Pasteurella Multocida and Immune Cells

Katharina F. Kubatzky

Abstract *Pasteurella multocida* was first discovered by Perroncito in 1878 and named after Louis Pasteur who first isolated and described this Gram-negative bacterium as the cause of fowl disease in 1880. Subsequently, *P. multocida* was also found to cause atrophic rhinitis in pigs, haemorrhagic septicaemia in cattle and respiratory diseases in many other animals. Among other factors such as lipopolysaccharide, outer membrane proteins and its capsule, the protein toxin (PMT) of *P. multocida* is an important virulence factor that determines the immunological response of the host's immune system. However, the exact molecular mechanisms taking place in cells of the innate and adaptive immune system are largely unknown for any of these virulence factors. Due to the obvious function of PMT on cells of the porcine skeletal system where it causes bone destruction, PMT was regarded as an osteolytic protein toxin. However, it remained unclear what the actual benefit for the bacteria would be. Recently, more attention was drawn to the osteoimmunological effects of PMT and the interplay between bone and immune cells. This review summarises the knowledge of effects of *P. multocida* virulence factors on the host's immune system.

Abbreviations

PMT	*Pasteurella multocida* toxin
IL	Interleukin
NFκB	Nuclear factor κB
CD	Cluster of differentiation

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1 Introduction

1.1 How the Immune System Fights Bacterial Infections

The immune system is a highly regulated network of cellular interactions that protects the organism from potential dangers that can be caused by the entrance of non-self particles such as bacteria, viruses and fungi. Haematopoietic stem cells in the bone marrow give rise to two lineages; the lymphoid lineage, which consists of B cells, T cells and NK cells and the myeloid lineage. B and T lymphocytes take part in the adaptive immune system. B cells play a role in humoral immunity as they give rise to plasma cells which produce antibodies against soluble antigens. Binding of an antigen to the B cell receptor initiates maturation to an antibody-producing plasma cell. T cells on the other hand differentiate in the bone marrow as well, but later on reside in the thymus where they need to undergo a selection process in order to fulfil their function in cell-based immunity, including recognition of antigens presented by antigen-presenting cells (APC).

The myeloid lineage can be further subdivided into two main branches, one originating from a granulocyte/monocyte precursor and the other from erythroid/megakaryocyte precursor cells. The latter differentiate into erythrocytes and platelets of the blood system. Several important immune cells are differentiated from the granulocyte/monocyte precursor cell: granulocytes, consisting of basophils, eosinophils and neutrophils, monocytes that further differentiate into macrophages and dendritic cells, and mast cells. Each of these immune cells has a specific task in the detection and clearing of an invading pathogen. Apart from their ability to act as effector cells, these cells also have the ability to communicate with each other in order to coordinate an efficient immune response.

In the presence of an infection, granulocytes are generally the first cells recruited to the site of the infection by stimuli from chemoattractants such as chemokines from the inflamed tissue or bacterial formyl peptides released by the invading microorganism itself (Muller 2011). They will then, at the site of infection, transmigrate through the epithelium and invade the inflamed tissue.

Granulocytes are effector cells that produce bactericidal substances and can phagocytose bacteria. Neutrophils also assist in maintaining this local inflammatory response through the production of reactive oxygen species (ROS), proteases and antimicrobial peptides (Stuart and Ezekowitz 2005). This then attracts macrophages, localised close to the inflamed tissue to the site of infection.

Digestion of pathogens through phagocytosis allows the subsequent presentation of the digested peptides via major histocompatibility complex II (MHC II) to T cells, a process that bridges the innate and the adaptive immune system. Phagocytosis involves the uptake of microbial particles through formation of a phagosome and the subsequent fusion with a lysosome to the phagolysosome in order to degrade the ingested material. The lysosome is a low pH milieu and contains bactericidal substances. Additionally, ROS as well as NO are produced that kill the pathogen. This process is often initiated by the complement cascade system that resides in the plasma. In the classical pathway, specific antibodies recognise an antigen, bind to it and thus coat the bacterium, which renders it more visible for phagocytes. In the alternative pathway, complement protein directly binds to the microbial pathogen. This opsonisation of bacteria is recognised by complement receptors on immune cells, which activates them to phagocytose the opsonised invader.

Phagocytosis is also associated with dendritic cells that can leave the tissue and migrate to regional lymph nodes. Once they are activated, dendritic cells are the most powerful antigen presenting cell type. Depending on the antigen detected, immature dendritic cells differentiate under the influence of paracrine cytokine stimulation to mature dendritic cells and are capable of inducing the polarisation of naïve T-cells (Sabatte et al. 2007). Polarisation of naïve T-cells into the helper T cells (Th) Th1 or Th2 depends on the kind of costimulatory molecules and cytokines that are produced by myeloid dendritic cells (Guermonprez et al. 2002). Cytokines are important mediators of the cellular immune response and can act in an autocrine as well as in a paracrine fashion. Cytokines play a predominant role in haematopoiesis and also affect the regulation of the immune response of mature haematopoietic cells. Many cytokines have a proliferative function that allows the immune system to specifically produce more effector cells in response to the activation of a target cell. In addition, the cytokine pattern released by innate immune cells stimulates naïve T cells to develop into helper T cells and determines the ultimate immune response given by these cells. While Th1 cells secrete IL-2, IFN-γ, TNF-α and participate in cell-mediated immune responses, Th2 cells are important helper cells for B cells through the production of IL-4, IL-5, IL-6, IL-10.

Immune cells are known to express a variety of so-called pattern recognition receptors that specifically recognise pathogen-associated molecular patterns (PAMPs). Recognition and binding of a microbial stimulus leads to the subsequent activation of intracellular signalling cascades, eventually resulting in clearance of the threat. Innate immune receptors can be transmembrane receptors such as the toll-like receptors (TLRs), as well as soluble components that mark microbial substances for detection by complement receptors, integrins or Fc receptors (Underhill 2003). TLRs are pattern recognition receptors as they are not activated

by specific ligands, but through recognition of more general pathogen-associated PAMPs such as lipids (lipopolysaccharides and lipoproteins), proteins (flagellin) or DNA and double-stranded RNA (Akira et al. 2006). The expression pattern of these receptors depends on the type of immune cell and their expression is not restricted to the haematopoietic system (Anders et al. 2004). While TLRs 1, 2, 4, 5 and 6 are primarily located at the cell surface to detect microbial PAMPs, TLRs 3, 7, 8 and 9 are localised in endosomal compartments which allows efficient recognition of viral or bacterial nucleic acids. Eventually, TLR signalling events result in the activation of transcription factors such as NFκB, Map kinases or IRFs (Brown et al. 2011). A central task of TLR signalling is the NFκB-dependent production of inflammatory cytokines as an initial defence mechanism. However, novel functions are emerging and TLRs were shown to play a substantial role in phagocytosis, antigen presentation and crosstalk with T cells as well as clearance of microbes through autophagy (Blander and Medzhitov 2004).

1.2 Bacterial Strategies of Immune Evasion

Many bacteria live in close relationships with their host and sometimes they even benefit the host. They have thus developed strategies in a co-evolutionary process to overcome the above-mentioned arsenal of mechanisms that help the immune system fight the microbe. Interestingly, many commensals can turn into pathogens, for example upon a change in the health of the host or due to changes in the pathogenicity of the microbe. The word pathogenicity describes the sum of all the characteristics of a microorganism that play a role in infection. The term virulence on the other hand describes the degree of pathogenicity of a specific microorganism. There are for example several strains of a given microorganism that due to the absence of virulence factors have a different degree of virulence. These virulence factors can be produced as an integral part of the bacterium, such as its cell wall or it can be synthesised upon a certain trigger and then cause an infection. In general it can be said that the pathogenicity of a microorganism depends on its capability to adhere to host cells, to colonise at the site of infection and then to further invade the organism. Additionally, bacteria can produce toxins that help to destroy host cells.

Strategies to evade the host's immune response can therefore include the manipulation of the immune system, for example through targeting specific signalling pathways that result in dampening the immune response, or by changes in the bacterium itself or its localisation (Srikumaran et al. 2008).

The extracellular matrix is an important interaction platform between bacteria and their host. It therefore participates in many mechanisms of virulence and evasion (Fig. 1). Bacterial surface components help the microorganism in the process of bacterial adhesion and invasion but on the other hand provide characteristic molecular patterns that can be recognised by immune cells. Factors that help the bacteria to adhere and to prevent being cleared from the mucosa or

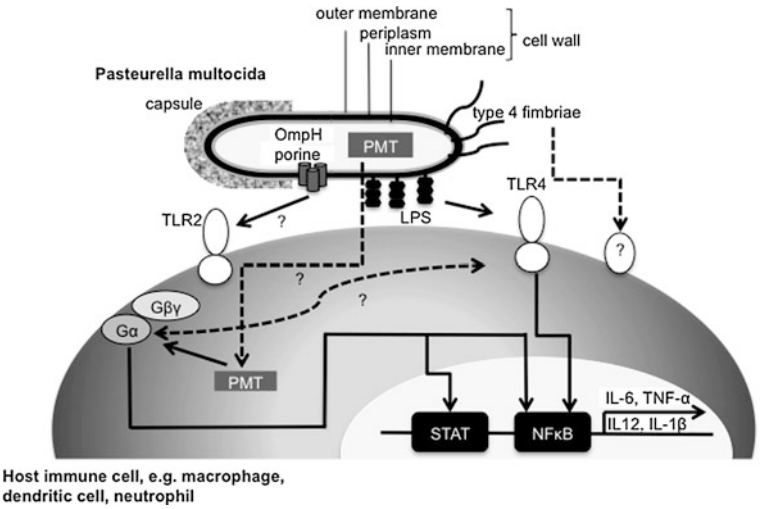

Fig. 1 *Strategies of bacterial host colonisation*. Gram-negative bacteria such as *P. multocida* express various virulence factors that determine their pathogenicity. The most important virulence factors of *P. multocida* are the presence of a polysaccharide capsule, fimbriae, lipopolysaccharide and outer membrane proteins, e.g. the porin OmpH. PMT is an AB toxin and probably encoded by a bacteriophage. PMT acts intracellularly and deamidates G proteins eventually causing the activation of signal transducer and activator of transcription factors (STAT) or NFκB. Toll-like receptors present on the host's immune cells detect LPS (TLR4) and probably also proteins of the outer membrane. TLR signalling cascades result in the activation of the transcription factor NFκB and the subsequent release of inflammatory cytokines. Known interactions are depicted using *solid arrows*, hypothetical pathways are shown using *dotted arrows*

epithelium include lectins, fimbriae, as well as non-fimbrial adhesins and glycosaminoglycan-binding proteins.

To avoid recognition through the host, bacteria have developed ways to alter their surface structures through antigenic hypervariability of structures that are easily detected by the immune system (Finlay and McFadden 2006). Factors that can undergo variation include the carbohydrates that are used to build up a capsule surrounding and masking the bacterial cell wall, pili and proteins being expressed in the outer membrane (Omp). Lipopolysaccharide (LPS), a typical constituent of the outer membrane of the cell wall of Gram-negative bacteria that causes the typical symptoms of a bacterial infection such as fever, is varied for example through addition of side groups causing the development of different serotypes of a bacterial strain. This variation can affect antigenicity, serum sensitivity but also adhesion (van der Woude and Baumler 2004).

Additionally, bacteria protect themselves by attacking parts of the complement system or immunoglobulins through specifically acting bacterial proteases. Even if these mechanisms fail and the pathogen is phagocytosed, bacteria have means to act intracellularly, for example by inhibiting phagolysosomal fusion (Finlay and

McFadden 2006; Frehel et al. 1986). Subversion of intracellular pathways by toxins and peptides or proteins released from bacteria has come to attention in recent years. Eventually this mechanism influences gene transcription through post-translational modifications of cellular targets or inhibition of degradation processes (Finlay and McFadden 2006; Hildebrand et al. 2010; Ribet and Cossart 2010).

Strains capable of producing bacterial toxins are usually more virulent. While the word endotoxin means substances that are integral components of the bacterium, such as the cell wall, bacterial DNA or RNA, exotoxins are proteins produced by the bacteria that are either actively secreted or will be released during bacterial lysis. AB toxins contain a receptor-binding domain that facilitates uptake of the bacterial protein by a host cell surface receptor. Bacterial protein toxins can have an enzymatic activity that very specifically modifies intracellular host target proteins. Often their targets are members of the Rho GTPase family (Aktories 2011), but they can also be other proteins that play a central role in host viability or regulation of the immune system (Lax and Thomas 2002; Oswald et al. 2005).

2 *Pasteurella multocida* and Immune Cells

2.1 Pasteurella multocida-Associated Diseases

Pasteurella multocida is a Gram-negative, facultatively anaerobic coccobacillus that can be found on many animals worldwide where it can cause a variety of infections. The *P. multocida* species can be further subdivided into four subspecies, namely multocida, gallicida, septica and tigris (Harper et al. 2006). Serologically, five types (A, B, D, E and F) can be defined according to differences of the capsular antigen (Arumugam et al. 2011; Carter 1955) and 16 types based on differences in LPS composition (Harper et al. 2011; Heddleston et al. 1972). Although *P. multocida* infections are zoonotic diseases, humans are rarely affected and bacteria are usually transferred from animals for example through cat or dog bites and scratches. For humans it can be considered an opportunistic infection as it is mainly found in immune-compromised patients. This can also be true for animals, where *P. multocida* is usually a commensal that can become pathogenic due to an underlying infection or changes in the expression pattern of virulence factors. Clinically, *P. multocida* infections in humans are mostly associated with skin and soft tissue infections. However, respiratory tract infections as well as bone and joint infections are also known and the latter can result in osteomyelitis and septic arthritis (Zurlo 2005).

Pasteurella multocida infections such as fowl cholera, bovine haemorrhagic septicaemia, enzootic calf pneumonia, swine atrophic rhinitis or rabbit snuffles are economically relevant problems and the mechanisms of pathogenesis have therefore been extensively studied in various animal models. Details of these disease

syndromes are described in "Host-Pathogen Interactions.". Interestingly, different serotypes are associated with specific types of diseases. While fowl cholera is associated with serotype A:1 and A:3 strains (Adler et al. 1999), haemorrhagic septicaemia is associated with B:2 and E:2 strains (Shivachandra et al. 2011) while atrophic rhinitis of pigs is primarily caused by toxigenic type D and A strains (Amigot et al. 1998).

A number of virulence factors have so far been defined and will be discussed below, but the molecular basis for the interference between *P. multocida* strains and the host's immune system is only incompletely understood. Thus the application of new methods such as proteomic analysis, more refined detection methods and the availability of the complete genomic sequence of *P. multocida* (May et al. 2001; "The Key Surface Components of *Pasteurella multocida*: Capsule and Lipopolysaccharide") are likely to enable us to define other factors that contribute to the disease or are important factors in the interaction between host and pathogen.

2.2 Major Virulence Factors of Pasteurella multocida

2.2.1 LPS

LPS is a component of the bacterial cell wall typical for Gram-negative pathogens. Detection of LPS by TLR4 expressed on cells of the innate immune system ultimately leads to a humoral response characterised by the secretion of antibodies. Due to its prominent role, LPS acts as both a virulence factor and a serovar-specific immunogen. Of the 16 different serotypes known for *P. multocida* (Heddleston et al. 1972), at least some are genetically related, meaning that they share the same outer core locus. Distinct point mutations in one of the enzymes involved in attachment of sugar modifications such as the phosphoethanolamine (PEtn) transferase gene or phosphocholine synthase generate a modified LPS (Harper et al. 2011). Changes in the outer core of the LPS oligosaccharides can be the result of bacterial phase-variation strategies; however, this has not been shown for *P. multocida* (Harper et al. 2007). An unusual finding was, however, that *P. multocida* strains simultaneously express two LPS glycoforms, which differ in their inner core (Harper et al. 2007). This highlights the fact that detailed knowledge of the genetic background and structural studies will be a prerequisite in order to identify suitable targets to generate efficient vaccines. Details of LPS structure are described in "Pathogenomics of *Pasteurella multocida*".

Recognition of LPS occurs via binding to TLR4 and eventually results in the release of NFκB-induced inflammatory cytokines and expression of adhesion molecules that attract leukocytes to the site of infection. The work of Galdiero et al. (2000) showed that whole *P. multocida* bacteria as well as LPS preparations isolated from *P. multocida* enabled efficient adhesion of neutrophils to isolated bovine endothelial cells, presumably supported by the upregulation of adhesion

markers such as VCAM-1 (Richardson et al. 1997) and followed by subsequent transmigration through the endothelial cell layer 2–4 h after stimulation. *P. multocida* LPS was also able to induce the expression and the subsequent release of proinflammatory and immunomodulatory cytokines such as IL-1α, IL-6, TNF-α, IFN-γ and IL-12 from a mix of murine splenocytes (Iovane et al. 1998). This result is in line with the many reports using *E. coli* or *Salmonella* LPS as a stimulating agent to induce TLR4-dependent signalling cascades; however, no data are available that would directly compare *P. multocida* LPS with commercially available LPS preparations or evaluate different *P. multocida* LPS compositions for TLR4 signalling.

The endotoxin LPS is an exogenous pyrogen and LPS-initiated release of cytokines eventually induces fever. Whether the endotoxic activity of *P. multocida* LPS is comparable to that of other microbes is controversially discussed in the available literature and the susceptibility might be species specific (Dabo et al. 2007; Harper et al. 2006). However, from studies employing bacteria with mutations in the sugar core of LPS or mutants with truncated LPS, it is also clear that LPS is required to cause disease, as these mutant strains show decreased viability in animals (Fernandez de Henestrosa et al. 1997; Harper et al. 2004).

From the side of the host, LPS can generally be considered a protective antigen that can be detected efficiently. It has therefore been tested whether *P. multocida* LPS might also be a good immunogen. Passive immunisation using monoclonal antibodies against LPS from a serotype A strain indeed killed bacteria and protected mice from infection. However, this was true only for homologous infections with the same mice strain, suggesting that protection is serovar specific (Wijewardana and Sutherland 1990). Other examples include an opsonic monoclonal antibody that was able to deliver opsonized bacteria for phagocytosis by macrophages. This was demonstrated in an experimental setting were peritoneal macrophages were prepared and incubated with *P. multocida*, monoclonal antibodies against LPS and complement to facilitate uptake of bacteria (Ramdani and Adler 1991). However, passive immunisation of mice using affinity-purified anti-LPS serum did not protect mice efficiently against infection, thus suggesting that LPS plays a minor role as a protecting immunogen (Lu et al. 1991). One method to enhance the effect of LPS antibodies is the use of an LPS-protein mix or ribosomes, which achieved complete protection in a mouse model system under homologous infection conditions (Dabo et al. 2007; Rimler and Phillips 1986; Ryu and Kim 2000).

2.2.2 Capsule

The antigenicity of the capsule is used to identify the five known serogroups A, B, D, E and F (Carter 1955), suggesting that the capsule gives rise to B cell-mediated antibody production, although this notion remains controversial. Details of capsule composition, biosynthesis and regulation and role in pathogenesis are presented in "Pathogenomics of *Pasteurella multocida*".

2.2.3 Adhesins

Pili or type IV fimbriae are widespread among Gram-negative bacteria but can also be found in Gram-positive bacteria (Proft and Baker 2009). They are built up by long filaments consisting of app. 20 kDa fimbrial subunits that facilitate adhesion to host cells. Initially, adherence occurs at epithelial cells and this represents a crucial step in successful colonisation of the host and subsequent infection. While epithelial cells do not belong to haematopoietic immune cells, they present an important physical barrier with effective antimicrobial defence mechanisms through production of antimicrobial peptides and the activation of innate immune receptors (Marques and Boneca 2011). Although type I fimbriae were recently shown to be recognised by TLR4 proteins expressed on macrophages (Mossman et al. 2008), nothing is known about type IV fimbriae and the resulting response of the innate immune system.

Pili are important virulence factors that facilitate colonisation of the host; on the other hand, type IV fimbriae have been used successfully as immunogenic targets to develop vaccines, e.g. against *Yersiniae pestis*, *Salmonella enteriditis*, *Pseudomonas aeruginosa*, *Dichelobacter nodosus* and *Moraxella bovis* (Adler et al. 1999; Proft and Baker 2009). The fimbrial subunit protein of *P. multocida*, PtfA, is a 15 kDa protein with a high degree of similarity to other fimbrial proteins, except for its prolonged signal sequence (Adler et al. 1999; Doughty et al. 2000). The protein is found on serotype A, B and D strains (Ruffolo et al. 1997) and is often associated with increased virulence (Harper et al. 2006). Due to the significant divergence in the C-terminal part between various *P. multocida* strains, development of a vaccine might be difficult (Doughty et al. 2000). There are also other *P. multocida* proteins with similarity to proteins encoding for fimbriae, fibrils or filamentous haemagglutinins in other bacteria, but no details are available concerning their interaction with immune cells.

2.2.4 Outer Membrane Proteins and Porins

To date, some outer membrane proteins (Omps) of *P. multocida*, including OmpH, OmpA, P6-like protein, PlpB, GlpQ, Lpp and Oma78 have been characterised experimentally concerning their structure and immunogenicity; recently, proteomic studies revealed the existence of 28 other proteins and ten of these are predicted to be porins (Boyce et al. 2006). Several Omps were shown to act as virulence factors with OmpH being the best characterised among them. It was found to be present in basically all bovine isolates (Dabo et al. 2007) and is discussed as a vaccine candidate as it was shown to protect mice and chicken against *P. multocida* challenge (Luo et al. 1999; Vasfi Marandi and Mittal 1997).

OmpH is a trimeric porin that is also known as Protein H, with a molecular mass of 37.5 kDa (Chevalier et al. 1992). So far it is the only porin that has been tested in more detail for its ability to stimulate immune cells. In a study comparing the effects of *P. multocida* LPS with recombinant and reconstituted porin, OmpH

was shown to modulate the response of the immune system by inducing the expression and production of proinflammatory cytokines such as IL-1α, IL-6, TNF-α or IFN-γ and IL-12 from monocytes and macrophages of murine splenocytes (Iovane et al. 1998). None of the typical B cell-produced Th2 cytokines such as IL-4 and IL-10 were detected. It can therefore be hypothesised that OmpH does not directly influence the adaptive immune system but that it can influence the secretion of cytokines by innate immune cells. The release of the cytokines was delayed compared to LPS and the observed gene induction was less efficient, at least at low concentrations. It is currently unknown how *P. multocida* porins and OmpH interact with the host cell. Porins from other Gram-negative microorganisms however, were shown to be detected by TLR2 receptors (Wetzler 2010). Why this would cause differences in the kinetics of cytokine production compared to the LPS-mediated initiation of TLR4 signalling is unclear.

Bacteria can act as chemoattractants and it was therefore tested whether OmpH alone was sufficient to trigger neutrophil migration (Galdiero et al. 1998). Indeed, the presence of porin-induced neutrophil migration and actin polymerisation in a concentration-dependent manner, eventually resulting in an oxidative burst (Galdiero et al. 1998). As *P. multocida* suspensions induced the adhesion of neutrophils to endothelial cells, it was tested whether the same could be achieved using isolated membrane components (Galdiero et al. 2000). Adhesion could be induced using either 200 nM of isolated LPS or 50–200 nM Protein H and both substances were able to stimulate bacterial transmigration; however, maximum transmigration for porin was reached at a later time point. Experiments investigating the ability of OmpH to trigger the production of the immunoregulatory messenger molecule NO from murine macrophages showed that the porin itself was unable to do so. However, the presence of IFN-γ increased the induction of iNOS (inducible nitric oxide synthase) expression and subsequent NO release from macrophages compared to the stimulation with IFN-γ alone (Marcatili et al. 2000).

2.2.5 *Pasteurella multocida* Toxin

Toxigenic *P. multocida* serotype A and D strains are able to produce a 146 kDa protein toxin (PMT). In swine, these toxigenic strains are widespread and the reduced weight gain of animals presents an economically relevant problem (Foged 1992; Harper et al. 2006). PMT is the causative agent of atrophic rhinitis, which causes osteoclastic bone resorption at the nasal turbinates (Felix et al. 1992) and isolated PMT is able to induce all the symptoms of the disease (Chrisp and Foged 1991; Dominick and Rimler 1986). On the genetic level, the toxA gene sequence encoding for PMT has a lower G + C content compared to the rest of the *Pasteurella* genome, suggesting that it is phage-encoded (Pullinger et al. 2004). In addition, no specific sequence provides for a mechanism to secrete PMT. Using electron microscopy, PMT was found to reside in the cytoplasm and to be absent in undamaged bacteria (iDali et al. 1991). Therefore, it is possible that PMT is only released from the cells when the temperate phage is induced (Pullinger et al. 2004).

In vitro, PMT acts as a potent mitogen for a variety of cell types such as fibroblasts, bladder epithelial cells and osteoclasts (Hoskins et al. 1997; Martineau-Doize et al. 1993; Rozengurt et al. 1990). PMT is taken up by host cells through receptor-mediated endocytosis, presumably via binding to gangliosides (Dudet et al. 1996; Pettit et al. 1993). The heterotrimeric G proteins $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_i$ (Orth et al. 2005, 2008; Wilson et al. 1997; Zywietz et al. 2001), which PMT renders constitutively active through deamidation of a conserved glutamine residue to glutamate (Orth et al. 2009), are the specific cellular targets of PMT. This leads to the activation of intracellular signalling pathways such as Rho GTPase signalling, phospholipase Cβ (PLCβ), activation of mitogen activated protein (Map) kinases, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway or the activation of phosphoinositol (PI) 3 kinases (Wilson and Ho 2011). PMT-modulated signalling cascades have been identified using cell lines and not primary immune cells, thus it is still under investigation whether these signalling changes eventually modify the immune response of the host.

One major pathway of haematopoietic cells including immune cells is the activation of JAK-STAT signalling. As PMT is able to activate this pathway (Orth et al. 2007), it can be hypothesised that the toxin might have a function in immune signalling. Dendritic cells are the most potent antigen-presenting cells and the only APC capable of initiating immune responses of the adaptive immune system. Therefore, several groups have investigated the effect of PMT on these cells (Bagley et al. 2005; Blocker et al. 2006). Maturation of human monocytes into dendritic cells is monitored measuring the expression of specific surface markers, such as CD80, 83, 86 and HLA-DR. The PMT-mediated upregulation of these markers was concentration dependent and detectable at 10 pM, but was highest at 7–20 nM. Bacterial protein toxins such as cholera toxin, pertussis toxin, heat-labile enterotoxin (*E. coli*) or adenylate cyclase (*Bordetella pertussis*) have been shown to trigger monocyte maturation through elevation of the intracellular cAMP levels either directly or indirectly through ADP-ribosylation of G proteins and subsequent constitutive adenylate cyclase activation (Bagley et al. 2002a, b). Additionally, the production of IL-12, which induces proliferation and cytokine production in T cells, is suppressed (Bagley et al. 2002a, b; Kubin et al. 1994). PMT on the other hand activates PLCβ followed by an increase in intracellular calcium levels. Interestingly, calcium is known to induce maturation of dendritic cells and to inhibit IL-12 production (Faries et al. 2001). It was therefore investigated whether PMT would have similar effects as other protein toxins on DC reactivity, though using a different pathway.

In contrast to the endotoxin LPS that is an efficient activator of cytokine secretion, due to the elevation in cAMP levels, bacterial protein toxins do not induce the production of cytokines (Zidek 1999) and are able to downregulate LPS-induced production of IL-12 (Bagley et al. 2002b; Gagliardi et al. 2000). This was also verified for PMT (Bagley et al. 2005). Nevertheless, due to the activated phenotype induced by PMT a co-culture of PMT-treated dendritic cells with naive T cells increased T cell proliferation comparable to LPS or cholera toxin treatment. The ability of toxins to induce maturation of DCs in vitro usually correlates

well with their ability to boost an antibody response in vivo due to efficient antigen presentation and T cell-supported B cell activation (Pulendran and Ahmed 2011). However, when used as an antigen for vaccination, PMT was repeatedly shown to downregulate the antibody response and to even suppress the production of antibodies in response to the injection of cholera toxin (Bagley et al. 2005; Nielsen et al. 1991; van Diemen et al. 1994). These findings suggest that other mechanisms in addition to the activation and maturation of dendritic cells exist, rendering the organism unresponsive to active toxin.

The migration of dendritic cells to regional lymph nodes is an important factor in generating an efficient adaptive immune response. Using murine dendritic cells, PMT was found to induce RhoA-mediated actin polymerisation (Blocker et al. 2006). Morphologically, this translated into altered cell morphology with long needle-like extensions instead of the usual irregularly formed shape of immature dendritic cells. As a consequence, complement-induced actin re-organisation was impaired, leading to the inhibition of cell migration in response to chemoattractants such as complement C5a or the chemokines CCL5 and CCL9. In contrast to *P. multocida* factors such as LPS or Protein H localised at the bacterial cell wall, PMT itself did not act as a chemoattractant. However, measuring the uptake of the dye texas red macropinocytosis, i.e. the formation of large endocytic vesicles for the uptake of pathogens or antigens, was found unchanged. While macropinocytosis also requires an active cytoskeleton, mechanistically, Rac activation might be more important here and was shown to be unaffected by PMT (Blocker et al. 2006; Nobes and Marsh 2000).

2.2.6 Aspects of PMT in Osteoimmunology

In mammals bone cells regulate the skeleton while the immune system deals with the detection and destruction of invading pathogens. Interestingly, there is strong crosstalk between these two systems that led scientists to define the emerging field of osteoimmunology (Arron and Choi 2000; Mensah et al. 2009). Osteoclasts are multinucleated cells that break down bone. They are however generated through fusion of mononuclear precursor cells developed from macrophages via a macrophage colony-stimulating factor (M-CSF) and RANKL-mediated differentiation process and therefore belong to the haematopoietic lineage. To investigate the effects of PMT on bone marrow cells in more detail, haematopoietic murine progenitor cells were cultivated with PMT. When the ability of haematopoietic cells to take up fluorescently labelled PMT was tested, all of the cells tested took up PMT to varying degrees (Fig. 2). However, in an experimental setup to test the ability to survive without additional cytokines, only two types of cells were found to survive (Hildebrand et al. 2011) (Fig. 3a). Subsequent characterisation of the two cell types by FACS analysis showed that the surviving cells were B cells and macrophages, which, after prolonged incubation with the toxin, further differentiated into osteoclasts as shown by fluorescence staining for the osteoclast-specific enzyme tartrate-resistant acidic phosphatase (TRAP) and the presence of multiple

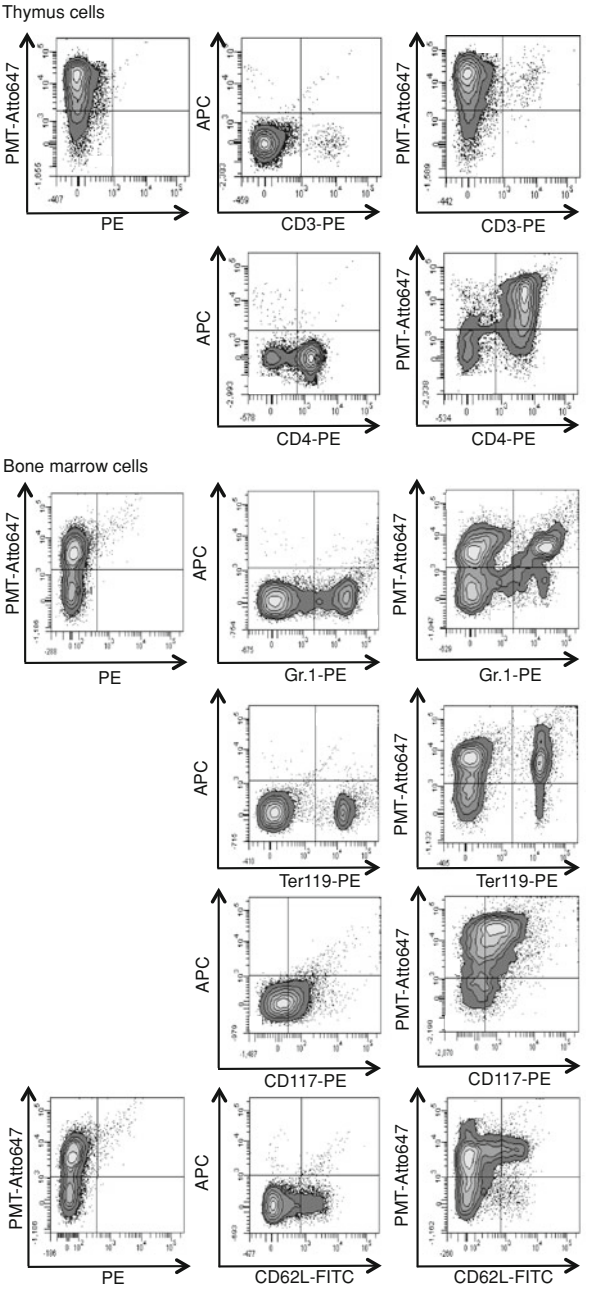

Fig. 2 *Uptake of PMT by immune cells*. Murine bone marrow cells or thymus cells were prepared from BL6 mice. The cells were incubated for 4 h with Atto647-labelled PMT (6.5 nM) at 37°C. Subsequently, cells were stained for cell type specific surface markers [T cells (CD3, CD4), monocytes (Gr.1), erythroid progenitors (Ter119), stem cells (CD117) and platelets (CD62L)] and analysed by FACS analysis on a FACS Canto (Beckton Dickinson)

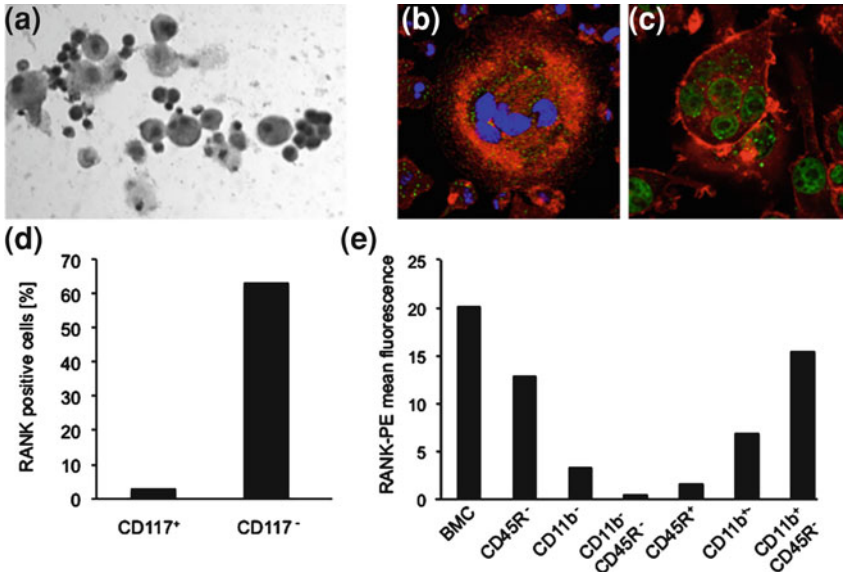

Fig. 3 *PMT induces the differentiation of murine haematopoietic bone marrow cells into B cells and macrophages*. **a** Morphological analysis of bone marrow cells (BMCs). Cytospins of BMCs stimulated with PMT (6.5 nM) were paraformaldehyde fixed, stained with hematoxylin and eosin, and mounted with glycerine-PBS for microscopy. **b** TRAP staining of BMCs differentiated with PMT (6.5 nM) for 5 days. The actin cytoskeleton was stained using Phalloidin-TRITC (*red*). Nuclei were counterstained with SYTOX Green (shown in *blue*). Osteoclasts or pre-osteoclasts were characterised by detecting the expression of TRAP using the ELF 97 phosphatase substrate (*yellow-green*). Osteoclasts additionally display multi-nuclearity. **c** TRAP staining of RAW 276.4 macrophages differentiated with PMT (6.5 nM) for 6 days. The actin cytoskeleton was stained using Phalloidin-TRITC (*red*). Nuclei were counterstained with SYTOX Green (shown in *green*). Osteoclasts or pre-osteoclasts were characterised by detecting the expression of TRAP using the ELF 97 phosphatase substrate (*yellow-green*). Osteoclasts additionally display multi-nuclearity. **d** Purification of $CD117^+$ stem cells. Stem cell receptor (CD117) expressing cells were purified from a mix of total bone marrow cells by MACS purification technology, double stained for CD117 and CD11b, and analysed by FACS. Quantification of macrophages and osteoclasts in $CD117^+$ and $CD117^-$ cells. After 9 days of stimulation with PMT (6.5 nM), the number of $RANK^+$ osteoclasts from the $CD117^+$ and $CD117^-$ fractions were quantified by FACS analysis with specific antibodies. **e** PMT-induced osteoclast generation is B-cell dependent. Using MACS cell purification of $B220^+$ cells or $CD11b^+$ cells or purification of both cell types, five different fractions (BMC mix, $CD45R^-$ $CD11b^-$, $CD45R^+$, $CD11b^+$, and $CD45R^+$ $CD11b^+$ cells) were generated. After 14 days of PMT stimulation (6.5 nM), cells were stained for the osteoclast marker RANK and analysed by FACS

nuclei (Fig. 3b). This was also verified by the ability of PMT to differentiate a murine macrophage cell line RAW 264.7 into osteoclasts (Fig. 3c). Although stem cells were able to take up PMT, CD117-positive stem cells were unable to differentiate into receptor activator of NFκB (Rank) positive osteoclasts, suggesting that PMT triggers immature macrophages to further mature into osteoclasts (Fig. 3d). While it is possible that intracellular, PMT-mediated signalling

pathways are able to induce this maturation process at least for mature macrophages, subsequent experiments support the hypothesis that B cells play a central role in PMT-derived osteoclast differentiation. Generating isolated macrophages (CD11b) and B cells (CD45R) through cells MACS-assisted cell sorting, it was shown purified macrophages had a decreased ability to differentiate into osteoclasts in the presence of PMT when compared with a mix of bone marrow cells (Fig. 3e). However, the addition of purified B cells to the macrophage population restored osteoclastogenesis, suggesting that the B cell population plays an essential role in PMT-mediated differentiation of macrophages into osteoclasts. Indeed, PMT-treated B cells produce large amounts of osteoclastic cytokines such as TNF-α, IL-6, RANKL and IL-1β. However, it is also possible that direct cell–cell-contacts are also required for this process and transwell experiments would be required to investigate this question in more detail. Apart from its function as an inducer of osteoclastogenesis, IL-1β is an endogenous pyrogen that causes fever. On a molecular level, pro-IL-1β production causes a cell to activate the inflammasome, which allows the cleavage of the inactive zymogen through the cysteine protease caspase-1 into its active mature form of cleaved IL-1β (Broz and Monack 2011; Gross et al. 2011). The process of inflammasome activation is associated with the activation of apoptotic pathways (Miao et al. 2011). This however is in great contrast to our findings that PMT protects cells from apoptosis through activation of the survival kinases Pim and Akt (Preuss et al. 2010), suggesting that the cleavage of pro-IL-1β in the absence of an inflammasome activation by yet unknown mechanisms might be possible.

3 Discussion

Pasteurella multocida is a commensal microorganism for many animals and its colonisation, with the exception of toxA producing strains of serotype A and D, does usually not harm its host (Biberstein 1990). However, it can turn into a pathogen causing severe diseases. It is not well understood how the transition from commensal into a pathogen occurs, but it is possible that an underlying infection that weakens the immune system triggers the development of a secondary opportunistic infection. While in this scenario a change in the immune status of the host is the pivotal event, the ability of *P. multocida* to express a variety of virulence factors is likely to play a role as well. In particular, the continual adaption to the host through genetic exchange is likely to contribute to both successful commensalism as well as pathogenesis (Michael et al. 2012; Redfield et al. 2006). It is also conceivable that inherent differences exist between pathogenic versus commensal populations that have not yet been described (Dabo et al. 2007). With proteomic approaches becoming a technology that is used more frequently and conveniently to address issues regarding differences at the protein level, this question may be answered within the next years.

Indeed, many of the data available on *P. multocida* virulence factors were generated at a time when studies were restricted to simple experimental setups. Additionally, at that time only a limited amount of information was available regarding signalling pathways of the immune system such as the TLR pathway (Medzhitov et al. 1997). Therefore, many publications investigating the influence of *P. multocida* virulence factors were limited to a phenotypic description without providing molecular details on the mechanism of action. It will be of great interest to return to these early findings regarding the function of virulence factors such as LPS, adhesins and outer membrane proteins and to elucidate the molecular details now. Additionally, the availability of more sophisticated immunological and biochemical techniques and the feasibility of proteomic studies at the level of the host as well as of the microorganism will give us new insights into the process of mutual regulation between the two and will ultimately help to develop tools to prevent or control *P. multocida* related animal diseases. Using these techniques it will also be of interest to identify and characterise new proteins and to evaluate their function in pathogenicity. Another conundrum is the finding that although *P. multocida* is an important pathogen for many wildlife and domestic animals where it causes severe diseases, for humans *P. multocida* related infections are rare and often associated with immune compromised patients (Harper et al. 2006; Zurlo 2005). Whether this is due to differences in the immune system that allows better detection or more efficient clearing is currently unknown.

Bacteria do not only interact with cells of the immune system, but they also play an important role in the pathology of bone-related conditions. Often, a combination of inflammation and loss of bone is detected in an on-going bacterial infection. Examples of that type include periodontal disease, osteomyelitis or bacterial arthritis (Nair et al. 1996). Considering the fact that osteoclasts are differentiated from macrophages through M-CSF and RANKL-mediated differentiation process (Fig. 4a), an excess in osteoclast differentiation might dampen the innate immune response by macrophages. However, it is not clear whether bacteria cause pathological bone loss through direct destruction of bone components, for example, through manipulation of cellular processes that regulate bone resorption, or through activation of the host's immune system, eventually leading to the production of osteolytic factors and proinflammatory cytokines. A prime example of bacterially induced osteoclastogenesis is represented by PMT. While the detailed mechanism of PMT on osteoclast activity remains largely unknown, phenotypically PMT was shown to induce the differentiation of pre-osteoclasts into osteoclasts (Jutras and Martineau-Doize 1996; Martineau-Doize et al. 1993), eventually causing increased bone resorption. Additionally, PMT seems to inhibit osteoblasts which impedes effective bone regeneration (Mullan and Lax 1998).

While numerous reports discuss the possibility of a close interaction or even relationship between B-cells and osteoclasts (Blin-Wakkach et al. 2006; Calvani et al. 2004; Horowitz et al. 2005; Miyaura et al. 1997), little is known on this kind of crosstalk with respect to the influence of bacterial infections. Additionally, it is not clear why bacteria target the bone system at all and if this might represent a new mechanism of immune evasion. Due to the close connection between the bone

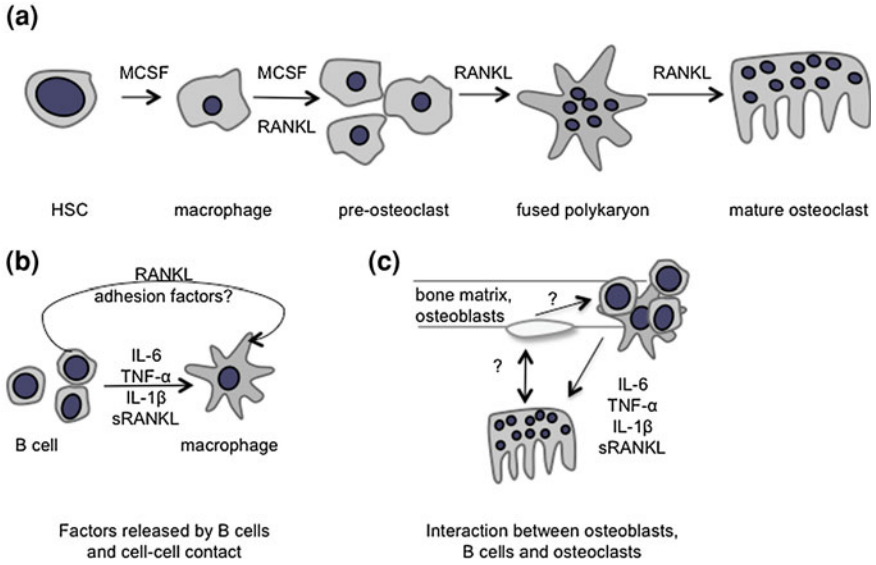

Fig. 4 *Osteoclast differentiation*. Classical osteoclastogenesis **a** involves the cytokine-induced differentiation of haematopoietic progenitors from the myeloid lineage and subsequent differentiation into macrophages and osteoclasts via the two factors M-CSF and RANKL. **b** The recently described model of PMT-induced osteoclastogenesis involves the cooperation between B cells and macrophages to efficiently generate osteoclasts (Hildebrand et al. 2011). In addition to cytokines secreted by B cells, direct cell–cell contact is likely to play a role. **c** In a hypothetical model of PMT-mediated osteoclast formation the interaction of osteoblasts and osteoblasts as well as the interaction between immune cells and cells of the bone system are likely to contribute to the differentiation process

and immune system, manipulating the response of immune cells might allow bacteria to shift the equilibrium from bone homeostasis to increased bone resorption as a side effect. Some bacterial products directly stimulate bone resorption, for example LPS, teichoic acid, lipid A associated proteins, as well as cell wall and cell surface components of a number of bacterial strains (Nair et al. 1996). Recently, we showed that PMT influences the activity of B cells and that B cells are important helper cells that stimulate PMT-mediated osteoclast differentiation from isolated murine macrophages (Hildebrand et al. 2011) (Fig. 4b). We hypothesise that PMT will be a valuable tool to investigate osteoimmunological questions and to identify signalling pathways that connect signals from osteoblasts, osteoclasts and immune cells that play a role in the regulation of osteoclast formation (Fig. 4c).

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