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Review

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Toll-like receptor agonist-based nanomedicines as veterinary immunotherapies

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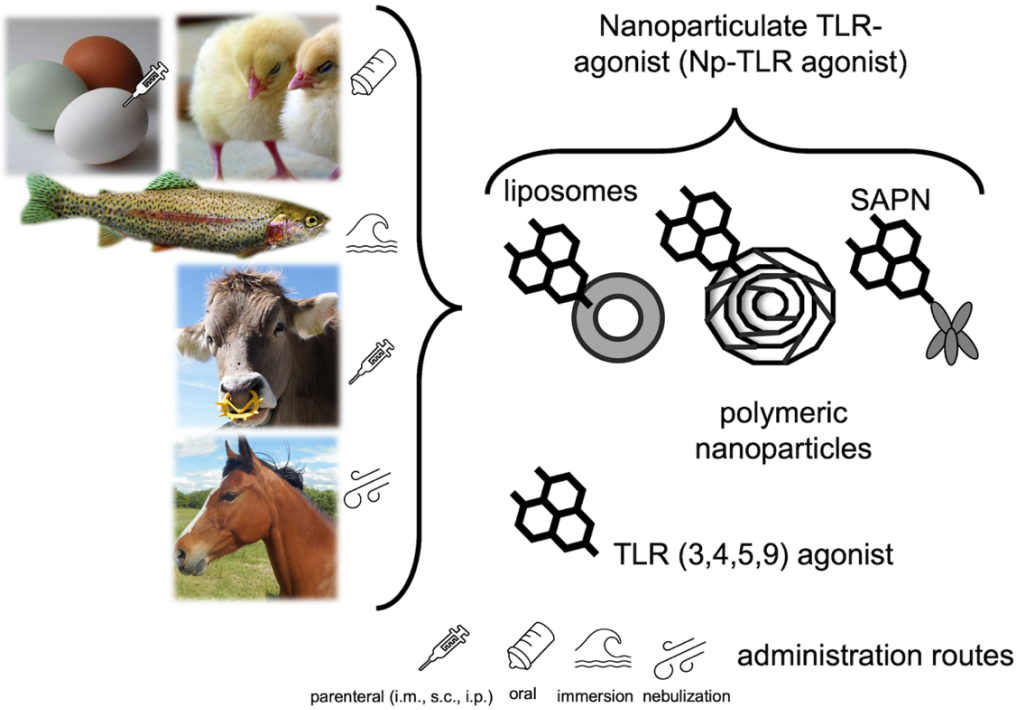
Graphical abstract



Abstract

Many infections affecting animals enter across mucosa, needing of secretory immunity to reject the disease; protection against some pathogens must be early, fast, and specifically elicited, while from others must be wide enough to fight against variable serotypes. Today, however, most veterinary vaccines are injectable (not recommended for chicken and small fishes), aimed to control clinical signs instead of eradicating the disease and poor inducers of secretory immunity. These drawbacks are compensated by vaccination with live attenuated agents, by using potentially toxic oily adjuvants and with indiscriminate use of antibiotics. In this review, the benefits of commercial and experimental nanomedicines acting as immunostimulants and vaccine adjuvants made of toll-like receptor (TLR) agonists loaded in nanoparticles (NP-TLR agonists), are presented. Np-TLR agonists induce a magnified, site-limited triggering of innate immunity; also allow modifying the administration route from injectable to mucosal or nebulized, provide structural protection to TLR agonists and avoid their diffusion far from the administration site. Future implementation of immunotherapies based on Np-TLR agonists will be discussed as a function of scale production feasibility.

Keywords: polyIC, CpG, flagellin, vaccine, immunotherapeutic, liposomes

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Purpose and rationale

The field of veterinary immunotherapeutic is wide and heterogeneous, in epidemiological and economics terms, demographic context, and species-specific needs [1]. In this paper, we will not address particular points from classical veterinary vaccinology, which have been extensively discussed in recent reviews [2], [3]. Instead, this review seeks to provide readers interested in innovative veterinary immunotherapeutic agents, with a concise overview of the performance of toll-like receptors (TLR) agonist-based nanomedicines, newly tested against selected animal diseases. The manufacture of human or veterinary nanomedicines presents several hurdles absent in classical medicines that make their development more complex and deserve to be highlighted in advance. The first is that structural features of nanomedicines must be rationally designed, to provide pharmacodynamics tailored to the needs of each pathology. The second is the challenging production of nanoparticles (NPs) – particularly those made of polymers – on an industrial scale. The third is the fact that NP structures will differ according to the production method employed. In this situation, the performance of the latest experimental formulations of NP-TLR agonists already in the market will be described and discussed together with their structure-function relationship according to their scaling-up feasibility.

Overview of the relevant literature

Nanotechnology in veterinary

The near future will bring a steady rise in the number of global livestock and poultry (predominantly in developing countries such as China, India, and Brazil), and at the same time, a significant increase in the global incidence of zoonotic and food-borne diseases. In addition, the global population growth, estimated to be 8 billion by 2025 and 9 billion people by 2050 [5], is accompanied by new consumer demands for healthy and high-quality food [6]. North America is the greatest region for the veterinary market (lead by the USA); Western Europe and Asia Pacific are second and third. It is estimated that 80% of antibiotics in the USA are used in animals to improve yields and meat quality [7]. A claim for the reduced use of antibiotics and hormones, however, has been included in the recent demands for enhanced public health. Veterinary immunotherapies may satisfy the need for reduced use of antibiotics, by minimizing their environmental impact, side effects, and residues in food chain products, and by improving animal health and productivity (Figure 1) [8], [9], [10].

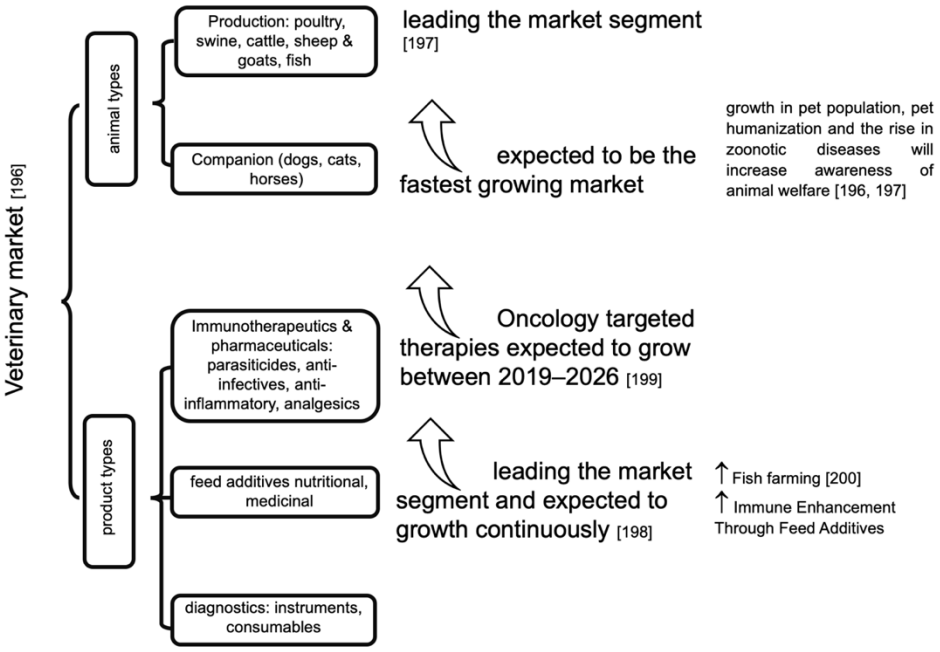

Figure 1. The veterinary market

Finally, despite being commonly administered as a response to infectious diseases [11], and as partly due to changes in societal perceptions on animal welfare, there is an increased interest in vaccines and other immunotherapies for companion animals [10] suffering from cancer (chemotherapy is the currently preferred treatment for pets tumors, notwithstanding its negative effect on the patient's health and their often limited benefits) [12] and allergic disorders. This dynamic new landscape for veterinary medicine is a unique opportunity for the intervention of nanotechnologies [13]. Nanotechnologies have already revolutionised several industrial markets, particularly that of human medicine [14], but are relatively new in veterinary therapeutics and animal production [15]–[17].

Customarily, the veterinary sector of therapeutics has leaned toward pharmaceuticals instead of immunotherapies. This is because of high costs and lower profit margins (the economic returns of veterinary vaccines are nearly 50 folds lower than those for humans) and challenges to clinical veterinary trials [18], [19]. Active or passive immunotherapies are mediated by targeted moieties engaging immune receptors [20]. Not all immunotherapies, however, are equal, and some of them could be better suited than others for veterinary use, a field where cost effectivity is essential [21]. For instance, immunotherapies based on monoclonal antibodies are predicted to be excessively expensive and of uncertain success, because of physiological differences between animal of different gender and species, combined with incomplete knowledge of each immune system (a fact reflected in the poor results obtained in pets) [18], [22]. Moreover, tough nanotechnology may improve the performance of certain active pharmaceutical ingredients (APIs), and antibody-targeted NPs. Besides not meeting the expected success in humans [23], the resulting complex nanoscale structures of difficult characterization and scaling, **are** facts that may hinder their access to the veterinary field. However, immunotherapies performed with molecules less complex and cheaper than antibodies and loaded in NPs May overcome these hurdles. For instance, TLR agonists are small synthetic molecules and short sequences of nucleic acids used to treat either companion or feed animals against pathologies of diverse origin. NP-TLR agonists present advantages compared to free TLR agonists, the most impressive being their higher ability to stimulate innate immunity or elicit antitumoral, antimicrobial, or anti-allergic activities.

Using innate immunity to trigger adaptive immune responses.

Currently, the knowledge gained in the last decade on the mechanisms underlying human innate immune responses has focused the interest of many research groups worldwide on developing new immunotherapies based on TLR agonists. It is acknowledged that innate immunity is the key to control and augment adaptive immune responses [24], [25]. Innate immunity, represented by leucocytes (macrophages and dendritic cells [DC] natural killers [NK]) and complement, is the first line of defence against pathogens. Innate responses are faster (minutes/hours) than the adaptive ones (days/weeks), which are mediated by antibodies and T cells. Innate responses also decay faster, limiting the tissue damage resulting from its potency and non-specificity. One of the main mechanisms to trigger innate immunity is mediated by the engagement of structurally preserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) to pattern-recognition receptors (PRRs) on antigen-presenting cells (APCs). PAMPs are lipo- and peptide glycans, glucans, proteins, and nucleic acids found in bacteria, viruses, parasites, or fungi, specific to the microorganism and considered as "non-self" by the host [26]. DAMPs are self-molecules that include multiple heat-shock proteins, S100, and high-mobility group box 1 (HMGB1) that are released in response to injury or any other anomaly in the cells [27]. There are several types of PRRs, including the cytoplasmic retinoic acid-inducible gene (RIG)-like helicases (RLHs), the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the TLRs. The TLRs are a family of highly conserved PRRs in mammals, and consist of transmembrane proteins located in the cell surface and endosomal compartments [28]–[30] whose activation leads to the release of inflammatory mediators (Figure 2).

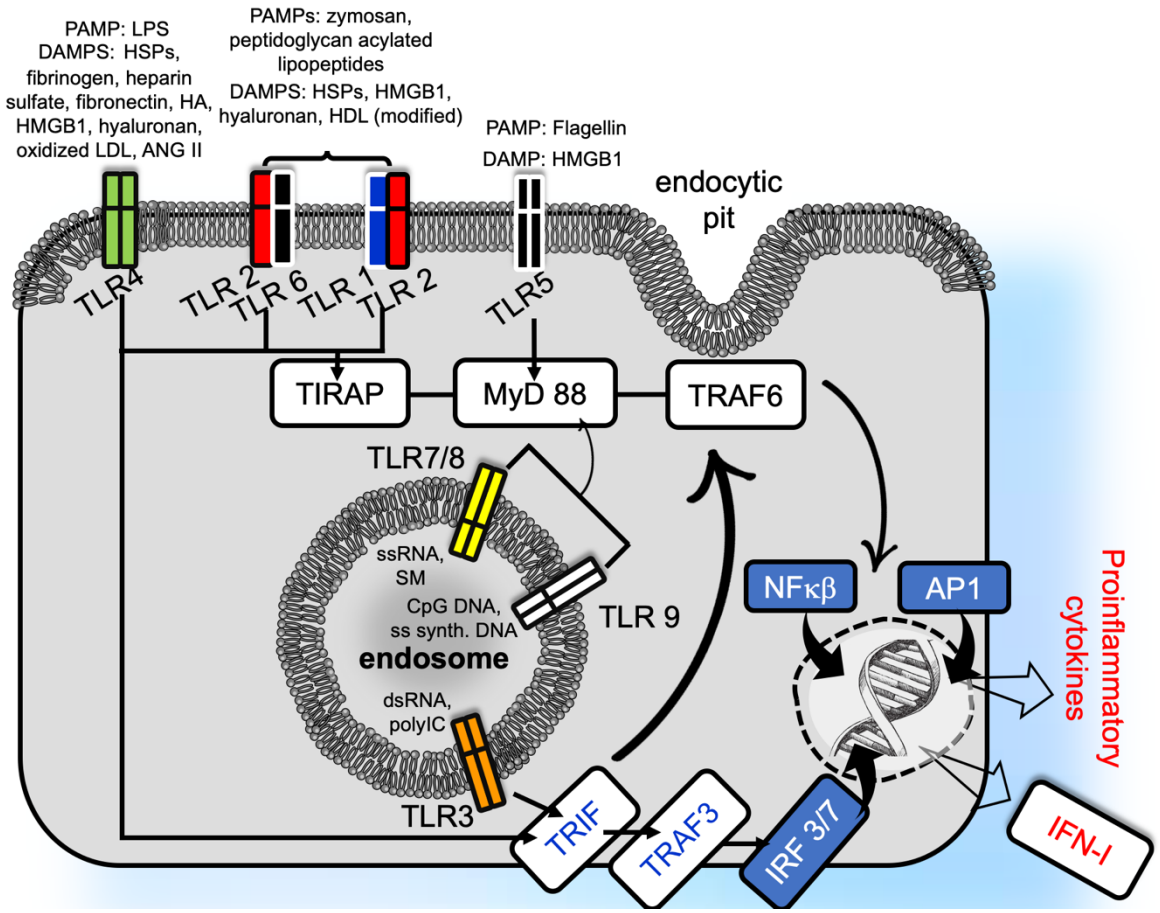

Figure 2. *Simplified scheme of TLR activation pathways. Upon recognition, the activation of TLR triggers downstream signals mediated by its intracellular domain Toll/IL-1 receptor (TIR), enabled by adaptor proteins such as TIR domain-containing adapter-inducing interferon-b (TRIF), myeloid differentiation primary response 88 (MyD88), tumor necrosis factor (TNF) receptor–associated factor (TRAF) 3 or 6 [29]. The downstream signals end up in the translocation of NF-kB and the interferon transcription regulator factor (IRF3/7) to the nucleus, with the subsequent production of proinflammatory cytokines and type I IFN, useful to combat bacterial, viral and parasitic infections [24], [28-30]. Specifically, TLR3, TLR7, TLR8, and TLR9 are endosomal receptors, whose ligand binding domain faces the endosomal lumen and thus, different from surface TLR, would only be activated by endocytosed material [31]. These TLR recognise different classes of bacterial, viral and endogenous nucleic acids: TLR3 is a receptor of double strand RNA (dsRNA), TLR7 and 8 recognize single strand RNA (ssRNAs) and small molecule (SM), imidazoquinolines, whereas TLR9 is activated by unmethylated simple strand DNA (unmethylated ssDNA) [31]*

TLRs are typically expressed in macrophages, neutrophils, lymphocytes, dendritic cells, dermal endothelial cells, mast cells, and mucosal epithelial cells [31]–[33] as well as in tissues associated with the external environment such as the gastrointestinal tract, trachea, and lungs, as well as in organs such as the adrenal glands, testes, thymus, liver, and spleen [34]. The TLRs are the most often studied PRRs in relation with adjuvant, immunostimulatory or immunomodulatory activity [35]. Besides endogenous agonists, many immunostimulants and adjuvants consist mostly of artificial sequences of nucleic acids or small synthetic molecules that mimic PAMPs, and that act as TLR agonists [36]. TLR agonists are known to evocate powerful adaptive cellular immune responses. Many of them are in preclinical development or human clinical trials acting as antitumoral or adjuvants against intracellular pathogens [37]. In humans, TLR3 and TLR7 agonists are considered as the immunotherapeutic agents with the highest potential to boost antitumoral therapies [38].

Systemic toxicity limits parenteral administration of TLR agonists

The ability to trigger an intense pro-inflammatory response upon TLR binding is just one side of the coin of the immunostimulant activity of TLR agonists. The other side, responsible for limiting their broader application, is the dangerousness of their parenteral administration. In

humans, parenteral TLR agonists may cause lethal septic toxic-shock-type reactions and contribute to the dysfunction of remote organs non-physically related to the treated area [39]–[43]. The oldest example is the complete Freund's Adjuvant (CFA), consisting of agonists of TLR2, TLR4, and TLR9, present in heat-killed *Mycobacterium tuberculosis* suspended in non-metabolizable (paraffin and mannide monooleate) oils, and trehalose 6,6' dimycolate (TDM), a stimulant of the macrophage-inducible C-type lectin (Mincle). Injection of water-in-oil (w/o) emulsions of antigens and CFA induces a Th1-dominated response when compared to injection in Incomplete Freund's Adjuvant (IFA), which lacks mycobacterial components and induces a Th2-dominated response [44]. CFA is associated not only with higher antibody responses [45], [46], but also with higher toxicity and delayed-type hypersensitivity reactions compared with IFA [47]. The toxicity of parenteral TLR agonists is known mainly from humans and laboratory animals' data. In experimental animals (rats and Guinea pigs), local (subcutaneous [SC]) or systemic (intraperitoneal) injection of 10–100 μg/kg TLR4 agonist LPS (lipopolysaccharide) or TLR2/6 agonists MALP-2 (macrophage-activating lipopeptide-2) or FSL-1 (fibroblast-stimulating lipopeptide-1), induce fever, anorexia, adipsia, and reduction of volunteer locomotion [48]-[54]. On the other hand, despite the lack of extreme toxicity caused by subcutaneously administered TLR7 and TLR3 agonists such as the imidazoquinoline imiquimod (IMQ) and polyIC [55], the reactions are intense enough in humans to be approved only as a topical formulation for clinical use as occurs with IMQ [56]. SC administration of 5 mg/kg IMQ, induces not only local inflammatory cytokines but also influences distant organs such as the liver and spleen and includes the expression of inflammatory genes in the brain. The toxicity of SC IMQ is ascribed to its diffusion out from the air *pouch* of the SC injection [57].

Moreover, even topical administration of high IMQ doses induces systemic toxicity in humans [58]. In human trials, high doses of parenterally administered polyIC induced poor levels of interferon but had highly toxic effects [59] such as fever and anaemia. Similar doses in dogs generated severe adverse effects [60]. Such toxicities can be reduced by formulating the TLR agonists as nanomedicines like Np-TLR agonists [61].

Advantages of NP-TLR agonists versus free TLR agonists: Lower toxicity and endosomal targeting

Over the last 25 years, the portfolio of human nanomedicines has grown, including accurately characterized products manufactured under good manufacturing practices (GMPs), with improved therapeutic effects and adme-tox profiles than conventional medicines [62]. Pharmacokinetics, biodistribution, and pharmacodynamics of APIs from nanomedicines, are profoundly modified upon loading in ad-hoc-designed NPs. Such NPs also protect the structure of labile APIs against enzymatic or physicochemical insults and eventually provide extensive internalization by selected cells plus targeted delivery to intracellular compartments. These unique abilities of nanomedicines have proven useful in the design of less toxic and more efficient antimicrobial and antitumoral agents [63]. The same key principles may be used to design nanomedicines to prevent and control animal diseases and to make animal rearing more profitable for farmers. In addition, formulated as nanomedicines, injectable APIs can be administered by a topical or mucosal route. Nanomedicines account for a reduced amount of drug needed to get a given clinical effect, making treatments cost-effective and safer results that are convenient for the animal welfare sector [64]–[67].

NP-TLR agonists have met three important goals. The first was limiting the release of agonists from the administration site to the systemic circulation. Loaded into NPs, the agonists are released to their close environment, minimizing the risk of toxic cytokinemia. Relatively heavy and labile agonists such as polyIC are reported to suffer rapid degradation by ubiquitous ribonucleases in plasma and tissues of humans and primates (but not in mice) [68]. The off-target degradation of polyIC may be reduced if loaded in NPs. Besides, off-target, non-TLR-mediated toxicity of TLR agonists, such as the antagonist activity of IMQ on adenosine receptors, may also be reduced by loading IMQ in NPs [69]. Overall, NPs may be either in situ depots of TLR agonists, enhancing their delivery in the intact form to surface TLR or be internalized by neighbour cells. The second goal takes

place upon NP internalization and is the massive endocytic delivery of TLR agonists [70]. Upon NP endocytosis, agonists are delivered to endosomal TLR, magnifying the activity of low doses [71]. The third goal is avoiding the access of TLR agonists to the cytoplasm. This is beneficial for polyIC, which not only activates endosomal TLR3 in DC or macrophages but also to the ubiquitously distributed cytoplasmic receptor MDA5 (Figure 3).

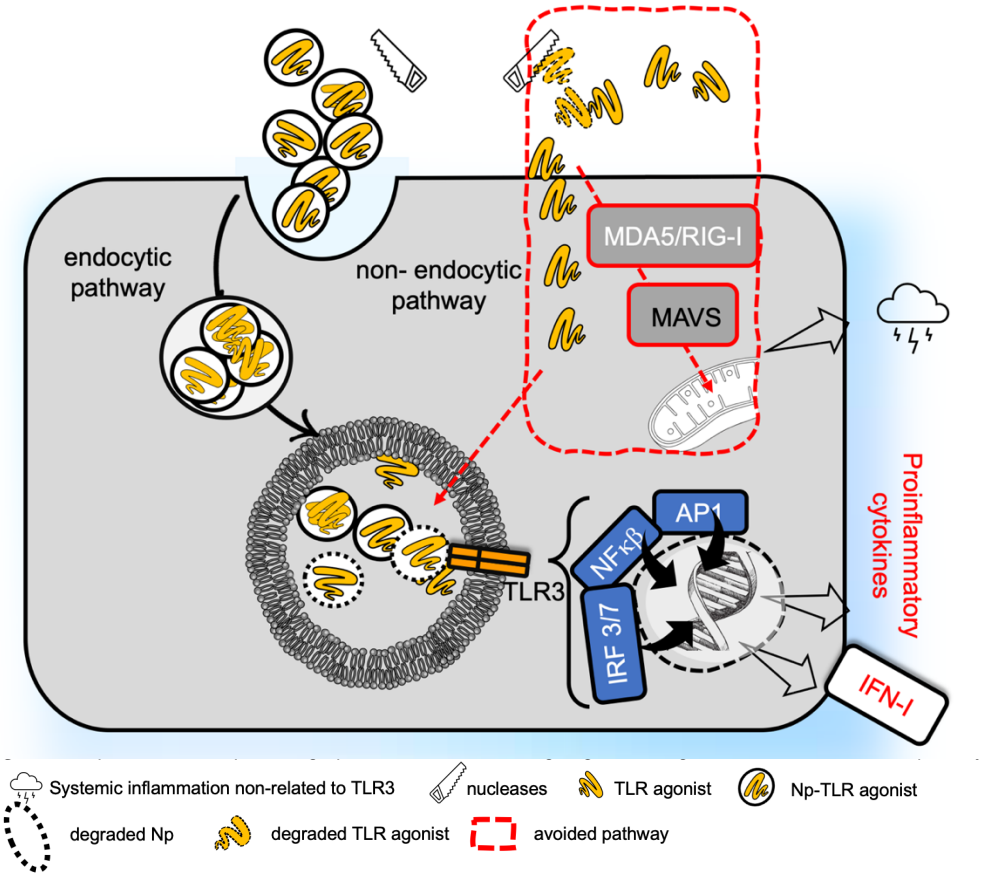

Figure 3. Simplified scheme representing Np-mediated endosomal targeting of TLR3-agonist and avoidance of MAV pathway.

In general, RIG-I and MDA5 engagement converge in the critical adaptor protein mitochondrial antiviral signalling protein responsible for systemic inflammation and cytokinemia observed in viral infections. The cytoplasmic access of polyIC therefore, may cause toxicity like viral dsRNA infections. The immunostimulant in current clinical trials ARNAX is a DNA/RNA hybrid molecule that differs from polyIC in that it does not induce systemic inflammation in mice, upon parenteral administration [72]. The safety of ARNAX is owed to its endocytosis and its subsequent delivery to endosomes to engage the TLR3, which provokes the avoidance of the MAV cytoplasmic route. Loading polyIC in NPs would thus provide exclusive delivery of polyIC to endosomes and avoiding cytoplasmic access. Finally, loading agonists in NPs enables the simultaneous activation of two TLR. For instance, in mouse macrophages, TLR3 and 7 are expressed in the same endosomal compartment [73]. Loading 3 and 7 TLR agonists into the same NPs provides double endosomal targeting. It was recently reported that upon endocytic uptake of phospholipid micelles containing IMQ complexed with polyIC, a synchronic and synergic activation of TLR7 and TLR3 is caused, resulting in potent antitumoral adjuvants in the absence of systemic inflammation [74]. Additional examples of endosomal targeting for TLR 7 and 8 agonists are reviewed elsewhere [75], [76]

Opportunities for Np-TLR agonists in veterinary immunotherapy

Veterinary immunotherapies differ from human treatments in that they must be cost-effective enough, effective in large scale, and applied according to the specific needs of each animal species. In this light, immunotherapies pursue the following goals: (A) To reduce the use of antibiotics. The indiscriminate use of antibiotics is responsible for the emergence of resistant strains of pathogens and has the potential for increased environmental contamination and residual content in meat or eggs [77]–[79]. The global use of antibiotics in livestock production and agriculture (estimated to be 63 000–240 000 metric tons per year) is expected to increase by 67% from 2010 to 2030, especially in emerging economies [80]; (B) To avoid the use of live attenuated vaccines. Live attenuated vaccines, although efficacious and providing mucosal immunity, need cold-chain storage and transportation, present short shelf life, safety concerns (in pregnant animals), and may cause immunosuppression. In addition, wild type viruses may revert to virulence by mutation or genetic recombination [81], [82]; (C) To replace potent adjuvants accompanying inactivate or subunit vaccines. Vaccination with inactivated microorganisms or subunits are safer but induce mainly humoral systemic immunity and fail to recall mucosal response (essential to impair the entrance of pathogens across mucosal surfaces) and fetal protection. Besides, it requires frequent boosters with potent adjuvants, usually based in mineral oils, which are accepted for veterinary use, despite their toxicity (and being suspected of causing deleterious effects). Such adjuvants have to be injected, which makes it difficult to administer to large numbers of small animals such as chickens or fish; (D) To provide cross-serotype protection. In particular, this is important for minor serovars for which live attenuated vaccines are not available and in instances of vaccination against *Salmonella, E. coli*, and viruses.

Since preventive vaccination and immunostimulants have recently been recognized as the best alternative to the use of antibiotics [83] [84] (Figure 4), vaccine adjuvants, and immunostimulants based on NP-TLR agonists are attractive because of their potential to overcome the issues mentioned previously.

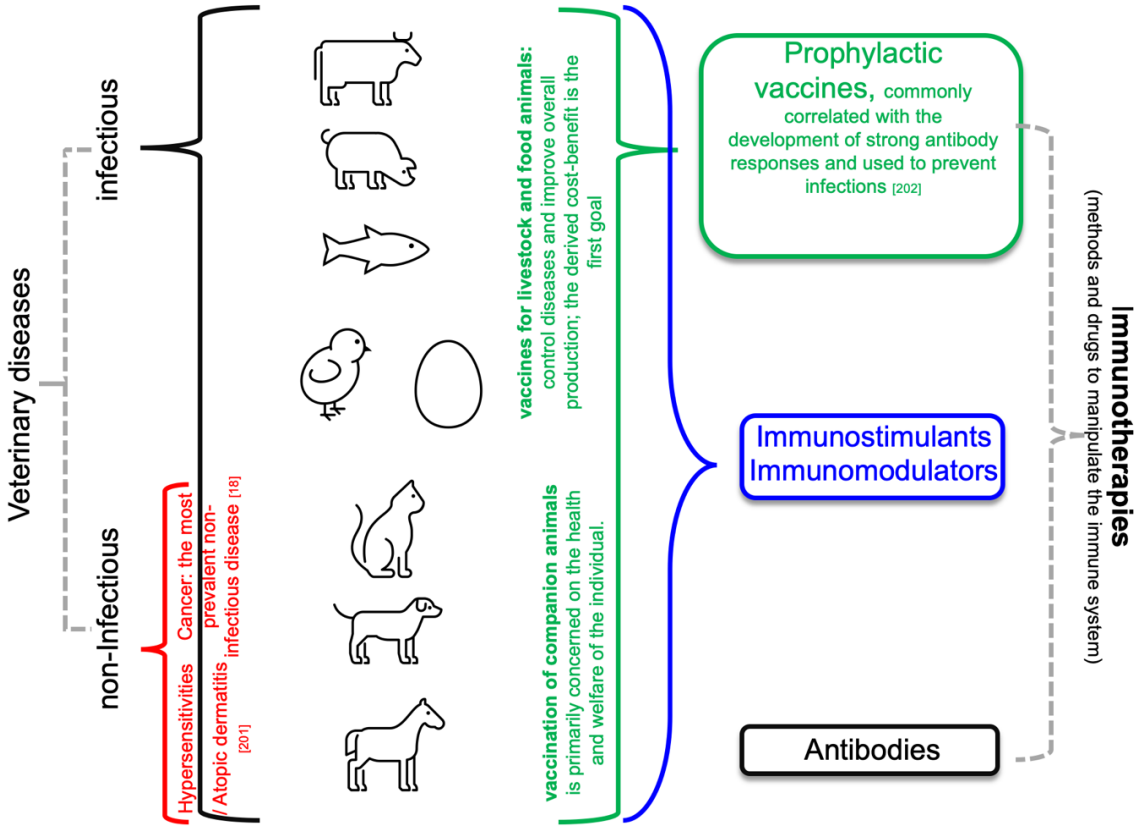

Figure 4. Veterinary diseases vs immunotherapies

As explained in Section 4, NP-TLR agonists magnify the innate immune response in a safe and site-selective fashion. Additionally, by combining mucoadhesivity/mucopenetration with the protection of APIs against physicochemical stress suffered across the gastrointestinal transit, NPs are known to be efficient inducers of mucosal secretory immunity [85]. The same structural protection allows implementing the use of immersion or nebulization routes without losing key structural features needed to trigger immune activation. Finally, the immunostimulation induced by Np-TLR agonists shifts the local cytokine profile from allergenic to pro-inflammatory in the resulting immunomodulatory agents.

We have grouped the performance of NP-TLR3, -TLR4, -TLR5 and -TLR9 agonists as immunostimulants or as vaccine adjuvants, as a function of animal species, each one with different and subtle patterns of TLR expression and affected by different infectious or non-infectious diseases. The structural features of each NP-TLR agonist and treatment scheme are shown in Table1.

In Cattle:

Despite being highly conserved in mammals, different animal species may present different TLR expression patterns [86] as well as different TLR specificity for agonists [87]. The type of microbial ligand recognized by TLR homologs in varied species is generally conserved, although subtle species-specific differences in ligand recognition and signalling exist between TLR orthologs [88]. Homologues of human TLRs 1-10 have been identified both in cattle and sheep [89].

NP-TLR9 agonist (ZelNate®) as immunostimulant against bovine respiratory disease (BRD):

BRD is caused by different etiological agents and despite existent vaccination, antimicrobial drugs, environmental and management strategies, BRD is a leading cause of morbidity and death in the cattle industry worldwide. Stressful events are recognized as the primary insult leading to the eventual compromise of the animal's pulmonary immune status. In such situations, the commensal bacteria of the upper respiratory tract *Mannheimia haemolytica* invades and infects the lower respiratory system inducing lung lesions and inflammation, a substantial reduction in average daily gain compared to cattle with healthy lungs and possibly death. Protection against *M haemolytica* is mediated by antibodies. In 2015, the first commercial immunostimulant veterinary nanomedicine Zelnate® [90] (Bayer HealthCare, MO, USA) was introduced to the market. Zelnate® is aimed to reduce the use of antibiotics against BRD, and is indicated in 4-month-old cattle or older when administered at the time of or within 24 hours after a perceived stressful event [90]. Zelnate® consists of lyophilized cationic liposomes (DOTIM/cholesterol) loaded with bacterial plasmid DNA rich in non-methylated CpG motifs. Field studies demonstrated that intramuscularly (IM)-administered Zelnate® was equally as effective as the currently approved SC antibiotic Tilmicosin for the control of BRD in feedlot cattle in medium risk conditions [91]. Zelnate® constitutes a potent inductor of type I INF via IRF3 cGAS and STING pathways, resulting in lung anti-inflammatory action, mitigation of lung lesions, and decreased mortality in cattle exposed to *M. haemolytica* [92].

NP-TLR3 or 9 agonists as vaccine adjuvant against foot and mouth disease (FMD):

FMD is caused by an aphthovirus that generates high fever, blisters, and lameness. FMD is endemic in many countries of the Middle East, Africa, Asia, and parts of South America. The antigenic diversity of FMD is a major concern, and current vaccines do not protect against different strains of the same viral serotype unless strong adjuvants are used [93]. Protection against FMD is mostly mediated by antibodies. Current vaccinations confer protection, but subclinical colonization in the pharynx is not avoided allowing for the spreading of diseased carriers. Adjuvants providing long-term humoral and cellular protection are fundamentally required [94]. In 2014, a first report on the efficacy of polyIC or CpG plus virus antigens associated to VacciMax®, a vaccine delivery platform made of liposomes formulated in a water-in-oil emulsion developed by ImmunoVaccine Technologies Inc (not approved for veterinary or human use yet) against FMD, carried out in India was published [95]. TLR agonists- VacciMax®, formulations can be prepared by mixing an equal volume of aqueous-phase-containing liposomes and different oil phases such as IFA

[96], Montanide™ ISA51 (adjuvants consistent of water-in-mineral-oil emulsion), [97] or mineral oil-mannide oleate (8.5:1.5 vol/vol) [98]. The report, despite not specifying the nature of the used oil emulsion, showed that administered either by IM or SC routes to bull calves, TLR agonists VacciMax®, induced protective immunity against FMD. PolyIC-Vaccimax induced higher antibody titres and longer protective immunity than CpG-Vaccimax. After a first dose that induced a slow response, one boost produced a faster 100% protective response compared to the 75% protection response induced by a Montanide™ ISA206 adjuvanted commercial vaccine. No further studies were published in this area, despite finding out that NP-TLR9 agonists may provide faster and longer responses than conventional vaccination against FMD.

NP-TLR3 agonist as vaccine adjuvant against Mycobacterium avium subsp. paratuberculosis (MAP)

Aerosol inhaled MAP is the causative agent of the worldwide spread of Johne's disease, which affects cattle and sheep [99]. The resultant chronic inflammation of the intestinal tract is responsible for decreased milk production, premature culling, and reduced carcass value, leading to considerable economic losses to the livestock and associated industries. Vaccination is not widely used because of the risk of interference with intradermal testing for bovine tuberculosis. Besides safety concerns, vaccination induces limited protection in young animals and reduces losses, but not the incidence of the disease. The host defence against intracellular mycobacterial pathogens is mounted on CD4 T cells that ends up on the induction of IFNγ and bactericidal activity in macrophages. Cationic adjuvant liposomes (CAFs) developed by the Statens Serum Institute (Copenhagen, Denmark) combine DDA (dimethyl-dioctadecylammonium) with different immunostimulants, that induced minimal side effects and good immune responses in human clinical trials [100]. CAF01, for instance, contains the synthetic mycobacterial TDB (α, α′ trehalose dibehenate), an agonist of the Mincle surface receptor that activates APC through the TLR-independent Syk-CARD9 pathway [101]. In CAF09, TDB is replaced by the much better stimulator and easier to manufacture monomycoloyl glycerol (MMG)-1, a synthetic analogue of a mycobacterial cell-wall lipid that signals via the Mincle receptor [102]. A recent study showed that SC vaccination of calves with polyIC electrostatically adsorbed to the surface of CAF09 along with MAP recombinant proteins (polyIC-Map-CAF09) induced higher cell-mediated and humoral immune response than with CAF01 [103]. Despite needing further protection assessment against a challenge, the repeated immunization with polyIC-MAP-CAF09 maintained a higher frequency of CD4+ T cells inducing IFN-γ, TNF-α, and IL-2 than Montanide™ ISA 61 VG adjuvanted MAP vaccination [103].

Interestingly, intraperitoneal (IP) or nasal vaccination of mice with CAF09 is observed to induce higher CD8+ T-cell response than upon SC or IM routes [102]. The oral route for cattle immunization, however, is less advantageous than for other non-ruminant animal species because of antigen degradation in the rumen before reaching the intestines. In general terms, the production of antibodies tends to be transient since antibodies do not persist on the mucosal surface in the absence of a booster. An additional issue is the difficult assessment of protection against a lethal experimental challenge that differs from natural infection [104].

NP-TLR9 agonist as vaccine adjuvant against bovine mastitis

Bovine mastitis is a potentially fatal mammary gland inflammatory infection mostly caused by coliforms, streptococci and *Staphylococcus aureus* and is responsible for the greatest economic losses for dairy farmers and industry worldwide [105]. Diverse pathogens that cause mastitis induce different immune responses in the mammary gland, and therefore, the host requires highly specific pathogen-dependent responses for protection on the bases of serum IgG2 and in the udder as well as the activity of polymorphonuclear cells. Mastitis management is costly and includes antibiotic therapy, culling of chronically affected cows, post-milking teat disinfection, as well as ensuring routine maintenance of milking machines. The few worldwide commercially available vaccines based on lysates or inactivated organisms adjuvanted with mineral oil have shown limited efficacy in preventing intramammary infections [106], [107]. A recent study suggests that NP-TLR9 agonist-mediated adjuvancy

may induce earlier responses, needed to protect the animals during the critical period of challenge. Seeking to prevent bovine intramammary infections before the beginning of lactation, two recombinant antigens of *S. aureus* adjuvanted with CpG mixed with cationic liposomes (CpG-cationic liposomes) were recently reported for vaccination against bovine mastitis caused by *S. aureus* [108]. A humoral immune response similar to that of traditional $Al(OH)_3$ used as a control adjuvant was recalled, excepting that CpG-cationic liposomes and not $Al(OH)_3$ induced IgG2 specific responses for both recombinant proteins. Vaccination with CpG-cationic liposomes also stimulated the highest antibody levels in milk 30 and 45 days after a pre-calving booster. A single booster 21 days before parturition was enough to quickly increase specific IgG levels in the serum during a period of high susceptibility to intramammary infections.

NP-TLR3 agonist as vaccine adjuvant against bovine viral diarrhea (BVD):

Pestivirus induced BVD is the most prevalent infectious disease of cattle. BVD is endemic around the world and has a profound impact on the cattle industry because of production and reproductive losses. The disease presents different clinical outcomes, including reproductive and immunosuppressive effects. Live attenuated and inactivated vaccines are used to control BVD. Both types of vaccines present concerns regarding potential reversion and the use of powerful adjuvants, such as Procision-A™, an adjuvant made of Quil A, cholesterol, amphigen base, and liquid paraffin present in PregSure® BVD (Pfizer, Karlsruhe, Germany). Such an adjuvant was suspected of playing a role in the induction of alloantibodies transmitted via colostrum to the calves and was retracted from the market in 2010 [109]. A vaccine made of two antigens of BVD virus (structural envelope glycoprotein E2 and NS3 protein) and adjuvanted with polyIC loaded within poly(lactic-co-glycolic acid, PLGA) NPs, was recently tested against cattle BVD [110]. E2 was adsorbed on the surface, and NS3 was encapsulated in the NPs core of the polyIC-PLGA NPs, a spatial arrangement claimed to induce both B- and T-cell responses. The protection induced by polyIC-PLGA NPs was comparable to that of a commercial inactivated BVD virus vaccine employing Quil A as an adjuvant. The results suggest that polyIC-PLGA NPs could replace powerful toxic adjuvants, and that helped to induce specific responses.

NP-TLR9 agonist as vaccine adjuvant against Trueperella pyogenes:

T. pyogenes is part of the biota of skin and mucous membranes of the upper respiratory and urogenital tracts of animals, and also an opportunistic pathogen that can cause mastitis, liver abscesses, and pneumonia. There is no vaccine available, and the current antibiotic therapy may induce drug-resistant isolates constituting a potential threat to veterinary health. A Th1 type immune response is needed to contain *T. pyogenes* infection. Recently a vaccine made of a DNA construct including the nucleic acid sequences of various virulence factors of *T. pyogenes,* adjuvanted with CpG and loaded in chitosan NPs (CpG-DNA-chi NPs) was tested on mice against *T. pyogenes* [111]. CpG-DNA-chi NPs markedly increased the synthesis and release of IFN-γ, IL-2, and IL-4, inducing strong humoral and cellular immune responses that protected mice against a challenge with the highly virulent *T. pyogenes* TP7. The efficacy of this strategy in cattle remains to be determined.

In Chickens:

Chicken TLR21 (chTLR21) is absent in the human species but has homologs in fish and frogs and displays similarity with mouse TLR13. chTLR21 activates NF-kB in response to unmethylated CpG DNA, typically recognized by mammalian TLR9 [88].

NP-chTLR21 agonist (Victrio®) as in ovo immunostimulant against E.coli:

Egg transmission of *E. coli* is common and can lead to omphalitis, colibacillosis, coliseptic-emia, or even death in chickens. Mortality oscillates between 5–20% with more severe infections occurring in young chicks. Current vaccines and antibiotics against *E coli* are not effective. Displaying the same nanostructure, Zelnate™, Victrio® [112] was recently launched as *in ovo* immunostimulant *against E. coli* in embryonated eggs and new-born chicks to reduce mortality and the use of antibiotics as a priority treatment. In the current broiler chicken industry, birds can reach a 2-kg body

weight by 30–35 days post-hatch. *In ovo* immunotherapies may provide good health during the first week of broiler chicken growth, supplying better performance and profitability alternatives to antibiotics. *In ovo* injected Victrio® (18-day-old embryonated eggs) one day prior to *E. coli* challenge (19-day-old embryonated eggs), showed significantly lower mortality (6.6%) than in the non-treated group (17.2%) and in the group treated with the commercial antibiotic gentamicin sulphate (8%) [113].

Np-chTLR21 agonist as immunostimulant against E coli:

The safety and immunostimulant effects of CpG formulated with single-wall carbon nanotubes (SWCNTs) or liposomes containing gemini surfactant (LG) were recently determined against *E. coli* septicemia in neonatal broilers [114]. Gemini surfactants contain two hydrophobic tails (12 C) and two quaternary ammonium groups as polar head groups linked by a spacer group (3 C), that depending on molecular modifications, can self-assemble into a range of structures from micellar to inverted micellar or bilayer structures [115]. Birds receiving both CpG formulations showed a significantly higher survival rate (60–80%) compared to the saline control group (20–30%). Bacterial loads and clinical scores were significantly lower in groups treated with CpG-SWCNTs or -LG compared to the groups receiving free CpG. Despite data on SWCNT biodistribution (worth to be known in animals destined to human consumption) was lacking, no evidence of any adverse effects of these formulations was found in the organ growth rates of birds until 42 days of age.

Np-chTLR21 agonist as nebulized immunostimulant against E coli:

The immunostimulant effect of CpG- gemini lipid-coated NPs, formulated into the bio adhesive polymer polyvinylpyrrolidone (CpG-PVPG NP) and administered by compressor nebulizer, was recently determined in neonatal chicks [116]. Nebulization is a cost-effective immunization method in poultry because of its easily accessible, highly vascularised, and highly permeable lung mucosa. Besides, needle-free is safer than parenteral vaccination due to decreased risk of contamination from infected needles and potential irritation from injections [117]. Nebulized droplets smaller than 3 μm, can bypass the mucociliary transport and reach deep lung sites to access the BALT (bronchus-associated lymphoid tissue) at the junctions of the primary and caudal secondary bronchi in the avian lung, aiding in immune stimulation and lymphocyte recruitment [118]. Upon nebulization, the whole structure of CpG-PVPG NPs was found in the trachea, tracheal bifurcation, and diffusing through the connective lung tissue, and was available to trigger a response at the site of infection. Overall, nebulized CpG-PVPG NPs induced improved protection against *E. coli* compared to naked CpG when challenged on day 2 or 3 post application but showed similar low protection when the challenge was performed on day 4 [116]. Such a transient response would require repeated doses to protect against real challenges.

NP-chTLR21 agonist as IM nebulized vaccine adjuvant against avian influenza (AI):

The mucosal pathogen orthomyxovirus type A, AI virus (AIV), causes respiratory symptoms and systemic disease (multiorgan failure), a high percentage of morbidity and mortality, and is associated with significant economic losses worldwide due to mortality, morbidity, culling of birds, lost trade markets [119], and public health implications [120]. Vaccination is used to help control AIV and limit losses in areas where the virus is endemic. Most of the current vaccines, however, are inactivated whole viruses adjuvanted with oil emulsions [121]. Such vaccines are considered safe, and relatively inexpensive for poultry, particularly in areas where labor costs are low. Individual inoculation can ensure a higher coverage within the vaccinated population but has to be applied to each bird individually and is not very capable of inducing local, mucosal antibodies [122]. Recently, two studies reported the effect of vaccination with IM or *jet*-nebulized CpG-PLGA NPs plus inactivated H9N2 AIV against AIV on chickens [123], [124]. The first study revealed that IM-administered CpG-PLGA NPs plus free H9N2 AIV, reduced virus dissemination by inducing higher systemic and mucosal antibodies as well as hemagglutination inhibition antibody titres, compared to CpG-PLGA NPs containing encapsulated H9N2AIV [123]. In the following assay, CpG-PLGA NPs plus free AIV was nebulized, to find the induction of higher mucosal responses than IM CpG-PLGA NPs plus free AIV, or than nebulized or IM-free

CpG plus free AIV [124]. The results showed a higher efficacy of nebulized nanomedicines to induce mucosal immunity than if intramuscularly administered. The effectivity of this approach against a challenge remains to be tested.

NP-TLR 5 agonists as vaccine adjuvant against AI:

Salmonella enterica serovar Typhimurium flagellin, linked to Self-Assembling Protein Nanoparticles (SAPN) (flagellin-SAPN) was tested as vaccine adjuvant against AIV [125]. SAPN are NPs made of self-assembled protein monomers. Each SAPN monomer contains two coiled-coil oligomerization domains held together by a linker. On both the N and C terminus of the monomer, antigens can be liked with conserved native conformation [126]. In the study, flagellin-SAPN (24 self-assembled monomers in octahedral symmetry) was linked to two conserved influenza antigens, M2e and Helix C (M2e/Helix C-flagellin-SAPN) and intramuscularly administered to chickens. M2e/Helix C-flagellin-SAPN induced significantly higher titres of neutralizing antibodies in comparison to flagellin lacking SAPN. Tough induction of mucosal immunity was not measured, and it was observed that M2e/Helix C-flagellin-SAPN protected mice against a lethal AIV challenge, to the same extent than a commercial inactivated virus human vaccine, but with a higher degree of cross-neutralization activity. The effectivity of this approach against challenged chickens remains to be tested.

NP-TLR5 agonist as vaccine adjuvant against infectious bronchitis virus (IBV):

IB is caused by an inhaled coronavirus, that exhibits multiple serotypes in constant change. IB is a highly contagious avian disease that brings significant economic losses due to decreased weight gain and poor egg quality and production. Both live attenuated and inactivated virus vaccines for IBV present limitations either of safety or weak protection against a limited number of serotypes. Broader protection and a safe and rapid response to outbreaks of new strains of viruses are needed. The protection should be induced within one week after application, preferably by a mass application method, that could be administered at any age, including *in ovo*. Recent work showed that flagellin-SAPN adjuvanted vaccines administered by a parenteral route might induce broad protection against different IBV strains [127]. Flagellin-SAPN linked to a highly conserved antigenic sequence of the spike glycoprotein S from the M2118 strain of IBV in its native trimeric conformation and known to induce cross-protection against various serotypes (IBV- flagellin-SAPN), was IM-administered to chickens challenged with M41 strain of IBV. While mucosal immunity was not measured, a significant reduction of tracheal virus shedding and lesser tracheal lesion scores than those immunized with buffer or flagellin-SAPN alone were reported together with a high systemic antibody response [127].

NP-TLR5 agonist as vaccine adjuvant against Salmonella:

Salmonella enterica serovar *enteritidis* is the most common infectious agent causing animal and human salmonellosis [128]. Importantly, *S. enteritidis*-infected poultry meat and contaminated eggs, are primary sources of human salmonellosis [129]. Salmonellosis reduces egg production in chickens and is responsible for economic losses in the poultry industry [130]. *S. enterica* resistant to clinically important antibiotics causes at least 100 000 food-borne human infections per year [131]. Vaccines only marginally decrease rather than eliminate *Salmonella* colonization and shedding in the chicken intestine [132]. Furthermore, commercially available killed *Salmonella* vaccines must be parenterally injected into each bird making it difficult for farmers and highly stressful to chickens. An oral vaccine against *Salmonella* inducing robust mucosal IgA and cell-mediated immune responses should be an effective control approach for poultry salmonellosis. Recently, the efficacy of mucoadhesive vaccine containing immunogenic *Salmonella* outer membrane proteins (OMPs) adjuvanted with surface adsorbed flagellin-polyanhydride NPs (f-OMP-OH NPs), was tested against *Salmonella* on chickens [133]. Chickens orally immunized with f-OMPs-OH NPs showed higher OMP-specific IgG response and serum IFN-γ, enhanced CD8+ /CD4+ cell ratio in the spleen and increased OMP-specific lymphocyte proliferation than the group receiving soluble antigens. Additionally, f-OMPs-OH NPs were better targeted to chicken immune cells in peripheral blood and splenocytes and intestinal Peyer's patch sites, than NPs without surface

coated flagellin. Importantly, the vaccine cleared *Salmonella* cecal colonization in 33% of challenged chickens. In a further work, chickens were orally immunized with cationic chitosan NPs, electrostatically complexed with negatively charged OMPs and flagellin (f-OMPs-Chi NPs) [134]. f-OMPs-Chi NPs were found to adhere to the mucosa, enter in the lamina propria and Peyer´s patch sites of the ileum, and displayed significantly higher OMPs specific mucosal IgA production and lymphocyte proliferation response, compared to groups vaccinated with a mixture of OMPs plus flagellin and soluble antigens. Vaccination also increased the expression of TLR2, TLR4, IFN-γ, TGF-ß, and IL-4 mRNA expression in chicken cecal tonsils. The response on challenged chickens remains to be determined.

NP-chTLR21 agonist as immunostimulant against Salmonella enterica serovar typhimurium:

Recently, low doses of CpG adsorbed to multi-walled carbon nanotubes (MWCNTs) via 1-pyrenebutanoic acid, succinimidyl ester (PySE) linker (CpG-MWCNTs), were used as immunostimulants against lethal septicemia caused by *S enterica typhimurium* on chickens [135]. CpG-MWCNT provided extensive intracellular delivery of CpG to chickens' cells, compared to free CpG, and were equally effective in cell priming at a 1000-fold lower concentration than free CpG. Such high intracellular uptake of MWCNTs may play a role in the magnification of the immune response induced by CpG since significantly small doses of subcutaneously injected CpG-MWCNTs were able to attract immune cells lymphocytes and macrophages. Administered 1 day after birth, it induced survival against those challenged day 5 with SC injected *Salmonella*.

NP-chTLR21 as an oral vaccine adjuvant against Campylobacter jejuni:

C. jejuni is a major cause of bacterial food-borne illness in humans, although chickens are usually not clinically affected by its colonization. The risk of human campylobacteriosis, thus, can be reduced by decreasing the *C. jejuni* load in poultry products and the intestinal *C. jejuni* burden in poultry flocks. From 2005, broiler meat contaminated with *C. jejuni* has been the most reported food-borne infection-causing human bacterial gastroenteritis in Europe [136]. IgA is critical to avoid the infection, but no commercial vaccines to induce cross-protection against *C. jejuni* are yet available [137]. A recent study showed that CpG-PLGA-NPs co-administered with *C. jejuni* lysate by oral route to chickens, synergistically reduced *C. jejuni* cecal colony-forming unit counts better than CpG-PLGA NPs or *C. jejuni* lysate alone, in layer and broiler chicken. [138].

NP-TLR4 or chTLR21 agonists as in ovo immunostimulant against Marek's disease virus (MDV):

MDV infects chickens via the respiratory route and causes Marek's disease, characterized by T-cell lymphoma and immunosuppression. Half of the countries in the world have reported cases of MD. Current vaccines prevent the disease but not the transmission of infection, leading to the emergence of increasingly virulent strains. During the first days of life, chickens are exposed to MDV, an increased innate response during the neonatal period before maturation of the adaptive immune response is therefore required to achieve protection. Recently, LPS or CpG encapsulated into PLGA NPs (LPS- or CpG-PLGA NPs) were tested as *in ovo* immunostimulants against MDV. In chicken embryos, free and encapsulated LPS- or CpG-PLGA NPs were observed to induce similar immune responses (including the induction of IFN-γ, IFNα, IL-1β, and IL-10 in spleen, lungs, and bursa of Fabricius) than in mature chickens. It was also observed that the expression of cytokine genes was different and tissue-dependent, either for LPS- or CpG-PLGA NPs, with no deleterious effects on embryo viability [139]. In a further report, LPS- or CpG-PLGA NPs administered *in ovo* and to post-hatch chickens, was shown to protect against MDV. The reduction of tumour incidence in the CpG-PLGA NP-treated groups (60% vs. control and 42% LPS-PLGA NPs) was associated with the upregulation of IL-18 and IL-1β [140].

In Pigs:

The cell-specific pattern of TLR9 expression varies between species, and these differences might be involved in determining variability in species-specific immune responses to infection [141]. In horses and pigs but not in humans and mice, TLR9 is expressed in pulmonary intravascular macrophages (PIMs) [142], [143].

Such differences in TLR9 expression make it difficult to extrapolate between research findings in laboratory rodents or humans and responses in production animal species [144].

NP-TLR9 agonist as intranasal adjuvant against swine influenza:

Pigs are considered as the "mixing vessel" for the generation of novel influenza A virus reassortants of zoonotic and pandemic potential. Swine influenza A virus (SIV) is a constant threat in the global pig industry and one of the most prevalent respiratory pathogens in swine worldwide. SIV causes the sudden onset of highly contagious broncho-interstitial pneumonia, resulting in a substantial economic burden to producers. Available vaccines are monovalent or multivalent whole inactivated SIV for IM administration, that does not protect against unrelated heterologous influenza viruses and induces inefficient cell-mediated immunity and mucosal antibody responses [145], [146]. Further, vaccine-associated enhanced respiratory disease (VAERD) and maternal antibody interference in piglets, are linked to adjuvants containing mineral oils such as emulsigen D and amphigen that accompany inactivated vaccines [147], [148]. Thus, there is a need for vaccines providing effective mucosal and cellular immune responses that override maternal antibodies, protect pigs against broad-spectrum of SIV, and that avoid the induction of VAERD. Compared to oral delivery, the nasal route offers a larger and highly vascularized surface mucosa with reduced chances of enzymatic and chemical degradation of the up-taken antigen. Vaccination by the nasal route presents antigens to the immune cells like natural infection, eliciting local mucosal IgA antibodies as the first line of defence. Recently, CpG co-encapsulated with soluble antigens of the H1N2OH10 influenza virus into polyanhydride NPs (CpG-H1N2OH10 OH NPs), was tested as an intranasal vaccine against SIV [149]. CpG-H1N2OH10-OH NPs induced higher mucosal IgA antibodies than five-times higher amounts of antigens alone. It also induced enhanced cell-mediated immune response, generating better lymphoproliferative response and higher IFN-γ secretion. Upon challenged with a virulent heterologous virus (H1N1OH7), CpG-H1N2OH10-OH NPs provided better protective efficacy, through a significant reduction in influenza-induced fever, a 16-fold reduction of nasal virus shedding, and an 80-fold reduction in lung virus titres compared to pigs immunized with a five-fold higher amounts of antigens alone.

In Fish:

Fish TLRs have high structural similarity to the mammalian TLR system, but also exhibit specific characteristics and large diversity. For instance, double-stranded (ds)-RNA of viruses are recognised both by endosomal TLR3 (that responds to polyIC producing IFN) of fish [150] and by TLR22, another endosomal TLR. Additionally, LPS recognition and sensitivity in fish are different from mammals [151]. TLR4 was lost from the genomes of most fish; the TLR4 genes found in zebrafish do not recognize the mammalian agonist LPS and are likely paralogous and not orthologous to mammalian TLR4 genes [152], [153].

On the other hand, because of their life in an aquatic environment, smaller size (compared to mammals), and a higher number, fish vaccination presents peculiar features and challenges absent in larger animals. For instance, fish have large mucosal surfaces (skin, gills, gut, and nasal mucosae); mucosal vaccination, performed either by immersion or by the oral route, results in more practical and affordable outcomes than injection. Immersion vaccination involves immersion of fish in water containing vaccine antigens. It can be performed by dip, which is rapid, as the fish are immersed in water containing a relatively high dose of vaccine antigen(s) for one or several minutes, or by bath where the fish receives a more diluted vaccine antigen preparation for a more extended period [154]. Most of the commercially available vaccines against fish viruses, however, are IP injected [155]. The development of the more convenient mucosal vaccines is handicapped by the lack of effective adjuvants and basic knowledge on the immune response of fish. A drawback of vaccination by immersion is related to its poor efficiency to elicit effective local and systemic immune responses since the vaccine structure needs to be protected against degradation before reaching the sites where immune induction occurs. Other inconveniences are the challenges associated with the production of massive quantities of antigens and the potential induction of tolerance. Nonetheless, vaccination by immersion is preferable to parenteral route,

which is more efficient, but is time-consuming, labour-intensive, and may be stressful for the handled fish [156]. Injections are problematic in small fish, and fish as small as 0.5 g may be immersion vaccinated when they are considered adaptively immunocompetent.

NP-TLR3 agonists as vaccine adjuvant against viral hemorrhagic septicemia (VHS):

VHS is a systemic infection of several salmonids, marine and freshwater fish. It causes lethargy, darkening of the skin, exophthalmia, anemia, hemorrhages, abnormal swimming, and a rapid onset of mortality. There are no commercial vaccines or antiviral treatments against VHS available yet. A recent study reported the effect of parenteral vaccination with recombinant glycoprotein G antigens or with inactivated whole hemorrhagic virus adjuvanted with polyIC loaded in chitosan NPs (polyIC-Chi-NP), on a zebrafish model of VHS infection. Upon IP injection, most of the polyIC-Chi-NP remained at the administration site, where nearly ~ 50% of its structure was degraded after 7 days. Vaccination induced the survival of treated animals, indicating the induction of protection against VHS [157]. These promising results require testing of challenges in relevant fish species.

NP-TLR3 agonist and LPS as mucosal immunostimulant against Pseudomonas aeruginosa (PAO1) and Aeromonas hydrophila:

PAO1 and *A. hydrophila* infections cause symptoms similar to VHS: hemorrhages, ulcers, fin and tail rot, "mouth fungus," "saddleback lesions," ascites, exophthalmia, and colour changes. Fish frequently may appear lethargic and inappetent [158] In humans, *A. hydrophila* mainly causes gastroenteritis, septicaemia, and tissue infections. Because of the high costs of injectable fish vaccines, in some developing countries, the use of antibiotics over preventative measures against bacteria is preferred [159]. In such settings, improved vaccines that could efficiently induce mucosal immunity would significantly reduce the need for antibiotics against *PAO1* and *A. hydrophila*. Recently, LPS and polyIC co-loaded into cationic liposomes (LPS-polyIC-liposomes) were tested as parenteral or mucosal immunostimulants against otherwise lethal PAO1, *A. hydrophila* and spring viraemia of carp virus infections in zebrafish [160] [161]. Upon IP injection in adult rainbow trout, LPS-polyIC-liposomes were internalized by macrophages and accumulated in immune relevant tissues (spleen and head kidney). LPS-polyIC-liposomes were also administered to zebrafish by the IP route and by bath immersion. Administered by the two routes, the liposomes were found in the liver; administered by IP injection or immersion, liposomes were found in the spleen and intestine, respectively. In addition, those animals immunized by the IP route were protected against lethal challenges of PAO1 or spring viraemia of carp virus, while no protection was induced by empty liposomes or free LPS and polyIC used as controls. Fish immunized by bath immersion were protected against a lethal challenge of spring viraemia of carp virus [160]. A subsequent work reported the biodistribution of LPS-polyIC-liposomes and expression of innate immune-related genes, in zebrafish larvae immersion vaccinated with LPS-polyIC-liposomes and submitted to a lethal challenge of *A. hydrophila*. Liposomes were found in the intestine and the expression of TNFα, IL1β, nos2a, irf1a gig2e [markers of an immune response to viral infection] and ptgs2a [proinflammatory]) was upregulated, together with higher survival compared to those vaccinated with empty liposomes, LPS, and polyIC alone [161]. Zebrafish larvae are important fish models since their innate immune system is active by the first day of embryogenesis, and their body is transparent at the early stage or can be depigmented, allowing real-time visualization. Larvae are easy to breed and handle, and there is a rising number of markers for immune cells and transgenic lines allowing further identification of the cell types involved in the immunisation process [162]. Despite promising results, further field tests in fish other than zebrafish are required.

In Horses:

Horses are highly susceptible to LPS-induced cardiopulmonary distress and lung inflammation [163]. Such sensitivity has been ascribed to the expression of TLR2, TLR4 [164], in particular of TLR4 in pulmonary intravascular macrophages (PIMs) [164]. Additionally, TLR9 is expressed in a wide variety of cells, particularly in PIMs (a major source of TLR9 in the equine lung), alveolar macrophages, bronchial epithelial cells, and type-II cells among others. TLR9 mRNA expression is increased upon LPS treatment [143].

NP-TLR9 agonist as a nebulized immunomodulator against equine asthma:

Equine asthma comprises inflammatory diseases of the lower airways including inflammatory airway disease, recurrent airway obstruction (RAO), and summer pasture-associated obstructive airway disease, caused by allergic triggers such as organic and inorganic substances from hay dust and straw. Up to 80% of horses in different populations may suffer from mild to moderate asthma, while 11–17% may acquire severe asthma [165]. Equine asthma can have a significant impact on a horse's performance and quality of life. Over time, severely asthmatic horses may develop changes in the lung, which make it difficult to breathe, even at rest. The avoidance of allergens currently constitutes the most important therapeutic measure, together with symptomatic therapy with glucocorticoids and bronchodilators. As absolute avoidance of allergens is difficult to impossible, and treatment with long-term medications may be associated with adverse effects, there is a great need for new therapeutic concepts. In such sense, inhaled therapeutics are reaching the market as shown by the approval in 2020 of the first inhalation therapy based on the corticosteroid prodrug ciclesonide for severe equine asthma by Boehringer Ingelheim, aimed to reduce lower airway inflammation. A recent publication showed that CpG electrostatically bound to cationized gelatin NP surface (CpG-gNP) induced regulatory anti-inflammatory and anti-allergic cytokine IL-10 expression and significantly reduced clinical symptoms of RAO upon inhalation employing a vibrating mesh nebulizer [166]. Nebulized CpG-G NPs decreased the respiratory effort, nasal discharge, tracheal secretion, mucus viscosity, and neutrophil infiltration and increased arterial oxygen pressure up to 4 weeks post-treatment in horses [167]. In a further study, asthma-affected horses receiving nebulized CpG-G NPs alone or combined with relevant allergens, experienced clinical improvement of nasal discharge, breathing rate, amount of mucus secretion and viscosity, neutrophil percentage and partial oxygen pressure directly after and 6 weeks after treatment [168].

In Cats:

NP-TLR3 and 9 as an immunomodulator against feline herpesvirus 1 (FHV-1):

FHV-1 is a common cause of ocular and upper respiratory infections in cats that can be a major cause of morbidity and sometimes mortality, especially in young kittens [169]. Current vaccination provides incomplete immunity [170]. Clinical signs of FHV-1 infection can be reactivated with repeated exposure, after induction of stress, or after administration of immunosuppressive drugs. Local activation of innate immune responses, however, may create an environment that is less susceptible to viral entry and replication or to bacterial invasion and colonization. PolyIC and noncoding plasmid DNA (as TLR9 agonist) complexed to cationic liposomes (liposome TLR receptor complex, LTC) mixed with carboxymethylcellulose to increase mucosal adhesion, were reported to induce local mucosal immune responses in the upper respiratory tract of kittens, that may protect, either partially or entirely from infections or decrease the severity and duration of clinical signs of illness, along with decreased viral or bacterial shedding [171, 172]. LTC administered to the mucosa of healthy cats (a combination of 0.2 mL in each nostril and 0.6 mL orally) were internalised by immune and epithelial cells in the upper airways, inducing the recruitment and activation of feline leukocytes, as well as upregulation of the expression of costimulatory molecules (OX40 and MHCII). Cytokines associated with innate immune responses, including INF-α, INF-γ, TNF-α, and IL-12, were also produced. LTC treatment significantly reduced conjunctivitis and decreased shedding of FHV-1 DNA in kittens on some post-inoculation days. Unfortunately, the immunostimulation was performed only 24 hours before the FHV-1 challenge, and despite clinical scores that were consistent with a positive effect with LTC treatment, the statistical significance was lost after adjusting for repeated kitten observations over time [172].

In Dogs:

NP-TLR9 agonist as an immunomodulator against atopic dermatitis (AD):

Canine AD is a congenital, inflammatory allergic skin disease associated with distinctive clinical signs and only treated with allergen im-

munotherapy (AIT). AIT is a lengthy, not completely succeeding, and costly procedure as it requires identifying and formulating the allergens that contribute to the disease for each dog [173]. An efficacious immunomodulation not requiring allergen identification would thus be desirable. Recently, CpG bound to gelatin NPs (CpG-G NP) was used to treat dogs with AD [174]. SC administration of CpG-G NPs significantly decreased IL-4 mRNA expression, and after 18 weeks, an improvement in at least 50% of patients was seen. This result was comparable to that obtained with traditional allergen immunotherapy but with fewer collateral effects.

NP-TLR3 and 9 as immunomodulator against canine herpesvirus:

Canine herpesvirus is a severe viral infection in puppies worldwide and is often lethal to affected litters. It can be transmitted by lifelong latently infected adults. LTC treatment was recently reported to elicit effective antiviral immunity in dogs following a canine herpesvirus outbreak [175]. LTC administered to the mucosa of healthy dogs (a combination of 0.5 mL in each nostril and 2 mL orally) induced key innate immune cytokines, including IL-8, MCP-1, IL-12p40, IFNγ and TNFα on peripheral blood mononuclear cell (PBMC) cultures and oropharyngeal cells. LTC treatment resulted in immune cell infiltration (monocytes and lymphocytes peaking at 72 hours), activation of the upper airway, and oropharyngeal tissues. The production of TNFα was triggered, the expression of MHCII was upregulated, and macrophage bactericidal activity was increased. Overall, cellularity remained elevated in the nose for at least 7 days, whereas cell counts returned to normal in the oropharynx by 7 days. A single administration of LTC induced a therapeutic response (decreased conjunctivitis observation days) consistent with induction of early antiviral immunity, without perturbing the microbiome of the oropharynx.

NP-TLR4 agonist as vaccine adjuvant against Echinococcus granulosus:

E. granulosus is an important pathogen for several domestic animal species and causes a considerable impact on both human and animal health with important socioeconomic loss in endemic areas [176]. Echinococcosis is a chronic parasite disease distributed worldwide, mostly in developing countries with no vaccine available. A recent report showed that SC immunization with MPLA co-encapsulated with a recombinant antigen of *E. granulosus* in liposomes (MPLA-Eg-lipo) induced higher levels of specific IgG and IFN-γ and generated 95% of protective immunity in mice challenged with *E. granulosus* as evidenced by the number of hydatid cysts in comparison with mice receiving PBS [177]. These results point a hopeful path toward an anti-echinococcus vaccine for dogs.

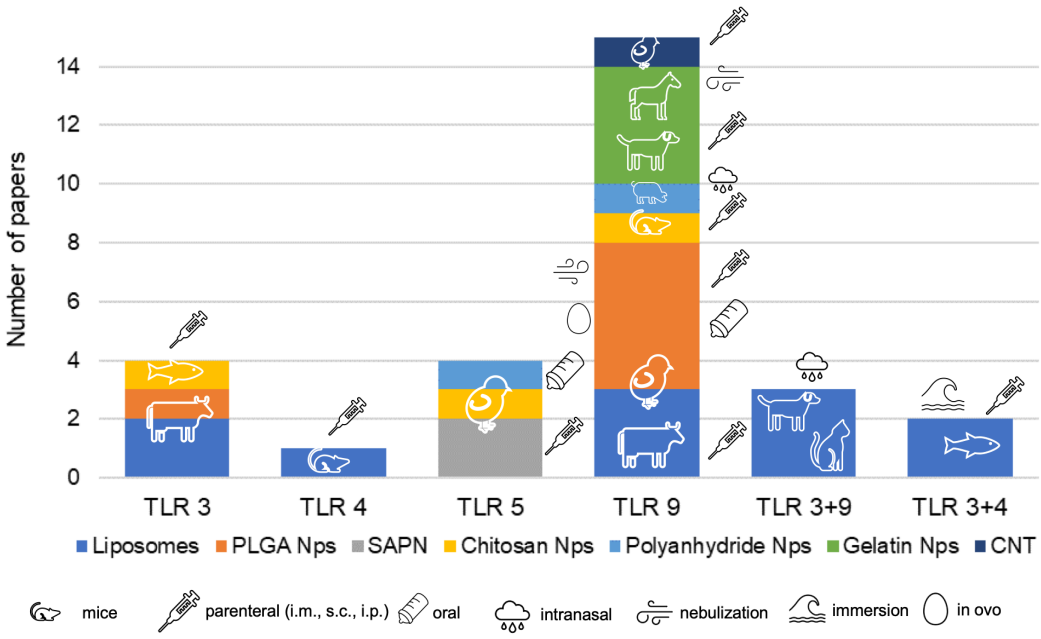

Figure 5. Number of papers vs type of NP-TLR agonist, administration route and animal species.

Unknowns behind experimental approaches to NP-TLR agonists: the need for nano instead of microparticles and scaling-up techniques

As pictured in Figure 5, this review gathered the available data on the performance of commercial and experimental NP-TLR agonists, aimed at reducing the use of antibiotics by providing mucosal immunity, inducing a broader response, or accelerating the onset of protection on feed animals and intended to have anti-allergic and antiherpetic effects on companion animals.

Most of the data published in the last 5 years were based on NP-TLR prepared with lab-scale methods and tested on a small number of animals. With few exceptions, mostly clinical, microbiological, or immunological aspects were reported, but mechanistic or pharmacodynamic features of NP-TLR agonists were poorly addressed. Such issues raise two important uncertainties. One is about the true need for immunotherapies performed with nanoscale, instead of micro- or macro-sized particulate agents. Excepting for cationic liposomes injected or administered by bath immersion for fish, LTC instilled to cats, MWCNTs injected to chickens, and SAPN regarded as endocytosable particles because of their small size, the reports did not specify whether NPs acted as depots of TLR agonists or if the entire structure of NP-TLR agonist was internalized by target cells. A comparison between NP-TLR agonist action with that of free agonists mixed with Np lacked in most reports. Those data are key to decide if the use of NPs is or is not essential: if immunotherapies are mediated by TLR agonists released from particulate depots, the size of the NPs would not be relevant since microparticles or even macroscopic depots could be used for delivery of agonists of surface TLR receptors. The size of particles becomes relevant only if their activity is mediated by endocytic internalisation.

The other major uncertainty is related to the former question and is about the technical feasibility of producing NPs at an industrial scale and according to good manufacturing practices. The reviewed nanomedicines were prepared with materials of different chemical nature. However, only those based on lipids made it to market in 2015. This fact suggests that veterinary nanomedicines would follow the same trend, in terms of chemical nature of raw materials, as human nanomedicines which are made of few biomaterials, lipids mostly. This is because the challenges of industrial production of nanoscale-sized liposomes, in accordance with astringent quality control rules, were solved in the early 90 [178]. Accordingly, their complex structural characterization, because of their character of non-biological complex drugs, can be performed following available international guidelines [178]. Liposomes are made of GRAS (generally recognized as safe) materials (ranging from animal, vegetal or microbial, to synthetic origin) that lack toxicity for humans if administered by routes other than intravenous. Liposomes admit all routes of administration, and, presumably, the same lack of toxicity will be found in veterinary medicine. The commercial liposomal immunostimulants Zelnate® and Victrio®, sharing identical structure, were IM-administered to cattle and *in ovo* injected; while experimental liposomal formulations such as Vaccimax and CAF09 were SC administered to cattle; nebulized to chickens, administered by the mucosal route to cats and dogs and IP or by bath immersion to fish.

The situation is far from being solved for NPs made of materials other than lipids, which are less characterized in terms of structure, production, and toxicity. For instance, the industrial production of polymeric NPs seems to be more challenging than that of liposomes. For human use, there are only commercially available PLGA microparticles, microspheres [179], [180], and polyanhydride macroscopic matrices [181]. There is an intrinsic difficulty in scaling up PLGA NPs, particularly those of diameter below 100 nm. Robust characterization studies correlating product-critical quality attributes, such as number average molecular weight and size, across a wide design space, are imperative for clinical translation and commercialization of polymer NPs [182]. Many formulations show poor drug loading, and these systems often exhibit burst release kinetics [183]. On the other hand, the absence of methods providing batch to batch consistency, the heterogeneous mixing conditions required for self-assembly, and additional purification steps difficult the GMP processes. Up to 2009 (no further data available), the preparation of polymeric NPs by the emulsification-solvent diffusion (evaporation), nanoprecipitation (potentially scalable)

and the salting-out methods have only been translated from the laboratory scale (volume of 60 mL) to pilot scale (volume of 2 L), far from the volumes needed for field use [184], [185]. Microfluidics is a technique that may enable the industrial scale-up of polymeric NPs, increasing the possibilities of endocytic targeting and magnifying the immunostimulant activity [180]. Products made with such technology, however, are not available in the market yet.

PLGA is a poly(α-hydroxy ester) that suffer bulk degradation mediated by bonds cleavage throughout the particle structure. This shortens the polymer molecular weight, increases water content, and releases soluble monomers, chain fragments, and encapsulated actives. An important feature of PLGA particles is the lack of need for cross-linking agents. Most of the ~ 500–700 nm-sized PLGA NPs formulated the CpG as mucosal adjuvants (oral, aerosol) or *in ovo* immunostimulant. To avoid a burst release, CpG was complexed with PEI; however, no data on stability or release profile along the gastrointestinal (GI) tract or nebulization was provided.

SAPN are agents known to provide intracellular delivery [186]. The production of SAPN at an industrial scale in GMP conditions for human vaccines, based on a system of expression in *E. coli,* has recently been described [187]. Curiously, in the studies of Karch et al., 2017 and Li et al., 2018, SAPN were linked to flagellin, an agonist of the non-endocytic TLR 5 located on the cell surface. The IM administration of SAPN flagellin induced a systemic reaction against AIV (protecting mice against lethal challenge) and against IBV in chickens (reducing clinical symptoms of disease). Whether or not mucosal immunity (needed to cope with AIV and IBV diseases) was raised, was not yet explored.

Polyanhydrides are mucoadhesive copolymers of methylvinyl and maleic anhydride; polyanhydride NPs display highly labile anhydride linkages and hydrophobic backbone; experience superficial degradation showing a typical zero-order release profile [181]. Polyanhydrides are used as macroscopic controlled release implants in clinics (Gliadel and Septicin), but no production methods of polyanhydride NPs at an industrial scale have been developed. On the other hand, chitosan, a polysaccharide with antimicrobial and mucoadhesive properties, is a linear copolymer of β-(1→4)-linked monosaccharides (2-acetamido-2-deoxy-β-d-glucopyranose and 2-amino-2-deoxy-β-d-glucopyranose), obtained by the deacetylation of chitin under alkaline conditions or by enzymatic hydrolysis with chitin deacetylase. However, currently, no chitosan particles have made it to market, not even in the microscale [188]. Due to their mucoadhesive character, ~ 200-500 nm-sized chitosan or polyanhydride NPs were used to induce mucosal immunity upon oral and nasal administration. However, food products that contain insoluble, indigestible, and potentially bio-persistent NPs, such as those made of cross-linked chitosan NPs is an area of major concern [189]. Uncertainties on the feasibility of chitosan NPs industrial scaling-up, together with poor biodegradability and biocompatibility of NPs cross-linked with TPP and glutaraldehyde [190] are compensated by the fact that polyanhydride NPs do not suffer from comparable safety issues.

Gelatin NPs of ~ 250 nm bound to TLR9 agonists, were administered by nebulization to asthmatic horses. Gelatin NPs derived from bovine or porcine bones or skins, with a wide range of molecular weights, display heterogeneous NPs size distribution. The use of recombinant human gelatin, to avoid the risk of contamination with transmissible spongiform encephalopathy and the replacement of cross-linking agents (glutaraldehyde and carbodiimide) by genipin, would be safer options [191], [192].

Discussion

NP-TLR agonists discussed above are nanomedicines and, as such, require a more accurate structural characterization, and to be prepared with materials well suited to be sized in the nanoscale by industrial methods. Nanomedicines are known to provide controlled pharmacokinetics and pharmacodynamics of APIs which benefit humans and probably other animal species. Potentially, the enabling character of nanotechnologies and the ability to increase the added value of products may revert the poor interest of biotech companies in veterinary immunotherapy. Historically, however, the individual value of farm animals has been neglected, and the use of immunotherapies has been promoted purely as objects of human safety and in

support of the global economy [193], [194]. For veterinary nanomedicines to succeed, their advantages must be perceived as valuable, as has already occurred in the field of human medicine. However, the assessment of veterinary nanomedicines will be difficult, since veterinary pharmacovigilance (that explores safety and effectiveness of anti-infective vaccines) and socioeconomic evaluations of veterinary products (cost-effectiveness under field conditions) are in general, poorly addressed. In addition, the adverse effects of veterinary vaccines are underreported [1], [195].

Conclusions

There are immediately visible benefits, different from improved efficacy or lower toxicity, that veterinary nanomedicines can provide, and that could attract the interest of veterinary markets. For instance, the avoidance of injectables and of unnecessary handling provided by inhaled or bath-immersion nanomedicines should be attractive for the fish and chicken industries. In addition, mucosal immunomodulators for companion animals is a convenient pathway to overcome classical desensitization strategies. There are no available data, however, of current business ventures focused on veterinary nanomedicines administered by routes other than parenteral. Future social changes toward increased animal welfare that are already underway will likely increase the use of nanomedicines in the veterinary field.

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Conflict of interest

The authors declare there is no conflict of interest. For a signed statement, please contact the journal office: editor@precisionnanomedicine.com

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