

# Transcriptional reprogramming of dendritic cells by differentiation stimuli

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Immature and mature dendritic cells (DC) have been well characterized functionally and phenotypically. Microorganisms or bacterial products such as lipopolysaccharide (LPS) and inflammatory molecules, including tumor necrosis factor (TNF- $\alpha$ ), are both believed to activate the DC maturation program which allows DC to initiate and amplify innate and adaptive immune responses. However, there is increasing evidence that the functional state of DC, induced by different stimuli, may be relevant for the immune response outcome. Thus, we compared the transcriptional program of mature, transitional and immature DC, after either LPS or TNF- $\alpha$  stimulation. GeneChip<sup>®</sup> oligonucleotide microarrays, representing approximately 6,500 murine genes and ESTs, were used for this analysis. A very diverse modulation of gene expression was observed with the two stimuli. Only LPS-treated cells showed a pattern of expression of genes compatible with a definitive growth arrest and with a suitable activation and control of the immune response.

**Key words:** Dendritic cell / Microarray / Transcription analysis / Inflammatory stimulus

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## 1 Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC) able to initiate the innate and adaptive immune responses [1, 2]. Immature DC are strategically located in tissues that represent pathogen entry routes (the skin and mucosal surfaces), where they continuously monitor the environment through the uptake of particulate and soluble products. DC maturation is associated with reduced endocytic and phagocytic capacities, enhanced production of inflammatory cytokines and chemokines, and acquisition of migratory functions allowing antigen-loaded DC to move from non-lymphoid to lymphoid tissues or, within lymphoid tissues, to migrate from the marginal zones to the T cell areas. Migrated and mature DC have acquired high cell surface major histocompatibility complex (MHC) and costimulatory protein expression, have the ability to activate CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses [3, 4], and are programmed for apoptotic death [5]. However, beside their physiological role, there are evidences that DC can also participate in uncontrolled immune responses that have been associated with diseases such as the development of chronic inflammation [6]. It has been proposed that the functional

state of DC, induced by different stimuli, may be relevant for the immune response outcome [7].

The transcription profile, transcriptome, is a major determinant of cellular phenotype and function [8]. Differences in gene expression are indicative of morphological, phenotypical and functional changes induced in a cell by environmental factors and perturbations. Microarrays have been successfully applied to identify genes that discriminate between Th1 and Th2 functions in humans [9] and anergic and activated B cells in mice [10]. Microarray technology is, thus, a valid approach to investigate possible differences induced in particular cell types by diverse external factors. Concerning DC, a transcriptional profile of immature and LPS-matured human monocyte-derived DC has been carried out revealing 225 differentially expressed genes in the two situations out of a total of 10,962 genes screened [11]. These genes mainly consisted of chemokines (RANTES, ELC, PARK, MDC and TARC) and chemokine receptors (CCR7), enzymes (such as germinal center kinase-related protein kinase), IFN-inducible proteins, lipase A, CD52, CD11b, CD23, and glucose 6 phosphatase. Nevertheless a comparison between different stimuli in their efficiency in inducing DC maturation has never been performed. The most common stimuli used to activate DC are TNF- $\alpha$  and LPS. Moreover TNF- $\alpha$  has been widely used to generate *in vitro* bone marrow-derived DC. This procedure is also followed to obtain large

[1 21796]

**Abbreviations:** DC: Dendritic cells EST: Expressed sequence tag

amounts of DC for cell-based therapies of cancer. Thus, to evaluate the differences in DC response to LPS and TNF- $\alpha$  we performed a genome-wide transcriptional analysis of activated DC and we compared it with the expression analysis performed on immature DC. We observed that LPS only was able to induce the transcription of genes responsible for DC growth arrest and it was much more effective than TNF- $\alpha$  in activating the expression of genes involved in antigen processing and T cell stimulation. Moreover LPS- but not TNF- $\alpha$ -stimulated DC expressed genes able to control the inflammation during the immune response. The transcriptional program analysis suggests that TNF- $\alpha$  is an ineffective stimulus for terminal DC differentiation. The observation that the expression of several genes found with the GeneChip utilization corresponds to a number of functional characteristics of DC validates the applicability of the oligonucleotide microarray technology for monitoring gene expression in mouse cells.

## 2 Results and discussion

### 2.1 Genomic-scale gene expression analysis

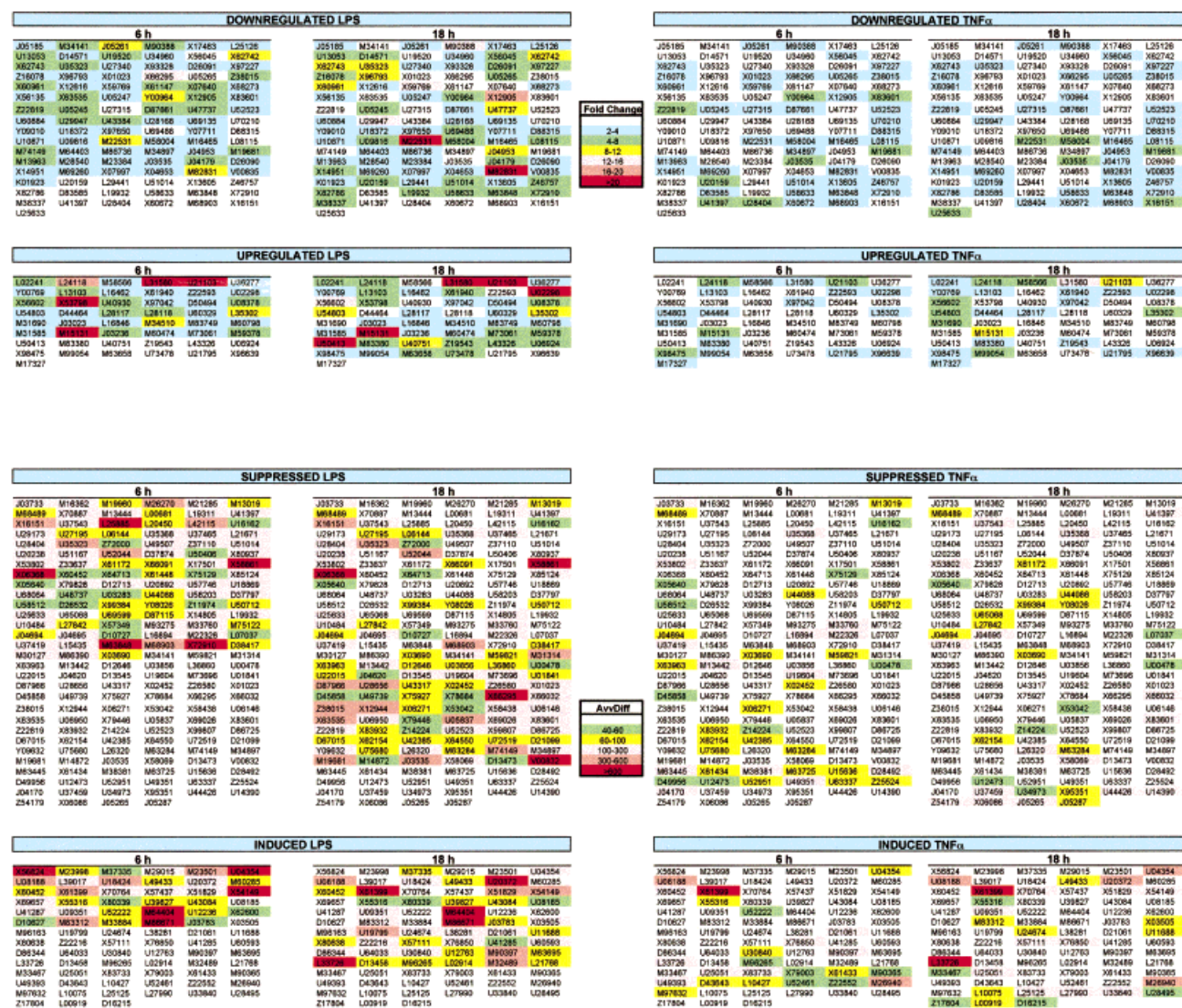
To investigate the differential effects of TNF- $\alpha$  versus LPS on DC priming we have used the Affymetrix GeneChip technology [12–15], that permits the simultaneous analysis of the expression of thousands of genes. These analyses require homogeneous cell populations to avoid dilution and contamination of information. Bone marrow-derived mouse DC are extremely unstable and it is not possible to obtain homogeneous immature DC without contamination with mature and intermediate DC. Cell lines that closely parallel fresh DC functions are a valid alternative. Thus we took advantage of the previously described mouse DC line, D1 [5]. D1 cells are a splenic, myeloid and growth factor-dependent DC line that can be maintained indefinitely in culture in the immature state. This cell line can be driven to full maturation using different stimuli. In particular, D1 cells reach a mature state 18 h after LPS or TNF- $\alpha$  stimulation, as assessed by phenotypical (up-regulation of class II and costimulatory molecules) and functional characteristics, like antigen presentation, inhibition of migration, block of antigen uptake, cytoskeleton rearrangements [5, 16]. Gene expression analysis was performed on immature, transitional (6 h LPS and TNF- $\alpha$ -activated) and mature (18 h LPS- and TNF- $\alpha$ -activated) D1 cells using GeneChip oligonucleotide probe arrays representing approximately 6,500 distinct mouse genes and ESTs. The 6,500 probe sets are subdivided in four individual chips, A–D, each containing oligonucleotide probes for about 1,600 genes and ESTs. The hybridized arrays were analyzed using the GeneChip Expression Analysis Program 3.3. Data analy-

sis protocols, sensitivity and quantitative aspects of the method have been previously precisely described [12]. In brief, gene probes are represented by 20 perfectly matched 20-mer oligonucleotides (PM) and 20 control oligonucleotides with a single mismatch (MM). Gene expression levels are defined averaging the differences (AvgDiff) between the PM and MM of every probe pair over the entire probe set:

$$\text{Avg Diff} = \frac{\Sigma(\text{PM} - \text{MM})}{n \text{ of pairs}}$$

An AvgDiff of 20 approximates the lower value of fluorescence intensity for detected genes. An absolute (individual) analysis of each probe array was performed to determine gene expression levels in each target cRNA analyzed. Signal intensities were then normalized among hybridized arrays by measuring the average value of signal intensities for each chip-array and scaling to a fixed arbitrary value (target intensity). We used a target intensity of 100, calculated considering average values of signal intensities for mouse GeneChips. This particular procedure has been developed in order to minimize the variability of GeneChip performance and sample preparation [17, 18]. Subsequently, probe arrays hybridized with TNF- $\alpha$ - and LPS-activated D1 cell samples were compared to the same baseline sample (non-stimulated cells) to determine differences in expression levels between treated and untreated cells. Among the genes and ESTs displayed on the chip 25% were called present in non-stimulated and 6 h and 18 h LPS- and TNF- $\alpha$ -activated DC. Hybridization efficiency was assessed for each array by measuring the signal intensities of the three control bacteria BioB, BioC, BioD and one phage cre gene cRNA (see Sect. 4). BioB “spikes” at 1.5 pM were usually detected. Thus, under these hybridization conditions the detection limit was around 1.5 pM.

Genes modulated in treated cells with respect to untreated cells were divided into four main groups: induced (not detected in non-activated but detected in activated cells), up-regulated, down-regulated, suppressed (detected in non-activated and not detected in activated cells). For up-regulated and down-regulated genes we have considered only those ones that showed at least a twofold change in the level of mRNA expression in two independent experiments (fold change  $\geq 2$ ; Fig. 1). Differences in expression are calculated by dividing the intensity values (AvgDiff) of genes from treated cells by intensity values of genes from non-stimulated cells. Since not-detected genes do not have reliable intensity values, we could not use the fold change as a parameter for the expression analysis of induced and suppressed genes. Thus, the former were selected on the basis of the level of expression reached after stimulation (a minimum AvgDiff of 40 in two independent exper-



**Fig. 1.** Known genes differentially expressed in LPS- and TNF- $\alpha$ -activated DC versus unstimulated cells. Genes were divided in four groups (induced, suppressed, up-regulated, down-regulated) and represented by their GenBank accession numbers. Fold change values are indicated for up-regulated and down-regulated genes. Intensity values before and after stimulation are shown for suppressed and induced genes, respectively. Genes in white boxes are not indicated under the indicated conditions.

iments), and the latter on the basis of the level of expression they had in unstimulated cells (a minimum AvgDiff of 40, suppressed in two independent experiments) (Fig. 1).

Although LPS and TNF- $\alpha$  were considered equivalent factors for DC maturation, D1 cells activated with these two different stimuli showed a very diverse gene expression program (Fig. 1). In Table 1 we have listed an example of the gene families differentially expressed or modulated in mature DC compared to non-stimulated cells.

## 2.2 Genes involved in cell cycle control and survival

Terminal differentiation results in the growth arrest of proliferating cells. There are evidences that the cell cycle control in immature, splenic, mouse DC is not stringently regulated and small increase in cell number can be observed when they are plated over an irradiated stromal cell monolayer of fibroblasts and endothelial cells [19]. Mature DC completely lose the proliferation capacity and die by apoptosis 8–9 days after activation. A very similar differentiation process can also be induced in



**Table 1.** Differential gene expression analysis in LPS- and TNF- $\alpha$ -stimulated versus unstimulated D1 cells

ID <sup>1</sup>	Gene name	LPS 6 h	LPS 18 h	TNF $\alpha$ 6h	TNF $\alpha$ 18 h	ID <sup>1</sup>	Gene name	LPS 6 h	LPS 18 h	TNF $\alpha$ 6h	TNF $\alpha$ 18 h
<b>Cell surface and membrane proteins</b>						<b>Antigen processing and presentation</b>					
L09754	CD30L	NC	NC	I 36*	I 42*	U60329	PA28	U 3.2	U 3.2	NC	NC
U12763	OX40L	NC	I 70	NC	NC	X97042	UBC4M4	U 2.3	U 2.6	U 2.2	U 2.6
M83312	CD40	I 345*	I 201*	NC	I 4.3*	M55637	TAP-1cas	NC	U 2.8	NC	NC
M34510	CD14	U 11.6	U 3.2	NC	NC	U35323	H-2M $\beta$ 2	D 12.5	D 9.2	D 2.2	D 2.2
Y08026	IAP38	S 73**	S 73**	NC	S 73**	U35323	H-2M $\alpha$	S 304**	S 304**	NC	NC
U10484	Jaw1	S 242**	S 242**	NC	D 2.6	U35323	H-2M $\beta$ 1	D 6.9	S 149**	NC	NC
X93328	F4-80	D 2.6	D 2.4	NC	NC	D83585	proteasome Z subunit	NC	NC	D 2.5	D 2.2
Z16078	CD53	D 3.9	D 7.3	D 2	D 2.3	K01923	I-A $\alpha$	D 2.8	D 3.5	NC	NC
X68273	Macrosialin	D 2.3	D 2.1	NC	NC	V01527	I-A $\beta$	NC	D 3.7	NC	NC
U47737	TSA1	D 4.1	D 9.9	NC	NC	<b>Secreted molecules</b>					
U18372	CD37	D 3.8	D 2.9	NC	NC	J03783	IL-6	I 45*	I 78*	NC	NC
U05265	gp49	NC	D 4.9	D 2.9	D 3.7	M86671	IL-12p40	I 820*	I 759*	NC	NC
L08115	CD9	NC	D 4.3	D 2.9	D 2.4	M64404	IL-1RA	U 13.4	U 9	NC	NC
X72910	HSA-C	NC	D 5.5	D 2.8	D 2.5	X03505	serum	NC	I 256*	NC	I 67*
U25633	TMP	S 165**	S 165**	NC	D 7.4						
<b>Cell cycle and apoptosis</b>						M15131	amyloid IL-1 $\beta$	U 82	U 34.8	U 7.4	U 11.8
D86344	TIS	NC	I 273*	NC	NC	M73061	MIP-1 $\alpha$	U 3.9	U 5.5	NC	NC
L49433	c-IAP-1	I 71*	I 68*	NC	I 77*	X53798	MIP-2	U 20.2	U 5.7	NC	NC
L16846	BTG1	U 3.2	U 2.4	NC	NC	U02298	RANTES	U 3.7	U 28	U 3	U 3.4
M83749	cyclin D2	U 3.7	U 3.7	NC	NC	X58861	C1q $\alpha$	S 628**	S 628**	NC	NC
U19860	GAS	NC	U 2.8	NC	NC	X66295	C1qC	D 15	S 656**	D 3.7	D 3.5
D50494	RCK	U 3	U 2.2	NC	NC	X16151	ETA1	S 485**	S 485**	NC	D 7.9
M64403	CYL-1	D 2.4	D 3.8	NC	NC	M19681	JE	D 5.9	S 348	D 6	D 4.6
U70210	TR2L	NC	NC	D 2.4	D 3.5	X06086	MEP	D 2.6	D 2.9	D 2	S 126**
U58633	p34CDC2	NC	D 5.5	D 2.2	D 3.2	U50712	MCP5	S 83**	S 83**	S 83**	S 83**
X82786	Ki-67	NC	D 6.4	NC	D 2.6	X83601	PTX3	D 3.8	S 211**	D 3.6	S 211**
Z26580	cyclin A	NC	S 147**	NC	NC	M22531	C1qB	D 8.7	D 42.8	D 2.4	D 4.2
X66032	cyclin B2	NC	S 108**	NC	S 108**	M58004	C10	D 3.1	D 6.5	D 2.6	D 5.2
X64713	cyclin B1	NC	S 47**	NC	S 47**	L19932	$\beta$ ig-h3	NC	D 5.1	NC	NC
D86725	mMCM2	NC	S 125**	D 2.7	NC	X12905	properdin	D 4.7	D 16.2	D 2.2	NC
Z72000	BTG3	S 46**	S 46**	NC	S 46**						

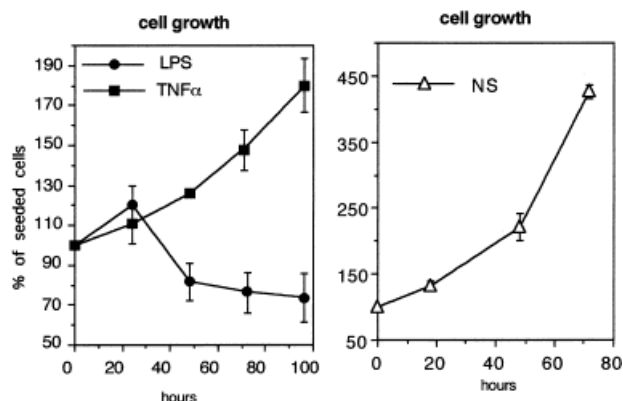
1, accession number; NC: no change in the level of expression; I: induced (detected only in stimulated cells); U: up-regulated; D: down-regulated; S: suppressed (detected only in unstimulated cells), \*AvgDiff reached after stimulation,

\*\* AvgDiff in the baseline, values without asterisks represent the fold change in stimulated versus unstimulated cells.

immature D1 cells that undergo growth arrest and terminal differentiation *in vitro*. An important difference between LPS- and TNF- $\alpha$ -activated D1 cells resided in the complexity of the pattern of genes involved in the control of cell cycle progression (Table 1). Cyclins are usually expressed during mitogenesis and they control cell proliferation. Cyclins A and B are involved in the control of G1/S and G2/M transition, respectively. LPS induced the suppression of type A and B cyclins, including genes like MCM2 [20], which allow the initiation of DNA replication. In contrast, TNF- $\alpha$ -activated D1 cells, showed exclusive suppression of cyclins necessary to pass the G2 phase (cyclin B1, cyclin B2), suggesting that they can still initiate DNA replication and that they could be arrested at a cell cycle checkpoint between S and G2. D-type cyclins have a different pattern of expression with respect to the other type of cyclins. They are induced in many different cell types by mitogens and are essential for G1 phase progression, but they have also been described to be indu-

ced by differentiation stimuli in megakaryocytes facilitating their differentiation process [21]. Moreover, cyclin D2 is induced in macrophages following the anti-mitogenic stimulus of LPS [22]. Interestingly, LPS but not TNF- $\alpha$  induces the up-regulation of D type cyclins (Table 1). In addition, anti-proliferative genes, such as BTG1, GAS, RCK were exclusively up-regulated in LPS-activated D1 cells (Table 1). This particular pattern of expression could suggest that only LPS was able to induce a definitive DC growth arrest. LPS-activated DC underwent irreversible growth arrest already 24 h after stimulation [23], whereas TNF- $\alpha$ -activated D1 cells showed only a strong proliferation slow down but not a definitive growth arrest (Fig. 2), with a doubling time of 130 h. These results imply that only LPS drives complete terminal differentiation of DC and that TNF- $\alpha$  induces only partial DC activation.

As is consistent with the observation that LPS is able to promote both DC maturation and survival [23] of termi-



**Fig. 2.** LPS but not TNF- $\alpha$  induces definitive growth arrest of DC. D1 cells were stimulated either with LPS or TNF- $\alpha$  for the indicated time. The number of viable cells at the indicated time points is expressed as percentage of seeded cells. LPS, LPS-stimulated cells; TNF- $\alpha$ , TNF- $\alpha$ -stimulated cells; NS, non-stimulated cells. Standard deviations of five independent experiments are reported.

nally differentiated DC, LPS-activated D1 cells also expressed anti-apoptotic genes, at late time points, such as TIS (topoisomerase-inhibitor suppressed, [24]). Moreover, mRNA coding for the c-IAP-1 protein (required together with TRAF-1 (up-regulated) and TRAF-2 (expressed) to inhibit TNF- $\alpha$ -induced apoptotic cell death, [25]) was observed in both TNF- $\alpha$ - and LPS-activated D1 cells.

### 2.3 Genes involved in antigen processing and peptide loading on MHC molecules

The survival of growth-arrested DC after LPS stimulation would be necessary to allow mature DC to migrate into lymphoid tissues and prime naive CD8<sup>+</sup> and CD4<sup>+</sup> T cells. To accomplish this task, DC should magnify their capacity to produce peptides from native protein antigens to be loaded on MHC class I and class II molecules. Interestingly, genes involved in the antigen presentation function showed a distinctive pattern of expression in LPS or TNF- $\alpha$ -activated D1 cells. Only LPS up-regulated the PA28 proteasome activator and the TAP-1 molecule mRNA (Table 1). The PA28 protein dramatically increases the spectrum of peptides produced and the efficiency of the 20S proteasome, whereas the TAP-1 molecule is required to transfer proteasome-produced peptides from the cytosol to the endoplasmic reticulum [26]. The proteasome activator was up-regulated already at 6 h after LPS activation, while TAP-1 up-regulation was measurable only at late time points. This pattern of mRNA up-regulation in D1 cells after LPS stimulation correlates

well with the kinetic of MHC class I new biosynthesis in D1 cells, which peaks at 18 h after bacteria [16] or LPS stimulation. In addition, LPS is more efficient than TNF- $\alpha$  in inducing the up-regulation of MHC class I molecules and the stability of peptide-MHC complex expression at the surface of DC can be extended from 24 to 72 h when DC are pretreated with LPS but not TNF- $\alpha$  [27]. Thus, LPS-stimulation induces a remarkable activation of the entire intracellular apparatus necessary for class I antigen presentation function. We did not observe any class I mRNA up-regulation with the GeneChip analysis. In fact, the class I oligonucleotide probes are specific for the H-2D molecule and the surface class I up-regulation observed in D1 cells, 18 h after challenge, was relative to the H-2K protein (data not shown, [16]).

Concerning MHC class II genes, we have previously shown that the up-regulation of class II protein synthesis is very rapid, peaking as early as 1 h after DC activation and this is followed by a striking down-regulation [16]. The GeneChip approach validated the previous analysis of MHC protein expression during DC maturation, showing that class II molecule mRNA were down-regulated in LPS-stimulated D1 cells. Class II molecule mRNA are also down-regulated in LPS-stimulated human DC [10]. Moreover, in agreement with the increased stability observed in peptide-MHC class II complexes in activated DC, the H-2M molecules, which regulate MHC class II loading with antigenic peptides, were either down-regulated or suppressed and, again, the level of down-regulation was more pronounced in LPS-treated compared to TNF- $\alpha$ -stimulated D1 cells. The down-regulation was already evident 6 h after LPS stimulation (Table 1). This gene expression pattern implicates that LPS is more effective than TNF- $\alpha$  in inducing the reprogramming of presentation activity typical for mature DC. Taken as a whole, these data indicate that LPS but not TNF- $\alpha$  is sufficient to drive DC toward a stage of maturation appropriate for the immune response activation. Our observations are also supported by the finding that TNF- $\alpha$ -activated DC are, actually, rather inefficient in activating T cells in MLR assays *in vitro* and in conferring tumor protection in mouse models *in vivo* [28]. This is also supported by the strong up-regulation of the mRNA coding for the well known leukocyte chemo-attractants RANTES, MIP1 $\alpha$  and MIP2, in LPS-activated but not in TNF- $\alpha$ -treated D1 cells (Table 1). A similar pattern of chemokines up-regulation at early and late time points after LPS stimulation has been also observed using RNase protection and chemotactic assays [29]. Furthermore, LPS-but not TNF- $\alpha$ -stimulated D1 cells expressed interleukin (IL)-6 (Table 1 and Fig. 3). This cytokine is known to be involved in increasing the spectrum of peptides presented by DC [8], in leukocyte recruitment [30] and in B cell differentiation [31].

## 2.4 Gene involved in the control of inflammatory responses

A physiological immune response originates from a well-controlled inflammatory response. Genes involved in activating and controlling the inflammation process are differentially regulated in DC matured in the presence of the two different stimuli (Table 1). In LPS-matured DC and in much lesser extent in TNF- $\alpha$ -treated cells is observed suppression or strong down-regulation of the complement molecule C1q (Table 1). This protein is known to play a role in inflammatory responses by increasing phagocytic activity and microbial killing of macrophages and neutrophils, by enhancing B cell secretion of immunoglobulins and by inducing the expression of adhesion molecules on platelets. In addition the C1q molecule has been described as being the basis for fibroblast attachment and growth at sites of chronic inflammation [32]. Recently a strong inflammatory role in the central nervous system has been attributed to C1q [33]. Thus, the down-regulation of C1q transcription at late time points may be a way of controlling inflammatory processes.

Numerous *in vivo* studies indicate that the balance between IL-1 and IL-1RA is important in influencing the response to pathogens. A definite anti-inflammatory role has, indeed, been attributed to the IL-1RA molecule that has an important function in limiting organ damage subsequent to the host response to infection [34]. In LPS-matured D1 a strong up-regulation of IL-1 $\beta$  together with IL-1 receptor antagonist (IL-1RA) mRNA expression is observed, both at early and late time points. Whereas in TNF- $\alpha$ -stimulated D1 cells only little IL-1 $\beta$  production and not IL-1RA expression is observed (Table 1). Thus, LPS-matured DC are likely to have an important role not only in stimulating but also in controlling the inflammatory response.

Another cytokine differentially expressed in LPS-activated compared to TNF- $\alpha$ -treated D1 cells is IL-

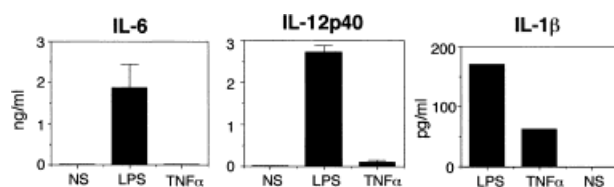


Fig. 3. Cytokine production after LPS and TNF- $\alpha$  treatment. Supernatants of unstimulated, TNF- $\alpha$ - and LPS-treated DC were collected 18 h after stimulation and tested by ELISA for the presence of IL-6, IL-12p40 and IL-1 $\beta$

12p40 (Table 1, Fig. 3). The IL-12p40 homodimer is an IL-12p75 antagonist *in vitro* [35] and it acts as a potent immune-suppressant of Th1 response [36] *in vivo*. It has been shown to induce a deviation of pancreas infiltrating CD4<sup>+</sup> T cells to the Th2 phenotype in NOD mice as well as to reduce the onset of spontaneous diabetes [37].

## 3 Concluding remarks

Together, the above data show clear differences in DC activation induced by distinct stimuli. TNF- $\alpha$  comes out as a mild alert stimulus unable to drive DC to terminal differentiation. The relevance of the stimuli used to induce DC maturation should be taken into account for DC-based therapies, since it is likely that the quality of activation may affect the final outcome of the clinical response.

Microarray approach allows quantitative and simultaneous analysis of gene expression of a large amount of genes. Many cellular processes are regulated by changes in mRNA levels. Thus systematic studies of gene expression patterns have proven to be extremely useful for studying cellular effects of natural stimuli and to be a powerful tool to identify molecular events and key pathways involved in specific cellular functions [34].

## 4 Materials and methods

### 4.1 Cells and reagents

The D1 cells were derived from murine splenic DC and maintained *in vitro* in IMDM supplemented with 30% R1 conditional medium as described [5]. LPS (*Escherichia coli* serotype 026:B6) was purchased from Sigma Chemical Co. and used at 10  $\mu$ g/ml. Murine rTNF- $\alpha$  (Genetech Inc., San Francisco, CA) was used at 100 U/ml. Cells were grown and harvested at the same time.

### 4.2 RNA extraction, amplification and labeling for hybridization

Antisense cRNA was prepared following Affymetrix (Santa Clara, CA) recommendations. Briefly, mRNA was directly extracted from frozen pellets using the Direct Oligotex kit from Qiagen (Chatsworth, CA) and converted to double-stranded cDNA using a modified oligo dT primer with a 5' T7 RNA polymerase promoter sequence [12] and the Superscript Choice System for cDNA synthesis (Life Technologies, Gaithersburg, MD). Double-stranded cDNA (0.5  $\mu$ g) was transcribed to cRNA with the T7 RNA polymerase (T7 Megascript kit; Ambion, Austin, TX) in the presence of a mixture of unlabeled ATP, CTP, GTP, UTP and biotin-labeled CTP and UTP (ENZO Diagnostics, Farmingdale, NY). cRNA was purified on an affinity column (RNeasy; Qiagen).

### 4.3 Probe array hybridization and scanning

The Mu6500 GeneChip array consists of a set of four individual chips, A-D, collectively representing 6,500 murine genes and ESTs. Analysis of the D1 samples was performed by hybridizing the cRNA to the GeneChip arrays A-D. Probe array hybridizations were carried out as described [13]. cRNA was fragmented to an average size of 50–200 bases, by incubation for 30 min at 94 °C in 40 mM Tris-acetate pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate. Samples were then diluted in the hybridization solution (1 M NaCl, 10 mM Tris pH7.6, 0.005% Triton X-100, 0.1 mg/ml herring sperm DNA, BioB-, BioC-, BioD-, cre-control cRNA at a concentration of 1.5, 5, 25, 100 pM, respectively) at a final concentration of 0.05 µg/ml heated at 94 °C for 5 min and placed in the hybridization cartridge (200 µl/chip). Hybridizations were performed at 40 °C for 16 h. Following hybridization the chips were rinsed with 6× SSPE-T (0.9 M NaCl, 60 mM NaH<sub>2</sub>PO<sub>4</sub>, 6 mM EDTA, 0.005% Triton X-100 adjusted to pH 7.6) and 0.5× SSPE-T and stained by incubating with 2 µg/ml streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and 1 mg/ml acetylated BSA (Sigma, St. Louis, MO).

The arrays were read at a resolution of 7.5 µm, using a confocal scanner (Affymetrix) and analyzed with the GeneChip 3.3 Gene Expression analysis program (Affymetrix).

Genes, that showed, in two independent experiments, a fold change of at least 2 in stimulated cells over the baseline were considered differentially expressed.

### 4.4 ELISA

IL-1β, IL-12p40 and IL-6 were quantified using DuoSET ELISA Development System (R&D Systems, Minneapolis, MN).

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