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Expression profilings of 39 genes selected by ANOVA could separate precursors of murine dendritic cells and macrophages

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Abstract

Dendritic cells (DCs) and macrophages share some stages in the development and function of antigen presentation. But it is difficult to separate them from their precursors. We used one-way ANOVA (analysis of variances) on murine expression profilings of several hematopoietic cells associated with DCs and macrophages to find the genes with great differences across the cell groups. These groups were the DCs from spleen, cultivated DCs, DC precursors, DC progenitors, DC progenitor cell lines, hematopoietic stem cell (HSC), and bone marrow-derived macrophages. The data of expression profilings were all downloaded from GEO and ArrayExpress database. After the normalization of 11 housekeeping genes across 42 arrays, we got 39 genes (44 probesets) by analysis of one-way ANOVA (Bonferroni step-down) with p values cutoff of 0.05. These genes (probesets) could separate the hematopoietic cells well by the methods of unsupervised hierarchical clustering and principal component analysis (PCA). The class prediction also indicated that these genes could separate the precursors of DC and macrophages with 20 arrays composed of 5 cell types with the same normalization. The accuracy rate of class prediction was 90% (18/20). The genes selected by one-way ANOVA included those of MHC (major histocompatibility complex) and defense of immunity, cell adhesion, chemokine or its receptors, and transcription factors. The results indicated that these 39 genes could separate precursors of DC and macrophages very clearly. It was suggested that these genes might represent some important molecules that related with the precursors of DCs and macrophages, and were worthy for further study.

Keywords: Expression profilings; Dendritic cells; Macrophages; One-way ANOVA; Class prediction

Dendritic cells (DCs) and macrophages have multiple functions including phagocytosis, transportation, processing and antigen presentation to T lymphocytes. Thus, both DCs and macrophages are referred to as antigen-presenting cells (APCs). Compared with macrophages, DCs are sparsely populated (less than 1% composed cells of total lymphocytes), but strong antigen presenting [1,2]. During hematopoiesis, DCs are considered to be differentiated cells derived from lymphoid and myeloid precursors or monocytes [3]. Macrophages are part of the mononuclear phagocytic (reticuloendothelial) system and also originate from myeloid stem cell or progenitors in bone marrow. When monocytes leave the bloodstream and enter the tissue, they become activated and differentiate into macrophages. The mouse macrophages that we discuss here are derived from bone marrow. We call them precursors of macrophage.

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The differences between the precursors of DCs and macrophages are not fully understood. The surface markers of murine macrophages were also expressed in some subsets or the precursor of DCs, such as CD14, CD11b, and CD45 [1,4].

In this study, we studied the gene expression profilings of mouse hematopoietic cells associated with DC development and macrophage precursors. We also validated the use of expression profilings to identify different groups of hematopoietic cells.

All the expression profilings that associate with DC development and macrophages are downloaded from Gene Expression Omnibus (GEO) at NCBI [5] and ArrayExpress at EBI [6]. The need for normalization arises naturally when dealing with experiments involving multiple arrays. Affymetrix has approached the normalization problem by proposing that intensities should be scaled so that each array has the same average value [7]. Here, we used 11 housekeeping genes to normalize each array because of their stable expressions and reflecting endogenetic changes across these samples (arrays) [8,9].

After normalization of these housekeeping genes, we used one-way analysis of variance to select the significant probesets (genes) according to the expression profilings among various cell groups. This method could filter out genes that did not vary significantly across different groups with multiple samples and select those genes that exhibit great changes of the experiment. This comparison was performed for each probeset or gene, and the genes with sufficiently small p values were returned [10,11]. The probesets (genes) that selected by one-way ANOVA were further processed with unsupervised hierarchical clustering, principal component analyses (PCA), and class prediction to observe whether they could separate these cell groups correctly.

Materials and methods

The downloaded expression profilings of microarray. Altogether 42 expression profilings of DC development associated cells and macrophage precursors were downloaded from Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/). These data included dendritic cells which were sorted by CD11c (high+), CD4(+/−) or CD8(+/−) population from spleen (GSM4697–GSM4711) and the same sorted cell cultivated for 2 h (GSM4757–GSM4775), dendritic cell precursors from bone marrow (GSM8435–GSM8440), dendritic cell progenitors from bone marrow (GSM10877–GSM10881), hematopoietic stem cells from bone marrow (GSM10882–GSM10883), DC progenitor cell lines (MHH0083–MHH0089), and bone marrow-derived macrophages (GSM6072–GSM6074, GSM6076, GSM6078–GSM6080, GSM6082, GSM6084, and GSM6085). The data of these cells served as the training set (Table 1). The test set was also downloaded from Gene Expression Omnibus database. The cells included Lin-Sca-1 + c-kit + HSCs from different murine BXD recombinant inbred strains (GSM36673–GSM36708), DC precursors (GSM8641–GSM8643) from murine bone marrow, erythroid progenitors (GSM9604, GSM9638, GSM9639), side population in murine bone marrow (GSM26999–GSM27002), and bone marrow-derived macrophages (GSM6075–GSM6088) (Table 3). All datasets of these cells were

Table 1
The downloaded expression data of DC developmental cells and macrophage precursor (training set)

Cell groups	Samples	Sources	Descriptions
Dendritic cells from spleen	6	Alexander D. Edwards et al. [12]	Sorted CD11c high population from mice spleen
Cultivated dendritic cells	6	Alexander D. Edwards et al. [12]	Sorted CD11c high population cultivated for 2 h
Dendritic cell progenitors from bone marrow	5	Christine Hacker et al. [13]	$FLT3^{+}/CD11b^{+}$ Dendritic cell progenitor sorted from bone marrow
Dendritic cell precursors	6	Athena W. Wong et al. [14]	Wild-type B6, CIITA−/− and IAB−/−, day 10 dendritic cells from bone marrow
Dendritic cell progenitor cell lines	7	Chozhavendan Rathinam et al. [15]	This FDCP-mixed cell line is permissive for DC differentiation in the presence of GM-CSF. 0, 6, 24, and 48 h induction by GM-CSF (5 ng/mL)
Hematopoietic stem cells	2	Christine Hacker et al. [13]	Lin-c-kit + Sca1 + sorted hematopoietic stem cells from bone marrow
Bone marrow-derived macrophages	GSM6072	Roland Lang and Peter Murray et al. [16]	Unstimulated macrophages, WT_IL-6_0_rep1
	GSM6078		Unstimulated macrophages, WT_IL-6_0_rep2
	GSM6073		Stimulation for 100 min with 10 ng/ml IL-6, WT_ rep1
	GSM6079		Stimulation for 100 min with 10 ng/ml IL-6, WT_ rep2
	GSM6074		Stimulation for 400 min with 10 ng/ml IL-6, WT_ rep1
	GSM6080		Stimulation for 400 min with 10 ng/ml IL-6, WT_ rep2
	GSM6076		SOCS3−/−, for 100 min with 10 ng/ml IL-6, KO_IL-6_rep1
	GSM6082		SOCS3−/−, for 100 min with 10 ng/ml IL-6, KO_IL-6_rep2
	GSM6084		Stimulation for 100 min with 10 ng/ml IL-6, WT_ rep3
	GSM6085		Stimulation for 400 min with 10 ng/ml IL-6, WT_ rep3

Notes. CIITA−/−, deficient of the major histocompatibility complex (MHC) class II transactivator; IAB−/−, deficient of the major histocompatibility complex (MHC); SOCS3−/−, deficient of the suppressor of cytokine signaling 3.

performed by the Affymetrix microarray named mouse MG-U74av2 (12,488 probesets) except for DC precursors (GSM8641–GSM8643) of test set that were performed by the array of mouse MG-U74a (12,654 probesets). The two arrays were well matched for the most probesets that overlapped between them. These two types of arrays (U74av2 and U74a) included 16–20 probe pairs that interrogated different parts of the gene sequence, which was known as a probeset.

Data preprocessing, normalization, and filtering. We truncated each negative value of 42 arrays to 0.01. Then the expression values of training and test sets were normalized by the average values of 11 housekeeping genes across the arrays [8,9]. These housekeeping genes included mouse β-actin, GAPDH, β-glucuronidase, β-2 microglobulin, hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1), phosphoglycerate kinase 1, ribosomal protein L14, transferrin receptor, adenosine deaminase, ATP synthase 5c1, and 18S ribosomal RNA genes.

We used GeneSpring 7.2 (Silicon Genetics) to filter out the probesets whose normalized values were all less than 0.1 in the 42 arrays as well as the probesets (genes) with 36 "Absent Call" of 42 arrays. There were 8862 probesets after filtering out low values and "Absent Call" from the total 12,488 probesets. The values of the remaining probesets were transformed to logarithm (base 2). Both the arrays (samples) and probesets (genes) were normalized to median.

One-way ANOVA. We used analysis of variances (one-way ANOVA) of GeneSpring 7.2, to find the set of genes (probesets) which showed statistically significant differences in the normalized expression levels across the 42 arrays of 7 cell groups. One-way ANOVA was performed on the parameter of "cell groups"; using parametric test and all available error estimates. The variances were calculated using cross-gene error model. The method of multiple testing corrections was Bonferroni step-down (Holm). The p value cutoff was 0.05 [10,11]. Even if the gene was found significant in the first step, it would perhaps be removed from the multiple-testing, so the multiple-testing adjustment was based on the number of probesets N. This process was continued as long as genes pass the successive tests after the number of $(N-1)$ adjustment [9]. Here, the number of probesets N was 8862.

Hierarchical clustering, principal component analysis, and SOMs. Unsupervised hierarchical clustering analysis was carried out by Cluster3.0 [17, updated by Michiel de Hoon] and Treeview software using median-centered Pearson correlation and complete linkage. The principal component analysis (PCA) was performed by GeneSpring 7.2. Clustering of Self-Organizing Maps (SOM) was performed by GeneCluster 2.0 package and constructed 4 class (2×2) SOMs [18].

Class prediction. We used GeneSpring 7.2 to perform the class prediction. The training set was 42 arrays that associated with DC development and macrophage precursors. The test set was 20 arrays of 5 hematopoietic cell groups that included HSCs, DC precursors, erythroid progenitors, side populations in bone marrow- and bone marrow-derived macrophages. Classification was generated by the 'K-Nearest Neighbors' algorithm using the training set of 42 arrays and the test set of 20 arrays for the parameter 'cell groups'. We selected the top 44 probesets (39 genes) by the one-way ANOVA of 'Bonferroni step-down' for the judging gene lists. The prediction was done by Fisher's exact test. The number of neighbors was 3. The decision cutoff for p value ratio was 0.05 [10].

Results

One-way ANOVA on DC developmental cells and macrophage precursors

Significant differences among the seven hematopoietic cell groups in the training set were found in 44 probesets, which represented 39 genes (Table 2). These genes included those of MHC class II molecules, inflammatory responses and phagocytosis, cell adhesion, transcription factors, metabolism, growth, and apoptosis.

Clustering of self-organizing maps on DC developmental cells and macrophage precursors

The 44 probesets (39 genes) that were selected by one-way ANOVA were further analyzed by clustering of 2×2 self-organizing maps (SOMs). The clusters were classified into C_1 to C_4. The genes of class C_1 were up-regulated in splenic DCs and cultivated DCs and down-regulated in other cell groups (Fig. 1 and Table 2) These genes were H2-Eb1, H2-Aa, H2-Ab1, Ii, and Pscdbp. Class C_2, comprised of 17 genes, was overexpressed in DCs and their precursors and was of lower expression in macrophage precursors. The genes included chemokines (CCL17, CCL22) and receptor (CCR7), antigen presentation (CD83, Igk-V8), and transcription factors (Fos, Nfkbia, and Basp1). Six genes of class C_3 were only up-regulated in DC progenitor cell lines after GM-CSF induction. Class C_4, including 11 genes, was overexpressed in both macrophages and DC progenitors. These genes included S100 calcium-binding proteins A4, A8, and A9, cathepsin (Ctsb, Ctsl) and phagolysosome (Lzp-s), desmoyokin (Ahnak), chitinase (Chi3L3), neutrophilic granule protein (Ngp), colony-stimulating factor receptor (Csf1r), and secreted phosphoprotein (Spp1 or Opn, osteopontin). Most of these genes were associated with cell adhesion, migration, inflammatory responses, and phagocytosis that matched the function of macrophages (Table 2).

Principal component analysis on DC developmental cells and macrophage precursors

We used the set of 44 probesets to perform principal component analysis (PCA). The cumulative percent of variances of the largest three components which were used to draw 3D plot were more than 78%. The first three components were used to make scatter plot in three-dimension. From the 3D plot of PCA on 44 probesets (Fig. 2), the 42 arrays of hematopoietic cells could be clearly separated into seven main groups. They were HSCs from bone marrow, DCs from spleen, cultivated DCs from spleen, DC precursors from bone marrow, DC progenitors from bone marrow, DC progenitor cell lines, and macrophages.

Unsupervised hierarchical clustering on DC developmental cells and macrophage precursors

The results of unsupervised hierarchical clustering showed that there were six main classes across 42 arrays of hematopoietic cells using 44 probesets selected by one-way ANOVA. These six classes were the sorted and cultivated DCs from spleen, DC precursors from bone marrow, DC progenitors from bone marrow, DC progenitor cell lines, HSCs, and macrophage precursors. DC precur-

Table 2
Forty-four probesets (39 genes) selected by one-way ANOVA

Probesets	Genes	*p* values	Annotations	SOM classes
94285_at	H2-Eb1	0.0007	histocompatibility 2	C1
92866_at	H2-Aa	0.0017	histocompatibility 2	C1
100998_at	H2-Ab1	0.0024	histocompatibility 2	C1
101054_at	Ii	0.0002	Ia-associated invariant chain	C1
104257_g_at	Pscdbp	0.0483	pleckstrin homology binding protein	C1
103040_at	Cd83	0.0012	IgSF, APC signaling	C2
94085_at	Prg1	0.0023	proteoglycan 1	C2
102156_f_at	Igk-V8	0.0029	immunoglobulin kappa chain	C2
95673_s_at	Basp1	0.0041	brain abundant, membrane	C2
95674_r_at		0.0071	attached signal protein 1	C2
102914_s_at	Bcl2a1d	0.0044	B-cell leukemia/lymphoma 2	C2
93869_s_at		0.0092	related protein A1d	C2
102310_at	Ccl22	0.0096	chemokine (C-C motif) ligand 22	C2
97783_at	Ccl17	0.0106	chemokine (C-C motif) ligand 17	C2
104443_at	Ccr7	0.0395	chemokine (C-C motif) receptor	C2
161666_f_at	Gadd45b	0.0017	growth arrest and	C2
102779_at		0.0168	DNA-damage-inducible 45 β	C2
95338_s_at	Mmp12	0.0361	matrix metalloproteinase 12	C2
95339_r_at		0.0483		C2
100584_at	Anxa4	0.0164	calcium-dependent phospholipid binding	C2
101554_at	Nfkbia	0.0274	nuclear factor of kappa gene enhancer in B-cells inhibitor	C2
160901_at	Fos	0.0276	FBJ osteosarcoma oncogene	C2
104598_at	Dusp1	0.0348	dual specificity phosphatase 1	C2
161522_i_at	Cst3	0.0439	cystatin C	C2
100946_at	Hspa1b	0.0472	Heat shock protein 1B (HSP70)	C2
101787_f_at	Ccrn4l	0.0406	carbon catabolite repression 4-like	C2
92778_i_at	Superantigen	0.003	membrane glycoprotein	C3
98092_at	Plac8	0.0088	placenta-specific 8	C3
92780_f_at	Mtv7	0.0116	mammary tumor virus locus 7	C3
103049_at	Mycn	0.0123	v-myc viral related oncogene	C3
102877_at	Gzmb	0.0242	granzyme B	C3
101209_at	Fcer1a	0.0474	Fc receptor, alpha polypeptide	C3
97519_at	Spp1	0.0004	secreted phosphoprotein 1 (Opn)	C4
101753_s_at	Lzp-s	0.0005	P lysozyme structural	C4
100611_at		0.0176		C4
94831_at	Ctsb	0.0043	cathepsin B	C4
103887_at	S100a9	0.0127	S100 calcium-binding protein A9	C4
99051_at	S100a4	0.0136	S100 calcium-binding protein A4	C4
103448_at	S100a8	0.0245	S100 calcium-binding protein A8	C4
96153_at	Ngp	0.0381	Neutrophilic granule protein	C4
160255_at	Ahnak	0.04	nucleoprotein, desmoyokin	C4
101963_at	Ctsl	0.0455	cathepsin L, epitope	C4
92694_at	Chi3l3	0.0479	chitinase 3-like 3	C4
104354_at	Csf1r	0.0495	colony stimulating factor 1 receptor	C4

Notes. SOM classes were from the results of (2×2) SOMs. There were 5 genes that each gene comprised of 2 probesets (see Fig. 1).

sors and macrophages formed a larger group. The gene clustering showed the genes of colony-stimulating factor 1 receptor (Csf1r) and phagocytosis, such as P lysozyme structural (Lzp-s), cathepsin B (Ctsb), and cathepsin L (Ctsl), were highly up-regulated in macrophages and moderately up-regulated in DC progenitors. The mouse MHC genes (histocompatibility 2) were overexpressed in the splenic DCs, such as H2-Aa, H2-Ab1, and H2-Eb1, as well as antigen presenting genes (Ii and CD83). The array names are abbreviated by the first letter and last four figures. For an example, G0877 and M0084 represent for GSM10877 and MHH0084 arrays, respectively (Fig. 3).

Class prediction

We chose 20 arrays to predict 5 groups of hematopoietic cells, including HSCs (4 samples), DC precursors (3 samples), erythroid progenitors (4 samples), side populations in bone marrow (3 samples), and bone marrow-derived macrophages (7 samples). The values of BM1 were the average of GSM36707 and GSM36708 (repeated experiment) arrays. Also, BM2, BM5, and BM6 represented the combinations of GSM36703 and GSM36704, GSM36711 and GSM36712, and GSM36673 and GSM26674 arrays, respectively. The gene expression profilings correctly identified 18 of 20 of the hematopoietic cell samples (90%

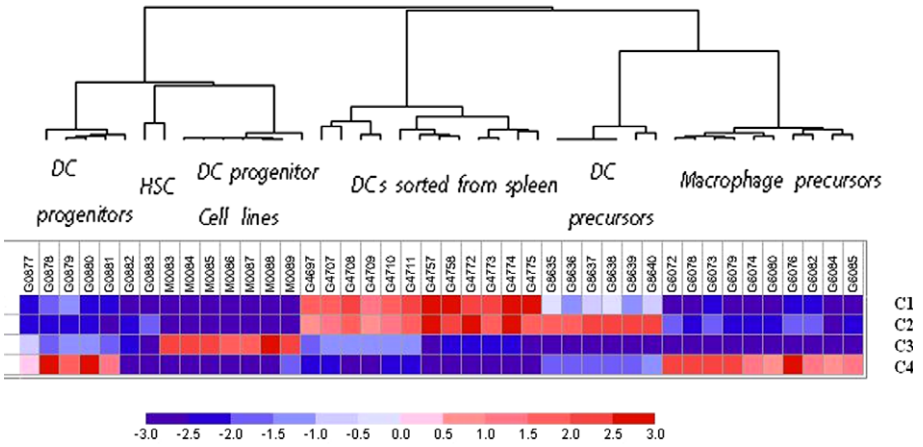

Fig. 1. SOMs clustering of (2 × 2) classes on 42 arrays. The red color represents the higher values of gene expression and the blue represents for lower values. The colors at bottom of the figure represent the relative expression levels. Top of the figure is the tree of hierarchical clustering. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

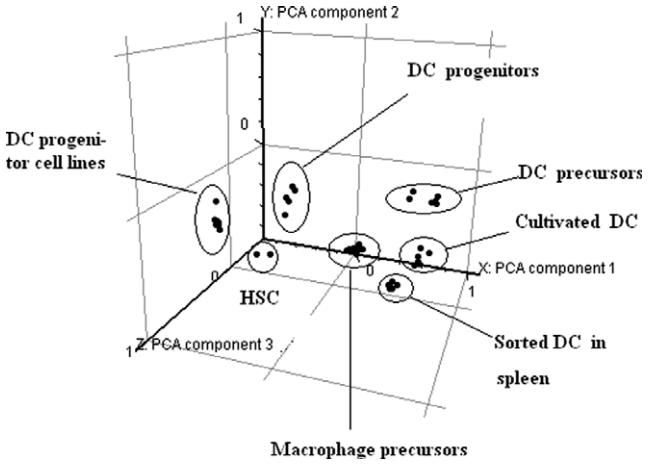

Fig. 2. PCA on 42 arrays of DC the developmental cells and macrophages. From the 3D graph, these DC associated cells can be divided into 7 groups clearly. The names of cell groups are marked in the figure.

accuracy). Two samples of side populations from bone marrow were mistaken as DC progenitors (Table 3).

Discussions

During the development of hematopoietic cells, it was reported that some DCs and macrophages originated from progenitors of myeloid cells or monocytes in bone marrow. Both DCs and macrophages exhibit similar functions, including phagocytosis, antigen presentation, as well as the secretion of some growth factors, cytokines or chemokines. They also produce both MHC-I and MHC-II molecules [22]. We used one-way ANOVA and tried to find different gene expression between DCs and macrophages. We chose "Bonferroni step-down (Holm)" as the method of one-way ANOVA. The reason we chose this method was it could compute the probeset or gene with the most significant p value and the gene set was selected after passing the successive test. The left 44 probesets (39 genes) after filtering of "Bonferroni step-down" had significantly different expression profilings across cell groups as a whole.

The method of unsupervised hierarchical clustering tried to find internal structure or relationships in the dataset of hematopoietic cell groups instead of determining the known samples or conditions [23]. The result showed the groups of varied cells could be clustered because of the similar distance by 44 probesets (Fig. 3). Furthermore, the DC precursors and macrophages could form a larger tree. This result indicated that they were similar in expression profilings than other hematopoietic cells.

SOM is a clustering algorithm where a grid of two-dimensional nodes is iteratively adjusted to reflect the global structure in the expression dataset [24]. SOM can extract biologically meaningful groups of genes and provides a survey of expression patterns. Both SOMs and unsupervised hierarchical gene clustering showed that the murine MHC class II genes (H2-Aa, H2-Ab1 and H2-Eb1, and Ii) associated with antigen presentation were highly expressed in the relative mature DCs from spleen, but the genes related with phagocytosis (Lzp-s, Ctsb, Ctsl, and Chi3L3) were mainly overexpressed in macrophages and moderately elevated in DC progenitors. In the development of hematopoietic cells, when DC progenitors differentiate to the mature DCs (DCs sorted in spleen), these phagocytosis-related genes are down-regulated and the function of phagocytosis becomes weaker. The analysis result matched the function of the mature DCs and macrophages.

PCA is used commonly for reducing the dimensionality of complex data and one of the visualization techniques. This analysis can be considered as a combined biological sample and represents the largest variation in gene expression [25]. We used the first three principal components representing 78% variances of 44 probesets to separate the 7 groups of hematopoietic cells including the DCs and macrophages. Both the results of hierarchical clustering and PCA indicated that these 44 probesets (39 genes) were

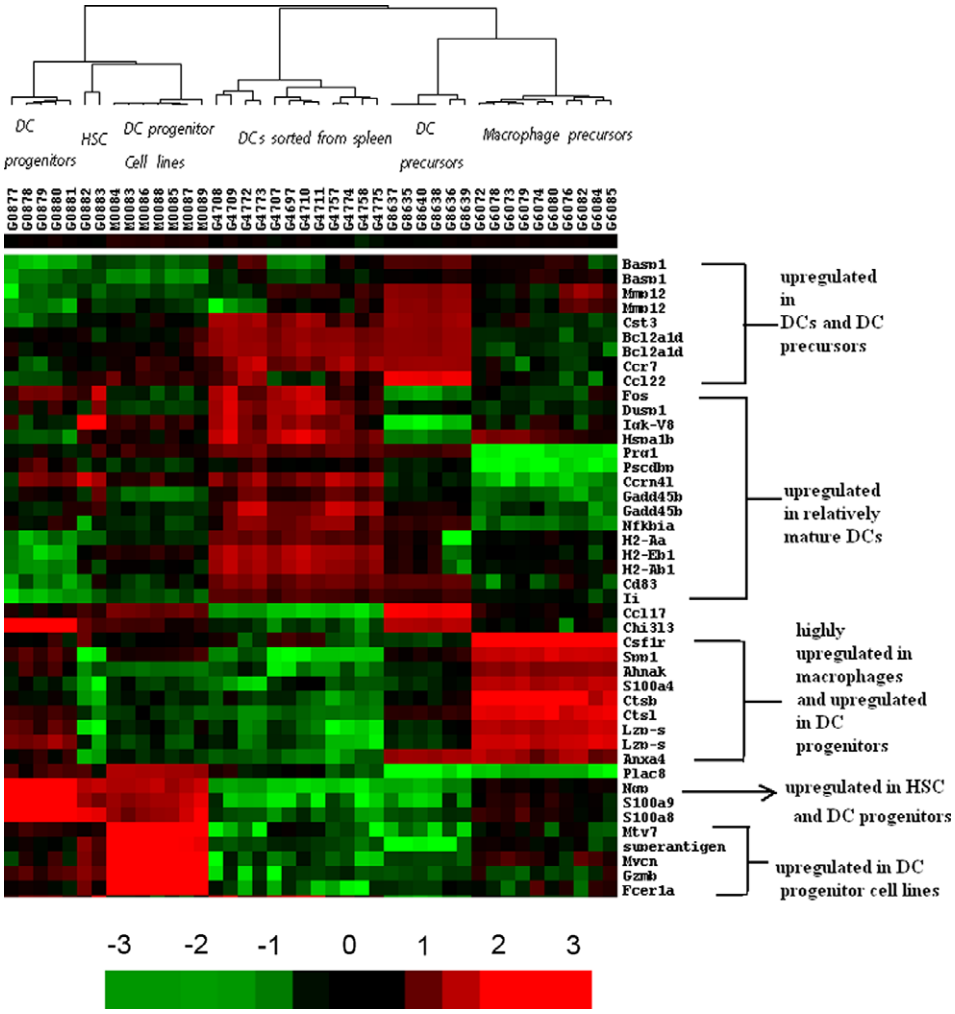

Fig. 3. Unsupervised hierarchical clustering analysis of macrophages and DC developmental cells. The gene-expression values are represented by using a red-green color scheme, with the red corresponding to higher than median expression values, the black corresponding to equal to median, and the green corresponding to lower than median expression values. The array names in the top of the figure are combined with the first letter and the last four numbers. The gene symbol names are listed in the right of the figure. The folds indicated by color scheme were logarithm scales (base 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

the potential key genes for the feature of DCs and macrophages.

The result of class prediction showed that these 39 genes (44 probesets) could predict correctly for the precursors of DC (3 samples) and macrophages (7 samples) in bone marrow. There was no error in judging these 10 samples. The side populations from bone marrow are regarded as progenitor cells or HSCs, and they are of a mixed cell population [21]. This might explain why two samples from this group were misclassified by gene expression profilings.

Most genes selected by one-way ANOVA are known as the function-associated genes for the DCs and macrophages. On the other hand, some selected genes do not have well-defined function. For example, spp1 (secreted phosphoprotein 1) or opn (osteopontin) was only up-regulated in macrophages (Fig. 3). This gene is an extracellular matrix protein of pleiotropic properties and has been recently recognized as a potential inflammatory cytokine [26]. Does this gene possess unique function in macrophages or their precursors? Pscdbp (pleckstrin homology binding protein, or Cytip), which was reported a regulator of lymphocyte adhesion [27], was up-regulated in the mature spenic DCs (Figs. 1 and 3). Basp1 (brain abundant, membrane attached signal protein 1, or Cap23), which was a major cortical cytoskeleton-associated and calmodulin-binding protein [28], was mainly up-regulated in the DC precursors and some macrophages. These genes perhaps are the potential key regulators for the DCs and macrophages, and are worthy of further research.

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Table 3
Class prediction for 20 arrays composed of 5 hematopoietic cell groups

Samples	Predicted class	*p* values	True/false	Descriptions
BM1	HSC	0.011	True	Lin-Sca-1 + c-kit + HSC of recombinant inbred strains [19]
BM2	HSC	0.011	True	
BM5	HSC	0.011	True	
BM6	HSC	0.011	True	
GSM8641	DC precursor	0.002	True	Wild type, mouse day 10 DC's [14]
GSM8642	DC precursor	0.002	True	CITA−/− DC
GSM8643	DC precursor	0.002	True	IAb−/− DC
GSM9638	None	0.481	True	Erythroid precursors [20]
GSM9639	None	0.481	True	
GSM9604	None	0.481	True	
GSM26999	DC progenitor	0.001	False	Side populations from bone marrow [21]
GSM27000	None	0.238	True	
GSM27001	DC progenitor	0.001	False	
GSM6075	Macrophage	0.01	True	Donor derived cells from the bone marrow were differentiated to macrophages [16]
GSM6081	Macrophage	0.01	True	
GSM6083	Macrophage	0.01	True	
GSM6086	Macrophage	0.01	True	
GSM6087	Macrophage	0.01	True	
GSM6088	Macrophage	0.01	True	
GSM6077	Macrophage	0.01	True	

Notes. BM1, BM2, BM5, and BM6 combined with GSM36707 and GSM36708, GSM36703 and GSM36704, GSM36711 and GSM36712, and GSM36673, and GSM26674, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.03.125.

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