Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 443–448
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Gene expression profiles in dendritic cells conditioned by 1α,25-dihydroxyvitamin D_3 analog☆

Matthew D. Griffin[a], Nianzeng Xing[a,1], Rajiv Kumar[a,b,*]

[a] *Department of Internal Medicine, Division of Nephrology, Mayo Clinic and Foundation, 200 First Street SW, Medical Sciences 1–120, Rochester, MN 55905, USA*

[b] *Departments of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, 200 First Street SW, Medical Sciences 1–120, Rochester, MN 55905, USA*

Abstract

Inhibition of dendritic cell (DC) maturity is an important immunomodulatory effect of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and related analogs (D_3 analogs). The mechanisms underlying 1α,25$(OH)_2D_3$-mediated DC modulation are Vitamin D receptor (VDR)-dependent and likely involve direct or indirect regulation of multiple genes. Gene expression profiles of bone marrow-derived DCs (BMDCs) generated in the absence or presence of a potent D_3 analog were analyzed using microarray technology. Results for D_3 analog-conditioned DCs were also compared with glucocorticoid-conditioned BMDCs and with BMDCs conditioned with D_3 analog and glucocorticoid combined. Of ∼12,000 gene products assayed, 52% were considered to have detectable expression in unconditioned BMDCs. Based on relative expression levels, 5.3% of these expressed genes were "silenced" or "suppressed" in D_3 analog-conditioned BMDCs and 2.1% were "augmented". In addition, 1.7% of gene products undetectable in control BMDCs were "induced" by D_3 analog. Functional grouping of modulated genes demonstrated important effects of D_3 analog on immunoreceptors, on chemokines and chemokine receptors, on growth factors/cytokines and related receptors, and on neuroendocrine hormones and related receptors. Many of these gene products were unaffected or differently regulated by glucocorticoid suggesting specific VDR-mediated regulatory effects. Confirmation of microarray analysis results for two differentially regulated chemokines (MIP-1α and RANTES) was obtained by RT-PCR and ELISA. The methodology provides novel insights into DC gene regulation by 1α,25$(OH)_2D_3$ agonists.

Keywords: Dendritic cells; Vitamin D; Gene chip array; Glucocorticoids; Chemokines; Antigen presentation

1. Introduction

The immunomodulatory effects of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) are well recognized and data from multiple animal models of immune-mediated disease support the therapeutic potential of "non-calcemic" 1α,25$(OH)_2D_3$ analogs (D_3 analogs) in autoimmunity and transplantation [1]. A key target for 1α,25$(OH)_2D_3$-mediated immunosuppression is the dendritic cell (DC)—a subtype of antigen presenting cell (APC) with unique functional capabilities in initiating cellular immune responses in vivo as well as in maintaining immune tolerance to self [2,3]. Along with a number of other investigators, we have reported that DCs, generated in vitro from bone marrow precursors, are inhibited in their ability to stimulate T-cell activation and proliferation following exposure to relatively low concentrations 1α,25$(OH)_2D_3$ and D_3 analogs [4–7]. "D_3-analog-conditioned" bone marrow-derived DCs (D_3-BMDCs) exhibit phenotypic similarities to immature DCs which have been reported to induce antigen-specific immune tolerance in vivo [2,3]. In keeping with this similarity, inoculation of female mice with male D_3-BMDCs was associated with subsequent prolonged survival of male skin grafts [5].

Immature and mature DCs may be distinguished on the basis of their morphology, of their expression levels of immunostimulatory surface proteins and secreted factors (MHC proteins, co-stimulatory receptors, cytokines), or of their functional potency (ability to stimulate resting T-cell). Nonetheless, the mechanisms underlying DC modulation by various physiologic or pharmacologic interventions are generally not well characterized and it is likely that important regulators of DC differentiation and maturation have

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

* Corresponding author. Tel.: +1-507-284-0020; fax: +1-507-266-4710.
E-mail address: rkumar@mayo.edu (R. Kumar).
[1] Present address: Department of Urology, Beijing Chaoyang Hospital, Capital University of Medical Science, Beijing, PR China.

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.039

yet to be identified. Furthermore, the mechanisms by which immature DCs promote regulatory T-cell activity remain poorly understood. In this study we describe results of gene chip microarray analysis carried out in order to obtain a broad profile of the modulatory effects of a D_3 analog on BMDCs. Gene expression profiles from BMDCs generated in the absence or presence of an optimized concentration of D_3 analog were compared. Because we had previously observed distinct inhibitory effects of glucocorticoid and $1\alpha,25(OH)_2D_3$ agonists on specific DC proteins [8], the analysis also included populations of BMDCs generated in the presence of dexamethasone or combined D_3 analog and dexamethasone. The results identify categories of gene product that are selectively modulated in DCs by the Vitamin D system and may play important functional roles in mediating immunomodulatory effects of $1\alpha,25(OH)_2D_3$ and D_3 analogs in vivo.

2. Materials and methods

2.1. Generation of bone marrow-derived dendritic cells

Female C57BL/6 mice, aged 8–12 weeks were purchased from Jackson Laboratories, Bar Harbor, ME, USA and maintained in a specific pathogen-free housing facility. Murine bone marrow cultures were initiated as previously described [4] using recombinant murine GM-CSF and IL-4 (Peprotech Inc., Rocky Hill, NJ, USA) at 10 ng/ml each. Cultures were carried out for 7 days with cytokines replenished on days 2, 4, and 6. For generation of conditioned BMDCs, the D_3 analog $1\alpha,25(OH)_2$ 19-ene, 23-yne, 26,27-hexafluoro, 19-nor-Vitamin D_3 (kindly provided by Dr. Milan Uskokovic, Hoffman La Roche, Nutley, NJ, USA) and/or dexamethasone (Sigma–Aldrich Chemicals, St. Louis, MO, USA) were added at final concentrations of 10^{-10} and 10^{-8} M, respectively, on day 2 of culture and were replenished on days 4 and 6.

2.2. RNA preparation and gene chip array

BMDC populations were lifted for RNA preparation on day 7 of culture. Total RNA was prepared using the RNeasy mini kit® (Qiagen Inc., Valencia, CA, USA). Sample quality was assessed with an Aligent 2100 Bioanalyzer® (Aligent Technologies, Palo Alto, CA, USA). All samples contained 18S and 28S rRNA peaks with no degradation. A minimum of 8 μg of RNA from each sample was subsequently processed. Synthesis and cleaning of double-stranded cDNA followed by synthesis of biotin-labeled cRNA and fragmentation of biotinylated cRNA into 100–150 nt fragments were all carried in the Mayo Cancer Center microarray core facility according to protocols described in the Affymetrix GeneChip® Expression Analysis manual (Affymetrix Inc., Santa Clara, CA, USA). For each sample, 15 μg of fragmented, biotinylated cRNA was hybridized to a U74A microarray (Affymetrix Inc.) then washed with buffers of varying stringencies, stained with streptavidin-PE and scanned with a Hewlett-Packard GeneArray® Scanner (Hewlett-Packard, Santa Clara, CA, USA). The GeneChip® Microarray Suite v4.01 software program (Affymetrix Inc.) was then used to generate data for subsequent statistical analysis. The "average difference" in fluorescence between positive match and mismatch (negative control) probes for each gene product on the array was used as the primary index of gene expression and relative expression level. An average difference >500 was chosen as indicating detectable expression of a given gene product. RT-PCR and ELISA for RANTES and MIP1α were carried out as previously described.

3. Results

3.1. Basic profile of modulated gene expression in D_3 analog-conditioned BMDCs

Gene microarray results for unconditioned (control) and D_3 analog-conditioned day 7 murine BMDCs were compared as shown in Fig. 1. Using an average difference of >500 as cutoff for detectable expression, 52% of gene products tested were designated as being expressed by control BMDCs. Using two-fold change in average difference as cutoff for significantly modulated expression, the effect of D_3 analog-conditioning on specific genes was grouped into four categories: (a) "silenced" (detectable expression in control BMDCs, undetectable expression in D_3 analog BMDCs—1.2% of expressed genes); (b) "suppressed" (detectable expression in control BMDCs, two-fold lower expression in D_3 analog BMDCs—4.1% of expressed genes); (c) "induced" (undetectable expression in control BMDCs, detectable expression in D_3 analog BMDCs—1.7% of non-expressed genes); (d) "augmented" (detectable expression in control BMDCs, two-fold higher expression in D_3 analog BMDCs—2.1% of expressed genes).

3.2. Functional clustering of D_3 analog-modulated genes

Examination of the individual genes that were significantly modulated in D_3 analog BMDCs revealed a number of functionally-related clusters that are summarized in Table 1(A) and (B). A cluster of immunoreceptors (surface receptors with recognized functional roles in DC antigen acquisition and presentation) was predominantly inhibited (Table 1(A)), in keeping with the reduced capacity of D_3 analog BMDCs to stimulate antigen-specific T-cells [4–8]. Potent modulation of multiple chemokines and chemokine receptors was also evident (Table 1(A)), suggesting an important ability of $1\alpha,25(OH)_2D_3$ agonists to modify DC migration and capacity to recruit additional cell populations. Interestingly, while chemokine receptors

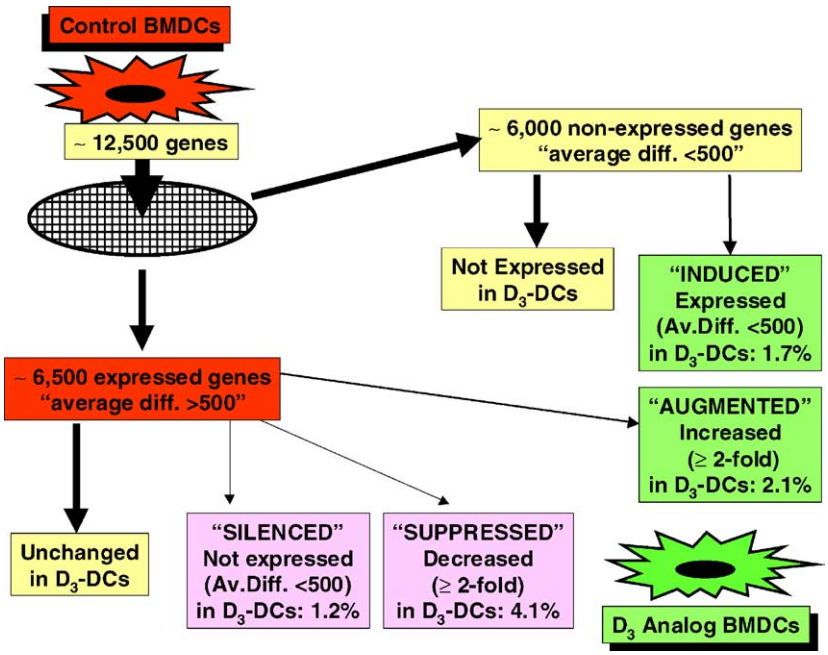

Fig. 1. The comparative analysis of gene expression profiles in unconditioned BMDCs and D_3 analog-conditioned BMDCs is illustrated schematically. For unconditioned BMDCs, approximately 6500 of 12,500 tested probes sets were designated as expressed based on average difference >500 between positive match and mismatch probes for each gene product. Based on the corresponding average difference values for D_3 analog-conditioned BMDCs, genes with significantly altered expression were categorized as "silenced" (1.2% of expressed genes), "suppressed" (4.1% of expressed genes), "augmented" (2.1% of expressed genes), and "induced" (1.7% of non-expressed genes).

were predominantly inhibited, expression of a number of pro-inflammatory chemokines was augmented in D_3 analog BMDCs. A number of growth factors and cytokines as well as related receptors demonstrated significant modulation under the influence of D_3 analog (Table 1(B)). The majority of these gene products, including PDGF, TGFβ3 and FGFR I were strikingly up-regulated although their role in regulating DC immune functions has not been characterized to date. Finally, a number of neuroendocrine hormones and related receptors (Table 1(B)) were shown to be abundantly expressed by BMDCs and to be regulated by D_3 analog. It is noteworthy that inhibition of DC immunostimulatory function has been reported for such hormones and may be an important tissue-specific paracrine or autocrine regulatory mechanism [9].

3.3. Glucocorticoid effects on D_3 analog-modulated genes

The influence of glucocorticoid (dexamethasone) conditioning alone or in combination with D_3 analog is also summarized in Table 1(A) and (B) for individual genes. As shown, many of the genes that were modulated by D_3 analog were unaffected by dexamethasone or were regulated in an opposing fashion. Furthermore, combined D_3 analog and dexamethasome conditioning resulted in a variety of profiles including additive effects, counteractive effects or a dominant effect of one of the two hormone pathways. Strikingly, a number of chemokine receptors underwent potent additive inhibition under the influence of combined conditioning. Although complex, the comparative analysis confirmed that the $1\alpha,25(OH)_2D_3$/VDR pathway imparts discreet modulatory effects on multiple genes in DCs.

3.4. Confirmation of gene microarray results for individual gene products

The results of microarray analysis suggested differential regulation of two pro-inflammatory chemokines—RANTES and MIP-1α—by D_3 analog and dexamethasone. As shown in Fig. 2, these expression profiles could be confirmed at the mRNA level (by RT-PCR) and protein level (by ELISA of culture supernatants). RANTES expression is shown to be inhibited by both D_3 analog and glucocorticoid alone with additive inhibition when the two steroid agents are combined. In contrast, MIP-1α expression is inhibited by glucocorticoid but augmented by D_3 analog and an intermediate effect is observed with combined conditioning. Additional confirmatory RT-PCR data for multiple chemokine receptors has been obtained [8].

4. Discussion

Microarray experiments provide a wealth of gene expression data and are particularly suited to the comparison of cellular samples subjected to specific physiologic or

Table 1
Gene products with significantly altered RNA expression levels in D_3 analog-conditioned compared to control BMDCs

Functional group	Gene product	GenBank accession	Silenced	Suppressed	Fold decrease	Induced	Augmented	Fold increase	Glucocorticoid effect	Combined glucocorticoid + D_3 analog effect
(A)										
Immuno-receptors	CD86	L25606	X						Similar	Same as D_3 analog
	CD83	AF001036	X						Similar	Same as D_3 analog
	DEC205	U19271		X	−2.2				Unaffected	Additive suppression
	OX40L	U12763		X	−3.5				Similar	Same as D_3 analog
	CD1d	M63695		X	−2.5				Similar	Same as D_3 analog
	FcR I (high affinity)	X70980	X						Similar	Same as D_3 analog
	Mannose receptor C	Z11974		X	−3.1				Opposing	D_3 analog and GC effects neutralized
	CD84	D13695		X	−2.3				Unaffected	Same as D_3 analog
	Ly6	X04653					X	5.8	Opposing	GC effect dominant
Chemokines and chemokine receptors	CCR1	U29678		X	−2.1				Unaffected	Additive suppression
	CCR2	U56819		X	−18.0				Similar	Additive suppression
	CCR5	AF022990		X	−5.2				Unaffected	Additive suppression
	CCR7	L31580		X	−4.3				Unaffected	Additive suppression
	CX3CR1	AF074912					X	2.2	Unaffected	Same as D_3 analog
	RANTES	AF065947		X	−5.1				Similar	Additive suppression
	MIP-2	X53798		X	−2.1				Opposing	D_3 analog effect dominant
	MIP-1α	J04491					X	2.0	Opposing	D_3 analog and GC effects neutralized
	MIP-1β	X62502					X	2.1	Opposing	D_3 analog and GC effects neutralized
	MIP-1γ	U49513					X	2.3	Unaffected	Same as D_3 analog
(B)										
Growth factors + cytokines + related receptors	PDGFα	M29464					X	10.1	Unaffected	Same as D_3 analog
	TGFβ3	M32745					X	8.2	Unaffected	Same as D_3 analog
	IL-6	X54542		X	−2.2				Similar	Same as D_3 analog
	Heparin-binding EGF-like GF	L07264					X	4.7	Opposing	D_3 analog effect dominant
	IFNα2	K01238				X			Unaffected	Same as D_3 analog
	Lymphotoxin β	U16985				X			Unaffected	GC effect dominant
	IL-4R (secreted)	M27960					X	2.1	Unaffected	GC effect dominant
	FGFR I	U22324					X	2.4	Similar	Additive increase
	IL-1R I	M20658		X	−2.0				Opposing	D_3 analog and GC effects neutralized
	IL-1R II	X59769					X	2.6	Similar	Same as D_3 analog
	IL-11R	U69491		X	−2.3				Unaffected	GC effect dominant
Neuro-endocrine hormones and related receptors	Adrenomedullin	U77630				X			Similar	Same as D_3 analog
	Cysteine-rich intestinal peptide	M13018					X	2.6	Unaffected	Same as D_3 analog
	Preproencephalin 2	M55181		X	−9.5				Similar	Additive decrease
	Natriuretic peptide precursor C	D28873				X			Unaffected	Same as D_3 analog
	Pancreatic polypeptide receptor	U40189	X						Similar	Same as D_3 analog
	RAMP 1	AJ250489					X	5.8	Similar	Additive increase
	RAMP 3	AJ250491	X						Unaffected	Same as D_3 analog

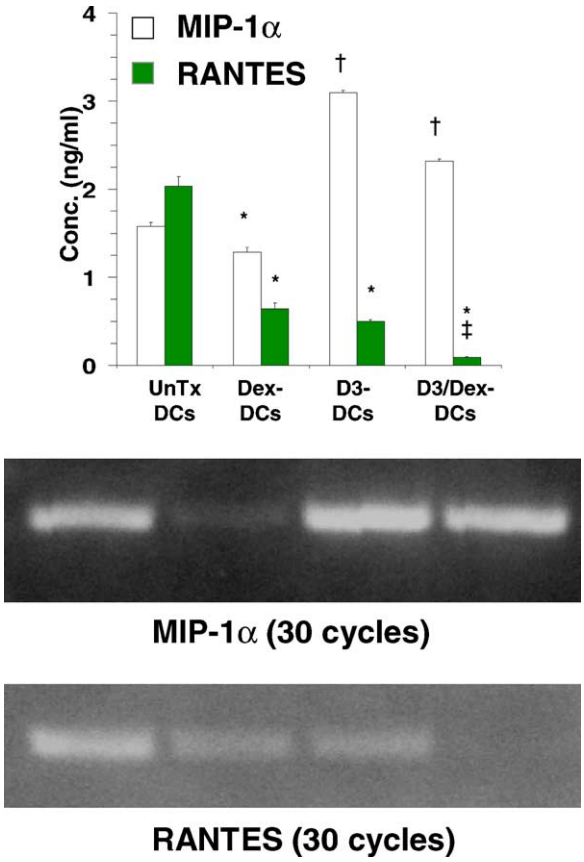

Fig. 2. Results of RT-PCR (lower panels) and ELISA of culture supernatant (upper panel) are shown for chemokines RANTES and MIP-1α in four populations of BMDCs: unconditioned (UnTx DCs), glucocorticoid-conditioned (Dex-DCs), D_3 analog conditioned (D_3-DCs), and D_3 analog + glucocorticoid-conditioned (D_3/Dex-DCs). RANTES expression was inhibited by both D_3 analog and glucocorticoid alone with additive inhibition when the two steroid agents are combined. In contrast, MIP-1α expression was inhibited by glucocorticoid but augmented by D_3 analog and an intermediate effect was observed with combined conditioning. For both chemokines, the results were consistent with those of microarray analysis. $^{*}P < 0.05$ for results lower than that for UnTx DCs, $^{\dagger}P < 0.05$ for results higher than that for UnTx DCs, $^{\ddagger}P < 0.05$ for results lower than those for Dex-DCs and D_3-DCs.

pharmacologic modifications [10]. In contrast to expression studies involving single or small numbers of gene products, the use of microarray may reveal regulation of individual genes that might not be otherwise appreciated. In addition, profiling of gene expression patterns may be used to distinguish different functional states among apparently similar cell populations [11] or to identify clusters of genes undergoing concurrent regulation [12]. In the current study, we identify four such clusters in DCs generated from bone marrow in the presence of an analog of $1\alpha,25(OH)_2D_3$ that we have previously found to potently retard DC immunostimulatory capacity [4]. In addition, we show distinct variations between the effects of the Vitamin D pathway and those of the glucocorticoid pathway which is also well recognized as an inhibitor of DC maturation [13]. The most immediately striking of these D_3 analog-regulated groups of gene products is the chemokine/chemokine receptor group which is increasingly recognized as a key element of DC immune function and as a bona fide target for immunomodulatory therapy [14]. In contrast to the broad inhibitory effect of glucocorticoid on chemokine gene expression (possibly mediated through inhibition of the NF-κB signaling pathway [15]), we find that individual chemokines may be up-regulated in the presence of D_3 analog. A number of other unexpected observations from the current survey, such as potent induction of a number of growth factors and cytokines, and modulated expression of neuroendocrine hormonal systems may provide opportunities for investigating alternative mechanisms involved in the functional properties of immature DCs.

The limitations of gene array data also merit brief consideration [10]. Absolute determinations of the presence or absence of a given transcript as well as of altered expression among different samples must involve arbitrary choices of cutoff points. For genes with low expression level or minor alterations in expression, the analysis strategy employed may substantially skew the final interpretation. For the current study we have used relatively conservative criteria for data analysis. The second major limitation relates to the lack of information concerning the effect of posttranscriptional and posttranslational events on the actual expression and function of the final protein products. For this reason, the confirmation of altered RNA levels by specific assays such as RT-PCR or Northern blotting and correlation with protein expression assays such as Western blotting or immunoassay must be considered essential steps in pursuing the physiologic importance of microarray-derived data. Despite such limitations, the results presented here provide a number of novel insights into the gene regulatory profile underlying DC-specific effects of $1\alpha,25(OH)_2D_3$ and related analogs. We anticipate that further investigation of the gene groups identified by this analysis will contribute to a broader understanding of the mechanisms underlying immunomodulatory properties of steroid hormones.

Acknowledgements

Supported by NIH grant DK59505 and by a Mayo Foundation CR75 award. The authors wish to acknowledge the technical contributions of Lori Bachman and of the staff of the Mayo Cancer Center Microarray Core Facility.

References

[1] M.D. Griffin, N. Xing, R. Kumar, Vitamin D and its analogs as regulators of immune activation and antigen presentation, Ann. Rev. Nutr. 23 (2003) 117–145.
[2] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, K. Palucka, Immunobiology of dendritic cells, Ann. Rev. Immunol. 18 (2000) 767–811.
[3] R.M. Steinman, D. Hawiger, K. Liu, L. Bonifaz, D. Bonnyay, K. Mahnke, T. Iyoda, J. Ravetch, M. Dhodapkar, K. Inaba, M.

Nussenzweig, Dendritic cell function in vivo during the steady state: a role in peripheral tolerance, Ann. N. Y. Acad. Sci. 987 (2003) 15–25.

[4] M.D. Griffin, W.H. Lutz, V.A. Phan, L.A. Bachman, D.J. McKean, R. Kumar, Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs, Biochem. Biophys. Res. Comm. 270 (3) (2000) 701–708.

[5] M.D. Griffin, W.H. Lutz, V.A. Phan, L.A. Bachman, D.J. McKean, R. Kumar, Dendritic cell modulation by 1α,25-dihydroxyvitamin D_3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo, Proc. Natl. Acad. Sci. U.S.A. 98 (12) (2001) 6800–6805.

[6] G. Penna, L. Adorini, 1α,25-dihydroxyvitamin D_3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T-cell activation, J. Immunol. 164 (5) (2000) 2405–2411.

[7] L. Piemonti, P. Monti, M. Sironi, P. Fraticelli, B.E. Leone, E. Dal Cin, P. Allavena, V. Di Carlo, Vitamin D_3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells, J. Immunol. 164 (9) (2000) 4443–44451.

[8] N.L. Xing, M.L. Maldonado, L.A. Bachman, D.J. McKean, R. Kumar, M.D. Griffin, Distinctive dendritic cell modulation by vitamin D_3 and glucocorticoid pathways, Biochem. Biophys. Res. Commun. 297 (3) (2002) 645–652.

[9] S. Dunzendorfer, C.J. Wiedermann, Neuropeptides and the immune system: focus on dendritic cells, Crit. Rev. Immunol. 21 (6) (2001) 523–557.

[10] R.A. Young, Biomedical discovery with DNA arrays, Cell 102 (1) (2000) 9–15.

[11] T.R. Golub, D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H. Coller, M.L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, E.S. Lander, Molecular classification of cancer: class discovery and class prediction by gene expression monitoring, Science 286 (5439) (1999) 531–537.

[12] Q. Huang, Q.D. Liu, P. Majewski, L.C. Schulte, J.M. Korn, R.A. Young, E.S. Lander, N. Hacohen, The plasticity of dendritic cell responses to pathogens and their components, Science 294 (5543) (2001) 870–875.

[13] M. Moser, T. De Smedt, T. Sornasse, F. Tielemans, A.A. Chentoufi, E. Muraille, M. Van Mechelen, J. Urbain, O. Leo, Glucocorticoids down-regulate dendritic cell function in vitro and in vivo, Eur. J. Immunol. 25 (10) (1995) 2818–2824.

[14] S. Sozzani, P. Allavena, A. Vecchi, A. Mantovani, Chemokines and dendritic cell traffic, J. Clin. Immunol. 20 (3) (2000) 151–160.

[15] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 270 (5234) (1995) 286–290.