# Transcriptome of Hypoxic Immature Dendritic Cells: Modulation of Chemokine/Receptor Expression

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## Abstract

**Hypoxia is a condition of low oxygen tension occurring in inflammatory tissues. Dendritic cells (DC) are professional antigen-presenting cells whose differentiation, migration, and activities are intrinsically linked to the microenvironment. DCs will home and migrate through pathologic tissues before reaching their final destination in the lymph node. We studied the differentiation of human monocytes into immature DCs (iDCs) in a hypoxic microenvironment. We generated iDC *in vitro* under normoxic (iDCs) or hypoxic (Hi-DCs) conditions and examined the hypoxia-responsive element in the promoter, gene expression, and biochemical KEGG pathways. Hi-DCs had an interesting phenotype represented by up-regulation of genes associated with cell movement/migration. In addition, the Hi-DC cytokine/receptor pathway showed a dichotomy between down-regulated chemokines and up-regulated chemokine receptor mRNA expression. We showed that *CCR3, CX3CR1*, and *CCR2* are hypoxia-inducible genes and that *CCL18, CCL23, CCL26, CCL24,* and *CCL14* are inhibited by hypoxia. A strong chemotactic response to CCR2 and CXCR4 agonists distinguished Hi-DCs from iDCs at a functional level. The hypoxic microenvironment promotes the differentiation of Hi-DCs, which differs from iDCs for gene expression profile and function. The most prominent characteristic of Hi-DCs is the expression of a mobility/migratory rather than inflammatory phenotype. We speculate that Hi-DCs have the tendency to leave the hypoxic tissue and follow the chemokine gradient toward normoxic areas where they can mature and contribute to the inflammatory process.**
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## Introduction

Dendritic cells (DC) are a heterogeneous group of cells derived from myeloid or lymphoid precursors, which populate peripheral tissues and lymphoid organs and play an important role as antigen-presenting cells. Their main functions include antigen uptake and processing, migration from peripheral tissues to lymphoid organs, antigen presentation in a MHC class I– and class II–restricted manner, production of cytokines, and expression of costimulatory molecules (1). DCs reside in nonlymphoid tissues as immature DCs (iDCs), which are highly adapted for the uptake of antigen via receptor- and nonreceptor-mediated mechanisms (2). After antigen engulfing and activation by proinflammatory cytokines, iDCs differentiate into mature DCs, which have a reduced potential for antigen uptake but a higher capacity for antigen presentation (3) and migrate to the regional lymph nodes to stimulate the specific immune response (4).

iDCs are widely distributed throughout the body and occupy sentinel positions in many nonlymphoid tissues. One important source of iDCs is the circulating monocyte that, following extravasation, differentiates in response to cytokines, such as interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor. Once in the tissue, the redistribution of the cell is determined by the microenvironment and specifically by local production of chemotactic mediators, activation of chemokine receptors, and regulation of adhesion molecules (5, 6). Chemokines are small, secreted, chemotactic proteins that regulate cell migration under steady-state and inflammatory conditions (7). iDCs express a unique repertoire of receptors that bind a pattern of ''inflammatory'' chemokines and are themselves an important source of chemokines (6, 8). The migratory ability of DC is intrinsically linked to its maturation state and, thus, to the inflammatory (6) or physiologic (9) microenvironment. Hypoxia, a low partial oxygen pressure, is the common denominator of many pathologic processes (10), which creates a unique microenvironment by regulating the expression of metabolic enzymes and transcription factors. Hypoxic areas can be intermixed with normoxic tissues as

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observed in most solid tumors (11). Mononuclear phagocytes are exquisitely sensitive to hypoxia or other inhibitory and stimulatory signal. They are programmed to respond and adapt to different physiologic or pathologic conditions (10, 12-15). Differentiation of monocytes into iDCs may occur under hypoxia, and we were interested in determining whether this microenvironment modified the phenotype of iDCs.

The response to hypoxia is associated with changes in gene expression (16-19). Transcriptional activation by hypoxia is mediated primarily by the hypoxia-inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix transcription factor composed of the constitutive subunit HIF-1β and the oxygen-sensitive subunits HIF-1α, HIF-2α, or HIF-3α. The α subunits are posttranslationally stabilized by hypoxia, translocate to the nucleus where they dimerized with HIF-1β, and transactivate the hypoxia-responsive elements (HRE) present in the promoter of many oxygen-sensitive genes (20, 21). Induction of HIF-1 is a tightly regulated process, mediated by the activity of several oxygen-dependent enzymes and transcription factors (16, 22). The gene expression profile of human DCs relative to activation, differentiation, and lineage has been reported (23-27). We have previously shown that hypoxia has a profound effect on the transcriptome of primary human monocytes (17, 18, 28), but its effect on monocyte differentiation into iDCs is largely unknown.

We studied the transcriptome of iDCs, generated under hypoxic conditions (Hi-DCs), and show that Hi-DCs are characterized by a distinct gene expression pattern. We observed a characteristic dichotomy between down-regulated chemokine and up-regulated chemokine receptor mRNAs and showed a different chemotactic response of Hi-DCs relative to iDCs. We speculate that Hi-DCs have the tendency to leave the hypoxic tissue and follow the chemokine gradient toward normoxic areas where they can mature and contribute to the inflammatory process.

## Results

### *Gene Expression Profile of Hi-DC*

DCs can originate from the differentiation of monocytes following extravasation in the tissue where they exist as iDCs or mature DCs. We studied the effects of the hypoxic microenvironment on the differentiation of monocytes into iDCs in response to cytokine stimulation. Blood monocytes from healthy donors were cultured for 4 days in medium containing IL-4 and granulocyte macrophage colony-stimulating factor under normoxic or hypoxic environment obtaining iDCs and Hi-DCs, respectively.

Fluorescence-activated cell sorting analysis was used to assess the iDC and Hi-DC phenotype. Figure 1A shows the membrane marker expression of a representative iDC population characterized by high CD1a and HLA-II, intermediate CCR5, and low CD80. The phenotype of Hi-DCs was similar (Fig. 1B), although a higher percentage of CD80 (from 20% to 57%) and CCR5 (from 64% to 94%) was consistently observed.

Experiments were done to evaluate the gene expression profile of Hi-DCs. Monocytes from three independent donors were differentiated into iDCs or Hi-DCs and the individual gene expression profile was determined. Hypoxia caused major changes in the gene expression profile and induced modulation of 2,148 mRNA species by at least 2-fold (1,686 up-regulated and 462 down-regulated). These results provided the first indication that iDCs and Hi-DCs had a different phenotype with regard to the gene expression signature.

To gain insights into the nature of the changes induced by hypoxia, modulated genes were selected and clustered on the bases of KEGG pathways and sorted according to their biological function using a *P* value cutoff of 0.05. We highlight 14 pathways containing a statistically significant proportion of genes modulated by hypoxia (Fig. 2, *dotted columns*). Up-regulation was the most common change in the gene expression that differentiated Hi-DCs from iDCs. We

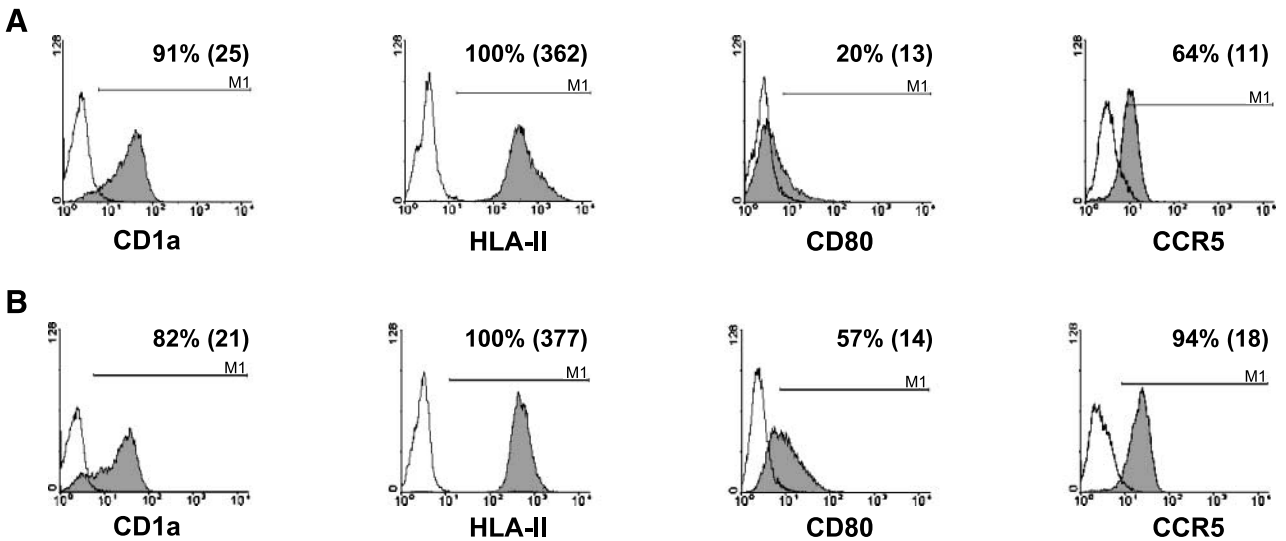


**FIGURE 1.** iDC and Hi-DC phenotypes. Monocytes were cultured for 4 d with IL-4 and granulocyte macrophage colony-stimulating factor in normoxic (**A**) and hypoxic (**B**) conditions and tested for the expression of CD1a, HLA-II, CD80, and CCR5 by fluorescence-activated cell sorting analysis. Open histograms, negative control; solid histograms, marker expression. In each histogram, we show the percentage of positive cells and, in parenthesis, the mean fluorescence intensity. The data are plotted as fluorescence intensity in a logarithmic scale on the abscissas versus the number of positive cells on the ordinates. One representative experiment is shown.

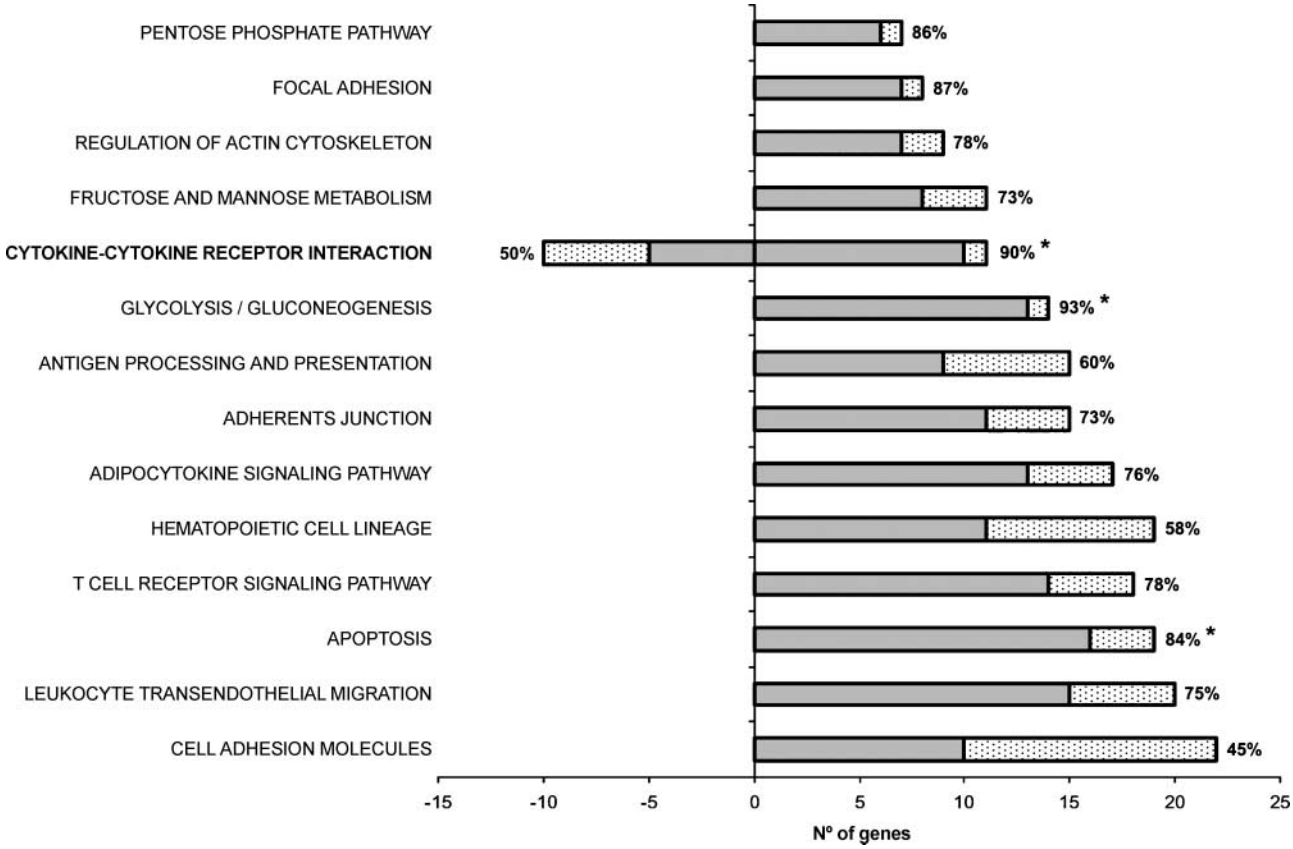


**FIGURE 2.** Functional analysis of gene expression. The genes modulated by hypoxia were clustered in different KEGG pathways using the Database for Annotation, Visualization and Integrated Discovery software. The categories listed on the histogram represent the pathways in which we identified several modulated genes significantly different from the total number of genes printed on the chip belonging to that pathway (confidence of 95%). Dotted columns, number of genes up-regulated or down-regulated by at least 2-fold in every pathway. The promoter of the genes modulated in Hi-DCs was analyzed for the presence of members of the HRE family. The definitions of promoter and "membership to the HRE family" are detailed in Materials and Methods. Gray columns, number of genes whose promoter contained at least one member of the HRE family. The percentage of the $HRE^+$-modulated genes is shown at the tip of the columns. *, pathways in which the percentage of $HRE^+$ genes is significantly different ($P < 0.05$) from the background defined as the frequency of $HRE^+$ gene promoters among those spotted on the chip ( ~ 60%). The cytokine-cytokine receptor interaction class is highlighted in bold because it is the only one in which there were a significant number of down-regulated genes.

found an increase in the expression of genes belonging to the glycolysis/gluconeogenesis and pentose phosphate pathways (Fig. 2). These pathways are induced by hypoxia in many cell types, such as human macrophage, monocytes, endothelial cells, and cancer cells (10, 16, 19, 22, 29). These results extend to Hi-DCs the characteristic response to hypoxia of defined pathways. Furthermore, we observed up-regulation of genes belonging to the antigen processing and presentation pathways, a typical function of DCs (Fig. 2).

HRE is a promoter element controlling hypoxia-induced gene transcription. We investigated whether there was a relationship between inducibility and presence of HRE in the promoter ($HRE^+$ genes). The number of genes positive for HRE in each pathway, the relative percentage, and the significance of the enrichment in $HRE^+$ genes are shown in Fig. 2. The frequency of $HRE^+$ genes spotted on the chip was ~60%, and it represented the background of HRE containing genes in our gene population. The overall frequency of $HRE^-$ genes among those up-regulated by hypoxia was 35%, showing that a substantial number of genes are induced in Hi-DCs independently of a direct activation by the HIF pathway. However, we found a statistically significant enrichment in $HRE^+$ genes in three pathways, apoptosis, glycolysis/gluconeogenesis, and cytokine-cytokine receptor interaction (Fig. 2), showing a positive selection of genes containing the HRE in the promoter in selected hypoxia-inducible pathways. The majority of the genes up-regulated in Hi-DCs fell into several pathways associated with cell movement/migration, including regulation of actin cytoskeleton, focal adhesion, adherens junction, and leukocyte transendothelial migration (Fig. 2), suggesting an improved motility of Hi-DCs over that of iDCs. The list of the 3-fold up-regulated genes belonging to the above categories is shown in Table 1. The cytokine-cytokine receptor interaction pathway had a very interesting pattern. First, it was the only one in which up-regulated and down-regulated genes were concomitantly represented (Fig. 2). Second, a close inspection of the individual genes present in these clusters (Table 2) revealed the existence of a clear dichotomy between chemokine and chemokine receptor mRNA expression. The chemokine receptor mRNAs *CCR3, CXCR4, CX3CR1,* and *CCR2* were all up-regulated. In contrast, the expression of the chemokine coding genes *CCL26, CCL24, CCL23, CCL18, CCL14*, and

*CCL13* was inhibited (Table 2). The opposite modulation of receptors and ligands was restricted to the chemokine gene family because hypoxia increased mRNAs coding for macrophage migration inhibitory factor, platelet-derived growth factor, and tumor necrosis factor (TNF) family ligands and down-regulated the expression of cytokine receptor mRNAs, such as *IL17RB, IL12RB1,* and *LIFR* (Table 2). These results show the existence of a characteristic pattern of Hi-DC gene expression that involves the chemokine/receptor cluster. Some of the genes listed in Table 2 are induced by hypoxia in different cell types, such as neuroblastoma cells, human macrophage, monocytes, cancer cells, and endothelial cells. The appropriate references are indicated. However, the majority of them are novel hypoxia-responsive genes not previously known to be induced by low oxygen tension in DCs.

### *The Chemokine/Receptor Pathway*

Migration is an essential component of the DC biology, and we studied in detail the chemokine/receptor system. We validated the modulation of chemokine/receptor genes by reverse transcription-PCR (RT-PCR; Fig. 3) and confirmed the dichotomy in mRNA modulation consisting in inhibition of cytokine mRNAs *CCL24, CCL23, CCL26, CCL18, CCL14,* and *CCL13* and up-regulation of cytokine receptor mRNAs *CXCR4, CX3CR1, CCR2,* and *CCR3* in Hi-DCs. The up-regulation of *macrophage migration inhibitory factor, TNFSF14,* and *vascular endothelial growth factor* (*VEGF*) was also validated (Fig. 3).

To determine the relationship between chemokine receptor mRNAs and protein expression, we studied the membrane expression by fluorescence-activated cell sorting analysis. iDCs had a low expression of surface CCR2, CCR3 (Fig. 4), and intermediate CCR5 (Fig. 1), whereas CXCR4 and CX3CR1 were absent. In contrast, Hi-DCs were characterized by high expression of CCR2, CXCR4 (Fig. 4), and CCR5 (Fig. 1) and a minimal expression of CX3CR1 and CCR3. We concluded that the up-regulation of chemokine receptor mRNAs in Hi-DCs was associated with parallel changes in receptor membrane expression only in the case of CCR2 and CXCR4, whereas the levels of membrane CX3CR1 and CCR3 were not changed by hypoxia.

Decrease of chemokine production that we observed in Hi-DCs implies their constitutive expression in iDCs. We studied chemokine production by ELISA (Fig. 5A). We found that iDCs produced very high levels of CCL18 in the nanogram range and significant amounts of CCL13 and CCL26 in the picogram range. Hypoxia inhibited completely the secretion of CCL26 and CCL13 and caused a major reduction (from 17,225 to 374 pg/mL) of CCL18 production. These results paralleled the changes in specific mRNAs and showed that hypoxia inhibited the production of CCL18, CCL13, and CCL26

**Table 1. Induced Hypoxia Genes Clustered in Several KEGG Pathways Involved in Cell Migration**

| Gene ID* | Gene Symbol † | Gene Description | Fold Change ‡ |
|---|---|---|---|
| Leukocyte transendothelial migration ($P < 3.39E-02$)§ | | | |
| NM_000584 | IL-8 (CXCL8) | IL-8 | 23.47 |
| NM_002872 | RAC2 | Ras-related c3 botulinum toxin substrate 2 | 14.63 |
| AA670344 | VIL2 | Villin 2 (ezrin) | 14.46 |
| AI826881 | CTNNA1 | Catenin (cadherin-associated protein), α1 | 6.50 |
| R64130 | PPBP | Pro-platelet basic protein (chemokine (c-x-c motif) ligand 7) | 6.15 |
| NM_000265 | NCF1 | Neutrophil cytosolic factor 1 | 5.58 |
| *L01639* | *CXCR4* | *Chemokine (c-x-c motif) receptor 4* | 5.43 |
| AF101051 | CLDN1 | Claudin 1 | 4.22 |
| Cell adhesion molecules ($P < 3.64E-02$)§ | | | |
| NM_002162 | ICAM3 | Intercellular adhesion molecule 3 | 9.28 |
| NM_000889 | ITGB7 | Integrin, β7 | 9.06 |
| AA700015 | CD58 | CD58 antigen | 4.54 |
| M90684 | HLA-G | HLA-G histocompatibility antigen, class I, G | 4.25 |
| AF101051 | CLDN1 | Claudin 1 | 4.22 |
| U62824 | HLA-C | MHC, class I, C | 3.80 |
| AI741056 | SELPLG | Selectin P ligand | 3.51 |
| NM_004385 | CSPG2 | Chondroitin sulfate proteoglycan 2 | 3.12 |
| Regulation of actin cytoskeleton ($P < 8.50E-10$)§ | | | |
| NM_002872 | RAC2 | Ras-related c3 botulinum toxin substrate 2 | 14.63 |
| NM_005022 | PFN1 | Profilin 1 | 12.21 |
| NM_000177 | GSN | Gelsolin | 3.69 |
| Adherens junction ($P < 2.83E-02$)§ | | | |
| NM_002872 | RAC2 | Ras-related c3 botulinum toxin substrate 2 | 14.63 |
| BG330076 | CTNNA1 | Catenin (cadherin-associated protein), α1, 102 kDa | 6.50 |
| BE872974 | ACP1 | Acid phosphatase 1, soluble | 3.27 |
| Focal adhesion ($P < 1.00E-09$)§ | | | |
| NM_002872 | RAC2 | Ras-related c3 botulinum toxin substrate 2 | 14.63 |
| NM_005163 | AKT1 | v-akt murine thymoma viral oncogene homologue 1 | 8.38 |

NOTE: Genes were ordered by fold change. RIKEN and EST are excluded. We indicated the genes validated by quantitative PCR in italic.
*Genbank accession number.
† Common gene symbol, a brief gene description, and the fold change value are specified for each gene.
‡ Fold change is calculated as a ratio of hypoxic/normoxic signals (average of expression level of three experiments). Genes modulated by >3-fold are shown.
§ $P$ value of each pathway is shown. $P$ value is a measure of the statistical significance of the overlap between the hypoxia modulated and total number of genes included in the given KEGG pathway. Significant level, $P < 0.05$.

**Table 2. Hypoxia-Modulated Genes Belonged to Cytokine-Cytokine Receptor Interaction KEGG Pathway**

| Gene ID* | Gene Symbol † | Gene Description | Fold Change ‡ | HRE Positions§ | References‖ | Cell Type |
|---|---|---|---|---|---|---|
| Up-regulated¶ ($P < 4.2E-14$)** | | | | | | |
| *NM_001837* | *CCR3* | *Chemokine (c-c motif) receptor 3* | *7.42* | — | | |
| *L01639* | *CXCR4* | *Chemokine (c-x-c motif) receptor 4* | *5.43* | *−51/−650/−1276/−1369/−1470* | (19, 22, 48) | *NBC, MDM, Mn, EC, TAM* |
| *U20350* | *CX3CR1* | *Chemokine (c-x3-c motif) receptor 1* | *4.38* | *−1803* | — | — |
| *NM_000648* | *CCR2* | *Chemokine (c-c motif) receptor 2* | *3.29* | *−1091* | — | — |
| NM_006140 | CSF2RA | Colony-stimulating factor 2 receptor, α | 3.12 | −742/−1772 | — | — |
| NM_005211 | CSF1R | Colony-stimulating factor 1 receptor | 3.01 | −75 | — | — |
| *NM_003807* | TNFSF14 | TNF (ligand) superfamily, member 14 | 47.55 | −127/−1251/−1787 | — | — |
| *NM_002415* | MIF | Macrophage migration inhibitory factor | 24.96 | −421 | (16, 29) | *CC, M* |
| AB033831 | PDGFC | Platelet-derived growth factor C | 11.40 | −354 | (30, 68) | *EC, HPASMC* |
| AW518486 | TNFSF8 | TNF (ligand) superfamily, member 8 | 5.07 | −642 | — | — |
| NM_016639 | TNFRSF12A | TNF receptor superfamily, member 12a | 3.69 | −219/−314/−431/−816/−1185 | — | — |
| Down-regulated † † ($P < 1.72E-03$)** | | | | | | |
| *U58913* | *CCL23* | *Chemokine (c-c motif) ligand 23* | −73.0 | −1374 | — | — |
| *AB000221* | *CCL18* | *Chemokine (c-c motif) ligand 18* | −48.6 | — | — | — |
| *AF096296* | *CCL26* | *Chemokine (c-c motif) ligand 26* | *−18.8* | −1571/−1855 | — | — |
| *NM_002991* | *CCL24* | *Chemokine (c-c motif) ligand 24* | *−8.8* | −709 | — | — |
| *NM_004166* | *CCL14* | *Chemokine (c-c motif) ligand 14* | −7.3 | — | — | — |
| *NM_005408* | *CCL13* | *Chemokine (c-c motif) ligand 13* | −8.9 | — | — | — |
| AF208111 | IL17RB | IL-17 receptor β | −4.5 | — | — | — |
| AI637915 | IL12RB1 | IL-12 receptor β1 | −3.9 | −600/−787/−912/−1240 | — | — |
| NM_002310 | LIFR | Leukemia inhibitory factor receptor | −3.4 | −619 | — | — |
| NM_003839 | TNFRSF11A | TNF receptor superfamily, member 11a | −3 | — | — | — |

NOTE: Cytokine-cytokine receptor interaction pathway is shown. We indicated the genes validated by quantitative PCR in italic. RIKEN and EST are excluded.
Abbreviations: CC, cancer cells; NBC, neuroblastoma cells, Mn, monocytes; TAM, tumor-associated macrophage; EC, endothelial cells; MDM, monocyte-derived macrophage; M, macrophage; HPASMC, human pulmonary artery smooth muscle cell.
*Genbank accession number.
† Common gene symbol, a brief gene description, and the fold change value are specified for each gene.
‡ Fold change is calculated as a ratio of hypoxic/normoxic signals (average of expression level of three experiments). Genes modulated by >3-fold are shown.
§ Position(s) of HRE sequences in the promoter.
‖ Representative references describing the induction of the correspondent gene by hypoxia.
¶ Statistically significant ($P < 0.01$, $\chi^2$).
** $P$ value of cytokine-cytokine receptor interaction pathway. $P$ value is a measure of the statistical significance of the overlap between the hypoxia modulated and total number of genes included in the given KEGG pathway. Significant level, $P < 0.05$.
† † Not significant ($P > 0.05$, $\chi^2$).

secretion. The evaluation of the *VEGF* mRNA and protein was included in each experiment to verify the correspondence between protein and mRNA. Expression of VEGF in Hi-DCs was about six times greater than that of iDCs (130 pg/mL) as shown by the representative experiment of Fig. 5B in agreement with the levels of *VEGF* mRNA (Fig. 3).

These results raised the question as to whether the chemokine receptors were functional, and we measured the ability of Hi-DCs and iDCs to migrate *in vitro* in response to specific ligands. The following chemokines were used: CXCL12 (agonist of CXCR4), CCL2 (agonist of CCR2), CCL24 (agonist of CCR3), and CX3CL1 (agonist of CX3CR1; Fig. 6). iDCs migrated in response to CCL2 and CCL24, as expected from the surface expression of the specific receptors. The high expression of CCR2 in Hi-DCs caused a major increase in the chemotactic response of the cells to CCL2. Moreover, Hi-DCs acquired the ability to migrate in response to the CXCR4 agonist CXCL12. Microarray results show that these two chemokines have a similar mRNA expression in iDCs and Hi-DCs (accession number GSE6863). The low CCR3 expression is sufficient to trigger a chemotactic response to CCL24 in iDCs but not in Hi-DCs. No migration in response to CX3CL1, the CX3CR1 agonist, was observed either in iDCs or in Hi-DCs. Antibodies to chemokine receptors inhibited the migration of iDCs and Hi-DCs, showing the specificity of the response (Fig. 6). These results show that Hi-DCs are more responsive to chemokines than iDCs, showing a functional characteristic of these cell populations.

## Discussion

We studied the effects of the hypoxic microenvironment on the differentiation of circulating monocytes into iDCs. We showed that Hi-DCs express a characteristic phenotype defined by induction of *CCR2, CCR3, CXCR4,* and *CX3CR1* mRNA; by inhibition of *CCL13, CCL14, CCL18, CCL23, CCL24,* and *CCL26* mRNA; and by up-regulation of genes belonging to the cell motility and migration classes. The high chemotactic response to CCL2 and CXCL12 is one of the functional properties that differentiates Hi-DCs from iDCs.

Despite the growing interest on the hypoxic microenvironment (12, 13, 16-18, 28), there is little information on the effects of hypoxia on the differentiation and maturation of DCs. This issue is important because DCs home and migrate through hypoxic areas represented by inflammatory, necrotic, poorly vascularized pathologic tissues before reaching their final destination in the lymph node. Adaptation of the cell to the hypoxic environment is associated with profound changes in gene expression (10, 30). Accordingly, we found that Hi-DCs differ from iDCs in the expression of ~2,000 genes, 78% of which were up-regulated and 22% were down-regulated. Changes in the Hi-DC transcriptome did not affect the characteristic iDC membrane marker expression represented by CD1a, HLA-II, CCR5, and CD80, although an increase of HLA-II, CCR5, and CD80 expression was consistently observed. Clustering the modulated genes according to the KEGG pathways was instrumental to reveal the characteristic features of Hi-DCs. This procedure identified the genes whose

expression was representative, in a statistically significant way, of the biological pathways defined by KEGG.[5]

Hypoxic cells share the need for alternative energy sources to compensate the inhibition of oxidative metabolism (10, 16), and we found that Hi-DCs had an increased expression of genes belonging to the glycolysis/gluconeogenesis and pentose phosphate pathways. Up-regulation of these genes is characteristic of the hypoxic status in various cell types, showing that Hi-DCs had the predicted response to the hypoxic environment and validating our results on the bases of the literature. These conclusions are supported by the observation that VEGF mRNA and protein, consistent markers of the hypoxic response, were up-regulated in Hi-DCs.

The response to hypoxia goes beyond the quest for energy and involves genes controlling cell behavior and function. We found that 37% of the metabolic pathways induced by hypoxia were related with cell motility, such as those controlling actin cytoskeleton, adherens junction, and focal adhesion, and included genes such as *RAC2*, which regulates cytoskeletal reorganization, and *ICAM3*, which regulates adhesion molecules (31, 32). This pattern suggested that Hi-DCs have the potential for being mobile cells ready for redistribution within the tissues or migration to the lymph nodes.

The cytokine-cytokine receptor KEGG category was uniquely changed in Hi-DCs. This pathway was characterized by up-regulated and down-regulated genes, whereas all the others comprised only up-regulated ones. There was a clear partition between chemokines and chemokine receptors, the former being down-regulated and the latter being up-regulated by hypoxia. *IL-8* (*CXCL8*), a multivalent cytokine (33) with chemokine activity, is not included in the chemokine/receptor category of the KEGG classification; it is inducible by hypoxia and by other stimuli, such as IFN-γ (34, 35), and *IL-8* mRNA is augmented in Hi-DCs (Table 1). The down-regulation of the chemokine coding genes is probably to be limited to those comprised in the KEGG category but it is still indicative of a biological trend. We confirmed the microarray results by RT-PCR, providing definitive evidence of inhibition of *CCL18, CCL23, CCL26, CCL24, CCL14,* and *CCL13* mRNA expression and increase in *CCR2, CCR3, CXCR4,* and *CX3CR1* mRNA expression in Hi-DCs. Collectively, these features represent valuable markers for Hi-DC definition.

We were intrigued by the unexpected observation of down-regulation of these cytokine mRNAs by hypoxia, and we studied the production of CCL13, CCL18, and CCL26 in iDCs and Hi-DCs. We confirmed the data of the literature showing that CCL18 is constitutively produced by iDCs at very high levels (36, 37). In contrast, Hi-DCs produced very little CCL18 as predicted by the mRNA expression. CCL18 down-regulation was previously shown in iDCs exposed to differentiating stimuli, including lipopolysaccharide, TNF-α, CD40L, or IFN-γ (37-39), and we extend this observation to hypoxia. CCL13 is a constitutive chemokine produced by iDCs that can be increased by proinflammatory stimuli (40-42). Our demonstration that hypoxia inhibits CCL13 release clearly differentiates the response to inflammatory and hypoxic microenvironment of iDCs. CCL26 belongs to the eotaxin group that recruits Th2 lymphocytes, basophiles, and eosinophils via CCR3 receptor. CCL26 is expressed by several tissue (43-45), and we show that iDCs are among them. Interestingly, this chemokine can antagonize the CCR2 receptor (38, 43) and its down-regulation by hypoxia could be required to allow the response of Hi-DCs to CCR2 ligands. In general, the inhibition of many endogenous chemokine production suggests that hypoxia targets the proinflammatory function of DCs, reducing their capacity to recruit other inflammatory leukocytes, and prevents loss of responsiveness by receptor down-regulation or desensitization dependent on autocrine chemokine production.

Up-regulation of chemokine receptor mRNAs is a functionally relevant modulation of gene expression that characterizes Hi-DCs. Up-regulation of *CXCR4* mRNA by hypoxia was previously reported in several cell types, including mononuclear phagocytes (17, 19, 22, 46-48), and we extend this observation to Hi-DCs. We now report that *CCR2, CCR3*, and *CX3CR1* are also hypoxia-inducible genes. The increase in *CCR2* and *CXCR4* mRNA was associated with a parallel change of membrane protein. Increase of membrane CCR5 levels in Hi-DCs above those of iDCs was also observed

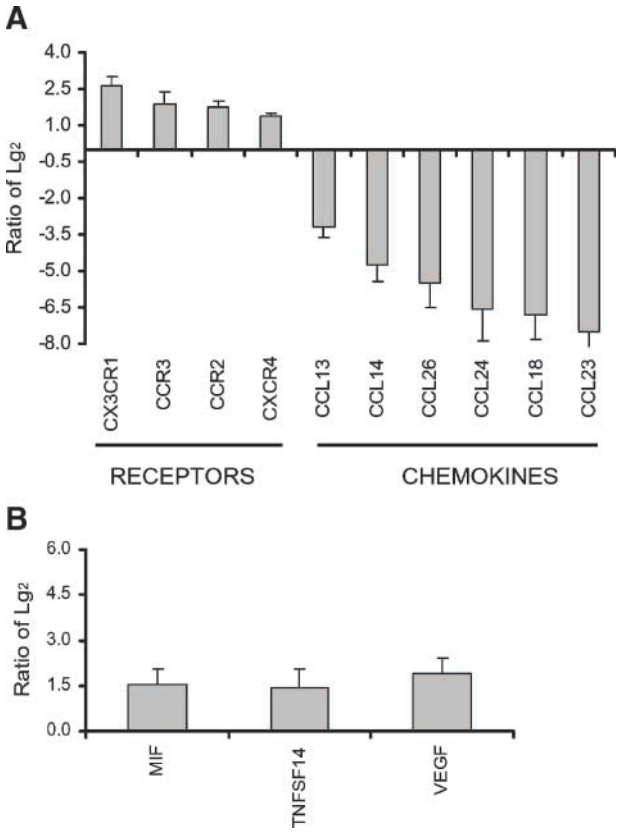


**FIGURE 3.** Validation of gene expression by RT-PCR. iDC mRNAs were tested by RT-PCR for the expression of the genes listed in the figure. **A.** Validation of the chemokine/receptor gene cluster modulated in Hi-DCs (Table 2). **B.** Validation of *macrophage migration inhibitory factor* (*MIF*), *TNFSF14,* and *VEGF* mRNAs. The results are expressed as log2 ratios of fold changes. Columns, mean of triplicate determinations with independent donors; bars, SE.

[5] http://www.genome.jp/kegg/pathway.html

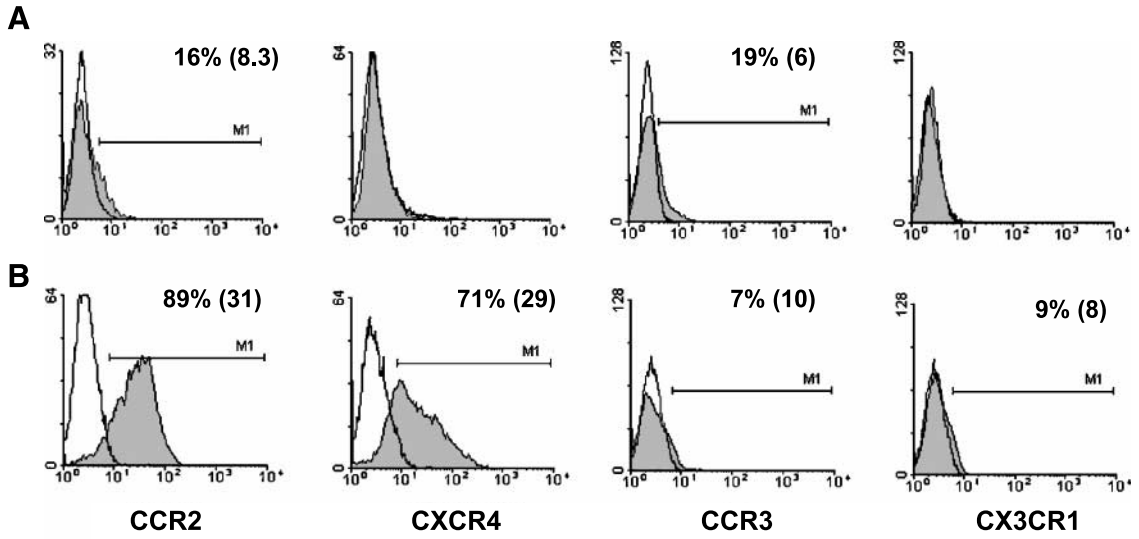


**FIGURE 4.** Chemokine receptor expression on Hi-DC surface. The membrane expression of CCR2, CXCR4, CCR3, and CX3CR1 was measured by fluorescence-activated cell sorting analysis in iDCs (**A**) or Hi-DCs (**B**). Open histograms, negative control; solid histograms, marker expression. In each histogram, we show the percentage of positive cells and, in parenthesis, the mean fluorescence intensity. The data are plotted as fluorescence intensity in a logarithmic scale on the abscissas versus the number of positive cells on the ordinates. One representative experiment out of three independent determinations done is shown.

(Fig. 1), supporting the concept of a general trend of up-regulation of chemokine receptors in Hi-DCs. In contrast, surface expression of CCR3 or CX3CR1 was similar in iDCs and Hi-DCs. Lack of correlation between mRNAs and protein expression was observed in cytokine-stimulated human airway epithelial cells, and the possibility of translational regulation of CCR3 by hypoxia was suggested (49). The increase in CCR2 surface expression was a novel finding and raised the question of the functional response of Hi-DCs to the chemokine receptor–specific ligands. The results showed that Hi-DCs were highly responsive to CXCL12, the ligand of CXCR4, and to CCL2, the ligand of CCR2, in accordance with the high membrane expression. The response to CCR2 and CXCR4 agonists is a functional discriminator of Hi-DCs because iDCs were insensitive to CXCL12 and much less responsive to CCL2. In conclusion, these results show that one of the functional characteristics of Hi-DCs is the preferential migratory response to CCR2 and CXCR4 agonists, which may take them into different compartments than the iDCs.

The transcriptional response to hypoxia involves several regulatory elements in the promoters of the inducible genes (22), and HRE family is one of the most prominent. HRE sequences are rather common and ~60% of the promoters contain at least one member of the family. Activation of HRE can be sufficient to drive gene transcription, as in the case of erythropoietin (22), or it may require the collaboration of other transcription factors, as in the case of inducible nitric oxide synthase (20, 21, 50). About 35% of the hypoxia-inducible genes in Hi-DCs lack a HRE sequence in the promoter, showing that a remarkable portion of the hypoxia transcriptome does not require a direct involvement of HIF binding. There was a statistically significant association between presence of HRE and induction by hypoxia of genes belonging to cytokine-cytokine receptor interaction, glycolysis/gluconeogenesis, and apoptosis pathways. These results depict an interesting situation in which a functional clustering based on responsiveness to hypoxia associates with the presence of HRE, supporting the idea that HRE is important for the response to hypoxia of selected pathways in Hi-DCs. One remarkable exception is represented by the lack of HRE in the promoter of *CCR3,* which is induced by hypoxia. There may be a functional HRE outside the operational promoter that we have defined or other transcription factors mediate the transcriptional response to hypoxia (30) as shown for the induction of IL-8 (34, 35). Alternatively, stabilization of the message or a secondary response to other hypoxia-inducible genes in an autocrine type of mechanism could explain these results.

It was reported that hypoxia inhibited the migratory capacity of DCs through extracellular matrix by reducing matrix metalloproteinase-9 expression and augmenting the protease inhibitor tissue inhibitor of metalloproteinase 1 (51, 52). We observed that there is no intrinsic defect in the migration of Hi-DCs, which respond more than iDCs to an appropriate stimulus in the absence of an extracellular matrix barrier as it occurs in the Transwell assay. Taken together, these results show that the Hi-DC motility is positively regulated by induction of CCR2 and CXCR4 but is counteracted by a decreased extracellular matrix destructive capacity.

## Conclusion

We show that the hypoxic microenvironment changes the differentiation program of monocytes into iDCs by modifying the gene expression profile and promoting the expression of motility-related genes. The resulting Hi-DCs differ from iDCs by increase of surface expression of CCR2 and CXCR4 and by decreased secretion of constitutively produced CCL18, CCL13, and CCL26 chemokines. Functionally, Hi-DCs acquire the ability of a strong migratory response to CCR2 or CXCR4 agonists. We conclude that Hi-DCs are a distinct group of DCs endowed with high sensitivity to chemoattractant, ready to leave

the hypoxic tissue and migrate toward a normoxic area and finally to the lymph nodes to orchestrate the immune response.

The hypoxic tumor microenvironment generates immunosuppressive circuits (53-55) and hypoxia-inducible factors, such as VEGF, contribute to the immunosuppression by inhibiting the generation of mature DCs and the development of an effective T-cell–mediated response (56-59). Therefore, Hi-DCs must leave the VEGF-rich hypoxic microenvironment to complete their maturation and fulfill the antigen-presenting functions. The migratory program may be an important way for Hi-DCs to settle in a differentiation-permissive environment and a gradient of CXCR4 and CCR2 ligands may be a way to drag the Hi-DCs out of the hypoxic areas. Inhibition of chemokine production may be needed to free the chemokine receptors for optimal performance in driving the cells to a normoxic area. These considerations suggest that strategies to generate chemokine gradients in the pathologic tissue could boost the immune response by mobilizing Hi-DCs and promoting the immunoreactivity.

## Materials and Methods

### *Generation and Culture of Monocyte-Derived iDCs*

Human peripheral blood mononuclear cells were isolated from venous blood of voluntary healthy donors by HistoPaque (Sigma) density gradient centrifugation. All cultures were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 50 μg/mL gentamicin (Schering-Plough). Monocytes were purified with a monocyte isolation kit II (Miltenyi Biotech) by negative selection. The resulting preparations were consistently >90% $CD14^+$ as determined by FACSCalibur (BD Biosciences).

To generate iDCs, the monocytes were incubated in six-well culture plates ($1.5 \times 10^6$ cells/mL) in complete medium supplemented with 100 ng/mL granulocyte macrophage colony-stimulating factor and 100 ng/mL IL-4 (PeproTech, Inc.) for 4 days under hypoxic and normoxic conditions (60, 61). Cells were maintained at 37°C in a humidified incubator containing 20% $O_2$, 5% $CO_2$, and 75% $N_2$ (normoxia) or under hypoxia using an anaerobic work station incubator (BUG BOX, Ruskinn) flushed for 20 min at a dynamic pressure of 35 p.s.i. and a flow rate of 25 L/min with a mixture of 1% $O_2$, 5% $CO_2$, and 94% $N_2$ and then sealed at a positive pressure to reduce atmospheric leaks.

### *Microarray Processing and Statistical Analysis*

Total RNA was independently isolated from DCs derived from the monocytes of three different donors using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The quality of RNA was checked using Agilent Bioanalyzer 2100 (Agilent Technologies), and the RNA was quantified by NanoDrop (NanoDrop Technologies). The RNA was reverse transcribed into cDNA and biotin labeled according to the Affymetrix instructions. Biotin-labeled cRNA was cleaned up with the Qiagen RNeasy Mini kit and ethanol precipitation, checked for quality with Agilent Bioanalyzer 2100, and fragmented by incubation at 94°C for 35 min in 40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L potassium acetate, and 30 mmol/L magnesium acetate. Fragmented cRNA was used 'for hybridization to Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix). GeneChips were scanned using an Affymetrix GeneChip Scanner 3000. All microarrays were examined for surface defects, grid placement, background intensity, housekeeping gene expression, and a 3′:5′ ratio of probe sets from genes of various lengths.

Expression values were quantified and array quality control was done with the statistical algorithms implemented in Affymetrix Microarray Suite 5.0. The resulting data were analyzed by GeneSpring Expression Analysis Software GX 7.3 (Silicon Genetics). The raw data were normalized as follows: (*a*) per microarray sample, by dividing the raw data by 50th percentile of all measurement, and (*b*) per gene, by dividing the normalized data by the median of the expression level for the gene in all samples. We analyzed the normalized data using Significance Analysis of Microarrays (Stanford University, Stanford, CA; ref. 62). Fold change was calculated as the ratio between the average expression level in the hypoxic and normoxic conditions. We selected a modulated gene list of 2-fold induction or inhibition with a false discovery rate of 0%. We calculated the inverse ratio for down-regulated genes represented as a fold change preceded by a minus sign. These list was analyzed and clustered in different KEGG pathways sorted by $P < 0.05$, according to their biological function using the Database for Annotation, Visualization and Integrated

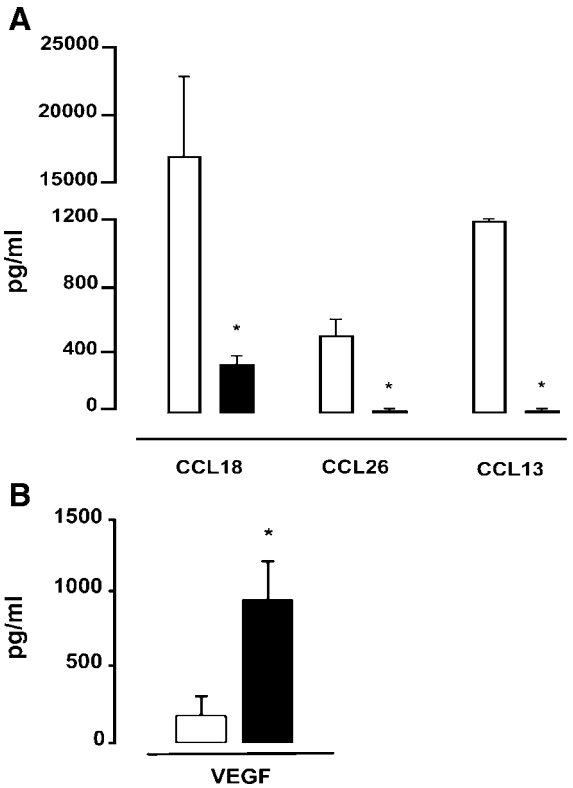


**FIGURE 5.** CCL13, CCL18, CCL26, and VEGF production by Hi-DCs. Cell-free supernatants collected from 4-d-old cultures of iDCs (*white columns*) or Hi-DCs (*black columns*) were assayed for CCL13, CCL18, and CCL26 content (**A**) and VEGF content (**B**) by ELISA. Cells from three different donors were used in independent experiments. Columns, mean of CCL13, CCL18, and CCL26 production (**A**) and VEGF production (**B**) measured as pg/mL; bars, SD. *, $P < 0.05$, significant difference in VEGF production in Hi-DCs relative to iDCs.

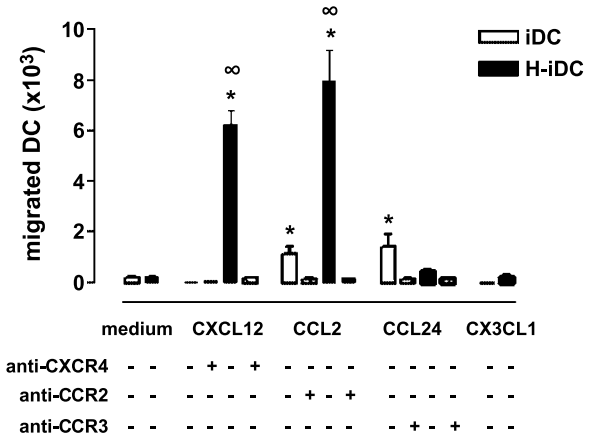


**FIGURE 6.** Chemotactic response of Hi-DC and iDC. iDC (*white columns*) and Hi-DC (*black columns*) chemotactic response was measured in triplicate using the Transwell system. Hi-DC generation and chemotaxis were done under hypoxic condition without any exposure to normoxia. The stimuli used were as follows: medium, CXCL12 (200 ng/mL), CCL2 (100 ng/mL), CCL24 (100 ng/mL), and CX3CL1 (500 ng/mL). Part of cells was pretreated with 10 μg/mL of anti-CXCR4, CCR2, or CCR3 neutralizing monoclonal antibody as indicated by the + sign in the figure. Columns, average number of cells $\times 10^{-3}$ that migrated in the lower chamber of the Transwell; bars, SD. The results of one representative experiment out of three done are shown. *, $P < 0.05$, chemotactic response significantly different from the medium control; ∞, $P < 0.05$, chemotactic response of Hi-DCs significantly different from iDCs.

Discovery 2.0 program (63). The complete data set for each microarray experiments (accession number GSE6863) was lodged in the Gene Expression Omnibus public repository at National Center for Biotechnology Information.[6] We mapped the HRE elements in the promoter regions of the genes represented in the Affymetrix HG-U133_Plus_2 chip genes of the Affymetrix probe sets. We downloaded the annotation file for the HG-U133_Plus_2 from NetAffx Analysis Center,[7] and the data set was restricted to the known mRNA sequences listed in the RefSeq database. The mapping information of the RefSeq genes in the human genome hg18, National Center for Biotechnology Information build 36.1, was obtained through the University of California at Santa Cruz genome Web site.[8] A total of 24,054 RefSeq genes were mapped to 42,120 Affymetrix probe sets. We operationally defined as "promoter" the first 2,000 bases upstream the transcription initiation site and generated a data set containing the promoters of the genes coding for the mRNAs spotted on the chip. All promoter sequences were extracted from the human genome. The HRE consists of a four-nucleotide core (*CGTG*) flanked by degenerated sequences. The HRE sequences were obtained from the Transfac eukaryotic transcription factor database.[9] HRE consensus elements [also referred to as "HRE family"; (T|G|C)(A|G)(*CGTG*)(C|G|A)(G|C|T)(G|T|C)(C|T|G)] were searched in the promoter sequences with a customized PERL script. About 60% of the promoters contain at least one HRE consensus element. $\chi^2$ was used to evaluate the significance of the HRE frequency in the promoter regions of genes belonging to the different pathways. $P < 0.05$ was considered significant.

### *Real-time Quantitative RT-PCR*

Real-time quantitative PCR was done as detailed previously (17) starting from 2 μg of total purified RNA. Quantitative RT-PCR was done in triplicate for each target gene. Expression data were normalized using the BestKeeper software (64) to the values obtained in parallel for *ribosomal protein S18, actin-related protein 2/3 complex, subunit 1B*, and *thrombospondin 1*. These genes were taken as reference because they were not modulated under hypoxic exposure. Relative expression values were calculated using QGene software (65).

### *Flow Cytometry*

Flow cytometry was done as described previously (66). The following monoclonal antibodies were used: anti-CD1a, anti-CD80, anti-HLA-DP, anti-HLA-DQ, and anti-HLA-DR (Vinci-Biochem); anti-CCR5, anti-CXCR4, and anti-CCR3 (R&D Systems); and anti-CX3CR1 (Eppendorf). Samples were analyzed using a FACSCalibur CellQuest (BD Biosciences). Cells were electronically gated according to light scatter properties to exclude cell debris and contaminating lymphocytes.

### *Determination of Cytokine and Chemotaxis*

Cell-free supernatants were collected and frozen until use. CCL18, CCL13, CCL26, and VEGF cytokines were quantified by ELISA (R&D Systems) in accordance with the manufacturer's instructions. Migration was measured under normoxic or hypoxic conditions in duplicate using a Transwell system (24-well plates; 8.0-μm pore size; Costar). A total of 600 μL of RPMI 1640 with or without 200 ng/mL of recombinant human CXCL12, 100 ng/mL of recombinant human CCL2, 100 ng/mL of recombinant human CCL24, or 500 ng/mL of recombinant human CX3CL1 (all from PeproTech) were added to the lower chamber. Wells with medium only were used as a control. A total of $2.5 \times 10^5$ cells in 100 μL were added to the upper chamber and incubated at 37°C for 2 h in normoxic or hypoxic incubator. Cells that migrated into the lower chamber were harvested and counted by flow cytometry acquiring events for the fixed time of 60 s. Part of cells was pretreated with anti-CXCR4, anti-CCR2, or anti-CCR3 neutralizing monoclonal antibody (10 μg/mL; all from R&D Systems) for 30 min at 4°C. The cells were tested for migration as described before without washing step. The counts fell within a linear range of the control titration curves obtained by testing increasing concentrations of cells. The results are expressed as mean number of migrated cells ± SE (67). Unpaired Student's *t* test was used to determine the differences in migration.

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# Transcriptome of Hypoxic Immature Dendritic Cells: Modulation of Chemokine/Receptor Expression

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