

Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays

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Abstract

Understanding the whole process of dendritic cell (DC) activation might help in the development of more efficient immunotherapeutic strategies for tumor patients. Part of this process is cytokine secretion, which has important effects on innate and adaptive immune response. Here, we cultured circulating monocytes for five days with interleukin-4 and GM-CSF followed by two-day culture with or without CD40 ligand and LPS to create a mature DC (mDC) and an immature DC (iDC) phenotype, respectively, characterized by differential expression of co-stimulatory molecules (CD80, CD83). We then compared the cytokine expression profile of the mDC and iDC using two protein platform arrays. Twelve supernatants from mDC paired with 12 from iDC were compared. The mDC protein expression profile showed significant increases in 16 out of 34 factors tested, including TNF α , IL-10, IL-12, IFN γ , MIP1 α , MIP1 β , IL-8, MDC, RANTES, and IL-6, which play a crucial role in the regulation of the innate immune response as well as the recruitment and activation of adaptive immune effectors. Interestingly, some of the cytokines expressed during maturation were also found in the gene expression profile identified in tumor metastases following IL-2 therapy using cDNA arrays. This finding suggests a possible role for resident DC maturation as a mediator of systemic IL-2 effects. Most important, the array of cytokines secreted during DC maturation may be considered an important component during adoptive transfer. Further characterization of the kinetics and persistence of their secretion should be undertaken in the future.

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Abbreviations: APC, antigen presenting cells; DC, dendritic cells; iDC, immature dendritic cells; mDC, mature dendritic cells

1. Introduction

Understanding the process of dendritic cell (DC) activation might be a key to the development of more efficient immunotherapeutic strategies for treating patients with cancer [1]. In the last decade, interest in the biology of DC resident in tumor deposits or adoptively transferred for vaccination purposes has consistently increased. Resident DC might influence the development of a natural immune response and mediate the intensity of

therapy-induced immune responses. In previous research, we studied the gene expression profile of metastatic deposits in patients with melanoma undergoing high-dose interleukin-2 (IL-2) therapy using an 8000-gene cDNA microarray [2]. Comparative analysis of the transcriptional changes occurring at tumor sites and in peripheral blood mononuclear cells (PBMC) pre- and post-IL-2 administration demonstrated predominant up-regulation of DC-associated cytokines, chemokines, and respective receptors, suggesting their central role in the target organ during systemic IL-2 therapy.

DC also offer great potential in the context of active-specific vaccination against cancer [1,3]. Several early-phase clinical studies show effectiveness of DC-based vaccination [4,5]. Most studies suggest that DC maturation is a requirement for the development and maintenance of tumor-directed T cell responses [6–8]. While

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immature DC (iDC) induce regulatory, IL-10-producing CD4⁺ and CD8⁺ T cells, mature DC (mDC) induce functionally active IFN- γ -producing CD8⁺ T cells [1]. There is also preliminary evidence that mDC are able to induce stronger T cell responses than iDC in tumor patients [9].

Gene expression profiling of DC during different maturation steps identified the transcription of various cytokines and chemokines including IL-10, IL-12, IL-6, TNF α , TARC, RANTES, MCP-1, CCR7, MIP-3 β , and MIG [10–12]. These results open the question of whether a corresponding gene product could be observed in similar conditions. However, to our knowledge no comprehensive protein-based investigation of the secretory response of DC in response to maturation stimuli has been reported. Secretion of a few cytokines during DC activation has been characterized including IL-6, IL-10, IL-12, IL-2, and TNF α [11,13–15]. In addition, using a traditional ELISA, MIP-1 α , MIP-1 β , and IL-8 were observed in response to maturation stimuli [16]. Since cytokines might serve an important role in linking innate and adaptive anti-tumor-antigen immune responses [17], we performed this explorative study to identify the most consistently expressed immune mediators that accompany DC maturation. Innovation in high throughput proteomic platforms allows the analysis of a large number of molecules in one setting using relatively small amounts of material. Using such tools, we compared the cumulative secretion of cytokines during 48 h of DC maturation with CD40L and LPS using two multiprotein platform arrays.

2. Results

2.1. Maturation of DC

Phenotypic analyses of mDC and iDC consistently demonstrated that HLA-DR antigens and CD86 were expressed at high levels, while mature DC expressed consistently and significantly more CD80 and CD83 than iDC. Thus, the present treatment with CD40L and LPS generated two subsets of DC in distinct stages of activation. A representative example is given in Fig. 1.

2.2. Differential cytokine/chemokine expression by iDC and mDC

IL-4 and GM-CSF were highly expressed in both mature and immature DC supernatants due to the fact that they had been added for generation of DC and, therefore, served as internal controls for both methods of detection. Out of 34 proteins analyzed, 16 factors were significantly more expressed by mDC compared with iDC (Table 1). Using the Search Light Proteome ArrayTM, 12 proteins were found to be secreted in significantly larger amounts by mDC with cumulative concentration ratios of iDC and mDC between 1.25 and 101 (Table 2). The Protein Profiling BiochipTM detected seven proteins in mDC supernatants with fluorescence intensities significantly higher than in iDC supernatants (fluorescence ratios between mDC and iDC ranging from 2.7 to 144 [Table 3]). No direct concentration measurement could be obtained with the Protein

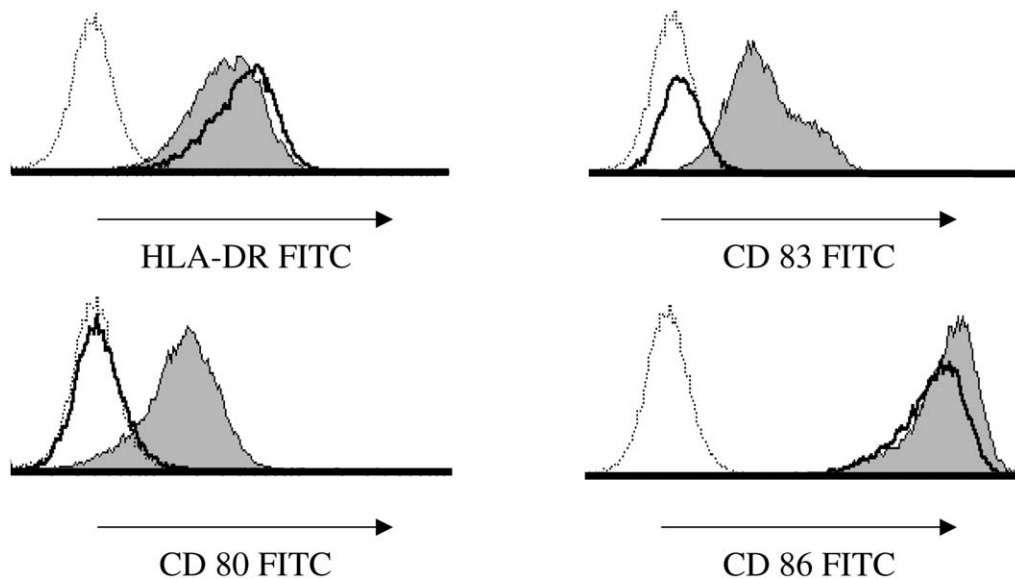


Fig. 1. Monocytes derived from PBMC were cultured for five days in the presence of IL-4 and GM-CSF to generate dendritic cells (DC), followed by a two-day culture with either only IL-4 and GM-CSF or with additional CD40L and LPS. DC cultured with CD40L and LPS (filled gray histogram) show an up-regulation of CD80 and CD83 whereas cells cultured without CD40L and LPS (solid black line) remain negative for these maturation markers. HLA-DR and CD86 are expressed in both cell types. Representative histograms are given with a dashed line showing the isotypic control.

Table 1
Panel of 34 factors in alphabetical order analyzed using Search Light Proteome Array™ and Protein Profiling Biochip™

Eotaxin	IL-4	IP-10	sCD23
G-CSF	IL-5	MCP-1	sICAM-1
GM-CSF	IL-6	MCP-3	TARC
I-309	IL-7	MDC	TGFβ
IFNγ	IL-8	MIG	TNFα
IL-1α	IL-10	MIP1α	TNFβ
IL-1β	IL-12	MIP1β	TRAIL
IL-2	IL-13	RANTES	
IL-3	IL-15	sCD95 (sFAS)	

Profiling Biochip™ since only relative fluorescence signals are provided by this method, which are not linearly proportional to the concentration. Nevertheless, the factors shown in Table 3 were all significantly more released by mDC and are comparable to the results obtained with the Search Light Proteome Array™. In particular, results for three proteins present in both arrays (TNF, IL-10, IL-12) were comparable. None of the tested proteins was significantly more produced by iDC compared to mDC.

3. Discussion

This work confirms previous reports that documented the release of several cytokines and chemokines during DC maturation and at the same time broadens the information to a larger set of putative immune effectors [11,13,14,16]. Thus, in addition to the up-regulation of co-stimulatory molecules, cytokine release is a major component of DC activation. This is not surprising as cytokines and chemokines may play an important role in activating effectors of the innate immune response, which may be necessary for the

Table 2
Proteins produced at significantly higher concentrations by maturing DC as detected by Search Light Proteome Array™, median concentration, *p*-value (paired sign *t*-test level of significance < 0.05), and concentration ratio between maturing DC and iDC

	Maturing (pg/ml)	Immature (pg/ml)	<i>p</i> -Value (sign <i>t</i> -test)	Ratio
TNFα	10,001	99	0.002	101
MIP1α	45,738	1135	0.000	40
IL-10	92.5	3.8	0.021	24
MIP1β	19,679	3273	0.000	6.01
RANTES	5613	1773	0.033	3.17
IL-8	207,961	81,303	0.000	2.56
MCP-1	42,326	17,648	0.002	2.40
IFNγ	11.5	5.1	0.037	2.25
IL-12	19.3	9.5	0.003	2.03
MDC	239,859	127,634	0.014	1.88
Eotaxin	117	78	0.003	1.50
IL-2	19.2	15.4	0.007	1.25

Data and *p*-values are presented without correction for the number of tests and should be considered exploratory.

Table 3
Proteins produced by maturing DC at significantly higher levels of fluorescence intensities than proteins secreted by iDC as detected by Protein Profiling Biochip™, median fluorescence intensity (FI), *p*-value, and fluorescence ratio between maturing DC and iDC

	Maturing FI	Immature FI	<i>p</i> -Value (sign <i>t</i> -test)	Ratio
TNFα	10,990	76.1	0.006	144
IP-10	17,439	326	0.039	53
IL-12	5331	138.4	0.006	39
IL-10	2330	121.9	0.006	19
MIG	6434	1237	0.039	5.20
IL-7	791	229.6	0.000	3.45
IL-6	57,996	21,421	0.006	2.71

Data and *p*-values are presented without correction for the number of tests and should be considered exploratory.

destruction of damaged cells and subsequent uptake of antigen. In addition, release of cytokines and chemokines may attract adaptive immune effectors within the target organ or in the draining lymph nodes. This step may be a requirement for the initiation of T cell immune responses through epitope engagement and further potentiation by soluble or membrane-bound co-stimulatory signals. In the case of DC maturation induced with CD40L and LPS, a specific pattern of protein release is observed. During maturation, DC release factors for the regulation of antigen uptake and the activation of the innate immune system (TNFα, IL-10, IL-12, IFNγ); chemo-attractants for various immune cells including monocytes, NK cells, and T cells (MIP1α, MIP1β, IL-8, MCP-1, MDC, RANTES, MIG, IP-10, Eotaxin); and cytokines stimulating activation and proliferation of T cells (IL-2, IL-6, IL-7).

Interestingly, we noted that some of the up-regulated factors (IL-6, IL-8, MIG, MIP1α, MIP1β, IP-10, MCP-1, Eotaxin) are also induced by the systemic administration of immune-stimulators such as IL-2 to patients with advanced cancer. In a previous study, we had analyzed the changes in transcriptional profile of circulating monocytes and tumor deposits induced by the systemic administration of high-dose IL-2 [2]. The common up-regulation of cytokines possibly substantiates our hypothesis that the salient down-stream effect of IL-2 in vivo might be a potent maturation of resident DC within the target organ.

The portrait depicted here is also relevant to strategies for preparing DC as reagents for potent vaccinations. It is possible that the array of immune modulatory products resulting from the DC maturation process may be the main modulators of the DC's effectiveness. Contrary to co-stimulatory molecules and other differentiation markers, however, cytokine production by DC is transient and DC can extinguish the production of cytokines (IL-6, IL-10, IL-12, TNFα) and chemokines (MIP-1α, MIP-1β, IL-8) within a few hours from the maturation stimulus [11,16]. This may have

major implications during the delivery of antigen as exhausted DC are more likely to elicit a Th2 than a Th1 response [11]. Thus, further studies should address the kinetics of expression of this large set of cytokines relative to the application of the clinically relevant maturation stimulus. In most circumstances DC are “matured” 48 h before their administration. At that point most of the secretory repertoire is exhausted, and their effectiveness may be only dependent on the expression of co-stimulatory molecules; a relatively narrow component of their potential.

In conclusion, this study shows that maturing DC release a large number of factors that may influence the innate and adaptive immune response. A set of cytokines up-regulated in both the present study and previous genetic data [2] supports the hypothesis that DC activation possibly has a role in the target organ after systemic immune stimulation.

4. Materials and methods

4.1. Generation and maturation of dendritic cells and collection of supernatants

PBMC were collected from eight healthy donors (one donor: three samples, three donors: two samples, and four donors: one sample) in the Department of Transfusion Medicine, Clinical Center, Bethesda, MD. All cells were maintained in complete medium (CM) consisting of Iscove's medium (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated human AB-serum (Gemini Bioproducts, Inc, Calabasas, CA), 10 mM hepes buffer (Cellgro, Mediatech, Inc. Herndon, VA), 0.03% L-glutamine (Biofluids), 100 U/ml penicillin/streptomycin (Biofluids), 10 µg/ml ciprofloxacin (Bayer, West Haven, CT), and 0.5 mg/ml amphotericin B (Biofluids). PBMC were thawed and plated into a T175 flask. After 4 h, nonadherent cells and medium were removed. The remaining adherent cells were further cultured in CM. IL-4 and GM-CSF (PeproTech, Rocky Hill, NJ) were added at a concentration of 1000 U/ml on days 0, 2, and 4. After five days of culture, CM was removed and DC were separated into two parallel cultures in fresh CM containing IL-4 and GM-CSF (each at 1000 U/ml) with or without the addition of CD40L (500 ng/ml, PeproTech) and LPS (5 µg/ml, Sigma, St. Louis, MO) to induce mDC or maintain iDC, respectively. After 48 h, supernatants were collected and immediately frozen at −80 °C while DC were simultaneously collected and analyzed by flow cytometry.

4.2. Flow cytometric analysis of dendritic cells

DC were washed and suspended at a concentration of 2×10^5 cells per 50 µl FACS buffer (phosphate buffer

plus 5% FCS; Biofluids) and incubated at 4 °C in the dark for 30 min with 10 µl of the fluorochrome-labeled antibodies. The antibodies used were anti-CD80-FITC, anti-CD83-FITC, anti-CD86-FITC, and anti-HLA-DR-FITC (all antibodies were purchased from Becton Dickinson, San Jose, CA). Cells were then washed in 4 ml of FACS buffer and analyzed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson). At least 30,000 events were acquired for analysis.

4.3. Cytokine platform assays

Twelve supernatants of maturing DC paired with 12 supernatants of iDC were analyzed. Immediately after thawing, all supernatants were analyzed using two multiprotein ELISA platforms: Search Light Proteome Array™ (Perbio Pierce, Boston, MA) and Protein Profiling Biochip™ (Zyomyx, Hayward, CA). Both methods were used according to the manufacturer's instructions. In brief, after a typical sandwich ELISA on these specific arrays, signals were generated and read by cameras using either chemiluminescence (Search Light Proteome Array™) or fluorescence (Protein Profiling Biochip™). Taken together, both methods allowed analysis of 34 proteins (Table 1).

4.4. Statistical analysis

Twelve mDC and iDC samples from eight donors (one donor: three samples, three donors: two samples, and four donors: one sample) were analyzed in paired conditions. Statistical analyses were performed using the sign *t*-test for paired samples. A result was considered significant ($p < 0.05$) if mDC released differential amounts of the respective protein compared with iDC. Data and *p*-values are presented without correction for the number of tests and should be considered exploratory.

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