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Original Article

Uptake of Autologous and Allogenic Tumor Cell Antigens by Dendritic Cells

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Abstract

Background: Dendritic cells (DCs) are professional antigen presenting cells (APCs), and there is considerable interest in their application as a cellular adjuvant for cancer immunotherapy. Previous studies indirectly demonstrated that DCs were able to take up tumor lysate (crude soluble tumor antigens) and also cross-present tumor associated antigens (TAA) which elicits anti-tumor immune response.

Objective: To provide direct evidence that demonstrates the uptake of tumor lysate by DCs and to find out whether this capability is restricted to allogenic or autologous tumor lysate preparation.

Methods: DCs were generated from magnetic bead-isolated monocytes of B-CLL patients as well as healthy donors. Proteins of tumor lysate were conjugated with FITC. Their uptake by autologous as well as allogenic DCs was analyzed using FACS flowcytometry system.

Results: In both autologous and allogenic experiments, green fluorescence intensity (FL1) of immature DCs incubated with FITC-labeled tumor lysate was clearly higher than unpulsed counterparts, which were considered as background.

Conclusion: Immature DCs are able to efficiently take up FITC-labeled tumor lysate of autologous as well as allogenic sources. This finding confirms the results of previous studies, which have demonstrated that tumor lysate-pulsed DCs were able to elicit cytotoxic anti-tumor response and concluded that DCs could take up tumor lysate.

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Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs). Their interaction with T cells is a key event in the early stages of a primary immune response. DCs express high levels of MHC and co-stimulatory molecules such as CD40, CD80, and CD86, and they also produce high levels of cytokines, including IL-6, IL-8, IL-10, and IL-12[1]. These properties, combined with the efficient capture of antigens by immature DCs (imDC), allow them to efficiently present antigenic peptides and stimulate

Table 1: Clinical characteristics of B-CLL patients

Patients	Gender	Stage	Age	$CD19^+$%*	$CD3^+$ %	$CD14^+$%	$CD56^+$%	$CD3/CD56^+$%
1	Female	Non-progressive	65	86	10.6	1	2	2.3
2	Male	Non-progressive	68	53	26	6.4	7.6	5
3	Male	Non-progressive	66	62.5	18	2	9	2.5

* : % is considered in PBMC

Ag-specific naïve T cells. Due to their potency as APCs there is considerable interest in using these cells as adjuvant to enhance immunity against cancer[2].

Large numbers of DCs can be generated from human monocytes by culturing them in the presence of suitable cytokines.[3,4] During this process DCs pass through an immature stage in their differentiation pathway when they efficiently capture surrounding antigens and present them to naive T cells. Immature DC capture antigens by several pathways such as: 1) macropinocytosis; 2) receptor-mediated endocytosis via C-type lectin receptors (mannose receptor, DEC-205) or Fcγ receptors Type I (CD64) and Type II (CD32) (uptake of immune complexes or opsonized particles); 3) phagocytosis of particles such as latex beads, apoptotic and necrotic cell fragments (involving CD36 and avb3 or avb5 integrins), viruses, bacteria as well as intracellular parasites such as Leishmania major, and 4) internalization of heat shock proteins gp96 and Hsp70 via as yet unknown surface receptors. Captured antigens are directed to endosomal compartments for MHC class II loading and presentation[5,6]. Recent studies demonstrated the ability of imDC to capture apoptotic cells and to elicit CTL response.[7,8] None of the previous studies have directly demonstrated uptake of tumor lysate. In the present study, using a novel method for fluorescence labeling of protein, we have shown that imDC can take up autologous as well as allogenic tumor cell lysate.

Material and Methods

Patients and healthy donors

Three B-chronic lymphocytic leukemia (B-CLL) patients (2 males and 1 female) with a mean age of 66.3 were studied (Table 1). All patients had non-progressive disease and had not previously received any therapy. The diagnostic and staging criteria as well as criteria for progressive and non-progressive disease have been described earlier[9,10]. Six age-matched healthy donors were also included in this study.

Culture medium

Complete culture medium (CM) consisted of RPMI 1640 with L-glutamine (Sigma), 100 IU/ml Penicillin (Sigma), 100 µg/ml Streptomycin (Sigma), and 10% heat-inactivated pooled human AB^+ serum (Karolinska Hospital Blood Transfusion Center, Sweden).

Cell separation

Peripheral blood of CLL patients was collected in sterile heparinzed tubes. Buffy coats from healthy donors were provided by Karoliska hospital blood transfusion center. Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation at 750g on Ficoll-paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) for 20 min. PBMC were harvested from the interphase and washed three times in phosphate buffered saline (PBS) to remove platelets.[11] $CD14^+$ cells were positively selected using anti-CD14 antibody conjugated magnetic microbeads (Miltenyi Biotech, Germany) according to the manufacturer's instruction. In brief, PBMC were incubated with saturating concentration of anti-CD14 conjugated colloidal microbeads (MiniMACS®) for 15 min at 4°C. Magnetically labeled cells were and passed through MACS®-columns under strong magnetic field and positively enriched.

To purify B cell of CLL patients, PBMC were washed three times with PBS, placed on nylon wool columns (Biotest, Breiech, Germany) to elute B cells from the column.[12,13] Effluent B cells were collected and cell purity was determined by flowcytometry on FACScan (Becton-Dickinson, Mountain-View, CA, USA) .

Generation of dendritic cells

DCs were generated from $CD14^+$ cells as previously described.[14] Briefly, the isolated $CD14^+$ cells were applied on MidiMACS (Miltenyi Biotech, Germany) columns for second time to obtain pure $CD14^+$ cells. Enriched $CD14^+$ cells at a density of 10^6 /ml were cultured in CM supplemented with 100 ng/ml rhGM-CSF (Leukomax ®, Novartis, Switzerland) and 50 ng/ml rhIL-4 (SPRI; 10 ng/ml) for 5 days. On day 3, CM was replaced with fresh CM

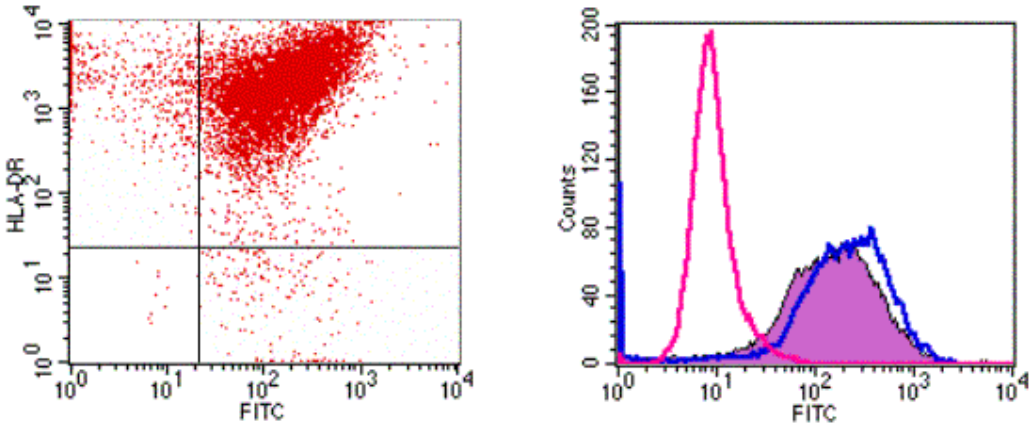

Figure 1. Flowcytometry analysis of CLL patients imDC taken up FITC-conjugated tumor lysate. Day 5 imDC were loaded with FITC-conjugated autologous tumor lysate derived from malignant B cell at 37°c for 4h. **Left**) Demonstration of high HLA-DR expression of FITC positive cells indicating DC identity. **Right**) FACS analysis of imDC, before and after (dark gray) uptake of fluorescence-labeled tumor lysate. Fluorescence intensity of FITC-lysate pulsed mDC is obviously increased in compare to unpulsed controls. Overlaid open histogram demonstrates fluorescence intensity of labeled DCs before the surface fluorescence quencher applied.

and cytokines. Cells with typical morphology of DC and $CD3^-$, $CD14^-$, $CD19^-$, $CD83^-$, $CD80^+$, $CD86^+$, $HLA\text{-}DR^+$ were defined as imDC as described earlier.[15] On day 5, imDCs were used for phenotyping and functional assay. $CD1a^+/HLA\text{-}DR^+$ double positive cells were used as imDC in all experiments.

Immunophenotyping

Cells were analyzed by flowcytometry on FACScan using fluorochrome-conjugated CD3, CD5, CD14, CD19, CD80, CD86 and HLA-DR monoclonal antibodies (mAb) and their negative isotype controls (Becton-Dickinson, CA, USA). Anti-CD19, CD1a and CD83 were obtained from DAKO A/S (Glostrup, Denmark). Surface marker staining was performed according to the protocol described earlier.[14] Briefly, 0.5-1×10^6 purified cells were stained with fluorochrome-conjugated mAb or isotype non-labeled antibodies. Cells were incubated on ice for 20 min followed by two washings with ice-cold PBS and analyzed by FACScan using the CELLQuest software (Becton Dickinson, CA, USA).

Preparation of tumor cell lysate

Purified B cells were resuspended at a density of 4x10^7/ml in serum-free medium and subjected to four freeze-thaw cycles using dry ice and 37°C cell culture incubator. For the removal of crude cell debris, the lysate was centrifuged for 10 min at 300g at 4°C and the supernatant was collected. The protein concentration of the lysate was determined by a commercial protein assay kit (Bio-Rad, Munich, Germany).

Florescence labeling of tumor lysate

Conjugation of tumor lysate proteins with FITC was performed taking advantage of FITC molecule's tendency to make covalent binding with lysine amino acid molecules in protein structure[16]. Tumor lysate was mixed with FITC solution (Sigma, 10 μg/ml), at 1:1 (V/V) ratio and incubated over night at 4°C. To elute free FITC molecules, the FITC-protein solution was dialyzed using dialysis filter with molecular weight cut off (MWCO) of 3500 (Spectrum, CA, USA) against dialysis buffer at 4°C for 24h. Protein concentration of the FITC-conjugated lysate was determined at a wavelength of 595nm. The conjugate was added to immature DCs at a final concentration of 120 μg/ml on day five of DC generation.

Uptake of FITC-labeled lysate by imDC

Immature DCs (5 days) were incubated with fluorescence labeled lysate (120 μg/ml) at 37°C for 4h, and then washed twice and their fluorescence intensity determined using FACS system. Unpulsed imDC were used to determine background fluorescence. After dialysis, the dialyzing buffer was used to determine the amount of free FITC in conjugated lysate solution. DCs were incubated with dialyzing buffer for 4 hours at 37°C followed by determination of fluorescence intensity of DCs. To quench the probable fluorescence produced by

non-specific attachment of FITC-labeled proteins to DCs surface, DCs were initially incubated with FITC-conjugated tumor lysate for 4 h at 37 °C and then with Trypan blue (0.5mg/ml in Tris Hanks buffer) for 5 min at 4°C followed by twice washing with cold PBS. Cells were analyzed by flowcytometry using CELLQuest software.

Results

Purity of monocyte, B cell and identity of DCs
Monocyte purity in six cell isolation experiments of healthy donors (97.3±0.9%) was confirmed after FACS analysis. The purity of $CD19^+/CD5^+$ B cells in the three B-CLL patients ranged from 89% to 95% (91.3±1.4; mean±SEM) while the purity of monocytes ($CD14^+$ cells) ranged from 92% to 98% (93% ±2; mean ±SEM). Immature DCs were generated from peripheral blood monocytes of non-progressive B-CLL patients and healthy control donors. The cells displayed a characteristic morphology and phenotype of DCs ($CD14^-$, $CD1a^+$, $CD80^+$, $CD86^+$, $CD83^-$, HLA-DR^{++}) in either normal or B-CLL individuals.

Uptake of FITC-labeled tumor lysate by immature DCs
I-Autologous experiments
Five days immature DCs of CLL patients could take up FITC-labeled tumor lysate prepared from autologous B-CLL tumor cells. Using flowcytometry to determine the mean fluorescence intensity (MFI) of pulsed DCs (180±12, mean±SEM), considerable increase was detected compared with unpulsed imDC, which were used as background control (4.3±0.7, mean±SEM). Application of surface fluorescence quencher (Trypan blue) had a minor effect on mean fluorescence intensity of pulsed DCs (92±6, mean±SEM). Simultaneous anti-HLA-DR staining of FITC positive cells confirmed the identity of DCs analyzed for green fluorescence intensity as HLA-$DR^+/FITC^+$ cells (Fig 1).

II-Allogenic experiments
Immature DCs generated from monocytes of healthy donors could efficiently take up allogenic B-CLL tumor lysate. The results of six independent experiments show considerable increase in mean fluorescence intensity (MFI) of DCs pulsed with FITC-labeled B-CLL tumor cell lysate (360.6±17.7, mean±SEM) in comparison with unpulsed control dendrititc cells (3.9±1.1, mean±SEM).

Discussion

The nature of the tumor associated antigens (TAA) and the optimal methods for DC loading are likely to constitute the most crucial parameter in DC-based tumor immunotherapy which needs to be analyzed. The most commonly used, clinically approved, approach is based on loading of empty MHC class-I molecules with exogenous peptides.[17-19] This is however limited by: (I) peptide restriction to a given HLA type; (II) induction of CTL responses only; and (III) limitation of the induced responses to defined TAA. These TAA have been identified based on the T cell responses in individual tumor-bearing patients which introduces another level of limitation as the individual T cell repertoire may be biased[5]. Furthermore, aside from the possibility that the repertoire is tolerized, these TAA are unlikely to represent tumor rejection antigens. Indeed, a current and common drawback to DC-based immunotherapy protocols is that it remains to be determined whether, or which, of the defined TAA peptides represent rejection antigens in vivo[7].

In contrast to the peptide-based approach, unfractionated tumor material may provide both MHC class I and MHC class II epitopes and does not require the identification of TAA. Furthermore, antigen presentation by MHC class I and class II leads to the diversification of immune responses and engage other effectors.[5,20] Recent studies demonstrate that DCs can capture apoptotic tumor cells and elicit MHC class I-restricted secondary CTL responses against tumor antigens.[8,20,21] This approach could provide a very attractive strategy for DC-based vaccination protocols whereby tumor-derived epitopes could be presented without the need for molecular characterization of TAA.

Several studies regarding capability of DCs to take up tumor cell lysate, present TAA and elicit effective immune response both in vitro[22-24] and in vivo[25-30]. Although direct evidence for capture of apoptotic tumor cells by DCs has already been shown[8], none has directly demonstrated the uptake of tumor cell lysate. Herein, we have directly shown that immature DCs efficiently take up soluble tumor antigens via a pathway non-specific for autologous or allogenic proteins. The results of this study confirm the indirect evidences that show the activation of T cells against tumor antigens using DCs loaded with tumor cell lysate[22-30].

The goal of vaccination approaches in human cancer is to induce tumor-specific, long-lasting immune response that leads to tumor elimination. The induction of tumor immunity can be viewed as a three-step process that includes: (I) presentation of TAA; (II) selection and activation of TAA-specific T-cells as well as non-Ag-specific effectors; and (III) homing of TAA-specific T-cells to the tumor site and recognition of restriction elements leading to the elimination of tumor cells.[5,31] In the present study, DCs have been generated from monocystes of CLL patients using magnetic bead-isolated

monocytes which confirms the results of another study regarding DCs generation in vitro using B-CLL patient's monocytes.[22] We have also shown that the generated DCs display morphologic and phenotypical characteristic similar to DCs generated from healthy donors under the same condition. Furthermore, DCs generated from B-CLL patient's monocytes had the same capability in antigen uptake from surrounding microenvironment. These are in agreement with results of previous study, which demonstrated no significant deficiency in functional properties of DCs in B-CLL patients compared with normal controls.[14] Feasibility of this method and easy access to tumor cell samples (B lymphocytes) pave the way for the application of DCs in B-CLL patient's immunotherapy.

Direct demonstration of tumor cell lysate uptake by mouse DCs has been previously reported.[25] However, the technique applied to lable the lysate was not protein specific as the lipophilic dye (PKH-2) has a high tendency to stain cell membrane fragments and lipoproteins. We have specifically conjugated tumor lysate proteins with FITC, forming covalent bonds at lysine sites in protein structure.[16] Non-specific binding of FITC-labeled proteins to the cell membrane was ruled out by using cell surface fluorescence quencher that leaves only fluorescence of intracellular origin detectable.

Taking advantage of simultaneous fluorescence staining of FITC/lysate-loaded DCs with one or more non-FITC conjugated mAbs would provide an easy technique to analyze for assessment of functional properties of different human DCs subpopulations.

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