On/Off TLR Signaling Decides Proinflammatory or Tolerogenic Dendritic Cell Maturation upon CD1d-Mediated Interaction with Invariant NKT Cells

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On/Off TLR Signaling Decides Proinflammatory or Tolerogenic Dendritic Cell Maturation upon CD1d-Mediated Interaction with Invariant NKT Cells

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Invariant NKT (iNKT) cells play an effector/adjuvant function during antimicrobial and antitumoral immunity and a regulatory role to induce immune tolerance and prevent autoimmunity. iNKT cells that differentially modulate adaptive immunity do not bear a unique phenotype and/or specific cytokine secretion profile, thus opening questions on how a single T cell subset can exert opposite immunological tasks. In this study, we show that iNKT cells perform their dual roles through a single mechanism of action relying on the cognate interaction with myeloid dendritic cells (DCs) and leading to opposite effects depending on the presence of other maturation stimuli simultaneously acting on DCs. The contact of murine purified iNKT cells with immature autologous DCs directly triggers the tolerogenic maturation of DCs, rendering them able to induce regulatory T cell differentiation and prevent autoimmune diabetes in vivo. Conversely, the interaction of the same purified iNKT cells with DCs, in the presence of simultaneous TLR4 stimulation, significantly enhances proinflammatory DC maturation and IL-12 secretion. The different iNKT cell effects are mediated through distinct mechanisms and activation of different molecular pathways within the DC: CD1d signaling and activation of the ERK1/2 pathway for the tolerogenic action, and CD40–CD40L interaction and NF-κB activation for the adjuvant effect. Our data suggest that the DC decision to undergo proinflammatory or tolerogenic maturation results from the integration of different signals received at the time of iNKT cell contact and could have important therapeutic implications for exploiting iNKT cell adjuvant/regulatory properties in autoimmune diseases, infections, and cancer. The Journal of Immunology, 2010, 185: 7317–7329.

N atural killer T cells represent an important innate T cell subset that perceives signals of danger and cellular distress and modulates the adaptive immune response accordingly (1–3). In the presence of danger signals, NKT cells acquire an adjuvant function that is fundamental to boost antimicrobial and antitumor immunity (4). Conversely, in the absence of pathogens, NKT cells perform a negative regulatory role to restore peripheral T cell tolerance toward self-Ags and prevent autoimmune diseases (5–9). Both effects of NKT cells involve the cell–cell interaction and downstream modulation of dendritic cells (DCs) (10–15). Because of the pivotal role of DCs in shaping adaptive immunity, the crosstalk between NKT cells and DCs has been a field of extensive study, but the effect and mechanism of this interaction remain controversial. Some reports have shown that NKT cell activation induces rapid maturation of DCs followed by increased IL-12 secretion and enhancement of Th1 immune responses (16, 17). In contrast, in vivo studies on experimental models of autoimmune diseases demonstrated that NKT cell activation promotes differentiation of tolerogenic DCs and immune tolerance (14, 15). These controversial findings open questions as to how a single cell subset, the NKT cells, can play opposite effects on DC differentiation (2).

The existence of two classes of NKT cells, type I or invariant NKT cells and type II noninvariant NKT cells, originally raised the idea that the two different NKT cell subsets exert distinct immune functions. However, recent findings indicate that a specific adjuvant or regulatory function cannot be selectively ascribed to one of the two subsets. For example, CD1d-restricted, Vα14+ invariant NKT (iNKT) cells enhance T cell immunity against pathogens (18–20) and tumors (21–23), but they can also inhibit T cell response in autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) (5, 7) and type 1 diabetes (6, 9). An alternative hypothesis holds that iNKT cells with functionally distinct cytokine profiles exist and play opposite immune functions by favoring either proinflammatory or tolerogenic DC maturation (24). It has been proposed that the exclusive secretion of Th1-type cytokines, such as IL-12 and IFN-γ by iNKT cells, favors proinflammatory DC maturation during microbial infections and in anti-tumoral responses (17, 25, 26), while the predominant secretion of Th2-type cytokines promotes iNKT cell regulatory function (5, 27, 28) and the capacity to induce tolerogenic DCs (14, 15). In sharp contrast with that view, iNKT cells do not have a biased Th1 or Th2
cytokine phenotype like classical T helper cells do and, regardless of their role, they secrete a large and diverse array of cytokines of the Th1 and Th2 type. For example, iNKT cells that counterregulate autoimmunity and promote immune tolerance secrete Th2-type cytokines, such as IL-4, IL-5, IL-13, TGF-β, and IL-10, but also large amounts of the proinflammatory Th1 cytokine IFN-γ (6, 9, 29, 30). Hence, the mechanisms by which iNKT cells differentially modulate DCs to either enhance or suppress T cell immunity remains elusive.

In this study, we clarify how the same iNKT cell subset can play either regulatory or adjuvant function, and we highlight the events that regulate the fate of DCs upon iNKT cell contact. Specifically, we demonstrate that in steady-state conditions, that is, in the absence of pathogen-derived signals, cell–cell contact with purified iNKT cells directly triggers the tolerogenic maturation of DCs by engaging the CD1d receptor molecule and activating the ERK1/2 signaling pathway. Importantly, cytokine secretion by iNKT cells is completely dispensable for such modulatory effect. iNKT cell–modulated DCs show the classical features of tolerogenic DCs, such as intermediate expression levels of MHC class II and costimulatory molecules, a cytokine profile characterized by high secretion of IL-10, and minimal release of proinflammatory cytokines IL-12, IL-6, and TNF-α, as well as capacity to prime regulatory T (Treg) cells and prevent autoimmune diabetes in vivo. Strikingly, we found that the same iNKT cells that promote tolerogenic DC maturation play a completely opposite and strongly adjuvant effect when they contact DCs that are simultaneously stimulated through TLR4 and enhance their proinflammatory maturation and IL-12 secretion. In these conditions, iNKT cells interact with mature DCs through CD40-CD40L and transmit a signal that synergizes with the TLR4-induced NF-κB pathway.

The iNKT cell–DC interaction is central for modulation of adaptive immune responses and represents a critical crossroad between T cell immunity and immune tolerance. Our data indicate that the iNKT cell–DC communication does not produce a single adjuvant or regulatory immune effect but that it is integrated in a complex network of concomitant stimuli and intercellular interactions that orchestrate the final DC decision to undergo proinflammatory or tolerogenic maturation.

Materials and Methods

Mice

C57BL/6, SJL/J, and NOD mice were purchased from Charles River Laboratories (Calco, Italy). BDC2.5/NOD mice were a gift from Dr. Jonathan Katz (Cincinnati Children’s Research Foundation, University of Cincinnati, Cincinnati, OH), and CD1d<sup>−/−</sup> and CD40<sup>−/−</sup> C57BL/6 mice were a gift from Dr. Paolo Dellabona and Dr. Giulia Casorati (San Raffaele Scientific Institute, Milan, Italy). All mice were maintained under specific pathogen-free conditions in the animal facility at San Raffaele Scientific Institute, Milan, Italy. Bone marrow-derived immature DCs were incubated with 10<sup>5</sup> iNKT cells (DC/NKT cell ratio, 1:2), or left untreated (immature DCs). For cytokine profiling with microarrays, after 4 h of iNKT-DC coculture, the nonadherent cell fraction was removed and the adherent fraction containing DCs or iNKT cell-matured DCs (nktDCs) previously irradiated and loaded with GalCer; 100 ng/ml) in combination with TLR agonists was used for experiments after two rounds of antigenic stimulation with GalCer-loaded DCs. Expansion the iNKT cell population within the cell line was assessed by FACS analysis with PE-labeled GalCer/CD1d dimers and T cell surface markers before each experiment. For cytokine assays, aliquots of culture supernatants were removed after 72 h and assayed for the presence of IL-4, IL-10, IL-17, and IFN-γ. For iNKT cell purification, 6 d after the last Ag stimulation of the iNKT cell line, the cell suspensions were labeled with GalCer/CD1d complex (containing a portion of murine IgG1) followed by PE- or biotin-conjugated anti-mouse IgG1 mAbs and microbead-conjugated anti-PE or anti-biotin mAbs and sorted through magnetic columns (Miltenyi Biotec).
antigenic stimulation, the BDC2.5 T cells were analyzed for their pro-liferative and suppressive capacity. Briefly, the BDC2.5 T cells were Ag-
stimulated with BDC2.5 peptide-loaded LPS-DCs and their proliferation
was measured by means of [H]thymidine incorporation in the last 18 h of
a 3-d period. To assess the capacity of nktDC-induced Treg cells to sup-
press proliferation of naive effector T cells, BDC2.5 CD4+ T cells were
freshly isolated from BDC2.5 mice, labeled with 5 μM CFSE following the
manufacturer’s protocol, stimulated with BDC2.5 peptide-loaded LPS-
DCs in the presence of increasing numbers of nktDC-induced Treg cells,
and their proliferation was evaluated by means of CSFE dilutions. For
cytokine secretion profiling by intracellular staining, after 4 d of antigenic
and their proliferation was evaluated by means of CSFE dilutions. For
Flow cytometric analysis
Cells were stained with fluorochrome-conjugated mAbs, washed, and an-
alyzed on a FACSCalibur (BD Biosciences). For intracellular cytokine
staining of BDC2.5 T cells, cells were rested for 7 d after antigenic stim-
ulation, stained with Leukocyte Activation Cocktail for 4–6 h, and then
subjected to intracellular staining with BD Cytofix/Cytoperm kit (BD
Biosciences) following the manufacturer’s protocol. For intracellular
 Czech. For intracellular cytokine
flow cytometry, cells were fixed with 4% paraformaldehyde (PFA), permeabilized
with ice-cold 95% methanol, and subsequently incubated with Alexa 647-
conjugated anti–p-ERK1/2 mAb and FACS analyzed.
Endocytosis
DCs were incubated in complete RPMI 1640 plus 5% FBS containing FITC-
dextran or Lucifer Yellow. After 60 min of incubation, cells were collected,
washed extensively in FACS buffer (PBS plus 1% BSA and 0.01% sodium
azide), stained with allophycocyanin-conjugated anti-CD11c mAb, and
FACS analyzed.
Cytokine determination
IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17, IFN-γ, and TNF-α in cell culture
supernatants were quantitated by BD Cytometric Bead Array (CBA from
BD Biosciences) and FACS analysis following the manufacturer’s pro-
tocol. Data were analyzed with FCAP Array software v1.0.1 (Soft Flow, St.
Louis, MN).
In vivo induction of regulatory BDC2.5 CD4+ T cells and
immunity tolerance
For in vivo tolerance induction, 6-wk-old SJL/J mice were injected i.v. with
10^6 LPS-DCs or nktDCs and pulsed in vitro with the PLP peptide or with PBS
alone. Two days later mice were immunized s.c. with 100 μg PLP peptide in
CFA. Twelve days later, 10^7 cells from pooled draining lymph nodes were
incubated with increasing concentrations of peptide in a 96-well plate.
Proliferation of T cells was measured by incorporation of [H]thymidine
during the last 18 h of a 96-h incubation. For in vivo induction of regulatory
CD4+ T cells, 2.5 × 10^6 naive BDC2.5 CD4+ T cells isolated from spleno-
cytes of TCR transgenic BDC2.5 NOD mice (with anti-Vβ4 mAb and
magnetic bead separation) were injected i.v. into the lateral tail vein of 4-wk-
old NOD mice. Twenty-four hours later, 10^6 DCs, pulsed with the BDC2.5
peptide 1040–1051, were injected into the same mice; as a control a group,
BDC2.5 mice were injected with PBS alone. Seven days after the DC in-
jection, peripheral lymph nodes (PLNs) were collected and previously
injected BDC2.5 CD4+ T cells were isolated with microbead-conjugated
anti-Vβ4 mAb and magnetic separation and stimulated in vitro with BDC2.5
peptide-loaded or unloaded LPS-DCs (DC/T cell ratio, 1:2.5).
 Supernatants were collected after 72 h and different cytokine concentration
was determined as described above. Cell proliferation was measured by means of
H3thymidine incorporation during the last 18 h of a 72-h period.
Diabetes protection
Three groups of 4-wk-old female NOD mice received one i.v. injection of
LPS-DCs, nktDCs (10^6 cells/mouse), or PBS vehicle alone. Diabetes was
monitored by testing blood glucose levels with an Accu-Check glucometer
(Roche Diagnostics, Indianapolis, IN). The animals were considered di-
abetic after two consecutive blood glucose measurements >250 mg/dl.
Western blotting and immunoprecipitation
CD11c+ DCs were homogenized for 30 min in ice-cold RIPA buffer.
Total cell lysates (30–60 μg of total proteins) were boiled in Laemmli
buffer and subjected to 12% SDS-PAGE before being immunoblotted with
phospho-specific mAbs. For immunoprecipitation, total cell lysate was
blocked with normal rat IgG protein G-agarose and subsequently subject-
ted to immunoprecipitation with anti-murine CD1d or isotype-matched
Ab at 4°C for 18 h. Immunoprecipitates were collected, washed extensively in
PBS, boiled in Laemmli buffer, and subjected to 15% SDS-PAGE before
being immunoblotted with anti-FcRγ mAb.
Real-time RT-PCR
Total RNA was extracted with TRIzol reagent from purified CD11c+ DCs
(purified by magnetic separation, purity >98%). RNA was reverse-
transcribed by using a SuperScript III kit (Invitrogen) according to the
manufacturer’s instructions. Quantitative real-time RT-PCR was carried
out with the DNA Engine Opticon 2 real-time detection system and SYBR
Green system. Data were normalized according to expression levels of β-
actin.
Immunofluorescence analysis
Cells were fixed with 4% PFA and permeabilized with 0.2% Triton X-100
followed by 30 min each with primary Ab and secondary Ab, labeled with
appropriate fluorochrome, before mounting in Fluoromount-G solution
(SouthernBiotech, Birmingham, AL). Confocal microscopy was performed
with an UltraView ERS microscope (PerkinElmer, Wellesley, MA) with a
63× oil immersion objective. Images were processed with Adobe Pho-
toshop CS.
Statistics
Statistical significance of differences in means ± SD of cytokine release
and proliferation by cells from different groups was calculated using the
unpaired Student t test. The log-rank test was used for the comparison of
diabetes incidence rates between the different groups; p values of <0.05
was considered statistically significant.
Results
iNKT cells induce tolerogenic DC maturation
In vivo activation of iNKT cells by administration of their Ag,
αGalCer, induces immune tolerance through DC modulation (13–
15). Although DCs of αGalCer-treated mice show tolerogenic features,
such as intermediate expression of maturation markers, capacity to prime Treg cells, and to transfer immune tolerance, it
remains unclear whether iNKT cells are directly responsible for
triggering tolerogenic DC maturation (14, 15). Although different
lymphocyte subsets, including NK cells, NKT cells, and γδ T cells,
interact with DCs and cause their proinflammatory maturation and
function (10, 30–33), so far there is no evidence that a specific cell
subset can directly elicit the tolerogenic maturation of DCs. To
assess whether iNKT cells promote maturation of tolerogenic DCs
(tolDCs), we performed coculture experiments with purified iNKT
cells and bone marrow-derived immature DCs (CD11c+CD11b+,
CD8a− myeloid DCs; Supplemental Fig. 1A). iNKT cell lines were
established in vitro as previously described through repeated anti-
genetic stimulations with αGalCer-pulsed mature DCs (34, 35), and
iNKT cells were purified prior to the addition to immature DCs
through staining with αGalCer-loaded CD1d dimers* and magnetic
sorting. The expansion and purification procedure resulted in a
highly pure iNKT cell population (>98%; Supplemental Fig. 1B)
that retained the phenotypical and functional features of ex vivo
iNKT cells, including a mixed and unbiased CD4+ and double-
negative (DN) phenotype (Supplemental Fig. 1C) and secretion of
a large and diverse array of cytokines upon TCR stimulation
(Supplemental Fig. 1D). As early as 4 h after the beginning of the
iNKT-DC coculture, a transcriptional analysis with genome-wide
microarrays performed on purified CD11c+ DCs revealed their
significant activation with >350 genes differentially regulated (Supplemental Fig. 2A). After 20 h of iNKT cell coculture, DCs showed all the morphological and functional features of mature DCs. These included the redistribution of MHC class II molecules from the lysosomal compartment to the cell surface (Fig. 1A, 1B), the upregulation of costimulatory molecules CD80, CD86, CD200, and PVR (Fig. 1B, Supplemental Fig. 2B), and the acquisition of Ag-presenting capacity (Supplemental Fig. 2C). Notably, the expression levels of costimulatory molecules were intermediate between those of immature DCs and LPS-DCs, a feature that is typical of myeloid DCs with tolerogenic properties (36, 37). The most stringent tolerogenic feature of nktDCs was represented by their cytokine profile that was completely skewed toward a tolerogenic type with high secretion of IL-10 and minimal release of proinflammatory cytokines, such as IL-12, TNF-α, and IL-6 (Fig. 1C). To exclude that iNKT cells were exclusively responsible for the high IL-10 levels detected in the supernatants of the iNKT cell-DC cocultures, we performed a quantitative real-time PCR for IL-10 on CD11c+ DCs purified after iNKT cell modulation. Supplemental Fig. 2D shows that DCs strongly upregulated IL-10 gene expression upon iNKT cell-induced maturation. The tolerogenic maturation that we observed was integrally related to the regulatory capacity of iNKT cells. In fact, the addition of T cells other than iNKT cells (CD3+ αGalCer-CD1d dimer− T cells) to immature DCs under the same culture conditions did not trigger tolerogenic DC maturation (Supplemental Fig. 2E, 2F).

The primary function of steady-state tolerogenic DCs is to transport self-Ags from peripheral tissues to draining lymph nodes where they prime T cells in a tolerogenic fashion (38). Hence, to carry out their tolerogenic function, tolDCs must be able to reach peripheral tissues and upload soluble Ags similar to immature DCs, and, at the same time, they should acquire the capacity of mature DCs to migrate into lymph nodes. The homeostatic migration of tolDCs is regulated by expression of specific chemokine receptors, such as CCR5, drives tolDCs into peripheral tissues, and CCR7, which guides them through the afferent lymph into lymph nodes (39). To further support the hypothesis that iNKT cell-modulated DCs were tolerogenic, we evaluated their capacity to upload soluble Ags and to acquire the migratory properties of tolDCs. First, we compared the endocytic capacity of nktDCs with that of immature and LPS-matured DCs by measuring the uptake of two fluorescent compounds: FITC-dextran to assess mannose receptor-mediated endocytosis, and Lucifer Yellow for fluid phase endocytosis. DCs matured by iNKT cells exhibited a strong immunosuppressive capacity and prevented autoimmune diabetes

DCs matured through coculture with iNKT cells (nktDCs) shared many characteristics with tolerogenic mature DCs, such as intermediate expression of MHC class II and costimulatory molecules, ability to process and present Ags to naive T cells, and a predominant IL-10–secreting cytokine profile. However, the hallmark of tolDCs is their capacity to induce differentiation and/or expansion of Treg cells from naive T cell precursors and to induce immune tolerance in vivo (40). To demonstrate that nktDCs play such

**FIGURE 1.** iNKT cells induced tolerogenic DC maturation. **A,** After 20 h of coculture with iNKT cells, DCs exhibit MHC class II molecule redistribution from the lysosomal compartment toward the cell surface typical of mature DCs. Immature DCs (immDCs), nktDCs, and LPS-matured DCs were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, labeled with CD11c (blue), MHC class II (green), and LAMP2 (red) and analyzed by confocal microscopy. Original magnification ×40. **B,** nktDCs expressed intermediate levels of maturation markers. Different DC samples were analyzed by FACS for the expression of maturation markers as indicated. Histograms were gated on CD11c+ DCs. Dotted lines represent staining with isotype controls. C, nktDCs showed tolerogenic cytokine profile with high secretion of IL-10 and minimal release of IL-12, IL-6, and TNF-α. Supernatants were collected from the different DC types after 20 h of maturation with LPS or coculture with iNKT cells and analyzed for the presence of the indicated cytokines. Results are the mean ± SD from one triplicate experiment out of four. *p < 0.0001 for IL-10; †p < 0.005 for IL-12. D, nktDCs retained the phagocytic capacity of immature DCs. CD11c+ cells from iNKT cell-DC cocultures were assayed for phagocytosis of FITC-dextran or Lucifer Yellow by flow cytometry. Dotted lines represent cells incubated with medium alone. E, nktDCs acquired the chemokine receptor expression pattern of tolDCs. The different DC types were stained with CD11c and CCR5 or CCR7 mAbs and FACS analyzed. Dotted lines represent staining with isotype controls. All data are representative of three to four independent experiments. *p < 0.0001.
inhibited proliferation of BDC2.5 CD4+ effector T cells in a dose-dependent manner (Fig. 2B). Although those nktDC-induced regulatory T cells expressed several markers of Treg cells, such as GITR, FR-4, and OX-40, they did not belong to the classical FoxP3+CD25+ regulatory T cell subset and were FoxP3- and LAG3-negative (Fig. 2C). The analysis of the cytokine profile of nktDC-induced Treg cells revealed a predominant IL-10-secreting phenotype (Fig. 2D, 2E) similar to that of regulatory Tr1 cells (41). However, we found that the addition of anti–IL-10 mAb did not inhibit the suppressive capacity of nktDC-induced Treg cells (data not shown), thus suggesting that these Treg cells were not bona fide Tr1 cells (42).

Next, we determined whether nktDCs induce Treg cell differentiation and immune tolerance in vivo. We injected SJL/J mice
with PLP peptide-loaded nktDCs or LPS-DCs and then challenged them with the same peptide Ag in CFA 2 d after the DC injection. When we recalled the Ag-specific T cell response in vitro, we observed that the PLP peptide-specific T cells from mice immunized with nktDCs proliferated poorly compared with T cells from untreated control mice or mice immunized with LPS-DCs. To be sure that CD4+ T cell tolerance was related to injected nktDCs, we tested the tolerogenic properties of nktDCs in a different immune tolerance setting. TCR transgenic BDC2.5 CD4+ T cells (from BDC2.5 NOD mice) were transferred into nontransgenic NOD mice 24 h before the injection of untreated cells, LPS-DCs, or nktDCs previously pulsed with the BDC2.5 peptide Ag. Because naive CD4+ BDC2.5 T cells injected into NOD mice normally reach the PLNs (43), we isolated CD4+ BDC2.5 T cells from PLNs of untreated NOD mice or mice previously challenged with peptide-pulsed LPS-DCs or nktDCs and tested their Ag-specific response in vitro. We again observed that CD4+ T cells from mice immunized with nktDCs were anergic and proliferated significantly worse than did CD4+ T cells from mice immunized with LPS-DCs (Fig. 3B). Additionally, BDC2.5 CD4+ T cells from nktDC-treated mice showed a regulatory cytokine profile with a predominant secretion of IL-10 and minimal release of IFN-γ and IL-2 in sharp contrast with that of CD4+ T cells from LPS-DC–treated mice that mostly secreted IFN-γ and no IL-10 (Fig. 3C). We concluded that nktDCs were able to induce IL-10–secreting Treg cells in vivo. As an independent measure of immune tolerance, we asked whether injection of nktDCs could prevent autoimmune disease, specifically spontaneous autoimmune diabetes, in the NOD mice. As shown in Fig. 3D, administration of nktDCs into 4-wk-old NOD mice completely prevented the onset of clinical diabetes. While 70–80% of NOD mice left untreated or injected with LPS-matured DCs developed autoimmune diabetes by 32 wk of age, none of their NOD littermates treated with nktDCs showed clinical signs of diabetes. We concluded that the interaction with iNKT cells drives immature DCs through a tolerogenic maturation pathway enabling them to induce differentiation of IL-10–secreting regulatory T cells, restore immune tolerance toward self-islet Ags, and prevent autoimmune diabetes.

**FIGURE 3.** nktDCs induced immune tolerance in vivo and prevented autoimmune diabetes. A, Peripheral tolerance was induced in vivo by treatment with nktDCs. Mice were injected i.v. with PLP peptide-pulsed LPS-DCs, nktDCs (10^6 cells/mouse), or PBS and after 48 h challenged with PLP peptide in CFA. Two weeks later peptide-specific T cell response was recalled in vitro by adding increasing concentrations of PLP peptide to lymphocytes from draining lymph nodes. The proliferation was measured by means of [3H]thymidine incorporation during the last 18 h of a 3-d period. Results are means ± SD from one representative triplicate experiment. *p < 0.05; **p < 0.001. B and C, DCs induced differentiation of anergic Tr1-like cells in vivo. Mice were injected i.v. with PBS (Untreated), BDC2.5 peptide-loaded LPS-DCs, or nktDCs (10^6 cells/mouse) after adoptive transfer of naive BDC2.5 CD4+ T cells (2 × 10^5 cells/mouse). One week later, BDC2.5 CD4+ T cells were isolated from pancreatic lymph nodes and proliferation was measured by mean of [3H]thymidine incorporation (B) and cytokine secretion by CBA (C). Results are means ± SD of triplicates representing three independent experiments. *p < 0.001; **p < 0.0001. D, nktDCs protected NOD mice from autoimmune diabetes. Four-week-old NOD female mice received a single i.v. injection of PBS (n = 5), LPS-DCs (10^5 cells/mouse; n = 7), or nktDCs (10^6 cells/mouse; n = 7). Diabetes was diagnosed after two consecutive measurements of glycemia >250 mg/dl.

Simultaneous TLR signaling and iNKT cell modulation abolished tolerogenic maturation and enhanced proinflammatory features of myeloid DCs

Our data supported previous findings showing that iNKT cells induce tolerogenic DC differentiation (13–15). However, there is also clear indication that iNKT cells trigger proinflammatory DC maturation, that is, IL-12 secretion (10, 11). One possible explanation for such controversial findings is that distinct iNKT cell subsets with opposite phenotype and function exist. According to that view, it is possible that the iNKT cells that we used in our cocultured experiments carried a specific regulatory phenotype and/or function that enabled them to selectively promote tolerogenic DC maturation. An alternative possibility is that the differ-
ent iNKT cell actions depend on the activation/maturation state of DCs at the time of iNKT cell modulation. To distinguish between those two possibilities, we purified iNKT cells from a single iNKT cell line and cocultured them with immature DCs with and without LPS. As shown in Fig. 4, the presence of iNKT cells at the time of LPS-induced DC activation sustained TLR4-induced maturation and significantly enhanced the secretion of the proinflammatory cytokine IL-12 (Fig. 4B). We found that the enhancement of IL-12 secretion was completely blocked when iNKT cells were added to LPS-stimulated CD40 knockout DCs (Fig. 4C). These findings are in accordance with previous studies and reinforce the idea that the CD40–CD40L interaction is crucial for the adjuvant effect of iNKT cells on myeloid DCs.

**FIGURE 4.** iNKT cells synergized with LPS and enhanced TLR4-induced proinflammatory DC maturation. A, iNKT cells enhanced TLR4-induced expression of maturation markers on DCs. DCs were left immature (immDCs), stimulated with LPS alone (LPS-DCs), iNKT cells alone (nkDCs), or iNKT cells plus LPS (NKT + LPS-DCs), collected after 20 h, stained with FITC-conjugated anti-CD11c and PE-conjugated anti-MHC class II, CD80, CD86, and CD40 and analyzed by flow cytometry. Histograms were gated on CD11c+ DCs. B, iNKT cells increased IL-12p70 secretion on TLR4-stimulated DCs. DCs were treated as in A, and supernatants were analyzed for the presence of IL-12p70, TNF-α, and IL-6. Results are means ± SD from one triplicate experiment out of two. C, The absence of CD40 significantly impaired the synergistic effect of iNKT cells on TLR4-stimulated DCs. DCs from wild-type (wt DCs) or CD40 knockout mice (CD40 ko DCs) were treated as described in A, and the levels of IL-12p70 were assessed. Results are means ± SD from one triplicate experiment out of two. *p < 0.0001; **p < 0.01.

iNKT cell-driven tolerogenic maturation of DCs required cell–cell contact and the CD1d molecule

While the mechanism underlying the adjuvant effect of iNKT cells on DCs is well characterized and requires CD40–CD40L interaction (10, 44), the mechanism and molecular pathway involved in the iNKT cell-induced tolerogenic DC maturation are unknown. Previous studies postulated that the protolerogenic iNKT cell action on DCs is mediated through cytokine secretion (15). Alternatively, iNKT cells could promote tolerogenic DC maturation through a cell–cell contact mechanism similar to that played for their adjuvant function (10, 45). To assess whether tolerogenic DC maturation is triggered by cytokines or cell–cell contact, we performed iNKT cell-DC coculture experiments in Transwell plates. In those experiments, iNKT cells were placed in the upper chamber and physically separated from immature DCs residing in the lower chamber of the Transwell. Strikingly, the tolerogenic maturation of DCs, that is, upregulation of maturation markers MHC class II, CD80, and CD86 (Fig. 5A) and secretion of IL-10 (Fig. 5B), was completely inhibited when cell–cell contact between immature DCs and iNKT cells was prevented in the Transwell culture system. We exclude that the cell–cell contact was required for iNKT cell activation rather than DC maturation since iNKT cell were already activated at the time of being cocultured with DCs. Moreover, to furtherly exclude that possibility, we activated iNKT cells by adding αGalCer-pulsed LPS-matured DCs in the upper well and measured tolerogenic maturation of immature DCs placed in the lower well. Although iNKT cells were certainly activated in those experimental conditions, we did not observe tolerogenic maturation, that is, acquisition of an IL-10–secreting phenotype, in the immature DCs placed in the lower well (data not shown).

To further demonstrate that the cytokine release by iNKT cells was completely unnecessary for their protolerogenic action, we added anti–IFN-γ, anti–IL-4, and anti–IL-10 mAbs to the iNKT cell-DC cocultures and found that blocking iNKT cell cytokine secretion did not abolish the tolerogenic DC maturation, even when all three mAbs were added together (Fig. 5C, 5D).

Our next question regarded the molecules and receptors involved in the adjuvant and protolerogenic iNKT cell–DC communication. Our data on the adjuvant iNKT cell action on DCs confirmed previous reports showing that the CD40–CD40L interaction is crucial for the iNKT cell action on DCs. However, when we performed the iNKT cell-DC coculture experiment by using CD40 knockout DCs, we observed that the CD40–CD40L interaction was not required for the tolerogenic DC maturation (Fig. 5A, 5B). Also, the blocking of the OX40–OX40L interaction, a mechanism involved in the adjuvant modulation of iNKT cells on plasmacytoid DCs (45), had no effect on tolerogenic DC maturation (data not shown). On the contrary, the absence of the CD1d molecule on immature CD1d knockout DCs completely inhibited iNKT cell-induced tolerogenic maturation, that is, upregulation of MHC class II and costimulatory molecules (Fig. 5A) as well as IL-10 secretion (Fig. 5B). Because iNKT cells were Ag-stimulated with αGalCer-loaded mature DCs prior to the addition to immature DCs, we could exclude that the CD1d molecule was required for iNKT cell activation and concluded that it functioned as receptor molecule and transmitted the tolerogenic maturation signal within the DC. The crucial role of CD1d as receptor molecule to induce tolerogenic DC maturation was definitively demonstrated by the finding that cross-linking of CD1d molecules on the surface of immature DCs was sufficient to trigger their tolerogenic maturation, that is, IL-10 secretion (Fig. 5E). The occurrence of direct cell–cell contact between immature DCs and activated iNKT cells was confirmed by immunofluorescence analysis (Fig. 5F).
CD1d is an MHC class I-like molecule responsible for glycolipid Ag presentation to iNKT cells. The short cytosolic domain and the absence of a signaling motif in the CD1d structure is apparently inconsistent with its capacity to trigger the complex signaling pathway that regulates tolerogenic DC maturation upon iNKT cell contact. One possible explanation is that membrane-associated signaling components, such as ITAM-containing adapters, might provide CD1d molecule with the capacity to transmit intracellular signals within the cell. A Western blot analysis performed on total cell lysates of immature CD11c+ DCs demonstrated that, at the steady-state, DCs express all ITAM-bearing adapters proteins (data not shown) and the FeRγ coprecipitated with the CD1d molecule (Supplemental Fig. 4A). These data indicate that, under steady-state conditions, the CD1d molecule is associated with the ITAM-bearing FeRγ and forms a stable receptor complex that can transmit activation signals within the immature DC. Next, we tested whether TCR and LAG3, two T cell surface molecules actively involved in T cell–DC contact, were responsible for CD1d triggering. The addition of blocking Abs against those molecules in our iNKT cell–DC cocultures did not inhibit tolerogenic DC maturation (Supplemental Fig. 4B), thus suggesting that a yet unidentified iNKT cell ligand is responsible for triggering CD1d-mediated DC activation.
Tolerogenic or proinflammatory DC maturation induced by iNKT cell contact involved different intracellular signaling pathways

The proinflammatory DC maturation through TLR signaling is normally associated with the activation of the transcription factors NF-κB and the p38 MAPK pathway (46). The activation of that signaling pathway promotes DC maturation and acquisition of a strong proinflammatory phenotype with high IL-12 production and capacity to prime Th1-type immune responses. Conversely, the intracellular cell signaling events generated within DCs that undergo tolerogenic maturation are profoundly different and preferentially involve the activation of the ERK1/2 signaling pathway (37). We analyzed the signaling events induced within the DC by the iNKT cell contact in steady-state conditions or in the presence of simultaneous TLR4 stimulation. As expected, TLR4 stimulation by LPS induced the phosphorylation of both IκBα (a hallmark of NF-κB activation) and p38 MAPKs (Fig. 6A). In contrast, p38 MAPKs were not phosphorylated after iNKT cell contact (Fig. 6A). The IκBα was partially phosphorylated in iNKT cell-matured DCs but not as much as in the LPS-stimulated DCs. This finding permitted us to exclude that the iNKT cell-induced DC maturation was due to contaminating LPS in our cell preparation and suggests that an alternative signaling pathway is involved in the iNKT cell-induced tolerogenic DC maturation. A Western blot analysis revealed that CD11c+DCs expressed significantly higher levels of phosphorylated ERK1/2 compared with immature DCs (Fig. 6B). Coculture of immature DCs with iNKT cells in the presence of a selective inhibitor of MEK1/2 kinases (responsible for ERK1/2 phosphorylation) completely blocked both IL-10 secretion and upregulation of maturation marker CD86 on DCs (Fig. 6C), thus indicating that, although the IκBα and the NF-κB signaling pathway may be partially activated, the ERK1/2 signaling pathway is crucial for iNKT cell-induced tolerogenic DC maturation. The finding that CD1d cross-linking was sufficient to induce ERK1/2 phosphorylation suggests that the CD1d molecule engagement was responsible for iNKT cell-induced tolerogenic DC maturation (Fig. 6D).

Next, we asked what are the intracellular signaling events generated within the DC by the simultaneous stimulation with iNKT cells and LPS. We detected activation of both the NF-κB and ERK1/2 pathways after TLR4 stimulation regardless of the presence of iNKT cells (Fig. 6E) in accordance with previous observations (47). We concluded that the ERK1/2 pathway induces tolerogenic DC maturation, upon iNKT cell contact, only when activated in the absence of simultaneous NF-κB activation.

Discussion

The DC interaction with T cells represents a critical crossroad between immunity and tolerance. During infections and antitumoral immunity, effector T cells favor DC maturation and proinflammatory function (48). Conversely, in steady-state conditions or when T cell responses are no longer needed, T cells with a regulatory phenotype and function inhibit proinflammatory DC maturation and promote DC differentiation toward a tolerogenic phenotype to actively maintain immune homeostasis and prevent autoimmunity (49, 50).

iNKT cells, a unique T cell subset that recognizes self-Ags and microbial glycolipid Ags presented by the CD1d restriction molecule, behave similar to conventional T cells playing both effector

FIGURE 6. Distinct tolerogenic or proinflammatory effects of iNKT cells on DC maturation required different signaling pathways. A. The p38 MAPK and NF-κB signaling pathways were not activated within the immature DCs by the iNKT cell contact. Total cell lysates from LPS-matured or iNKT cell-matured DCs were analyzed for phospho-p38 MAPK (upper panel) or phospho-IκBα (lower panel). B. iNKT cell contact induced the activation of ERK1/2 pathway through CD1d engagement. The expression of p-ERK1/2 was evaluated by Western blot analysis on immature DCs cocultured with iNKT cells. C. The activation of the ERK1/2 signaling pathway was necessary for the iNKT cell-induced tolerogenic DC maturation. Immature DCs were cocultured with iNKT cells in the presence of ERK inhibitor U0126 (10 μM), and IL-10 gene expression was evaluated on purified CD11c+ DCs (left inset) while CD86 expression on CD11c+ DCs was measured by FACS analysis (right inset). Results are from one representative experiment out of three independent determinations. *p < 0.0001. D. CD1d cross-linking with anti-CD1d mAb and secondary anti-rat IgG was sufficient to trigger ERK1/2 activation, that is, tolerogenic DC maturation. p-ERK/GAPDH ratio was calculated by densitometric analysis of the intensity of the two bands. E. Simultaneous iNKT cell contact and LPS stimulation of DCs activated both the ERK1/2 and NF-κB signaling pathways. Data are representative of two independent experiments.
and regulatory immune functions in both cases through DC modulation. However, while conventional CD4+ T cells with regulatory function represent a well distinct immune cell subset that expresses specific cell markers such as CD25high, FoxP3, and GITR, secrete modulatory cytokines such as IL-10 and TGF-β, and suppress T cell proliferation, so far distinct iNKT cell subsets with a unique regulatory or adjuvant phenotype/function have not been found. It was originally proposed that the two functional iNKT cell phenotypes exist and are characterized by secretion of a completely different set of cytokotines: modulatory cytokotines such as IL-10, IL-4, IL-5, IL-13, and TGF-β for the regulatory iNKT cells, and proinflammatory cytokotines IL-12 and IFN-γ for the adjuvant iNKT cells (51). Several findings weakened this hypothesis, indicating that secretion of modulatory cytokotines, such as IL-10 and IL-4, is dispensable in different models of iNKT cell-induced tolerance (52–54). Moreover, in most cases, regulatory iNKT cells that suppress T cell immunity and prevent autoimmune diseases do not show a biased regulatory cytokine profile and secrete a diverse array of cytokotines, including large amounts of the proinflammatory cytokotine IFN-γ (6, 9, 55, 56). In this study, we demonstrated that the direct cell–cell contact is crucial for the tolerogenic effect of iNKT cells on DCs whereas iNKT cell-secreted cytokotines are dispensable.

It was also proposed that iNKT cells bearing a CD4+ phenotype play adjuvant/infiammatory function while DN iNKT cells are Treg cells able to suppress immunity. Such a hypothesis was contradicted by reports showing that CD4+ iNKT cells counter-regulate autoimmunity (6). Our data are in line with those findings and support the idea that the dual adjuvant/Suppressor iNKT cell function, that is, capacity to induce inflammatory/tolerogenic DC maturation, is not related to a specific phenotype and/or cytokine profile, but it is the result of the integration between different stimuli simultaneously acting on DCs. In fact, the same iNKT cell line regardless of its cytokine profile and with a mixed CD4+ and DN phenotype induces opposite immunological effects according to the maturation state of DCs. In steady-state conditions, the iNKT cell contact provokes tolerogenic DC maturation via engagement of the CD1d receptor complex and activation of the ERK1/2 signaling pathway. Those DCs acquire classical tolerogenic features, including upregulation of MHC class II and costimulatory molecules CD80 and CD86 and secretion of IL-10 (37, 42). The tolerogenic properties of iNKT cell-matured DCs were fully demonstrated by their capacity to induce Treg cells and, most importantly, promote immune tolerance in vivo and prevent autoimmune disease (i.e., diabetes) in the NOD mice. The ability of iNKT cell-modulated DCs to prevent autoimmune diseases, such as EAE (15) and type 1 diabetes (13, 14), has been previously demonstrated and appears to be IL-10–dependent (15). In this study, we assessed that iNKT cells induce tolerogenic DC maturation with a mechanism that is IL-10–independent. One possible explanation is that, in the aforementioned in vivo models, IL-10 was not directly responsible for iNKT cell-induced tolerogenic DC maturation but rather was involved downstream in the induction of peripheral tolerance by nktDCs. However, we collected preliminary evidence that IL-10 secretion, although a marker of DC tolerogenic maturation, was not directly involved in the differentiation of Treg cells. In fact, nktDCs were able to trigger differentiation of Treg cells when used as APCs, even in the presence of anti–IL-10 mAb (Supplemental Fig. 3).

Another important implication of our results is that the same iNKT cells that provoke tolerogenic DC maturation play an adjuvant effect when added to TLR4-matured DCs and enhance their proinflammatory maturation through CD40–CD40L interaction. Previous studies showed that iNKT cells require simultaneous TLR-induced stimulation of DCs to exert their adjuvant effect. For example, coculture experiments demonstrated that CD1d-restricted iNKT cell clones increase proinflammatory DC maturation (i.e., upregulation of MHC class II and CD86 molecules, as well as IL-12p70 secretion) only when DCs are concomitantly stimulated by LPS (10). In vivo studies showed that iNKT cell activation through αGalCer administration induced DC maturation (i.e., upregulation of CD86 molecule), but iNKT cell-matured DCs required simultaneous CD40 stimulation (12). Similarly, adjuvant properties of iNKT cells in vivo require the injection of their Ag, αGalCer, in CFA, a reagent that contains pathogen-derived molecules engaging TLRs at the time of iNKT cell activation (12, 16, 57, 58). Collectively, these findings suggest that iNKT cells exert opposite effects on DC function according to the concomitant presence/absence of “danger” signals at the time of their activation.

Such a conclusion could help in clarifying some paradoxical results on the role of iNKT cells in autoimmune diseases. In fact, according to the timing, route, and frequency of αGalCer administration, iNKT cells played opposite effect on the pathogenesis of autoimmune diseases. For example, a single i.p. injection of αGalCer before the induction of EAE was effective in preventing the disease, but the same treatment exacerbated autoimmune when αGalCer was injected at the time of immunization with the self-Ag (7). One possible explanation, in line with the aforementioned hypothesis, is that EAE immunization with the self-Ag in CFA, at the time of αGalCer administration, provided a maturation signal that synergized with the iNKT cell contact and favored proinflammatory DC maturation. Conversely, the preliminary administration of αGalCer in the absence of CFA triggers iNKT cell activation in the steady-state and provokes tolerogenic DC maturation. This idea is further supported by previous observation that stimulation of iNKT cells with αGalCer and simultaneous blockade of DC maturation inhibits EAE, whereas stimulation of iNKT cells with αGalCer-pulsed, CD40-activated (mature) DCs exacerbates the autoimmune disease (59).

Also, in a virus-induced model of autoimmune diabetes, the iNKT cell interaction with DCs in the context of a viral infection (i.e., in the presence of “danger” signals) enhances proinflammatory DC maturation (45). Overall, these data support the idea that the iNKT cell interaction with immature DCs favors the generation of immune tolerance, whereas the interaction between mature DCs and iNKT cells promotes T cell immunity.

In our experimental setting, the same iNKT cell line was able to play both a tolerogenic and adjuvant effect regardless of the phenotype and based on the presence/absence of DC stimulation through TLR4. However, we cannot exclude that, in vivo, iNKT cells with a strongly biased cytokine profile preferentially induce either protolerogenic or proinflammatory DC maturation. For example, high levels of IFN-γ and IL-12 released by adjuvant iNKT cells could synergize with the maturation signal transmitted by iNKT cells through cell–cell contact and favor proinflammatory DC maturation (60).

The signals that regulate the adjuvant or protolerogenic effect of iNKT cells on DCs are completely different. In fact, while iNKT cells transmit a proinflammatory maturation signal through the CD40–CD40L or OX40–OX40L interaction (58, 61, 62), we found that those molecular contacts are dispensable whereas the engagement of the CD1d molecule is necessary for tolerogenic DC maturation. We exclude that, in our experimental setting, the CD1d molecule was required to activate iNKT cells rather than to convey a tolerogenic maturation signal within the DC. First, iNKT cells added to the immature DC cultures were previously activated by stimulation with αGalCer-pulsed DCs. Furthermore, the presence/absence of the iNKT cell Ag, αGalCer, in the iNKT cell-DC signaling pathway. Those DCs acquire classical tolerogenic features, including upregulation of MHC class II and costimulatory molecules CD80 and CD86 secretion and secretion of IL-10 (37, 42). The tolerogenic properties of iNKT cell matured DCs were fully demonstrated by their capacity to induce Treg cells and, most importantly, promote immune tolerance in vivo and prevent autoimmune disease (i.e., diabetes) in the NOD mice. The ability of iNKT cell-modulated DCs to prevent autoimmune diseases, such as EAE (15) and type 1 diabetes (13, 14), has been previously demonstrated and appears to be IL-10–dependent (15). In this study, we assessed that iNKT cells induce tolerogenic DC maturation with a mechanism that is IL-10–independent. One possible explanation is that, in the aforementioned in vivo models, IL-10 was not directly responsible for iNKT cell-induced tolerogenic DC maturation but rather was involved downstream in the induction of peripheral tolerance by nktDCs. However, we collected preliminary evidence that IL-10 secretion, although a marker of DC tolerogenic maturation, was not directly involved in the differentiation of Treg cells. In fact, nktDCs were able to trigger differentiation of Treg cells when used as APCs, even in the presence of anti–IL-10 mAb (Supplemental Fig. 3).

Another important implication of our results is that the same iNKT cells that provoke tolerogenic DC maturation play an adjuvant effect when added to TLR4-matured DCs and enhance their proinflammatory maturation through CD40–CD40L interaction. Previous studies showed that iNKT cells require simultaneous
cocultures did not modify the iNKT cell capacity to induce tolerogenic DC maturation (data not shown). Most importantly, iNKT cells, similar to conventional T cells, require costimulatory signals to be activated (63), signals that cannot be provided by immature DCs in our iNKT cell-DC cocultures. An alternative possibility is that the CD1d molecule clustering through interaction with the TCR is necessary to sustain the immunological synapse and the cell–cell contact between the iNKT cell and the DC while the activation signal is transmitted through a different receptor molecule. However, the addition of anti-TCRβ mAb and blockage of the TCR–CD1d interaction did not abolish tolerogenic DC maturation, thus reinforcing the idea that CD1d functions as a receptor molecule.

It is not surprising that the dual action of iNKT cells on DCs is mediated through distinct mechanisms. In fact, CD40 and OX40L are only expressed by fully mature DCs and they cannot be involved in the iNKT cell contact with immature DCs. Hence, the protolerogenic action of iNKT cells most likely involves receptor molecules, such as CD1d, that are expressed by steady-state immature DCs (55). CD1d is an MHC class I-like molecule primarily known for its role in glycolipid Ag presentation. Similar to other restriction molecules, CD1d plays additional biological functions, including cell signaling. In fact, several studies demonstrated that CD1d ligation produces activating signals within human monocytes and intestinal intraepithelial cells (60, 64). We demonstrated that the CD1d engagement alone produced a tolerogenic signal and induced IL-10 release by immature DCs. Other reports have shown that CD1d ligation induces IL-10 secretion, specifically on intestinal epithelial cells (64). In some cases, CD1d ligation on human monocytes and immature DCs induced IL-12p70 secretion (60). In that report, the inducible form of IL-12 (IL-12p40) was not released upon CD1d ligation and required addition of suboptimal doses of LPS, thus supporting the idea that the CD1d-transmitted signal integrates with other maturation stimuli. Further studies are necessary to clarify whether CD1d ligation alone is sufficient to induce tolerogenic DC maturation or requires different and concomitant signals provided, in our experimental setting, by iNKT cells.

The short cytosolic domain and the absence of a signaling motif in the CD1d structure are apparently inconsistent with the capacity of CD1d to trigger the complex signaling pathway required to induce tolerogenic DC maturation (65). Previous evidence indicated that the short cytoplasmic tail of CD1d must be directly involved in cell signaling since its blockage by a tyrosine kinase inhibitor inhibits signaling within the intestinal epithelial cells (64). Our data extended that finding and demonstrated that the cytoplasmic portion of the CD1d molecule physically associates with an ITAM-containing adapter, the FcRγ. That adapter contains tyrosine residues that can be phosphorylated by Src family kinases and can transmit activating signal within the cell. Blocking either LAG3 or the TCR, two surface molecules that regulate conventional T cell–DC contact (39, 66), does not inhibit iNKT cell-induced tolerogenic DC maturation, thus suggesting that a yet unidentified ligand on iNKT cell surface engages the CD1d receptor complex.

Not only the receptor molecules but also the intracellular signaling pathways that regulate the different iNKT cell actions on DCs are very distinct. Previous studies linked the ERK1/2 signaling pathway with induction of tolerogenic DC maturation through different mechanisms, such as intercellular contact with splenic stromal cells, yeast zymosan, and E-cadherin disruption (37, 67, 68). However, because the ERK1/2 signaling pathway is also activated during LPS-induced proinflammatory DC maturation, it is still unclear whether it is effectively responsible for the signaling cascade leading to tolerogenic DC maturation. In this study, we demonstrated that ERK1/2 activation is necessary for iNKT cell-induced tolerogenic DC maturation. However, the presence of LPS in our iNKT cell-DC cocultures renders ineffective the ERK1/2 signal induced by the iNKT cell contact, thus suggesting that activation of the NF-κB pathway overshadows the ERK1/2-mediated signal that remains silent during TLR4 stimulation (47).

Our data demonstrate that the contact between activated iNKT cells and DCs results in immunogenic or tolerant immune responses depending on the DC maturation state. During infection, the iNKT cell contact with DCs concomitantly stimulated by pathogen-driven signals generates an adjuvant effect to boost T cell immunity and favor the clearance of microbes. However, once the pathogen is cleared, iNKT cells encounter immature DCs and promote their tolerogenic maturation to induce Treg cell differentiation and restore immune tolerance against self-Ags. According to this scenario, DCs integrate different signals, including iNKT cell modulation, and then appropriately orchestrate immune responses to either promote resistance to infections or tolerate self-Ags.

Preclinical studies suggested that the protolerogenic function of iNKT cells, similar to that of conventional Treg cells (69), can be exploited to silence immune responses in autoimmune diseases through αGalCer administration (5, 6, 9, 54). Our data indicate that the presence of danger signals, promoting proinflammatory DC maturation at the time of αGalCer administration, could shift the role of iNKT cells in autoimmune diseases from protection toward contribution to the pathogenic process. This suggests that a better understanding of the molecular pathways that regulate iNKT cell-induced tolerogenic DC maturation is absolutely necessary before we can take advantage of the therapeutic potential of iNKT cells to treat autoimmune diseases, allergies, and to prevent transplant rejection.

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Disclosures

The authors have no financial conflicts of interest.

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Figure S1. Phenotypical and functional features of DCs and iNKT cells used in the co-culture experiments. (A) FACS analysis of bone-marrow derived DCs used in co-culture experiments. (B) Analysis of iNKT cell percentage before (left panel) or after (right panel) magnetic sorting with αGalCer-loaded CD1d dimers. (C) Cytokine profile of iNKT cell lines was determined on supernatants after 72 hours of antigenic-stimulation with αGalCer-loaded LPS-DCs.
Figure S2. iNKT cells directly trigger DC activation and maturation. (A) 354 genes were differentially expressed between immature DCs and iNKT cell-matured DCs \((p<0.0001)\). Data are representative of two independent triplicate experiments. (B) Mean percentage ± SD of CD11c+ DCs that expressed the indicated maturation markers \(*, p<0.05 \text{ and } **, p<0.01\). (C) DCs matured by iNKT cells acquired antigen-presenting capacity. The different DC types were pulsed with the BDC2.5 peptide and used as APCs to stimulate TCR transgenic BDC2.5 T cells in a proliferation assay. (D) The DC origin of IL-10 was verified by quantitative RT-PCR for IL-10 on CD11c+ cells. (E-F) CD4+ T cells did not induce DC maturation. BDC2.5 CD4+ T cells were purified and added in parallel with iNKT cells to immature DCs as in figure 1A. Maturation was assessed by mean of CD86 expression and IL-10 secretion by CBA. Data are representative from three independent experiments.
Figure S3: BDC2.5 T cells antigen-stimulated with nktDCs+aIL-10 maintained their suppressive capacity. TCR-transgenic BDC2.5 T cells were repeatedly stimulated with BDC2.5 peptide-pulsed nktDCs with/out monoclonal antibody against IL-10 (10ng/ml). T<sub>reg</sub> cells induced by nktDCs or nktDCs+αIL-10 were added at 1:1 ratio to BDC2.5 T effector cells (CD4<sup>+</sup>CD25<sup>-</sup>: Teff) stimulated with peptide-loaded LPS-DCs as APC. T<sub>eff</sub> cells proliferation was determined by <sup>3</sup>H-thymidine incorporation. Results are means ± SD from one triplicate experiment.
Figure S4. CD1d formed a receptor complex in steady state immature DCs by associating with the ITAM-bearing adapter FcRγ. (A) CD1d physically associate with the ITAM-bearing adapter FcRγ on immature DCs. Total cell lysate of immature DCs was immunoprecipitated with anti-CD1d or isotype matched monoclonal antibodies IgG2b. Immunoprecipitates were resolved on 15% SDS-PAGE and immunoblotted with anti-FcRγ mAb. (B) The CD1d receptor complex did not trigger tolerogenic DC maturation through binding with LAG3 or TCR on iNKT cells. Monoclonal antibodies against LAG3 or TCRβ or isotype controls were added to the iNKT cell-DC co-cultures and the DC maturation was evaluated after 20 hours by mean of expression of MHC II, CD40 and CD86 with FACS analysis.