Control of Mycobacterial Replication in Human Macrophages: Roles of Extracellular Signal-Regulated Kinases 1 and 2 and p38 Mitogen-Activated Protein Kinase Pathways

Antje Blumenthal,1 Stefan Ehlers,1 Martin Ernst,2 Hans-Dieter Flad,2 and Norbert Reiling1*

Department of Immunochemistry and Biochemical Microbiology,1 and Department of Immunology and Cell Biology,2 Research Center Borstel, D-23845 Borstel, Germany

Received 10 January 2002/Returned for modification 25 March 2002/Accepted 11 June 2002

Intracellular persistence of mycobacteria may result from an intricate balance between bacterial replication and signaling events leading to antimicrobial macrophage activities. Using human monocyte-derived macrophages, we investigated the relevance of mitogen-activated protein kinase activation for the growth control of Mycobacterium avium isolates differing in their abilities to multiply intracellularly. The highly replicative smooth transparent morphotype of M. avium strain 2151 induced significantly less p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation than the smooth opaque morphotype of the same strain, which was gradually eliminated from macrophage cultures. Inhibition of the p38 pathway by highly specific inhibitors did not significantly affect mycobacterial replication within macrophages, regardless of the in vitro virulence of the M. avium strain. However, repression of the ERK1/2 pathway further enhanced intracellular growth of highly replicative M. avium strains, although it did not increase survival of the poorly replicating M. avium isolate. Inhibition of the ERK1/2 pathway resulted in decreased tumor necrosis alpha (TNF-α) secretion irrespective of the virulence of the M. avium isolate used for infection, revealing that TNF-α could have been only partially responsible for the control of intracellular M. avium growth. In conclusion, ERK1/2- and TNF-α-independent pathways are sufficient to limit intramacrophage growth of less-virulent M. avium strains, but early ERK1/2 activation in infected macrophages is critically involved in controlling the growth of highly replicative M. avium strains.

Macrophages play a central role in the first line of defense against pathogenic microorganisms because they are critically involved in the activation of both innate and acquired immune responses. Following phagocytosis, macrophages become activated to initiate defense mechanisms, e.g., production of nitric oxide and phagosome acidification, that ultimately lead to the degradation of many microbial species (33; reviewed in reference 19). Paradoxically, macrophages are also the key target cells of a variety of pathogens, e.g., mycobacteria, that have developed strategies to invade macrophages and replicate intracellularly.

Infections with mycobacteria, such as tuberculosis, are characterized by their chronic course. Both human and mouse studies have provided ample evidence that even in the face of an adequate immune response, mycobacteria like Mycobacterium tuberculosis and Mycobacterium avium are able to persist inside macrophages (13; reviewed in reference 20). Of interest, several strains and distinct morphotypes (smooth transparent, smooth opaque) of M. avium differ with respect to virulence and persistence in an in vivo infection model (25). One potential mechanism by which virulent mycobacterial strains, as opposed to avirulent strains, may achieve a state of long-term persistence is the modulation of signaling cascades leading to macrophage activation (reviewed in reference 23).

Diverse signaling cascades are involved in triggering cellular responses to pathogenic organisms (reviewed in reference 22). One essential branch of cell signaling in eucaryotic organisms is the ubiquitously expressed family of mitogen-activated protein (MAP) kinases (reviewed in reference 7). These serine/threonine kinases are critically involved in cell proliferation, differentiation, and cell death, as well as the inflammatory response (reviewed in reference 17). In mammals there are three subfamilies of MAP kinases that can be activated independently and simultaneously: p46 and p54 c-Jun-NH2-terminal kinases, p42 and p44 extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAP kinase (reviewed in reference 5). MAP kinases mediate cellular responses to a variety of extracellular stimuli, such as physical stress (e.g., osmotic changes), inflammatory cytokines, growth factors, and bacterial components (e.g., lipopolysaccharide [LPS]) (reviewed in reference 18). Highly specific, cell-permeable inhibitors of MAP kinase activity have been useful tools in identifying some physiological functions of these signaling cascades in terms of infectious processes. By using these inhibitors, the life cycles of some viruses, e.g., human immunodeficiency virus, were determined to depend on ERK1/2 and p38 MAP kinase activity (29, 32, 34). ERK1/2 activity was also shown to be critically involved in invasion of the facultative intracellular bacterium Listeria monocytogenes, but not Salmonella enterica serovar Typhimurium (31). Even growth of some tumors in vivo was successfully blocked by MAP kinase inhibitors (28).

The activation of MAP kinase signaling cascades by mycobacterial components (6, 15) as well as viable M. avium (26) has only recently been described. However, the functional rel-
evance of MAP kinase activity with respect to uptake and intracellular persistence of mycobacteria has remained unexplored. In particular, it is unknown whether there is a direct correlation between the magnitude of MAP kinase activation and the magnitude of intracellular replication of different \textit{M. avium} strains or morphotypes. In this study, we investigated whether highly specific MAP kinase inhibitors would interfere with intramacrophage growth and cytokine induction of \textit{M. avium} strains differing in their in vitro replication rates.

**MATERIALS AND METHODS**

- **Bacteria.** \textit{M. avium} strains 2151 (morphotypes smooth transparent and smooth opaque) and SE91 were originally isolated from AIDS patients (1, 13). Mycobacterial strains were grown in Middlebrook 7H9 medium (Difco, Detroit, Mich.) containing 10% OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson) and 0.05% Tween 80 (Sigma, Deisenhofen, Germany) until mid-log phase. Absence of contaminating microorganisms was verified by plating culture material on brain heart infusion agar (Difco) and Ziel-Neelsen staining. The suspension was frozen in aliquots at −70°C until use. For calculation of CFU, aliquots were serially diluted in sterile, distilled water containing 0.05% Tween 80 and plated on 7H10 agar containing 0.05% pyruvate (Sigma). After incubation for 3 weeks at 37°C, CFU were calculated. For infection, bacterial aliquots were thawed and centrifuged for 10 min at 835 × g. Bacteria were resuspended in phosphate-buffered saline and sonicated (3 to 5 min, 35 kHz; Bandelin, Berlin, Germany) to disrupt aggregates. Bacterial LPS of \textit{S. enterica} serotype Friedenau 4909 was kindly provided by H. Brade (Research Center Borstel, Borstel, Germany).

- **Inhibitors.** PD98059 and SB203580 were purchased from Calbiochem (Schwalbach, Germany) and used at concentrations of 3 or 10 μM. Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (vol/vol) as solvent control.

- **Isolation and cultivation of human monocyte-derived macrophages.** Mononuclear cells were isolated from the peripheral blood of healthy volunteers by density gradient centrifugation (4). Lymphocytes and monocytes were separated clear cells were isolated from the peripheral blood of healthy volunteers by density gradient centrifugation (4). Lymphocytes and monocytes were separated

- **Human macrophages were infected with either the smooth transparent (SmT) or the smooth opaque (SmO) morphotypes of \textit{M. avium} and SmO show different growth kinetics in human macrophages in vitro.** Human macrophages were infected with either the smooth transparent (SmT) or the smooth opaque (SmO) morphotype of \textit{M. avium} 2151. After 4 h, both morphotypes were phagocytosed at comparable numbers (Fig. 1A) with a multiplicity of infection averaging 1 mycobacterium per 12 to 14 macrophages. Mycobacterial growth in human macrophages 7 days after infection is shown in Fig. 1B. The SmT morphotype replicated extensively compared to the SmO morphotype, which was gradually eliminated during infection. In the absence of macrophages, both morphotypes replicated to a comparable extent in cell culture medium (data not shown). Although the two morphotypes differentially persisted in infected cultures, macrophage numbers were identical 7 days postinfection (Fig. 1B). When depicted as CFU related to macrophage number (relative CFU, as described in Materials and Methods), a 774-fold increase in CFU of SmT above that of SmO was observed (Fig. 1C).

**RESULTS**

\textit{M. avium} 2151 SmT and SmO show different growth kinetics in human macrophages in vitro. Human macrophages were infected with either the smooth transparent (SmT) or the smooth opaque (SmO) morphotype of \textit{M. avium} 2151. After 4 h, both morphotypes were phagocytosed at comparable numbers (Fig. 1A) with a multiplicity of infection averaging 1 mycobacterium per 12 to 14 macrophages. Mycobacterial growth in human macrophages 7 days after infection is shown in Fig. 1B. The SmT morphotype replicated extensively compared to the SmO morphotype, which was gradually eliminated during infection. In the absence of macrophages, both morphotypes replicated to a comparable extent in cell culture medium (data not shown). Although the two morphotypes differentially persisted in infected cultures, macrophage numbers were identical 7 days postinfection (Fig. 1B). When depicted as CFU related to macrophage number (relative CFU, as described in Materials and Methods), a 774-fold increase in CFU of SmT above that of SmO was observed (Fig. 1C).
ERK1/2, but not p38, activity is involved in control of intracellular M. avium replication in human macrophages. In order to investigate whether MAP kinase activity was directly involved in mycobacterial survival, infection experiments were performed in the presence or absence of highly specific pharmacologic inhibitors of MAP kinase signaling cascades. PD98059 is a well-established inhibitor of the ERK1/2 pathway that acts by repressing activation of MEK-1, the kinase upstream of p42 and p44. The pyridinyl imidazole SB203580 specifically inhibits p38 activity by binding to the ATP-binding site and inhibiting enzyme activity but not p38 phosphorylation (reviewed in reference 8 and references therein).

The presence of MAP kinase inhibitors did not significantly affect mycobacterial uptake into macrophages 4 h postinfection compared to cultures with no additive or with DMSO as solvent control (data not shown). After 7 days, CFU of the highly replicative SmT morphotype had substantially increased in cultures containing PD98059 (10 μM), whereas the presence of SB203580 did not have any effect even at concentrations up to 10 μM. The gradual elimination of the SmO morphotype was unaffected after coincubation with either PD98059 or SB203580 (Fig. 3A). In order to validate the observed growth-enhancing effect of PD98059 on the highly replicative variant 2151 SmT, parallel cultures were infected with another strain of M. avium (SE01) which also showed extensive growth in macrophages. Again, the presence of PD98059 led to an increase in mycobacterial CFU relative to macrophage numbers, while SB203580 did not alter intracellular growth of this strain (Fig. 3B). DMSO alone had no influence on mycobacterial growth. In addition, a direct effect of PD98059 and SB203580 on mycobacterial growth was excluded by cultivation of mycobacteria in the presence of MAP kinase inhibitors but in the absence of macrophages (data not shown).

The same set of experiments was performed independently for six different donors. Although there was substantial donor-dependent variation with regard to the extent of mycobacterial proliferation within macrophages, the presence of PD98059 led to a significant increase in colony counts of the highly proliferative strains 2151 SmT (Fig. 3C) and SE01 (Fig. 3D). These results were confirmed by using a second, more potent...
MEK-1 inhibitor, U0126 (data not shown). In contrast, the presence of PD98059 did not significantly alter CFU counts of the SmO morphotype, and mycobacterial growth was repressed in all cases (Fig. 3C). The presence of SB203580 consistently had no significant effect on intramacrophage replication rates of any of the M. avium strains tested (Fig. 3C and D). Therefore, activation of the ERK1/2 pathway seems to be critically involved in controlling intracellular mycobacterial replication of highly replicative M. avium strains.

Cytokines are only partially involved in control of intramacrophage growth of M. avium. To gain further insight into the mechanisms by which inhibition of the ERK1/2 pathway might exert its intramacrophage growth-promoting effect on certain M. avium strains, the magnitude of cytokine secretion induced by M. avium strains differing in their virulence was analyzed in the presence and absence of MAP kinase inhibitors. To this end, human macrophages were infected with M. avium 2151 SmT or SmO and the levels of the macrophage-activating cytokine TNF-α and the macrophage-deactivating cytokine IL-10 into the supernatant were analyzed. Both M. avium morphotypes induced the release of TNF-α and IL-10 in a dose-dependent manner (Fig. 4). The highly replicative SmT morphotype, which led to only a weak MAP kinase activation, induced the release of significantly smaller amounts (up to three times less than SmO) of TNF-α and IL-10. In contrast, the SmO morphotype, a strong activator of MAP kinases, led to a strong induction of cytokine release. Therefore, M. avium-induced TNF-α and IL-10 release reflected the extent of MAP kinase activation by morphotypes differing in their intracellular replication rates in vitro.

Since inhibition of p38 and ERK1/2 activities had different effects on the intracellular replication rates of the M. avium strains examined, it appeared possible that inhibition of p38 and ERK1/2 activities in macrophages infected with these strains would have strain- or virulence-dependent effects on TNF-α and IL-10 secretion. Our data (Fig. 5) clearly show, however, that the effect of MAP kinase inhibition on cytokine expression was not virulence specific. In fact, a differential effect of MAP kinase inhibition on TNF-α and IL-10 secretion, previously demonstrated by our labo-

FIG. 3. PD98059, but not SB203580, enhances intracellular mycobacterial growth. Cultures of human monocyte-derived macrophages were preincubated with 3 or 10 μM PD98059 (●) or SB203580 (○) or 0.1% (vol/vol) DMSO as solvent control for 60 min. Macrophages were infected with M. avium 2151 smooth transparent (SmT) or smooth opaque (SmO) (A) or M. avium strain SE01 (B). The number of intracellular viable bacteria correlated to macrophage numbers (relative CFU) was determined 7 days postinfection. Values represent means of duplicates ± SD of one representative experiment out of six. (C and D) Results obtained from different donors. Macrophages were preincubated with 10 μM MAP kinase inhibitor or 0.1% (vol/vol) DMSO. Cells were infected with 2151 SmT or 2151 SmO (C) or with SE01 (D). For each donor, CFU counts 7 days postinfection were correlated to macrophage numbers and are shown as relative CFU. The boxes indicate the 25th and 75th percentiles. Whiskers above and below the boxes indicate the 90th and 10th percentiles. Medians are marked. All outlying points are graphed. Data shown were obtained from six independent experiments (six different donors) performed in duplicate. *, P < 0.05; n.s., not significant.
ratory for *M. avium* strain SE01 (26), was seen for both morphotypes of strain 2151 and was, therefore, independent of the virulence of the *M. avium* isolate. TNF-α release was significantly reduced by PD98059 in a dose-dependent manner after stimulation with either *M. avium* strain. In contrast, IL-10 formation was not inhibited in the presence of PD98059 (Fig. 5A). Inhibition of p38 activity by cocultivation with SB203580 did not reduce TNF-α formation in macrophages induced by either morphotype. Although statistically not significant, *M. avium*-induced TNF-α release was usually enhanced. Strain 2151 SmT always induced little IL-10 formation (Fig. 4). Usage of SB203580 further reduced IL-10 release, although effects were not found to be statistically significant because all measured IL-10 amounts were near the lower detection limit of the ELISA (Fig. 5B). The highly activating smooth opaque morphotype induced the formation of higher amounts of IL-10. In this case, IL-10 release was significantly reduced in the presence of SB203580 (Fig. 5B).

**DISCUSSION**

The aim of this study was to analyze whether the extent of MAP kinase signaling induced by various *M. avium* strains and morphotypes would be a determinant for their subsequent intracellular survival. We found that *M. avium*-induced activation of ERK1/2 and p38 MAP kinases was inversely correlated to the virulence of the infecting mycobacterial variant in vitro. Inhibition of ERK1/2 activation enhanced intracellular multiplication of highly replicative strains, whereas p38 activation was not involved in controlling intracellular growth or survival of either high-level or low-level replicating variants of *M. avium*. However, the regulation of *M. avium*-induced cytokine release was independent of mycobacterial virulence. We found ERK1/2 activation essential for TNF-α formation, whereas p38 activity, but not the ERK1/2 pathway, was involved in IL-10 release.

Infection with *M. avium*, a facultative intracellular opportunistic pathogen in humans, is a well-studied model for mycobacterial infections. A major phenotypic characteristic of *M.
avium isolates is their ability to appear in different colony morphotypes: smooth (either transparent or opaque) or rough (reviewed in reference 14). Although morphotypes of the same strain are very closely related, previous studies demonstrated that the smooth transparent variant is usually better able to survive and grow intracellularly than the opaque morphotype (10, 21). With regard to the M. avium morphotypes used in this study (2151 SmT and SmO), this pattern has been confirmed both in murine macrophages and in a mouse model of infection (1, 2). Our results extend these findings to human monoocyte-derived macrophages. This is not trivial, because in the case of the M. tuberculosis strain H37Rv and its avirulent variant H37Ra, growth patterns in mice and murine macrophages are not reflected by their in vitro growth characteristics in human macrophages (24). Why different morphotypes of M. avium either possess or lack the capacity for intracellular persistence is entirely unknown, but the responsible mechanisms likely involve modulation of intracellular signaling pathways (reviewed in reference 23).

Focusing on MAP kinase signaling events, we observed activation of both ERK1/2 and p38 in response to the two closely related morphotypes of M. avium 2151. Since the magnitude of MAP kinase activation was inversely correlated to the intramacrophage replication potential of these strains, we hypothesized that ERK1/2 and p38 signaling might indeed play a direct role in controlling the growth of these strains.

A variety of commercially available compounds have proven to be powerful tools for functional analysis of particular cell signaling elements (8). The highly specific inhibitors PD98059 and SB203580 have previously been used to demonstrate involvement of ERK1/2 and p38 MAP kinase pathways in uptake and persistence of pathogenic viruses and bacteria (16, 29).

Regarding intracellular mycobacterial replication, inhibition of the ERK1/2 pathway led to an increased growth of M. avium 2151 SmT (and also of another, highly replicative strain, SE01) within human macrophages. This growth-enhancing effect was, however, not observed for the related SmO morphotype of the same strain. These data indicate that replication of some mycobacterial strains, such as M. avium 2151 SmO, can easily be controlled by macrophages, even in the presence of drugs interfering with essential signaling pathways. In this respect, it is interesting that ERK1/2 activity also seems not to be involved in controlling the replication of another facultative intracellular bacterium, L. monocytogenes (16). In contrast, other mycobacterial variants, like M. avium 2151 SmT and SE01, are more resistant to growth control by the macrophage. In macrophages infected with these strains, additional repression of the ERK1/2 pathway might unbalance innate defense mechanisms to an extent more favorable to mycobacterial replication. Notably, inhibition of p38 activity in human macrophages did not significantly affect intracellular replication of any of the M. avium variants used. From these results, we conclude that p38 activity is not critically involved in the control of mycobacterial growth in infected macrophages in vitro. Parallel infection studies performed in bone marrow-derived macrophages of C57BL/6 mice (A. Blumenthal et al., unpublished observations) corroborate our data and show that our results are not restricted to the human system.

One possible mechanism by which the magnitude of MAP kinase activity may influence intracellular mycobacterial replication could be the induction of cytokines. With regard to TNF-α, several previous studies have shown an inverse correlation between the extent of TNF-α release in reaction to M. avium and the virulence of the infecting strain or morphotype. Therefore, the hypothesis was put forth that certain M. avium isolates are more virulent because they induce only little TNF-α in vitro (10, 27). With respect to the two morphotypes of M. avium 2151, our data confirm this hypothesis and, in addition, provide a plausible explanation at the signal transduction level. Thus, the virulent morphotype induced less MAP kinase phosphorylation than the avirulent morphotype. Virulence may therefore simply be a reflection of an intrinsic inability of certain mycobacterial strains to stimulate macrophages. However, it is intriguing to speculate that virulence may also reside in the capacity of some mycobacterial strains to inhibit early signal transduction events, such as MAP kinase phosphorylation.

PD98059 and SB203580 were used to analyze whether MAP kinase activation would affect M. avium-induced cytokine release in a virulence-dependent way. As shown, independent of the morphotype used, TNF-α production was critically dependent on the activation of the ERK1/2 pathway, whereas p38 activity, and not ERK1/2, was involved in IL-10 release, which corroborates earlier studies using another well-replicating M. avium strain, SE01 (26).

We suggest that differential regulation of pro- and anti-inflammatory mediators at the level of MAP kinase activity may represent a general macrophage response to M. avium (independent of the strain or morphotype) and, possibly, to other virulent and avirulent mycobacterial species.

Previous studies described contradictory effects of neutralization of endogenous IL-10 in vitro (9, 30). In our system, inhibition of endogenous IL-10 by SB203580 was not accompanied by enhanced growth of any M. avium strain tested. We conclude that IL-10 release by infected macrophages may not be critical in intracellular growth control of M. avium. Inhibition of the ERK1/2 signaling cascade in M. avium 2151 SmT- or SE01-infected macrophages was followed by diminished TNF-α release and enhanced intracellular mycobacterial replication. An important role for TNF-α in the control of M. avium replication in vitro has previously been demonstrated (1). Furney et al. (10) showed a significant inhibitory effect of TNF-α on intracellular replication only for extensively replicating M. avium variants, whereas the effect on low-level-replicating mycobacteria was minimal. Thus, early inhibition of TNF-α release may be one of the mechanisms by which PD98059 promotes intramacrophage growth of some highly replicative strains of M. avium.

A number of arguments, however, strengthen the view that control of M. avium replication inside macrophages involves more than stimulation by TNF-α. First, infection of macrophages with M. avium SE01 induces TNF-α levels comparable to those achieved by the easily controlled 2151 SmO (26; also data not shown). Yet, SE01 is a highly replicative M. avium strain and, in this respect, quite similar to 2151 SmT, which induces only low amounts of TNF-α. Second, inhibition of the ERK1/2 pathway markedly reduced TNF-α secretion induced by 2151 SmO, yet intracellular survival rates stayed low for this isolate.

Since the growth-enhancing effect of ERK1/2 pathway inhibition cannot be entirely explained by modulation of TNF-α,
levels, it would appear that M. avium-induced activation of the ERK1/2 signaling cascade is involved in other, TNF-α-independent cellular processes that ultimately lead to restriction of mycobacterial growth. Alternatively, it is entirely possible that M. avium strains simply differ in their susceptibility to TNF-α-induced bacteriostatic mechanisms and that, for some strains, higher TNF-α levels are required to achieve growth inhibition than for others.

Our present study demonstrates that, for M. avium infection, the relationship between macrophage activation (as reflected by MAP kinase phosphorylation), TNF-α and IL-10 secretion, and bacteriostasis is more complex than previously thought. We have identified ERK1/2 signaling as a critical pathway for growth restriction of some M. avium strains. We further uncovered that ERK1/2-independent mechanisms, as well as TNF-α-independent mechanisms, must exist to curtail the growth of other M. avium variants. It would appear that novel strategies for antitymocobacterial immunotherapy will require a more thorough understanding of the subtleties of the host-pathogen relationship.

ACKNOWLEDGMENTS

We thank Stefan Uhrig for helpful discussions. We gratefully acknowledge the expert technical assistance of R. Bergmann, E. Kaltenhäuser, S. Kroger, and J. Siwinski.

This work was supported in part by the Deutsche Forschungsgemeinschaft, SFB 415, project C7.

REFERENCES