Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10[1]

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IL-10 regulates inflammation by reducing cytokine and chemokine production from activated macrophages. We performed microarray experiments to identify possible effector molecules of IL-10 and to investigate the global effect of IL-10 on the transcriptional response induced in LPS-activated macrophages. To exclude background effects of endogenous IL-10, macrophages from IL-10-deficient mice were used. IL-10 up-regulated expression of a small number of genes (26 and 37 after 45 min and 3 h, respectively), including newly identified and previously documented targets such as suppressor of cytokine signaling-3 and IL-1 receptor antagonist. However, the activation program triggered by LPS was profoundly affected by IL-10. IL-10 repressed 62 and further increased 15 of 259 LPS-induced genes. For all genes examined, the effects of IL-10 were determined to be STAT3-dependent. These results suggest that IL-10 regulates STAT3-dependent pathways that selectively target a broad component of LPS-induced genes at the mRNA level. *The Journal of Immunology,* **2002, 169: 2253–2263.**

Interleukin-10 is an essential anti-inflammatory cytokine produced primarily from T cells and activated macrophages. IL-10 is capable of blocking or reducing the output of numerous proinflammatory agents from macrophages including cytokines such as TNF-α, IL-6, and IL-12, chemokines, and prostaglandins via blocking cyclooxygenase 2 expression (1, 2). Because of these broad effects, IL-10 has elicited considerable clinical interest to treat chronic inflammatory conditions such as Crohn's disease (3) and hepatitis C-induced fibrosis (4).

No consensus has emerged as to how IL-10 inhibits production of cytokines and chemokines from macrophages. Inhibition of TNF-α production by IL-10, for example, has been attributed to effects on NF-κB activation (5), mitogen-activated protein kinase (MAPK)[4] signaling pathways (6), rate of transcription (7), mRNA stability (8), translational efficiency (6), cleavage from the membrane, and uptake via TNF receptors (9) (for reviews, see Refs. 1 and 2). Also, conflicting results have been reported for most of these effects. In contrast, genetic and biochemical analysis has elucidated the membrane proximal events of IL-10 signaling. The functional IL-10R consists of the ligand binding IL-10R1 and the accessory subunit IL-10R2 (1, 2). Although IL-10R2 is expressed on most cells and tissues, IL-10R1 is expressed on hemopoietic cells and up-regulated on macrophages upon activation (1, 2). This fact, combined with genetic evidence from a variety of animal model systems, supports the notion that macrophages are the primary target of IL-10 (10, 11). Binding of IL-10 to its receptor initiates signaling via the Janus kinase-STAT pathway. Limited studies using fetal liver-derived Janus kinase 1-deficient macrophages (12) suggest this kinase is essential for early IL-10 signaling. STAT3 plays a pivotal role because the conditional inactivation of STAT3 in myeloid lineage cells results in abrogated IL-10 responses of macrophages and development of chronic enterocolitis similar to IL-10-deficient (IL-10$^{-/-}$) mice (11).

It is not known how IL-10 attenuates macrophage activation downstream of STAT3. Experimental evidence suggests that new protein synthesis is required for IL-10 to inhibit LPS-induced cytokine production, since IL-10 does not appear to inhibit IL-12 p40 or TNF-α expression in LPS-stimulated macrophages when cycloheximide is added (8, 13). To date, few genes have been described to be induced by IL-10. We reasoned that a gene expression screen for IL-10-induced genes in macrophages should identify a range of targets potentially involved in deactivation of macrophages. We took advantage of macrophages from mice deficient in IL-10 or STAT3 and microarray techniques to identify and validate genes induced and repressed by IL-10 in resting and activated macrophages.

Materials and Methods

Reagents, mice, and macrophages

IL-10 and IL-4 were purchased from BD PharMingen (San Diego, CA), LPS was obtained from Sigma-Aldrich (St. Louis, MO). IL-10$^{-/-}$ mice (14) on a C57BL/6 background and controls were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT3$^{flox/-}$ and LysMcre breeding pairs were a gift from I. Förster (Technical University of Munich, Munich, Germany). Peritoneal-derived macrophages (PDM) and bone marrow-derived macrophages (BMDM) were isolated as previously described (10). Detection of IL-10 and TNF-α in culture supernatants was by ELISA using Ab pairs from BD PharMingen.

Affymetrix gene chip analysis

IL-10$^{-/-}$ BMDM were stimulated with IL-10 (10 ng/ml), LPS (100 ng/ml), or IL-10 + LPS for 45 min or 3 h. For both timepoints, two completely independent experiments were performed. Total RNA was prepared using TRIzol (Life Technologies, Gaithersburg, MD), processed, and hybridized to MG-U74Av2 gene chips according to Affymetrix protocols (Santa Clara, CA). Chips were scanned and analyzed using Affymetrix Microarray

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Received for publication April 8, 2002. Accepted for publication June 18, 2002.

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[1] This work was supported by American Heart Association Grant 0151039B to P.J.M., Cancer Center CORE Grant P30 CA 21765, and by the American Lebanese Syrian Association of Charities. R.L. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (LA-1262/1).

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[4] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; PDM, peritoneal-derived macrophages; BMDM, bone marrow-derived macrophages; IL-1ra, IL-1R antagonist; SOCS, suppressor of cytokine signaling; GADD, growth arrest and DNA damage; EST, expressed sequence tag; JE/MCP, JE/monocyte chemoattractant protein; Tpl, tumor progression locus; TLR, Toll-like receptor; C/EBP, CCAAT/enhancer binding protein; AMBP, α_1-microglobulin/bikunin precursor.

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Suitev4.0 software. Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to constant target intensity (2500) for all arrays used. The signal intensity for each gene was calculated as the average intensity difference, represented by (Σ(PM$-$MM)/(number of probe pairs)), where PM and MM denote perfect-match and mismatch probes.

Data analysis

Data sets of 12,488 probe sets per array were compared using Microsoft Excel (Microsoft, Redmond, WA) and Spotfire software. To avoid negative ratios, average intensity differences < 5 were first set to 5 (15). Data were normalized by mean using the untreated sample as the baseline within each experiment. To identify differentially expressed genes, we excluded all genes from the analysis that were scored absent in the test sample (for up-regulated genes) or absent in the baseline sample (for down-regulated genes) in one or both experiments. Fold changes were calculated separately for both experiments as the ratio of normalized average intensity difference (test sample) divided by normalized average intensity difference (baseline sample). Thresholds were set for fold change (2-fold and greater unless otherwise indicated) and absolute difference (at least 500) between normalized average intensity differences. Consistency between experiments varied between samples depending on treatment and timepoint. For example, of the genes induced $>$3-fold by IL-10 in the first experiment, 50.0% for the 45 min and 55.6% for the 3 h timepoint were up-regulated at least 2-fold in the second experiment. When LPS treatment was compared with untreated, the corresponding numbers were 87.7 and 78.0%, respectively. To minimize the number of false-positives, only those genes that reproducibly met all the thresholds described above in both independent experiments were considered differentially expressed.

Northern blotting and real-time quantitative RT-PCR

For Northern analysis, 10–15 μg total RNA were separated on 1% formaldehyde-agarose gels and blotted onto Hybond N (Amersham, Piscataway, NJ). Probes were prepared from plasmids containing either full-length cDNAs (IL-12p40, GAPDH, IL-1R antagonist (IL-1ra), suppressor of cytokine signaling (SOCS)3, junB, growth arrest and DNA damage (GADD)45γ, GADD45α) or expressed sequence tags (ESTs) (NFIL-3, JE/monocyte chemoattractant protein (MCP)-1, tumor progression locus (Tpl)-2). For real-time quantitative RT-PCR, 1 μg of total RNA was reverse-transcribed using Superscript II (Life Technologies) and a mix of random hexamer and oligo(dT) primers. Primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA). For β-actin, TNF-α, arginase-1 and arginase-2, internal TaqMan probes were designed and included in the PCR. Sequences of primers and probes are available from the authors upon request. For all other target genes, the SYBR-green master mix was used to detect accumulation of PCR product during cycling on the SDS7700 (Applied Biosystems). Expression of target genes was normalized to β-actin and displayed as fold-change relative to the untreated 45-min sample used as the calibrator (set to 1).

Results

Choice of IL-10$^{-/-}$ BMDM as experimental system

We reasoned that experimental parameters, especially the choice of macrophage type and timepoints after stimulation, would be critical determinants of the results obtained by gene expression profiling. We analyzed the influence of these variables on inhibition of TNF-α production by IL-10 to establish conditions optimal to identify IL-10-induced genes and investigate its overall impact on the transcriptional response to LPS. In response to LPS, both BMDM and PDM rapidly produced TNF-α that was inhibited by IL-10 as expected (Fig. 1). However, PDM made more TNF-α and showed a stronger inhibitory effect of IL-10. This difference was inversely correlated with much higher production of IL-10 in BMDM, suggesting that endogenous IL-10 blunted TNF-α production. We concluded that a system devoid of endogenous IL-10 would be advantageous for uncovering the full spectrum of IL-10-induced changes in gene expression. BMDM from IL-10$^{-/-}$ mice produced high amounts of TNF-α and were fully responsive to inhibition by IL-10. TNF-α production was down-regulated by IL-10 as early as 1 h after stimulation, but this effect was increased at 2–4 h. Therefore, we chose two timepoints for global expression profiling. After 45 min, mRNAs encoding IL-10-induced inhibitors of inflammatory responses should be present at detectable levels. A timepoint of 3 h was chosen to visualize later IL-10-induced differences in the transcriptional response to LPS. This later timepoint is expected to contain both directly and indirectly IL-10-induced genes that may play a role in the anti-inflammatory effects of this cytokine.

Changes in gene expression induced by IL-10 in resting and activated macrophages

Stimulation with LPS induced a $>$2-fold induction of 149 genes after 45 min, and of 402 genes after 3 h, whereas 40 and 752 genes were repressed $>$2-fold by LPS at these timepoints (Fig. 2). This substantial reprogramming of gene expression is consistent with other studies examining the impact of Toll-like receptor (TLR) ligands and pathogens on the macrophage transcriptome (16–18). In contrast, treatment with IL-10 induced 26 and 37 and repressed 25 and 22 genes $>$2-fold after 45 min and 3 h, respectively (Fig. 2). A comparison of gene expression profiles in macrophages treated with LPS or IL-10 + LPS showed few differences after 45 min, but after 3 h the expression of 194 genes differed $>$2-fold (125 induced, 69 repressed by addition of IL-10) (Fig. 2).

Of the 259 genes induced $>$3-fold by LPS after 3 h, 62 were repressed at least 1.5-fold by IL-10, whereas 15 genes were further up-regulated by IL-10 (Fig. 3). As expected, a number of cytokines and chemokines previously described to be down-regulated by IL-10 (1, 2) were repressed in the microarray analysis confirming the robustness of this system. In addition, many transcripts belonging to various functional categories were identified as suppressed

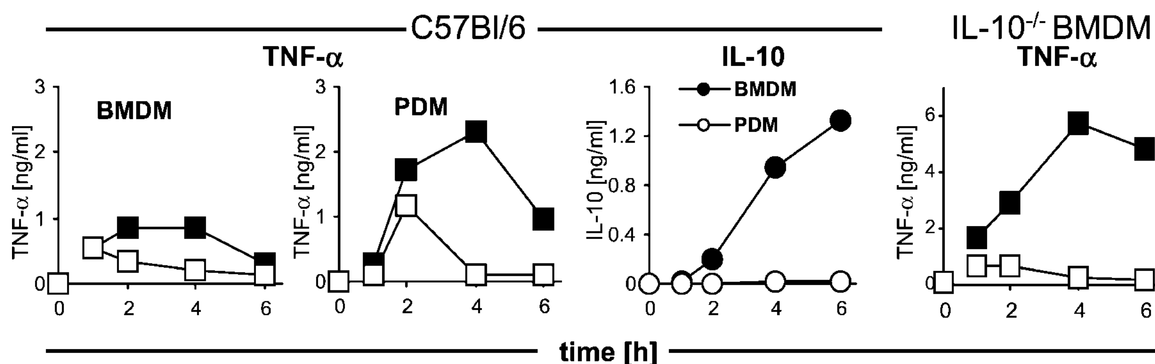


FIGURE 1. Inverse correlation between TNF-α and IL-10 production by PDM and BMDM. IL-10 (10 ng/ml) was added (□) or not (■) to macrophages, followed by stimulation with LPS (100 ng/ml) for the indicated time. Cytokine levels in the supernatant were measured by ELISA. In the case of TNF-α production, similar results were obtained when brefeldin A-treated macrophages were analyzed for accumulation of intracellular cytokine by flow cytometry (data not shown).

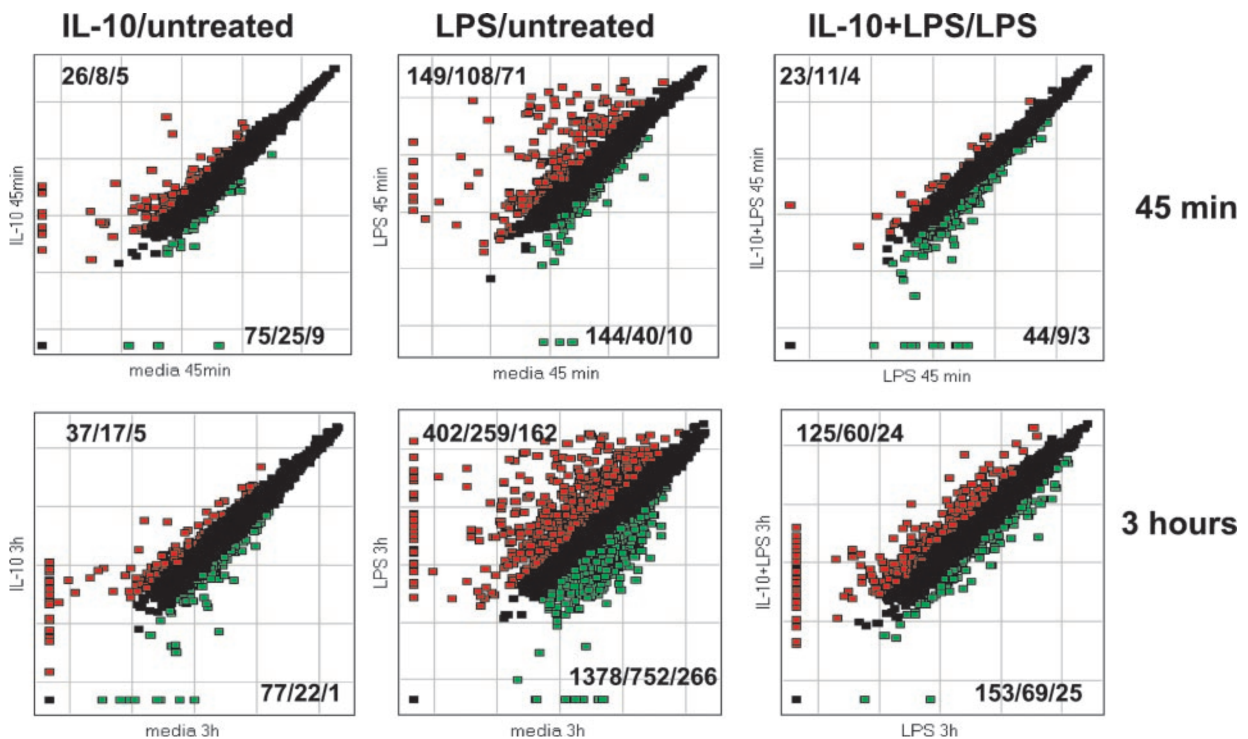


FIGURE 2. Time-dependent changes in gene expression induced by IL-10 and/or LPS. IL-$10^{-/-}$ BMDM were left untreated or stimulated with IL-10 and/or LPS for 45 min (*upper panels*) and 3 h (*lower panels*). RNA was prepared and processed for hybridization to MG-U74Av2 microarrays. Data were analyzed with Spotfire software, using the algorithm described in *Materials and Methods*. Genes that are induced >2-fold are shown in red, while those repressed >1.5-fold appear green. For each comparison, the number of genes induced >2/>3/>5-fold is presented in the upper left corner and the number of genes repressed >1.5/>2/>3-fold is presented in the lower right corner.

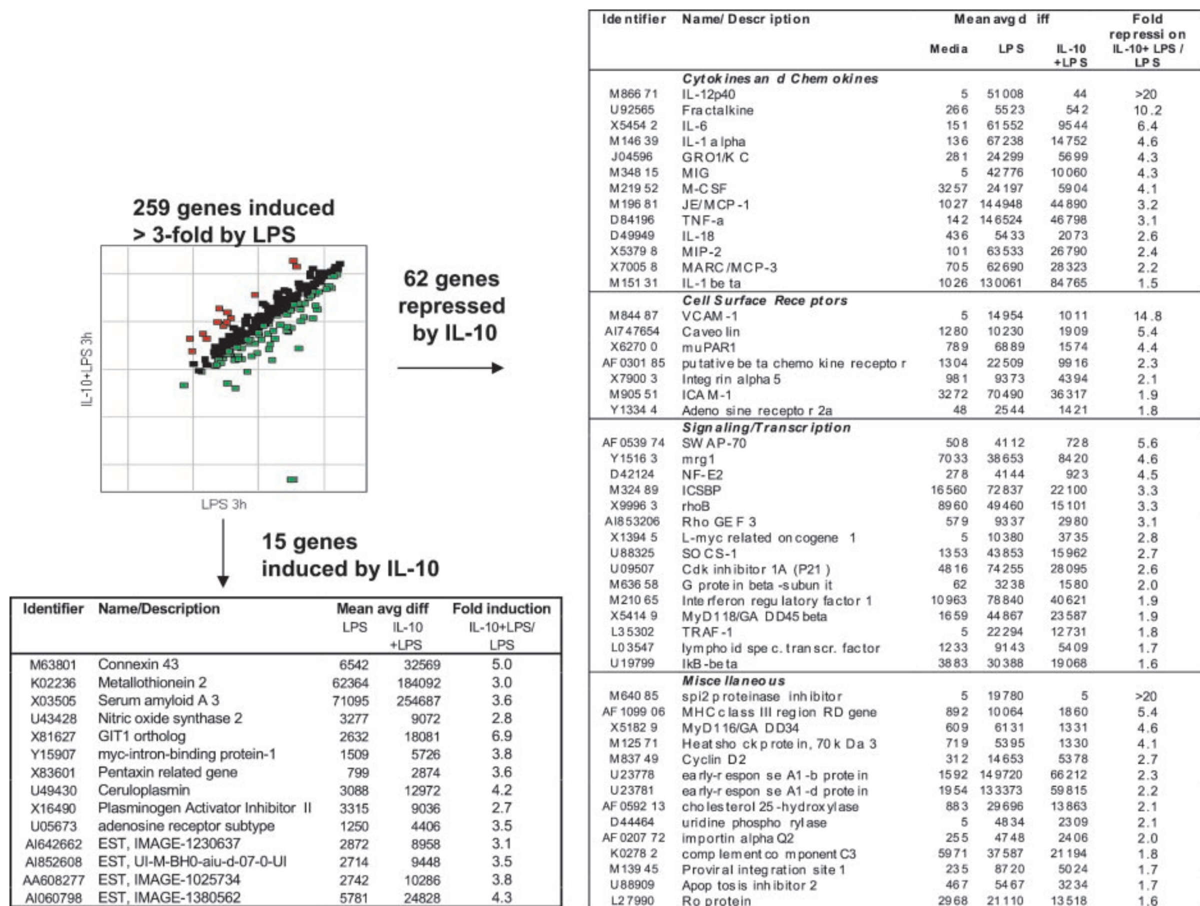
by IL-10 for the first time (Fig. 3). It should be noted that 104 of the 259 LPS-induced genes were not regulated by IL-10 in both independent experiments.

Genes and ESTs induced by IL-10 in resting and LPS-activated macrophages at 45 min are listed in Table I. Results from the 3 h timepoints are listed in Table II. Of the known target genes of IL-10 (1, 2), a significant fraction was confirmed in the microarray analysis including IL-1ra, CD32, CCR5, CCR1, scavenger receptor, and arginase-2. Some genes reported to be induced by IL-10 were not confirmed because they were not represented on the microarray (tissue inhibitor of metalloproteinase 1), not regulated (CD14, TNFR2, CD16, and CD64), or did not pass one or more of the thresholds in the stringent analysis criteria ($p19^{INK4D}$, fMLPR). One of the genes most strongly induced by IL-10 was SOCS3, which has been reported as a target gene common to IL-10 and LPS, and implicated in inhibition of macrophage responses to IFN-γ (19–21). Around half of the genes induced by IL-10 were independently up-regulated in response to LPS (Table I). For some of these genes, the combination of IL-10 and LPS resulted in synergistic induction (IL-1ra, Bcl-3, metallothionein-2, NFIL-3). Some genes were up-regulated specifically by IL-10 (e.g., GADD45γ, connexin 43, CCAAT/enhancer binding protein (C/EBP) δ, IL-4Rα) or the combination of IL-10 + LPS, but not by LPS (e.g., B-ATF) (Table I). Independent confirmation of the results was obtained by Northern analysis or real-time quantitative RT-PCR for a subset of 15 genes scored as induced by the microarray analysis (Fig. 4).

One of the longer-term goals of this work is to identify molecular mediators of the attenuating effect IL-10 has on macrophages. In addition to SOCS3 and IL-1ra, both previously implicated in inhibition of macrophage activation, several of the target genes of IL-10 identified in this study could play a role in macrophage deactivation. Previous studies suggest IL-10 may regulate NF-κB and MAPK pathways, both essential for the initiation and propagation of proinflammatory gene expression (5, 6). We found several genes regulated by IL-10 that have been implicated in controlling NF-κB activation, such as metallothionein-2 (22) and Bcl-3 (23). Other IL-10 targets are involved in MAPK pathway regulation, e.g., GADD45γ (24) and Tpl-2, a MAPK kinase kinase (25) that can also activate IκB kinases (26). Mice deficient in Tpl-2 have a selective defect in TNF-α production due to impaired nucleocytoplasmic transport of the TNF-α mRNA (27). Because IL-10 inhibits TNF-α production, it is not clear whether induction of Tpl-2 by IL-10 is related to the anti-inflammatory effect of IL-10. To resolve this question, it will be important to test macrophages from mice lacking Tpl-2 for IL-10-induced deactivation. Further, several genes for transcriptional regulators were also induced by IL-10. One example of this group is the basic region leucine zipper transcription factor NFIL-3 that can function as transcriptional activator (28) or repressor (29).

STAT3 dependence of gene induction and repression by IL-10

Macrophages deficient in STAT3 are hyperresponsive to LPS and fail to respond to IL-10 with down-regulation of cytokines and inhibition of proliferation (11). To determine whether STAT3 is required for the induction and repression of the IL-10-dependent target genes described in this study, we used BMDM deficient in STAT3 (11). Expression of selected genes was analyzed by Northern blotting (Fig. 5*A*) or real-time quantitative RT-PCR (Fig. 5*B*). Inhibition by IL-10 of LPS-induced expression of IL-12 p40, TNF-α, and JE/MCP-1 was dependent on functional STAT3. The reduced levels of these mRNAs in the LPS-stimulated wild-type BMDM compared with IL-$10^{-/-}$ and STAT$3^{-/-}$ BMDM illustrates the attenuation of inflammatory responses by endogenous



Identifier	Name/Description	Mean avg diff LPS	Mean avg diff IL-10 +LPS	Fold induction IL-10+LPS/ LPS
M63801	Connexin 43	6542	32569	5.0
K02236	Metallothionein 2	62364	184092	3.0
X03505	Serum amyloid A 3	71095	254687	3.6
U43428	Nitric oxide synthase 2	3277	9072	2.8
X81627	GIT1 ortholog	2632	18081	6.9
Y15907	myc-intron-binding protein-1	1509	5726	3.8
X83601	Pentaxin related gene	799	2874	3.6
U49430	Ceruloplasmin	3088	12972	4.2
X16490	Plasminogen Activator Inhibitor II	3315	9036	2.7
U05673	adenosine receptor subtype	1250	4406	3.5
AI642662	EST, IMAGE-1230637	2872	8958	3.1
AI852608	EST, UI-M-BH0-aiu-d-07-0-UI	2714	9448	3.5
AA608277	EST, IMAGE-1025734	2742	10286	3.8
AI060798	EST, IMAGE-1380562	5781	24828	4.3

Identifier	Name/Description	Mean avg diff Media	Mean avg diff LPS	Mean avg diff IL-10 +LPS	Fold repression IL-10+LPS / LPS
	Cytokines and Chemokines				
M86671	IL-12p40	5	51008	44	>20
U92565	Fractalkine	266	5523	542	10.2
X54542	IL-6	151	61552	9544	6.4
M14639	IL-1 alpha	136	67238	14752	4.6
J04596	GRO1/KC	281	24299	5699	4.3
M34815	MIG	5	42776	10060	4.3
M21952	M-CSF	3257	24197	5904	4.1
M19681	JE/MCP-1	1027	144948	44890	3.2
D84196	TNF-a	142	146524	46798	3.1
D49949	IL-18	436	5433	2073	2.6
X53798	MIP-2	101	63533	26790	2.4
X70058	MARC/MCP-3	705	62690	28323	2.2
M15131	IL-1 beta	1026	130061	84765	1.5
	Cell Surface Receptors				
M84487	VCAM-1	5	14954	1011	14.8
AI747654	Caveolin	1280	10230	1909	5.4
X62700	muPAR1	789	6889	1574	4.4
AF030185	putative beta chemokine receptor	1304	22509	9916	2.3
X79003	Integrin alpha 5	981	9373	4394	2.1
M90551	ICAM-1	3272	70490	36317	1.9
Y13344	Adenosine receptor 2a	48	2544	1421	1.8
	Signaling/Transcription				
AF053974	SWAP-70	508	4112	728	5.6
Y15163	mrg1	7033	38653	8420	4.6
D42124	NF-E2	278	4144	923	4.5
M32489	ICSBP	16560	72837	22100	3.3
X99963	rhoB	8960	49460	15101	3.3
AI853206	Rho GEF 3	579	9337	2980	3.1
X13945	L-myc related oncogene 1	5	10380	3735	2.8
U88325	SOCS-1	1353	43853	15962	2.7
U09507	Cdk inhibitor 1A (P21)	4816	74255	28095	2.6
M63658	G protein beta-subunit	62	3238	1580	2.0
M21065	Interferon regulatory factor 1	10963	78840	40621	1.9
X54149	MyD118/GADD45 beta	1659	44867	23587	1.9
L35302	TRAF-1	5	22294	12731	1.8
L03547	lymphoid spec. transcr. factor	1233	9143	5409	1.7
U19799	IkB-beta	3883	30388	19068	1.6
	Miscellaneous				
M64085	spi2 proteinase inhibitor	5	19780	5	>20
AF109906	MHC class III region RD gene	892	10064	1860	5.4
X51829	MyD116/GADD34	609	6131	1331	4.6
M12571	Heat shock protein, 70 kDa 3	719	5395	1330	4.1
M83749	Cyclin D2	312	14653	5378	2.7
U23778	early-response A1-b protein	1592	149720	66212	2.3
U23781	early-response A1-d protein	1954	133373	59815	2.2
AF059213	cholesterol 25-hydroxylase	883	29696	13863	2.1
D44464	uridine phosphorylase	5	4834	2309	2.1
AF020772	importin alpha Q2	255	4748	2406	2.0
K02782	complement component C3	5971	37587	21194	1.8
M13945	Proviral integration site 1	235	8720	5024	1.7
U88909	Apoptosis inhibitor 2	467	5467	3234	1.7
L27990	Ro protein	2968	21110	13518	1.6

FIGURE 3. Effect of IL-10 on LPS-induced genes. The 259 genes induced >3-fold by LPS compared with untreated macrophages in two independent experiments are displayed in the scatter plot to analyze changes in expression induced by treatment with IL-10 + LPS compared with LPS alone. Genes that are further induced >2-fold by the addition of IL-10 are shown as red squares, ones that are repressed >1.5-fold as green squares. These remaining genes were grouped into four categories as shown excluding 14 ESTs. Fold change values were obtained by dividing mean average differences (from two experiments) of IL-10 + LPS-treated by LPS sample (for induction by IL-10) and of LPS treated by IL-10 + LPS-treated sample (for repression by IL-10).

IL-10. For the IL-10-induced targets identified in this study including Tpl-2, NFIL-3, GADD45γ, IL-1ra, α_1-microglobulin/bikunin precursor (AMBP), protein C receptor, MT-2, and B-ATF, the absence of STAT3 completely abrogated inducibility by IL-10 and reduced induction of IL-4Rα, Bcl-3, and connexin 43 mRNAs (Fig. 5). This side-by-side comparison of wild-type macrophages with cells incapable of producing or responding to IL-10 also revealed that the expression of Tpl-2, AMBP, IL-4Rα, and B-ATF after stimulation with LPS is mediated indirectly by IL-10 or other factors signaling via STAT3 (Fig. 5).

Up-regulation of IL-4Rα by IL-10 correlates with increased IL-4-dependent expression of arginase-1 (Fig. 6)

The finding of increased IL-4Rα expression in macrophages treated with IL-10 (Tables I and II, Figs. 4 and 5), suggested an enhanced sensitivity to IL-4 as a functional consequence of exposure to IL-10. Importantly, both cytokines are known to promote “alternative activation” of macrophages, a functional state characterized by high phagocytic capacity but a reduced ability to kill pathogens (30). A hallmark of “alternative activation” is high arginase activity, which competes with inducible NO synthase for L-arginine, the common substrate of both enzymes (31, 32), and can be due to the expression of either one of two isoforms. Arginase-2 was shown to be induced by LPS (33), and we found in this study that IL-10 synergized with LPS in increasing arginase-2 expression (Fig. 4, Table II). Expression of arginase-1 is induced by the Th2 cytokines IL-4 and IL-13 (34) in a STAT6-dependent manner (32). IL-10 strongly synergizes with IL-4 to induce arginase-1 (35), but the mechanistic basis for this effect has been unknown. Therefore, we evaluated whether the magnitude of arginase-1 induction after IL-4 stimulation is linked to the IL-10-mediated increase in IL-4Rα expression we observed in the array analysis. IL-4 strongly induced arginase-1 expression, which was further increased 10-fold by addition of IL-10 (Fig. 6), correlating with the increased levels of IL-4Rα in macrophages treated with IL-10 alone or in combination with IL-4. In contrast, LPS down-regulated IL-4Rα expression and potently inhibited expression of arginase-1 in response to IL-4 (Fig. 6). Combined addition of IL-10 and LPS restored high level IL-4Rα expression and brought back synergistic induction of arginase-1 in macrophages exposed also to IL-4. Taken together, increased expression of the IL-4Rα may represent the basis for the synergistic effect of IL-10 on IL-4-induced arginase-1 expression in macrophages.

Discussion

In the 13 years since the discovery of IL-10, major advances have been made in understanding the function of this important cytokine (1, 2). We now recognize that IL-10 plays an essential role in the endogenous anti-inflammatory response of the host. IL-10 acts primarily on activated macrophages, where IL-10R expression is highest. The IL-10R activates STAT3 and loss of this transcription factor in macrophages mimics loss of IL-10 itself (11), suggesting

Table I. *Genes induced after 45 min by IL-10 in resting and LPS-stimulated macrophages*[a]

Identifier	Name/Description	Mean Average Intensity Difference[b]				Fold Change[c]		
		Media	IL-10	LPS	IL-10 + LPS	IL-10[d]	LPS[d]	IL-10 + LPS[e]
	Transcriptional regulators							
U89411	E4BP4	4,940	4,415	2,673	11,488	0.9	0.5	**4.3**
AF017021	B-ATF	773	1,155	529	3,119	1.5	0.7	**5.9**
X60831	UBF	1,683	1,836	1,188	3,344	1.1	0.7	**2.8**
X61800	C/EBPδ	4,688	11,039	3,829	7,087	**2.4**	0.8	1.9
U83148	NFIL-3/E4BP4	1,586	5,373	4,369	19,003	**3.4**	2.8	**4.3**
M90397	Bcl-3	290	6,785	2,397	7,342	**23.4**	8.3	**3.1**
U20735	junB	4,209	11,483	72,678	70,053	**2.7**	17.3	1.0
	Signal transduction							
AF055638	GADD45γ	1,267	6,501	440	5,816	**5.1**	0.3	**13.2**
U14103	RGL protein	157	68	166	947	0.4	1.1	**5.7**
D13759	Tpl-2/COT	1,020	3,542	1,636	9,113	**3.5**	1.6	**5.6**
U00937	GADD45α	1,695	4,572	9,584	9,279	**2.7**	5.7	1.0
AV138783	GADD45β	2,235	6,345	29,492	34,943	**2.8**	13.2	1.2
AV374868	SOCS-3	684	23,962	41,364	75,027	**35.0**	60.4	1.8
	Secreted mediators							
M37897	IL-10	1,130	3,267	26,730	40,828	**2.9**	23.7	1.5
X62502	Macrophage-inflammatory protein 1β	2,183	10,319	105,950	114,945	**4.7**	48.5	1.1
L32838	IL-1ra	82	916	11,075	48,731	**11.2**	134.8	**4.4**
U50712	MCP-5	5	5	2,053	5,021	1.0	410.5	**2.4**
	Others							
X68680	AMBP	389.9	265.8	37.8	1,112.2	0.7	0.1	**29.4**
AV327201	Mannosidase 2, αb1	1,149	5	74	1,400	0.0	0.1	**18.9**
AF030169	UNC-119	2,171	1,211	453	1,285	0.6	0.2	**2.8**
AV364086	Thrombomodulin	265	509	55	2,069	1.9	0.2	**37.6**
AV326295	Glutamate receptor, ionotropic	947	2,083	291	1,574	**2.2**	0.3	**5.4**
L39017	Protein C receptor, endothelial	674	550	396	2,803	0.8	0.6	**7.1**
M27960	IL-4Rα	9,914	29,812	6,335	18,652	**3.0**	0.6	**2.9**
U43320	Frizzled homolog 7	701	1646	571	783	**2.3**	0.8	1.4
L07264	Epidermal growth factor-like growth factor	659	426	603	2,106	0.6	0.9	**3.5**
D31863	Phosphatidylinositol glycan, class A	309	422	284	1,025	1.4	0.9	**3.6**
M63801	Connexin 43	1,166	3,166	1,635	10,228	**2.7**	1.4	**6.3**
Z18278	Serotonin receptor 5A	681	2,394	1,206	2,001	**3.5**	1.8	1.7
K02236	Metallothionein 2	13,679	28,430	33,137	108,739	**2.1**	2.4	**3.3**
X03505	Serum amyloid A3	1,460	2,347	6,174	28,674	1.6	4.2	**4.6**
AV374010	Bone morphogenetic protein-2 homolog	105	872	588	248	**8.3**	5.6	0.4
AI849193	EST	809	1,897	337	508	**2.3**	0.4	1.5
AA189677	EST	2,016	646	848	1,780	0.3	0.4	**2.1**
AI596360	EST	206	945	140	317	**4.6**	0.7	**2.3**
AI840413	EST	1,455	3,844	1,863	469	**2.6**	1.3	0.3
AA980618	EST	470	1,243	908	1,124	**2.6**	1.9	1.2
AI550400	EST	296	1,247	720	543	**4.2**	2.4	0.8
C81072	EST	157	966	442	145	**6.1**	2.8	0.3
AA710132	EST	415	1,381	1,320	1,438	**3.3**	3.2	1.1
AI849679	EST	68	1,247	317	1,689	**18.4**	4.7	**5.3**
AA645293	EST	18	1,163	376	577	**64.4**	20.8	1.5

[a] Data are from two independent experiments and were analyzed as described in *Materials and Methods.* Genes shown were induced >2-fold in both experiments.
[b] Mean of normalized average differences from two experiments.
[c] Ratio of mean average differences; numbers in bold represent genes induced by IL-10 or IL-10 + LPS >2-fold.
[d] Relative to media sample.
[e] Relative to LPS sample.

that most, if not all, IL-10 signal transduction proceeds through STAT3. Despite this knowledge, major gaps remain in understanding how IL-10 exerts its anti-inflammatory effects. For example, we do not know how STAT3 mediates the IL-10 signal, nor whether the effects of STAT3 are direct or via the synthesis of other proteins. We do not know how IL-10 regulates the large number of physiologically relevant targets that have been described and whether this is through a common mechanism or if diverse pathways are involved. Our study was designed to address two fundamental questions concerning IL-10 action. First, what spectrum of genes are induced by IL-10 with or without a concomitant inflammatory stimulus and second, what is the range of inflammatory genes regulated by IL-10?

Genes induced by IL-10

We found that IL-10 induced a highly restricted number of genes in resting macrophages. In LPS-activated macrophages, IL-10 induced a different set of genes. It is tempting to speculate that key anti-inflammatory genes may be represented in this latter group because the effects of IL-10 on macrophages are most clearly demonstrated with concomitant activation. Two independent signals from the IL-10R and a TLR may be necessary to engender the full range of anti-inflammatory mechanisms.

Several genes regulated by IL-10 with or without concomitant LPS stimulation warrant further investigation for their potential role in IL-10 signaling. One obvious candidate is SOCS3, a member

Table II. *Genes induced after 3 h by IL-10 in resting and LPS-stimulated macrophages*[a]

Identifier	Name/Description	Mean Average Intensity Difference[b]				Fold Change[c]			
		Media	IL-10	LPS	IL-10 + LPS	IL-10[d]	LPS[d]	IL-10 + LPS/LPS	IL-10 + LPS/media
	Cell surface molecules								
U05673	Adenosine receptor subtype	81	115	1,250	4,406	1.4	15.4	**3.5**	54.4
U29678	CCR1	511	1,953	346	1,012	**3.8**	0.7	**2.9**	2.0
X94151	CCR5	3,706	9,111	2,270	3,592	**2.5**	0.6	1.6	1.0
AF022990	CCR5	6,180	13,936	2,937	5,261	**2.3**	0.5	1.8	0.9
M31312	FcRII	4,323	15,731	8,993	38,122	**3.6**	2.1	**4.2**	8.8
U05894	G-CSFR	1,291	2,480	1,881	8,019	1.9	1.5	**4.3**	6.2
M58288	G-CSFR	2,812	4,260	2,354	16,341	1.5	0.8	**6.9**	5.8
M27960	IL-4R	12,887	64,870	5,136	100,141	**5.0**	0.4	**19.5**	7.8
M59446	Scavenger receptor	2,152	3,135	5,172	10,961	1.5	2.4	**2.1**	5.1
M72332	Selectin, platelet	5	1,908	5	15,816	**381.5**	0.9	**3,464.8**	3,163.2
Y09864	Type I IFN receptor, IFNaR2b	10,295	12,915	10,147	24,672	1.3	1.0	**2.4**	2.4
	Signal transduction-related								
M90397	Bcl-3	864	7,045	5,891	20,388	**8.2**	6.8	**3.5**	23.6
M72394	Calcium-dependent phospholipid binding protein (PLA2)	926	1,429	2,425	5,725	1.5	2.6	**2.4**	6.2
X52191	Fgr oncogene homolog	1,073	2,281	1,551	3,696	**2.1**	1.4	**2.4**	3.4
X81627	GIT1	508	895	2,632	18,081	1.8	5.2	**6.9**	35.6
AI046826	Growth factor receptor bound protein 2-associated protein	3,149	3,277	5	2,565	1.0	0.0	**561.9**	0.8
AF001871	Guanine nucleotide exchange factor and integrin binding protein homolog GRP1	282	463	344	1,354	1.6	1.2	**3.9**	4.8
U43187	MEK kinase 3	2,535	2,704	1,110	2,524	1.1	0.4	**2.3**	1.0
U88984	Nck-interacting kinase	1,305	1,820	1,325	3,484	1.4	1.0	**2.6**	2.7
AI846534	Noninherited maternal Ag-related expressed kinase 6	1,708	1,992	1,870	9,766	1.2	1.1	**5.2**	5.7
U57686	p130 Cas-related protein Sin	741	381	450	1,510	0.5	0.6	**3.4**	2.0
U87814	Proline-glutamic acid-serine-threonine phosphatase interacting protein	13,746	18,455	3,048	8,256	1.3	0.2	**2.7**	0.6
U18310	Stress-activated protein kinase/extracellular signal-regulated kinase/kinase 1	815	1,361	2,194	5,266	1.7	2.7	**2.4**	6.5
U58885	Src homology 3 domain protein 2B	3,583	4,728	1,818	9,601	1.3	0.5	**5.3**	2.7
U10531	Ski/sno related	44	847	2,589	1,713	**19.4**	59.2	0.7	39.2
U88328	SOCS-3	82	6,265	62,180	106,493	**76.0**	754.6	1.7	1,292.4
AV374868	SOCS-3	1,115	4,567	32,356	53,464	**4.1**	29.0	1.7	47.9
M97590	Tyrosine phosphatase (PTP-1)	17,006	35,503	8,341	53,019	**2.1**	0.5	**6.4**	3.1
X64361	*vav*	11,037	17,194	2,093	14,602	1.6	0.2	**7.0**	1.3
D83266	*vav*-T	11,105	17,060	2,389	13,600	1.5	0.2	**5.7**	1.2
	DNA binding, transcription								
AF017021	B-ATF	617	1,260	4,055	17,689	2.0	6.6	**4.4**	28.7
U41465	Bcl-6	5,594	6,949	2,947	9,782	1.2	0.5	**3.3**	1.7
M61007	C/EBPβ	13,415	22,224	14,486	70,386	1.7	1.1	**4.9**	5.2
X58250	H2.0-like homeo box gene	2,438	2,851	532	7,276	1.2	0.2	**13.7**	3.0
Y15907	*myc*-intron-binding protein-1	193	595	1,509	5,726	**3.1**	7.8	**3.8**	29.6
U60593	*N-myc* downstream regulated 1	5,249	16,126	5,400	7,289	**3.1**	1.0	1.3	1.4
U83148	NFIL3/E4BP4 transcription factor	2,130	3,584	2,650	6,441	1.7	1.2	**2.4**	3.0
U52073	TDD5	5,638	16,884	5,271	7,942	**3.0**	0.9	1.5	1.4
AF062567	Transcription factor Sp3	5,092	5,410	1,015	2,898	1.1	0.2	**2.9**	0.6
AJ245617	TAT-binding protein-related factor-proximal protein homolog (*Drosophila*)	1,743	1,663	219	797	1.0	0.1	**3.6**	0.5
AF062071	Zinc finger protein 216	8,195	9,974	5,564	21,379	1.2	0.7	**3.8**	2.6
	Cytokines, chemokines, growth factors								
M13926	G-CSF	497	1,537	366	2,673	**3.1**	0.7	**7.3**	5.4
V00755	IFN-β, fibroblast	5,859	7,687	6,385	49,603	1.3	1.1	**7.8**	8.5
AB023418	MCP-2	606	1,908	4,040	3,566	**3.1**	6.7	0.9	5.9
M29464	Platelet-derived growth factor α	4,476	4,708	2,300	7,948	1.1	0.5	**3.5**	1.8
	Enzymes								
X58077	2-5 A synthetase L2	1,234	1,760	145	2,871	1.4	0.1	**19.7**	2.3
D28941	α-2,3-sialyltransferase	1,813	4,499	646	3,412	**2.5**	0.4	**5.3**	1.9
AF032466	Arginase II	593	645	628	4,251	1.1	1.1	**6.8**	7.2
M25944	Carbonic anhydrase II (CAII)	588	430	601	1,607	0.7	1.0	**2.7**	2.7
U48403	Glycerol kinase	2,745	6,951	9,744	15,201	**2.5**	3.5	1.6	5.5

(*Table continues*)

[a] Data are from two independent experiments and were analyzed as described in *Materials and Methods.* Genes shown were induced >2-fold in both experiments.
[b] Mean of normalized average differences from two experiments.
[c] Ratio of mean average differences; numbers in bold represent genes induced by IL-10 or IL-10 + LPS >2-fold.
[d] Relative to media sample.

Table II. *Continued*

Identifier	Name/Description	Mean Average Intensity Difference[b]				Fold Change[c]			
		Media	IL-10	LPS	IL-10 + LPS	IL-10[d]	LPS[d]	IL-10 + LPS/LPS	IL-10 + LPS/media
M85153	Glycoprotein galactosyltransferase α 1,3	15,278	20,347	2,251	33,421	1.3	0.1	**14.8**	2.2
AI021573	Inducible 6-phosphofructokinase	475	624	1,526	8,738	1.3	3.2	**5.7**	18.4
AF040094	Inositol polyphosphate-5-phosphatase, 75 kDa	585	3,866	10,768	10,334	**6.6**	18.4	1.0	17.7
U43428	NO synthase 2	116	5	3,277	9,072	0.0	28.3	**2.8**	78.4
AB013912	RuvB-like protein (DNA-helicase)	2,102	2,212	189	1,283	1.1	0.1	**6.8**	0.6
L32836	S-adenosyl homocysteine hydrolase	1,572	2,064	493	1,920	1.3	0.3	**3.9**	1.2
	Others								
Z22661	Apolipoprotein CI	172	708	581	522	**4.1**	3.4	0.9	3.0
X75926	ATP-binding cassette 1	3,706	3,943	1,318	4,398	1.1	0.4	**3.3**	1.2
AF032459	BimEL	250	1,032	2,520	3,225	**4.1**	10.1	1.3	12.9
U04827	Brain fatty acid-binding protein	884	742	401	2,956	0.8	0.5	**7.4**	3.3
Y13087	Caspase-6	536	2,089	42	802	**3.9**	0.1	**19.3**	1.5
U49430	Ceruloplasmin	702	339	3,088	12,972	0.5	4.4	**4.2**	18.5
D45889	Chondroitin sulfate proteoglycan 2	5	808	3,773	5,022	**161.5**	754.6	1.3	1,004.3
M12660	Complement component factor h	3,027	4,593	419	1,855	1.5	0.1	**4.4**	0.6
M63801	Connexin 43	1,536	11,391	6,542	32,569	**7.4**	4.3	**5.0**	21.2
X75888	Cyclin E	5	437	385	1,777	**87.3**	77.0	**4.6**	355.5
U80227	ELL homolog	2,334	2,797	467	1,693	1.2	0.2	**3.6**	0.7
X77952	Endoglin	9,078	8,690	5,892	14,405	1.0	0.6	**2.4**	1.6
X60671	Ezrin	2,737	2,143	3,530	13,096	0.8	1.3	**3.7**	4.8
U18975	Ganglioside expression 2	4,717	5,379	1,366	3,558	1.1	0.3	**2.6**	0.8
X67644	gly96	18,715	29,093	16,430	43,316	1.6	0.9	**2.6**	2.3
X93035	gp39	5	5	45	3,839	1.0	8.9	**86.2**	767.7
X61940	Growth factor-inducible immediate early gene (3CH134)	2,879	4,655	4,088	36,307	1.6	1.4	**8.9**	12.6
AB028272	hsp40	1,899	1,417	406	1,388	0.7	0.2	**3.4**	0.7
D12645	Kinesin family member 3a	1,027	2,272	797	811	**2.2**	0.8	1.0	0.8
J03484	Laminin, γ1	863	431	275	1,348	0.5	0.3	**4.9**	1.6
AF004874	Latent TGFβ binding protein 2	308	932	349	325	**3.0**	1.1	0.9	1.1
V00835	Metallothionein 1	27,567	45,696	34,920	147,598	1.7	1.3	**4.2**	5.4
K02236	Metallothionein 2	13,559	37,167	62,364	184,092	**2.7**	4.6	**3.0**	13.6
U18869	Mitogen-responsive 96 kDa phosphoprotein p96	22,519	54,412	17,378	46,969	**2.4**	0.8	**2.7**	2.1
AV206059	Motilin-related peptide	14	60	616	4,700	**4.3**	43.7	**7.6**	333.3
AV373134	Mpv17 transgene, kidney disease mutant-like	276	1,822	1,418	1,039	**6.6**	5.1	0.7	3.8
X83601	Pentaxin-related gene	5	84	799	2,874	**16.8**	159.8	**3.6**	574.8
X16490	Plasminogen activator inhibitor, type II	324	134	3,315	9,036	0.4	10.2	**2.7**	27.9
U74079	Protein cofactor	15,688	14,753	8,289	29,658	0.9	0.5	**3.6**	1.9
X92410	RAD23a homolog (*Saccharomyces cerevisiae*)	466	1,302	950	856	**2.8**	2.0	0.9	1.8
AF036585	Semaphorin VIb	130	1,348	160	222	**10.3**	1.2	1.4	1.7
U60438	Serum amyloid A2	801	866	4,884	23,821	1.1	6.1	**4.9**	29.7
X03505	Serum amyloid A3	2,559	4,708	71,095	254,687	1.8	27.8	**3.6**	99.5
M22998	Solute carrier family 2 (facilitated glucose transporter), member 1	1,843	1,463	2,212	6,872	0.8	1.2	**3.1**	3.7
AF004100	Zinc transporter 4	115	1,078	2,491	2,059	**9.3**	21.6	0.8	17.9
	ESTs								
AA178600	EST IMAGE-621302	8,580	9,311	772	2,315	1.1	0.1	**3.0**	0.3
AA575098	EST IMAGE-893535	1,436	1,207	306	899	0.8	0.2	**2.9**	0.6
AA608277	EST IMAGE-1025734	152	547	2,742	10,286	**3.6**	18.1	**3.8**	67.8
AA608387	EST IMAGE-1052539	763	696	707	1,686	0.9	0.9	**2.4**	2.2
AA673486	EST IMAGE-1080031	566	3,217	2,055	1,947	**5.7**	3.6	0.9	3.4
AA690863	EST IMAGE-1164753	1,907	2,812	2,500	6,771	1.5	1.3	**2.7**	3.6
AA762522	EST IMAGE-1229091	1,883	2,230	2,521	9,806	1.2	1.3	**3.9**	5.2
AA763368	EST IMAGE-1247550	8,061	11,104	14,112	33,404	1.4	1.8	**2.4**	4.1
AA763950	EST IMAGE-1225256	254	735	1,018	3,212	**2.9**	4.0	**3.2**	12.6
AA815503	EST IMAGE-1069161	3,831	4,497	1,102	2,695	1.2	0.3	**2.4**	0.7
AA874490	EST IMAGE-1263336	2,775	5,944	2,475	15,847	**2.1**	0.9	**6.4**	5.7
AA983101	EST IMAGE-1348693	5	5	5	3,681	1.0	0.9	**806.4**	736.2
AI060798	EST IMAGE-1380562	318	719	5,781	24,828	**2.3**	18.2	**4.3**	78.1
AI286698	EST IMAGE-1395161	3,412	4,391	2,743	7,399	1.3	0.8	**2.7**	2.2
AI317205	EST IMAGE-1853344	9,571	11,122	1,065	3,147	1.2	0.1	**3.0**	0.3
AI429868	EST IMAGE-1228437	453	1,298	233	145	**2.9**	0.5	0.6	0.3
AI642662	EST IMAGE-1230637	432	1,021	2,872	8,958	**2.4**	6.7	**3.1**	20.7
AI647003	EST IMAGE-1382920	1,492	1,203	4,125	12,080	0.8	2.8	**2.9**	8.1
AI787183	EST IMAGE-1889276	4,033	2,743	1,494	5,193	0.7	0.4	**3.5**	1.3
AW214298	EST IMAGE-2644954	3,183	4,646	2,591	6,803	1.5	0.8	**2.6**	2.1

(*Table continues*)

Table II. *Continued*

Identifier	Name/Description	Mean Average Intensity Difference[b]				Fold Change[c]			
		Media	IL-10	LPS	IL-10 + LPS	IL-10[d]	LPS[d]	IL-10 + LPS/LPS	IL-10 + LPS/media
AI837621	EST	328	1,750	371	913	**5.3**	1.1	**2.5**	2.8
AI838320	EST	2,844	6,772	1,137	5,117	**2.4**	0.4	**4.5**	1.8
AI838360	EST	78	619	344	2,457	**7.9**	4.4	**7.1**	31.5
AI838388	EST	2,670	3,723	711	2,044	1.4	0.3	**2.9**	0.8
AI841159	EST	5,439	2,453	94	966	0.5	0.0	**10.3**	0.2
AI841410	EST	206	233	359	1,277	1.1	1.7	**3.6**	6.2
AI843287	EST	19,734	21,818	24,345	76,837	1.1	1.2	**3.2**	3.9
AI843709	EST	2,965	5,066	976	2,445	1.7	0.3	**2.5**	0.8
AI844507	EST	432	1,478	902	1,228	**3.4**	2.1	1.4	2.8
AI846077	EST	1,370	2,493	888	3,280	1.8	0.6	**3.7**	2.4
AI846938	EST	1,907	4,353	1,102	2,826	**2.3**	0.6	**2.6**	1.5
AI848522	EST	400	1,546	109	362	**3.9**	0.3	**3.3**	0.9
AI849082	EST	17,046	27,135	8,270	48,721	1.6	0.5	**5.9**	2.9
AI849939	EST	42	690	5	1,803	**16.4**	0.1	**395.0**	42.9
AI852608	EST	216	540	2,714	9,448	**2.5**	12.6	**3.5**	43.8
AV245111	EST	1,395	1,185	706	1,594	0.8	0.5	**2.3**	1.1
AV245978	EST	4,030	5,924	1,425	4,227	1.5	0.4	**3.0**	1.0
AV343395	EST	352	1,262	241	1,054	**3.6**	0.7	**4.4**	3.0
AW046452	EST	4,089	2,577	738	1,852	0.6	0.2	**2.5**	0.5
AW048233	EST	1,999	2,292	534	1,514	1.1	0.3	**2.8**	0.8
AW049619	EST	1,347	1,886	349	973	1.4	0.3	**2.8**	0.7
AW049806	EST	9,863	12,920	2,572	9,964	1.3	0.3	**3.9**	1.0
AW060401	EST	3,201	5,754	1,457	5,417	1.8	0.5	**3.7**	1.7
AW060951	EST	1,240	4,610	492	5,756	**3.7**	0.4	**11.7**	4.6
AW121051	EST	383	1,703	1,096	1,324	**4.5**	2.9	1.2	3.5
AW121294	EST	120	174	409	3,309	1.5	3.4	**8.1**	27.6
AW121304	EST	265	867	793	813	**3.3**	3.0	1.0	3.1
AW121353	EST	3,840	4,556	2,243	7,203	1.2	0.6	**3.2**	1.9
AW122572	EST	6,279	7,004	2,158	4,665	1.1	0.3	**2.2**	0.7
AW123191	EST	1,728	16,478	3,968	13,930	**9.5**	2.3	**3.5**	8.1
AW123694	EST	3,983	4,230	1,102	3,329	1.1	0.3	**3.0**	0.8
AW123921	EST	14,505	27,744	5,780	19,461	1.9	0.4	**3.4**	1.3
AW124785	EST	3,804	5,770	629	2,604	1.5	0.2	**4.1**	0.7
AW124874	EST	3,281	3,549	1,448	3,691	1.1	0.4	**2.5**	1.1
AW124933	EST	3,080	2,334	507	1,221	0.8	0.2	**2.4**	0.4

of the SOCS family that inhibits cytokine receptor signaling by binding phosphotyrosine residues on key signaling molecules and targeting them for destruction via its ubiquitin E3 ligase activity (36). SOCS3 binds to gp130 (37, 38), the signaling component of IL-6 family cytokine receptors (39), but may have additional targets, as overexpression studies have shown that it can inhibit

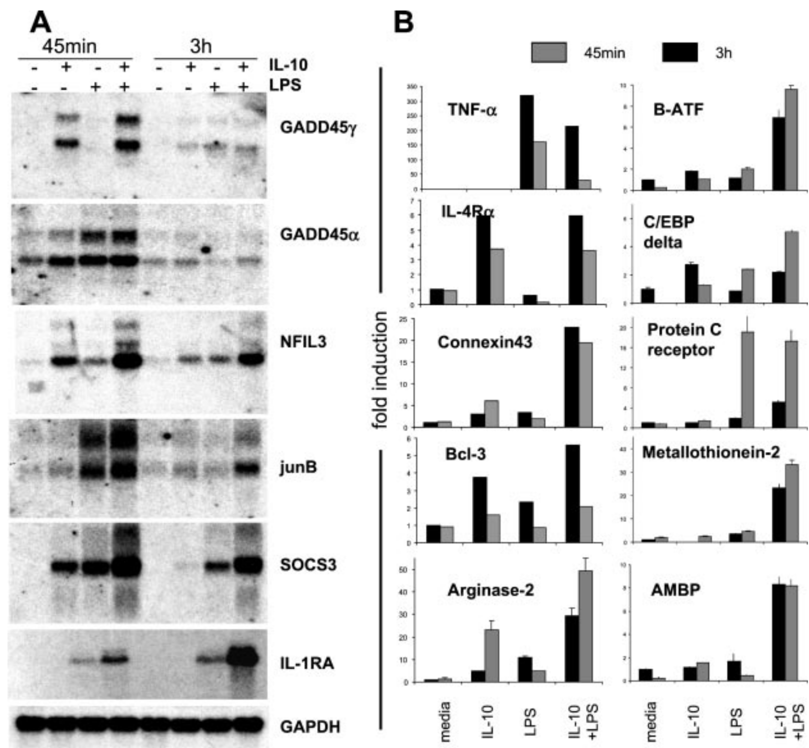


FIGURE 4. Validation of differential gene expression induced by IL-10 and/or LPS by Northern analysis and real-time quantitative RT-PCR. IL-$10^{-/-}$ BMDM were stimulated for 45 min and 3 h as indicated, followed by preparation of RNA. Northern blotting (*A*) and real-time quantitative RT-PCR (*B*) were done as described in *Materials and Methods*. Data are mean + SD of duplicate samples. Note the differing scales of the ordinate in each case.

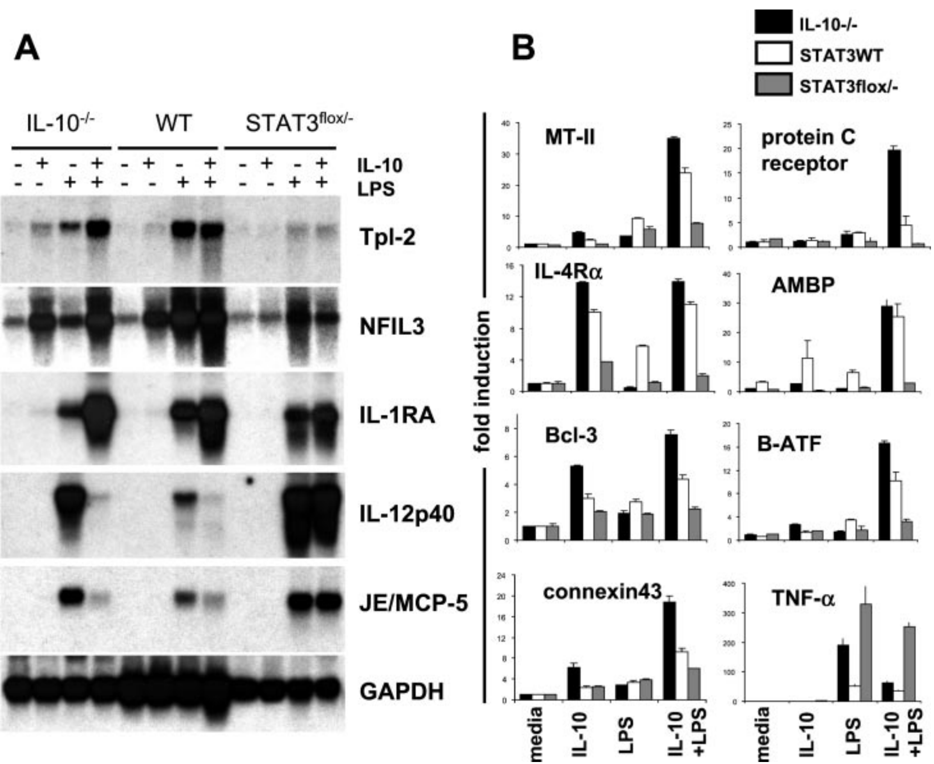


FIGURE 5. Role of STAT3 in the regulation of selected target genes by IL-10 and LPS. BMDM from IL-$10^{-/-}$, $STAT3^{flox/-}$ LysMcre and $STAT3^{+/+}$ LysMcre mice were stimulated for 2 h with IL-10 (10 ng/ml) and/or LPS (100 ng/ml), followed by preparation of RNA. Samples were analyzed by Northern blotting (*A*) or real-time RT-PCR (*B*) as described in *Materials and Methods*. Data are mean + SD of duplicate samples.

IFN-γ signaling (20). It has even been speculated that IL-10-induced SOCS3 might inhibit LPS-induced p38 MAPK signaling and thereby interfere with TNF-α mRNA translation (6). The question of whether SOCS3 indeed plays a role in the control of macrophage activation by IL-10 could best be answered using SOCS3-deficient macrophages. Because SOCS3-deficient embryos die in mid-gestation (40), we are in the process of generating radiation chimeras to perform such experiments in the near future. Other IL-10 targets also give potential clues to the anti-inflammatory effects of IL-10. Among these, three genes encoding proteins involved in MAPK and related pathways, GADD45α, GADD45γ, and Tpl-2, suggest that a focus of IL-10 signaling research should be on these pathways. The role of IL-10 in regulating MAPK and related pathways is presently a controversial topic and requires clarification at the molecular level (1, 6, 41).

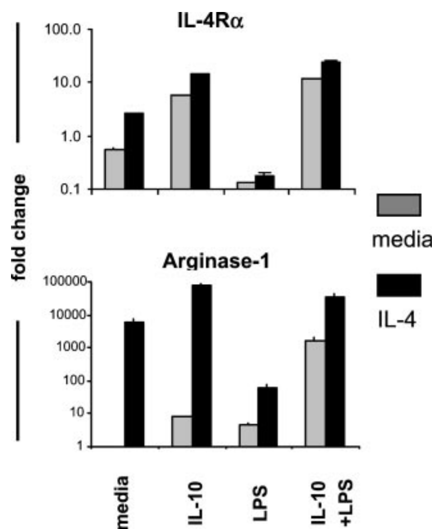


FIGURE 6. IL-10- and LPS-induced changes in IL-4Rα expression correlate with inducibility of arginase-1 expression by IL-4. IL-$10^{-/-}$ BMDM were pretreated with IL-10 and/or LPS for 30 min, followed by addition of IL-4 (1 ng/ml) (■) or media (▨). Total RNA was prepared 3 h and 8 h after addition of IL-4. After reverse transcription, cDNA was subjected to real-time quantitative RT-PCR for β-actin, IL-4Rα, and arginase-1. Fold changes relative to the 8 h media sample were calculated as stated in *Materials and Methods*. Data for IL-4Rα and arginase-1expression are from 3 and 8 h timepoints, respectively. Shown are mean + SD of duplicate samples.

Global effects of IL-10 on proinflammatory gene expression induced by LPS

We found that IL-10 regulates a large number of LPS-induced genes. Our study confirmed the inhibition by IL-10 of many previously reported proinflammatory gene products (Fig. 3) and extended this finding at the genomic level with the identification of numerous new IL-10-repressed genes. Although IL-10 repressed the expression of a large fraction of LPS-induced genes, a further fraction (~40%) remained unchanged. This result suggests two important interpretations that contribute to understanding the anti-inflammatory effects of IL-10. First, the large number of LPS-induced genes inhibited by IL-10 suggests a common mechanism is operative. It is unlikely that IL-10 induces a different mediator for each inflammatory target. We favor the interpretation that IL-10 regulates a limited group of gene products (transcriptionally and/or posttranscriptionally) that subsequently regulate the inflammatory targets. Second, it is unlikely that IL-10 targets more global cell processes such as transcriptional initiation, because of the large fraction of LPS-induced genes unaffected by the addition of IL-10. Therefore, the IL-10-induced anti-inflammatory mechanism is specific enough to target a fraction of LPS-induced genes while leaving others unaffected.

STAT3 is essential for all observed effects of IL-10

Previous work has suggested that STAT3 is crucial for IL-10 signaling (11, 42). This is most clearly shown in mice lacking STAT3 in macrophages and neutrophils which have a strikingly similar

phenotype to IL-10-deficient mice (11). Other work using dominant-negative versions of STAT3 in macrophage cell lines suggested that IL-10 signals via STAT3-dependent and -independent pathways (43). Using STAT3-deficient macrophages, we show that all IL-10-induced genes tested require STAT3 and that inhibition of gene expression of proinflammatory targets also requires STAT3 signaling (Fig. 5). Although we cannot rule out some STAT3-independent effects for IL-10, our results suggest that STAT3 is essential for most, if not all, IL-10 signaling.

IL-10 controls macrophage arginase expression in response to IL-4 and LPS

In addition to identifying candidate mediators of IL-10′s deactivating effects, we also expected to find genes affecting macrophage function in other ways. The up-regulation of IL-4Rα expression by IL-10 in a STAT3-dependent manner caught our attention, because it implied a possibly enhanced responsiveness to IL-4 as a functional consequence of exposure to IL-10. In fact, we observed that opposing changes in IL-4Rα expression induced by IL-10 or LPS in IL-10-deficient macrophages were linked to corresponding changes in the expression level of arginase-1 in response to IL-4 (Fig. 6). These observations offer a mechanistic explanation for the previously described synergistic induction of arginase-1 expression by IL-4 and IL-10 (35). Further, the microarray experiments also showed that expression of the extrahepatic isoform arginase-2 in LPS-stimulated macrophages (33) is controlled by IL-10 (Table II, Fig. 4). Because the ability to make IL-10 also determines expression of arginase-1 in response to LPS (Fig. 6) or the combination of TNF-α and IFN-γ (34), IL-10 increases total arginase levels in macrophages in multiple ways.

Concluding remarks

Certain caveats are evident in a microarray study of this nature. The most significant are the timepoints chosen for data analysis. In this study, the two timepoints chosen were based upon the well-recognized effects of IL-10 on proinflammatory mediator production. Thus, we focused on 45 min and 3 h as a representative window where the expression of TNF-α and several other cytokines and chemokines is substantially reduced in in vitro macrophage culture and in in vivo models where mice are challenged with TLR agonists. However, it is clear that IL-10 can have later effects that may be mediated by distinct mechanisms (41) and even have proinflammatory effects (44). A second caveat is that we can only observe changes in mRNA levels and IL-10 may induce a plethora of cellular changes at the proteome level that also contribute to its anti-inflammatory effects. Despite these limitations, our study has revealed several aspects of IL-10 function not previously appreciated. Understanding the rules that govern the IL-10-mediated shaping of the macrophage transcriptome and its subsequent influence on the proteome will provide insights into the endogenous anti-inflammatory response.

Acknowledgments

We thank Irmgard Förster for the gift of STAT3$^{flox/-}$ LysMcre mice, Andrew Pappas and Deepak Kaushal for help with software, and Martine Roussel for critical appraisal of the manuscript.

References

1. Donnelly, R. P., H. Dickensheets, and D. S. Finbloom. 1999. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J. Interferon Cytokine Res. 19:563.*
2. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol. 19:683.*
3. Fedorak, R. N., A. Gangl, C. O. Elson, P. Rutgeerts, S. Schreiber, G. Wild, S. B. Hanauer, A. Kilian, M. Cohard, A. LeBeaut, and B. Feagan. 2000. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease: the interleukin 10 inflammatory bowel disease cooperative study group. *Gastroenterology 119:1473.*
4. Nelson, D. R., G. Y. Lauwers, J. Y. Lau, and G. L. Davis. 2000. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. *Gastroenterology 118:655.*
5. Schottelius, A. J., M. W. Mayo, R. B. Sartor, and A. S. Baldwin, Jr. 1999. Interleukin-10 signaling blocks inhibitor of κB kinase activity and nuclear factor κB DNA binding. *J. Biol. Chem. 274:31868.*
6. Kontoyiannis, D., A. Kotlyarov, E. Carballo, L. Alexopoulou, P. J. Blackshear, M. Gaestel, R. Davis, R. Flavell, and G. Kollias. 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J. 20:3760.*
7. Donnelly, R. P., S. L. Freeman, and M. P. Hayes. 1995. Inhibition of IL-10 expression by IFN-γ up-regulates transcription of TNF-α in human monocytes. *J. Immunol. 155:1420.*
8. Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-β and interleukin-10. *J. Biol. Chem. 267:23301.*
9. Dickensheets, H. L., S. L. Freeman, M. F. Smith, and R. P. Donnelly. 1997. Interleukin-10 upregulates tumor necrosis factor receptor type-II (p75) gene expression in endotoxin-stimulated human monocytes. *Blood 90:4162.*
10. Murray, P. J., L. Wang, C. Onufryk, R. I. Tepper, and R. A. Young. 1997. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *J. Immunol. 158:315.*
11. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of stat3 in macrophages and neutrophils. *Immunity 10:39.*
12. Rodig, S. J., M. A. Meraz, J. M. White, P. A. Lampe, J. K. Riley, C. D. Arthur, K. L. King, K. C. Sheehan, L. Yin, D. Pennica, et al. 1998. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell 93:373.*
13. Aste-Amezaga, M., X. Ma, A. Sartori, and G. Trinchieri. 1998. Molecular mechanisms of the induction of IL-12 and its inhibition by IL- 10. *J. Immunol. 160: 5936.*
14. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263.*
15. Schroder, A. J., P. Pavlidis, A. Arimura, D. Capece, and P. B. Rothman. 2002. Cutting edge: Stat6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. *J. Immunol. 168:996.*
16. Boldrick, J. C., A. A. Alizadeh, M. Diehn, S. Dudoit, C. L. Liu, C. E. Belcher, D. Botstein, L. M. Staudt, P. O. Brown, and D. A. Relman. 2002. Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc. Natl. Acad. Sci. USA 99:972.*
17. Ehrt, S., D. Schnappinger, S. Bekiranov, J. Drenkow, S. Shi, T. R. Gingeras, T. Gaasterland, G. Schoolnik, and C. Nathan. 2001. Reprogramming of the macrophage transcriptome in response to interferon-γ and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J. Exp. Med. 194:1123.*
18. Nau, G. J., J. F. Richmond, A. Schlesinger, E. G. Jennings, E. S. Lander, and R. A. Young. 2002. Human macrophage activation programs induced by bacterial pathogens. *Proc. Natl. Acad. Sci. USA 99:1503.*
19. Ito, S., P. Ansari, M. Sakatsume, H. Dickensheets, N. Vazquez, R. P. Donnelly, A. C. Larner, and D. S. Finbloom. 1999. Interleukin-10 inhibits expression of both interferon α- and interferon γ-induced genes by suppressing tyrosine phosphorylation of Stat1. *Blood 93:1456.*
20. Stoiber, D., P. Kovarik, S. Cohney, J. A. Johnston, P. Steinlein, and T. Decker. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-γ. *J. Immunol. 163:2640.*
21. Dalpke, A. H., S. Opper, S. Zimmermann, and K. Heeg. 2001. Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J. Immunol. 166:7082.*
22. Papouli, E., M. Defais, and F. Larminat. 2002. Overexpression of metallothionein-II sensitizes rodent cells to apoptosis induced by DNA cross-linking agent through inhibition of NF-κB activation. *J. Biol. Chem. 277:4764.*
23. Brasier, A. R., M. Lu, T. Hai, Y. Lu, and I. Boldogh. 2001. NF-κB-inducible Bcl-3 expression is an autoregulatory loop controlling nuclear p50/NF-κB1 residence. *J. Biol. Chem. 276:32080.*
24. Lu, B., H. Yu, C. Chow, B. Li, W. Zheng, R. J. Davis, and R. A. Flavell. 2001. Gadd45γ mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector Th1 cells. *Immunity 14:583.*
25. Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan, and S. C. Ley. 1996. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO J. 15:817.*
26. Lin, X., E. T. Cunningham, Jr., Y. Mu, R. Geleziunas, and W. C. Greene. 1999. The proto-oncogene cot kinase participates in CD3/CD28 induction of NF-κB acting through the NFκB-inducing kinase and IκB kinases. *Immunity 10:271.*
27. Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias, and P. N. Tsichlis. 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell 103:1071.*
28. Zhang, W., J. Zhang, M. Kornuc, K. Kwan, R. Frank, and S. D. Nimer. 1995. Molecular cloning and characterization of NFIL3a, a transcriptional activator of the human interleukin-3 promoter. *Mol. Cell. Biol. 15:6055.*
29. Cowell, I. G., A. Skinner, and H. C. Hurst. 1992. Transcriptional repression by a novel member of the bZIP family of transcription factors. *Mol. Cell. Biol. 12:3070.*

30. Goerdt, S., and C. E. Orfanos. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity 10:137.*
31. Modolell, M., I. M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by Th1 and Th2 cytokines. *Eur. J. Immunol. 25: 1101.*
32. Rutschman, R., R. Lang, M. Hesse, J. N. Ihle, T. A. Wynn, and P. J. Murray. 2001. Stat6-dependent substrate depletion regulates nitric oxide production. *J. Immunol. 166:2173.*
33. Salimuddin, A. Nagasaki, T. Gotoh, H. Isobe, and M. Mori. 1999. Regulation of the genes for arginase isoforms and related enzymes in mouse macrophages by lipopolysaccharide. *Am. J. Physiol. 277:E110.*
34. Munder, M., K. Eichmann, J. M. Moran, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol. 163:3771.*
35. Munder, M., K. Eichmann, and M. Modolell. 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by $CD4^+$ T cells correlates with Th1/Th2 phenotype. *J. Immunol. 160:5347.*
36. Kile, B. T., B. A. Schulman, W. S. Alexander, N. A. Nicola, and D. J. Hilton. 2002. Opening Pandora's SOCS box–a tale of destruction and degradation. *Trends Biochem. Sci. 27:235.*
37. Nicholson, S. E., D. De Souza, L. J. Fabri, J. Corbin, T. A. Willson, J. G. Zhang, A. Silva, M. Asimakis, A. Farley, A. D. Nash, et al. 2000. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc. Natl. Acad. Sci. USA 97:6493.*
38. Schmitz, J., M. Weissenbach, S. Haan, P. C. Heinrich, and F. Schaper. 2000. Socs3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP-2 recruitment site of gp130. *J. Biol. Chem. 275:12848.*
39. Heinrich, P. C., I. Behrmann, G. Muller-Newen, F. Schaper, and L. Graeve. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/Stat pathway. *Biochem. J. 334:297.*
40. Marine, J. C., C. McKay, D. Wang, D. J. Topham, E. Parganas, H. Nakajima, H. Pendeville, H. Yasukawa, A. Sasaki, A. Yoshimura, and J. N. Ihle. 1999. Socs3 is essential in the regulation of fetal liver erythropoiesis. *Cell 98:617.*
41. Denys, A., I. A. Udalova, C. Smith, L. M. Williams, C. J. Ciesielski, J. Campbell, C. Andrews, D. Kwaitkowski, and B. M. Foxwell. 2002. Evidence for a dual mechanism for IL-10 suppression of TNF-α production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-κB in primary human macrophages. *J. Immunol. 168:4837.*
42. Riley, J. K., K. Takeda, S. Akira, and R. D. Schreiber. 1999. Interleukin-10 receptor signaling through the Jak-Stat pathway: requirement for two distinct receptor-derived signals for anti-inflammatory action. *J. Biol. Chem. 274:16513.*
43. O'Farrell, A. M., Y. Liu, K. W. Moore, and A. L. Mui. 1998. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. *EMBO J. 17:1006.*
44. Lauw, F. N., D. Pajkrt, C. E. Hack, M. Kurimoto, S. J. van Deventer, and T. van der Poll. 2000. Proinflammatory effects of IL-10 during human endotoxemia. *J. Immunol. 165:2783.*