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Review article

How *Mannheimia haemolytica* defeats host defence through a kiss of death mechanism

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Abstract – *Mannheimia haemolytica* induced pneumonias are only observed in goats, sheep and cattle. The bacterium produces several virulence factors,whose principal ones are lipopolysaccharide and leukotoxin. The latter is cytotoxic only for ruminant leukocytes, a phenomenon that is correlated with its ability to bind and interact with the ruminant β2-integrin Lymphocyte Function-associated Antigen 1. This paper globally reviews all the information available on host-pathogen interactions underlying respiratory mannheimiosis (formerly pasteurellosis), from the stable and the Petri dish to the biochemical cascade of events triggered by the leukotoxin inside ruminant leukocytes. One conclusion can be made: the most widespread cattle respiratory disease with the most important impact on beef production worldwide, is probably due to a tiny ruminant-specific focal variation in the CD18- and/or CD11a-expressing genes.

***Mannheimia haemolytica* / leukotoxin / β2–integrin / ruminant**

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1. INTRODUCTION

Man understood many centuries ago that it would be more valuable to breed rather than to hunt animals like cattle, sheep and goats but he quickly realised the difficulty in warranting good animal health. Several practices such as sanitary slaughtering, quarantining animals, importing restrictions, vaccination and medical treatments have been intended to control, if not eradicate, illnesses. In the same way, antibiotics are used to fight against pathogens that have found in highly concentrated breeding conditions a choice niche extremely favourable for the contagion. Nevertheless, it is now clearly established that prophylaxy and metaphylaxy create a selective pressure towards the emergence of resistant strains which in turn could lead to new pathologies [55].

2. BOVINE PNEUMONIAS

The decrease of animal diseases (and bovine diseases in particular) has thus become an absolute priority since it is well-known that medical cost has the main impact on farm profitability, independently of market prices [60]. These costs are unequivocally brought by respiratory diseases [65, 119, 157] since about 25% of the calves experience at least one episode of respiratory disease during the first year of life, with frequencies over six birth years ranging from 14 to 38%. The incidence of bovine respiratory diseases is greater in male calves than in female calves during both preweaning and feedlot periods [133].

On the morbidity level, pneumonias do exert, by far, the most severe impact: they are responsible for about 75% of clinically visible diseases [52, 85] with average respiratory morbidity rates ranging from 15 to 45% [95].

On the mortality level, pneumonias are directly incriminated in about 45 to 55% of the cases [52, 174].

On the production costs level, medical treatments generate about eight percent of total production costs, without making an allowance for losses due to lower zootechnical performances [65, 66].

The main biological causes of bovine pneumonias are (i) the lungworm *dictyocaulus viviparus*, (ii) the viruses – the Herpes Virus-1, the Respiratory Syncytial Virus, the Parainfluenza-3 virus, the Viral Diarrhea-Mucosal Disease, the adenovirus, the coronavirus and (iii) the bacteria – *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis* and *Arcanobacterium pyogenes* [51, 86, 87, 111, 131, 189].

Most authors consider that, whatever the causative factor (environment, virus, parasite), the bacterium *M. haemolytica* is systematically found as a complicating agent. Consequently, we will further focus on this bacterium and the way it acts.

3. *MANNHEIMIA HAEMOLYTICA*

3.1. Diversity

Mannheimia haemolytica is a weakly hemolytic, gram-negative coccobacillus with the following complete taxonomy: superkingdom *Bacteria*; phylum *Proteobacteria*; class

Gammaproteobacteria; order *Pasteurellales*; family Pasteurellaceae; genus *Mannheimia* [166].

The bacterium has been the subject of extensive reclassification in the past: first called *Bacterium bipolare multocidum* by Theodore Kitt in 1885 [96], it was renamed *Pasteurella haemolytica* in 1932 [135] and classified into two biotypes, A and T, based on its ability to ferment arabinose and trehalose, respectively [154]. There were 13 A serotypes and four T serotypes identified [194], the latter being reclassified as *Pasteurella trehalosi* in 1990 [19, 155]. Nine years later, studies based on DNA-DNA hybridisations and 16S RNA sequencing led to renaming previous A serotypes (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16 and A17) as *Mannheimia haemolytica* while the remaining A11 serotype became *M. glucosida* [7, 194]. The name *Mannheimia* was given in tribute to Walter Mannheim, a German biologist whose research has improved the understanding of the taxonomy of the Pasteurellaceae family [7].

From the twelve serotypes described, A1 and A2 are prevailing all over the world. A1 is known as the major causative agent of bovine mannheimiosis (formerly pasteurellosis, also known as the shipping fever), even if other serotypes like A6, A7, A9, A11 and A12 have also been reported [144]. A1 and A2 are both able to colonise the upper respiratory tract of cattle and sheep but they are often species-specific. So, healthy cattle frequently carry serotype A2 in their upper respiratory tract but following a stress or a coinfection, A1 quickly takes the place of A2 as the main serotype [58], probably by horizontal transfer from ill animals [70]. However, recent surveys have shown that serotype A6 is increasingly prevalent in the United Kingdom [49] and in the USA [3, 143] with about 30% of strains serotyped. Nevertheless, based on lipopolysaccharide profiles and outer membrane proteins within each serotype, it has been concluded that, apart from the nature of their capsules, serotypes A1 and A6 are extremely similar [39, 130].

3.2. Antibiotic resistance

Due to the often ineffective immunoprophylactic measures taken, antimicrobials are used to a large extent for prophylaxy, metaphylaxy or growth-stimulation. Moreover, the delay of analysing isolates from ill animals in a diagnostic lab makes it difficult to choose a suitable antibiotic and that is why it is commonly required to start the therapy immediately. Consequently, the result is that *M. haemolytica* exhibit increasing resistance to a large number of antimicrobial agents [177].

Besides, isolates are investigated for their antimicrobial resistance properties in the national monitoring programmes of only five European countries: France, Germany, the United Kingdom, the Netherlands and Portugal [25, 94, 118]. Data obtained from Germany and France in 1997 illustrate that there is a high degree of variability among isolates originating from the same animal source and that resistance rates may also vary over time [94].

Molecular analysis has provided insight into the variety of resistance genes so far known to be present in *Mannheimia* isolates. Most of these resistance genes are associated with mobile genetic elements and can thus easily be exchanged between bacteria. The occurrence of these resistance genes in a wide range of bacteria implies that *Mannheimia* isolates have access to large gene pools within which an interchange of resistance genes takes place [94].

The most frequent resistances are found against beta-lactams, tetracyclines, sulfonamids and aminoglycosides [76, 94, 177]. A few resistance genes have already been cloned and sequenced [70].

3.3. Pathogenesis

Mannheimia haemolytica plays a major role as a secondary pathogen in the final

progression of severe pleuropneumonias in cattle, sheep and goats. Its pathogenesis involves many predisposing agents such as viruses (Parainfluenza virus 3, Bovine Herpes virus 1, Bovine Respiratory Syncytial virus), bacteria (*Pasteurella multocida, Mycoplasma bovis, Arcanobacterium pyogenes*), environment (excessive temperatures, change of feed, dust, …) or stress associated to weaning, dehorning and shipping [51, 111, 189–191]. These factors seem to alter the upper respiratory tract epithelium allowing *M. haemolytica* to colonise it, escaping clearance, and to move from the nasopharynx to the lungs, leading to a broncho-alveolar kind of pneumonia which is accompanied by high morbidity [52, 85] and mortality [52, 174].

3.4. Clinical signs and lesions

The severity of the clinical signs can vary from unapparent to rapidly fatal disease but a few characteristic features could be mentioned: there is always some degree of depression and anorexia, fever as high as 42 °C, increased heart rate, a substantial weight loss and rhinitis resulting in a mucopurulent nasal discharge or a dry, encrusted muzzle. Increased lacrymation and a cough are often present. The respiratory rate increases in the early stages, followed by dyspnea of such severity as to cause oral breathing and expiratory grunting in some cases. Auscultation reveals increased vesicular and bronchial sounds anteroventrally, progressing to rales that are at first moist but later dry; pleuritic friction rubs may be heard. Calves may stand with elbows abducted and neck extended and diarrhea occurs in some animals [189].

Pulmonary lesions are lobar, anteroventrally distributed and are characterised by extensive infiltration of neutrophils (that fail to combat infection) [153, 175] and exudation of fibrin into airways and alveoli. In histological descriptions, the pleura was seldom mentioned, presumably because it did not differ from expectations suggested by the gross lesions of fibrinous exudation. The interlobular septa are distended with gelatinous material and contain edema, fibrin, leukocytes, and distended lymphatics which are frequently thrombosed. The bronchi have normal walls although there may be some necrosis and desquamation of epithelial cells. They often contain the products of deeper inflammatory processes: necrotic debris, leukocytes, fibrin, etc. [51, 189]. The cut surface usually consists of several colours due to the changes described above, plus hemorrhage, infarction, necrosis and solidification of tissue; either in the acute, fluid, congestive stages of the disease process (red hepatisation) or in the subacute stages where exudates had become more purulent (grey hepatisation) [51]. The term consolidation is used more commonly today for such exudative changes. The smaller airways are inflamed, starting at the terminal bronchioles. The alveoli contain oedema, fibrin, and occasionally haemorrhages in variable proportions, but the more interesting aspects are the inflammatory cells (neutrophils and macrophages) and the areas of coagulation necrosis. These latter are multifocal and may involve whole or confluent lobules, but not whole lobes. The necrosis importance is in fact wrought by the cytolysis of many neutrophils and macrophages that pour a variety of toxic compounds (enzymes, histamine, prostaglandins, etc.) in situ which in turn aggravate pulmonary damage [51, 189].

3.5. Known host-pathogen interactions

Several virulence factors have been described for *M. haemolytica*: they include the capsule that plays a great role in adherence and invasion, outer membrane proteins that are important in eliciting the protective immune response, adhesins implicated in colonisation, the neuraminidase that reduces the viscosity of respiratory mucus and allows closer bacterial apposition to the cell surface, the lipopolysaccharide (LPS) and the leukotoxin (LKT).

These factors allow *M. haemolytica* to escape or exceed clearance and host defences,

to proliferate in the lung and to cytolyse alveolar macrophages and neutrophils, which further enhances the lung injuries [31, 91, 183].

The roles of the main actors of the illness, that is to say LKT, LPS, neutrophils and macrophages, will be further described below.

4. LEUKOTOXIN

4.1. The main virulence weapon

Leukotoxin appears to be the main virulence factor. Indeed, inactivation of *M. haemolytica* leukotoxin by a gene knockout hardly causes any further pulmonary lesions although the wild-type and mutant strains were equally capable of colonising the upper respiratory tracts of the calves [165]. Moreover, necrosis of neutrophils can be reproduced in vitro with purified LKT [5, 34, 40, 46, 88, 89, 161, 162, 176]. Biologically, it is worth noting that even if LKT is able to bind leukocytes from various animal species, it is only cytotoxic for ruminant leukocytes, suggesting that the interaction specificity between LKT and ruminant leukocytes could be responsible for the ruminant-specificity of *M. haemolytica* [92, 149, 161]. Amongst leukocytes, macrophages are more resistant than neutrophils to the lytic effect of LKT, and alveolar macrophages from adult cattle are more resistant than alveolar macrophages from calves under 16 weeks of age [137].

4.2. Production and activation

The leukotoxin is a 102 kDa-protein which is secreted in the logarithmic-phase of growth. It belongs to the RTX (repeats in toxin) family of multidomain exotoxins, which includes amongst others the *Escherichia coli* hemolysin, *Actinobacillus pleuropneumoniae* hemolysins, *Actinobacillus actinomycetemcomitans* leukotoxin, *Pasteurella aerogenes* Pax toxin, *Bordetella pertussis* adenyl cyclase hemolysin, *Actinobacillus equuli* hemolysin, *Actinobacillus suis* hemolysins and maybe a secreted protein from *Haemophilus paragallinarum* [16, 91, 126, 147, 180]. All these pore-forming toxins contain near the C-terminal ends of the protein highly conserved regions containing glycine-rich nonapeptide repeats, the number of which ranges from six (LKT in this case) to 41, and seems to be related to their mechanism of activation and secretion [32, 91, 110]. LKT is synthesised as an inactive form (proLKT) that needs acylation to become active. This process is thought to be necessary in order to remove charges from the toxin and to increase its hydrophobicity [159]. Following Westrop et al., a domain between amino acids 379 and 616 of LKT is required for recognition of the protoxin by the acylase and Lys-554 is a likely activation site [182], although it may not be the only one [140].

The study of biological effects induced by two genetically defined leukotoxin mutants has demonstrated that neither acylation nor the amino terminal 344 amino acids are required for LKT binding to the CD18 subunit of LFA-1, but are essential for LKT-induced $[Ca^{2+}]_i$ elevation, generation of reactive oxygen metabolites, production of IL-8 and cytolysis in target cells [167].

4.3. Gene organisation and transcriptional regulation

A four-gene polycistronic operon codes for the synthesis, the activation and the secretion of LKT: in the order of their genetic organisation, *lktC* is needed for LKT acylation and hence activation, *lktA* codes for LKT itself, and *lktB* and *lktD* code for proteins involved in secretion [71, 160]. Two kinds of transcripts are produced, the main of which (90%) is ~ 3.5 kb long and solely encodes for *lktCA*. Rarer transcripts span the entire four-gene cluster (~ 7.5 kb long) via antitermination within the *lktA–lktB* intergenic region [73, 160].

The promoter region appears to be complex and LKT expression is regulated at the transcriptional level by various *cis*- and *trans*-acting factors [70, 72, 73, 116, 160]. The operon promoter activity reaches its maximum during the early logarithmic phase of bacterial growth and declines as the cells enter late logarithmic and stationary phases [54], which is well-correlated with the maximum production of LKT [9, 14, 178]. LKT is produced and secreted into the culture supernatant by all of the *M. haemolytica* strains, although some strain-to-strain variation in the amount of produced or secreted leukotoxin could be observed [40].

Bacterial growth and LKT production are co-regulated by factors such as iron, temperature [117] and oxygen. The role of O_2 in the expression of LKT was studied by Uhlich et al. and it was demonstrated that FnrP, homologous to Fnr, the global transcriptional regulator of anaerobic respiration in *Escherichia coli*, suppresses and increases LKT transcription respectively under aerobic and anaerobic conditions [171, 172]. Furthermore, similar results of anaerobic regulation of toxin production have been obtained with *Actinobacillus actinomycetemcomitans* [97]. At first sight, an increase in toxin production under anaerobic conditions seems astonishing for a respiratory pathogen. This could nevertheless appear as an evolutionary advantage since the lung lesions are anteroventrally distributed, a region which is known to be less oxygenated as the disease progresses. Thus, increasing lesions lead to less oxygenated areas; which in turn cause an increase in LKT production that amplify the disease.

4.4. Natural diversity

Robert Davies and his team have studied the *Mannheimia haemolytica* leukotoxin diversity in cattle and sheep. Sequence analysis of the *lktA* gene from 31 ovine and bovine strains has allowed the identification of eight main allelic variants. The substitution rates differ across the entire structural gene, suggesting a variation in the degree of selection imposed on different segments of the protein [42]. In 2002, the study of polymorphism and molecular divergence of the entire leukotoxin operon in 23 bovine and ovine isolates of *M. haemolytica*, six strains of *Mannheimia glucosida* and three of *Pasteurella trehalosi* has confirmed the complex mosaic structure of the operon, suggesting that it has been derived from a series of inter- and intra-species horizontal DNA transfers between distinct lineages of *M. haemolytica*. The most conserved gene seems to be *lktD*, while *lktA* contains overall more substitutions than the other operon genes [43], even leading to leukotoxin diversity. For example, serotype A2 strains are associated with at least four different leukotoxin types (LktA2, LktA3, LktA8 and LktA10), whereas serotype A1, A5, A6, A8, A9 and A12 isolates are much less diverse and associated with very similar leukotoxins (LktA1.2 and LktA1.3) [40, 42]. Accordingly, Davies and Baillie have studied the effect of this amino acid diversity on leukotoxin cytotoxicity against bovine and ovine cell types [40], previously investigated on the basis of their genetic relationships [41]. Some leukotoxins associated with bovine (LktA1.1) and ovine (LktA1.2 and LktA1.3) strains differ in their cytotoxicity against the same cell type, i.e. against bovine or ovine neutrophils but overall, the leukotoxin structure and function are highly conserved in *M. haemolytica*. Therefore, the data support the hypothesis that the most likely advantage of the recombinational exchanges to the pathogen is the generation of antigenic variation which will provide an adaptative advantage against the host antibody response [40].

5. THE LEUKOTOXIN TARGETS(S)

5.1. Macrophages and neutrophils

The central role of macrophages and neutrophils in the development of fulminating pneumonic mannheimiosis is well supported.

Experimental aerosol exposure to *M. haemolytica* induces rapid infiltration of neutrophils into the lung and a marked increase in the neutrophil/macrophage ratio in pulmonary lavage fluid of calves [175]. These changes correlate well with characteristic reported histologic features in which (i) small airways become plugged with purulent exudate [112] and (ii) clustered inflammatory cells with elongated or streaming nuclei, referred to as "oat cells", are commonly found within inflamed alveoli [51]. Furthermore, there is reliable evidence indicating that mobilisation of neutrophils does not effectively fight infection but contributes to the development of lung lesions; as a matter of fact, neutrophil depletion prior to inoculation with *M. haemolytica* protected calves from the development of gross fibrinopurulent pneumonic lesions [153, 179], although less severe inflammatory changes still occurred [20]. Thus, the host-pathogen interaction centrally involved is between LKT and polymorphonuclear leukocytes (PMN) and the neutrophil-mediated inflammatory response itself appears to be a major determinant of *M. haemolytica* pathogenesis.

5.2. β2-integrins

Another amazing observation has led to set a hypothesis concerning the nature of the interaction between LKT and ruminant neutrophils: LKT do not induce leukocyte cytolysis from BLAD animals, an acronym designating the genetic illness called Bovine Leukocyte Adhesion Deficiency, which is characterised by a deficit in PMN trafficking. This shortage significantly decreases, nay, abolishes their ability to self-extract from the bloodstream by diapedesis, resulting in the recurrent apparition of infectious disorders in these animals.

At the molecular level, the BLAD phenotype is due to the D128G mutation in the CD18 beta subunit of β2-integrins which results in a very important decrease of their membrane expression [151, 152]. Indeed, this family of integrins is precisely responsible for the leukocyte fixation at the endothelium surface – the initial step of diapedesis – through their interaction with intercellular adhesion molecules (ICAM). Therefore, the resistance exhibited by BLAD neutrophils towards LKT suggests that β2-integrins are the ruminants Achille heel for *M. haemolytica*'s LKT.

5.3. Lymphocyte Function-associated Antigen 1

Integrins are transmembrane receptors that play an important role in cellular adhesion even if their recognition as a surface receptor family only dates back to 1987 [81]. Since, they were extensively studied (more than 26 000 articles to date) and appear to be implicated in many biological, physiological and pathological processes. All integrins consist of a 120 to 180 kDa alpha subunit and a 90 to 110 kDa beta subunit that are non-covalently associated single-pass transmembrane proteins [158]. The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counterreceptor for surface proteins of apposed cells [82]. Approximately 20 integrins are described to date, which are classified into eight sub-families, named according to their beta subunit, for example β2-integrins. CD18 is the constant beta-subunit of the β2-integrin family. It is found associated with CD11a-d alpha-subunits, leading to the heterodimers CD11a/CD18 or LFA-1 (Lymphocyte Function-associated Antigen 1) that predominates, CD11b/CD18 or Mac-1, CD11c/CD18 or CR4 and CD11d/CD18 [15, 168]. The CD11a-d/CD18 heterodimers are expressed on all leukocytes and mediate high affinity adhesion to a variety of cell types that express one or more of the β2-integrin ligands, the intercellular adhesion molecules (ICAM-1 to -5) [8, 59, 129, 168].

The expression level of β2-integrins is regulated by several factors, including mediators of inflammation, cytokines (interleukins-1 and -4, interferon α and tumour

necrosis factor-β) and the formation of Fc-g-receptor complexes. For example, phorbol myristate acetate (PMA), a mimetic of diacylglycerol that activates the protein kinase C [50, 132, 136], increases the expression of LFA-1 from normal bovines but not from BLAD animals, because the mutant CD18 is no more able to bind any CD11. These findings indicate that the expression of CD18 by bovine neutrophils is a dynamic system, capable of rapidly responding to inflammatory stimuli by increasing surface expression of CD18 [33, 62].

In the context of the interaction between LKT and ruminant β2-integrins, several studies have demonstrated that when bovine leukocytes are incubated with antibodies directed against CD11a or CD18, the cytotoxic effect of LKT is decreased, nay, abolished. These data suggest that the binding of LKT on ruminant LFA-1 is liable for the virulence specificity of *M. haemolytica* against ruminants. The precise identification of the subunit that binds LKT appears controversed [88], even if CD18 seems probable [5, 46, 109]. Fortunately, the recent cloning, sequencing and characterisation of the *Bos taurus* CD11a [56] will give the first opportunity to express homologous and heterologous LFA-1 in vitro to definitely answer the question.

6. THE LIPOPOLYSACCHARIDE

Bacterial lipopolysaccharides (LPS), derived from gram-negative microorganisms, typically consist of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating "core" oligosaccharide and a distal polysaccharide (or O-antigen) [145].

The presence of LPS in RTX toxin preparations, as well as the harsh conditions required to remove it, suggests that LPS may complex with RTX toxins. More, concentrated culture supernatant preparations of *M. haemolytica* contain LKT and LPS as the most prominent components, with an LPS/LKT molar ratio around 60:1. Complexes result in enhanced and stabilised leukolytic activity [100, 108]. It could then be postulated that the reproduction of disease in vitro with purified LKT would not be attributed to LKT alone. This statement can be disproved since (i) purified LKT is generally preincubated with the LPS inhibitor polymyxin B and (ii) LPS could be quantified with the Limulus amebocyte lysate test.

Furthermore, a recent study has indicated an association between the incidence and severity of ovine pneumonic mannheimiosis and the LPS chemotype, suggesting an important role for the LPS chemotype in determining host-species susceptibility to lung infection [75]. Indeed, the LPS chemotype varies both between and within serotypes of *M. haemolytica* and is predominantly smooth in bovine isolates and rough in ovine isolates [4, 39, 98].

The LPS receptors and signal pathways in mononuclear phagocytes have been reviewed by Chen et al.; many investigators have reported that binding of LPS to many cell types is nonsaturable. Many other studies have on the other hand provided evidence for a role of receptors as potential targets in LPS stimulation [27]. For example, it is amazing to know that β2-integrins have been described as transmembrane signalling receptors for LPS [83, 185]. It has furthermore been concluded that CD18 molecules are not essential for cellular responses to LPS [186].

Some experiments have shown that LPS complexes with an LPS-binding acute phase protein that is rapidly synthesised in vivo following an inflammatory response. This protein, termed LPS binding protein (LBP), binds with high affinity to all chemotypes of LPS via lipid A [169, 170]. Complexes of LPS and LBP interact with human monocytes via specific binding to the CD14 molecule [187]. It has, however, been shown that LPS can activate mononuclear phagocytes in the absence of LBP, leading to the conclusion that the CD14-dependent pathway may not be unique for LPS interaction with

and stimulation of macrophages and monocytes.

Most types of lipid A bind a membrane-spanning receptor identified as toll-like receptor 4 (TLR4) present on macrophages and endothelial animal cells via an interaction that involves other proteins, including LBP, CD14 and MD-2 [2, 77, 124, 141, 148, 150]. In macrophages, lipid A activation triggers the biosynthesis of diverse mediators of inflammation such as TNF-α and IL-1β [17, 48] and activates the production of costimulatory molecules required for the adaptative immune response [124, 125], events that are desirable for clearing local infections. The reader who wants to know more about LPS endotoxin is invited to read the complete review of Raetz and Whitfield [145].

Several transmembrane signalling mechanisms appear to be involved in LPS-induced activation of alveolar macrophages [27, 163, 173, 188]. We will further describe those involved in *M. haemolytica*'s pathogenesis.

7. MODES OF ACTION

7.1. Apoptosis/necrosis

Low and high concentrations of LKT induce respectively apoptosis and cell lysis in bovine leukocytes. The ability of low LKT concentrations to induce apoptosis in host leukocytes may allow bacteria to escape host immune surveillance by destroying the actors of innate response (macrophages and neutrophils) and enhancing the inflammatory process. At higher concentrations, the apoptotic mechanisms would be exceeded and necrosis occurs, leading to lung lesions. Nevertheless, it is not really easy to distinguish the effects caused by LKT and LPS since it is likely that they could act separately or together (when they form complexes) via common or distinct pathways that act as a complex network, transmitting many messages inside and outside the cell, controlling cell life and leading to cell lysis [34, 162].

Table I. Main biological effects triggered by LKT and LPS in leukocytes. LFA-1: Lymphocyte Function-associated Antigen-1; LKT: leukotoxin; LPS: lipopolysaccharide; NF-κB: Nuclear Factor-kappa B.

Biological effects	LKT	LPS
LFA-1 synthesis	•	
NF-κB activation and cytokines release	•	•
Intracellular calcium elevation	•	•
Release of arachidonic acid metabolites	•	

However, activation of bovine neutrophils by *M. haemolytica* leads in bulk to elevation of intracellular calcium [139], oxidative burst [114] and production of several lipid mediators [30, 69] and proinflammatory cytokines [193] (Tab. I).

7.2. Death pathways

Upon binding to LFA-1, LKT induces tyrosine (Y735) phosphorylation of the CD18 tail via a nonreceptor tyrosine kinase (NRTK) signalling cascade involving PI3-kinase and Src kinases in bovine (Figs. 1 to 4), but not in porcine leukocytes (LKT binds to porcine LFA-1 without eliciting any effects). This binding is known to involve G proteins [79, 90] and to cause, in a dose dependent way, sustained elevation in intracellular calcium in bovine leukocytes [36, 89, 139] that results mainly from an incoming flux from the extracellular medium via voltage-gated channels (Fig. 2) [61, 79, 80, 139]. This calcium entrance is clearly involved in cytolysis [61] and is essential for triggering the NF-κB translocation into the nucleus (detectable after five minutes of exposure), as well as the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-8, since calcium chelation blocks both phenomena (Fig. 1) [80, 99, 192, 193]. It should be noted that NF-κB is known to be exploited by some pathogens [164] and that its activation and calcium elevation by LKT have only been observed

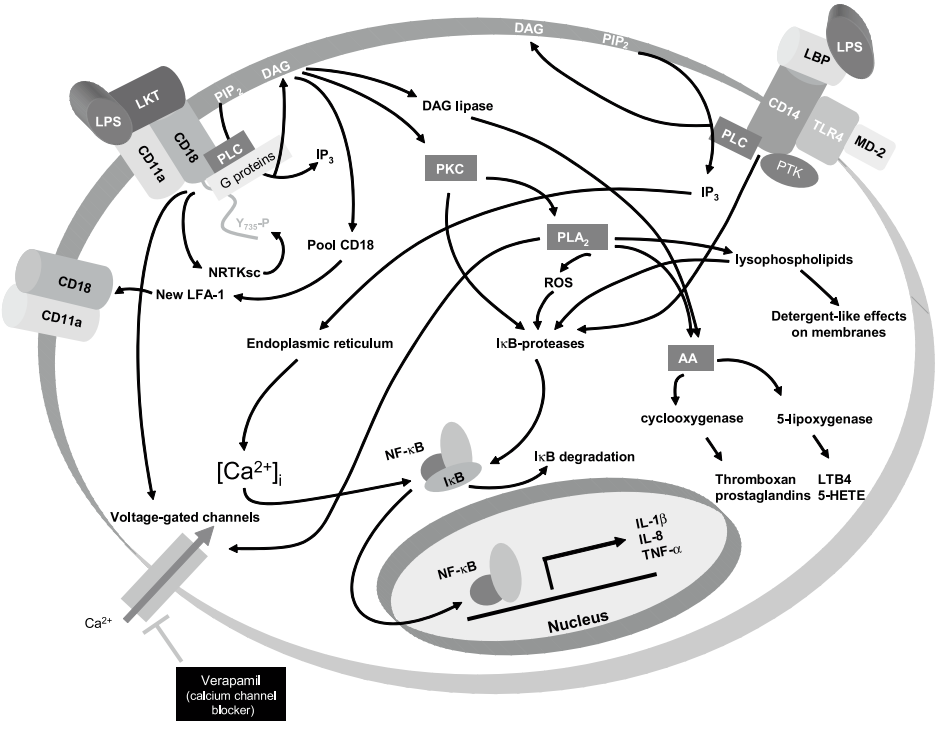

Figure 1. Leukocyte signalling pathways triggered by LKT and LPS. AA: arachidonic acid; DAG: diacylglycerol; 5-HETE: 5-hydroxy-eicosatetraenoic acid; IκB: Inhibitor kappa B; IL-1β: interleukin-1 beta; IL-8: interleukin-8; IP_3: inositol triphosphate; LBP: LPS binding protein; LFA-1: Lymphocyte Function-associated Antigen-1; LKT: leukotoxin; LPS: lipopolysaccharide; LTB4: leukotriene B4; NF-κB: Nuclear Factor-kappa B; NRTKsc: nonreceptor tyrosine kinase signalling cascade; PIP_2: phosphatidylinositol 4,5-bisphosphate; PLA_2: phospholipase A2; PLC: phospholipase C; PKC: protein kinase C; PTK: protein tyrosine kinase; ROS: reactive oxygen species; TNF-α: Tumour Necrosis Factor-alpha; TLR4: toll-like receptor 4.

in bovine alveolar macrophages (BAMs) but not in porcine alveolar macrophages (PAMs) or bovine pulmonary artery endothelial cells (BPAECs), suggesting cell-type and species-specific activation mechanisms. On the other hand, LPS effects are demonstrable in BAMs, PAMs and BPAECs [80]. On the contrary to LKT, LPS induces at very low concentrations (1 to 10 ng/mL) an elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, suggesting a release from intracellular stores (Fig. 2). The requirement of an LPS binding protein (LBP)-CD14-coupled signalling mechanism involving tyrosine phosphorylation through a non Gi-Go coupled activation of PLC activation seems to be necessary, even if the role of other G proteins cannot be ruled out [79].

The expression of proinflammatory cytokine genes is differentially regulated by tyrosine kinase-dependent and -independent pathways in BAMs in response to LKT and LPS, since tyrosine kinase inhibitor herbimycin A blocks the expression of TNF-α, IL-1β and IL-8 genes in BAMs stimulated with LKT, while only the expression

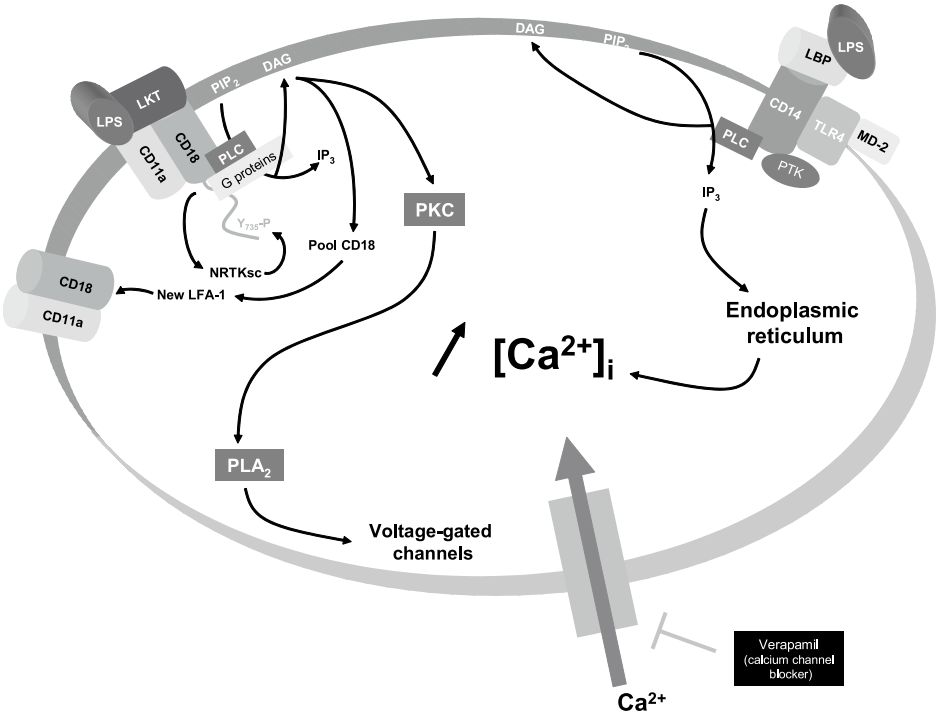

Figure 2. Leukocyte signalling pathways triggered by LKT and LPS leading to elevation of intracellular calcium. DAG: diacylglycerol; IP_3: inositol triphosphate; LBP: LPS binding protein; LFA-1: Lymphocyte Function-associated Antigen-1; LKT: leukotoxin; LPS: lipopolysaccharide; NRTKsc: nonreceptor tyrosine kinase signalling cascade; PIP_2: phosphatidylinositol 4,5-bisphosphate; PLA_2: phospholipase A2; PLC: phospholipase C; PKC: protein kinase C; PTK: protein tyrosine kinase; TLR4: toll-like receptor 4.

of IL-1β is blocked in BAMs stimulated with LPS [80].

Activation of phospholipases A2 (PLA_2) by LKT and C (PLC) by LKT and LPS has also been reported (Figs. 1 to 4) [64, 79, 90, 142].

PLA_2 are a diverse class of enzymes with regards to function, localisation, regulation, mechanism, sequence, structure and role of divalent metal ions. They play a central role in diverse cellular processes including phospholipid digestion and metabolism, host defence, and signal transduction by catalysing the hydrolysis of the sn-2 fatty acyl bond of many different phospholipids which may themselves serve as intracellular second messengers or can be further metabolised as precursors in the production of specific proinflammatory lipid mediators such as leukotrienes, prostaglandins and hydroxyecosatetraenoic acids (HETES) via arachidonic acid (AA) formation (Fig. 4) [123]. Mammalian leukocytes contain several types of PLA_2 enzymes, the type most commonly involved in arachidonic acid production being high-molecular-mass (85 kDa) cytosolic PLA_2 ($cPLA_2$) [10, 11] which requires micromolar concentrations of Ca^{2+} for translocation from the cytosol to the nuclear

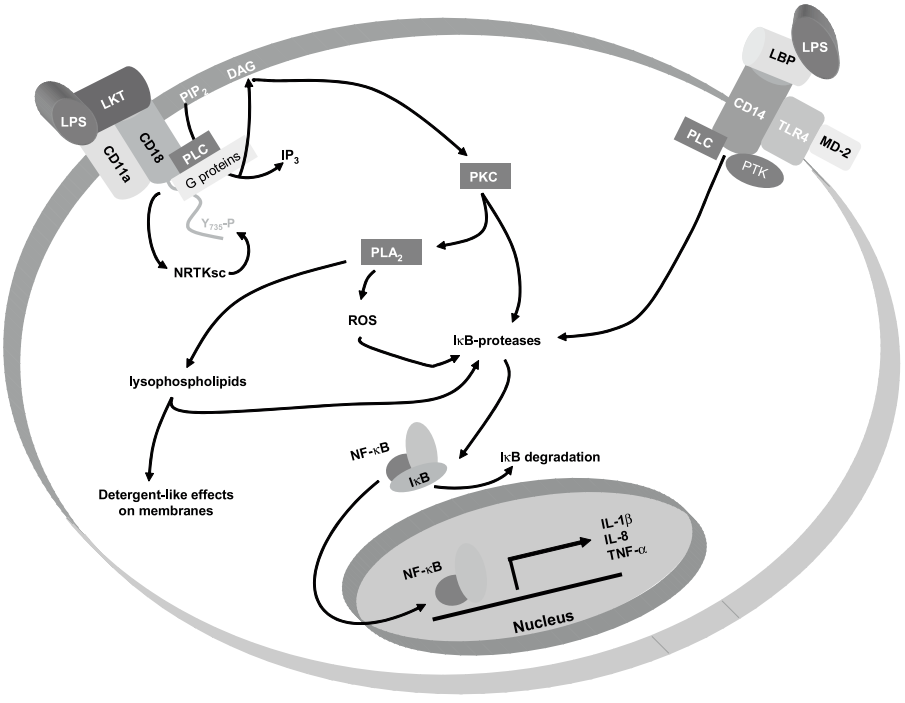

Figure 3. Leukocyte signalling pathways triggered by LKT and LPS leading to NF-κB activation. DAG: diacylglycerol; IκB: Inhibitor kappa B; IL-1β: interleukin-1 beta; IL-8: interleukin-8; IP_3: inositol triphosphate; LBP: LPS binding protein; LFA-1: Lymphocyte Function-associated Antigen-1; LKT: leukotoxin; LPS: lipopolysaccharide; NF-κB: Nuclear Factor-kappa B; NRTKsc: nonreceptor tyrosine kinase signalling cascade; PIP_2: phosphatidylinositol 4,5-bisphosphate; PLA_2: phospholipase A2; PLC: phospholipase C; PKC: protein kinase C; PTK: protein tyrosine kinase; ROS: reactive oxygen species; TNF-α: Tumour Necrosis Factor-alpha; TLR4: toll-like receptor 4.

envelope [44, 45, 47, 63, 123]. The $cPLA_2$ activity seems to be regulated by G proteins and by protein kinase C [79, 90]. Although $cPLA_2$ is clearly implicated, one must also assume that other phospholipases, including $sPLA_2$ (secretory PLA_2), may also be involved in the molecular pathogenesis of *M. haemolytica* LKT [176].

The 5-lipoxygenase products of AA, leukotriene B4 (LTB4) and 5-hydroxy-eicosatetraenoic acid (5-HETE), are implicated (Fig. 4) as important chemotactic agents for bovine neutrophils and mediators of inflammation in *M. haemolytica* infection [28, 57, 68, 176]. LTB4 and 5-HETE may in fact serve as biological amplifiers in the inflammatory process by inducing a further accumulation of polymorphonuclear leukocytes (PMN) at the site of injury [69, 120]. Moreover, the hydrolysis of phospholipids by PLA_2 leads to the elaboration of lysophospholipids, which are known to cause detergent-like effects on membranes [181] and to induce apoptosis (via NF-κB) as well as necrosis (Fig. 3) [78, 121, 122].

Phospholipase C-induced hydrolysis of phosphatidylinositol bisphosphate (PIP_2) releases inositol triphosphate (IP_3) and

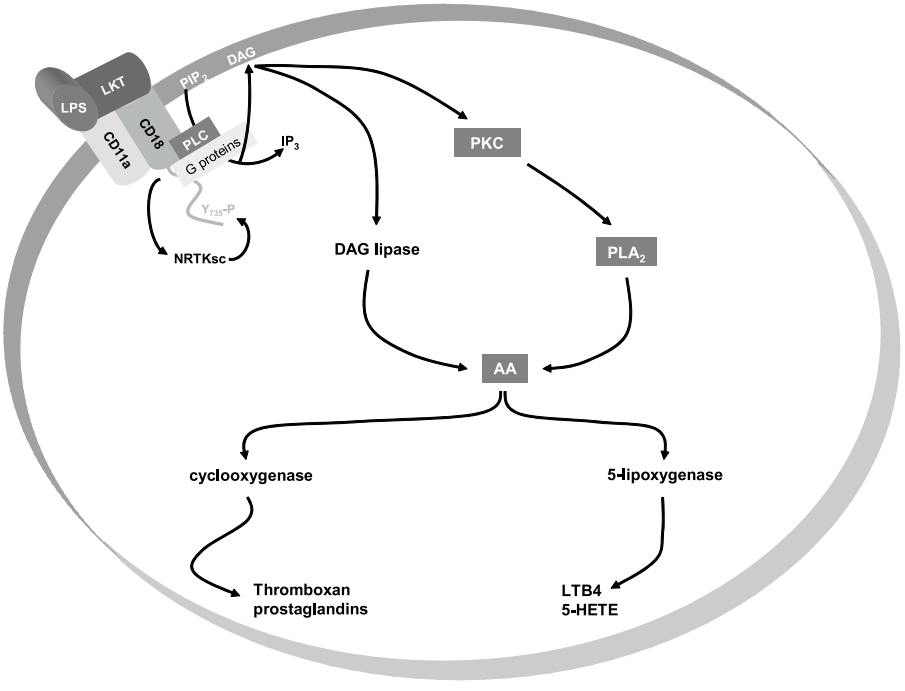

Figure 4. Leukocyte signalling pathways triggered by LKT and LPS leading to arachidonic acid metabolites. AA: arachidonic acid; DAG: diacylglycerol; 5-HETE: 5-hydroxy-eicosatetraenoic acid; IP_3: inositol triphosphate; LFA-1: Lymphocyte Function-associated Antigen-1; LKT: leukotoxin; LPS: lipopolysaccharide; LTB4: leukotriene B4; NRTKsc: nonreceptor tyrosine kinase signalling cascade; PIP_2: phosphatidylinositol 4,5-bisphosphate; PLA_2: phospholipase A2; PLC: phospholipase C; PKC: protein kinase C; TLR4: toll-like receptor 4.

diacylglycerol (DAG). It has been shown in murine and rat macrophages that IP_3 mediates intracellular Ca^{2+} mobilisation from endoplasmic reticulum stores by LPS [107, 142]. In BAMs, this release does not involve AA [79], even if a DAG lipase pathway could convert DAG to AA [6], most probably in LKT stimulation (Fig. 4). DAG is also known to activate the protein kinase C (PKC) family in a variety of cell systems [12, 132, 136]. Phorbol esters, such as phorbol myristate acetate (PMA), can substitute for DAG in activating PKC [132] in a prolonged action, since phorbol esters are not readily metabolised. PMA rapidly induces NF-κB translocation into the nucleus [104] and increases the expression of LFA-1 from bovine neutrophils, leading to enlarge the disease by increasing the number of LKT binding sites (Fig. 1) [62]. Dore et al. have shown that in PMA-non-stimulated bovine neutrophils, most PKC activity was detected in the cytosolic fraction and was dependent on the presence of added calcium and phospholipids whereas membrane-associated PKC did not have such a dependence. Stimulation with PMA caused redistribution of PKC activity in the cell consisting of a decrease in cytosolic PKC activity and an increase in membrane-associated PKC activity. As in non-stimulated cells, the former was dependent on the presence of calcium and phospholipids and the latter did not have such a requirement [50].

Oxygen-derived free radicals are also generated following LKT stimulation (Fig. 3) [114]. At the molecular level, they could act as second messengers and activate several factors and genes involved in the immune response and in apoptosis [35]. When overproduced, they exceed the antioxidant defence systems i.e. nonenzymatic (vitamin A, C and E) and enzymatic (superoxyde dismutase) mechanisms, which leads to oxidative stress that entail several biological effects on proteins [37, 38], DNA and lipids [21]. For its part, *Mannheimia haemolytica* may be able to resist at least to a certain level of free radical damage, since serotypes A1 and A2 produce superoxide dismutase [1, 146].

In summary (Tab. I), several factors that lead to cell lysis could be produced or activated following LKT and LPS stimulation.

7.3. Pore formation

LKT belongs to the RTX toxin family whose members are known to lyse their target cells through the formation of pores that lead to the efflux of K^+, influx of Ca^{2+}, colloidal osmotic swelling and eventual cell lysis. The pore size varies among bacterial species from 0.6–1 nm (LKT in this case) [29, 53, 84] to 2–3 nm in diameter [18, 102]. The LKT pore formation mechanism has not yet been described but we could approach it by analysing the way the other RTX toxins act.

Thus, the characterisation of *Actinobacillus actinomycetemcomitans* leukotoxin (LTX) pore formation in HL60 cells (a promyelocytic cell line) has shown that rapid cell death ensues with large conductance increases within seconds following high concentrations of toxin exposure. Cells undergo morphological changes consistent with rapid cell death [93]. When adding LTX to the bathing solution of an artificial bilayer, no channel activity was seen. However, if LTX was added to the lipid monolayer before forming the bilayer, large conductance fluctuations were seen in the bilayer. The authors interpreted this result by implying that the aqueous form of the toxin will not spontaneously incorporate into a bilayer, but if the toxin is partially unfolded, as likely happens at the lipid monolayer-water interface, insertion into the membrane occurs and channels are formed. These are also consistent with LTX being required to interact with a cell surface receptor in order to facilitate toxin activation [93, 103]. It has been shown that this receptor is the human β2-integrin LFA-1, expressed on immune cell surfaces matching the profile of cytolytic targets [101].

A model for the pore forming structure of the *E. coli* hemolysin HlyA has been proposed which assumes that the hydrophobic N-terminal domains make up eight membrane-spanning α-helical sequences, four of which are hydrophobic segments of 21 amino acids each and four are amphipathic with the polar side of the helices providing the hydrophilic, negatively charged interior of the pore that may explain the reported cation selectivity of the pore. The N-terminal amphiphilic portion of hemolysin does not directly participate in the pore structure but may compete with the insertion of the α-helical amphipathic sequences assumed to be part of the pore structure and may thereby regulate the lifetime of the hemolysin pore [113]. Conflicting data on the number of toxin molecules required for pore formation have been published. However, it has been confirmed that HlyA creates a cation-selective ion channel of high conductance [13, 127, 128]. Moreover, the data suggest that a receptor is needed for the lytic activity of the toxin [13] in a two-stage process: first, the target cell binding requires glycine-rich repeat regions and modification of the toxin by the C gene product at an adjacent site and, afterwards, the N-terminal hydrophobic regions allow pore formation [32]. The target cell specificity could therefore be due either to a specific binding on the receptor, to the toxin ability to interact with the membrane or even to both parameters.

Interestingly, Ana Soloaga and her collaborators have studied the perturbation produced by purified α-hemolysin on pure phosphatidylcholine bilayers in the form of large unilamellar vesicles, under conditions in which the toxin has been shown to induce vesicle leakage. The bilayer systems containing bound protein have been examined by differential scanning calorimetry, fluorescence spectroscopy, differential solubilisation by Triton X-114, and freeze-fracture electron microscopy. The results obtained, complemented by structure prediction studies, have led to the conclusions that (i) α-hemolysin, under conditions leading to cell lysis, becomes inserted in the target membrane in the way of intrinsic or integral proteins and that (ii) inserted α-hemolysin occupies only one of the membrane phospholipid monolayers, i.e. it is not a transmembrane protein. Consequently, the insertion of one or more of these molecules in the outer monolayer of the membrane could induce an increase in the lateral pressure of the monolayer lipids and, beyond a certain increase, the monolayer will reach a point of transient breakdown (perhaps repaired by a net transfer of lipids to the inner monolayer) and subsequent leakage of contents. Upon the whole, these experiments and calculations are against the idea of *E. coli* hemolysin acting as a pore-forming toxin [156].

Moreover, it is intriguing to note that the overall pore formation mechanism resembles that of other toxins of bacterial origin such as colicins, diphtheria, tetanus and botulinum toxin [127].

7.4. Molecular synergies with other pathogens

As already stated above, *Mannheimia haemolytica*'s pathogenesis involves many predisposing viral and bacterial agents that could break down the antimicrobial barrier consisting of beta defensins and anionic peptides found in epithelial cells, resident and inflammatory cells, and serous and mucous secretions of the respiratory tract, then allowing *Mannheimia haemolytica* to be released from its usual commensal status [22].

On the other hand, impaired neutrophils and lymphocyte functions are observed in bovine viral diarrhea (BVD) virus [23], bovine respiratory syncytial (BRS) virus [184] and bovine herpes virus-1 (BHV-1) infected cattle. The latter is known to decrease host defence amongst others by diminishing the activities of T lymphocytes, B lymphocytes, monocytes and macrophages [24, 67] and by interfering with the host's antigen presentation machinery to evade the host's immune response in vivo [74, 134]. Moreover, it has been shown that leukocyte exposure to inflammatory cytokines released in response to BHV-1 infection (interleukin-1 beta, interleukin-8, tumour necrosis factor alpha and interferon gamma) can modulate the migration and functional activation of bovine leukocytes [26, 105, 106]. So, when *Mannheimia haemolytica* enters a BHV-1 infected lung, it encounters leukocytes whose recruitment and LFA-1 expression (and hence the leukotoxin susceptibility) are increased [26, 106]. In contrast, interleukin-8 expression was minimal in lesions of BRSV pneumonia [26]. Since inflammatory cytokines (tumour necrosis factor-alpha, interleukin-1 beta and interleukin-8) are also produced in response to *Mannheimia haemolytica* infection [80, 99, 100, 192, 193], they may, therefore, represent therapeutic targets to be modulated in order to treat or prevent mannheimiosis, as recently demonstrated in vitro [115].

8. CONCLUSION AND PERSPECTIVE

M. haemolytica's pneumonias are known to be one of the main diseases in the cattle industry. Several virulence factors have been described, with the most important being leukotoxin and lipopolysaccharide, that could in fact be compared to "the lips that deliver the kiss of death". They could act together (because they form complexes)

or separately through distinct or common pathways, leading to the production of several factors that are able to damage the cell and to amplify the disease. Among these actors, Ca^{2+} signals play a crucial role by governing a host of vital cell functions and so are necessary for cell survival. However, more recently, it has become clear that cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartimentalisation, can cause cytotoxicity and trigger either apoptotic or necrotic cell death [138].

Consequently, we can say that *M. haemolytica* could be considered among the pathogens that have reached the summum of evolution, being able to cooperate with other microbes to use the innate immune response against its host by promoting neutrophils and macrophage cell lysis. In this way, LKT seems to be very important by conferring species-specificity through specific interaction with the β2-integrin LFA-1. The accurate study of this binding at the molecular level will unambiguously represent a future step in the struggle against *M. haemolytica* and could open the way to the selection of naturally resistant animals.

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