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ORIGINAL RESEARCH

Uncarinic Acid C Isolated from Uncaria rhynchophylla Induces Differentiation of Th1-Promoting Dendritic Cells Through TLR4 Signaling

Kyu Sik Kim3, Thanh Nhan Nguyen Pham1, Chun-Ji Jin1,5, Akemi Umeyama4, Noboru Shoji4, Toshihiro Hashimoto4, Je-Jung Lee1,2 and Masao Takei1

1Research Center for Cancer Immunotherapy, Chonnam National University Hwasun Hospital, 160 Ilsim-ri, Hwasun-eup, Hwasun-gun, Jeollanam-do 519-809, South Korea. 2Department of Hematology-Oncology, Chonnam National University Medical School, Gwangju. 3Department of Pulmonary Medicine, Chonnam National University Medical School, Gwangju. 4Faculty of Pharmaceutical Sciences, Tokushima University, Yamashiro-cho, Tokushima, 770-8514, Japan. 5Department of Surgery, Chonnam National University Medical School, Gwangju. Corresponding author email: mtakei@pep.ne.jp

Masao Takei and Je-Jung Lee are corresponding authors equally.

Abstract: Uncarinic acid C (URC) is triterpene isolated from Uncaria rhynchophylla and is a pharmacologically active substance. The induction of dendritic cells (DC) is critical for the induction of Ag-specific T lymphocyte responses and may be essential for the development of human vaccines relying on T cell immunity. DC might be a potential target for URC. We demonstrate that URC activates human DC as documented by phenotypic and functional maturation, and altered cytokine production. The expression of CD1a, CD38, CD40, CD80, CD83, CD86, HLA-DR and CCR7 on URC-primed DC was enhanced. The production of IL-12p70 by URC-primed DC was higher than that of lipopolysaccharide (LPS)-primed DC. The production of IL-12p70 by URC-primed DC was inhibited by the anti-Toll-like receptor 4 (TLR4) monoclonal antibody (mAb), but partially abolished by anti-TLR2 mAb. mRNA coding for TLR2 and TLR4 was expressed in URC-primed DC. URC-primed DC induced the NF-κB transcription factor. Naïve T cells co-cultured with URC-primed DC turned into typical Th1 cells that produced large quantities of IFN-γ depending on IL-12 secretion. URC enhanced the T cell stimulatory capacity in an allo MLR. In the cytotoxic T-lymphocyte assay (CTL) assay, DNA fragmentation assay and 51Cr release on URC-primed DC were more augmented than that of TNF-α-primed DC. DC matured with URC had an intermediate migratory capacity towards CCL19 and CCL21. These results suggest that URC modulates DC function in a fashion that favors Th1 polarization via the activation of IL-12p70 dependent on TLR4 signaling, and may be used on DC-based vaccine for cancer immunotherapy.

Keywords: Dendritic cells, Uncarinic acid C, Th1, CTL

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Introduction
The immune system is confronted with antigens and proteins that have not been encountered previously. Due to their unique antigen processing and presentation and high-level expression of costimulatory and cytokine molecules, DC are highly specialized antigen-presenting cells (APC) with a unique ability to activate resting T lymphocytes and initiate primary immune responses as to induce peripheral tolerance.1–3 DC orchestrate a variety of immune responses by stimulating the differentiation of naïve CD4 T cells into helper T effectors such as Th1, Th2 or Th17 type.4–7 Cytokines, secreted by DC at the time of initial T cell stimulation, play an important role in the subsequent differentiation of effector T cells. Th1 cells, through interferon gamma (IFN-γ) production, regulate antigen presentation and immunity against intracellular pathogens. Although different DC subsets may have some intrinsic potential to preferentially induce Th1, Th2 or Th17 cells, DC also display considerable functional plasticity in response to signals from microbes and the local microenvironment.8 Multiple reagents have been reported to induce DC maturation and the known DC maturation stimuli include the CD40 ligand (CD40-L), IFN-α, TNF-α, oligo CpG nucleotides1 or Toll-like receptor ligands.9 Toll-like receptors (TLRs) are expressed mainly on macrophages and DC, triggering results in the development of effector DC that promote Th1 responses.10 Recently, several studies proposed the significance of TLR signaling in the induction of anti-cancer immunity. Important aims of DC research are the development of DC-based strategies for enhancing immune responses against tumor, infectious agents or autoimmune diseases. DC are being used in a number of clinical immuno-therapy protocols in the cancer setting. However, to date, the results of these trials seem to suggest that only modest clinical benefits may be attributed to this modality. Therefore, it is important to identify factors that might affect the differentiation, maturation and function of DC.

The hooks of Uncaria sp. are contained in Choto-san as the main component herb and have been widely used as an important component of many Chinese prescriptions in Asia. URC is isolated from Uncaria rhynchophylla and phytochemically classified as triterpene. Triterpenes have been identified as a unique class of natural products possessing diverse biological activities. We have recently reported that some terpenes induce DC from human monocytes and drive Th1, Th2 or IL-10-producing Treg cells.11–14 A number of alkaloids have been reported as antihypertensive principles from the genus Uncaria. URC showed potent inhibitory activity against phospholipase Cγ1 (PLCγ1) and inhibited the growth of cancer cells at high doses.15 Thus, although terpene compounds have pharmacological activity, little is known about how URC influence the initiation of the specific immune response at the level of DC, the highly professional APC for T cells. DC might be a potential target for URC. In this study, we investigated the ability of URC on human DC differentiation (surface molecule up-regulation), function (cytokine productions) and their activation (NF-κB translocation to the nucleus) detail.

Materials and Methods
Culture medium, reagents and monoclonal antibodies
The culture medium used in this study was serum-free AIM-V medium (Life Technologies, Paisley, UK). Recombinant human IL-4 (IL-4), recombinant human granulocyte-macrophage colony-stimulation factor (GM-CSF), anti-TLR2 mAb and anti-TLR4 mAb purchased from R&D systems (Minneapolis, MN). For flow cytometry, monoclonal antibodies (mAbs) toward the following antigens were purchased from Becton-Dickinson (San Jose, CA): anti-CD14-FITC (fluorescent isothiocyanate), anti-CD1a-PE (phycoerthrin), anti-CD38-PE, anti-CD40-FITC, anti-CD54-PE, anti-CD83-PE, anti-CD86-PE, anti-HLA-DR-FITC, anti-CCR7 and anti-DC-Lamp-PE. Endotoxin levels in all agents were below 1.0 EU/ml.

Isolation of URC from Uncaria rhynchophylla
URC was prepared as described previously.15 The purity of URC was >99%. The endotoxin in URC was removed using End Trap 5/1 (Profos AG, Regensburg, Germany). Endotoxin levels in URC were below 0.05 EU/ml.

Generation of Monocyte-Derived DC
All cell subsets were isolated from human peripheral blood of normal healthy donors. Peripheral blood mononuclear cells (PBMC) were first isolated from
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heparinized wholeblood by Ficoll/Isopaque/1.077 g/ml (Pharmacia, Freiburg, Germany) density gradient centrifugation (465 × g, 30 min, 22 °C) as described previously.11 PBMC were further separated into CD14+ monocytes using the magnetic activated cell sorter (MACS) according to the manufacturer’s instructions (Miltenyi Biotec; Auburn, CA). The purity of CD14+ monocytes was always more than 90%. Monocytes were cultured with GM-CSF (50 ng/ml) and IL-4 (25 ng/ml) in serum-free AIM-V medium for 6 days. After 6 days, cells (>95% CD1a+, CD14−) were harvested and re-cultured in serum-free AIM-V medium containing GM-CSF and IL-4 for additional 2 days with various concentrations of URC, but with LPS (100 ng/ml) or TNF-α (25 ng/ml). All subsequent tests were performed after harvesting the cells at day 8 and after removing the above factors by extensive washing. The medium was replenished with cytokines every 2 days. To determine the production of IL-10 and IL-12p70 by URC-primed DC, LPS-primed DC or TNF-α-primed DC, DC (2 × 10^4 cell/well) were stimulated with CD40-L transfected J558 cells (5 × 10^4 cell/well, kindly provided by Prof. Kalinski at University of Pittsburgh, PA) for 24 h. The cell-free supernatants were collected and frozen at –20 °C until measurement of cytokines using enzyme-linked immunosorbent assay (ELISA).

Immunophenotype Studies

Dual-Color immunofluorescence flow cytometry was performed using the following panel of monoclonal antibodies: PE-conjugated antihuman CD1a, FITC-conjugated antihuman CD14, PE-conjugated antihuman CD38, FITC-conjugated antihuman CD40, PE-conjugated antihuman CD54, PE-conjugated antihuman CD80, PE-conjugated antihuman CD83, PE-conjugated antihuman CD86, FITC-conjugated antihuman HLA-DR, PE-conjugated antihuman DC-Lamp and FITC-conjugated antihuman CCR7. Negative controls were isotype-matched with irrelevant monoclonal antibodies (Becton-Dickinson). Cells were re-suspended in staining medium containing PBS and 0.1% NaN₃ and then fixed with 1.0% paraformaldehyde. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. The samples were analyzed on FACSCalibur (Becton-Dickinson) with CellQuest software (Becton-Dickinson). Ten thousand cells were analyzed per sample. The results were expressed as mean fluorescence intensity (MFI).

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed with the Gel Shift assay system (Promega, E1, USA). Briefly, oligonucleotides with the consensus sequence for NF-κB (5′-AGTTGAGGGGACTTCCCAGG-3′) were end-labeled with [γ-32P] adenosine triphosphate (3 mCi/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the T4 polynucleotide kinase. The labeled oligonucleotide was then purified in microspin G-25 columns (Sigma) and used as a probe for EMSA. Nuclear extract protein (10 μg) was pre-incubated with the binding buffer for 5 min, followed by incubation with the labeled probe for 15 min at room temperature. Each sample was electrophoresed in a 5% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer (pH7.4) at 120 V for 5 h. After electrophoresis, the gel was dried and exposed to autoradiography film.

RT-PCR and Real-Time PCR

Total cellular RNA was extracted using RNeasy Mini kits (Qiagen GmbH, Germany), according to manufacturer’s recommendations. Equal amounts of RNA from all samples were analyzed. Reverse transcription of 1 μg of RNA into cDNA was performed using PrimeScriptTM 1st strand cDNA Synthesis Kit (Takara Bio Inc, Shiga, Japan). Primers used for real-time polymerase chain reaction (PCR) were purchased from Bioneer (South Korea). The primers for the TLRs were designed using the Primer-BLAST program from PubMed (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index). (hTLR2-forward: GCCTCTCCAAGGAAGAATCC; hTLR2-reverse: TCCTGTTGTTGGACAGGTCA; hTLR4-forward: AAGCCGAAAGGTGATTGT-TG; hTLR4-reverse: CTGAGCAGGGTCTTCTCACC). The realtime PCR was performed using the Corbett Research Rotor-Gene 3000 (Corbett Life Science, Germany). The reaction mixture contained 12.5 μl 2X QuantiTect SYBR Green PCR Master Mix (Qiagen GmbH, Germany), 0.5 μmol/l of forward and reverse primer and 2 μl of cDNA in a total volume of 25 μl. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by dsDNA-binding SYBR Green. After the PCR was
performed, a melting curve was constructed in the range of 55 °C to 95 °C. Data were analyzed using the Rotor Gene 6 analysis software. As a first step in data analysis β-actin was used for normalization of all samples (normalizer). As second step in data analysis we used the comparative Ct method (ΔΔCt method) with the following formula: ΔCt = Ct (target, TLR)−Ct (normalizer, β-actin). The comparative calculation involved finding the difference between ΔCt of treated-samples and the mean value of the ΔCt from normal ones.

**Determination of Naïve T Cell Polarization by DC**

DC were co-cultured with naïve T cells (2.5 × 10^5 cells/200 µl) at 1:5 DC/T cells ratio in 96-well U-bottomed tissue culture plates (Costar, Cambridge, MA). Briefly, naïve CD4+ T cells were isolated from allogeneic PBMC using naïve CD4+ T cells isolation kit (Miltenyi Biotec). The purity of isolated cells was >95% for naïve CD4+ T cells as determined by flow cytometry using FACSCalibur. Some cultures were supplemented with neutralizing Abs to block endogenous cytokines: anti-IL12 mAb (10 µg/ml, R&D systems). On day 5, cells were washed out completely and expanded with fresh medium containing 10 U/ml of rIL-2 (R&D Systems). One hundred microliters of culture supernatant was replaced with medium containing 10% FBS and 1% penicillin-streptomycin supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 for 10 days. After 10 days, CD8+ T cells were re-stimulated with irradiated (25Gy) autologous monocytes (2:1) which were loaded with WT-1 peptide and then cells were cultured in 2 ml of medium containing 10% FBS and 1% penicillin-streptomycin supplemented with 10 ng/ml IL-2 and 5 ng/ml IL-7 for another 10 days. The T2 cells (HLA-A0201+) were used as target cells. T2 cells (5 × 10^5 cells/ml) were loaded with 20 µg/ml of WT-1 peptide or of HIV-1 peptide (unrelated peptide) and incubated for 2 h at 37 °C in 5% CO₂.

**DNA Fragmentation Assay**

To analyze target-induced CTL apoptosis, 8 h [³H]-methylthymidine DNA fragmentation (JAM) assay was performed as described previously. CTL were labeled with 5 µCi of [³H]-methylthymidine per 10⁶ cells 18 h prior to the assay in the absence of IL-2 and served as the target for tumor cells. The CTL (3 × 10⁴ cells/well) were co-cultured with tumor cells (T2) at 37 °C in 5% CO₂ at various tumor-to CTL ratios for 8 h, then harvested and analyzed in a liquid scintillation counter (Beckman). Data are expressed as a percentage of DNA fragmentation based on the percentage decrease in cpm as compared with untreated controls using the following formula: % DNA fragmentation = (Control cpm-Sample cpm/Control cpm) × 100. This assay was done in triplicate.

**Cytotoxic T-lymphocyte (CTL) Assay**

Details of the method used in this assay have been described previously. Briefly, DC (2 × 10^6) cultured from monocytes of HLA-A0201 donors were loaded with 20 µg/ml WT-1 peptide for 2 h at 37 °C in 5% CO₂. WT-1 peptide can bind to HLA-A0201 as reported by Pinifla-Ibarz et al. In 24 well plates, autologous CD8+ T cells were co-cultured as effector cells 5:1 with DC in 2 ml of medium (RPMI1640:AIM-V = 1:1) containing 10% FBS and 1% penicillin-streptomycin supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 for 10 days. After 10 days, CD8+ T cells were re-stimulated for 24 h with Dynabeads CD3/CD28 T cell expander (Invitrogen). The cell-free supernatants were collected and frozen at −20 until measurement of cytokines using ELISA.
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**Chemotaxis**
DC migration was performed in 24-well transwell cell culture chambers (5-µm pore; Costar) as described previously.12 10^5 cells were washed, suspended in RPMI 1640 supplemented with 10% FBS medium and loaded in the upper chamber compartment, CCL19 (250 ng/ml; Peprotech), CCL21 (250 ng/ml; Peprotech) or without chemokines (medium) were added to the lower compartment. After 4 h of incubation at 37 °C, cells that had migrated into the lower compartment were collected and counted. The results were expressed as net migration percent calculated as: (the number of cells that migrated into the lower chamber containing chemokine-number of cells that migrated in medium alone)/total number of cells loaded in the upper chamber × 100.

**Statistical Analysis**
Statistical analysis of the results was performed by ANOVA. Differences were considered statistically significant when P value were less than 0.05.

**Results**
Urc induced human monocyte-derived DC
In order to study the direct effect of URC on the function of human DC, immature monocyte-derived DC were exposed to URC, and phenotypical and functional DC maturation was analyzed. Human monocytes were cultured with GM-CSF and IL-4 for 6 days under standard conditions, followed by an additional 2 days in the presence of various concentrations of URC (0.001–10 µM). Under these conditions, we found that CD1a, CD38, CD40, CD54, CD80, CD83, CD86, HLA-DR and DC-Lamp expression levels on URC-primed DC were enhanced. Typical phenotype results are shown in Figure 1. The viability of cells treated with a concentration of 10 µM of URC was 95%. As a positive control, human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by an additional 2 days in the presence of LPS (100 ng/ml) or TNF-α (50 ng/ml). LPS and TNF-α are a known DC maturation-enhancing factor. The expression levels of CD1a, CD38, CD40, CD54, CD80, CD83, CD86, HLA-DR, CCR7 and DC-Lamp determined by MFI on LPS-primed DC and TNF-α-primed DC were also found to be strongly enhanced (Fig. 1). Immature DC (with medium) were generated by cultivating human monocytes with GM-CSF and IL-4 for 8 days served as a control. The expression level of CD14 as expressed by MFI on day 8 was found to be low and even undetectable in some samples (data not shown).

**Effects of Anti-TLR2 mAb and Anti-TLR4 mAb on Cytokine Production by URC-primed DC**
As DC serve as the professional antigen-presenting cells and that their secretion of immunoregulatory and proinflammatory cytokines plays a crucial role in T cell priming, we next investigated whether cytokine production by human monocyte-derived DC was affected by treatment with URC. It has been reported

![Figure 1](image-url)
that the level of IL-12 production by DC is a major factor driving the development of Th1 cells. Therefore, we measured IL-12p70 production in immature DC and in DC matured for 2 days in the presence of the above factor following stimulation with CD40-L transfected J558 cells for 24 h. Major enhancements of IL-12p70 production were caused by 0.1 µM of URC (Fig. 2A), whereas with LPS-primed DC or TNF-α-primed DC only small increases can be seen (Fig. 2A). The concentrations of URC were used at 0.1 µM for subsequent experiments. In contrast, the production of IL-12p70 by immature DC was low (Fig. 2A).

Toll-like receptor (TLR) agonists are potent activators of innate immune responses, activating DC maturation and inflammatory cytokine secretion by innate cells. We next examined whether the ability of IL-12 production by URC-primed DC depended on TLRs signaling. Before stimulation with URC, monocyte-derived DC were incubated in the presence of the TLR2 mAb or TLR4 mAb or an isotype control (IgG). The anti-TLR4 mAb inhibited IL-12p70 production by URC-primed DC or LPS-primed DC by 65%–80% (Fig. 2B), whereas anti-TLR2 mAb partially influenced IL-12p70 production by URC-primed DC (Fig. 2B). In contrast, the anti-TLR2 mAb did not affect IL-12p70 production by LPS-primed DC (Fig. 2B). The response to TNF-α was not inhibited in the presence of the anti-TLR2 mAb and anti-TLR4 mAb (Fig. 2B). IL-12p70 production by URC-, LPS- or TNF-α-primed DC was not influenced when IgG1 was added instead of anti-TLR2 mAb or anti-TLR4 mAb (Fig. 2B).

**URC-primed DC Induced NF-κB Activation and TLR Expression**

DC activation and maturation driven by TLR agonist such as LPS has been clearly associated to NF-κB activation. To determine whether URC uses similar activation pathways, we monitored its ability to activate of the NF-κB translocation into the nucleus. DC were cultured in the presence of URC for 30 min, 1 h and 2 h, and nuclear extracts were analyzed for NF-κB content. As shown in Figure 3A, URC was able to induce NF-κB translocation and activation. Similar results were obtained with LPS-primed DC (Fig. 3A). The expression of TLR2 and TLR4 on monocytes, immature DC, URC-primed DC and LPS-primed DC was further analyzed by real-time quantitative RT-PCR, because of their different selective expression on DC. Monocytes and immature DC expressed the highest levels of TLR2 and TLR4 on the cell population examined. mRNA expression of these two receptors was dramatically down-regulated upon differentiation of monocytes into DC after 8 days of culture with GM-CSF, IL-4 plus LPS or URC (Fig. 3B). URC-primed DC

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**Figure 2.**

**A** IL-12p70 production by CD40-L transfected J558-stimulated DC. DC were generated by stimulating immature DC with URC (0.01–1 µM), LPS (100 ng/ml) or TNF-α (25 ng/ml). Cells were stimulated with the CD40-L transfected J558 cells for 24 h. After 24 h, the production of IL-12p70 was measured by ELISA in culture supernatants. iDC, immature DC. Data are the mean ± S.E.M. of four independent experiments. *P < 0.05 compared with immature DC.

**B** IL-12p70 production by URC-primed DC was inhibited anti-TLR2 mAb and anti-TLR4 mAb. DC were generated by stimulating immature DC with URC-primed DC (0.1 µM), LPS-primed DC (100 ng/ml) or TNF-α-primed DC (25 ng/ml). Cells were stimulated with the CD40-L transfected J558 cells for 24 h. After 24 h, the production of IL-12p70 was measured by ELISA in culture supernatants. iDC, immature DC. Data are the mean ± S.E.M. of five independent experiments. *P < 0.05 compared with without mAb.
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expressed considerable levels of TLR2 and TLR4. In URC-primed DC, TLR4 expression seemed to be more prominent than TLR2 (Fig. 3A and B). The expression of TLR4 on URC-primed DC was higher than that of LPS-primed DC. These data suggest that URC might activate by DC via a TLR4 and/or TLR2 signaling.

**URC-primed DC Promote the Differentiation of Naïve T Cells into Th1 Cells**

We next evaluated the nature of primary allogeneic T cell responses stimulated by URC-primed DC. Allogeneic URC-, LPS- or, TNF-α-primed DC induced a substantial increase in the secretion of IFN-γ by T cells (Fig. 4A), but had little effect on IL-4 secretion (Fig. 4B) and IL-13 secretion (data not shown). DC differentiated with URC co-cultured with naïve T cells turned into typical Th1 cells. This pattern of IFN-γ production by naïve T cell co-cultured with URC-primed DC were similar to the production of IL-12p70 by URC-primed DC. On the other hand, the production of IFN-γ and IL-4 by naïve T cells co-cultured with TNF-α-primed DC was not influenced by anti-IL-12 mAb (Fig. 4A and B).

To analyze the contribution of DC-derived IL-12 on the development of Th1 cells, we investigated the effect of a neutralizing anti-IL-12 mAb in co-cultured experiments, where naïve T cells were co-cultured with URC-primed DC. In URC-primed DC, neutralization of IL-12 increased the development of IL-4 producing T cells and decreased the development of IFN-γ producing T cells (Fig. 4A and B). Similar results were obtained with LPS-primed DC (Fig. 4A and B). On the other hand, the production of IFN-γ and IL-4 by naïve T cells co-cultured with TNF-α-primed DC was not influenced by anti-IL-12 mAb (Fig. 4A and B).

**Immunostimulatory Capacity in an Allogeneic Mixed Lymphocyte Reaction**

The ability to induce allogeneic T cell proliferation is a functional hallmark of DC in vitro. Changes in surface marker expression were also observed at a functional level, when analyzing the allostimulatory capacity of DC in an allo MLR. URC-primed DC demonstrated a higher stimulatory efficiency in an allo MLR than immature DC, LPS-primed DC or TNF-α-primed DC (Fig. 5). Co-culture of T cells with URC-primed DC resulted in an enhancement of T cell proliferation, indicating that URC potentiate the Ag-presenting activity of DC in an allo MLR.
DC Generated with URC Augment the Cytotoxicity of CD8+ T Cells Against T2 Target Cells

We compared the CTL responses of autologous CD8+ T cells supported by DC differentiated with URC (0.1 µM) or TNF-α. In an 8 h JAM assay to measure CTL apoptosis by tumor cells, T2 cells loaded with WT-1 peptides strongly induced DNA fragmentation of CTL that were generated with URC-primed DC pulsed with WT-1 peptides (Fig. 6A). Percentage of DNA fragmentation was dependent on the increased number of CTL cells. Similar results were obtained with a 51Cr release assay to measure lysis of target cells (Fig. 6B). On the other hand, percentage of DNA fragmentation in JAM assay and 51Cr release were low or undetectable when T2 loaded with HIV-1 peptide (unrelated peptide) and T2 (without peptides) were the target cells (negative control) (Fig. 6A and B).

URC-primed DC are Capable to Migrate

Since the other important requirement for DC efficient immunotherapy is the capacity to migrate towards the lymph node, we also studied the migratory capacity of DC toward CCL19 and CCL21 in vitro. As expected from the result of CCR7 expression, we observed migration response towards CCL19 and CCL21 in the URC-primed DC (Fig. 7). In contrast, the observed migration response towards CCL19 and CCL21 in TNF-α-primed DC and immature DC was found to be low (Fig. 7). URC-primed DC induced the expression of CCR7, the differences observed in the migration assay stress the importance of assaying the functionality of the expressed CCR7 in a migration assay, in order to assess the actual migratory capacity of DC.

Discussion

In this study, we investigated whether URC was able to alter the phenotype and function of DC derived from human monocytes in vitro. Here, we demonstrated that culture of immature DC with URC revealed up-regulation of the surface expression of costimulatory and HLA molecules. URC-primed DC functionally enhanced T cell stimulatory activity in an allo MLR. Furthermore, DC generated with URC induced Th1 cells. In addition, URC showed a good capacity to migrate towards CCL19 and CCL21, and showed...
Figure 6. Autologous CD8+ T cells with URC-primed DC showed higher cytolytic activity against T2 target cells loaded with WT-1 peptide at a high effector-to-target ratio than against T2 target cell without WT-1 peptide. A) The CTL were labeled with [3H]-methylthymidine and served as the target for tumor cell lines. T2 cells induced more DNA fragmentation of CTL that were generated with URC-primed DC pulsed with WT-1 peptides than those that were generated with immature DC or TNF-α-primed DC pulsed with WT-1 peptides. B) Specific lysis was measured by 51Cr release assay. Data are the mean ± S.E.M. of three independent experiments. Note: *P < 0.05 compared with T2.
hereby both in vitro characteristics that are important for optimized DC for immunotherapy, the capacity to migrate and to produce IL-12p70 upon CD40 ligation. The differentiation of monocytes into DC has a critical impact on the immune response and DC plays a critical role in directing different effector T cell responses.1,3 The interaction of T cells with DC is crucial for directing T cell differentiation towards the Th1, Th2 or Th17 cell type.4,20 Th1 and Th2 cells represent terminally differentiated effector cells characterized by a different cytokine production and homing capacity.21,22 It showed that URC-primed DC polarized into Th1 via high IL-12p70 secretion upon CD40-L (T cells engagement) stimulation, since the production of IFN-γ from Th cells by URC-primed DC was affected by the presence of neutralizing anti-IL-12 mAb. IL-12 production by DC is a major inducer of IFN-γ and IL-12p70 is the cytokine responsible for DC-mediated Th1 polarization, and for the enhancement of antitumor responses of T lymphocytes. The Th1 cells that produce IFN-γ have been shown to exert a powerful antitumor effect. Similar results were obtained with LPS-primed DC. The production of IL-12p70 and IFN-γ by URC-primed DC was more strongly enhanced than that of LPS-primed DC and TNF-α-primed DC. IL-12 plays a central role in the immune system, not only by augmenting the cytotoxic activity of T cells and NK cells and regulating IFN-γ production, but also by the capacity of IL-12 to promote the development of Th1 cells.23-25 Therefore, it suggests that the effect of URC on the production of IL-12p70 by DC and strengthening of the Th1 response by naïve T cells might contribute to a potential antitumor effect of URC. The rationale for selecting IL-12 production as a potency assay for DC generated for human therapy is based on IL-12 properties and its confirmed role in host defense against pathogens and cancer. The generation of effective antitumor immunity involves the production of Th1 cytokines such as IL-12 and IFN-γ that might facilitate the induction and/or activation of tumor Ag-specific CD4 and CD8 cells. URC-primed DC provide the proinflammatory cytokines needed for T cell activation and Th1 development. It seems that TNF-α-primed DC drive the differentiation of naïve T cells towards Th1 cells via an unknown factor, because TNF-α-primed DC did not increase the IL-12 production upon CD40-L.

The understanding of mechanisms controlling IL-12 induction by adjuvants in general and URC in particular may contribute to improving their impact on cellular immune responses in human therapies. Recent studies have demonstrated that the signaling via TLR which are newly identified receptor molecules recognizing many pathogens, is involved in the induction of anti-cancer immunity.30 An important immunomodulatory property of TLR agonists is their capacity to enhance the IL-12 production from DC and other innate immune cells and consequently their ability to promote Th1-type responses, which play a key protective role in immunity to tumor.26 TLR2 and TLR4 signaling frequently enhances the production of IL-12, a major Th1-inducing cytokine, on DC. We evaluated the role for TLRs in the development of Th1 cells induced by URC-primed DC. In this study, we found that anti-TLR2 mAb and anti-TLR4 mAb inhibited the production of IL-12p70 by URC-primed DC and influenced the production of Th1 cytokines from naïve T cells co-cultured with URC-primed DC. The inhibitory effect of anti-TLR2 mAb in IL-12p70 production by URC-primed DC was weak. We detected mRNA expression of TLR2 and TLR4 on URC-primed DC. However, the magnitude of TLR4 expression on URC-primed DC seemed to be higher than the TLR2 expression. Interestingly, we found that upon URC and LPS stimulation, mRNA expression of TLR2 and TLR4 was down-regulated with overall mRNA transcript detection levels being lower than in immature DC. It has been reported that monocyte-derived immature DC down-regulate TLR2 and TLR4 upon maturation with the corresponding

![Figure 7. Migration in response to CCL19 and CCL21 by URC-primed DC, LPS-primed DC or TNF-α-primed DC. URC-primed, LPS-primed DC or TNF-α-primed DC were prepared and recovered and their migratory abilities in response to CCL19 (250 ng/ml) and CCL21 (250 ng/ml) were determined in vitro.](image-url)
cognate ligand LPS.27 Our experimental data support their observations showing that LPS in human DC and monocytes express different mRNA TLR transcripts. One might speculate that URC-induced regulation of TLR2 and TLR4 expression may be involved in positively or negatively modulating the recognition of URC motifs by TLR2 and TLR4. URC and LPS were, in part, involved in down regulation of human TLR. It has been reported that TLR2 and TLR4, when exposed to LPS, can lead to NF-κB activation and DC maturation.28 Our experiments showed that common signaling pathways are activated by URC and LPS, as they both induce NF-κB activation and modulate TLR expression. From an antibodies experiment results, it appears likely, that URC-primed DC drive Th1 cell polarization via the activation of IL-12p70 dependent on TLR4 and/or TLR2 signaling.

DC are professional APC that are required for the initiation of immune responses. In the immunotherapy against malignant diseases, it has been suggested that the induction of tumor antigen-specific CTL is most important for eliminating tumor cells. Antitumor immunity has classically been measured by the quantity of tumor-antigen-specific CD8+ T cells.29 In this context, it is important to know whether URC-primed DC enhanced specific CTL responses. As expected from their Th1-polarizing effect, the DC differentiated with URC induced a stronger CTL response than immature DC or TNF-α-primed DC. DC activated with TLR agonists, especially TLR4 and TLR8 agonists stimulate IL-12-producing human myeloid DC, which activate CD8 CTL against tumors.10 Moreover, Kawasaki et al30 have reported that TLR4 mediates LPS-mimetic signal transduction by an anti-cancer agent Taxol, a plant-derived diterpene, in mice but not human. More recently, synthetic ligands for TLR4, TLR7 or TLR9 have been through preclinical evaluation and clinical trials against cancer.2 It suggests that TLR ligands are useful applications for immunotherapy for cancer patients. Therefore, our results suggest that URC is a promising approach for the treatment of cancer.

The ability of DC to migrate to mature local lymph nodes and their subsequent presentation of antigen to T cells plays an essential role in the initiation of adaptive immunity. In tissue mature DC must be responsive to lymph node derived signals, but must also be able to down-regulate tissue anchoring proteins including E-cadherin that would be to detrimental DC migration and antigen presentation to naïve T cells. URC-primed DC had up-regulated the expression of CCR7 and an intermediate migratory capacity towards CCL19 and CCL21, suggesting that URC-primed DC have the potential to migrate in vivo. In most clinical trials using DC-based immunotherapy, immature monocyte-derived DC pulsed with tumor antigen peptides were used. Recent studies reported that mature DC could be a better antitumor adjuvant.31 However, there is no clear answer, yet.

Because Polymyxin B is LPS inhibitor, we investigated the effect of Polymyxin B on IL-12p70 production by URC or LPS. Polymyxin B completely inhibited the LPS-induced IL12p70 (data not shown). In contrast, the same treatment did not affect the production of IL-12p70 by URC-primed DC, ruling out the possible contamination with LPS.

Several mechanisms have been proposed to explain the apparent adjuvant effects of TLR agonists on antitumor immunity. TLR trigger the secretion of critical cytokines, and TLR can stimulate the proliferation of CD4+ T cells and CD8+ T cells. Moreover, TLR signaling frequently enhances the production of IL-12 on DC. Thus, it is strongly suggested that DC matured by TLR stimulation may induce T cell differentiation toward Th1 by presenting antigens to T cells while promoting a Th1-leading situation in the local environment. Several TLR agonists have been developed as anticancer drugs. We expect to be able to correlate the in vitro DC product attributes with in vivo immunologic and clinical end points.

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**Abbreviations**

URC, Uncarinic acid C; DC, dendritic cells; LPS, lipopolysaccharide; CD40-L, CD40ligand; MLR, allogeneic mixed lymphocyte reaction; TNF-α, tumor necrosis factor-alpha;
GM-CSF, granulocyte-macrophage colony-stimulation factor; TLR, Toll-like receptor.

Disclosure
This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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