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Definition of an *in vitro* model of human monocyte activation representative of the defensive inflammatory response

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SOMMARIO

La reazione di difesa innata/infiammatoria è attivata in risposta a patogeni esterni o a segnali provenienti dal tessuto danneggiato. I monociti/macrofagi hanno un ruolo chiave nell'inizio e risoluzione della infiammazione per mezzo di differenti programmi di attivazione. Infatti i macrofagi possono adottare in vivo una varietà di fenotipi diversi che dipendono dai cambiamenti del microambiente tissutale, esibendo un continuum di stati funzionali diversi. Inoltre i monociti del sangue periferico non sono una popolazione omogenea ma differiscono nei loro fenotipi e funzioni. Nonostante l'esplosivo aumento di informazioni sull'argomento, molte questioni sono ancora aperte riguardo la caratterizzazione fenotipica e funzionale dei monociti/macrofagi, e il loro ruolo durante l'omeostasi e l'infiammazione. La maggior parte dei dati provengono da studi sul topo e molti immunologi fanno ancora affidamento su modelli di topo malgrado la distanza evolutiva e le differenze tra i sistemi immuni murino e umano. Nel tentativo di capire le questioni di cui sopra e di dirigere gli sforzi verso una immunobiologia basata sull'uomo, il fine di questo lavoro è stato quello di costruire e validare un modello umano della risposta di difesa innata/infiammatoria in vitro che ricapitolasse le differenti fasi della reazione infiammatoria, dal reclutamento e inizio, allo sviluppo e risoluzione dell'infiammazione e conseguente ripristino della omeostasi. Il modello è basato su monociti umani primari del sangue esposti in coltura a cambiamenti sequenziali delle condizioni microambientali (chemiochine, citochine, temperatura, molecole di derivazione batterica, ecc.) per 48 h. L'analisi al citofluorimetro ha dimostrato che la popolazione monocitaria utilizzata era rappresentativa dell'eterogeneità monocitaria così come presente nella circolazione sanguigna. Tutte le fasi della risposta infiammatoria sono state definite mediante analisi trascrittomica effettuata con U133Plus 2.0 GeneChip (Affymetrix). I risultati sono stati confrontati e integrati con profili trascrizionali pubblicamente disponibili di monociti/macrofagi, raccolti e annotati in un database ad hoc. Il profilo trascrittomico di alcuni fattori trascrizionali e fattori correlati con l'infiammazione sono stati confermati e validati mediante qPCR e ELISA. La "cluster analysis" ha rivelato cluster ampi e distinti che comprendono geni con un chiaro andamento che ben descrivono le differenti fasi dell'infiammazione. Per ottenere maggiori indicazioni sul ruolo biologico dei geni differenzialmente espressi durante la risposta infammatoria, ciascun cluster è stato analizzato con la GSEA (Gene Set Enrichment Analysis). I set di geni identificati dalla GSEA correlati con il profilo di espressione dei differenti cluster ha rivelato che la fase infiammatoria era arricchita di pathway infiammatorie mentre la fase anti-infiammatoria, così come quella di risoluzione, di pathway relative al metabolismo, al ciclo cellulare e al riarrangiamento genico. Inoltre confrontando le liste dei geni differenzialmente espressi tra monociti e macrofagi M1 e tra monociti e macrofagi M2 estratte dal meta-database, è stato dimostrato che i monociti trattati in vitro secondo il modello mostrano un profilo M1 durante la fase infiammatoria e M2 durante la risoluzione. L'espressione genica dei fattori trascrizionali e di quelli relativi alla infiammazione rispecchiavano il profilo di espressione ottenuto con microarray. In conclusione i dati di microarray e l'analisi cinetica dei fattori infiammatori e anti-infiammatori validano il modello in vitro proposto, modello che consente di descrivere la sequenza tempo-dipendente e coordinata degli eventi relativi alla infiammazione.

ABSTRACT

The innate/inflammatory defensive reaction is activated in response to foreign pathogens or signals from damaged tissue. Monocytes/macrophages are key players in the initiation and resolution of inflammation by different activation programmes. Indeed in vivo macrophages can adopt a variety of different phenotypes depending on changes in the tissue microenvironment displaying a continuum of diverse functional states. Moreover peripheral blood monocytes are not a homogeneous population but differ in their phenotypes and functions. In spite of the explosive growth of data, many issues are still open about the phenotypic and functional characterization of monocytes/macrophages, and their role during the homeostasis and in inflammatory conditions. The great majority of the data originates from studies in mice and many immunologists still rely on mouse models despite the evolutionary distance and the differences between the murine and human immune systems. In an attempt to understanding the above issues, and to direct efforts in human immunobiology, the aim of this work was to build and validate a human model of innate/inflammatory defence response in vitro that recapitulates the different phases of the inflammatory reaction, from recruitment and initiation, to development and resolution of inflammation, and re-establishment of homeostasis. The model is based on human primary blood monocytes exposed in culture to sequential changes of microenvironmental conditions (chemokines and cytokines, temperature, bacterial-derived molecules, etc.) for 48 h. The flow cytometrical analysis has shown that the monocyte population used is representative of the monocyte heterogeneity as present in the circulation. All phases of the inflammatory response were profiled by transcriptomic analysis carried out with U133Plus 2.0 GeneChip (Affymetrix). Results were compared and integrated with publicly available transcriptional profiles of monocyte/macrophages, collected and annotated in an ad hoc database. The transcriptomic profiling of some transcriptional and inflammatory-related factors were confirmed and validated by qPCR and by ELISA. The "cluster analysis" revealed broad distinct clusters comprising genes with a clear behaviour that well described the different phases of inflammation. To gain more insight into the biologic role of the genes that are differentially expressed during the inflammatory response, each cluster was subjected to gene set enrichment analysis (GSEA). The gene sets identified by GSEA correlated with the expression profile of different clusters revealed that the inflammatory phase was enriched in inflammatory pathways while the anti-inflammatory phase, as well as the resolution phase, in pathways related to metabolism, cell cycle, and gene rearrangement. Moreover, by comparing the lists of differentially expressed gene between monocytes vs. M1 macrophages and vs. M2 macrophages extracted from the meta-database, it was shown that monocytes treated in vitro according to model resemble M1 during the inflammatory phase and M2 during the resolution. The gene expression of transcriptional and inflammatory-related factors matched with the expression profile obtained with microarrays. In conclusion the microarray data and the kinetical analysis of inflammatory and anti-inflammatory factors validate the proposed in vitro model of the inflammatory response, and allowed describing the time-dependent and coordinated sequence of inflammation-related events.

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1. INTRODUCTION

1.1 Overview of the inflammatory response

In the healthy organism the innate immune system provides the first line of defence against external or internal danger signals, and functions by triggering a protective inflammatory response that develops during time through different phases: from initiation to full inflammation to resolution and re-establishment of tissue integrity. So the first phase of an inflammatory response is aimed at destroying pathogens, removing dead and dying cells, damaged extracellular matrix (ECM) material, and cellular debris, followed by a recovery phase in which the tissue is restored to a healthy, fully functional condition. Different ensembles of signalling molecules are utilized during each of these phases. These signals guide the recruitment into the tissue of cells needed to effect the removal and repair phases and within the microenvironment instruct the cells which of several states of differentiation are the appropriate ones to assume at that particular time.

Briefly, when in a tissue occurs an infection or any potential dangerous event such as trauma, the innate immune system is activated by PAMPs (Pathogen-associated molecular patterns) or DAMPs (Damage-associated molecular patterns) [1, 2] respectively, which in turn activate receptors of innate immune system, PRR (Pattern-Recognition Receptor, such as Toll-like receptors and NOD-like receptor) [3], setting in motion a local inflammatory response that includes the recruitment of leukocytes (*i.e.* neutrophils and monocytes) [4] from blood vessels and the production of a series of pro-inflammatory molecules (including chemokines, cytokines, vasoactive amines, eicosanoids and product of proteolytic cascade) by local immune cells (e.i. mast cells and resident macrophages). The most important signalling routes that generate inflammatory response are TNF

and Interleukin 1(IL-1)/Toll pathways. These pathways are a central component of the innate immune response to bacterial lipopolysaccharides (LPS), a main component of the outer membrane of Gram-negative bacteria such as *Escherichia coli*. Examples of genes up-regulated through activation of these pathway include IL-1β, IL-6, TNF-α, the chemokine IL-8, and the cell adhesion molecules ICAM-1 and E-selectin. These highly potent effectors together with reactive oxygen and nitrogen intermediates (ROIs and RNIs, respectively) releasing by neutrophils and macrophages, do not discriminate between microbial and host targets, so collateral damage to host tissue in unavoidable. Thus the activation of innate immune system has side effects collectively known as inflammation, mainly owing to tissue damage to the host, and the innate immune response and inflammatory response are two ways to call the same biological process. In the late phase of inflammation, T cells appear in the tissue by means of chemokines responsible for their recruitment, and may influence the inflammation progress before and resolution then. In fact, the lymphocytes and natural killer cells (NK) produce IFN-γ which keeps the innate immune cells in an active state but when the injurious stimulus is cleared and the inflammation is resolved with inflammatory cytokines catabolism, they produce anti-inflammatory cytokines IL-4, IL-13, and IL-10. These cytokines in turn induce innate immune cells to produce growth factors, tissue factors and anti-inflammatory cytokines, including TGF-β, responsible for the reconstruction and tissue remodelling. The inflammatory mechanisms are potentially harmful to the host, and so the inflammation has to be tightly controlled to avoid excessive tissue damage [5]. It is generally thought that a controlled inflammatory response, occurring for a short period of time (acute inflammation), has a therapeutic effect or physiological purpose in proving protection to the body against infection and injury, but if lasts too long as in the case of enhanced or deregulated reactions (chronic

inflammation) can itself become harmful and detrimental, and degenerate into a series of pathological conditions, from auto-inflammatory or chronic inflammatory to autoimmune diseases with deleterious consequences to the host. The chronic inflammatory state does not seem to be caused by classic inducers of inflammation, such as infection and injury. Instead, it seem to be associated with malfunction of tissue, that is, with the homeostatic imbalance of one of several physiological system that are not directly functionally related to host defence or tissue repair. Maintaining homeostasis, *i.e.* maintaining tissue morphology as well as tissue function, is the ultimate goal of tissue in multicellular organisms [6]. From this perspective inflammation also presumably evolved as an adaptive response to tissue malfunction or homeostatic imbalance [7]. Thus, while the disease state is a displacement from this homeostasis, the inflammation is an adaptive response for restoring homeostasis.

The mononuclear phagocyte system (MPS) plays major roles in development, scavenging, inflammation, and anti-pathogen defences. Under the term MPS are grouped lineage-committed bone marrow precursors, circulating monocytes, resident macrophages and dendritic cells (DC) [8]. The issue of heterogeneity in the MPS still leads to a confusion and debate about DC as truly distinct cells from macrophages [9][1], but a review of this issue is beyond the scope of this essay, which focuses only on monocytes/macrophages. While the development and classification of monocytes and macrophages is very complex [10], here a simple scheme is shown (Figure 1). The monocytes/macrophages are involved in the host defence both by the direct elimination of foreign agents and as organizers of each different phases of the inflammatory process (see below).

[1] Macrophages and myeloid DC allegedly represent alternative differentiation options of bone marrow progenitors and blood monocytes.

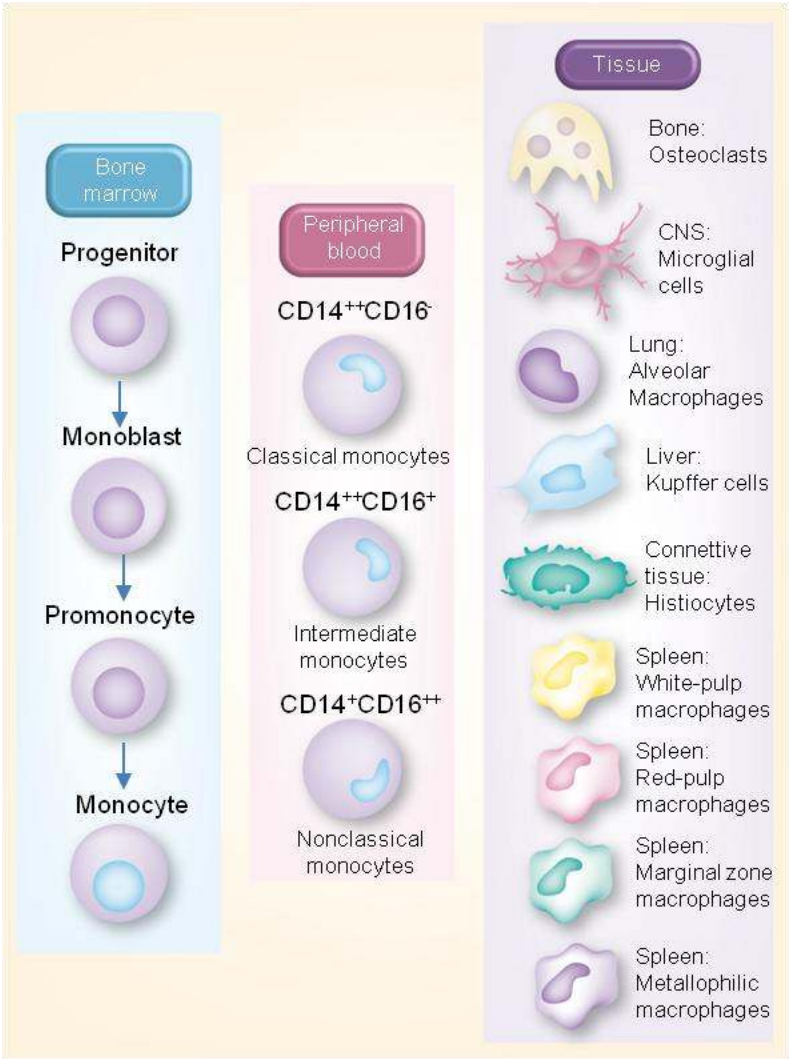

Figure 1. *Genealogy and nomenclature of monocyte/ macrophage lineage. The picture is taken from Ref. 11.*

1.2 Human blood monocyte subsets

Monocytes are a group of cells constituting 5-10% of the total circulating leukocytes in humans. They have some typical morphological features such as irregular cell shape, oval- or kidney-shaped nucleus, cytoplasmic vesicles, and high cytoplasm-to-nucleus ratio. Monocytes can remain in the circulation for up to 3 days, after which time, if they have not been activated, they die and are removed. Monocytes originate in the bone marrow from the common monocyte, macrophage and DC precursor (MDP) [12], circulate in the bloodstream and enter tissues, where they differentiate into macrophages, in order to replenish the pool of tissue macrophages. Monocytes have been considered as the systemic reservoir of myeloid precursors for the renewal of tissue macrophages and antigen-presenting DC. However, many DC and macrophage subpopulations (for example, lymphoid organ DC, plasmacytoid DC, skin Langherans cell and brain microglia) originate from the MDP independently of monocytes [12, 13], and in some cases they can even develop directly from the bone marrow [14]. Abundant experimental evidence indicates that monocytes are innate effectors of the inflammatory response to microbes [15], killing pathogens via phagocytosis, production of reactive oxygen species (ROS), nitric oxide (NO), myeloperoxidase and inflammatory cytokines. In some circumstances they can trigger and polarize T-cell responses [15, 16] and may also contribute to tissue repair and neovascularisation. In addition, monocytes can both stimulate and suppress T-cell responses in infectious and autoimmune diseases [17]. Studying the biology of monocytes is useful for the understanding of susceptibility to infection, providing ideas and tools to control, delay, or alleviate the long-term detrimental side effects of the inflammatory response. It has long been recognized that human peripheral blood monocytes are not a homogeneous population but rather differ in

their phenotypes and functions. In recent years investigators have identified three functional subsets of human monocytes, the characterization of which is still in its infancy and is a matter of intense investigation, as well as the specific roles that they exert in homeostasis and inflammation *in vivo*, reminiscent of those of the previously described classically and alternatively activated macrophages (see below). The new nomenclature that groups monocytes into three subsets, based on the expression of the surface markers CD14 and CD16, has recently been approved by the Nomenclature Committee of the International Union of Immunologic Societies [18]. Based on this new nomenclature, the major population of human monocytes (90%) with high CD14 but no CD16 expression ($CD14^{++}CD16^{-}$ or $CD14^{+}CD16^{-}$) are now termed *classical* monocytes, whereas the minor population (10%) of human monocytes is further subdivided into the *intermediate* subset, with low CD16 and high CD14 ($CD14^{++}CD16^{+}$ or $CD14^{+}CD16^{+}$), and the *non-classical* subset, with high CD16 but with relatively lower CD14 expression ($CD14^{+}CD16^{++}$ or $CD14^{dim}CD16^{+}$) [18][2].

Over the recent years, an increasing amount of knowledge has been gained in the field of monocyte subpopulations. Many authors demonstrated that the three subsets express different transcriptomes [22-28], although discrepancy between studies were evident. These discrepancies may be due to differences in cell isolation methodology and in the purity of the cell populations isolated, the use of negative versus positive selection, and the microarray methodologies which use different

[2] Recent data, hovewer, indicate the this classification may be inappropriate and has led to confusion in functional studies, in part because CD16 expression is shared by many cell types and does not define a unique functional monocyte subset, and may mask heterogeneity the may be spread across some or all subsets. In fact, based on current evidence, there seems to be at least two distinct functional populations within the $CD16^{+}$ monocyte population, defined according to Tie-2 and slan expression [19, 20]. The expression of these surface markers does not follow the current definition of monocytes subsets based on CD14 and CD16 expression [21].

amounts of total RNA for the hybridization and different probes to identify the genes, and even distinct solid supports for the probes [29]. However, it seems there is stronger agreement for the proximity of relationship between the intermediate and non-classical monocyte subset, while the classical subset is the most distant subset [21]. This close relationship suggests a direct developmental relationship between these two subsets, although this has yet to be formally proven, as well as how characteristics previously ascribed to $CD16^+$ monocytes are distributed between intermediate and non-classical subsets [21].

The physiological roles of monocyte subsets *in vivo* are not fully defined and the subsets might have different roles during the homeostasis, immune defense/inflammation, and tissue repair. In general terms, both human $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ monocytes have inflammatory properties reminiscent of the murine $Gr1^+Ly6C^+$ monocytes, while $CD14^+CD16^{++}$ monocytes display patrolling properties similar to those of murine $Gr1^-Ly6C^-$ monocytes.

Moreover it is worth remembering that to date transcriptome analysis of monocyte subsets has been done at the basal unstimulated level and this has shown dramatic differences that are consistent with a different functional repertoire of the three types of monocytes. Since much of the function of monocytes involves their gene expression after activation, it will be important to analyze the stimulus-induced transcriptome of these cells in order to asses pro- and anti-inflammatory properties of the monocyte subsets.

The three monocyte subsets are different in gene expression patterns, in their capacity to become activated and secrete key inflammatory cytokines in response to different stimuli, in antigen processing and presentation, in pro-angiogenic and patrolling behaviour. The phenotypic and functional differences between three monocyte subsets are summarized in Table I,

and recently discussed in an exhaustive review [21]. Moreover the authors of this review report a complete and referenced list of studies on bacterial and viral infections, autoimmune diseases and inflammatory conditions, in which the frequencies of the three monocyte subsets have been evaluated. Briefly, within the $CD16^+$ cells, it appears that the intermediate subset is the main population to be perturbed in almost all disease conditions irrespective of their aetiology, while in bacterial and viral infections (*e.g.* sepsis, tubercolosis, dengue fever, hepatitis B, C and HIV) most studies observed a concurrent expansion of both the intermediate and non-classical subsets. In the few studies on autoimmune disease (*e.g.* Crohn's disease, rheumatoid arthritis) it has been reported expansion only in the intermediate subset, and for other inflammatory condition (*e.g.* asthma, coronary artery disease) excluding autoimmune disease, the expansion was either the intermediate or the non-classical subset.

To date a relevant question that still needs to be elucidated concerns the origin of the various monocyte subpopulations. It is unknown if they are end-stages of different paths of differentiation of a common precursor, or they represent subsequent maturation stages in a common path of differentiation, where the intermediate subset could be a phenotypical and/or developmental intermediate between the classical and non-classical subsets.

In the centre of this issue there are the differences in monocyte subset trafficking observed during the acute and chronic inflammation in studies on mice. In a model of *Listeria monocytogenes* infection, non-classical monocytes ($Gr1^-Ly6C^-$) extravasate rapidly within 1 h, invade the surrounding tissue, and develop a very early inflammatory response producing chemokines involved in the recruitment of the other effectors cells such as granulocytes, NK cells, and T cells, and cytokines, such as TNF-α a cytokine central to macrophage-mediate inflammation and the

innate response [31]. However this inflammatory response is only transient, and at 8 h after infection classical monocytes ($Gr1^{+}Ly6^{+}$) are the main producers of inflammatory cytokines. Moreover it has been observed that in the presence of *Listeria monocytogenes* pathogen *in vivo*, the two subsets of monocytes differentiate into two distinct cells types: $Gr1^{-}Ly6C^{-}$ patrolling monocytes initiate a macrophage differentiation program that resembles that of M2 macrophages (see below), while $Gr1^{+}Ly6^{+}$ monocytes differentiate into DC-like cells that resemble Tip-DC [31]. On the other hand, only classical monocytes migrate to injured tissue in a model of skeletal muscle injury and determinate early inflammatory responses [32]. Generally, they infiltrate inflamed tissues more robustly than their non-classical counterparts, and are specially increased in the circulation during systemic or chronic infection [15]. After engulfing dying cells, they differentiate into cells that resemble non-classical monocytes, which mediate tissue repair mechanism [32]. By contrast, after myocardial infarction, both monocyte subsets appear to home to the same tissue at different stages of inflammation [33]. Specifically, although the classical subset of monocytes first infiltrates the infracted heart and exhibits inflammatory functions, the non-classical subset is recruited at a later stage and promotes tissue healing by expressing high amounts of vascular endothelial growth factor [33]. The two subsets are under the control of distinct trafficking mechanisms, with the classical subset being recruited via CCR2 and the non-classical one utilizing a CXCR1-dependent pathway [33]. So, some studies conclude that classical monocytes had differentiated into non-classical monocytes [32], while others concluded that distinct populations of monocytes are recruited from the blood [31, 33]. Together, these observations reveal an unsuspected dichotomy of the differentiation potential and functions of blood monocytes subsets during *Listeria monocytogenes* infection and myocardial infarction. Moreover, in

atherosclerosis as a model of chronic inflammation, both monocyte subsets are recruited in the same time and healing is correlated with a reduction in total monocyte recruitment [34].

Finally, these findings focus the attention on the fact that a specific subset of monocytes is committed to become a specific type of macrophage. In summary, in mouse it seems that the non-classical monocytes contribute to resident macrophage populations [35], and it is possible that when they are recruited in the inflamed tissue may differentiate into alternatively activated macrophages [31, 32, 33], while classical monocytes give rise to classically activated macrophages [31, 33, 36]. However the developmental relationship between the different monocyte subsets and different macrophage phenotypes has yet to be fully and formally proven and there is not yet strong supporting evidence in man.

Table I. Phenotypic and functional differences between the three monocytes subsets [21, 30]

	Classical $CD14^{++}$ $CD16^{-}$	**Intermediate** $CD14^{++}$ $CD16^{+}$	**Non-classical** $CD14^{+}$ $CD16^{++}$
Approximate proportions to total monocyte	85%	5%	10%
Surface markers expressed	CD62L, CCR2, CLEC4D, CLEC5A, IL13Rα1, CXCR1, CXCR2	CD74, HLA-DR, Tie-2 (CD202B), ENG (CD105)	Siglec10, CD43, CX3CR1, SLAN (subpopulation)
Surface markers not expressed	CX3CR1, CD123, P2RX1, Siglec10	CD62L, CXCR1, CXCR2, CLEC4D, IL13Rα1	CD62L, CCR5, CCR2, CXCR1, CXCR2, CLEC4D, CD163, IL13Rα1
Preferential responses to LPS	IL-10, G-CSF, CCL2, RANTES (CCL5), IL-6, IL-8 (CXCL1), IL-1α, TNF	IL-6, IL-8 (CXCL1)	TNF-α, IL-1β, IL-6, IL-8 (CXCL1), CCL3
Defined gene signatures	Wound healing and coagulation S-100 proteins Scavenger receptors C-type like receptors Anti-apoptosis Response to stimuli	MHC class II presentation and processing	Cytoskeletal rearrangement Complement components Pro-apoptosis Negative regulation of transcription
Described functions	**Antimicrobial activity** -superior phagocytosis - ROS, NO, MPO, Type I interferon **Inflammation and Leukocyte recruitment** - response to cell surface TLRs (TLR2 and TLR4)	**T cell proliferation and activation** **Inflammation** -superior ROS production **Angiogenesis** (Tie-2+ subpopulation) **Tissue repair**	**T cell proliferation and activation** (SLAN+ subpopulation) **"Patroling" behaviour *in vivo*** - Sensing tissue damage **Scavenging and response to viruses/nucleic acids via endosomal TLRs (TLR7 and TLR8)** **Scavenge Immune Complex**

1.3 Macrophage polarization

Macrophages are very heterogeneous and versatile cells that are present in virtually all tissues. They originate from the differentiation of circulating peripheral blood monocytes that migrate into tissues under a variety of stimuli, including inflammation, infections and cell damage, to become resident tissue macrophages. The traditional role of these cells has been linked to the phagocytosis of pathogens or cellular debris, and the host defence and tissue repair [37, 38]. However, independent of inflammation and tissue damage, macrophages also play a central role in tissue homeostasis by clearing apoptotic or senescent cells. Resident macrophages constitute 5-15% of the total cell number of most organs and their number is increased further in response to inflammatory stimuli. The specialization of macrophages in particular microenvironments explains their heterogeneity. They can be classified, according to their tissue location, into osteoclasts (bone), alveolar macrophages (lungs), microglial cells (CNS), histiocytes (connective tissues), Kupffer cells (liver), Langerhans cells (skin) and so on. Moreover macrophages can undergo different activation processes and gain different functional phenotypes, as a consequence of tissue-derived (damaged tissue) or cell-derived signals (from microbes or activated lymphocyte) in surrounding microenvironment [8, 39, 40]. The macrophage polarization defines the different typologies of the activation programs to which the cells answer to carry out their defensive functions. In this way macrophages become able to response with appropriate functions in distinct contexts, and the functional diversity becomes the key feature of these cells. Although the microenvironmental stimuli and the resulting functional phenotypes are varied, two main macrophage phenotypes have been suggested, mirroring the Th1/Th2 polarization scheme. In fact, a useful and dominant nomenclature for $CD4^+$

T cells reflects the roles that these helper cells play in a given scenario of inflammation. Type 1 immune response, mediated by Th1 cells, refers to the inflammatory response that clears viral, bacterial, and protozoan infections. Type 2 immune response, mediated by Th2 cells, refers to a response that is more efficacious in clearing multicellular parasites. Since it has been shown that distinct populations of macrophages facilitate and control type 1 and type 2 immune responses not surprisingly they have been termed M1 or classically activated macrophages, and M2 or alternative activated macrophages, respectively [41, 42]. Th1-related cytokines like IFN-γ, alone or in concert with microbial stimuli (*e.g.* LPS) or cytokines (*e.g.* TNF-α), activate macrophages towards the functional M1 program. M1 macrophages are characterized by an IL-12^{hi}IL-23^{hi}IL-10^{lo} phenotype; are efficient producers of effector molecules (ROI and RNI) and inflammatory cytokines (IL-1β, TNF, IL-6); participate as inducers and effector cells in polarized Th1 responses; mediate resistance against intracellular parasites and tumors [43, 44]. Conversely, anti-inflammatory cytokines can induce the M2 activation program (alternative activation). M2 macrophages are regarded as a continuum of functionally and phenotypically related cells generated in response to a variety of stimuli. In fact, M2 macrophages are generally divided into a, b, and c subtypes [41, 42]. They appear to perform separate tasks in inflammation with variable capacity to produce inflammatory cytokines depending on the signal, and are designated by different monikers in different publications. M2a macrophages are the alternatively activated or profibrotic macrophages, elicited by Th2-related cytokines IL-4 or IL-13 [45]. M2b are regulators or Th2-related macrophages, activated by triggering of Fcγ receptors in presence of a Toll-like receptor (TLR) stimulus. M2c are deactivated cells, involved in remodeling, or anti-inflammatory cells, elicited by glucocorticoids, IL-10 or TGF-β. Some researchers also regarded the M2c

as regulatory macrophages [45, 46, 47]. M2 cells are characterized by an IL-12^{lo}IL-23^{lo}IL-10^{hi} phenotype and generally have high levels of scavenger, mannose and galactose-type receptors, and their arginine metabolism is shifted to ornithine and polyamines, while in M1 is shifted to NO and citrulline. In addition, differential regulation of components of the IL-1 system occurs in alternatively polarized macrophages, with low levels of IL-1β and caspase-1, and high levels of IL-1Ra and decoy IL-1 type II receptor (sIL-1RII) [48]; the opposite regulation occurs in M1 cells [44]. IL-33, another cytokine of the IL-1 family, is associated with Th2 and M2 polarization [49, 50]. In general, alternative macrophages take part in polarized Th2 responses, allergy, parasites clearance, the dampening of inflammation, the promotion of tissue remodeling, angiogenesis, immunoregulation, and tumor promotion [52].

Macrophage taxonomy is an attempt to rationally categorize an extended variety of cell functions. Indeed the M1/M2 paradigm is limiting to define the complexity and plasticity of mononuclear phagocytes. *In vivo* macrophages can adopt a variety of functional phenotypes depending on changes in the tissue microenvironment. So, the polarization of macrophage functions should be viewed as an operationally useful, simplified, conceptual framework describing a continuum of diverse functional states, of which M1 and M2 activation states are not ontogenically defined subsets but represent the extremes [42, 47, 50, 52]. The classification M1/M2 persists despite a growing body of evidence indicating that M2 designation encompasses cells with dramatic differences in their biochemistry and physiology [53]. In this regard Mosser and Edward [47] have suggested a macrophage classification taking into account the three functions of these cells in maintaining homeostasis: host defence, would healing, and immune regulation. Classifying macrophages according to these functions provides three basic macrophage population: classically activated macrophages,

wound-healing macrophages and regulatory macrophages. The authors believe that this classification also helps to illustrate how macrophages can evolve to exhibit characteristics that are shared by more than one macrophage population [47]. Though agreeing with the concept of this classification, in general terms this essay will continue to refer to M1 and M2 macrophages, as the most authors still do. In fact, while M1, M2a, M2b, M2c do not necessarily represent distinct populations of cells, they do represent a useful functional nomenclature.

M1 and M2 macrophages have distinct chemokinome profiles, with M1 macrophages expressing Th1 attracting chemokines such as CXCL9 and CXCL10, and M2 macrophages expressing the chemokines CCL17, CCL22 and CCL24 [54, 55]. Chemokines can also influence macrophage polarization, with CCL2 and CXCL4 driving macrophages to an M2-like phenotype [56, 57].

M1 and M2 polarized macrophages have distinct features in terms of metabolism of the iron, folate and glucose [58, 59], and it has long been known that macrophages and metabolism are connected [58]. Indeed, recent evidence shows the importance of metabolism in shaping the functional phenotype of macrophages in response to distinct polarizing stimuli in the tissue microenvironment, under normal as well as pathological settings. The macrophage-metabolism connection has two faces: on one hand, macrophages exert an “extrinsic” regulatory function on metabolic functions, via release of soluble mediators such as inflammatory cytokines; on the other hand, “intrinsic” metabolic functions of these cells contribute to shaping their activation state [58, 59]. Polarized macrophages show a distinct regulation of glucose metabolism. Macrophages in response to M1 stimuli display a metabolic shift towards the anaerobic glycolytic pathway, while exposure to M2 stimuli such as IL-4 show a minor effect [60]. The use of specific metabolic pathways can be functionally related to different

purposes. M1 activated macrophages are often associated with acute infection: these cells need to quickly acquire microbicidal activity as well as keep up with the hypoxic tissue microenvironment [61]. In this context, an anaerobic process like glycolysis is best suited to meet their rapid energy requirements. In contrast, M2 polarization-related functions like tissue remodelling, repair and healing require a sustained supply of energy. This request is fulfilled by oxidative glucose metabolism (oxidative phosphorylation), which is believed to be the metabolic pathway of choice in M2 macrophages [62]. Moreover M2 macrophages show a significant up-regulation of fatty acid uptake and fatty acid oxidation, which are suppressed in M1 macrophages [63]. Lipid metabolism also contributes to macrophage phagocytosis by fulfilling its energetic needs and regulating membrane fluidity necessary for this process. In fact, saturated and unsaturated fatty acids differentially modulate macrophage pahgocytosis. Also the amino acid metabolism is closely linked to the functional phenotype of myelomonocytic cells. M1 macrophages are characterized by the expression of NO-synthase 2 (NOS2) and production of NO, which is an important effector for their microbicidal activity [64]. In contrast, M2 macrophages do not produce NO, but express high levels of arginase-1 (ARG-1), which catalyses polyamine production which is necessary for collagen synthesis, cell proliferation, fibrosis and other tissue remodeling functions [65]. Interestingly polyamine production *per se* has been reported to be a driver of M2 polarization [66]. Moreover, evidence supports a critical role for the metabolism of various aminoacids in regulating different steps of both innate and adaptive immunity, and catabolitic enzymes, such as indoleamine 2, 3-dioxygenase (IDO), ARG1 and NOS2, have acquired novel functions [67]. L-arginine-derived metabolites, cysteine/cysteine, and tryptophan metabolism (via IDO) are important mediators of the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs).

Similarly, intracellular nicotinamide adenine dinucleotide (NAD), an end product of tryptophan metabolism, has been demonstrated as an important regulator of inflammatory cytokines like TNF and IL-6 in mononuclear phagocytes, with implications in various pathologies [68].

Recent studies in mouse as well as human macrophages show striking differences in iron metabolism between M1 and M2 polarized cells [69, 70]. M1 macrophages express high levels of proteins involved in iron storage, such as ferritin while expressing low levels of ferroportin, an iron exporter. In contrast, M2 macrophages show low levels of ferritin but high levels of ferroportin. This divergent iron metabolism can be related to functional outcomes. Sequestration of iron by M1 cells would have a bacteriostatic effect (since iron is essential for supporting growth) and thus support host protection from infection. Conversely, iron release from M2 cells would favour tissue repair as well as tumor growth, consistent with the functional phenotype of these cells. Based on the facts presented above, it is clear that divergent iron management seems to be an important metabolic signature in polarized macrophages [71]. Collectively, these facts highlight that metabolic adaptation is an integral aspect of macrophage polarization and their functional diversity.

In physiological and pathological conditions macrophages are confronted with an oxygen gradient and contribute to the orchestration of the tissue response to hypoxic conditions [72]. They adapt to hypoxia by shifting their metabolic setting to glycolysis [40]. In addition, activation of hypoxia inducible factor (HIF) 1 and 2 orchestrates profound functional changes, including expression of chemokines and chemokine receptors (CXCR4 and CXCL12) [73] and angiogenic factor (VEGF).

In summary, the initial inflammatory response is carried out by activated macrophages in classical or alternative modality, eliminating invading microbes and promoting the inflammatory response, whereas the resolution

phase is carried out by macrophages in deactivated modality, unresponsive to inflammatory stimuli and active in the elimination of the injured cells and tissues, in promoting angiogenesis, cell proliferation, matrix deposition and in general in tissue remodeling (Figure 2). The mechanisms that account for macrophage deactivation play key roles in maintaining homeostasis and keeping the immune response under control [74]. Both innate and adaptive signals can influence macrophage phenotype alterations, which can have potentially dangerous consequences if not appropriately regulated. For example, classically activated macrophages can cause damage to host tissues, predispose surrounding tissue to neoplastic transformation and influence glucose metabolism by promoting insulin resistance (see later). Macrophages that are normally involved in wound healing can promote fibrosis, exacerbate allergic responses and be exploited by pathogens for intracellular survival. Regulatory macrophages can contribute to the progression of neoplasia (see later), and the high levels of IL-10 that these cells produce can predispose the host to infection.

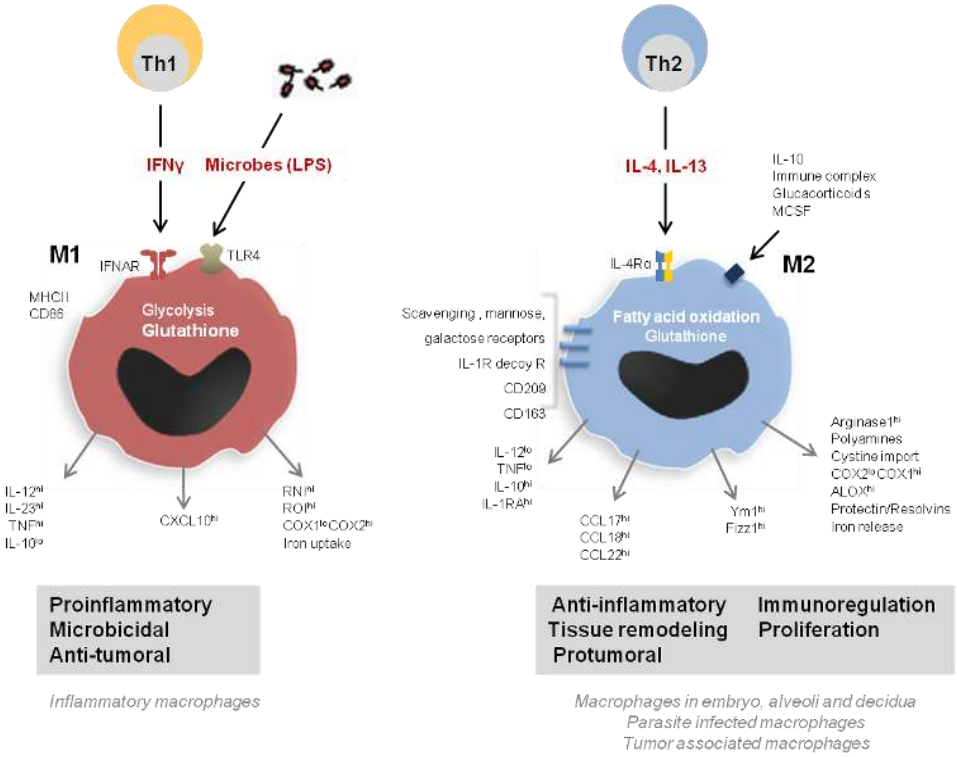

Figure 2. *Schematic representation of the M1- and M2-polarized macrophages. The polarizing signals and major molecular, metabolic and functional characteristic of these macrophages are indicated. Subtypes of M2-polarized macrophages are not distinguished in this figure. The figure is taken from [59].*

1.4 Macrophage plasticity

Plasticity and flexibility are key features of macrophages and of their activation states. A controversial issue is whether a phenotypic and functional evolution of macrophages occurs *in vivo*. Several studies suggest that the phenotype of polarized M1-M2 macrophages can change, to some extent, and reverse *in vitro* and *in vivo* [75, 76]. As mentioned above, in mice it has been observed that the M1 to M2 switch during the progression of the inflammatory response enables the dual role of macrophages in orchestrating the onset of inflammation and subsequently promoting healing and repair [31, 32, 33]. The controversy refers to the mechanism underlying this switch is whether M1 and M2 macrophages consist of phenotypically distinct subpopulations that can serve different functions [31, 33] or the same cells can shift from one to another functional phenotype based on microenviromental signals [32]. So it is not clear whether this phenotypic alteration is the result of the de-differentation of the original macrophages back to the resting state or of the migration of a new subpopulation of macrophages into the tissue site where they replace the first cells. Regardless of the mechanisms, there are some cases in which a phenotypic switch in the macrophages population occurs over time and often is associated with pathology. Three specific examples of this phenotypic switch are reported: 1. endotoxin tolerance, an altered state of responsiveness to secondary stimulation with LPS, resulting in a global and sustained switch of the gene expression program from a pro-inflammatory M1 signature to an M2-like anti-inflammatory phenotype [77]; 2. obesity-induced insulin resistance or type-2 diabetes, and atherosclerosis lesions are metabolic syndromes that can lead to a switch in the phenotype of adipose tissue macrophages from wound-healing (as in healthy, non-obese humans) to classically activated macrophages [78, 79]; 3. cancer, where

the original classically activated macrophages have the potential to contribute to the earliest stages of neoplasia [80], and then, as tumor progresses, can progressively differentiate to a regulatory phenotype and eventually become cells that share the characteristics of both regulatory and wound-healing macrophages [47]. Although the pathology provides proof of principle that macrophages can undergo dynamic transitions between different functional states, the stability of M1 and M2 phenotypes in a physiological setting is still unclear and requires further investigation. However, it is now apparent that specialized or polarized T cells (Th1, Th2, Treg) that are key orchestrators of macrophage polarized activation [51] also exhibit previously unsuspected flexibility and plasticity [81]. The commonly held view is that macrophage polarization is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and micro-organism associated molecular patterns. These signals are thought to dictate a transcriptional response that shapes the phenotype and function of macrophages on the basis of the physiological or pathophysiological context. This model is based on a large number of independent experimental studies. However the data are still incomplete and far from being systematic, and our knowledge of the molecular determinants (mechanistic basis) of macrophage diversity in different tissues or in response to changing environment is to a large extent unknown. Progress has been made in defining the molecular mechanism underlying macrophage polarization, including signalling pathways, miRNA, epigenetic modification, posttranscriptional regulators, and transcriptional factors [52, 59, 74, 82, 83]. Briefly it is worth to remember in this essay the recent advances/progress in understanding of the transcriptional regulation of macrophage polarization, *i.e.* of inflammatory response. The transcriptional factors can translate signals from the microenvironment into a polarized macrophage phenotype imposing different requirements for

gene activation in response to stimulation [82, 83]. For example the induction of the transcriptional response by LPS in macrophages is orchestrated by many transcription factors, consistent with the complexity of the response. These transcriptional factors can be divided into three categories on the basis of their mode of activation and function. The first category consists of transcriptional factors that are constitutively expressed and that are activated by signal-dependent post-translational modifications (*e.g.* NF-kB, IFN-regulatory factors - IRFs- and cAMP-responsive-element-binding protein 1 - CREB1). The second category of transcription factors are synthesized *de novo* after LPS stimulation, and they could enable the reprogramming of macrophage functions (*e.g.* CCAAT/enhancer-binding protein-δ – C/EBPδ). The third category of transcriptional factors consists of lineage-specific transcriptional regulators, the expression of which is turned on during macrophage differentiation (*e.g.* PU.1, runt-related transcription factor 1 – RUNX1) and their combined expression specifies the macrophage phenotype. The transcription factors of the three categories do not act independently, but function coordinately to effect the LPS-induced transcriptional response [84, 85, 86].

A network of signalling molecules and transcription factors underlies the different forms of macrophage activation. Canonical IRF/STAT signalling pathways are activated by IFN and TLR signalling to skew macrophages function towards the M1 phenotype via STAT1; or by IL-4/IL-13 and IL-10 to skew towards the M2 phenotype via STAT6 and STAT3 [87, 88]. The balance between activation of STAT1 and STAT3/STAT6 finely regulates macrophage polarization and activity. A predominance of NF-kB and STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and inflammatory function, while a predominance of STAT3 and STAT6 activation results in M2 macrophage polarization, associated with immune suppression and tumor progression. STAT-mediated activation of

macrophages is regulated by members of SOCS family. IL-4 and IFN-γ, the latter in concert with TLR stimulation, up-regulate SOCS1 and SOCS3, which in turn inhibit the action of STAT1 and STAT3, respectively [89, 90]. Downstream of, or in parallel with, the IRF/STAT/SOCS pathway, a panel of transcription factors orchestrates polarized macrophage activation, and some of these are described hereafter.

NF-kB is a key transcription factor related to M1 macrophage activation that regulates the expression of a large number of inflammatory genes like TNFA, IL1B, COX2, IL6 and IL12p40, characteristic of the M1 polarization state [91]. However, NF-κB activation also activates a genetic program essential for resolution of inflammation [92] and for M2 polarization of tumor-associated macrophages (TAMs) [93].

IRF5 is up-regulated in M1 macrophages, in which it is essential for induction of cytokines (IL-12, IL-23, TNF) involved in eliciting Th1 and Th17 responses [94].

PPARγ [95] and PPARδ [96, 97] control distinct subsets of genes associated with M2 macrophage activation and oxidative metabolism. PPARγ is constitutively expressed by adipose tissue macrophages, but its expression can also be induced by IL-4 and IL-13 [98], which indicates that M2 polarization in the context of Th2 cell responses might also involve PPARγ. Moreover PPAR receptors are involved in inflammatory responses [99, 100] and monocyte-macrophage differentiation [101-103].

A number of reports have demonstrated the role of Kruppel-like factor 4 (KLF4) in both monocyte differentiation and macrophage activation [104, 105, 106]. Another recent study using a myeloid-specific knockout for the KLF4 demonstrated its role in regulating M2 polarization of macrophages as well as in protecting from obesity-induced insulin resistance [107]. Similarly, IRF4 has been implicated in regulating M2 genes in macrophages and in mediating lipolysis functions [108]. Moreover IRF4 was shown to

specifically regulate M2 macrophage polarization in response to parasites or the fungal cell wall component chitin [109].

The CREB-C/EBPβ axis specifically regulates M2-associated genes [110] and is crucial for wound-healing [111]. Moreover C/EBP proteins have specific functions during macrophage development, with C/EBPα that is mainly expressed in undifferentiated pluripotent myeloid cell and gradually decreased with macrophages maturation. Conversely, expression of C/EBPβ and C/EBPδ is up-regulated during macrophage maturation [112]. In addition C/EBPα regulates myeloid development and interacts with NF-kB to regulate inflammation [113].

Finally, PU.1 is a transcriptional factor which must be constantly expressed at high levels to induce and then maintain macrophage differentiation [114], but it is able to interface with other transcription factors that are known to be relevant for macrophage differentiation, such as IRF8 [115], or for functional specialization, such as IRF4 and IRF5, and C/EBPβ [102]. By contrast moderate levels of PU.1 and high expression of MafB, an inducer of monocyte differentiation [116, 117], are able to drive monocyte differentiation towards macrophages as opposed to DC [118, 119].

Other transcription factor involved in the control of macrophages phenotype under physiological and pathological conditions is the hypoxia-inducible factor (HIF) and its two isoforms HIF-1α and HIF-2α [120]. Their effects are mediated by hypoxia that is an important microenvironmental signal of the inflamed tissue. Gene expression profiling of monocytes and macrophages has characterized profound changes in response to hypoxia [121, 122], such as the expression of angiogenesis- and metastasis-related genes (*e.g. VEGF*, *FGF2*, *MMP7* and *MMP9*) and pro-inflammatory (*e.g. TNFA*, *IL1B*, *MIF*, *CCL3* and *COX2*) [123] as well as M2 markers like IL-10 and arginase 1 [124]. The two isoforms seem to be implicated in driving these different responses in line with the fact that HIF-1α is expressed in M1-

polarized macrophages and HIF-2α in M2-polarized macrophages [125]. Moreover, recently it has been demonstrated that in monocytes, unlike in macrophages, it is NF-kB1, and not HIF-1α, which is of central importance for the expression of hypoxia-adjusted genes. These new data demonstrate that during differentiation of monocytes into macrophages crucial cellular adaption mechanisms are decisively changed [126].

In the last years gene expression profiling techniques and genetic approaches have been used to cast light on the understanding of the plasticity of macrophage activation, but the mechanisms underlying the program must still be clarified. Elucidating the molecular basis of macrophage activation is a fundamental step to understand inflammatory disorders and develop new therapeutic strategies.

1.5 Surfing data tsunami: the macrophages.com website

Large-scale genomic analysis related to innate immune responses of mammalian species have generated large sets of heterogeneous genomic data. A growing need to store, retrieve and analyse these datasets has led to the emergence of various on line data repositories, some of these listed here:

- Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) [127] provides a vast amount of gene expression data;
- Kyoto Encyclopedia of Genes and Genomes (KEGG, at http://www.genome.jp/kegg/), Biocarta, (at http://www.biocarta.com/) and Reactome (at http://www.reactome.org/) databases provide the biological processes or pathways which are "hidden" in the gene expression profiles;
- Innate DB [128] provides manually curated protein-protein interaction data to help system-levels analysis of immune responses in human and mice, and provides access to the visualization of interactome-based pathways relevant to innate immunity;
- Immunome Knowledge Base (IKB) [129] that integrate Immunome, ImmTree, Immunome Database, species-specific databases that have been developed for the investigation of immune systems in specific model organisms;
- Immunology Database and Analysis Portal resource (www.immport.org) provides human data with a comprehensive list of immune-related genes and differential gene expression information, including single nucleotide polymorphisms (SNPs) data and a specialised section to analyse polymorphism in the human major histocompatibility complex;
- ImmGen (Immunological Genomic Project) [130] focused on the mouse immune system;

- IIDB repository [131] focused on Toll-like receptor (TLR) genes and TLR signalling pathways in the mouse genome.

Despite the large amount of data accessible through these and other on-line portals with immunity-driven database backends, these available innate immunity-based resources do not specifically focus on monocyte/macrophage systems. As mentioned in previous paragraphs, monocytes/macrophages have a fundamental role in many aspects of biological functions from tissue remodelling during development, wound healing and tissue homeostasis, to innate immunity and to pathology of tissue injury and inflammation. With the escalation of genome-scala data, an enormous wealth of information has been accumulated on these cells in the literature on their functional activity, signalling pathways, and their role in health and disease. So, in recent years, a centralized portal on macrophage biology is born, macrophages.com (http://www.macrophages.com/), a resource that integrates macrophage-related data, provide bioinformatics tools to facilitate data analysis and allow comparative analysis of functional motifs and evolution in macrophage-active promoters, and centralises links to other research and teaching materials relevant to study of macrophages. Briefly, the website is composed by following sections: (1) data analysis screenshots from the bioinfoweb gene-centric portal, (2) collection of gene expression datasets and clustering analysis with Biolayout Express3D, (3) large publication and reviews section, (4) comprehensive macrophage image library divided in two main categories (tissue macrophage and transgenic animals), (5) protein expression data with access to HPA resource, and (6) macrophage pathway resource. An accurate description of website is available in “Macrophages.com: An on-line community resource for innate immunity research” [132]. Researchers interested in studying the biology of macrophages can usefully refer to this website as a comprehensive online resource.

1.6 Man is not a mouse. The importance of human immunology

Immunobiology has advanced tremendously over the last 50 or so years. In this time, a whole system of innate immune receptors and CD antigens have been discovered, different hematopoietic cell subsets have been discriminated, dozens of cytokines and chemokines have been identified as mediators of cellular response and cell-to-cell communication. Nevertheless almost none of these advances in basic immunology have been incorporated into standard medical practice. A reason of that is the overreliance on the mouse model. The mouse has been so successful at uncovering basic immunologic mechanisms that many immunologists rely on it to answer every question. In fact the use of animal models in immunological research has proven useful for investigating and assessing mechanisms resulting in autoimmune and inflammatory diseases [133] and mouse models will continue to provide important information for many years to come. However, the question is still open of how the animal data can be translated to the human situation. In fact mice are lousy models for clinical studies and this is readily apparent in autoimmunity [134] and in cancer immunotherapy [135] where of dozens of protocols that work well in mice, very few have been successful in humans. Moreover, despite conservation between human and mouse genome (to date only 300 or so genes appear to be unique to one species or the other) there exist a sheer evolutionary distance (65 million years) that raised significant differences between the two species in immune system development, activation, and response to challenge, in both the innate and adaptive arms [136]. In this regards, Mestas and Hughes [137] have carefully examined the many differences between mice and humans with respect to various immune markers, suggesting that the potential limitations of extrapolating data from mice to humans should be taken in account. Moreover Mark. M. Davis [138] thinks

that the mouse models are not the answer to everything in immunology and underlines that we need to make greater efforts in human immunology if we are to realize the potential health benefits. After all, it is worth remembering that humans live “in natura” more or less, outbred and exposed to many more diseases than laboratory mice [139]. This topic has been recently discuss in depth in a symposium entitled “Wild Immunology”, where the central question was the importance to understand infection and immunity in wild systems [140].

In several cases no strict molecular correlation has been found between immune reactions in mice versus humans, and there is evidence of alternative molecular pathway usage [141]. These issues arise also in the case of the more conserved reactions involving the innate/inflammatory response and monocyte/macrophage activation mechanisms. First of all in the blood of a healthy human adult, monocytes represent about 10% of the total peripheral blood leukocyte pool against 1.5% in the mouse blood. The human blood contains three main monocytes subsets while mouse blood only has two. Moreover, the monocyte subsets described in humans do not fully correspond to those identified in the mouse [141, 142] and there are still uncertainties regarding distinct expression patterns of cell-surface markers. Although general properties are retained between mouse and man, differences are apparent for instance in the types of pathogens that infect humans and the effector molecules that are deployed by macrophages to control infections. To make some examples: phenotypic markers of M2 polarization such as chitinase 3-like-3 lectin (CHI3L3, also known as Ym-1) and the transcription factor found in inflammatory zone 1 (Fizz-1 or Relmalpha or Retnla) have been identified in the mouse but are not expressed in human macrophages [45, 54]. Moreover, Fizz1 inhibits Th2-driven inflammation in the lung of mice but not in humans [143]. Neither Arginase-1 (ARG1) nor nitric oxide synthase (iNOS) are expressed

by *in vitro* polarized human macrophages stimulated with IL-4 or IFN-γ, respectively, in amounts comparable with those expressed by mouse macrophages. This discrepancies have fueled an intense debate on similarities between human and mouse macrophages subsets and their expected function [144, 145], since a number of the commonly used phenotypic subset marker are not implicitly conserved across species [146]. In general, mice are highly resilient to induction of inflammation in many experimental models, as compared with humans, which are for instance much more sensitive to the inflammatory effects of bacterial LPS [147]. Another example is IL-37, a new member of the IL-1 family that is a potent anti-inflammatory cytokine in humans but is absent in the mouse [148]. The p47 immunity-related GTPase (IRG) family, involved in the protective anti-mycobacterial autophagy response, has 20 members in mice but only two in humans (IRGM and IRGC) [149, 150]. Human and mice have different number and functional Toll-like Receptors (TLR), which have a major role in pathogen recognition and activation of the innate immune response. Humans encode eleven TLRs but only ten (TLR1-TLR10) are functional, while mice express also TLR12 and TLR13, but TLR10 is not functional [151]. The fact that mice may express TLRs that are not found in humans and vice versa can make it challenging to generalize findings about the fine mechanisms of innate/inflammatory response regulation between humans and mouse model systems. Acute and chronic inflammatory conditions such as sepsis and many autoimmune diseases occur spontaneously in humans (*i.e.* without deliberate exposure/induction), but do not occur in mice and are hard to induce without genetic or experimental manipulation to alter host response. Moreover it is often not made clear that most data on allergy, an innate immune response as inflammation, derive solely from experiment in mice or rats, species that obviously never suffer from allergic human diseases [152].

1.7 Aim of work: an in vitro human model of the inflammatory reaction

Many studies on monocyte/macrophage activation in inflammation have relied on murine models *in vivo* and on isolated primary mouse cells (mainly peritoneal or bone marrow derived macropahges), and on *in vitro* models based on immortalized monocytic cell lines (either human or murine). Experiments with monocytic cell lines have limitations due to the fact these are transformed/tumor cells, which differ from primary monocytes/macrophages certainly in terms of regulation of cell cycle and in most cases also in terms of differentiation and activation state [153-156].

More recently, a wealth of information has become available that has been obtained with human normal/primary monocytes/macrophages *ex vivo* or *in vitro* (primary macrophages isolated from tissues, *in vitro* differentiated myeloid precursors, *in vitro* matured macrophages, peripheral blood monocytes). These studies have investigated the activation of monocytes/macrophages in response to different kinds of challenges, either administered in culture or upon *in vivo* pathological conditions, and have provided information about the modes of type I *vs*. type II inflammatory activation *vs*. deactivation of macrophages in the human being. However, there is no information at present on the features of the entire course of the inflammatory reaction and on the possibility that the same cell population could be first polarized towards an effector inflammatory programme and subsequently re-polarized to the deactivation programme.

In this context, the aim of this study is to set up a reliable and representative model, based on human primary cells, that could allow us to study the development of the inflammatory reaction during its entire course, thus opening the possibility of accurately characterizing the development and regulation of human macrophage functions.

We propose here an *in vitro* model of the type I inflammation that reproduces the different phases of the inflammatory defence response occurring *in vivo*, from recruitment of inflammatory monocytes to the site if inflammation, to the onset and development of the inflammatory reaction, until resolution of inflammation and re-establishment of tissue homeostasis. The model is based on human primary blood monocytes exposed in culture to sequential changes in the microenvironmental conditions (chemokines and cytokines, temperature, bacterial-derived molecules) for 48 h. Macrophage activation has been assessed by transcriptomic profiling, data validated for some inflammation-related genes by real-time PCR and protein production, and representativeness of the findings confirmed by comparison with an *ad hoc* constructed and annotated database of gene expression in human monocytes/macrophages. Robustness and reproducibility of the model was demonstrated by the homogeneity of gene and protein profiles in monocytes from 12 individual donors. Although simplified, this model thus provides an accurate description of the inflammatory reaction in humans, from initiation to conclusion, in a totally primary system.

2. MATERIALS AND METHODS

2.1 Monocyte isolation from peripheral blood and in vitro activation

Human monocytes were obtained from peripheral blood buffy coats of healthy donors (n=12) by two-step gradient density centrifugation with Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and then separated using Monocyte Isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol.

The purity of isolated cells (> 98%) was determined microscopically after cytocentrifugation and staining with a modified Wright-Giemsa dye (Diff Quik®, Medion Diagnostics AG, Düdingen, Switzerland). Viability was determined by trypan blue dye exclusion. Monocytes were also analyzed for cell-surface CD14 and CD16 antigen expression by flow cytometry (FACScan, Becton Dickinson, Rutherford, NJ, USA). The $CD14^{dim}CD16+$ subset of purified monocytes was < 8% (Figure 3).

Monocytes were cultured at a density of $5x10^6$ cells/well in 6-well culture plates (Corning Incorporated, Costar®, NY, USA) in 2 ml of RPMI 1640+Glutamax-I Medium (GIBCO® by Life Technologies, Paisley, UK) supplemented with 50 µg/ml Gentamicin (GIBCO®), 5% heat-inactivated human AB serum (Sigma-Aldrich Inc., St. Louis, MO, USA) in moist air with 5% CO_2. During culture, monocytes were sequentially exposed to inflammatory and anti-inflammatory stimuli at different time points to mimic the micro-environmental conditions of an inflamed tissue. All human recombinant cytokines were obtained from R&D Systems (Minneapolis, MN, USA), while LPS (from *E.coli* serotype 055:B5) was from Sigma-Aldrich Inc..

Briefly, the *in vitro* stimulation was performed as follows: at time 0 monocytes were exposed to CCL2 (20 ng/ml) at 37°C. After 2 h, CCL2 was removed, cells were washed with PBS, fresh medium was added

containing LPS (5 ng/ml) and the temperature was increased to 39 °C. TNF-α (10 ng/ml) and IFN-γ (25 ng/ml) were added at times 3 and 7 h, respectively. Temperature was maintained at 39°C. At time 14 h, all inflammatory stimuli were removed, monocytes were washed with PBS, fresh medium was added containing IL-10 (20 ng/ml), and temperature brought back to 37°C until the end of experiment. At time 24 h, medium containing IL-10 was removed, fresh medium containing TGF-β (10 ng/ml) was added, and the culture prolonged until 48 h.

Freshly isolated monocytes were taken as time 0. Cells were harvested in 700 µl of Qiazol (Qiagen, Hilden, Germany) at times 2, 2.5, 3, 3.5, 4, 14, 24, and 48 h. Supernatants were collected at times 4, 14, 24, 48 h. Samples were stored at -80°C until analysis.

2.2 Staining procedure

The immunostaining were performed on peripheral blood monocytes from three donors, isolated and stimulated in culture as described in paragraph 2.1. After *in vitro* stimulation, the cells were collected and centrifuged at 300xg for 10 min, the supernatant fluid was discarded and the cell pellet was resuspended in cold PBS (Lonza, Verviers Belgium) plus 1% BSA (Sigma-Aldrich). An adequate volume of cells was added to four different tubes ($1x10^6$ cells/tube) and incubated with the following labeled antibodies in a total final volume of 100 µl: tube 1, isotype control labeled with phycoerythrin (PE) (5 µl) + isotype control labeled with peridin chlorophyll protein (PerCP) (1 µl); tube 2, anti-CD14-PerCP (10 µl) + isotype control-PE (5 µl); tube 3, anti-CD16-PE (10 µl) + isotype control-PerCP (1 µl); tube 4, anti-CD14-PerCP (10 µl) + anti-CD16-PE (10 µl). Isotype controls and specific antibodies were used at the same final concentration. All antibodies were from BD Biosciences (San Jose, California, USA). Tubes were incubated for 30 min on ice in the dark, then diluted with 1 ml PBS/BSA 1%

and centrifuged at 300xg for 10 min. Finally, samples were resuspended with 0.5 ml PBS/BSA 1% and immediately analyzed by flow cytometry.

2.3 Flow cytometric analysis

Monocytes were analyzed for identification of the three subsets ($CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{+}CD16^{++}$) by flow cytometry (FACScan, Becton Dickinson, Rutherford, NJ, USA) with BD Cell Quest software. Monocytes were initially gated using a morphological selection (gate) based on forward scatter (FSC, cellular size) and side scatter (SSC, cellular complexity) parameters. The subsequent evaluation of CD14 and CD16 expression was performed by quantification of FL3 (red) and FL2 (orange) fluorescence emissions, which represent the specific binding of antibodies conjugated to PerCP and PE fluorochromes, respectively. Amplified settings for FSC and SSC were used in linear mode and those for fluorescence channels were used in logarithmic mode. A threshold was fixed on FSC to exclude cellular debris. The analysis of CD14 and CD16 expression was performed both in PBMC and in purified monocyte suspension, with acquisition of 30,000 morphologically gated events per tube. The percentage of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{+}CD16^{++}$ monocyte subsets in the monocyte preparations purified by magnetic sorting fully reflected the percentage of the same subpopulations found in PBMC (Figure 3). Figure 5 reports the single antibody histograms of a representative experiment. The percentages of the single and double positive populations were calculated by fluorescence histogram analysis.

2.4 RNA isolation and microarray hybridization

For the “early time” series (0, 2, 2.5, 3, 3.5 h) total RNA was extracted from monocytes of 3 donors. For the “long time” series (0, 4, 14, 24, 48 h) RNA was collected from monocytes of 9 other donors. Extraction was performed

using Qiagen miRNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA samples were quantified by ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was checked by microcapillary electrophoresis on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) on the basis of the ratio between 28S and 18S rRNA peak areas and of the RIN (RNA integrity number) index. Only good quality RNA was used (28S/18S ratio ≥ 1.7, RIN index ≥ 7). RNA samples were stored at -80°C until use.

For high-throughput gene expression profiling, RNA samples were prepared from 100 ng total RNA using the GeneChip® 3' IVT Express kit (Affymetrix) for "early times" samples, and from 1 µg total RNA using the GeneChip® One Cycle cDNA Synthesis kit (Affymetrix) for "long times" samples, following the manufacturer's protocols. Biotinylated cRNAs (15 µg) were fragmented and hybridized for 16 h at 45°C onto GeneChip® HG-U133 Plus 2.0 Arrays (Affymetrix). After washing and staining, arrays were scanned with the GeneChip® Scanner 3000 7G (Affymetrix) and fluorescent images were acquired and analyzed using GCOS software (Affymetrix) to generate a total of 60 raw intensity files (CEL files).

2.5 Data analysis

Data analysis was performed in R using Bioconductor libraries and R statistical packages. Probe level signals were converted to expression values using robust multi-array average procedure (RMA; [157]) and HG-U133 Plus 2.0 custom Chip Definition Files (CDF) based on GeneAnnot ([158]; CDF Version: 2.2.0, GeneCards Version: 3.04, GeneAnnot Version: 2.0). Briefly, intensity levels were background-adjusted and normalized using quantile normalization, and log2 expression values calculated using median polish summarization. Raw data are available with the author.

Genes showing different expression profiles along the time-course experiments were identified using the microarray Significant Profiles method coded in the maSigPro R package [159]. maSigPro first applies a least-square technique to estimate the parameters of a general regression model for each gene (*make.design* function) and then uses the regression coefficients of the model to identify genes with statistically significant changes in their expression profiles (*p.vector*, *T.fit* and *get.siggenes* functions). Since the time-course was composed of 9 points, we computed a regression fit for each gene using a polynomial with a degree of 3 (cubic regression model) and selected those regression models with an associated corrected p-value ≤0.05. P-values have been corrected for multiple comparisons using the false discovery rate procedure (FDR), *i.e.* setting the parameter Q=0.05 in the *p.vector* function. Once the statistically significant gene models were determined, the regression coefficients were used to identify genes showing statistically significant expression changes over time. To do this, a second model was constructed using only significant genes and applying a variable selection strategy based on stepwise regression. Specifically, we selected the backward stepwise regression and, at each iteration, retained those variables with a p-value ≤0.01 (*i.e.*, set the *T.fit* parameters at step.method=backward and alfa=0.01). Finally, we generated the list of significant genes by setting an additional selection criterion based on the R-squared value of the second regression model (*i.e.*, set the *get.siggenes* parameters rsq=0.6 and vars=all). Results have been visualized clustering genes into k=9 groups, using maSigPro k-mean clustering and default value for k.

2.6 Collection and processing of publicly available gene expression data

We retrieved datasets of monocytes, macrophages, and dendritic cells (DC) from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), which contains information about cell treatment and gene expression data obtained with Affymetrix arrays. Specifically, 24 series comprising 474 samples of human normal monocytes, macrophages, and DC were downloaded from GEO and 303 samples organized in a proprietary database using the software A-MADMAN (Supplementary Table S1). A-MADMAN is an open source web application that allows the automatic download and organization of GEO and proprietary raw data and annotations, the automatic import of metadata from GEO records into a local relational database, the subsequent manual annotation and selection of samples through user-defined tags, and the selection of samples to be analyzed using a complex logical query on tags [160]. All samples have been manually re-annotated and tagged based on the meta-information provided by GEO and by the original publications. In particular, we labeled 62 samples as untreated monocytes and 46 and 20 samples as M1 and M2 activated monocytes/macrophages, respectively (Supplementary Table S2).

Raw expression data (*i.e.*, CEL files) obtained from different platforms have been integrated using an approach inspired by the generation of custom Chip Definition Files (CDF; [161]). In custom CDF, probes matching the same transcript, but belonging to different probes sets, are aggregated into putative custom-probe sets, each one including only those probes with a unique and exclusive correspondence with a single transcript. Similarly, probes matching the same transcript but located at different coordinates on different type of arrays may be merged in custom-probe sets and arranged in a virtual platform grid. As for any other microarray geometry, this virtual

grid may be used as a reference to create the virtual-CDF file, containing the probes, shared among the platforms of interest, and their coordinates on the virtual platform, and the virtual-CEL files containing the intensity data of the original CEL files properly re-mapped on the virtual grid. Once defined the virtual platform through the creation of its custom-CDF and transformed the CEL files into virtual-CEL files, raw data, originally obtained from different platforms, are homogeneous in terms of platform and can be preprocessed and normalized adopting standard approaches, as RMA or GCRMA. Here, expression values were generated from intensity signals using the combined HG-U133A/HG-U133Av2/HG-U133 Plus2.0 virtual-CDF file, the custom definition files for human GeneChips based on GeneAnnot, and the transformed virtual-CEL files. Intensity values for a total of 12167 meta-probesets were background-adjusted, normalized using quantile normalization, and gene expression levels calculated using median polish summarization (RMA algorithm; [157]).

This expression matrix has been analyzed to identify differentially expressed genes in the comparisons between subsets of monocytes tagged as untreated, M1, and M2 (128 samples, see Supplementary Table S2) using the Significance Analysis of Microarray method (SAM; [162]) coded in the *samr* R package (http://cran.r-project.org/web/packages/samr/index.html). Specifically, in the comparison between untreated monocytes and samples labeled as M1 (or as M2), we used the two-class procedure, estimated the percentage of false positive predictions with 1000 permutations, and selected those transcripts whose q-value (*i.e.*, False Discovery Rate, FDR) was equal to 0. This selection was further refined setting the lower limit for fold change induction (or reduction) to 5 and 8, when considering the comparison between untreated monocytes and samples M1 or untreated monocytes and samples M2, respectively.

2.7 Over-representation analysis

Over-representation analysis was performed using Gene Set Enrichment Analysis software (GSEA; http://www.broadinstitute.org/gsea/index.jsp; [163]) and the gene sets of the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). GSEA was applied on log2 expression data of the entire time course. The median expression profiles of the 9 groups of genes identified by maSigPro was used as continuous phenotype labels, and the Pearson's correlation as the metric to select gene sets with expression patterns resembling those encoded in the phenotype labels. As gene sets we used KEGG, Biocarta, and Reactome lists of the *C2: curated gene sets* collection. Finally, gene sets were defined as significantly enriched if the False Discovery Rate (FDR) was < 5% when using Signal2Noise as metric and 1,000 permutations of phenotype labels.

2.8 Gene expression validation by qPCR

For gene expression validation by qPCR we used two different methods (ΔΔCt and Comparative Quantitation) as described hereafter:

1. cDNAs were reverse-transcribed from total RNA (100 ng) using High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. Taqman polymerase chain reaction was performed by an ABI PRISM 7900 sequence detection system (Applied Biosystems) and using Taqman Universal PCR Master Mix (Applied Biosystems) in 50 µl reaction volume. Primers and probes were supplied by Applied Biosystems as pre-made solutions, both for targets (*IL6*, *TNFA*, *IL7R*, *CD163*, *MMP9*, *MAFB*, *KLF4*, *PPARG*, *PPARD*, *C/EBPA*) and for endogenous control (*GAPDH*). Each cDNA sample was run in triplicate. Thermal cycling was started with an initial denaturation at 50°C for 2 min and 95°C for 10 min, followed by 40 thermal cycles of 15 sec at 95°C and 1 min at 60°C. Statistical analysis of the QRT-PCR signals was performed

using the ($2^{-\Delta\Delta Ct}$) method [164, 165], which calculates relative changes in gene expression of the target gene normalized to the endogenous control and relative to a calibrator sample (0 h). QRT-PCR reactions were carried on six independent samples and then the data obtained were represented, in terms of relative quantity (RQ) of mRNA level variations, as mean ± SEM value.

2. cDNAs were reverse transcribed from total RNA (100 ng) according to the QuantiTect Reverse Transcription Kit (Qiagen) instructions, with oligo-dT and random primers, to allow for high cDNA yield. Three separate reverse transcriptions were performed for each samples and an identical reaction without the reverse transcriptase was run, as negative control. Taqman polymerase chain reaction was performed by an Rotor-Gene™ 3000 (Corbett Research, Doncaster Victoria, Australia), using the QuantiTect SYBR Green PCR master Mix (Qiagen). The final reaction contained 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix, 0.3 µM of each primer and 2.5 µl of cDNA in a total volume of 25 µl. PCR conditions were 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 50-60°C for 30 s and 72°C for 30 s. Primer sequences were supplied by Qiagen both for target (*IL1B*, *IL1RN*, *IL1R2*, *IL1F9*, *IL18* and *IL18BP*) and housekeeping (*ACTB*) genes. Relative gene expression was calculated using the efficiency correction method, which calculates the relative expression ratio from the qPCR efficiencies and the Ct between the target gene and the endogenous control, relative to a calibrator sample (0 h) [166].

2.9 Protein detection by ELISA

IL-6, CXCL8, CCL5, IL-1β, IL-1Ra, sIL-1RII, IL-18, IL-18BP and IL-1F9 proteins were measured on supernatants collected at different stimulation times (4, 14, 24, and 48 h), by enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-6, CXCL8, CCL5, IL-1β, IL-1Ra, sIL-1RII, and IL-

18BP were purchased from R&D Systems (Minneapolis, USA), while the kit for IL-18 was obtained from MBL (Nagoya Aichi, Japan), and two kits for IL-1F9 were obtained from USCNK Life Science Inc. (Wuhan, China) and from Innovative Research (Novi, MI, USA). ELISA assays were performed according to the manufacturers' instructions. Each sample was assayed in duplicate, and detection carried out with a JUPITER microplate spectrophotometer (Asys Hitech GmbH, Eugendorf, Austria).

After measuring the concentration of both IL-18 and IL-18BP in each sample, the law of mass action was used to calculate free-IL-18 (*i.e.*, the fraction of cytokine not bound to its inhibitor IL-18BP) as previously described [167, 168]. Briefly, the calculation was based on a 1:1 stoichiometry in the complex IL-18 and IL-18BP, a molecular weight of 18.4 kDa for IL-18 and 17.6 kDa for IL-18BP, and a dissociation costant (Kd) of 0.4 nM [169].

2.10 Statistical data analysis

The qPCR and ELISA results are expressed as mean values ± SEM. Differences between groups were analyzed using ANOVA and Fisher 's test. A *P* value of less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1 The in vitro monocyte-based model of inflammation

To build an *in vitro* model of inflammation, blood monocytes from 12 individual healthy donors were exposed in culture to a temporal sequence of different micro-environmental conditions that mimic the *in vivo* development of the inflammatory reaction. Monocytes were isolated from buffy coats by magnetic selection of $CD14^+$ cells. The recovered cells were viable and >98% monocytes, as judged by morphological examination on cytosmears and by flow cytometrical analysis of scattering and CD14 positivity. It should be noted that the percentage of $CD14^{dim}CD16^+$ monocytes after magnetic purification fully reflected the percentage of the same monocyte subpopulation in total blood and in PBMC (about 8-12% of total monocytes, Figure 3). Therefore, the monocyte population we used in our experiments is representative of the monocyte heterogeneity as present in the circulation. The experimental procedures were carried out in the presence of 5% human pooled AB serum (as opposed to autologous serum or plasma), in order to avoid a putative source of variability. As shown in the Figure 4, freshly isolated monocytes were exposed to the chemokine CCL2 for 2 h at 37°C, to represent the CCL2 driven efflux of inflammatory monocytes from circulation to the site of inflammation. At 2 h, monocytes were exposed to the TLR4 agonist LPS, to mimic the encounter of inflammatory monocytes with infectious agents at the tissue site of reaction, and the temperature was raised to 39°C (as in an inflamed tissue). Coating of tissue culture plates with collagen and fibronectin, to reproduce the presence of extracellular matrix in the tissue microenvironment, was avoided after preliminary experiments, due to the potent direct macrophage activation of the collagen/fibronectin coated plastic surfaces (probably “seen” as a foreign entity). The development of the inflammatory reaction

was reproduced by keeping the temperature at 39°C until 14 h and by adding in sequence TNF-α (at 3 h, representing the tissue/resident cell reaction) and IFN-γ (at 7 h, representing the reaction of the later influx of Th1 cells). To reproduce the destruction of the inflammation-inducing infectious agent and the resolution of the inflammatory response, at 14 h all the inflammatory stimuli were washed off, the temperature was brought down to 37°C and fresh medium containing IL-10 was added (representing the activation of anti-inflammatory mechanisms). As conclusive phase of the resolution, at 24 h monocytes were exposed to TGF-β to reproduce macrophage deactivation towards re-establishment of tissue integrity and homeostasis.

Transcriptomic analysis was performed on monocytes from each individual donor at five different stages of activation: freshly isolated monocytes (time 0); cells at the early and late phases of inflammation (collected after 4 h and 14 h of culture, respectively), corresponding to different stages of classically activated macrophages (M1 polarization; [41, 43]); and cells during the resolution of inflammation (collected at 24 and 48 h), corresponding to different stages of macrophage “deactivation” (M2c polarisation; [45]). In addition, a series of samples were collected at very early times (2.0, 2.5, 3.0 and 3.5 h), in order to better analyse the first phases of inflammatory activation.

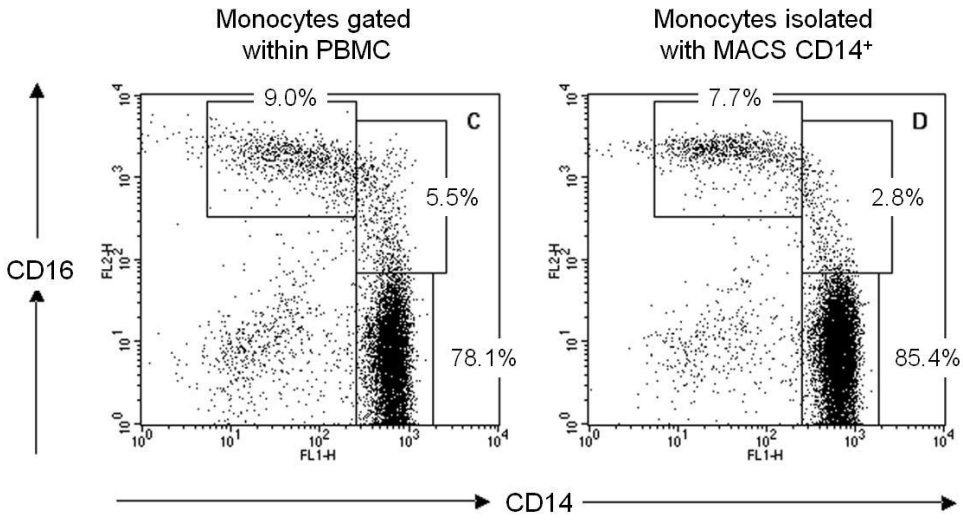

Figure 3. *Scatter analysis of suface markers CD14 and CD16 on monocyte subsets in PBMC (left panel) and after magnetic purification (right panel). Shown is a representative staining for identification of monocyte subsets in a blood sample from a healthy donor. The percentage of* $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{dim}CD16^{+}$ *monocytes after magnetic purification fully reflected the percentage of the same monocyte subpopulation in PBMC, showing that the monocyte population used in our experiments is representative of the monocyte heterogeneity as present in the circulation.*

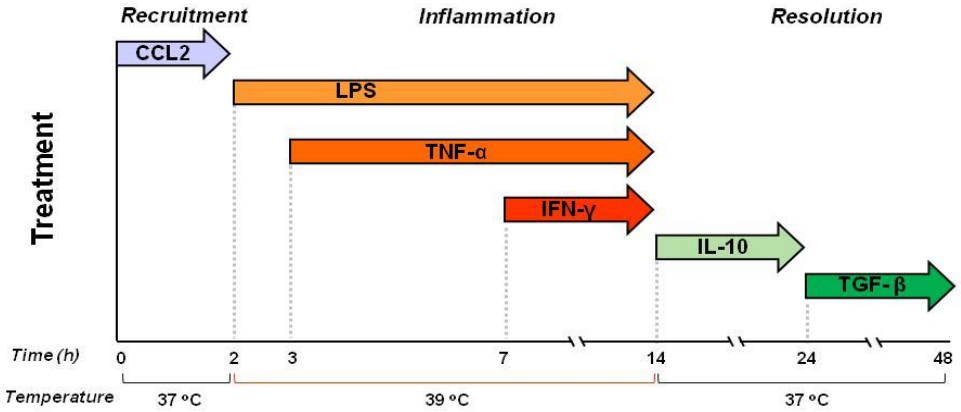

Figure 4. *Graphic representation of the in vitro model of inflammation based on human primary monocytes. Freshly isolated monocytes are first exposed to the chemokine CCL2 for 2 h at 37°C. At 2 h, monocytes are exposed to LPS and the temperature is raised to 39°C. Temperature is then kept at 39°C until 14 h while TNF-α and IFN-γ are added at 3 and 7 hours, respectively. At 14 h all the inflammatory stimuli are washed off, the temperature brought down to 37°C and fresh medium containing IL-10 added. Finally, monocytes are exposed to TGF-β at 24 hours.*

3.2 Changes of monocyte subsets during the inflammatory reaction

As previously mentioned, the flow cytometrical analysis shows that the monocyte population obtained after magnetic purification fully reflected, in terms of heterogeneity of monocyte subpopulations, the heterogeneity of monocytes present in total blood and in PBMC preparations (Figure 3, and Figure 5 panels A and B). Preliminary data show that during the different phases of the inflammatory reaction the $CD14^{++}CD16^{-}$ monocytes were reduced starting from late inflammation (14 h) to resolution phases (Figure 5, right panels C, D, E, F). The $CD14^{++}CD16^{-}$ subset is the monocyte subpopulation preferentially recruited by CCL2 to an inflamed tissue soon after an infection *in vivo* [38, 170].

On the other hand, the $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ subsets entirely disappeared during the early inflammation (4 h) after stimulation with LPS and TNF-α, with $CD14^{++}CD16^{+}$ monocytes growing back to baseline percentage (8.14%), and $CD14^{+}CD16^{++}$ monocytes increasing over baseline (from 2% to 6.19%) during the resolution phase. These findings are in agreement with the role in tissue remodeling and angiogenesis attributed to $CD16^{+}$ cells during the resolution of inflammation [26]. Taken together these observations could validate: 1. the hypothesis that CD16 may be a marker of activation among $CD14^{+}$ monocytes [30]; 2. the direct developmental relationship between the three subsets, with $CD14^{+}$ monocytes developing into $CD16^{+}$ cells [32]. In fact in the model the same monocyte population goes through all inflammatory phases and is polarised to M2 [31, 33], although there is no evidence of the intermediate subset being more closely related to classical or non-classical subsets [27-29].

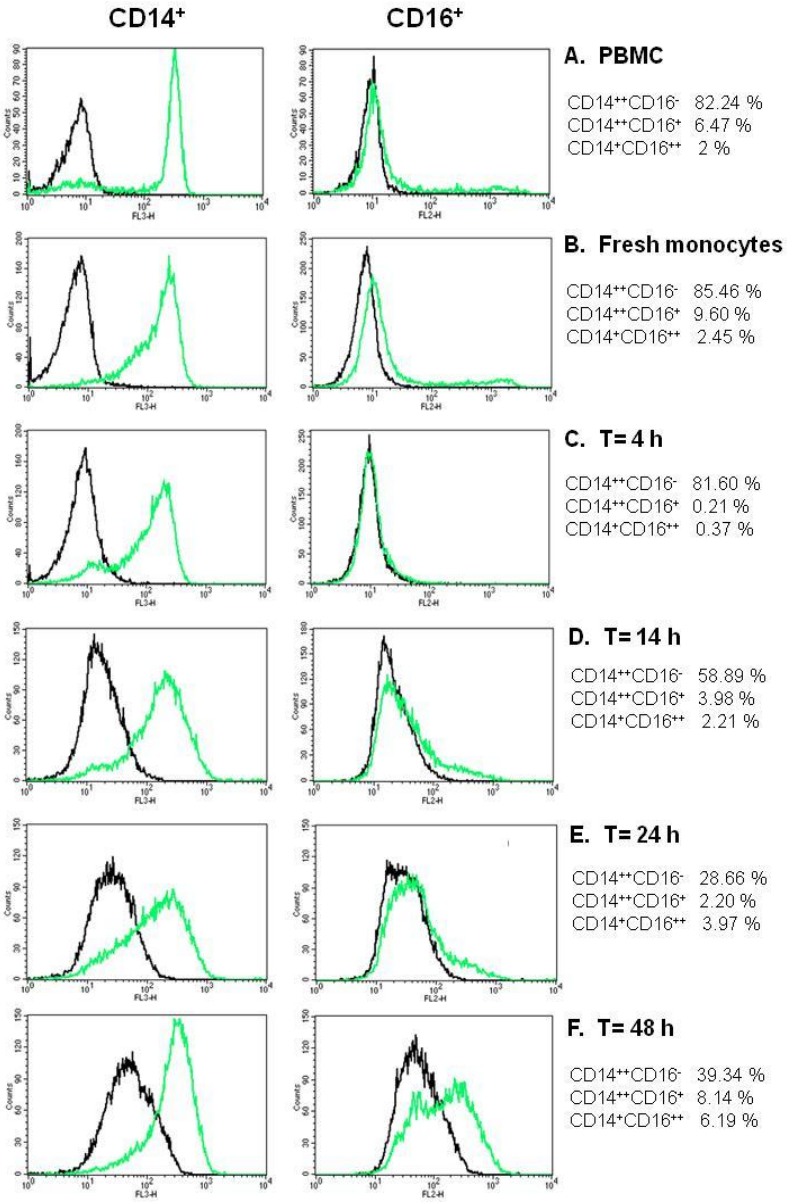

Figure 5. *Fluorescence histogram analysis of the monocyte subpopulations observed during the different phases of the inflammatory reaction (C, D, E, F). A and B show the monocyte subpopulations in PBMC and in fresh monocytes (after magnetic isolation), respectively. Concomitant analysis of expression of CD14 (right) and CD16 (left) was performed. The percentage of* $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{dim}CD16^{+}$ *monocytes are reported for a single representative experiment, although the histograms only show the single stainings.*

3.3 Transcriptional profiling and cluster analysis identify distinct gene signatures during the inflammatory response

After mRNA isolation and retrotranscription, cRNA corresponding to different stages of monocyte activation was hybridized onto Affymetrix microarrays to generate gene expression profiles. To investigate the effect of the different stimuli on the transcriptional levels along the time-course experiment, we started by identifying genes showing statistically significant expression changes over time. For this, we used the microarray Significant Profiles method coded in the maSigPro R package [159] with default parameters. Results of the maSigPro analysis revealed quite dramatic changes in gene expression during the different phases of the inflammatory reaction, and during the concomitant monocyte-to-macrophage differentiation. Indeed, a total of 3995 genes were differentially expressed during the course of inflammation at a 95% confidence level (false discovery rate procedure (FDR) ≤ 0.05). Crucially, using k-means clustering method and maSigPro default parameters (*i.e.*, 9 clusters), significant genes were shown to have distinct expression profiles during the inflammatory reaction (Supplementary Figure 1). To gain insights into the mechanisms by which these genes are linked to the inflammation processes, the 9 clusters generated by maSigPro have been merged into five major functional groups of genes characterising the different phases of inflammation (Figure 6). In particular, we defined as belonging to the functional group *inflammation* genes included in clusters 1 and 2, as *early anti-inflammation* and *anti-inflammation* genes of clusters 3 to 5, as *inflammation driven differentiation* genes of cluster 6, and as *positive* and *negative differentiation* genes of cluster 7 and of clusters 8 and 9, respectively.

The *inflammation* phase, corresponding to monocyte-to-M1 macrophages differentiation, is associated with the modulation of 392 transcripts. Of these, 218 are transiently up-regulated during the first four hours of the inflammatory process (Supplementary Figure 1, cluster 1), while 174 remain highly expressed during the late phases, *i.e.*, until 14 h (Supplementary Figure 1, cluster 2). In both clusters, transcriptional levels decrease during the resolution phase. Genes included in these two groups are the typical effectors of classical activation, such as inflammatory cytokines (*e.g.*, *IL1B*, *IL6*, *TNFA*, *IL12B*), chemokines (*e.g.*, *CCL4*, *CCL5*, *CCL20*), extracellular mediators (*e.g.*, *PTX3*, *EDN1*, *APOL2*), and enzymes (*e.g.*, *PTGS2*, *PLA1A*) (Figure 6). The *early anti-inflammatory* (Supplementary Figure 1, cluster 3) and *anti-inflammatory* clusters (Supplementary Figure 1, clusters 4 and 5) contain 850 and 1021 genes, respectively, and basically include genes down-regulated in M1 polarized monocytes. Their median expression levels rapidly decrease during the stimulation with LPS/TNF-α (4 h), increase or remain stably low during the stimulation with IFN-γ (14 h), and return to basal level in the resolution phase. *Early anti-inflammatory* and *anti-inflammatory* include genes coding for transcriptional factor such as CCAAT/enhancer binding protein alpha (*C/EBPA*), innate receptors (such as *TLR5*, *TLR7* and *TLR8*), purinergic receptors (*e.g.*, *P2RX7*), and Fc receptor (*e.g.*, *FCER1A*, *FCRLB*). We hypothesize that the decreased expression of some receptors involved in the inflammatory response may be related to loss of responsiveness following activation (similar to tolerance), which is restored at the end of inflammatory process when inflammatory monocytes have become tissue-regulating macrophages and should be ready to respond at a new danger signal.

Moreover, these clusters include genes for the highly conserved metal-binding proteins metallothioneins (specifically *MT1G*, *MT4*, *MT1E*, *MT1M*,

MT1F, and *MT1X*) involved in metal homeostasis, detoxification, modulation of inflammation, control of the oxidative stress, cell proliferation [171] and strongly up-regulated in endotoxin tolerance [172].

Genes associated to the *inflammation driven differentiation* are characterized by an expression signal rapidly increasing upon the inflammatory reaction and then remaining at elevated levels throughout all phases of the reaction (Supplementary Figure 1, cluster 6, 457 genes). This group comprises genes needed for the inflammatory response and also critical for the process of monocyte differentiation into deactivating and tissue-repairing macrophages. Although the cluster includes a number of inflammatory genes and M1 polarization markers (*e.g.*, *IL7R*, *CCR7*, *CCL19*, *CXCL11*; [54]) nevertheless they present expression profiles markedly different from those of the *inflammation* or of the *positive differentiation* clusters. Notably, this cluster also includes several genes that are allegedly highly expressed in M2c polarization, such as *IL10*, *CCL24*, and *CCL22* [54].

The last three clusters group genes that may be important for the differentiation of monocytes to macrophages. The *positive differentiation* cluster includes 214 genes (Supplementary Figure 1, cluster 7), which are not expressed in fresh monocytes and during the early phases of inflammation, but are progressively up-regulated along the time course with a transcriptional peak during the repair phase. These include genes for transcriptional factors such as *MAFB* and some extracellular mediators (*e.g.*, *C1Q*, *APOE*). Conversely, the *negative differentiation* clusters comprise a total of 1061 genes (Supplementary Figure 1, clusters 8 and 9), which are highly expressed in fresh monocytes and in the early inflammation phase, and then reduce their transcriptional levels during the subsequent phases. Among these genes, there are transcription factors of the Krüppel-like family (*e.g.*, *KLF4*) and of the peroxisome proliferation-

activated receptor family (*e.g.*, *PPARG*), c-type lectin members (*e.g.*, *CLEC3B*, *CLEC7A*, *CLEC10A*, *CLEC11A*), adhesion (*e.g.*, *SELL*, *ICAM3* and *AMICA1*), and signalling molecules (MAP kinases).

It is possible that these genes may define the state of differentiation of monocytes to macrophages independently of the concurring inflammatory reaction. Indeed, it should be noted that the fresh monocytes used in these experiments are an heterogeneous population as present in the blood and could therefore include both “inflammatory” monocytes that differentiate into effector cells in the tissue, and “homeostatic” monocytes that replenish the pool of tissue macrophages in physiological conditions.

Finally, the clustering reported in Figure 6 highlights the striking homogeneity and reproducibility of the gene expression profiles in the different donors at the different time points, thus reinforcing the robustness of the model.

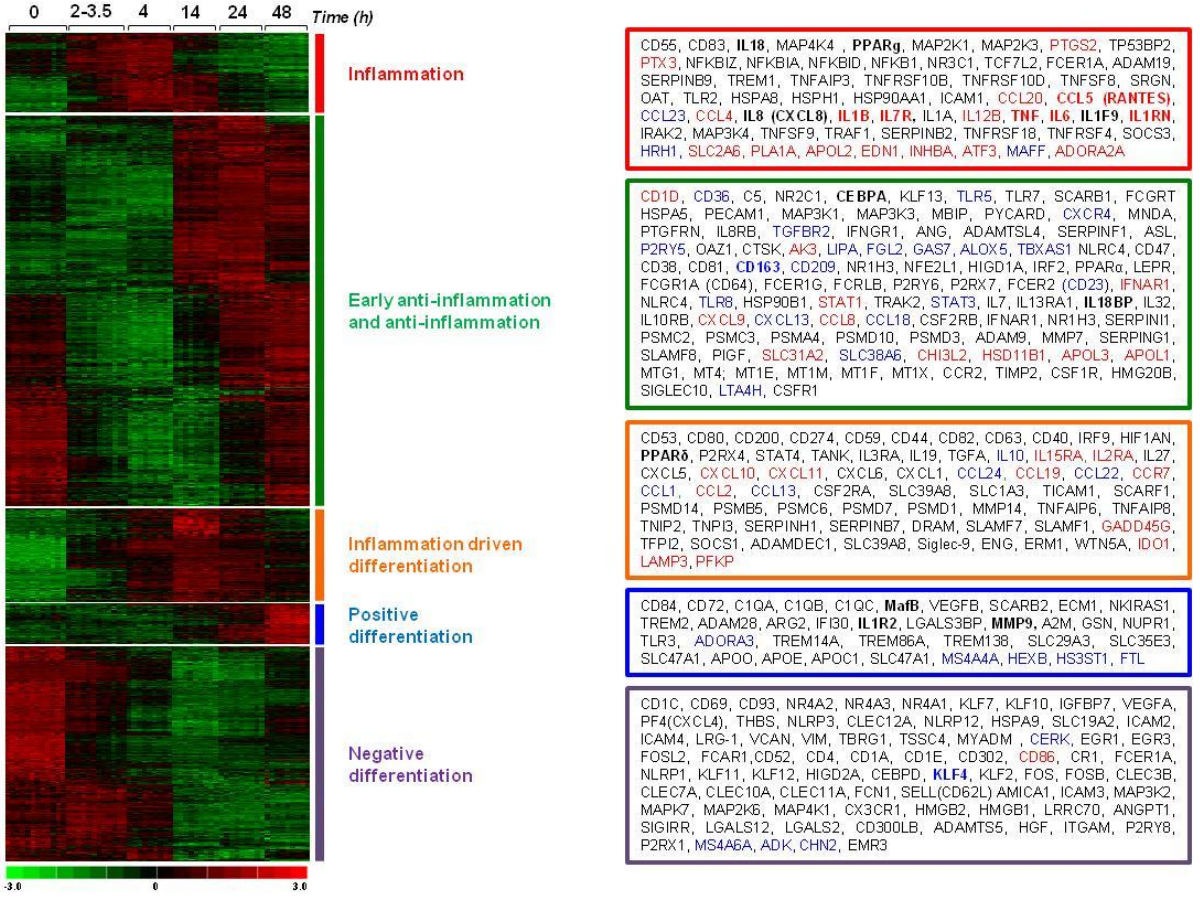

Figure 6. *Heat-map indicating the fold-expression levels of the genes that were identified by maSigPro as coherently down-regulated (green) or up-regulated (red) during the time course experiment. Genes are organized into five major functional groups characterizing the different phases of the inflammatory model: inflammation, early anti-inflammation and anti-inflammation, inflammation driven differentiation, positive and negative differentiation. The representative genes from each clusters are shown. The red and blue genes are a selection of genes strictly associated with M1 and M2 macrophage polarization, respectively, as reported in literature [54], while bold genes are those validated by qPCR and/or ELISA.*

3.4 Pathway analysis reveals that monocyte activation and macrophage differentiation are closely related biological processes

To investigate the biologic role of the genes differentially expressed during the development of the inflammatory response, each cluster was subjected to Gene Set Enrichment Analysis (GSEA) [163]. GSEA is a computational method that determines whether an *a priori* defined set of genes shows statistically significant differences between two biological states. Specifically, we searched in the samples of the *in vitro* model for statistical associations between expression profiles of distinct clusters and other gene 'signatures' that register elevated activity of various signalling pathways or dysregulated cellular processes derived from KEGG, Biocarta, and Reactome. We identified a total of 155, 358, 55, 149, and 66 pathways most strongly associated with the median expression profile of the *inflammatory*, *early anti-* and *anti-inflammatory*, *inflammation driven differentiation*, *positive differentiation* and *negative differentiation* clusters, respectively. The most representative gene sets associated with the *inflammatory* and *early anti-* and *anti-inflammatory* clusters are listed in Table II. The complete lists of pathways for each cluster are reported in the Supplementary Table S3. While some of the identified pathways/gene sets are not readily interpretable in the context of the specific functional activation/differentiation phase, other pathways are clearly related to the various phases of the inflammatory process. We found that the majority of gene sets associated to the *inflammation* clusters are classical inflammatory pathways involved in innate immune activation as the NFkB, MAPK and JAK-STAT signalling, NOD-like receptor and Toll-like receptor signalling, cytokine/chemokine receptor interaction, and the IL-1 receptor pathway. These typical inflammatory pathways were not found associated

to other clusters (with the exception of *inflammation driven differentiation*, see below), and they are mainly involved in type I inflammatory response carried out by M1 macrophages [173]. On the other hand, the *early anti-* and *anti-inflammatory* clusters are enriched in pathways associated to lipid, protein, and carbohydrate metabolism, and regulation of gene expression (*i.e.*, RNA splicing and miRNA biogenesis), and cell cycle. The modulation of genes involved in cellular metabolic activities is a prominent feature of M2 macrophage polarization/differentiation [173, 174], and it is conceivable that the up-regulation of these pathways occurs during the phases of resolution and repair, when major rearrangements of cellular functions are required, from inflammation to anti-inflammation and to synthesis of tissue repair-promoting factors.

The *inflammation driven differentiation* group is associated to signalling cascades that are in common with both inflammatory and anti-inflammatory phases, while pathways enriched in the *positive differentiation* and *negative differentiation* clusters are similar to those found during the anti-inflammatory phase. Moreover, the expression profiles of all these three clusters statistically resembles that of pathways associated with cell cycle.

Globally, the functional enrichment analysis indicates that genes involved in inflammatory response and monocytes activation present transcriptional profiles that are statistically similar to those of genes involved in the control of the different cellular processes (as cell growth/proliferation and metabolism) during the monocyte-to-macrophage differentiation *in vitro*. These results establish a transcriptional link between monocyte activation and differentiation, inflammation and metabolism on one side and inflammation, resolution and cell differentiation on the other.

Table II. Most representative gene sets associated with the inflammatory and early anti- and anti-inflammatory clusters. KEGG, Biocarta, and Reactome gene sets have been obtained from the C2: *curated gene sets collection* of the Molecular Signatures Database. Gene sets were defined as significantly enriched if the False Discovery Rate (FDR) was < 5% when using Signal2Noise as metric and 1,000 permutations of phenotype labels.

Cluster	FDR q-val
Inflammation	
BIOCARTA_NFKB_PATHWAY	0.003
BIOCARTA_IL-1R	0.010
BIOCARTA_IL-10_PATHWAY	0.013
BIOCARTA_INFLAM_PATHWAY	0.021
BIOCARTA_CD40_PATHWAY	0.033
BIOCARTA_CYTOKINE_PATHWAY	0.044
KEGG_MAPK_SIGNALING	0.000
KEGG_CYOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.000
KEGG_NOD_LIKE_RECEPTOR_SIGNALING	0.000
KEGG_ECM_RECEPTOR_INTERACTION	0.001
KEGG_CELL_ADHESION_MOLECULES_CAMS	0.005
KEGG_PATHWAYS_IN_CANCER	0.008
KEGG_JAK_STAT_SIGNALING	0.011
KEGG_NOTCH_SIGNALLING	0.015
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING	0.037
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	0.000
REACTOME_GPCR_LIGAND_BINDING	0.000
Early anti- and Anti-inflammation	
KEGG_OXIDATIVE_PHOSPHORYLATION	0.000
KEGG_RNA_DEGRADATION	0.001
KEGG_FATTY _ACID_METABOLISM	0.014
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	0.000
REACTOME_ELECTRON_TRANSPORT_CHAIN	0.000
REACTOME_INTEGRATION OF ENERGY METABOLISM	0.000
REACTOME_METABOLISM _OF _CARBOHYDRATES	0.000
REACTOME_PYRUVATE_METABOLISM_AND_TCA_CYCLE	0.000
REACTOME_METABOLISM_OF_PROTEIN	0.000
REACTOME_DIABETES_PATHWAYS	0.000
REACTOME_METABOLISM_OF _RNA	0.003
REACTOME_FORMATION_AND_MATURATION_OF_MRNA_TRANSCRIPTS	0.004
REACTOME_MRNA_SPLICING	0.009
REACTOME_METABOLISM_OF_MRNA	0.011
REACTOME_GENE_EXPRESSION	0.016
REACTOME_MICRORNA_BIOGENESIS	0.040
REACTOME_CELL_CYCLE_MITOTIC	0.000
REACTOME G1_S_TRANSITION	0.000
REACTOME G2_M_CHECKPOINTS	0.031

3.5 Monocytes display an M1 gene signature during inflammation that develops into an M2 gene signature during the resolution phase

To assess the capacity of the *in vitro* model of inflammation of representing the transition from M1 to M2 phenotype polarization, we merged 24 publicly available microarray studies into a meta-dataset using the software A-MADMAN [160], and extracted gene expression signals for 62 fresh human unstimulated monocytes, 46 M1 and 20 M2 polarized macrophages.

Samples were labeled as M1 if the meta-information provided by GEO or by the original publications referred to monocytes treated with either LPS/TNF-α or IFN-γ, and as M2 if describing monocytes treated with glucocorticoids or IL-10 or TGF-β (M2c). Gene expression signals of the meta-dataset were generated using the *Virtual-chip* approach, which allows integrating raw expression data (*i.e.*, CEL files) obtained from different Affymetrix arrays. Specifically, expression values were generated from intensity signals using the combined HG-U133A/HG-U133Av2/HG-U133 Plus2.0 virtual-CDF file, the custom definition files for human GeneChips based on GeneAnnot [158], and the transformed virtual-CEL files. Intensity values for a total of 12167 meta-probesets were background-adjusted, normalized using quantile normalization, and gene expression levels calculated using median polish summarization (RMA algorithm; [157]).

The meta-dataset was analyzed by the Significance Analysis of Microarrays (SAM; [162]) algorithm to identify a list of genes differentially expressed in unstimulated monocytes, M1 and M2 polarized macrophages. The statistical comparison returned that monocyte-to-M1 differentiation is associated with modulation of 98 genes, of which 85% are highly expressed in M1 and 15% in monocytes (Figure 7A; Supplementary Table S4), while monocyte-to-M2 differentiation results in 107 genes, 62% highly

expressed in M2 and 38% in monocytes (Figure 7B; Supplementary Table S5). Transcripts that are up-regulated in M1 cells as compared to monocytes included cytokines and chemokines, while those up-regulated in M2 cells included enzymes and extracellular mediators. After excluding those genes that are modulated in both M1 and M2 *vs*. monocytes, the two signatures of M1 and M2 polarization were used to cluster samples of the *in vitro* model of inflammation. As shown in the Figure 7 (panels C and D), these signatures recapitulate the behavior of monocytes during the development of the *in vitro* inflammatory response. Fresh monocytes in panel C show a gene expression profile fully overlapping with that of the fresh monocytes in the meta-database, *i.e.*, they express the M1-like expression profile during the inflammatory phases, to return to a monocyte-like profile in the resolution phase (panel C). On the other hand, when considering the gene set that distinguish monocytes from M2 cells, fresh monocytes have the same profile as monocytes from the meta-database, this profile gradually changing during the progression of inflammation, to become comparable to the profile of M2 cells during the end (48 h) of resolution phase (panel D).

Moreover, when comparing the list of genes differentially expressed during the entire inflammation process (Figure 6) with the list of genes differentially expressed in monocytes *vs*. M1 (Figure 7A), the majority of genes expressed in M1 cells (34%) belong to *inflammation* cluster, *i.e.* their expression signals rapidly grow during the inflammatory process, are steady during the late inflammation, and return to basal levels thereafter. Some of these transcripts correspond to cytokines (*e.g.*, *IL12B*, *TNF*, *IL6*) and chemokines (*e.g.*, *CCL4*, *CCL20*), signalling molecules (*e.g.*, *NFKB1*), and extracellular proteins functionally related to the innate inflammatory response (*e.g.*, *PTX3*) (Table III). On the other hand, the comparison between the list of genes differentially expressed during the inflammatory

process and those differentially expressed in monocytes *vs*. M2 (Figure 7B) indicates that 21% of genes expressed in M2 cells belongs to the cluster termed *positive differentiation* and are expressed only during the resolution phase. Among these transcripts there are extracellular mediators such as *APOE*, *APOC1* (Table III). In both comparisons, the majority of genes up-regulated in fresh monocytes and in common with genes of Figure 6 belongs to the *anti-inflammation* cluster (26%) for monocytes *vs*. M1, and to *negative differentiation* cluster (51%) for monocytes *vs*. M2. The *negative differentiation* cluster includes genes that are up-regulated in fresh monocytes and during inflammation, and down-regulated during the resolution phase, and some of them are involved in immune response (*e.g.*, *FCER1A*, *NLRP3*) and in cell adhesion (*e.g.*, *ICAM3*, *VCAN*) (Table III). Moreover, among genes related to both M1 and M2 polarization, some belong to the *inflammation driven differentiation* cluster (14% and 20% respectively). In this cluster, we find genes encoding for chemokines and chemokine receptors (*e.g.*, *CXCL1*, *CCL2*, *CCL13*, *CCR7*) (Table III). The notion that this cluster is apparently related to both M1 and M2c polarization suggests that inflammatory activation is strictly connected to M2c development and eventual macrophage differentiation.

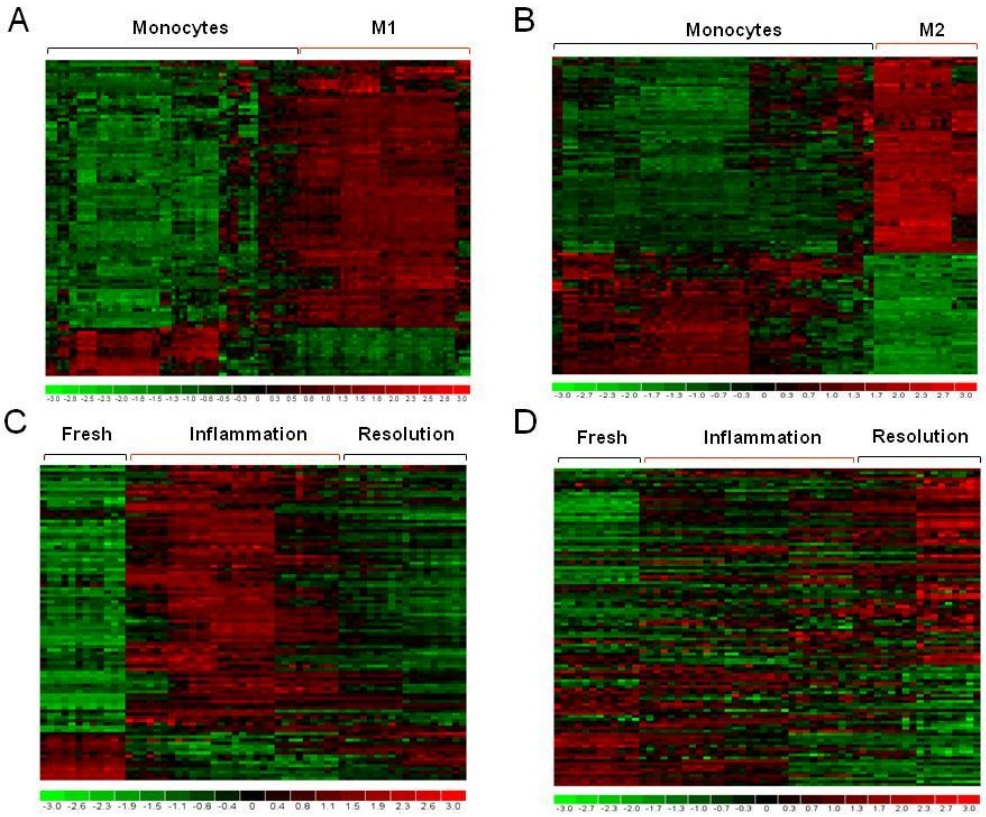

Figure 7. *Heat-map representing the fold-expression levels of gene lists identified by SAM as statistically down-regulated (green) or up-regulated (red) in M1 and in M2 samples once compared to fresh unstimulated monocytes. A. Fold-expression levels of 98 genes associated to monocyte to M1 differentiation in unstimulated monocytes and M1 samples. B. Fold-expression levels of 107 genes associated to monocyte to M2 differentiation in unstimulated monocytes and M2 samples. C. Fold-expression of the 98 genes associated to monocyte to M1 differentiation in samples of the in vitro model of inflammation. D. Fold-expression of the 107 genes associated to monocyte to M2 differentiation in samples of the in vitro model of inflammation.*

Table III. Association of genes differentially expressed in the comparisons between fresh monocytes and M1 and M2 samples to the functional groups defined from the analysis of the *in vitro* model of inflammation.

Gene Symbol	Cluster
Genes up-regulated in M1 polarization	
IL12B, PTX3, CCL4, IL1RN, TNF, IL6, CCL20, IL1A, ICAM1, NFKB1, TRAF1, SERPINB9, IL1F9, MAFF	*Inflammation*
CXCL1, DRAM, TNIP3, CCL2, SLAMF7, CCR7, TNFAIP6	*Inflammation driven differentiation*
Genes down-regulated in M1 polarization	
P2RY5, FGL2, CD1D	*Anti-inflammation*
Gene up-related in M2 polarization	
TREM2, A2M, NUPR1, C1QA, C1QB, MS4A4A, APOE, APOC1, ADORA3	*Positive differentiation*
ADAMDEC1, CD59, TFPI, CCL13	*Inflammation driven differentiation*
Gene down-regulated in M2 polarization	
FCER1A, LGALS2, PF4, CD69, CD93, NR4A2, VCAN, CD62L, ICAM3, NLRP3, ERG1	*Negative differentiation*

3.6 Validation of gene expression by qPCR

In order to quantitatively validate the microarray results, a total of ten genes were examined by qPCR, employing the same RNA used to hybridize the Affymetrix arrays. These genes included five transcription factors (*C/EBPA*, *KLF4*, *PPARD*, *PPARG*, *MAFB*) as markers of monocyte differentiation, and five inflammation-related factors (*IL7R*, *IL6*, *TNFA*, *CD163*, *MMP9*) as markers of monocyte activation, and were selected within each functional group of Figure 6. The data from qPCR matched the patterns emerged from the microarray analysis (Figure 6 and Supplementary Figure 1). In particular, genes belonging to the *inflammation* cluster (*i.e.*, *PPARG*, *IL6*, *TNFA*) are up-regulated during the early phase (4 h) while *IL7R* during the late phase of inflammation (14 h; Figure 8). *CD163*, belonging to the *early anti-inflammation* group is highly up-regulated during the beginning of resolution phase (precisely during the stimulation with IL-10) while the transcription factor *C/EBPA*, belonging to *anti-inflammation* cluster, is up-regulated during the end of resolution phase (precisely during the stimulation with TGF-β). The levels of *PPARD*, belonging to *inflammation driven differentiation*, increase during the late inflammation (14 h) and, although a slight decrease, remain elevate during the resolution. MAFB and MMP9 associated to *positive differentiation* are up-regulated during the resolution phase. Finally, the transcriptional factor KLF4, belonging the *negative differentiation* group and highly expressed in fresh monocytes, decreases its expression level during the inflammation and resolution phases.

Since *CD163* expression significantly increases at 24 h and decreases at 48 h, while genes for *MMP9* and *C/EBPA* are maximally expressed at 48 h, we hypothesize that CD163 expression might be induced by IL-10, while expression of MMP9 and *C/EBPA* could be induced by TGF-β.

The gene expression pattern of inflammatory-related factors during the progress of the inflammatory reaction also reflect the polarization towards the functional M1 program when monocytes were stimulated with LPS/TNF-α/IFN-γ (with inflammation-dependent up-regulation of expression of the M1 markers *IL7R*, *IL6* and *TNFA*; [54]), and towards the functional M2 program when stimulated with IL-10/TGF-β (with up-regulation of the M2 markers *CD163* and *MMP9*; [41, 54]).

Our data confirm that the expression pattern of the transcription factor *MAFB*, a known myeloid differentiation marker, correlates with the expression patterns of its candidate target genes *CD163* and *MMP9* [117], which increased during the resolution phases (M2c functional differentiation).

The expression of the transcription factors *PPARG* and *PPARD* are strongly increased during early (4 h) and late (14 h) inflammation respectively, with *PPARD* maintaining a high expression level also during the resolution phases.

Taken together, these results highlight that, while transcription factors may contribute in different manner to macrophage polarization, down-regulation of *PPARG* and *KLF4* in parallel to up-regulation of *MAFB* seem to be critical for monocyte to M2c macrophage differentiation.

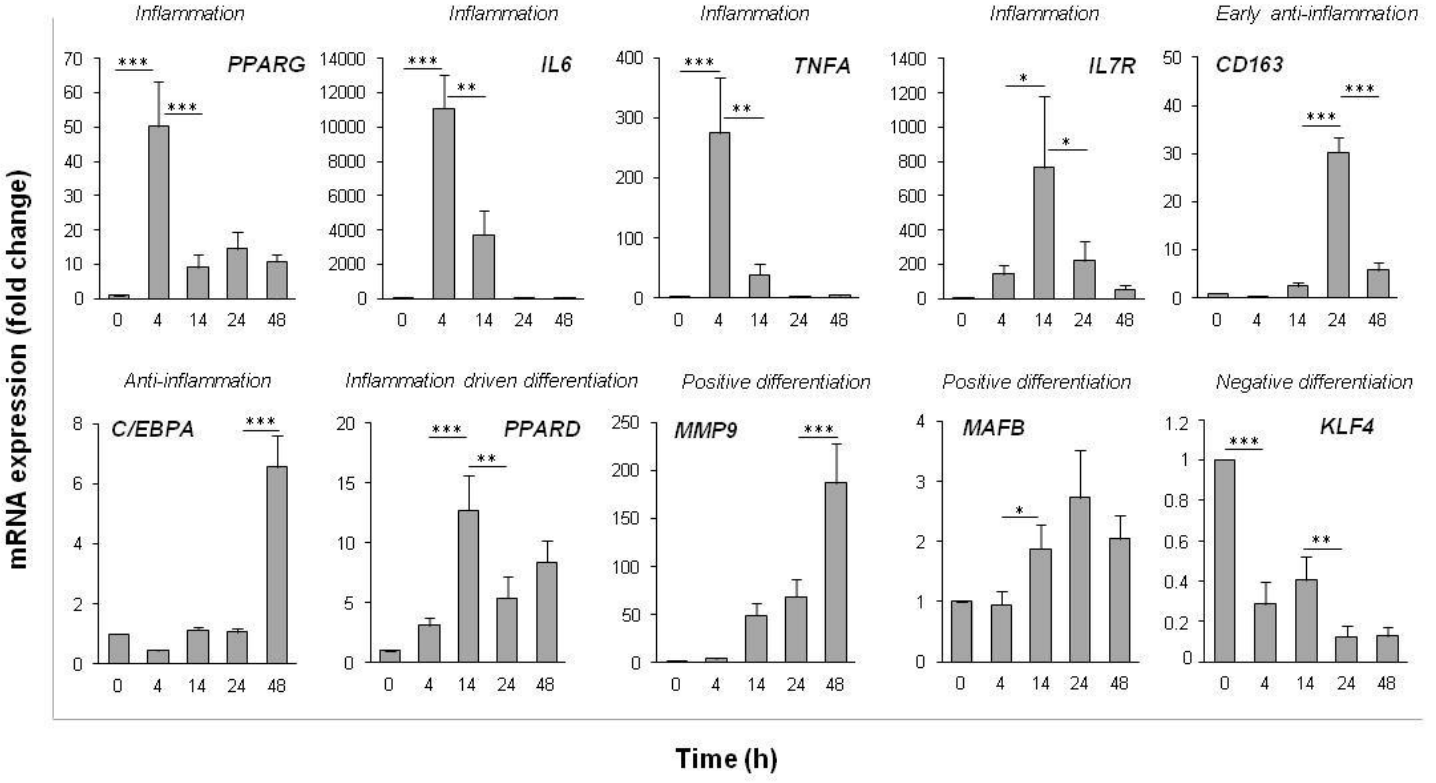

Figure 8. *Fold-expression levels determined by qPCR for genes of the inflammation (PPARG, IL-6, TNFA, IL7R), early anti-inflammation (CD163), anti-inflammation (C/EBPA), inflammation driven differentiation (PPARD), positive differentiation (MMP9 and MAFB), and negative differentiation (KLF4) groups.. The mean expression values ± SEM from six different donors are reported. Statistical significance was calculated with ANOVA followed by Fisher's test for significant differences between two consecutive experimental time-points.*
** P < 0.05 ; ** P < 0.001 ; ***P < 0.0001*

3.7 Kinetics of pro-inflammatory cytokine IL-6 and chemokines CXCL8 and CCL5 production during inflammatory reaction

To follow the progress of inflammatory reaction, protein production of pro-inflammatory mediator IL-6 and M1 polarization-associated chemokines CXCL8 (IL-8) and CCL5 was evaluated (Figure 9). IL-6 is one of cytokines mainly produced by monocytes/macrophages for initiating and driving acute inflammatory response [175]. So it is not surprising that in the model IL-6 was abundantly produced during the inflammatory phase, especially at 14 h, and it is completely absent during the resolution.

Also CXCL8 and CCL5 both significantly increased during the inflammation following TLR- and IFNγ-dependent induction, and were reduce after that the inflammation was turned off by an M2-inducing signal, such as IL-10, which generally inhibits the expression of M1 chemokines [42] (Figure 9). Moreover, unlike CXCL8, CCL5 already increases at 2 h, after stimulation with CCL2 and before stimulation with inflammatory stimuli. This is in agreement with CCL2 signalling *per se* able to mediate the recruitment of monocytes from the blood to the tissue inducing the production of other chemokines, such as CCL5 [170].

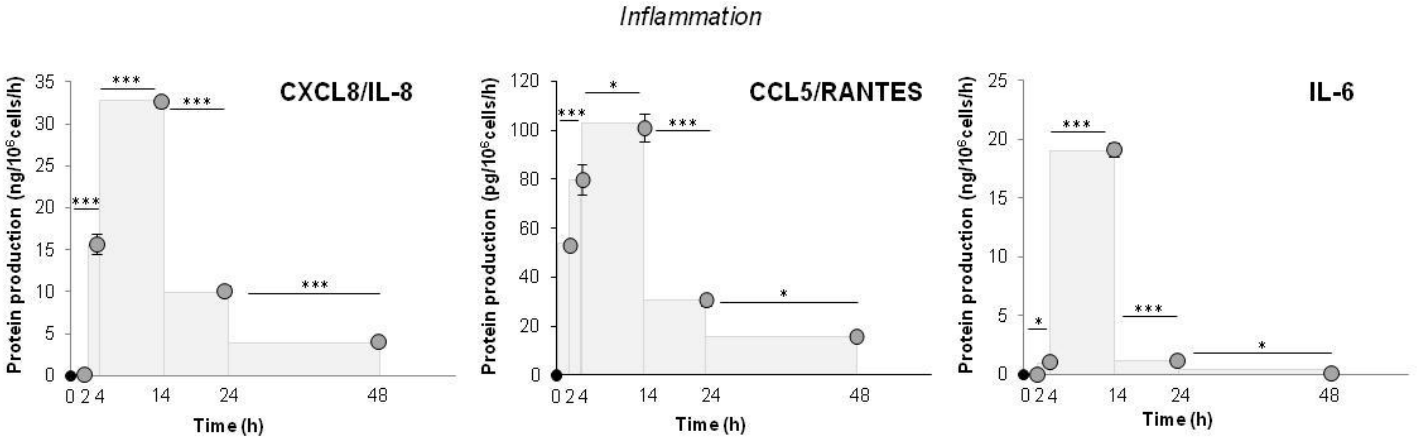

Figure 9. *Protein production of inflammatory chemiokines CXCL8 (IL-8) and CCL5 (RANTES), and cytokine IL-6 during the inflammatory reaction. Soluble proteins recovered in the supernatant production are reported in terms of velocity of production, e.i. the amount of protein produced per one million cells per hour. The mean production values ± SEM of triplicates from one representative donor are reported. Statistical significance was calculated with ANOVA followed by Fisher's test for significant differences between two consecutive experimental time-points.*
** P < 0.05 ; ** P < 0.001 ; ***P < 0.0001*

3.8 The modulation of IL-1 family members during the inflammatory reaction reflect the macrophage differentiation

We further focused on the study of gene expression (by qPCR) and protein production (by ELISA) of the members of IL-1 family that have important roles in innate immune response. In particular, we studied the inflammatory cytokine IL-1β (and its gene *IL1B*), and its two natural inhibitors, the IL-1 receptor antagonist (IL-1Ra, and its gene *IL1RN*) and the IL-1 receptor type II (in its soluble form sIL-1RII; and its gene *IL1R2*). In addition, we have examined the inflammatory cytokine IL-18 (gene *IL8*) and its inhibitor IL-18 binding protein (IL-18BP; gene *IL18BP*), and the orphan cytokine IL-1F9 (gene *IL1F9*). Referring to the functional groups depicted in Figure 6, *IL1B*, *IL1RN*, *IL1F9* and *IL18* are included in the *inflammation* cluster, while *IL18BP* and *IL1R2* belong to the *early anti-inflammation* and the *positive differentiation* cluster, respectively.

The exposure to inflammatory stimuli (LPS and TNF-α) induced an early (4 h) increase in gene expression of both inflammatory cytokines IL-1β and IL-18, while they were down-regulated during the late phase of inflammation (at 14 h, after the addiction of IFN-γ in culture). The expression of both cytokines returned to basal levels during the resolution phase (Figure 10, upper panels).

The expression of IL-1Ra was reduced at 14 h and remained unchanged thereafter, while IL-1RII was readily induced only at the end of resolution after treatment/stimulation with TGF-β (Figure 10, upper panels). The expression level of IL-18 inhibitor, IL-18BP, was induced in the late inflammation by IFN-γ as expected [176]. Despite the absence of IFN-γ after 14 h, the transcription of IL-18BP tends to remain high even after the addition of IL-10 and TGF-β (24 h and 48 h), cytokines involved in the resolution phase of the inflammatory response (Figure 10, upper panels).

The protein production reported as the velocity of production (pg or ng/hr/million cells), confirmed the inflammatory role of IL-1β highlighting its abundant presence during the full (14 h) development of the inflammatory response, with a significant decrease in the later phases (Figure 10, bottom panels). A constant high protein level was observed for the production of its receptor antagonist IL-1Ra until 24 h, *i.e.*, during the resolution phase (Figure 10, bottom panels). About sIL-1RII, the other IL-1 inhibitor, the maximal velocity of production was at 4 h while decreasing thereafter. This means that the cytokine is not absent but is not further produced. In fact, after the initial high production, the level of cytokine remained almost constant throughout the entire inflammatory reaction (data not shown).

The velocity of production of the IL-18 protein was calculated as the amount of biologically active cytokine, *i.e.*, that is not bound to its inhibitor IL-18BP (free IL-18), by concomitantly assessing the levels of both IL-18 and IL-18BP proteins. Free IL-18 is strongly increased in the initial phase of inflammation (4 h) and then diminished to almost disappear in the phase of resolution (48 h) (Figure 10, bottom panels).

The increase of IL-18 and IL-1β during the inflammatory phase and their decrease with the progress of the reaction is expected in a normal inflammatory response, in which the inflammatory factors must be depleted or neutralised after the elimination of the pathogen, to avoid tissue damage. In parallel, the increase of the IL-18 inhibitor IL-18BP and of the IL-1 inhibitors IL-1Ra and sIL-1RII respond to the same need to turn off the acute reaction to proceed to the stage of restoration of homeostasis. Moreover, the observed data of IL-1β expression and production, of IL-1Ra production, and IL-1RII expression also reflect what is expected in the process of macrophage polarization, which predicts low levels of IL-1β and high levels of IL-1RN and IL-1RII in M2 macrophages and the opposite trend in M1 [40, 42].

In addition, we have focused particular attention on the study of gene expression and protein production of a novel member of IL-1 signalling system, IL-1F9, a cytokine that appears to have pro-inflammatory activities though its physiological function remains unknown. The reason for our interest arises from the observation that *IL1F9* is one of genes differentially expressed during the inflammatory phase (belonging to *inflammation* cluster) and it appears among genes up-regulated in M1 polarization as detected by the comparison described in paragraph 3.5. This would suggest IL-1F9 as new candidate M1 marker. As shown in Figure 11, *IL1F9* was not expressed in fresh monocytes while it was significantly expressed in response to inflammatory stimulation, being maximal during the late phase of inflammation (14h) and during the first resolution phase (24h). The gene was completely down-regulated during late resolution. With regard to protein production, two different ELISA kits were used to detect the protein. Both assays had low sensitivity (lower detection limits were 78 and 15.6 pg/ml). We have observed (data not shown) that the protein seemed to be produced only during the inflammatory phase, but unfortunately it was not possible to obtain an accurate measure of the cytokine due to of the uncertain reliability of the kits.

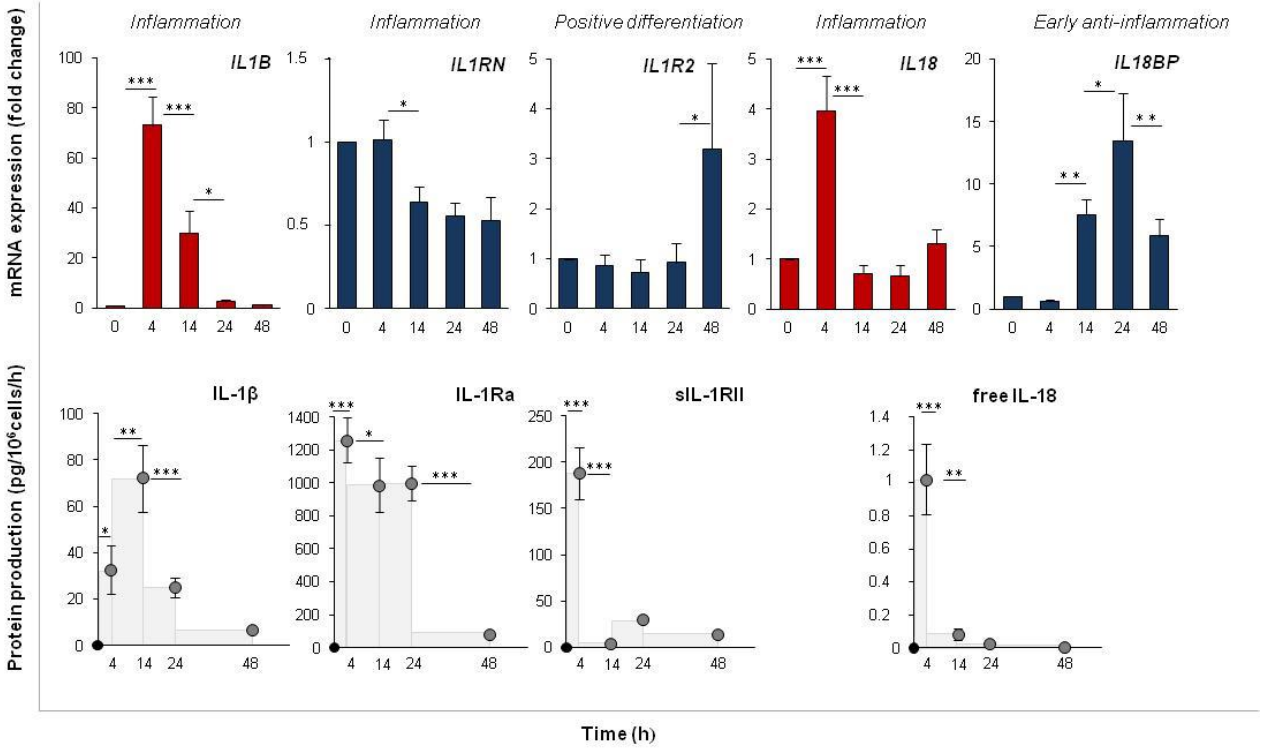

Figure 10. IL-1 family members. *Fold expression levels determined by qPCR (upper panels) and protein production (bottom panels) of IL-1 family cytokines and receptors during the different phases of inflammatory reaction in vitro. Soluble proteins recovered in the supernatant production are reported in terms of velocity of production, e.i. the amount of protein produced per one million cells per hour. IL-18 is reported as free-IL-18 [167, 168], e.i. the fraction of cytokine not bound to its inhibitor (IL-18BP) and therefore biologically active. The mean values ± SEM from six different donors are reported. Statistical significance was calculated with ANOVA followed by Fisher's test for significant differences between two consecutive experimental time points.* P < 0.05 ; ** P < 0.001 ; ***P < 0.0001.*

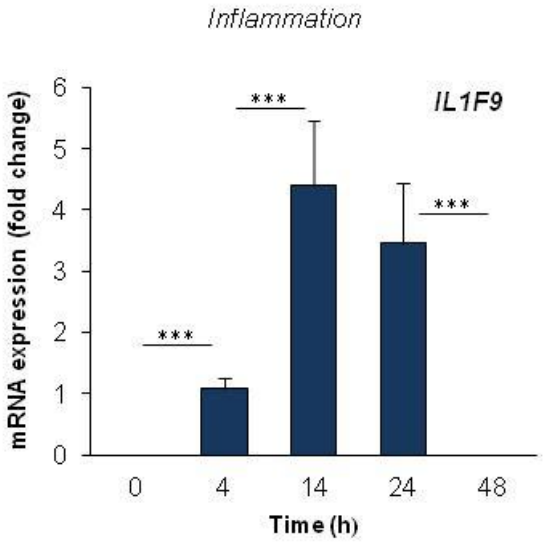

Figure 11. IL-1 family member IL-1F9*. Gene expression of IL-1 family cytokine IL-1F9 during the different phases of inflammatory reaction in vitro. The mean production values ± SEM of triplicates from one representative donor are reported. Statistical significance was calculated with ANOVA followed by Fisher's test for significant differences between two consecutive experimental time points.*
** P < 0.05 ; ** P < 0.001 ; ***P < 0.0001.*

4. DISCUSSION AND CONCLUSION

The aim of this work was to build a reliable and representative *in vitro* model, based on human primary cells, that simulates the *in vivo* development of the inflammatory reaction during its entire course, from recruitment of inflammatory monocytes to the site of inflammation, to the onset and development of the inflammatory reaction, until resolution of inflammation and re-establishment of tissue homeostasis.

During infection or under other inflammatory conditions, monocytes transmigrate the activated endothelium of blood vessels in response to chemotactic stimuli released by the underlying inflamed tissue [43, 47]. The monocyte chemoattractant protein CCL2 (previously known as MCP-1) is the most important chemokine for monocyte recruitment *in vivo*, even if its mechanism of action remains unclear [36]. CCL2 can be secreted by stromal cells (*e.g.*, mast cells, fibroblasts, resident macrophages, vascular smooth muscle cells, and endothelial cells) and mediates the influx of monocytes from blood to sites of injury or infection [177]. Thus, in the *in vitro* model human monocytes were at first exposed at CCL2 to simulate the recruitment to the site of infection.

After extravasation[3], the differentiation of monocytes into activated macrophages is mediated by exposure to pathogen-associated molecular pattern (PAMPs) from microorganisms or damage-associated molecular patter (DAMPs) from dying parenchymal cells, which trigger the inflammatory response by activating pathogen recognition receptor (PRR) on monocytes [178, 179, 180]. DAMPs have also been implicated in inflammatory and autoimmune diseases (*e.g.* rheumatoid arthritis and

[3] After transmigration, monocytes encounter the ECM molecules with which they certainly interact. However, in the *in vitro* model the coating of the culture vessel with collagen and fibronectin provided a non-natural surface that immediately activated the naïve monocytes. Thus, it was chosen to avoid ECM coating, also in light of the notion that these molecules do not play a crucial role on the polarization of macrophages *in vitro* [183].

systemic lupus erythematosus) where excessive and persistent inflammation plays a key role in pathogenesis [179]. Being the goal of our model to reproduce the sequence of phases of the inflammatory reaction, we have chosen to mimic a bacterial infection in the tissue by means of a typical activation of TLR4 with bacterial lipopolysaccharide (LPS), which is a major activator of monocytes [181]. Soon afterwards, monocytes were exposed to TNF-α, a key cytokine in the innate immune response, in order to simulate the early inflammatory reaction by other tissue cells, which in fact produce and release inflammatory cytokines in response to injury [182]. Monocytes at the inflammatory site achieve full inflammatory activation upon interaction with IFN-γ, a Th1-type cytokine. IFN-γ can be produced by natural killer cells (NK), achieving a significant but transient activation of monocytes, or by adaptive immune cells (Th1 cells), usually necessary to maintain activated macrophages [47]. Thus, few hours after exposure to TNF-α, monocytes in culture were exposed also to IFN-γ, to reproduce the late inflammatory phase that *in vivo* involves T lymphocyte infiltration. As consequence of stimulation with LPS and IFN-γ, monocytes undergo "classical" M1 activation [44]. Throughout the inflammatory phase, the cells were maintained at 39°C to simulate the temperature increase in the inflammatory microenvironment during an infection [184].

The inflammatory reaction resolves spontaneously when the pathogen is destroyed or the inflammatory stimulus is eliminated. This requires a series of tuned events, from macrophage transition from an acute inflammatory into a deactivated state, to up-regulation of anti-inflammatory cytokines, and to epigenetic changes in chromatin [185, 186]. Furthermore, the microenvironmental changes due to apoptosis of activated neutrophils and T cells, and the modulation of macrophage activation upon enhanced phagocytic activity, induce macrophages to release anti-inflammatory and immune-regulatory cytokines such as IL-10 and TGF-β [187, 188]. The

phenotype of macrophages exposed *in vitro* to TGF-β and IL-10 (referred to as M2c type) shares similarities with anti-inflammatory macrophages [44, 47], despite there are no *in vitro* studies that use apoptotic cells as a stimulus of macrophage differentiation [189]. Moreover, the presence of anti-inflammatory cytokines such as IL-10 and TGF-β is one of mechanisms that account for macrophage deactivation as illustrated by inhibition of LPS-induced TNF-α production [82, 190], and both these cytokines are responsible of attenuation of the inflammatory response [190].

Thus, in order to simulate the inflammatory resolution and to induce M2 polarization, we removed the inflammatory stimuli, brought the temperature back to 37°C, and exposed cells to the anti-inflammatory cytokine IL-10 first and, after removal of IL-10, to TGF-β. We have sequentially separated the exposure to IL-10 and TGF-β, because the former is the most important anti-inflammatory cytokine responsible of the deactivation of monocytes [173], while the latter is the cytokines most involved in the tissue repair phases [191].

In this *in vitro* model of inflammation, blood monocytes from 12 individual healthy donors were used. The use of individual healthy donors has allowed us to study the mechanisms of innate immune system in a normal population exposed to a variable environment, avoiding all issues of representativeness related to the use of inbred models and supporting the relevance of directing laboratory-based immunology towards wild immunology [140]. We also wanted to avoid using an animal model, such as mouse, which has important differences from man in the immune system in general [137] and in the innate effector cells in particular [141]. It is important to acknowledge that, after a half-century of mouse-dominated research, now human immunology is both advancing and providing insights into basic biology [192].

Another very important aspect of this model, as compared to other models based on human primary cells, is that it allows us to follow the changes within the same cell population during the entire course of the inflammatory reaction, from the initial polarization towards an effector inflammatory program to the subsequent re-polarization to the deactivation program. In the isolated monocytes used in the model, the percentage of the three different monocyte subsets ($CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, $CD14^{dim}CD16^{+}$) fully reflected the percentage of the same monocyte subpopulations in the blood, indicating that the monocyte population we use in our experiments fully represent the monocyte heterogeneity as present in the circulation *in vivo*.

An issue not yet resolved regards the plasticity of the phenotypic and functional commitment of monocyte subpopulations, *i.e.* if they are terminally differentiated (excluding the possibility to switch from one subpopulation to another), or if they maintain a certain level of plasticity that allows them to transdifferentiate from one subpopulation to another depending on the physiological *vs*. pathological microenvironmental conditions. Our observations (decrease of the $CD14^{+}$ subpopulation and increase of the $CD16^{+}$ subpopulation) indicate that during the inflammatory reaction there is a direct relationship between the three subsets, with $CD14^{+}$ monocytes that probably become $CD16^{+}$ [32], although it is not clear if the intermediate subset is more closely related to the classical or non-classical subsets [27-29]. Moreover, we have observed that the $CD16^{+}$ subset entirely disappeared during the early inflammation after stimulation with LPS and TNF-α. This could support the hypothesis that CD16 may be a marker of activation among $CD14^{+}$ monocytes [30], and confirms the need to identify new markers to better discriminate between different monocyte subsets [21]. These observations are therefore worth of further investigation.

We observed that a total of almost 4000 genes were differentially expressed during the course of inflammation, and during the concomitant monocyte-to-macrophage differentiation, consistent with the complexity of these processes. Supervised hierarchical clustering revealed six broad clusters comprising genes with a distinct behaviour that well describe the different phases of inflammation: *Inflammation*, *Early Anti-inflammation*, *Anti-inflammation*, *Inflammation driven differentiation*, *Positive differentiation,* and *Negative differentiation*.

The *Inflammation* phase, corresponding to monocyte-to-M1 differentiation, includes genes for the typical effectors of classical activation, such as the inflammatory cytokines *IL1B*, *IL6*, and *TNFA*, the chemokines *CXCL8* and *CCL5*, soluble innate immune mediator such as *PTX3*, and enzymes such as *PTGS2* and *PLA1A* [54]. The *early anti-inflammation* and *anti-inflammation* clusters include the genes down-regulated in M1 polarized cells. These include transcription factors such as CCAAT/enhancer binding protein alpha (*C/EBPA*), receptors such as *TLR5*, *TLR7* and *TLR8*, purinergic receptors (*e.g.*, *P2RX7*), Fc receptor (*e.g.*, *FCER1A*, *FCRLB*). We reasoned that the decreased expression of some receptors involved in inflammatory response may be related to loss of responsiveness following activation (similar to tolerance), which is restored at the end of inflammatory process when inflammatory monocytes have become tissue-regulating macrophages and should be ready to respond at a new dangerous signal.

Moreover, to these clusters belong the highly conserved metal-binding proteins metallothioneins (*e.g.*, *MT1G*, *MT4*, *MT1E*). These are involved in metal homeostasis, detoxification, modulation of inflammation, control of the oxidative stress, cell proliferation [171], and are strongly up-regulated in endotoxin tolerance that represents a distinctive state of alternative polarization [172].

A cluster that we have defined *inflammation driven differentiation* included genes whose expression rapidly increased as soon as the inflammatory reaction began and remained up-regulated throughout all the phases of the reaction, therefore we reasoned that they may represent genes needed for the inflammatory response and also critical for the process of monocyte differentiation into deactivating and tissue-repairing macrophages. In fact, this cluster includes both a number of inflammatory genes and M1 polarization markers and genes that are allegedly highly expressed in M2c polarization. *Positive* and *negative differentiation* clusters identify genes that are important for the differentiation of monocytes to macrophages such as transcription factors *MAFB*, *KLF4* and *PPARG*, and c-type lectin members, adhesion and signalling molecules.

It is possible that these genes may define the state of differentiation of monocytes to macrophages independently of the concurring inflammatory reaction. Indeed, it should be noted that the fresh monocytes used in these experiments are an heterogeneous population as present in the blood and could therefore include both “inflammatory” monocytes that differentiate into effector cells in the tissue, and “homeostatic” monocytes that replenish the pool of tissue macrophages in physiological conditions.

When investigating the biological role of the genes that are differentially expressed during the development of the inflammatory response, GSEA analysis has shown that the majority of pathways belonging to the *inflammation* cluster are classical inflammatory pathways mainly involved in innate immune activation and in type I inflammatory response carried out by M1 macrophages (*e.g.*, NFkB, MAPK and JAK-STAT signalling, NOD-like receptor and TLR signalling, cytokine/chemokine receptor interaction, IL-1 receptor pathway [173]). On the other hand, the *early anti- and anti-inflammation* clusters are enriched in pathways associated to lipid (fatty acid oxidation), protein, and carbohydrate metabolism (oxidative glucose

metabolism), and regulation of gene expression (*i.e.*, RNA splicing and miRNA biogenesis) and cell cycle. The same pathways were found in the *positive differentiation* cluster. The modulation of genes involved in these cellular metabolic activities is a prominent feature of M2 macrophage polarization/differentiation [58, 173, 174], and it is conceivable that the up-regulation of these pathways occurs during the phases of resolution and repair, when major rearrangements of cellular functions are required, from inflammation to anti-inflammation and to synthesis of tissue repair-promoting factors. Moreover, the observed enrichment of pathways associated with cell cycle agrees with the fact that macrophages involved in the resolution of inflammation showed up-regulation of several genes related to cell cycle and proliferation [193]. Proliferation seems to emerge as an important property of M2-polarized macrophages [194], although its *in vivo* relevance needs further investigation.

By comparing the lists of differentially expressed gene between monocytes *vs*. M1 and *vs*. M2 macrophages, extracted from the meta-database, it is evident that monocytes treated *in vitro* in our model of inflammation resemble the M1 transcriptome during the inflammatory phase and that of M2 during the resolution phase. In addition, by comparing the list of genes differentially expressed during the entire inflammatory process with the list of genes differentially expressed during monocytes *vs*. M1 differentiation, and monocytes *vs*. M2 differentiation, it can be observed that most of the genes expressed in M1 cells belong to the *inflammation* cluster, while those in M2 cells belong to the *positive differentiation* cluster. Moreover, among genes related with M1 and M2 polarization, several belong to the cluster *inflammation driven differentiation*. The notion that this cluster is apparently related to both polarizations suggests that the inflammatory activation is a process strictly connected to macrophages differentiation, considering that

in the model the same monocytes differentiate into M1 then to M2 in response to inflammatory and anti-inflammatory stimuli, respectively.

Thus, the fact that the genes involved in inflammatory monocyte activation belong at the same biological pathways involved in the control of different cellular processes (such as cell growth/proliferation and metabolism) during monocyte-to-macrophage differentiation *in vitro* establishes a connection between monocyte activation and differentiation, inflammation and metabolism at the transcriptional level. Therefore, the resolution of inflammation is strictly connected to the progress of cell differentiation in the tissue.

Taken together, all these findings (supervised hierarchical clustering, GSEA, comparison between model and database) demonstrate that monocytes entering an inflammatory environment first polarized into M1 in presence of LPS/TNF-α/IFN-γ, and then switch to M2-polarized macrophages in presence of IL-10/TGF-β. The fact that, in this *in vitro* model, the same monocyte population (which however is heterogeneous) goes thought all the phases of the inflammatory process by changing its phenotype and function, and eventually polarize into M2, was never before demonstrated for human cells, and only suggested by mouse studies [31, 32, 33]. Thus, our results demonstrate that the phenotype of polarized human M1 and M2 macrophages can change, and reverse *in vitro* [75].

The M1 and M2 polarization of monocytes has been validated by data of quantitative gene expression and protein production. A series of inflammation-related factors and chemokines, chosen as M1 markers or monocyte activation markers, such as IL-6, TNF-α, IL-7R, IL-1β, IL-18, IL-1F9, CXCL8, CCL5 were found to be expressed, and their gene products synthesized, during the inflammatory phases (4 and 14 h), while those chosen as M2 markers, such as CD163, MMP9, IL-1Ra, sIL-1RII, IL-18BP were expressed during the resolution phases (24 and 48 h). Regarding the

IL-1 family, we have focused our attention on a less known member, IL-1F9, since we observed that *IL1F9* is one of the genes differentially expressed during the inflammatory phase and appears among genes up-regulated in M1, thus appearing as a good candidate as a new M1 marker. We have confirmed by real-time PCR its mRNA expression only during the inflammatory reaction, but we could not detect the protein. Thus, either the protein detection methods are not sensitive enough for detection, or mRNA up-regulation is not followed by significant protein production and it represent a non-functional signal of monocytes activation, a sort of "predisposing" condition favouring subsequent responsiveness to inflammatory challenges.

In addition, a series of transcription factors were examined as markers of monocyte/macrophage differentiation, all being factors involved both in monocyte differentation and macrophage polarization. Our data confirm that the expression pattern of the transcription factor gene *MAFB*, a known myeloid differentiation marker, correlated with the expression patterns of its candidate target genes *CD163* and *MMP9* [117] which increase during the resolution phases. Expression of *PPARG* and *PPARD* increased during the inflammation phases and only *PPARD* maintained an high expression level also during the resolution phases, confirming their role in inflammation [195], and in the control of monocyte-to-macrophage differentiation [101, 102], respectively. Moreover, we observed that the transcription factors *KLF4* and *C/EBPA*, both critical regulators of monocyte differentiation, seem to have an opposite gene expression profile, the former being significantly down-regulated during all phases of inflammatory reaction, while the latter showing a strong increase during the repair phase.

The observed expression profile of *PPARG* and *KLF4* does not seem to be in agreement with the fact that these transcription factors appear to be linked to M2 polarization [196, 197]. However, it should be underlined that

the present study is exclusively focused on M2c polarization, while those relating *PPARG* and *KLF4* to M2 were addressing M2a polarization, which is functionally very different.

Taken together, these results highlight that, while transcriptional factors may contribute in different manner to macrophage polarization, down-regulation of *PPARG* and *KLF4* in parallel to up-regulation of *MAFB* seem to be critical for monocyte to M2c differentiation.

The up-regulation or down-regulation of transcriptional factors is important for determining macrophage differentiation, and the same transcriptional factors might drive the expression of genes involved in monocyte activation. For this reason, the transcriptional factors can become the connecting link between the two processes.

In conclusion, the transcriptional data and the kinetical analysis of production of inflammatory and anti-inflammatory factors validate the proposed *in vitro* model of the inflammatory response, thus allowing us to describe the time-dependent and coordinated sequence of inflammation-related events. This model could therefore open the possibility of accurately characterize the development and regulation of human monocyte/macrophage differentiation and polarization.

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6. SUPPLEMENTARY DATA AND TABLES

Table S1: Complete list of the datasets used in this study and their sources. Genome-wide expression levels and meta-information of 303 samples were organized in a proprietary database using A-MADMAN.

GEO series	Platform	Total samples in series	Samples used in this study	Reference
GSE4984	HG-U133 Plus2.0	12	6	Fulcher et al., 2006
GSE5099	HG-U133A	30	14	Martinez et al., 2006
GSE5547	HG-U133 Plus2.0	24	6	Humphrey et al., 2007
GSE6965	HG-U133 Plus2.0	4	4	Mezger et al., 2008
GSE7509	HG-U133 Plus2.0	26	26	Dhodapkar et al., 2007
GSE7568	HG-U133 Plus2.0	25	25	Gratchev et al., 2008
GSE7807	HG-U133 Plus2.0	8	4	Woszczek et al., 2008
GSE8286	HG-U133A	9	9	Liu et al., 2008
GSE8515	HG-U133A	15	15	Jura et al., 2008
GSE8608	HG-U133 Plus2.0	6	1	Hofer et al., 2008
GSE8658	HG-U133 Plus2.0	63	30	Szatmari et al., 2007
GSE9080	HG-U133Av2	6	3	---
GSE9874	HG-U133A	60	11	Hägg et al., 2008
GSE9946	HG-U133A	12	12	Popov et al., 2008
GSE9988	HG-U133 Plus2.0	62	58	Dower et al., 2008
GSE10856	HG-U133 Plus2.0	4	4	Chang et al., 2008
GSE11393	HG-U133Av2	9	3	Llaverias et al., 2008
GSE11430	HG-U133 Plus2.0	10	10	Maouche et al., 2008
GSE11864	HG-U133 Plus2.0	10	10	Hu et al., 2008
GSE12108	HG-U133 Plus2.0	14	13	Butchar et al., 2008
GSE12773	HG-U133 Plus2.0	10	5	Rate et al., 2009
GSE12837	HG-U133A	24	3	Coppe et al., 2009
GSE13762	HG-U133 Plus2.0	15	15	Széles et al., 2009
GSE14419	HG-U133Av2	16	16	---

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Table S2: Complete list of 128 samples labeled as untreated monocytes and as M1 and M2 activated monocytes and their sources.

GEO series	Platform	GEO samples
		Untreated monocytes
GSE5099	HG-U133A	GSM115051; GSM115046; GSM115047; GSM115048; GSM115049; GSM115050
GSE7807	HG-U133 Plus2.0	GSM189447; GSM189448; GSM189449; GSM189450
GSE8286	HG-U133A	GSM205587; GSM205588; GSM205590; GSM205591; GSM205592; GSM205594
GSE8658	HG-U133 Plus2.0	GSM214749; GSM214734; GSM214737; GSM214738; GSM214739; GSM214740; GSM214741; GSM214742; GSM214743; GSM214744; GSM214745; GSM214746
GSE9080	HG-U133Av2	GSM230145; GSM230149; GSM230147
GSE9988	HG-U133 Plus2.0	GSM252476; GSM252478; GSM252479; GSM252480; GSM252481; GSM252484; GSM252485
GSE11393	HG-U133Av2	GSM287664; GSM287665; GSM287666
GSE11430	HG-U133 Plus2.0	GSM257664; GSM257666; GSM257668; GSM257670; GSM257672
GSE11864	HG-U133 Plus2.0	GSM299556; GSM299557; GSM299561; GSM299562
GSE12108	HG-U133 Plus2.0	GSM305434; GSM305436; GSM305438; GSM305440; GSM305430; GSM305432
GSE12837	HG-U133A	GSM15431; GSM321582; GSM15430
GSE13762	HG-U133 Plus2.0	GSM346564; GSM346577; GSM346553
		M1 activation
GSE5099	HG-U133A	GSM115055; GSM115057
GSE9988	HG-U133 Plus2.0	GSM252423; GSM252424; GSM252425; GSM252427; GSM252428; GSM252429; GSM252431; GSM252432; GSM252433; GSM252434; GSM252435; GSM252436; GSM252437; GSM252438; GSM252439; GSM252440; GSM252441; GSM252442; GSM252443; GSM252444; GSM252445; GSM252447; GSM252448; GSM252449; GSM252450; GSM252451; GSM252453; GSM252454; GSM252455; GSM252456; GSM252457; GSM252458; GSM252459; GSM252460; GSM252461; GSM252462; GSM252463; GSM252464; GSM252430; GSM252426
GSE14419	HG-U133Av2	GSM360141; GSM360145; GSM360184; GSM360188
		M2 activation
GSE7568	HG-U133 Plus2.0	GSM183464; GSM183465; GSM183466; GSM183467; GSM183482; GSM183483; GSM183484; GSM183485; GSM183486; GSM183487; GSM183217; GSM183305; GSM183306; GSM183315; GSM183316; GSM183392; GSM183393; GSM183394; GSM183462; GSM183463

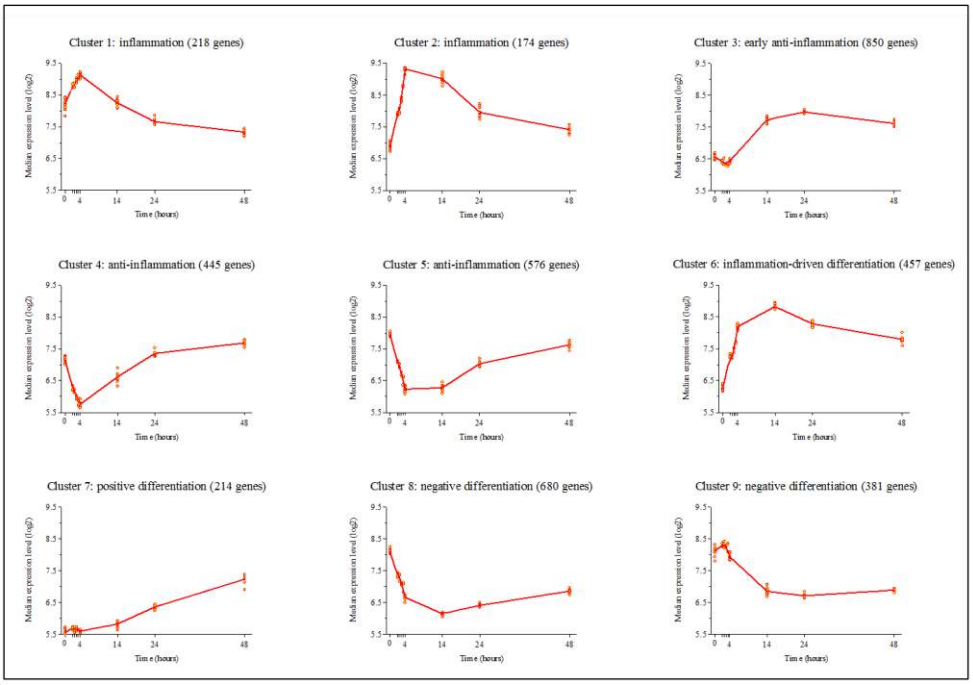

Figure 1. *Data visualization by cluster analysis. Nine separated clusters are show. Solid red lines have been drawn joining the average value of gene expression at each time point for each donor (dots). In the text the clusters are reported as follows: 1 and 2 as Inflammation (218 and 174 genes, respectively), 3 as Early-anti-inflammation (850 genes), 4 and 5 as Anti-inflammation (445 and 576 genes respectively), 6 as Inflammation driven differentiation (457 genes), 7 as Positive Differentiation (234 genes), 8 and 9 as Negative Differentiation (680 and 381 genes, respectively). The complete list of the genes differentially expressed is available with the author.*

Table S3: Complete list of the Gene sets identified by GSEA as correlated with the expression profiles of clusters

Cluster	FDR q-val
Cluster 1	
BIOCARTA_IL1R_PATHWAY	0,00955
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	0
KEGG_RIBOSOME	0
KEGG_OLFACTORY_TRANSDUCTION	0
KEGG_MAPK_SIGNALING_PATHWAY	0
KEGG_CALCIUM_SIGNALING_PATHWAY	0,00473
KEGG_HEDGEHOG_SIGNALING_PATHWAY	0,00506
KEGG_ECM_RECEPTOR_INTERACTION	0,00887
KEGG_FOCAL_ADHESION	0,00813
KEGG_MATURITY_ONSET_DIABETES_OF_THE_YOUNG	0,00873
KEGG_PATHWAYS_IN_CANCER	0,00803
KEGG_NOTCH_SIGNALING_PATHWAY	0,01536
KEGG_SMALL_CELL_LUNG_CANCER	0,01408
KEGG_BASAL_CELL_CARCINOMA	0,01684
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0,01637
KEGG_TIGHT_JUNCTION	0,01585
KEGG_WNT_SIGNALING_PATHWAY	0,01886
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	0,02123
KEGG_PROXIMAL_TUBULE_BICARBONATE_RECLAMATION	0,02955
KEGG_GLYCOSPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	0,03132
KEGG_CELL_ADHESION_MOLECULES_CAMS	0,03723
KEGG_NITROGEN_METABOLISM	0,04622
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,04816
REACTOME_GPCR_LIGAND_BINDING	0
REACTOME_GTP_HYDROLYSIS_AND_JOINING_OF_THE_60S_RIBOSOMAL_SUBUNIT	0
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	0
REACTOME_REGULATION_OF_BETA_CELL_DEVELOPMENT	0
REACTOME_OLFACTORY_SIGNALING_PATHWAY	0
REACTOME_PEPTIDE_CHAIN_ELONGATION	0
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	0
REACTOME_NUCLEAR_RECEPTOR_TRANSCRIPTION_PATHWAY	0
REACTOME_REGULATION_OF_GENE_EXPRESSION_IN_BETA_CELLS	0
REACTOME_G_ALPHA_S_SIGNALLING_EVENTS	0
REACTOME_CLASS_B2_SECRETIN_FAMILY_RECEPTORS	0,00060
REACTOME_VIRAL_MRNA_TRANSLATION	0,00086
REACTOME_AMINE_LIGAND_BINDING_RECEPTORS	0,00090
REACTOME_TRANSLATION	0,00100
REACTOME_DOWNSTREAM_EVENTS_IN_GPCR_SIGNALING	0,00118
REACTOME_TRANSLATION_INITIATION_COMPLEX_FORMATION	0,00120
REACTOME_AMINE_COMPOUND_SLC_TRANSPORTERS	0,00165

REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	0,00189
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_THE_43S_COMPLEX	0,00187
REACTOME_NCAM1_INTERACTIONS	0,00311
REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	0,00310
REACTOME_PHOSPHOLIPASE_CMEDIATED_CASCADE	0,00302
REACTOME_GAP_JUNCTION_ASSEMBLY	0,01105
REACTOME_ACTIVATION_OF_BH3_ONLY_PROTEINS	0,01232
REACTOME_SLC_MEDIATED_TRANSMEMBRANE_TRANSPORT	0,01221
REACTOME_ADHERENS_JUNCTIONS_INTERACTIONS	0,01365
REACTOME_G_ALPHA_Q_SIGNALLING_EVENTS	0,01320
REACTOME_NEUROTRANSMITTER_RELEASE_CYCLE	0,01510
REACTOME_FRS2MEDIATED_CASCADE	0,01486
REACTOME_NA_CL_DEPENDENT_NEUROTRANSMITTER_TRANSPORTERS	0,01446
REACTOME_GLUCOSE_AND_OTHER_SUGAR_SLC_TRANSPORTERS	0,01407
REACTOME_SHCMEDIATED_CASCADE	0,01509
REACTOME_FGFR_LIGAND_BINDING_AND_ACTIVATION	0,01505
REACTOME_CELL_CELL_ADHESION_SYSTEMS	0,01654
REACTOME_CELL_JUNCTION_ORGANIZATION	0,01721
REACTOME_INTRINSIC_PATHWAY_FOR_APOPTOSIS	0,01901
REACTOME_TRANSMISSION_ACROSS_CHEMICAL_SYNAPSES	0,02268
REACTOME_NCAM_SIGNALING_FOR_NEURITE_OUT_GROWTH	0,02424
REACTOME_XENOBIOTICS	0,02757
REACTOME_MYOGENESSIS	0,03350
REACTOME_INORGANIC_CATION_ANION_SLC_TRANSPORTERS	0,03516
REACTOME_REGULATION_OF_INSULIN_LIKE_GROWTH_FACTOR_ACTIVITY_BY_INSULIN_LIKE_GROWTH_FACTOR_BINDING_PROTEINS	0,03538
REACTOME_PHASE_1_FUNCTIONALIZATION_OF_COMPOUNDS	0,04208
REACTOME_PHASE_1_FUNCTIONALIZATION	0,04527
Cluster 2	
BIOCARTA_TNFR2_PATHWAY	0,00193
BIOCARTA_NFKB_PATHWAY	0,00379
BIOCARTA_RELA_PATHWAY	0,00480
BIOCARTA_NTHI_PATHWAY	0,00380
BIOCARTA_IL10_PATHWAY	0,01368
BIOCARTA_IL1R_PATHWAY	0,01404
BIOCARTA_DEATH_PATHWAY	0,01580
BIOCARTA_INFLAM_PATHWAY	0,02143
BIOCARTA_HIVNEF_PATHWAY	0,02317
BIOCARTA_TID_PATHWAY	0,02365
BIOCARTA_CD40_PATHWAY	0,03357
BIOCARTA_AMI_PATHWAY	0,04390
BIOCARTA_CYTOKINE_PATHWAY	0,04493
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	0
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	0
KEGG_ECM_RECEPTOR_INTERACTION	0,00109

KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,00087
KEGG_GLYCOSPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	0,00319
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,00567
KEGG_GRAFT_VERSUS_HOST_DISEASE	0,00521
KEGG_PATHWAYS_IN_CANCER	0,00738
KEGG_CYTOSOLIC_DNA_SENSING_PATHWAY	0,00833
KEGG_JAK_STAT_SIGNALING_PATHWAY	0,01130
KEGG_SMALL_CELL_LUNG_CANCER	0,01215
KEGG_FOCAL_ADHESION	0,01392
KEGG_TYPE_I_DIABETES_MELLITUS	0,01332
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	0,01458
KEGG_MAPK_SIGNALING_PATHWAY	0,01530
KEGG_PROTEASOME	0,01614
KEGG_NOTCH_SIGNALING_PATHWAY	0,01530
KEGG_APOPTOSIS	0,01543
KEGG_TIGHT_JUNCTION	0,01563
KEGG_BASAL_CELL_CARCINOMA	0,01910
KEGG_ALLOGRAFT_REJECTION	0,02005
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	0,02176
KEGG_LINOLEIC_ACID_METABOLISM	0,02290
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	0,03234
KEGG_AUTOIMMUNE_THYROID_DISEASE	0,04691
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	0
REACTOME_GPCR_LIGAND_BINDING	0
REACTOME_OLFACTORY_SIGNALING_PATHWAY	0,00098
REACTOME_AMINE_LIGAND_BINDING_RECEPTORS	0,00150
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	0,00138
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	0,00416
REACTOME_RNA_POLYMERASE_I_PROMOTER_OPENING	0,00565
REACTOME_AMINO_ACID_AND_OLIGOPEPTIDE_SLC_TRANSPORTERS	0,00596
REACTOME_PACKAGING_OF_TELOMERE_ENDS	0,00606
REACTOME_CELL_JUNCTION_ORGANIZATION	0,00606
REACTOME_AMINO_ACID_TRANSPORT_ACROSS_THE_PLASMA_MEMBRANE	0,00568
REACTOME_REGULATION_OF_INSULIN_LIKE_GROWTH_FACTOR_ACTIVITY_BY_ INSULIN_LIKE_GROWTH_FACTOR_BINDING_PROTEINS	0,00521
REACTOME_CELL_CELL_ADHESION_SYSTEMS	0,00671
REACTOME_SLC_MEDIATED_TRANSMEMBRANE_TRANSPORT	0,00630
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	0,00640
REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	0,00649
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	0,00697
REACTOME_CLASS_B2_SECRETIN_FAMILY_RECEPTORS	0,00679
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	0,00707
REACTOME_XENOBIOTICS	0,00947
REACTOME_INORGANIC_CATION_ANION_SLC_TRANSPORTERS	0,01158
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	0,01182
REACTOME_NCAM1_INTERACTIONS	0,01135
REACTOME_AMINE_COMPOUND_SLC_TRANSPORTERS	0,01285
REACTOME_NA_CL_DEPENDENT_NEUROTRANSMITTER_TRANSPORTERS	0,01257

REACTOME_G_ALPHA_S_SIGNALLING_EVENTS	0,01243
REACTOME_REGULATION_OF_APC_ACTIVATORS_BETWEEN_G1_S_AND_EARLY_ANAPHASE	0,01219
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	0,01186
REACTOME_ADHERENS_JUNCTIONS_INTERACTIONS	0,01303
REACTOME_TOLL_LIKE_RECEPTOR_3_CASCADE	0,01458
REACTOME_GLUCOSE_AND_OTHER_SUGAR_SLC_TRANSPORTERS	0,01436
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	0,01479
REACTOME_FORMATION_OF_TUBULIN_FOLDING_INTERMEDIATES_BY_CCT_TRIC	0,01581
REACTOME_STEROID_HORMONES	0,01648
REACTOME_DOWNSTREAM_EVENTS_IN_GPCR_SIGNALING	0,02319
REACTOME_RNA_POLYMERASE_I_PROMOTER_CLEARANCE	0,02369
REACTOME_CYTOCHROME_P450_ARRANGED_BY_SUBSTRATE_TYPE	0,02387
REACTOME_TRAF6_MEDIATED_INDUCTION_OF_THE_ANTIVIRAL_CYTOKINE_IFN_ALPHA_BETA_CASCADE	0,02403
REACTOME_STABILIZATION_OF_P53	0,02418
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0,02610
REACTOME_APOPTOSIS	0,02850
REACTOME_PHASE_1_FUNCTIONALIZATION_OF_COMPOUNDS	0,03593
REACTOME_GAP_JUNCTION_ASSEMBLY	0,03712
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	0,03665
REACTOME_NCAM_SIGNALING_FOR_NEURITE_OUT_GROWTH	0,04031
REACTOME_UNFOLDED_PROTEIN_RESPONSE	0,04171
REACTOME_AXON_GUIDANCE	0,04109
REACTOME_ZINC_TRANSPORTATION	0,04317
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	0,04418

Cluster 3

BIOCARTA_PROTEASOME_PATHWAY	0,00103
BIOCARTA_ATRBRCA_PATHWAY	0,00104
BIOCARTA_FAS_PATHWAY	0,01474
BIOCARTA_RAC1_PATHWAY	0,01183
BIOCARTA_CASPASE_PATHWAY	0,01013
BIOCARTA_TNFR1_PATHWAY	0,01006
KEGG_PROTEASOME	0
KEGG_LYSOSOME	0
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	0
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0,00082
KEGG_DNA_REPLICATION	0,00066
KEGG_STEROID_BIOSYNTHESIS	0,00113
KEGG_MISMATCH_REPAIR	0,00225
KEGG_N_GLYCAN_BIOSYNTHESIS	0,00197
KEGG_BASE_EXCISION_REPAIR	0,00175
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL_GPI_ANCHOR_BIOSYNTHESIS	0,00376
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0,00385
KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS	0,00484
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	0,00725
KEGG_PEROXISOME	0,00712

KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	0,00664
KEGG_SELENOAMINO_ACID_METABOLISM	0,00960
KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	0,00965
KEGG_HOMOLOGOUS_RECOMBINATION	0,01115
KEGG_PROPANOATE_METABOLISM	0,01244
KEGG_VIBRIO_CHOLERAE_INFECTION	0,01293
KEGG_PYRIMIDINE_METABOLISM	0,01384
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	0,01574
KEGG_PYRUVATE_METABOLISM	0,02247
KEGG_RNA_DEGRADATION	0,02247
KEGG_PENTOSE_PHOSPHATE_PATHWAY	0,02263
KEGG_OXIDATIVE_PHOSPHORYLATION	0,03021
KEGG_CITRATE_CYCLE_TCA_CYCLE	0,03171
KEGG_PROTEIN_EXPORT	0,04312
KEGG_VASOPRESSIN_REGULATED_WATER_REABSORPTION	0,04457
REACTOME_SYNTHESIS_OF_DNA	0
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	0
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	0
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	0
REACTOME_S_PHASE	0
REACTOME_DNA_REPLICATION_PRE_INITIATION	0
REACTOME_STABILIZATION_OF_P53	0
REACTOME_CELL_CYCLE_CHECKPOINTS	0
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	0
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC	0
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	0
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	0
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	0
REACTOME_M_G1_TRANSITION	0
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0
REACTOME_DNA_REPAIR	0
REACTOME_REGULATION_OF_APC_ACTIVATORS_BETWEEN_G1_S_AND_EARLY_ ANAPHASE	0
REACTOME_CELL_CYCLE_MITOTIC	0
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	0
REACTOME_SIGNALING_BY_WNT	0
REACTOME_G1_S_TRANSITION	0
REACTOME_MITOTIC_M_M_G1_PHASES	0
REACTOME_LAGGING_STRAND_SYNTHESIS	0
REACTOME_DNA_STRAND_ELONGATION	0
REACTOME_GLOBAL_GENOMIC_NER	0
REACTOME_DOUBLE_STRAND_BREAK_REPAIR	0
REACTOME_HIV_INFECTION	0
REACTOME_APOPTOSIS	0
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	0
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	0,00004
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	0,00004
REACTOME_TELOMERE_MAINTENANCE	0,00004

REACTOME_G2_M_CHECKPOINTS	0,00004
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	0,00015
REACTOME_MEMBRANE_TRAFFICKING	0,00015
REACTOME_CENTROSOME_MATURATION	0,00026
REACTOME_REPAIR_SYNTHESIS_OF_PATCH_27_30_BASES_LONG_BY_DNA_POLYMERASE	0,00025
REACTOME_EXTENSION_OF_TELOMERES	0,00045
REACTOME_DUAL_INCISION_REACTION_IN_GG_NER	0,00047
REACTOME_LOSS_OF_NLP_FROM_MITOTIC_CENTROSOMES	0,00077
REACTOME_TRANSCRIPTION_COUPLED_NER	0,00085
REACTOME_G2_M_TRANSITION	0,00135
REACTOME_FANCONI_ANEMIA_PATHWAY	0,00150
REACTOME_MICRORNA_BIOGENESIS	0,00153
REACTOME_POST_TRANSLATIONAL_PROTEIN_MODIFICATION	0,00175
REACTOME_CLATHRIN_DERIVED_VESICLE_BUDDING	0,00236
REACTOME_CHOLESTEROL_BIOSYNTHESIS	0,00307
REACTOME_SYNTHESIS_OF_GPI_ANCHORED_PROTEINS	0,00317
REACTOME_SYNTHESIS_OF_GLYCOSYLPHOSPHATIDYLINOSITOL	0,00313
REACTOME_PEROXISOMAL_LIPID_METABOLISM	0,00409
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	0,00619
REACTOME_GOLGI_ASSOCIATED_VESICLE_BIOGENESIS	0,00609
REACTOME_BASE_EXCISION_REPAIR	0,00660
REACTOME_MITOTIC_PROMETAPHASE	0,00929
REACTOME_METABOLISM_OF_AMINO_ACIDS	0,01074
REACTOME_TOLL_LIKE_RECEPTOR_9_CASCADE	0,01077
REACTOME_LYSOSOME_VESICLE_BIOGENESIS	0,01148
REACTOME_MITOCHONDRIAL_TRNA_AMINOACYLATION	0,01231
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION	0,01224
REACTOME_PHOSPHORYLATION_OF_THE_APC	0,01224
REACTOME_HIV_LIFE_CYCLE	0,01208
REACTOME_FORMATION_OF_THE_EARLY_ELONGATION_COMPLEX	0,01237
REACTOME_MRNA_PROCESSING	0,01315
REACTOME_PACKAGING_OF_TELOMERE_ENDS	0,01369
REACTOME_HIV1_TRANSCRIPTION_INITIATION	0,01487
REACTOME_ACTIVATED_AMPK_STIMULATES_FATTY_ACID_OXIDATION_IN_MUSCLE	0,01542
REACTOME_POST_CHAPERONIN_TUBULIN_FOLDING_PATHWAY	0,01554
REACTOME_APCDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B	0,01604
REACTOME_LATE_PHASE_OF_HIV_LIFE_CYCLE	0,01706
REACTOME_INACTIVATION_OF_APC_VIA_DIRECT_INHIBITION_OF_THE_AP_COMPLEX	0,01843
REACTOME_RNA_POL_II_CTD_PHOSPHORYLATION_AND_INTERACTION_WITH_CE	0,02147
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	0,02177
REACTOME_CONVERSION_FROM_APC_CDC20_TO_APC_CDH1_IN_LATE_ANAPHASE	0,02190
REACTOME_ACTIVATED_TLR4_SIGNALLING	0,02939
REACTOME_METABOLISM_OF_CARBOHYDRATES	0,03284
REACTOME_TRNA_AMINOACYLATION	0,03315
REACTOME_RNA_POLYMERASE_I_III_AND_MITOCHONDRIAL_TRANSCRIPTION	0,03526

REACTOME_REGULATION_OF_LIPID_METABOLISM_BY_PEROXISOME_PROLIFERATOR_ACTIVATED_RECEPTOR_ALPHA	0,03749
REACTOME_TRANSCRIPTION_OF_THE_HIV_GENOME	0,03873
REACTOME_ENERGY_DEPENDENT_REGULATION_OF_MTOR_BY_LKB1_AMPK	0,03980
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION	0,03995
REACTOME_TOLL_LIKE_RECEPTOR_4_CASCADE	0,04292
REACTOME_CHAPERONIN_MEDIATED_PROTEIN_FOLDING	0,04544
REACTOME_DUAL_INCISION_REACTION_IN_TC_NER	0,04883
REACTOME_GLUCOSE_METABOLISM	0,04923
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	0,04997
REACTOME_GLUCOSE_TRANSPORT	0,04966

Cluster 4

BIOCARTA_ATRBRCA_PATHWAY	0,00300
BIOCARTA_MCM_PATHWAY	0,00200
BIOCARTA_CREB_PATHWAY	0,03000
BIOCARTA_RAC1_PATHWAY	0,04500
KEGG_LYSOSOME	0
KEGG_OXIDATIVE_PHOSPHORYLATION	0
KEGG_DNA_REPLICATION	0
KEGG_PARKINSONS_DISEASE	0
KEGG_BASE_EXCISION_REPAIR	0
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL_GPI_ANCHOR_BIOSYNTHESIS	0
KEGG_PROPANOATE_METABOLISM	0
KEGG_PEROXISOME	0
KEGG_HUNTINGTONS_DISEASE	0
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	0
KEGG_PYRIMIDINE_METABOLISM	0
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	0
KEGG_ALZHEIMERS_DISEASE	0
KEGG_MISMATCH_REPAIR	0
KEGG_CITRATE_CYCLE_TCA_CYCLE	0
KEGG_RNA_DEGRADATION	0
KEGG_PENTOSE_PHOSPHATE_PATHWAY	0
KEGG_N_GLYCAN_BIOSYNTHESIS	0,00100
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	0,00100
KEGG_PYRUVATE_METABOLISM	0,00200
KEGG_RNA_POLYMERASE	0,00400
KEGG_LYSINE_DEGRADATION	0,00600
KEGG_HOMOLOGOUS_RECOMBINATION	0,01300
KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	0,01300
KEGG_FATTY_ACID_METABOLISM	0,01400
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	0,02200
KEGG_RIBOFLAVIN_METABOLISM	0,02300
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	0,02200
KEGG_SELENOAMINO_ACID_METABOLISM	0,03600

KEGG_GLYCOSAMINOGLYCAN_DEGRADATION	0,03600
KEGG_VASOPRESSIN_REGULATED_WATER_REABSORPTION	0,04900
REACTOME_ELECTRON_TRANSPORT_CHAIN	0
REACTOME_DNA_REPAIR	0
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	0
REACTOME_DNA_STRAND_ELONGATION	0
REACTOME_GLOBAL_GENOMIC_NER	0
REACTOME_LAGGING_STRAND_SYNTHESIS	0
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_SYNTHESIS_OF_DNA	0
REACTOME_EXTENSION_OF_TELOMERES	0
REACTOME_TRANSCRIPTION_COUPLED_NER	0
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	0
REACTOME_CELL_CYCLE_MITOTIC	0
REACTOME_S_PHASE	0
REACTOME_INTEGRATION_OF_ENERGY_METABOLISM	0
REACTOME_BASE_EXCISION_REPAIR	0
REACTOME_CENTROSOME_MATURATION	0
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	0
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	0
REACTOME_REPAIR_SYNTHESIS_OF_PATCH_27_30_BASES_LONG_BY_DNA_POLYMERASE	0
REACTOME_DUAL_INCISION_REACTION_IN_GG_NER	0
REACTOME_PEROXISOMAL_LIPID_METABOLISM	0
REACTOME_G2_M_CHECKPOINTS	0
REACTOME_PYRUVATE_METABOLISM_AND_TCA_CYCLE	0
REACTOME_DNA_REPLICATION_PRE_INITIATION	0
REACTOME_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_CELL_CYCLE_CHECKPOINTS	0
REACTOME_SYNTHESIS_OF_GPI_ANCHORED_PROTEINS	0
REACTOME_LOSS_OF_NLP_FROM_MITOTIC_CENTROSOMES	0
REACTOME_SYNTHESIS_OF_GLYCOSYLPHOSPHATIDYLINOSITOL	0
REACTOME_MITOTIC_M_M_G1_PHASES	0
REACTOME_METABOLISM_OF_CARBOHYDRATES	0
REACTOME_MICRORNA_BIOGENESIS	0
REACTOME_FANCONI_ANEMIA_PATHWAY	0,00100
REACTOME_CITRIC_ACID_CYCLE	0,00100
REACTOME_GLYCOGEN_BREAKDOWN_GLYCOGENOLYSIS	0,00100
REACTOME_GLUCOSE_METABOLISM	0,00100
REACTOME_G2_M_TRANSITION	0,00100
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION	0,00100
REACTOME_DOUBLE_STRAND_BREAK_REPAIR	0,00100
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	0,00100
REACTOME_HIV_INFECTION	0,00100
REACTOME_METABOLISM_OF_RNA	0,00100
REACTOME_HIV_LIFE_CYCLE	0,00100
REACTOME_M_G1_TRANSITION	0,00100
REACTOME_G1_S_TRANSITION	0,00100

REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION	0,00200
REACTOME_MITOCHONDRIAL_TRNA_AMINOACYLATION	0,00200
REACTOME_FORMATION_OF_THE_EARLY_ELONGATION_COMPLEX	0,00200
REACTOME_POST_TRANSLATIONAL_PROTEIN_MODIFICATION	0,00200
REACTOME_METABOLISM_OF_MRNA	0,00200
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	0,00200
REACTOME_DUAL_INCISION_REACTION_IN_TC_NER	0,00300
REACTOME_STABILIZATION_OF_P53	0,00400
REACTOME_TOLL_LIKE_RECEPTOR_9_CASCADE	0,00400
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION_FROM_TYPE_2_PROMOTER	0,00400
REACTOME_MITOTIC_PROMETAPHASE	0,00500
REACTOME_LATE_PHASE_OF_HIV_LIFE_CYCLE	0,00500
REACTOME_TELOMERE_MAINTENANCE	0,00600
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	0,00700
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	0,00700
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC	0,00700
REACTOME_TRNA_AMINOACYLATION	0,00800
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	0,00900
REACTOME_GLUCONEOGENESIS	0,01000
REACTOME_ABORTIVE_ELONGATION_OF_HIV1_TRANSCRIPT_IN_THE_ABSENCE_OF_TAT	0,01100
REACTOME_RNA_POL_II_CTD_PHOSPHORYLATION_AND_INTERACTION_WITH_CE	0,01100
REACTOME_HIV1_TRANSCRIPTION_INITIATION	0,01200
REACTOME_ACTIVATED_AMPK_STIMULATES_FATTY_ACID_OXIDATION_IN_MUSCLE	0,01200
REACTOME_NEP_NS2_INTERACTS_WITH_THE_CELLULAR_EXPORT_MACHINERY	0,01300
REACTOME_SNRNP_ASSEMBLY	0,01500
REACTOME_ENERGY_DEPENDENT_REGULATION_OF_MTOR_BY_LKB1_AMPK	0,01500
REACTOME_MYD88_CASCADE	0,01500
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	0,01600
REACTOME_CLATHRIN_DERIVED_VESICLE_BUDDING	0,01900
REACTOME_REV_MEDIATED_NUCLEAR_EXPORT_OF_HIV1_RNA	0,02000
REACTOME_DIABETES_PATHWAYS	0,02100
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	0,02100
REACTOME_LYSOSOME_VESICLE_BIOGENESIS	0,02100
REACTOME_MTOR_SIGNALLING	0,02200
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION_FROM_TYPE_3_PROMOTER	0,02300
REACTOME_SIGNALING_BY_WNT	0,02300
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	0,02300
REACTOME_VPR_MEDIATED_NUCLEAR_IMPORT_OF_PICS	0,02300
REACTOME_GLUCOSE_TRANSPORT	0,02600
REACTOME_TRANSCRIPTION_OF_THE_HIV_GENOME	0,02800
REACTOME_HIV1_TRANSCRIPTION_ELONGATION	0,03100
REACTOME_NUCLEAR_IMPORT_OF_REV_PROTEIN	0,03100
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	0,03100
REACTOME_CELL_DEATH_SIGNALLING_VIA_NRAGE_NRIF_AND_NADE	0,03100
REACTOME_PYRUVATE_METABOLISM	0,03200

REACTOME_TOLL_LIKE_RECEPTOR_4_CASCADE	0,03400
REACTOME_ACTIVATED_TLR4_SIGNALLING	0,03700
REACTOME_MEMBRANE_TRAFFICKING	0,04000
REACTOME_RHO_GTPASE_CYCLE	0,04300
REACTOME_TRANSPORT_OF_RIBONUCLEOPROTEINS_INTO_THE_HOST_NUCLEUS	0,04300
REACTOME_GOLGI_ASSOCIATED_VESICLE_BIOGENESIS	0,04400
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0,04400
REACTOME_MRNA_PROCESSING	0,04300
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	0,04700

Cluster 5

KEGG_OXIDATIVE_PHOSPHORYLATION	0
KEGG_PARKINSONS_DISEASE	0
KEGG_DNA_REPLICATION	0
KEGG_ALZHEIMERS_DISEASE	0
KEGG_HUNTINGTONS_DISEASE	0
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0
KEGG_BASE_EXCISION_REPAIR	0
KEGG_PROPANOATE_METABOLISM	0
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0
KEGG_CITRATE_CYCLE_TCA_CYCLE	0
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	0
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL_GPI_ANCHOR_BIOSYNTHESIS	0
KEGG_SPLICEOSOME	0
KEGG_LYSINE_DEGRADATION	0,00063
KEGG_LYSOSOME	0,00076
KEGG_PYRIMIDINE_METABOLISM	0,00076
KEGG_PENTOSE_PHOSPHATE_PATHWAY	0,00082
KEGG_PEROXISOME	0,00092
KEGG_RNA_DEGRADATION	0,00175
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	0,00171
KEGG_PYRUVATE_METABOLISM	0,00183
KEGG_RIBOSOME	0,00187
KEGG_MISMATCH_REPAIR	0,00276
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	0,00321
KEGG_N_GLYCAN_BIOSYNTHESIS	0,00347
KEGG_BUTANOATE_METABOLISM	0,00378
KEGG_FATTY_ACID_METABOLISM	0,01352
KEGG_GLYCEROPHOSPHOLIPID_METABOLISM	0,01400
KEGG_RIBOFLAVIN_METABOLISM	0,01794
KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM	0,01898
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	0,01938
KEGG_STARCH_AND_SUCROSE_METABOLISM	0,02426
KEGG_ONE_CARBON_POOL_BY_FOLATE	0,03324
KEGG_CYSTEINE_AND_METHIONINE_METABOLISM	0,04505
KEGG_MTOR_SIGNALING_PATHWAY	0,04863
KEGG_INSULIN_SIGNALING_PATHWAY	0,04903

REACTOME_ELECTRON_TRANSPORT_CHAIN	0
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_INTEGRATION_OF_ENERGY_METABOLISM	0
REACTOME_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_DNA_STRAND_ELONGATION	0
REACTOME_PYRUVATE_METABOLISM_AND_TCA_CYCLE	0
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	0
REACTOME_EXTENSION_OF_TELOMERES	0
REACTOME_DNA_REPAIR	0
REACTOME_LAGGING_STRAND_SYNTHESIS	0
REACTOME_GLOBAL_GENOMIC_NER	0
REACTOME_TRANSCRIPTION_COUPLED_NER	0
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	0
REACTOME_CITRIC_ACID_CYCLE	0
REACTOME_METABOLISM_OF_CARBOHYDRATES	0
REACTOME_GLUCOSE_METABOLISM	0
REACTOME_REPAIR_SYNTHESIS_OF_PATCH_27_30_BASES_LONG_BY_DNA_POLYMERASE	0,00067
REACTOME_DIABETES_PATHWAYS	0,00064
REACTOME_BASE_EXCISION_REPAIR	0,00092
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	0,00129
REACTOME_DUAL_INCISION_REACTION_IN_GG_NER	0,00133
REACTOME_TRANSLATION	0,00178
REACTOME_GLYCOGEN_BREAKDOWN_GLYCOGENOLYSIS	0,00196
REACTOME_PEPTIDE_CHAIN_ELONGATION	0,00252
REACTOME_METABOLISM_OF_RNA	0,00319
REACTOME_ELONGATION_AND_PROCESSING_OF_CAPPED_TRANSCRIPTS	0,00349
REACTOME_PEROXISOMAL_LIPID_METABOLISM	0,00366
REACTOME_GLUCONEOGENESIS	0,00365
REACTOME_SYNTHESIS_OF_GPI_ANCHORED_PROTEINS	0,00369
REACTOME_HIV_LIFE_CYCLE	0,00409
REACTOME_FORMATION_AND_MATURATION_OF_MRNA_TRANSCRIPT	0,00412
REACTOME_METABOLISM_OF_PROTEINS	0,00402
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	0,00402
REACTOME_MITOCHONDRIAL_TRNA_AMINOACYLATION	0,00568
REACTOME_VIRAL_MRNA_TRANSLATION	0,00679
REACTOME_MRNA_SPLICING	0,00904
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	0,00916
REACTOME_MRNA_SPLICING_MINOR_PATHWAY	0,01004
REACTOME_CENTROSOME_MATURATION	0,00996
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	0,01109
REACTOME_METABOLISM_OF_MRNA	0,01096
REACTOME_GTP_HYDROLYSIS_AND_JOINING_OF_THE_60S_RIBOSOMAL_SUBUNIT	0,01269
REACTOME_PYRUVATE_METABOLISM	0,01439
REACTOME_GENE_EXPRESSION	0,01555
REACTOME_DUAL_INCISION_REACTION_IN_TC_NER	0,01643
REACTOME_SNRNP_ASSEMBLY	0,01643
REACTOME_SYNTHESIS_OF_GLYCOSYLPHOSPHATIDYLINOSITOL	0,01781

REACTOME_TRNA_AMINOACYLATION	0,01869
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_THE_43S_COMPLEX	0,02172
REACTOME_INFLUENZA_LIFE_CYCLE	0,02391
REACTOME_TAT_MEDIATED_HIV1_ELONGATION_ARREST_AND_RECOVERY	0,02356
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION	0,02476
REACTOME_LATE_PHASE_OF_HIV_LIFE_CYCLE	0,02657
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	0,03094
REACTOME_G2_M_CHECKPOINTS	0,03039
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION	0,03359
REACTOME_PI3K_AKT_SIGNALLING	0,03461
REACTOME_DEADENYLATION_OF_MRNA	0,03441
REACTOME_MICRORNA_BIOGENESIS	0,03972
REACTOME_POST_TRANSLATIONAL_PROTEIN_MODIFICATION	0,03909
REACTOME_CELL_CYCLE_MITOTIC	0,03903
REACTOME_FORMATION_OF_THE_EARLY_ELONGATION_COMPLEX	0,03971
REACTOME_TRANSLATION_INITIATION_COMPLEX_FORMATION	0,04106
REACTOME_MTOR_SIGNALLING	0,04235

Cluster 6

BIOCARTA_PROTEASOME_PATHWAY	0
BIOCARTA_NFKB_PATHWAY	0,02000
BIOCARTA_RELA_PATHWAY	0,01500
BIOCARTA_INFLAM_PATHWAY	0,03900
BIOCARTA_DEATH_PATHWAY	0,04800
BIOCARTA_IL10_PATHWAY	0,04300
KEGG_PROTEASOME	0
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0
KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,00200
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,00200
KEGG_JAK_STAT_SIGNALING_PATHWAY	0,00200
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	0,00300
KEGG_STEROID_BIOSYNTHESIS	0,00200
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	0,00800
KEGG_ALLOGRAFT_REJECTION	0,00900
KEGG_GRAFT_VERSUS_HOST_DISEASE	0,01000
KEGG_CYTOSOLIC_DNA_SENSING_PATHWAY	0,01000
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	0,01500
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	0,01800
KEGG_ALPHA_LINOLENIC_ACID_METABOLISM	0,02800
KEGG_ECM_RECEPTOR_INTERACTION	0,02800
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	0
REACTOME_REGULATION_OF_APC_ACTIVATORS_BETWEEN_G1_S_AND_EARLY_ANAPHASE	0
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC	0
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	0
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	0
REACTOME_STABILIZATION_OF_P53	0

REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	0
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	0
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	0
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	0
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	0
REACTOME_M_G1_TRANSITION	0
REACTOME_SIGNALING_BY_WNT	0
REACTOME_SYNTHESIS_OF_DNA	0
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	0
REACTOME_CELL_CYCLE_CHECKPOINTS	0
REACTOME_DNA_REPLICATION_PRE_INITIATION	0
REACTOME_APOPTOSIS	0
REACTOME_S_PHASE	0,00100
REACTOME_PACKAGING_OF_TELOMERE_ENDS	0,00100
REACTOME_G1_S_TRANSITION	0,00600
REACTOME_CHOLESTEROL_BIOSYNTHESIS	0,00600
REACTOME_MITOTIC_M_M_G1_PHASES	0,00900
REACTOME_AMINO_ACID_AND_OLIGOPEPTIDE_SLC_TRANSPORTERS	0,00900
REACTOME_AMINO_ACID_TRANSPORT_ACROSS_THE_PLASMA_MEMBRANE	0,01100
REACTOME_RNA_POLYMERASE_I_PROMOTER_OPENING	0,01100
REACTOME_METAL_ION_SLC_TRANSPORTERS	0,03300
REACTOME_MEMBRANE_TRAFFICKING	0,03400
REACTOME_RNA_POLYMERASE_I_PROMOTER_CLEARANCE	0,03800
REACTOME_STEROID_HORMONES	0,04200
REACTOME_METABOLISM_OF_AMINO_ACIDS	0,04100
REACTOME_FORMATION_OF_TUBULIN_FOLDING_INTERMEDIATES_BY_CCT_TRIC	0,04200
REACTOME_STEROID_METABOLISM	0,04100

Cluster 7

BIOCARTA_MCM_PATHWAY	0,00069
BIOCARTA_ATRBRCA_PATHWAY	0,00209
BIOCARTA_PROTEASOME_PATHWAY	0,01733
BIOCARTA_COMP_PATHWAY	0,02160
KEGG_LYSOSOME	0
KEGG_DNA_REPLICATION	0
KEGG_OXIDATIVE_PHOSPHORYLATION	0
KEGG_BASE_EXCISION_REPAIR	0
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	0
KEGG_PROPANOATE_METABOLISM	0
KEGG_N_GLYCAN_BIOSYNTHESIS	0
KEGG_PARKINSONS_DISEASE	0
KEGG_MISMATCH_REPAIR	0
KEGG_PYRIMIDINE_METABOLISM	0
KEGG_PEROXISOME	0

KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	0
KEGG_PROTEASOME	0
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	0
KEGG_RNA_DEGRADATION	0
KEGG_CITRATE_CYCLE_TCA_CYCLE	0
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL_GPI_ANCHOR_BIOSYNTHESIS	0,00055
KEGG_PYRUVATE_METABOLISM	0,00062
KEGG_SELENOAMINO_ACID_METABOLISM	0,00099
KEGG_HOMOLOGOUS_RECOMBINATION	0,00104
KEGG_HUNTINGTONS_DISEASE	0,00116
KEGG_PENTOSE_PHOSPHATE_PATHWAY	0,00135
KEGG_FATTY_ACID_METABOLISM	0,00137
KEGG_ALZHEIMERS_DISEASE	0,00239
KEGG_RNA_POLYMERASE	0,00295
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	0,00291
KEGG_RIBOFLAVIN_METABOLISM	0,00552
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	0,00628
KEGG_GLUTATHIONE_METABOLISM	0,00746
KEGG_VIBRIO_CHOLERAE_INFECTION	0,00990
KEGG_SPHINGOLIPID_METABOLISM	0,00977
KEGG_BETA_ALANINE_METABOLISM	0,01098
KEGG_STEROID_BIOSYNTHESIS	0,01538
KEGG_CYSTEINE_AND_METHIONINE_METABOLISM	0,01779
KEGG_GLYCOSAMINOGLYCAN_DEGRADATION	0,01731
KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS	0,02031
KEGG_LYSINE_DEGRADATION	0,02257
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	0,02627
KEGG_HISTIDINE_METABOLISM	0,03284
REACTOME_DNA_REPAIR	0
REACTOME_SYNTHESIS_OF_DNA	0
REACTOME_DNA_STRAND_ELONGATION	0
REACTOME_S_PHASE	0
REACTOME_LAGGING_STRAND_SYNTHESIS	0
REACTOME_ELECTRON_TRANSPORT_CHAIN	0
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	0
REACTOME_DNA_REPLICATION_PRE_INITIATION	0
REACTOME_EXTENSION_OF_TELOMERES	0
REACTOME_CELL_CYCLE_CHECKPOINTS	0
REACTOME_CELL_CYCLE_MITOTIC	0
REACTOME_GLOBAL_GENOMIC_NER	0
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	0
REACTOME_REPAIR_SYNTHESIS_OF_PATCH_27_30_BASES_LONG_BY_DNA_POLYMERASE	0
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	0
REACTOME_M_G1_TRANSITION	0
REACTOME_G2_M_CHECKPOINTS	0
REACTOME_TRANSCRIPTION_COUPLED_NER	0
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	0

REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	0
REACTOME_MITOTIC_M_M_G1_PHASES	0
REACTOME_STABILIZATION_OF_P53	0
REACTOME_G1_S_TRANSITION	0
REACTOME_BASE_EXCISION_REPAIR	0
REACTOME_VIF_MEDIATED_DEGRADATION_OF_APOBEC3G	0
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	0
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC	0
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	0
REACTOME_METABOLISM_OF_RNA	0
REACTOME_FANCONI_ANEMIA_PATHWAY	0
REACTOME_MICRORNA_BIOGENESIS	0
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_SNRNP_ASSEMBLY	0
REACTOME_DOUBLE_STRAND_BREAK_REPAIR	0
REACTOME_REGULATION_OF_APC_ACTIVATORS_BETWEEN_G1_S_AND_EARLY_ANAPHASE	0
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	0
REACTOME_PYRUVATE_METABOLISM_AND_TCA_CYCLE	0
REACTOME_CLATHRIN_DERIVED_VESICLE_BUDDING	0
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0
REACTOME_HIV_INFECTION	0
REACTOME_PEROXISOMAL_LIPID_METABOLISM	0
REACTOME_CENTROSOME_MATURATION	0
REACTOME_BRANCHED_CHAIN_AMINO_ACID_CATABOLISM	0,00056
REACTOME_HIV_LIFE_CYCLE	0,00054
REACTOME_METABOLISM_OF_CARBOHYDRATES	0,00062
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	0,00060
REACTOME_TRNA_AMINOACYLATION	0,00059
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION	0,00070
REACTOME_SYNTHESIS_OF_GLYCOSYLPHOSPHATIDYLINOSITOL	0,00071
REACTOME_DUAL_INCISION_REACTION_IN_GG_NER	0,00086
REACTOME_MITOTIC_PROMETAPHASE	0,00087
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	0,00085
REACTOME_MEMBRANE_TRAFFICKING	0,00084
REACTOME_INTEGRATION_OF_ENERGY_METABOLISM	0,00093
REACTOME_SIGNALING_BY_WNT	0,00096
REACTOME_NEP_NS2_INTERACTS_WITH_THE_CELLULAR_EXPORT_MACHINERY	0,00100
REACTOME_GLUCONEOGENESIS	0,00105
REACTOME_MITOCHONDRIAL_TRNA_AMINOACYLATION	0,00103
REACTOME_POST_TRANSLATIONAL_PROTEIN_MODIFICATION	0,00111
REACTOME_TELOMERE_MAINTENANCE	0,00111
REACTOME_SYNTHESIS_OF_GPI_ANCHORED_PROTEINS	0,00119
REACTOME_GOLGI_ASSOCIATED_VESICLE_BIOGENESIS	0,00117
REACTOME_REV_MEDIATED_NUCLEAR_EXPORT_OF_HIV1_RNA	0,00132
REACTOME_NUCLEAR_IMPORT_OF_REV_PROTEIN	0,00141
REACTOME_VPR_MEDIATED_NUCLEAR_IMPORT_OF_PICS	0,00152
REACTOME_G2_M_TRANSITION	0,00154

REACTOME_LATE_PHASE_OF_HIV_LIFE_CYCLE	0,00164
REACTOME_GLUCOSE_METABOLISM	0,00162
REACTOME_TRANSPORT_OF_RIBONUCLEOPROTEINS_INTO_THE_HOST_NUCLEUS	0,00234
REACTOME_LOSS_OF_NLP_FROM_MITOTIC_CENTROSOMES	0,00249
REACTOME_PYRUVATE_METABOLISM	0,00264
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	0,00292
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	0,00288
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION	0,00456
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	0,00491
REACTOME_DUAL_INCISION_REACTION_IN_TC_NER	0,00534
REACTOME_REGULATION_OF_INSULIN_SECRETION	0,00577
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION_FROM_TYPE_2_PROMOTER	0,00582
REACTOME_GLUCOSE_TRANSPORT	0,00628
REACTOME_REGULATION_OF_GLUCOKINASE_BY_GLUCOKINASE_REGULATORY_PROTEIN	0,00637
REACTOME_INITIAL_TRIGGERING_OF_COMPLEMENT	0,00688
REACTOME_TRANSPORT_OF_THE_SLBP_INDEPENDENT_MATURE_MRNA	0,00716
REACTOME_METABOLISM_OF_AMINO_ACIDS	0,00736
REACTOME_METABOLISM_OF_MRNA	0,00828
REACTOME_HIV1_TRANSCRIPTION_INITIATION	0,00826
REACTOME_CITRIC_ACID_CYCLE	0,00859
REACTOME_RNA_POLYMERASE_III_TRANSCRIPTION_INITIATION_FROM_TYPE_3_PROMOTER	0,00897
REACTOME_REGULATION_OF_LIPID_METABOLISM_BY_PEROXISOME_PROLIFERATOR_ACTIVATED_RECEPTOR_ALPHA	0,00901
REACTOME_TOLL_LIKE_RECEPTOR_9_CASCADE	0,01016
REACTOME_LYSOSOME_VESICLE_BIOGENESIS	0,01055
REACTOME_FORMATION_OF_THE_EARLY_ELONGATION_COMPLEX	0,01047
REACTOME_ABORTIVE_ELONGATION_OF_HIV1_TRANSCRIPT_IN_THE_ABSENCE_OF_TAT	0,01765
REACTOME_TOLL_LIKE_RECEPTOR_4_CASCADE	0,01764
REACTOME_APCDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B	0,02139
REACTOME_GLYCOLYSIS	0,02216
REACTOME_ACTIVATED_AMPK_STIMULATES_FATTY_ACID_OXIDATION_IN_MUSCLE	0,02250
REACTOME_SYNTHESIS_OF_BILE_ACIDS_AND_BILE_SALTS_VIA_7ALPHA_HYDROXYCHOLESTEROL	0,03006
REACTOME_COMPLEMENT_CASCADE	0,03900
REACTOME_RNA_POL_II_CTD_PHOSPHORYLATION_AND_INTERACTION_WITH_CE	0,03879
REACTOME_INACTIVATION_OF_APC_VIA_DIRECT_INHIBITION_OF_THE_AP_COMPLEX	0,04023
REACTOME_MRNA_PROCESSING	0,04046
REACTOME_ACTIVATED_TLR4_SIGNALLING	0,04354
REACTOME_CYTOSOLIC_TRNA_AMINOACYLATION	0,04775
REACTOME_PHOSPHORYLATION_OF_THE_APC	0,04758

Cluster 8

BIOCARTA_EIF_PATHWAY	0,00200
KEGG_RIBOSOME	0

KEGG_SPLICEOSOME	0
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	0,00100
KEGG_OLFACTORY_TRANSDUCTION	0,00100
KEGG_CALCIUM_SIGNALING_PATHWAY	0,04100
KEGG_MATURITY_ONSET_DIABETES_OF_THE_YOUNG	0,03800
REACTOME_PEPTIDE_CHAIN_ELONGATION	0
REACTOME_GTP_HYDROLYSIS_AND_JOINING_OF_THE_60S_RIBOSOMAL_ SUBUNIT	0
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	0
REACTOME_TRANSLATION	0
REACTOME_VIRAL_MRNA_TRANSLATION	0
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	0
REACTOME_REGULATION_OF_GENE_EXPRESSION_IN_BETA_CELLS	0
REACTOME_TRANSLATION_INITIATION_COMPLEX_FORMATION	0
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_T HE_43S_COMPLEX	0
REACTOME_REGULATION_OF_BETA_CELL_DEVELOPMENT	0
REACTOME_GENE_EXPRESSION	0
REACTOME_INSULIN_SYNTHESIS_AND_SECRETION	0
REACTOME_INFLUENZA_LIFE_CYCLE	0
REACTOME_METABOLISM_OF_PROTEINS	0
REACTOME_MRNA_SPLICING	0
REACTOME_ELONGATION_AND_PROCESSING_OF_CAPPED_TRANSCRIPTS	0
REACTOME_FORMATION_AND_MATURATION_OF_MRNA_TRANSCRIPT	0
REACTOME_OLFACTORY_SIGNALING_PATHWAY	0,00100
REACTOME_G_ALPHA_S_SIGNALLING_EVENTS	0,00100
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	0,00300
REACTOME_NUCLEAR_RECEPTOR_TRANSCRIPTION_PATHWAY	0,00800
REACTOME_AMINE_LIGAND_BINDING_RECEPTORS	0,01000
REACTOME_MRNA_3_END_PROCESSING	0,01400
REACTOME_GPCR_LIGAND_BINDING	0,01600
REACTOME_PHOSPHOLIPASE_CMEDIATED_CASCADE	0,01600
REACTOME_CLASS_B2_SECRETIN_FAMILY_RECEPTORS	0,03200

Cluster 9

KEGG_RIBOSOME	0
KEGG_PARKINSONS_DISEASE	0
KEGG_OXIDATIVE_PHOSPHORYLATION	0
KEGG_SPLICEOSOME	0,00100
KEGG_ALZHEIMERS_DISEASE	0,00100
KEGG_HUNTINGTONS_DISEASE	0,00200
KEGG_CARDIAC_MUSCLE_CONTRACTION	0,01200
REACTOME_PEPTIDE_CHAIN_ELONGATION	0
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	0
REACTOME_GTP_HYDROLYSIS_AND_JOINING_OF_THE_60S_RIBOSOMAL_ SUBUNIT	0
REACTOME_TRANSLATION	0
REACTOME_VIRAL_MRNA_TRANSLATION	0

REACTOME_REGULATION_OF_GENE_EXPRESSION_IN_BETA_CELLS	0
REACTOME_TRANSLATION_INITIATION_COMPLEX_FORMATION	0
REACTOME_REGULATION_OF_BETA_CELL_DEVELOPMENT	0
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_THE_43S_COMPLEX	0
REACTOME_ELECTRON_TRANSPORT_CHAIN	0
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	0
REACTOME_REGULATION_OF_INSULIN_SECRETION	0
REACTOME_METABOLISM_OF_PROTEINS	0
REACTOME_INTEGRATION_OF_ENERGY_METABOLISM	0
REACTOME_GENE_EXPRESSION	0
REACTOME_DIABETES_PATHWAYS	0
REACTOME_ELONGATION_AND_PROCESSING_OF_CAPPED_TRANSCRIPTS	0,00200
REACTOME_MRNA_SPLICING	0,00200
REACTOME_INSULIN_SYNTHESIS_AND_SECRETION	0,00300
REACTOME_GLYCOGEN_BREAKDOWN_GLYCOGENOLYSIS	0,00500
REACTOME_FORMATION_AND_MATURATION_OF_MRNA_TRANSCRIPT	0,00600
REACTOME_PYRUVATE_METABOLISM_AND_TCA_CYCLE	0,00700
REACTOME_INFLUENZA_LIFE_CYCLE	0,00700
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	0,00800
REACTOME_CITRIC_ACID_CYCLE	0,01400

Table S4: Complete list of the genes differentially expressed between untreated monocytes and M1 macrophages, extracted from database

Gene Id	Symbol	Description
GC08P019841_at	LPL	lipoprotein lipase
GC04P089115_at	SPP1	secreted phosphoprotein 1
GC08P081561_at	ZBTB10	zinc finger and BTB domain containing 10
GC05M158674_at	IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
GC08P086563_at	CA2	carbonic anhydrase II
GC08M105570_at	LRP12	low density lipoprotein-related protein 12
GC19M006615_at	TNFSF14	tumor necrosis factor (ligand) superfamily, member 14
GC05M147184_at	SPINK1	serine peptidase inhibitor, Kazal type 1
GC09M116591_at	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
GC11M102146_at	MMP10	matrix metallopeptidase 10 (stromelysin 2)
GC04P074974_at	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GC12M010202_at	OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
GC05P149320_at	SLC26A2	solute carrier family 26 (sulfate transporter), member 2
GC12P027288_at	STK38L	serine/threonine kinase 38 like
GC12M088484_at	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
GC19P054067_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A
GC03P158637_at	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta
GC20M043387_at	SDC4	syndecan 4
GC11M002906_at	PHLDA2	pleckstrin homology-like domain, family A, member 2
GC01P239781_at	KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
GC02P187163_at	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
GC01P078182_at	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
GC06P012120_at	HIVEP1	human immunodeficiency virus type I enhancer binding protein 1
GC02P191222_at	NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)
GC03M195606_at	ATP13A3	ATPase type 13A3
GC07P065308_at	TPST1	tyrosylprotein sulfotransferase 1
GC01M094706_at	F3	coagulation factor III (thromboplastin, tissue factor)
GC01M177339_at	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
GC07M041695_at	INHBA	inhibin, beta A
GC17P031421_at	CCL4	chemokine (C-C motif) ligand 4
GC02P113591_at	IL1RN	interleukin 1 receptor antagonist
GC07P100558_at	SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
GC17P029621_at	CCL7	chemokine (C-C motif) ligand 7
GC08M095330_at	GEM	GTP binding protein overexpressed in skeletal muscle
GC12M074707_at	PHLDA1	pleckstrin homology-like domain, family A, member 1
GC11M008960_at	NRIP3	nuclear receptor interacting protein 3
GC16M086421_at	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
GC09P101623_at	NR4A3	nuclear receptor subfamily 4, group A, member 3
GC0XP149282_at	MAMLD1	mastermind-like domain containing 1
GC06P031652_at	TNF	tumor necrosis factor (TNF superfamily, member 2)

GC07P022732_at	IL6	interleukin 6 (interferon, beta 2)
GC16P082737_at	LRRC50	leucine rich repeat containing 50
GC09P000461_at	KANK1	KN motif and ankyrin repeat domains 1
GC08M080838_at	HEY1	hairy/enhancer-of-split related with YRPW motif 1
GC02P228386_at	CCL20	chemokine (C-C motif) ligand 20
GC02M113247_at	IL1A	interleukin 1, alpha
GC11M064376_at	EHD1	EH-domain containing 1
GC22P022997_at	ADORA2A	adenosine A2a receptor
GC19P010247_at	ICAM1	intercellular adhesion molecule 1
GC01P037712_at	ZC3H12A	zinc finger CCCH-type containing 12A
GC06M143114_at	HIVEP2	human immunodeficiency virus type I enhancer binding protein 2
GC04P103641_at	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
GC09M122704_at	TRAF1	TNF receptor-associated factor 1
GC02P151922_at	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
GC08M072916_at	MSC	musculin (activated B-cell factor-1)
GC17P071890_at	SPHK1	sphingosine kinase 1
GC20M055657_at	PMEPA1	prostate transmembrane protein, androgen induced 1
GC01M207854_at	LAMB3	laminin, beta 3
GC06M002832_at	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9
GC16M065513_at	RRAD	Ras-related associated with diabetes
GC01P190871_at	RGS13	regulator of G-protein signaling 13
GC01P160797_at	UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1
GC20M010566_at	JAG1	jagged 1 (Alagille syndrome)
GC04M100046_at	EIF4E	eukaryotic translation initiation factor 4E
GC17P065677_at	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2
GC12P100795_at	DRAM	damage-regulated autophagy modulator
GC14M050170_at	SAV1	salvador homolog 1 (Drosophila)
GC04M122332_at	TNIP3	TNFAIP3 interacting protein 3
GC04P160409_at	RAPGEF2	Rap guanine nucleotide exchange factor (GEF) 2
GC02P113451_at	IL1F9	interleukin 1 family, member 9
GC10P027027_at	PDSS1	prenyl (decaprenyl) diphosphate synthase, subunit 1
GC04M139304_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
GC04M103401_at	SLC39A8	solute carrier family 39 (zinc transporter), member 8
GC01M094066_at	GCLM	glutamate-cysteine ligase, modifier subunit
GC05M077816_at	LHFPL2	lipoma HMGIC fusion partner-like 2
GC17P029606_at	CCL2	chemokine (C-C motif) ligand 2
GC17P015788_at	ADORA2B	adenosine A2b receptor
GC22P036922_at	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
GC08M029249_at	DUSP4	dual specificity phosphatase 4
GC06P151653_at	AKAP12	A kinase (PRKA) anchor protein 12
GC19M044913_at	CLC	Charcot-Leyden crystal protein
GC01P158975_at	SLAMF7	SLAM family member 7
GC17M035963_at	CCR7	chemokine (C-C motif) receptor 7
GC01M024044_at	FUCA1	fucosidase, alpha-L- 1, tissue
GC07M149953_at	GIMAP6	GTPase, IMAP family member 6
GC01M097255_at	DPYD	dihydropyrimidine dehydrogenase

GC14M059132_at	RTN1	reticulon 1
GC06P088239_at	SLC35A1	solute carrier family 35 (CMP-sialic acid transporter), member A1
GC04M164668_at	MA01	membrane-associated ring finger (C3HC4) 1
GC01M016821_at	CROCCL1	ciliary rootlet coiled-coil, rootletin-like 1
GC13M047884_at	P2RY5	purinergic receptor P2Y, G-protein coupled, 5
GC13M047962_at	RCBTB2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2
GC07M076662_at	FGL2	fibrinogen-like 2
GC11M059695_at	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
GC03M152526_at	P2RY13	purinergic receptor P2Y, G-protein coupled, 13
GC01P156416_at	CD1D	CD1d molecule
GC08M048812_at	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
GC14P074815_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog

Table S5: Complete list of the genes differentially expressed between untreated monocytes and M2 macrophages, extracted from database

Gene Id	Symbol	Description
GC08P024297_at	ADAMDEC1	ADAM-like, decysin 1
GC03M058153_at	DNASE1L3	deoxyribonuclease I-like 3
GC12M045755_at	AMIGO2	adhesion molecule with Ig-like domain 2
GC01M160219_at	OLFML2B	olfactomedin-like 2B
GC01M111827_at	ADORA3	adenosine A3 receptor
GC05M042835_at	SEPP1	selenoprotein P, plasma, 1
GC0XM065158_at	VSIG4	V-set and immunoglobulin domain containing 4
GC17M015073_at	PMP22	peripheral myelin protein 22
GC14M092239_at	LGMN	legumain
GC19P040465_at	HAMP	hepcidin antimicrobial peptide
GC14P092720_at	C14orf109	chromosome 14 open reading frame 109
GC06M003667_at	C6orf145	chromosome 6 open reading frame 145
GC18M019365_at	NPC1	Niemann-Pick disease, type C1
GC11M033681_at	CD59	CD59 molecule, complement regulatory protein
GC05M039408_at	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
GC19M011546_at	ACP5	acid phosphatase 5, tartrate resistant
GC07M024704_at	DFNA5	deafness, autosomal dominant 5
GC07P023252_at	GPNMB	glycoprotein (transmembrane) nmb
GC06M041234_at	TREM2	triggering receptor expressed on myeloid cells 2
GC12M009103_at	A2M	alpha-2-macroglobulin
GC01P158063_at	SLAMF8	SLAM family member 8
GC19P018358_at	GDF15	growth differentiation factor 15
GC08M082553_at	FABP4	fatty acid binding protein 4, adipocyte
GC02M216516_at	MREG	melanoregulin
GC12M026165_at	BHLHE41	basic helix-loop-helix family, member e41
GC11P059804_at	MS4A4A	membrane-spanning 4-domains, subfamily A, member 4
GC02M188039_at	TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
GC0XM154158_at	CLIC2	chloride intracellular channel 2
GC04M157902_at	PDGFC	platelet derived growth factor C
GC16M028457_at	NUPR1	nuclear protein, transcriptional regulator, 1
GC12M067531_at	CPM	carboxypeptidase M
GC05M101597_at	SLCO4C1	solute carrier organic anion transporter family, member 4C1
GC11P047236_at	NR1H3	nuclear receptor subfamily 1, group H, member 3
GC01M056671_at	PPAP2B	phosphatidic acid phosphatase type 2B
GC04P166468_at	SC4MOL	sterol-C4-methyl oxidase-like
GC14P060517_at	SLC38A6	solute carrier family 38, member 6
GC09P019281_at	DENND4C	DENN/MADD domain containing 4C
GC07M091579_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1
GC05P036642_at	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3
GC11M061323_at	FADS1	fatty acid desaturase 1
GC17P019378_at	SLC47A1	solute carrier family 47, member 1
GC14P058174_at	DACT1	dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)

GC19P050100_at	APOE	apolipoprotein E
GC19P050109_at	APOC1	apolipoprotein C-I
GC01M055027_at	DHCR24	24-dehydrocholesterol reductase
GC02P238432_at	RAMP1	receptor (G protein-coupled) activity modifying protein 1
GC03P053855_at	IL17RB	interleukin 17 receptor B
GC17P031415_at	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
GC0XP043400_at	MAOA	monoamine oxidase A
GC10P102096_at	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
GC20M043960_at	PLTP	phospholipid transfer protein
GC16P022732_at	HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
GC13P097593_at	FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)
GC15P078232_at	FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)
GC01P022835_at	C1QA	complement component 1, q subcomponent, A chain
GC17P029707_at	CCL13	chemokine (C-C motif) ligand 13
GC17M031364_at	CCL23	chemokine (C-C motif) ligand 23
GC17P007883_at	ALOX15B	arachidonate 15-lipoxygenase, type B
GC16P029597_at	QPRT	quinolinate phosphoribosyltransferase
GC17M075513_at	TBC1D16	TBC1 domain family, member 16
GC01P022852_at	C1QB	complement component 1, q subcomponent, B chain
GC0XM037893_at	SRPX	sushi-repeat-containing protein, X-linked
GC11M087666_at	CTSC	cathepsin C
GC01M149035_at	CTSK	cathepsin K
GC11P086427_at	TMEM135	transmembrane protein 135
GC18M019996_at	OSBPL1A	oxysterol binding protein-like 1A
GC11M005203_at	HBB	hemoglobin, beta
GC04M084507_at	HPSE	heparanase
GC01P157526_at	FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
GC02M229597_at	PID1	phosphotyrosine interaction domain containing 1
GC15P037660_at	THBS1	thrombospondin 1
GC07M141273_at	CLEC5A	C-type lectin domain family 5, member A
GC04M075092_at	PPBP	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)
GC01M032573_at	MARCKSL1	MARCKS-like 1
GC04M075086_at	PF4	platelet factor 4
GC22M036290_at	LGALS2	lectin, galactoside-binding, soluble, 2
GC01P078858_at	IFI44L	interferon-induced protein 44-like
GC03M173706_at	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
GC11P000303_at	IFITM1	interferon induced transmembrane protein 1 (9-27)
GC05P137829_at	EGR1	early growth response 1
GC12M009796_at	CD69	CD69 molecule
GC01M151629_at	S100A8	S100 calcium binding protein A8
GC06M112089_at	FYN	FYN oncogene related to SRC, FGR, YES
GC03P144320_at	CHST2	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2
GC04P074845_at	IL8	interleukin 8
GC04P075470_at	EREG	epiregulin
GC12M088244_at	DUSP6	dual specificity phosphatase 6
GC20M023008_at	CD93	CD93 molecule
GC19P006838_at	EMR1	egf-like module containing, mucin-like, hormone

		receptor-like 1
GC14P020493_at	RNASE2	ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)
GC02M156889_at	NR4A2	nuclear receptor subfamily 4, group A, member 2
GC21M043659_at	SIK1	salt-inducible kinase 1
GC19P050663_at	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GC16P083412_at	CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2
GC01P065970_at	PDE4B	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)
GC02P069995_at	MXD1	MAX dimerization protein 1
GC05P082804_at	VCAN	versican
GC06M133106_at	VNN2	vanin 2
GC18P055718_at	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
GC09M136940_at	FCN1	ficolin (collagen/fibrinogen domain containing) 1
GC01P245648_at	NLRP3	NLR family, pyrin domain containing 3
GC01M159066_at	CD244	CD244 molecule, natural killer cell receptor 2B4
GC01M167926_at	SELL	selectin L
GC04M084305_at	PLAC8	placenta-specific 8
GC19M010305_at	ICAM3	intercellular adhesion molecule 3
GC01M151612_at	S100A12	S100 calcium binding protein A12
GC01P191044_at	RGS2	regulator of G-protein signaling 2, 24kDa

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