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Mycobacterium avium Infection and Modulation of Human Macrophage Gene Expression

Teresa Greenwell-Wild,* **Nancy Vázquez,*** **Davis Sim,*** **Marco Schito,†** **Delphi Chatterjee,‡** **Jan M. Orenstein,§** **and Sharon M. Wahl[1]***

***Mycobacterium avium* is a facultative intracellular pathogen cleared rapidly via intact host defense mechanisms. In the absence of adequate T cell function, as occurs in HIV-1-induced immunodeficiency, *M. avium* becomes an opportunistic infection with uncontrolled replication and reinfection of macrophage hosts. How *M. avium* infects, survives, and replicates in macrophages without signaling an effective microbicidal counterattack is unresolved. To address whether *M. avium* signals the expression of molecules, which influence mycobacterial survival or clearance, human monocyte-derived macrophage cultures were exposed to *M. avium*. Within minutes, *M. avium,* or its cell wall lipoarabinomannan, binds to the adherent macrophages and induces a spectrum of gene expression. In this innate response, the most abundant genes detected within 2 h by cDNA expression array involved proinflammatory chemokines, cytokines including TNF-α and IL-1, and adhesion molecules. Associated with this rapid initial up-regulation of recruitment and amplification molecules was enhanced expression of transcription factors and signaling molecules. By 24 h, this proinflammatory response subsided, and after 4 days, when some bacteria were being degraded, others escaped destruction to replicate within intracellular vacuoles. Under these conditions, inducible NO synthase was not up-regulated and increased transferrin receptors may facilitate iron-dependent mycobacterial growth. Sustained adhesion molecule and chemokine expression along with the formation of multinucleated giant cells appeared consistent with in vivo events. Thus, in the absence of T lymphocyte mediators, macrophages are insufficiently microbicidal and provide a nonhostile environment in which mycobacteria not only survive and replicate, but continue to promote recruitment of new macrophages to perpetuate the infection.   *The Journal of Immunology,* 2002, 169: 6286–6297.**

Mycobacterium avium is an environmental microorganism which also exists in multiple hosts, including humans. Typically nonpathogenic and readily cleared via innate and adaptive host defense pathways, *M. avium* can invade and replicate in immunocompromised hosts with impunity. *M. avium* complex (MAC)[2] prevalence increased dramatically with the emergence of HIV-1-induced immunodeficiency (1, 2), becoming a common opportunistic infection. In a cyclic fashion, *M. avium* also enhances permissiveness for HIV-1 infection (3–6). With the advent of highly active antiretroviral therapies and restoration of CD4^{+} T cell levels, MAC incidence continues to decline (7–9).

However, highly active antiretroviral therapy is not universally available nor always successful, and reports of MAC infection in non-HIV-1 infected individuals, although rare, are increasing (10). HIV-1-immunocompromised individuals may acquire *M. avium* through the intestinal tract where it translocates across the mucosa to enter submucosal macrophages in the lamina propria (11, 12). From the mucosa, dissemination may then occur to a variety of susceptible tissues.

In HIV-1-negative individuals, susceptibility to pulmonary infections often occurs in the context of predisposing lung conditions (10, 13). MAC has been shown to present as pulmonary infections in immunocompetent patients including elderly women and smokers, as well as immunocompromised individuals who have undergone organ transplantation or with inheritable defects in IFN-γ (10, 14). Cutaneous manifestations of MAC in immunocompetent individuals also occur, especially in children (15, 16). All of these manifestations of MAC have in common infiltration by mononuclear cells and granuloma formation.

Although enhanced susceptibility to *M. avium* infection generally reflects reduced T cell numbers and/or functional deficiencies, it is the macrophage that represents the primary host. The interaction between *M. avium* and macrophages, initiated via cell surface receptors, results in subsequent internalization and residence as an intracellular pathogen. The evidence that multiple macrophage receptors may be involved in the initial bacterium-host cell encounter (17), including complement receptors, vitronectin receptors, CD14, mannose receptors, CD43, and Toll-like receptors (TLR) (13, 18–23), underscores the need for *M. avium* to be internalized to survive. Once within the macrophage, this facultative intracellular pathogen must commandeer the host cell machinery to enable its own multiplication and survival. However, the mechanisms by which this is accomplished are largely unknown.

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[2] Abbreviations used in this paper: MAC, *M. avium* complex; TLR, Toll-like receptor; iNOS, inducible NO synthase; MAg, *M. avium* Ag; LAM, lipoarabinomannan; GFP, green fluorescent protein; RPA, ribonuclease protection assay; MAP, mitogen-activated protein; GNB, guanine nucleotide-binding protein; IRF, IFN regulatory factor; MIP, macrophage-inflammatory protein; PLGF, placenta growth factor; MMP, matrix metalloproteinase; MRP, migration inhibitory factor-related protein; MCP, monocyte chemoattractant protein; TIMP, tissue inhibitor of MMP; CAP, cytoplasmic antiproteinase; ADORA, adenosine A1 receptor; PKC, protein kinase C; PDGF, platelet-derived growth factor; PP, protein phosphatase; ITG, integrin; HSP, heat shock protein; GST, glutathione S-transferase; ERBB, epidermal growth factor; IEX, immediate early gene X.

Whereas only a few mycobacteria may initiate the infection, over time, enormous numbers of microorganisms may be found locally or at dissemination sites as a consequence of intracellular replication and reinfection in susceptible macrophages. Little is known regarding how macrophages either kill *M. avium* or become its breeding ground. For example, controversy exists over whether reactive oxygen or nitrogen intermediates are toxic to *M. avium* (24, 25), although inducible NO synthase (iNOS) is considered essential in defense against experimental *Mycobacterium tuberculosis* (26). However, many of these studies have been performed using rodent macrophages or cell lines which do not necessarily parallel human macrophage responses to mycobacteria. To explore the mechanisms whereby *M. avium* enters macrophages and takes up residence in the absence of molecular signals derived from T lymphocytes, notably IFN-γ, we have infected human peripheral blood monocyte-derived macrophages with *M. avium* in vitro, and evaluated host cell gene expression by cDNA expression arrays from 2 h to 7 days postinfection. Multiple genes encoding transcription factors, signal transduction molecules, and proteins involved in regulation of inflammatory and immune responses were differentially expressed. The immediate early response was consistent with immune activation, but this response was reversed once the mycobacteria were safely internalized. By comparing intact mycobacteria, *M. avium* Ag, and purified cell wall lipoarabinomannan (LAM), a TLR2 agonist (23), we identify a complex profile of overlapping gene expression, reflecting involvement of a TLR2 signal cascade.

Materials and Methods

Tissue analysis

Tissue biopsies were obtained with consent from HIV-1-seropositive patients with MAC and from HIV-1-seronegative donors and processed as described (3, 4). *M. avium* was visualized following staining with acid-fast New Fuchsin (Sigma-Aldrich, St. Louis, MO) (3). Glutaraldehyde-fixed tissues or cells were postfixed in OsO_4, dehydrated through graded ethanol and propylene oxide, embedded in Spurr's epoxy, and thick- and thin-sectioned. Thin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and viewed in a Zeiss EM10 microscope (LEO electron microscope; Oberkochen, Germany) (3).

Purification of human monocytes by counterflow centrifugal elutriation

Human peripheral blood cells were obtained by leukapheresis of normal volunteers at the Department of Transfusion Medicine (National Institutes of Health, Bethesda, MD), diluted in endotoxin-free PBS without Ca^{2+} and Mg^{2+} (BioWhittaker, Walkersville, MD) and density-sedimented on lymphocyte separation medium (LSM; ICN Pharmaceuticals, Aurora, OH). The monocytes in the mononuclear cell layer were purified as described (4), suspended in DMEM (BioWhittaker) with 2 mM L-glutamine and 10 μg/ml gentamicin (complete medium), and plated in 6-well (6×10^6 cells/well) or 48-well (1.5×10^6 cells/well) plates (Corning, Cambridge, MA). After an initial adherence for 4–6 h at 37°C in 5% CO_2, 10% human AB⁻ serum (Department of Transfusion Medicine) was added to the culture medium. Cells were cultured 7 days to enable differentiation into macrophages.

Macrophage M. avium infection or treatment with M. avium Ag (MAg) and LAM

Adherent macrophages were infected with viable *M. avium* at a ratio of 5:1 for 2 h at 37°C (4). The *M. avium* is a virulent smooth transparent strain passaged in immune-deficient animals to maintain virulence (27). Unbound bacteria were removed by washing the cells three times with PBS and refeeding with complete medium containing 10% FCS. Control populations of adherent macrophages were mock-infected and cultured in parallel. For visualization of mycobacteria-macrophage binding, green fluorescent protein (GFP)-tagged mycobacteria (28) were added to macrophage monolayers for indicated times and evaluated by confocal microscopy. Macrophages from five different donors were infected or not with *M. avium,* and cells and supernatants were harvested at indicated times from 2 h to 7 days after infection. In additional cultures, 0.5–50 μg/ml MAg prepared from a *M. avium* lysate (4) or 0.1–10 μg/ml *M. avium* LAM (29) was added for 2 h at 37°C. After incubation with MAg or LAM, cells were washed three times with PBS, and fresh complete medium with 10% FCS was added before culture for the indicated times.

cDNA expression array

Total cellular RNA was extracted from adherent control or infected macrophages using the Qiagen RNeasy minikit (Chatsworth, CA) (4). The Atlas cDNA Expression Array (Clontech Human Array 1.2I, catalog no. 7850-1; Clontech Laboratories, Palo Alto, CA; complete 1200 gene list at http://atlasinfo.clontech.com) was performed using 5 μg of DNase-digested total RNA. The RNA was converted into first-strand cDNA, labeled with [^{32}P]dATP, and purified by column chromatography (NucleoSpin Extraction Spin Column; Clontech Laboratories). The labeled probe was mixed with denaturing solution (1 M NaOH, 10 mM EDTA) for 20 min, then C_0t-1 DNA and neutralizing solution (1 M NaH_2PO_4, pH 7.0) were added for 10 min at 68°C. The nylon membranes were hybridized in a solution of Express Hyb (Clontech Laboratories) and sheared salmon testes DNA for 30 min at 68°C. The probe was added to the membrane and hybridized overnight at 68°C. The membranes were then washed, exposed to phosphor screens, and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Arrays were analyzed using AtlasImage 1.01a software (Clontech Laboratories). From each donor, macrophage cultures were mock-infected and incubated in parallel with the cells exposed to *M. avium* and harvested at the same time intervals. The gene expression in infected cells was then compared with the corresponding control population from the same donor expressed as a ratio (fold change) after normalization to housekeeping genes.

Ribonuclease protection assay (RPA)

Total cellular RNA was extracted using the RNeasy minikit (Qiagen) from 6×10^6 control or treated macrophages per well in a six-well plate. Total RNA (3 μg) was used with the hCK-2 template of the Riboquant Multi-Probe RPA system (BD PharMingen, San Diego, CA). Band densities were normalized to the GAPDH housekeeping gene using ImageQuant (Molecular Dynamics).

Cytokine ELISA

The supernatants from the *M. avium*-infected LAM- or MAg-stimulated macrophages were analyzed for TNF-α production by ELISA (R&D Systems, Minneapolis, MN).

Results

M. avium *infection in vivo and in vitro*

In immunocompromised hosts, *M. avium* invades the intestinal mucosa and is initially taken up by macrophages in the lamina propria (Fig. 1*A*), from where it can disseminate to lymph nodes (Fig. 1*B*) and other tissues. Enormous numbers of acid fast, deep red-stained mycobacteria can be readily identified within tissue macrophages. Once inside macrophages, many, but not all mycobacteria survive and replicate within phagocytic vacuoles (Fig. 1*C*) to eventually be released to reinfect a new macrophage host. As evident, large numbers of macrophages accumulate at these sites of infection (Fig. 1, *A* and *B*), largely due to induction of chemokine synthesis by infected macrophages (30). In the absence of adequate T cells, this cycle of recruitment and infection appears to progress unchecked.

To assess the impact of *M. avium* and its constituents on macrophage phenotype and function, peripheral blood monocytes were cultured as adherent monocyte-derived macrophages and exposed to viable mycobacteria, mycobacterial Ags (MAg), or purified *M. avium* cell wall glycoprotein, LAM. In these isolated primary macrophage cultures, live *M. avium* follows a pattern similar to that observed in vivo, entering the cells and in many cases, escaping mycobactericidal actions. Within minutes, mycobacteria adhere to the macrophage surface (Fig. 2*A*), initiate signal transduction (N. Vázquez, T. Greenwell-Wild, and S. M. Wahl, manuscript in preparation), are internalized, and take up residence. Following infection, the macrophages undergo morphological changes, often assuming an elongated configuration and demonstrating highly
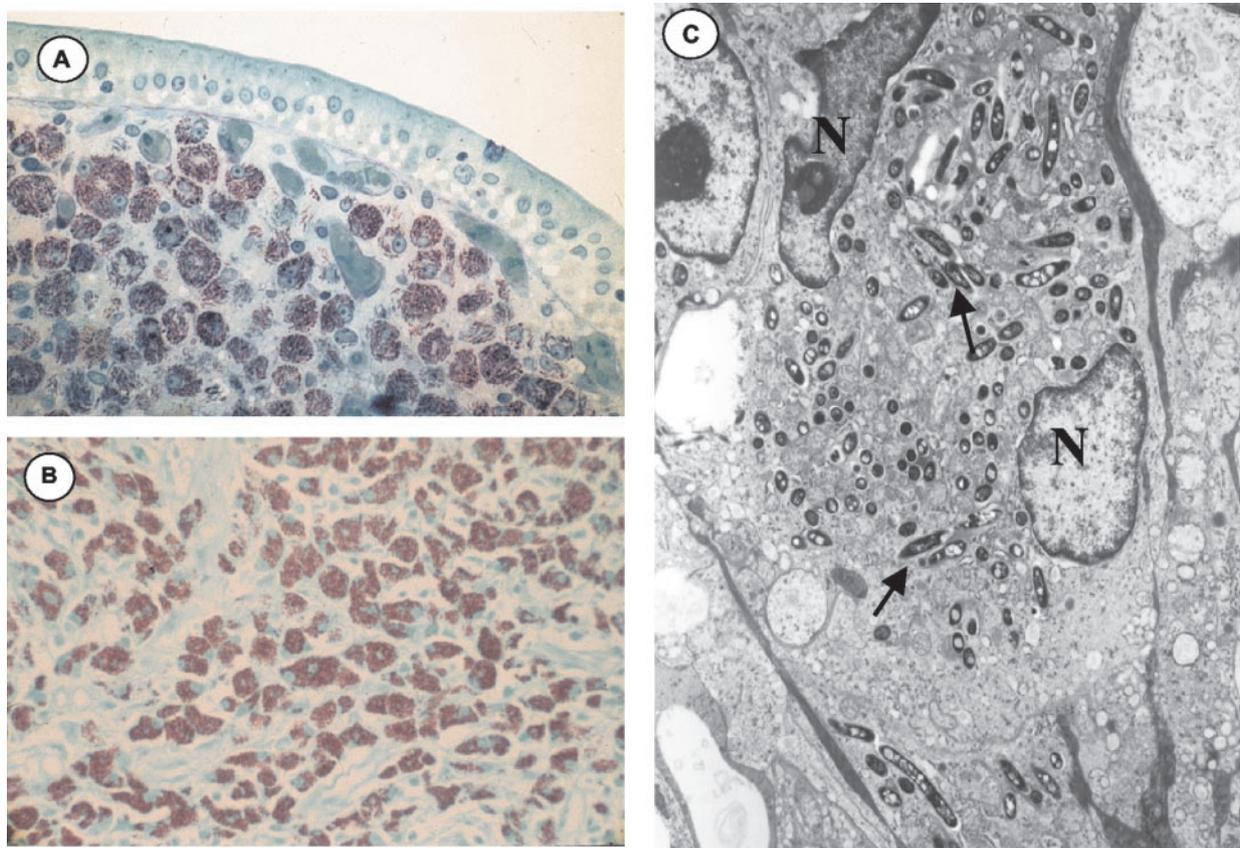
FIGURE 1. *M. avium* infection of tissue macrophages. *A*, In immunodeficiency states, *M. avium* invades mucosa, infects and replicates within macrophages and drives recruitment of monocytes which accumulate in the tissue and provide additional hosts for the mycobacteria. Basic Fuchsin with methylene blue and azure II stain of a semi-thin plastic section of the small intestine of an AIDS patient reveals dramatic numbers of infected macrophages in the mucosa. *B*, *M. avium* dissemination to the lymph node in an AIDS patient. Large numbers of infected macrophages are identified by acid-fast staining of mycobacteria in a lymph node paraffin section. *C*, Electron micrograph of binucleated (N) tissue macrophage with multiple intracellular mycobacteria in phagosomes (arrows) (original magnification ×10,000).

interactive membranes (Fig. 2*B*), compared with control uninfected cells (Fig. 2*D*). Mycobacteria are able to replicate in these lymphocyte-depleted macrophage cultures (Fig. 2*C*), as they do in macrophages in immunodeficient hosts (Fig. 1*C*). Not only are intact bacteria then released to interact with proximal and/or distant cells but, as some bacteria are killed by the macrophages,
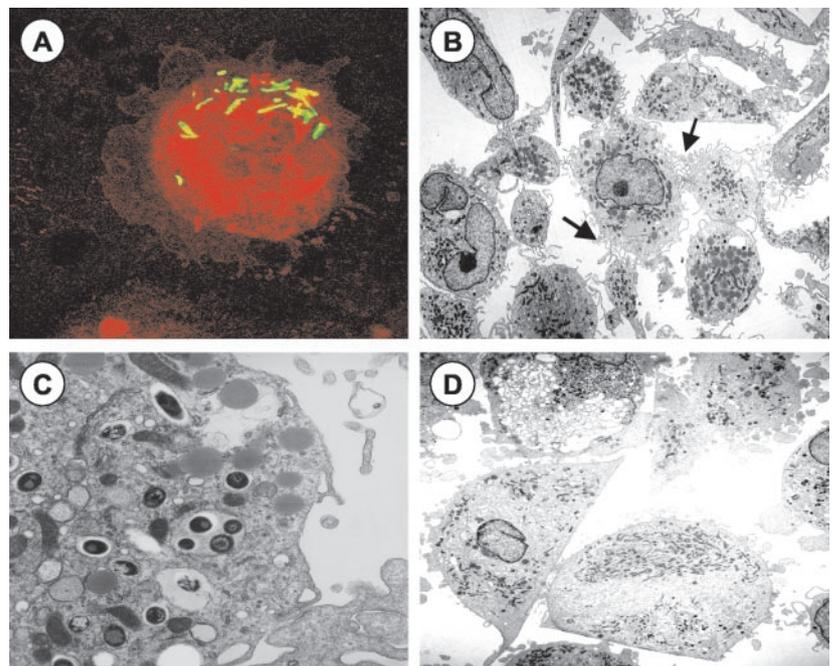
FIGURE 2. *M. avium* infection of macrophages in vitro. *A*, Exposure of adherent monocyte-derived macrophages to GFP-tagged mycobacteria revealed rapid (5 min) bacteria binding to the cell surface. *B*, Following infection, the macrophages exhibit morphological changes, including elongation and extensive cell-cell membrane interactions (arrows). *C*, In 7 day-infected macrophages, replication of mycobacteria occurs in cytoplasmic vacuoles. *D*, Parallel mock-infected control macrophages.

Table I. *Macrophage genes up-regulated within 2 h by* M. avium[a]

GenBank Accession Number	Gene Description	Average Fold Increase
Signal transduction		
M29366; M34309	ERBB-3R protein tyrosine kinase	23.4
M36430	GNB1; transducin β 1	15.2
X63465	rap1 GTPase-GDP dissociation stimulator 1	13.5
X75621	Tuberin; tuberous sclerosis 2 protein	7.4
D10495	PKC-δ	3.9
D21878	Bone marrow stromal Ag 1	3.7
M95678	Phospholipase C β 2 (PLCB2)	3.2
L36719	Dual-specificity MAP kinase kinase 3	3.1
X08004	*ras*-related protein RAP-1B	3.1
M63960	Serine/threonine protein phosphatase (PP) 1-α 1	3.0
U12779	MAP kinase-activated protein kinase 2	2.9
M19922	Fibroblast growth factor-3 integration site 2 proto-oncogene	2.8
X02751	*N-ras*; transforming p21 protein	2.8
M31724	Protein-tyrosine phosphatase 1B	2.7
U10550	Gem; *ras* family member (*ras*-like protein KIR)	2.6
L14778	Serine/threonine PP 2B catalytic subunit α	2.5
M29870; M31467	Rat sarcoma-related C3 botulinum toxin substrate 1	2.4
U17032	GTPase-activating-associated protein	2.4
AF068920	Leucine-rich repeat protein suppressor human homolog of CLR-1 -2; *ras*-binding protein sulphonylurea receptor-8	2.4
U51004	Histidine triad nucleotide-binding protein; PKC inhibitor 1	2.4
L41816	Calcium/calmodulin-dependent protein kinase 1	2.3
S87759	PP2C-α	2.2
U24166	EB1 protein	2.1
V00568	c-*myc* oncogene	2.0
M28213	*ras*-related protein RAB2	2.0
X57346	14-3-3 protein $\beta\alpha$; PKC inhibitor protein-1	2.0
X15014	Ral A; guanosine triphosphate-binding protein	2.0
M19722	c-*fgr* proto-oncogene (p55-FGR)	2.0
L32976	Protein kinase mixed linege kinase-3; sprk	2.0
Receptors/receptor associated proteins		
X01057; X01058; X01402	IL-2Rα	6.1
AF060231	Herpes virus entry protein C	4.9
M32315 + M55994	TNFR superfamily member 1B	3.7
X60592	CDw40 Ag; CD40 ligand receptor	3.3
M33294	TNFR1	3.0
M73482	Neuromedin B receptor	3.0
M20566	IL-6Rα subunit precursor; CD126 Ag	2.6
D11086	IL-2Rγ	2.5
X01060	Transferrin receptor; CD71 Ag	2.4
L11695	TGF-β I receptor	2.2
AF076974	Transformation/transcription domain-associated protein	2.2
Transcription		
X14454	IRF1	8.5
X59738	Zinc finger X-chromosomal protein	5.2
X61498	NF-κ-B p100 subunit	4.5
D90209	Activating transcription factor 4	4.1
M92843	Tristetraproline; growth factor-inducible nuclear protein 475	3.7
U08015	Transcription factor NF-ATc	3.0
L34673	DNA-binding protein HIP116; ATPase	2.7
U12979	Activated RNA polymerase II transcriptional coactivator p15	2.6
M26679	Homeobox protein HOX-A5; HOX-1C	2.5
D21090	UV excision repair protein RAD23 homolog B (HHR23B)	2.5
M14631	Adenylate cyclase-stimulating G α protein; (guanine nucleotide-binding protein G-sα)	2.4
M23619	High mobility group protein	2.4
U22431	Hypoxia-inducible factor 1 α	2.3
D28468	Albumin D box-binding protein; D-binding protein; TAXREB302	2.3
M34079	26S protease regulatory subunit 6A; TAT-binding protein 1	2.2
M58603	NF-κB DNA binding subunit	2.2
M96684	Purine-rich ssDNA-binding protein α	2.2
M76541	Transcriptional repressor protein yin and yang	2.2
X69391	60S ribosomal protein L6	2.1
L11672	Zinc finger protein 91 (ZNF92)	2.1
AF084199	Transcription repressor protein PR domain containing 1-binding factor 1	2.1
M28372	Cellular nucleic acid-binding protein	2.0
M59079	CCAAT-binding transcription factor subunit B	2.0
		(Table continues)

[a] Total cellular RNA from adherent macrophages infected 2 h with virulent *M. avium* was interrogated by cDNA expression array. Enhanced genes (average $\geq$2-fold above control) in three to five donors are shown.

Table I. *Continued*

GenBank Accession Number	Gene Description	Average Fold Increase
Adhesion molecules		
J03132	ICAM1	14.3
X74295	Integrin α_{7B} (IGA7B)	8.2
M73780	Integrin (ITG) β_8	3.9
M59040	CD44 Ag; phagocytic glycoprotein I	3.9
M81695; Y00093	Leukocyte adhesion receptor p150, 95; CD11C Ag	3.1
M15395	LFA-1/CR3/p150,95 β-subunit precursor; CD18 Ag	2.9
M14648; J02826; M18365	Vitronectin receptor α subunit (VNRA)	2.9
X07979	ITG β_1	2.9
D13866; D14705; L23805; L22080	α1 catenin; cadherin-associated protein	2.8
Y00796	ITG α_L	2.7
X53586	ITG α_6 precursor (ITGBI)	2.4
J04145	Leukocyte adhesion receptor m01; CD11B Ag	2.3
J04536	Leukosialin precursor	2.2
M59911	ITG α_3; galactoprotein B3 (GAPB3)	2.2
Chemokine/cytokines/growth factors		
X01394	TNF-α; cachectin	143.4
Y00787	IL-8	33.3
J04130	MIP1-β	23.0
X53799	MIP2-α	19.5
K02770	IL-1β	18.6
M23452	MIP1-α	13.4
X13967; M63420	Leukemia inhibitory factor	8.1
M92357	B94 protein	6.7
M21121	Small inducible cytokine A5 (SCYA5); RANTES	5.4
M19154; M22045; M22046; Y00083	TGF-β2	4.7
M62302	Embryonic growth/differentiation factor 1	4.4
U14407	IL-15	4.4
X54936	PLGF1 + PLGF2	4.2
X17543; M30134	IL-9; T-cell growth factor p40	4.1
X79929	OX40 ligand	3.7
M14743; M17115	IL-3	3.6
M31145	Insulin-like growth factor binding protein 1	3.1
X04602; M14584	IL-6	3.0
X56692	C-reactive protein	2.9
M17733	Thymosin β-4	2.9
D16431	Hepatoma-derived growth factor	2.8
Y00809	Eosinophil granule major basic protein	2.7
X06374	PDGFA	2.5
M62505	C5a anaphylatoxin receptor; CD88 Ag	2.5
M92381	Thymosin β-10	2.3
X02811; X02744; M12783; M16288	PDGFB	2.3
M57627	IL-10	2.2
M24398	Parathymosin	2.2
Cell cycle/apoptosis		
AF039067; AF071596	IEX-1L anti-death protein	7.9
U29680; Y09397	Bcl-2-related protein A1 (Bcl2A1)	5.2
AF013263	Apoptotic protease-activating factor 1	4.8
L08246	Induced myeloid leukemia cell differentiation protein myeloid cell leukemia-1	3.6
U09579	Cyclin-dependent kinase inhibitor 1A	3.3
L22474	Apoptosis regulator *bax*	3.1
Z23115; L20121; L20122	Apoptosis regulator *bcl*-x	2.6
U60520; U8143; X98172; AF00962	Caspase-8; IL-1 converting enzyme-like apoptotic protease 5	2.5
AF060515	Cyclin K	2.4
U01160	Transmembrane 4 superfamily protein	2.4
M87338	Activator 1 40-kDa subunit	2.2
D15057	Defender against death	2.0
M23254	Calpain 2 large (catalytic) subunit	2.0
M77234	*fte*-1; yeast mitochondrial protein import homolog	2.0
U28014 + U28015	Caspase-4	2.0
Protease/protein turnover		
X07819	MMP7; matrilysin	3.9
X57766	MMP11; stromelysin 3	3.2
X63187	Major epididymis-specific protein E4 (hE4)	2.5
D45248	Proteasome activator HPA28 subunit β	2.4
		(*Table continues*)

Table I. *Continued*

GenBank Accession Number	Gene Description	Average Fold Increase
X03124	Metalloproteinase inhibitor 1 (TIMP1)	2.3
L40377	CAP2; protease inhibitor 8	2.2
X12451	Cathepsin L; major excreted protein	2.2
D00761	Proteasome β1 subunit	2.2
J05070	MMP9	2.2
M11233	Cathepsin D	2.1
U14575	Activator of RNA decay	2.0
Z81326	Neuroserpin precursor	2.0
M74524	Ubiquitin-conjugating enzyme E2 17-kDa	2.0
Homeostasis and detoxification		
M11717	70-kDa heat shock protein (HSP) 1 (HSP70.1)	5.4
L19185	NK-enhancing factor B	2.9
X67951	Thioredoxin-dependent peroxide reductase 2	2.7
U03688	Dioxin-inducible cytochrome P450 1B1 (CYP1B1)	2.6
X76648	Glutaredoxin	2.6
M34664	60-kDa heat shock protein (HSP60)	2.5
J03746; B28083	Microsomal glutathione S-transferase (GST) 12	2.4
P09488; X68676; S01719	GST mu1 (GSTM1; GST1); HB subunit 4; glutathione 4	2.3
M25627	GST A1 (glutathione1; GSTA1)	2.3
M13267	Cytosolic superoxide dismutase 1	2.2
X15722	Glutathione reductase	2.1
Metabolism		
X58022	Corticotropin-releasing factor-binding protein	4.5
AB008775	Aquaporin 9	3.7
D10232	Renin-binding protein	3.5
M33374	NADH-ubiquinone dehydrogenase 1β subcomplex 7 18-kDa subunit	2.9
U18840	Myelin-oligodendrocyte glycoprotein precursor	2.5
U08098	Estrogen sulfotransferase 1	2.4
M68867	Cellular retinoic acid-binding protein II	2.0

released MAg and LAM may activate additional cells. Similar to infected cells, MAg exposed cells appeared activated with inter-active cell membranes (not shown) in contrast to control cell populations (Fig. 2*D*).

Gene expression in M. avium-*infected macrophages*

The morphological and structural changes of macrophages early after exposure to *M. avium* and during the evolution of infection demonstrated a bacterial influence on phenotype and function. To determine the transcriptional impact of the initial interaction between *M. avium* and macrophages which may underlie mycobacterial persistence, the cells were exposed to *M. avium* at a ratio of 5:1 for 2 h when RNA was collected and processed for cDNA expression array. Although all isolated monocytes were adhered and differentiated under parallel conditions for 7 days before infection, and individual cultures were synchronously exposed to mycobacteria, heterogeneity in donor macrophage responses to *M. avium* was noted (not shown). As data from individual donors were reproducible, the heterogeneity, at least in part, stems from the level of constitutive activation/differentiation of the control macrophages from each donor upon which the fold change was based, but may also reflect differential donor susceptibility to the pathogen. We present data (Table I) in which macrophages from five separate donors were infected with *M. avium*. Only those genes which were reproducibly up-regulated in a minimum of three donors with a mean $\geq$2.0-fold increase above parallel donor control macrophages are represented in Table I. For assessment of transcriptional suppression, we set our cutoff as a mean decrease of 2-fold in infected compared with uninfected macrophage cultures from the same donor, again in at least three replicate experiments.

We selected 2 h after exposure to *M. avium* as our first time point for evaluation (Table I) to detect genes that might be regu-lated following initial *M. avium*-receptor interactions as compared with later time points (1–7 days) which may reflect indirect or intracellular regulation (Table II). Within the first 2 h after exposure to *M. avium*, many of the differentially expressed genes encoded transcription factors and signal-related molecules, including components of the mitogen-activated protein (MAP) kinase and G protein pathways. Among the most highly up-regulated early response signaling genes were epidermal growth factor (ERBB)-3 receptor protein tyrosine kinase, the guanine nucleotide-binding protein β (GNB) subunit 1, and the rap1 GTPase-GDP dissociation stimulator, as well as kinases, phosphatases, and other signal regulatory molecules (Table I). This heterogeneity in signaling genes may reflect multiple mycobacteria-recognizing receptors (17). In addition, a number of receptors, including IL-2R α and γ components, TNFR1, CD40 ligand receptor, IL-6Rα, the Herpes virus entry protein C, and the transferrin receptor were increased. Associated with enhanced receptors and signaling molecules, transcription-related genes were also increased, particularly those involved in the NF-κB pathway, as well as NF-AT cytoplasmic, activating transcription factor 4, and IFN regulatory factor (IRF)1.

Consistent with an effect on NF-κB pathway constituents (4), a dramatic increase in downstream proinflammatory gene expression occurred. With this initial engagement of an apparent host defense response, increased expression of genes for recruitment of immune cell reinforcements, cell-cell interactions, and cell activation were observed (Table I). The striking cell-cell membrane interactions (Fig. 2*B*) likely reflect *M. avium*-induced expression of multiple adhesion molecules, as represented by integrins β_1, β_8, α_3, α_6, α_7, α_v, and CD11b and c, in addition to ICAM1 and CD44. In addition to adhesion molecules, up-regulated chemokines (IL-8, macrophage-inflammatory protein (MIP)1β, MIP1α, MIP2α, RANTES)

Table II.   *Kinetics of* M. avium *enhanced macrophage gene expression*

Day 1		Day 4		Day 7	
M. avium enhanced genes (fold increase)					
IL-8	88	GNB1	43	MRP 8	52
GNB1	19	MRP 8	30	α1 antitrypsin	14
MRP 8	16	α1 antitrypsin	17	IL-8	9.6
IL-1β	14	MMP11	6.4	PDGFA	7.6
ERBB-3	12	MMP7	5.6	B94	6.2
CRF	11	RANTES	5.1	MRP14	5.4
α1 antitrypsin	9.3	MRP14	4.5	ICAM1	4.5
MMP14	8.2	TIMP 1	4.1	CD44	4.3
CDW40 Ag	7.5	ICAM1	4.1	MAP kinase-activated protein kinase 2	4.0
MCP1	7.4	Angiotensin-converting enzyme	4.1	IL-4	3.7
TIMP1	6.4	Transferrin receptor	4.1	Mut L homolog	3.1
c-*myc*	5.9	Methionine amino peptidase 2	4.1	INT-2 proto-oncogene	3.1
MMP7	5.8	Corticotropin-releasing factor receptor I-R	4.1	MCP-1	3.1
ras-related C3 botulinum toxin substrate 1	5.5	IL-1β	3.9	NADH-ubiquinone dehydrogenase-1β	3.0
HSP70	5.3	CD44	3.8	Cyclin-dependent kinase inhibitor 1A (p21)	3.0
Vascular endothelial growth factor	5.2	ERBB-3	3.6	Proteasome activator HPA28β	2.7
Platelet activating factor receptor	5.0	NADH-ubiquinone dehydrogenase-1β	3.6	Yamaguchi sarcoma viral-related oncogene homolog	2.7
ezrin	4.8	subcomplex 7			
PRKAR1B	4.5	V-ral simian leukemia viral oncogene homolog A	3.5	*ras*-related protein RAB2	2.7
CYP1B1	4.3	Macrophage CSF I receptor 1-R	3.3	MMP14	2.6
		MMP14	3.1	CYP1B1	2.6
M. avium suppressed genes (fold decrease)					
CD40R-associated factor 1	4.7	Cathepsin D	2.8	cAMP-dependent protein kinase	2.6
ADORA	2.6	Inhibitor of DNA binding 2 protein	2.7	Type 1β regulatory subunit	
Caspase 10	2.2	NDP kinase B	2.7	Transducer of *erb*B2	2.3
Cathepsin D	2.1	ADORA	2.7	ITG α₃	2.2
		RNA polymerase II transcriptional coactivator p15	2.3	DNase II	2.1
		ras homolog gene family member A	2.1	Integrin αL	2.0
		Proteasome inhibitor HPI31	2.1		
		Mut L homolog	2.1		
		Integrin α3	2.0		
		Caspase 10	2.0		
		PKC inhibitor 1	2.0		

(30) and TGF-β would foster leukocyte recruitment and granuloma formation. Multiple cytokines were also increased early after exposure to *M. avium.* Although levels of gene expression were variable between monocyte donors ($n = 5$), IL-1β (average 18-fold) and TNF-α (average 143-fold) were consistently highly elevated within 2 h. Besides IL-1β and TNF-α, the cytokines, B94, LIF, placenta growth factor (PLGF), IL-15 and IL-6, were typically enhanced (Table I), and other cytokines were also modulated by contact with mycobacteria. IL-10 was transiently transcriptionally increased (Table I), but not sustained (Table II). Several apoptosis regulatory genes were also influenced in these *M. avium*-infected cells (Table I). An increased expression of proteases, particularly matrix metalloproteinases (MMP)7 and MMP11 (stromelysin 3) and cathepsins, was also evident in this early response. Additional genes involved in detoxification and/or associated with bactericidal activity were also modulated in infected cells (Table I). Although numerous genes were enhanced within 2 h after exposure to *M. avium*, only a single gene (DNase II, an acidic deoxyribonuclease) of nearly 1200 interrogated was reproducibly suppressed within this interval, and many genes were unaffected including iNOS, IL-12p40, and IFN-γ. At later intervals (see below), additional genes were observed to be down-regulated during mycobacterial growth within the macrophages.

Macrophage activation by mycobacterial components

Following the initial infection and in the absence of T cell activation, *M. avium* replicates within the cultured macrophages, although some are killed, degraded, and/or released (Figs. 1 and 2). To establish whether bacterial constituents such as MAg or the *M. avium* LAM stimulated a similar profile of cytokine genes, cultured macrophages were treated with intact microorganisms, MAg or purified LAM for 2–3 h and gene induction was compared with parallel unstimulated cell populations (Fig. 3). With one exception (Rap1), the most highly up-regulated genes in macrophages exposed to live mycobacteria and to purified LAM were identical at 2 h. Both TNF-α and IL-8 were dominant genes, whether triggered by *M. avium* or LAM. Chemokines and adhesion molecules maximally increased by purified LAM paralleled those induced by viable bacteria interactions with the macrophage. MAg, representing a complex mycobacterial lysate, also enhanced expression of these genes, but in a less defined pattern (Fig. 3). However, by RPA, both LAM and MAg, similar to live mycobacteria, significantly elevated IL-1β in a dose-dependent fashion (Fig. 4, *A* and *C*) as determined by densitometric analysis of the blots (Fig. 4, *B* and *D*) with smaller increases in IL-1Ra and IL-6. A higher concentration of the crude lysate (MAg) was required compared with the purified
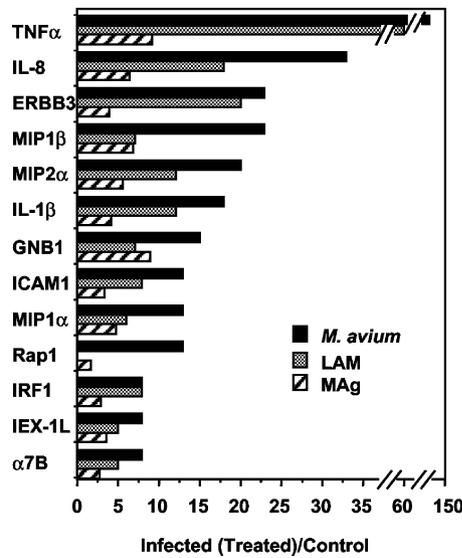
FIGURE 3. Gene expression profile for macrophages exposed to viable mycobacteria, MAg and LAM. Adherent macrophages were infected with *M. avium* (5:1) for 2 h or treated with LAM (10 μg/ml) or MAg (25 μg/ml) and total RNA processed for cDNA expression array. The 13 most highly expressed genes for *M. avium*-infected and LAM-stimulated cells are shown.

glycolipid to induce a comparable response. IL-1α, IL-12p40, and IL-10 were nominally affected in this RPA from a representative-donor within the 2–3 h interval following exposure and no induction

of IFN-γ was detected, confirming the array results with viable microorganisms.

TNF-α protein

To further compare the macrophage response to *M. avium*, MAg, and LAM, we monitored expression of TNF-α as a representative gene product. Supernatants from *M. avium*-infected macrophages expressed very high levels of TNF-α protein within hours after exposure to the mycobacteria (4), which declined precipitously, but remained minimally elevated above uninfected controls after infection (Fig. 5A). Both LAM and MAg induced a dose-dependent release of TNF-α, although purified LAM was typically a 50–100 times more potent stimulus (Fig. 5B). Thus, *M. avium*-induced gene regulation in infected macrophages identified by cDNA expression array, as represented by TNF-α, is consistent with and reproducible by RPA (4) and protein assessment (Fig. 5), and may represent a key mycobacterial-induced transient mediator associated with host defense.

Kinetics of M. avium-*modulated gene expression*

The transient release of TNF-α by macrophages infected with viable microorganisms prompted a kinetic analysis of *M. avium*-induced genes beyond the initial binding-signal transduction event registered at 2 h. In this regard, we monitored macrophage gene expression 24 h and 4 and 7 days after infection with *M. avium* as
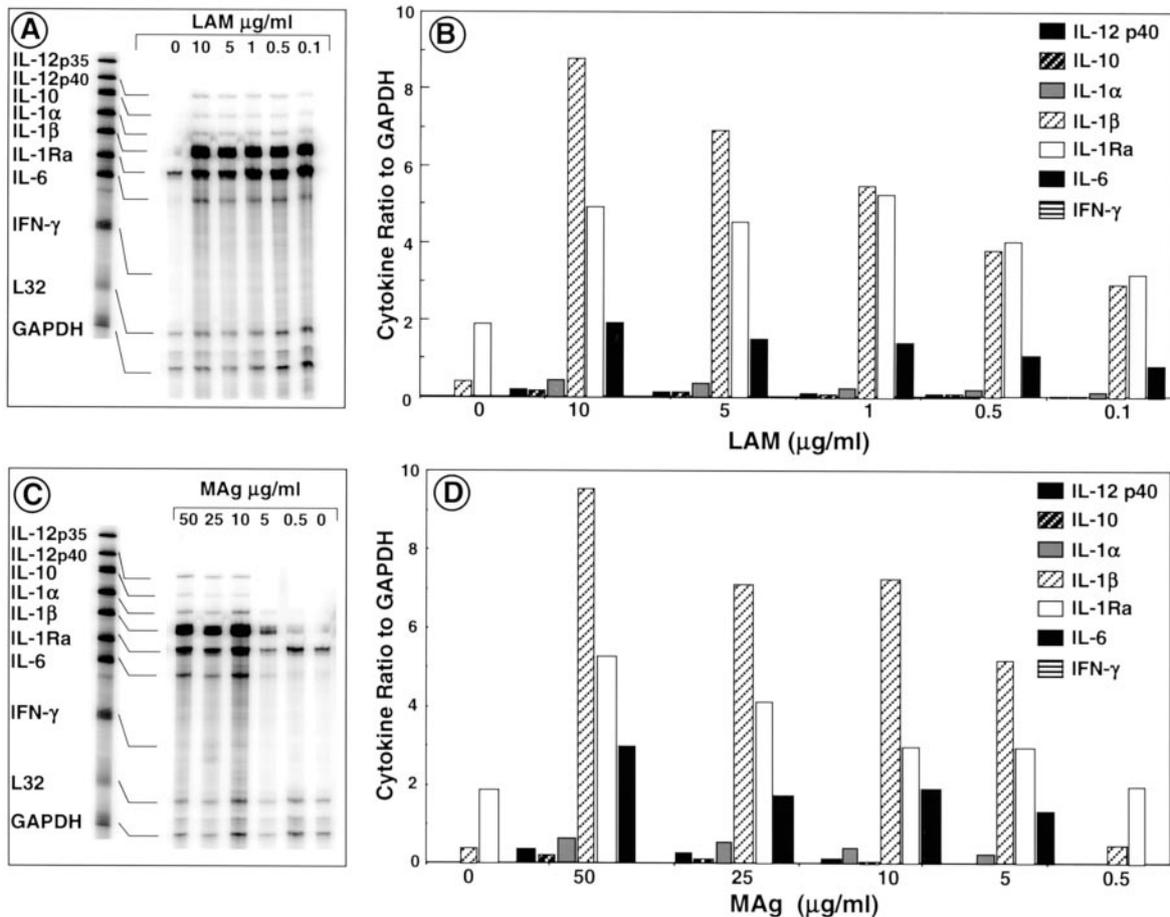

FIGURE 4. Mycobacterial constituents increase cytokine mRNA. *A* and *C*, Macrophages treated with *M. avium* lysate (MAg) or cell wall LAM at indicated concentrations for 3 h exhibit enhanced cytokine gene expression as detected by RPA. *B* and *D*, Graphs represent normalization of cytokine genes to GAPDH.
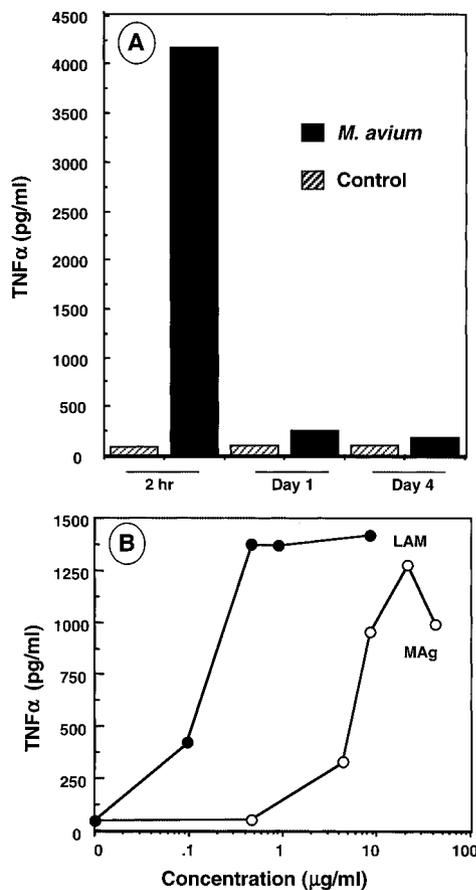
FIGURE 5. TNF-α protein expression by infected macrophages. *A*, Adherent macrophages were infected with *M. avium* for 2 h to 4 days and TNF-α levels in the supernatants were monitored by ELISA. *B*, Adherent macrophages were incubated with indicated concentrations of MAg or LAM for 3 h and supernatants were assayed for TNF-α by ELISA. Representative experiment, $n = 3$.
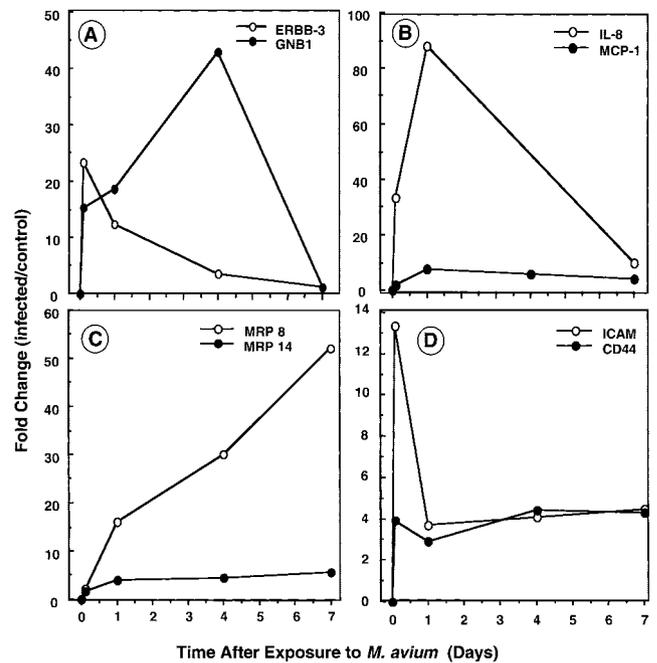

FIGURE 6. Kinetics of *M. avium*-modulated macrophage gene expression. Total cellular RNA from adherent macrophages from 2 h to day 7 post infection was interrogated by cDNA expression array for the indicated genes. *A*, Kinetics of ERBB-3R protein tyrosine kinase and GNB1. *B*, Expression of the chemokines, IL-8 and MCP-1, from 2 h until 7 days after infection. *C*, Macrophage inhibitory factor-related proteins 8 (calgranulin A) and 14 (calgranulin B) were increased throughout the 7 days after infection evaluated. *D*, ICAM and CD44 were adhesion molecules increased following *M. avium* infection. Average fold increases compared with mock-infected controls are represented from three to five donors.

compared with mock-infected parallel cultures from the same donors. The initial macrophage response, which appears to favor signaling and transcription factors, in addition to mediators of recruitment and activation of the cellular components of host defense (Table I), changes strikingly with time. Over the next 7 days, genes associated with inflammation and immunity recede, although some were more stably induced (IL-1β, GNB1, migration inhibitory factor-related protein (MRP)8/14, and MMPs) based on the means of individual donors (Table II, Fig. 6). Moreover, a new spectrum of genes was impacted during days 1–7 postinfection as shown in Table II (top 20 up-regulated genes are shown for days 1, 4, and 7 postinfection). Signal-related GNB1 and ERBB3, so strikingly up-regulated within 2 h, remained elevated for 4 days after infection (Fig. 6*A*). IL-8, which increased >30-fold within 2 h, increased further by 24 h and although it declined thereafter, remained elevated during the subsequent week. Monocyte chemoattractant protein (MCP)-1 levels, albeit donor-dependent (30), were elevated from day 1 through 7 (Fig. 6*B*). The migration inhibitory factor-related S100 proteins or calgranulins, particularly MRP8/calgranulin A, progressively increased through the 7 days evaluated (Fig. 6*C*). Sustenance of adhesion molecule expression (Fig. 6*D*) may facilitate formation of multinucleated giant cells. Several of the MMPs rapidly increased and remained elevated after infection with *M. avium*, while antiproteases, such as tissue inhibitor of MMP (TIMP)1 (Table II), cytoplasmic antiproteinase (CAP)2 and α1 antitrypsin (Fig. 7*B*) also increased. Notable is a lack of induc-

tion of iNOS or defensins at any time point evaluated after infection in this lymphocyte-depleted population.

The initial dramatic proinflammatory gene profile appears short-lived. Once the bacteria have been internalized, they apparently no longer transduce signals via surface receptors, and multiply within the cell (Fig. 2, days 4–7 postinfection). At this point, there appears to be a shift to a less inflammatory profile, which may be influenced by IL-10, the levels of which are maximal in the supernatants at 24 h (not shown). Failure to sustain an antimicrobial defense (Figs. 1 and 2*C*) did not appear to be the consequence of large scale down-modulation of macrophage genes. By 24 h, only CD40R-associated factor 1, caspase 10, adenosine A1 receptor (ADORA), and cathepsin D (Table II, Fig. 7, *C* and *D*) were reproducibly decreased >2 fold. Over the next 4 days, the negative transcriptional regulatory impact of *M. avium* on macrophages remained surprisingly minimal. ADORA, cathepsin D, and caspase 10 remained suppressed, in addition to decreases in nucleoside diphosphate kinase B, *ras* homolog gene family member A, histidine triad nucleotide-binding protein (protein kinase C (PKC) inhibitor 1), and several other regulatory molecules (Table II). Whether the continued suppression of ADORA, α3, cathepsin D and DNase through day 7 (Fig. 7), as well as additional genes (Table II), influences *M. avium* activity is unknown. Although additional genes might be down-regulated in individual donors, they were not always reproducibly inhibited in multiple donors. Collectively, the decrease in gene expression from 2 h to 7 days after exposure to *M. avium* is unimpressive and how, or if, these modulated genes impact on mycobacterial growth and survival remains to be determined.
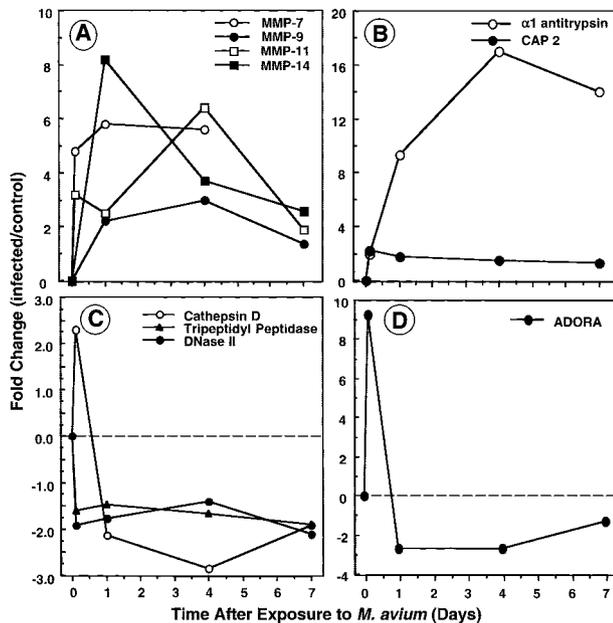
FIGURE 7. Kinetics of *M. avium*-enhanced/suppressed macrophage genes. Total cellular RNA from infected macrophages (2 h to 7 days after infection) was interrogated by cDNA expression array. Data for indicated genes represent average fold increases or decreases in infected compared with mock-infected macrophages from three to five donors. *A*, MMPs 7, 9, 11, and 14 were increased rapidly and sustained after infection. *B*, The protease inhibitors α1-antitrypsin and CAP2 were increased 2- to 16-fold above uninfected control cultures. *C*, Cathepsin D was rapidly increased (2 h) and then decreased along with tripeptidyl peptidase and DNase II. *D*, The adenosine receptor increased transiently and then was suppressed following *M. avium* infection.

Discussion

The innate response to mycobacteria is initiated when the microorganisms interact with cell surface pattern receptors, including TLR, to signal and/or become internalized. Recognition occurs largely through the mycobacterial cell wall constituent LAM, and *M. avium* envelope LAM binds toTLR2 and also may bind to the glycosylphosphatidylinositol-linked protein CD14 (20, 21, 23, 31, 32). Based on our comparison of gene expression mediated by intact mycobacteria, mycobacterial Ags, and *M. avium* cell wall LAM, it appears that a shared ligand mediating *M. avium*-induced intracellular signaling is LAM. Nonetheless, multiple noncongruent genes were represented at lower transcriptional levels between viable mycobacteria and its cell wall constituent (not shown), consistent with the specificity of LAM, the potential engagement of additional receptors by intact bacteria, the transient LAM signal, and persistence of activation by the intact organisms. Whereas the bacteria may bind to the same TLRs as does LAM to orchestrate a shared signaling cascade, the uptake of the bacteria appears to engage additional pathways involving cytoskeleton and signal transduction. Agonist engagement of TLR2 (31) in primary monocyte-derived macrophages triggers a signaling cascade within minutes, including phosphorylation of MAP kinase family members and activation of the transcription factor NF-κB (4, 31, 33, 34) (N. Vázquez, T. Greenwell-Wild, and S. M. Wahl, manuscript in preparation). In our transcriptional analyses, multiple MAP kinase and NF-κB-dependent genes were rapidly and dramatically elevated by 2 h after infection. Up-regulation of Rac1 is consistent with recent reports that this Rho family GTPase is involved in TLR2 signaling (35), as well as cytoskeletal changes (36) and possibly, autocrine IL-6 signaling linked to STAT3 (37).

Signal transduction leads to rapid up-regulation of a panoply of chemokine mRNAs and proteins (30), similarly up-regulated by other bacteria (38, 39), and likely effective in mediating leukocyte recruitment and initiation of the adaptive immune response. The rapidity with which some of these chemokines are released (30) and their known interactions with cognate seven transmembrane domain G protein-coupled receptors suggests the possibility that the increased gene expression of G protein-associated signaling molecules might be a secondary consequence of engagement of this pathway. Also evident is the persistence of these molecules, particularly IL-8, a week after infection, which is consonant with granulomagenic congregation of inflammatory cells, essential as new bacterial hosts (30). Beyond recruitment of leukocytes, chemokines, in recent times, have been shown to have multiple additional activities in angiogenesis, matrix deposition, and proliferation (reviewed in Ref. 40).

Pathogenicity may also be fostered via enhanced transcription of adhesion molecules to facilitate migration through the endothelial barrier to the site of infection, promote cell-cell interactions, and formation of multinucleated giant cells (4, 5, 41–43). The regulation of macrophage trafficking may also be a function of elevated MRP8 and MRP14. The production of these myeloid S100 calcium-binding proteins has been associated with fast migrating cells that express high CD11b and preferentially use ICAM-1-dependent mechanisms of transendothelial migration (44, 45). Recruitment and activation of inflammatory cells at the site of infection would not only involve the orchestrated expression of leukocyte and vascular adhesion molecules, and the generation of chemotactic gradients, but also the production of multiple MMPs, essential to dissolution of basement membrane and matrix components.

Along with *M. avium*-mediated NF-κB activation (4) and abundant gene expression of TNF-α and IL-1β, an extensive repertoire of cytokines and other mediators transiently escalate subsequent to infection. Early, but unsustained increases in IL-10 and TGF-β may share in dampening the initial activation response (46, 47). TNF-α and IL-1β, proinflammatory cytokines that activate multiple signal transduction pathways to influence both immune and nonimmune cell function, also inhibit macrophage apoptosis (48). This is in keeping with the observations that infection with *M. avium* appears to protect macrophages from apoptosis, thereby maintaining the pool of infected and infectible targets (5, 6, 30). Avirulent mycobacteria reportedly more effectively promote macrophage apoptosis than virulent strains (49), consistent with our observations that phagocytic uptake and internalization of *M. avium* induced expression of apoptosis regulatory genes. The enhanced expression of immediate early gene X (IEX)-1L, Bcl-2A1, and Bcl-x may counteract apoptotic protease-activating factor 1 and Bax with the balance of pro- and antiapoptotic pathways favoring survival. During the 7-day postinfection interval monitored, morphologic integrity and function appeared uncompromised, and apoptosis was not frequently encountered (assessed by TUNEL staining; H. Hale-Donze, unpublished observation) in these infected monocyte-derived macrophages, consistent with the mycobacterial need for prolonging the functional longevity of their hosts.

The meek IL-12 response observed in this study does not conjure up an image of a robust Th1 response considered essential to eliminate the mycobacteria (50). IL-12 is reportedly a key cytokine in host defense against mycobacteria (51) and the absence of IL-12 increases infectibility in animals and humans (39, 52, 53). TLR2 signaling in murine macrophages was also recently shown to not induce IL-12p40, IFN-γ, or IL-6 mRNA compared with a TLR4 agonist (54). And in fact, the initial *M. avium* signaling response

and generation of inflammatory mediators by macrophages is transient, suggesting that once internalization occurs, the mycobacteria suppress the response which allows them to establish an infectious niche before recognition by the adaptive immune response. The virulence of strains of *M. tuberculosis* is considered inversely proportional to its efficacy in inducing proinflammatory cytokines (55) such as TNF-α, and the massive induction of TNF-α by *M. avium* and LAM could fit this pattern in an immune competent host. Further insight into the mechanisms of mycobacterial subversion may be found in the suppression of certain macrophage genes by the pathogen. In the second phase, when many of the initially enhanced genes have returned to control levels, evidence of microbicidally relevant genes implies an attempt to limit growth of ingested mycobacteria, but macrophages continue to harbor the *M. avium. M. avium* is transported to phagosomes, which in the absence of IFN-γ-mediated activation (31), do not fuse with protease-bearing lysosomes, enabling mycobacterial survival. Although lysosomal cathepsins D and L transcripts are initially increased, mycobacteria suppress those lysosomal enzymes which may otherwise contribute to their demise. Cathepsin D, an acidic protease up-regulated within hours in response to *M. avium*, is synthesized as an inactive 51-kDa proform and typically processed to a 30-kDa mature form in acidic lysosomal compartments (56). However, viable *M. avium*-harboring phagosomes, with retarded acidification, do not process procathepsin D (56, 57), blocking its proteolytic potential. *M. avium* inhibition of cathepsin D activity plus suppression of its transcription, together with enhanced expression of α1 antitrypsin, may contribute to mycobacterial escape from degradation. In contrast, MMP7 (matrilysin), MMP11, MMP14, and MMP9, which increase early, remain variably elevated through 7 days after infection. MMP9 has previously been shown to be up-regulated in *M. avium*-infected PBMC and to facilitate HIV-1 replication (58). At least at the transcriptional level, the corresponding modest increase in TIMPs may implicate a proteolytic imbalance. Whether persistence of MMPs subserves some mycobacterial need, in addition to facilitating recruitment, cytokine processing, and granuloma formation (58, 59), is under evaluation.

Increased virulence may also be associated with increased gene expression of the transferrin receptor for requisite iron transport (60), and transient increases in the free radical scavenging enzyme superoxide dismutase (2 h) may represent a potential mode of macrophage self-preservation, but also protect the mycobacteria. Decreased ADORAs, which reportedly regulate TNF-α and IL-6 (61) and suppress IL-12 production (62), could also support a less hostile environment. It is conceivable that the mycobacteria may incorporate multiple cellular mechanisms for their survival, enabling replication within a safe haven and eventual release by apoptotic or necrotic macrophages to begin the reinfection process. Depending on whether pro- or anti-inflammatory-driven responses prevail, the mycobacteria will be cleared or protective granuloma will evolve. Evidence for increased platelet-derived growth factor (PDGF), observed early after infection and elevated again after a week may be contributory to fibrogenesis.

Whereas *M. tuberculosis* infects individuals with apparently normal immune function, *M. avium* is an opportunistic pathogen. Our findings with *M. avium* may provide insight into unique and/or shared inducible host factors involved in the differential virulence between *M. avium* and *M. tuberculosis*. Comparing our data for 1200 potential *M. avium*-regulated genes in primary macrophages with that for 375 immunoregulatory genes evaluated for *M. tuberculosis* in a cell line (63) and in macrophages (39) revealed not only considerable overlap, but also differences at early time points. Our study also uniquely monitors gene expression at later time

points consonant with mycobacterial survival and intracellular growth. Moreover, analysis of *M. tuberculosis*-induced changes in gene expression in murine macrophages (64) revealed both shared and unique transcriptional pathways. Comparison of *M. avium*- and *M. tuberculosis*-induced gene expression in parallel macrophage cultures will provide important insight into virulence-related genes. In addition to host factors, environmental factors and bacterial genotype and phenotype all influence the outcome of infection and evolution of disease. These *M. avium* opportunists, to which many individuals are exposed, but which typically cause no symptoms or pathology as they are rapidly cleared, can become life-threatening in the context of HIV-1 infection or by other modes of immune suppression (3–5). Engagement of adaptive immunity and T cell function is essential to bacterial containment (65). The requisite IFN-γ signal is deficient in HIV-1-induced immunodeficiency as well as in our purified macrophage cultures. Whether due to a lack of IFN-γ or an inability to respond to IFN-γ, as occurs in macrophages infected with virulent *M. tuberculosis* (66), the microorganisms have a survival advantage. Unraveling the intricacies of *M. avium* entry and intracellular cohabitation with macrophages provides new insights into disarming mycobacterial invasion and evasion of host defense.

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