# Macrophage nutriprive antimicrobial mechanisms

Rui Appelberg[1]

*Laboratory of Microbiology and Immunology of Infection, IBMC-Institute for Molecular and Cell Biology and ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal*

**Abstract: In addition to oxidative and antibiotic mechanisms of antimicrobial activity, macrophages are able to deprive intracellular pathogens of required nutrients. Thus, microbial killing may not rely only in the toxic environment the microbe reaches but also may result from the scarcity of nutrients in the cellular compartment it occupies. Here, we analyze evidence for such nutriprive (from the latin *privare*, to deprive of nutrients), antimicrobial mechanisms. Although the direct analysis of nutrient availability is most often not feasible, indirect evidence of lack of nutrients in the microbial organelles has been inferred from the study of mutants, the analysis of gene expression, and the consequences of changing the intracellular location of the pathogen. We propose that according to the microbe and its survival strategy, different mechanisms to impede access to nutrients may be constitutively present or may be induced by cytokines and other pathways. Thus, membrane transporters may remove nutrients from vacuolar compartments, and enzymes may degrade some growth factors. A series of diverse compounds may sequester other molecules required for microbial growth, as exemplified by the action of iron chelators. Modulation of vesicular trafficking may prevent the fusion of certain vesicles containing nutrients with those containing the pathogen, counteracting the evasion strategies of the pathogen. The understanding of these mechanisms will certainly help in designing new therapeutic and prophylactic approaches to preventing infectious diseases. *J. Leukoc. Biol.* 79: 1117–1128; 2006.**

***Key Words:*** *nutrient · phagosome · iron · intracellular parasites · immunity*

## INTRODUCTION

The research into the mechanisms used by professional phagocytes to control microbial growth has been centered on the identification of toxic molecules or "toxic environments" that lead to their demise. This was started with the discovery of the phagocytes' oxidative burst and the study of the role of oxygen radicals in the 1960s [1]. Soon after, oxygen-independent mechanisms were suspected [1], and in the 1980s, nitric oxide (NO) production by a phagocyte synthase was identified as a major pathway of microbial elimination [2]. Even today, NO and its derivatives are considered the major players in the killing of intracellular parasites. However, numerous reports highlighted the existence of antimicrobial activity, which is not dependent on NO generation. NO-independent mechanisms of control of *Listeria monocytogenes* [3], *Legionella pneumophila* [4, 5], *Mycobacterium avium* [6, 7], *Trypanosoma cruzi* [8], and *Anaplasma phagocytophilum* [9] have been reported in the literature. Even when NO has been shown to play an important role in the control of microbial proliferation such as in the case of *Mycobacterium tuberculosis*, NO production by itself does not explain the ability of a host to control this pathogen [10]. Using double-knock mice, deficient in the generation of oxygen or nitrogen-reactive species, Shiloh et al. [11] concluded for the existence of additional antimicrobial mechanisms active against salmonellae and listeriae.

The lifestyles of intracellular pathogens vary considerably. Some appear to block vacuole maturation, arresting it at a certain stage (e.g., mycobacteria), some allow full maturation (e.g., *Coxiella* and certain *Leishmania*), and others appear to "construct" their own replicative organelle (e.g., *Legionella*, *Brucella*, and *Salmonella*). Mycobacteria and Salmonellae prevent the fusion of the pathogen-containing vacuole with the lysosome. Whereas the former remain in a compartment with early/late endosomal characteristics and continuously interact with endosomes [12–14], the latter only transiently inhabit such a compartment (containing Rab5 and early endosomal antigen 1), moving onto a more modified, microtubule-asssociated vesicular system, the *Salmonella*-induced filaments, which acidify through the action of vacuolar adenosinetriphosphatases (ATPases) and contain Rab7, its effector Rab-interacting lysosomal protein, and lysosomal-associated membrane protein-1 (LAMP-1) [15]. In contrast, parasitophorous vacuoles containing certain species of *Leishmania* mature into lysosomal-like compartments rich in cathepsins and contain Rab7, vacuolar ATPases, LAMP-1 and -2, and the mannose-6-phosphate receptor [16, 17]. These parasitophorous vacuoles are extremely fusogenic, acquiring material obtained by fluid-phase endocytosis [16, 18], from autophagic vacuoles [17], and even large structures such as phagocytosed zymosan [19, 20]. In contrast, the *L. pneumophila* phagosome has been shown to interact with the endoplasmic reticulum (ER) [21], but as replication of the bacteria occurs, replication vacuoles become acidic and begin interacting with the lysosomal compartment

[1] Correspondence: IBMC, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. E-mail: rappelb@ibmc.up.pt

Received February 3, 2006; revised March 6, 2006; accepted March 12, 2006; doi: 10.1189/jlb.0206079.

[22]. Clearly distinct is the vacuole occupied by *Toxoplasma gondii*: It does not interact with endosomes or lysosomes, does not express the vacuolar ATPases, and has unclear associations with the ER and mitochondria, likely mediated by the parasite rhoptry protein (ROP) 2 [23, 24]. The *Brucella* vacuole, conversely, avoids fusion with the lysosome and interacts with, or at least acquires markers from, the ER [25, 26], whereas the *Chlamydia* replication compartment, named inclusion, does not fuse with lysosomes and resides close to the Golgi apparatus and mitochondria [23]. Some pathogens use a more dramatic approach of lysing the vacuole membrane and invading the cytoplasm. Examples of the latter include *L. monocytogenes* [27], *T. cruzi* [28], and *Shigellae* [29].

Much of the information regarding how a microbe accesses nutrients inside the macrophage is still lacking: What are those nutrients? How are they obtained inside the host cell? Do the subversion tactics used by intracellular microbes, namely those that affect the normal trafficking of an ingested particle, play a role in the acquisition of nutrients? Conversely, the antimicrobial machinery of the phagocytes leading to restricted access to nutrients (nutriprive mechanisms) is still unclear: Does the macrophage limit access to nutrients at all? What are the mechanisms involved? Are they constitutive, or can they be induced upon activation by the immune system? Does the macrophage play an active role in sabotaging the subversion strategies of the ingested microbe, namely after activation?

Here, we will analyze some of these questions. We will define nutrient in a relatively broad sense—any molecule acting as a source of energy and reducing power as a source of basic elements (carbon, sulfur, nitrogen, iron, and others) or as a required growth factor, such as any organic molecule the microbe is unable to synthesize, namely vitamins, amino acids, or nucleotide bases. Electron acceptor molecules (in most instances, oxygen) and physicochemical properties such as the pH will also affect microbial growth and may be manipulated by the phagocyte or the structures they form in vivo (e.g., the granulomas). As we need to understand which nutrients are accessed by proliferating intracellular pathogens to evaluate whether they are limiting in a situation of restricted microbial growth, the first part of this article will review what is known about the nutritive potential of the macrophage habitat.

## IDENTIFICATION OF THE NUTRITIVE REQUIREMENTS OF INTRACELLULAR PATHOGENS AND OF THE SMORGASBORD SUPPLIED BY THE MACROPHAGE: EVIDENCE FOR THE DEPRIVATION OF SPECIFIC NUTRIENTS

Assessing the presence of specific nutrients in a defined compartment of a cell inhabited by a particular microbe is a daunting task, which is yet to be performed for most candidate molecules. This is made more complicated by the fact that different intracellular parasites reside in different compartments of the cell, many of which they custom-tailor themselves. It is clear today that this subversion of the vacuolar trafficking by many microbes is accomplished, not only to avoid the final lysosomal destiny and thus the escape from a toxic and possibly deadly environment but also to provide access to nutrients required for the intracellular replication [30]. Some nutrients, such as iron, can be more easily studied, and for those, there is already a large body of information available. Other molecules are much more difficult to study, and for those, we may have to rely on indirect evidence. The following will review evidence, direct or indirect, of access to nutrients by intramacrophage pathogens.

### Direct analysis

Wagner et al. [31] used the hard X-ray microprobes to detect X-ray fluorescence and to directly estimate the presence of 10 elements inside phagosomes containing mycobacteria. They paid particular attention to iron and showed an enrichment in vacuoles with time as well as a decrease following activation with interferon-γ (IFN-γ) or tumor necrosis factor (TNF). Furthermore, they showed that a mutant deficient in the synthesis of the siderophore, mycobactin, was defective in its ability to accumulate iron, showing that iron was supplied to the mycobacterial phagosome as it was used by the microbe, as expected. The study of iron is also amenable to analysis by ultrastructural autoradiography [31] or by scintillation counting on isolated bacterial cells [32] following the addition of $^{59}Fe$ to infected cells. The presence of a labile iron pool was also detected in the cytoplasm using an iron-sensitive fluorescent probe, calcein [33]. The pH in vesicular compartments can be determined by the accumulation of certain weak bases upon protonation in the low pH compartments followed by immunochemical or fluorescence detection of the amount of probe concentrated in such cellular compartments. This technique was used to determine the lack of acidification in mycobacterial vacuoles [34]. The pH can also be assessed in vacuoles by using fluorescent probes with a pH-sensitive emission spectrum coupled to the microbial cells, and in this way, reduced acidification of mycobacterial phagosomes has been confirmed [35].

### Indirect indications of the presence of nutrients

One indirect way to look at the availability of nutrients is to study gene expression signatures. Another is to analyze the natural requirement for growth factors (natural auxotrophy) or to compare the growth of auxotrophic mutants with the growth of wild-type microorganisms.

### Study of auxotrophy

*Salmonella* does not assimilate folate. Instead, it synthesizes it from para-amino-benzoic acid (PABA). As the host cannot provide PABA, the microbe will not synthesize folate if the synthesis of PABA is inhibited, hence the efficacy of sulfonamides in chemotherapy. In addition, *Salmonella* requires 2,3-dihydroxybenzoate (DHB) for enterochelin synthesis, and again, this precursor cannot be provided by the host. Chorismate is a precursor for PABA and DHB. Hoiseth and Stocker [36] produced a *Salmonella* mutant (*aro*A), which was unable to synthesize chorismate. This model system is thus the ideal situation to analyze how the lack of a nutrient (in this case, two, none of which can be supplied by the host and thus represent a situation of nutrient deprivation) will affect growth of a

pathogen inside its host. These authors reported an increase in 50% lethal dose ($LD_{50}$) from less than 20 bacteria for the wild-type strain to more than $3 \times 10^5$ bacteria for the *aro*$^-$ strain. Therefore, we can postulate that limitation of a nutrient in a host would make an auxotroph for that nutrient to increase its $LD_{50}$ to such an extent and thus set the read-out for the assessment of the presence of a specific nutrient or the existence of putative, nutriprive mechanisms in the host in the context of salmonellosis. It should be kept in mind, however, that other explanations may underlie an increase in $LD_{50}$ of a particular mutant and that even intracellular pathogens may at some point be extracellular and thus have different nutrient requirements. **Table 1** summarizes the impact of auxotrophy on the ability of intracellular pathogens to proliferate in vivo or in cultured macrophages.

Fields et al. [37] screened 9516 *Salmonella typhimurium* mutants for lack of survival in mouse macrophages and found that the avirulent mutants included 12 auxotrophs (four purine, four pyrimidine, one aromatic amino acid pathway, two histidine, and one methionine auxotrophs). Most auxotrophs had $LD_{50}$ of $10^3$ or greater. Leung and Finlay [38] produced a series of mutants of *S. typhimurium*, which they analyzed for their ability to proliferate inside the host. They found that purine, uracil, and isoleucine/valine auxotrophs were unable to proliferate in mice but survived in cultured macrophages. This suggests that the intracellular niche occupied by *Salmonella* does not provide the nutrients, which these auxotrophs require, and furthermore, the lack of growth is likely not a result of accrued susceptibility to the antimicrobial mechanisms of the macrophage but rather the lack of nutrients. The fact that the selection of the mutants was done on the basis of their inability to grow after random mutagenesis also suggests that nutrients other than purines, pyrimidines, and certain amino acids may be accessible to the intravacuolar salmonellae. More careful microbiological analysis shows that these mutants survive in vivo in the infected organs for quite a long time (2–3 weeks before the number slowly decreases over a 2-month period), again making the point that the basic limitation to growth is the lack of nutrients and not an increase in susceptibility to the host's toxic armamentarium [51]. In contrast, it has been shown that only 3.2% of human clinical isolates of the typhoid bacillus are prototrophic [39]. Indeed, most of the isolates were auxotrophic for tryptophan, suggesting that at least this amino acid is not limiting in the salmonella vacuole; otherwise, this pathogen would not cause infection. The fact that glutamine synthetase mutants of *S. typhimurium* were still virulent, and only the simultaneous destruction of the genes encoding this enzyme and that of the genes encoding the transporters of glutamine could increase the $LD_{50}$ to $>10^5$ bacteria [40] suggests that glutamine may be available to salmonellae in macrophages.

TABLE 1. The Effect of Auxotrophy on the Ability of Pathogens to Proliferate in vivo or in Cultured Macrophages

| Pathogen | Auxotrophy | Effect on growth | References |
|---|---|---|---|
| *Salmonella* spp. | Purines | Reduction | 37, 38 |
| | Pyrimidines | Reduction | 37, 38 |
| | Aromatic amino acids | Reduction | 37 |
| | Histidine | Reduction | 37 |
| | Methionine | Reduction | 37 |
| | Isoleucine/valine | Reduction | 38 |
| | Tryptophan | None | 39 |
| | Glutamine | None | 40 |
| *M. tuberculosis* | Purines | Reduction | 41, 42 |
| | Leucine | Reduction | 43 |
| | Lysine | Reduction | 44 |
| | Panthotenate | Reduction | 45 |
| *Mycobacterium bovis* BCG | Leucine | Reduction | 46, 47 |
| *L. pneumophila* | Tryptophan | None | 48 |
| | Thymidine | Reduction | 48 |
| *Brucella melitensis* | Purines | Reduction | 49 |
| *L. monocytogenes* | Several amino acids | None | 50 |
| | Adenine | None | 50 |
| | Uracil | None | 50 |
| | Niacin | None | 50 |

BCG, Bacillus Calmette-Guérin.

*M. tuberculosis*, albeit its slow replication rate, has no known growth factor requirements [52]. Mycobacteria auxotrophs have also been characterized, and the data are similar to those obtained with Salmonella, inasmuch as a lack of purines and certain amino acids appears to make the *M. tuberculosis* vacuole inhospitable to auxothophs, such as the *pur*C [41, 42], the *leu*D [43], and the *lys*A [44]. However, when proline and tryptophan auxotrophs were compared [53], the latter appeared to be less able to survive than the former, suggesting that the deficiency in certain amino acids is more severe than others or that the mutations may in some way affect the fitness of the mycobacteria to survive in the macrophage. In the case of mycobacteria, it is not clear whether the auxotrophy leads to an increase in susceptibility to antimicrobial (e.g., oxidative) mechanisms or to stress or if an inability to run salvage pathways for the required nutrient does not compensate for its presence in the vacuole. Similar caution must be used in interpreting the low virulence of panthotenate auxotrophs [45]. Diminished amounts of panthotenic acid may lead to defects in fatty acid metabolism and polyketide synthesis with possible deficiencies at the level of resistance to oxidative damage and reduced synthesis of virulence factors. When comparing the growth characteristics of a leucine BCG auxotroph, Jacobs and colleagues [46, 47] found that the lack of in vivo virulence exhibited by the mutant was related to its inability to proliferate inside a macrophage cell line and thus, concluded that leucine was not available to the mycobacteria inside the host cell. It is thus safe to speculate that the concentration of amino acids, purines, and vitamins is likely to be reduced severely in the mycobacterial vacuole.

*L. pneumophila* is quite demanding in regards to its nutritional requirements. However, it was shown that tryptophan auxotrophs are still able to proliferate in human monocytes, suggesting no limitation of this nutrient in the pathogen vacuole [48]. In contrast, thymidine auxotrophs did not grow in monocytes [48], supporting the notion that phagosomes lack this nucleotide. Likewise, *B. melitensis* mutants, deficient in purine biosynthesis, exhibit reduced virulence in mice [49]. An extensive analysis of *Brucella suis* mutants unveiled 15 amino

acid biosynthetic genes and five purine/pyrimidine biosynthetic genes whose mutation would lead to attenuation of the pathogen in cultured macrophages, suggesting that the *Brucella*-containing vacuole is poor in such nutrients [54]. It should be noted that the *Legionella* and the *Brucella* vacuoles possess markers of the ER, and yet, only the latter appears to be limiting in amino acids, showing that they are indeed distinct compartments.

In contrast, the cytoplasm probably presents no such restrictions, and even naturally auxotrophic *L. monocytogenes* can proliferate in this locale. In fact, this bacterium was shown to be quite demanding in regards to its growth-factor requirements: Seven amino acids and the vitamins riboflavin, thiamine, biotin, and thioctic acid are required for in vitro growth, and it is quite dependent on glucose (or some substituting sugar) for proliferation [55]. If on top of this natural auxotrophy, auxotrophic mutants are induced for additional amino acids, adenine, uracil, or niacin, listeriae are still able to proliferate intracellularly [50], suggesting no restriction of all these nutrients in the cytoplasm of macrophages. As already mentioned, the cytoplasm also has iron available as a labile iron pool [33]. It is not surprising that when made unable to egress the vacuole, *L. monocytogenes* loses its ability to replicate [56, 57], probably as it no longer obtains the required nutrients.

Toxoplasma requires cholesterol [58, 59] and purines [60] to proliferate. It is thus fair to suppose that the isolated vacuole it inhabits has access to these two groups of molecules. Cholesterol is obtained from low-density lipoproteins obtained through receptor-mediated endocytosis and recycled through the lysosomal compartment [58], whereas purines may reach the parasitophorous vacuole (PV) from the cytosol through functional pores in the PV membrane [61]. Similarly, *T. cruzi*, a cytosol inhabitant, and *Leishmania*, an inhabitant of an almost mature phagosome, also require purines [62] and must obtain them from these locations. Whereas no restrictions are apparent in a cytosolic location, the intravacuolar *Leishmania* parasites should resort to some strategy to obtain such metabolites. It has been reported that these parasitophorous vacuoles are extremely fusogenic and thus prone to obtain cargo from many intracellular vesicular compartments [16, 18–20], as they also contain an organic anion transporter, which pumps small anions from the cytoplasm [17].

The data discussed above strongly suggest that intracellular pathogens encounter macrophage niches, which can supply selected nutrients, and as might be expected from the variety of interactions these microbes establish with their host cell, this supply of nutrients varies according to the pathogen under study. These studies do not always allow us to tell whether certain nutrients are available to the pathogen, as they subverted the function of the phagocyte, or whether they are not restricted at all, as they would also not be to nonpathogenic microbes. Additional studies enlarge this picture as will be discussed next.

## Study of (other) metabolic mutants

Following the identification of the enzyme isocitrate lyase, an enzyme involved in the anaplerotic pathway known as the glyoxylate cycle, as a major protein produced during mycobacterial infection of macrophages [63], it was shown that this enzyme is required for the virulence of *M. tuberculosis* [64]. Today, it has also been shown to be essential for the replication of *Salmonella* [65] and fungi such as *Candida albicans* [66] inside their hosts. The current interpretation of these mutants states that the lack of a functional glyoxylate cycle impedes the survival on a one- or two-carbon source and therefore, that the vacuole inhabited by the microbes only has access to these carbon sources, namely fatty acids. In the case of mycobacteria and salmonellae, this was shown to occur late in infection in a chronic phase where granulomas are expected to be fully organized. It should be stressed, however, that other interpretations are possible as well to explain the phenotype of the isocitrate lyase mutants. This enzyme is induced in anaerobic conditions and may participate in recycling reduced nicotinamide adenine dinucleotide (NADH) into $NAD^+$ with production of glycine from the reductive amination of glyoxylate by glycine dehydrogenase [67].

Mutations affecting iron acquisition were shown to reduce virulence of different intracellular pathogens: *L. pneumophila* mutants with defective iron acquisition or assimilation were impaired in their ability to proliferate in a macrophage cell line [68], and mutations in *M. tuberculosis* leading to disruption of the mycobactin synthesis [69] or to overexpression of an iron-dependent repressor [70] reduced considerably the proliferative capacity of this microbe in vivo. Thus, although iron is available inside vacuoles inhabited by intracellular pathogens, it is likely present in suboptimal concentrations or must be removed from host chelating molecules such that these pathogens rely on efficient iron acquisition systems for proliferation.

*S. typhimurium* mutants deficient in the *MgtCB* genes are unable to proliferate in macrophages, a defect that can be mimicked in axenic cultures by inoculating these mutants in low $Mg^{2+}$ media [71]. Similarly, the analysis of *M. tuberculosis* *Mgt*C mutants has led to the suggestion that the vacuole harboring this bacterium is mildly acidic and with limiting levels of magnesium [72]. Recently, it was also shown that *MgtC* is required for intra-macrophage growth of *B. suis* [73], illustrating the possibility that $Mg^{2+}$ is in general limiting inside macrophage vacuolar compartments.

The reduction in the ability to proliferate in mice of a *narG* mutant of *M. bovis* BCG suggests that mycobacteria, residing in granuloma macrophages, use nitrate respiration and therefore, that oxygen is limiting in these cells [74].

The mutation of the gene encoding an amino acid transporter *Pht*A in *L. pneumophila* has allowed the identification of threonine as an essential nutrient acquired by this pathogen inside macrophages [75]. Conversely, the blocking of the function or the gene silencing of a neutral amino acid transporter SLC1A5 in macrophages was shown to severely reduce the proliferation of *L. pneumophila* [76]. The expression of this transporter is highly up-regulated upon infection of the host cell [76]. Thus, it appears that this pathogen, relying on amino acids as growth-supporting nutrients, recruits host cell machinery to satisfy its nutritive requirements.

## Expression profiling

Many genetic techniques have been designed to determine the genes that are regulated upon phagocytosis, namely in vivo expression technologies (IVET) and signature-tagged mutagen-

esis (STM). For example, using an IVET approach, it was shown that upon infection, *S. typhimurium* faces an environment poor in iron, magnesium, and copper [77]. However, with ways to analyze the expression of hundreds of genes through the use of high-throughput analysis technologies, namely microarrays, it is becoming possible to look for signatures in expression related to particular growth conditions. One can, for example, study the up- or down-regulation in expression of many different genes in response to hypoxia, nutrient deprivation, oxidative stress, and other cultural conditions and then compare the changes observed with those that occur once the microbe is ingested by macrophages in culture or even when they infected the host. Several studies have thus defined a series of regulons and compared them with the expression profile induced in macrophages, cultured in vitro or from in vivo experiments. One such study using *Salmonella* observed changes in the expression of 919 genes (approximately one-fifth of the genome-coding capacity), and changes suggested that the *Salmonella*-containing vacuole in macrophages cultured in vitro is poor in phosphate (given the strong up-regulation of the phosphate-starvation loci) and magnesium (there was an increase in expression of the genes *mgt*BC) and is acidic (some of the genes involved in the acid-tolerance response are indeed induced) [78]. Curiously, data in that report suggest no limitation in amino acids (namely the aromatic ones), iron [although a macrophage with a defective natural resistance-associated macrophage protein 1 (*Nramp1*)/*Slc11a1* transporter was used in the study], or oxygen (albeit the oxygen pressure in the culture medium was likely not the one found in the tissues of the animal). Potassium levels were likely high, and data suggested that gluconate was the carbon and energy source available to vacuolar salmonellae. The differences between this study and the STM analysis may highlight inherent limitations in using a particular method (e.g., low sensitivity regarding the levels of gene expression taking place, existence of redundant or alternative pathways, competition or complementation in the heterogeneous populations analyzed in the STM assays).

Schnappinger et al. [79] looked at the gene expression of *M. tuberculosis* and found 601 genes differentially regulated in intraphagosomal microorganisms, accounting for approximately 15% of the mycobacterial genome. It is interesting that a selected number of the genes, whose expression was up-regulated by the phagosomal environment, were also induced in vivo in the mycobacteria that infected the mouse lung. The genes that were induced upon phagosomal residence included those involved in fatty acid catabolism, the glyoxylate cycle, and mycobactin synthesis, suggesting that the mycobacterial phagosome is poor in iron and that mycobacteria rely on lipids for a source of carbon and energy. When they compared the phagosome-induced response with other regulons, they found that many genes were co-regulated in those in vitro conditions and in the intraphagosomal environment. Specifically as to what concerns this analysis, 35 genes were induced in IFN-γ-activated macrophages or in broth culture by exposure to low iron concentrations. It is interesting that the magnitude of induction of these genes was higher in macrophages that were able to produce NO than in inducible NO synthase (iNOS)$^{-/-}$ cells, suggesting that the response could be partly related to NO damage and not to iron deprivation or alternatively, that NO exacerbates iron deprivation. Many hypoxia-induced genes were also induced inside the macrophage in *M. tuberculosis*. Finally, there was also a series of genes that was up-regulated by phagosomal residence and nutrient starvation, and these were clearly independent of the presence of NO in the cell. Much of this picture can apply to in vivo-growing *M. tuberculosis*. Timm et al. [80] used a quantitative, real-time polymerase chain reaction approach to analyze gene expression in tubercle bacilli isolated from infected mice or human patients. Again, up-regulation of genes responsive to iron deprivation, glucose starvation, hypoxia, and fatty acid metabolism was found in mycobacteria extracted from mouse-infected lungs. The picture for samples taken from human patients was not so clear-cut, and there was less evidence of iron deficiency or use of the glyoxylate cycle. A different approach using a promoter trap strategy confirmed the use of fatty acids in vivo as a carbon source by *M. tuberculosis* infecting the lungs of mice, as well as the likely iron-restrictive environment faced by the mycobacterium in vivo [81].

Despite the generation of extremely valuable and extensive information, the regulons defined by microarray techniques for different stimuli overlap. For example, of the 36 genes induced by reactive nitrogen intermediates, 27 were also induced by hypoxia [82]. Thus, Voskuil et al. [83] have defined a "dormancy regulon", which can be induced by low NO concentrations or by hypoxia and whose expression can be detected in vivo in *M. tuberculosis* extracted from the lungs of infected mice. High-throughput analysis of gene expression in many model systems will continue to supply extensive amounts of raw data, which will be useful in addressing the questions discussed in this paper.

## Manipulation of the intracellular habitat

The manipulation of the intracellular trafficking of certain microbes has shown that the change in the habitat affects the survival or proliferative capacity of the microbe. Thus, *Sif*A mutants of *Salmonella*, forced into the cytoplasm of macrophages, fail to proliferate [84]. Curiously, that is not the case if the *Sif*A mutants infect epithelial cells where the cytoplasm can support bacterial growth [85]. In contrast, when the saprophyte *Bacillus subtilis* was made to express listeriolysin O, targeting it to the cytoplasm of a macrophage cell line, it became able to proliferate in this cell type [86]. Using a similar approach, it was shown that *B. suis* expressing listeriolysin O failed to proliferate after lysing the membrane vacuole in human monocytes [87]. Lack of proliferation in the cytoplasm might be seen as evidence for the presence of some restricting factor. This might be simply the lack of some nutrient or the presence of some toxic antimicrobial factor. In the latter case, it would obviously have to be specific for some but not all microbes.

*Brucella* mutants deficient in the *vir*B genes fail to target their final replication niche in nonphagocytic cells, a compartment with ER characteristics, remaining in an intermediate organelle between the ER and autophagic vacuole and losing their ability to replicate [88]. Whether these observations can be explained by the lack of suitable, nutritive conditions for the bacteria in the new cellular compartment is yet unclear. How-

ever, *Brucella* do not reach autophagic vacuoles in macrophages [89].

Promoting the fusion of phagosomes with lysosomes has different consequences according to the microbe. *M. avium* vacuoles made fusogenic with the lysosomes by co-infecting macrophages with this mycobacterium and *Coxiella* remain hospitable to the mycobacteria, despite the acidification and the acquisition of lysosomal characteristics [90]. This can be used as an argument to suggest that the subversion tactics of *M. avium* were selected, not to prevent exposure to the toxic lysosomal environment but to keep the microbe in a nutrient-favorable site. In the case of the co-infection, such nutrient-favorable conditions are likely maintained. In contrast, a similar strategy applied to *M. tuberculosis* leads to reduced proliferative capacity, suggesting that indeed, the lysosomal environment is too harsh for this mycobacterial species. In this regard, it has been shown that *M. avium* is more resistant to low pH than *M. tuberculosis* [91]. However, using a different strategy, Anes et al. [92] showed that promotion of phagosome/lysosome fusion with specific fatty acids led to reduced proliferation of *M. tuberculosis* or *M. avium*. Also, in the case of *Salmonella*, the promotion of phagosome-lysosome fusion leads to an inability to proliferate [93, 94].

## MECHANISMS OF NUTRIENT DEPRIVATION (NUTRIPRIVE MECHANISMS)

The many different styles of intracellular life make it likely to expect as many different ways of nutrient acquisition by the microbe inside the host cell. A major dichotomy can be established between those microbes that reside within a membrane vesicle, whatever its nature, and those that inhabit the cytoplasm. Evidence reviewed in the previous section already highlighted the existence of nutrient deprivation, and Table 1 summarized evidence, suggesting among other things that such deprivation may not be so severe in the case of the cytoplasm. At first glance, the latter milieu might sound more appealing, given the presumed, reduced level of toxicity and the bountiful supply of nutrients. However, not every microbe might feel at home in such locale of the cell, as exemplified by the *Sif*A mutants of *Salmonella* or the listeriolysin-expressing *Brucella*, unable to thrive in the cytoplasm of macrophages [86, 87]. Conversely, feeding inside vesicles may sound more difficult but might not be so. Vesicles harboring parasites may fuse with other vesicular compartments and thus, acquire nutrients: The endosomal route may bring iron in the form of holo-transferrin [95], and it is not unlikely that other nutrients may be transported through this organelle; fusion with other intracellular membrane systems has been described, such as with the ER [25], where access to proteic material might be at hand; fusion with autophagic vacuoles, albeit an attractive way to access cytosolic material, has yet to be shown to be a mechanism used by pathogens to acquire nutrients. One of the markers often used to determine the lysosomal nature of a microbial vacuole is the molecule LAMP-2 (also known as LGP96); this is a membrane protein associated with the transport of proteins for subsequent lysosomal degradation [96]. Although speculative, it is likely that microbes replicating inside LAMP-2-positive vesicles might use those proteins doomed for degradation for their own nutritional satisfaction. Pathogens can even subvert the host cell to use their own transporters, such as is the case of SLC1A5 [76].

Not much is known about the pathways leading to the putative deprivation of specific nutrients. One can envision mechanisms relying on degradative or withholding systems (enzymes that destroy particular nutrients such as amino acids, transporters that remove specific compounds from defined intracellular compartments, scavengers of specific substances such as iron chelators), as well as cellular mechanisms regulating the access of certain molecules to different compartments within the cell or even supracellular mechanisms regulating the access of nutrients to particular cells (e.g., within structures such as granulomas or abcesses). **Figure 1** presents a summary of some of the possible mechanisms able to lead to nutrient deprivation.

### Constitutive (in-built) mechanisms

Once a particle is ingested by a macrophage, it will be enveloped in a vacuole destined to fuse with lysosomes. Many successful macrophage parasites subvert that default pathway, not only to escape from the harsh lysosomal environment but presumably also to gain residence in a nutrient-rich milieu. We can thus speculate that the killing of a nonsuccessful microbe may rely not only in the toxic environment it will reach but also in the scarcity of nutrients of the phagolysosome. One likely transporter devoted to the depletion of iron and possibly other divalent cations is the molecule encoded by the gene *Nramp*1 (now classified as *Slc11a1*). This transporter is present in the endosomal compartment of macrophages and can be found in the phagosomal membrane around ingested *M. avium* and *Leishmania major* [97, 98]. Macrophages with mutant forms of the transporter are more susceptible to mycobacteria such as *M. avium* and *M. bovis* BCG, to salmonellae, and certain species of *Leishmania* [97]. The resistance to *M. avium* of mice expressing the functional form of NRAMP1 is overcome by overloading these animals with iron [99], thus suggesting a role for iron deprivation in the activity of this transporter. Curiously, *M. tuberculosis* appears to escape control by this system [100, 101]. It must be stressed, however, that the effects of NRAMP1 are pleiotropic and may impinge on other antimicrobial systems, namely the iNOS [102]. Infection by *M. avium* or loading with iron induced an increase in the *Nramp*1 gene expression [103, 104], highlighting the importance of this transporter in the interaction among this pathogen, the macrophage, and iron. Thus, macrophages are constitutively equipped with nutriprive mechanisms.

### Induced mechanisms

A major inducer of macrophage antimicrobial functions is the cytokine IFN-γ. It is well known that IFN-γ-activated macrophages produce oxidative radicals such as those dependent on the phagocyte NADH phosphate oxidase and the iNOS (type 2). Nevertheless, many reports suggest that the control of microbial growth is not fully explained by the activity of these pathways. Indeed, bacteria such as *L. pneumophila*, *M. avium*, *A. phagocytophilum*, and *L. monocytogenes* and the protozoa *T.*

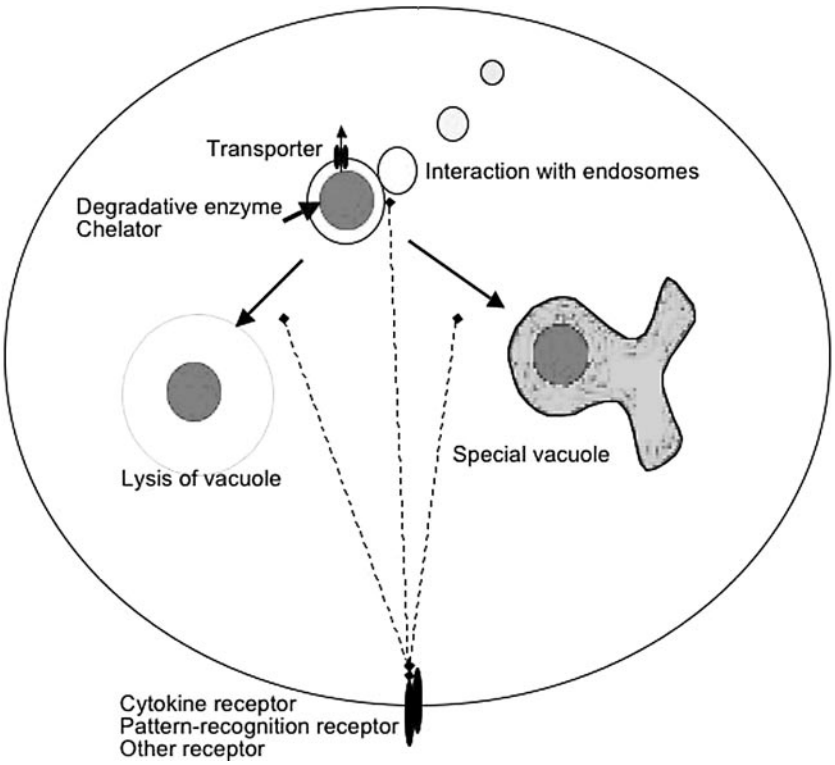


**Fig. 1.** Summary of the possible mechanisms leading to nutrient deprivation in infected macrophages. The vacuole containing the pathogen (gray circle) may acquire transporters that remove nutrients (e.g., NRAMP1 may remove iron), enzymes that degrade specific molecules [e.g., indoleamine 2,3-dioxygenase (IDO) may metabolize tryptophan], or chelators that sequester certain compounds (e.g., iron chelators). Signaling initiated by cytokine, pattern recognition, or other receptors may also affect the maturation of the vacuole, namely by preventing its interaction with endosomes (a source of nutrients such as transferrin-bound iron), its interaction with other subcellular compartments with the generation of special replicative vacuoles or the lysis of the vacuolar membrane.

*cruzi* resist the toxicity of NO and may even depend on its production for survival inside mice [3–9, 105, 106]. Thus, it is clear that additional antimicrobial mechanisms dependent on IFN-γ are triggered, and one of the possibilities is a series of nutriprive mechanisms.

One of the best-studied of such mechanisms is the effect of IFN-γ on iron metabolism. The control of *L. pneumophila* proliferation in human monocytes induced by IFN-γ was associated with its ability to restrict the access to iron [107]. Activated monocytes were shown to have reduced expression of the receptor for transferrin, a mechanism that can, at least in part, account for the phenomenon of iron withholding [107, 108]. Levels of expression of the transferrin receptor on mouse macrophages were also shown to correlate with their ability to support the growth of *L. monocytogenes* [109], and mRNA levels for the transferrin receptor were seen to decrease following activation with IFN-γ [104]. Similar iron limitation mechanisms may underlie the anti-*Histoplasma capsulatum* activity of IFN-γ-activated mouse macrophages [110]. Treatment with the cytokines IFN-γ and TNF leads to a decrease in the access to iron by mycobacteria [31], although acquisition through other chelates such as lactoferrin might increase following IFN-γ activation [32]. However, *T. gondii*, *Chlamydia psittaci*, and *Leishmania donovani* were shown to avoid these antimicrobial mechanisms [111], and given the lack of iron requirements of *Borrelia burgdorferi* [112], it is expected that they will not play a role against this pathogen. Cytokines also affect the expression of the putative iron transporter NRAMP1: It was shown that IFN-γ activates macrophages to increase its expression [98, 103, 113], and TNF enhances the expression of the *Nramp*1 gene [104], a phenomenon that is consistent with the ability of these cytokines to restrict access to iron and to exert antimicrobial activity.

It is curious that in the study of Schnappinger et al. [79], it was found that the up-regulation of genes in *M. tuberculosis*, which is specifically found in IFN-γ-activated macrophages and not in nonactivated cells, was strictly dependent on the production of NO by the phagocytic cell. The lack of a specific RNA signature for a putative nutriprive mechanism is not, however, an argument against the existence of such a mechanism. The well-documented restriction of iron, present in nonactivated cells but made more intense upon IFN-γ activation, is such a case. Conversely, the change in the requirement for the glyoxylate cycle enzymes, which occurs during the chronic stages of the infection by mycobacteria and salmonellae [64, 65], suggests that there are metabolic alterations induced by the immune response such that the availability of carbon sources changes.

A second well-known pathway triggered by IFN-γ and leading to the deprivation of a nutrient is the activation of the enzyme IDO, which degrades L-tryptophan into N-formylkynurenine and has been shown to play a role in the control of *Toxoplasma* [114–116], *Leishmania* [115], and *Chlamydia* infections [115–117]. However, the in vivo significance of the tryptophan-metabolizing pathways is confounded by the multiple effects that tryptophan catabolism has on microbes and the immune system: from simple depletion of the amino acid to the generation of immunomodulators (e.g., picolinic acid, shown to promote antimycobacterial activity in macrophages [118]) and from the deprivation of the microbe to the deprivation of the immune cell [119].

Toll-like receptors (TLRs) may convey antimicrobial signals to the macrophage following ligation of microbial molecules. It was shown that engagement of TLR2 on macrophages led to killing of *M. tuberculosis* [120]. Although macrophages triggered through TLRs can be induced to produce antimicrobial

molecules such as NO [120], additional antimicrobial mechanisms are being sought. Blander and Medzhitov [121] described deficient phagocytosis of microorganisms in macrophages defective in TLR2 and -4 or their signal transducer myeloid differentiation primary-response protein 88 (MyD88). It is more interesting that such deficient macrophages showed impaired maturation of phagosomes, and pathogens such as salmonellae ended up in a vacuolar compartment different from the one they normally occupy. These data have, however, not been reproduced in an independent study using alternative technical approaches [122]. Also, not every microbe may be susceptible to this pathway, such as in the case of *L. monocytogenes* [123].

### Counteracting the subversion pathways of parasites

Most of the known mechanisms used by the different microbes to survive inside macrophages have been described in nonactivated phagocytes. It is thus likely that once cytokine-activated macrophages are studied, it will be found that many of these microbial strategies are overcome by the host cell. Specifically, the inhibition of those subversion pathways that lead to acquisition of nutrients will fall under our definition of nutriprive mechanisms of the macrophage. It is known that the inhibition of acidification of mycobacterial phagosomes is reverted upon activation of macrophages with IFN-γ [124, 125]. In addition to stimulating the secretion of toxic radicals, IFN-γ acts on macrophages to induce the expression of a 47-kDa guanosinetriphosphatase, LRG-47, which promotes the maturation of the mycobacterial phagosome [126]. In the absence of this effector molecule, the mycobacterial phagosome fails to acidify in response to IFN-γ, as there is reduced assembly of the proton v-ATPase on its membrane, while interacting with the recycling endosomes and thus acquiring transferrin. The role of LRG-47 extends to other intracellular microbes such as *T. gondii* and *L. monocytogenes* [127].

The activation of macrophages with IFN-γ prevents the normal escape of *L. pneumophila* through the interaction with the ER, forcing the *Legionella*-containing phagosome to follow the default route of fusion with the lysosome [128]. This is associated with restriction of growth of the bacterium. What happens to the use of the SLC1A5 transporter is not yet known. Also, IFN-γ activation of macrophages has been shown to prevent the escape of *L. monocytogenes* into the cytoplasm of the macrophage [129] and can thus be seen as activating a nutriprive mechanism toward this bacterium.

*M. tuberculosis* dwells in endosomal compartments with access to transferrin and thus to iron [95]. The effect of activation, namely by cytokines, on this mechanism has hardly been studied. *M. avium* phagosomes interact actively with the endosomal compartment [12–14]. We have shown that activation of *M. avium*-infected macrophages with IFN-γ plus picolinic acid effectively restricts mycobacterial growth [118] and at the same time, severely restricts the fusion of endosomes with the mycobacterial vacuole [130], thus suggesting a way of restricting access to nutrients via the endosomal pathway. In this system, macrophages exhibit some of the alterations typical of apoptosis and raise the possibility that apoptotic macrophages limit the access of nutrients to the infecting parasites. It is interesting to note that apoptosis can be induced through the TLR signaling pathways, which at least in some cases, were shown to lead to antimicrobial activity [131–134]. An alternative way to seclude the intracellular pathogen from the access to nutrients may involve the induction of autophagy, recently described to be associated with BCG and *M. tuberculosis* killing [135]. It is interesting to note in this regard that a vitamin D analog can induce autophagic cell death through the phosphatidylinositol-3 kinase subunit, beclin-1 [136]; it has been known for a long time that vitamin D3 has an antimycobacterial effect, but the pathways involved are yet unknown.

*Leishmania mexicana* acquires material (presumably nutrients such as the purines they require to grow) from the cytosol through two different pathways: One uses an organic anion transporter assembled in many intracellular vacuoles, namely those that harbor other pathogens such as *H. capsulatum* or a listeriolysin-deficient mutant of *L. monocytogenes* or even vacuoles containing inert particles such as immunoglobulin G-covered latex beads; a second pathway relies on the fusion of vesicles deriving from autophagic events with the parasitophorous vacuole [17]. Like mycobacteria, the parasitophorous vacuole interacts with other membrane compartments of the cell, thus acquiring cargo from these vesicular compartments [16, 18, 20]. These materials can then be ingested by the parasite [16]. Again, little is known about how the activation of the macrophages affects these events.

## PERSPECTIVES

The study of nutriprive mechanisms is clearly more difficult than the search for specific toxic and antimicrobial molecules. These mechanisms are likely to differ from pathogen to pathogen according to its survival strategy inside the phagocytic cell. Nevertheless, there is already some evidence for constitutive and induced nutriprive mechanisms. Awareness of the possibility for these mechanisms will help the identification of additional instances, where they will be relevant in the control of pathogens. Most of the studies about intracellular trafficking of pathogens have been performed on resting macrophages. Assuming that some of the remodeling of intracellular trafficking induced by intramacrophagic pathogens aims at allowing the residence in a nutrient-rich niche of the cell, more work about the inhibition of such evasion tactics following activation signals, which will lead to microbial growth control (e.g., after activation by cytokines), will be needed. Experimental interference with the normal pathways of membrane trafficking will also help in providing clues about the mechanisms of nutrient acquisition by pathogens. Solute transporters may play an important role by removing essential nutrients, as in the case of SLC11A1/Nramp1, or by being subverted by the microbes themselves and providing them with the required nutrients, as in the case of SLC1A5. More work in this area will certainly aid in providing a clearer view about the trafficking of nutrients inside the infected macrophage. Manipulation of the microbes, inducing new nutritive requirements or alternatively, complementing their deficiencies, may provide new tools to study the mechanisms of nutrient deprivation. Finally, the accumulation of data about expression profiling expected to occur at increas-

ing rates will create huge amounts of information, which may be available to those studying these issues.

The understanding of the nutritive requirements of microbial pathogens and the nutriprive mechanisms induced in the macrophage will provide a framework for the identification of new targets for chemotherapy, namely inhibitors of the biosynthetic pathways essential for microbial proliferation and survival. Harth and Horwitz [137] presented proof of the concept that interfering with nitrogen metabolism leads to the identification of antimicrobial drugs. Particularly interesting candidates are the anaplerotic pathways turned on by limiting carbon sources. The identification of microbial products turned on after induction of nutrient deprivation and their use as antigenic targets in new vaccines can also be envisioned. Dietary manipulation of phagosome maturation and as we postulate, of access to nutrients may provide yet another singular approach as suggested by Anes and collaborators [92].

In summary, nutriprive mechanisms, acting in infected macrophages, exist and need to be better understood so that we gain better insight into infectious diseases caused by intracellular parasites and may one day be able to control them more effectively.

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