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*J Immunol* 2014; 193:2994-3002; Prepublished online 13 August 2014;
doi: 10.4049/jimmunol.1302600
http://www.jimmunol.org/content/193/6/2994

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/08/12/jimmunol.1302600.DCSupplemental

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The Natural Product Phyllanthusmin C Enhances IFN-γ Production by Human NK Cells through Upregulation of TLR-Mediated NF-κB Signaling

Youcai Deng,*††1 Jianhong Chu,‡†1 Yulin Ren,‡†1 Zhijin Fan,‡†1 Xiaotian Ji,‡ Jinhong Mundy-Bosse,‡ Shunzong Yuan,‡ ‡‡ Tiffany Hughes,‡ Jianying Zhang,‡ Baljash Cheema,§ Andrew T. Camardo,‡ Yong Xia,** Li-Chu Wu,** Li-Shu Wang,†‡ Xiaoming He,** A. Douglas Kinghorn,§ Xiaohui Li,‡ Michael A Caligiuri,‡*† and Jianhua Yu*‡‡

Natural products are a major source for cancer drug development. NK cells are a critical component of innate immunity with the capacity to destroy cancer cells, cancer-initiating cells, and clear viral infections. However, few reports describe a natural product that stimulates NK cell IFN-γ production and unravel a mechanism of action. In this study, through screening, we found that a natural product, phyllanthusmin C (PL-C), alone enhanced IFN-γ production by human NK cells. PL-C also synergized with IL-12, even at the low cytokine concentration of 0.1 ng/ml, and stimulated IFN-γ production in both human CD56bright and CD56dim NK cell subsets. Mechanistically, TLR1 and/or TLR6 mediated PL-C’s activation of the NF-κB p65 subunit that in turn bound to the proximal promoter of IFNG and subsequently resulted in increased IFN-γ production in NK cells. However, IL-12 and IL-15Rs and their related STAT signaling pathways were not responsible for the enhanced IFN-γ secretion by PL-C. PL-C induced little or no T cell IFN-γ production or NK cell cytotoxicity. Collectively, we identify a natural product with the capacity to selectively enhance human NK cell IFN-γ production. Given the role of IFN-γ in immune surveillance, additional studies to understand the role of this natural product in prevention of cancer or infection in select populations are warranted.

The online version of this article contains supplemental material.

Abbreviations used in this article: AML, acute myeloid leukemia; ChIP, chromatin immunoprecipitation; HER293T, human embryonic kidney 293T; IKKβ, inhibitory κ-B kinase β; PL-C, phyllanthusmin C; shRNA, short hairpin RNA; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.
many reasons, such as induction of regulatory T cells by IL-2 (22, 23), impairment of cytokine signaling via STAT-4 as a result of autologous hematopoietic stem cell transplantation or chemotherapy (24–26), and the systemic toxicity associated with the exogenous delivery of these cytokines that can, in some instances, activate a multitude of immune effector cells (27, 28).

There are multiple signaling pathways to control IFN-γ gene expression and its protein secretion. These include positive signaling pathways, such as the MAPK signaling pathway, the JAK–STAT signaling pathway, the T-BET signaling pathway, and the NF-kB signaling pathway, as well as negative regulation via the TGF-β signaling pathway (29). Activation of the MAPK pathway involves induction of ERK and p38 kinase, in part through the activation of Fos and Jun transcription factors (29). Binding of IL-12 to its receptor activates the JAKs-tyrosine kinase 2 and JAK2, leading to phosphorylation and activation of STAT-4 and other STATs as well (30). In human NK cells, IL-15 activates the binding of STAT1, STAT3, STAT4, and STAT5 to the regulatory sites of the IFNG gene (18). The activation of numerous transcription factors, including NF-kB, may be critical for achieving a maximal activation of IFNG transcription. Many of the synergetic stimuli that enhance IL-12–mediated IFN-γ production by NK cells share the ability to activate the transcription factor NF-kB (31). NF-kB is also an important downstream mediator of TLR signaling, which becomes activated in immune cells during injury and infections (32–34).

Small-molecule natural products have been the single most productive source for the development of drugs. By 1990, >50% of all new drugs were either natural products or their analogs (35, 36), including those which act through immune modulation (37). This proportion has decreased in recent years, perhaps because the proportion of synthetic small molecules has increased, while performing the isolation of natural products from crude extracts is time-consuming and labor-intensive; however, natural products and their analogs still account for >40% of newly developed drugs (38, 39). The popularity of developing drugs from natural products and their analogs is at least in part due to their relatively low side effects. Natural products provide enormous structural diversity, which also facilitates new drug discovery (40).

In this study, we screened natural products for their ability to enhance NK cell production of IFN-γ. We found that phyllanthusmin C (PL-C), a small-molecule ligan glycoside from plants, can induce NK cell IFN-γ production in the presence or absence of monokines such as IL-12 and IL-15. The induced NK cell activity resulted from enhanced TLR-NF-κB signaling. Interestingly, PL-C negligibly activated T cell IFN-γ production and also did not activate NK cell cytotoxicity. This selectivity of PL-C in immune activation should make it more suitable for development of a new clinically useful immune modulator.

Materials and Methods
Isolation of PBMCs and NK cells
Human PBMCs and NK cells were freshly isolated from leukopaks (American Red Cross, Columbus, OH) as described previously (41). PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA) density gradient centrifugation. NK cells (CD56+CD3–) were enriched with RosetteSep NK cell enrichment mixture (StemCell Technologies, Vancouver, BC, Canada). The purity of enriched NK cells was ≥80% (data not shown), assessed by flow cytometric analysis after staining with CD56-allophycocyanin and CD3-FITC Abs (BD Bio-Sciences, San Jose, CA). These enriched NK cells were further purified with CD56 magnetic beads and LS columns (Miltenyi Biotec, Auburn, CA). The purity of magnetic bead-purified NK cells was ≥99.5% (data not shown), as determined by the aforementioned flow cytometric analysis.

CD56bright and CD56dim NK cell subsets were sorted by a FACSAria II cell sorter (BD Biosciences) based on CD56 cell surface density after staining with CD56-allophycocyanin and CD3-FITC Abs. The purity of CD56bright and CD56dim subsets was ≥99.0% (data not shown). All human work is approved by The Ohio State University Institutional Review Board.

Cell culture and treatment
Primary NK cells, the NKL cell line (a gift from Dr. M. Robertson, Indiana University) and PBMCs were cultured or maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10% FBS (Invitrogen) at 37°C in 5% CO2. The NKL cell line is IL-2–dependent, and therefore, 150 IU/ml recombinant human IL-2 (Hoffman-LaRoche, Pendergrass, GA) was included in the culture, but cells were starved for IL-2 for 24 h prior to stimulation. For stimulation, cells were suspended at a density of 2.5 × 10⁶ cells/ml and seeded into a 6-well culture plate and rested for 1–2 h, followed by addition of stimuli. Cells were treated with PL-C in the presence or absence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml) (R&D Systems, Minneapolis, MN) for 18 h or the indicated time. Cells were harvested for flow cytometric analysis or for RNA extraction to synthesize cDNA for real-time RT-PCR or for protein extraction to perform immunoblotting. Cell-free supernatants were collected to determine IFN-γ secretion by ELISA with commercially available mAb pairs (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer’s protocol as described previously (42, 43). To determine whether PL-C acts on non–IL-12–enhanced IFN-γ production when IL-12 or IL-15 were given at lower concentrations, purified primary NK cells were treated with 1, 0.1 ng/ml IL-12 or 10, 0.1 ng/ml IL-15 with or without 10 μM PL-C for 24 h. Supernatants were then harvested for IFN-γ ELISA. To study NF-kB involvement in PL-C–mediated enhancement of NK cell IFN-γ production, 10 μM NF-kB inhibitor N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) was used to treat both purified primary NK cells and NKL cells together with PL-C in the presence of IL-12, compared with no TPCK treatment. For TLR blocking assays, the purified NK cells were pretreated with 10 μg/ml anti-TLR1 (InvivoGen), anti-TLR3 (Hycult Biotech), anti-TLR6 (InvivoGen), or 10 μg/ml anti-TLR1 plus 10 μg/ml anti-TLR6 for 1 h prior to PL-C and/or IL-12 stimulation. Cells treated with the same concentration of nonspecific anti-IgG were used as control. The blocking Abs were also kept in the culture during the stimulation. For studying the effect of PL-C combined with TLR agonist, cells were treated with or without various concentration of Pam3CSK4 (TLR1/2 agonist) or FSL-1 (TLR6/2 agonist) for 18 h.

PL-C was isolated in chromatographically and spectroscopically pure form from the aboveground parts of plant Phyllanthus poliainae or synthesized (to be reported elsewhere).

Intracellular flow cytometry
Intracellular flow cytometry was performed as described previously (42, 43). Briefly, 1 μM GolgiPlug (BD Biosciences) was added 5 h before cell harvest. After surface staining with CD3-FITC and CD56-allophyocyanin human Abs (BD Biosciences), the cells were then washed and resuspended in Cytofix/Cytoperm solution (BD Biosciences) at 4°C for 20 min. Fixed and permeabilized cells were stained with anti-IFN-γ–PE Ab (BD Biosciences). Labeled cells were used for a flow cytometric analysis. NK cells were gated on CD56+CD3– cells, and CD4+ or CD8+ T cells were gated on CD56+CD3+CD4+ or CD56+CD3+CD8+ cells, respectively. Data were acquired using an LSRII (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

Real-time RT-PCR
Real-time RT-PCR was performed as described previously (42, 43). Briefly, total RNA from purified primary NK cells or NKL cells was isolated with RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 to 3 μg total RNA with random hexamers (Invitrogen). Real-time RT-PCRs were performed as a multiplex reaction with the primer/probe set specific for IFNG, GZMA (granzyme A), GZMB (granzyme B), PRF1 (perforin), Fasl (Fas ligand), and an internal control 18S rRNA (Applied Biosystems, Foster City, CA). mRNA expression of IL-12Rβ1 (IL-12Rβ1), IL-12Rβ2 (IL-12Rβ2), IL-15Rα (IL-15Rα), IL-15Rβ (IL-15Rβ), and HPRT1 was detected by SYBR Green Master Mix (Applied Biosystems). The primers used are shown in Supplemental Table I. Expression levels were normalized to an 18S or HPRT1 internal control and analyzed by the ∆∆Ct method.

Cytotoxicity assay
Cytotoxicity assay was performed as described previously (42, 43). Briefly, multiple myeloma cell line ARH-77 target cells were labeled with 51Cr and cocultured with purified primary NK cells, which were pretreated with or
without 10 μM PL-C for 8 h in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml) prior to the coculture, at various E/T ratios in a 96-well V-bottom plate at 37°C for 4 h. At the end of coculture, 100 μl supernatants were harvested and transferred into scintillation vials with a 3-ml liquid scintillation mixture (Fisher Scientific). The release of 51Cr was counted on a TopCount counter (Canberra Packard, Meriden, CT). Target cells incubated in 1% SDS or complete medium were used to determine maximal or spontaneous 51Cr release. The standard formula of 100 × cpm (cpm experimental release − cpm spontaneous release)/cpm maximal release was used to calculate the percentage of specific lysis.

Immunoblotting

Immunoblotting was performed as described previously (42, 43). The equal number of cells from each sample was directly lysed in 2× Laemmli buffer (Bio-Rad, Hercules, CA) supplemented with 2.5% 2-ME, boiled for 5 min, and subjected to immunoblotting analysis as described previously (42). Abs against p65, p-p65, p-STAT3, p-STAT4, p-STAT5, STAT3, STAT4, STAT5 (Cell Signaling Technology, Danvers, MA), and T-BET (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoblotting. Immunoblotting with Abs against β-actin (Santa Cruz Biotechnology) served as an internal control.

EMSA

Nuclear extracts were isolated using a nuclear extract kit, according to the manufacturer’s instruction (Active Motif, Carlsbad, CA). EMSA was performed as described previously (44). Briefly, a 32P-labeled double-stranded oligonucleotide, 5′-GGGGGTTACAAAAAAATTTCCAGTCC-3′, containing an NF-κB binding site C3–3P (−278 to −268) of the IFNG promoter (45), was incubated with nuclear extracts (2 μg) for 20 min before resolving on a 6% DNA retardation gel (Invitrogen). After electrophoresis, the gel was transferred onto filter paper, dried, and exposed to x-ray films. In Ab gel supershift assays, p65 Abs (Rockland Immunochemicals, Gilbertsville, PA) were added to the DNA–protein binding reactions after incubation at room temperature for 10 min, followed by an additional incubation for 20 min before gel loading.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out with a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY), according to the

FIGURE 1. PL-C enhances IFN-γ production in human primary NK cells. (A) Chemical structure of PL-C. (B) Healthy donor PBMCs (left panel) or enriched NK cells (right panel) were treated with DMSO vehicle control or 10 μM PL-C for 18 h in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml). The cells were harvested and analyzed by intracellular flow cytometry to determine the frequency of IFN-γ−cells in CD56−CD3−NK cells (n = 8 for PBMC and n = 5 for enriched NK). (C) Highly purified (≥99.5%) human primary NK cells were treated with 10 μM PL-C for 18 h to determine the levels of IFN-γ secretion by ELISA. IFN-γ secretion from treatment with PL-C alone (left panel) or in combination with IL-12 (10 ng/ml, middle panel) or IL-15 (100 ng/ml, right panel) is shown. (D) Cells were treated as described in (C) and harvested at 12 h. IFNG mRNA expression was assessed by real-time PCR, and the relative IFNG mRNA expression of each treatment was normalized to untreated vehicle control in the same donor. Data are shown as mean ± SEM (n = 6 in each treatment; error bars represent SEM). *p < 0.05, **p < 0.01, which denote statistical comparison between the two marked treatment groups (B–D). (E) Highly purified (≥99.5%) primary human NK cells were treated with 10 μM PL-C in combination with various concentrations of IL-12 (10, 1, and 0.1 ng/ml) or IL-15 (100, 10, and 1 ng/ml) for 24 h to determine the levels of IFN-γ secretion. Representative data from one of three donors with the similar data are shown. *p < 0.05, **p < 0.01, which denote statistical comparison between the two marked treatment groups and are calculated from data of all tested donors. Error bars represent SD. (F) NKL cells were treated with 10 μM PL-C in the presence of IL-12 or IL-15 for 18 or 12 h to determine the levels of IFN-γ secretion (left panel) or IFNG mRNA expression (right panel), respectively. Data shown represent at least three independent experiments. *p < 0.05, **p < 0.01, respectively, compared with vehicle control. Error bars represent SD.
manufacturer’s protocol. An equal amount (10 μg) of rabbit monoclonal anti-p65 Abs or normal rabbit IgG Abs (Cell Signaling Technology) was used to precipitate the cross-linked DNA/protein complexes. The sequences of primers spanning the different NF-κB sites on the IFNG promoter have been described previously (31). DNA precipitated by the anti-p65 or the normal IgG Abs was quantified by real-time PCR, and values were normalized to input DNA.

**TLR activation assessment**

Human embryonic kidney 293T (HEK293T) cells were cotransfected with TLR1 or 6 expression plasmids (0.5 μg for each) for 24 h along with pGL3-3xB-Luc (1 μg), which contains three tandem repeats of κB site (46), and pRL-TK renilla-luciferase control plasmid (5 ng; Promega). The cells were then treated with various concentrations of PL-C for additional 24 h after replacing old medium with fresh medium. Firefly and renilla luciferase activities were detected by using Dual-Luciferase Reporter Assay System (Promega), and the ratio of firefly/renilla luciferase activities was used to determine the relative activity of NF-κB.

**TLR1 short hairpin RNA knockdown in NKL cells**

A TLR1 short hairpin RNA (shRNA) plasmid was constructed by inserting an RNA interference sequence (5′-GTCTCATCCACGTCTCAAT-3′) into GFP expressing pSUPER-retrovirus vector. Viruses were prepared by transfecting the shRNA plasmid and packaging plasmids into phoenix cells. Infection was performed as follows: NKL cells were cultured in virus-containing medium and centrifuged at 1800 rpm at 32°C for 45 min and then incubated for 2–4 h at 32°C. This infection cycle was repeated twice. GFP-positive cells were sorted on a FACSaria II cell sorter (BD Biosciences). Knockdown of TLR1 in the sorted NKL cells was confirmed by real-time RT-PCR.

**Statistical analysis**

An unpaired Student t test was used to compare two independent conditions (such as PL-C versus control) for continuous endpoints. Paired t test was used to compare two conditions with repeated measures from the same donor. A one-way ANOVA model was used for multiple comparisons. A two-way ANOVA model was used to evaluate the synergistic effect between IL-12 or IL-15 and PL-C. The p values were adjusted for multiple comparisons using Bonferroni method. All tests are two-tailed. A p value ≤ 0.05 was considered statistically significant.

**Results**

**PL-C selectively enhances IFN-γ production in human NK cells**

We were initially interested in >50 candidate compounds (e.g., curcumin, β-glucan, and so on) isolated from edible or nonedible plants, which we screened for their capacity to enhance human NK cell activity. We found that a diphyllin lignan glycoside, PL-C (Fig. 1A), which can be isolated from both edible (e.g., Phyllostachys reticulatus) and nonedible (e.g., Phyllostachys pooniana) plants of the Phyllostachys genus collected from parts of Asia (Supplemental Fig. 1A) (47, 48), was able to enhance IFN-γ production by NK cells. We first demonstrated that, when total PBMCs from healthy donors were cultured with PL-C in the presence of the cytokines IL-12 or IL-15 [stimulators of IFN-γ in NK cells, and each constitutively expressed in vivo (49)], intracellular staining for IFN-γ protein assessed via flow cytometric analysis indicated that NK cell IFN-γ production was significantly increased (Fig. 1B, left panel). In addition, PL-C also significantly enhanced IFN-γ production in enriched NK cells in the presence of IL-12 or IL-15 (Fig. 1B, right panel). To determine whether PL-C directly or indirectly acts on NK cells to enhance their IFN-γ production, we purified NK cells (purity ≥ 99.5%) from total PBMCs via FACS and measured the level of IFN-γ secretion from the purified NK cells using ELISA. Interestingly, PL-C induced NK cell secretion of IFN-γ even in the absence IL-12 or IL-15 (Fig. 1C, left panel). PL-C also enhanced NK cell IFN-γ secretion in the presence of IL-12 or IL-15 stimulation (Fig. 1C, middle and right panels). Statistical analysis indicated a synergistic effect of IL-12 and PL-C on IFN-γ expression (Supplemental Fig. 1B). Our data also showed that PL-C induced IFN-γ gene (IFNG) expression at the transcriptional level regardless of whether it was added alone or in the presence of IL-12 or IL-15 (Fig. 1D). When tested at a much lower concentration of IL-12 (1 and 0.1 ng/ml) or IL-15 (10 and 1 ng/ml), PL-C also could promote IFN-γ production in purified primary NK cells (Fig. 1E). Increased IFN-γ secretion and IFNG mRNA transcription also were found in the IL-2–dependent NK cell line, NKL (Fig. 1F). When PBMCs were used, we found that the majority of IFN-γ–producing cells were NK cells, whereas there were few if any CD4+ or CD8+ T cells responding to PL-C stimulation in combination with IL-12 or IL-15 (Supplemental Fig. 2A). Of note, PL-C showed no effect on NK cell cytotoxicity against the K562 cell line (data not shown) or multiple myeloma cell lines, ARH-77 (Supplemental Fig. 2B) and MM.1S (data not shown), regardless of whether cells were incubated in media alone, with IL-12, or with IL-15. Consistent with this, expression of cytotoxicity-associated genes such as granzyme A, granzyme B, perforin, and Fas ligand were also unaffected by PL-C when costimulated with IL-12 or IL-15 (Supplemental Fig. 2C).

**PL-C induces IFN-γ production in both CD56bright and CD56dim human NK cell subsets**

On the basis of the relative density of CD56 surface expression, mature human NK cells can be phenotypically divided into CD56bright and CD56dim subsets. Human peripheral blood NK cells are composed of ~10% CD56bright NK cells and ~90% CD56dim NK cells (50). Cytokine-activated CD56bright NK cells proliferate and secrete abundant IFN-γ but display minimal cytotoxic activity at rest; in contrast, CD56dim NK cells have little proliferative capacity and produce negligible amounts of cytokine-induced IFN-γ but are highly cytotoxic at rest (50). We found that during costimulation with IL-12 or IL-15, IFN-γ secretion from both CD56bright and CD56dim NK cells was enhanced by PL-C when compared with parallel cultures treated with a vehicle control (Fig. 2A, 2B). Noticeably, when costimulated with PL-C and IL-12, in some donors, CD56dim NK cells even produce more IFN-γ.

![FIGURE 2.](http://www.jimmunol.org/ Downloaded from)
IFN-γ than CD56bright NK cells, as previously reported when NK cells recognize tumor cells (51, 52).

Induction of IFN-γ production by PL-C in human NK cells is correlated with activation of NF-κB signaling

Cytokine-induced IFN-γ production occurs mainly through the JAK-STATs, T-BET, MAPK, or NF-κB signaling pathways (29). Transcription factors in these signaling pathways associate with corresponding binding sites in the regulatory elements of the IFNG gene, subsequently enhancing IFNG mRNA synthesis (29). Therefore, we determined which of these signaling pathways participate in the PL-C–mediated IFN-γ induction in NK cells. We found that NF-κB p65 phosphorylation increased upon stimulation of primary NK cells and the NKL cell line with PL-C alone, whereas the level of total p65 was less or negligibly changed (Fig. 3A, 3B, upper panels). Similarly, the substantial increase of p65 phosphorylation but not total p65 also was observed when primary NK cells or NKL cells were treated with PL-C in the presence of IL-12 or IL-15 (Fig. 3A, 3B, middle and bottom panels). No significant change in the level of p65 transcript was observed in primary NK cells and NKL cells (data not shown).

Next, we assessed whether PL-C affects IL-12R or IL-15R and their downstream STAT signaling pathways. When cotreated with IL-12, PL-C downregulated mRNA expression of IL-12Rβ1, IL-12Rβ2, and IL-15Rα. However, when cotreated with IL-15, PL-C had no obvious effect on all IL-12R or IL-15R, except for a moderate downregulation of IL-12Rβ1 (Supplemental Fig. 3A). We did not observe any upregulation of total or phosphorylated STAT3, STAT4, and STAT5 in either purified primary NK or NKL cells. There was no significant change of T-BET in either purified primary NK cells or NKL cells being treated with PL-C (Supplemental Fig. 3B, 3C). To further explore NF-κB involvement in PL-C–mediated enhancement of NK cell IFN-γ production, we used the NF-κB inhibitor TPCK, because it has been shown to directly modify thiol groups on Cys-179 of inhibitory κ-B kinase (IKKβ) and Cys-38 of p65/RelA, thereby inhibiting NF-κB activation (53). In purified primary NK cells and the NKL cell line, we found that TPCK indeed inhibited PL-C–induced p65 phosphorylation, and this was correlated with an inhibition of PL-C–induced IFN-γ secretion (Fig. 3C).

PL-C augments binding of p65 to the IFNG promoter in NK cells following PL-C stimulation

Since above we showed that PL-C induced NF-κB activity and also enhanced IFN-γ production in NK cells, we next investigated whether PL-C would facilitate the binding of NF-κB to the promoter of the IFNG gene in these cells. Four different NF-κB binding sites at the IFNG locus—κB, C3-1P, C3-3P, and C3 first

**FIGURE 3.** PL-C increases the phosphorylation of p65 in human primary NK and NKL cells. (A) Purified primary human NK cells were treated with 5 and 10 μM PL-C for 18 h. The cells were harvested and lysed for immunoblotting using p65 and p-p65 Abs. β-Actin immunoblotting was included as the internal control. Data shown are for treatment with PL-C alone (top panel) or in combination with IL-12 (10 ng/ml) (middle panel) or IL-15 (100 ng/ml) (bottom panel) and are the representative plots of four donors with similar results. Numbers under each lane represent quantification of p-p65 or p65 via densitometry, after normalizing to β-actin. (B) NKL cells were treated, and data are presented as described in (A). Data from one of three independent experiments with similar results are shown. (C) Purified primary human NK (left panel) or NKL cells (right panel) were cotreated with 10 μM PL-C and IL-12 (10 ng/ml) in the presence or absence of the NF-κB inhibitor TPCK (10 μM) for 18 h. Supernatants were assayed for IFN-γ secretion by ELISA (top panel), and cells were harvested and lysed for immunoblotting of p-p65 (bottom panel). Representative data from one of three donors with the similar data (left panel) and the summary of three independent experiments with similar results (right panel) are shown. **p < 0.01. Error bars represent SD.
Primary NK cells significantly reduced PL-C–mediated induction of IFN-γ, whereas blockade of TLR3 had no significant effect on NK cell activation. Combined blockade of TLR1 and TLR6 reduced PL-C–enhanced NK cell IFN-γ expression to levels lower than those seen with blockade of either TLR1 or TLR6 (Fig. 5A, top panel). To determine whether the effect of blocking Abs on PL-C–induced IFN-γ production is likely mediated through the NF-κB signaling pathway, we examined the phosphorylation level of p65 induced by PL-C in the presence and absence of the TLR blocking Abs. Consistently, blockade of TLR1 and/or TLR6 also inhibited PL-C–induced phosphorylation of p65, suggesting that PL-C–induced IFN-γ production occurs at least in part through the TLR1/6–NF-κB signaling pathway (Fig. 5A, bottom panel). We next examined whether PL-C could affect the expression of TLR1 and TLR6. No obvious changes in TLR1 or TLR6 gene expression were observed after treatment with PL-C alone or in the presence of IL-12 (data not shown). To further explore whether PL-C would augment TLR-mediated IFN-γ induction, NK cells were treated with 10 μM PL-C combined with a ligand of each of the two aforementioned TLRs in the presence of IL-12. PL-C enhanced IFN-γ production induced by Pam3CSK4 (TLR1/2 ligand) and FSL-1 (TLR6/2 ligand) in the presence of IL-12 when the ligands were at the concentration of 1 μg/ml (Fig. 5B). We also found that PL-C enhanced Pam3CSK4 and FSL-1–induced IFN-γ production in the presence of IL-12 when the ligands were added at various concentrations <1 μg/ml (Fig. 5C). To further confirm that PL-C activates the TLR-NF-κB signaling pathway, we cotransfected TLR1 or TLR6 with pGL-3κB-Luc and control plasmid pRL-TK renilla-luciferase plasmids. PL-C treatment was found to induce luciferase reporter activity in a dose-dependent fashion, suggesting an increase of NF-κB binding to the κB binding sites (Fig. 5D). Finally, we knocked down TLR1 expression in NKL cells by using TLR1 shRNA to validate that TLR1 signaling participated in PL-C–mediated enhancement of NK cell IFN-γ production. After confirming TLR1 was successfully knocked down in TLR1 shRNA NKL cells with ∼50% TLR1 mRNA inhibition (Fig. 5E), we found the increase in IFN-γ production mediated by PL-C vanished when cotreated with IL-12 or IL-15 in these cells (Fig. 5F). These data suggest that PL-C directly activates TLR-NF-κB signaling pathway to enhance IFN-γ production in NK cells.

**Discussion**

NK cells are an important lymphocyte subset with a capacity to destroy tumor cells and clear viral infection upon first encounter (50). Enhancement of NK cell activity for prevention or treatment of cancer and viral infection is a central goal in the field of immunology. NK cell activation can be achieved through exposure to cytokines such as IL-2 (55) and IL-12 (56, 57). However, this approach has had limited success in part because of the toxicity resulting from the systemic administration of these cytokines (58) and the pleotropic effects of these agents. One example of the latter is that IL-2 induces expansion of regulatory T cells (22, 23), which in turn dampen NK cell functions (59). What seems to be most useful for prevention of cancer or infection in those susceptible individuals would be an agent that produced a modest induction of NK function with relative specificity among immune effector cells.

In this study, we found that PL-C, a diphyllin lignan glycoside, which can be isolated from both edible and nonedible plants of the *Phyllanthus* genus, was able to specifically enhance IFN-γ production by human NK cells. Mechanistically, we show that PL-C may sense TLR1 and/or TLR6 on human NK cells, which in turn activates the NF-κB subunit p65 to bind to the proximal region of the *IFNG* promoter. PL-C has only negligible effects on T cell

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**FIGURE 4.** PL-C augments the binding of p65 to the *IFNG* promoter in human NK cells. (A) Schematic of *IFNG* promoter potential binding sites for p65 (45). (B) NK cells purified from healthy donors were treated with 10 μM PL-C or DMSO vehicle control in the presence of IL-12 (10 ng/ml) for 12 h. Cell pellets were harvested for nuclear extraction, followed by EMSA with a 32P-labeled oligonucleotide containing the C3-3P NF-κB p65 binding site of the *IFNG* promoter. Data shown represent one of three donors with similar results. (C) Cells were treated as described in (B), and the cell pellets were harvested to extract protein for ChIP assay of p65 binding to the *IFNG* promoter locus C3-3P. Mean of relative association of p65 at the *IFNG* promoter locus C3-3P from three independent experiments is shown. *p < 0.05, compared with cells treated with IL-12 alone. Error bars represent S.D.
effector function, which is consistent with higher expression of TLR1 and TLR6 in NK cells than in T cells (54). This increases the likelihood that pleiotropic effects on immune activation and systemic toxicity of the agent might be limited.

Targeting NK cells, especially by natural products, appears to be a rational approach for the prevention of cancer. In support of this, an 11-y follow-up population study of 3625 people $\geq 40$ y of age demonstrated that the potency of peripheral blood NK cells for lysing tumor cell targets was inversely associated with cancer risk (60). Moreover, as cancer susceptibility increases with age, NK cell potency subsides with age (61, 62). NK cell activity is correlated with relapse-free survival in some cancer patients (e.g., those with acute myeloid leukemia [AML]) (63). NK cells should be excellent tools to control tumor development at the early stage.

**FIGURE 5.** TLR1 and/or TLR6 mediate IFN-γ induction by PL-C in human NK cells. (A) Human NK cells were purified and pretreated with a non-specific IgG or anti-TLR1, anti-TLR3, anti-TLR6 or the combination of TLR1 and TLR6 blocking Abs (α) for 1 h. Cells were then treated with PL-C and IL-12 (10 ng/ml) for another 18 h, and supernatants were harvested to assess for IFN-γ secretion by ELISA (top panel) and cell pellets for p-p65 immunoblotting (bottom panel). Data shown are representative of one of six different donors with similar results. *p < 0.05, **p < 0.01, respectively, which denote statistical comparison between the two marked treatment groups and are calculated from data of all tested donors. Numbers underneath each lane represent quantification of protein by densitometry, normalized to β-actin. (B) Purified NK cells were treated with Pam3CSK4 (1 μg/ml; TLR1/2 ligand) or FSL-1 (1 μg/ml; TLR6/2 ligand) in the presence of IL-12 (10 ng/ml), with or without PL-C (10 μM) for 18 h, and then, supernatants were harvested to assay for IFN-γ secretion by ELISA. Data shown are representative of one of six donors with similar results. *p < 0.05, **p < 0.01, which denote statistical comparison between the two marked treatment groups and are calculated from data of all tested donors. (C) Purified primary NK cells were treated with various low concentration of Pam3CSK4 or FSL-1 with or without PL-C (10 μM) in the presence of IL-12 (10 ng/ml), and then, supernatants were harvested to assay for IFN-γ secretion by ELISA. Data shown are representative one of three donors with similar results. Error bars represent SD. (D) 293T cells were transfected with TLR1 (0.5 μg) or TLR6 (0.5 μg) expression plasmid along with pGL-3×κB-luc (1 μg) and pRL-TK renilla-luciferase control plasmids (5 ng; Promega). Cells were then treated with various concentration of PL-C for another 24 h with fresh medium, and DMSO was included as vehicle control. The ratio of the firefly to the renilla luciferase activities was used to show the relative luciferase activity, which corresponded to NF-κB activation. *p < 0.05, **p < 0.01, compared with vehicle control. Error bars represent SD. (E) NKL cells were infected with pSUPER or pSUPER-shTLR1 retroviruses and sorted based on GFP expression. (F) Both the vector-transduced cells (pSUPER) and the TLR1 knockdown (pSUPER-shTLR1) NKL cells were treated with or without PL-C in the presence or absence of IL-12 or IL-15. Cell pellets were harvested at 12 h for real-time RT-PCR. The relative IFNG mRNA expression induced by PL-C in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml) was shown in the upper or lower panel, respectively. The summary of three independent experiments with similar results are shown. **p < 0.01. Error bars represent SD.
as they play a critical role of tumor surveillance. Once cancer is established, tumor cells can inactivate immune cells, including NK cells, which can result in an immunosuppressive microenvironment (64). Indeed, NK cell function is found to be anergic or impaired in various types of cancer (65, 66). Moreover, at the late stage of cancer development, the immune system including NK cells and IFN-γ may edit tumor cells and facilitate their escape from immune destruction (6, 67, 68). Therefore, we think NK cells may play a more critical role in prevention than for treatment of cancer, and their selective modulation is important in this scenario. However, this is hindered by the lack of safe and effective NK cell stimulators.

Our findings provide a new avenue to prevent or treat cancer using natural products through enhancing NK cell immune-surveillance. Like many other natural products, PL-C is likely relatively safe compared with cytokines, and this can be supported by the lack of substantial toxicities observed in mice treated with up to 500 mg PL-C/kg body weight (data not shown). Developing less toxic drugs is important for preventing or treating some cancers, especially for those which are dominant in children or in elderly populations. One example for this is AML. AML is a disease that primarily affects older adults: the median age at diagnosis is >65 y (69, 70). The 5-y survival rate of AML in older adults remains under 10% (71). Elderly AML patients are less able than younger patients to tolerate effective therapies such as intensive chemotherapy and allogeneic stem cell transplantation. Therefore, PL-C may present a new approach to prevent or treat this disease.

PL-C selectively activates NK cells through regulating production of cytokines, especially IFN-γ. Therefore, in vivo, PL-C will most likely achieve its cancer prevention or treatment effects through increasing NK cell IFN-γ secretion to activate other innate immune components such as macrophages (72) as well as adaptive immune components such as CD8+ T cells (11, 12). Unlike cytokine stimulation, which usually induces both IFN-γ production and cytotoxicity, the selective induction of NK cell IFN-γ production by PL-C also provides a good opportunity to separate the two major functions of NK cells, cytokine production and cytotoxicity, especially when cytotoxicity may cause damage to normal tissues (e.g., in the graft-versus-host disease and pregnancy contexts). Interestingly, this separation naturally exists in our immune system as CD56bright and CD56dim NK cells have differential functions in terms of IFN-γ production and cytotoxicity, and some tissue or organs predominantly have only one of these subsets. For example, the lymph nodes (73) and the uterus (74) almost exclusively contain CD56bright NK cells.

Mechanistically, we found that PL-C may sense TLR1 and TLR6 to activate NF-κB signaling in NK cells, leading to the enhancement of IFN-γ production. In support of this, knockdown of TLR1 and TLR6 to activate NF-κB signaling in NK cells, leading to the enhancement of IFN-γ production. In support of this, knockdown of TLR1 and TLR6 by shRNA eliminated the effect of PL-C on IFN-γ production in NK-L cells. Others and we have previously demonstrated that TLR1 and TLR6 are substantially expressed in human NK cells (41, 54). Interestingly, TLR1 and TLR6 share 56% amino acid sequence identity (75) and both complex with TLR2 to recognize bacterial lipoproteins and lipopeptides (76). TLR1 and TLR6 may thus possess a common binding site for PL-C. It is unknown whether PL-C’s ability to sense TLR1 and TLR6 requires binding to their partner TLR2, because TLR2 has very low expression in NK cells (54). It is possible that triggering TLR1 and TLR6 signaling may only require a minimal expression level of TLR2, as we found for NK precursor cells, which respond to IL-15 but lack expression of the IL-15R chains responsible for signaling at a level detectable by flow cytometry (77). Regardless, our current study may have identified a potential novel agonist for TLR1 and/or TLR6. In support of this, we found that PL-C also activates TLR1 and TLR6 downstream NF-κB signaling in NK cells, and transfection of TLR1 or TLR6 induces NF-κB reporter activity. Moreover, our data suggest that PL-C either lowers the threshold for or synergizes with TLR1 and TLR6 ligands to activate NK cells.

In summary, we identified a natural product, PL-C, which effectively stimulates NK cells to secrete IFN-γ. PL-C appears to act through TLR1 and TLR6, which subsequently activate NF-κB signaling to induce binding of p65 to the proximal region of the IFNG promoter in NK cells. Our work would suggest that additional studies to understand the role of this natural product in prevention of cancer or infection in select populations are warranted.

Acknowledgments
We thank Hsiaoyin C. Mao for flow cytometry technical and scientific support.

Disclosures
The authors have no financial conflicts of interest.

References
The Natural Product Phyllanthusmin C Enhances IFN-γ Production by Human Natural Killer Cells through Upregulation of TLR-Mediated NF-κB Signaling

Youcai Deng *,†,‡, Jianhong Chu *,†, Yulin Ren *,†, Zhijin Fan *,‡, Xiaotian Ji †, Bethany Mundy ‡, Shunzong Yuan ‡∥, Tiffany Hughes ‡, Jianying Zhang #, Baljash Cheema ‡, Andrew T. Camardo ‡, Yong Xia ‡*, Lai-Chu Wu ‡*, Li-Shu Wang †, Xiaoming He ††, A. Douglas Kinghorn †, Xiaohui Li †, Michael A Caligiuri *‡, and Jianhua Yu *§

*Division of Hematology, Department of Internal Medicine, College of Medicine, The Ohio State University, Columbus, Ohio 43210, USA; †Institute of Materia Medica, College of Pharmacy, Third Military Medical University, Chongqing 400038, China; ‡Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, USA; ††State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, China; ∥PLA 307 Hospital, Beijing 100071, China; #Center for Biostatistics, The Ohio State University, Columbus, Ohio 43210, USA; **Department of Molecular and Cellular Biochemistry, The Ohio State University College of Medicine, Columbus, OH 43210, USA; ††Division of Hematology and Oncology, Medical College of Wisconsin, Milwaukee, WI 53226, USA; ‡‡Department of Biomedical Engineering, The Ohio State University, Columbus, Ohio 43210, USA; §The Ohio State University Comprehensive Cancer Center, Columbus, Ohio 43210, USA;

Supplementary Information
Supplemental Table 1: Primers for real-time RT-PCR used in this article

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Probe</th>
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<tr>
<td><strong>IFNG</strong></td>
<td>For : 5’-GAAAAGCTGACTAATTATTCGGAAGTCGTAAG-3’&lt;br&gt;Re: 5’-TGTCAGCCATCCTGGAGATGAG-3’</td>
<td>5’-FAM-CTTGAATGTCCAACGCAAGAAATCATGAG-3’&lt;br&gt;TAMRA-3’</td>
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<td><strong>GZMA</strong></td>
<td>For : 5’-TCCTATAGTTTCTGGCATCCTCTC-3’&lt;br&gt;Re: 5’-TTCTTCCATAATTTTTACAGACA-3’</td>
<td>5’-FAM-&lt;br&gt;CAGTTTGCAGTTCTCCTGCTGAATC-3’&lt;br&gt;TAMRA-3’</td>
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<td><strong>GZMB</strong></td>
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<td>5’-FAM-TGCTACTGCAGTGAGAAAGAC-3’&lt;br&gt;TAMRA-3’</td>
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<td><strong>Perforin</strong></td>
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<td>5’-FAM-&lt;br&gt;CGCTTCTACATTCCATGTGCTACACTC-3’&lt;br&gt;TAMRA-3’</td>
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<td><strong>FasL</strong></td>
<td>For : 5’TCAATGACCAGCTTCTCC-3’&lt;br&gt;Re: 5’-AAA GCA GGACAATTC CAT AGG TG-3’</td>
<td>5’-FAM-TCC AAC TCA AGG TCCATG CCT CTG G-3’&lt;br&gt;TAMRA-3’</td>
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<tr>
<td><strong>18S</strong></td>
<td>18S rRNA, PE Applied Biosystems, Foster City, CA</td>
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<tr>
<td><strong>HPR1</strong></td>
<td>For : 5’-CTTCCTCCCTGAGGAGGTC-3’&lt;br&gt;Re: 5’-CCGACAAAGGAAAGGCAAGA-3’</td>
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<td><strong>IL12Rβ1</strong></td>
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<td><strong>IL12Rβ2</strong></td>
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<td><strong>IL15Ra</strong></td>
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<td><strong>IL15Rβ</strong></td>
<td>For : 5’-GCAACAAGCTGGGATAACTCTC-3’&lt;br&gt;Re: 5’-CGCACACCTGAAACAGTTCAGTG-3’</td>
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Supplemental Figure Legends

Fig. S1

A

B

Supplemental Figure 1. Representative photographs of PL-C source plants Phyllanthus reticulates and Phyllanthus poilanei and synergistic effect of IL-12 and PL-C on IFN-γ induction by primary human NK cells. (A) Photographs of representative source plants, *Phyllanthus reticulates* (left) and *Phyllanthus poilanei* (right), courtesy of Dr. Wei Wang (Kunming Medical University, China) and Dr. Djaja Djendoel Soejarto (University of Illinois at Chicago, USA), respectively. (B) Purified primary human NK cells were treated as described in Figure 1C. The increase of IFN-γ in each case is presented as percent increase above treatment with vehicle control (untreated with PL-C or cytokines). In each donor, the paired bars compare the additive effect of IL-12 and PL-C-treated alone (left, composite bar) versus the effect of the co-stimulation with IL-12 and PL-C (right, black bar). Representative data of 3 out of 14 donors are shown. *P* < 0.001, additive effect of IL-12 and PL-C versus co-stimulation with IL-12 and PL-C.
Supplemental Figure 2. PL-C does not affect T cell IFN-γ production and primary NK cells cytotoxic activity. (A) Human PBMCs were treated with DMSO vehicle control or 10 μM PL-C for 18 hours in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml), as described in Figure 1C. The cells were harvested for intracellular flow cytometry to determine the frequency of IFN-γ+ cells in CD56−CD3−CD4+ or CD56−CD3−CD8+ T cells. Representative data from 1 out of 5 donors are shown. (B) Purified primary NK cells were treated with IL-12 (10 ng/ml) or IL-15 (100 ng/ml) with or without 10 μM PL-C for 8 hours and were sequentially co-cultured with 51Cr labeled ARH-77 cells at various effector/target cell ratios for additional 4 hours. 51Cr release was measured by TopCount counter. Data shown are the means of 3 donors. There is no statistically significant difference between vehicle control and PL-C treatment group in all conditions. Error bars represent S.D. (C) Purified primary NK cells were treated with 10 μM PL-C in the presence of IL-12 (10 ng/ml) (top) or IL-15 (100 ng/ml) (bottom) for 12 hours, and cell pellets were harvested for detecting Granzyme A (GZMA), Granzyme B (GZMB), Perforin (PRF1) and Fas ligand (Fasl) mRNA expression level by Real-time RT-PCR. Data shown are the means of 6 donors. P > 0.05, vehicle control versus PL-C in all panels. Error bars represent S.D.
Supplemental Figure 3. Effects of PL-C on IL-12 and IL-15 signaling pathways. (A) Purified human primary NK cells were treated with 10 μM PL-C in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml) for 12 hours. DMSO-treated cells were served as vehicle controls. Cell pellets were harvested to extract total RNA for real-time RT-PCR to determine mRNA expression levels of *IL-12β1*, *IL-12β2*, *IL-15Rα* and *IL-15β*. Data are shown as means of 3 donors. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, which denote statistical comparison between the two marked treatment groups. Error bars represent S.D. (B, C) Purified primary NK cells (B) or NKL cell (C) were treated with 5 or 10 μM PL-C in the presence of IL-12 (10 ng/ml) or IL-15 (100 ng/ml) for 4 hours. DMSO-treated cells were served as vehicle controls. Cell pellets were harvested for subsequent immunoblotting of T-BET, phosphorylated STAT3 (p-STAT3), p-STAT4, p-STAT5, STAT3, STAT4, and STAT5. Data represent 1 out of 3 donors with similar data (B) and 3 independent experiments with similar results (C). Numbers
underneath each lane represent quantification of detected protein by densitometry, after normalizing to β-actin.