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Gene expression in IFN-γ-activated murine macrophages

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Abstract

Macrophages are critical for natural immunity and play a central role in specific acquired immunity. The IFN-γ activation of macrophages derived from A/J or BALB/c mice yielded two different patterns of antiviral state in murine hepatitis virus 3 infection, which were related to a down-regulation of the main virus receptor. Using cDNA hybridization to evaluate mRNA accumulation in the cells, we were able to identify several genes that are differently up- or down-regulated by IFN-γ in A/J (267 and 266 genes, respectively, up- and down-regulated) or BALB/c (297 and 58 genes, respectively, up- and down-regulated) mouse macrophages. Macrophages from mice with different genetic backgrounds behave differently at the molecular level and comparison of the patterns of non-activated and IFN-γ-activated A/J or BALB/c mouse macrophages revealed, for instance, an up-regulation and a down-regulation of genes coding for biological functions such as enzymatic reactions, nucleic acid synthesis and transport, protein synthesis, transport and metabolism, cytoskeleton arrangement and extracellular matrix, phagocytosis, resistance and susceptibility to infection and tumors, inflammation, and cell differentiation or activation. The present data are reported in order to facilitate future correlation of proteomic/transcriptomic findings as well as of results obtained from a classical approach for the understanding of biological phenomena. The possible implication of the role of some of the gene products relevant to macrophage biology can now be further scrutinized. In this respect, a down-regulation of the main murine hepatitis virus 3 receptor gene was detected only in IFN-γ-activated macrophages of resistant mice.

Key words
- Gene array
- mRNA
- Mouse hepatitis virus 3 (MHV3)
- IFN-γ
- BALB/c mouse
- A/J mouse
- Macrophages

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Introduction

The mononuclear phagocyte system constitutes the second major cell population of the immune system, with varied morphologic forms and functions in most of the tissues of the organism. Upon stimulation, these cells undergo striking physiological changes playing important active roles.

Knowledge of macrophage functions depends to a large extent on the identification of gene products exerting specific functions under given conditions and may constitute a decisive step towards the understanding of both innate and specific mechanisms of immunity. Until recently most of our knowledge about the molecules expressed by various cell types was based on the study of their

presence on the membranes of the cells under investigation. This was convenient, since various techniques permitted raising antibodies against the surface molecules, and the worldwide effort of identifying them led to the powerful molecular definitions of the "clusters of differentiation". Now that we possess various cDNA libraries, as well as assays derived from them, we have on hand a useful tool to probe the expression profile of transcribed molecules. By clusters of differentiation-molecular definition, important structural entities on the cell membranes have been identified, and it is expected that expression profiling will permit us to focus on intracellular compartments and reveal meaningful regulatory polypeptides.

A/J mice have been described to be resistant to experimental infection with mouse hepatitis virus 3 (MHV3), developing a mild disease that disappears after a few days. On the other hand, BALB/c mice develop acute hepatitis after infection and die some days later (1). Attempts to identify the mechanisms involved in the resistance or susceptibility of mouse strains to MHV3 infection have led different groups to demonstrate the involvement of virus replication in macrophages (2-4), the antiviral state induced by IFN-γ only in macrophages from resistant animals (1), and the expression of a monokine with procoagulant activity (5,6).

Studying the molecular basis of the virus resistance induced by IFN-γ in macrophages, we have recently shown that down-regulation of a viral receptor gene with consequences on the gene product synthesis may be implicated in the resistance shown by A/J mice (7). In a previous study, by means of proteomic analysis of proteins extracted from A/J or BALB/c macrophages, we were able to tag several gene products that were synthesized at elevated or diminished levels (8), indicating that macrophages from resistant and susceptible strains behave differently at the molecular level upon IFN-γ activation.

The gene profiling technology adopted for the present study (9-11) using an array constructed from 1536 individual cDNA clones allowed us to study the mentioned defined portion of expressed genes. The choice of the library is of high relevance to the problem under study since in our library there are mainly immunologically relevant gene probes originating from an immunologically active organ, i.e., the fetal thymus. Not only can changes be detected at the quantitative and qualitative level of gene expression, but it is also possible to identify them by recognizing their protein product since the expressed sequence tags have been established for the individual entities of the cDNA library used for the evaluation of a given mRNA sample by hybridization, and protein products have been analyzed on 2D-SDS gels (10).

By using gene expression profiling to evaluate the mRNA content of IFN-γ-activated macrophages we attempted to provide detailed information about up- or down-regulated gene products to be in turn linked to the modulation of biological macrophage functions. As an example using a classical approach, we previously identified the regulation of the main MHV3 receptor gene expression in IFN-γ-activated macrophages as a central feature of resistance (7). The gene expression profiling shown here confirmed these previous data. Several new regulatory molecules can be considered for further scrutiny.

Material and Methods

Macrophage cultures

Bone marrow-derived macrophages from A/J and BALB/c mice were prepared as previously described (12) from bone marrow cells collected from femurs of 6- to 8-week-old A/J and BALB/c mice from the mouse colony of the Max-Planck Institute for Immunobiology, Freiburg, Germany, in gas-permeable Teflon bags. Cells were de-

tached by repeated careful stretching of the bags, washed once with medium and used in the experiments. They were cultivated in Dulbecco's modified Eagle's medium containing 10% FCS at a concentration of 10^5 cells/well in 96-well plates.

MHV3 replication and receptor expression

Experiments were carried out in order to evaluate in the macrophages the induction of anti-MHV3 state and the expression of virus receptors mediated by exogenous IFN-γ. BALB/c or A/J mouse bone marrow-derived macrophage cultures were activated for 18 h with 50 U/ml of IFN-γ (Genentech Inc., South San Francisco, CA, USA) and for the virus replication assay they were infected with MHV3 at multiplicity of infection of 0.1. Cell supernatants were then collected at different times and virus titers determined by a plaque assay (13). The data, reported as plaque-forming units per milliliter, were measured in triplicate cultures. For virus receptor expression, total cellular RNA was extracted from IFN-γ-activated macrophage cultures and reverse transcribed and the cDNAs were submitted to the polymerase chain reaction containing specific primers. Samples were then submitted to agarose gel electrophoresis for visualization (7).

RNA extraction from macrophages and reverse transcription

Total cellular RNA was extracted from macrophage cultures (10^7 cells/5 ml) on 6-cm diameter Petriperm dishes (Sartorius, Goettingen, Germany) treated or not for 18 h with 50 U/ml of IFN-γ by the isothiocyanate method (Trizol reagent; Invitrogen GmbH, Karlsruhe, Germany). Briefly, 20 ml Trizol was added to the frozen pellets and solubilization was achieved by passing the lysate through the pipette. After addition of chloroform, vigorous shaking, and standing on ice for 5 min, the suspension was centrifuged at 12,000 *g* for 15 min. The upper aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. The RNA precipitate was spun at 12,000 *g* for 15 min and the pellet was washed with 75% alcohol and solubilized in water. Reverse transcription was performed using reverse transcriptase SuperScript II (Invitrogen GmbH) and applying non-radioactive dNTP components.

Analysis of gene expression

A fetal thymus library was chosen for this study because: a) the mRNA population of the fetal thymus encompasses a much wider spectrum of expressed entities than any other source of immunocompetent cells, and, b) the availability of collections of proteomic data base entries concerning the "translability" of the gene products into protein molecules. So, a murine fetal thymus cDNA library was prepared from fetal thymuses of BALB/c mice using cytoplasmic RNA samples, which were reversely transcribed into cDNAs. The resulting preparations were amplified using cDNA packaging into infectious phage particles and, upon subsequent infection with *Escherichia coli* (LE 392/P2), plaques were collected. The original lambda ecc phage library was transformed into a plasmid-based library in order to be manageable by a robot. The array was configured as a stack of sixteen 96-well microplates providing a total of 1536 clonal positions. The geometry of the stack of sixteen microplates was carefully chosen so that it would form a 3-D ordered library, which was sampled for proteomic analysis on three coordinated axes. A clonal address is provided as a six-digit number leading to identification of the physical localization of the clone. Since this library was not normalized it contains several redundant clones. Nucleotide sequences from all the clones were established and are available to the scientific community upon request (9-11).

In order to identify modified gene expression in IFN-γ-activated macrophages the cDNA was hybridized to the arrayed DNA, and the relative hybridization intensity was established by comparing the intensity of relevant spot pairs using chemoluminescence as a readout. The procedure was carried out by labeling the probe with horseradish peroxidase and cross-linking with glutaraldehyde, followed by the light emitting reaction of luminol, which produces blue light upon oxidation. Relative hybridization intensity was established by comparison to the intensity of control spots.

Nylon sheets with 1536 clones in duplicate were prepared as already described (10). Briefly, the robot first merged four 96-well microtiter plates into a single 386-well plate, and each of the 386-well plates was spotted on the nylon sheet in duplicate. This was done with all original microtiter plates. For good visual orientation the duplicates were spaced with diagonal, horizontal and vertical orientation. The probe quantitation was performed via an image analysis system originally implemented for analysis of protein spots. The image analysis system is called Kepler (version 8.0; Large Scale Biology Corporation, Rockville, MD, USA), developed at Argonne National Laboratory, and the spot intensity was modeled as a 3-D Gaussian curve. No spot normalization was performed within one hybridization sheet. Differences in intensity within the duplicates were attributed either to experimental variations or to robotic deposition of the spots on the nylon sheet. The nylon sheets were used to expose X-ray films (30 s to 6 min) and those showing comparable cytochrome c spot intensity (cytochrome c control spots served as a measure of chemiluminescence homogeneity) were subjected to image analysis (Kepler). After signal quantification, the data were processed for calculation of arithmetic means. Only those expression changes based on reliable modeling were considered. A cut-off limit at 300 units (since artifacts were present in low intensity spot modeling) and minimal ratios of 0.5 and 2.0 for up- and down-regulation of genes, respectively, were applied. As a frame reference we used the expression of the elongation factor α gene, which is present in six copies in our ordered library. Each data set refers to a clonal position for which in most instances the molecular identity of the cDNA is known. A/J and BALB/c samples were hybridized in parallel separately on two different nylon filters, and the repetition was performed upon stripping on the other filter (cross-wise). Gene expression analysis was repeated several times, showing low experimental variation.

Some genes identified as being differentially expressed in IFN-γ-activated A/J and BALB/c mouse macrophages were submitted to database queries in order to investigate their possible involvement in macrophage regulatory processes. This topic is addressed in the discussion of the biological functions of selected genes.

Results

The approaches used to elucidate the cellular and molecular basis of resistance against MHV3 infection are indicated in Table 1. A/J mice were shown to be resistant and BALB/c mice were shown to be susceptible to experimental infection with MHV3 (1). IFN-γ activation of macrophage cultures from A/J mice led to a partial restriction of MHV3 growth, in contrast to the BALB/c macrophage activation (1). Our control experiments showed at least 10 times lower virus titers in supernatants of IFN-γ-activated A/J macrophages than in the other cultures (data not shown). Studies on the expression of genes coding for virus receptors and on virus binding to membrane proteins from IFN-γ-activated macrophages showed down-regulation of virus receptor expression only in macrophages from the resistant A/J mice (7). Thus, the *in vitro* ability of activated A/J

macrophages to restrict MHV3 growth correlated with the diminished virus binding and virus receptor gene expression, leading us to suggest that these experimental observations reflect the contribution to the *in vivo* resistance expressed by these mice following experimental virus infection (7).

Proteomic studies including computer-aided image comparison of gels obtained from 2-D SDS-PAGE of extracted proteins from IFN-γ-activated A/J and BALB/c macrophages revealed the up- and down-regulation of several gene products (Table 1). By using a similar approach, now focused on mRNA expression, in the present paper we report gene expression profiling data showing that several up- and down-regulated genes were detected in IFN-γ-activated macrophages from A/J and BALB/c mice (Table 1).

In Table 1 we also present the DNA hybridization data. The data were selected by arbitrarily applying a cut-off limit at 300 units and minimal ratios of 0.5 and 2.0 for up- and down-regulation of genes, respectively. In this way we observed 266 and 58 down-regulated genes (including 175 and 50 signals only detectable in controls) in IFN-γ-activated macrophages from A/J and BALB/c mice, respectively. On the other hand, IFN-γ activation of A/J and BALB/c macrophages led to up-regulation of 267 and 297 genes (including 174 and 96 signals only detectable upon activation), respectively. Selected up- or down-regulated genes, listed in Tables 2 and 3, respectively, may serve as a basis for evaluating physiological changes promoted by IFN-γ in macrophages. Table 4 presents a list of 42 differentially expressed genes and their function in macrophage biology, as indicated by recent publications (7,14-55). One can observe modulation of genes coding for proteins involved in enzymatic reactions such as adenosine monophosphate deaminase, pyruvate kinase, acetoacetyl-CoA synthetase, TNF-α converting enzyme, serine protease inhibitor, or involved in the control of nucleic acid synthesis and transport such as DNA ligase, RNA polymerase II, zinc finger protein or protein synthesis, transport

Table 1. Cellular and molecular basis of resistance against MHV3 obtained by biological assays and expression profiling.

Mouse strain	*In vivo* viral infection	Biological assays			Proteomic analysis		Gene expression profile		
		Virus replication	Virus receptor		Cell lysate (2-D gel spots) (8)		mRNA (hybridization spots)		
					Up-regulation	Down-regulation	C/IFN-γ ratio	Profile	Number of spots
A/J	Resistant	C	+++	+++			∞	Down-regulated	175
							>2 (7.13 to 2.02)	Down-regulated	91
		IFN-γ	+	–	4	26	0	Up-regulated	174
							<0.5 (0.45 to 0.139)	Up-regulated	93
BALB/c	Susceptible	C	++++	++++			∞	Down-regulated	50
							>2 (4.61 to 2.01)	Down-regulated	8
		IFN-γ	++++	++++	13	16	0	Up-regulated	96
							<0.5 (0.49 to 0.108)	Up-regulated	20

Data for gene expression profiling in macrophages from A/J or BALB/c mice activated or not with IFN-γ *in vitro* (control, C) and IFN-γ-activated macrophage cultures (IFN-γ). For the gene expression profile, extracted RNA samples were copied by reverse transcription, and the relative hybridization intensity was established by comparing the intensity of relevant spot pairs by chemoluminescence. An arbitrary limit of minimal intensity of 300 units and of a minimal ratio of up- and down-regulation of 0.5 and 2, respectively, was utilized. Using these criteria, we identified hybridization spots that, upon IFN-γ activation, decreased (C/IFN-γ >2) or increased (C/IFN-γ <0.5) in intensity, as well as spots below the thresholds 0.5 and 2.0 in control macrophages (C/IFN-γ = 0) or in IFN-γ-activated macrophages (C/IFN-γ = ∞). The assays are described in the Material and Methods section and in detail in the original publications (1,7,8). MHV3 = murine hepatitis virus 3.

Table 2. Selected genes up- or down-regulated by IFN-γ in macrophages from A/J mice.

Up-regulated selected genes

ca	A/J C	A/J IFN-γ	C/IFN-γ	Accession	Gene
60914	0	5737	0	MMUGTN:023998-30	high-glucose-regulated protein 8
10602	0	3685	0	U50078	guanine nucleotide exchange factor p53
51210	0	3421	0	MMUGTN:037962-2	exportin (nuclear export receptor for tRNAs)
31216	0	3212	0	EM_HUM9:HSRPII140	RNA polymerase II 140-kDa subunit
60804	0	2936	0	NM_013843	zinc finger protein
80111	0	2768	0	EM_MUS:MMGPIP137	gpi-anchored protein
51115	0	2490	0	EM_MUS:MMCOF	cofilin
60905	0	2075	0	M92933	lymphocyte common antigen (Ly5)
81211	0	1879	0	EM_MUS:BC003861	hydroxymethylbilane synthase
61202	0	1813	0	MMUGTN:056337-12	platelet-activating factor acetylhydrolase (PAF-AH)
10901	0	1758	0	U87240	lysosomal alpha-mannosidase
51102	0	1293	0	M32599	glyceraldehyde-3-phosphate dehydrogenase
10913	0	1279	0	EM_MUS:MMNPTCC	nuclear pore-targeting complex
21003	0	1040	0	X14194	entactin
21112	0	1016	0	AF205079	palladin, actin-associated protein
41216	0	943	0	EM_MUS:AF322193	cleavage and polyadenylation specificity factor 1 (CPSF1)
10604	0	873	0	X53416	filamin, actin-binding protein
11214	0	774	0	EM_MUS:BC006945	DNA polymerase α 2
60214	0	749	0	EM_MUS:BC007133	apoptosis inhibitory protein 5
10810	0	712	0	X84014	laminin-5, α 3B chain
71215	0	619	0	EM_MUS:MM16741	capping protein α 2 subunit
11016	0	492	0	EM_MUS:AB021709	tumor necrosis factor (TNF) α converting enzyme
61204	0	309	0	X57024	glutamate dehydrogenase
70514	2841	20439	0.139	EM_MUS:AF323958	prostaglandin transporter (PGT)
60811	706	4836	0.146	EM_MUS:MMBCATA	beta-catenin
10902	531	2829	0.188	MMUGTN:194525-2	serine protease inhibitor
51009	2671	9851	0.271	EM_MUS:AY004877	cytoplasmic dynein heavy chain
60712	978	3430	0.285	J05503	carbamoyl-phosphate synthetase
81208	1316	3951	0.33	EM_MUS:AF210433	glucocorticoid modulatory element binding protein 1 (GMEB-1)
10313	3196	9419	0.34	BC002270	ubiquitin-conjugating enzyme E2 variant 1
71010	5778	13697	0.42	EM_MUS:AF127033	fatty acid synthase
51203	546	1260	0.43	U20780	ubiquitinating enzyme
21004	932	2130	0.44	M64098	high density lipoprotein binding protein (HBP)
10511	2033	4491	0.45	AF143956	coronin-2
70716	18243	40359	0.45	EM_MUS:AB024538	immunoglobulin superfamily containing leucine-rich repeat (ISLRI)
10312	1270	2756	0.46	D16250	bone morphogenetic protein (BMP) receptor
71014	1701	3690	0.46	EM_MUS:MM19604	DNA ligase I
71005	1446	3033	0.48	AF111102	major histocompatibility complex (MHC) class I region
61215	11446	23581	0.49	EM_MUS:MMADAPA1	α-adaptin

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Table 2 continued

Down-regulated selected genes

ca	A/J C	A/J IFN-γ	C/IFN-γ	Accession	Gene
70303	4558	0	INF	MMUGTN:031192-14	protein O-mannosyltransferase 1
50308	4462	0	INF	MMUGTN:036418-4	nucleolar RNA-associated protein
40713	2314	0	INF	EM_MUS:BC011412	splicing factor 3b
80708	2000	0	INF	EM_HUM4:BC007711	adenosine monophosphate deaminase 2
80405	1860	0	INF	M83196	microtubule-associated protein
40909	1565	0	INF	EM_MUS:MMTENASC	tenascin
40308	1443	0	INF	EM_MUS:BC006722	heat shock protein 70 (HSP70)
40303	1406	0	INF	M77196	mouse hepatitis virus receptor
81101	1397	0	INF	Y16414	exportin (tRNA)
51113	1261	0	INF	EM_HUM9:HSU50078	guanine nucleotide exchange factor
40304	1073	0	INF	Z12173	glucosamine-6-sulfatase
80411	1053	0	INF	EM_MUS:AB042528	helicase protein-like 4
60512	894	0	INF	X17124	virus-like (VL30) retrotransposon
60504	873	0	INF	U94479	integrin binding protein kinase
40614	868	0	INF	EM_MUS:BC015304	S-adenosylhomocysteine hydrolase
60509	802	0	INF	EM_MUS:AF374267	sterol regulatory element binding protein 2 (SREBP2)
70814	741	0	INF	EM_MUS:MMARPPO	acidic ribosomal phosphoprotein
60713	709	0	INF	EM_MUS:AB047820	ubiquitin C-terminal hydrolase
70808	673	0	INF	EM_MUS:AF039840	mitogen-activated protein kinase
50704	481	0	INF	Z12173	glucosamine-6-sulfatase
70205	317	0	INF	MMUGTN:044079-4	tyrosine phosphatase
30703	304	0	INF	X80754	guanosine triphosphate (GTP)-binding protein
30712	9804	2197	4.46	U42327	vascular cell adhesion molecule (VCAM-1)
40815	5299	1249	4.24	EM_MUS:BC005549	system acquired resistance (SAR1) protein (angiotensin II)
30708	10109	3006	3.36	EM_MUS:BC016619	pyruvate kinase 3
70508	3115	952	3.27	EM_MUS:MMRECEP	mouse receptor for advanced glycosylation end products (RAGE)
70312	6612	2029	3.26	MMUGTN:001687-2	topoisomerase (DNA) II binding protein
50612	5309	1697	3.13	AB017105	DNA helicase Q1
40509	6342	2099	3.02	EM_MUS:BC004745	tubulin α 6
30410	3294	1187	2.78	EM_MUS:AF033276	kinase anchor protein (AKAP-KL)
30608	5318	1937	2.75	J04487	aspartyl-tRNA synthetase α 2
60609	7437	2793	2.66	EM_MUS:MMSTYKIN	serine threonine tyrosine kinase (STY)
60513	4304	1835	2.35	EM_MUS:MMTALINR	talin
60312	8423	3992	2.11	AB026291	acetoacetyl-CoA synthetase
80109	33557	16639	2.02	EM_MUS:MMMAGPA	microfibril-associated glycoprotein (MAGP)

The data, organized in decreasing order of hybridization spot intensity, show the clonal address (ca) of each spot, the intensity units of control non-activated (A/J C) and IFN-γ activated (A/J IFN-γ) macrophage cultures, the respective intensity ratios (C/IFN-γ), the gene accession code (Accession) and the gene product description (Gene).

Table 3. Selected genes up- or down-regulated by IFN-γ in macrophages from BALB/c mice.

Up-regulated selected genes

ca	BALB/c C	BALB/c IFN-γ	C/IFN-γ	Accession	Gene
71010	0	15880	0	EM_MUS:AF127033	fatty acid synthase
51213	0	12732	0	EM_RO:RNFIBRON	fibronectin
60914	0	10402	0	MMUGTN:023998-30	high-glucose-regulated protein 8
60309	0	9452	0	MMUGTN:078718-20	dynactin 4
10312	0	8197	0	D16250	bone morphogenetic protein (BMP) receptor
30913	0	8067	0	EM_MUS:MMPGK1PS1	phosphoglycerate kinase (PGK1-ps1)
71005	0	6849	0	AF111102	major histocompatibility complex (MHC) class I region
10204	0	6600	0	AB021709	tumor necrosis factor (TNF) α converting enzyme
61111	0	6385	0	EM_MUS:BC003329	makorin, ring finger protein 1
80114	0	6322	0	EM_HUM1:AB071698	Np95-like ring finger protein
61014	0	6199	0	EM_MUS:AF210433	glucocorticoid modulatory element binding protein 1 (GMEB-1)
51012	0	5831	0	L07918	GDP-dissociation inhibitor
40509	0	5704	0	EM_MUS:BC004745	tubulin α 6
10411	0	5119	0	EM_MUS:AF322193	cleavage and polyadenylation specificity factor 1 (CPSF1)
40903	0	4969	0	M64085	spi2 proteinase inhibitor (spi2/eb1)
20502	0	4935	0	MMUGTN:033090-1	katanin p60
50405	0	4885	0	EM_MUS:AF230878	transcriptional co-repressor tif1 ß
50710	0	4688	0	EM_MUS:AF035117	rasgap-associated protein p56dok-2 (p56dok-2)
10602	0	4479	0	U50078	guanine nucleotide exchange factor p53
81008	0	4372	0	EM_MUS:MMCYCM	cyclophilin
51115	0	4292	0	EM_MUS:MMCOF	cofilin
51210	0	4242	0	MMUGTN:037962-2	exportin (nuclear export receptor for tRNAs)
20116	0	4110	0	EM_HUM8:HSLTGFBP4	latent transforming growth factor-ß binding protein-4
60712	778	6314	0.123	J05503	carbamoyl-phosphate synthetase
71014	1056	6742	0.157	EM_MUS:MM19604	DNA ligase I
40515	938	4892	0.192	EM_HUM3:AK056661	vacuolar protein sorting (VPS8)
10902	675	3281	0.206	MMUGTN:194525-2	serine protease inhibitor
60409	987	4319	0.229	AF006010	progestin-induced protein
10710	2156	9020	0.239	MMTFS3	mouse transcription factor s-ii
60909	6776	27783	0.244	MMU72634	rostral cerebellar malformation protein (RCM)
51010	975	3938	0.248	MMUGTN:031192-14	protein O-mannosyltransferase 1
20410	2721	9965	0.273	EM_MUS:MM65KDA	65-kDa macrophage cytosolic protein
10812	573	2092	0.274	MMUGTN:016783-15	phosphoglycerate mutase 1
40303	303	1046	0.290	M77196	mouse hepatitis virus (MHV) receptor
81211	1101	3716	0.296	EM_MUS:BC003861	hydroxymethylbilane synthase
20303	1750	5684	0.308	AF257711	proton-dependent high affinity oligopeptide transporter (PEPT2)
60609	1963	6091	0.322	MMSTYKIN	serine threonine tyrosine kinase
21010	1657	5121	0.324	EM_MUS:MMPDIA	disulfide isomerase (ERP59)
40104	1546	4398	0.352	U02082	guanine nucleotide regulatory protein
20906	2902	7782	0.373	AF098077	nuclear respiratory factor-1
10313	3984	10144	0.393	BC002270	ubiquitin-conjugating enzyme E2 variant 1
41215	3059	7781	0.393	EM_RO:RN10699	G-protein coupled receptor pH218
30410	1520	3514	0.433	EM_MUS:AF033276	kinase anchor protein (akap-kl)
70508	3199	7160	0.447	MMRECEP	receptor for advanced glycosylation end products (RAGE)
10108	3663	8057	0.455	AF155373	nuclear factor κ B subunit
30116	3050	6355	0.48	EM_MUS:BC003887	uridine monophosphate synthetase
30901	8403	17471	0.481	U51167	isocitrate dehydrogenase

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Table 3 continued

Down-regulated selected genes

ca	BALB/c C	BALB/c IFN-γ	C/IFN-γ	Accession	Gene
21214	19919	0	INF	MMUGTN:003842-11	nicotinamide nucleotide transhydrogenase
50313	5886	0	INF	MMUGTN:168786-1	sperm associated antigen
71205	5433	0	INF	AF111102	major histocompatibility complex (MHC) class I region
60305	2290	0	INF	Y13620	B cell CLL/lymphoma 9 (BCL9)
50308	2262	0	INF	MMUGTN:036418-4	nucleolar RNA-associated protein
80203	1752	0	INF	M19141	heat shock protein 70 (HSP70)
80714	1555	0	INF	EM_MUS:BC005770	beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)
20714	1187	0	INF	EM_MUS:BC007483	growth factor receptor bound protein 2-associated protein 1
50604	1184	0	INF	Y13622	latent transforming growth factor ß binding protein-4
60804	1141	0	INF	NM_013843	zinc finger protein
80611	1138	0	INF	EM_MUS:AY013811	protocadherin γ c3
80815	1007	0	INF	EM_MUS:MMALPA	α catenin
10316	927	0	INF	EM_MUS:MM05809	LAF1 transketolase
21203	757	0	IFN	AF058797	14-3-3 protein ß
30615	609	0	IFN	EM_MUS:MM36220	FK506 binding protein
50613	497	0	INF	EM_MUS:BC003300	ATP-binding cassette, sub-family F (GCN20)
81203	496	0	INF	EM_MUS:BC007158	procollagen, type I, α 2
80102	444	0	INF	NM_011602	talin
50514	334	0	INF	EM_MUS:MMMEK4	eph-related receptor tyrosine kinase (MEK4)
80204	17748	8018	2.21	U28322	Krueppel-type zinc finger protein
70308	3839	1775	2.16	NM_031397	bicaudal C homolog 1
30912	8823	4388	2.01	AF073879	myotubularin homologous protein 1

The data, organized in decreasing order of hybridization spot intensity, show the clonal address (ca) of each spot, the intensity units of control non-activated (BALB/c C) and IFN-γ-activated (BALB/c IFN-γ) macrophage cultures, the respective intensity ratios (C/IFN-γ), the gene accession code (Accession) and the gene product description (Gene).

and metabolism such as platelet-activating factor, gpi-anchored protein, ubiquitin C-terminal hydrolase, vacuolar protein sorting (VPS8) or in cytoskeleton arrangement and extracellular matrix such as coronin 2, entactin, VCAM1, tenascin, fibronectin or receptor expression such as capping protein α2 subunit, growth factor receptor (Gab3), eph-related receptor tyrosine kinase (MEK4), mouse hepatitis virus receptor or phagocytosis such as tubulin α6, talin, cofilin or in resistance and susceptibility to infections and tumor cytotoxicity such as pyruvate kinase, heat shock protein 70, gpi-anchored protein or in inflammation and cell activation and differentiation such as apoptosis inhibitory protein, tyrosine phosphatase, topoisomerase II, bone morphogenic protein receptor. For some genes, such as nuclear pore targeting complex, splicing factor ß, cyclophilin, fatty acid synthase, hydroxymethylbilane synthase, CPSF1, and 14-3-3 protein ß, the relationship with macrophage biology is yet to be established. Some of these investigated genes were found to be up- or down-regulated only in A/J macrophages (10 up-regulated and 8 down-regulated), some others only in BALB/c macrophages (5 up-regulated and 4 down-regulated), some were up- or down-regulated in cells from both strains (8 up-regulated and 2 down-regulated), one was up-regulated in A/J macrophages and down-regulated in BALB/c macrophages, and three were down-

Table 4. List of 42 differentially expressed genes in IFN-γ-activated A/J and/or BALB/c mouse macrophages and their relationship to key functions in macrophage biology as reported in recent publications.

ca	Gene	Expression*		Enzymatic mediation	Nucleic acid synthesis/Transport	Protein synthesis/Transport/Metabolism	Cytoskeleton arrangement/Extracellular matrix	Receptor expression	Phagocytosis	Resistance/Susceptibility to infection/Tumor	Inflammation/Differentiation/Cell activation	To be established	References
010511	coronin 2	▲A/J					*		*				14
010913	nuclear pore targeting complex	▲A/J										*	
021003	entactin	▲A/J					*						15
031216	RNA polymerase II 140-kDa subunit	▲A/J			*								16
051102	glyceraldehyde-3P-dehydrogenase	▲A/J		*		*							17
060214	apoptosis inhibitory protein	▲A/J				*					*		18
060811	ß-catenin	▲A/J										*	19
061202	PAF-AH	▲A/J		*		*							20
071215	capping protein α 2 subunit	▲A/J					*	*	*		*		21
080111	gpi-anchored protein	▲A/J				*				*	*		22
030708	pyruvate kinase	▼A/J		*						*	*		23
030712	VCAM1	▼A/J					*				*		24,25
040713	splicing factor 3b	▼A/J										*	
040909	tenascin	▼A/J				*	*						26
060312	acetoacetyl-CoA-synthetase	▼A/J		*		*							27
060713	ubiquitin C-t hydrolase	▼A/J		*		*							28
070205	tyrosine phosphatase	▼A/J		*		*					*		29,30
070312	topoisomerase II binding protein	▼A/J		*							*		31
080708	adenosine-MP deaminase	▼A/J		*						*			32
020410	65-kDa macrophage cytosolic protein		▲BALB/c				*				*		33,34
040104	guanine nucleotide regulatory protein		▲BALB/c									*	35
040515	vacuolar protein sorting (VPS8)		▲BALB/c			*							36
081008	cyclophilin		▲BALB/c									*	
051213	fibronectin		▲BALB/c				*		*	*	*		37
020714	growth factor receptor (Gab3)		▼BALB/c					*			*		38
021203	14-3-3 protein ß		▼BALB/c									*	
030912	myotubularin homologous protein 1		▼BALB/c	*			*						39
050514	MEK4		▼BALB/c					*				*	
010312	BMP receptor	▲A/J	▲BALB/c		*			*			*		40
010902	serine protease inhibitor	▲A/J	▲BALB/c	*				*			*		41,42
011016	TNF-α converting enzyme	▲A/J	▲BALB/c	*		*		*			*		43
041216	CPSF1	▲A/J	▲BALB/c									*	
051115	cofilin	▲A/J	▲BALB/c				*		*		*		44
071010	fatty acid synthase	▲A/J	▲BALB/c									*	
071014	DNA ligase 1	▲A/J	▲BALB/c	*	*						*		45
081211	hydroxymethylbilane synthase	▲A/J	▲BALB/c									*	
040308	heat shock protein 70	▼A/J	▼BALB/c			*				*	*		46,47
060513	talin	▼A/J	▼BALB/c				*		*				48,49
060804	zinc finger protein	▲A/J	▼BALB/c		*			*			*		50,51
040303	mouse hepatitis virus receptor	▼A/J	▲BALB/c					*		*			7
040509	tubulin α 6	▼A/J	▲BALB/c			*	*		*	*			52,53
070508	RAGE	▼A/J	▲BALB/c		*			*					54,55

Gene expression modulated (triangle = increase; inverted triangle = decrease) by IFN-γ activation in A/J and BALB/c mouse macrophages. ca = clonal address; CPSF = cleavage and polyadenylation specificity factor; BMP = bone morphogenic protein; RAGE = receptor for advanced glycation end products; MEK4 = eph-related receptor tyrosine kinase; PAF-AH = platelet-activating factor acetylhydrolase; VCAM1 = vascular cell adhesion molecule 1.

regulated in A/J macrophages and up-regulated in BALB/c macrophages.

In Figure 1 we present a selected area of the hybridization filter showing gene expression profiles of control and IFN-γ-activated macrophages from A/J and BALB/c mice. The area was chosen so that a pair of hybridization spots defining a given clonal address (in this particular case 040303) could be highlighted. The clonal address corresponds to the MHV receptor gene. It can be seen that some dots are larger than others. A quantitative representation of its average relative intensity is provided in the histogram, indicating that, upon IFN-γ activation, A/J mouse macrophages do not express the MHV receptor gene, whereas BALB/c mouse macrophages do express this gene. These data correlate with and confirm our previous observations made in studies based on biological assays (Table 1).

The reproducibility of the results was adequate for high expressions and less satisfactory for weak ones. Standard deviations are not provided because the validity scoring was based not only on measured values but also on comparison of subsequent hybridization upon stripping (other samples and even other projects). As a result, the scores for some spots were more reliable than others.

Discussion

The experiments planned and executed in this project are meant to be a continuation of our earlier efforts, in which, using a proteomic approach, we attempted to identify gene products involved in the regulation of IFN-γ activation of macrophages derived from mice resistant and susceptible to experimental infection with MHV3 (8). We have acquired knowledge on nucleic acids and protein functions while developing proteomic/genomic approaches that allow the identification of the genetic inheritance and the modulation of gene expression (9-11). Since there is no definite time point characterizing gene expression, we have chosen 18 h of IFN-γ macrophage activation since proteomic and biological studies are usually done at this time.

Samples of cDNA preparations from A/J and BALB/c mice (activated or not with IFN-γ) were hybridized with arrayed cDNA clones on nylon sheets. Each nylon sheet

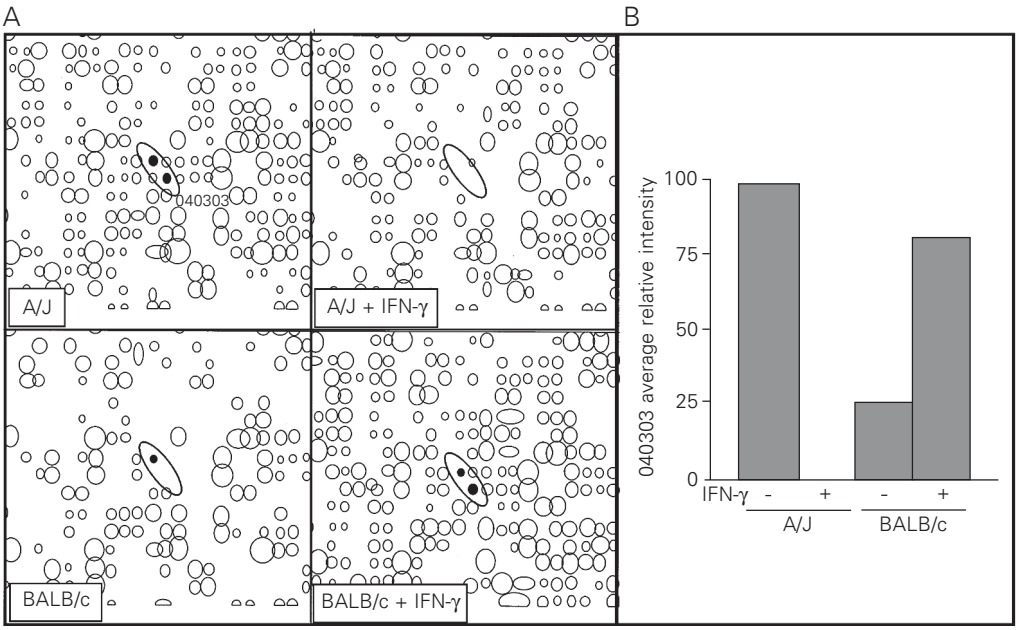

Figure 1. Selected areas of hybridization patterns generated from probes of control and IFN-γ-activated macrophages from A/J and BALB/c mice (A), showing the duplicated highlighted spots corresponding to the murine hepatitis virus (MHV) receptor gene expression (clonal address 040303), and the histogram of its average relative intensity (B).

contained 1536 cDNA clones of fetal thymus origin in duplicate, as well as several control dots of cytochrome c origin. A chemiluminescence readout followed by image analysis yielded data which indicated the relative intensity of hybridized entities.

As shown in Table 1, based on the observation that A/J mice were resistant and BALB/c mice were susceptible to experimental infection with MHV3, classical biological assays for the study of the cellular and molecular basis of resistance of mice to MHV3 led us to hypothesize and experimentally demonstrate that IFN-γ activation could partially restrict viral multiplication only in the resistant A/J macrophages and that the molecular basis of this restriction relied on the IFN-γ induced down-regulation of the main viral receptor (7). Taken together, proteomic experiments and gene expression profiling provided us not only with a confirmation of these predictions but with the possibility of a much deeper comprehension of the biology of IFN-γ activation of macrophages. We have demonstrated a panel of up- and down-regulated genes as well as identified their relationships to key functions in macrophages.

By expression profiling, we have evaluated the degree of up- or down-regulation of several genes in macrophages from A/J and BALB/c mice upon activation by IFN-γ. Since this technology allows us to identify the expression of genes that are modified upon activation by IFN-γ, we can further examine the biological role of the gene products and categorize the possible modulation of biological functions induced by IFN-γ activation in macrophages from both mouse strains. We have on hand, in terms of hybridization spots, several identified molecular entities of the cDNA library, and our semi-quantitative data show the overall influence of IFN-γ activation on macrophage gene expression. Contrary to the proteomic approach, in which every polypeptide species (if present in adequate amounts, if within the resolution limits of the separation, and if it contains methionine in the amino acid sequence) present in the 2-D SDS gel matrix shows up on the radiofluorogram, the hybridization readout is constrained to the portion of cDNA molecules present in the library. Among the 1536 clonal entities, there are about one thousand different molecular clones, encompassing about 470 low abundance ones. Since all of these clones are transcribable and translatable the number is satisfactory for adequate analysis. It represents about 20% of the messages present in a typical cell (the lymphocyte being the model) (56). Note that a cell has altogether about 40,000 mRNA molecules, some present in a relatively large copy number, others with only 3-5 copies per cell. There are approximately 5,000 different mRNA molecules in a cell (56). Our experiments revealed only those up- and down-regulated which have members present among the 1536 entities tested.

In Figure 1 we show the hybridization patterns of a selected area of the gene expression array obtained from control and IFN-γ-activated A/J and BALB/c macrophages, with the duplicated gene spots corresponding to the main MHV receptor (clonal address 040303) highlighted, as well as its histograms of average relative intensity. One can observe that, as predicted by classical assays performed in the past (7), there is a down-regulation of this receptor gene only in IFN-γ-activated macrophages from resistant A/J mice. Also, as previously shown (7), the receptor gene expression in IFN-γ-activated BALB/c macrophages was high, although its basal expression in control macrophages was found to be not always reproducible, possibly due to variations in the physiological state of the cells.

Since most of the genes available in our cDNA library have already been decoded and many of their protein products identified, we now know the gene products likely to be up- or down-regulated and have the possibility of deducing modulation of bio-

logical functions induced by IFN-γ activation in macrophages. As it is preliminarily shown in Table 4, we could identify genes coding for proteins participating in processes of macrophage biology like enzymatic reactions, nucleic acid synthesis and transport, protein synthesis, transport and metabolism, cytoskeleton arrangement and extracellular matrix, receptor expression, phagocytosis, resistance/susceptibility to infection or tumors and inflammation or cell differentiation. The data indicate that the overall gene regulation by IFN-γ can be quite different in macrophages originating from mice with different genetic backgrounds and this panel of IFN-γ-regulated genes may serve as a starting point for general and specific studies of macrophage biology.

This paper reveals a large assembly of genes differentially expressed in macrophages of two murine genetic backgrounds (A/J and BALB/c) upon IFN-γ activation. These data will turn out to be very useful for general studies of macrophage biology and can be an alternative strategy to confirm hypothetical as well as already defined features of macrophages, such as that of MHV receptor gene modulation upon IFN-γ activation.

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