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

Jianxiong Wang, Lu Cheng, Zenebech Wondimu, Mark Swain, Pere Santamaria and Yang Yang

*J Immunol* 2009; 182:6644-6647; doi: 10.4049/jimmunol.0804050
http://www.jimmunol.org/content/182/11/6644

References

This article cites 21 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/182/11/6644.full#ref-list-1

Why *The JI*? Submit online.
- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: CD28 Engagement Releases Antigen-Activated Invariant NKT Cells from the Inhibitory Effects of PD-1

Jianxiang Wang,2* Lu Cheng,2* 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 effect function 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.

*Department of Biochemistry and Molecular Biology, †Department of Microbiology and Infectious Diseases, and ‡Department of Medicine, Julia McFarlane Diabetes Research Centre, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

Received for publication December 8, 2008. Accepted for publication April 2, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by grants from the Canadian Diabetes Association (to Y.Y.) and the Canadian Institutes of Health Research (to Y.Y. and P.S.). The Julia McFarlane Diabetes Research Centre is supported by the Diabetes Association (FootBall). P.S. is a Scientist of the Alberta Heritage Foundation for Medical Research and a Juvenile Diabetes Research Foundation Scholar.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0804050
were almost identical in WT and gene-deficient mice (Fig. 1). A, Representative profiles of cytokine-producing CD1d tetramer^+ iNKT cells 3 h after αGalCer (αGC) treatment. B, Frequencies of IFN-γ- and IL-4-producing splenic iNKT cells in WT, CD28^-/-, and CD80/CD86^-/- mice. C, Serum IFN-γ and IL-4 levels in WT, CD28^-/-, and CD80/CD86^-/- 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 CD80 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 Biocision and BioExpress. DuoSet kits for ELISA were from R&D System. Biotec) were incubated with αGalCer (100 ng/ml) for 4 h at room temperature, followed by incubation with anti-PD-L1 mAb (9G2; 40 μg/ml) for 20 min. To activate iNKT cells in vitro, we incubated Thy1.2^+ or total splenocytes (5 × 10^6/ml) with αGalCer-pulsed DCs (2 × 10^5/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 monensin (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 FoxP3 stain kit (eBioscience) 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 iNKT cells synthesize but fail to release cytokines in the absence of CD28 signaling
To define the role of CD28 in iNKT cell function, we injected αGalCer into wild-type (WT) and CD28^-/- or CD80/CD86^-/- B6 mice and compared the frequencies of splenocyte cytokine-producing iNKT cells and serum cytokine levels 3 h later. In all of these mice, iNKT cells were activated to produce both IFN-γ and IL-4 (Fig. 1a). The frequencies of IL-4-producing iNKT cells in CD28^-/- and CD80/CD86^-/- mice were low, but the frequencies of IFN-γ-producing iNKT 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 iNKT 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 iNKT 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 iNKT cells
The reduced serum cytokine levels in αGalCer-treated, CD28-deficient mice might be the result of impaired development of iNKT cells (14, 15). Therefore, we determined the percentages and absolute numbers of iNKT cells in the thymus and spleen of WT and CD28^-/- or CD80/CD86^-/- mice. The percentages of thymic and splenic iNKT cells, as well as the absolute numbers of thymic iNKT cells, were significantly lower in CD28^-/- and CD80/CD86^-/- mice vs WT mice. Surprisingly, the absolute numbers of splenic iNKT 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 iNKT 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 iNKT 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 iNKT cell-derived cytokines in sera from the latter cannot be accounted for by differences in iNKT cell numbers.

The development of innate-like T cells, including iNKT 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 iNKT cells and innate-like CD8^+ T cells were arrested at an immature CD24^+ stage (18, 19), the majority of thymic iNKT cells in adult CD28 mice expressed a CD24low/CD44high 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 efficiently 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 stimulatory 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−/− or TAP1−/− mice. The recipients were WT or CD80/CD86−/− mice. Data correspond to the mean ± SD of two or three mice per group.

FIGURE 3. Cytokine secretion by activated iNKT cells from WT, CD28−/−, and CD80/CD86−/− mice. a, IFN-γ secretion by iNKT cells. Splenocytes were isolated from WT (lanes 1 and 3) and CD28−/− (lanes 2 and 4) mice 3 h after treatment with vehicle or αGalCer (αGC) and cultured for an additional 8 h in the presence or absence of ionomycin (1 μM). Data correspond to the mean ± SD of three independent experiments. b, Serum IFN-γ (left panel) and frequencies of IFN-γ-producing splenic iNKT cells in WT mice treated with αGalCer 4 h earlier. Some mice received 6-thio-GTP i.p. for two consecutive days (0.5 mg/kg daily) before αGalCer injection. Data correspond to mean ± S.D. of four mice per group in two separate experiments. c, Secretion of IFN-γ and IL-4 by splenocytes from WT mice. Splenocytes were stimulated for 48 h with αGalCer-pulsed (100 ng/ml) DCs (2 × 105/ml) from WT or CD80/CD86−/− mice in the presence or absence of anti-CD28 mAb (2 μg/ml). Representative data correspond to the mean ± SD of two independent experiments. d, Serum IFN-γ 16 h after injection of αGalCer-bearing splenic DCs (0.5 × 106 DCs per mouse) from WT and CD80/CD86−/− or TAP1−/− mice. The recipients were WT or CD80/CD86−/− mice. Data correspond to the mean ± SD of two or three mice per group.
PD-1 blockade restores the cytokine secretion of CD28−/− iNKT cells

We found that the activated iNKT cells rapidly up-regulated PD-1 and that DCs up-regulated the levels of PD-L1 upon αGalCer treatment (Fig. 4a). To investigate the contribution of PD-1 signaling on cytokine secretion by αGalCer-activated iNKT cells, we inhibited PD-1 signaling by injecting a blocking anti-PD-1 mAb into CD28−/− mice 1 h before αGalCer injection. Significant amounts of IFN-γ were detected in the serum of these mice, and IFN-γ levels peaked at ~16 h (Fig. 4b). IFN-γ was not detected in the serum of control Ig-treated counterparts. Furthermore, spleenocytes from these anti-PD-1 mAb-treated CD28−/− mice secreted large amounts of IFN-γ into the culture supernatant (Fig. 4c), spleenocytes from control Ig-treated mice did not, even in the presence of αGalCer (Fig. 4c).

We next cultured spleenocytes from untreated WT and CD28−/− mice with αGalCer-pulsed WT DCs. WT, but not CD28-deficient spleenocytes, produced IFN-γ in response to αGalCer/DCs, and IFN-γ production by WT spleenocytes was increased when PD-L1 on DCs was blocked by an anti-PD-L1 mAb (Fig. 4d). CD28−/− spleenocytes also produced IFN-γ when stimulated with αGalCer/DCs in the presence of a PD-L1-blocking mAb (Fig. 4d). Similarly, defective IFN-γ production by liver iNKT cells from CD28−/− mice was also augmented by PD-L1 blockade of DCs (data not shown). These results indicated that engagement of PD-1 on iNKT cells by PD-L1 on DCs inhibits cytokine secretion by αGalCer-activated iNKT cells, and this inhibitory effect can be largely removed by a CD28 signal.

DCs are the primary APCs for iNKT cells, and αGalCer stabilizes iNKT/DC conjugates for hours (2, 3). We postulate, based on our results, that the engagement of up-regulated PD-1 on iNKT cells by PD-L1 on DCs suppresses the release of cytokine deops from activated iNKT cells. However, coengagement of CD28 on activated iNKT cells by up-regulated CD80 and CD86 on DCs (5) would serve to release iNKT cells from inhibitory PD-1 signals, enabling iNKT 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 iNKT cells.

Disclosures

The authors have no financial conflict of interest.

References