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Intracellular survival of mycobacteriawithin macrophages : Role of Ca^{2+}-dependent signal in *Mycobacterium avium*pathogenesis?

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ABSTRACT

Background: Pathogenic *Mycobacterium avium*are able to survive intracellularly withinhost cells defenses after phagocytosis, whereas nonpathogenic strainssuch as*M. smegmatis*are efficiently killed.In the present study, we attempted to explore the molecular mechanisms by which *M. avium* escape the intracellular killing. We also tried to investigate the effect of changes in cytosolic Ca^{2+}levels on *M. avium* survival, the phagosome-lysosome fusion and the NFκB activation. **Methods:** To explore the invasion mechanism by which the pathogenic bacteria survive inside phagocytic cells as confirmed by killing assays, live and dead*M. avium* were testedby fluorescence microscopy techniques. The hypothesis that calcium levelscould playa keyrole in regulating the immune responseduring mycobacterial infections, was verified by using Ca^{2+} inhibitors. **Results:** Wehave shown that following phagocytosis,virulent *M. avium*invade successfully and replicate within host cells.The intracellular survival of this bacteriumis not affected by the nitric oxide production.Our results suggest that *M. avium* escape the intraphagosomal killingby blocking the phagosome-lysosome fusion.Furthermore, the NFκB activation is strongly inhibited by *M. avium* by a mechanism which might be in a part, regulated by the intracellular Ca^{2+}-signaling. **Conclusion:**these findingsargue that the virulent*M. avium*strainmight escape the intracellular killingwithin host phagocytes by a mechanism that inhibits the phagosome-lysosome fusion. This process seems to be a Ca^{2+}-signaling dependent. Furthermore, the NFκB is efficiently blocked in bacteria-infected macrophages. This should be regulated by a Ca^{2+}-dependent pathway.Interestingly, we conclude that mechanisms involved in*M. avium* resistance during mycobacterial infections could be linked and/or regulated by the intracellular Ca^{2+}pathways.

KEYWORDS : Mycobacteria, *M. avium*, Macrophages, Phagocytosis, Fluorescence microscopy.

RESUME :

Introduction : Les mycobactéries pathogènes telles que *Mycobacterium avium* sont des bactéries pathogènes capables de survivre à l'intérieur des cellules cibles de la défense immunitaire après phagocytose. Cependant, les souches non pathogènes telles que *M. smegmatis* sont efficacement tuées. Dans ce présent travail, nous avons essayé d'explorer les mécanismes moléculaires par lesquels *M. avium* échappent la mort intracellulaire. Nous avons également tenté d'étudier l'effet des variations intracellulaires de Ca^{2+} sur la capacité des bactéries de survivre, sur la fusion des phagosomes-lysosomes et enfin sur l'activation du facteur de transcription NFκB. **Méthodes :** afin d'étudier les mécanismes d'invasion par lesquels les bactéries virulentes survivent à l'intérieur des phagocytes, les bactéries vivantes aussi bien que les bactéries mortes sont testées par la technique d'immunofluorescence. L'hypothèse émise suggérant l'éventuel rôle clé du Ca^{2+} dans la régulation des réponses immunes au cours des infections à mycobactéries, a été vérifié en utilisant différents inhibiteurs spécifiques pour le Ca^{2+}. **Résultats :** nous avons montré que, après le processus de phagocytose, les souches virulentes de *M. avium* se multiplient efficacement à l'intérieur des cellules cibles infectées. La survie de ces bactéries n'est pas affectée par la production des dérivés toxiques tels que le NO. Nos résultats suggèrent que *M. avium* échappent la dégradation

intraphagosomale en inhibant la fusion des phagosomes-lysosomes. D'autre part, l'activation du NFκB dans les cellules infectées est fortement bloquée par *M. avium* par un mécanisme qui serait en partie, dépendant de la signalisation intracellulaire du Ca^{2+}. **Conclusion :** Dans l'ensemble, nos résultats indiquent que les *M. avium* pathogènes sont capables d'échapper la mort intracellulaire par les phagocytes en inhibant la formation des phago-lysosomes ; ce mécanisme semble être Ca^{2+} dépendant. De plus, l'activation du NFκB est efficacement altérée par les souches virulentes, et qui serait probablement régulée par les voies de signalisation intracellulaire du Ca^{2+}. En conclusion, le Ca^{2+} joue un rôle critique au cours des infections à mycobactéries ; il semble réguler le mécanisme de résistance développé par les souches virulentes au cours de l'activation des macrophages infectés.

MOTS CLÉS : *Mycobacterium avium*, mort intracellulaire, phago-lysosomes, Ca^{2+}

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1. Introduction :

Mycobacterium avium (*M. avium*) has been one of the most prevalent MAC (*M. avium* complex) species of nontuberculous mycobacteria (NTM) that causes infectious diseases[1]. Mycobacterial infections remain a major cause of morbidity and mortality worldwide in 2013 (WHO) an estimated number of 9.0 million people developed Tuberculosis and 1.5 million people died of this chronic inflammatory disease[2]. Independently, non-tuberculous mycobacterial infections mainly caused by the group of the *M. avium* complex including *M. avium*, *M. intracellulare*, *M. abscessus* and *M. chelonae*are increasing[3]. Infections with atypical mycobacteria are an important health problem for patients with patients with acquired immunodeficiency syndrome (AIDS) [4].HIV infection predisposes patients to the development of opportunistic infections by virulent mycobacteria. Previous studies [5-6]have shown that the *M. avium* infection may remain latent until the immun system of the individuals has been seriously compromised. *M. avium* bacteria may also infect individuals without apparent predisposing conditions causing chronic lung diseases in non-AIDS infections [7-8-9]such as bronchiectasis. Interstingly, a recent study [10] suggests that MAC infection is an important etiology in patients who present with nodular skin lesions, diarrhea, and fever and patients with MAC infection can have no pulmonary manifestations even in disseminated cases. Recently, it also has been shown [10] that *M. avium* skin and soft tissue infection complicated with scalp osteomylitis possibly secondary to anti-interferon-γ autoantibody formation. The authors suggest that when NTM infection is detected in an immunocompetent patient, IFN- autoantibodies-associated immunodeficiency should be considered

The bacterium can be acquired both through the intestinal route following ingestion then tranlslocation across the intestinal mucosa and respiratory route by aerosol transmission similar to tuberculosis. *M. avium*is capable of invading mucosal epithelial cells and translocating across the mucosa to infect macrophages interfering with several functions of the host cell[12]. The mechanism by which *M. avium*can invade resting macrophages and resist to antimicrobials are currently unknown. It could be attributed in part, to the structure of a cell wall, which is surrounded by a capsule of noncovalently attached polysaccharides, proteins and a small amount of lipids, which include GPLs and phenolic glycolipids[1]. Another mechanism of resistance observed in *M. avium* is the development of biofilms in the environment, such as in water distribution systems, in medical devices and possibly in human airways [13]. Furthermore, multiple invasion mechanisms can enhance mycobacterial invasion *in vivo* and the absence of one pathway can be compensated by others pathways. A previous work [14] suggested the role of complement in *M. avium* pathogenesis.

The pathways and mechanisms used by activated macrophages to kill *M. avium* are not well known. However, the majority of *M. avium* strain resist to intracellular killing by altering immune responses of host cells such as production of nitrates such as nitric oxide (NO)[15-16].Subsequent studies have shown that nitrogen intermediates are not involved in the intracellular killing of virulent mycobacteria. Furthermore, among the responses triggered by *M. avium* when invading macrophages are the production of suppressor cytokines growth factor β (TGF-β) such as transforming and IL-10 [17-18]. Consequently, macrophages infected with *M. avium*

become incapable to eliminate the infecting bacteria and fight the infection.

Inflammatory signaling is a central mechanism controlling host defenses against intracellular pathogenswhich develop several mechanisms to survive in host macrophages. Howeover,how theses mechanisms are regulated remains poorly undergetsrtoud. The phagocytosis plays an essentiel and critical role in host defense strategiesand since this event is often triggered by the interaction of target-bound opsonins with specific receptors on the surface of phagocytes, it would activate the host cell by increasing the cytosolic Ca^{2+} levels. The enhanced Ca^{2+} signals were accompagnied by enhanced phagolysosome formation[19]. Ca^{2+} is a ubiquitous intracellular messenger controlling a diverse range of cellular processes, such as gene transcription, cell proliferation and apoptosis[20]. Cytosolic Ca^{2+} signaling occurs through both Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular environment.In this context, we tried in the present work to explore the molecular mechanisms involved in*M. avium*pathogenesis. We also attempted to ascertain whether the Ca^{2+} signaling contributed in promoting the intracellular survival,phagolysosome fusion and factor transcription activation.

2. MaterialsandMethods

Reagents

The following antibodies were used for immunofluorescence microscopy : Anti-mouse LAMP-1 (lysosomal-associated membrane protein 1) was purchased from the lowa Hybridoma bank. The rabbit iNOS antibody was kindly provided Dr. Michael Marletta (University of Berkeley). For colocalization experiments of LAMP-1, the secondary antibodies were linked with Cy3. The following inhibitors were used SB20358 (Calbiochem), the i-NOS inhibitor L-NAME (N(G)-nitro-L-arginine methyl ester hydrochloride, SKF, 2-ABP (2-aminoethoxydiphenyl borate) were from Sigma. The lipopolysaccharide (LPS) from *Klebsiella* and *E. coli*, ionomycin and INF-γ iwere from (Sigma).The Oregon-Green was from Molecular Probes.

Cell line and bacterial culture conditions

The mouse macrophages cell line J774A.1 was cultured as described previously [21]. *M. avium**MAC101*and *M. smegmatis* mc2155 harboring a p19 (long-live)-EGFP plasmid were grown in the Middlebrook 7H11 culture medium (BD Life Sciences) supplemented with 0.5% glucose and 0.05% Tween 80 at 37°C on a shaker at 220 r.p.m until the culture reached an optimal density (OD_{600}) = 0.1,as previously described [22].

Macrophage infection

Bacterial cultures in exponential growth phase were pelleted, washed in PBS pH 7.4, and re-suspended in DEMEM medium. Clumps of bacteria were removed by ultrasonic treatment of bacteria suspensions in an ultrasonic water bath for 15 min followed by a lowed speed centrifugation for 2 min. A single cell suspension was verified by light microscopy as described previously [22].

J774 cells were seeded onto 24 well tissue culture plates at a density of 0.5 x 10^5cells per well and incubated for 2-3 days until 70% of confluency. Confluent cells were infected with bacteria at a multiplicity of infection (MOI)of 10:1 (bacteria/cell)in acomplete medium without antibiotics. Cells were allowed to interact with bacteria for 1h infection, then washed extensively with PBS and chased for the different, indicated time points at 37°C.

Colony-forming units (CFU) assays

Macrophages were plated in 24-well plates and infected with live *M. avium*bacteria at different time points.Cells were washed with PBS and lysed with sterilized water containing Triton X100 (0.1%). Quantitative cultures of *M. avium* were performed by10-fold serial dilutions inoculated on 7H10 agar plates. Ten microliters was plated by triplicate and the number of colonies was counted after 48h and referred as number of colonies (CFU) per milliliter.

Epifluorescence and confocal fluorescence microscopy

For the immunofluorescence confocal microscopy, the non-GFP *M. avium* strain, both live and dead bacteria were labelled with Oregon Green fluorescent probe, as described previously [23]. Cells were fixed with 3% paraformaldehyde (PFA) in PBS at 4°C for 15 min followed by a45 min incubation at a room

temperature. Subsequently, cells permeabilization, when required, was achieved with a 15 min treatment with 0.1% Triton X100 in PBS containing 0.2% BSA. After blocking with 2% fetal calf serum in 40 mM glycine-PBS, cells were incubated with the primary and secondary antibodyas described previously [21][24]. Fluorescence labelling and viability of mycobacteria were performed as described previously [21]. Cells were mounted with DAKO mounting medium and analyzed by confocal microscopy (Zeiss LSM510).

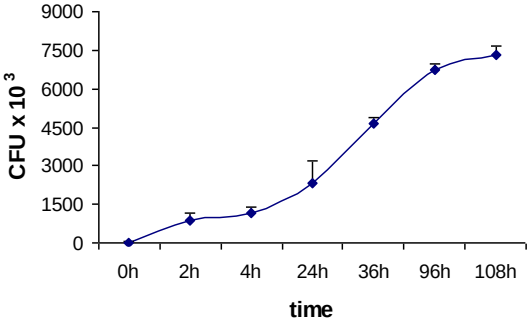
Nitric oxide experiments

Nitric oxide production by infected macrophages was measured by the Griess reaction following the supplier(s) (Sigma) instructions as previously described [22].

3. Results

Intracellular survival of M. avium in macrophages

M. avium is a pathogenic bacterium which is efficiently phagocytosed by macrophages (24). To evaluate its survival and replication inside host cells, we infected J774 cells and followed the colony-forming units (cfu) over the time course of the infection.We found that, in contrast to *M. smegmatis* which were killed within 48h [22], *M. avium*could replicate and survive up to 5 daysinside macrophages as observed in Figure 1a.This suggests that the pathogenic bacteria could escape the intraphagosomal killing by a mechanism which could be regulated by cytosolic Ca^{2+} leves, as it has been reported[19]. To verify this hypothesis, we tested the effect of ionomycin on the survival assays. Interestingly, we found that the ability of the pathogenic bacterium to survive and multiplicate inside macrophages was significantly decreased as shown in Figure 1b. They were efficiently killed within 2-3 days. It seems that pathogenic bacteria after invasing host cells, they alter the Ca^{2+} signal triggered in activated macrophages.



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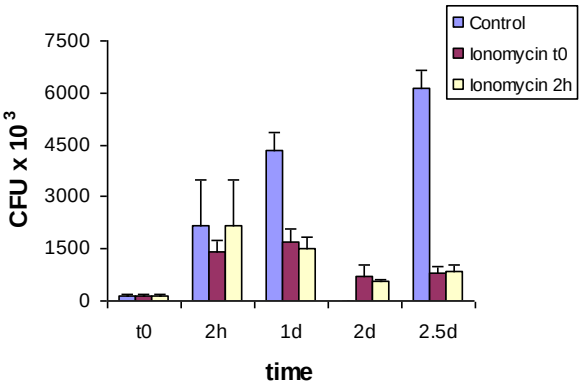


(b)

Figure 1: Intracellular survival of *M. avium* in J774 macrophages. J774 macrophages were infected with *M. avium* at MOI=10 :1 (bac/cell) for 1h and chased at the indicated time points (a). Ionomycin (0.1 nM) was added to infected cells at To and 2h of chasing (b).

Actin polymerization during phagocytosis

By the use of fluorescence confocal microscopy techniques, we attempted to verify the phagocytosis and follow the actin polymerization during the phagocytosis processusing rhodamine-phalloidin [21]. For this experiment, *M. avium* bacteria were labelled with Oregon green () before we allowed their interaction with J774 macrophages. As shown in Figure 2, the labelled actin could be clearly seen around the phagosomes containing bacteria. The assembly of actin is required for the phagosome formation to ingest either pathogenic or non-pathogenic bacteria by macrophages [22].

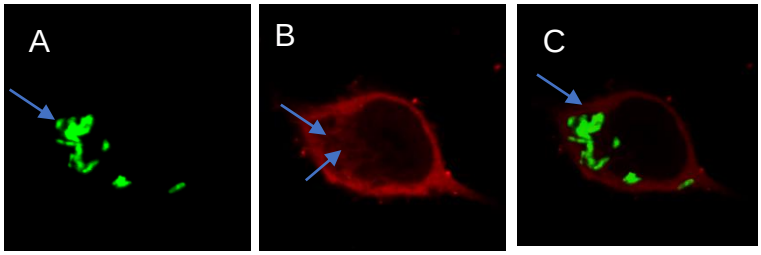
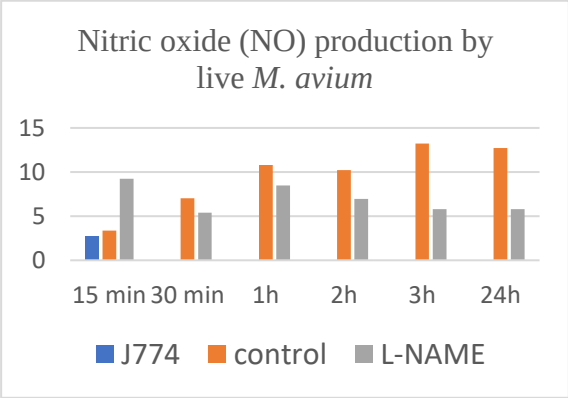


Figure 2 : Actin polymerization in infected J774 macrophages with *M. avium*.J774 macrophages were infected with oregon-green labelled bacteria(A). Cells were pretreated with rhodamine-phalloidin (B) before their interaction with bacteria. The colocalization of phagosomes surrounded by actin (red) with bateria (green) (C) is observed after 1h infection.

Nitric oxide production by M. avium-infected cells

The ability of *M. avium*-infected macrophages to produce the nitric oxide (NO) was verified at different timepoints of infection. As shown in Figure 3a, the rate of NO production was increasing over the time course of infection comparing to non-infected J774 macrophages. The NO production was significantly inhibited by adding L-NAME, a potent blocker of the inducing NO synthase (iNOS), responsable of NO production. Despite the NO production by activated macrophages, *M. avium* could resist to the intra-phagosomal killing by NO. The latest was induced by the iNOS, an active cytosolic enzyme which was detected by immunofluorescence using a specific anibody as shown in Figure 3b.



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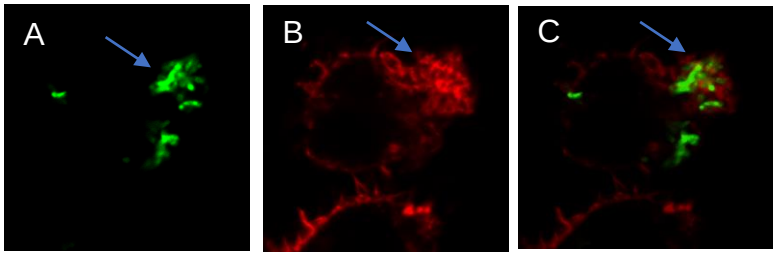


(b)

Figure 3 : Nitric oxide production by infected J774 macrophages with *M. avium*. Rate of NO production byinfected J774 macrophages with bacteria (10:1) in the absence and presence of L-NAME (500 µM) (a). Theactivated iNOS is localized within cytosol (red) around the ingested Oregon-Green labeled *M. avium* (green) (b).

M. avium inhibits phagosome-lysosome fusion

We have previously [22]demonstrated that the non-pathogenic *M. smegmatis*bacteriaare efficiently killed once they are inside macrophages. This was enhanced by the phagosome-lysosome fusion as detected by the colocalization of both markers red/green (merge) by indirect immunofluorescence technique (Figure 4 B-C). In parallel, we tried in the present study to verify whether the pathogenic *M. avium* bacteria affect this event during phagocytosis process. For these experiments, we infected J774 macrophages with live Oregon-Green *M. avium* (10:1) for 1h incubation then chased for 2h. After fixation and permeabilization, infected cells were labeled with antibody against LAMP1 used as a marker for lysosomes (red). As results, we do confirm that in contrast to non-pathogenic bacteria, *M. avium* bacteria block the phagosome-lysosome fusion as detected.

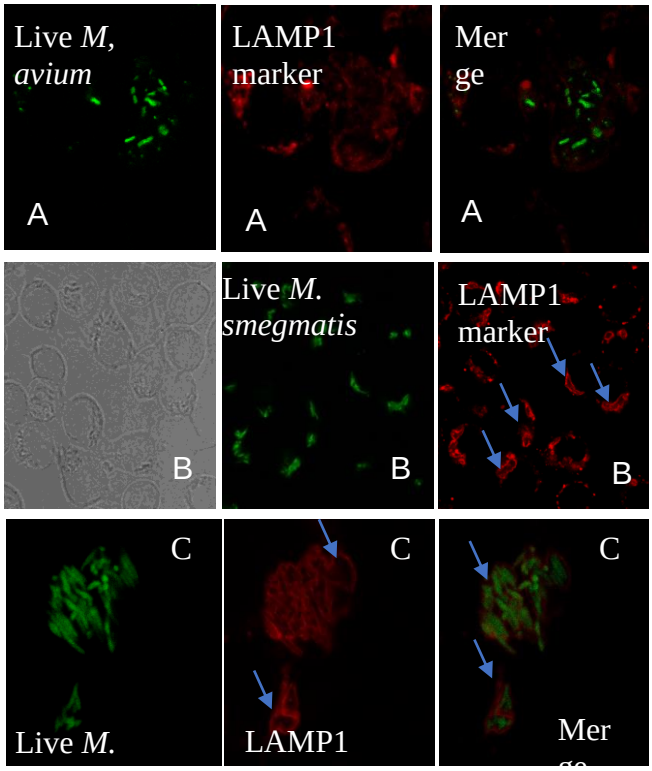


Figure 4 : Phagosome-lysosome fusion in infected J774 macrophages with mycobacteria. (A)J774 macrophages were infected for 1h and chased for 2h with live Oregon-Green labelled *M. avium* (green) (10:1). Indirect immunofluorescence assay confirms the phagosome (green)-lysosome (red) inhibition ; no colocalization observed (merge). (B)(C-zoom) J774 cells were infected with live GFP*-*M. smegmatis*(10:1)(green). Indirect immunofluorescence assays confirm the colocalization (merge) of phagosome containing non-pathogenic bacteria with lysosomes (LAMP1 marker, red).

Ca^{2+} effect on phagosome-lysosome fusion

Since the intracellular survival of *M. avium*within macrophages was modulated by cytosolic Ca^{2+} levels, we attempted to verify whether the phagosome-lysosome fusion is a mechanism Ca^{2+}-dependent. This was achieved by testing the effect of 0.1 nM ionomycin on both dead and live bacteria-infected macrophages. Interestingly, our results are consistent with previous reports[19], which strongly suggestedakey role of [Ca^{2+}] in enhancing the phagolysosome maturation

As shown in Figure 5, it seems that only live bacteria were able to inhibit the Ca^{2+}-signal, and consequently, the phagosome-lysosome fusion was blocked.
. Whereas, dead bacteria did not affect the Ca^{2+}-signal but the latest was significantly abolished when the Ca^{2+} blocker (2-ABP) was added during the macrophages infecion with bacteria.

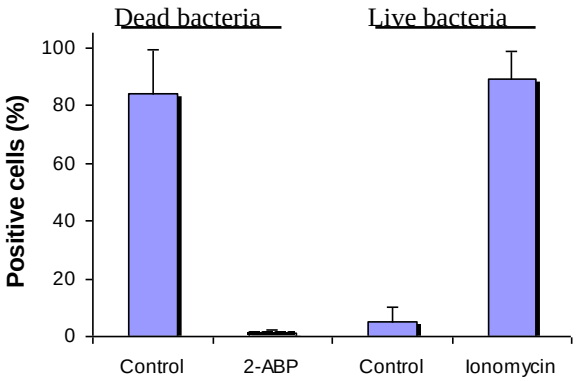


Figure 5 : Phagosome-lysosome fusion in J774 macrophages infected with dead or live bacteria. J774 cells were infected with either dead or live Oregon-Green labelled *M. avium* (10:1) for 1h then chased for 2h. Positive cells are expressed by the (%) of cells having both marker (phagosome-lysosome) colocalized.

M. avium infection inhibits the NF-κB activation

Previous work[24] reported the key role of NFκB during mycobaterial infections. According to the authors, pathogenic *M. avium* bacteria blocked significantly the activation of NFκB by inhibiting it's translocation into the nucleous.This was verified in the present study by indirect immunofluorescence technique. As shown in Figure 6, live Oregon-Green labelled *M. avium*blocked totally the translocation of NFκB from the cytosol into the nucleous (B-C). However, a clear translocation of NFκB into the nucleous was observed (A) when the LPS from *E. coli* wasused to activated J774 macrophages.

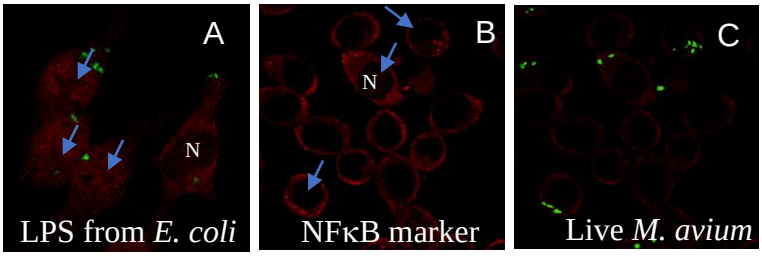
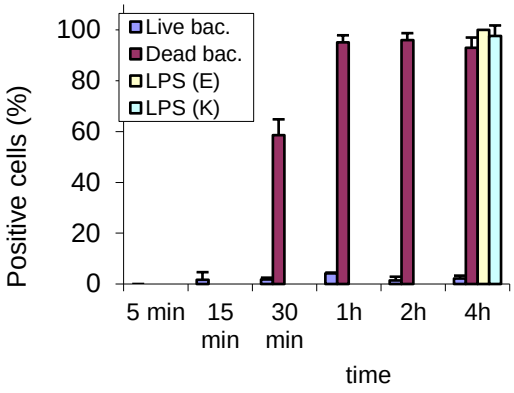


Figure 6 : Activation/inhibition of NFκB in macrophages. Translocation of NFκB into the nucleous is detectedwith a specific antibody (red)

Regulation of NFKB activation : role of Ca^{2+}-signal.

The activation or inhibition of NFκB during mycobacterial infections was investigated by fluorescence microscopy technique as previously described[22][24]. In Figure 7a, we observe that only live pathogenic *M. avium*bacteria were able to inhibit the translocation on NFκB into the nuclous. As results, the activation of NFκB is repressed only by live bacteria which were able to block the Ca^{2+}-signal in previous experiments. In comparison, the activity of NFκB was greater when macrophages were infected with dead bacteria or activated with LPS from *E. coli* or *Klebsiella*. In contrast to live bacteria, dead bacteria were able to activate the NFKB in macrophages which was significantly blocked by the potent inhibitor, SN50, as shown in Figure 7b. Furtheremore, when macrophages were infected with dead bacteria, the higher activity of NFKB obtained at different time of incubation with Ca^{2+} blockers such as 2-ABP and SKF, was almost completely inhibited (Figure 7c).

These findings are consistent with our results suggesting the key role of Ca^{2+} in regulating the cellular functions of infected macrophages. Finally, the role of Ca^{2+} levels in mediating cellular responses against pathogens, was confirmed by using live *M. avium* bacteria to infect macrophages in the presence of ionomycin.As presented in Figure7d, a strong increase in NFKB activity when ionomysin increases the cytosolic Ca^{2+} level. These findings argue the hypothesis that a C^a2^+-promotes signal tranduction which might increase phagocytosis, phagolysosome fusion in cascade activity of NFκB activation.



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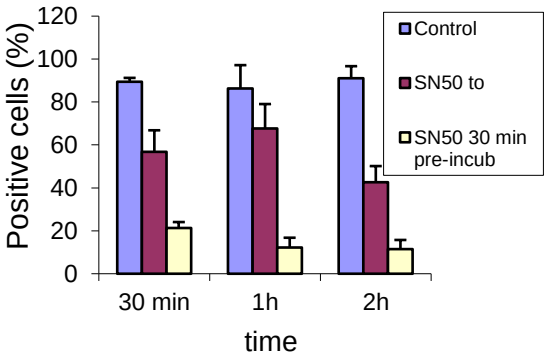

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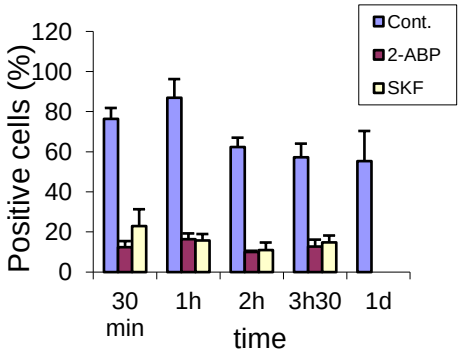

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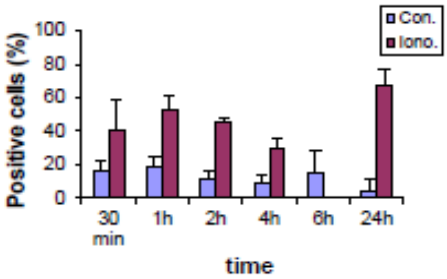

(d)

Figure 7: Regulation of NFκB activation: Role of Ca^{2+} signal. Percentage (%) of positive cells with NFκB translocation once macrophages are activated with (a) different activators (b) dead bacteria in the absence and the presence of SN50, a potent inhibitor of NFκB (c) dead bacteria in the absence and the presence of potent Ca^{2+} blockers (2-ABP, SKF) (d) live bacteria in the absence and the presence of ionomycin at different time points

4. Discussion

We have previously reported[21] that are efficiently killed after macrophages infection. The mechanism of killing involves the phagosome-lysosome fusion, the nitric production and NFκB activation. Another mechanism of killing by human macrophages has been reported [25], providing evidence that cell autophagy plays a key role in the control of mycobacterial infections. Thisprocess may prove a critical mycobactericidal effector mechanism utlilized by phagocytes.In contrast, the pathogenic*Mycobacterium tuberculosis*is a potenthuman pathogen by evading the host cellular immune system. They preferentially infect tissue macrophages and blood monocyteswhere they can replicate intracellularly within phagosomes after internalization. They inhibit efficiently acidification of the phagosome and subsequently prevent the phagosome-lysosome fusion[26-27-28].This process, is critical for *M. tuberculosis* persistence in human populations. Previous reports[29] have demonstrated that the mechanism responsible for the failure of Ca^{2+}-dependent phagosome-lysosome fusion by *M. tubersulosis* involved mycobacterial inhibition of human macrophagesshingosine kinase. Thus, inhibition of sphingosine kinase directly contributes to the intracellular survival and represent a novel molecular mechanism of *Mtb* pathogenesis.Furthermore, the hallmark feature of *M. tuberculosis* is its complex lipid rich cell wall that plays a key role in structural stability and permeability of the cell wall. It is also involved in inhibiting phagosome-lysosome fusion during the pathogenic process [30]. In this context, we were interested in the present study toexplore the mechanismbywhich the virulent *Mycobacterium avium*straincould escape the intracellular killing within host cells. In accordance with previous studies [15][17][24], wefoundthat *M. avium*bacteria were internalized efficiently into host cells by phagocytosis. During this processus, an actin polymerization was observed by immunofluorescence microscopy. A processus which is highly activated during phagocytosis [21].Interestingly,*M. avium* was able to survive and multiply within macrophages after infectiondespite the iNOS activation and NO production by infected *M. avium* macrophages. It is well known[22]that toxic reactive oxygene intermediates like nitrite oxide are very efficient to eradicate and kill a number of bacteria species once they are into phagosomes.In addition, the killing assays results obtained in the present work indicated clearly, that the intracellular survival of the pathogenic strain was significantly blocked when the cytosolic Ca^{2+} concentration was increased in the presence of ionomycin. The latest was used as an activator for Ca^{2+} influx [31] Consequently, we thought that a Ca^{2+}-dependent mechanism could be involved during the phagocytosis processin mycobacteria infectedmacrophages. It has already been reported [19]that the enhanced Ca^{2+}-signals were accompagnied by enhanced phagolysosome fusion, leading to a better understanding of the role of increased Ca^{2+} signals in promoting phagocytosis andphagolysosomal fusion.

The ability of *M. avium* bacteria to escape the intracellular killing is likely due to the inhibition of their maturation into phagolysosomes in which they reside. This was confirmed by fluorescence confocal microscopy data. The hypothesis that the phagosome-lysosome fusion could be a Ca^{2+}-dependent signaling was supported by several reports[32-33] suggesting suggesting that intracellular Ca^{2+} participates in phagolysosome formation. During phagocytosis process, the host phagocytes were rapidly activated and significant changes in intracellular free calcium levels were observed which were associated with signal transduction events and multiple functions ; including activation of cellular kinases and phosphatases, degranulation, phagosome-lysosome fusion and transcription control [20][34]. In this context, we have demonstrated that the phagosome-lysosome fusion was completly blocked by 2-ABP, a potent Ca^{2+} blocker, when macrophages were infected with heat-killed *M. avium*. These findings which are consistent with previous observations, strongly suggest that cytosolic Ca^{2+} might interfere with the virulence of *M. avium*. This evidence is supported by previous work [26-27][29] indicating that [Ca^{2+}] levels regulate phagosome-lysosome fusion in *M. tb*-infected macrophages and that mycobacteria viability is a primary determinant of the [Ca^{2+}] level.

It has been reported[24][35-36] that the NFκB is mainly involved in the cellular mechanism activation during the immune proinflammatory response against

pathogens. The putative role of this transcription factor was verified during phagocytosis by testing dead and live *M. avium*bacteria. In accordance with a previous study [24] we have found that the NFκB translocation into the nucleous was efficiently blocked by the live virulent strain as confirmed by immunofluorescence assays. Whereas, dead bacteria were able to induce the NFκB activation, a process which was significantly inhibited in the presence of the SN50;a potent blocker of NFκB activation.Furthermore, the NFκB pathway seems to be regulated by changes in cytosolic Ca^{2+} levels, since blocking this event by pharmacological Ca^{2+} blockers, SKF and 2-ABP, strongly reduced the translocation of NFκB into nucleous when dead bacteria were tested. Taken all together, these results support our hypothesisthat the NFκB activation by *M. avium*could be a Ca^{2+}-dependentpathway as already reported [37]. This was confirmed when live bacteriawere tested and compared to the results obtained in the presence of ionomycin, a potent activator of Ca^{2+}. Interstingly, the significant inhibition of the NFκB activation by live *M. avium* was restored when ionomycin was added to increase the level of cytosolic Ca^{2+} levels. Taken all together, the findingstrongly support the hypothesis that pathogenic *M. avium* might affect the Ca^{2+} signaling pathways after macrophages infection. Finally, we conclude that cytosolic Ca^{2+} levels play a critical role in regulating the cellular and molecular mechanisms activated by the innate and adaptative immune response during mycobacterial infections. Consequently, the host cellular functions which are Ca^{2+}-dependent events,were affected in particular, the phagosome-lysosome fusion and the NFκB activation, leading to the sustained intraphagosomal survival of virulent *M. avium*. The mechanism of pathogenic mycobacteria evasion to Ca^{2+}-mediated antimicrobial defensesis still poorly understood. Further work shoud be explored to a better understand.

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