APPLICATIONS

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Contents
APPLICATIONS..........................................................................................................................3
Principles of immunomodulation and immune stimulation in animals .....................................3
What is immune stimulation?........................................................................................................3
Rationale for targeting the innate immune response ......................................................................3
Immunostimulants in animals: mechanisms of action .................................................................4
Modulating the immune response via receptors, cytokines and enzymes ..................................4
Nucleic acids in immunostimulation ............................................................................................5
Bacterial cell wall components ......................................................................................................6
Viral immunostimulants in animals ...............................................................................................6
Other immunostimulants ...............................................................................................................7
Immune stimulants as preventative, therapeutic or adjuvant measures in animal health ..........8
Immunostimulants as adjuvants ....................................................................................................8
Immunostimulation and disease prevention ................................................................................8
Responsible use of therapeutic immunomodulation ....................................................................8
Summary of immunostimulation in animals ...............................................................................9
References ......................................................................................................................................9
Applications

Principles of immunomodulation and immune stimulation in animals

What is immune stimulation?
Animals are protected from pathogenic infection and the resulting damage by their immune system, which comprises a network of cells and molecules that detect and then eliminate invading pathogens in a coordinated response.\(^1\)

Sometimes, it may be necessary to use a therapeutic agent to assist with the elimination of an infection. For example, some types of pathogens can modulate the way in which the immune system works, impairing its ability to fight the infection. This may be a result of the pathogen evading detection or modifying immune effector responses.\(^2\) The use of antibiotics – chemical drugs that selectively induce toxicity in bacteria, leading to their death – is one way to help overcome this problem.\(^3\) However, this is not always appropriate, and recent research has been concerned with leveraging the animal’s own immune system to generate a response to the infection. Once an effective immune response has been mounted, a pathogen stands very little chance of surviving within the animal body.\(^4\) This could constitute a new approach in the management of infectious diseases, possibly contributing to reductions in dependence on antibiotics.

Immunomodulation encompasses all therapeutic interventions aimed at modifying the immune response, either up or down, until a desired level is reached. This is largely achieved by targeting components of the immune system to modify their actions and promote improved health.\(^4\)

Immunostimulants are substances that stimulate the immune system by inducing activation or increasing activity of any of its components. They vary according to their origin, mechanism of action and application (preventative, therapeutic or adjuvant).\(^4\)

Rationale for targeting the innate immune response
Immunity is often categorized into two interacting phases of response that operate via distinct effector cells. The innate immune response acts rapidly upon infection, and importantly, recognizes molecular patterns that are non-specific, i.e. can be found on many types of pathogen but not on the animal’s own cells. It does this using receptors called Toll-like receptors (TLRs). In this way, it acts as a gatekeeper, only permitting a defensive immune response to proceed if a pathogen is present. The second phase of response is called the adaptive immune response and it relies upon innate immune cells to signal the presence of a pathogen.\(^5\)

Due to these characteristics, stimulation of the innate immune system could be used therapeutically. As the innate immune system can respond to any type of pathogen, there is no need to identify and prioritize specific pathogens. Conventional therapies, such as antibiotics or vaccines are usually only effective against one or a few types of pathogens, and antibiotics do not work against viruses. The innate immune system’s capacity to react to a broad range of pathogens is particularly valuable in conditions where there are multiple causative agents.
There is also the opportunity to enhance the adaptive immune response as this develops downstream from the innate response. Thus, there is potential for a rapid response but with the enduring protection offered by a vaccine (which acts via the adaptive immune system).

Innate immune stimulation has the potential for prophylactic and perhaps therapeutic use, without the risk of promoting antibiotic resistance because it is the animal’s own immune system that is responsible for removing the pathogen.

**Immunostimulants in animals: mechanisms of action**

**Modulating the immune response via receptors, cytokines and enzymes**

One way to trigger an immune reaction is to introduce part of a pathogen into the animal body. Vaccines work in this way by supplying an antigen derived from a specific type of pathogen, which triggers an adaptive immune response. However, if an animal is immunosuppressed then it may fail to respond to vaccination.

Immunostimulants work by supplying a small molecule called a PAMP. These molecules contain molecular patterns found on many pathogens that can be recognized by the TLRs of innate immune cells. TLR activation launches a series of intracellular signaling events that ultimately lead to activation of immune cells and production of effector molecules of the innate immune response (including various chemokines, cytokines and antimicrobial products) (Figure 1). Significant efforts have begun to develop TLR agonists as therapeutic agents to stimulate the innate immune response.

Cytokines can also be administered to modulate the immune response. Cytokines are the signaling molecules of the immune system, and they are secreted by activated immune cells and detected by other immune cells. Cytokines instruct the immune cells on how to respond to an infection. They may have stimulatory or inhibitory effects, depending on the type of cytokine.

A further way of modulating the immune system is to change the activity of mediators or enzymes produced downstream from the initial TLR signaling. For example, COX-2 is an enzyme that is produced in response to pathogens, and inhibition of this enzyme may enhance antiviral responses.
Figure 1. TLRs are receptors that recognize non-specific molecular markers on animal pathogens (known as pathogen-associated molecular patterns [PAMPs]).

TLRs bind to bacterial or viral products.

dsRNA, double-stranded ribonucleic acid; LPS, lipopolysaccharide; ssRNA, single-stranded ribonucleic acid; TLR, Toll-like receptor.

Nucleic acids in immunostimulation

A major difference between bacterial DNA, which has potent immunostimulatory effects, and vertebrate DNA, which does not, is that bacterial DNA contains a higher frequency of unmethylated CpG dinucleotides. Short synthetic DNA sequences containing unmethylated CpG motifs, known as CpG oligodeoxynucleotides (ODNs), have been shown to induce activation of various immune cells, including B cells, natural killer (NK) cells and antigen-presenting cells such as monocytes and macrophages. CpG ODNs can also enhance the production of cytokines that are known to be involved in the development of an active immune response, including tumor necrosis factor-α, interleukin (IL)-12 and IL-6.

In mammals, unmethylated CpG sequences in DNA molecules are recognized by TLR9. In chickens, TLR21 acts as a functional homolog to mammalian TLR9 in the recognition of CpG ODNs. Viral double-stranded RNA (dsRNA) and synthetic dsRNA analogs are PAMPs that act via TLR3 and have been shown to induce antiviral immunity in fish. TLR3 and TLR7 recognize single-stranded RNA.

In addition to TLRs, there are a number of other pattern-recognition receptors, including NOD-like receptors, RIG-I-like receptors and other helicases. For example, DHX9 and DHX36 are enzymes that have also been proposed to play a role in the sensing of CpG DNA in the cytosol.

Recently, a protein, known as cyclic-GMP-AMP synthase (cGAS) has been found to detect DNA that is present in the cytosol of cells. This triggers a cascade which signals through the adaptor protein, stimulator of interferon genes (STING), and culminates in the production of interferon and other cytokines.
**Bacterial cell wall components**

Lipopolysaccharide (LPS) is an endotoxin that is contained in the cell membrane of certain types of bacteria, such as *Escherichia coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus influenza*, *Bordetella pertussis* and *Vibrio cholerae* (Figure 2). LPS is a PAMP and is specifically recognized by TLR4.\(^\text{19}\)

LPS is a large molecule consisting of a lipid component and a polysaccharide that contains repetitive glycan polymers called O-antigens. The polysaccharide component elicits a strong immune response in animals, including the production of cytokines by phagocytes and complement activation.\(^\text{19}\) LPS can also cause changes in the physical condition of an animal, including piloerection, inactivity, loss of thermoregulation and hypotension in a condition known as endotoxic shock.\(^\text{7,19}\)

LPS has been investigated as an immunostimulant used at low doses. In fish, it has been shown to increase the phagocytic activity of innate immune cells, and in addition, to increase the levels of lymphocytes, which are adaptive immune cells.\(^\text{20}\)

Thrombocytes from poultry that were stimulated *in vitro* with LPS resulted in the production of proinflammatory cytokines and nitric oxide, and increased phagocytosis.\(^\text{21}\)

Although mycobacteria do not contain LPS, mycobacterial cell wall extracts (MCWEs) contain other PAMPs, including lipoarabinomannan, which acts via TLR2 in mammals to stimulate the production of proinflammatory cytokines.\(^\text{22}\)

**Figure 2. Bacterial cell wall containing lipopolysaccharide\(^\text{19}\)**

**Viral immunostimulants in animals**

Virus-derived technologies make use of the immunostimulatory properties of viruses. *Poxviridae* are a family of viruses that have evolved features that allow them to evade immune detection. The use of biotechnology allows the virus to be modified, removing its ability to evade the immune system, and in addition, attenuating or inactivating it. When this is done, the virus is able to stimulate an immune response, and for over 35 years, it has been known that inactivated poxviruses can induce
Parapoxvirus ovis activates immune cells, stimulates phagocytosis and induces expression of cytokines, leading to the production of gamma interferon (IFN-γ), which has antiviral effects, and immune cell activation in a number of species.

Inactivated Parapoxvirus ovis has been used as an immunomodulator, demonstrating efficacy and safety in stimulating the equine immune response to aid in the reduction of equine upper respiratory disease associated with equine herpes virus types 1 and/or 4 infection(s). In cattle, administration of a biological response modifier obtained from Parapoxvirus ovis significantly reduced the rate of intramammary Staphylococcus aureus infection after calving. Treatment of cattle with a Parapoxvirus ovis immunomodulator protected against infection, reduced clinical manifestations and limited the spread and virus shedding of bovine herpesvirus-1. Compared to chickens who received a La Sota Newcastle disease vaccine only, those who received concomitant inactivated Parapoxvirus ovis had a lower mortality rate during the first few weeks of life.

Nanoparticles have also been developed that resemble the Papaya mosaic virus, which is a plant virus, and they have been shown to induce adaptive immunity and enhance the immune response to antigens, such as those contained in vaccines. It is not yet known how these effects are mediated or which pathways the nanoparticles act through.

**Other immunostimulants**

The production of cytokines by recombinant DNA technology has permitted the investigation of these molecules as immunostimulants, e.g. IL-1, IL-2 and IL-12; however, most of these have had minimal impact on disease processes and may have toxic side effects. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine produced by immune cells to stimulate the production of granulocytes, which are a class of innate immune cell that includes neutrophils. GM-CSF is ordinarily secreted in response to inflammatory stimuli, such as LPS. In horses, GM-CSF was shown to normalize low neutrophil counts.

The type I interferons are a family of antiviral proteins. The main natural source of IFN-α is dendritic cells, while IFN-β is derived from virus-infected fibroblasts, and IFN-ω is produced by lymphocytes. Biological responses to type I IFN include the inhibition of viral replication, increased activity of NK cells, increased expression of molecules for antigen-presentation, and enhanced activity of T cells of the adaptive immune system. IFN-ω is licensed for the treatment of certain viral infections in dogs and cats.

A fine balance is required between the different cytokines to ensure appropriate cell signaling that enhances immunity without inducing adverse events. This has led to a preference for measures that stimulate innate immunity, leading to regulated and natural cytokine production. Apart from microbial components and cytokines, synthetic compounds, such as imidazaoquinolines, poly I:C (a form of synthetic dsRNA) and CpG ODNs, can also stimulate the immune cells to mount an antiviral response through the production of endogenous IFN in fish.
Immune stimulants as preventative, therapeutic or adjuvant measures in animal health

Immunostimulants as adjuvants
Adjuvants are used to increase the speed and magnitude of the immune response to a vaccine, and they are used with vaccines that have poor immunogenicity. Immunostimulatory adjuvants act by enhancing the production of cytokines that contribute to the immune response. Many of the immunostimulants described above have been investigated as adjuvants by administering concomitantly with an antigen (in a vaccine) in order to boost the immune response.

- CpG ODN-adjuvanted vaccines have shown enhanced adaptive responses to many pathogens. Several studies have shown that vaccine-induced immune responses occur more rapidly with the use of CpG ODN adjuvants. The addition of CpG ODNs significantly boosts vaccine immunogenicity in aged mice, where defects in adaptive immune responses may be present.
- In chickens, the presence of flagellin, a PAMP that binds to TLR5, as a vaccine adjuvant enhanced cytokine expression and reduced mortality.
- In a mouse model, vaccination with an influenza vaccine administered with poly I:C induced a strong antibody response, whereas vaccination alone had little effect.
- Administration of a modified, non-toxic form of LPS as a vaccine adjuvant boosts the level of antibody production compared with vaccine alone.

Immunostimulation and disease prevention
Immunostimulation may also offer benefits independently from vaccination of livestock. This can be assessed by measuring the levels of immune cells and cytokines, or by comparing mortality rates in immunostimulated and non-immunostimulated animals.

- In macaques, a type of primate, molecules that activate TLR4, TLR7/8 and TLR9 mediated a range of stimulatory immunological effects, including increasing neutrophil levels, cytokine production and innate immune cell activation.
- CpG ODNs administered to chicks in ovo stimulated the innate immune responsiveness of heterophils and increased the resistance of young chickens to colonization by *Salmonella enterica* serovar Enteriditis.
- LPS or CpG ODNs administered to chickens increases the levels of nitric oxide, which mediates a number of biological reactions and contributes to the host defense against intracellular pathogens.
- Subcutaneous injection of CpG ODNs induced an acute-phase immune response (transient fever, mild transient increase in circulating neutrophils and elevated serum haptoglobin) in cattle.

Responsible use of therapeutic immunomodulation
With better understanding of basic biology and immune mechanisms, it is possible to exploit the potential of immunostimulants for animal welfare. However, it is important to acknowledge that sustained enhancement of the innate immune response may not be appropriate and that the implications of therapeutic intervention with TLR signaling must be considered.
polymorphisms within TLR genes have been associated with altered susceptibility to infectious, inflammatory and allergic diseases, and they have been found to play a role in the generation of tumors. An ‘overshooting reaction’ could lead to undesired outcomes such as septic shock syndrome or autoimmunity.

When considering any regimen to prevent or combat infection in feed animals, it is important to note key stages at which animals are at increased risk of infection, for example during the first week of life and during molting in chickens, and during shipping and the peripartum period in cattle.

**Summary of immunostimulation in animals**

- Immunomodulation is the modification of immune responses in animals, either to increase or decrease them, and it is usually achieved by targeting components of the immune system.
- Immunostimulants are substances that enhance the activity of the immune system.
- TLRs on innate immune cells can stimulate innate immunity through binding to pathogen-associated molecular patterns (PAMPs).
- Benefits of targeting innate immunity:
  - It can respond to any type of pathogen
  - It can trigger adaptive responses downstream
  - It can have prophylactic and therapeutic effects
- Immunostimulants include nucleic acids (CpG ODN and dsRNA), bacterial cell wall components (LPS and lipoarabinomannan), viral components (*Parapoxvirus ovis*) and small molecules (cytokines).
- Immunostimulants can be used as adjuvants to enhance vaccines, efficacy or in their own right to increase immune activity and protect against infection.

Bayer is actively exploring the science of immunology and innate immunity. For more information about products related to these areas from Bayer, visit www.Zelnate.com or www.BayerLivestock.com.

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