

Mice. Wild-type (WT) C57BL/6 (B6) mice were obtained from Charles River Japan (Yokohama, Japan). CD28-deficient ($CD28^{-/-}$) or ICOS-deficient ($ICOS^{-/-}$) B6 mice were kindly provided by Dr. Ryo Abe, Research Institute of Biological Science, Science University of Tokyo [12]. All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University.

Reagents. A synthetic form of α-GalCer was obtained from Kirin Brewery (Gunma, Japan). PE-conjugated tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared as described [13]. Anti-mouse CD80 mAb (RM80, rat IgG2a), anti-CD86 mAb (PO.3, rat IgG2b), and anti-B7RP-1 mAb (HK5.3, rat IgG2a) were prepared as described previously [14,15]. Control rat IgG was purchased from Sigma (St. Louis, MO).

Flow cytometric analysis. Mononuclear cells (MNC) were prepared from spleen and liver as described [3]. After pre-incubation with anti-mouse CD16/32 (2.4G2) mAb to avoid non-specific binding of mAbs to FcγR, surface molecules expressed on iNKT cells were analyzed on electronically gated α-GalCer/CD1d tetramer^{+} cells by four-color flow cytometry using a FACSCaliber (BD Bioscience, San Jose, CA). Surface molecules were stained with FITC-conjugated anti-NK1.1 mAb (PK136), PE-Cy5-conjugated anti-mouse ICOS mAb (7E.17G9), APC-conjugated anti-mouse CD28 mAb (37.51), or FITC-, PE-Cy5-, or APC-conjugated isotype-matched control mAbs. All these reagents were purchased from eBioscience (San Diego, CA).

Cell preparation and in vitro stimulation. Freshly isolated splenic MNC (5×10^5) from naïve or α-GalCer-primed mice were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 25 mM $NaHCO_3$ in humidified 5% CO_2 at 37 °C in 96-well U-bottomed plates (Coster, Cambridge, MA) as previously described [3]. Cells were stimulated with 100 ng/ml α-GalCer or vehicle (0.1% DMSO) in the presence (10 μg/ml) or absence of control rat Ig, anti-CD80 mAb, anti-CD86 mAb, and/or anti-B7RP-1 mAb. After 48 h, the cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by ELISA.

Coculture of iNKT cells and DC. Freshly isolated hepatic MNC were stained with PE-conjugated α-GalCer/CD1d tetramer, and the positive cells were enriched by autoMACS using anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The enriched iNKT cells were then sorted on a FACS Vantage (BD Bioscience, San Jose, CA) to obtain highly purified (98–99%) iNKT cells. Splenic dendritic cells (DC) were prepared according to the reported method [16]. Purified iNKT cells (1×10^5) and DC (5×10^4) were cocultured as previously described [4,5] with 100 ng/ml α-GalCer or vehicle (0.1% DMSO) in the presence or absence of 10 μg/ml control rat Ig, anti-CD80 mAb, anti-CD86 mAb, and/or anti-B7RP-1 mAb. After 48 h, the cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by ELISA.

In vivo treatment with α-GalCer and mAb. In most experiments, mice were i.p. injected with 2 μg α-GalCer in 200 μl PBS containing 0.1% DMSO. 0.1% DMSO in PBS was used as the vehicle control. Some mice were primed by i.p. injection of α-GalCer 3 days before. Some mice were i.p. administered with 300 μg control Ig, anti-CD80 mAb, anti-CD86 mAb, and/or anti-B7RP-1 mAb 1.5 days before the last α-GalCer injection. Sera were periodically obtained at 0–24 h after the last α-GalCer injection. IFN-γ and IL-4 levels in the sera were determined by ELISA. Hepatic and splenic MNC at 24 h were subjected to cytotoxic activity.

ELISA. IFN-γ and IL-4 levels in the culture supernatants or the sera were determined by using mouse IFN-γ or IL-4-specific ELISA kits (Ready-SET-Go!, eBioscience) according to the manufacturer's instructions.

Cytotoxicity assay. Cytotoxic activity of hepatic and splenic MNC was tested against NK-sensitive YAC-1 cells and NK-resistant P815 target cells by a standard 4-h ^{51}Cr release assay as previously described [3].

Experimental lung metastases. Log-phase cultures of B16 melanoma cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free RPMI 1640, and re-suspended to an appropriate concentration in PBS. Syngeneic B6 mice were i.p. injected with 2 μg α-GalCer or vehicle (0.1% DMSO) on day −1 and then i.v. inoculated with B16 cells (5×10^4) on day 0. Some mice were primed with α-GalCer on day −4. Some mice were i.p. administered with 300 μg control rat Ig, anti-CD80 mAb, anti-CD86 mAb, and/or anti-B7RP-1 mAb 1.5 days before the last α-GalCer injection. On day 14, the number of tumor colonies in the lungs was counted under a dissecting microscope.

Statistical analysis. Data were analyzed by a two-tailed Student's *t* test. *P* values less than 0.05 were considered significant.

Results

ICOS expression on naïve and primed iNKT cells

Constitutive ICOS expression on iNKT cells was demonstrated by flow cytometric analysis of freshly isolated hapatic MNC (Fig. 1A) and splenic MNC (data not shown) from WT B6 mice. Although NK cells but not T cells constitutively expressed ICOS as reported [17], ICOS expression was more remarkable on iNKT cells compared to that on NK cells. ICOS expression was independent of CD28 since a similar level of ICOS expression was observed on iNKT cells isolated from $CD28^{-/-}$ mice, and CD28 expression was also independent of ICOS expression since a similar level of CD28 expression was observed on iNKT cells isolated from $ICOS^{-/-}$ mice (Fig. 1B). As ICOS was induced on conventional T cells by TCR ligation [6], ICOS expression on iNKT cells was up-regulated 2–3 days after α-GalCer treatment and then returned to the basal level by 5–7 days (Fig. 1C). Similar results were obtained with spleen MNC in vivo and after in vitro stimulation of liver MNC with α-GalCer (data not shown).

Involvement of ICOS costimulatory pathway in IFN-γ and IL-4 production by α-GalCer-stimulated iNKT cells

We next investigated whether iNKT cells require IOCS-mediated costimulation for producing cytokines