CD1d ligation on human monocytes directly signals rapid NF-κB activation and production of bioactive IL-12

Simon C. Yue, Angela Shaulov, Ruojie Wang, Steven P. Balk, and Mark A. Exley*

Cancer Biology Program, Division of Hematology and Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215

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Natural killer T cells (NKT cells) expressing a semiinvariant CD1d-reactive T cell receptor (invariant NKT, iNKT) can be rapidly activated by monocytes or immature dendritic cells (iDCs) bearing a CD1d-presented glycolipid antigen and can in turn stimulate these myeloid cells to mature and produce IL-12. Previous studies have shown that iNKT-produced IFN-γ and CD40 ligand contribute to this dendritic cell maturation. This study demonstrates that CD1d ligation alone, in the absence of iNKT, could rapidly (within 24 h) stimulate production of bioactive IL-12p70 by CD1d+ human peripheral blood monocytes as well as iDCs. IFN-γ alone had no effect, but it markedly enhanced CD1d-stimulated IL-12 production. Monocyte differentiation, as assessed by CD40 and CD1a up-regulation, was also accelerated by CD1d stimulation, consistent with this representing a physiological response. CD1d ligation on the human monocytic cell line THP-1 similarly specifically stimulated IL-12 production. Biochemical studies showed that IL-12 activation can be enhanced by B7 ligation of CD28 and that IL-12 provided by DCs can further enhance iNKT IFN-γ production.

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Abbreviations: NKT cell, natural killer T cell; iNKT, invariant NKT cell; ogalcer, α-galactosylceramide; DC, dendritic cell; iDC, immature DC; ILM, N-acetyl-α-L-fucosyl-L-lysinyllactosamine; APC, antigen-presenting cell; TCR, T cell receptor; pI, phospho-I.

*To whom correspondence should be addressed at: Harvard Institute of Medicine, 330 Brookline Avenue, Boston, MA 02215. E-mail: exley@bidmc.harvard.edu.

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alent amounts of protein were run on SDS-PAGE and transferred to nitrocellulose. CD1d was detected with C3D5, a mouse mAb generated against a CD1d-GST fusion protein (23). Phospho-IκB (pIκB) was detected with a pIκB-specific mAb (Cell Signaling Technology, Beverly, MA). Secondary anti-mouse IgG-horseradish peroxidase and chemiluminescence substrate were from PerkinElmer Life Sciences.

Results

Reciprocal CD1d-Mediated Activation of Human iNKT, Monocytes, and iDCs. Previous studies have shown that CD1d is constitutively expressed by human monocytes as well as at somewhat elevated levels by DCs (23, 24). We confirmed this pattern of CD1d expression (Fig. 1A). Interaction of CD1d+ APC pulsed with galcer and iNKT stimulates them to release IL-12 and IFNγ, respectively (18, 23–25). Murine iNKT similarly induce APC to produce IL-12 through IFNγ and CD40L-dependent mechanisms (11, 12, 20). To determine whether there were functional differences between CD1d+ monocytes versus iDCs in their stimulation of iNKT, iNKT lines were prepared from healthy donors with a mAb against complementarity-determining region 3 of the invariant TCR (21). More than 90% of these iNKT lines from multiple donors expressed the invariant CD1d-reactive CD8 TCR and Vα24-Jα18 TCR and Vγ11 and were a mixture of CD4+CD8– and CD4–CD8– cells (data not shown).

Rested iNKT lines were stimulated with CD1d+ peripheral blood monocytes or iDCs in the presence or absence of galcer and then assessed for cytokine production as shown in Fig. 1B. There was no significant production of IFNγ when iNKT were cultured with monocytes or iDCs in the absence of galcer, but addition of galcer resulted in substantial IFNγ production after 1 day in both the monocyte and iDC cultures (Fig. 1B). IFNγ production was ~4-fold higher in the iDC cultures (Fig. 1B), consistent with the greater amount of IL-12 produced by the iDCs (see below). IL-4 production was also stimulated by galcer-pulsed monocytes and iDCs and was greater in the iDC cultures (Fig. 1C). These results demonstrated that both mono-

Flow Cytometry and Immunoblotting. FACS was performed with 1 × 10^6 cells after blocking with 10% human serum. iNKT lines were analyzed with anti-Vα24-phycoerythrin (PE) (Coulter) and 6B11-FITC (anti-invariant TCR) (21) or anti-Vγ11-FITC (Coulter). Expression of APC CD1 molecules and CD40 were assessed by flow cytometry. For single-color FACS, primary mAb were used at 10 μg/ml followed by anti-mouse IgG-FITC (Kirkegaard & Perry Laboratories). The primary mAb for CD1a–c were V6/6, 4A7/6, and M241, respectively (22), anti-CD40, and isotypes (BD Pharmingen), trace lymphocytes being gated out for CD1d FACS. Two-color FACS used lineage markers CD1a-PE, CD1d 42.1-PE, CD14-FITC, and CD40-FITC (BD Pharmingen). The CD1d and mock-transfected C1R cells were described in ref. 22.

For immunoblotting, cells were lysed in 1% SDS and equivalent amounts of protein were run on SDS/PAGE and transferred to nitrocellulose. CD1d was detected with C3D5, a mouse mAb generated against a CD1d-GST fusion protein (23). Phospho-IκB (pIκB) was detected with a pIκB-specific mAb (Cell Signaling Technology, Beverly, MA). Secondary anti-mouse IgG-horseradish peroxidase and chemiluminescence substrate were from PerkinElmer Life Sciences.
cytes and iDC could stimulate iNKT to produce IFNγ and IL-4, although iDCs were more potent inducers of both cytokines.

Previous studies showed that iDCs were stimulated to produce IL-12 when cultured with iNKT and galcer (11, 12, 18). IL-12 production was next assessed to determine whether iNKT could stimulate IL-12 production from human monocytes as well as iDCs. Significantly, both monocytes and iDC were rapidly (day 1) stimulated to produce bioactive IL-12p70 when cultured with iNKT and galcer (Fig. 1D). The higher levels of IL-12 produced by the iDCs were consistent with relative CD1d levels (Fig. 1A) as well as with the increased potency of iDC as APC in general and in stimulating iNKT cytokine production (Fig. 1B and C).

Finally, experiments were performed to determine whether iNKT-induced human monocyte and iDC IL-12 responses were dependent on iNKT IFNγ as previously shown in mice (11, 12, 20). Similar coincubations of iNKT with galcer and monocytes were performed in the presence or absence of neutralizing mAb to IFNγ. The results showed that anti-IFNγ could substantially inhibit monocyte IL-12 production (Fig. 1E). Furthermore, additional exogenous IFNγ could enhance iNKT-dependent monocyte IL-12 production, whereas IFNγ without galcer had no effect (Fig. 1E).

Based on these data showing that CD1d+ human monocytes (as well as iDCs) could rapidly stimulate and be activated by iNKT, we initiated studies to further define the molecular mechanisms mediating monocyte activation by iNKT.

**CD1d Ligation Induces IL-12 Production and Maturation of Monocytes.** Murine studies have shown that CD40L expressed by iNKT can stimulate DCs through CD40 ligation and that IL-12 production by the activated DCs can be enhanced by iNKT-produced IFNγ (11, 12, 20). Stimulation of human iDCs by iNKT was similarly found to be CD40L-dependent (18). Significantly, DC activation by iNKT in these studies was also galcer-dependent, suggesting that ligation of CD1d by the invariant TCR might directly contribute to DC activation (in addition to the clear role of TCR ligation by CD1d in activating the iNKT). The data above (Fig. 1E) further supported a similar IFNγ-dependent mechanism. Therefore, the potential roles of CD1d and CD40 ligation and of IFNγ in stimulating IL-12 production by monocytes and iDC were directly assessed.

CD40 and CD1d ligation by iNKT were modeled by using plate-bound CD40 and CD1d mAb, and iNKT-produced IFNγ was replaced by exogenous IFNγ. To minimize potential inhibitory effects mediated by Fc receptors and to optimize mAb orientation, plates were first coated with protein G followed by specific or control mAb. Peripheral blood monocytes cultured on these plates for up to 8 days in the absence of IFNγ did not produce significant amounts of IL-12p40, but in the presence of IFNγ CD1d ligation resulted in IL-12p40 production (Fig. 2A). In contrast, IFNγ alone had no effect, and monocyte IL-12p40 production was not stimulated by CD40 mAb.

Although the transcriptional control of IL-12 is primarily through IL-12p40, this chain associates with IL-23p25 to generate the bioactive IL-12p70. Therefore, IL-12p70 production by CD40- and CD1d-stimulated monocytes was also assessed. Monocytes cultured with CD1d mAb, even in the absence of IFNγ, produced substantial IL-12p70 (Fig. 2B). This CD1d-stimulated IL-12p70 production was further enhanced by the addition of IFNγ. CD40 mAb also stimulated IL-12p70 production, but only in the presence of IFNγ, and it was less potent than CD1d (Fig. 2B).

Based on these results, we compared monocyte production of IL-12 in response to CD1d ligation versus LPS stimulation, the latter being a well established mediator of myeloid cell maturation and IL-12 production that functions through Toll-like receptor 4. LPS rapidly stimulated the production of IL-12p70. Significantly, CD1d mAb stimulated comparable levels of IL-12p70 production after 1 or 3 days (Fig. 2C).

We also compared the activity of CD1d mAb 51.1, as used above, to several distinct mAb recognizing at least three CD1d epitopes (22–24). Both 51.1 and 42.1 recognizing distinct but overlapping CD1d epitopes showed marked induction of IL-12 with a dose-dependent enhancement by IFNγ, whereas IFNγ alone had no effect (Fig. 2D). As shown in Fig. 2E, the other CD1d mAb were also able to stimulate some monocyte IL-12p70 production. These results showed that CD1d ligation with any of several mAb directed at multiple epitopes could alone provide a rapid and potent stimulus for monocyte production of bioactive IL-12p70, which could be further enhanced by IFNγ.

Next, we addressed whether CD1d ligation could also influence monocyte differentiation. CD40 is minimally expressed by monocytes but is markedly up-regulated on iDC, and CD1a is expressed only by iDC. As shown in Fig. 2F, CD40 and CD1a up-regulation by cultured monocytes was specifically enhanced by CD1d ligation. This effect was most pronounced at day 3. By
CD1d mAb to stimulate IL-12 production (Fig. 3 included heat inactivation, which abrogated the ability of the bound CD1d mAb was mediating the THP-1 cell stimulation. Plate-bound CD1b and CD1c mAb similarly had no effect. stimulation also depended on protein G coating (Fig. 3 with BSA or FBS as indicated. (A) mAb against CD1a-d or CD40 were used as stimuli. (B) CD1d mAb with or without heat inactivation (HI) or LPS were used as stimuli. (C) CD1d mAb were used either bound via protein G (PG) or directly applied to plates blocked with BSA or FBS as indicated.

CD1d Ligation Stimulates IL-12 Production by the THP-1 Monocytic Cell Line. To investigate the mechanism of CD1d-stimulated IL-12 production, we screened human cell lines and found that THP-1, a human monocytic leukemia cell line, expressed CD1d at levels similar to those observed on peripheral blood monocytes (~1% of the level of C1R.CD1d cells), confirmed by immunoblotting (data not shown). In addition to CD1d, the THP-1 cells expressed low levels of CD1a but did not express detectable CD1b, CD1c, or CD40 (data not shown).

We next assessed IL-12 production in response to CD1d ligation on the THP-1 cells. There was no detectable production of the bioactive IL-12p70 under any conditions, including LPS, and no significant stimulation of IL-12p40 in the absence of exogenous IFNγ (data not shown). However, with the addition of IFNγ, the plate-bound CD1d mAb specifically stimulated IL-12p40 production at levels that were comparable to LPS stimulation (Fig. 3A). Consistent with the lack of detectable CD40 expression on the THP-1 cells, there was no stimulation by CD40 mAb. To further determine whether the IL-12 stimulation was specific to CD1d, the effect of CD1a ligation on THP-1 cells was also assessed. In contrast to CD1d, the plate-bound CD1a mAb did not stimulate IL-12p40 release (Fig. 3A). Plate-bound CD1b and CD1c mAb similarly had no effect.

Additional controls confirming that crosslinking by the plate-bound CD1d mAb was mediating the THP-1 cell stimulation included heat inactivation, which abrogated the ability of the CD1d mAb to stimulate IL-12 production (Fig. 3B). CD1d stimulation also depended on protein G coating (Fig. 3C). These results confirmed that THP-1 stimulation was directly mediated by CD1d mAb.

THP-1 Stimulation by CD1d Ligation Is Mediated Through NF-κB. THP-1 cells were next used to investigate the mechanism of CD1d-mediated activation. Because NF-κB plays a major role in regulating myeloid expression of IL-12p40 in response to activation by LPS and other Toll-like receptors, we determined whether the NF-κB pathway was activated in response to CD1d ligation on THP-1 cells. An obligate initial step in NF-κB activation is phosphorylation of IkB by IkB kinase, with subsequent ubiquitination and proteosome-mediated degradation of pIkB and release of NF-κB. To assess this activation step, we carried out immunoblotting for pIkB. THP-1 cells were preincubated with LLM (10 μM), a proteosome inhibitor to block pIkB degradation, and then added to CD1d mAb or control-coated wells. An increase in pIkB was observed within 15 min specifically in wells coated with protein G and CD1d mAb (Fig. 3B). A further increase was seen at 45 min, comparable to the activation observed with LPS at 15 min.

We next carried out inhibitor studies to determine whether the production of IL-12p40 in response to CD1d ligation depended on NF-κB activation. CD1d-stimulated production of IL-12 could be blocked by a number of relatively nonspecific antagonists of NF-κB, including proteosome inhibitors and pyrrolidine dithiocarbamate (not shown). Parthenolide is a relatively specific NF-κB antagonist that inhibits activity of the IkB kinase complex and phosphorylation of IkB (26). To enhance specificity, we first carried out parthenolide dose–response studies to determine the minimal concentration of drug needed to suppress LPS-mediated phosphorylation of IkB in THP-1 cells. As shown in Fig. 4B, parthenolide at a concentration of 10 μM (in the

![Fig. 4](image-url)
This study addressed the molecular interactions mediating activation of human CD1d\(^+\) monocyctic lineage cells by CD1d-reactive NKT cells. We initially showed that cultured human iNKT could stimulate the rapid production of IL-12 from both \(\text{gal}+\) monocytes and iDCs. Ab blocking implicated IFN\(\gamma\) in the IL-12 response of monocytes, consistent with murine data (11, 12). Possible mechanisms mediating this stimulation, including CD40L ligation of APC CD40, TCR ligation of the CD1d-galcer complex, and iNKT-produced IFN\(\gamma\), were then further dissected. Remarkably, CD1d ligation alone, through any of multiple independent mAb against at least three CD1d epitopes, could rapidly stimulate CD1d\(^+\) monocytes as well as iDC to produce bioactive IL-12\(\gamma70\). Furthermore, CD1d ligation enhanced monocyte differentiation, consistent with this representing a physiological response. Notably, IFN\(\gamma\) by itself had no effect, but it markedly increased the CD1d-mediated stimulation of IL-12 production.

CD1d-dependent IL-12 production was confirmed in the human THP-1 monocyctic leukemia cell line and was found to be mediated by rapid activation of the NF-\(\kappa\)B signal transduction pathway. Finally, we found that both CD40 and LPS, two known physiological stimuli of iDC and monocytes, respectively, could act synergistically with CD1d to stimulate IL-12\(\gamma70\) production. Taken together, these studies support a direct role for CD1d ligation in the rapid activation of the innate immune response by CD1d-reactive NKT cells.

A major function of conventional activated CD4\(^+\) T cells is to interact with iDCs bearing cognate MHC class II-presented antigens and stimulate their further maturation through CD40L ligation of CD40. Autoreactive T cell clones specific for group 1 CD1 proteins (CD1a, b, or c) were similarly found to stimulate the maturation of human iDCs, but distinct mechanisms including TNF\(\alpha\) appeared to be involved and IL-12 production was observed only when iDCs were also stimulated with LPS (18). In contrast to previous studies, the data reported here demonstrate that CD1d ligation can directly mediate monocyctic cell activation. The ability to directly stimulate monocytes through CD1d ligation may be a unique property of CD1d-reactive NKT cells and reflect an important physiological role for iNKT–monocyte interactions in the initiation of innate immune responses. The hypothesis that monocytes are physiologically activated by iNKT through CD1d ligation is consistent with the constitutive expression of CD1d on monocytes as well as DCs (23, 24), versus the regulated expression of proteins mediating interactions with conventional T cells (MHC class II, CD1a–c, CD40, CD80, and CD86).

CD1d has not been shown to associate with adaptor proteins that might link it to NF-\(\kappa\)B, and previous biochemical studies of CD1d have focused on endosomal targeting and its role in antigen presentation (27, 28). However, there are data suggesting that the CD1d cytoplasmic tail may mediate other interactions. An early study found that human T cell lines could be activated by MHC class II–invariant chain complex in the endoplasmic reticulum and associate with this complex on the cell surface, interactions that were independent of the CD1d cytoplasmic tail (31). This MHC class II association is intriguing, because TCR-mediated ligation of MHC class II proteins can provide a coactivation signal in B cells (32). However, this B cell signal is transduced through MHC class II association with the B cell receptor and its signal transduction complex, which are not present in monocytes. One study has shown that human monocytes can be activated by MHC class II crosslinking through mitogen-activated protein kinase pathways (33). Although we have not observed consistent mitogen-activated protein kinase pathway activation in response to

**Discussion**

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CD1d ligation (data not shown), a link between MHC class II and CD1d signaling in monocyctic cells remains possible. The established role of NF-κB in activating IL-12p40 transcription in response to other stimuli suggests that CD1d ligation functions similarly upstream of NF-κB. Further studies are underway to determine whether CD1d is linked to NF-κB by AP-2 or MHC class II proteins or by a distinct mechanism.

Recently, physiologically relevant endogenous and pathogen-derived relatively high-affinity CD1d-presented antigens recognized by the invariant TCR have been defined (34–36). This also is significant with respect to CD1d-mediated signal transduction, because the invariant TCR may require a high-affinity antigen to achieve a threshold level of CD1d crosslinking. However, an alternative to a high-affinity antigen is a lower-affinity multivalent antigen on the monocyte cell surface. In this context, CD1d is known to accumulate in lipid rafts, and their disruption can inhibit iNKT recognition (37). An interaction between CD1d and MHC class II on the cell surface may provide a further mechanism for CD1d clustering through CD4 binding of MHC class II/CD1d complexes in the case of CD4+ iNKT. In any case, even with a relatively high-affinity CD1d ligand it is not clear whether the invariant TCR can mediate the level of CD1d crosslinking achieved with CD1d Abs. Indeed, it seems likely that the signal generated in vivo by CD1d ligation will in many cases not be strong enough to by itself trigger monocyte activation and will instead be integrated with other signals. In addition to CD40 ligation, this includes at least one Toll-like receptor-mediated signal (LPS) functioning through NF-κB, which would provide a molecular mechanism for iNKT modulation of innate immune responses.

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