CXC Chemokine Ligand 4 Induces a Unique Transcriptome in Monocyte-Derived Macrophages

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In atherosclerotic arteries, blood monocytes differentiate to macrophages in the presence of growth factors, such as macrophage colony-stimulation factor (M-CSF), and chemokines, such as platelet factor 4 (CXCL4). To compare the gene expression signature of CXCL4-induced macrophages with M-CSF–induced macrophages or macrophages polarized with IFN-γ/LPS (M1) or IL-4 (M2), we cultured primary human peripheral blood monocytes for 6 d. mRNA expression was measured by Affymetrix gene chips, and differences were analyzed by local pooled error test, profile of complex functionality, and gene set enrichment analysis. Three hundred seventy-five genes were differentially expressed between M-CSF– and CXCL4-induced macrophages; 206 of them overexpressed in CXCL4 macrophages coding for genes implicated in the inflammatory/immune response, Ag processing and presentation, and lipid metabolism. CXCL4-induced macrophages overexpressed some M1 and M2 genes and the corresponding cytokines at the protein level; however, their transcriptome clustered with neither M1 nor M2 transcriptomes. They almost completely lost the ability to phagocytose zymosan beads. Genes linked to atherosclerosis were not consistently upregulated or downregulated. Scavenger receptors showed lower and cholesterol efflux transporters showed higher expression in CXCL4- than M-CSF–induced macrophages, resulting in lower low-density lipoprotein content. We conclude that CXCL4 induces a unique macrophage transcriptome distinct from known macrophage types, defining a new macrophage differentiation that we propose to call M4. ***The Journal of Immunology*****, 2010, 184: 4810–4818.**

The mononuclear phagocyte system is essential to the innate immune response and encompasses various types of constitutive tissue macrophages (e.g., Kupffer cells in the liver or alveolar macrophages in the lung). Under inflammatory conditions, macrophages can differentiate from peripheral blood monocytes under the influence of various growth factors, cytokines, or infectious agents (1). In atherosclerosis, macrophage differentiation is critically related to disease progression. During atherogenesis, blood monocytes are thought to enter the arterial wall and differentiate into macrophages, which sustain an inflammatory milieu and promote plaque formation (2–5).

As demonstrated by in vitro and in vivo data, macrophages present in chronically inflamed tissues can assume different phenotypes. The best defined polarization types are M1 and M2 (6). According to the classical paradigm, M1 macrophages can be obtained through activation by IFNγ, TNF-α, or LPS, whereas the alternative M2 macrophages can be induced through activation by IL-4, IL-10, or IL-13 (7, 8). The phenotypes of macrophages in vivo are incompletely described, and M1 and M2 are probably not the only macrophage phenotypes present in vivo.

In atherosclerosis, there is evidence for the presence of several different macrophage phenotypes within atherosclerotic plaques, some with features of M1 and M2 (9). In addition, other differentiation types like $CD14^{-}CD68^{+}$ and $CD14^{+}CD68^{-}$ macrophages have been identified in coronary artery lesions (10). In vivo, differentiation of macrophages toward different phenotypes has been associated with certain drugs, growth factors, and other mediators. M2 differentiation is induced by PPARγ agonists (9), whereas M-CSF preferentially induces $CD14^{+}CD68^{+}$ macrophages (10) and hemoglobin-haptoglobin promotes differentiation toward $CD163^{high}HLA\text{-}DR^{low}$ macrophages (11).

Only two growth factors are known to promote differentiation of monocytes into macrophages in vitro: M-CSF (12) and platelet factor-4 (CXCL4) (13). M-CSF has been shown to induce a transcriptome that is similar to that of M2 macrophages (14). The physiologic role and function of M-CSF has been thoroughly studied. Knockout mice lacking M-CSF (*CSF1*) or its receptor (*CSF1R*) are protected from atherogenesis (15, 16). By contrast, the role of the platelet chemokine CXCL4 is far more enigmatic. CXCL4 strongly suppresses megakaryocyte differentiation (17), inhibits monocyte apoptosis, and promotes macrophage differentiation (13). CXCL4 is released from platelets upon activation in micromolar concentrations and has a broad range of biologic functions, including induction of respiratory burst in human monocytes accompanied by secretion of several chemokines such as CCL3, CCL4, and CXCL8 (18–20). In vivo, the presence of CXCL4 within atherosclerotic lesions has been shown to correlate with clinical parameters (21). Eliminating the *PF4* gene coding for CXCL4 by homologous recombination has been shown to reduce lesion formation in a mouse model of atherosclerosis (22).

Although the transcriptomes of M-CSF–induced macrophages and their M1 or M2 polarizations have been extensively studied (14), the published data on the phenotype of CXCL4-induced macrophages are scarce. CXCL4 has been shown to induce macrophages expressing CD86, but not HLA-DR on the cell surface (13). We recently showed that CXCL4 strongly suppresses

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Received for publication April 30, 2009. Accepted for publication February 9, 2010.

This work was supported by Grant GL599/1-1 from the Deutsche Forschungsgemeinschaft (to C.A.G.) and National Institutes of Health Grant 58108 (to K.L.).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: acLDL, acetylated LDL; FDR, false discovery rate; GO, gene ontology; GSEA, gene set enrichment analysis; LDL, low-density lipoprotein; LPE, local pooled error; oxLDL, oxidized LDL; PCA, principal compenent analysis; SR-A, scavenger receptor-A.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901368

expression of the hemoglobin-haptoglobin receptor CD163 (23). Both findings suggest that the CXCL4 macrophage is distinct from its M-CSF counterpart. However, thus far a comprehensive transcriptome analysis of the CXCL4-induced macrophage phenotype has not been undertaken. Furthermore, it remains unclear whether the CXCL4 macrophage is relevant for atherogenesis and can be related to any of the known polarization patterns.

We hypothesized that the transcriptome of CXCL4-induced macrophages may be unique and different from M-CSF or other known polarization types. Therefore, we conducted a comprehensive analysis of the CXCL4 macrophage transcriptome and compared it to its M-CSF counterpart, speculating that this analysis might give insight into mechanisms by which CXCL4 macrophages promote disease progression in atherosclerosis.

Materials and Methods

Monocyte-derived human macrophages

With approval from the institutional review board, PBMCs were isolated from human peripheral blood using Histopaque (Sigma-Aldrich, St.Louis, MO) followed by negative isolation with magnetic beads (Stem Cell, Vancouver, Canada). Monocyte purity was 96.2 ± 0.2% as assessed by CD14 expression. After RBC lysis and several wash steps with 1 mM EDTA, monocytes were essentially free from platelet contamination as demonstrated by virtual absence of CD41 positivity in flow cytometry (data not shown). Monocytes were cultured in macrophage serum-free medium (Life Technologies, Carlsbard, CA) supplemented with Nutridoma SP (Roche, Indianapolis, IN) and penicillin/streptomycin (Sigma-Aldrich) for 6 d in the presence of 100 ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ) or 1 μM rhCXCL4 (Peprotech). The concentration of 1 μM rhCXCL4 was chosen because this concentration was previously demonstrated to be sufficient to induce macrophage differentiation from monocytes (13). Furthermore, our own preliminary experiments confirmed that after 6 d, this concentration induced expression of typical macrophage markers like CD11b or CD68 to a similar extent as M-CSF (Fig. 1 and data not shown).

Oxidized low-density lipoprotein–induced foam cell formation and phagocytosis assays

For foam cell formation assays, macrophages were exposed to 10 μg/ml DiI-labeled acetylated or oxidized low-density lipoprotein (oxLDL; Biomedical Technologies, Stoughton, MA) for 4 h at 37°C. Subsequently, cells were washed and fluorescence intensity was assessed in a flow cytometer (FACScalibur, BD Biosciences, San Jose, CA). Untreated macrophages served as negative controls.

Phagocytosis was assessed using M0 and M4 macrophages as phagocytes and zymosan beads (Alexa Fluor 488; Invitrogen, Carlsbad, CA) as targets, at a ratio of 10 zymosan beads to one macrophage. Macrophages were incubated at 37°C for 1 h with opsonized or nonopsonized zymosan beads. Opsonization was performed by incubation at 37°C for 1 h with

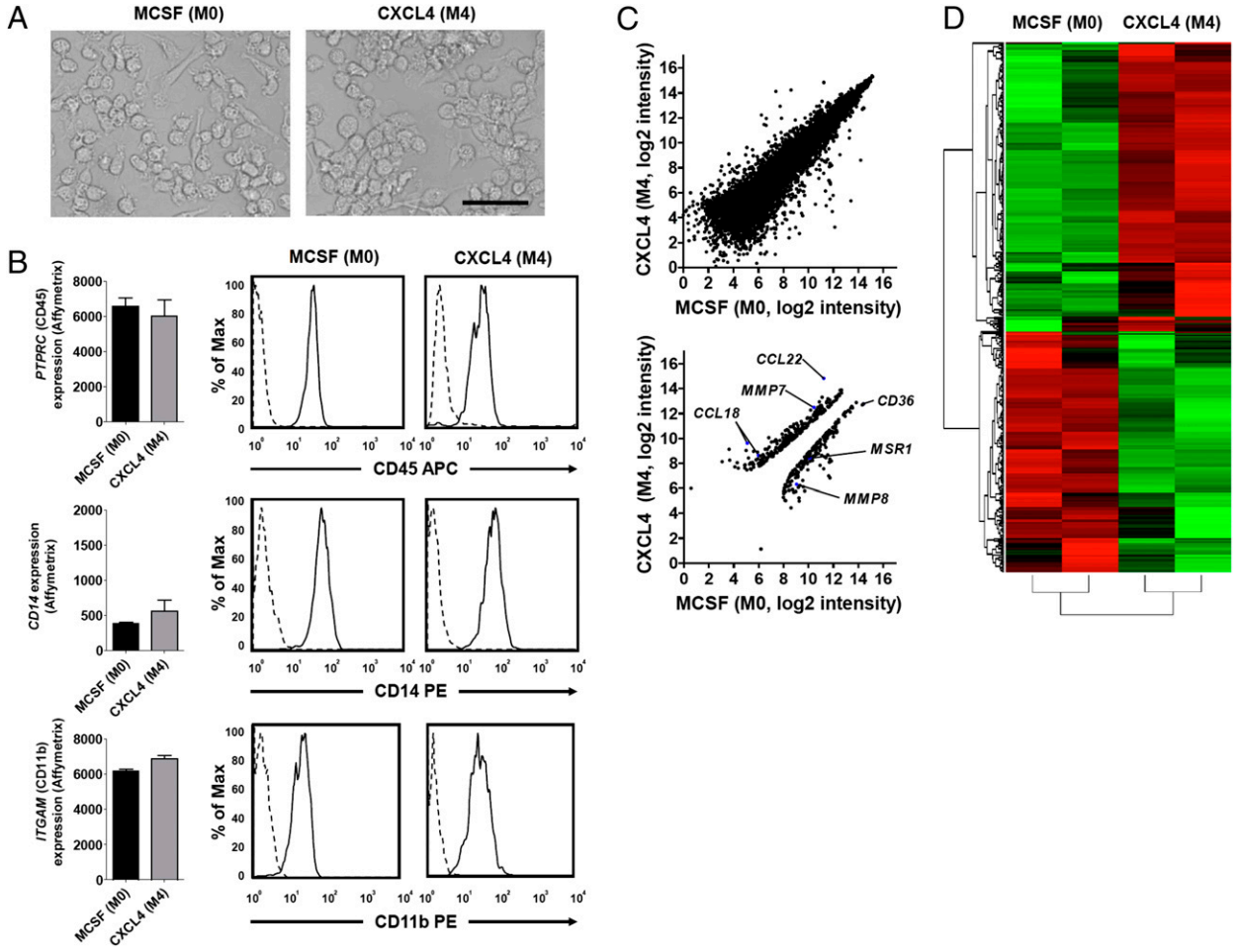

FIGURE 1. Primary human monocyte-derived macrophges differentiated with 100 ng/ml M-CSF (M0) or 1 μM CXCL4 (M4). *A*, Morphology of macrophages after 6 d in culture. Bar indicates 50 μm. *B*, Gene and protein expression of lineage marker genes *PTPCR* (CD45), *CD14* (CD14), *ITGAM* (CD11b) in both macrophage types (differences not significant by LPE test). *C*, The upper graph shows transformed (log_2) intensity of all expressed genes in M0 macrophages plotted against the intensity in M4 macrophages ($r = 0.934$; $p < 0.0001$). The lower plot shows the same data including only genes with FDR < 0.05 as determined by LPE test. *D*, Heat map showing all significantly differentially regulated genes (FDR < 0.05, by LPE test). Gene expression was normalized and standardized (gene list in Supplemental Table 1). Red indicates high, and green indicates low gene expression. Genes and conditions were allowed to freely cluster in the *y*- and *x*-axes, respectively.

autologous serum, followed by three PBS washing steps using low-speed centrifugation (1500 × *g*, 15 min). The extent of phagocytosis was analyzed by flow cytometry, using untreated macrophages (no beads) as controls.

Affymetrix gene chip experiments

For each condition RNA was isolated from macrophages derived from two donors using columns including a DNAse-step followed by reverse transcription (all reagents from Qiagen, Valencia, CA). RNA was labeled and hybridized to Affymetrix (Santa Clara, CA) HG133 Plus 2.0 arrays as described previously (24). For each donor, RNA was hybridized to a separate gene array. Signal intensity values were obtained from the Affymetrix MicroArray Suite software (MAS 5.0). The data set and technical information according to the *Minimum Information about a Microarray Experiment* requirements are available at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo), accession number GSE20484.

ELISA and cytokine bead arrays

Protein concentration of selected cytokines was measured in cell culture supernatants using ELISA (CCL18 [R&D Systems, Minneapolis, MN] and CCL22 [Cell Sciences, Canton, MA]) or cytokine bead arrays (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF; BD Biosciences) according to the manufacturers' instructions. Supernatants were pooled over 6 d and diluted when necessary to obtain concentrations within the range of the assays.

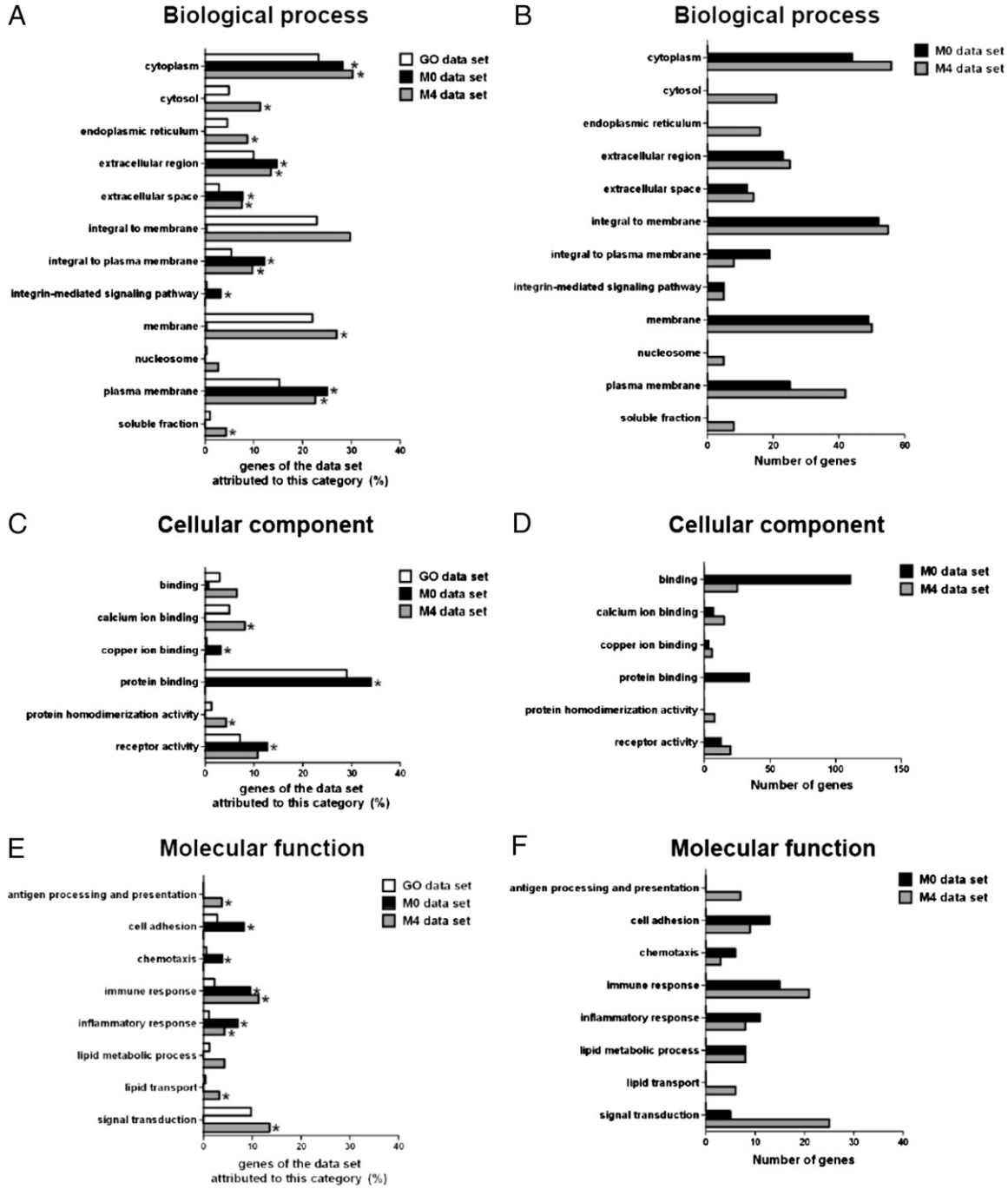

FIGURE 2. GO categories of regulated genes in M0 and M4 macrophages as determined by ProfCom analysis. Bars indicate the percentage (*A*, *C*, *E*) or the absolute number (*B*, *D*, *F*) of genes attributed to a certain GO category within all genes of the GO data set (empty bars), genes overexpressed in M0 macrophages (black bars), or genes overexpressed in M4 macrophages (gray bars). Data are arranged by biologic process (*A*, *B*), cellular component (*C*, *D*), and molecular function (*E*, *F*). $*p < 0.05$, adjusted for multiple testing.

Flow cytometry

For flow cytometry, cells were treated with Fc block (Miltenyi Biotec, Auburn, CA) and subsequently stained with antibodies against CD36 (clone CB38; BD Biosciences) and SR-A (clone 351615; R&D Systems). For SR-A staining, an FITC-labeled secondary Ab was used. Appropriate isotype controls were used in all experiments. Fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences). Fluorescence was assessed as background-corrected mean fluorescence.

Local pooled error test

For statistical analysis, the open source statistical software package R (www.r-project.org) was used including the local pooled error (LPE) test for differential expression discovery between two conditions (25). Gene chip data were analyzed as described previously (24). After exclusion of nonexpressed genes, data were normalized and log2 transformed to achieve normal distribution. The LPE test is statistically powerful for identifying differentially expressed genes between low-replicated microarray data. It pools probe sets with similar expression levels providing a statistic for each probe set. The absolute value of the LPE-statistic is larger for more significantly differentially expressed probe sets. A false discovery rate (FDR) was calculated to discover probe sets differentially expressed with FDR < 0.05 (26). Heat maps were constructed using *R* in a way that allows all conditions and genes to freely cluster both in the *x*- (condition) and the *y*-axes (gene).

Profile of complex functionality

To assess functional networks regulated in each macrophage type, profiled complex functionality was analyzed using the ProfCom software (National Institutes of Health), a Web-based tool for the interpretation of genes that were identified to be functionally linked by experiment (27). This tool corrects for multiple testing and compares the proportion of genes related to specific gene ontology (GO) categories among the genes found regulated to the proportion of genes related to the same category within the gene ontology reference genes.

Gene set enrichment analysis

Gene set enrichment was analyzed using an open access software for gene set enrichment analysis (GSEA) (28) to assess potential similarities between the CXCL4-induced gene expression profile and the known M1 and M2 signatures. The latter were extracted from the gene expression data of monocyte–macrophage differentiation and polarization as published by Mantovani et al. (14) (GEO data set 2430). Using the LPE test, genes differentially expressed between the M1 and M2 data set were identified and used as M1 and M2 gene sets, respectively. Overexpression of the M1 and M2 gene sets was tested by GSEA in the M-CSF and CXCL4 gene expression data. GSEA calculates an enrichment score, which indicates the degree of overrepresentation of these gene sets and estimates its significance with adjustment for multiple hypothesis testing.

Modified principal component analysis and hierarchical clustering

A modified principal component analysis (PCA) was performed on the previously published M1 and M2 (14) as well as on the new CXCL4 gene expression data normalized to the corresponding M-CSF gene expression sets. This normalization step avoided bias owing to interexperimental

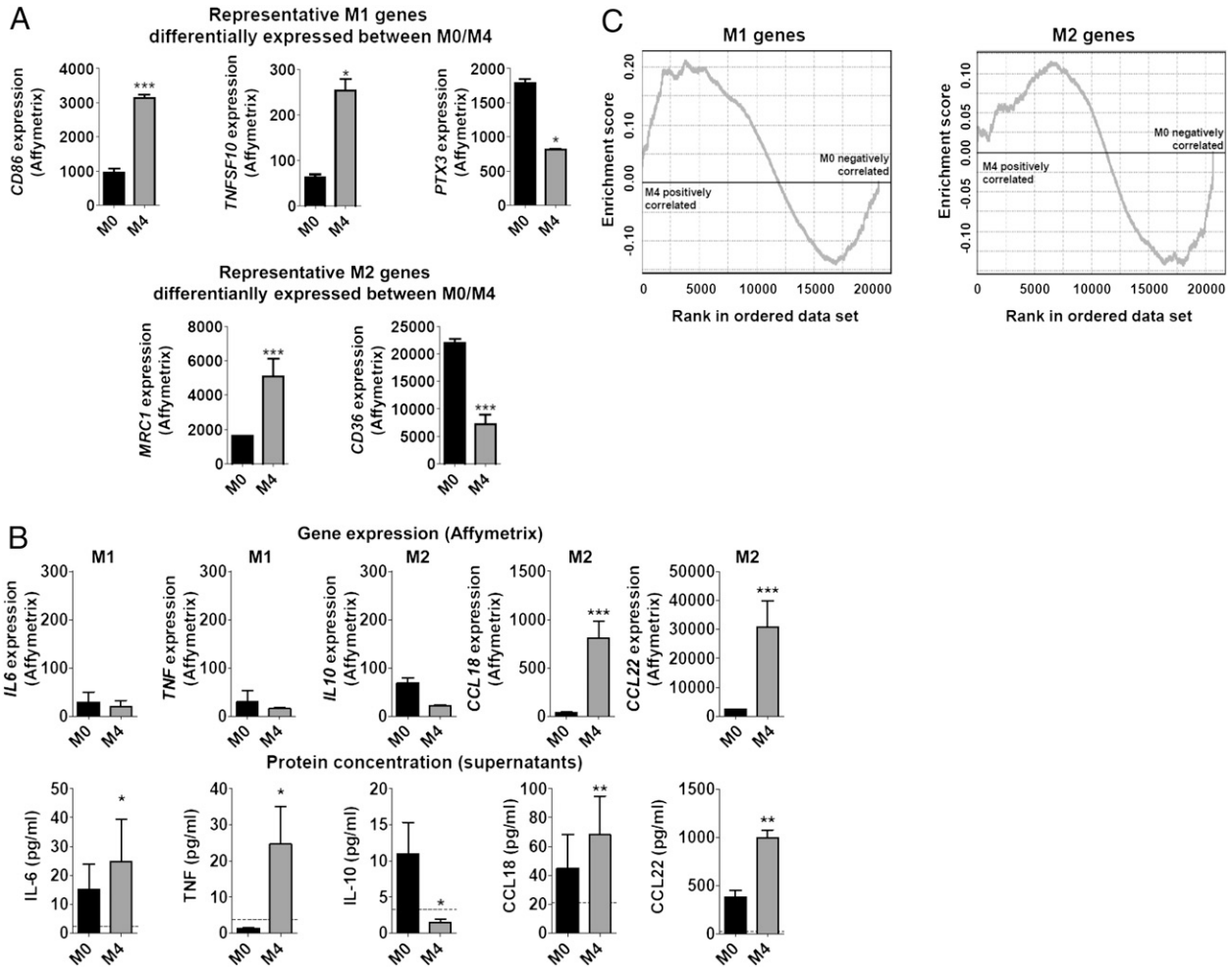

FIGURE 3. M4 macrophages do not display a clear M1 or M2 transcriptome pattern. *A*, Gene expression of selected markers for M1 and M2 polarization (*TNFSF10*, TRAIL; *PTX3*, pentraxin-3; *MRC1*, mannose receptor). ***FDR < 0.001, by LPE test. *B*, Gene expression (*upper row*) and protein levels (*bottom row*) of cytokines related to M1 or M2 polarization. ***FDR < 0.001, by LPE test (gene expression); $*p < 0.05$ by paired *t* test (protein levels), dotted line indicates detection limit of the assay. *C*, Enrichment plot of M1 or M2 gene sets in M0 versus M4 macrophages. All genes were ranked using the GSEA difference of class metric. For each gene in the M1 or M2 gene set, the enrichment score was calculated and plotted against the position of the genes within the rank ordered data set. In both cases, no significant enrichment was found.

Table I. *Probe sets and genes attributed to M1 polarization or M2 polarization (22) with significantly differential expression between M0 and M4 macrophages, according to LPE test: log_2 normalized expression data, z stats, and FDR as calculated by LPE test*

Probe Set	Gene Symbol	Annotation	M0 #1	M0 #2	M4 #1	M4 #2	z Stats	FDR
Probe sets/genes related to M1 polarization								
205686_s_at	*CD86*	CD86	9.72	10.07	11.66	11.57	−6.97	<0.0001
210895_s_at	*CD86*	CD86	11.27	11.66	12.89	13.01	−6.74	<0.0001
209728_at	*HLA-DRB4*	MHC, class II, DR β 4	11.86	11.57	12.90	13.29	−6.28	0.0017
205685_at	*CD86*	CD86	8.93	9.34	10.64	10.82	−5.84	0.0039
213831_at	*HLA-DQA1*	MHC, class II, DQ α 1	9.86	2.03	10.77	5.92	−5.62	0.0075
204670_x_at	*HLA-DRB1*	MHC, class II, DR β 1	12.41	11.94	13.09	13.58	−5.22	0.0105
204972_at	*OAS2*	2′-5′-oligoadenylate synthetase 2	8.14	7.64	9.23	9.52	−4.72	0.0188
202688_at	*TNFSF10*	TNF-related apoptosis inducing ligand TRAIL	6.12	5.83	7.83	8.12	−4.63	0.0202
208306_x_at	*HLA-DRB1*	MHC, class II, DR β 1	12.66	12.65	13.34	13.98	−4.39	0.0284
215193_x_at	*HLA-DRB1*	MHC, class II, DR β 1	12.45	12.16	13.14	13.38	−4.37	0.0298
206157_at	*PTX3*	Pentraxin 3	10.76	10.85	9.65	9.69	4.32	0.0322
Probe sets/genes related to M2 polarization								
207861_at	*CCL22*	CCL22	11.32	11.14	14.40	15.28	−11.47	<0.0001
32128_at	*CCL18*	Pulmonary and activation-regulated chemokine (PARC)	5.34	4.84	9.29	9.94	−7.92	<0.0001
204438_at	*MRC1*	Mannose receptor	10.64	10.67	11.99	12.58	−7.13	<0.0001
223280_x_at	*MS4A6A*	Membrane-spanning 4-domains, subfamily A, member 6A	7.55	5.53	9.68	8.58	−7.02	<0.0001
224356_x_at	*MS4A6A*	Membrane-spanning 4-domains, subfamily A, member 6A	7.78	5.93	9.80	8.81	−6.98	<0.0001
204112_s_at	*HNMT*	Histamine N-methyltransferase	12.05	12.45	10.51	10.77	6.84	<0.0001
201427_s_at	*SEPP1*	Selenoprotein P, plasma, 1	10.16	9.03	8.33	3.89	7.64	<0.0001
209555_s_at	*CD36*	CD36	14.32	14.50	13.02	12.54	9.08	<0.0001
206488_s_at	*CD36*	CD36	14.37	14.47	13.13	12.42	9.15	<0.0001
211719_x_at	*FN1*	Fibronectin 1	11.35	12.01	9.45	6.72	12.59	<0.0001
212464_s_at	*FN1*	Fibronectin 1	11.62	11.88	10.06	6.25	12.69	<0.0001
210495_x_at	*FN1*	Fibronectin 1	11.56	12.13	9.63	6.72	12.99	<0.0001
216442_x_at	*FN1*	Fibronectin 1	11.42	12.01	9.46	4.17	14.06	<0.0001
209924_at	*CCL18*	Pulmonary and activation-regulated chemokine	6.25	5.71	7.93	9.33	−6.31	0.0017
219666_at	*MS4A6A*	Membrane-spanning 4-domains, subfamily A, member 6A	7.14	6.29	9.31	8.30	−5.73	0.0039
208422_at	*MSR1*	Scavenger receptor-A	7.93	12.27	5.76	11.11	5.72	0.0039
227265_at	*FGL2*	Fibrinogen-like protein 2	8.66	7.25	10.55	8.80	−5.55	0.0075
204834_at	*FGL2*	Fibrinogen-like protein 2	8.50	7.86	10.42	9.26	−5.49	0.0075
211732_x_at	*HNMT*	Histamine N-methyltransferase	10.11	10.63	8.79	9.51	4.44	0.0259
228772_at	*HNMT*	Histamine N-methyltransferase	8.42	8.81	7.07	7.33	4.00	0.0463

variance. First, PCA was performed including all genes that were significantly overexpressed (as determined by LPE) in M1 relative to M2. Subsequently, a second PCA was performed including all genes that were overexpressed in M2 relative to M1. The first principal components from each of these analyses (independent by definition) were used to define a new coordinate space in which CXCL4 gene expression data were plotted.

Hierarchical clustering was used to determine the level of similarity between the three normalized groups (29). All genes were included in the analysis, and the results are displayed in a dendrogram. Distance was determined by average linkage, wherein the distance between two groups, A and B, is determined according to the equation

$$d_{AB} = \frac{1}{n_A n_B} \sum_{i \in A} \sum_{j \in B} d_{ij}, \quad (1)$$

where n_i is the number of members in group i and d_{ij} is the Euclidean distance between two points, i and j.

Results

M-CSF and CXCL4 induce macrophages with a similar transcriptome

Preliminary experiments confirmed that after 6 d in culture both M-CSF– as well as CXCL4-induced macrophages (which we suggest calling M0 and M4, respectively) displayed a morphology characteristic of macrophages (Fig. 1*A*). Accordingly, gene and protein expression of the classical lineage markers (CD45, CD14, and CD11b) were comparable in both macrophage types, indicating that the cells studied were fully differentiated macrophages (Fig. 1*B*).

When comparing the overall gene expression signature of M0 and M4 macrophages, the two gene expression patterns were found to be similar and highly correlated ($r = 0.934$; $p < 0.0001$; Fig. 1*C*, *upper panel*). Of 26,051 probe sets expressed above the detection limit in at least one macrophage sample, 460 annotated probe sets were significantly upregulated or downregulated with $FDR < 0.05$ corresponding to a total number of 375 regulated genes (Fig. 1*C*, *lower panel*). Two hundred six of these genes displayed higher and 169 displayed lower expression levels in M4 macrophages as compared with M0 macrophages (Fig. 1*D*). A list of differentially expressed genes is given in Supplemental Table I.

CXCL4-induced macrophages overexpress genes implicated in the immune response, Ag processing and presentation, and lipid metabolism

Based on the genes found to be differentially expressed between M0 and M4 macrophages by LPE test, we sought to identify functional processes as defined by gene ontology that were transcriptionally overrepresented in M4 macrophages. Applying profiling of complex functionality analysis (27), we found a number of biologic processes that were associated with genes expressed in M0 or M4

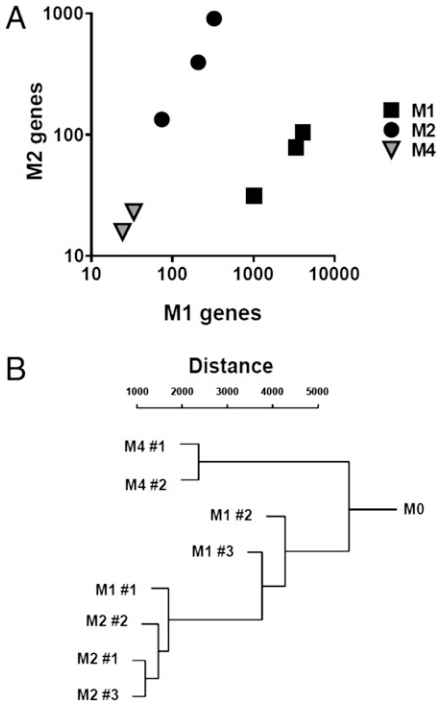

FIGURE 4. The M4 macrophage transcriptome is distinct from the M1 or M2 transcriptomes. *A*, Modified principal components analysis of M1 and M2 gene expression data (as described in *Materials and Methods*). M4 gene expression data were plotted into a coordinate space defined by M1 and M2 gene expression data. *B*, Hierarchical clustering of the normalized M1, M2, and M4 gene expression data. All genes were included in the analysis, and the results are displayed as dendrogram. #1, #2, and #3 indicate donor-specific replicates for each condition.

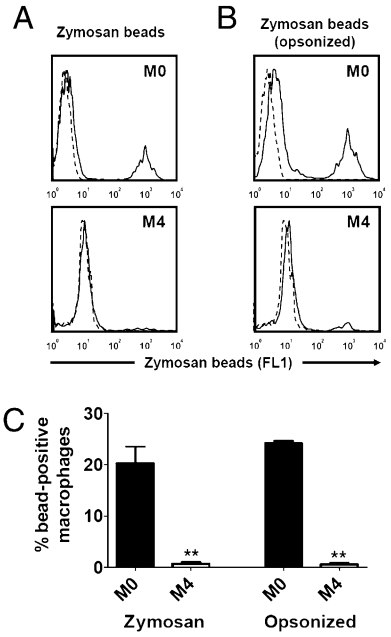

FIGURE 5. M0 and M4 macrophages display differential phagocytotic capacity. Macrophages differentiated with M-CSF (M0) or CXCL4 (M4) were exposed to zymosan beads (*A*) or zymosan beads opsonized with FCS (*B*) as described in *Materials and Methods*. The phagocytotic capacity of M0 and M4 macrophages was assessed by flow cytometry. Representative histograms are shown in *A* and *B*, and results of two independent experiments are presented as bar graphs in *C*. $**p < 0.01$.

macrophages (Fig. 2*A*–*F*). Most prominently, both M0 and M4 macrophages overexpressed genes related to the inflammatory and the immune response. In M4 macrophages, *CCL18* and *TNFSF10* (TRAIL) were overexpressed, whereas in M0 macrophages *AIF1*, *ALOX5*, and *IL1RN* were found in the inflammation and immune response gene sets (in all cases, $p < 0.05$).

Genes involved in Ag processing and presentation were significantly overrepresented in M4 macrophages ($p < 0.05$), including *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4*, and *HLA-DQA1* (coding for MHC class II), as well as the costimulatory surface molecule *CD86*. Interestingly, several genes implicated in lipid metabolism and transport were also found overexpressed ($p < 0.05$), including *APOC2*, *APOE*, and *SORL1*. By contrast, genes overexpressed in M-CSF–induced macrophages were more likely to be implicated in chemotaxis (represented by the chemokines *CCL3*, *CCL7*, and the chemokine receptor *CCR1*; $p < 0.05$) or cell adhesion, as indicated by the integrin genes *ITGAV*, *ITGA6*, *ITGB8B*, and the *COL6A* gene coding for the extracellular matrix component collagen 6A; $p < 0.05$.

M4 macrophages do not display a clear M1 or M2 pattern, and their transcriptome is distinct from the M1 or M2 transcriptomes

As reported previously, M0 macrophages display a gene expression pattern similar to that of M2 macrophages, whereas the gene signature of M1 macrophages is distinct (14). To better understand the characteristics of M4 macrophages, we sought to assess whether the M4 macrophage transcriptome is comparable to either of these polarization types. When examining selected genes' characteristics for M1 or M2 polarization, it became clear that a large number of polarization marker genes were not differentially expressed between M0 and M4 macrophages. This finding was true for a number of cytokines (*IL6*, *IL12*, *TNF* [TNF-α, all M1], *IL10* [M2]), many chemokines (*CCL2*, *CCL5* [both M1], *CCL1* [M2]), several surface receptors (*CCR7*, *TLR2, TLR4* [all M1]), or specific enzymes (*NOS2* [iNOS, M1], *ARG1* [arginase-1, M2]) (8). Alternatively, a small number of marker genes displayed significant differential expression between M0 and M4 macrophages; however, there was no clear pattern for preferential expression of M1 or M2 markers in either of the macrophage types (Fig. 3*A*). Table I shows all M1 and M2 genes significantly overexpressed in either M0 or M4 macrophages. Measuring protein levels of cytokines released into cell culture supernatants largely confirmed this pattern with IL-6, TNF (both M1), CCL18, and CCL22 (both M2) levels being higher in M4 macrophages, and IL-10 (M2) levels being higher in M0 macrophages (Fig. 3*B*). No differences were seen for the levels of IL-1β, IL-8, and IL-12p70 (data not shown).

To generate larger gene sets for M1 or M2 polarization, we compared gene array data sets for M1 and M2 polarized macrophages as published by Mantovani et al. (14). These gene expression data were derived from human monocyte-derived M-CSF macrophages, which were treated with either LPS and IFN-γ (M1) or IL-4 (M2) (14). Genes with FDR < 0.05, as determined by LPE testing between M1 and M2 (Supplemental Table 2), were included in the gene sets (Supplemental Table 3). Using these gene sets, we performed GSEA for M1 and M2 genes. This demonstrated no significant overexpression of either of the gene sets in M0 or M4 macrophages (FDR = 0.98 [M1 gene set] and 1.0 [M2 gene set], respectively; Fig. 3*C*). This finding establishes that M4 macrophages are neither M1 nor M2 but represent a distinct phenotype.

To test whether the M4 macrophage transcriptome is similar to any of the classical polarization patterns (M1 and M2), we used a modified PCA on the normalized M1 and M2 data (14). At first,

PCA was performed including all genes that were significantly overexpressed (as determined by LPE) in M1 relative to M2 (n = 2431). A second PCA was performed that included all genes overexpressed in M2 relative to M1 (n = 3944). Based on the principal components from each of these analyses, a new coordinate space was defined in which the M4 gene expression data were plotted. The M4 macrophages did not cluster with either M1 or M2 macrophages (Fig. 4*A*). To corroborate this finding, we used hierarchical clustering, including all genes of the M1, M2, and M4 macrophage expression data. This analysis confirmed that the M4 transcriptome significantly differs from M1 or M2 macrophages and represents a unique macrophage phenotype. In fact, M1 and M2 are more similar to each other than to M4 (Fig. 4*B*).

Phagocytotic function

One function of macrophages is to phagocytose pathogens and foreign materials (29). Phagocytosis was recently shown to be inhibited in M2-polarized macrophages (30). To test the phagocytosis function, we incubated M0 and M4 macrophages with zymosan beads with and without serum opsonization. Although ~20% of M0 macrophages phagocytosed zymosan beads, this function was almost completely suppressed in M4 macrophages (Fig. 5*A*, 5*B*).

Atherosclerosis-related genes in M4 macrophages and potential functional implications

To understand the potential relevance of CXCL4-induced macrophages in atherogenesis, we further investigated the list of differentially expressed genes. As indicated by GO analysis, these included several chemokines and matrix metalloproteases and two members of the cathepsin family, but also some genes implicated in lipid metabolism and foam cell formation. Most of these gene groups did not display a consistent pattern, indicating that both proatherogenic and anti-atherogenic genes were expressed in M4 macrophages. Whereas *MMP7* and *MMP12* showed higher expression in M4 than in M0 macrophages, *MMP8* expression was higher in M0 macrophages (Fig. 6*A*).

Strikingly, genes implicated in foam cell formation (i.e., the scavenger receptors *CD36* and *MSR1* [SR-A] and the cholesterol efflux transporter *ABCG1*) displayed highly differential expression between M0 and M4 macrophages (*ABCA1* mRNA was expressed in neither M0 nor M4 macrophages). Although M0 macrophages expressed higher levels of *CD36* and *MSR1* mRNA, *ABCG1* mRNA expression was higher in M4 macrophages. This gene expression pattern suggested that M4 macrophage would be less likely to take up modified LDL and more likely to promote cholesterol efflux (Fig. 6*A*, 6*B*). At the protein level, CD36 expression was slightly lower in M4 macrophages, whereas no significant difference was seen for SR-A expression (Fig. 6*C*).To assess the functional relevance of this finding, we studied uptake of DiI-labeled acetylated LDL (acLDL) or oxLDL by M0 and M4 macrophages. After 4 h exposure to 10 μg/ml DiI-labeled acLDL or oxLDL, M4 macrophages displayed a significantly reduced content of modified LDL as assessed by flow cytometry (Fig. 6*D*, 6*E*). This finding suggests that the gene signature actually translates into cellular function and that the prevailing gene expression pattern of M4 macrophages tends to result in reduced foam cell formation.

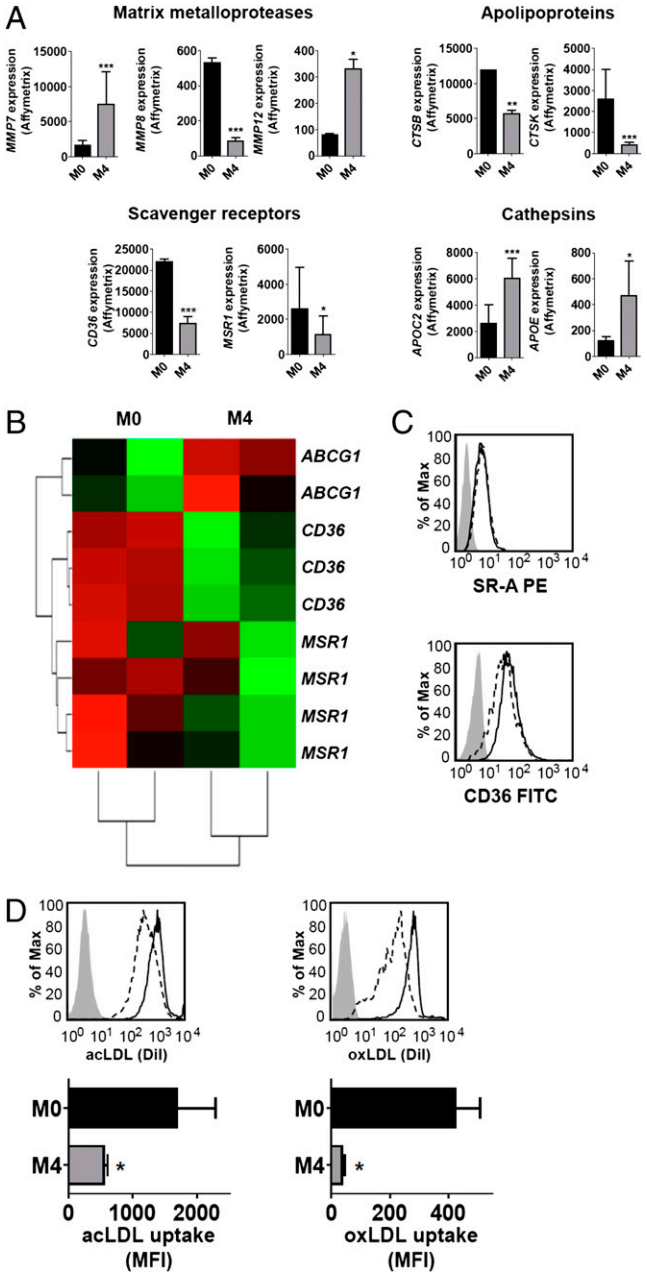

FIGURE 6. Atherosclerosis-related genes in M4 macrophages and potential functional implications. *A*, Expression of atherosclerosis-related genes with significantly different expression level in M0 and M4 macrophages. *FDR < 0.01; ***FDR < 0.001. *B*, Heat map of genes implicated in foam cell formation. Red indicates high gene expression, and green indicates low gene expression. Genes and conditions were allowed to cluster freely in the *y*- and *x*-axes, respectively. *C*, Representative histograms of surface expression of scavenger receptor-A (SR-A) and CD36 in M0 (solid line) and M4 (dotted line) macrophages derived from the same donor. Isotype control shown in gray. *D* and *E*, Mean fluorescence intensity of DiI-labeled acetylated (*D*) or oxidized (*E*) LDL in M0 (solid line) and M4 (dotted line) macrophages after 4 h exposure to 10 μg/ml LDL, as determined by flow cytometry. Representative histograms and a bar graph summarizing the flow cytometric data are shown. $*p < 0.05$ by t test, mean ± SEM; n = 3–6.

Discussion

We report the first comprehensive analysis of the transcriptome of monocyte-derived macrophages induced by the chemokine CXCL4. Our study demonstrates that (1) CXCL4 induces a macrophage phenotype that is distinct from that induced by M-CSF (2), the CXCL4-induced transcriptome shares similarities with, but is also distinct from, each of the classical M1 and M2 phenotypes, and (3) the transcriptome of CXCL4-induced macrophages is not clearly pro- or anti-atherogenic. Based on its unique properties, we suggest referring to the macrophage polarization induced by CXCL4 as M4 macrophages.

Our knowledge about heterogeneity of polarized macrophages has increased significantly. Thus, in addition to the classical M1 macrophages (characterized by high expression of proinflammatory cytokines, iNOS expression, and production of reactive oxygen species) and M2 macrophages (expressing high levels of mannose receptor, dectin-1, and arginase), a number of M2 subsets have been characterized (1). These subsets include M2 macrophages activated by IL-4 or IL-13 (now termed M2a), macrophages activated by immune complexes (termed M2b), and macrophages polarized with glucocorticoids or IL-10 (M2c) (31). The M-CSF–induced transcriptome and the corresponding M1 and M2 (more specifically, M2a) transcriptomes have been studied by Mantovani et al. using Affymetrix gene chips (14). These experiments showed a close similarity between M-CSF–induced and M2a macrophages. Furthermore, they demonstrated differences between M1 and M2a in genes involved in metabolic activities as well as genes coding for chemokines (14).

Similar to M-CSF, the platelet chemokine CXCL4 has been demonstrated to prevent monocyte apoptosis and promote macrophage differentiation from human peripheral blood monocytes (13). Surprisingly, the phenotype of these CXCL4-induced macrophages has not been studied in detail. Our data suggest that CXCL4 induces a macrophage phenotype that shares similarities with both M1 and M2 macrophages. Thus, some M1- and M2-related genes are overexpressed in M4 macrophages as compared with M-CSF–induced macrophages. This finding was confirmed for a number of cytokines on the protein level. Most importantly, an unbiased analysis using different approaches such as gene set enrichment, modified principal component, and hierarchical clustering analysis all confirmed the uniqueness of the CXCL4-induced macrophage transcriptome.

Platelets, as well as monocytes and monocyte-derived macrophages, are present within atherosclerotic lesions, and it is now clear that both contribute to lesion formation (5). The platelet chemokine CXCL4 is known to promote atherosclerosis as demonstrated in CXCL4-deficient *PF4*$^{-/-}$ mice. On the *Apoe*$^{-/-}$ background, the *PF4*$^{-/-}$ mice showed ~60% reduction of lesion size in the aorta (32). One way by which CXCL4 may contribute to atherogenesis is by promoting macrophage differentiation from monocytes present in the arterial wall. It had been speculated that CXCL4 could induce a macrophage polarization favorable for the development of atherosclerotic lesions. Our in vitro data suggest that CXCL4 alone is not sufficient to promote atherosclerosis, because compared with M-CSF–induced M0 macrophages, CXCL4-induced M4 macrophages express a number of atherosclerosis-related genes at higher and others at lower levels.

Thus, compared with M0 macrophages, CXCL4 induced high expression of the matrix metalloproteases 7 and 12, whereas MMP-8 was expressed only at low levels. Although all three MMPs have been clearly implicated in atherosclerosis (33), the gene expression data need to be interpreted with caution, because the activity of MMPs is regulated by complex mechanisms involving proteolytic cleavage by cathepsins, MMPs, and serine proteases (33). Accordingly, changes in gene expression do not necessarily indicate changes in activity in vivo (33). Although genes coding for the apolipoproteins *APOC2* and *APOE* were expressed only at low levels in CXCL4 macrophages, two members of the proteolytic cathepsin family (B and K) showed high gene expression levels. Several cathepsins have been found to be overexpressed in atherosclerotic lesions and contribute to atherogenesis through different mechanisms, including effects on lipid metabolism, inflammation, and MMP activity (34).

Most strikingly, when examining expression levels of genes implicated in foam cell formation, CXCL4 macrophages showed low levels of scavenger receptors necessary for uptake of modified LDL and at the same time higher levels of the cholesterol efflux transporter ABCG1. The finding that exposure of M4 macrophages to acLDL or oxLDL resulted in less intracellular cholesterol content than in M0 macrophages from the same donor suggests that these findings on the gene expression level translate into relevant functional differences.

In vivo, entire platelets with their granule contents are present in atherosclerotic lesions and not isolated CXCL4. Thus, CXCL4 can synergize with other platelet elements to induce a proatherosclerotic macrophage phenotype that is believed to be lacking in *PF4*$^{-/-}$ mice. In fact, it has been demonstrated in *Apoe*$^{-/-}$ mice that pharmacologic inhibition of heterodimerization of CXCL4 with CCL5, which is also released from activated platelets, resulted in significant reduction of lesion formation (35). We recently showed that CXCL4-induced macrophages lack expression of the hemoglobin scavenger receptor CD163. CXCL4 and CD163 expression are inversely correlated in human atherosclerotic lesions (23). This finding supports the notion that the M4 macrophage phenotype can actually be identified within human atherosclerotic lesions and might have pathophysiologic relevance in atherosclerosis.

Our data provide new insight into the process of macrophage differentiation. By comparing the transcriptome of M-CSF– and CXCL4-induced macrophages in vitro, we identify M4 macrophages and provide novel starting points for further atherosclerosis- and other disease-related research.

Acknowledgments

We thank Keely Arbenz-Smith for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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