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Synergistic effect of Toll-like receptor 4 and 7/8 agonists is necessary to generate potent blast-derived dendritic cells in Acute Myeloid Leukemia

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ABSTRACT

Leukemic cells from AML patients can be differentiated to dendritic cells (DCs). Such DCs have potential for immunotherapy of patients. Blasts from 15 AML patients were differentiated into DCs and matured by different TLR agonists. We could generate AML-DCs from 73% of patients mostly with M4 or M5 subtypes. The DC recoveries ranged from 28% to 50%. The results showed that concomitant use of TLR4 and TLR7/8 agonists induced proficient DCs. Therefore, a combination of TLR4 and 7/8 agonists can be considered as an appropriate maturation cocktail for AML-DC production in order to use in the immunotherapy of AML patients.

1. Introduction

Despite intensive consolidation chemotherapy in AML patients, relapses occur in 50% of patients due to the presence of minimal residual disease (MRD) [1–4]. Based on the major role of the immune system in the prevention and control of leukemia, alternative treatments have been explored to modulate the immune system [5,6]. Dendritic cells (DCs) are professional antigen presenting cells and are recognized as key regulators of the immune system. Recently, DC vaccination has been developed as a promising immunotherapy for cancer. Culture of DCs ex vivo circumvents the immunosuppressive features of tumor microenvironments, and can result in the eradication of residual tumor cells [3,7–9].

AML blasts can be differentiated to DCs (AML-DC) in vitro. These converted blasts display typical DC markers (e.g., CD40, CD86, CD1a, CD83 and CCR7) [8,10,11]. Leukemic DCs differentiated from AML blasts can induce anti leukemic T-cell responses [12,13].

Pathogen-associated Toll-like receptor (TLR) agonists are critical in the induction of immune responses to pathogens [14]. A number of studies have been conducted using different synthetic TLR agonists to modulate DC function to favor the generation of Th1 responses, considered necessary for effective cancer immunotherapy [15–17].

Several artificial TLR agonists have been synthesized so far. Bacterial lipopolysaccharide (LPS), is recognized by TLR4. TLR4 employs both MyD88 and TRIF pathways to activate NFκB and IRF3 transcription factors respectively [13,14].

R848 is a low molecular weight synthetic imidazoquinoline compound which mimics the natural ligand ssRNA. R848 as a TLR7/8 ligand, exerts its activating function via the MyD88-dependent signaling pathway and triggers NF-κB activation [17–19]. TLR3-mediated activation can be carried out by Poly(I:C) (Polyinosine–polycytidylic acid) which is a synthetic analog of double-stranded RNA (dsRNA). TLR3 activation is TRIF dependent and the only TLR which acts completely independent of MyD88 [20]. However, treatment of DCs with a single TLR agonist is inadequate to induce an effective Th1 response. Simultaneous targeting of TLR3, TLR4 and TLR7/8 synergistically induces a strong polarized Th1 response from human monocyte-derived dendritic cells [19,21].

Here, we demonstrate that a combination of TLR agonists has a noticeable synergistic effect on maturation and function of AML blast derived dendritic cells.

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2. Materials and methods

2.1. Cell culture medium and reagents

The cell culture medium (hereafter named DC medium) was liquid RPMI-1640 Glutamax medium (Gibco, BRL Life Technologies, Paisley, UK) supplemented with 5% heat-inactivated pooled human AB serum (Iranian Blood Transfusion Organization, Tehran, Iran), 1% penicillin-streptomycin solution (Biosera, East Sussex, UK), 1% NEAA (non-essential amino acid solution) (Sigma, st. Louis, MO, USA) and 1 mM sodium pyruvate (Sigma, st. Louis, MO, USA). DC medium without AB serum is referred to as incomplete medium. Human recombinant GM-CSF (hrGM-CSF) and human recombinant IL-4 (hrIl-4) were obtained from R&D Systems.

The following conjugated mouse anti-human monoclonal antibodies were obtained from eBioscience (San Diego, CA, USA): FITC conjugated anti-CD14 (IgG1, Clone 61D3), FITC-conjugated anti-CD83 (IgG1, Clone HB15e), PE-conjugated anti-CD1a (IgG1, Clone HI149), FITC-conjugated anti-CD40 (IgG1, Clone 5C3), PE-CY5 conjugated anti-CD86 (IgG2b, Clone: IT2.2), PE-CY5 conjugated anti-HLA-DR (IgG2b, Clone: LN3), PE-CY5-conjugated anti-CD3 (IgG1, Clone UCHT1), eZFluor™ FITC anti-CD4 and PE anti-CD8 Cocktail (IgG1, Clone: RPA-T4/RPA-T8), PE anti-CD197 (CCR7) (Rat IgG2a, Clone: 3D12) and their respective isotype controls. PE-conjugated anti-hACE/CD143 (mouse IgG1, Clone: 171417) was provided by R&D Systems. Cell culture tested Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma–Aldrich, Dorset, UK) was reconstituted in RPMI-1640 at a concentration of 1 mg/ml. Poly(I:C) and R848 (Resiquimod) were from InvioGen (San Diego, CA, USA) and solubilized according to the manufacturer's instructions. Aliquots of TLR ligands were frozen at −20 °C and thawed before use.

2.2. Patients' characteristics and sample collection

Heparinized peripheral blood samples were drawn from 15 AML patients (10 males and 5 females) referred to Shariati Hospital oncology center in Tehran following informed consent. The patients' characteristics are shown in Table 1. The subtypes of AML were determined according to the French-American-British (FAB) classification criteria [22,23]. Blood was diluted 1:1 with phosphate buffered saline (PBS), and layered onto Ficoll/Hypaque (Lymphoflot, Bio-Rad, Germany) density gradient. After centrifugation at 2300 rpm for 23 min at room temperature, the mononuclear cell (MNC) fraction containing the malignant cells could be harvested from the buffy coat layer. This fraction was washed twice to minimize platelet contamination and then cryopreserved using freezing container (Mr. Frosty, Sigma–Aldrich, USA) or used immediately for culture experiments.

2.3. Generation of mature AML-DCs from leukemic blasts

AML derived dendritic cells were generated from blasts as described in other studies with some modifications [24–27]. Fresh or thawed leukemic blasts were resuspended and seeded in 24-well culture plates (Orange Scientific, Braine-l'Alleud, Belgium) for 6 days at 0.7×10^6 cells in 0.7 ml of DC medium per well in the presence of 20 ng/ml rhIL-4 and 50 ng/ml GM-CSF. Cytokines were replenished on day 3. On day 6 of the culture, immature AML-DCs were stimulated separately with Maturation Factors (MF) MF1: LPS (2 μg/ml), MF2: LPS + R848 (2 μg/ml) and MF3: LPS + R848 + poly(I:C) (12 μg/ml) for 24 h. The optimized doses of MFs and the time of stimulation were determined through culturing of the cells in mediums supplemented with graded concentrations of stimulators (data not shown).

Matured cells were harvested at day 7 for subsequent experiments. Cultured AML-DCs were immunophenotyped by flow cytometry on day 7 and evaluated for their viability by staining with trypan blue exclusion.

2.4. Immunophenotyping by flow cytometry

To confirm the differentiation and maturation of AML-DCs, the expression of cell surface markers were analyzed using conjugated antibodies to CD14, CD1a, CD11c, CD83, HLA-DR, CD40, CD86, CCR7 and their respective isotype controls. At the end of the culture period, cells were harvested, centrifuged, and resuspended in ice cold FACS buffer (PBS containing 0.1% NaN3 and 2% FCS). The cells were then aliquoted at 10^5 cells per FACS tube (Falcon 2054, Becton Dickinson, NJ, USA). The optimized amount of conjugated mAbs were added to the cell pellets and the tubes were incubated on ice in the dark for 45 min. Cells were then washed and resuspended in 400–600 μl of FACS buffer and analyzed immediately or fixed by the addition of 1–2% paraformaldehyde.

Data acquisition was carried out on a FACS Calibur flow cytometer (Becton-Dickinson, CA, USA) and analyzed using CellQueste software. Data were expressed as percentage of positive cells in comparison to the cells stained with isotype controls.

2.5. Allogeneic mixed lymphocyte reaction (MLR)

The allostimulatory potential of DC (day 7 immature and mature AML-DCs) and controls (primary blast cells) was compared using the allogeneic mixed leukocyte reaction (MLR). Responder PBMCs were prepared from one unrelated healthy and fixed donor. After washing with phosphate-buffered saline (PBS) the PBMCs were resuspended at $5–10 \times 10^6$ cells/ml in incomplete medium and labeled with 2.5 μM CFSE (Molecular Probes Europe, Invitrogen Detection Technologies, Leiden, Netherlands) for 15 min at 37 °C. The reaction was stopped by the addition of an equal volume of fetal calf serum (FCS), followed by a 10-min incubation at 4 °C. After 3 washes with incomplete medium to remove the free CFSE, 10^5 CFSE-labeled-PBMCs in 100 μl DC medium were seeded in a 96-well plate and co-cultured with the graded numbers of irradiated (3000 rad) AML blasts, immature and mature AML-DCs (Stimulator/Responder ratios: 1:20, 1:10 and 1:5) for 5 days at 37 °C and 5% CO_2. T cells alone were used as background controls. Maximal stimulation of the T-cell population was also determined by culturing of T cells with 4% PHA (Gibco, BRL Life Technologies, Paisley, UK). Following five days of co-culture, recovered lymphocytes were analyzed by flow cytometry. Allogeneic T cells in the ratio of 1:10 were stained with anti-CD3 conjugated antibody to specify the features of proliferated cells.

2.6. Phagocytosis assay using FITC-labeled dextran beads

Immature and mature AML-DCs were incubated with 1 mg/ml FITC-dextran beads (MW: 40 kDa; Sigma) prepared in RPMI containing HEPES at 37 °C for 1 h, with control cells set at 4 °C (controls for adherence of beads to the cell surface). Cells were then washed three times with ice-cold FACS buffer and analyzed by flow cytometer.

3. Results

3.1. DCs can be efficiently generated from leukemia blasts of AML patients

PB or BM samples from fifteen AML patients were cultured in a medium supplemented with hrGM-CSF and hrIL-4 for 6 days. The cells showed a typical DC morphology with an increased diameter and developed cytoplasmic protrusions. The viability of AML-DC preparations was >95% by trypan blue staining (data not shown). Blasts from 11 patients could be converted to leukemic DCs and exhibited a DC-like morphology. When 0.7×10^6 leukemic blasts were cultured in 0.7–1 ml of DC medium containing 5% AB serum, the median numbers of mature leukemic DCs harvested were 0.3×10^6 (range, $0.2–0.35 \times 10^6$). Thus, recoveries ranged from 28% to 50% (median: 42%). There was no difference in the quantity of DCs matured in the presence of different TLR agonists.

3.2. Phenotypic analysis of AML-DCs by flow cytometry

To evaluate the differentiation of primary blasts, AML-DCs were analyzed and compared with fresh blasts for expression of key surface markers such as CD1a, CD83, CD40, CD86, CD11c and HLA-DR. On FACS analysis, 6-day cultured DCs showed a typical phenotype of immature DCs with low level of CD14 and high level expression of CD1a and HLA-DR, intermediate level expression of CD11c, CD40 and CD86. As shown in Fig. 1, increased expression of CD83, CD86 and CD40 was observed for all three different conditions of maturation, whereas HLA-DR expression was slightly up regulated. Although, there was significant difference in the expression of CD83, CD86 and CD40 ($P < 0.05$) between fresh primary blasts and AML-DCs, there was no significant difference between different TLR agonists stimulation. In addition, we could generate a mature DC phenotype from 73% (11/15) of patients mostly with M4 or M5 subtypes.

3.3. Leukemic derivation of dendritic cells

In order to find out whether the cultured DCs were originated from the malignant blasts, a phenotypic analysis based on CD143 (ACE) expression was performed. The mean percentage of positive cells for ACE molecule in four separate experiments on immature AML-DCs was $10 \pm 3\%$ (Fig. 2). We also compared ACE expression in DCs derived from patient's blasts and DCs derived from normal monocytes of healthy volunteers. In contrast to AML-DCs, DCs obtained from monocytes of healthy donors produced high levels of ACE ($50 \pm 4.5\%$, $n = 3$). Thus, the slight expression of ACE on AML-DCs proved their leukemic origin.