Granulocyte-Macrophage Colony-Stimulating Factor Induces an Expression Program in Neonatal Microglia That Primes Them for Antigen Presentation[1]

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Neonatal microglial cells respond to GM-CSF and M-CSF by acquiring different morphologies and phenotypes. To investigate the extent and consequences of this process, a global gene expression analysis was performed, with significant changes in transcript levels confirmed by biochemical analyses. Primary murine microglial cells underwent substantial expression reprogramming after treatment with GM-CSF or M-CSF with many differentially expressed transcripts important in innate and adaptive immunity. In particular, many gene products involved in Ag presentation were induced by GM-CSF, but not M-CSF, thus potentially priming relatively quiescent microglia cells for Ag presentation. This function of GM-CSF is distinct from its primary function in cell proliferation and survival. ***The Journal of Immunology,*** **2002, 169: 2264–2273.**

Microglial cells originate from bone marrow elements that migrate into the brain in two successive waves. The first wave enters during the fetal period, whereas the second one colonizes the brain during the neonatal period, a few days after birth and before the blood-brain barrier is formed. Once settled in the brain parenchyma, microglial cells are forged under the influences of the microenvironment into a resident population whose characteristics are still largely unknown (1–3). Upon closure of the blood-brain barrier, any further transmigration into the parenchyma and exchange with circulating blood elements is restricted. An exception to this rule is a specific population of perivascular and submeningeal microglial cells, which continue to be replenished by infiltrating monocytes (4, 5). Once in the brain, the parenchymal microglial cells are shaped under the influence of growth factors and cytokines released by astrocytes, as well as neurochemicals released by neurons (1, 6–8).

Two hemopoietic CSFs, M-CSF and GM-CSF, are thought to play important roles in neonatal and postnatal microglial differentiation (9–11). M-CSF and GM-CSF both stimulate proliferation of neonatal microglia (12), but differently affect their morphology, phenotype, and Ag-presenting function. Morphologically, neonatal microglial cells cultured in M-CSF appear amoeboid, whereas those cultured in GM-CSF assume a dendriform shape (13). Phenotypically, neonatal microglial cells treated with M-CSF display some characteristics of macrophage (mϕ)[3] whereas those cultured in GM-CSF assume some of the markers associated with an immature dendritic cell (DC)-like phenotype (11, 13, 14). Importantly, the cytokine production capacity and competence as APCs are also differentially regulated by exposure to M-CSF and GM-CSF (13–17). Priming of microglial cells with GM-CSF enables them to become fully competent as APCs, upon contact with activated T cells or proinflammatory cytokines (18, 19). Although the competency of microglial cells for Ag presentation under noninflammatory conditions is still a matter of debate; it is apparent that GM-CSF endows both neonatal and adult microglial cells with an enhanced ability to process and present Ags (18, 19).

M-CSF is constitutively produced by astrocytes from embryo to adulthood (20). However, although GM-CSF is produced during the neonatal period, it is scarcely present in the adult CNS (20, 21). In the normal adult CNS, the only source of GM-CSF is from peripherally primed, infiltrating T cells, which patrol the brain parenchyma (22, 23). Because extravasation of activated T cells into the CNS is non-Ag dependent, the majority of T cells will exit without meeting their specific Ag. In contrast, if the T cells encounter a cognate self ligand, a potential autoimmune process could begin (23) with massive production of proinflammatory mediators. Under such conditions, microglial cells, already primed by T cell-released GM-CSF, will become fully competent APCs. Activated astrocytes and endothelial cells could also function as an additional source of GM-CSF (23, 24). Thus, it appears that M-CSF plays an important role in shaping the adult resident parenchymal microglia, whereas GM-CSF could have an important role in promoting their proinflammatory function. In this respect, the functional states of microglial cells elicited by M-CSF or GM-CSF have been proposed to correspond to the resting or "activated" form of microglia, respectively (11).

DNA microarray analysis can provide a very broad transcriptional profile of cells under different culture conditions or differentiation states. In this study, the transcript levels of ~6000 genes

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[3] Abbreviations used in this paper: mϕ, macrophage; DC, dendritic cell; hsp, heat shock response; MIP, mϕ inflammatory protein; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; EAE, experimental allergic encephalitis.

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have been analyzed, in primary murine neonatal microglial cells either untreated or cultured with GM-CSF or M-CSF. Both cytokines led to large-scale changes in the microglia expression profile. M-CSF treatment primarily induced transcription of genes involved in tissue organization and remodeling, maintenance, and regulation of brain homeostasis. By contrast, GM-CSF treatment induced transcription of genes important for T cell stimulation, chemotaxis, Ag processing, innate immunity, and immunosuppression, thus readying microglia for Ag presentation.

Materials and Methods

Preparation of microglia

Neonatal microglia was prepared from newborn SJL/J mice as previously described (13). Briefly, after careful removal of the meninges, brains were mechanically disrupted and passed through a 100-μm filter. Cells were seeded in MEM (Life Technologies, Rockville, MD) with the addition of 10% FCS, 5 μg/ml insulin (Life Technologies), and 2.0 mg/ml L-glucose (Sigma-Aldrich, St. Louis, MO) for 12–14 days. Mixed glial cultures were shaken overnight on an orbital shaker (first shake). Adherent glial cells were trypsinized, split, and reseeded for an additional 10–12 days of culture. The procedure was repeated twice (second and third shakes). The purity of each preparation was assessed by CD11b staining and was always >93%. GM-CSF (10 ng/ml) or M-CSF (5 ng/ml) were added at the beginning of the culture and again every 3 days. Microglial cells from the second shake were used for global gene expression analysis. In some experiments (Fig. 3, *a* and *b*), IFN-γ was added to the untreated control for 48 h. Surface staining was performed as previously described (13) using the following mAbs, hamster anti-mouse CD11c (clone HL-3), rat anti-mouse CD11b (clone M1/70), rat anti-mouse CD45 (clone 30-F11), rat anti-mouse CD24 (clone 30-F1), hamster anti-mouse B7-1 (clone 16-10A1), and rat anti-mouse B7-2 (clone GL1), all from BD PharMingen (San Diego, CA); rat anti-mouse DEC-205 (clone NLDC-145; American Type Culture Collection, Manassas, VA); goat anti-Tweak (Research Diagnostics, Flanders, NJ); and rat anti-mouse MARCO (clone ED31; a gift from P. Ricciardi-Castagnoli).

RNA extraction, cDNA biotinylation, gene chip hybridization

Total RNA was isolated from 10 million cells using TRIzol reagents (Life Technologies), and biotinylated cDNA was prepared and hybridized to Affimetrix microarrays using the manufacturer's protocols. Transcripts exhibiting an average difference (between specific and control oligonucleotides) of 1500 were considered to be expressed: the percentages calculated using the Affimetrix "absolute call" were similar. Transcripts with average difference below this level were considered below the level of detection.

RNase protection assay

Total RNA was isolated using TRIzol reagents (Life Technologies). RNase protection assay was performed using 4–6 μg of total RNA using BD PharMingen Riboquant kit rCK-2 multiprobe template set following the manufacturer's recommendations.

Immunostaining

Spinal cord frozen sections (10 μm thick) were fixed in acetone at −20°C, washed in PBS, and blocked with BSA. The following primary Abs diluted in PBS/1% BSA were added to the slides and incubated overnight at 4°C: goat anti-Tweak, used at 5 μg/ml (Research Diagnostics), in combination with anti-mouse CD45-FITC conjugated (BD PharMingen). Sections were washed in PBS and incubated with rabbit anti-goat IgG conjugated to Alexa 594 (Molecular Probes, Eugene, OR) to detect Tweak. To amplify the signal from the green fluorescence, the amplification kit, Alexa Fluor 488 signal amplification system and Oregon Green Dye-conjugated probes (Molecular Probes) was used. Fluorescent microscopy was performed on an Olympus IX70 (Olympus, Melville, NY) with ×60 N.A. 1.4 infinity corrected optics and a PXL cold CCD camera (Roper Scientific, Tucson, AZ).

Western blot analysis

Microglial cells untreated or treated with GM-CSF (10 ng/ml), M-CSF (5 ng/ml), or IFN-γ (10 ng/ml) were pelleted and lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 8), and supplemented with the protease inhibitor mixture (Complete Mini Roche), for 1 h at 4°C. Supernatants were collected by centrifugation and protein content was normalized. Proteins were run on a SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. After transfer, the membrane was probed with CD74 (clone In-1; BD PharMingen) specific for the MHC-associated invariant chain or the anti-MHC class II mAb KL-304 (ATCC), followed by 1/5000 dilution of the secondary HRP-conjugated mAb and chemiluminescence detection.

Mixed lymphocyte reaction

MLR was performed combining different amount (from 25 to 200 × 10^3 cells) of microglial cells either untreated or treated with GM-CSF or M-CSF, previously irradiated at 600 rad (Cs^{137}), with 200 × 10^3 splenocytes from either B10BR, C57BL/6, or SJL/J (The Jackson Laboratory, Bar Harbor, ME). Proliferation was tested by [^{3}H]thymidine incorporation. Bone marrow-derived DCs prepared as previously described (25) were used as positive control in each MLR.

Results

Global expression profile

Microglial cells were isolated from neonatal brain tissue of SJL mice, and cultured in the presence or absence of GM-CSF (10 ng/ml) or M-CSF (5 ng/ml) (13). Expression of RNA transcripts was analyzed for ~6000 murine genes of known or predicted function from the National Center for Biotechnology Information Unigene database, using Affimetrix U74A microarrays. Marked differences were observed in the transcriptional profiles of the microglia after treatment with GM-CSF or M-CSF, as compared with each other or as compared with untreated cells. Over 600 transcripts were uniquely expressed under each of these conditions, with more than one-third of the transcripts exhibiting differential expression in largely nonoverlapping sets (Fig. 1*a*). Large

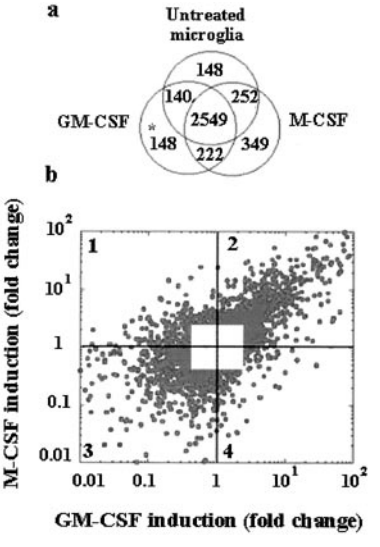


FIGURE 1. Differential gene expression in microglia after treatment with M-CSF or GM-CSF. *a*, A total of 3808 of 6000 mRNA transcripts of known or predicted function are expressed in untreated microglia or after GM-CSF or M-CSF treatment. For a transcript to be considered positive, the average difference between specific hybridization and mismatch control hybridization must be >1500. Of the 3808 transcripts expressed in microglial cells, 2549 are not affected by either GM-CSF or M-CSF treatment, 148 are only expressed in untreated microglial cells, 140 are expressed in both untreated and GM-CSF treated cells, 148* are expressed uniquely in GM-CSF treated cells, 222 are expressed in both GM-CSF and M-CSF treated cells, 252 are expressed in both untreated and M-CSF treated cells, 349 are expressed uniquely in M-CSF-treated cells (Venn Diagram). *b*, Changes in expression level induced by GM-CSF or M-CSF. The blank central rectangle defines genes whose expression does not change >2.5-fold. Approximately 16% of genes are up-regulated or down-regulated by at least 2.5-fold after treatment with either GM-CSF or M-CSF. Quadrant 1 genes are up-regulated by M-CSF and down-regulated by GM-CSF, quadrant 2 genes are up-regulated by both GM-CSF and M-CSF, quadrant 3 genes are down-regulated by both GM-CSF and M-CSF, and quadrant 4 genes are up-regulated by GM-CSF and down-regulated by M-CSF.

Table I. *Functional categories of genes differentially regulated by GM-CSF or M-CSF treatment of microglial cells*[a]

	GM-CSF	M-CSF	gi No.
Binding proteins and receptors			
Neuropeptide Y receptor	+	−	1679633
41BB	+	+	1117783
Opioid RΔ1	+	+	348246
Opioid RΣ1	0	−−	3493173
Chemokine orphan R1	+	+	2439995
Cadherin-related neural receptor	0	+	3253078
Thrombin receptor	0	+	202027
CD44	+	+	53679
AA4	+	0	3695112
AC133 Ag	++	0	2789657
Serotonin R 5A	+	+	49758
Brain fatty acid binding protein	++++	++	507169
IL-18 binding protein	+	0	4586396
Insulin-like growth factor binding protein 2	0	+	550378
Neutral amino acid transporter	0	+	2459560
Glycoprotein-associated amino acid transporter	0	+	5824164
Retinol binding protein	0	+	50547
Fatty acid transport protein	+	0	563828
Insulin-like growth factor binding protein 3	−	+++	550380
Ceruloplasmin	0	+	1224107
Annexin I	++	0	198844
Annexin XI	+	0	1815638
Annexin 8	0	+	2612794
CD47	++++	−	3036964
CD68	0	−	52988
Integrin β_5	−−−−	−−	3478696
Apolipoprotein E	−−−−	0	220334
Apolipoprotein CI	−−	−	410495
Growth factors/neuropeptides			
EGF factor 8	++	0	199142
EGF-like growth factor	+	+++	192999
Mast cell growth factor	0	+	199151
NGF-β	0	+	193494
Preproenkephalin	0	+	201032
Endothelial monocyte-activating polypeptide I	+++++	++	1150723
Insulin-like growth factor I	−−−	−	51801
Enzymes			
Liver arginase	++++	++	1293092
Eosinophil secondary granule ribonuclease	++++	0	1695898
Leucine arylaminopeptidase	++	+	1674500
Lysine-ketoglutarate reductase	+	+	4107273
Carnitine palmytoyltransferase	+	+	425266
Phospholipase D2	+	+	2088544
Arginase II	+	0	2642321
Pyruvate dehydrogenase	+	+	200276
Metallothionein	+	0	53247
Lysosomal acidic lipase	+	0	4456670
Adenilate cyclase 7	+	0	602411
Aminolevulinis acid synthase 2	+	+	191857
HK ATPase	+	+	596067
Carboxypeptidase H	0	+	50312
Sodium potassium ATPase	−	+	51111
Carbonic anhydrase	0	+	199078
Arachidonate 15-lipoxygenase	−	+	509607
Cytosine-5 methyltransferase	0	+	2689717
Phospholipase D2	+	+	2088544
Ecto-5 nucleotidase	+	+	3046874
Glutathione transferase Gt8.7	+	+	193687
Cytochrome *c* oxidase	+	+	459880
Cholesterol 25 hydroxylase	0	+	4038305
Deoxycytidine kinase	0	+	456676
Topoisomerase II α	0	+	404643
Adenylsuccinate synthetase	0	+	404056
S-adenosylmethionine decarboxylase	0	+	220330
CGMP phosphodiesterase α	0	+	53587
β-1,3-*N*-acetylglucosamintransferase	0	+	4191391
Geralylgeranyl transferase	0	+	1345081
Chromodomain helicase	0	+	455014
Phosphatase 1B	0	+	961469
Fatty acid synthase	−	+	50947
p170 phosphatidylinositol 3-kinase	0	+	1305537
Extracellular superoxido dismutase	0	+	1915962
Cu-Zn superoxido dismutase	−	0	192929
Stearoyl-coenzyme A desaturase 1	0	+	200949
Plasminogen activator	0	++	202109
PAM	0	++	1711198
G6pd-2	0	+	1806125
Galactosyltransferase α	+++++	++	193563
Diacylglycerol acyltransferase	+++	+	3859933
ADAM 10	+	++++	2282607
Metalloprotease-disintegrin	−−	−	3273477
Metallothionein III	0	+	199133
Metallothionein II	+	0	199131
Tissue organization and remodelling			
Tenascin C	0	+++	54768
α1 type I procollagen	+	++	424103
Procollagen type V α2	0	+	309180
Procollagen type IV	0	+	192282
Amyloid A3	0	+	54028
Procollagen type XI	0	+	1212743
Procollagen type VI	0	+	50478
Dystroglycan	+	+	1155350
Ryudocan	+	+++	2373476
Biglycan	0	+	53666
Thrombospondin 2	0	+	340421
SPARC glycoprotein	0	+	54168
Connexin 43	0	+	191773
Transgelin	0	+	1160197
Structural proteins			
Lamin A	+	+	1838920
Gelsolin	+	0	193463
α-Actin	0	++	49861
Trafficking			
Vamp 4	+	+	3108178
Syntaxin 3A	+	+	924267
Rab 23	+	++	438161
Rab 3D	+	+	200631
Chaperones			
hsp47	0	++	51449
hsp105	0	+	840651
Transcription factors			
Jun	0	+	52758
Δ EF1	0	+	1027499
NF-Atc	0	+	3643194
TRA 1	+	+	2662352
Krox 20	++	0	198599
NFIL3/EABP4	+	0	2076877
UBF	+	0	55115
GATA-binding protein 4	+	0	293344
Sint 1	+	0	6165418
Cortactin	0	+	414990
RGS-r	+	+	2605641
RAM14-1	0	+	2894671
COP 9 complex subunit 7a	0	+	3309173
Apoptosis			
CPP32 apoptotic protease	0	+	4097524
Apoptotic protease activating factor	0	+	3694812
TIAP	0	+	3135206

(*Table continues*)

Code: 0 genes, which are not up- or down-regulated as compared to the untreated control. +, Genes up-regulated between 2.5- and 10-fold. ++, Genes up-regulated between 10- and 20-fold. +++, Genes up-regulated between 20- and 50-fold. ++++, Genes up-regulated >50-fold. −, Genes down-regulated between 2.5- and 10-fold. −−, Genes down-regulated between 10- and 20-fold. −−−, Genes down-regulated between 20- and 50-fold. −−−−, Genes down-regulated >50-fold.

differences were observed in the transcript levels of expressed genes, with ~16.5% of expressed genes exhibiting up- or down-modulation of at least 2.5-fold after cytokine treatment (Fig. 1*b*). Separate experiments using independent primary microglia isolates showed a good correlation in expression levels for both GM-CSF and M-CSF treatments ($R = 0.85$, data not shown). In addition, many gene products were analyzed individually by flow cytometry or Western blotting to validate the transcript analysis (see Figs. 2–4 and 6). The high percentage of transcripts differentially expressed after treatment suggest that neonatal microglial cells undergo true lineage differentiation in response to M-CSF or GM-CSF, as opposed to simply a change in activation state.

Overall, the majority of genes substantially up-regulated in M-CSF-treated cells involved functions related to maintenance and regulation of brain homeostasis (Table I). These include genes involved in myelination growth and development, such as tenascin C (24-fold), ryudocan (21-fold), biglycan (9-fold), nerve growth factor (8-fold), and epidermal growth factor-like growth factor (36-fold) (26–29); in processing of neuropeptides, such as peptidylglycine-α-amidating monooxygenase (11-fold) and carboxypeptidase H (10-fold) (30, 31); in tissue remodeling, such as collagens type I, IV, V, VI, and XI (from 3- to 11-fold); or in the heat shock response (hsp), for example, hsp47 (11-fold) and hsp105 (4-fold; Table I). In contrast, many of the gene products up-regulated in GM-CSF-treated cells appear to be involved in cellular processes related to immune cell activation, Ag presentation, and innate immunity. These are discussed in detail.

mϕ and DC markers

Although no proteins are known to be expressed uniquely on mϕ or DCs, the pattern of expression of the integrins CD11b (Mac-1), CD11c, and the lectin-like receptor DEC-205 is considered characteristic, with expression of $CD11c^+$, $CD11b^{low}$, and $DEC\text{-}205^+$ associated with an immature DC phenotype, and a higher surface expression of $CD11b^{high}$ observed for mϕ. As previously reported (13), treatment of neonatal microglial cells with GM-CSF or M-CSF alters the expression of these markers toward a DC-like or mϕ-like phenotype, respectively. These results matched well with the levels of the corresponding transcripts from the microarray analysis (data not shown).

Low-affinity receptors for GM-CSF are present on untreated microglial cells and are slightly up-modulated by GM-CSF and down-modulated by M-CSF (Table II). Interestingly, the high-affinity GM-CSFR (common β-chain among IL-3, IL-5, and GM-CSFR) is absent on untreated cells, but is up-regulated >26-fold upon GM-CSF treatment.

Table II. *Cytokine and growth factor receptors*[a]

	Untreated	M-CSF	GM-CSF	gi No.
GM-CSFR low affinity	21.5	10.6	41.0	192594
GM-CSFR high affinity	–[b]	–	26.0	191821
M-CSFR	112.7	57.2	23.9	50980
IL-6 R	9.8	3.3	3.5	49725
IFN-γR	1.1	.6	1.3	194131
p75 TNFR	7.5	4.2	8.1	809043
p55 TNFR	11.1	6.0	8.2	54848
IL-10R	16.0	7.5	23.0	1305488
IL-4R secreted form	30.1	9.8	22.7	198365
IL-7R	–	–	7.2	198377
IL-11R	–	–	2.9	1916003
IL-17R	7.9	8.3	4.2	1161342
Lymphotoxin-βR	9.7	5.1	7.9	600222

[a] Data are reported as average differences between specific and control hybridization for each transcript ($\times 10^{-3}$); gi No. refers to gene bank accession number.
[b] Transcripts below the level of detection.

Cytokine and chemokine receptors

Consistent with their key role as sensors of CNS injury, neonatal microglia express mRNA encoding receptors for several proinflammatory cytokines including IL-6R, IFN-γR, and TNF-αR (Table II). Neither of the receptors appeared to be regulated by M-CSF or GM-CSF (Table II) with the exception of IL-6R, which is slightly down-regulated by both the hemopoietic factors.

Microglial cells also expressed receptors for anti-inflammatory cytokines, particularly the IL-10R and the secreted form of the IL-4R (Table II); both of which are slightly down-regulated by M-CSF but not GM-CSF.

The role of cytokine in the CNS goes beyond the regulation of microglia immune function. In this respect, cytokines like IL-7 and IL-11 have been shown to induce proliferation on cultured astroglia (32) as well as to induce glial differentiation (33).

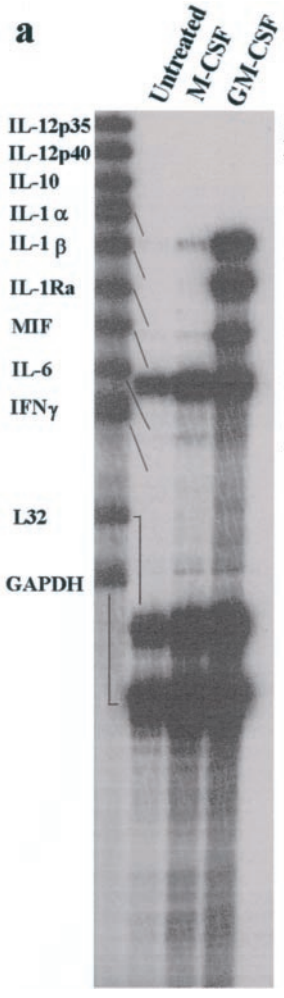
Overall, the cytokine receptors profile of microglial cells do not appear to be significantly changed by treatment with both hemopoietic factors, with exception of IL-7R and IL-11R, absent in untreated microglial cells, but up-regulated by GM-CSF (Table II).

Cytokines and chemokines

Under inflammatory degenerative and traumatic conditions in the brain, microglial cells are the resident primary source of both pro- and anti-inflammatory cytokines. Untreated microglial cells display transcripts for strongly immunosuppressive and/or proapoptotic cytokines like TGF-β1 and Tweak (Fig. 2, *b* and *c*), reflecting a strategy that appear to limit immune-mediated events within the CNS. Transcripts for TGF-β and Tweak were both decreased by M-CSF treatment. Tweak was also down-regulated by GM-CSF. Transcripts encoding for several chemokines were detected in untreated microglia, including mϕ inflammatory protein (MIP)-1α, MIP-1β, MIP-1γ, MIP-2, monocyte chemoattractant protein (MCP)-1, MCP-3, and MCP-5. Treatment with GM-CSF markedly increased transcripts for chemokines C10, MIP-1α, MIP-1γ, and MIP-2, while at the same time down-regulating MCP-5. C10, MIP-1γ, and MIP-2 were also up-regulated by M-CSF treatment, albeit to a lower degree. MCP-1 was up-regulated only by M-CSF. Both global transcript analysis (Fig. 2*b*) and RNase protection assay (Fig. 2*a*) demonstrated induction of the proinflammatory IL-1α, IL-1β, and the IL-1R antagonist upon treatment with GM-CSF but not M-CSF. Both hemopoietic factors up-regulate mRNA encoding for IFN-β (Fig. 2*b*).

Ag presentation

Microglial cells are the primary APCs resident in the brain. In the resting condition, they express very low levels of class II MHC protein and the class-II-associated invariant chain, but these can be up-regulated by treatment with the inflammatory cytokines such as IFN-γ. Previously, we have reported that GM-CSF skewing of microglial cells made them more inclined to up-regulate class II MHC expression in response to proinflammatory cytokines (13). Due to a mismatch between the MHC haplotype of the microglia cells ($H\text{-}2^s$) and the microarray target mRNA ($H\text{-}2^b$), MHC expression could not be monitored by the global gene analysis. Therefore, class II MHC levels were monitored by Western blotting. Class II MHC protein, which is almost undetectable in untreated cells, was increased to a barely detectable level after treatment with GM-CSF, but not M-CSF, although class II MHC surface staining was not increased (data not shown). As expected, IFN-γ was a much better inducer of class II MHC proteins (Fig.



b

	Untreated	M-CSF	GM-CSF	gi #
IL-1α	3.2	6.2	25.0	200756
IL-1β	-	2.0	2.9	198293
IL-1Ra	-	-	11.7	487864
IL-11	-	5.4	2.5	415653
IFN- β	-	17.0	8.0	51554
KC	-	-	2.9	201042
C10	-	57.5	86.6	192243
Mip 1α	5.4	6.3	18.1	201524
Mip 1β	5.1	8.0	9.7	53126
Mip 1γ	4.1	16.8	173.0	1234834
Mip 2	2.4	8.7	8.5	127100
MCP1	27.7	87.2	31.5	193486
MCP 3	7.5	7.2	5.3	437872
MCP 5	27.5	20.3	1.0	1477581
TGF-β1	22.2	4.2	23.0	3688423
Tweak	7.3	-	-	2707220

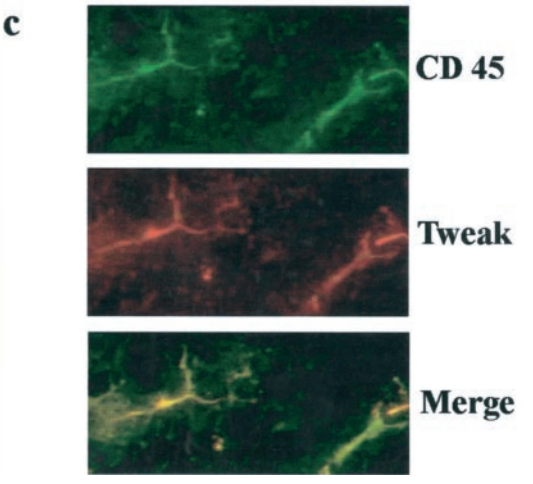


FIGURE 2. Cytokines and chemokines. *a*, RNase protection assay for several cytokines performed on untreated or GM-CSF- and M-CSF-skewed microglial cells. *b*, Global gene expression analysis. Data are reported as fluorescent units of average differences between specific hybridization and mismatch control hybridization ($\times 10^{-3}$); gi # refers to the gene bank accession number. *c*, Immunohistochemistry performed on brain section reveals the colocalization between the microglial cell marker CD45 and Tweak.

3*a*). Similar results were observed for the class II-associated invariant chain chaperone Ii, for which both alternately spliced p31 and p41 variants as well as partially processed p10 and p12 forms were observed after GM-CSF but not M-CSF treatment (Fig. 3*b*). This pattern for invariant chain was observed also by transcript analysis (Fig. 3*c*). Transcript levels for the class II MHC-associated peptide exchange factor H2-M were low but detectable in each of the samples, and were not changed by either treatment. No significant change in class I MHC protein was observed after treatment with either M-CSF or GM-CSF (data not shown).

Activation of T cells requires costimulatory interactions in addition to generation and recognition of MHC-peptide complexes. Several costimulatory receptors are present on microglial cells. As previously reported, neonatal microglia express CD80 (B7-1) and CD86 (B7-2; Fig. 4, *a* and *b*), which provide a CD28-dependent costimulatory signal (18). The expression of B7-1 was slightly up-regulated by GM-CSF, whereas B7-2 profile was not affected. The heat-stable Ag, CD24, has recently been added to the list of costimulatory molecules involved in T cell activation (34). CD24 play an important role in CNS immunity since its targeted mutation completely abrogated development of experimental allergic encephalitis (EAE), although its mutation does not prevent the induction of autoreactive T cells (35). In this study, we report that CD24 was dramatically increased (122-fold) in GM-CSF-treated cells (Fig. 4, *a* and *b*). CD24 up-regulation on microglial cells could represent a novel, CD28-independent, costimulatory signal for expansion of autoreactive T cells.

Altogether, GM-CSF-skewed cells were endowed with enhanced T cell stimulatory function, as assessed in an MLR assay (Fig. 3*d*).

Proteases

Cathepsins are a large family of cysteine and aspartic proteases implicated in endosomal protein degradation and generation of peptides for Ag processing. Using global gene expression analysis, differential expression of many of the cathepsins was found. Cathepsin F, L, and S, which are involved in invariant chain degradation and peptide loading, are differently distributed among APCs. Cathepsin S is present in B cells, DCs, and mϕ, whereas cathepsin F is more selectively expressed in tissue mϕ. Neonatal microglial cells have been reported to express cathepsin S and L, but not F (13), while resting microglial cells have been reported to express cathepsin E (36). Global gene expression analysis confirms these observations. Interestingly, both cathepsins L and F were up-regulated by GM-CSF (Fig. 5*a*), while cathepsin E was down-regulated (Fig. 5*a*). Neonatal microglial cells expressed cathepsin B, C, D, and S, with levels not significantly regulated by either GM-CSF or M-CSF (Fig. 5*a*).

Matrix metalloproteinases (MMP) are a family of proteases that have been implicated in processing of extracellular matrix during a variety of pathological conditions. In DC and Langerhans cells, MMPs enhance cell migration (37, 38). Treatment of microglia with GM-CSF strongly up-regulated expression of MMP-12 (mϕ metalloelastase), MMP-9, and MMP-11 (stromelysin-3; Fig. 5*b*),

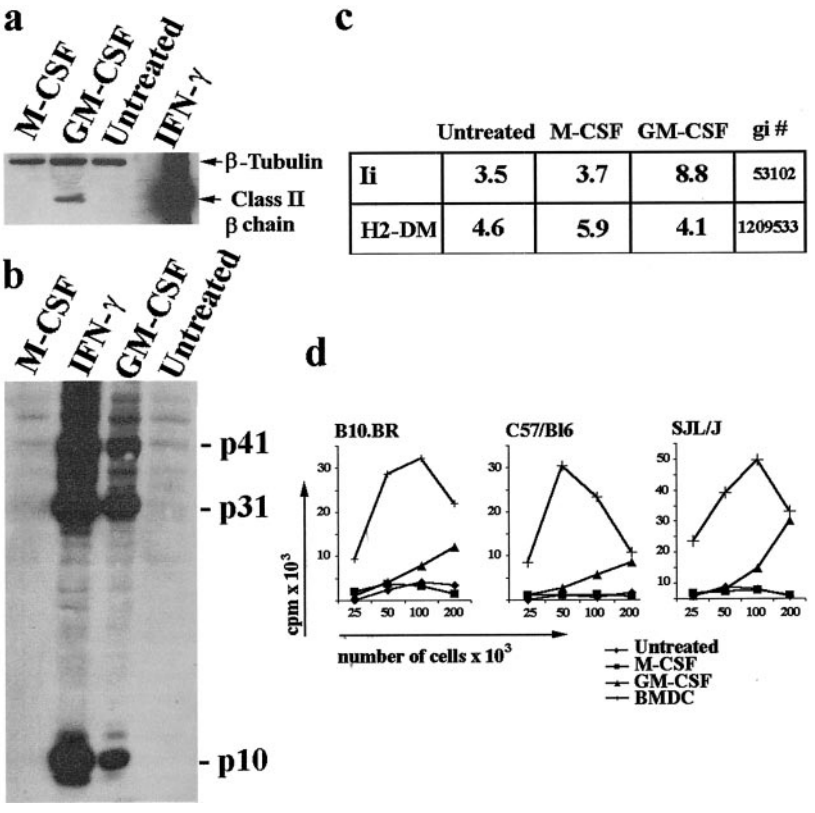


FIGURE 3. MHC class II proteins and invariant chain. *a*, Detection of MHC class II protein by Western blot analysis. The same amount of protein was loaded in each lane as visualized by the β-tubulin internal control. *b*, Detection of invariant chain protein by Western blot analysis. *c*, Global gene expression analysis. *d*, MLR performed using untreated or GM-CSF and M-CSF-treated microglial cells as APCs (from 25 to 200 $\times$ 10^3) and 2 $\times$ 10^5 splenic cells from B10.BR, C57BL/6, or SJL/J as effector cells. Bone marrow-derived DCs (from 25 to 200 $\times$ 10^3) were used as positive controls. A total of 1 μCi of [^{3}H]thymidine was added to the cultures after 3 days, and cells were harvested 24 h later.

whereas M-CSF selectively up-regulates MMP-9. These proteases are potent collagenase, and may play an important role in remodeling the brain microenvironment during disease processes as well as affecting cell trafficking into the brain (39, 40). It is also tempting to speculate that these proteases may mediate extracellular Ag processing. Notably, antigenic determinants derived from myelin basic protein have been reported to be presented in the CNS through a pathway that does not involve internalization and endosomal processing (41).

Antimicrobial response

Microglial cells respond to foreign pathogens by phagocytosis after receptor-mediated recognition of microbial Ags as well as producing several antimicrobial factors. Untreated microglial cells express mRNA encoding for several endocytic receptors, including Fcγ and complement receptors, the mϕ scavenger receptor, and CD14, a receptor for LPS (Fig. 6*a*). Among these, both FcγRI and RIII appeared to be down-modulated by the treatment with GM-CSF (Fig. 6*a*). MARCO is a receptor expressed on both mϕ and DC (F. Granucci and P. Ricciardi-Castagnoli, manuscript in preparation), which is important in DC for clearance of bacteria. MARCO transcripts are expressed in untreated microglial cells and up-regulated >15-fold in GM-CSF, but not in M-CSF-treated cells (Fig. 6*a*). Surface staining with an anti-MARCO Ab confirmed the up-regulation observed by global transcript analysis (Fig. 6*b*).

Microglia can produce antimicrobial peptides, which act as endogenous antibiotics, and are important contributors to regional innate immunity. Untreated microglial cells express mRNA encoding for natural resistance-associated mϕ protein 1 and 2, as well as Cryptidin and cathelin-like peptides. The majority of these antimicrobial peptides are down-regulated by GM-CSF treatment, but unaffected by M-CSF.

Discussion

The neonatal microglial cell phenotype closely resembles that of circulating monocytes (2, 11–13), and can be skewed to a more mϕ-like or DC-like phenotype by M-CSF or GM-CSF, respectively (13). Using global gene expression analysis, we now show that upon treatment of microglial cells with either GM-CSF or M-CSF, a large proportion of expressed genes is up-regulated or down-regulated >2.5-fold (>16%). Such a large-scale shift in gene expression indicates that neonatal microglial cells undergo a marked transformation by either GM-CSF or M-CSF, and is further evidence that neonatal cells exist in a relatively undifferentiated state. It has been previously shown that in T cells activated by a specific super Ag, the number of genes differentially expressed is ~4.4% at best (42). Also, in the DC response to different pathogens, the number of regulated genes is always <10% (43). Thus, it is likely that microglial cell skewing with either GM-CSF or M-CSF induces a true lineage differentiation as opposed to a different state of activation.

Although skewing of microglia toward a mϕ-like phenotype occurs following exposure to M-CSF within the CNS, both growth factors are needed for full maturation of other tissue mϕ. M-CSF$^{-/-}$ mice are largely devoid of alveolar mϕ and bone osteoclasts (although interestingly, they do retain near normal CNS microglial populations; Refs. 44 and 45). In contrast, alveolar mϕs from GM-CSF$^{-/-}$ animals are normal in number but exhibit deficits in function. GM-CSF$^{-/-}$ alveolar mϕ are unable to effectively degrade surfactant and are defective in their ability to clear pathogens (46).

The biological activity of GM-CSF is initiated by its binding to a specific receptor formed by an α and β subunit. The α subunit binds GM-CSF with low affinity (47). The β subunit does not bind GM-CSF alone, but in combination with the α subunit will form a

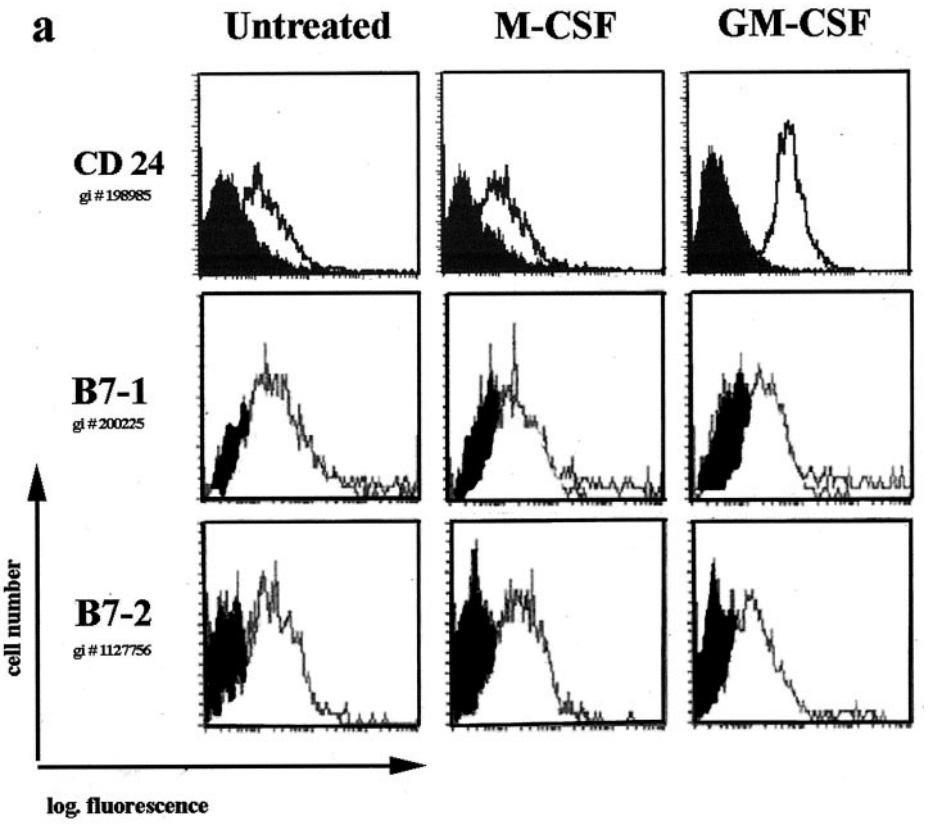


FIGURE 4. Proteins involved in T cell stimulation. *a*, Surface expression of CD24 (mAb 30-F1), B7-1 (mAb 16-10A1), and B7-2 (mAbGL1) analyzed by flow cytometry. *b*, Global gene expression analysis.

b

	Untreated	M-CSF	GM-CSF
CD 24 gi # 198985	2.5	41.3	122.5
B7-1 gi # 200225	38.2	20.0	20.0
B7-2 gi # 1127756	2.4	1.7	1.9

high-affinity GM-CSFR (48). The interplay between the two GM-CSFRs currently is incompletely understood, although it is thought that in humans both receptors mediate myeloid lineage differentiation, but only the high-affinity one mediates cell growth (49). The low-affinity GM-CSFR was present on untreated microglial cells and was down-regulated by M-CSF, consistent with a skewing toward a mϕ phenotype. Interestingly, the high-affinity receptor appeared only after GM-CSF treatment, again consistent with a differentiation away from the resting phenotype. Overall, the majority of transcripts regulated by M-CSF were involved in lipid, carbohydrate, or protein metabolism (Table I). Of particular interest was the up-regulation of a series of proteoglycans involved in tissue remodeling, as well as molecules important in glial-neuron interaction. In general, it appeared that M-CSF regulates functions already evident in untreated cells, and related to the role of microglial cells in brain homeostasis under physiological conditions. This is in accord with the fact that M-CSF is constitutively present in the brain parenchyma.

a

	Untreated	M-CSF	GM-CSF	gi #
Cathepsin B	212.6	165.8	204.4	309151
Cathepsin C	66.1	45.0	60.5	2145015
Cathepsin D	194.3	180.5	216.6	50302
Cathepsin E	6.2	5.3	-	3392946
Cathepsin F	-	-	4.3	4826564
Cathepsin H	8.2	2.2	3.2	454100
Cathepsin L	23.6	31.5	95.8	53046
Cathepsin S	154.0	67.3	182.5	4138249
Cathepsin Z	27.6	19.9	38.8	5019547

b

	Untreated	M-CSF	GM-CSF	gi #
Mϕ Metalloelastase	-	-	51.2	199127
Metalloproteinase 9	-	14.2	4.9	433434
Stromelysin 3	-	-	33.0	6678893

FIGURE 5. Cathepsins and metalloprotease. Global gene expression analysis.

GM-CSF up-regulated a large number of transcripts strongly implicated in CNS immunity. Interestingly, both neonatal and adult microglial cells require a multistep activation process to reach full competency as APCs, wherein a preactivation with GM-CSF is followed by a secondary activation with a proinflammatory cytokine (19) or CD40 ligand (18). Under physiological conditions in the adult brain, the only source of GM-CSF is activated T cells,

a

	Untreated	M-CSF	GM-CSF	
Fc γ R I	22.2	10.7	8.6	200752
Fc γ R III	3.4	-	-	1835127
Mannose R	13.4	8.9	12.3	52997
CR 1	31.8	16.8	14.6	2130534
CR 3	38.8	20.4	55.1	198434
CD14	172.3	145.9	146.4	50338
Ficolin-A	-	8.5	-	2957011
N-Formylpeptide R	1.1	1.6	3.8	3549279
PIRA 1	-	-	1.3	2138352
PIRA 3	2.3	-	19.1	2138356
Scavenger Receptor Type I	4.8	3.6	3.5	1167551
Scavenger Receptor Type II	36.0	34.3	14.7	192739
Protein C Receptor	2.9	6.7	3.0	728480
MARCO	1.3	-	16.2	682722
Cryptidin 6	4.1	4.5	1.9	2182214
Cathelin-like	5.7	4.9	-	1177533
Natural resistence-associated macrophage protein 2	15.8	19.3	14.9	755040
Natural resistence-associated macrophage protein 1	45.8	19.4	5.9	474903
Arginase	1.2	49.8	113.0	1293092

b

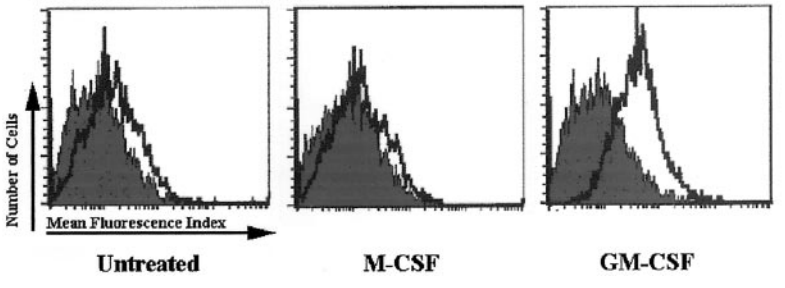


FIGURE 6. Antimicrobial receptors and peptides. *a*, Global gene expression analysis. *b*, Surface expression of MARCO (mAb ED31) analyzed by flow cytometry.

which survey the brain parenchyma (22, 23). The amount of GM-CSF released by these cells could be sufficient to induce a preinflammatory state or precompetency of local microglial cells. If this is followed by a sufficient number of infiltrating T cells, which either specifically or by cross-reaction recognize a cognate ligand, an autoimmune process could ensue. In contrast, if no productive interaction is generated, the activated T cells will leave the brain without inciting inflammation (23). The interaction between infiltrating T cells and microglia has been studied in a model of host vs graft disease, without interference from infiltrating mϕ/monocytes. In this model, when a sufficient number of T cells crossed the blood brain barrier, the cytokine milieu produced was sufficient to activate resident microglial cells (50).

Costimulatory molecules play a fundamental role in the interaction between T cells and APCs. CD24, expressed on APC, provides a CD28-independent costimulation by interacting with either p-selectin, or in a homotypic interaction, with another CD24 molecule expressed on T cells (51). The functional significance of this newly described costimulatory pathway is as yet not understood, although it appears that CD24, different from members of the B7 family, is not required for the induction phase of an immune response (52). In the animal model of EAE, it was shown that targeted mutation of CD24 does not affect the priming and proliferation of self-reactive T cells; however, it does effect EAE development (35). Also, passive transfer of myelin oligodendrocyte glycoprotein 35-55-specific $CD24^+$ T cells in $CD24^{-/-}$ mice is not sufficient for EAE induction, because a still not well understood homotypic CD24 interaction must occur for extravasation of self-reactive T cells into the CNS (35). Because CD24 expression on microglial cells is dramatically up-regulated by GM-CSF, it seems likely that upon entry of activated GM-CSF-secreting T cells into the brain, CD24 would be readily up-regulated on microglial cells, which in turn would acquire higher costimulatory capacity to sustain T cell expansion.

Chemokines are small chemoattractant molecules, which regulate cell trafficking and homing in every organ. Microglial cells produce a basal level of several chemokines essential for regulating T cell infiltration and patrolling of the brain parenchyma. GM-CSF, much more than M-CSF, up-regulated the transcripts for several of these chemokines, further establishing its role as priming factor for brain immunity.

Microglial cells play a pivotal role in protein degradation and tissue remodeling, which occurs during degenerative, traumatic, and inflammatory CNS conditions. A series of aspartic proteases, cysteine proteases, and metalloproteinases are involved in this process. MMP-9, MMP-11, and MMP-12 are strongly up-regulated with GM-CSF and may participate in tissue remodeling and microglial cell migration. Cathepsin S, L, and F are involved in Ag processing and invariant chain degradation (53–55), both important for the Ag-presenting role of microglial cells. Both cathepsin F and L were up-regulated by GM-CSF. However, neither GM-CSF nor M-CSF altered the expression of cathepsin S, which was previously shown to degrade myelin basic protein at both neutral

and acidic pH, and to be up-regulated by neurotrophic factors in microglial cells (56). Other proteins important for Ag processing and presentation also were up-regulated by GM-CSF, including both class II MHC protein and its associated chaperone invariant chain. Although up-regulation of class II MHC proteins by GM-CSF does not result in a large increase in surface expression, the small degree of up-regulation that was observed was sufficient to promote much stronger T cell proliferation in an MLR assay.

Several substances that are produced in the normal brain account for the maintenance of an immunosuppressive environment. Normal neuronal activity and secreted neurotrophin inhibit the microglia expression of MHC class II and costimulatory molecules, and this tends to counteract the activation of microglia and astrocytes by IFN-γ and LPS (7, 8). As observed in this study, microglial cells also produce TGF-β and Tweak, immunosuppressive cytokines that have been shown to be strongly apoptotic toward activated T cells. Consistent with this observation, activated T cells entering the brain parenchyma are known to be more likely to undergo apoptosis than to proliferate. Consistent with its role as preactivator of microglial cells, GM-CSF decreases expression of Tweak, and up-regulates proinflammatory cytokines like IL-1α and β along with several chemokines that can promote leukocyte migration into the brain.

In summary, the global gene expression analysis reported in this study establishes the molecular basis for the previously recognized role of GM-CSF in promoting microglial participation in adaptive immune responses. GM-CSF treatment leads to up-regulation of molecules involved in T cell costimulation (CD24), chemotaxis (C10, MIP-1α, MIP-1γ), Ag processing class II MHC, invariant chain, cathepsin F and L), innate immunity (MARCO), and to down-regulation of apoptosis-inducing factors (Tweak, TGF-β), which together constitute a differentiation process that prime microglia cells for Ag presentation.

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