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Advances in Swine Immunology Help Move Vaccine Technology Forward

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Abstract

In veterinary animal species, vaccines are the primary tool for disease prevention, a key tool for treatment of infection, and essential for helping maintain animal welfare and productivity. Traditional vaccine development by trial-and-error has achieved many successes. However, effective vaccines that provide solid cross-protective immunity with excellent safety are still needed for many diseases. The path to development of vaccines against difficult pathogens requires recognition of uniquely evolved immunological interactions of individual animal hosts and their specific pathogens. Here, general principles that currently guide veterinary immunology and vaccinology research are reviewed, with an emphasis on examples from swine. Advances in genomics and proteomics now provide the community with powerful tools for elucidation of regulatory and effector mechanisms of protective immunity that provide new opportunities for successful translation of immunological discoveries into safe and effective vaccines.

Keywords

adaptive immunity; innate immunity; T-cell; B-cell; PRRSV; cholera toxin; mucosal immunity

Introduction

For the purpose of protecting animal health, vaccinology is the application of immunology to solve infectious disease problems. Infectious diseases exert a profound burden on animal health and welfare, cause economic injury to farmers and producers, and threaten human populations through zoonotic transmission of endemic and epidemic disease due to established and newly emergent pathogens. More recent non-disease applications of

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immunology include the use of vaccination to induce immunological castration of boars for elimination of boar taint.

Innate Immune Response to Infection

In the case of infectious disease, the immunological principles that guide current thinking on vaccine development and efficacy can be grouped into categories related to antigen detection, stimulation of an appropriate adaptive response, and establishment of memory. Detection of a pathogen is mediated by innate danger signals in cells that sense infection by the presence of molecular structures, including viral double-stranded RNA, CpG motifs of bacterial DNA, bacterial lipopolysaccharide (LPS), and repetitive structures such as viral capsids, that are not present in healthy cells. The danger signals elicit a robust intracellular immune response with sufficient plasticity to guide intracellular immune responses that are appropriate for the invading pathogen (Beutler et al., 2007). A striking demonstration of this plasticity is the varying patterns of gene expression induced in human dendritic cells exposed to *Escherichia coli*, influenza virus, or *Candida albicans* (Huang et al., 2001). In this microarray expression profiling experiment, 289 genes were pathogen-regulated. Interestingly, a unique set of 118 genes were specific for *E. coli*, and 58 were specific for influenza, but no genes were specifically induced in response to the fungus (Huang et al., 2001). This result suggests that a multitude of signals are triggered and integrated early in an immune response to finely modulate host responses to infection.

Viral infection of permissive cells induces, most prominently, a mRNA expression profile dominated by type I interferons (α and β) and interferon-responsive genes. These pathways are activated by viral components binding to cell surface and intracellular sentinel proteins including various Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG-I), and melanoma differentiation-associated protein 5 (MDA-5), to initiate signaling cascades through a variety of pathways that trigger type I interferons and interferon-responsive effectors. Similarly, bacterial infection also induces gene expression cascades through interactions of cell wall components, CpG and other conserved bacterial features with TLRs. Signaling cascades through NF-κB and cyclic AMP-dependent protein kinases, in particular, induce inflammatory cytokine production. The molecular sensors and signaling pathways for other common pathogen classes, including fungi, nematodes, cestodes, amoeba, apicomplexa and so on, are less well characterized. It may reflect the fact that rapidly dividing organisms such as viruses and bacteria require rapid responses to overcome the high rates of replication that these pathogens can achieve and which may result in incapacitation or death. Therefore, there is a powerful selective force to evolve efficient innate sensors, response pathways, and effector molecule countermeasures. By contrast, pathogens that do not produce an immediate threat to host well-being may have evoked a lower selective pressure, or the animal hosts may have evolved immunological strategies that tolerate the pathogen at the cost of reduced energy partitioning for growth and reproduction, in which case more time is available to develop physiological and immunological survival mechanisms.

The general molecular features of innate anti-viral and anti-bacterial responses that have been elucidated primarily in murine host-pathogen models provide useful models for

investigation of host defense in veterinary species. In swine, for example, respiratory infection by the gram-negative bacteria, *Actinobacillus pleuropneumoniae*, induces an acute, robust lung cytokine response characterized by IL-1, IL-6 and IL-8 production (Baarsch et al., 2000; Baarsch et al., 1995; Morrison et al., 2000; Myers et al., 2002; Van Reeth et al., 2002). However, despite the presence of bacterial LPS and CpG DNA, there is no induction in vivo of tumor necrosis factor (TNF), the classical inflammatory cytokine molecule and effector product of NF-κB activation, even though in vitro incubation of alveolar macrophages with boiled extracts of *A. pleuropneumoniae* readily induces TNF (Baarsch et al., 2000; Baarsch et al., 1995). The lesson learned here is that laboratory models provide guidance for hypothesis-driven studies in veterinary animal species, but the details must be verified by experimental analysis.

A contemporary idea in anti-viral immunity is that the plasmacytoid dendritic cell, a rare cell type in blood, produces prodigious amounts of interferon α upon viral stimulation, and thus plays a key role in antiviral immunity (Liu, 2005). Nevertheless, direct evidence in support of this key antiviral role has been difficult to obtain (Reizis et al., 2011). Indeed, an interesting example in swine relates to the role of type I interferon in development of immunity to porcine reproductive and respiratory syndrome virus (PRRSV). An early, influential study concluded that PRRSV does not result in appreciable interferon α production (van Reeth et al., 1999). Since other studies indicated that PRRSV infection was persistent and adaptive immune responses were slow to develop, it was widely assumed that lack of interferon production was the key immunological defect (Murtaugh et al., 2002). However, direct examination of adaptive immune responses to PRRSV infection, in comparison to simultaneous responses to an irrelevant antigen, indicated no delay in the antigen-specific adaptive response (Mulupuri et al., 2008). It was subsequently shown that type I interferon induction is a variable, strain-dependent feature of PRRSV infection (Lee et al., 2004). The role of type I interferons in PRRSV immunity and vaccinology is confusing at present and serves as a cautionary tale against relying on untested assumptions about immunological mechanisms in veterinary species (Murtaugh and Genzow, 2011).

The discovery of TLRs and other innate sensors of microbial infection in the 1990's appeared to present a convenient mechanistic explanation for the activity of adjuvants. It held the promise that development of TLR ligands would provide a rational route to improved adjuvants for subunit and inactivated microbial vaccines. While it is clear that TLR ligands enhance adaptive B-cell and T-cell responsiveness (Khoruts et al., 1998; St Paul et al., 2013), these actions may not be mediated by TLR signaling. A well-known property of adjuvants is the enhancement of antibody responses, but genetic ablation of TLR signaling pathways did not affect the level of antibodies raised to various T-cell dependent antigens administered with a variety of classical adjuvants (Gavin et al., 2006). Likewise, administration of type I interferon or poly ICLC (a synthetic complex of polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose), a TLR-3 agonist, with attenuated PRRSV vaccination did not enhance protection against virulent challenge, and may have exacerbated disease (Charerntantanakul et al., 2006; Murtaugh and Genzow, 2011; Zhu et al., 2007). Although the molecular mechanisms by which adjuvants potentiate antigen-specific immune responses are not completely elucidated, the

investigations stimulated by the discoveries of innate sentinels of danger or non-self have firmly established the role of innate responses to infection in initiation of productive antigen-specific B-cell and T-cell responses that are the foundation of vaccination.

Antigen-Specific Adaptive Immunity

Prior to the discovery of innate molecules that sensed different classes of pathogens, Coffman and Mosmann and colleagues recognized that individual helper T cell clones displayed cytokine secretion patterns that fell into two classes based on production of interleukin-2 (IL-2), interferon γ, and IL-4 (Mosmann et al., 1986; Mosmann and Coffman, 1989b). They and others further determined that these cytokine expression patterns were related to differences in functional properties and in vivo responses to infectious agents, leading to the Th1-Th2 paradigm (Coffman, 2006; Mosmann and Coffman, 1989a, b). Now, veterinary immunologists seemed to have a roadmap for design and development of highly effective vaccines to a broad array of disease agents. Simply stated, one could find a known or predicted TLR ligand associated with a particular pathogen, formulate it with a candidate vaccine material, and determine efficacy by the presence of a cytokine profile in serum or cell culture that was linked to humoral or cell-mediated immune outcomes that might protect against intracellular or extracellular pathogens. The concept that Th1-type responses optimized immunity to intracellular pathogens, whereas Th2-type responses biased toward extracellular pathogens, provided vaccine developers with hypothesis-based strategies intended to maximize immunological efficacy. Moreover, the paradigm highlighted specific cytokines that appeared to play key roles in directing T-cell development toward Th1 (IL-12) or Th2 phenotypes (IL-4), suggesting that selected cytokines could further enhance vaccine efficacy. Challenge experiments in swine combining IL-12 treatment with viral vaccines indicate an enhancing effect, but these findings have not been substantiated further (Foss et al., 2002; Lawson et al., 2010; Zuckermann et al., 1998).

The symmetry and beauty of the Th1-Th2 paradigm stimulated a flurry of studies, primarily in mice and humans, that described additional phenotypic populations of CD4+ helper T cell subsets, subsets of CD8+ T cells and even macrophages. The various subpopulations were characterized by cytokine expression patterns and were aligned according to dichotomous functional criteria, such as inflammatory versus cytotoxic macrophages, or further divided intovarious lymphoid subpopulations of increasingly refined functional and molecular properties. These studies have provided a wealth of hypothesis-driven research in immunological mechanisms of autoimmunity, transplantation phenomena, allergy and infectious disease responses. However, the impact on vaccinology remains to be determined; in both the veterinary and human worlds, there is little evidence that the impressive expansion of immunological knowledge has benefited vaccine development.

Notwithstanding the challenge of applying immunological principles to veterinary vaccine development, there still is a compelling need to elucidate the molecules and mechanisms of protective immunity to key pathogens in animal species. Dissection and characterization of these regulatory and effector functions, in most cases, requires an expanded toolkit for quantitative measurement of cytokine presence in relevant cell populations and tissues in which immune responses are initiated and evolve. Thus, analysis of cytokine levels in

lymphoid tissues is required, rather than in serum or blood. Assuming that the Th1-Th2 model is relevant to individual animal species, it is essential to determine which cytokines predict functionally important immune responses. For example, IL-4 in mice not only differentiates CD4+ T-cells to the Th2 phenotype, it also is important for B-cell development and antibody production (Kopf et al., 1993; Kuhn et al., 1991). The convergence of IL-4 association with antibody effector function that is wholly consistent with molecular regulation of B-cells argues strongly that IL-4 is a useful correlate of Th2-type immunity. In swine, a reliable assay for IL-4 was not available for many years. Therefore, IL-10 was used as a surrogate indicator of Th2 responses or, even worse, absence of interferon γ was inferred to indicate a Th2-type response. Investigation of the role IL-4 plays in porcine B-cell differentiation and function indicated that, rather than acting as a stimulatory factor, IL-4 inhibited proliferation, suppressed IL-6 secretion, and blocked inducible antibody production (Murtaugh et al., 2009). Hence, the absence of a mechanistic role for IL-4 in B-cell function in swine creates doubt about its value as a type 2 marker. It is possible that IL-13 fulfills this role in swine, since its gene resulted from a duplication of the IL-4 gene (Murtaugh et al., 2009), and it has many functional and molecular characteristics in common with IL-4 (Bautista et al., 2007; Kelly and Locksley, 2000). Again, it is important to establish the validity of key immunological models for specific host-pathogen interactions in veterinary species.

A key aspect of immunology as applied to vaccine development, and the hallmark of vaccine efficacy, is the memory response encoded in antigen-specific B-cells and T-cells. In a primary immune response, antigen-specific lymphocytes proliferate and differentiate into short-lived effector cells whose functional activity can be monitored in blood by the level of specific antibodies or responding T-cells that peak early and wanes over time. An alternative differentiation pathway results in a phenotypically distinct population of quiescent, long-lived memory cells that persist in lymphoid and peripheral tissues even after evidence of the previous infection, i.e. antigen and possibly detectable immunity, is gone. The presence of memory cells is demonstrated in vivo by antigenic challenge that induces an anamnestic, geometric, increase in antigen-specific antibodies and T-cells. The equivalent response can be demonstrated in vitro in a recall response assay. Rigorous application of the memory response principle, either in vivo or in vitro, does not appear to be a standard feature of veterinary immunology or vaccinology research, at least recently in swine, even though great effort has been expended on newer vaccines, including vaccines for PRRSV.

PRRSV is an interesting case. It was thought to be a persistent infection (Wills et al., 1997), which would imply that true memory responses would be difficult to demonstrate in vivo due to the continual presence of antigen that could simply be eliciting active immunity. However, it is now apparent that PRRSV infection, though prolonged, is not persistent; within 200 to 300 days pigs show no sign of infection and lose the ability to transmit infection to susceptible animals (Corzo et al., 2010; Murtaugh and Genzow, 2011; Schaefer and Morrison, 2007; Torremorell et al., 2002). During the course of acute, viremic and prolonged, lymphoid tissue infection, PRRSV-specific memory B-cells peak in number at 40–100 days of infection then decline (Mulupuri et al., 2008). However, re-challenge with genetically similar or unrelated viruses at about 200 days of infection does not elicit consistent changes in antibody titers to various PRRSV proteins (Murtaugh, unpublished

data). B-cells responsive to recall antigens are abundant in a variety of lymphoid tissues, despite the lack of anamnestic response in vivo, suggesting that the absence of an anamnestic response in vivo may be due to the absence of B-cell exposure to antigen. This possibility is supported by the absence of evidence of re-infection upon challenge with live virus (Foss et al., 2002). Highly effective memory immunity in the absence of an anamnestic response is emerging in other cases as an effective form of immunological protection carried out by long-lived effector cells that control pathogens within hours of infection (Masopust and Picker, 2012). While the mechanisms of protective immunity against PRRSV are not fully elucidated, the implication for vaccine development is that standard immunological models of adaptive immunity must not be applied blindly to individual host-pathogen interactions. Rather, the parameters of infection and host immunity need to be determined and immunological effector responses evaluated for usefulness as surrogate measures of protection against future infectious challenge.

Mucosal Immunity

The majority of immunological models are built on systemic infections involving primary lymphoid tissues and spleen, whereas the majority of infections occur at mucosal surfaces, including the gastrointestinal tract, reproductive, and respiratory tissues. Delivery of effective immunological protection at mucosal surfaces has been challenging in all species, including humans. Yet, in swine, bacterial and viral pathogens, including enterotoxigenic and exotoxigenic *Escherichia coli* and rotavirus persist as serious disease threats to neonatal swine, *Brachyspira* is currently re-emerging with new species and disease syndromes, porcine epidemic diarrhea virus appeared in the United States for the first time in 2013, and reproductive disease caused by PRRSV continues to appear epidemically. Various animal and tissue models have been established in swine for investigation of mucosal immune mechanisms and induction of protective memory. They include respiratory models of bacterial and viral infection (Baarsch et al., 2000; Baarsch et al., 1995; Saif, 1996; Saif et al., 1994; VanCott et al., 1994), enteric viral infection and defined antigen models (Brim et al., 1995; Foss and Murtaugh, 1999a, 2000; Hyland et al., 2004; Hyland et al., 2006b; Lanza et al., 1995; Saif, 1999; VanCott et al., 1993), ex vivo secretory tissue preparations in Ussing chambers, and epithelial transwell cell culture systems (Gookin et al., 2004; Hyland et al., 2006a; Schmidt et al., 2007).

Induction of protective immune responses at mucosal surfaces, particularly intestinal mucosa, is a substantial challenge to vaccine development, due to the hostile environment of the stomach, proteolytic activity in the intestine, and an immunologically tolerant state mediated by IL-10 (Kuhn et al., 1993; Rennick et al., 1997). However, studies with the model mucosal antigen, cholera toxin, showed that highly efficient oral immunization can be accomplished, and that oral immunization in swine can deliver B-cell and T-cell immunity to local mucosal sites, distant mucosal sites, and systemically (Foss, 1998; Foss and Murtaugh, 1999a, 2000; Hyland etal., 2004). Importantly, the mucosal immunogenic properties inherent in the cholera toxin B subunit can be transferred to an irrelevant antigen, though the magnitude of the effect needs to be increased substantially for vaccinal applications (Hyland et al., 2004). The cholera toxin model in swine revealed that oral immunization produced a strongly biased IgA secretory response both locally in small

intestine and distantly in saliva (Foss and Murtaugh, 1999a, b). The same antigen and adjuvant formulation administered parenterally induced a largely systemic response dominated by IgG isotypes (Foss and Murtaugh, 1999a, b). Oral immunization also produced systemic T-cell mediated responses shown in vivo by delayed-type hypersensitivity reactions upon skin testing (Foss, 1998).

Conclusion

Going forward, veterinary immunologists are better equipped that at any time in the past to elucidate basic immunological processes in animal species to relevant disease agents, and to apply this knowledge to vaccine development. Direct characterization of immunological processes in relevant host-pathogen interactions is critical since translational approaches involving mouse and other model systems are poor predictors of efficacious vaccine development. Thus, unbiased experimental approaches embracing high throughout characterization of gene expression patterns, elucidation of protein profiles by mass spectrometry, and efficient methods for genetically engineering animals provides opportunities to understand mechanisms of immune induction in mucosal and systemic lymphoid tissues, effector function at the periphery, and regulatory interactions that facilitate maximum protection.

Major challenges still exist in veterinary immunology and vaccinology, particularly with respect to in vitro systems for antigen-specific T-cell culture and cloning, but these obstacles are surmountable. In swine, recent completion of the porcine genome is likely to stimulate a wave of research and publications that build on previous work to greatly expand our molecular and genetic understanding of porcine immunology, and provide a critical framework for new tool development (Dawson et al., 2013; Groenen et al., 2012; Haverson et al., 2001; Lunney et al., 1994; Saalmuller et al., 1998). Purified protein and antibody reagents are increasingly available to elucidate immune developmental and regulatory pathways in animal species, as are molecular tools and sequence data for characterization of humoral and cellular immune effector functions (Butler, 1998; Butler et al., 2006; Butler et al., 2001; Butler et al., 2009; Butler et al., 2004; Eguchi-Ogawa et al., 2009; Eguchi-Ogawa et al., 2012; Eguchi-Ogawa et al., 2010; Honma et al., 2003; Schwartz et al., 2012a, b; Sinkora et al., 2002; Uenishi et al., 2009). Methods for recombinant antibody expression and tetramers for antigen-specific T-cell responses in swine have been developed (Butler et al., 2013; Patch et al., 2011). These advances significantly enhance vaccine development by providing mechanistic models for interpretation of experimental data, and use of surrogate immunological endpoints that predict in vivo outcomes.

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