Polarized monocyte response to cytokine stimulation

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Abstract

**Background:** Mononuclear phagocytes (MPs) stand at the crossroads between the induction of acute inflammation to recruit and activate immune effector cells and the downmodulation of the inflammatory process to contain collateral damage. This decision is extensively modulated by the cytokine microenvironment, which includes a broad array of cytokines whose direct effect on MPs remains largely unexplored. Therefore, we tested whether polarized responses of MPs to pathogens are related to the influence of selected cytokines or represent a mandatory molecular switch through which most cytokines operate.

**Results:** Circulating CD14+ MPs were exposed to bacterial lipopolysaccharide (LPS) followed by exposure to an array of cytokines, chemokines and soluble factors involved in the immune response. Gene expression was studied by global transcript analysis. Two main classes of cytokines were identified that induced a classical or an alternative pathway of MP activation. Expression of genes affected by NFκB activation was most predictive of the two main classes, suggesting that this pathway is a fundamental target of cytokine regulation. As LPS itself induces a classical type of activation, the most dramatic modulation was observed toward the alternative pathway, suggesting that a broad array of cytokines may counteract the pro-inflammatory effects of bacterial components.

**Conclusions:** This analysis is directly informative of the primary effect of individual cytokines on the early stages of LPS stimulation and, therefore, may be most informative of the way MP maturation may be polarized at the early stages of the immune response.

**Background**

Resident and recruited mononuclear phagocytes (MPs) display a versatile phenotype that reflects the plasticity of these cells in response to microenvironmental signals. This heterogeneity spans a continuous spectrum that can be polarized into two extremes recently described by Mantovani et al. [1]. Pathogen stimulation exemplified, for instance, by lipopolysaccharide (LPS) stimulation in the presence of interferon (IFN)-γ induces M1 MPs through engagement of Toll-like receptors (TLRs). M1 MPs are true antigen-presenting cells capable not only of killing invading organisms but of concomitantly recruiting and activating immune effector cells [2,3].
Treatment of MPs with type II cytokines such as interleukin (IL)-4, IL-13 and, partly, IL-10 [4], polarizes their function towards tissue repair, angiogenesis and containment of collateral damage through reduction of inflammation (the M2 macrophage phenotype). This alternative mode of macrophage activation accounts for a distinct phenotype with a key role in humoral immunity and tissue repair [5].

It has been suggested that the extreme dichotomy between a classical M1 and an alternative M2 polarization of macrophage function may not take into account intermediate regulation by cytokines such as IL-10, transforming growth factors (TGF-α and TGF-β), macrophage colony-stimulating factor (M-CSF), IFN-α and IFN-β and tumor necrosis factor (TNF) [5]. Most important, a rigid dichotomy in MP function may not directly apply to physiological and/or pathological conditions in which these cells are exposed to an array of cytokines produced by innate or adaptive immune mechanisms during infection, tissue damage or in other conditions. Indeed, a comprehensive overview of the modulatory properties of cytokines on the MP reaction to a pathogen is missing. In addition, little is known about the transcriptional changes occurring in MPs on exposure to pathogen components such as LPS.

A recent study analyzed the transcriptional profile induced by the exposure of circulating MP conditioned in vitro for 7 days with IL-4 and GM-CSF (immature dendritic cell, (DC)) to pathogen components [6]. Bacterial, viral and fungal components elicited distinctive pathways that were, however, largely overlapping. The predominant response of these DCs to most pathogen components encompassed a rapid upregulation of genes associated with the innate arm of the immune response followed by induction of adaptive immune response genes.

The response of circulating MP-derived DCs is short-lived as these cells can exhaust their production of effector molecules (cytokines and chemokines) within a few hours of LPS stimulation [7]. The transience of mRNA and protein expression can cause DCs to redirect the immune response in different ways at different time points. For instance, soon after stimulation, DCs elicit T-cell responses of the Th-1 type, whereas at later stage of activation they prime T-cell responses of the Th-2 type, suggesting that their function is strongly dependent on the timing and duration of exposure to individual and/or combined stimulatory conditions in the surrounding microenvironment. At the transcriptional level, the dual function of DCs shifted from an early pro-inflammatory phase occurring within 3 hours to a later regulatory phase occurring approximately 8 hours following LPS exposure. During these evolving stages of DC activation, cytokines play a dominant role in shaping the function of DCs and other immune cells, providing a malleable link between the innate and adaptive immune responses [8]. In natural conditions, circulating or resident MPs may encounter a pathogen before the surrounding microenvironment has a chance to influence their maturation. Therefore, it is unknown whether non-conditioned circulating CD14+ MPs would react similarly to DCs on engagement with infectious agents. Thus, a preliminary aim of this study was to evaluate the kinetics of the response of non-conditioned circulating CD14+ MP to LPS. The results suggested that these cells respond to LPS similarly to immature CD14+ DCs, with a surge in transcriptional activity that peaks around 3 hours after stimulation and in which the activation of genes associated with a classical activation of innate immune mechanisms predominates [6].

The stringent dichotomy describing a classical activation of MPs into mature antigen-presenting cells caused by IFN-γ and an alternative induction into macrophages induced by IL-4 and IL-13 may not apply to physiological conditions in which the microenvironment responds to pathogen exposure with a broad array of cytokine secretion. We therefore investigated whether polarized responses of MPs to pathogens are extreme behaviors that can be observed in vitro by studying a few illustrative cytokines or whether they represent a mandatory molecular switch through which most cytokines operate. Thus, we stimulated non-conditioned CD14+ MPs with LPS for 1 hour. The MPs were then exposed to an array of different cytokines that may be expressed in distinct pathologic conditions by different immune-cell subsets. The 1-hour interval was empirically selected to induce a biphasic model in which the presumed modulation by cytokines occurred during an ongoing reaction to LPS. This allowed mapping of cytokines into conditional subclasses based on their effects on the global transcriptional changes responsible for MP activation and differentiation.

Two main classes of cytokines were identified that induced a classical or alternative pathway of MP activation, respectively. An intermediate class (including IL-10) was also identified, while TNF-α, TNF-β and GM-CSF displayed a quite distinct behavior from the other cytokines. Expression of genes affected by NFκB activation was most predictive of the two main classes, suggesting that in most cases the NFκB pathway is a central target of cytokine regulation that modulates the cascade of events following LPS stimulation. Overall, it seems that MP maturation/differentiation goes through a molecular switch that is partly independent of the fine differences in the stimulatory properties of the various cytokines and, with few exceptions, is pre-programmed towards a classical or an alternative route. As LPS itself induces a classical type of activation, the most dramatic modulation in this model was observed toward the alternative pathway, suggesting that a broad array of cytokines may counteract the pro-inflammatory effects of bacterial components.
Results

Effect of LPS stimulation on circulating CD14^+ MPs

Enriched MP preparations (about 90% CD14^+) were exposed to LPS. Total RNA extracts were obtained 4 and 9 hours after. These time points were selected to catch salient stages of the biphasic response of MP to LPS described by others [6,7]. Amplified antisense RNA (aRNA) [9] was hybridized to a custom-made 17K cDNA microarray chip enriched with genes relevant to immune function. The transcriptional profile of LPS-induced MPs was similar to that described by others in DCs [6]. In particular, genes associated with the innate response of CD14^+, immature DCs to pathogen components [6,10] were similarly upregulated in CD14^+ MPs (data not shown). This finding suggests that the differential expression of the LPS co-receptor CD14 between the two cell populations has a relatively minor impact on the transcriptional regulation of the innate immune response [11].

Kinetics of the response of CD14^+ MPs to LPS and its modulation by cytokines

Aliquots of CD14^+ MPs were stimulated in parallel with LPS and exposed 1 hour later to individual cytokines selected from a library of recombinant proteins possibly relevant to MP regulation. MPs were kept in culture for 4 and 9 hours, at which times aRNA was prepared for transcriptional analysis. Unsupervised Eisen’s clustering [12] was applied to the complete dataset (Figure 1). The kinetics of the response to LPS had the greatest influence on the global transcriptional profile of MP induction; samples preferentially clustered according to time of stimulation rather than type of treatment. This was underlined by the observation that MPs stimulated with LPS alone clustered with the cytokine-stimulated MPs according to the time elapsed after stimulation. In addition, a cluster containing most of the samples obtained 9 hours after stimulation (9') included three control samples consisting of CD14^+ MPs not exposed to LPS or cytokines (no stimulation). These three samples were prepared at times 0, 4 and 9 hours to parallel the culture conditions used for stimulation. This finding suggested that the transcriptional profile of CD14^+ MPs 9 hours after LPS stimulation and 8 hours after treatment with most cytokines converges toward a less reactive metabolic state closer to that of unstimulated MPs. Several cytokines, however, maintained a more active metabolic profile and after 9 hours retained a transcriptional footprint relatively close to that of samples treated for 4 hours (4'). This group included the genes for most IFN-α isoforms, IFN-β, vascular endothelial growth factor (VEGF), FLT-3 ligand, TGF-α, the chemokine RANTES (CCL5), IL-2, IL-4, IL-15 and the chemokines MIP1α (CCL3) and MIP1β (CCL4), suggesting that these cytokines may have relatively prolonged kinetics of MP activation.

The average number of genes whose expression was increased compared to unstimulated MPs was higher (420 genes) after 4 hours than after 9 hours (265 genes). About half of the genes upregulated after 4 hours remained upregulated at 9 hours (223 genes). This is in accordance with Huang’s observation [6] that transcriptional changes in DCs occur predominantly in the early phase of the response to pathogen components, with about half the genes displaying transitory expression and the other half sustained expression. For this reason, subsequent analyses were limited to the 4-hour time point.

Transcriptional modulation by cytokines 4 hours after LPS stimulation of CD14^+ MPs

Although LPS alone strongly affected the transcriptional program of MPs, it was possible to discern the contribution of individual cytokines. This analysis was limited to samples treated for 4 hours, when the most dramatic effects on gene modulation were noted. Genes differentially expressed in samples treated with cytokines compared to those treated with LPS alone were identified. Stringent criteria were applied to select genes expressed in at least 80% of samples with a threefold or greater increase or reduction in expression over LPS in at least one of the cytokine-treated samples. This gave 2,057 genes that were deemed most relevant to the analysis. Using these genes, all cytokine-treated samples were subjected to unsupervised clustering to evaluate their relatedness (Figure 2a).

Two main classes of cytokines were identified. One class (Figure 2a, blue horizontal line) included IL-4, IL-13 TGF-α, TGF-β and VEGF, which are unquestionably associated with
Figure 2 (see legend on next page)
Figure 2 (see previous page)
Definition of cytokine classes based on their modulatory effect on the response of CD14+ circulating MP to LPS. (a) Definition of cytokine classes based on their modulatory effect on the response of CD14+ circulating MPs to LPS. CD14+ MPs were stimulated with LPS and exposed after 1 h to 42 individual cytokine stimulations. The clusterogram represents 2,057 genes obtained by Eisen hierarchical clustering of the complete 17K dataset filtered for genes that are expressed in a minimum of 80% of the samples 4 h after LPS stimulation, and that, at least in one experiment, displayed a greater than threefold change in expression over stimulation with LPS alone. Two main classes of cytokines are distinguished: the blue bar indicates type II cytokines (for example, IL-4 and IL-13) and the red bar indicates type I cytokines (such as IFN-γ, CD40L and FLT-3L). A smaller third class including IL-10 is also shown (green bar). TNF-α (purple bar), TNF-β and GM-CSF (dark-gray bars) clustered separately from all other cytokines. (b) Unsupervised principal component analysis (PCA) of the unfiltered 17K gene dataset. Cytokine treatments are color-coded according to the groups classified in (a): blue circles, alternative type I cytokines; green circles, alternative type II cytokines; red circles, classical cytokines; purple circle (arrowed), TNF-α; dark-gray circles, TNF-β and GM-CSF; yellow circle (arrowed), LPS alone.

Cytokine-mediated modulation of LPS-stimulated CD14+ MPs predominantly affects pathways downstream of NFκB
Signatures associated with several pathways of immune-cell activation were constructed by selecting genes from the global pool of 17K clones according to literature information without pre-existing information about the association of their expression to either class of cytokines. Signature genes were then subjected to supervised clustering according to the cytokine classification shown in Figure 2a. This independent process identified virtual signatures, in some cases portraying opposite transcriptional regulation by the two classes. The signature that most strongly discriminated the two classes comprised 121 genes whose expression is closely dependent on NFκB modulation [11] (Figure 3a). This is not surprising as LPS acts through engagement of Toll receptor 4 (TLR4) and CD14, with resulting activation of NFκB [3]. It would, therefore, seem intuitive that the strongest modulation in the present experimental conditions would target this pathway. In particular, several TNF-α and IL-1-related genes classically modulated by NFκB during the acute phases of the innate immune response [11] were strongly and inversely modulated by the two cytokine classes.

The same 121 genes were used for unsupervised class prediction by reclustering cytokine-treated samples (Figure 3b). This independent analysis segregated cytokines into two classes that with the exception of one (IFN-α2b) matched the respective original classical and alternative classification (Figure 3a, red and blue horizontal bars, respectively). Interestingly, the cytokines that belonged to the alternative II class clustered with the alternative cytokines (Figure 3a, green horizontal bars) while TNF-α (Figure 3a, purple horizontal bar), TNF-β, and GM-CSF (Figure 3a, dark gray horizontal bar) clustered separately but in proximity of the classical group. Analysis of early signaling events occurring 1 hour and 30
Figure 3 (see legend on next page)
minutes after LPS stimulation and, therefore, 30 minutes after the additional cytokine exposure, demonstrated significantly increased levels of the free p50 subunit of NFκB in MP whole-cell extracts treated with alternative class cytokines (IL-4 and IL-13). In addition, IL-10 and TNF-α significantly upregulated p50, whereas no significant changes were caused by classical cytokines (IFN-γ, IL-6 and IL-3). Extracts from MPs stimulated only with LPS also failed to demonstrate changes in NFκB subunit release; this is probably related to the 90-minute period from stimulation that allowed a return of signaling molecules to baseline conditions (Figure 3c). The similarity of the IL-10 and TNF-α effects on p50 to those of alternative cytokines contrasts with the dramatic differences observed on the respective transcriptional profiles, suggesting that other pathways induced by these cytokines may prevail in the conditions tested here.

Among the additional pathways tested, those mediated through STATs, Janus kinases (JAKs), and interferon regulatory factor (IRF) did not appear consequential to the experimental conditions tested in this study (data not shown).

Cytokine-mediated modulation of metalloproteinase expression in LPS-stimulated CD14+ MPs

Matrix metalloproteinases (MMP) are tightly connected to MP activation. MMP released by MPs contribute to normal and pathological tissue remodeling and MP migration. In addition MMP function as regulatory proteins by promoting the activation or degradation of cytokines. Finally, MMP are susceptible to cytokine stimulation. Thirty-six MMP and MMP-related genes (filtered from a larger group of 184 MMPs, disintegrins, α-defensins, TGF-β, TNF-α, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), IL-1 and monocyte chemotactic protein-3 (CCL7/MCP-3) genes) [16,17] were clustered according to the alternative and classical group denomination and the 11 most representative are shown in Figure 4. The alternative group of cytokines induced the transcription of MMPs (MMP 7, 9, 10, 19), enzymes related to MMP function (disintegrin ADAM 9, pro-collagen proline dioxygenase, MEK1 kinase, serine protease inhibitor, cathepsin L) and structural proteins (gap junction connexin 26, laminin A/C). This confirms the role of alternative MP activation in promoting tissue remodeling, cell-cell interactions and local control of the inflammatory process through activation (via MMP-7, 9) [18-21] or degradation (via MMP-19) of cytokine activity [17,22]. In addition, these observations suggest a role for the cytokines in the alternative group (other than the well-documented IL-4 or IL-13) in polarizing MPs toward an M2 regulatory phenotype.

Characterization of the alternative and classical groups of cytokines

Comparison of gene-expression patterns induced by the two cytokine classes (classical and alternative) identified 2,007 genes that were differentially expressed at a less than 0.001 significance level (t-test, p<value). Genes associated with immune function were proportionally over-represented. A selection of genes relevant to MP function is shown in Figure 5. In most cases, the pattern of expression echoed the class allocation suggested by the literature [23]. Classical cytokines induced genes responsible for the cytotoxic and migratory properties of MPs such as those for CD95, TRAIL, granzymes, perforin, CD16 (stimulatory Fcγ receptor) and CD62L. In addition, antigen presentation was enhanced as suggested by the coordinate expression of several HLA class II genes. IFN-γ and the other classical cytokines inhibited the expression of the macrophage-derived chemokine CCL22/MDC, while upregulating the expression of CXCR3, as previously reported [23].

Alternative cytokines induced the expression of several cytokines and their respective receptors involved in the chemotaxis and activation of neutrophils, MPs, natural killer (NK) cells, DCs, helper T lymphocytes (Th2) and B lymphocytes. Several genes known to be associated with alternative MP activation [5,23] were consistently upregulated. These included the mannose receptor, the inhibitory Fc-IIIb receptor CD32, and the cell-surface molecule CD44 [24], which is associated with the disposal of inflammatory cell corpses without expansion of the inflammatory process. Several inducible chemokines were expressed in response to alternative stimulation such as CCL22/MDC, supporting the emerging role of this cytokine as an enhancer of polarized Th2 responses [25]. Other chemokines known to be induced by master type II cytokines and associated with the induction of Th2 responses were also induced by alternative activation; these included CCL11 (eotaxin), CCL1 (I-309), CCL2 (MCP-1) and CCL7 (MCP-3) [23]. Of interest was the relatively higher expression of IL-24, a cytokine belonging to the IL-10 family constitutively expressed by MPs [26]. Although the true role of this cytokine in inflammatory processes is not known, it is likely that its pro-apoptotic and angioregulatory properties have important roles in tissue repair and remodeling during
inflammation. Finally, various chemokine receptors responsible for MP trafficking and localization were differentially regulated by the two cytokine groups, including in particular CCR1 and CCR5, which were induced by alternative stimulation, and CCR2, induced by classical stimulation.

This early transcriptional profile underlines the primary role of MPs during the acute phases of the response to pathogen as effector cells that can kill pathogens, take up antigen and migrate to local regional lymph nodes to recruit adaptive immune responses (classical activation). In contrast, alternative cytokines may be produced in the microenvironment to maintain a resident MP phenotype rich in chemokine production, which can attract Th2-type immune responses while continuing pathogen clearance through retention of phagocyte properties and promoting tissue remodeling.

Surprisingly, genes of the IL-1 family and its receptors, TNF and IL-6 were consistently upregulated by the alternative class of cytokines, suggesting that LPS alters MP polarization with regard to these cytokines [5,10]. Particularly interesting is the alternative induction of IL-6 and its receptor, which may play a central role in mediating the transition from neutrophils to MP recruitment during progression from acute to chronic inflammation [27].

Validation of microarray analysis by TaqMan real-time PCR
To define the validity and accuracy of our global microarray analysis, quantitative TaqMan real-time PCR was performed on amplified RNA material isolated after stimulation of monocytes obtained from five additional normal donors with representative cytokines/soluble factors selected from the alternative and from the classical groups. Comparison of monocyte stimulation with a candidate cytokine from the alternative/M2 (IL-4) and the classical/M1 (IFN-γ) group, respectively, is shown in Figure 6. Ten genes whose expression

Figure 4
Effect of cytokines on the expression of genes for matrix metalloproteinases (MMPs) and MMP-related genes. MMP genes and MMP-related genes (such as disintegrins, IFN-γ and related genes, IFN-α, α-defensins, TGF-β, TNF-α, IGF-1, EGF, FGF, IL-1, MCP-3 - complete list available from the authors on request) were clustered according to the alternative and classical groups and filtered for 70% presence and at least one value equal to or greater than a 1.5-fold change in expression (a total of 36 genes). The clusterogram displays 11 of the most representative genes. Color coding is as Figure 2.

Figure 5 (see following page)
Selection of genes differentially expressed between classically and alternatively activated MPs based on previous annotations linking their function to MP activation. Eighty-one representative genes with known MP-associated function are shown among 2,007 genes differentially expressed (p_2 < 0.001, Student’s t-test). The nomenclature for chemokines and chemokine receptors follows the recommendations of the IU/s/WHO subcommittee on chemokine nomenclature [44]. Color coding as in Figure 2.
Figure 5 (see legend on previous page)
was upregulated by alternative cytokines were tested, as
the thrust of the analysis was the evaluation of the effect of
alternative cytokines on LPS-stimulated MPs. The relative
expression of five genes out of 10 (IL-1α, IL-1 receptor, man-
nose receptor, NFKb-p105/50 and TRAF) was significantly
higher after treatment with IL-4, as suggested by the array
data. Also, the expression profiles of the other genes tested
reproduced the pattern observed in the array experiments
even if they did not reach statistical significance for each indi-
vidual gene. This is not surprising because array experiments
summarize in signature fine differences in gene expression,
that often describe patterns rather than absolute significance
for individual genes.

Reproducibility of the estimates of mannose receptor, NFKb-
p105/50 and TRAF gene expression in MPs obtained from the
same five donors was evaluated after stimulation with four
cytokines/soluble factors selected from the alternative (IL-13,
IL-1α, IL-4, TGF-β) and four from the classical groups (FLT-
3 ligand, IFN-γ, IL-3, CD40L) (Figure 7). In all cases gene
expression significantly reflected the pattern of gene expres-
sion detected by microarray analysis (Figures 3, 5).

Discussion

It has been suggested that MP activation and maturation
progresses through a polarized mechanism whereby two
extreme products result that promote inflammation on one
side and tissue repair on the other [1,5]. The first mechanism
has been called the classical pathway of MP activation. It
induces M1 monocytes specialized for pathogen killing and
activation of innate and adaptive immune effector cells. A sec-
ond, alternative, pathway induces M2-type monocytes com-
mitted to clearing pathogen through internal metabolism
while reducing inflammation. This process limits the collat-
eral damage induced by an excessive immune response, and,
upon cessation of the pathogenic stimulus, promotes tissue
repair. This dichotomy is based on the study of a few
cytokines deemed representative of the classical mode (LPS,
IFN-γ) or the alternative mode (IL-4, IL-13) of MP activation.
Other cytokines such as IL-10, TGF, M-CSF, IFN-α/β and
TNF, although partially overlapping both pathways, display
functional effects that diverge significantly enough that they
would be inaccurately grouped in either class [5].

In this study we evaluated whether MP commitment is
dependent on an early bipolar switch through which most
cytokines operate. This was done by testing in parallel a
library of 42 stimulatory molecules possibly present in the tis-
sume microenvironment following a pathogenic insult. This
modulation was tested on MPs triggered by a pathogenic
stimulus, exemplified in this case by LPS. This was done on
the assumption that in most circumstances resident or migra-
tory MPs reaching an infected area are exposed concomi-
tantly to a pathogen and to the cytokine milieu resulting from
the infection. The transcriptional profile identified by this
study cannot distinguish between the direct effect of each sol-
uble factor analyzed and the downstream activation of tran-
scriptional pathways by secondary paracrine or autocrine
secretion of biological modifiers by MPs. However, the main
goal of this study was to identify the overall effect on MPs of
the exposure to individual cytokines. Future analyses,
focused on specific cytokine patterns described here, should
possibly include the addition of blocking antibodies to segre-
gate secondary from primary MP responses.

The results suggest that MP activation is in most cases a bi-
opolar process regulated by an internal switch through which
cytokines modulate the yin and yang of the MP transcrip-
tional program. In fact, most cytokines preferentially induced
one or other pattern of transcriptional activation. We, there-
fore, mapped most of the cytokines within a classical or an
alternative classification according to their effects on CD14+
LPS-induced MPs. In particular, it appeared that transcrip-
tional programs down-stream of NFκB activation [11] were
mostly associated with either class, suggesting that the NFκB
system is at the center of the switch regulating MP activation/
differentiation in the conditions tested in the present study.
This is not surprising as LPS signaling is mediated through
the TLR-4 and CD14, which in turn directly regulate the IκB
It appears that cytokine regulation modulates the release of the p50 subunit of NFκB, which is in turn responsible for the downstream effects on the transcriptional program. Interestingly, reclustering of cytokines based on NFκB-dependent genes consolidated the two classes of cytokines adding TNF-α and TNF-β, IFN-α, and GM-CSF to the classical group and the alternative type II cytokines with the alternative group, suggesting that NFκB may be central to MP polarization.

This information cannot of course be generalized to other conditions as the model tested is strongly biased by NFκB induction by LPS. This is underlined by the unexpected alternative upregulation of genes associated with IL-1, TNF and IL-6 [11]. This observation suggests that in different conditions, cytokines may differently modulate MP activation, possibly through various modulatory feedback mechanisms [5]. In addition, genes associated with the interleukinated arginine and tryptophan pathways that modulate nitric oxide induc-
tion and are indirectly associated with NFκB function were, at least in part, differentially regulated by the two classes of cytokines [30-32]. It has been suggested that inducible nitric oxide is produced rapidly after LPS stimulation of MPs and inhibits NFκB through the stabilization of IkB [16]. It is possible that cytokines may counteract this effect through modulation of this metabolic junction.

Cytokine and chemokinome effects on MP function are tightly intertwined with the enzymatic activities of MMP. Membrane-bound cytokine receptors and adhesion molecules can be released from the cell surface by MMPs acting as ‘shedases’ or ‘convertases’. This, in turn, can downregulate cell-surface signaling by removal of receptors, or induce paracrine activity by release of soluble proteins. Not surprisingly, IL-13 overexpression results in production of several MMPs [33]. For instance, MMP-9 activates latent TNF-α on the surface of MPs or soluble VEGF [19,34]. Furthermore, Yu and Stamenkovic [21] observed that gelatinase B/MMP9 bound to CD44 activates latent TGF-β stored in the pericellular matrix. MMP-7 can enhance tissue repair by facilitating migration of epithelial cells [16]. In agreement with these reports we observed that the transcription of MMP-7, MMP-9 and CD44 are coordinately induced (Figures 4, 6) by the alternative activation of MPs. Conversely, the downregulation of MMP-7 and MMP-9 by the classical cytokines confirms their inhibitory effects on MMP expression. Inhibition of MMP-9 production by IFN-β and IFN-γ has been recently reported by Sanseau et al. [35], who noted that interferons regulate MMP expression through IRF/NFκB interaction. Binding of NFκB p50/p65 to the MMP-9 promoter is competitively inhibited by IFN-β and IFN-γ-induced IRF-1. Possibly, NFκB regulation of MMP promoters through release of p50 (Figure 3) was responsible for the transcriptional activation of MMP expression by alternative cytokines observed in this study (Figure 4). These observations confirm the tight specificity of the relationship between cytokine and MMP regulation, which is finely tuned at several check points and strongly polarized, in these experimental conditions, toward an M2 phenotype.

Enhanced transcription of gap junction/connexin 26 by the alternative cytokines is also of particular interest in view of the hypothesized junctional communications among MPs or between MPs and endothelial cells [36,37]. The finding that MPs activated by alternative cytokines induce the transcription of genes for gap junction components is of physiological importance and may be the missing link in the identification of factors that regulate the expression of gap junction connexins in MPs. In addition, it opens up the possibility that in a milieu dominated by alternative cytokines, where the ultimate goal is to return to homeostasis, the induction of gap junctions increases the ability to transmit or receive regulatory signals [38] that could facilitate the return to normal housekeeping functions.

Conclusions

The early-phase transcriptional profile presented in this study may not comprehensively parallel the plethora of biological effects that a given cytokine can induce under in vitro or, most importantly, in vivo conditions. Secondary, autocrine and paracrine modulation through the cytokine network following a primary stimulation may introduce novel on and off switches that could override the original signal. Nevertheless, this analysis is directly informative on the primary effect of individual cytokines on the early stages of LPS stimulation and, therefore, may be most informative on the way MP maturation may be polarized at the early stages of the immune response. The clustering of most cytokines into two main groups suggests that their control of central switches (NFκB), or regulatory molecules (cytokines, MMPs, gap junctions, cytotoxic molecules, migratory markers) is essentially bimodal. This polarization program turns MPs to a ‘cytotoxic’ or a ‘symbiotic’ phenotype [18]. In physiologic conditions, this dualism is probably modulated by a multiplicity of factors: the extent and duration of the environmental insult and the conditions of the resulting microenvironment. Possibly, predominant and persistent stimulation by pathogen components (such as LPS) may polarize MP towards the cytotoxic phenotype. A predominantly regulatory response is then mounted by the host, mediated by alternative cytokines that would take over at a later stage to induce a symbiotic phenotype aimed at resuming homeostasis upon pathogen clearance.

Materials and methods

MP separation and FACS staining

Peripheral blood mononuclear cells (PBMCs) from an HLA-A*0201-positive healthy cauciasian male donor age 35 were collected at the Department of Transfusion Medicine, NIH. PBMCs were isolated by Ficoll gradient separation and frozen until analysis. After thawing, PBMCs were kept overnight in 175-cm² tissue-culture flasks (Costar) in complete medium (CM) consisting of Iscove’s medium (Biofluids) supplemented with 10% heat-inactivated human AB-serum (Gemini Bioproducts), 10 mM HEPES buffer (Cellgro; Mediatech), 0.03% l-glutamine (Biofluids), 100 U/ml penicillin/streptomycin (Biofluids), 10 µg/ml ciprofloxacin (Bayer), and 0.5 mg/ml amphotericin B (Biofluids). Adherent and non-adherent cells were gently removed from the flask and centrifuged. MPs were separated by negative selection using the MP isolation kit and an autoMACS system (Miltenyi). Before and after separation cells were stained with anti-CD14-FITC (Becton Dickinson), and analyzed using a FACSscilbur flow cytometer and CellQuest software (Becton Dickinson).

Stimulation of MPs and RNA isolation

Negatively selected CD14⁻ cells were washed twice with serum-free OPTI-MEM (OM) medium (Gibco-BRL) prepared similarly to CM. CD14⁻ cells were then seeded at a concentration of 1 x 10⁶/ml in 10 ml OM in 25 cm² flasks (Falcon) and
### Table 1
Concentration and doses of cytokines/soluble factors used for stimulation of monocytes

| Cytokine                  | Concentration in vitro | Source                                           |
|---------------------------|------------------------|--------------------------------------------------|
| Aldara                    | 3 µM                   | 3M Pharmaceuticals                                |
| BCA-1                     | 10 ng/ml               | Peprotech                                        |
| CD40 L                    | 500 ng/ml              | Peprotech                                        |
| FLT-3 ligand              | 100 ng/ml              | Peprotech                                        |
| GM-CSF                    | 1,000 IU/ml            | Peprotech                                        |
| IFN-γ                     | 1,000 U/ml             | Biogen                                           |
| IFN-α2                    | 1,000 U/ml             | Peprotech                                        |
| IFN-α2b (α2, α2b, )       | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αA (2a)               | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-α1                    | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αB2                   | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-α4b                   | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αC                    | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αF                    | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αG                    | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αH2                   | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αJ1                   | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αK                    | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-αWA                   | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IFN-β                     | 1,000 U/ml             | PBL Biomedical Laboratories                      |
| IL-1α                     | 10 ng/ml               | National Cancer Institute (NCI) Biological Research Branch |
| IL-1β                     | 10 ng/ml               | NCI Biological Research Branch                   |
| IL-2                      | 6,000 IU/ml            | Chiron                                           |
| IL-3                      | 15 ng/ml               | NCI Biological Research Branch                   |
| IL-4                      | 1,000 IU/ml            | Peprotech                                        |
| IL-5                      | 10 ng/ml               | Peprotech                                        |
| IL-6                      | 100 ng/ml              | Peprotech                                        |
| IL-8                      | 100 ng/ml              | Peprotech                                        |
| IL-10                     | 10 ng/ml               | Peprotech                                        |
| IL-12                     | 10 ng/ml               | Peprotech                                        |
| IL-13                     | 20 ng/ml               | Peprotech                                        |
| IL-15                     | 20 ng/ml               | Peprotech                                        |
| LPS only                  | 5 µg/ml                | Sigma Aldrich                                    |
| MIP-1α (CCL3)             | 100 ng/ml              | Peprotech                                        |
| MIP-4 (PARC, CCl18)       | 100 ng/ml              | Peprotech                                        |
| MIP-1β (CCL4)             | 100 ng/ml              | Peprotech                                        |
| RANTES (CCL5)             | 100 ng/ml              | Peprotech                                        |
| TARC (CCL17)              | 100 ng/ml              | Peprotech                                        |
| TGF-α                     | 10 ng/ml               | Peprotech                                        |
| TNF-α                     | 10 ng/ml               | Peprotech                                        |
| TNF-β                     | 20 ng/ml               | Peprotech                                        |
| TGF-β                     | 5 ng/ml                | Peprotech                                        |
| TNF-α                     | 100 ng/ml              | Peprotech                                        |
| VEGF                      | 10 ng/ml               | R&D Systems                                       |
stimulated with 5 µg/ml LPS (Sigma) for 1 h. LPS was used at 5 µg/ml to simulate maximal pathogen exposure as in [6]. No LPS was added to the non-stimulation control flask. After 1 h, 42 cytokines, chemokines and soluble factors were added individually to the MP suspensions (Table 1). Then, 4 and 9 h after LPS stimulation, MPs were harvested, washed twice in PBS and lysed for RNA isolation using 700 µl RNeasy lysis buffer (Qiagen) per 25 cm² flask, according to the manufacturer’s protocol.

**Probe preparation, amplification and hybridization to microarrays**

Total RNA was isolated using RNeasy minikits (Qiagen). Amplified antisense RNA (aRNA) was prepared from total RNA (0.5-3 µg) according the protocol previously described by us [9,39]. Test samples were labeled with Cy5-dUTP (Amersham) while the reference sample (pooled normal donor PBMCs) was labeled with Cy3-dUTP. Test-reference sample pairs were mixed and co-hybridized to 17K cDNA microarrays.

**Microarrays and statistical analyses**

Hybridized arrays were scanned at 10-µm resolution on a GenePix 4000 scanner (Axon Instruments) at variable PMT voltage to obtain maximal signal intensities with less than 1% probe saturation. Resulting jpeg and data files were analyzed via mAdb Gateway Analysis tool [40]. Data were further analyzed using Cluster and TreeView software [12] and Partek Pro software (Partek). The global gene-expression profiling of 4- and 9-h treated and untreated MP consisted of 98 experimental samples. Subsequent low-stringency filtering (80% gene presence across all experiments and removal of genes that did not have a log₂ ≥ 2.3 ratio in at least one of the samples) selected 10,370 genes for further analysis. Clustering of experimental samples according to Eisen et al. [12] was based on these genes. Gene ratios were average corrected across experimental samples and displayed according to the central method for display using a normalization factor as recommended by Ross [41].

**NFκB protein activation analysis**

MPs separated from peripheral blood by adherence were stimulated for 1 h with LPS and for an additional 30 min with cytokines selected from the alternative group (IL-13, IL-1α, IL-4, TGF-β) and four from the classical groups (FLT-3L, IFN-γ, IL-3, CD40L). Taq-Man real-time PCR was performed on amplified RNA material isolated after stimulation for 4 h in conditions identical to those applied for the cDNA array study to validate the expression of the following 10 genes: TRAF binding protein, NFkB-p105/50, MMP9, MMP19, MCP-1, mannose receptor, IL-24, IL-1αR, IL-1A and FADD-MORT. An ABI Prism 7900 HT sequence detection system with 384-well capability (Applied Biosystems) was used for detection. Primers and TaqMan probes (Biosource) were designed to span exon-intron junctions and to generate amplicons of less than 150 bp. TaqMan probes were labeled at the 5’ end with the reporter dye molecule TAMRA (6-carboxytetramethylrhodamine; emission λmax = 582 nm). The following are the sequences for forward (f) and reverse (r) primer and probe (p) pairs:

- **IL-1α f**: TGTATGTGACTGCCCAAGATGAA
  **IL-1α r**: ACTACCTGTGATGGTTTTGGGTATC
  **IL-1α p**: FAM-AGTGCTGCTGAAGGAGATGCCTG-TAMRA
- **IL-1 rec. f**: TGTCACCAGCCAGTTGAGT
  **IL-1 rec. r**: GCACTGGGTCTATCTCATCAATT
- **IL1 rec p**: FAM-ACATTGCTTACTGGAAGTGGAATGGGT-CAG-TAMRA
- **TRAF bp f**: TTGGTTCTAGAGGTGTCTCAACAAG
  **TRAF bp r**: CTCCGATTGTGTTCTGCTAGTTC
  **TRAF bp p**: FAM-AGCAAAGTGTTATCCGCAATGGGT-GTCC-TAMRA
- **MMP9 f**: TGGATCCAAAACTACTCGGAAGA
  **MMP9 r**: GAAGGCGCGGGCAAA
  **MMP9 p**: FAM-CGCGGGCGGTGATTGACGAC-TAMRA
- **MMP19 f**: GACGAGCTAGCCCGAACTGA
  **MMP19 r**: TTTGGCACTCCCGTAAACAAA
  **MMP19 p**: FAM-TCAGCAGCTACCCCAAACCAATCAAGG-TAMRA
- **Mannose receptor f**: CTAAACCTACTCATGAAATTACTTACAAACAAA
  **Mannose receptor r**: CTCCGACCAGGTGGA
Mannose receptor p: FAM-ACACAAGGAAGATGGACCTCT- TCTAAACCGTC-TAMRA
FADD-MORT f: GGTGGCTACCTGTGTAACAAGA
FADD-MORT r: ACATGGCCCACTCTGTT
FADD-MORT p: FAM-TTACGACAGGGCGGTACCCTCACA-TAMRA
NFkB p105/50 f: CTACACCGAAGCAATGAACT
NFkB p105/50 r: CAGCGAGTGGGCGTAGA
NFkB p105/50 p: FAM-CAGGCAGCCTCCAGGAGTCTAMRA
IL-24 f: AAGAAATGAGATGTTTTTTCATCAGA
IL-24 r: CTGCATTGTGCTTCCGGAAT
IL-24 p: FAM-ACAGTGACACAGGGGTTCGTC-TAMRA
MCP-1 f: CATGGTACTAGTGTTTTTTAGATACAGAGACTT
MCP-1 r: TAATGATTCCCTGGAAAGACCTCAA
MCP-1 p: FAM-AACCACAGCTCACCCTGGAGT TAMRA

Standards for the selected genes were amplified by reverse transcriptase primer-specific amplification of 6 ng antisense RNA obtained from PBMCs stimulated in vitro with IL-2 (300 IU/ml and Flu M1 peptide) and reverse transcribed using random dNTP primers (Boehringer Mannheim). Amplified cDNA standards were quantified by spectrometry and the number of copies was calculated using the Oligo Calculator software [42]. Six micrograms of test antisense RNA samples were converted to cDNA using random primers and were immediately used for quantitative real-time PCR (RT-PCR). RT-PCR reactions of cDNA samples were conducted in a total volume of 20 µl, including 1 µl cDNA, 1x TaqMan Master Mix (Applied Biosystems), 2 µl of 20 µM primers and 1 µl of 12.5 µM probe. Thermal cycler parameters included 2 min at 50°C, 10 min 95°C and 40 cycles involving denaturation at 95°C for 15 sec, annealing-extension at 60°C for 1 min.

Linear regression analyses of all standard curves were 0.98 or greater. Standard curve extrapolation of copy number and quantity means were performed using the ABI Prism SDS 2.1 software (Applied Biosystems). Normalization of samples was performed by dividing the quantity mean of the gene of interest run in duplicate by the quantity mean of reference actin filament associated protein (AFAP) gene × 105 [43].

Additional data files
The following additional data are available with the online version of this article. Additional data file 1 is a spread sheet containing microarray raw data (intensity ratios of test versus reference samples) subsequently subjected to cluster analysis.

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