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Activation of Plasmacytoid Dendritic Cells with TLR9 Agonists Initiates Invariant NKT Cell-Mediated Cross-Talk with Myeloid Dendritic Cells

Carlos J. Montoya,†‡ Hyun-Bae Jie,§ Lena Al-Harthi,* Candice Mulder,* Pablo J. Patiño,† María T. Rugeles,‡ Arthur M. Krieg,‡ Alan L. Landay,* and S. Brian Wilson*§

CD1d-restricted invariant NK T (iNKT) cells and dendritic cells (DCs) have been shown to play crucial roles in various types of immune responses, including TLR9-dependent antiviral responses initiated by plasmacytoid DCs (pDCs). However, the mechanism by which this occurs is enigmatic because TLRs are absent in iNKT cells and human pDCs do not express CD1d. To explore this process, pDCs were activated with CpG oligodeoxyribonucleotides, which stimulated the secretion of several cytokines such as type I and TNF-α. These cytokines and other soluble factors potently induced the expression of activation markers on iNKT cells, selectively enhanced double-negative iNKT cell survival, but did not induce their expansion or production of cytokines. Notably, pDC-derived factors licensed iNKT cells to respond to myeloid DCs: an important downstream cellular target of iNKT cell effector function and a critical contributor to the initiation of adaptive immune responses. This interaction supports the notion that iNKT cells can mediate cross-talk between DC subsets known to express mutually exclusive TLR and cytokine profiles. The Journal of Immunology, 2006, 177: 1028–1039.

A ctivation of the innate immune response is crucial to control the early invasion of pathogens and the subsequent establishment of adaptive responses. Myeloid dendritic cells (mDCs) capture Ags in peripheral tissues and migrate to secondary lymphoid nodes to instruct naive T cells, while plasmacytoid DCs (pDCs) enter to the lymphoid nodes directly via the blood (1–4). Resting DCs that capture self-Ags in the steady state are able to induce tolerance, (5, 6) whereas in the presence of inflammation, these same cells are able to initiate adaptive immune responses (7). A significant component of this discrimination is controlled by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (8, 9). The TLR family is the best-characterized class of PRRs. TLR recognition of PAMPs initiates inflammatory responses by orchestrating the recruitment of leukocytes and the activation of stromal and resident innate cells.

CD1d-restricted iNKT cells (iNKT) cells and dendritic cells (DCs) have been shown to play crucial roles in various types of immune responses, including TLR9-dependent antiviral responses initiated by plasmacytoid DCs (pDCs). However, the mechanism by which this occurs is enigmatic because TLRs are absent in iNKT cells and human pDCs do not express CD1d. To explore this process, pDCs were activated with CpG oligodeoxyribonucleotides, which stimulated the secretion of several cytokines such as type I and TNF-α. These cytokines and other soluble factors potently induced the expression of activation markers on iNKT cells, selectively enhanced double-negative iNKT cell survival, but did not induce their expansion or production of cytokines. Notably, pDC-derived factors licensed iNKT cells to respond to myeloid DCs: an important downstream cellular target of iNKT cell effector function and a critical contributor to the initiation of adaptive immune responses. This interaction supports the notion that iNKT cells can mediate cross-talk between DC subsets known to express mutually exclusive TLR and cytokine profiles. The Journal of Immunology, 2006, 177: 1028–1039.

Activation of Plasmacytoid Dendritic Cells with TLR9 Agonists Initiates Invariant NKT Cell-Mediated Cross-Talk with Myeloid Dendritic Cells

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neutralizing Abs against IL-6 and TNF-α. Cultures were incubated at 37°C in 5% CO₂. Culture supernatants for ELISA were collected, filtered with a 0.22-μm filter, and stored at −20°C until their use with fresh cells.

**Cytokine blockade**

Freshly isolated cells resuspended in complete medium (1 × 10⁶/ml) were cultured, stimulated with/without A-class CpG ODN (4 μg/ml), rh-IFN-α (5000 U/ml), or PBMC-conditioned supernatant (dilutions: 1/20, 1/10, 1/5, and 1/2). For blockade, neutralizing Abs were added 1 h before the addition of the stimuli: anti-IFN-α (10,000 U/ml), anti-IFN-β (3,000 U/ml), anti-IFN-IR (CD118, 10 μg/ml), anti-TNF-α (10 μg/ml), anti-IL-6 (0.1 μg/ml), and anti-IP-10 (10 μg/ml).

**Isolation of DC and iNKT cells**

BDCA-1 positive (mDC) or BDCA-2 positive (pDC) cells were magnetically isolated from 7 × 10⁶ PBMCs per the manufacturer’s specifications (BDCA-1 and BDCA-2 DC isolation kits; Miltenyi Biotec), using an AutoMACS (Miltenyi Biotec) set to run the POSSELD software program. Selection of pDCs and B cells was done with BDCA-2 and CD19 microbeads per the manufacturer’s recommendations. The number and viability of these purified DC subgroups were determined by trypan blue exclusion, and purity assessed by flow cytometry.

To isolate iNKT cells, 8 to 10 × 10⁶ PBMCs were incubated 20 min on ice with 10 μg of PE-labeled-i anti-invariant NKT cell mAb (clone 6B11; BD Pharmingen) and 100 μl of FeRy-b-blocking reagent (Miltenyi Biotec). The 6B11⁺ cells were then isolated using anti-PE microbeads (Miltenyi Biotec) as per manufacturers specifications and were magnetically isolated using the program Posset on an AutoMACS instrument. Viability and purity were determined as described for DCs.

**Coculture of purified iNKT cells, pDCs, and mDCs**

Purified iNKT cells (30K cells) were cultured alone, with either pDCs (20K cells) or mDCs (20K cells) in 0.5 ml of complete culture medium and incubated either with/without A-class or control ODN; after 24 h of culture, cells were analyzed by flow cytometry.

**Culture of purified pDCs with A-class CpG ODN**

Freshly isolated BDCA-2-positive cells were cultured in 24-well plates (2.5 × 10⁶ pDC/ml) and incubated with 4 μg/ml A-class CpG ODN during 24 h at 37°C/5% CO₂. Then, the pDC-conditioned supernatants were collected centrifuged 10 min/500 × g/RT, filtered with a 0.22-μm filter, and stored at −20°C until their use for incubation with purified iNKT cells and for analysis of cytokine production by ELISA.

**Incubation of purified iNKT cells with pDC-conditioned supernatant**

Purified iNKT cells (2.5 × 10⁶ cells/ml) were incubated for 24 h/37°C/5% CO₂ with or without pDC-conditioned supernatant (dilution 1/2 with complete culture medium). Cells were washed with RPMI 1640 medium and resuspended in complete culture medium before the coculture with α-GalCer-loaded mDCs.

**Coculture of preactivated iNKT cells and α-GalCer-loaded mDC**

Purified mDCs were resuspended in complete medium and incubated with or without α-GalCer (10, 20, 50, or 100 ng/ml) for 24 h/37°C/5% CO₂. In parallel, iNKT cells were preincubated with or without pDC-conditioned supernatants and then cocultured with the α-GalCer-loaded mDCs at a ratio of 25K:10K, respectively. After 24 h of incubation, 100 μl of culture supernatant was gently collected from each well and stored at −20°C until analysis by ELISA. Then, 100 μl of fresh complete culture medium was added to each well, and the plate was incubated for another 24 h at 37°C/5% CO₂. Afterward, 1 μCi per well of [3H]thymidine was added and the plate was again incubated overnight; finally, the culture was harvested after 24 h of culture, and incorporated counts were counted using a 1205 Beta Plate (Pharmacia).

**Flow cytometry**

Phenotypic analysis of iNKT cells, mDCs, and pDCs was performed by three- or four-color flow cytometry. For iNKT cell characterization, the following combinations of Abs were used: VJ11 FITC/Vα24PE, VJ11 FITC/β811 PE, or β811 PE/CD3 PEC. CD4-APC and 6B11-PE was used for iNKT cell subset analysis. Evaluation of MDC and pDC was done with the combinations LinFITC/CD11c PE/HLA-DR PEC and LinFITC/CD123 PE/HLA-DR PEC, respectively. PBMCs were labeled with CFSE per the functional contributions to these very same immune processes, including the CpG ODN-dependent responses, despite not expressing detectable TLRs. Almost all iNKT cells recognize glycolipid Ags presented by CD1d. The regulation of MDC maturation and cytokine secretion by iNKT cells is an important component of their effector function (28–33). Recently, Steinman and coworkers (34) have argued that this interaction is a major control mechanism for this process that is independent of TLR signaling. It is not known how TLR9-induced signals affect iNKT cells; however, the activation of iNKT cells in vivo clearly can lead to the subsequent activation of mDCs, B cells, monocytes, NK cells, and T cells (30, 35–37). Thus, it seems reasonable to speculate that the immune responses regulated either by activated iNKT cells or TLR agonist-activated pDCs would overlap.

Despite these overlapping regulatory activities between DCs and iNKT cells, their possible interaction during the human immune response has not been explored extensively. Moreover, most of the regulatory studies in vitro have been with human DCs derived from monocytes incubated with recombinant cytokines (GM-CSF, IL-4, and/or TNF-α), and it is unclear how immature DCs and iNKT cells, normally present in peripheral blood, may be activated by TLR agonists and interact with one another to modulate immune responses. Thus, using the model of innate immune activation with the TLR9 agonists CpG ODNs, we evaluated the interaction between CpG ODN-activated pDCs, iNKT cells, and mDCs.

**Materials and Methods**

**Abs and reagents**

Fluorochrome-labeled mAbs against human molecules 6B11, CD3, CD4, CD8, CD11c, CD19, CD25, CD38, CD40, CD69, CD86, CD45RO, CD123, CD154, HLA-DR, perforin, IFN-γ, IL-4, TNF-α, lineage markers (anti-CD3, CD14, CD16, CD19, CD20, and CD56) and isotype control Abs were obtained from BD Pharmingen.

FITC-labeled anti-Vβ11 and PE-labeled anti-Vα24 were obtained from Beckman Coulter/Immunotech. FcR blocking reagent, anti-PE magnetic beads, and isolation kits for mDCs and pDCs were obtained from Miltenyi Biotec. Recombinant human (h) IFN-α and neutralizing mAbs against IFN-α, IFN-β, and CD118 were obtained from PBL Biomedical Laboratories. Recombinant Abs against IL-6 and TNF-α were obtained from BD Pharmingen, and anti-IP-10 was obtained from R&D Systems. PMA and ionomycin were obtained from Sigma-Aldrich; α-galactosylceramide (α-GalCer) was from Kirin Brewery.

**CpG ODNs**

Synthetic endotoxin-free ODNs were provided by Coley Pharmaceutical Group (lowercase letters, phosphorothioate linkage; capital letters, phosphodiester linkage 3’ of the base; underlined letters, CpG dinucleotides): A-class CpG ODN 2216 5’-ggGGAACGATCTgggggG-3’; A-class control ODN 2245 5’-ggGGAGACGCTgggggG-3’; B-class CpG ODN 2006 5’-TCGTTGTTTGTCTTTGTTT-3’; C-class CpG ODN 2395 5’-TCGTTGTCTTTGGGgCGCCGGCC-3’; B- and C-class control ODN 2137 5’-TCGTTGTCTTTGGGgCGCCGGCC-3’.

**Isolation and culture of mononuclear cells**

Heparinized whole-blood samples were obtained from healthy adult donors after obtaining informed consent for an approved protocol Institutional Review Board ORA#0306110. PBMCs were isolated by Ficoll gradient (BioWhittaker). The viability of PBMCs was determined by trypan blue exclusion.

PBMCs (1 × 10⁶/ml) were suspended in complete culture medium (RPMI 1640 supplemented with 10% of heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine). ODNs were used at concentrations of 0.04–4 μg/ml; PMA at 50 ng/ml and ionomycin at 500 ng/ml. Cultures were incubated at 37°C in 5% CO₂. Culture supernatants for ELISA were collected and stored at −80°C until the measurement of cytokine levels.

Conditioned supernatants from PBMCs or cells incubated for 24 h either with or without 4 μg/ml A-class control ODN or A-class CpG ODN were collected, filtered with a 0.22-μm filter, and stored at −20°C until their use with fresh cells.
manufacturer’s specifications (Invitrogen Life Technologies/Molecular Probes).

Intracellular stainings were performed following the manufacturer’s recommendations (BD Pharmingen). Brefeldin A solution (1 × 40 μl per well; BD Biosciences) was added during the last 6–12 h of culture. After the staining for extracellular Ags, cells were incubated with 500 μl of 1× permeabilizing solution 2 (BD Biosciences) during 10 minroom temperature/dark. Isotype-matched control Abs were included for all experiments as controls for nonspecific binding. Because of the low frequency of iNKT cells and DCs in PBMCs, from 2.5 to 5 × 10⁶ total gated cells were analyzed for each data point. Dead cells were gated out by forward and side scatter. Flow cytometry was performed using the FACS Calibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

Detection of cytokines by ELISA
Commerially available kits were used to determine cytokine concentrations in culture supernatants, and assays were performed per the manufacturer’s specifications; IFN-α, TNF-α, IL-6, and IL-12 were obtained from Pierce Endogen; and IFN-γ, IP-10, IL-4, IL-7, IL-15, and IL-18 were obtained from R&D Systems.

Statistical analysis
Data are shown as a mean ± SD of three or more independent experiments. Statistical analysis for the comparison of different stimuli was performed using Student’s two-tailed t test. A value of p < 0.05 was considered significant.

Results
CpG ODN-stimulated PBMCs activate iNKT cells in culture
To evaluate the interaction of pDCs, mDCs, and iNKT cells during the development of TLR9-dependent innate responses, PBMCs were incubated with different classes of CpG ODNs or their respective ODN controls. CpG ODNs induce the expression of activation markers on particular cell types (4, 27, 38). Thus, the expression of CD40 on pDCs and mDCs, CD69 on iNKT cells, and CD86 on B lymphocytes was determined. Incubation of PBMCs with A-class CpG ODN induced the highest expression of CD40 on both pDCs and mDCs, while B and C classes of CpG ODNs led to the highest expression of CD86 on B cells (Table I). Unexpectedly, CpG ODN markedly stimulated CD69 expression on iNKT cells. When compared with A- and B-class ODNs, C-class CpG ODNs induced an intermediate level of expression of CD40 and CD69 on DCs and iNKT cells, respectively.

Because A-class CpG ODN was the most potent ODN for inducing activation markers on DCs and iNKT cells and is thought to be pDC selective, this ODN was selected for further study. Dose-response experiments indicated that the incubation of PBMCs with 4 μg/ml A-class CpG ODNs induced the highest secretion of IFN-α and expression of CD40 on the positive control pDC population and CD69 on iNKT cells (Fig. 1). Consequently, this concentration elicited equivalent maximal responses in both the iNKT and pDC populations; the A-class CpG ODN was selected to evaluate the interaction between pDC and iNKT cells.

To determine whether treatment of PBMCs with A-class CpG could expand the iNKT cell population, the frequency of these T cells in PBMCs was measured after 18, 24, 36, 48, and 72 h of cell culture. There were no significant differences in the percentage of iNKT cells observed over the time course of activation (data not shown). Because human iNKT cell subsets are CD4⁺, CD8α⁻, CD4⁺/CD8α⁻ (double negative, DN), it was important to determine whether the relative proportions and activation status of these subsets in response to A-class CpG ODN stimulation were subset specific. Treatment of PBMCs with A-class CpG ODNS did not significantly modify the proportion of CD4⁺, CD8⁺, DN and double-positive (DP) iNKT cells or selectively activate any particular subset (Fig. 2 and data not shown).

The combination anti-Vα24/anti-Vβ11 identifies a population of T cells whose TCR coexpression for both chains is highly enriched for iNKT cells but not specific for the invariant Vα24Jα18 TCR α-chain (39). To validate that the population of T cells identified for the donors used in this work were iNKT cells, the frequency of iNKT cells was determined in parallel using the combination anti-Vβ11 with the invariant chain CDR3 loop-specific mAb 6B11. For all donors used there were no significant differences between the frequencies of Vα24/Vβ11⁺ T cells and 6B11/Vβ11⁺ T cells (Fig. 3A). Hence, the frequency of iNKT cell subsets was unaffected by activation of PBMCs with CpG ODNS.

To further explore the effect of CpG ODNS on iNKT cells, the expression of other T cell activation markers was evaluated (Fig. 3B). When compared with basal conditions or control ODNS, A-class CpG ODNS significantly up-regulated the expression on iNKT cells of the early activation markers CD38 and CD69; the expression of CD25, CD154 and HLA-DR was unaffected (Fig. 3B and Table II). As expected, the T cell memory marker CD45RO was expressed on >80% of iNKT cells, independent of the condition of cell culture. The pattern of expression of these proteins after 18, 48, and 72 h of incubation with A-class control or CpG ODN, was identical with those at 24 h. Hence, CpG ODN treatment selectively induced iNKT cell activation markers, with A-class CpG ODNS being the most potent.

Table I. Effect of different classes of ODNs on the expression of activation markers by immune cells (n = 4)*

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No Stimulation</th>
<th>A-Class CpG ODN</th>
<th>B-Class CpG ODN</th>
<th>B and C Class CpG ODN</th>
<th>C-Class CpG ODN</th>
</tr>
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<tbody>
<tr>
<td>pDC CD40⁺</td>
<td>1.5 ± 2.2</td>
<td>19.7 ± 10</td>
<td>3.9 ± 3.9</td>
<td>0.8 ± 0.6</td>
<td>7.7 ± 5.9</td>
</tr>
<tr>
<td>mDC CD40⁺</td>
<td>2.3 ± 2.4</td>
<td>19.4 ± 13.7</td>
<td>5.8 ± 5</td>
<td>2.4 ± 1.8</td>
<td>9.6 ± 7.8</td>
</tr>
<tr>
<td>iNKT cells CD69⁺</td>
<td>7.7 ± 5.7</td>
<td>51.9 ± 14.9</td>
<td>19.9 ± 13.2</td>
<td>11.3 ± 10.3</td>
<td>33.1 ± 12</td>
</tr>
<tr>
<td>B cells CD86⁺</td>
<td>26.9 ± 4.8</td>
<td>48.4 ± 4.4</td>
<td>63.6 ± 10.7</td>
<td>47.9 ± 2.8</td>
<td>65.7 ± 12.1</td>
</tr>
</tbody>
</table>

*Expressed as the percentage (mean ± SD) of positive cells for each marker, detected by flow cytometry after 24 h of incubation. For pDCs and mDCs, cells analyzed were from a gate comprising all the mononuclear cells; for iNKT cells and B lymphocytes, the gate contained all the lymphocytes.

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FIGURE 1. Dose-response of innate immune cells to A-class CpG ODN. PBMCs were incubated for 24 h with different concentrations of A-class ODNs (control and CpG ODN), and the expression of activation markers and the secretion of IFN-α were evaluated by flow cytometry and ELISA, respectively.

A. During the analysis, mononuclear cells were included in a region (R1) created in the forward vs side scatter, and used to define the lineage-negative/HLA-DR-positive cells (R2); in the later region, the pDCs were determined as the cells bright for CD123 (R3) and mDCs as cells bright for CD11c. In the later subpopulation the expression of CD40 was analyzed (upper panel, representative dot plots for pDCs). For iNKT cells, the lymphocytes (R1) positive for both CD3 and 6B11 were defined (R2), and in this later region, the expression of CD69 was evaluated (lower panel).

B. The incubation with 4 μg/ml A-class CpG ODN induced the highest secretion of IFN-α and expression of CD40 on pDC and CD69 on iNKT cells. Data are reported as means ± SD (n = 4). * p < 0.05
of incubation, and there was no significant differences when compared with the expression observed after 24 h.

**CpG ODN-dependent activation of iNKT cells is mediated by soluble factors**

A-class CpG ODNs characteristically activate pDCs to produce massive amounts of IFN-1 (α, β, and ω) and stimulate their own maturation (4). This class of cytokines also is thought to be integral to the activation of other innate and adaptive immune cells such as NK cells, monocytes, and T lymphocytes (42–45). To test whether the activation of iNKT cells was dependent on soluble factors, PBMCs were incubated with CpG ODNs for 24 h, and the concentration of cytokines that are characteristic of CpG ODN activation was determined (46). When compared with unstimulated PBMCs, CpG ODN treatment markedly increased the secretion of IFN-1 and IP-10, and the production of TNF-α and IL-6 was modestly elevated. There was

**FIGURE 2.** Frequency of iNKT cell subgroups in culture of PBMCs with A-class ODNs. PBMCs were incubated for 24 h with 4 μg/ml A-class ODNs (control and CpG ODN) and the expression of CD4 and CD8 on iNKT cells was evaluated by flow cytometry. A, iNKT cells were detected in the lymphocyte region (R1) as cells DP for Vβ11 and Vα24 (R2); in this later subpopulation, the frequency of iNKT cell subgroups was defined according to the expression of the molecules CD4 and CD8 and the conditions of PBMC stimulation. B, Summary of the data showing that, in comparison to PBMCs not stimulated or incubated with A-class control ODN, the incubation with A-class CpG ODN did not significantly modify the proportion of CD4<sup>+</sup>, CD8<sup>+</sup>, and DN and DP iNKT cells. Data are reported as means ± SD (n = 5).

**FIGURE 3.** Flow cytometry detection of iNKT cells and impact of A-class ODNs on their expression of activation markers. A, For the analysis of iNKT cells in PBMCs, different combinations of mAbs were used; despite the combination of anti-Vβ11 and the clone 6B11 (anti-CDR3 loop of invariant chain) more specifically detects the iNKT cells, there were no significant differences regarding the use of the combination anti-Vα24/anti-Vβ11. B, PBMCs were incubated for 24 h with 4 μg/ml A-class ODNs (control and CpG ODN) and the expression of CD25, CD123, CD45RO, CD69, CD154 and HLA-DR was evaluated by flow cytometry. In these representative histograms from one of five healthy subjects, it is shown that the incubation with A-class CpG ODNs up-regulated the expression of CD38 and CD69, while the expression of CD45RO and HLA-DR was not up-regulated. More than 80% of iNKT cells express CD45RO, a phenotypical marker of memory cells.
no significant increase in the secretion of other possible activators of iNKT cells such as IL-7, IL-12, IL-15, IL-18, and IFN-γ (Table III). These results are consistent with other published findings (46).

To evaluate the potential role for IFN-I on iNKT cell activation, PBMCs were incubated with A-class CpG ODN with/without a mixture of mAbs to block the effect of IFN-I (neutralizing anti-IFN-α, neutralizing anti-IFN-β, and neutralizing anti-IFN-I receptor (CD118)). Although incubation with CpG ODNs alone significantly increased the expression of CD69 on iNKT cells, the coinubcation with CpG ODN and IFN-I blocking mixture reduced the expression of CD69 × 45% (p = 0.024) (Table IV). The effect of this blocking mixture was very heterogeneous in the healthy controls evaluated (range of CD69 reduction, 15–67%). As a positive control for these experiments, PBMCs were incubated with rh-IFN-α with/without the same blocking mixture. In all cases, rh-IFN-α up-regulated the expression of CD69 (56% ± 15%), while the blocking mixture was 95–100% effective at inhibiting iNKT cell activation by rh-IFN-α. This suggested that other cytokines/chemokines/soluble factors were potentially important for inducing activation markers on iNKT cells.

The contributions of IFN-I and other soluble mediators of CpG ODN-induced activation of iNKT cells were confirmed using PBMC-conditioned supernatants. Fresh PBMCs were incubated 24 h with/without A-class CpG ODN, rh-IFN-α, PBMC-conditioned supernatant, and the anti-IFN-I mixture. The conditioned medium and rh-IFN-α consistently induced higher expression of CD69 than did optimal doses of CpG ODNs (Fig. 5A). The anti-IFN-I blocking mixture completely inhibited the expression of CD69 induced by rh-IFN-α, and only partially blocked the expression induced by CpG ODN or PBMC-conditioned supernatant.

Because A-class CpG ODN stimulation of PBMCs also induced the secretion of IP-10, IL-6, and TNF-α in addition to IFN-α, neutralizing Abs against IP-10, IL-6, and TNF-α were used to evaluate the contributions these cytokines/chemokines in the activation of iNKT cells. Only neutralization of IFN-I and TNF-α had moderate yet equivalent inhibitory effects (Fig. 5B). Neutralization of IP-10 and IL-6 had no effect on expression of CD69 by iNKT cells. Simultaneous neutralization of all four cytokines had an additive effect but was only 40–50% effective at reducing CpG ODN-induced expression of CD69. This suggests that IFN-I and TNF-α were important for iNKT activation but other soluble factor(s) contribute to this process.

**A-class CpG ODN-mediated activation of purified iNKT cells**

Treatment of PBMCs with CpG ODNs activates several different subpopulations of leukocytes. To clarify the interaction mediated by soluble factors between CpG ODN-activated pDCs and iNKT cells, highly purified populations of cells were isolated using Miltenyi magnetic beads and an AutoMacs instrument. The purified pDCs were incubated for 24 h with A-class CpG ODN, and the pDC-conditioned supernatants were used to treat purified iNKT cells (Fig. 6A). The incubation of purified iNKT cells with pDC-conditioned supernatants resulted in the same up-regulated expression of CD69 (as well as CD38, data not shown), while the coinubcation with combinations of neutralizing Abs was again only partially effective at inhibiting this response (Fig. 6B). It is important to note that the concentration of cytokines in supernatants from CpG ODN-stimulated purified pDC is lower than in supernatants from PBMCs. This is likely a consequence of using the BDCA2 Ab for positive selection of pDC. Although highly selective for this cell subset, the Ab inhibits subsequent in vitro responses of pDC to TLR-dependent activation. However, the stimulation of purified pDC with CpG ODN still resulted in secretion of the same cytokines/chemokines as was observed with PBMC-derived supernatants (IFN-α, PBMC = 1,247 + 477 pg/ml, pDC = 449 + 64 pg/ml; IP10, PBMC = 1,681 + 310 pg/ml, pDC = 917 + 466 pg/ml; TNF-α, PBMC = 489 + 208 pg/ml, pDC = 263 + 79 pg/ml; IL-6, PBMC = 216 + 20 pg/ml, pDC = 167 + 50 pg/ml). Thus, the same concentration of neutralizing Abs used for the PBMC experiments was used for supernatants from purified pDC.

**FIGURE 4.** Impact of A-class ODNs on cytokine and perforin production by iNKT cells. PBMCs were incubated for 24 h with 4 μg/ml A-class ODNs (control and CpG ODN) and the expression by iNKT cells of IFN-γ, IL-4, TNF-α, perforin and CD69 was evaluated by intracellular staining and flow cytometry. As a control for positive activation, the PBMCs were incubated by 6 h with PMA (50 ng/ml) and ionomycin (500 ng/ml). It is shown that, despite the stimulation with A-class CpG ODN up-regulating the expression of CD69, the incubation with this CpG ODNs did not increase the expression IFN-γ, IL-4, TNF-α or perforin by iNKT cells. Data are reported as means ± SD (n = 3).
24 h of incubation with or without A-Class CpG ODNs, using commercial ELISA kits. The purity of iNKT cells was always >85%, while the purity of mDCs and pDCs was >90% (Fig. 7A). Consistent with the observation that iNKT cells do not express TLR9, treatment of purified iNKT cells with A-Class CpG ODNs did not result in the up-regulation of CD69 expression (Fig. 7B). Similar results were observed when purified iNKT cells and mDCs were cocultured and incubated without stimulation or with control and A-Class CpG ODNs (p = 0.437 and p = 0.382). When iNKT cells were cocultured with pDCs and incubated with A-Class CpG ODNs, the expression of CD69 on iNKT cells significantly increased in comparison with unstimulated cells or incubated with control ODNs (p = 0.020 and 0.007, respectively) (Fig. 7B). Thus, CpG ODN-mediated activation of iNKT cells is pDC dependent, and quiescent iNKT cells fail to respond to unstimulated mDCs when purified and cultured together in vitro.

iNKT cells are strongly activated by TCR-mediated CD1d-restricted signals, particularly when mDCs are used as APCs. It is also well known that α-GalCer is presented by CD1d and a potent activator of iNKT cells (49, 50). To determine whether pDC-derived conditioned medium could license iNKT cells to respond to mDCs, purified iNKT cells were treated with conditioned or control medium and then cocultured with mDCs plus increasing doses of α-GalCer. First, purified iNKT cells were incubated with/without pDC-conditioned supernatant (dilution 1/2 in complete culture medium), and after 24 h of incubation, iNKT cells were washed, and cocultured with purified mDC with/without α-GalCer (0, 10, 20, and 50 ng/ml). Strikingly, only those iNKT cells that had been primed with conditioned medium from CpG ODN-treated pDCs were able to respond to α-GalCer-loaded mDCs. These preactivated iNKT cells acquired the ability to respond to α-GalCer in a dose-dependent fashion by secreting IFN-γ, and to much lesser extent, IL-4, and vigorously proliferating (Fig. 8). In comparison, iNKT cells incubated with conditioned medium from control ODN-incubated pDCs did not secrete cytokines or proliferate. Thus, soluble factors produced by CpG ODN-stimulated pDCs enhance the CD1d-restricted activation of iNKT cells and licensed these T cells to interact with mDCs.

Because CpG ODN enhanced iNKT cell responses to α-GalCer-loaded mDC and TLR4 ligands have been shown to directly enhance CD1d-dependent Ag presentation to iNKT cells (33), the ability of ODNs to support expansion and survival of iNKT cells

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<thead>
<tr>
<th>Table III. Concentration of cytokines in supernatants from PBMCs cultured with A-class CpG ODNa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>IFN-α (n = 8)</td>
</tr>
<tr>
<td>IP-10 (n = 8)</td>
</tr>
<tr>
<td>TNF-α (n = 8)</td>
</tr>
<tr>
<td>IL-6 (n = 8)</td>
</tr>
<tr>
<td>IL-7 (n = 4)</td>
</tr>
<tr>
<td>IL-12 (n = 8)</td>
</tr>
<tr>
<td>IL-15 (n = 4)</td>
</tr>
<tr>
<td>IL-18 (n = 4)</td>
</tr>
<tr>
<td>IFN-γ (n = 4)</td>
</tr>
</tbody>
</table>

aCytokine concentrations (pg/ml) were measured in culture supernatants after 24 h of incubation with or without A-Class CpG ODNs, using commercial ELISA kits (detection limit, 3.5 pg/ml).

bMean ± SD.

Table IV. Role of IFN-1 on A-Class CpG ODN-mediated activation of iNKT cells

<table>
<thead>
<tr>
<th>Individual</th>
<th>Frequency of iNKT cells (%)</th>
<th>No stimulation</th>
<th>A-Class CpG ODN</th>
<th>A-Class ODN and Anti-IFN-1</th>
<th>Reduction of CD69 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>3.9</td>
<td>59.5</td>
<td>36.7</td>
<td>41.0%</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>9.5</td>
<td>60.1</td>
<td>35.9</td>
<td>47.8%</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>4.9</td>
<td>33.3</td>
<td>16.8</td>
<td>58.1%</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>3.6</td>
<td>41.8</td>
<td>19.6</td>
<td>58.2%</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>8.2</td>
<td>48.3</td>
<td>21.4</td>
<td>67.1%</td>
</tr>
<tr>
<td>6</td>
<td>0.20</td>
<td>4.6</td>
<td>30.4</td>
<td>15.2</td>
<td>58.9%</td>
</tr>
<tr>
<td>7</td>
<td>0.22</td>
<td>5.5</td>
<td>72.5</td>
<td>62.6</td>
<td>14.8%</td>
</tr>
<tr>
<td>8</td>
<td>0.27</td>
<td>2.2</td>
<td>27.2</td>
<td>21.1</td>
<td>24.4%</td>
</tr>
<tr>
<td>9</td>
<td>0.29</td>
<td>2.7</td>
<td>48.4</td>
<td>32.3</td>
<td>34.6%</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>0.17 (± 0.08)</td>
<td>4.9 (± 2.5)</td>
<td>46.8 (± 15.3)</td>
<td>29.1 (± 15)</td>
<td>44.9% (± 17.7)</td>
</tr>
</tbody>
</table>

The percentage of iNKT cells positive for CD69 was determined by flow cytometry. The iNKT cells were evaluated via the lymphocyte gate, using mAbs against Vβ11 and Vα24 or against CD3 and the invariant α-chain (clone B611). The reduction of CD69 expression by the IFN-1 blocking mixture was calculated with the following formula:

\[ \frac{1}{\frac{\% \text{ A-Class CpG ODN} \text{ alone} - \% \text{ No stimulation}}{\% \text{ A-Class CpG ODN} \text{ with anti-IFN-I} - \% \text{ No stimulation}}} \times 100 \]
FIGURE 5. Role of cytokines on the A-class CpG ODN-mediated activation of iNKT cells. A, PBMCs were incubated 24 h with A-class CpG ODN (4 µg/ml), rh-IFN-α (5,000 U/ml) or supernatant from CpG ODN-stimulated PBMCs (dilution 1/2 with complete culture medium); the activation mediated by IFN-I was blocked with a combination of anti-IFN-α (10,000 U/ml), anti-IFN-β (3,000 U/ml) and anti-CD118 (10 µg/ml). Then, the expression of CD69 on iNKT cells was evaluated by flow cytometry, gating on the iNKT cells with mAbs 6B11 and anti-CD3. Blocking the IFN-I partially inhibited the expression of CD69 induced by CpG ODNs or PBMC-conditioned supernatant. Data are reported as means ± SD (n = 3). B, PBMCs cultured with A-class CpG ODNs were coincubated with neutralizing Abs against IFN-α (10,000 U/ml), TNF-α (10 µg/ml), IL-6 (0.1 µg/ml), and IP-10 (10 µg/ml). After 24 h, the expression of CD69 on iNKT cells was determined by flow cytometry. The neutralization of IFN-α and TNF-α significantly decreased the CpG ODN-induced CD69 expression, while there was an additive effect observed when all the neutralizing Abs were used in combination. Data are reported as means ± SD (n = 3).

FIGURE 6. Neutralization of IFN-α and TNF-α partially inhibits the expression of CD69 on purified iNKT cells incubated with supernatants from CpG ODN-stimulated purified pDCs. A, The iNKT cells and pDCs were purified from PBMCs using anti-6B11 PE plus anti-PE microbeads or anti-BDCA-2 microbeads, respectively, and the POSSELD program of AutoMACS. In these representative dot plots, the percentage of pDCs and iNKT cells is shown in the positive fraction after the magnetic isolation. B, To obtain pDC-conditioned supernatants, pDCs were purified with anti-BDCA-2 microbeads and incubated by 24 h with A-class CpG ODN; these supernatants were collected, filtered (0.22 µm), and used for the coincubation with purified iNKT cells and neutralizing Abs against IFN-α (10,000 U/ml), TNF-α (10 µg/ml), IL-6 (0.1 µg/ml), and/or IP-10 (10 µg/ml). The incubation of purified iNKT cells with pDC-conditioned supernatants up-regulated the expression of CD69, which was partially blocked by the neutralization of IFN-α and TNF-α. This graphic shows representative data from one of three independent experiments.
was evaluated. PBMCs were stimulated with α-GalCer in the presence or absence of CpG ODN or LPS, with or without depletion of pDC and B cells (Fig. 9). Interestingly, activation of iNKT cells in the presence of CpG resulted in the preferential expansion and long-term survival of DN iNKT cells. After deletion of pDC and B cells, only CD4+ iNKT cells expanded in response to α-GalCer. And, as expected, the deletion of TLR9-responding cells had a significant impact on the ability of iNKT cells to respond to α-GalCer. It also should be noted that, consistent with the results of Brigl et al. (33), LPS induced the most robust expansion of CD4+ and DN iNKT cells (data not shown), but these cells failed to survive in long-term cultures (Fig. 9B).

Discussion

Freshly isolated resting pDCs express low levels of MHC class I and II and CD86 and do not express detectable levels of CD80 or CD86. In humans, these cells express CD4 and CD123 but lack the expression of myeloid markers, such as CD11b, CD11c, CD13, and CD33, and are found in blood and secondary lymph organs (4). These cells participate in innate responses to several different types of viruses, including influenza, HSV-family viruses, and HIV (24). (51, 52) pDCs rapidly produce vast amounts of IFN-I within the first 24 h of viral infection (21, 22) This burst of cytokine secretion is thought to be critical for activation of other cells of the innate and adaptive immune systems, including the induction of mDC maturation (4), (23, 24). Over the next 48–72 h, in a process that is dependent on autocrine stimulation by IFN-I, pDCs differentiate into mature DCs capable of stimulating T cells (16). The pDC-dependent T cell effects are thought to be dependent on IFN-I but independent of IL-12. In human DCs, the main source of IL-12 important for activating NK cells and inducing Th1-like T cell responses is thought to be mDCs (53, 54). Consequently, as is the case with TLR profiles, there appears to be clear species differences between human and murine pDC and mDC effector function (4, 9). However, the pDC lineage is unique in the capacity to secrete massive amounts of IFN-I.

The cross-talk between pDCs and mDCs and the ability of pDCs to induce adaptive T cell responses by activating mDCs have recently been demonstrated (23, 24, 51, 55). Treatment of immature mDCs with IFN-I leads to their activation and enhanced production of IL-12, IL-15, and IL-18 (56, 57). A similar IFN-I-dependent or CpG-dependent effect has been noted for NK cell activation (55, 58).

Interestingly, iNKT cell activation is important for the same mDC maturation, NK activation, and antiviral or antitumor responses (28, 29, 31, 32, 36, 59–63). Yet, iNKT cells do not express detectable levels of TLRs, do not respond directly to CpG ODNs even in the presence of highly purified mDCs (Fig. 7), and human
pDCs and/or lymphoid DCs do not express CD1d (14, 49, 64, 65). Activation of iNKT cells by CpG ODNs has been reported to be IL-12 and IL-15 dependent (60, 66, 67). However, despite the significant congruent involvement of iNKT cells and pDCs in these responses, the consequences of pDC activation on iNKT cell function has not been investigated and is poorly understood.

Thus, to determine whether pDC activation can control iNKT cell function, a study of TLR9-dependent activation with CpG ODNs was undertaken. Treatment of PBMCs with CpG ODNs strongly induced activation markers on iNKT cells. This effect was most pronounced for the pDC-selective A-class CpG ODNs (Tables I and II) (38, 68). Even though CpG ODN treatment resulted in the induction of activation markers on iNKT cells, and in this condition of stimulation it was observed a dose-response effect for the production of IFN-γ regarding the concentration of α-GalCer used for the incubation with purified mDCs. This graphic shows representative data from one of four independent experiments. B, Twenty-four hours after the collection of supernatants, 1 μCi of [3H]thymidine was added to each well and, after an incubation overnight, the cell culture plate was harvested and the proliferation of the iNKT cells was determined measuring the cpm in a beta counter. In this representative figure of four independent experiments, it is shown that, when purified iNKT cells were preincubated with pDC-conditioned supernatant, there was an increase in the proliferation of iNKT cocultured with α-GalCer-loaded mDCs.

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**FIGURE 8.** Role of CpG ODN-activated pDCs on the CD1d-restricted activation of iNKT cells. A, Purified iNKT cells were incubated by 24 h with/without supernatants from CpG ODN-stimulated pDCs (dilution 1/2); after, these preincubated iNKT cells were washed and cocultured by triplicate with purified mDCs previously incubated by 24 h with/without α-GalCer (0, 10, 20, and 50 ng/ml). After 24 h of coculture, supernatants (100 μl per well) were collected and replaced with fresh complete culture medium. The concentration of IFN-γ and IL-4 was measured in these supernatants by ELISA; there was an increase in the secretion of IFN-γ and IL-4 when the iNKT cells were preincubated with pDC-conditioned supernatants, and in this condition of stimulation it was observed a dose-response effect for the production of IFN-γ regarding the concentration of α-GalCer used for the incubation with purified mDCs. This graphic shows representative data from one of four independent experiments. B, Twenty-four hours after the collection of supernatants, 1 μCi of [3H]thymidine was added to each well and, after an incubation overnight, the cell culture plate was harvested and the proliferation of the iNKT cells was determined measuring the cpm in a beta counter. In this representative figure of four independent experiments, it is shown that, when purified iNKT cells were preincubated with pDC-conditioned supernatant, there was an increase in the proliferation of iNKT cocultured with α-GalCer-loaded mDCs.

**FIGURE 9.** Frequency of expanded iNKT cell subgroups in culture of PBMCs activated with α-GalCer ODNs. A, Effect of ODNs on iNKT cells subsets after α-GalCer activation in the presence or absence of pDC and B cells. Whole PBMC, or PBMC where pDC and B cells were removed using anti-BDCA-2 and anti-CD19 microbeads, were labeled with CFSE and cultured for 8 days with α-GalCer (100 ng/ml) with or without ODNs. Cells were stained with 6B11-PE and CD4-APC to enumerate iNKT cell subsets, and gated on 6B11+ cells to determine the extent of cell division by CFSE dilution. The data are representative of three separate experiments. B, Activation of iNKT cells with α-GalCer and ODNs results in selective long-term survival of DN iNKT cells. PBMC were activated with control IL-2 (20 U/ml), IL-2, and α-GalCer + ODNs, or IL-2 and α-GalCer + LPS (1 μg/ml) and cultured for 21 days. Cells were supplemented with IL-2 and fresh medium every seventh day. The data are representative of four separate experiments.
that direct cell contact with pDC, in part mediated by OX40/ OX40L, contributes to the activation of iNKT following CpG exposure (69). Marshner et al. (69) examined the consequences of pDC and iNKT interactions, and their results are in agreement with respect to licensing of iNKT cells, role of IFN-γ, and subsequent activation by mDCs. However, these authors found that cell-cell contact, including pDCs that do not express detectable CD1d, was necessary for the effect. In the results presented in this study, licensing of iNKT cells to recognize CD1d on mDCs did not require cell-cell contact of iNKT cells with pDC. These differences may be the result of different experimental protocols. Marshner et al. (69) admixed in semi pure iNKT cells, selected on the basis of Vα24 expression, into PBMC to levels of 10% whereas either PBMC or reconstitution experiments were used in this study.

It is unlikely that IL-6, IL-7, IL-12, IL-15, IL-18, or IFN-γ made any significant contribution to the induction of activation markers on iNKT cells, because these cytokines were absent or not induced when comparing A-class CpG with control ODN treatment (Table III and Figs. 5 and 6). Importantly, exposure of iNKT cells to on iNKT cells, because these cytokines were absent or not induced any significant contribution to the induction of activation markers.


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