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Research article

TRANSCRIPTIONAL PROFILES DURING THE DIFFERENTIATION AND MATURATION OF MONOCYTE-DERIVED DENDRITIC CELLS, ANALYZED USING FOCUSED MICROARRAYS

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Abstract: Dendritic cells (DC) are professional antigen-presenting cells capable of initiating primary immune responses. They have been intensively studied and are used in both basic immunology research and clinical immunotherapy. However, the genetic pathways leading to DC differentiation and maturation remain poorly understood. Using focused microarrays with oligonucletotide probes for 120 genes encoding co-stimulatory molecules, chemokines, chemokine receptors, cytokines, cytokine receptors, TLRs, and several other related molecules, we analyzed the kinetics of gene expression for the overall differentiation process of monocytes into mature DC. In parallel, we compared the transcriptional profiles in DC maturation in the presence of LPS, TNF-α or trimeric CD40L. We found similar transcriptional profiles for early immature DC and immature DC, respectively generated by culturing monocytes with GM-CSF and IL-4 for three or six days. We identified sets of common and stimuli-specific genes, the expression of which changed following stimulation with LPS, TNF-α or CD40L. A dynamic analysis of the entire DC differentiation and maturation process showed that some important inflammatory and constitutive chemokines are transcribed in both immature and mature DC. The correlative expression kinetics of the gene pairs *IL1R1/IL1R2*, *IL15/IL15RA*, *DC-SIGN/ICAM-2* and *DC-SIGN/ICAM-3* imply that they all play crucial roles in mediating DC functions. Thus, our analysis with focused microarrays shed

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Abbreviations used: CD40L – CD40 ligand; DC – dendritic cells; GADD45 – growth arrest and DNA damage gene 45; GM-CSF – granulocyte-macrophage colony-stimulating factor; LPS – lipopolysaccharide; MoDC – monocyte-derived dendritic cells; TLRs – toll-like receptors

light on the transcriptional kinetics of DC differentiation and maturation, and this method may also prove useful for identifying novel marker genes involved in DC functions.

Key words: Dendritic cells, Monocytes, Microarray, Transcriptional profile

INTRODUCTION

Dendritic cells (DC) are the most effective antigen-presenting cells (APC) for activating naïve T cells *in vivo* and initiating the primary immune responses [1]. They are derived from the bone marrow, and they reside in virtually every tissue in a relatively immature state. Immature DC (iDC) have the ability to discriminate self and non-self antigens using conserved pattern-recognition receptors, such as Toll-like receptors (TLRs), which recognize the pathogen-associated molecular patterns (PAMP) of microorganisms. iDC sense foreign antigens in peripheral tissues and take them up via fluid phase or receptor-mediated endocytosis when encountering them. The foreign antigens are then processed into peptides capable of binding to major histocompatibility complex (MHC) molecules. In the presence of inflammatory signals, iDC mature and migrate to draining lymph nodes, where they develop into potent, mature DC (mDC) and eventually localize to the T-cell zones of the lymph nodes. Naïve T lymphocytes that recirculate through the lymph nodes encounter mDC and become activated upon recognition of the MHC/peptide complexes displayed on the mDC, and through interaction with the co-stimulatory molecules and cytokines produced by the mDC. The co-stimulatory molecules provide a second signal that is required for T-cell activation, and the cytokines drive the proliferation and differentiation of T cells. Moreover, chemokines and their cognate receptors are essential for the migration of iDC through the lymphatics to the draining lymph nodes and toward sites of infection.

This process of DC differentiation has been successfully reproduced *in vitro* by culturing $CD34^+$ precursor cells isolated from cord blood [2] and bone marrow [3] in the presence of GM-CSF and TNF-α. Moreover, monocytes from human peripheral blood cells have become a major source for generating myeloid DC *ex vivo* following incubation with GM-CSF and IL-4 for use in immunotherapy [4, 5]. MoDC are phenotypically immature and can be matured *in vitro* following exposure to a variety of stimuli, such as LPS, TNF-α and CD40L [6, 7]. A growing body of evidence shows that DC respond differently to different stimuli, and that depending on the stimuli, the T cells that come into contact with the DC will acquire diverse effector functions [8, 9]. Thus, an increasing number of researchers are using high-density microarray techniques to try to clarify the underlying changes and differences in gene expression via separate or comparative analyses of DC matured with different stimuli [10-13]. Many highly parallel gene expression analyses of immature DC and monocytes have also been performed [14, 15].

Although microarray studies have identified many gene expression alterations, the results are often inconsistent because of the variation in the microarray platforms and DC-generating protocols used in different laboratories. In addition, very few kinetics analyses have been carried out on the gene expression kinetics during the entire differentiation process from monocytes to mature DC [10]. To better understand the potential role of specific genes with expression that is regulated during DC differentiation and during the DC response to various stimuli, we isolated and purified $CD14^+$ monocytes, and cultured them with GM-CSF and IL-4 for three or six days, respectively to obtain early immature DC or immature DC. Then we treated the cells for two days with three stimuli, LPS, TNF-α and trimeric CD40L, in order to generate mature DC. At the same time, we developed focused microarrays printed with oligonucleotide probes for 120 genes encoding co-stimulatory molecules, chemokines, chemokine receptors, cytokines, cytokine receptors, TLRs, and several other molecules, since these molecules have been reported to be important for DC differentiation and function [16-18]. We used these focused microarrays to systematically analyze the kinetics of gene transcription during DC differentiation and maturation in response to different stimuli.

MATERIALS AND METHODS

Generating immature and mature DC

The human subjects were two healthy adult volunteers (Suzhou Blood Center, China). Monocytes were isolated from peripheral blood mononuclear cells (PBMC) by magnetic sorting with anti-CD14 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) The purified cells were > 97% $CD14^+$ as determined by flow cytometry. Immature DC were prepared from these monocytes via culture for six days in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in the presence of 500 U/ml GM-CSF (Peprotech, Rocky Hill, USA) and 100 U/ml IL-4 (Invitrogen/Biosource, Carlsbad, USA). Every two days, 50% of the medium was removed and the same volume of fresh medium containing twice the amount of cytokines was added. To obtain mature DC, the immature DC were transferred to new 6-well plates and treated for two days with either 1 μg/ml LPS (Sigma, St Louis, USA), 350 U/ml TNF-α (Invitrogen/Biosource, Carlsbad, USA), or 2 μg/ml trimeric CD40L (prepared in our own laboratory).

Cell surface antigen analysis

Flow cytometry (EPICS Altra, Beckman Coulter, Fullerton, USA) was used to analyze the cell surface antigens of the DC. Cell staining was performed with the following fluorescently labeled anti-human monoclonal antibodies: FITC-CD14 (clone 61D3, mouse IgG1), FITC-CD80 (clone 2D10.4, mouse IgG1), PE-CD86 (clone IT2.2, mouse IgG2b), and PE-CD83 (clone HB15e, mouse IgG1). The flow cytometry data was interpreted with WinMDI software (version 2.9; Joseph

Trotter, Scripps Institute, San Diego, USA). The percentage of positive cells and the genometric mean fluorescence intensity (MFI) were automatically calculated from the flow cytometric histograms and used to compare the expression levels of surface antigens.

Custom-made, focused oligo-microarrays

A list of 120 genes with GenBank accession numbers was prepared based on our interest in co-stimulatory molecules, cytokines, chemokines, and other molecules potentially involved in DC development (Tab. 1). The focused microarrays, customized by SuperArray, Inc. (Frederick, USA), used one gene-specific 60-mer 3' biased oligonucleotide for each gene. The probes were designed through a rigorous design methodology developed by SuperArray, Inc. They were printed onto nylon membranes. A series of human housekeeping genes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal protein S27a (RPS27A) and beta-2 microglobulin (B2M), were included in each array as positive controls. Two concentrations of biotin-labeled artificial DNA probes were also spotted onto the microarray to serve as detection controls. Sequences not expected to be present in the cDNA, including the plasmid pUC18 and three artificial sequences (AS1R2, AS1R1 and AS1), were also printed on the array as detection and background hybridization controls (negative controls). The arrays were stored at -20ºC until needed.

RNA preparation and microarray hybridization

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, USA). 0.1-3.0 μg of RNA was converted to cDNA by reverse transcription using a TrueLabeling-AMP™ Linear RNA Amplification Kit (SuperArray, Inc.). Biotin-labeled cRNA was generated from the cDNA by an *in vitro* transcription reaction supplemented with biotin-16-dUTP (Enzo, Farmingdale, USA), and purified with ArrayGrade^{TM} cRNA Cleanup Kit (SuperArray, Inc.). Hybridization of the biotin-labeled cRNA to custom-made microarrays was performed as suggested by the manufacturer (SuperArray, Inc.). The hybridized arrays were washed with non-stringent wash buffer (300 mM sodium chloride, 30 mM sodium citrate, 1% SDS), followed by stringent wash buffer (15 mM sodium chloride, 1.5 mM sodium citrate, 0.5% SDS). The arrays were incubated with alkaline phosphatase-conjugated streptavidin solution (1:7,500 dilution, SuperArray) for 10 min at room temperature. After several washes, chemiluminescent signals were detected with CDP-Star reagent (SuperArray). The arrays were then exposed to X-ray films. The films were scanned with ScanJet (Hewlett Packard, Palo Alto, USA), and the acquired images were analyzed as grayscale TIFF files.

Microarray data processing

The spots in the TIFF files were digitized and analyzed with GEArray Analysis Suite software (SuperArray, Inc.). All the raw signal intensities were corrected for background using the software to subtract the signal intensity of the negative

control (plasmid pUC18 DNA), and were then normalized by dividing them by the corrected signal intensity of the housekeeping gene (β-actin) on the same array. The intensity value was unreliable when it was less than 0.05, so the corresponding gene transcript was considered undetectable. To identify transcript expression that was affected at two or more differentiation stages, we generated Venn diagrams using the Gene List Venn Diagram (http://mcbc.usm.edu/genevenn/genevenn.htm). In addition, normalized signal intensities were visualized by generating grayscale images displaying the transcript levels as a variation in gray intensity, and they were hierarchically or K-means clustered according to published methods [19, 20] using TIGER Mev 4.1 software (http://www.tm4.org/mev.html).

RESULTS

The generation and maturation of MoDC *in vitro*

Immature DC were produced *in vitro* from normal human monocytes (Mo), more than 97% of which were $CD14^+$ at the start of culture. The monocytes from two healthy blood bank donors were cultured in the presence of GM-CSF and IL-4. After 3 days in culture, dispersed, spherical and attached monocytes had formed a large number of non-adherent aggregates with short projections extending from their surfaces. These cells are considered to be early immature DC and were here designated iDC-3d. After 6 days in culture, most of the cells had become non-adherent and enlarged, and they appeared in clusters or dispersed, with long dendritic projections. These cells are conventionally regarded as immature DC, and they were designated iDC-6d in this study. To generate mature DC, immature DC were treated with LPS, TNF-α or CD40L for another two days, yielding cells respectively designated LPS-DC, TNF-α-DC or CD40L-DC. In addition to displaying the typical morphology of mature DC (clusters or large, single floating cells, both with long protruding veils), the mDC also possessed a mature surface phenotype detected by flow cyotmetry (Fig. 1). DC stimulated with LPS, TNF-α or CD40L showed significantly higher percentages of CD80, CD83 and MFI of CD86 than immature DC. CD14, a monocyte-specific marker, was barely detectable on either immature DC or stimulated DC. In agreement with a previous report [21], we found that TNF-α induced partial maturation of DC, since the expression levels of CD80, CD86 and CD83 on these cells were lower than on DC stimulated with LPS or CD40L. However, TNF-α was included in this study because it is a classic stimulus for DC maturation. In addition, to isolate the effect of TNF-α alone on DC maturation, we did not use the combination of TNF-α and IL-1β, which induces complete maturation of DC [8]. The above morphological and cytometric results suggest that we succeeded in generating DC with different maturation statuses.

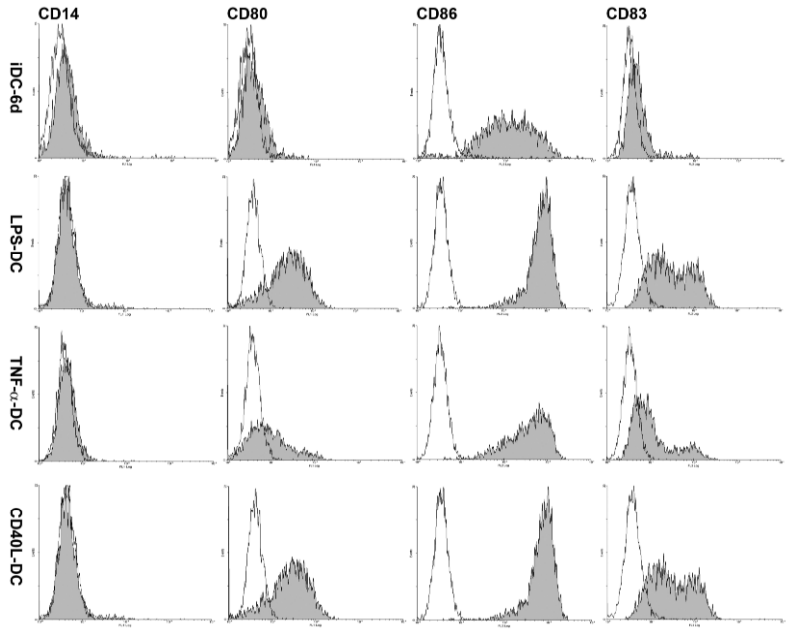

Fig. 1. The surface phenotypes of the studied dendritic cells (DC). $CD14^+$ monocytes derived from PBMC were cultured for six days in the presence of GM-CSF and IL-4 to generate iDC-6d, followed by an additional two days in culture with LPS (generating LPS-DC), TNF-α (generating TNF-α-DC) or CD40L (generating CD40L-DC). The DC were analyzed for the expression of the indicated surface molecules by staining with fluorescently labeled antibodies. Fluorescence was detected by flow cytometry. The solid curve in the histograms shows the specific expression of CD14, CD80, CD86 or CD83, whereas the blank curve shows the expression of the isotype control. The data is representative of two independent experiments.

Gene expression profiles in Mo, iDC-3d and iDC-6d

Of the 120 genes in the microarrays, 59 (49% of the total) were expressed in Mo, iDC-3d or iDC-6d (Fig. 2A and Tab. 1). Of these, 34 were commonly expressed at all three differentiation stages. Seven genes were exclusively expressed in Mo: *CCL4*, *CCL20*, *CXCL1*, *IL1B*, *IL10*, *IL15* and *TNFRSF8* (CD30). Two genes were specifically expressed in iDC-3d: *CCL21* and *IL8RB*. Only one gene, *CCL8*, was uniquely expressed in iDC-6d.

In order to better understand the expression profiles of the genes involved in the differentiation from monocytes to immature DC, we performed a hierarchical clustering analysis on the shared set of 34 genes expressed in Mo, iDC-3d and iDC-6d. The clustering analysis was carried out using Gene Tree and Sample Tree clustering within TIGER Mev 4.1 software (Fig. 2B). The Sample Tree showed that iDC-3d and iDC-6d clustered closely, suggesting that the expression levels of many more genes were altered during the differentiation of monocytes into immature DC than during the maturation of iDC-3d into iDC-6d. To reduce

the complexity of the Gene Tree and to view the gene expression profiles more clearly, we divided it into four distinct clusters (Clusters I, II, III and IV) according to the distance threshold (0.751) as shown in the centroid graphs in Fig. 2B.

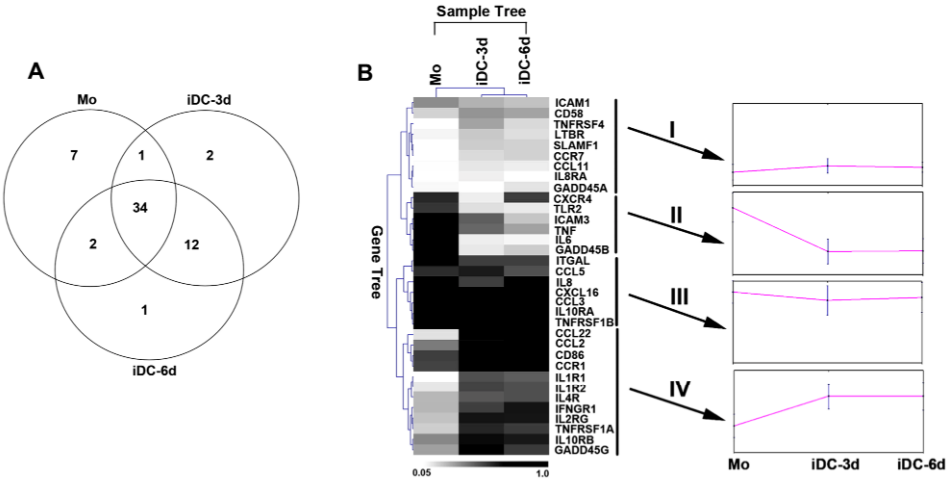

Fig. 2. The gene expression profiles in Mo, iDC-3d and iDC-6d. **A.** Venn diagram showing the number of genes expressed in Mo, iDC-3d and iDC-6d. A gene is considered to be expressed if the intensity values from both donors are more than 0.05. The numbers in the overlapping regions represent genes commonly expressed at different stages of differentiation and maturation, while the numbers in the non-overlapping regions represent genes expressed at a specific stage. **B.** Two-dimensional hierarchical clustering of commonly expressed genes (34 genes) in Mo, iDC-3d and iDC-6d. Each row represents the expression profile of a single gene, while each column represents a differentiation stage (Mo, iDC-3d or iDC-6d). The normalized gene expression values for the two donors are averaged and visualized in gray density according to the scale at the bottom. The Gene Tree shows the similarities in the expression patterns among the genes, and the Sample Tree shows the similarities in the expression patterns for the differentiation stages. The Gene Tree is further divided into four clusters on the basis of the distance threshold of 0.751, and the corresponding centroid graphs are plotted at right. The centroid graphs display the centroids for each cluster, and thus the expression profiles of the clustered genes can be viewed clearly.

The transcript levels for the nine genes in Cluster I [*ICAM1* (*CD54*), *CD58*, *TNFRSF4* (*CD134*), *LTBR*, *SLAMF1* (*CD150*), *CCL11*, *CCR7*, *IL8RA* and *GADD45A*] and the seven genes in Cluster III [*ITGAL* (*CD11a*), *CCL3*, *CCL5*, *IL8*, *CXCL16*, *IL10RA* and *TNFSF1B* (*TNFRII*)] remained relatively constant during the differentiation of the monocytes into immature DC (iDC-3d and iDC-6d). Therefore, we postulated that these genes do not play a central role in regulating DC differentiation. By contrast, our results suggest that the 12 genes in Cluster IV and 6 genes in Cluster II are important for DC differentiation. As found in earlier studies [14, 22, 23], the mRNA levels for *CD86*, *CCL22*, *CCR1*, *IFNGR1*,

Tab. 1. The transcriptional profiles of 120 genes in Mo, iDC-3d and iDC-6d. The data is the mean intensities (MI) of gene expression in Mo, iDC-3d and iDC-6d from two donors. MI is categorized into the following groups: MI < 0.05, -; 0.05 ≤ MI < 0.25, +; 0.25 ≤ MI < 0.50, ++; 0.50 ≤ MI < 0.75, +++; 0.75 ≤ MI < 1.00, ++++. +/- means that the gene was not detected in one of the two donors (intensity value < 0.05).

Gene symbol	GenBank accession No.	Mo	iDC-3d	iDC-6d	Gene symbol	GenBank accession No.	Mo	iDC-3d	iDC-6d
Costimulatory molecules and receptors (number: 37)					CCL19	NM006274	+/-	+/-	-
CD28	NM006139	-	-	-	CCL20	NM004591	++++	-	-
CD80	NM005191	-	+	+	CCL21	NM002989	+/-	+	+/-
CD86	NM006889	+++	++++	++++	CCL22	NM002990	+	++++	++++
CTLA4	NM005214	-	-	-	CCL25	NM005624	+/-	+	+
ICOS	NM012092	-	-	-	CCR1	NM001295	+++	++++	++++
ICOSLG	NM015259	-	-	-	CCR2	NM000648	-	+/-	+/-
PDCD1	NM005018	-	-	-	CCR3	NM001837	-	-	-
CD274	NM014143	-	-	-	CCR4	NM005508	-	-	-
PDCD1LG2	NM025239	-	-	-	CCR5	NM000579	+/-	++++	+++
CD276	NM025240	-	+/-	-	CCR6	NM004367	-	-	-
VTCN1	NM024626	-	-	-	CCR7	NM001838	+	+	+
TNFSF14	NM003807	-	+/-	-	CCRL1	NM016557	-	-	-
TNFRSF14	NM003820	+/-	+	+	CXCL1	NM001511	+++	-	-
LTBR	NM002342	+	++	+	IL8	NM000584	++++	+++	++++
BTLA	NM181780	-	-	-	CXCL9	NM002416	-	-	-
CD40LG	NM000074	-	-	-	CXCL10	NM001565	+/-	-	-
CD40	NM001250	-	++	+	CXCL11	NM005409	-	-	-
TNFRSF4	NM003327	+	++	+	CXCL12	NM000609	-	-	-
TNFSF4	NM003326	-	-	-	CXCL13	NM006419	-	-	-
TNFSF9	NM003811	-	-	-	CXCL16	NM022059	++++	++++	++++
TNFRSF9	NM001561	-	-	-	CX3CL1	NM002996	-	-	-
TNFRSF11A	NM003839	+/-	+	+	IL8RA	NM000634	+	+	+
TNFSF11	NM003701	-	+/-	+/-	IL8RB	NM001557	-	+	-
TNFRSF7	NM001242	-	-	-	CXCR3	NM001504	-	-	-
TNFSF7	NM001252	-	+	+	CXCR4	NM003467	+++	+	+++
TNFRSF8	NM001243	+	-	-	BLR1	NM001716	-	+/-	-
TNFSF8	NM001244	-	-	-	*Cytokines and cytokine receptors (number: 31)*				
ICAM1	NM000201	++	++	++	IFNG	NM000619	-	-	-
ITGAL	NM002209	++++	+++	+++	IL1A	NM000575	++++	+/-	+
ICAM2	NM000873	++	+/-	+	IL1B	NM000576	++++	+/-	+/-
ICAM3	NM002162	++++	++	++	IL2	NM000586	-	-	-
CD209	NM021155	-	++	++	IL4	NM000589	-	-	-
SLAMF1	NM003037	+	+	+	IL6	NM000600	++++	+	+
CD2	NM001767	-	+/-	+/-	IL10	NM000572	+	-	-
CD58	NM001779	+	++	++	IL12A	NM000882	-	-	-
FAS	NM000043	-	-	-	IL12B	NM002187	-	-	-
FASLG	NM000639	-	-	-	IL15	NM172175	+	-	-
Chemokines and chemokine receptors (number: 39)					TGFB1	NM000660	-	-	-
CCL2	NM002982	++	+++	++++	TNF	NM000594	++++	++	++
CCL3	NM002983	++++	++++	++++	LTA	NM000595	-	-	-

Gene symbol	GenBank accession No.	Mo	iDC-3d	iDC-6d	Gene symbol	GenBank accession No.	Mo	iDC-3d	iDC-6d
CCL4	NM002984	+	+/-	-	IL1R1	NM000877	+	+++	++
CCL5	NM002985	+++	+++	+++	IL1R2	NM004633	+	+++	+++
CCL7	NM006273	+	+	+/-	IL2RA	NM000417	+/-	+/-	+/-
CCL8	NM005623	-	+/-	++	IL2RB	NM000878	-	-	-
CCL11	NM002986	+	+	+	IL2RG	NM000206	++	+++	+++
CCL13	NM005408	-	++	+++	IL4R	NM000418	++	++	+++
CCL15	NM032965	-	-	+/-	IL6R	NM000565	+/-	+/-	+/-
CCL16	NM004590	-	-	-	IL6ST	NM002184	-	-	-
CCL17	NM002987	-	++++	++++	IL10RA	NM001558	++++	++++	++++
CCL18	NM002988	-	+++	+++	IL10RB	NM000628	++	+++	+++
IL12RB1	NM005535	-	-	-	TLR4	NM003266	+/-	-	+/-
L12RB2	NM001559	-	+/-	-	TLR5	NM003268	-	-	-
IL15RA	NM002189	+/-	-	-	TLR6	NM006068	-	+/-	-
IFNGR1	NM000416	++	+++	+++	TLR7	NM016562	+/-	-	-
TGFBR1	NM004612	-	-	-	TLR8	NM016610	-	-	-
TGFBR2	NM003242	-	-	-	TLR9	NM017442	-	-	-
TNFRSF1A	NM001065	+	+++	+++	TLR10	NM030956	-	-	-
TNFRSF1B	NM001066	++++	++++	++++					
TLRs (number: 10)				*GADD45 (number: 3)*					
TLR1	NM003263	+/-	+	+	GADD45A	NM001924	+	+	+
TLR2	NM003264	+++	+	+	GADD45B	NM015675	++++	+	+
TLR3	NM003265	-	-	-	GADD45G	NM006705	++	+++	+++

IL4R and *TNFRSF1A* were expressed at higher levels in immature DC than in purified monocytes. Interestingly, we found the transcription level of two of the IL-1 receptors, *IL1R1* and *IL1R2*, to be similarly up-regulated, by > 5-fold in immature DC compared to monocytes. As in [15], we found the transcription levels for *IL6*, *TNF* and *TLR2* to be significantly lower in immature DC than in monocytes. Unexpectedly, we observed that the mRNA level of *ICAM3* (*ICAM-3*) was 2-fold lower in immature DC than in monocytes, and the transcription level of *CXCR4* was 3.5-fold lower in iDC-3d than in monocytes. The transcription level of *CXCR4* in iDC-6d was comparable to that in monocytes.

The transcriptional responses of iDC to maturation stimuli

Genes with a transcription level that changed in moving from the immature (iDC-6d) to mature stage can be divided into four main groups: up-regulated, down-regulated, induced (not detected in iDC-6d, but detected in mature DC), and suppressed (detected in iDC-6d, but not detected in mature DC). A gene was defined as up- or down-regulated if its transcription levels showed at least a 2-fold change in both donors ($\geq$ 2-fold change).

Seventeen of the 120 genes (14%) were up-regulated or induced upon stimulation with LPS, TNF-α or CD40L compared to iDC-6d prior to stimulation (Tab. 2). We could not find any down-regulated or suppressed genes, suggesting that DC maturation is primarily a process of protein synthesis or accumulation, not protein disappearance or degradation.

Tab. 2. Differential expression analysis in LPS-, TNF-α- and CD40L-stimulated DC versus iDC-6d. The genes were divided into four groups: ND, not detectable in mature DC; NC, no change in the level of expression; +, ++ or +++, up-regulated; and I, induced. The fold change values (FC) for the up-regulated genes were grouped as follows: +, $2 \leq FC < 4$; ++, $4 \leq FC < 8$; +++, $8 \leq FC < 12$. The MI values from two donors are given in parentheses for the induced (I) genes.

Gene symbol	Gene name	GenBank accession No.	LPS-DC	TNF-α-DC	CD40L-DC
Costimulatory molecules and receptors					
CD80	CD28LG/CD28LG1	NM005191	++	+	+
TNFRSF4	ACT35/CD134	NM003327	+	+	NC
TNFRSF11A	EOF/FEO	NM003839	NC	+	+
Chemokines and chemokine receptors					
CCL19	CKb11/ELC	NM006274	I (0.629)	I (0.213)	I (0.770)
CCR6	BN-1/CKR-L3	NM004367	ND	ND	I (0.073)
CCR7	BLR2/CDw197	NM001838	++	+	++
CXCL1	GRO1/GROα	NM001511	I (0.325)	ND	ND
CXCL9	CMK/Humig	NM002416	I (0.068)	ND	ND
CX3CL1	ABCD-3/C3Xkine	NM002996	I (0.099)	I (0.081)	I (0.109)
Cytokines and cytokine receptors					
IL1A	IL-1A/IL1	NM000575	+	NC	NC
IL1B	IL-1/IL1-BETA	NM000576	+++	NC	NC
IL6	BSF2/HGF	NM000600	+	NC	NC
IL12B	CLMF/CLMF2	NM002187	ND	ND	I (0.170)
IL15	IL-15	NM172175	I (0.240)	I (0.204)	I (0.576)
IL15RA	MGC104179	NM002189	I (0.464)	I (0.139)	I (0.326)
GADD45					
GADD45A	DDIT1/GADD45	NM001924	+	NC	++
GADD45B	MYD118	NM015675	+	NC	+

Co-stimulatory molecules. Of the 37 co-stimulatory molecules included in the microarray, three were up-regulated at the mRNA level after stimulation, and in no case was this up-regulation specific for one stimulus. As expected, the gene expression of CD80 significantly increased upon maturation with all three stimuli, which correlated with cell surface expression of the CD80 molecule, as revealed by flow cytometry. Consistent with other researchers [23, 24], we found the transcript for *TNFRSF11A*, also known as *RANK*, to be > 2-fold higher in DC stimulated with TNF-α than in iDC-6d. We also found a significant up-regulation of RANK mRNA in CD40L-stimulated DC. The *TNFRSF4* (*OX40* or *CD134*) mRNA and protein levels have been shown to increase gradually during DC maturation [24]. Our results confirmed that stimulation with TNF-α or LPS increases the expression of *OX4O* mRNA in the DC.

Chemokines and chemokine receptors. Of the 39 chemokines and chemokine receptors probed by this microarray, the expressions of 6 were up-regulated or induced in the stimulated DC. *CCL19* mRNA was substantially induced in all

three stimulus groups. It is remarkable that stimulation with LPS and CD40L increased the transcription level for *CCL19* 3-fold more than stimulation with TNF-α. As in previous studies [23], we found that LPS was the only stimulus that induced the transcription of *CXCL1* and *CXCL9*, two members of the CXC chemokine family. *CX3CL1* is the only member of the CX3C chemokine family. Our findings that all three stimuli induced *CX3CL1* expression confirm the previous findings that the expression of *CX3CL1* increases during the maturation of human and murine DC [25, 26]. We found that the transcription of two chemokine receptors, *CCR6* and *CCR7*, differed between mature and immature DC, whereas the transcript for *CCR6* was only detectable at a marginal level in CD40L-stimulated DC. Our finding that all three stimuli strongly up-regulate the transcription of *CCR7* is consistent with the consensus results of multiple laboratories [23].

Cytokines and cytokine receptors. Of the 31 cytokines and cytokine receptors investigated in this study, we found that the transcription levels for 6 differed between stimulated DC and iDC-6d. The mRNAs encoding the pro-inflammatory cytokines IL-1A, IL-1B and IL-6 were significantly higher after maturation with LPS, but not after maturation with TNF-α or CD40L. Remarkably, the transcript level for *IL-1B* was ~9-fold higher in LPS-stimulated DC than in iDC-3d. In some reports, the gene expressions of *IL-1B* and *IL-6* were respectively up-regulated in response to CD40L and TNF-α [12, 13], suggesting that mature DC may be an important source of pro-inflammatory cytokines. As in previous studies [13], we failed to detect the transcription of the gene encoding the alpha subunit of IL-12 (IL-12A, p35) for any of the stimulus groups, although the gene coding for the beta subunit of IL-12 (IL-12B, p40) was found to be induced selectively in response to CD40L. We found that the transcript for *IL-15* was detectable in all three stimulus groups, but not in immature DC. Moreover, CD40L induced the greatest level of IL-15 mRNA compared with LPS and TNF-α. Our results correlate well with those of another study reporting up-regulation of *IL-15* mRNA in response to LPS and a cytokine cocktail containing TNF-α [13]. The gene encoding IL15RA, a subunit of the IL-15 receptor, was also demonstrated to be expressed in all the stimulus groups, but not in iDC-6d, and its level was > 2-fold greater in LPS- and CD40L-stimulated DC than in TNF-α-stimulated DC.

GADD45. As in previous reports [11, 12], two of the three GADD45 genes probed in our microarrays were found to be strongly up-regulated upon stimulation with LPS and CD40L.

TLRs. The expression levels of none of the 10 TLRs examined in this study changed significantly as a result of any of the stimuli. We therefore postulate that TLRs do not play essential roles in DC maturation.

The kinetics of gene expression during the differentiation and maturation of DC

To explore the universal dynamics of gene expression during the differentiation and maturation of DC, we selected the genes (34 genes) characteristically regulated in the development of DC according to the following criteria: (1) genes that were up-regulated or induced at the mRNA level in all three mature DC (LPS-DC, TNF-α-DC and CD40L-DC; Tab. 2); (2) genes that were specifically expressed at any one or two differentiation stages of Mo, iDC-3d and iDC-6d; and (3) genes that were commonly expressed in Mo, iDC-3d and iDC-6d, and the average expression levels for the two donors differed by > 2-fold between any two of the three differentiation stages. These genes were clustered using a K-means cluster algorithm integrated into TIGER Mev 4.1 software, and 10 separate clusters representing different kinetics of gene expression were defined (Fig. 3). We used data from mature DC (mDC) instead of LPS-DC, TNF-α-DC or CD40L-DC, because the expression level for one gene in mDC is the average intensity of this gene from all three stimulus groups, and it could therefore be taken as representative of any one stimulus. Thus, we were able to obtain the general characteristics of DC differentiation and maturation independently of the stimuli used.

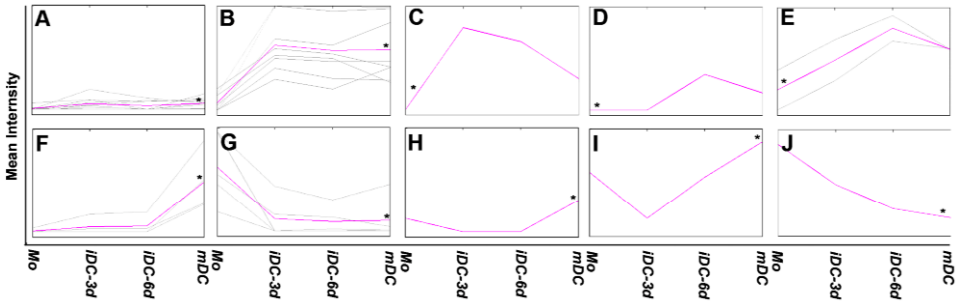

Fig. 3. The patterns of gene expression during DC differentiation and maturation based on a K-means clustering analysis. "mDC" represents mature DC, and the expression level of a gene in mDC is the average of the mRNA intensities of this gene in LPS-DC, TNF-α-DC and CD40L-DC. This clustering analysis includes: genes with expressions that were detectable, but not in all differentiation and maturation stages (Mo, iDC-3d, iDC-6d and mDC); genes with detectable expression at all four stages; and genes that show > 2-fold changes in their average intensity values for the two donors between any two of the four stages. Each line represents the expression profile of a single gene, and the asterisks indicate a line; below this line is the centroid graph of the cluster, which displays the mean expression level of the genes in the cluster. The centroid graph is the same as the expression line of the gene if one cluster includes only one gene.

The genes *TNFRSF14* (*HVEM*), *TNFRSF8* (*CD30*), *TNFSF7* (*CD70*), *CCL7*, *CCL21*, *CCL25*, *CX3CL1*, *IL8RB*, *IL10* and *TLR1* were expressed at marginal levels or were undetectable across all the stages of DC differentiation and maturation (Fig. 3A), suggesting that they may not be parts of the key genes

involved in DC development. Fig. 3B and C show genes rapidly up-regulated or induced after monocyte culturing with GM-CSF and IL-4 for three days. The expression levels of eight genes [*CD40*, *CD209* (*DC-SIGN*), *CCL17* (*TARK*), *CCL18* (*PARC*), *CCL22* (*MDC*), *IL1R1*, *IL1R2* and *TNFRSF1A* (*TNFRI*)] remained constant from iDC-3d through mDC (Fig. 3B). In particular, the transcription levels for *CCL17* and *CCL22* were nearly as high as that of β-actin, the internal positive control on the same microarray. CCL18 transcripts were also expressed at a high level in a sustained fashion. Our data confirms the results of previous research that found a constitutive and abundant presence of *CCL17*, *CCL18* and *CCL22* mRNAs in immature DC and in DC matured with LPS [23, 27]. Transcripts for the co-stimulatory molecules CD40 and DC-SIGN were markedly induced during the monocyte-immature DC transition, probably because these two molecules could respectively be induced by GM-CSF and IL-4 [28, 29]. The mRNAs for the two receptors of IL-1, IL1R1 and IL1R2 were strongly up-regulated in iDC-3d compared with monocytes, and remained at high levels through to the mDC stage. We observed a significant increase in *TNFRI* mRNA in iDC-3d compared to monocytes. Moreover, consistent with a previous report [30], we found the mRNA level for the *TNFRI* gene remained stable in immature and mature DC. The transcript for chemokine receptor CCR5 was detected at a high level in iDC-3d. However, its expression declined dramatically during the transformation of immature DC (iDC-6d) into mature DC (Fig. 3C). The expression profile of CCR5 mRNA correlated with the kinetics of the CCR5 protein during DC maturation, which was probably due to ligand-induced down-regulation [27]. Fig. 3D and E show genes whose expression peaked at the iDC-6d stage. The transcript for *CCL8* (*MCP-2*) was induced to a moderate level in iDC-6d (Fig. 3D), while the transcripts for the other two CC chemokine family members, *CCL2* (*MCP-1*) and *CCL13* (*MCP-4*), were progressively up-regulated until they reached their maximum in iDC-6d (Fig. 3E). The mRNAs for CD80, CCL19, CCR7 and IL15RA were up-regulated or induced specifically in mature DC (Fig. 3F). Among them, the proteins encoded by CD80, CCL19 and CCR7 are recognized as markers of mature DC [16]. Figs. 3G-J show genes that are down-regulated or suppressed at any given stage of DC differentiation and maturation. The transcription levels for *ICAM2* (*ICAM-2*), *CCL4*, *CCL20*, *TNF* (*TNF-α*), and *TLR2* were significantly lower in iDC-3d than in monocytes, and they remained at a low or an undetectable level during the subsequent stages (Fig. 3G). Our findings agree with those in the most recent report [15], except that those researchers detected a high level for the *CCL20* transcript in monocytes and DC using Northern blotting and quantitative real-time PCR [31, 32]. Fig. 3H and I show genes transiently down-regulated or suppressed at the iDC-3d stage. *IL-15* mRNA was reduced to an undetectable level in both immature stages of DC (iDC-3d and iDC-6d), after which, its expression returned to a level comparable to that of the monocytes in the mature DC (Fig. 3H). However, the mRNA level of *CXCR4* declined in iDC-3d and then gradually increased in iDC-6d and in mature DC (Fig. 3I). In addition,

we found that the mRNA encoding ICAM3 (ICAM-3), the specific ligand for DC-SIGN, is progressively down-regulated during DC differentiation and maturation (Fig. 3J).

DISCUSSION

Through microarray analysis of the transcripts in Mo, iDC-3d and iDC-6d, we found that the gene expression profiles of iDC-3d and iDC-6d are quite similar. Moreover, both iDC-3d cells and iDC-6d cells share dendritic morphology, i.e. they form non-adherent aggregates with protruding veils. Therefore, it is reasonable to presume that the time for generating DC could be shortened to three days or less. In fact, a fast protocol for producing mature DC within two days instead of the conventional seven days has already been published [33].

There is growing evidence that the maturation state of DC, which can be altered by different stimuli, may explain the different immune responses observed in studies with DC [8, 9]. Therefore, we performed a parallel analysis of gene expression in DC matured with LPS, TNF-α or CD40L. These are the stimuli most commonly used for activating DC. We found that LPS, TNF-α and CD40L induce the expression changes of a common set of maturation-associated genes, mainly consisting of *CD80*, *CCR7* and *CCL19* (Tab. 2), providing further evidence that these stimulated cells are mature DC. LPS, a strong inducer of pro-inflammatory cytokines and chemokines in various cell types [34, 35], is shown here to selectively increase the expression of genes involved in inflammatory responses, such as *IL1A* (*IL-1A*), *IL1B* (*IL-1B*), *IL6* (*IL-6*), *CXCL1* and *CXCL9* (Tab. 2). It has been proposed that the secretion of the pro-inflammatory cytokines IL-1 and IL-6 may act in an autocrine fashion to activate DC [24, 36]. However, this hypothesis was questioned in a recent report, which stated that inflammatory mediators originating from non-hematopoietic tissues or hematopoietic cells, such as TNF-α, IL-1 and IL-6, are neither sufficient nor necessary for DC activation *in vivo* in a mouse model [37]. The real function of these pro-inflammatory cytokines needs further investigation. The mRNA expression of the pro-inflammatory chemokines CXCL1 and CXCL9 in LPS-stimulated DC (Tab. 2) may result from the activation of the NF-κB signaling pathway [38, 39], whereas the functional roles of CXCL1 and CXCL9 secreted by mature DC have yet to be identified.

The *GADD45* family of genes encodes three small, evolutionarily conserved, highly acidic proteins: GADD45A, GADD45B, and GADD45G. These genes are rapidly induced in various types of cells by genotoxic or non-genotoxic stress, and are implicated in growth suppression, DNA repair, cell cycle arrest, and apoptosis [40-43]. Given the importance of the multifunctional activities displayed by *GADD45* genes, we incorporated these genes into this study to try to determine their expression profiles and potential involvement in DC differentiation and maturation. We found that, compared to the levels in iDC-6d, the mRNA levels of *GADD45A* and *GADD45B* were up-regulated in DC

matured with LPS and CD40L, but not in those matured with TNF-α (Tab. 2). The increase in *GADD45A* mRNA might be related to the Th1 polarization effects of LPS- and CD40L-stimulated DC [44, 45], because *GADD45A* expressed in DC was recently demonstrated to be essential for Th1 cell development using a knock-out mouse model [18]. GADD45B can not only induce apoptosis under certain conditions, but can also protect some cell lines from apoptosis [43, 46, 47]. Thus, the up-regulation of *GADD45B* mRNA in DC stimulated with LPS and CD40L remains obscure. Interestingly, the transcript for *RANK*, which is expressed on DC as an anti-apoptotic receptor and can enhance DC survival upon ligation with the RANK ligand (RANKL) [48, 49], was found to be higher in DC matured with CD40L or TNF-α than in iDC-6d (Tab. 2). Therefore, we propose that the increased expression of *GADD45B* and *RANK* contribute to the long-term survival of DC matured with different stimuli.

Our data demonstrates for the first time the dynamics of gene expression during the entire differentiation process of monocytes into mature DC by comparing the transcriptomes of cells at different developmental stages: monocytes, iDC-3d, iDC-6d, and mature DC. Chemokines can be broadly divided into constitutive chemokines, which are constitutively produced within lymphoid tissue, and inflammatory chemokines, which are differentially generated in response to inflammatory stimuli within peripheral tissue [27]. Interestingly, we found that both the genes encoding constitutive chemokines, including CCL17, CCL18 and CCL22, and the genes coding for inflammatory chemokines, including CCL2, CCL8 and CCL13, were up-regulated or induced to high levels in immature DC (iDC-3d or iDC-6d), and remained steady until the DC matured (Fig. 3B, D and E). This suggests that the constitutive and inflammatory chemokines secreted by DC may function both in peripheral tissues, where immature DC are located, and in secondary lymphoid organs, where mature DC are located, although they are supposed to be produced in different anatomic locations *in vivo*.

In addition, the transcriptional kinetics of the gene pairs *IL1R1/IL1R2*, *IL15/IL15RA* and *DC-SIGN/(ICAM2/ICAM3)* indicate their critical roles in the differentiation and biological function of DC. IL-1 (IL-1A and IL-1B), a multifunctional cytokine involved in a host of pro-inflammatory responses and diseases [50], affects target cells through two distinct types of transmembrane receptors, IL1R1 and IL1R2. IL1R1 transduces the signal from IL-1, while IL1R2 acts as a decoy receptor to subtract IL-1 from ILR1 [51, 52]. The ratio between the expression of IL1R1 and IL1R2 has been shown to be the central factor in determining the responsiveness of a cell to IL-1 [53]. Our finding of strong up-regulation of *IL1R1* and *IL1R2* mRNA in immature DC and mature DC (Fig. 3B) is likely to indicate that DC maintain a minimal sensitivity to IL-1 in normal individuals. Perturbation of the steady state of IL1R2 and IL1R2 in DC will lead to abnormal responsiveness of DC to IL-1 and result in hyper- or hypo-activation of T cells in diseases associated with IL-1 [54, 55]. IL-15 is another pleiotropic pro-inflammatory cytokine that plays various roles in innate and adaptive immunity. Membrane-bound IL-15 on DC has been shown to be

indispensable for NK cell proliferation and survival in human lymphoid organs, probably because of the trans-presentation of IL-15 by IL-15RA expressed on DC [56, 57]. Moreover, autocrine IL-15 is essential in activating DC and rendering DC resistant to apoptosis [58, 59]. Our data shows that the mRNAs for IL-15 and IL-15RA are both strongly induced in mature DC, but not detected in the immature stages (iDC-3d and iDC-6d; Fig. 3F and H). This suggests the key role of this cytokine/cytokine receptor pair in activating DC in an autocrine fashion and in regulating innate immunity in a "trans-presentation" mode. DC-SIGN is a type II membrane protein that is preferentially expressed by DC. It has been postulated that DC-SIGN servers as a receptor for ICAM-2 in the case of mediating DC trafficking through the endothelium [60], and a receptor for ICAM-3 in the case of initiating effective interaction between the MHC class II/peptide complex on DC and the T-cell receptor (TCR) on T cells [61]. Our finding that the strong induction of DC-SIGN mRNA is concomitant with the rapid down-regulation of ICAM-2 and ICAM-3 mRNAs in DC suggests that the formation of ligand-receptor complexes on DC is avoided in order to ensure the maximal function of DC-SIGN for interaction with T cells and endothelial cells.

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