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Differential Induction of Bone Marrow Macrophage Proliferation by Mycoplasmas Involves Granulocyte-Macrophage Colony-Stimulating Factor

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We have studied the ability of three different *Mycoplasma* species to induce proliferation of bone marrow-derived macrophages (BMM). We observed a significant mitogenic effect when BMM cells from BALB/c, DBA/2J, SJL, and C57BL/6 mice were incubated with membranes derived from *Mycoplasma arginini* or *M. arthritidis* but not when they were incubated with an equivalent amount of *M. pulmonis* membrane. We also determined that pretreatment of mycoplasma membrane preparations with papain eliminated the ability of these preparations to induce BMM proliferation. To determine whether these membrane fractions acted indirectly by stimulating the production of soluble factors known to stimulate proliferation of BMM cells, we performed blocking studies with antibodies directed against colony-stimulating factor 1 (CSF-1), interleukin-3 (IL-3), and granulocyte-macrophage colony-stimulating factor. Our results indicate that antibodies directed against either CSF-1 or IL-3 failed to block mycoplasma-initiated proliferation of BMM cells. However, when anti-GM-CSF was added to proliferative cultures at the time of initiation, we saw a dose-dependent reduction of mycoplasma-initiated proliferation. We conclude that the ability of mycoplasma membranes to initiate the proliferation of BMM is not shared by all species of mycoplasma and that it involves the production of GM-CSF by an as yet undetermined cell.

The animal mycoplasmas represent the smallest bacteria known and are distinguished by their lack of a cell wall and their requirement for sterol for growth. A very interesting property of the mycoplasmas from a host-parasite interaction point of view is that they are almost always found in close association with the outer surface of host cells (2). Several common species preferentially colonize the mucous membranes of the respiratory and genitourinary tract in addition to the synovial and cartilaginous tissues of the joint and have been implicated in diseases involving these tissues. These include pneumonia, inflammatory diseases of possible autoimmune origin, and a possible role in rheumatoid arthritis (8). Mycoplasmas have been demonstrated to be the causative agent of chronic arthritis in several animal species but have not been shown to play a direct role in human arthritis (8, 33).

Mycoplasmas have been shown by many investigators to have several effects on cells of the immune system. Various mycoplasma species have been shown to possess T-cell (5, 20) or B-cell (7, 20, 27) mitogenic properties. In addition, certain species of mycoplasmas have been shown to elicit natural killer cell (34), cytotoxic T lymphocyte (14), and cytolytic macrophage (14) activities. More recently, these organisms have been shown to increase Ia expression in B cells (25a), primary bone marrow-derived macrophage (BMM) cultures, and the myelomonocytic cell line WEHI-3 (31). Infections by these organisms have also been demonstrated to alter the expression of interleukin-1 (IL-1) in a cell-specific manner (9). Furthermore, it has been demonstrated that membranes from *Mycoplasma pneumoniae* will induce human lymphocytes to produce substances that have activity resembling that of colony-stimulating factor (16) and that other mycoplasmas stimulate rat lymphocytes to produce IL-2 (11).

In the present studies, we demonstrate that mycoplasma membranes are mitogenic for normal BMM. This mitogenic activity is not shared by all species of mycoplasma and appears to involve the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) within the BMM cultures.

MATERIALS AND METHODS

Mice. BALB/c, DBA/2J, SJL/J, C57BL/6, and (BALB/c × C57BL/6)F_1 mice, 3 to 4 months old, originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred in our animal facility, were used for these experiments.

Tissue culture media. The media used throughout these experiments consisted of Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 50 μM 2-mercaptoethanol.

BMM cultures. Bone marrow cells were obtained from femurs and tibias of mice and were cultured in DMEM containing the additives described above as well as 10% horse serum and 10% L929 cell-conditioned medium (LCM), as a source of colony-stimulating factor 1 (CSF-1), for 24 h (31). At this time, cells were washed to remove nonadherent cells, and the remaining adherent cells were grown for an additional 3 days in LCM. The medium was then removed, fresh medium without LCM was added, and the cells were incubated for 24 h. Macrophages were removed from flasks by scraping with a cell scraper. This technique yielded greater than 95% viable Mac-1^+ adherent cells as assessed by immunofluorescence. These cells were counted, and 4×10^4 cells were added to each well of a 96-well plate in 100 μl

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of culture medium and incubated overnight, at which time they were ready for experimental manipulation.

Mycoplasma cultures. Mycoplasma organisms were grown as described previously (4, 32). Once harvested, the cell pellet was washed twice in 0.25 M NaCl, suspended in phosphate-buffered saline, and stored at −70°C until use. For heat inactivation, the organisms were heated to 65°C for 30 min. Crude membrane fractions were produced by osmotic lysis of cell pellets in distilled water after the organisms were loaded with 2 M glycerol as described by Razin (24) and Rottem et al. (26). Unlysed organisms were pelleted by centrifugation at 4,000 × *g* at 4°C for 20 min, and membranes were harvested from the supernatants by centrifugation at 30,000 × *g* at 4°C for 1 h. Protein determinations were made on the membrane preparations by the BCA protein assay standard protocol (Pierce Chemical Co., Rockford, Ill.) (30). These preparations were divided into portions and stored at −70°C until use.

Antibodies. For the blocking studies described in this article, we used the following antibodies. The rat anti-mouse CSF-1 monoclonal antibody 5A1 (13) was the kind gift of Hsiu-San Lin, Washington University, St. Louis, Mo. Rat monoclonal anti-mouse IL-3 antibody 19B3.1 (1) was the kind gift of Joe Abrams, DNAX Corp., Palo Alto, Calif. Rabbit anti-mouse GM-CSF antibody 871-48 (18) was the kind gift of Diane Mochizuki of Immunex Corp., Seattle, Wash. The rat monoclonal antibody Mac-1 was the kind gift of Don Cohen, University of Kentucky, Lexington.

Lymphokines. Our source for CSF-1 was LCM (31), which was added at 10% of final volume. IL-3 was derived from supernatants of the WEHI-3 cell line and was used at 10% of final volume (21). Recombinant GM-CSF was obtained from Genzyme Corp., Boston, Mass., and used at a final concentration of 50 U/ml (10).

Proliferation assays. Four-day BMM cultures were harvested and washed three times to remove any residual CSF-1; cells were counted and suspended at 10^6/ml in medium without LCM. The proliferation assay is a modification of the CSF-1 [^{3}H]thymidine assay (19). Briefly, 4 × 10^4 BMM were incubated in a total volume of 0.2 ml in individual wells of a 96-well plate. To experimental wells, mycoplasma membrane (approx. 0.5 μg of protein) was added. As a positive control for BMM proliferation, 10% LCM (20 μl) was added to these wells. The cultures were incubated for 26, 50, or 74 h with the addition of 1 μCi of [^{3}H]thymidine (ICN, Irvine, Calif.) during the last 24 h of culture. The cultures were harvested onto glass fiber filters with a cell harvester (Skatron Inc., Sterling, Va.), and [^{3}H]thymidine incorporation was determined by liquid scintillation spectrometry.

RESULTS

We have previously shown that membrane preparations from mycoplasmas induce increased major histocompatibility complex (MHC) expression in BMM (31). While performing these experiments, we consistently isolated more BMM cells from cultures that were treated with mycoplasmas than from either untreated BMM cultures or those treated with interferon. This could have been the result of either a mitogenic effect by the mycoplasma or an increase in cell viability over control cultures. In order to test these possibilities, proliferation experiments were performed with various numbers of heat-killed *M. arginini*. Replicate cultures were harvested on days 1 through 5 to determine when maximal proliferation was achieved. Addition of *M. arginini*

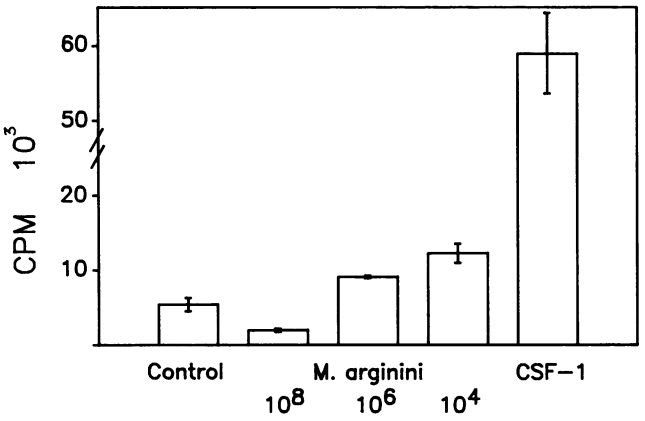

FIG. 1. Graphic representation of titration of proliferative capacity of *M. arginini* organisms that were inactivated by heating to 70°C for 30 min on BMM cultures compared with that obtained with LCM-derived CSF-1 and unstimulated BMM control cultures. Values represent means of quadruplicate determinations; bars indicate standard errors of the means.

to cultures led to two- to threefold increases in BMM proliferation compared with control cultures, and maximal responses were observed on days 3 and 4 when 10^4 organisms were used (Fig. 1). As a positive control, BMM cultures were stimulated with 10% LCM as a source of CSF-1 (31). As shown in Fig. 1, the magnitude of proliferation achieved with CSF-1 exceeded by two- to threefold that observed for mycoplasma-induced BMM cultures. This was a consistent observation throughout these studies with few exceptions.

Concurrent with our testing of intact, heat-killed mycoplasma organisms for inducing BMM proliferation, we also tested purified membrane preparations for mitogenic activity. The results in Fig. 2 demonstrate that significant proliferation of BMM was induced by membrane preparations containing 0.5 μg of total membrane protein. Interestingly, greater amounts of membrane resulted in decreased proliferative responses. The kinetics for the response to membrane preparations were the same as those observed when heat-killed organisms were used, with the maximal response occurring on either day 3 or 4 (data not shown). Comparison

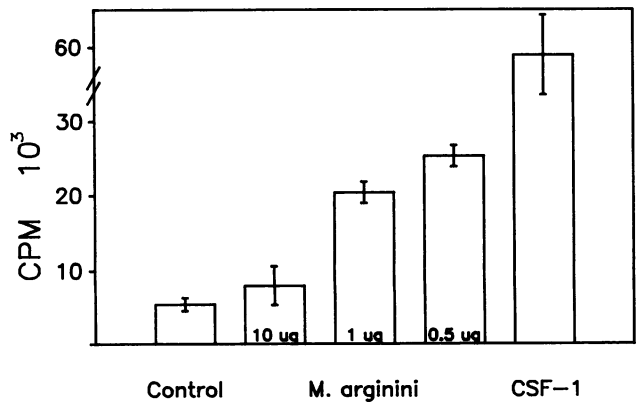

FIG. 2. Graphic representation of titration of proliferative capacity of membrane preparations from *M. arginini* on BMM cultures compared with that obtained with LCM-derived CSF-1 and unstimulated BMM control cultures. Values represent means of quadruplicate determinations; bars indicate standard errors of the means.

TABLE 1. Mitogenic activity of mycoplasmas on BMM

Strain	[^{3}H]thymidine incorporation[a] (cpm)				
	Control	CSF-1	*M. arginini*	*M. arthritidis*	*M. pulmonis*
Expt 1					
BALB/c	3,857	129,388	13,181	8,464	4,883
(C × B6)F_1[b]	5,025	122,133	11,899	13,056	3,546
Expt 2					
BALB/c	5,874	58,287	17,952	32,215	9,375
C57BL/6	14,635	98,391	20,600	31,348	16,023
DBA/2	3,271	26,409	12,096	13,113	3,172

[a] Control proliferation reflects values for cultures grown for 48 h and pulsed with [^{3}H]thymidine for the final 24 h. The standard error of the mean did not exceed 10% of the mean value for any of the determinations. Values for mycoplasma membranes alone ranged from 250 to 500 cpm for all three species.

[b] (BALB/c × C57BL/6)F_1.

of the magnitude of the response between Fig. 1 and 2 indicates that mycoplasma membranes induced stronger BMM proliferation than whole, heat-killed organisms. This has been a consistent observation throughout our studies. In other experiments, we have been unable to demonstrate mitogenic activity in culture supernatants of this organism (data not shown).

Since our previous report on the MHC-inducing capacity of mycoplasmas demonstrated that all species of *Mycoplasma* tested were capable of inducing increased MHC expression, we wondered whether the proliferative capacity of *M. arginini* was shared by other *Mycoplasma* species. To that end, we tested membranes from *M. arginini*, *M. arthritidis*, and *M. pulmonis* for the ability to induce BMM proliferation. Both *M. arginini* and *M. arthritidis* membranes induced three- to fourfold increases in BMM proliferation compared with control cultures (Table 1). On the other hand, *M. pulmonis* membranes were unable to stimulate proliferation of these BMM cultures. To determine whether our lack of induction with *M. pulmonis* membranes was due to a concentration effect, we added membrane protein ranging from 0.005 to 10 μg to separate BMM cultures and did not observe induction of BMM proliferation at any of the amounts tested (data not shown).

We next decided to determine whether the BMM mitogenic activity of mycoplasmas was unique to the BALB/c mouse strain. This possibility was examined by testing the abilities of membranes from *M. arginini*, *M. arthritidis*, and *M. pulmonis* to elicit proliferative responses in BMM cells from C57BL/6, (BALB/c × C57BL/6)F_1, and DBA/2J mice (Table 1). BMM cells from all three mouse strains proliferated in the presence of *M. arginini* and *M. arthritidis* but not in that of *M. pulmonis*, indicating that mycoplasma-induced proliferation was not unique to the BALB/c strain.

In order to better understand the chemical nature of the mycoplasma mitogen, membrane preparations were treated with various concentrations of the proteolytic enzyme papain for 2 h and compared with untreated membrane preparations on C57BL/6 BMM cultures. Treatment of membranes with papain led to a 75% reduction in the mitogenic activity of *M. arthritidis* membranes when results were corrected for background proliferation (Fig. 3). In contrast, mitogenic activity was not appreciably reduced following exposure of the membrane preparation to 90°C for 30 min (data not shown). Taken together, these data indicate that the mitogenic factor consists, at least in part, of a heat-stable protein.

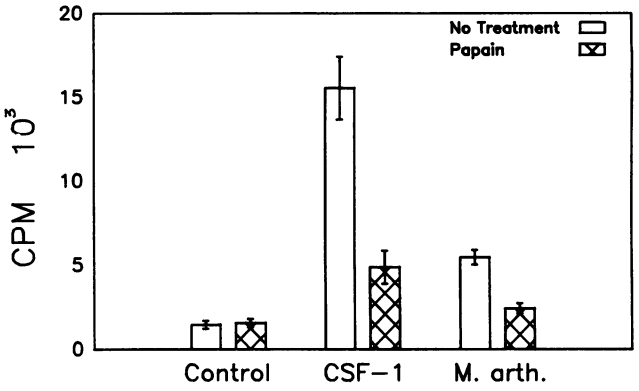

FIG. 3. Effect of papain treatment on the mitogenic activity of membrane preparations from *M. arthritidis* (M. arth.) compared with effect of papain treatment on CSF-1-induced BMM proliferation. Values are means of six determinations ± standard errors of the means.

Since it had been reported previously that membranes from *M. pneumoniae* and *M. neurolyticum* induce the production of some interleukins and/or colony-stimulating factors in lymphocyte cultures (11, 16), we decided to determine whether this was the mechanism by which *M. arginini* and *M. arthritidis* were stimulating the proliferation of BMM cultures. To address this issue, we used monoclonal antibodies specific for CSF-1, IL-3, and GM-CSF that had previously been shown to block the biological activity of these lymphokines (1, 13, 18).

We began these experiments by separately titrating each of the three colony-stimulating factors for the minimal amount of factor required to achieve maximal BMM proliferation. We next determined the concentration of each antibody required to neutralize the biological activity of the appropriate colony-stimulating factor when a fivefold excess of that factor was used (data not shown). Once the optimal neutralizing titer was determined, we tested the abilities of these antibodies to inhibit the mycoplasmal mitogenic activity. As shown in Fig. 4, antibodies specific for CSF-1 did not affect the mycoplasma-induced proliferation of BMM cells while blocking 95% of the CSF-1-induced proliferation.

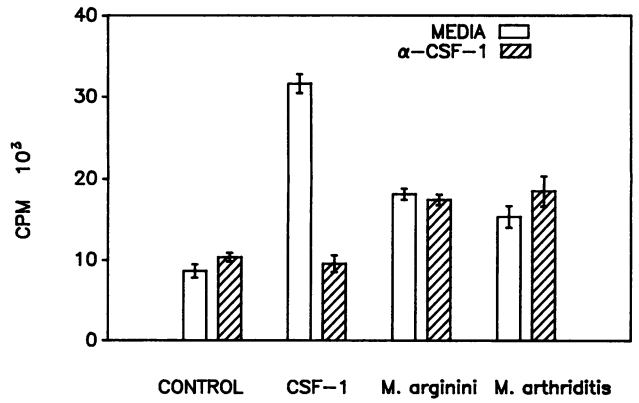

FIG. 4. Effects of medium alone and of monoclonal antibodies directed against CSF-1 (α-CSF-1) on the mitogenic activities of membrane preparations from *M. arginini* and *M. arthritidis* compared with their effects on CSF-1-induced BMM proliferation. Values represent means of six determinations ± standard errors of the means.

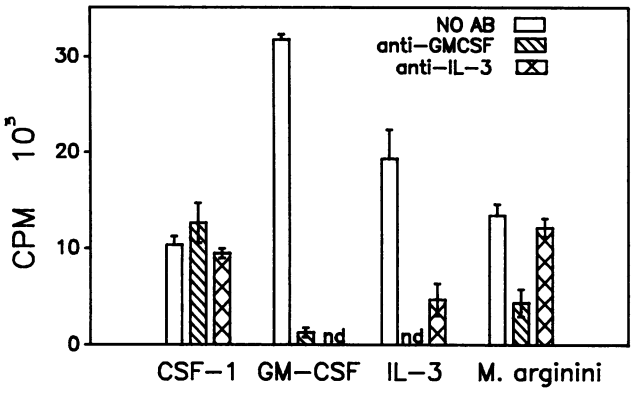

FIG. 5. Effect of monoclonal antibodies directed against GM-CSF and IL-3 on the mitogenic activity of membrane preparations from *M. arginini* compared with their effects on CSF-1, GM-CSF, and IL-3 on the proliferation of BMM cultures. Values represent means of six determinations ± standard errors of the means. nd, Not determined; AB, antibody.

Likewise, when antibodies to IL-3 were added to these cultures, the IL-3-induced response was reduced by 80% and the mycoplasma-induced proliferation was virtually unaffected (Fig. 5). However, when antibodies to GM-CSF were added to these cultures, the mycoplasma-induced proliferation was reduced by 67% (Fig. 5). While the magnitude of this reduction did not approach that of the antibody's ability to block recombinant GM-CSF (96%) (Fig. 4), it was nonetheless a very significant reduction of the proliferative activity normally seen with mycoplasma membranes. These data clearly demonstrate that the mechanism for induction of BMM cells by mycoplasmas involves, at least in part, the production of GM-CSF by a cell that we have yet to identify.

DISCUSSION

The data presented in this report demonstrate the ability of membrane preparations from mycoplasma organisms to induce the proliferation of primary BMM cells. We have presented data demonstrating mitogenic activity in membrane preparations from *M. arginini* and *M. arthritidis* but not in similar preparations from *M. pulmonis*, indicating that the factor that mediates this activity is not shared by all *Mycoplasma* species. In an effort to characterize the factor that mediates this activity, we have shown that it consists, at least in part, of a heat-stable protein. The evidence supporting this conclusion is its sensitivity to papain treatment (Fig. 3) and its ability to withstand heating to 90°C for 30 min (data not shown). Our preliminary studies using ultrafiltration membranes have also indicated that the factor is probably less than 60 kDa in size (data not shown). Finally, the mechanism of action of this factor does not involve the production of either IL-3 or CSF-1 but does involve the production of GM-CSF by an as yet undefined cell in our BMM cultures.

These findings are in agreement with a previously published report on the effects of *M. pneumoniae* on human peripheral blood mononuclear cells (16). It was reported that membrane preparations from this organism led to the production of a colony-stimulating factor similar to GM-CSF (16).

We have yet to identify the cell in our BMM culture that is responsible for the production of GM-CSF. Since numerous cell types have been shown to be capable of producing GM-CSF, the most likely cell would be one of the monocytic lineage, as better than 95% of the cells in the culture at the time of mycoplasma addition were Mac-1$^+$. Alternatively, it is possible that a contaminating T, B, or stromal cell might be responsible; however, since peak proliferative responses were observed between 3 and 4 days following addition of mycoplasmas, the cell must be relatively abundant. If that is the case, it suggests that mycoplasmas induce BMM cultures in a purely autocrine fashion. In this scenario, the mycoplasma-derived factor stimulates BMM cells to produce GM-CSF, which in turn stimulates the proliferation of BMM cells. Thus, GM-CSF is both made and utilized by the same or possibly nearby cells.

In an effort to determine whether the mitogenic activity of mycoplasmas is mediated by previously described mycoplasmal factors, two possibilities immediately come to mind. We have previously reported that mycoplasmas produce a factor, MIaF, which induces MHC class I and II expression in BMM and certain macrophage cell lines (31). Since GM-CSF has been reported to increase MHC class II expression in BMM cultures (10), one might conclude that the mechanism for MIaF's activity involves the production of GM-CSF. However, unlike the proliferative responses induced by mycoplasmas, MIaF activity was demonstrated for all species of *Mycoplasma* tested, including *M. pulmonis*, which did not induce BMM proliferation. This suggests that the factors that mediate these two activities are probably not the same. It also suggests that MIaF activity probably does not involve the production of GM-CSF unless the method by which *M. pulmonis* induces MHC expression is markedly different from that for either *M. arginini* or *M. arthritidis*. Additional support for the separation of these two activities was provided by recent experiments in our laboratory demonstrating the inability of GM-CSF to induce MHC expression in the WEHI-3 myelomonocytic cell line, which is MHC inducible by MIaF (data not shown).

Another candidate for this proliferative effect is the previously described *M. arthritidis* mitogen (MAM) (4). The fact that MAM is highly stimulatory for mouse T cells raises the possibility that this factor is stimulating residual T cells in our BMM cultures, leading to the production of GM-CSF, which in turn would stimulate the proliferation of BMM cells. While this is an attractive alternative, several findings argue against it. First, we assayed T-cell proliferation in the presence of membranes from *M. arthritidis* and *M. arginini*, and only *M. arthritidis* was able to stimulate T-cell proliferation regardless of the concentration of *M. arginini* membranes used (data not shown). Second, and probably most persuasive, is the fact that MAM is inactivated by heating for 1 h at 56°C (7), while the factor that leads to BMM proliferation is stable after treatment at 90°C for 30 min. It is thus probable that the mycoplasmal factor that initiates BMM cell proliferation is distinct from previously described mycoplasmal factors that have been shown to affect immune cells.

It has recently been reported that modified low-density lipoproteins (LDLs) can induce the production of GM-CSF, CSF-1, and G-CSF in endothelial cell cultures (23). They show that when LDLs are oxidized by exposure to Fe^{2+} or prolonged storage at 4°C, these oxidized LDLs are capable of inducing dramatic increases in mRNA for GM-CSF, CSF-1, and G-CSF. It is thus possible that mycoplasma membrane preparations contain similarly oxidized LDLs, leading to the production of GM-CSF.

The significance of this host response to mycoplasma products remains to be determined. However, there are numerous studies documenting the involvement of myco-

plasmas in several animal diseases, including atypical interstitial pneumonia (3), arthritis (8, 33), and probably ankylosing spondylitis (28) and Reiter's syndrome (29). The mechanism for these organisms' pathogenic effects has not been conclusively determined, but most investigators believe that it involves the activation of host immune cells. With the recent description of T-cell superantigens, which are possessed by some microorganisms, including *M. arthritidis* (6), it has been proposed that these antigens may be involved in the development of autoimmunity by the expansion of autoreactive T-cell clones (17). Since antigen presentation is an absolute requirement for superantigen activity, it is intriguing that mycoplasma organisms also possess factors that lead to the proliferation of such cells. In addition, it has been proposed that GM-CSF may also affect local immune responses by acting as a "trap" for migrating cells, leading to activation, proliferation, and retention of these cells at the site of infection and aggravating the inflammatory response (12, 15, 25, 35). It remains controversial whether this activation of host immune cells leads to the development of specific autoimmunity or whether it simply represents an aggravated inflammatory response.

While there is little solid evidence for the direct role of mycoplasmas in the cause or development of autoimmune disease, the induction of BMM proliferation probably has important implications in the mechanisms of mycoplasma-induced pathogenesis. The extent to which this activity is involved in vivo and to what degree this potential expansion of macrophage populations leads to pathogenesis remain to be determined.

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