

Gene Expression Profiling in Immune Cells Using Microarray

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T cell · T helper 1 · T helper 2 · Dendritic cell · DNA microarray

Abstract

The recent development of DNA microarray, which offers the opportunity to study the expression of thousands of individual genes simultaneously in different biological systems, has provided new insights into the immune system. Examples discussed in this review include molecular descriptions of the differentiation program of T helper (Th) cells into Th1 and Th2 pathways and the genetic program underlying maturation of dendritic cells. It is anticipated that this new information can be used to understand gene function in both physiological and pathological conditions of the immune system.

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Introduction

Complex interactions regulate the functions of living organisms. Understanding how the constituents of a biological system interact with each other is as important as

understanding their function in a reductionistic experimental model, which does not take into account biological complexity. The generation of simple and controlled experimental models allowed the acquisition of a large amount of data. Nevertheless, this information now needs to be integrated so that we can comprehend how the different constituents interact in the sequences of events that guarantee life. Interaction of diverse disciplines, such as biology, physics and informatics, is now necessary to manage this complexity. Gene expression analysis can represent the beginning of the chain process to completely solve cell functions at the molecular level. For example, a typical feature of T and B cells of the immune system is their replication capacity, i.e., the ability to duplicate the DNA to undergo mitosis and eventually to die by apoptosis. These activities require the intervention of a multiplicity of gene products. Since the genes of an organism code the programs for these activities and gene transcription is necessary to execute these programs, gene expression analysis at different stages of cell cycle might enable us to identify genes that are important for cell cycle regulation and genes responsible for inducing apoptosis. Cells of our organism are also extremely susceptible to environmental changes, such as changes induced by interleukins, hormones or drugs. Cellular responses that imply gene

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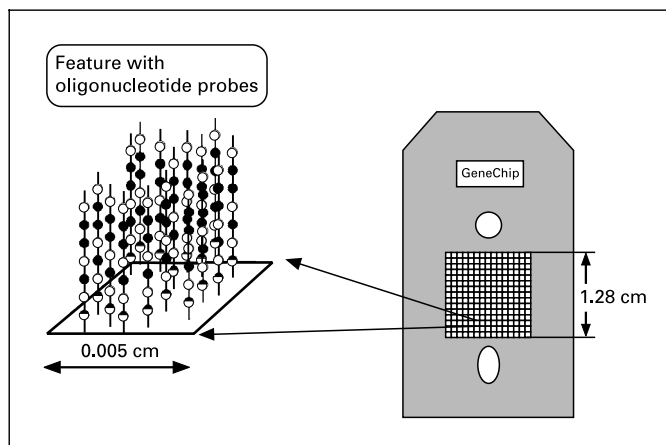


Fig. 1. Schematic GeneChip representation. GeneChips are constituted by a glass wafer of 1.28 cm $\times$ 1.28 cm containing oligonucleotide probes directly synthesized on it. GeneChips are subdivided in features of 0.005 cm $\times$ 0.005 cm, each containing millions of copies of a particular 20-mer oligonucleotide probe.

transcription and regulation can be investigated by comparing levels of gene expression of resting versus stimulated cells. For a long time molecular biologists have tried to develop systems to identify new genes and to associate those genes with particular cell functions. Technologies, such as subtractive libraries, RDA and differential display, have been set up to investigate expressed genes in different cell conditions. Even though these systems have led to the identification of important proteins, they are not efficient and cannot be automated. Until now, the only efficient approach has been the sequencing of all of the genes expressed in different tissues. This procedure is extremely long and expensive, and does not give any information concerning the modulation of gene expression levels.

Technology

GeneChip Development

In 1991, Stephen Fodor, a research scientist working at Affymax, an American company interested in the identification of new drugs by the screening of peptide libraries, conceived a new method that would allow the simultaneous comparative expression analysis of thousands of genes in different cell types. Stephen Fodor is now founder and CEO of a company, Affymetrix (Santa Clara, Calif., USA), which develops and commercializes this technology. Fodor's idea was to deposit thousands of oligonu-

cleotide probes of different genes on a solid glass support to analyze the expression of thousands of different genes at the same time.

Oligonucleotide probes are directly synthesized on a glass support which is subdivided into features, each containing millions of copies of a given oligonucleotide probe (fig. 1); 20 distinct features represent the entire gene probe. Each gene is represented by 20 distinct oligonucleotides chosen to distinguish members of closely correlated families or splicing variants of the same gene. Moreover, to minimize the effects of an unspecific hybridization, each feature is flanked by a control feature that contains control oligonucleotides, that is oligonucleotides identical to the oligonucleotide probes, except for a single mismatch in the central region. Typically, the correct nucleotide is replaced by the complementary nucleotide (fig. 2). A gene is considered expressed if it is possible to unequivocally identify hybridization with oligonucleotide probes, but not with control ones [1]. Genes and expressed sequence tags displayed on the chips are selected from public databases.

At present, GeneChips are available that display probes for the entire *Escherichia coli* and *Saccharomyces cerevisiae* genome, and for half of the human and mouse genomes [2]. GeneChips are produced by adapting the semiconductor photolithography technology to the chemical synthesis of oligonucleotides [3]. A surface of a solid support modified with photolabile protecting groups is illuminated through a photolithographic mask yielding reactive hydroxyl groups in the illuminated region. A 3'-O-phosphoramidite-activated deoxynucleoside, protected at the 5'-hydroxyl with a photolabile group, is then presented to the surface. Coupling occurs at sites that were exposed to light. After capping and oxidation, the substrate is rinsed and the surface is illuminated through a second mask to expose additional hydroxyl groups for coupling (fig. 3). The process is repeated until the entire number of oligonucleotide probes and control have been synthesized.

To measure gene expression levels, samples are prepared according to the following scheme: a cDNA, prepared starting from total RNA or mRNA, is retro-transcribed to produce a cRNA using biotinylated oligonucleotides. By this reaction, the mRNA is amplified from 20 to 200 times (fig. 4). cRNA mixture is then fragmented to improve efficiency of the hybridization reaction and exposed to the chip. Hybridized biotinylated cRNA is then visualized by using phycoerythrin-conjugated streptavidin. As a final result, some chip features will be highly fluorescent, some others partially fluorescent or nonflu-

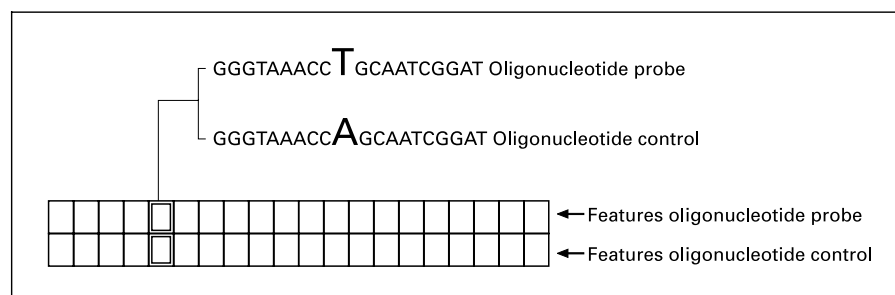


Fig. 2. 20 complementary 20-mers constitute a complete gene probe based on automated selection criteria. These criteria include tests for sequence uniqueness relative to the rest of the genome and the possibility to distinguish among different splicing variant of a given gene. To increase the sensitivity and specificity of detection, each comple-

mentary oligonucleotide (perfect match) is synthesized with a closely related mismatch partner in a physically adjacent position. The mismatch partner is identical to the perfect match except for a single base difference at the central position of the 20-mer.

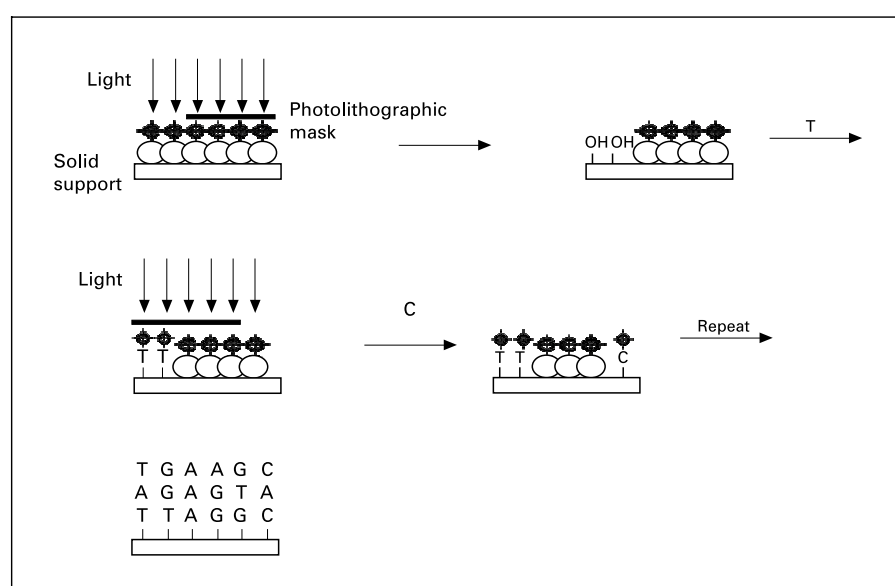


Fig. 3. A surface bearing photoprotected hydroxyls is illuminated through a photolithographic mask, generating free hydroxyl groups in the photodeprotected region. The hydroxyl groups are then coupled to a deoxynucleoside phosphoramidite. Cycles of illumination and coupling are repeated until the desired set of products is obtained.

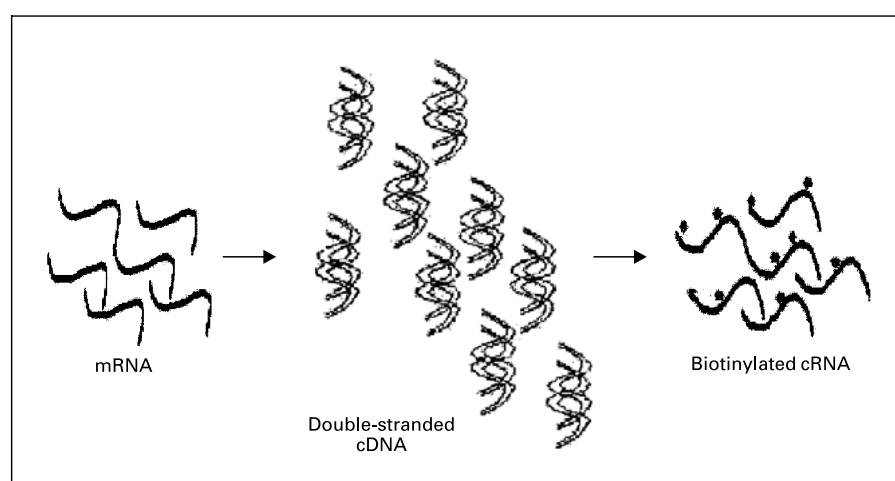
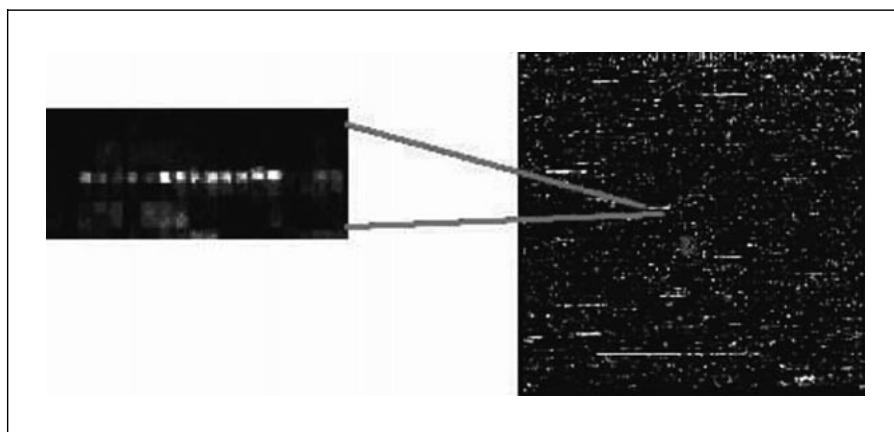


Fig. 4. mRNA is retrotranscribed to produce double-stranded cDNA. From the cDNA, a complementary cRNA is synthesized by using biotinylated nucleotides.

Fig. 5. GeneChip image after hybridization. Fluorescence intensity correlates directly with the frequency of a particular type of cRNA inside the reaction mixture. A Gene-Chip area is shown where probes, but not oligonucleotide controls, have hybridized with a particular type of cRNA.



rescent (fig. 5). The hybridized GeneChip is then scanned using a confocal system. Data are transferred to a computer that calculates gene expression by comparing the level of hybridization of oligonucleotide probes with the control ones. Moreover, since fluorescence intensity directly correlates with the concentration of a particular mRNA in the cell, the algorithm also provides quantitative evaluations of gene expression levels. GeneChips can identify genes with very low frequencies in the reaction mixture (1/300,000), which allows quantitative analysis in four orders of magnitude [1, 2].

Given the huge amount of information that can be obtained from a simple analysis, data interpretation is not a negligible task. A close collaboration between informatic and bioinformatic scientists is necessary for the realization of data mining software. These studies will allow us to obtain information concerning the integrated role of different proteins in the complexity of biological functions. Thus, storage analysis and interpretation of this information represent a major challenge.

Classification of Gene Expression Data

A classification scheme is a method for organizing large amounts of data in order to make the retrieval of information efficient. For gene expression studies, this involves finding similar gene expression patterns by comparing profiles. Cluster analysis, where genes are grouped according to their profile of expression, is the established approach of classification. Basically, two types of clustering are used, hierarchical and nonhierarchical. Differences in the diverse algorithms produce different interpretations of the data. Thus, it is recommended to use more than one algorithm to get the maximal information.

Usually, clusters obtained with hierarchical methods do not overlap with clusters obtained with stochastic methods, and one possibility is to apply two mathematically distinct methods in sequence to exclude possible bias towards one approach or the other.

In hierarchical clustering, data points are forced into a strict hierarchy of nested subsets: the closest pair of points is grouped and replaced by a single point representing their set average, the next closest pair of points is treated similarly, and so on. The data points are thus fashioned into a phylogenetic tree whose branch lengths represent the degree of similarity between the sets. Hierarchical clustering has recently been described for gene expression and has clearly proven valuable [4].

A further approach used is the self-organizing maps: gene expression data are transformed into vectors or coordinates in an n -dimensional space, where n represents the number of variables or time points. The vectors are displayed in a simple map (for example two-dimensional space), where similar vectors are placed next to each other and different ones on the opposite side [5].

Finally, principal component analysis represents another useful mathematical method that allows one to get an approximate visualization of the entire data set without losing experimental information (variance) [6]. Principal component analysis allows the dimensionality of complex data to be reduced and to globally describe the features of the kinetic points that best explain the corresponding gene expression profile.

Gene Expression Analysis of the Immune System

A high degree of functional specialization is a common feature of the multiple cellular components of innate and adaptive immunity. As adaptive immune responses develop to fight specific pathogens, B and particularly T cells show a striking level of functional adaptation in response to different pathogens. A first functional distinction among T lymphocytes identifies naïve and memory cells based on their antigen experience. A second functional distinction has been made among effector/memory T cells based on a mutually exclusive pattern of secreted cytokines. These two distinct types of CD4⁺ T helper (Th) cells control distinct and opposing immune effector functions. Th1 cells secreting IFN- γ and TNF induce phagocyte-dependent immune responses, exemplified by the delayed type hypersensitivity reaction. They play a critical role in fighting microbial and viral infections, but can also cause tissue damage if perturbed Th2 cells, secreting IL-4, IL-5 and IL-13, promote IgE production and eosinophil function, both of which play a role in the pathogenesis of allergic reactions.

Initially, naïve T cells must determine whether antigen is present and whether it poses a threat to the body. This information is provided by antigen-bearing dendritic cells (DC) inside secondary lymphoid organs. DC are the most potent antigen-presenting cells (APC) for Th cells and seem to be able to instruct the T cells via the secretion of cytokines, such as IL-12, to differentiate along the Th1 or the Th2 pathway. The environment in which the DC have been stimulated, the type of stimulus and the origin of the DC all play a part in the fate of the T cell response.

Transcript Imaging of Th Cell Development Using DNA Microarray

Recent experiments have yielded gene expression profiles of the development of Th cell subtypes [7–9]. Th1 and Th2 cells develop from naïve CD4⁺ T cells. The differentiation process is initiated by ligation of the T cell receptor and directed by cytokines present during the initiation of a T cell response. In recent years, details of the pathways leading to Th differentiation have begun to emerge. Transcription factors controlling type 1 and 2 differentiation include the type-2-associated factors GATA-3 [10], *c-maf* [11] and Stat6 [12], as well as the type 1 factors T-bet [13], Stat4 [14] and IFN regulatory factor 1 [15, 16]. What initiates transcription factor activity is unclear, but may relate to the type and dose of Ag [17, 18] or the nature of APC [19, 20]. However, the ulti-

mate determinant of T cell polarization is the cytokine milieu present at the time of T cell activation. IL-4 promotes Th2 development, whereas IL-12, produced primarily by macrophages and DC in vivo, drives Th1 development. The functional program of type 1 and type 2 T lymphocytes requires these cells to home to different sites. Th1 cells have been shown to preferentially express the chemokine receptor CCR5 and CXCR3, whereas Th2 cells were reported to preferentially express CCR3, CCR4, CCR8 and the chemoattractant receptor CRTh2 [21]. Other cell surface molecules also distinguish type 1 and type 2 T cells, including IL-12R β 2 [22, 23], ST2 [24], IL-18R [25] and P-selectin glycoprotein ligand 1 [26].

Because dysregulation of the Th cell differentiation process can result in Th1-driven inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis and psoriasis or in Th2-driven disease states, such as allergy and asthma [27], several studies have tried to elucidate the genetic program that controls the functional properties of Th1 versus Th2 cells using DNA microarray.

Recent gene expression profiling experiments have reported differences in gene expression between both human and mouse type 1 and type 2 cells [7–9]. Human cord blood or mouse spleen CD4⁺ T cells were expanded in vitro under conditions that would lead to differentiation to a Th1 phenotype (IL-12 plus neutralizing antibodies to IL-4) or to a Th2 phenotype (IL-4 plus neutralizing antibodies to IL-12). As expected, Th cell subsets did express their signature cytokines. Transcripts for IFN- γ and TNF- α were preferentially expressed on Th1 cells as compared to Th2 cells [7–9]. Higher levels of IL-4, IL-5 and IL-13 were found on Th2 cells [8, 9] instead. The simultaneous expression of the Th2 cytokines was expected, as they are all transcribed from a single gene cluster as a result of a coordinated regulation [28, 29]. In one study, two members of the TNF-receptor-associated factor (TRAF) family were found to be expressed differently in type 1 and type 2 cells [8]. TRAF4 was expressed at a higher level in type 1 cells, while TRAF5 was preferentially expressed in type 2 cells. Members of this family serve as adapter proteins that mediate cytokine signaling. In particular, they seem to play a role in TNF and IL-1 signaling, resulting in activation of transcription factors NF- κ B and AP-1 [30].

The gene expression profiles of type 1 and type 2 cells also revealed differential expression of genes encoding transcription factors, some of which (GATA2, *c-maf*, STAT4 and IRF-1) have been previously characterized [10, 14–16, 31]. In addition, several transcription factors that have not been associated with T cell polarization

were also identified, including ETS-1, ROR α 2, IRF-7A and *c-fos*. Although the target genes of these factors in regulating the gene expression patterns specific to each Th cell subset are not known, it is possible that some of these factors might control individual cytokine gene expression, as GATA3 and T-bet control IL-4 and IFN- γ production, respectively.

Th1 cells are more susceptible to activation-induced cell death (AICD) [32], a mechanism for downregulation of an immune response and maintenance of T cell tolerance. Results from gene expression analysis suggested a potential mechanism for Th1 AICD [7], as Th1 cells express higher levels of TRAIL than Th2 cells, an apoptosis-inducing molecule, of BAK, a proapoptotic Bcl-2 family member, and of proapoptotic caspase-8, perforin and granzyme B. Many of the apoptosis-related genes were further upregulated by additional IL-12 treatment. Indeed, incubation of Th1 cells with IL-12 resulted in an increased proportion of Th1 cells, but not Th2 cells, undergoing AICD. Thus, in addition to promoting Th1 differentiation, by acting on differentiating naïve precursors, IL-12 also affects the phenotype and expression profile of already differentiated Th1 cells [33].

Other gene expression changes defined in these studies were consistent with previous experiments, defining differential recruitment of Th1 and Th2 cells to sites of inflammation. One study [7] has reported an increased expression of mRNA for fucosyltransferase VII, which codes for an enzyme that mediates the fucosylation of selectin ligands on the surface of T cells. This fucosylation is required for the first step of lymphocyte adhesion to endothelial cells, i.e. 'rolling'. Recent *in vivo* observations have validated the biological relevance of this finding. Fucosyltransferase VII was, in fact, found to be upregulated on T cells, infiltrating the inflamed joints of patients affected by either rheumatoid arthritis [7] or juvenile idiopathic arthritis [De Benedetti et al., in preparation]. In both diseases, the T cells infiltrating the synovium have a clear Th1 phenotype.

IL-12 also induced two chemokine receptors, CCR5 and CCR1, both of which promote increased responsiveness of Th1, but not Th2 cells to MIP-1 α or RANTES. The activity of RANTES and other chemokines is regulated by CD26 (dipeptidyl-peptidase IV)-mediated cleavage [34]. The DPP4 (encoding CD26) mRNA was found upregulated in Th1 cells compared with Th2 cells. The inactivation of chemokines by CD26 may contribute to the fine control of chemotactic migration of Th1 cells by providing a stop signal that keeps cells at the site of inflammation. Finally, higher expression on Th1 cells,

and further upregulation by IL-12, or integrin α 6 β 1 [7, 35] suggest that adhesion and extravasation of Th1 cells into tissues triggered by inflammatory chemokines might then be mediated by higher surface levels of integrin α 6 β 1 binding to laminin in basal membranes and extracellular matrix. In contrast, mRNA for the integrin α 4 β 7 [7] was increased in Th2 in comparison to Th1 cells. As integrin α 4 β 7 mediates binding of lymphocytes to the mucosal vascular addressing MAdCAM-1, these data predict a preferential homing of Th2 cells to mucosal sites.

Analysis of DC Maturation Using the GeneChip Approach

DC are professional APC, which are able to initiate innate and adaptive immune responses [36, 37]. 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 nonlymphoid to lymphoid tissues or, within lymphoid tissues, to migrate from the marginal zones to the T cell areas. Migrated and mature DC have high cell surface major histocompatibility complex (MHC) and costimulatory protein expression, have the ability to activate CD8+ and CD4+ T cell responses [38, 39], and are programmed for apoptotic death [40]. The process of DC maturation can be reproduced *in vitro*. Immature monocyte-derived human DC (hMDC) or immature bone-marrow-derived mouse DC can be induced to mature *in vitro* using many different stimuli, including inflammatory cytokines, bacterial cell products, such as lipopolysaccharide (LPS) and lipoteichoic acid, bacterial DNA and double-stranded viral RNA, and live bacteria.

DC maturation has been investigated at the molecular level using microarray approaches. A transcription profile of immature and LPS-matured hMDC has been carried out, revealing 225 differentially expressed genes in the two situations out of a total of 10,962 genes screened [41]. These genes mainly consisted in 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 and CD23 glucose-6-phosphatase. Moreover, oligonucleotide microarrays have been used to measure gene expression of hMDC in response to different types of stimuli, such as gram-negative bacteria (*E. coli*), yeast

(*Candida albicans*) and viruses (influenza virus). Immediately after contact with any of the three pathogens, a rapid downregulation of gene associated with phagocytosis and pathogen recognition, and a transient increase in transcripts for cytokines, chemokines and receptors that contribute to the recruitment of leukocytes at the site of infection has been documented [42]. Together with a common response to the different pathogens, a microbe-specific response has also been observed in most functional category genes. Stimulus-specific activation and maturation of hMDC clearly indicate plasticity in their functions. Plasticity is an important DC feature, since immune response outcome depends on their functional state [43]. Pathogen-specific DC functional plasticity has been documented also in mice: at the yeast stage, the fungus *C. albicans* stimulates DC to produce IL-12 and induce Th1 responses while, at the hyphae stage, *C. albicans* stimulates DC to produce IL-4 and Th2 responses [44].

DC ability in diversifying T cell responses is not only related to the type of stimulus, but also to the DC maturation stage. Early-activated hMDC produce large amounts of IL-12 and induce Th1 responses. However, 'exhausted' DC, as a result of prolonged activation, lose the ability to produce IL-12 and preferentially induce Th2 responses [45].

In the mouse system, a kinetic study of DC gene expression following gram-negative bacteria and LPS activation has revealed that during the process of maturation, DC undergo a sequence of precise transitional stages [46, 47].

Genes involved in the antigen presentation function were regulated in LPS- or bacteria-activated DC. The PA28 proteasome activator and the TAP-1 molecule mRNAs showed a clear upregulation. 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 [48]. The proteasome activator was already upregulated at 6 h after LPS activation, while TAP-1 upregulation was measurable only at later time points. This pattern of mRNA upregulation correlates well with the kinetics of MHC class I new biosynthesis that peaks at 18 h after bacterial or LPS stimulation [49]. Thus, DC priming induces a remarkable activation of the entire intracellular apparatus necessary for class I antigen presentation function.

Concerning MHC class II genes, it has been shown that the upregulation of class II protein synthesis is very rapid, peaking as early as 1 h after DC activation, and this is

followed by a striking downregulation [49]. The microarray approach validated this observation, showing that class II molecule mRNAs were downregulated after activation. 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 downregulated as early as 6 h after LPS stimulation. Thus, activation stimuli are sufficient to drive DC toward a stage of maturation appropriate for the immune response activation. This is also supported by the strong upregulation of the mRNAs coding for the well-known leukocyte chemoattractants RANTES, MIP1 α and MIP2. Furthermore, LPS-stimulated DC expressed IL-6. This cytokine is known to be involved in increasing the spectrum of peptides presented by DC [50], in leukocyte recruitment [51] and in B cell differentiation [52].

The most unanticipated finding of global gene expression analysis on maturing mouse DC was that they produce IL-2 in a tightly regulated time frame following bacterial activation [46]. Thus, the adjuvant activity of bacteria in DC may be explained not only by the upregulation of costimulatory surface proteins and the maximization of the efficiency in presenting antigens [36], but also by the production of costimulatory molecules, such as IL-2. This seems to be a unique feature of DC, as it has been found that macrophages are unable to produce IL-2 upon bacterial activation. Two waves of IL-2 production by DC following bacterial encounter have been observed. The first wave is between 4 and 8 h after bacterial uptake and the second wave is between 14 and 18 h following activation. This timing is compatible with the appearance of MHC class II/peptide and MHC class I/peptide complexes at the cell surface [49]. Notably, DC are able to present exogenous captured antigens to CD4 T cells in a few hours, while at least 8 h are required to process and present bacterial antigens in association with MHC class I molecules [49]. Thus, early-activated DC are perfectly equipped to prime CD4 T cells, despite their relatively low levels of MHC and membrane-associated costimulatory molecules and expression of T cell inhibitory cytokines, such as IL-10 [45]. At later time points, IL-2 could represent a key costimulatory protein in activating CD8 T cells, even though DC have not yet reached their terminal maturation stage. These data could explain the ability of activated DC to prime CD8 T cell in a CD4-independent manner [38].

Exogenous sources of IL-2 for induction of T cell proliferation may also be required when the frequency of responder antigen-specific T cells or their affinity for

MHC-peptide complexes is low, as frequently happens in vivo during immune responses to microorganisms.

IL-2 is also thought to activate NK cells in vitro. However, this effect has never been considered to be relevant in vivo during immunocompetent responses. It was commonly believed that IL-2 is exclusively produced by T cells during the acquired immune response, while the activation of NK cells occurs previously during the innate response [53]. As DC can produce IL-2 early after activation, this assumption should be revised. It is well established that DC are able to activate NK cell responses in direct NK-DC interactions [54]. Thus, IL-2 is likely to be the relevant and obvious costimulatory factor. Bacterially or LPS-matured DC are likely to play an important role not only in stimulating but also in controlling the inflammatory response; in fact, physiological immune responses originate from a well-controlled inflammatory process. Genes involved in activating and controlling inflammation have been shown to be modulated during DC maturation. The complement molecule C1q is strongly downregulated. This protein is known to play a role in inflammatory responses by increasing both phagocytic activity and the 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 [55]. Recently a strong inflammatory role in the central nervous system has been attributed to C1q [56]. Thus, the downregulation of C1q transcription at later time points may be a way of controlling inflammatory processes.

Moreover, in LPS-matured DC, a strong upregulation of IL-1 β together with IL-1 receptor antagonist (IL-1RA)

mRNA expression was observed, both at early and late time points. 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 [57].

Concluding Remarks

In the past few years, there has been an extraordinary interest in gene expression technologies. Gene microarrays now allow us to study the expression of thousands of individual genes simultaneously in different biological systems. A comprehensive gene expression profile of a cell can be readily obtained. Gene expression profiles can be used as exemplified in this review to define the changes in cellular physiology that occur, as immune cells differentiate in vitro upon stimulation by cytokines. The findings that hundreds of specific genes are expressed at different levels and that some of these genes are also expressed differentially in pathological conditions should prove valuable in elucidating gene function, exploring the causes and mechanisms of disease and discovering potential therapeutic and diagnostic targets.

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