

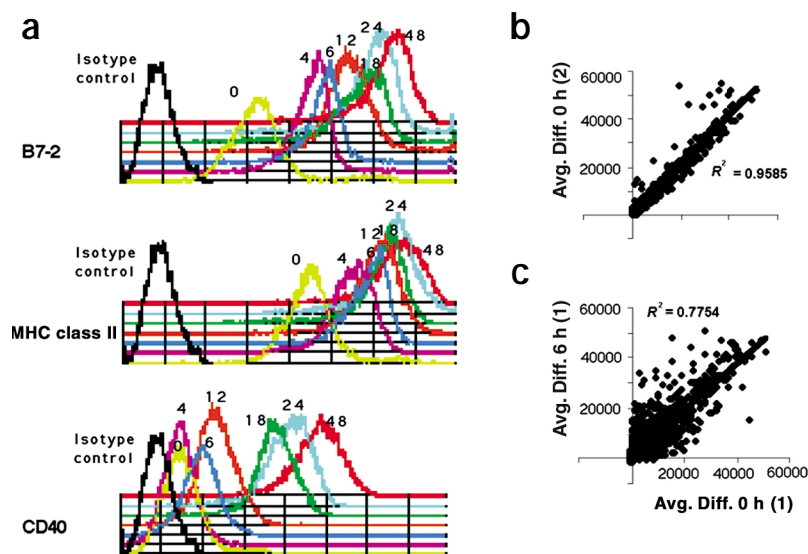
# Inducible IL-2 production by dendritic cells revealed by global gene expression analysis

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Dendritic cells (DCs) are strong activators of primary T cell responses. Their priming ability is acquired upon encounter with maturation stimuli. To identify the genes that are differentially expressed upon maturation induced by exposure to Gram-negative bacteria, a kinetic study of DC gene expression was done with microarrays representing 11,000 genes and ESTs (expressed sequence tags). Approximately 3000 differentially expressed transcripts were identified. We found that functional interleukin 2 (IL-2) mRNA, which gave rise to IL-2 production, was transiently up-regulated at early time-points after bacterial encounter. In contrast, macrophages did not produce IL-2 upon bacterial stimulation. Thus, IL-2 is an additional key cytokine that confers unique T cell stimulatory capacity to DCs.

Dendritic cells (DCs) are professional antigen-presenting cells that are able to initiate adaptive immune responses to invading pathogens. Key functions of DCs include uptake and processing of antigen and priming of naïve T cells<sup>1</sup>, functions that are segregated in time. Immature resting DCs located in nonlymphoid tissues, such as skin and mucosae, take up antigen. Mature DCs loaded with antigen<sup>1</sup> and capable of priming T cells migrate from nonlymphoid tissues to the T cell area of lymph nodes or spleen. Thus, when immature DCs come into contact with inflammatory stimuli, they undergo a maturation process that transforms them from phagocytic and migratory cells to nonphagocytic, highly efficient stimulators of naïve T cell responses<sup>2</sup>.

Mature DCs are programmed to undergo apoptotic death within 9–10 days<sup>3</sup>. Immature monocyte-derived human DCs (hMDCs) or immature bone marrow-derived mouse DCs (mBMDCs) can be induced to mature *in vitro* with many different stimuli, including inflammatory cytokines; bacteria cell products, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA); bacterial DNA; double-stranded viral RNA; and live bacteria. This last stimulus represents one of the most potent catalysts of the DC terminal differentiation process in the mouse: it induces a rapid and effective DC phenotypic and functional maturation<sup>4</sup>. A key part of the complexity of innate and adaptive immunity to microorganisms relies on how they affect DC activation<sup>5</sup>.



**Figure 1. Developmentally synchronized DCs.** (a) DCs were incubated with *E. coli* and activation tested by measuring surface expression of CD40, B7-2 and MHC class II at the indicated time-points by flow cytometry. RNA was extracted from synchronized cells. (b) Example of gene expression comparison in duplicate experiments (1 and 2 at time 0): 3340 genes present in the duplicates were compared. The  $R^2$  of duplicate linear regressions for each time-point was never lower than 0.9 (data not shown). (c) Gene expression comparison at two time-points (time 0 and time 6 of experiment 1), excluding genes that were consistently absent in both the analyses.  $R^2$  of comparison analysis linear regressions usually ranging between 0.7–0.8 (data not shown). The degree of gene expression is indicated by the average difference (Avg. Diff.) value.

**Table 1. Results of PCA on the entire dataset**

Independent variables <sup>a</sup>	PC1 (n=1)	PC2 (n=2)	PC3 (n=3)	PC4 (n=4)
0 h (1)	-0.26	0.53	-0.161	0.347
0 h (2)	-0.252	0.643	-0.337	-0.228
4 h (1)	-0.264	0.142	0.468	0.328
4 h (2)	-0.267	0.164	0.327	-0.441
6 h (1)	-0.268	-0.017	0.36	0.186
6 h (2)	-0.269	0.015	0.339	-0.351
12 h (1)	-0.272	-0.149	0.111	0.209
12 h (2)	-0.273	-0.163	0.043	-0.121
18 h (1)	-0.271	-0.185	-0.013	0.232
18 h (2)	-0.27	-0.223	-0.14	-0.289
24 h (1)	-0.268	-0.229	-0.246	0.093
24 h (2)	-0.27	-0.196	-0.252	-0.243
48 h (1)	-0.266	-0.139	-0.256	0.319
48 h (2)	-0.271	-0.121	-0.259	-0.036
Eigenvalue <sup>b</sup>	0.932	0.022	0.019	0.008
Cumulative (%) <sup>c</sup>	93.2	95.4	97.3	98.1

The values in the columns are the coefficients of the PC indicated at the top. <sup>a</sup>The independent variables are the seven different time-points analyzed in duplicate. Numbers 1 and 2 in parentheses indicate the two independent experiments. <sup>b</sup>The eigenvalues express the variance explained by a particular PC (overall variance=1). <sup>c</sup>The cumulative values are the percentage of information that is described by the sum of the first *n* PCs.

To study how DCs are conditioned by bacterial encounter, we carried out a kinetic analysis of gene expression in immature mouse DCs stimulated at different time-points with live Gram-negative bacteria. Many genes, which were differentially expressed during maturation, encoded transcription factors, signal transduction molecules and proteins involved in cytoskeletal rearrangements or in the activation and control of immune responses. We also observed induction of inter-

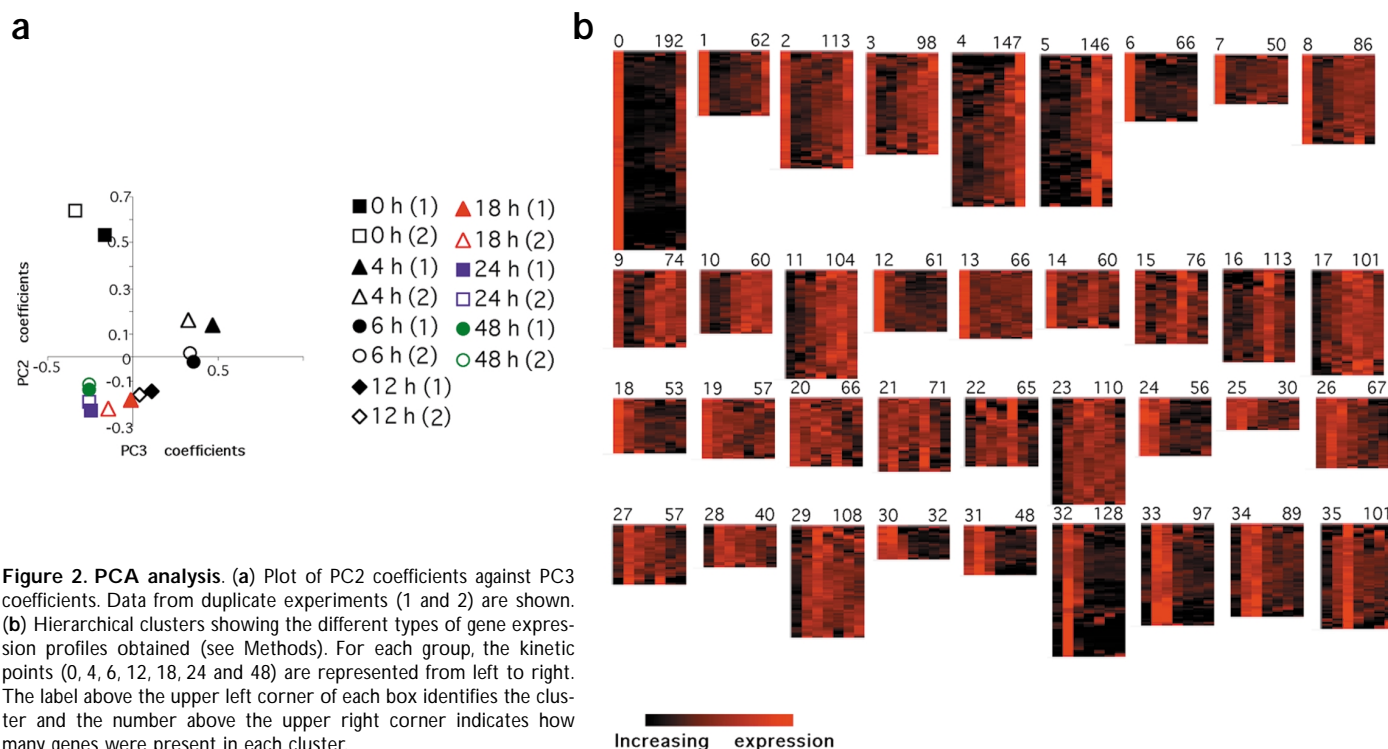
leukin 2 (IL-2) mRNA at early time-points after bacterial encounter and induction of IL-2 protein in culture supernatants. To test whether this cytokine could have a role in activating T cell responses, we compared the ability of wild-type and IL-2<sup>-/-</sup> DCs to stimulate T cells in primary mixed lymphocyte reactions (MLRs). The ability of IL-2<sup>-/-</sup> DCs to induce both CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation was severely impaired, which indicated that IL-2 is a key cytokine in conferring the unique T cell stimulatory capacity to DCs. Bone marrow-derived macrophages, activated with bacteria, did not produce any IL-2.

## Results

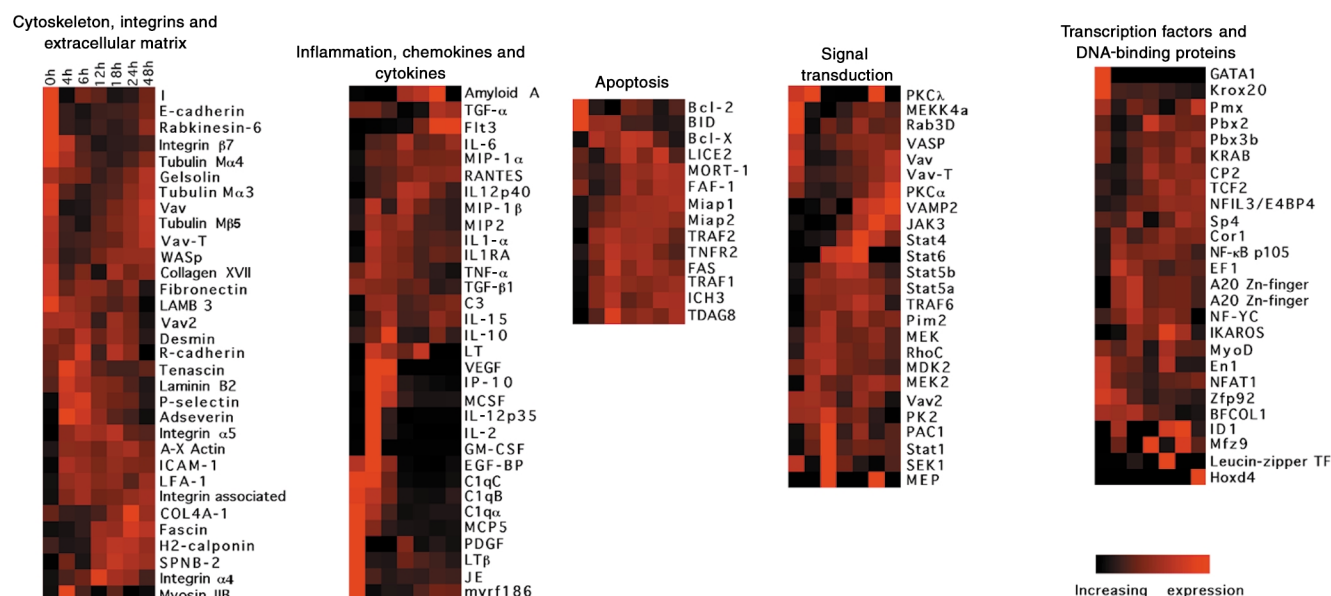
### Analysis of developmentally synchronized DCs

Transcription analysis requires homogeneous populations to avoid dilution and contamination of data. Due to their plasticity, mBMDCs are extremely heterogeneous, and it is not feasible to obtain homogeneous immature cells without contamination with mature and intermediate DCs<sup>6,7</sup>. There is a DC culture system that allows the propagation of homogeneous immature growth factor-dependent (granulocyte monocyte-colony-stimulating factor, GM-CSF) mouse DCs that can fully mature in response to bacteria, bacterial cell products or inflammatory cytokines<sup>3</sup>, mimicking the *in vivo* DC maturation process. Studies of a well characterized DC line, D1, done with this culture system show similar maturation to that seen with fresh splenic or bone marrow-derived DCs<sup>8-11</sup>.

We activated D1 cells with Gram-negative *Escherichia coli* and transcriptionally analyzed immature cells as well as mature cells that had been stimulated for 4, 6, 12, 18, 24 or 48 h with high-density oligonucleotide arrays that displayed probes for 11,000 genes and expressed sequence tags (ESTs). At each time-point after stimulation, D1 cells were phenotypically characterized for their state of developmental synchronization by analyzing surface expression of major histocompatibility complex (MHC) class II, B7-2 and CD40 (**Fig. 1a**). Biotin-labeled cRNA was generated and hybridized onto



**Figure 2. PCA analysis.** (a) Plot of PC2 coefficients against PC3 coefficients. Data from duplicate experiments (1 and 2) are shown. (b) Hierarchical clusters showing the different types of gene expression profiles obtained (see Methods). For each group, the kinetic points (0, 4, 6, 12, 18, 24 and 48) are represented from left to right. The label above the upper left corner of each box identifies the cluster and the number above the upper right corner indicates how many genes were present in each cluster.



**Figure 3. Transcription response of DCs to bacteria.** An example of 130 genes is shown. Genes were grouped according to their most likely function, deduced from data available in public databases and published literature.

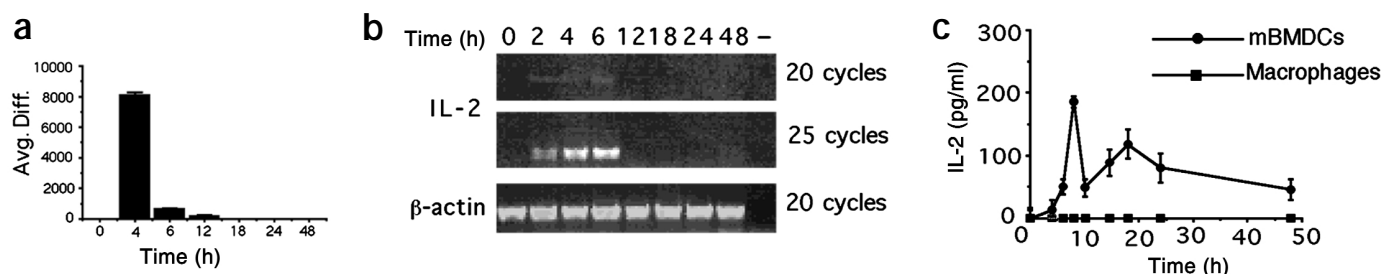
arrays. To assure the reproducibility of the assay, the experiment was done in duplicate and gene expression profiles compared (Fig. 1b). The variability observed never exceeded 5% of the genes and usually affected transcript expression to a degree that was close to the lower limit of detection (1.5 pM). Approximately 30% of the genes and ESTs displayed on the array were present at each time-point tested. Excluding genes that always remained below the detection limit during the entire kinetic assay, we obtained 9930 genes that were expressed at least at one time-point.

### Principal component analysis

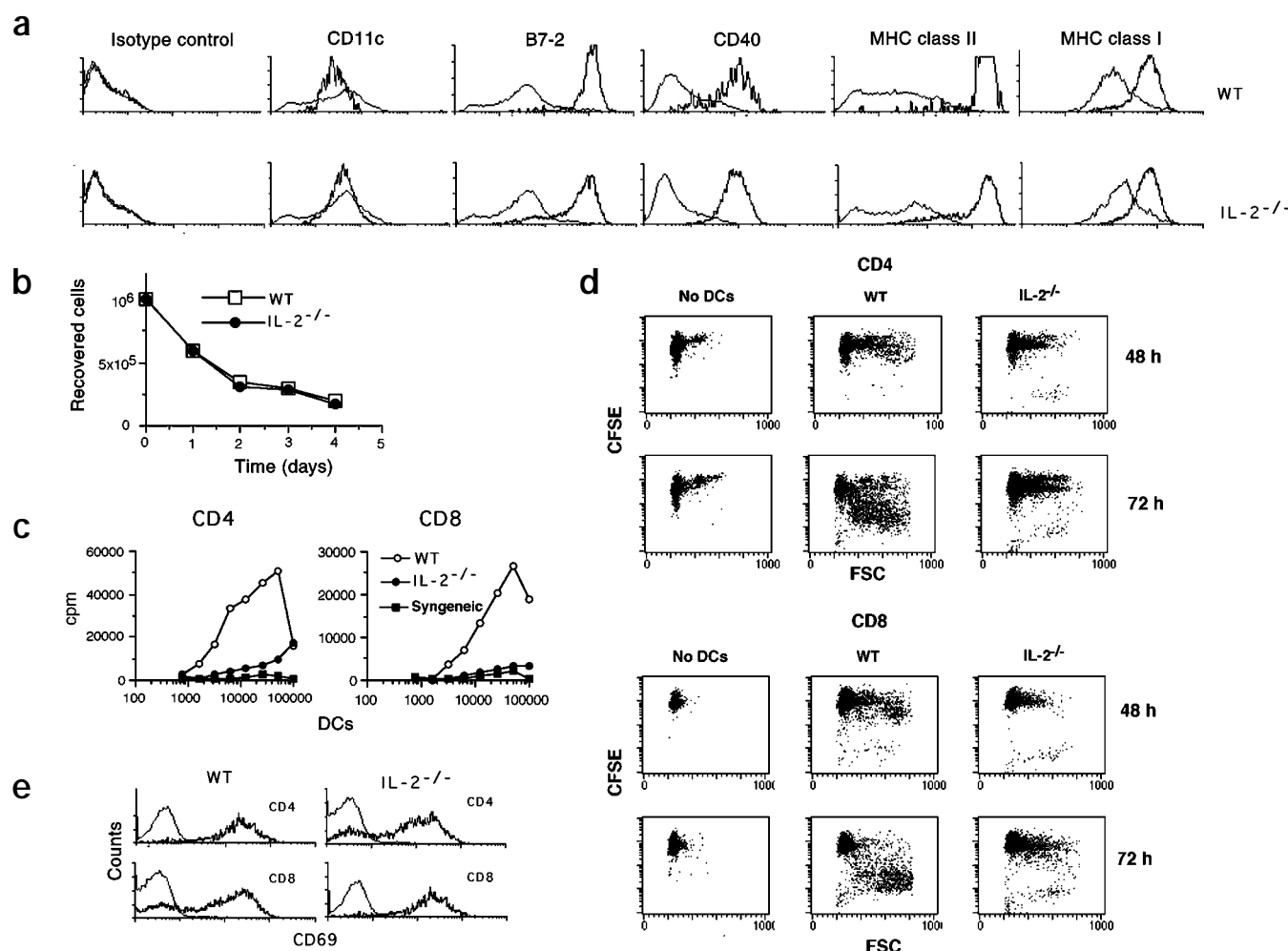
To get an approximate visualization of our entire dataset, without losing experimental information (variance), we first applied the principal component analysis (PCA) method, which allows the dimensionality of complex data to be reduced<sup>12</sup>. Thus, we were able to globally describe features of the kinetic points that best explained the corresponding transcriptome (Table 1). We analyzed only the first four principal components (PCs) because they globally explained >98% of the overall variance. Although PC1 was able to describe >93% of the

total variance, it did not contain any kinetic information, as it was simply a measure of the time-independent average expression. Conversely, PC2 and PC3 were two time-dependent parameters. PC2 represented the trend of expression over time, giving a measure of gene down-regulation; PC3 described the shape of this trend, indicating the concavity of the expression curve. PC2 versus PC3 coefficients are shown (Fig. 2a).

Qualitatively this analysis indicates that, after activation, the general organization of gene expression was immediately influenced. Cells then progressively returned to gene expression, which was similar to that of immature cells but clearly distinct from it. Thus, in addition to genes permanently expressed in either immature or terminally differentiated cells and that characterize these two stages, there is a group of genes that are transiently modulated during activation. The process of DC maturation differentiation stabilized 24 h after activation. PC4 was able to discriminate between two independent replicates (Table 1), which indicated the existence of a systematic experimental error that contributed to the overall variance of <1%. Given the systematic nature of the error and the correlation coefficient ( $R^2$ )



**Figure 4. IL-2 expression by DCs.** (a) IL-2 mRNA levels in D1 cells after activation with bacteria. Data represent average differences (Avg. Diff.) and standard deviations calculated from four probes distributed on four different chips (duplicates of chip A and chip B, see Methods). (b) Semi-quantitative PCR of 40 ng samples of cDNA, which showed IL-2 expression in mBMDs after bacterial stimulation (done as in Fig. 1) at the indicated time-points. mRNA was from mBMDs. Double-stranded cDNA was transcribed from mRNA, purified and quantified. β-actin acted as a loading control. (c) Quantification of the amount of IL-2 present in the supernatant of bacteria-activated DCs and macrophages. IL-2 was measured in the supernatants, by ELISA, at the indicated time-points. This experiment was repeated four times with similar results.



**Figure 5. DC-derived IL-2 was a key molecule for T cell activation.** (a) Activation profile of mBMDCs after stimulation with bacteria. Expression of the indicated molecules by unstimulated DCs (thin lines) or DCs that had been activated by bacteria for 15 h (thick lines) were analyzed by flow cytometry. (b) Survival curve of wild-type and IL-2<sup>-/-</sup> DCs after bacterial encounter. Bacteria-activated DCs (10<sup>6</sup>) were added in six-well plates and the number of viable cells remaining at the indicated time-points evaluated by trypan blue exclusion. (c) Proliferative response of alloreactive T cells measured by tritiated thymidine incorporation. Graded numbers of bacteria-activated wild-type and IL-2<sup>-/-</sup> DCs were incubated with 2x10<sup>5</sup> allogeneic T cells in 96-well plates. Background T cell proliferation was evaluated by coculturing syngeneic T lymphocytes with wild-type DCs. Proliferation was assessed after 72 h by 16 h of exposure to [<sup>3</sup>H]thymidine, expressed as the mean cpm of duplicates. (d,e) Alloreactive T cells activation. DCs (5x10<sup>5</sup>) were stimulated with bacteria in 24-well plates and 2x10<sup>6</sup> CFSE-labeled CD4<sup>+</sup> or CD8<sup>+</sup> allogeneic T cells were added to the culture. (d) Cycling cells were analyzed at the indicated time-points by FACS analysis. (e) After 48 h of culture, CD69 expression was evaluated on small noncycling T lymphocytes (thin lines) and large T cell blasts (thick lines).

of linear regression in correlation plots (**Fig. 1**), it was appropriate to average the replicates and analyze the mean values further.

## Gene clustering

To identify differentially expressed genes within the collection of 9930 genes and ESTs obtained, we first applied a clustering algorithm, which groups genes according to the similarity of their expression patterns, based on self-organizing maps (SOMs)<sup>13</sup>. Average differences (parameters that indicate the degree of expression<sup>14</sup>) between the duplicates were used and data filtered to exclude all the genes or ESTs that showed a fold change between maximum and minimum kinetic values lower than 3. With this filter, sequences to be clustered were reduced to 2951, about 50% of which were ESTs (**Fig. 2b**). Then, a second clustering method (hierarchical clustering) was applied to each SOM cluster to further investigate smaller profile differences and to verify that all the sequences were grouped correctly<sup>15</sup>. Using this approach, we obtained a good clustering of the genes, with

each hierarchical cluster having a correlation coefficient that was higher than 0.79 (**Fig. 2b**). (For further details on Fig. 2, see [www.btbs.unimib.it/DCgenes](http://www.btbs.unimib.it/DCgenes).)

To classify genes and divide them into functional families, different databases—GenBank, SwissProt, Kegg and Gene Ontology—were searched (**Fig. 3**). As expected, most of the differentially expressed genes encoded transcription factors and signal transduction molecules. They were distributed in different clusters, some transiently induced at different time-points and some stably induced or down-regulated. An example of the genes affected by bacterial induction is shown (**Fig. 3**).

To control the validity of this analysis, we checked, within the different clusters, whether well established markers for DC activation followed the described regulation. For example, inflammatory products such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , cluster 26), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ , also known as CCL3, cluster 23), MIP-1 $\beta$  (also known as CCL4, cluster 27) and MIP-2 (cluster 28) were modulated during the kinetic assay, as observed in other DC systems<sup>16,17</sup>.





They showed a peak of induction at early time-points and were progressively down-regulated towards the end of the kinetic assay. This suggested that the inflammatory activity of DCs mainly occurs early after activation, before DCs have left the inflammatory site (**Fig. 3**). In addition, IL-12p35 was transiently induced during activation (cluster 32) (**Figs. 2 and 3**), with expression peaking at 4 h. Temporary expression of IL-12p35 also occurs with LPS-activated hMDCs, but with delayed kinetics<sup>16</sup>. IL-12p40 mRNA (cluster 17) was strongly up-regulated at early time-points after bacterial encounter and down-regulated at later time-points (**Figs. 2 and 3**).

Activation with bacteria induces DC maturation and survival<sup>18</sup>. The anti-apoptotic genes that were up-regulated after bacteria encounter were mouse inhibitor of apoptosis protein 1 (MIAP-1, cluster 17), MIAP-2 (cluster 11), Bcl-x (cluster 22), TNF receptor-associated factor 1 (TRAF1, cluster 23) and TRAF2 (cluster 29). Conversely, Bcl-2 mRNA was already down-regulated 4 h after bacterial stimulation (cluster 0), which showed that it was not involved in maintaining mature DC survival (**Fig. 3**). Expression of members of the Vav family and WASp, proteins involved in actin reorganization downstream of specific signals<sup>19</sup>, was regulated during maturation (**Fig. 3**).

### Induction of IL-2 production by DCs

We observed that cluster 32 contained the transcript encoding the T cell growth factor IL-2. Bacterial encounter induced transient IL-2 mRNA up-regulation in D1 cells at early time-points (4–6 h) after activation (**Fig. 4a**). We therefore tested the hypothesis that this cytokine could be an important costimulatory molecule produced by DCs. IL-2 expression was confirmed by semi-quantitative polymerase chain reaction (PCR) analysis of fresh mBMDCs, which showed expression kinetics that were similar to those of D1 cells (**Fig. 4b**). Protein secretion was measured by enzyme-linked immunosorbent assay (ELISA). Accumulation of IL-2 in the supernatant was observed at 4–8 h and again 14–18 h after activation (**Fig. 4c**). To test whether IL-2 production after bacterial encounter was DC-specific, we did the same analysis on bone marrow-derived macrophages. After activation with bacteria, supernatants were collected at similar time-points and IL-2 measured. No IL-2 production by macrophages was observed (**Fig. 4c**). This indicated that, after bacterial activation, IL-2 was specifically induced only in DCs.

### DC-derived IL-2 mediates T cell activation

The role of IL-2 produced by early bacteria-primed DCs in T cell activation was investigated by analyzing the capacity of IL-2<sup>-/-</sup> and wild-type DCs to stimulate alloreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in a primary mixed lymphocyte reaction (MLR) assay. mBMDCs from wild-type and IL-2<sup>-/-</sup> mice<sup>20</sup> were activated with bacteria at a multiplicity of infection (MOI) of 10 (**Fig. 1**). Wild-type and IL-2<sup>-/-</sup> DCs were equally activated by bacterial stimulation (**Fig. 5a**) and did not show any difference in viability (**Fig. 5b**). CD4<sup>+</sup> or CD8<sup>+</sup> allogeneic T cells were added to early bacteria-primed DCs (4–7 h after activation) and DNA synthesis tested by thymidine incorporation after three days of culture. The ability of IL-2<sup>-/-</sup> DCs to induce T cell proliferation was severely impaired (**Fig. 5c**).

We also directly examined T cell division by flow cytometry analysis. CD4<sup>+</sup> or CD8<sup>+</sup> allogeneic T cells labeled with the cell dye carboxyfluorescein diacetate succinimidyl ester (CFSE) were added to early bacteria-activated wild-type and IL-2<sup>-/-</sup> DCs. The number of mBMDCs able to induce maximal T cell proliferation activity, as identified in the previous experiment, was used in this MLR assay. Very few cycling T lymphocytes were observed in IL-2<sup>-/-</sup> DC cultures (**Fig. 5d**). This was not due a nonspecific lack of DC function because IL-2<sup>-/-</sup> DCs were able to activate T cells, as judged by the increased number of blast cells

expressing the early activation marker CD69 after 48 h of culture (**Fig. 5e**). No CD69 up-regulation was observed on T cells cultured in absence of DCs (data not shown).

## Discussion

To identify genes that confer DCs with a high T cell stimulatory capacity, we examined, with kinetic global transcription analysis, immature DCs and mature cells stimulated by Gram-negative bacteria. The number of genes expressed at different stages of DC ontogeny was similar and encompassed ~30% of genes displayed on the arrays. Thus, approximately the same number of genes were induced and suppressed at different time-points after activation. The diversity of transcripts expressed in immature, transitional and mature DCs were similar in magnitude, as has already been suggested for resting and activated T cells<sup>21</sup>.

The entire dataset was visualized with the PCA method. In these types of studies, PCA allows one to describe and distinguish a process of cellular activation from a process of cellular differentiation. Activation is a reversible process: upon exposure to a given stimulus, the cells undergo transitional functional and phenotypic modifications and then return to their original state. In contrast, the differentiation event is an irreversible process that induces progression toward a new functional state. As the gene expression profile is a major determinant of cellular phenotype and function, PCA applied to a cell gene expression pattern in a kinetic study allows the visualization of similarities among different states within the same cell after stimulation. In the case of DCs as visualized by PCA, the cells undergo differentiation because their gene expression profile shows not only a profound reprogramming at early time-points, which would be consistent with activation, but it also progresses to a new, distinct steady-state. As indicated by PCA, the process of DC differentiation is quite rapid. During the 24 h after bacterial encounter, DCs experience all the transcription modification necessary to progress from immature to mature cells.

As expected, activation with bacteria induced the modulation of many genes involved in cytoskeleton rearrangements, antigen processing, control of migration and apoptosis and regulation of inflammatory responses. In particular, many factors that modulate the dynamic properties of actin filaments were differentially expressed during maturation. These factors were proteins involved in coupling actin filaments to the cell surface, for example, members of the VASP family or proteins involved in cross-linking actin filaments, such as fascin, or in severing them, such as gelsolin<sup>22</sup>. In addition, the Vav proteins that induce typical Rac-1 and RhoG-like cytoskeletal changes—including cell spreading, membrane ruffling and the formation of lamellipodia as a consequence of extensive reorganization of F-actin<sup>23</sup>—were also regulated through transcription. Constitutive, but modulated, expression of the transcript overlapping myelin, the transcript expressed outside the central nervous system, was observed in D1 cells (cluster 23). Thymic DCs express tissue-specific antigens to negatively select autoreactive T cells<sup>24</sup>; analogously, peripheral DC expression of sequestered antigens could be a mechanism for maintaining peripheral tolerance.

The most unanticipated finding of this study was that DCs produce IL-2 in a tightly regulated time-frame. Thus, the adjuvant property of bacteria is explained by induction, in DCs, not only of the up-regulation of costimulatory surface proteins and the maximization of the efficiency in presenting antigens<sup>5</sup>, but also by induction of the production of costimulatory molecules such as IL-2. This seems to be a unique feature of DCs because macrophages are unable to produce IL-2 upon bacterial activation. Two waves of IL-2 production by DCs after bacterial encounter were observed. The first was 4–8 h after bacterial uptake and



the second was 14–18 h after activation. This timing was compatible with the appearance of MHC class II<sup>+</sup>-peptide and MHC class I<sup>+</sup>-peptide complexes at the cell surface<sup>4,8,25,26</sup>. DCs are able to present exogenous captured antigens to CD4<sup>+</sup> T cells in a few hours, whereas at least 8 h are required to process and present bacterial antigens in association with MHC class I molecules<sup>4</sup>. Thus, early-activated DCs are perfectly equipped to prime CD4<sup>+</sup> T cells, despite their relatively low expression of MHC and membrane-associated costimulatory molecules and production of T cell-inhibitory cytokines, such as IL-10<sup>16</sup>. At later time-points, IL-2 could represent a key costimulatory protein in activating CD8<sup>+</sup> T cells, even though DCs have not yet reached their terminal maturation stage. This data could explain the ability of activated DCs to prime CD8<sup>+</sup> T cell in a CD4-independent manner<sup>27</sup>. When the frequency of responder antigen-specific T cells or their affinity for MHC<sup>+</sup>-peptide complexes is low, exogenous sources of IL-2 for induction of T cell proliferation may be also required, as can frequently happen *in vivo* during immune responses to microorganisms.

Signals induced by T cell receptors and IL-2 are involved in activating and maintaining transcription of the IL-2 receptor (IL-2R)  $\alpha$  chain, which forms, together with the  $\beta$  and  $\gamma$  chains, the high affinity IL-2R. The  $\alpha$  subunit is up-regulated *in vivo* at the surface of specific T cells 8 h after superantigen injection<sup>21</sup>. The reduction in IL-2 concentration in the supernatant, 8–10 h after activation, could be explained by reuptake of the cytokine by DCs expressing IL-2R<sup>28</sup>. It could be important that the release of IL-2 by DCs is efficiently controlled to avoid “bystander activation” of unrelated T cells. Secretion of IL-2 could also be necessary to counteract the effect of IL-10, which is produced by early-activated DCs<sup>16</sup> and which we also found at a high concentration (data not shown). In absence of IL-2, IL-10 has a well documented inhibitory function in alloantigen responses<sup>29</sup>.

IL-2 is also thought to activate natural killer (NK) cells *in vitro*. However, this effect has never been considered relevant *in vivo* during immunocompetent responses. It was commonly believed that IL-2 is exclusively produced by T cells during the acquired immune response, whereas activation of NK cells occurs prior to that, during the innate response<sup>30</sup>. As DCs can produce IL-2 early after activation, this assumption should be revised. DCs can activate NK cell responses in direct NK cell–DC interactions<sup>11</sup>; thus IL-2 is likely to be the relevant and obvious costimulatory factor. The finding that DCs can produce IL-2 at early time-points after bacterial uptake suggests a primary role of DCs in the activation of innate responses; it also helps explain the unique ability of these cells to prime T lymphocytes. This observation was made more than 20 years ago<sup>31</sup>, but, until now, the molecular events responsible for T cell priming were not understood.

## Methods

**DC, macrophages and culture medium.** D1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 100 IU of penicillin, 100  $\mu$ g/ml of streptomycin, 2mM L-glutamine (all from Sigma) and 50  $\mu$ M  $\beta$ -mercaptoethanol (in complete IMDM) with 30% supernatant from R1 medium (supernatant from NIH3T3 fibroblasts transfected with GM-CSF)<sup>3</sup>. Macrophages and DCs were derived from bone marrow cells collected from four different mice and divided in two separate cultures. mBMDCs were collected after 14 days of bone marrow culture in 30% R1 medium. Macrophages were collected after 14 days of bone marrow culture in presence of M-CSF. Wild type and IL-2<sup>−/−</sup> DCs were from mice from the same litter. mBMDCs were used for bacterial activation only if they were immature, as judged by low B7-2 expression and absence of CD40. Cells that showed a partial spontaneous activation were discarded.

**Infection with bacteria.** *E. coli* (DH5 $\alpha$  strain) were grown overnight in Luria-Bertani medium (LB). Samples (100  $\mu$ l) were inoculated in 10 ml of fresh LB and grown for an additional hour at 37 °C. Bacteria were added to cell cultures at a MOI of 10. Cultures were incubated for 1.5 h. DC and macrophage cultures were washed and supplemented with gentamycin and tetracycline at a final concentration of 50  $\mu$ g/ml and 30  $\mu$ g/ml, respectively.

**Sample preparation and array hybridization.** Antisense cRNA was prepared following Affymetrix (Santa Clara, CA) recommendations. Briefly, total RNA was extracted from frozen pellets with the Trizol procedure. The Oligotex kit from Qiagen (Chatsworth, CA) was used to purify mRNA. Double-stranded cDNA was retro-transcribed with a modified oligo(dT) primer with a 5' T7 RNA polymerase promoter sequence and the Superscript Choice System for cDNA synthesis (Life Technologies, Gaithersburg, MD). Double-stranded cDNA (1  $\mu$ g) was transcribed to cRNA with the ENZO kit (Affymetrix). cRNA was purified on an affinity column (Rneasy, Qiagen) and then fragmented to an average size of 50–200 bp, by incubation for 35 min at 94 °C in 40 mM Tris-acetate at pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate. Samples were diluted in the hybridization solution (1 M NaCl, 10 mM Tris at pH 7.6, 0.005% Triton X-100, 0.1 mg/ml of herring sperm DNA, BioB, BioC, BioD and cre control cRNAs at concentrations of 1.5, 5, 25 and 100 pM, respectively) at a final concentration of 0.05  $\mu$ g/ml and heated to 94 °C for 5 min. Analysis of the samples was done by hybridizing the fragmented cRNAs to the Affymetrix Mu1k GeneChip<sup>®</sup> array, which consisted of two individual chips (called A and B) that collectively represented ~11,000 murine genes and ESTs. Probe array hybridizations were carried out, as described<sup>32</sup>, by placing the samples in the hybridization cartridge at a final volume of 200  $\mu$ l/chip. Hybridizations were done under rotation at 45 °C for 16 h. After hybridization, the chips were rinsed with 6 $\times$ 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), 0.5 $\times$  SSPE-T and stained by incubation with 2  $\mu$ g/ml of phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) and 1 mg/ml of acetylated bovine serum albumin (Sigma). The arrays were read at a resolution of 7.5  $\mu$ m by a confocal scanner and analyzed with the MicroArray Suite 4.0 Gene Expression analysis program (both from Affymetrix).

**PCA analysis.** Briefly, we first rewrote our dataset in terms of a matrix in which the 9930 rows represented the genes and ESTs (data observations) and the 14 columns represented the seven different time-points in duplicate (independent variables). The  $a_{ij}$ th element of the matrix was the average difference, which represented gene expression<sup>14</sup>, of the  $i$ th gene at the  $j$ th experimental condition. The method proceeded through a unitary transformation (rotation) of the matrix, which returned two distinct matrices: the eigenvector matrix and the eigenvalue matrix. The columns of the eigenvector matrix were the PCs of the dataset and the rows were the time-points in duplicate. The eigenvalues represented the percentage of the overall variance that each component described. PC1, PC2, PC3 and PC4 together were able to describe >98% of the entire variance.

**PCR primers and IL-2 ELISA.** The sequences of the PCR primer pairs (5'→3') used were as follows. IL-2: sense, TCCTCACAGTGACCTCAAGTCC; antisense, TGACAGAAG-GCTATCCATCTCC.  $\beta$ -actin: sense, CATCGTGGGCGCGCTCTAGGCAC; antisense, CCG-GCCAGCCAAGTCCAGACGC. IL-2 ELISA was done with the DuoSet kit (R&D Systems, Minneapolis, MN), following the manufacturer recommendations.

**MLR.** Wild-type and IL-2<sup>−/−</sup> DCs (5 $\times$ 10<sup>5</sup>) were activated with bacteria and 4–7 h later incubated with 2 $\times$ 10<sup>6</sup> CFSE-labeled T cells. T cell division was assessed by FACS analysis 48 or 72 h later. Alternatively graded numbers of bacteria-activated DCs were incubated with 2 $\times$ 10<sup>5</sup> T cells and proliferation tested by [<sup>3</sup>H]thymidine incorporation 72 h later. CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were purified (to 99% purity) from BALB/c or C57BL/6 mice lymph nodes by negative selection of macrophages, DCs, B cells and CD4<sup>+</sup> or CD8<sup>+</sup> T cells. These cell populations were eliminated with the MiniMACS columns (Miltenyi Biotec, Auburn, CA, GmbH) after preincubation with Mac1, Cd11c, B220 and CD4 or CD8 antibodies (all from PharMingen, San Diego, CA).

**Hierarchical clusters.** Clusters showing the different types of gene expression profiles were obtained with the Xcluster program (<http://genome-www.stanford.edu/~sherlock/cluster.html>). A filter was used to examine genes that showed a difference in expression of at least 3 between the maximum and minimum kinetic expression values. Genes were first clustered with the SOM algorithm; then the hierarchical method was applied to each SOM cluster. After various attempts at clustering, 36 clusters was chosen as the most appropriate number: higher numbers resulted in the multiplication of clusters with very similar profiles and lower numbers did not give good correlation coefficients in the hierarchical clusters.

**Mice.** Pathogen-free C57BL/6 and BALB/c mice were from Harlan Italy (Milan, Italy). C57BL/6 IL-2<sup>−/−</sup> mice were kept in pathogen free conditions. All experiments were done in compliance with the relevant laws and institutional guidelines.

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1. Banchereau, R. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
2. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and downregulated by tumor necrosis factor  $\alpha$ . *J. Exp. Med.* **179**, 1109–1118 (1994).
3. Winzler, C. *et al.* Maturation stages of mouse dendritic cells in growth factor-dependent long-term



- cultures. *J. Exp. Med.* **185**, 317–328 (1997).
4. Rescigno, M. *et al.* Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Natl Acad. Sci. USA* **95**, 5229–5234 (1998).
  5. Banchereau, J. *et al.* Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18**, 767–811 (2000).
  6. Lutz, M. B. *et al.* An advance culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Meth.* **223**, 77–92 (1999).
  7. Pierre, P. *et al.* Development regulation of MHC class II transport in mouse dendritic cells. *Nature* **388**, 787–792 (1997).
  8. Rodriguez, A. *et al.* Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nature Cell Biol.* **1**, 362–368 (1999).
  9. Rescigno, M. *et al.* Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1 $\beta$ , and the production of interferon  $\gamma$  in the absence of IL-12 during DC-T cell cognate interaction. A new role for fas ligand in inflammatory responses. *J. Exp. Med.* **192**, 1661–1668 (2000).
  10. Singh-Jasuja, H. *et al.* Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J. Exp. Med.* **191**, 1965–1974 (2000).
  11. Fernandez, N. C. *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. *Nature Med.* **5**, 405–411 (1999).
  12. Raychaudhuri, S., Stuart, J. M. & Altman, R. B. Principal components analysis to summarize microarray experiments: application to sporulation time series. *Proceedings of the Pacific Symposium on Biocomputing* 455–466 (2000).
  13. Tamayo, P. *et al.* Interpreting pattern of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc. Natl Acad. Sci. USA* **96**, 2907–2912 (1999).
  14. Lockhart, D. J. *et al.* Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnol.* **14**, 1675–1680 (1996).
  15. Staudt, L. M. & Brown, P. O. Genomic views of the immune system. *Annu. Rev. Immunol.* **18**, 829–859 (2000).
  16. Langenkamp, A., Messi, M., Lanzavecchia, A. & Sallusto, F. Kinetics of dendritic cell activation: impact on priming of Th1, Th2 and nonpolarized T cells. *Nature Immunol.* **1**, 311–316 (2000).
  17. Sallusto, F. *et al.* Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* **29**, 1617–1625 (1999).
  18. Rescigno, M. *et al.* Dendritic cell survival and maturation are regulated by different signaling pathways. *J. Exp. Med.* **188**, 2175–2180 (1998).
  19. Bustelo, X. R. Regulatory and signaling properties of the Vav family. *Mol. Cell. Biol.* **20**, 1461–1477 (2000).
  20. Kramer, S. *et al.* Thymic selection and peptide-induced activation of T cell receptor-transgenic CD8 T cells in interleukin-2-deficient mice. *Eur. J. Immunol.* **24**, 2317–2322 (1994).
  21. Teague, T. K. *et al.* Activation changes the spectrum but not the diversity of genes expressed by T cells. *Proc. Natl Acad. Sci. USA* **96**, 12691–12696 (1999).
  22. Borisy, G. G. & Svitkina, T. M. Actin machinery: pushing the envelope. *Curr. Opin. Cell Biol.* **12**, 104–112 (2000).
  23. Movilla, N. & Bustelo, X. N. Biological and regulatory properties of Vav-3, a new member of the Vav family of oncoprotein. *Mol. Cell. Biol.* **19**, 7870–7885 (1999).
  24. Klein, L. *et al.* Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nature Med.* **6**, 56–61 (2000).
  25. Cella, M. *et al.* Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782–787 (1997).
  26. Lutz, M. B. *et al.* Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J. Immunol.* **159**, 3707–3716 (1997).
  27. Schuurhuis, D. H. *et al.* Immature dendritic cells acquire CD8 $^{+}$  cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. *J. Exp. Med.* **192**, 145–150 (2000).
  28. Kronin, V., Vremec, D. & Shortman, K. Does the IL-2 receptor  $\alpha$  chain induced on dendritic cells have a biological function? *Int. Immunol.* **10**, 237–240 (1998).
  29. Zeller, J. C. *et al.* Induction of CD4 $^{+}$  T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF- $\beta$ . *J. Immunol.* **163**, 3684–3691 (1999).
  30. Biron, C. A. *et al.* Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**, 189–220 (1999).
  31. Steinman, R. M. *et al.* Dendritic cells of the mouse: identification and characterization. *J. Invest. Dermatol.* **75**, 14–16 (1980).
  32. Wodicka, L. *et al.* Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nature Biotechnol.* **15**, 1359–1367 (1997).

