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Cutting Edge: CD28 Engagement Releases Antigen-Activated Invariant NKT Cells from the Inhibitory Effects of PD-1

Jianxiong Wang,1* Lu Cheng,1* Zenebech Wondimu,‡ Mark Swain,‡ Pere Santamaria,† and Yang Yang3*

Costimulatory and coinhibitory signals are important for the maintenance of immune homeostasis both in the steady state and during immune responses. In this study, we explore the relative contributions of these signals to the rapid production of large amounts of cytokines by activated invariant NKT cells (iNKT cells). We find that upon antigenic stimulation, iNKT cells rapidly up-regulate programmed death (PD)-1 and induce high levels of PD ligand 1 and costimulatory molecules on the surface of cognate Ag-presenting dendritic cells and that iNKT cells require a CD28 signal to secrete cytokines in the presence of a PD-1/PD ligand 1 interaction. CD28-deficient iNKT cells synthesized but failed to secrete cytokines during activation, and blockade of the PD-1 pathway restored the ability of CD28-deficient iNKT cells to secrete cytokines. The opposing functions of CD28 and PD-1 thus tightly regulate the unique effector function of iNKT cells. The Journal of Immunology, 2009, 182: 6644–6647.

Invariant NKT cells (iNKT cells)4 produce large amounts of cytokines in response to a lipid Ag/CD1d complex (1–3), contributing to mobilization of both innate and adaptive immunity against pathogens and to immune regulation (4–7). Cytokine production by iNKT cells is CD28 dependent (8). However, whereas conventional T cells primarily require CD28 to initiate cytokine gene transcription, iNKT cells constitutively express cytokine mRNAs (9, 10) and can synthesize cytokine proteins in response to TCR ligation in the absence of CD28 signaling (11). These observations suggest that CD28 may play distinct roles in the activation of T vs iNKT cells (i.e., transcriptional vs posttranslational regulation).

Alternatively, CD28 signaling may modulate the functional capacity of iNKT cells during thymic development. Whereas conventional T cells exit the thymus with transcriptionally silent cytokine gene loci, iNKT cells transcribe cytokine genes early on in development (10, 12). Evidence also shows that the cytokine production competency of iNKT cells is established during thymic development (13). CD28 signaling appears to contribute to positive selection/maturation of thymic iNKT cells and other innate-like T cells (14, 15), but it remains unclear whether the lack of CD28 signaling during thymic development fosters the generation of cytokine production-incompetent iNKT cells.

Engagement of CD28 on T cells by B7 on professional APCs induces signaling events that synergize with those emanating from the TCR. Other members of this family of costimulatory receptor/ligand pairs, including programmed death (PD)-1 and its ligand PD ligand (PD-L) 1, transduce inhibitory signals (16). It was found recently that activated iNKT cells expressed up-regulated PD-1 that played an important role in the induction of anergy of iNKT cells (17). However, it remains unknown whether the PD-1 signal regulates the function of activated iNKT cells.

In this study, we investigate the relative contributions of PD-1/PD-L1 and CD28/B7 interactions to the activation of iNKT cells. We show that CD28-deficient iNKT cells are cytokine production incompetent. This is not due to an intrinsic or developmental defect of iNKT cells, although CD28 signaling is required for the expansion of immature thymic iNKT cells. iNKT cells rapidly up-regulate PD-1 upon activation and induce PD-L1 expression on dendritic cells (DCs), suppressing cytokine secretion. In agreement with this, a PD-1/PD-L1 blockade restored cytokine secretion by CD28-deficient iNKT cells. These results indicate that CD28 signaling serves to release iNKT cells from the inhibitory effects of the PD-1/PD-L1 interaction.

Materials and Methods

Mice

Wild-type and CD28, CD80, CD86, CD80/CD86, or TAP1 gene-deficient B6 mice were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility at the University of Calgary (Calgary, Canada) according to the Institutional Animal Care Committee guidelines.

1 Department of Biochemistry and Molecular Biology, 2 Department of Microbiology and Infectious Diseases, and 3 Department of Medicine, Julia McFarlane Diabetes Research Centre, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

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*Address correspondence and reprint requests to Dr. Yang Yang, Department of Biochemistry and Molecular Biology, Julia McFarlane Diabetes Research Centre, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta T2N 4N1, Canada. E-mail address: yyang@ucalgary.ca

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However, whereas sera from were almost identical in WT and gene-deficient mice (Fig. 1b). In all of these mice, NKT cells in CD28−/− mice 3 h after αGalCer treatment. Data correspond to mean ± S.D. of 3-5 mice per group (*, p < 0.01 vs WT mice). d, Serum IFN-γ in WT, CD28−/−, and CD80/CD86−/− mice 3 and 16 h after αGalCer injection. Data correspond to mean ± S.D. of 3-4 mice per group at each time point. e, Expression of CD40 and CD80 on the surface of CD11c+ splenic DCs from control and αGalCer-injected mice (4 h after treatment). Profiles shown are representative of three independent experiments.

Reagents

α-Galactosylceramide (αGalCer) was provided by Kirin Brewery. CD1d tetramers were provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Abs for intracellular staining were from BD Pharmingen. Abs against PD-1 and PD-L1 were from eBioscience and BioExpress. DuoSet kits for ELISA were from R&D System. Abs for intracellular staining were from BD Pharmingen.

αGalCer treatment

Mice were injected i.p. with αGalCer (4 µg/dose) or PBS containing 0.5% polysorbate-20 (Sigma-Aldrich). Mice were bled and sacrificed to measure serum cytokines and harvest cells. To block PD-1, anti-PD-1 mAb (J43; 800 µg/mouse) was injected 1 h before αGalCer injection. To block PD-1 on DCs, CD11c+ splenic DCs purified with Ab-conjugated microbeads (Miltenyi Biotec) were incubated with αGalCer (100 ng/ml) for 4 h at room temperature, followed by incubation with anti-PD-1 mAb (9G2; 40 µg/ml) for 20 min. To activate NKT cells in vitro, we incubated Thy1.2+ or total splenocytes (5 × 10⁶/ml) with αGalCer-pulsed DCs (2 × 10⁶/ml) for 2 days.

Intracellular staining

Thy1.2+ splenocytes were cultured in RPMI 1640 culture medium supplemented with 10% FCS and containing 2.0 µM monesin (GolgiStop; BD Pharmingen) at 37°C for 2 h. Cells were then permeabilized, fixed, and stained with anti-IFN-γ-PE or anti-IL-4-PE Abs. Cells were further stained with a CD1d tetramer and an anti-TCR Ab and analyzed by FACS. Data were analyzed using FlowJo (Tree Star).

Statistics

All statistical analyses were performed with GraphPad Prism 5 software. The results of in vivo studies and intracellular staining studies were evaluated with a Mann-Whitney U test. In all cases, we considered p < 0.05 as statistically significant.

Results and Discussion

Activated NKT cells synthesize but fail to release cytokines in the absence of CD28 signaling

To define the role of CD28 in NKT cell function, we injected αGalCer into wild-type (WT) and CD28−/− or CD80/CD86−/− B6 mice and compared the frequencies of splenic cytokine-producing NKT cells and serum cytokine levels 3 h later. In all of these mice, NKT cells were activated to produce both IFN-γ and IL-4 (Fig. 1a). The frequencies of IL-4-producing NKT cells in CD28−/− and CD80/CD86−/− mice were low, but the frequencies of IFN-γ-producing NKT cells were almost identical in WT and gene-deficient mice (Fig. 1b). However, whereas sera from αGalCer-treated WT B6 mice contained high levels of both IFN-γ and IL-4, sera from CD28−/− and CD80/CD86−/− mice did not (Fig. 1c). In WT mice, serum IFN-γ levels peaked ~16 h after αGalCer injection. By this point, serum IFN-γ was hardly detectable in CD28−/− and CD80/CD86−/− mice (Fig. 1d).

We further investigated the effect of CD28 deficiency on NKT cell-induced maturation of DCs (5). Splenic DCs were isolated 4 h after αGalCer injection for phenotypic and functional analyses. αGalCer stimulation increased the expression of CD40 and CD80 on DCs in both types of mice (Fig. 1e). Furthermore, these DCs were able to elicit Ag-specific T cell responses when used as APCs (data not shown). Therefore, the CD28 deficiency does not impair the ability of activated NKT cells to mobilize DCs in vivo. We also obtained similar results in CD28-deficient nonobese diabetic mice (data not shown).

Impaired cytokine secretion in CD28−/− and CD80/CD86−/− mice cannot be accounted for by differences in absolute numbers of peripheral NKT cells

The reduced serum cytokine levels in αGalCer-treated, CD28−/− deficient mice might be the result of impaired development of NKT cells (14, 15). Therefore, we determined the percentages and absolute numbers of NKT cells in the thymus and spleen of WT and CD28−/− or CD80/CD86−/− mice. The percentages of thymic and splenic NKT cells, as well as the absolute numbers of thymic NKT cells, were significantly lower in CD28−/− and CD80/CD86−/− mice vs WT mice. Surprisingly, the absolute numbers of splenic NKT cells in WT and CD28−/− and CD80/CD86−/− mice were similar (Fig. 2a and data not shown). Further analyses revealed that the absolute numbers of splenic NKT cells in young CD28−/− and CD80/CD86−/− mice were much lower and reached the levels seen in WT mice only with age (Fig. 2b and c). These observations are compatible with homeostatic expansion of NKT cells in the periphery, an event that might be associated with the absence of FoxP3+ regulatory T cells in these mice. These results indicate that the absence of NKT cell-derived cytokines in sera from the latter cannot be accounted for by differences in NKT cell numbers.

The development of innate-like T cells, including NKT cells, requires specific signals mediated by Slamf receptor/SAP adaptor, as well as CD28 (14, 15, 18, 19). However, unlike in Slam/SAP deficient mice in which thymic NKT cells and innate-like CD8+ T cells were arrested at an immature CD24+ stage (18, 19), the majority of thymic NKT cells in adult CD28 mice expressed a CD24lowCD44high NK1.1high mature
phenotype similar to that seen in WT thymic iNKT cells (Fig. 2d). Taken together, these observations suggested that CD28 deficiency resulted in an impaired expansion but not maturation of thymic iNKT cells.

Cytokine secretion by activated iNKT cells is CD28 dependent

To ascertain whether the impaired cytokine secretion capacity of CD28−/− iNKT cells was caused by a cell-intrinsic developmental defect akin to that seen in Csf-2-deficient mice (13), we isolated splenocytes from WT and CD28−/− mice 3 h after αGalCer injection and incubated cells with 1 μM ionomycin for 8 h. As expected, splenocytes from vehicle-treated control mice produced no IFN-γ with/without ionomycin stimulation. Splenocytes from αGalCer-treated WT mice produced substantial amounts of IFN-γ before ionomycin challenge, and the levels of IFN-γ increased further with ionomycin stimulation. In contrast, splenocytes from αGalCer-treated CD28−/− mice did not produce IFN-γ in the absence of ionomycin but did so sufficiently in the presence of ionomycin (Fig. 3a), indicating that CD28-deficient iNKT cells are competent to secrete IFN-γ.

We then blocked CD28 signaling in WT iNKT cells by injecting a Vav1 inhibitor, 6-thio-GTP, into WT B6 mice for two consecutive days before αGalCer injection to disrupt CD28-dependent Vav1-Rac signaling cascades (20). The inhibitor completely abolished the ability of WT iNKT cells to secrete IFN-γ into serum despite the fact that most of the iNKT cells in these mice could produce IFN-γ in response to αGalCer (Fig. 3b). We then cultured enriched splenic iNKT cells from WT mice with αGalCer-pulsed CD11c+ DCs (αGalCer/DCs) isolated from either WT mice or CD80/CD86−/− mice. Splenic iNKT cells produced both IL-4 and IFN-γ in response to WT αGalCer/DCs, but little IL-4 and no IFN-γ in response to CD80/CD86−/− αGalCer/DCs. Importantly, addition of an anti-CD28 mAb into the cultures restored the ability of iNKT cells to secrete IL-4 and IFN-γ in response to CD80/CD86−/− αGalCer/DCs (Fig. 3c). The different levels of restored IL-4 and IFN-γ production, however, may reflect differentiated thresholds of individual cytokines for the costimulatory signal. Taken together, these results demonstrate that CD28 signaling controls the secretion of cytokines from intracellular stores in activated iNKT cells. This previously unrecognized role for CD28 signaling appears to be a peculiarity of iNKT cells, because CD28 signaling in conventional T cells controls events that lie upstream of cytokine secretion.

It has been shown that CD28 engagement can, at least partially, abrogate the inhibitory effect of certain NK receptors and enable iNKT cells to respond to endogenous lipid Ags (21). To investigate the contribution of NK receptors to cytokine secretion by iNKT cells, we injected αGalCer-pulsed DCs from WT or TAP1−/− mice into WT or CD80/CD86−/− mice and measured serum IFN-γ 16 h after. Whereas WT DCs elicited high levels of serum IFN-γ in WT hosts, CD80/CD86−/− αGalCer/DCs could not do so when injected into CD80/CD86−/− hosts, as expected. Injection of WT αGalCer/DCs into CD80/CD86−/− mice resulted in the secretion of IFN-γ into the serum. However, TAP1−/− αGalCer/DCs that did not express MHC class I on their surface did not result in higher levels of serum IFN-γ (Fig. 3d). Thus, MHC class I binding NK receptors...
Based on our results, that the engagement of up-regulated PD-1 on αNKT cells by PD-L1 on DCs suppresses the release of cytokine depot from activated αNKT cells. However, coengagement of CD28 on activated αNKT cells by up-regulated CD80 and CD86 on DCs (5) would serve to release αNKT cells from inhibitory PD-1 signals, enabling αNKT cells to respond rapidly when appropriately activated. Our results indicate that the opposing effects of PD-1 and CD28 play a fundamental role in the regulation of effector function of αNKT cells.

**Disclosures**

The authors have no financial conflict of interest.

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