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New approaches to innate immunity in livestock and the potential for manipulation - review

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NEW APPROACHES TO INNATE IMMUNITY IN LIVESTOCK AND THE POTENTIAL FOR MANIPULATION – REVIEW

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The Innate Immune System –sculpting the animal immune response

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The Innate Immune System –sculpting the animal immune response

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OVERVIEW

The innate immune response refers to the primitive, evolutionarily conserved, immune system that responds rapidly to a foreign stimulus. It has been long known to involve resident immune cells such as macrophages and dendritic cells (DC) and acts in consort with other "innate" protective mechanisms such as physical barriers (e.g. mucous), chemicals (e..g. free radicals), temperature controls, to form the body's front-line defence systems. Not only does the innate immune response provide rapid defence, but also produces cytokines that amplify more innate responses, and importantly sculpts the ensuing adaptive immune response. These responses are mediated by cytokines and chemokines that are responsible for the development, recruitment and activation of infiltrating cells.

Thus, the innate immune response is the basis of the inflammatory response. The cardinal signs of *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain) and *functio laesa* (loss of function), are the result of increased blood flow, vascular permeability and cell infiltration caused by the cytokines and chemical products released from stimulated innate immune cells. These signs have been known for centuries and the cellular and cytokine details elaborated in recent decades. However, is only in the last 14 years that our appreciation of the innate immune system has been shaken from the now apparently naïve notion of an essentially non-specific response, to one with a degree of specificity and structure, albeit not as elaborate as the adaptive immune response. The discoverers of this response won the 2011 Nobel Prize for this seminal contribution (*http://www.nobelprize.org/nobel_prizes/medicine/laureates/2011*).

This revolution in our understanding of the innate immune system began with the discovery of the Drosophila Toll mutant as a developmental anomaly and subsequent demonstration of the role of the Toll receptor in protection from infection [1]. This was quickly followed by noting homology of the Toll gene with the interleukin-1 (IL1) cytokine family implicating a role in immune response [2], identification of mammalian homologues, designated Toll-like

Receptors (TLRs) and demonstration of TLR4 as mediating the sensing and response to the prototypical inflammatory stimulus, lipopolysaccharide (LPS) [3]. Since these discoveries were occurring at a time of enormous technological advances such as high throughput DNA and RNA sequencing, high density microarrays and generation of gene targeted mice for *in vivo* validation of function, there was an explosion of discovery in the ensuing years [4]. These led to the identification of a family of up to 13 TLRs in mammals and the rapid identification of the signal transduction components such as the adaptor molecules, kinases, transcription factors and sets of genes involved in the innate response (Fig.1). The families of receptors that initiate innate immune responses to pathogens grew as it was realised that the TLRs could not explain responses to all pathogens. These included RIG-I-Like Helicases (RLHs), DNA sensors [4], Nod-Like Receptors (NLRs) [5] and C –type Lectins [6]. The expanded family of receptors which co-evolved with pathogenic microorganisms are commonly known as Pathogen Recognition Receptors (PRRs). Accordingly, the molecules that trigger these receptors are known as Pathogen Associated Molecular Patterns (PAMPs) [7, 8]. PAMPs are present in almost all invasive organisms, from bacteria to viruses and fungi, and are derived from the major families of molecules found in these organisms, including cell surface glycolipids,, nucleic acids, lipids, proteins and chemicals (Fig.1). The innate receptors or PRRs also recognise endogenous non-pathogen ligands such as extracellular matrix components, nucleic acids from dying or damaged cells, immune complexes, misfolded proteins and crystalline aggregates (e.g. uric acid, amyloid protein and cholesterol) which are referred to as Danger Associated Molecular Patterns (DAMPs) [9, 10].

The elaboration of components of the innate immune response has enabled a broader appreciation and definition of the innate immune system (Fig.1). While first thought to involve mainly resident immune cells such as macrophages and dendritic cells (DC), we now recognise it to also involve epithelial and other parenchymal cells that may form the first point of contact with the pathogen or stimulus. Our understanding of the innate signaling pathways that are upstream regulators of the cytokines that mediate immunity and inflammation, has opened the door to understanding the basis of diseases of the immune system, inflammatory diseases and cancers. Genes encoding innate immune signaling components are subjects of mutations in disease as well as targets for suppressive mechanisms evolved by pathogens to evade the host defences. The molecules within the innate pathways can be used as biomarkers of an immune response, genetic stratification for immune competence or disease susceptibility and as targets for therapeutics. Knowing and refining ligands to activate these pathways has informed vaccine and drug development efforts. Harnessing and expanding our knowledge of innate immune pathways and outcomes across species will be a crucial part of improved health in human, veterinary and agricultural endeavours [11].

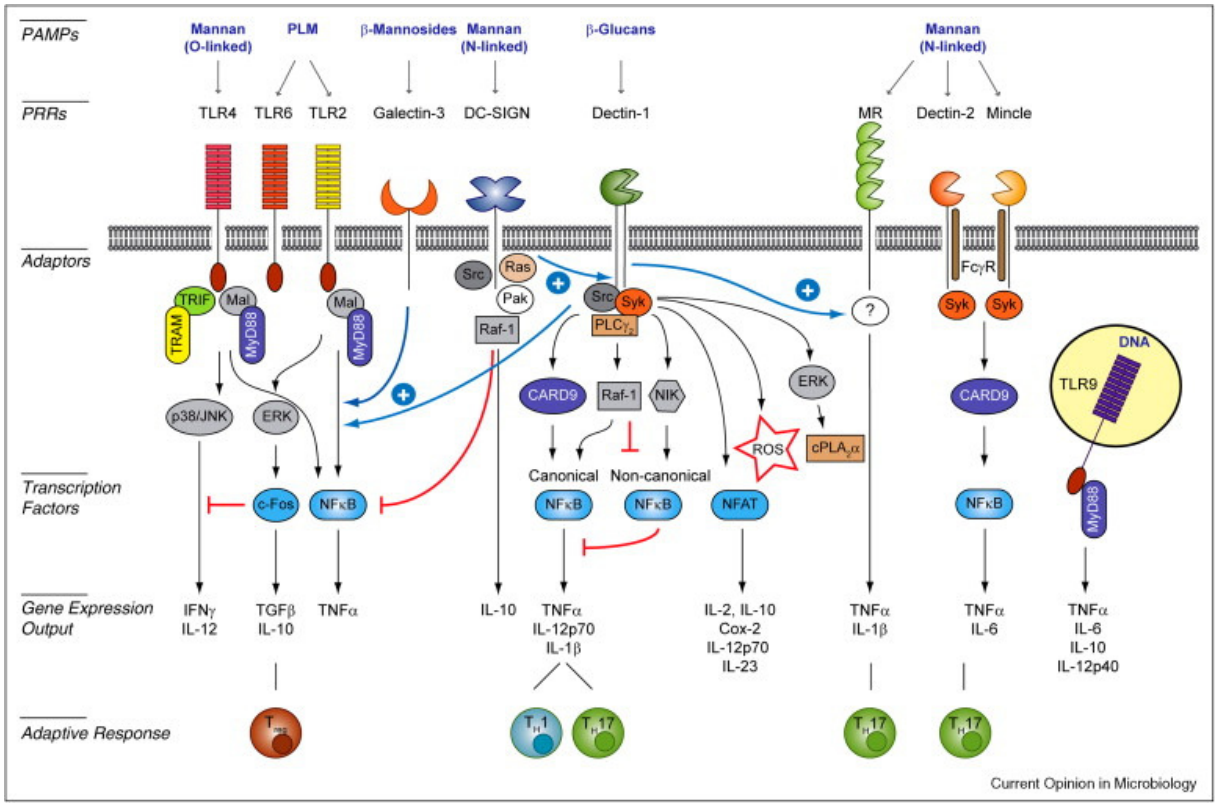

Figure 1. Schematic representation of the innate immune response to fungal pathogens, triggered by the recognition of fungal Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRR) expressed on host cells. The ensuing intracellular signaling events lead to defined adaptive immune response outcomes.

"*Surface receptors from the Toll-like (TLRs) and C-type lectin families as well as endosomal TLRs participate to the recognition of fungal PAMPs (e.g., O-linked and N-linked mannans, phospholipomannan (PLM), β-mannosides, β-glucans and DNA). Integration of simultaneously activated signaling pathways occurs at the level of signaling adaptors and transcription factors shared between overlapping pathways. The resulting cytokine responses, oxidative burst and arachidonic acid release following activation of cPLA2α, in turn shape the activation of the adaptive response and ultimately determine the outcome for the host. Abbreviations: ROS: reactive oxygen species; cPLA2α: cytosolic phospholipase A2α; MR: mannose receptor*" [12]

CYTOKINES MEDIATING INNATE IMMUNE RESPONSES

During the last 50 years the existence of soluble factors that mediate inflammatory and immune responses were described. These usually were given names that described the function by which they were first discovered, e.g. tumour necrosis factor (TNF), interferon (IFN), and colony stimulating factors (CSF). Many were characterised as contributing to the process of inflammation or regulating innate and/or adaptive immune responses. Since the primary pathways of innate immune recognition results in the production of different types or proportions of cytokines and thus different pathophysiological consequences, it was decided to first provide brief overviews of some of the major cytokines that mediate innate responses.

Inflammatory Cytokines. This term usually refers to the group of cytokines including tumour necrosis factor (TNFα), interleukin 6 (IL6), interleukin 1 (IL1) and assorted chemokines. The essential pro-inflammatory role of these cytokines has been verified in many models and antagonists (antibodies or soluble receptors) of these 3 cytokines have been used clinically to treat inflammatory disorders such as rheumatoid arthritis [13, 14].

TNFα is a cytokine (formerly known as cachectin) [15], that is produced mainly by activated macrophages but also by T cells and NK cells during inflammation. It is first produced in an inactive form that is anchored to the cell membrane; the active soluble form is generated by proteolytic cleavage by the protease enzyme ADAM [16]. TNFα can bind two receptors, TNFR1 (widely expressed and responds to both soluble and membrane-bound TNFα) [17] or TNFR2 (expressed mainly on immune cells and responds to membrane-bound TNFα). Binding of the TNFR activates the expression of effector genes involved in the inflammatory response and regulation of cell survival/death. TNFα can induce fever and cell death (apoptosis) and contributes to inflammation and septic shock [18].

IL6 is another proinflammatory cytokine produced by activated macrophages, T cells and other cell types. It acts through a receptor complex composed of a so-called IL6 Receptor α subunit which is the primary binding chain, and a signal transducing chain called gp130 which is shared by the related cytokines, IL11, LIF, CNTF and oncostatin M [19]. IL6 signals through a pathway used by many cytokines involving receptor-associated Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) which move to the nucleus and activate gene transcription [20]. IL6 can be pro- or anti-inflammatory depending on the cell context and stimulus and it is also involved in osteoclastogenesis. IL6 can also signal by binding to a soluble form of the receptor (sIL6Rα) and then signaling via gp130, a process called trans-signaling. The different types of signaling may help explain its occasionally opposite biological effects [21].

IL1 is a proinflammatory cytokine initially identified as two subcomponents, IL1α and IL1β [22]. They are produced by macrophages, fibroblasts and other immune cells. IL1β plays an important role in inflammation associated with infection as well as so-called sterile inflammation (refer below). It is an endogenous pyrogen as it induces fever. IL1β is produced as a pre-protein and similarly to TNF is cleaved into an active form, although the precursor protein is cleaved by Caspase1. There is now a family of IL1-like proteins including

IL1Receptor antagonist, IL18 and IL1F5, 6, 7, 8, 9 and 10 whose functions are just being characterised [23]. IL1F7 has been recently named IL37 and has an anti-inflammatory action [24].

Chemokines These are a family of small proteins 8-10kD which are produced during inflammatory or immune responses and attract immune cells from the blood or tissues [25]. The chemokines are a large family of genes with 20-50% amino acid homology and are now classified and named into four groups, according to the arrangement of the first two of their four cysteine residues: CCL, CXCL, XCLs and CX_3CL. Chemokines are produced at the site of inflammation or infection by innate immune cells and each type binds a specific receptor on one or more immune cells. This results in the cell moving towards the site of chemokine production which is usually also the site of infection. CCL2 (aka MCP1) is a chemokine that attracts monocytes and NK cells [26]. CCL5 (aka RANTES) attracts lymphocytes, eosinophils and basophils. CXCL8 (aka IL8) is responsible for the recruitment of neutrophils from the bloodstream into an inflammatory site [27]. CXCL13 recruits lymphocytes while XCL1 (aka lymphotactin 1α) and XCL2 (aka lymphotactin 1β) attract T cells. A more comprehensive review of matching chemokines and their receptors which are occasionally redundant can be found in [28]. Chemokine receptors are G protein coupled receptors (7 transmembrane proteins) which transduce signals via the intracellular G proteins that activate the lipid based secondary messengers inositol phosphate and diacylglycerol. Interestingly, the family of G-protein coupled receptors are considered one of the most "druggable" targets in the pharmaceutical industry. Chemokines are key products of the innate immune response because they not only recruit the first round of acute inflammatory cells such as macrophages and neutrophils, but also link to the initiation of an adaptive response by shaping the nature of T and B cells that are recruited and activated.

Anti-inflammatory cytokines: ***IL10*** is a cytokine that can be produced by macrophages, T cells and regulatory T cells. It can downregulate the production of inflammatory cytokines and inhibit immune responses by decreasing expression of MHC class II and co-stimulatory molecules. Gene knockout studies in mice have implicated a particularly important role of IL10 in inflammation in the gastrointestinal tract. Similar to other cytokines, IL10 binds two specific receptors and activates the JAK/STAT signaling pathway [29, 30].

TGFβ is a member of a super family of cytokines that also includes activins, inhibins, bone morphogenic proteins and others [31]. It is associated with many physiological, pathological and developmental processes [32]. Its role in controlling inflammation and innate immune responses is complex and can be pro- or anti- inflammatory depending on whether it is regulating adaptive (T) cells or innate cells, respectively [33].

IFNs are usually known as antiviral proteins, but like others they actually have pleiotrophic effects that are sometimes overshadowed by the name and means of discovery. IFN was discovered over 50 years ago as a factor that inhibited viral replication [34, 35]. We now know there are 3 types of IFNs which are distinguished by their amino acid sequence, production cell and stimulus and cognate receptors. Type I IFNs are a family of 14-20

cytokines (IFNs α, β, ω, κ τ in ruminants only, and ε) [36]. They are induced in response to virus and bacteria and some endogenous ligands (see below) [37]. Type II IFN is a single IFNγ whose production is stimulated by proliferating T cells and NK cells. It is has potent immunoregulatory activities (and relatively weak antiviral potency) and is associated with a Th1 type immune response. Type III IFN (aka IFNλ, IL28, 29) is produced in response to viral infection. All have the definitive antiviral action, but with different potencies. Most importantly, IFNs can affect diverse cell biologies including cell proliferation, growth, senescence, survival, motility, differentiation and other specialised functions [38]. IFNs signal through the JAK/STAT pathway which results in the activation and nuclear translocation of latent transcription factors, Signal Transducers and Activators of Transcription (STATs) [39]. These pathways modulate the expression of Interferon Regulated Genes (IRGs) which encode the effector proteins of the IFN response [40]. Most of these cytokine responses are designed to be transitory. There are about 2,000 potential IRGs according to a database of IRGs called the Interferome (i*nterferome.org*) [41]; however, usually only hundreds are regulated in a given experiment. This database is designed to identify sets of IRGs in different scenarios and their contribution to particular biological and pathological responses (examples include breast cancer metastases, HIV response, vaccine responses).

IFNs are used therapeutically to treat viral infections such as HCV and HBV [42], multiple sclerosis and various cancers. IFN induction is a key driver of vaccine adjuvants but to date the preference has been to include the inducer in formulations (see later). However, it can exhibit dose-limiting toxicities and contribute to diseases such as Systemic Lupus Erythematosus (SLE). Indeed IFN blocking antibodies are currently being trialled in the treatment of SLE [43].

PRIMARY CELL TYPES ASSOCIATED WITH THE INNATE IMMUNE SYSTEM:

The innate immune system is extremely broad and includes many cell types. Most cells that may encounter pathogens express innate immune receptors (PAMPs and DAMPs) however we will outline the main types of cells thought to mediate the predominant effects of the innate immune response. These are the cells of the immune system that are resident or recruited to tissues upon infection and mediate the duration and severity of inflammation (Fig. 2). They also travel to the draining lymph node to influence the development of the adaptive immune response.

Macrophages and monocytes: Macrophages are tissue resident cells that are highly phagocytic and express a broad array of PAMPs and DAMPs; they are critical in both the long term and short term response to an inflammatory stimuli. They can mature in response to pathogens to express MHC class II (MHCII) molecules and travel to draining lymph nodes where the MHCII-bound peptides can activate naïve T-cells. At the site of infection they typically transform into two functional types following stimulation: M1 (or classically

activated) macrophages and M2 (or alternatively activated macrophages). M1 macrophages are involved in the early response to pathogens, are proinflammatory producing cytokines such as TNF, IL1 and IL6 and typically promote Th1 adaptive immune responses. M2 macrophages are induced during the resolution phase of a response and are involved in tissue repair and produce anti-inflammatory cytokines such as IL10 and TGFβ [44]. Monocytes are found in the blood and sparsely within tissues, they are highly phagocytic and are rapidly recruited to sites of infection. They can mature into dendritic- or macrophage-like cells and travel to the draining lymph node only in response to pathogen stimuli. They also express inflammatory and immunomodulatory cytokines such as IL-8, TNF-alpha and IL-6.

Dendritic cells: Dendritic cells (DCs) are highly mature cells specialized to present antigen to naïve T-cells and initiate an adaptive immune response. They are found in an immature form within tissues and mature to express very high levels of MHCII and co-stimulatory molecules (required for efficient activation of naïve T-cells) before they migrate to the lymph node. If they encounter no pathogenic stimuli in their time within the tissue they are thought to "tolerise" T-cells and probably B-cells within the lymph node so they do not respond to the antigen which is likely to be a self antigen. This is known as peripheral tolerance and is likely what most DCs spend their lives doing. It is only after an inflammatory innate immune stimuli is received that DCs induce the antigen-specific proliferation of T-cells and B-cells that is so characteristic of an adaptive immune response. They present the pathogenic antigens to the naive T-cells in the context of MHCI and MHCII which is one essential for T-cells to become activated and proliferate; DCs are particularly efficient at this due to their high MHCI and MHCII expression. The other essential signals are delivered by DCs, monocytes, neutrophils and macrophages which all produce inflammatory cytokines within the lymph node which give the second signal for T- and B-cells to proliferate if they were not previously suppressed.

Neutrophils: Neutrophils are highly effective at capturing pathogens and can even chase down and "eat" mobile bacteria by following a trail of bacterial chemicals. They are highly motile and are rapidly recruited to the site of infection by chemokines and other mediators of inflammation. There they "hunt" for pathogens and rapidly degrade any they find before either dying or travelling to the draining lymph node. Neutrophils express very high levels or inflammatory cytokines and are critical mediators of early inflammatory responses. Little is understood about their role once they exit the site of infection.

NK-cells: NK-cells are a type of "innate" T-cell that, similarly to antigen-specific cytotoxic T-cells, can kill other cells. The major distinction is that they do this in an antigen independent manner; it is thought they recognize stressed cells within an inflammatory environment and kill these by releasing cytotoxic granules and other factors that induce cell death. NK-cells can also produce potent cytokines and mediators of immunity. Thus they lie at the interface of innate and adaptive cells; similarly to all "innate" recognition mechanisms they do not require specific recognition of antigen but rather act through gene encoded non-variant receptors to recognize target cells. However, they also can form a "memory"

phenotype where they proliferate and remain at the site ready to reactivate if the appropriate signals are received. They are being very actively studied for their role in cancer killing.

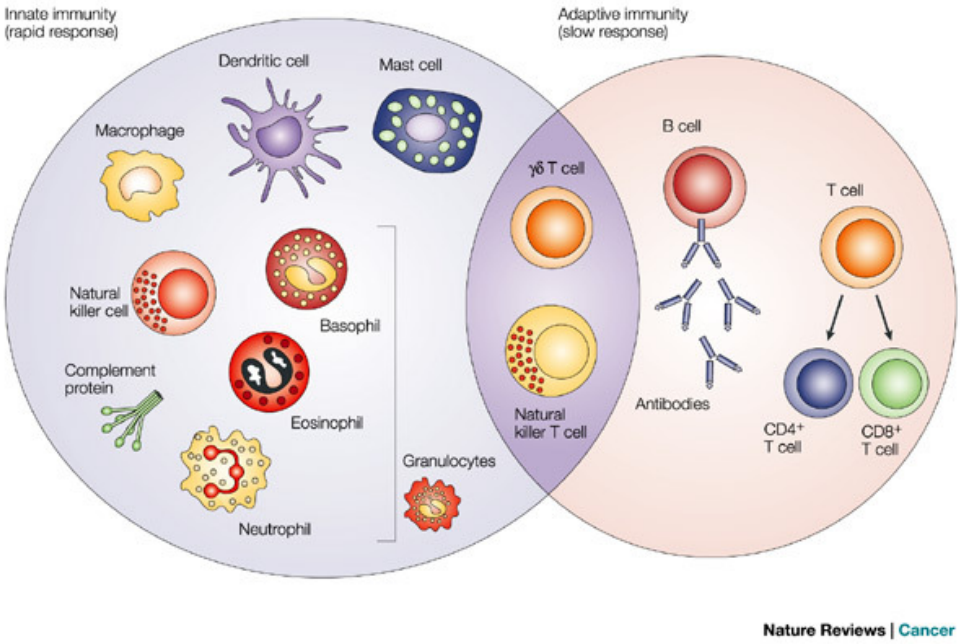

Figure 2: The innate immune response functions as the first line of defense against infection. It consists of soluble factors, and diverse cellular components . The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. [45]

INNATE SCULPTING OF ADAPTIVE IMMUNE RESPONSES.

The innate immune system that is described in detail below orchestrates the production of signaling molecules including the premier cytokines and chemokines outlined above, and these in turn determine the nature of the adaptive response that follows days or weeks later. The strength and duration of innate immune signaling will determine the nature and number of cells that infiltrate the lesion and later the draining lymph node. These begin as so-called acute inflammatory cells such as macrophages, monocytes and neutrophils which are rapidly recruited from the blood. Tissue resident cells such as tissue macrophages and dendritic cells can also participate in the establishment of immunity by collecting innate stimuli within the peripheral tissues and migrating to the lymph node where they can deliver potent activatory signals to naive T and B cells. Not only do the cytokine products of innate immunity determine the timing and numbers of cells, but also their state of differentiation and activation – all of which can be determined by well characterised cell surface markers (at least in murine and human systems). In summary, the nature and "quality" of the innate response determines the nature (success or failure) of the adaptive response.

PATTERN RECOGNITION RECEPTORS –INNATE RECOGNITION AND SIGNALLING

TOLL LIKE RECEPTORS (TLRs).

TLRs have a characteristic structure of an extracellular domain composed of multiple Leucine Rich Repeats (LRRs) whose composition and number convey a diversity of recognition properties to the individual TLR members. This is the principal reason why the TLR receptors are able to recognise such a diverse array of ligands present on or within highly divergent pathogens. The intracellular portion of the TLR proteins are characterised by a common Toll IL1 Receptor (TIR) domain which is also found in the TLR signaling adaptors and the IL1 family of receptors and is the region that determines which adaptor molecules bind with each TLR [46].

TLR4 was the first mammalian TLR to be identified, as a mutation in the gene in C3HEJ mice made them resistant to LPS [3]. It has been arguably the most extensively studied and will be covered in some detail here, as many of the signal transduction pathways and general principles also apply to other PRRs [4, 10, 47, 48]. TLR4 is localised at the plasma membrane where it recognises LPS bound to a protein called MD2 an interaction that is facilitated by soluble or surface bound CD14. Once engaged and dimerised, TLR4 recruits the TIR containing adaptors Mal (or TIRAP) and MyD88. This is followed by the recruitment of cellular kinases and signaling molecules which ultimately lead to the activation of the AP-1 and NFκB transcription factors which activate TLR induced gene expression. The target genes of this pathway include the proinflammatory cytokines, TNFα, IL6, IL1 and some chemokines [4, 10, 47, 48].

Once TLR4 has bound ligands and initiated the MyD88-Mal-NFκB signaling pathway from the cell surface, the receptor is internalised in an endosome at which point two additional TIR-containing adaptors, TRAM and TRIF are recruited. These subsequently activate the transcription factor IRF3 which then translocates to the nucleus and drives the transcriptional activation of IFNβ. These two transcription factor pathways of NFκB and IRF, driving inflammatory cytokines and type I IFNs, respectively are a common feature of many PRR sensing and signalling responses. The TLR4 response is unique in that IFNβ is the only type I IFN produced [49]. For other PRRs it is more common to activate the expression of several IFNα subtypes and some IFNβ –via activation of different combinations of IRF family members. Mice deficient in an IFN response (IFNAR, IRF3 or IFNβ null) are resistant to models of LPS induced septic shock [50, 51]. The absence of IL6, TNFα or IL1 signaling also reduces septic shock in mice demonstrating that many of these pathways act in consort to elicit pathology. TLR4-null mice do not develop septic shock when administered LPS, but can develop septic shock to other TLR ligands. TLR4 recognises LPS which is a major component of the cell wall of gram negative bacteria, as well as other ligands including endogenous molecules such as heat shock proteins and in injured lung, oxidised phospholipids.

TLR2 recognises a broad range of ligands from bacteria viruses and fungi usually in a heterodimeric complex with ***TLR1 or TLR6*** [48, 49]. The TLR 1 and 6 genes occur in a locus with ***TLR10*** which has also been shown to share ligands with TLR1 and the requirement of being a co-receptor with TLR2, but appears to lack similar downstream signaling pathways. Since the TLR10 gene in the mouse is a pseudogene, its function cannot be determined by the usual mouse knockout approach. The TLR2:1 complex recognises triacyl lipoproteins whereas the TLR2:6 complexes detect diacyl lipoproteins [52, 53]. Like TLR4, these TLRs are located on the cell surface recruit TIR adaptors MyD88 and Mal, but usually not TRAM/TRIF and thus activate the NFκB pathway (via similar adaptors and kinases as TLR4), but not the IRF/IFN pathway.

TLR5 is less well characterised than other TLRs and has one well characterised ligand in bacterial flagellin [54]. Mice studies have also implicated a protective role for TLR5 in intraperitoneal infections with *S.typhimurium, P. aeruginosa* and urinary tract infection with *E coli;* but it can enhance infection in mice orally innoculated with *S.typhimurium*. Thus the route of infection and likely the cell responding to the PAMP stimulation is important to the ensuing response and the impact on the host. It appears to signal via similar pathways as TLR2 and 4 to induce NFκB dependent genes. TLR 5 has some homology with ***TLR11*** which is present in mice but not in humans and recognises uropathic bacterium and a PAMP from protozoan *Toxoplasma gondii* [4, 48, 49].

TLR3 is located in intracellular endosomes and senses viral double stranded RNA from viruses. It is also involved in the recognition of the synthetic ligand poly I:C which has potent IFN inducing and immunoregulatory effects[55, 56]. It signals via the recruitment of the TIR adaptor, TRIF followed by TRAF3 and activation of Tbk/IKKε which activates IRF3 and drives type I IFN production. TLR3 is notable as the only TLR signaling pathway that does not utilise MyD88 in its signal transduction[4, 48, 49]..

TLR7 and TLR8 are also located in endosomes where they recognise viral single stranded RNA and small purine analogues (imidazolquinolines such as imiquimod which are used as wart therapies and as potential vaccine adjuvants) [57]. The genes for these two TLRs are collocated on the X chromosome and as a consequence they have been associated with sex-liked disease such as autoimmune diseases which have a component of aberrant activation of the innate immune system by nucleic acid complexes. These features have led to these two TLRs often being grouped together but there is emerging evidence that TLR8 which has been suspected to be non-functional has independent roles in the innate immune response. These TLRs recruit the TIR adaptor MyD88 to activate the NFκB pathway as described above. TRAF3 recruits and activates IKKα and IRF7 which is phosphorylated by IKK and IRAK then translocated to the nucleus to activate the expression of type I IFNs (mostly IFNα subtypes) and other IRF7 target genes including chemokines [4, 48, 49].

TLR9 is another endosomal TLR which recognises CpG motifs in unmethylated bacterial (and some viral) DNA. TLR9 induction of type I IFN and NFκB pathways are similar to those of TLR7/8 [4, 48, 49, 58].

TLR 13 has long been an orphan receptor that is expressed in mice but not humans. A recent study identified a conserved bacterial 23S ribosomal RNA (rRNA) sequence as a ligand for TLR13, and interestingly the sequence recognized by TLR13 when bound by certain antibiotics, cannot be detected by TLR1[59].

RIG-I LIKE HELICASES (RLHs).

While the TLRs 3,7,8 and 9 can sense microbial nucleic acids in endosomal compartments, many viruses replicate in the cytoplasm and here they are sensed by Retinoic acid Inducible Gene – I (***RIG-I***) [60] and its homologues Myeloid Differentiation Associated gene 5 (***MDA 5***) [61] and ***LGP2***. RIG-I is the prototype of this family and is composed of a helicase domain that is responsible for RNA recognition and a Caspase Recruitment Domain (CARD) that initiates downstream signaling. RIG-I recognises the genomic RNA of dsRNA viruses and dsRNA generated as a replication intermediate of ssRNA viruses. Since it recognises uncapped 5'-Triphosphate groups, RIG-I can distinguish between endogenous RNA which is capped with a 7 methyl guanosine group. MDA 5 recognises the long dsRNA which is produced during replication of sense-strand ssRNA viruses [61, 62]. LPG2 binds viral RNA, but by contrast has no CARD domain and its role in RNA sensing is controversial with both competitive inhibition and facilitation of RIG-I and MDA5 signaling reported[63]. Thus RIG-I is necessary for initiating innate responses to flaviviruses, paramixovirusesm orthomyxoviruses, whereas MDA5 is necessary for the responses to picornaviruses. Both appear to be required for optimal responses to rotaviruses and rhinovirus [5, 62, 63].

CYTOPLASMIC RNA SENSORS

NOD2 is well characterised as a sensor of bacterial peptidoglycan (see below) but has also been recently shown to sense ssRNA and then recruits the adaptor MAVS via its LRR domain rather than a CARD domain like the RLRs [64]. This is important in the induction of type I IFNs in response to VSV and RSV [65].

DDX1, DDX21 and DHX 36 These three members of the DEAD/H-box helicases which act as a triad to sense poly I:C. This complex then recruits TRIF which activates IRFs and consequent type I IFN production. Interestingly these DNA sensors are expressed at relatively high levels in cells, compared to the RLRs which are lowly expressed and induced by IFN signaling [63, 66].

CYTOPLASMIC DNA SENSORS

Cytoplasmic DNA sensors are indicated for DNA viruses, synthetic DNA (poly dA:dT rich) and DNA vaccines, and are able to elicit IFN responses independently of TLR9 [67, 68]. The first DNA sensor conclusively characterised was ***STING***, a dsDNA recognition, endoplasmic reticulum localised protein [67, 68]. Its activation is followed by translocation to other subcellular organelles and association with Tbk and activation of IRF3 and IFNβ transcription. STING has also been identified as the innate sensor of a relatively new class of bacterial signaling molecules, the cyclic dinucleotides such as c-diGMP which mediates IFN

production [69]. Evidence of NFκB pathway activation by STING is weak. Thus STING is involved in the sensing and activation of antiviral pathways in response to viruses such as as vesicular stomatitis virus (VSV), cytomegalo virus (CMV) and herpes simplex virus-1 (HSV1), as well as bacterial infections such as *Lysteria monocytogenes*. Recent studies cast doubt on whether STING actually binds to cytosolic DNA and suggest that upstream DNA sensors are required for initial DNA sensing [63, 70].

DAI was identified as a DNA sensor that triggered IRF3 driven IFN transcription, but the knockout mouse raised questions about whether it had a redundant or cofactor role. It stimulated IFN production in response to synthetic DNA and HSV1[71].

RNA polymerase III recognises cytosolic AT rich DNA but rather than behaving as a direct sensor and activator of signalling, it generates RNA which is sensed and signals via RIG-I [72].

LRRFIP1 is a Leucine Rich Repeat containing DNA sensor which activates IRF3 by interaction with β-catenin which subsequently results in recruitment of p300 and activation of type I IFN genes [73].

DHX36 and DHX9 are members of the DEAD/H-box helicases which sense CpG DNA motifs. These interact with MyD88 and DHX9 triggers nuclear translocation of the p50 subunit of NFκB, whereas DHX36 triggers IRF7 activation of IFNα transcription [74].

DDX41 is responsible for sensing cytosolic DNA in the early phases of infection by viruses such as HSV1 and adenovirus and activates the IFN antiviral pathway [75].

Ku70 a protein involved in DNA repair, senses dsDNA and triggers the transcription of type III IFN via IRF1 and 7 [76].

IFI16 is a member of the family designated pyhin because it possesses a pyrin domain as well as a HIN200 domain [63, 70]. It senses synthetic and viral DNA and has been suggested to act upstream of STING in the induction of type I IFNs via Tbk and IRF3. The closest murine homologue, p204 has also been shown to be essential for the activation of NFκB and IRF3 pathways driven by HSV1[77]. Another member of the PYHIN family, *AIM2,* is another cytosolic DNA sensor that forms an "inflammasome" with ASC to trigger caspase 1 activation and production of mature IL1 via a mechanism similar to the NLRP sensors described below [78].

NOD –LIKE RECEPTORS (NLRs).

Members of the NLR family of cytosolic receptors contain N terminal protein interacting domains such as CARD, Pyrin or bacculovirus inhibitor of apoptosis protein repeat (BIR); central nucleotide oligomerisation and binding domain (nod or Nacht) and C terminal LRRs [79]. The LRR domains are responsible for ligand recognition. The Nod domains are

involved in aggregation of NLRs which is necessary for their signaling. The CARD domains recruit signaling adaptors such as RIP2 which links Nod1 and 2 to activation of the NFκB and MAP kinase pathways. Nod Like Receptor Proteins (NLRPs or NLRs) do not contain CARDs signal by recruitment of an adaptor called apoptosis associated speck-like protein containing a CARD (ASC) which is a central adaptor for many NLR proteins. The pyrin domain of ASC binds the NLR while the CARD domain recruits a Caspase which is a potent protease more commonly associated with cell death pathways or apoptosis. The large complex formed by many copies of the NLRP, ASC and Caspase proteins is the so-called "inflammasome" (Fig. 3) which serves to activate the latent caspase[80]. This caspase then cleaves the inactive pro-form of IL1 (also IL18 and IL33) generating a strong IL1 inflammatory stimuli. Interestingly very few cells express the pro forms of IL-1, 18 or 33 proteins unless they are induced via another innate stimuli, this is typically supplied via a TLR pathway which rapidly upregulates pro-IL-1 but no cleavage to active IL-1 (Signal 1). Activation of the NLRP proteins then supply signal 2 to activate the newly produced IL-1 via the recruited caspase. This is a tightly coordinated process as IL-1 is a potent inflammatory cytokine. NLRs are particularly involved in inflammation in the gastrointestinal tract, whether it is in response to pathogens or controlling the homeostasis with commensal microorganisms [5].

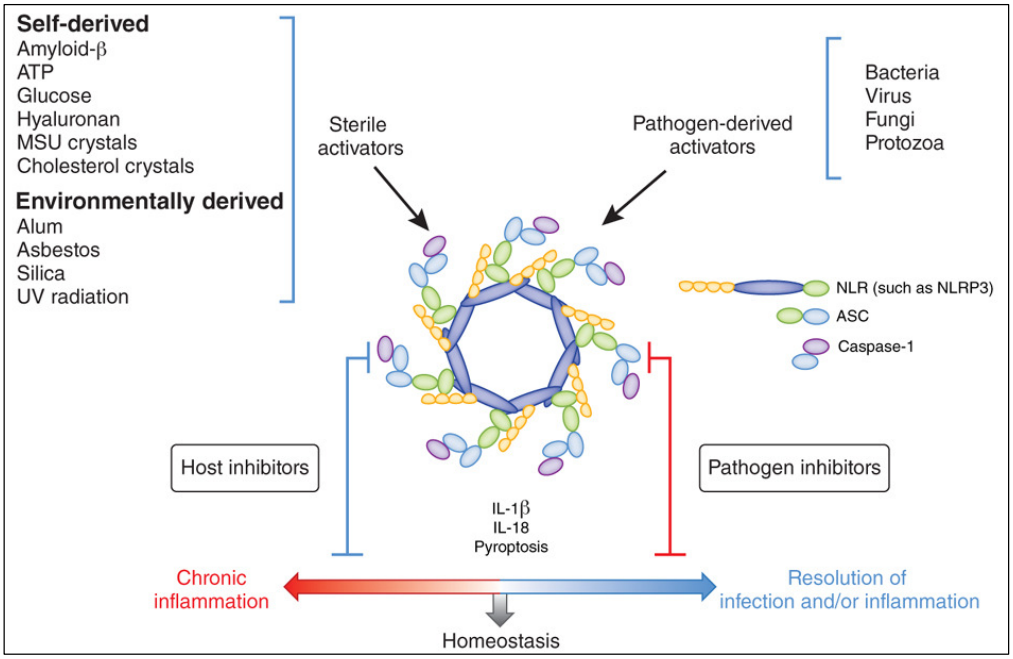

Figure 3: During infection or injury, inflammasomes are directly or indirectly activated by a wide array of danger-or pathogen-associated molecular patterns. The initial event leads to activation of caspase-1, release of IL-1b and IL-18 resulting in recruitment of effector cell populations of the immune response and tissue repair. Pathogen-derived inhibitors block inflammasome activation and thus the resolution of infection, whereas host-derived inflammasome inhibitors prevent the perpetuation of chronic inflammation. [81]

The NLR family of proteins mediate the activation of IL-1 proteins and subsequent innate immune responses by a hugely diverse array of ligands. It is somewhat unclear whether the NLR proteins are primary receptors for these ligands or are signaling molecules as there are no reported structures of NLR proteins associated with defined PAMP or DAMP molecules. They are probably not involved in direct recognition of these structures but rather assemble the complex needed to activate the caspase proteases while other pathways direct recognition of the diverse range of cellular stresses outlined below.

NOD1 Nod1 was identified as the gene responsible for recognition of *Shigella flexneri* in epithelial cells, independently of TLRs and responsible for NFκB driven production of the proinflammatory cytokines, IL6 and TNFα [82]. Nod1 recognises structures in bacterial cell wall proteoglycans. Since components of these are also recognised by TLRs, there is often synergy between TLRs and Nods in the response to bacterial infection. Nod 1-/- mice are more susceptible to *Chlostridium difficile,* correlating with reduced expression of the chemokine, CXCL1 and neutrophils infiltration [83]. Nod1 has also been shown to be responsible for the detection of peptidoglycan in *Helicobacter pylori* and driving the inflammatory response modulated by typical cytokines including CXCL8 (IL8) [84].

NOD2 Nod 2 was identified through its association with inflammatory bowel disease (IBD) [85]. It is, like Nod1, a cytoplasmic receptor that recognises muramyl dipeptide in bacterial cell wall peptidoglycans. The Nod 2 protein domain structure is similar to Nod 1 except it contains an additional CARD domain. Like Nod1, Nod2 recognises and responds to microbiota [5]. Many bacteria have ligands for both Nod1 and nod2 which suggests that they often act in consort to regulate early innate responses in the intestinal epithelium as well as local inflammatory responses including Th-17 dependent immune responses [86].

NLRP1 (Nalp1) The NLRP1 protein contains an N-terminal pyrin domain, a central NACH domain and a C terminal LRR domain. Nrlp1 has been associated with the susceptibility to the lethal toxin (LT) of *Bacillus anthracis* [87]. In humans it has recently been associated with Vitilago, lesions that surprisingly appear to have no associated inflammation [88].

NLRP3 The nlrp3 cytoplasmic sensor has a similar overall structure to nlrp1 and accordingly signals by forming an aggregated signaling inflammasome complex containing ASC and caspase 1 resulting in the secretion of mature, processed IL1 [89]. Nlrp3 detects pathogens including bacteria (*Listeria monocytogenes, Staphylococcus aureus)* their components and products including MDP and toxins (nigericin, aerolysin, listeriolysin O) and bacterial and viral RNA [5]. However, the standout feature of Nlrp3 is its ability to sense ligands that drive so-called “sterile” inflammation (inflammation associated with non-microbial stimuli). Collectively, these stimuli are called Danger Associated Molecular Patterns (DAMPs) or Danger Signals. Nlrp3 has been shown to drive inflammation in response to uric acid crystals, asbestos, amyloid plaques, silica crystals, alum (vaccine adjuvant), ATP, cholesterol and β amyloid. Obviously this data associated the nlrp3

inflammasome in a diverse range of pathologies that have long been known to have an association with inflammation. These include: gout, Alzheimer's disease, silicosis, asbestosis, type2 diabetes, atherosclerosis, vaccine adjuvant and macular degeneration. It is unlikely that nlrp3 directly binds to all of these diverse ligands. Rather, these stimuli are thought to activate generic processes such as K channel flux, oxidative stress, thioredoxin (TXNIP) intermediates, protease (cathepsin) activation, that subsequently activate the inflammasome. Mutations in NLRP3 are associated with Muckle Wells, Familial Cold Autoinflammatory Syndrome, Chromic Infantile Neurological and Cutaneous Articular syndrome which are very effectively treated by administration of anti-IL1beta therapies [81, 90-92].

NLRC4(IPAF) Nlrc4 can activate a caspase 1 inflammasome in response to bacterial flagellin or components of bacterial type III Secretion Systems (T3SS) [93]. It signals in association with NAIP which are also members of the NLR family. Studies in gene targeted mice have shown susceptibility to models of induced colitis, *S. typhimurium* infection and reduced CXCL1 levels [94], while in humans they are associated with increased cell death following *Legionella pneumophila* infection [95]. There may be some level of redundancy with nlrp3 in the regulation of intestinal immunity.

NLRP6 This NLR has a similar organisation and mechanism of action as nlrp3, involving ASC, caspase1 activation and IL1 secretion [5, 48]. Recent studies have demonstrated a crucial role of NLRP6 in the recognition of commensal organisms and fine tuning of systemic immune responses. $Nrlp6^{-/-}$ mice have altered composition of gut microbiome, spontaneous intestinal hyperplasia and susceptibility to disease. This is associated with regulation of CCL5 and IL18 [96].

NLRP12 Nlrp12 has a similar overall structure to the other Nlrp members described above, but unlike them appears to play an inhibitory role in innate immune stimulated inflammation. There is evidence that it suppresses NFκB and MAP kinase signaling pathways, but the mechanism is yet to be understood [97].

C-TYPE LECTINS (CTLs)

The CTLs are a large family of plasma membrane innate receptors composed of carbohydrate-binding domains that act as PRRs [98, 99]. The cytoplasmic domains of CTLs like Dectins contain Immunoreceptor Tyrosine based Activation Motifs (ITAMs) [4, 48]. These motifs signal via recruitment and activation of Syk kinase, MAP kinases, NFAT and NFκB pathways and subsequent production of proinflammatory cytokines. CLRs such as Mincle and CLEC9 recognise damaged cells [100]. These can trigger inflammation upon recognition of damaged cells and also enhance antigen presentation from damaged cells. In the case of CLEC9 it recognises actin fragments (an abundant intracellular cytoskeletal protein present in all cell types) and selects dead or dying cells with protruding actin for phagocytosis and destruction. CLEC9 is primarily expressed on dendritic cells and as such enhances antigen presentation of material contained within dead cells.

Dectin 1 and 2 and the mannose receptor recognise *Candida albicans* and sugar moieties in *M. tuberculosis* [101]. These sugar moities such as mannan and other mannose rich structures are very common in mycobacterial and fungal cell walls. CTLs are critical in protection from fungal pathogens where they mediate recognition of the fungi and phagocytosis of fungal cells [98]. Mutations within Dectin-1 and the signaling pathway molecule CARD9 are commmon in humans with susceptibility to fungal pathogens [102]. Thus C-type lectins have all the hallmarks of innate PRR as they activate specific intracellular signaling pathways in response to a broad class of ligands (glycans). Activation of various CTLs leads to proinflammatory and immunoregulatory cytokine expression that directs the adaptive immune system to produce IL-17 which is important for protection from tuberculosis and fungal infections.

PENTRAXINS –HUMORAL PRRs

The pentraxins as the name suggests are a family of humoral cytokines that share a pentomeric structure, whose monomers share a 200aa pentraxin signature domain containing the characteristic HxCxS/TWxS motif [103]. There are two main subfamilies: the short pentraxins and the long pentraxins. The best studied short pentraxins are C-reactive protein (***CRP)*** and (Serum Amyloid P component (***SAP***). They are induced by proinflammatory cytokines or TLR pathway activation. Short pentraxins bind to molecules on pathogen cell surfaces and coat the surface which promotes activation of the Complement pathway or binding of Fcγ Receptors (antibody receptors) which both lead to enhanced phagocytosis by innate immune cells. ***PTX3*** is the prototypic long pentraxin consisting of a unique N terminal sequence and a 200aa C terminal domain with homology to the short pentraxins. However, PTX3 lacks the calcium binding pocket of the short pentraxins which results in functional differences, together with its unique N terminus which binds a different spectrum of ligands. These include FGF2, extracellular matrix components, P Selectin, apoptotic cells, pathogens, the complement receptor C1q and the antibody receptor FcγRII [103, 104].

PREEXISTING CELLULAR IMMUNE ACTIVATORS OR "ALARMINS"

Cellular damage releases potent DAMPs such as uric acid and actin from dead or dying cells that activate PRRs on innate immune cells; however they also release soluble factors that directly activate innate immune pathways. These are cellular proteins that activate immune cells directly without engaging known PRR pathways and are called "alarmins". The canonical members are the High Mobility Group Protein-1 (HMGB-1) and interleukin-33 (IL-33) [105]. These proteins act directly on immune cells by binding specific receptors in a similar manner to cytokines, indeed many of them share key structural homology to classical proinflammatory cytokines. Many of the alarmins such as IL-33 appear to be involved in antiviral and parasitic immune responses [106]. This is likely because lytic viruses and large extracellular parasites often cause extensive cellular damage; indeed incubation of *T.colubriformis* with epithelial cells has been shown to release IL-33 [107] which typically

drives a type-2 immune response. This may be one of the critical triggers for development of type-2 immunity to most parasitic infections.

EFFECTOR TRIGGERED IMMUNITY

An important consideration in determining how mammals respond to pathogens is how the large numbers of commensal organisms, which also contain PAMPs, live within and upon most mammals without triggering immune responses. Certainly most organisms are constrained by effective barrier immunity, the skin and gut and lung mucosa are prime examples. However even in these areas commensal bacteria come into contact with immune cells. It has been shown within the gut that TLR receptors are differentially expressed on the underside of cells lining the gut wall and not on the luminal side [108]. Thus only bacteria crossing the mucosal and epithelial cell barrier should trigger PAMP pathways. Additionally specialised immune cells patrolling the region have minimal TLR expression levels with high expression of intracellular PRRs such as NODs and NALP proteins. This limits their ability to respond to extracellular PAMPs but still effectively monitor for invasive intracellular pathogens. Indeed most pathogenic bacteria within the gut are those that not only penetrate barrier defenses but also contain secretion systems that are capable of injecting molecules into the cell cytoplasm. A key feature of these secretion systems is the ability to inject cellular toxins that alter the host environment for the benefit of the bacteria. Cholera is a classical example, its toxin induces potent diarrhea that helps spread the pathogen.

Plants have a specialised system of activation of immunity following detection of cellular perturbations that is termed Effector Triggered Immunity (ETI), which is important in the resistance to pathogens [109]. It appears that this system of eliciting immune and proinflammatory pathways is also active in mammals [110]. ETI is manifest when an existing cellular pathway or protein that can trigger an immune response detects a pertubation caused by a toxins and not through direct recognition of the toxin itself. This can be caused by viral inhibition of protein synthesis causing a drop in the cellular levels of IkBa which is an inhibitor of NF-kB, ultimately leading to the activation of NF-kB and proinflammatory gene synthesis. The *E. coli* Cytotoxic Necrotizing Factor-1 (CNF-1) toxin modifies RAC2 to cause membrane ruffling, however modified Rac2 binds to both the receptor-interacting protein 1 (RIP1) and RIP2 which are part of many innate immune PRR driven pathways and cause NF-kB activation and subsequent immune activation [111]. Thus there is no direct recognition of the toxin itself, however the modification of a cellular target is recognised. While the role and importance of ETI is unclear in mammals it is likely that all the innate immune recognition mechanisms and pathways outlined above cooperate to determine how an individual organism responds to a pathogenic organism. Determining how all these innate pathways are integrated to generate the host immune response to a pathogen or commensal organism is an important question to be answered. Even more importantly for livestock farming and genetic selection will be determining how they cooperate to induce the complex resistance and susceptibility profiles seen within outbred animals.

GENERAL CONSIDERATIONS AND QUESTIONS ABOUT PRR SIGNALING

With an incredibly diverse and overlapping array of detectors, receptors, ligands and triggers, how does the innate immune system direct the adaptive immune system to produce specific immune outcomes that are often tailored to a particular pathogen. How is specificity achieved? How do normally harmless substances such as DNA and RNA induce potent innate immune responses when they are constitutively present within each cell and often in serum? Why do some pathogens induce very potent immune responses and others very poor immune responses? Why are commensal organisms mostly ignored?

SUBCELLULAR LOCALISATION

Several general patterns emerge from the discussion above on the importance of subcellular location in innate immune responses. Firstly, it is notable that PRRs exist at multiple sites within the cell: plasma membrane, endosomes, ER, mitochondria, mitochondrial associated membranes, other subcellular vesicles and tubules, cytoplasm and nucleus. Interestingly, this matches sites of pathogen attachment, translocation and replication - accordingly where different PAMPS will be exposed. Furthermore, it is clear that any one PRR signaling system may begin at one location and then move to other places during interaction with a pathogen and fire off different signals from different locations. For example, nucleic acid sensing TLRs are produced and located in the ER and are translocated to the endosomes after ligand stimulation [4, 47, 48]. These organelles are normally free of nucleic acid unless the cell is infected by a pathogen and nucleic acid is aberrantly produced. Additionally many of the PRRs that detect invasive bacteria are present within the cytoplasm, such as most of the NALP proteins and TLR5. These only trigger if cytoplasmic bacterial components are present; this is a fundamental process for the discrimination of commensal from pathogenic gut bacteria. Additionally, different adapters are recruited to different geographical locations within the cell; for example there is evidence that TLR9 activates the TRAF6-NFκB-IL12 pathway from early endosomes and the IRF7 IFN pathway after trafficking to lysosome related organelles. Once translocated TLR9 undergoes proteolytic processing by enzymes such as cathepsins and the processed form is what recognises DNA. This means that artificial agonist or antagonist strategies might take advantage of this knowledge for new therapeutic innate immune modulation approaches. It also means that only nucleic acid present in the lysosome triggers innate immunity, which is a mechanism to limit activation to viruses and bacteria that are actively phagocytosed and trigger endosomal maturation into lysosomes.

TISSUE /CELL SPECIFICITY – ORGAN SPECIFIC IMMUNITY

Whereas it was originally thought that PRR signaling was the domain of innate inflammatory cells such as macrophages, DC, etc, it is clear that the receptors and signaling machinery are broadly expressed. Tissue fibroblast and other stromal cells express PRR signaling components as do epithelial cells. PRRs in mucosal epithelial cells represent the key primary interface with the environment. Epithelial PRRs interact with commensal microorganisms

that constitute the microbiome and there is a symbiotic relationship of co-regulation between PRRs and organisms[112]. Inflammatory disease occurs when these systems are broken or fail and then the PRRs of immune cells come into play [113].

TLR signaling is different in different cells for many reasons. Firstly, expression may vary between cells. For example, TLR7 and 9 are highly expressed by plasmacytoid DCs (pDCs) which is partly responsible for their ability to produce high levels of type I IFNs rapidly in response to viral stimulation. Another factor in this rapid and large response is the high constitutive levels of the IFN inducing transcription factor, IRF7. This cell-specific expression of particular PRRs and associated signaling intermediates is thought to govern a lot of the cell and organism specificity seen during an immune response to a particular pathogen. Additionally there can be a hierarchy of PRRs expression with one sentinal receptor inducing the expression of other receptors which further refine the output. This effect is often termed priming as it "primes" the cells with further detection abilities. This is a common feature in most studies of NLRP or inflammasome family members in monocytes which have to be primed by cytokine or LPS stimulation before they express the inflammasome components required for signaling.

The basal levels of PRRs and signaling intermediates can also determine which cells respond as was shown in a recent elegant study by Zhao *et al.* [114]. In response to a (virus) stimulus, approximately 20% of the cells in a culture responded. Essentially, this was due to the fact that the levels of 8 PRR signaling components varied from cell-to- cell and only cells expressing high levels of all 8 components could respond. This not only exemplifies heterogeneity in cellular responses and the stochastic nature of the PRR response, but also explains why some situations need priming stimuli to ensure all components are at their optimal levels for signaling.

REGULATORS OF PRR SIGNALING

The PRR signal transduction system and all its pathways are a finely tuned network. It is the sum total of each pathway, the nature, magnitude, duration and cellular context of activation or suppression, which will determine the outcome. If all pathways are well balanced, then pathogen elimination or disease resolution occurs and there is a return to homeostasis. If not, the result can be acute severe disease (e.g. septic shock), chronic disease, autoimmunity, cancer, etc. Thus there are checks and balances at each stage of the PRR signaling pathway. There are numerous negative regulators that help keep the system tightly regulated and typically limit the duration of the proinflammatory response. For example SOCS proteins are inducible inhibitors of cytokine and innate immune pathways [115]. More recently they were shown to be regulators of primary TLR pathways by mediating degradation of signaling molecules within the pathway [116]. Phosphatases can remove critical phosphate residues from receptors which stops the recruitment of signaling molecules. Another example of positive and negative signal regulation is at the level of transcription. Both transcriptional activators (NFκB, IRF1, 3, 5, 7, STATs) and repressors (e.g. IRF2, 4) are activated at different stages of a response and in different cells. Another class of negative regulators of

primary and secondary PRR signaling pathways that will not be extensively covered here are the endogenous micro-RNAs (miRs) [117, 118]. These are transcriptionally activated by similar pathways as the mRNAs, but then act on mRNA to modulate their stability and hence the amount of proteins (e.g. proinflammatory cytokines and IFNs) that are produced. These also have therapeutic potential as innate immune response modifiers and as diagnostics since they are secreted into the bloodstream.

Key points of regulation of PRR signaling are common targets of virally encoded inhibitors of innate immunity [119]. These are key aspects of a successful virus that help it evade immunity and either set up a latent or chronic infectious state within the host. One key viral target is the production and action of Interferons. Viral proteins of HCV (NS3 protease), Influenza A (NS1) and HIV target IRF-driven type I IFN production as this is a key antiviral system responsible for limiting initial viral replication and spread. Pox viruses produce soluble IFN receptor mimics as well as intracellular inhibitors of TLR signaling molecules. An interesting question is why one virus would produce factors that target so many (apparently redundant) steps in the innate immune response? It may be because these factors are targeting different stages or times of the response, different subcellular or extracellular compartments or even different cell types. It must be remembered that most pathogens contains several PAMPS that will stimulate different PRRs at different locations, so a comprehensive approach may be required for immune evasion. This may be an important lesson for consideration in our exogenous therapeutic strategies. It may be essential to target many innate pathways to generate strong immunity and also inhibit many pathways to limit inflammation.

PRACTICAL OUTCOMES

It is evident from the preceding information that PRR pathways are key to the host response to infection, inflammation and many pathophysiological processes and diseases. This includes acute and chronic infection, inflammatory diseases, cancer, autoimmune diseases, metabolic syndrome, and other chronic diseases such as diabetes, Alzheimer's disease, and atherosclerosis. Certainly the latter mentioned diseases involving inflammasome activation have fuelled clinical trials of IL1 blockade by antibodies and soluble receptors. There appears to be plentiful opportunities for agonists or antagonists to modulate disease [120]. PRR pathway agonists have proven to be effective vaccine adjuvants and are being trialed as therapies for many diseases. The effectors produced following activation of innate immune pathways are frequently used as therapeutic agents; e.g. type I IFNs are used to treat multiple sclerosis and viral Hepatitis C. Blockade of inflammatory cytokines such as TNF is an effective treatment for reumatoid arthritis and the anti-IL-1beta therapies block familial fever diseases. Knowledge of these pathways and the factors involved are not only necessary to identify potential therapeutic targets, but also in order to understand disease susceptibility studies [121]. The required data now includes information on so-called "non-coding" RNAs (miRNA's and lncRNA's), which may necessitate "Next Generation" genomic and

transcriptomics studies. This knowledge is also important for design and interpretation of diagnostics. A full understanding of the genes and pathways involved in the ruminant innate immune system will enable the design of appropriate panels of biomarkers. The biomarker panels could be protein, mRNA or non-coding RNA (ncRNA) assays all of which contribute to immune and inflammatory outcomes.

Another practical outcome of a detailed knowledge of innate immune pathways is that this system can provide assays to measure the inflammatory potential and quality control of products that may be used within Veterinary or farming practices. The inflammatory potential of PAMPs could be screened for in ruminants and may offer new more powerful vaccine adjuvants or therapies for livestock use. Dietary supplements and other potential therapies could be screened for their ability to activate certain innate pathways which would offer insights into their inflammatory profile *in vivo*, without having to resort to expensive trials. Structures such as nanoparticles are of enormous clinical interest yet they also have the potential to activate inflammatory responses via the particulate-sensing NLR system. Therefore an NLR activation screen available for livestock would offer insights into the suitability of potential therapies for use within livestock and could be used to improve and inform the therapeutic manufacturing or development processes.

The dizzying array of innate immune receptors, pathways and cell types is indicative of an evolutionary arms race that mammalian organisms, including livestock have staged with pathogens over millions of years. The most fundamental force driving evolution of humanity and most mammals is the ability to combat infection within the early years of life, the most vulnerable period. Pathogens are much more dangerous to young organisms as this is likely to be the first time their immune system encounters a particular pathogen type. Initial resistance to the disease is therefore totally reliant on an efficient and flexible innate immune system. Until the extreme diversity and elegance of the adaptive immune system can be engaged to expel an invader and induce lasting immunity evolution has supplied a thorough catch all system of coordinated receptors and pathways to induce both broad and tailored immune responses. It is little wonder that mutations within genes and elements are responsible for the often subtle differences in genetic susceptibility found within a species.

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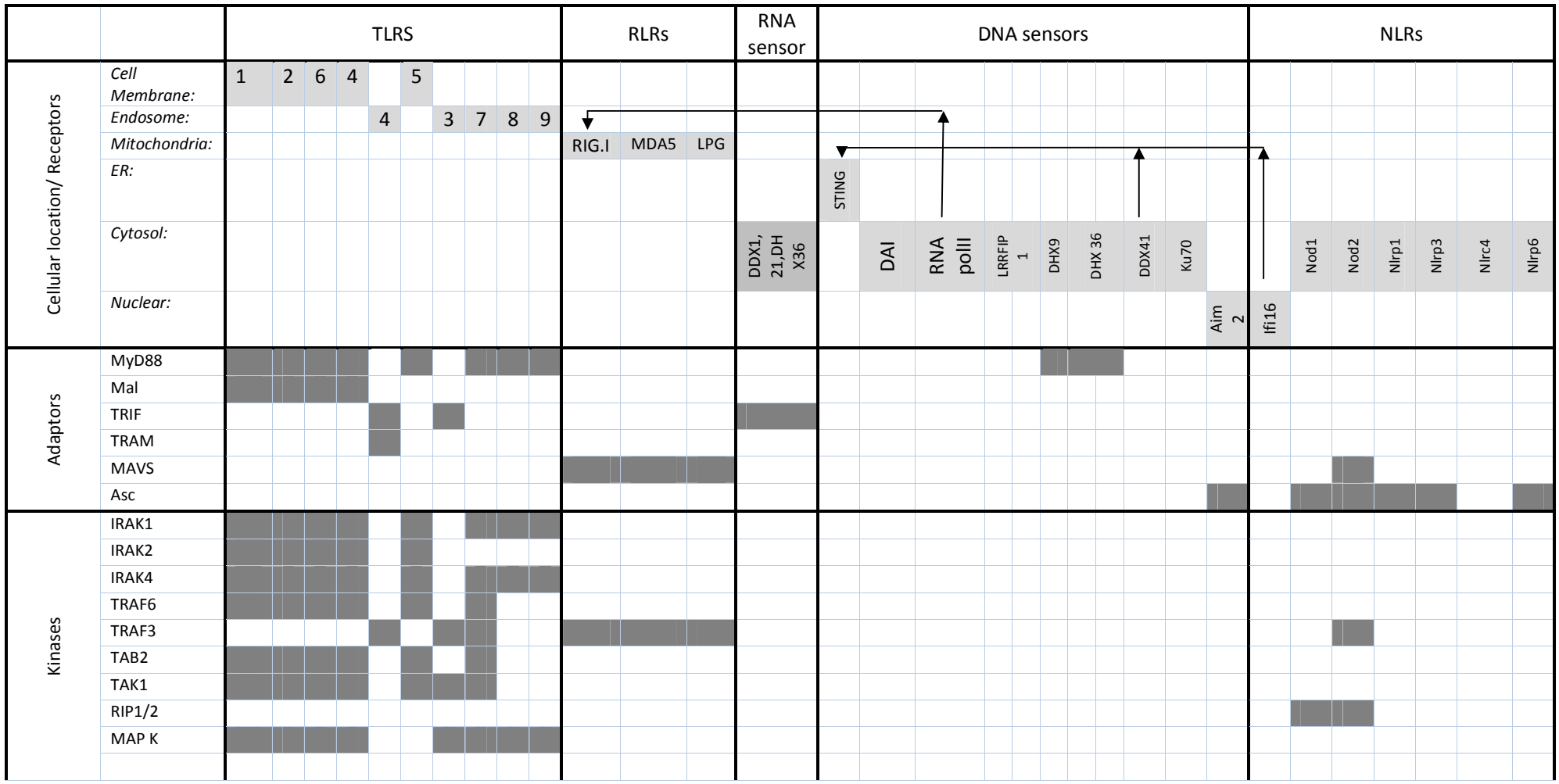
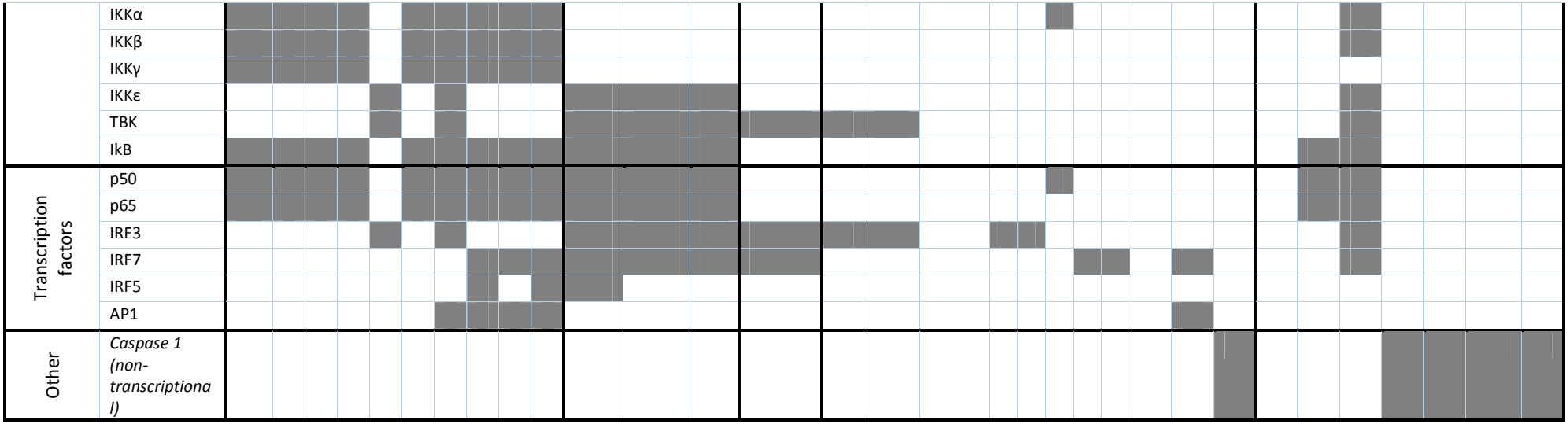
TABLE 1: Components of PRR Signal Transduction Pathways




IKKα
IKKβ
IKKγ
IKKε
TBK
IkB
Transcription factors
p50
p65
IRF3
IRF7
IRF5
AP1
Other
Caspase 1 (non-transcriptional)

Chapter 2

The relationship between the innate immune system versus the acquired immune system and their proportional contributions to an animal's response to disease and parasite exposure

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The innate immune system is conserved across evolution and present in all multicellular organisms. It provides the first line of immune defense against infectious agents whose unusual molecular structures (PAMPs) are recognized by fixed, germline encoded receptors (PRRs) as described in the previous sections. These PRRs include both soluble (e.g. complement) and cell-bound (e.g. TLR) proteins that, once activated, can result in cytokine- and chemokine-mediated inflammation, increased phagocytosis and/or lysis of the infectious agents. In vertebrates, an additional level of immune protection is provided by the adaptive or acquired immune system which is characterized by specific receptors on T-cells (TCR) and B-cells (Ig) that are *de novo* generated, through gene rearrangements, to recognize an almost unlimited number of foreign molecules (antigens). The innate immune system is mostly present and constant, independent of pathogen exposure. In contrast, the acquired immune system needs to be first activated by infection/vaccination and can then generate a memory of this exposure, allowing it to react more quickly and strongly to subsequent encounter with the antigenic molecules of the same pathogen or vaccine. The adaptive immune response can also develop into completely different directions which ultimately determine the type of immune effector response that will be generated to protect against infection. For example, a type 1 immune response is characterized by the production of IFNγ and cell-mediated immunity, important for protection against bacteria and viruses while a type 2 immune response promotes eosinophil and mast cell-mediated immunity suitable for protection against helminth parasites. This diversity is also reflected in the induction of different antibody isotypes appropriate for complement-mediated lysis, phagocytosis, mast cell activation, virus and toxin neutralisation or antibody-dependent cell-mediated cytotoxicity. Because of these observable consequences of the acquired immune response on pathogen killing or rejection and its role in vaccine-induced immunity, most of the genetic studies of disease resistance

have concentrated on finding a link with genes involved in driving the effector mechanisms, mostly with limited success.

Until recently, the two arms of the immune system (innate and acquired) were thought to act more or less independently, but the last few decades has seen a revolutionary new direction in immunology research, that puts the innate immune system as the main driver of the adaptive immune response. In particular, it is now thought that the first contact of a pathogen with the innate system *i.e.* the interaction of PAMPs with their respective PRRs, is what determines not only the strength of the ensuing adaptive immune response, but also the direction it will take and the ultimate effector response that will be generated. In this way it can be considered that the genes involved in the acquired immune response are not the determinants of genetic variability, but a read-out of the innate response genes activated at the start of an infection/vaccination. While the exact contribution of different innate response pathways to the varied outcomes of an acquired immune response are not yet fully understood, there are clear examples of how innate receptor interactions can determine adaptive outcomes. For example, the soluble PRR, dectin-1, recognizes β-glucans (PAMPs) present in the cell walls of fungi and its activation results in the generation IL-17-producing T cells which are critical for clearing fungal infections [1]. Another example is the recognition of a viral ssRNA (PAMP) by the host's cytoplasmic PRR, Nod2, triggers activation of interferon-regulatory factor 3 (IRF3) and production of interferon-beta (IFN-beta) which is important for antiviral defense [2]. Type-2 adaptive immune responses seem to have a variety of different innate stimulation pathways [3], and neither the PAMPs or PRRs activated during helminth infections have yet been identified; it does however seem likely that, as in other infections, the innate immune system and its receptors can account for most of the host variability in resistance/susceptibility to parasite infections. Discovering the innate pathways leading to effective immune outcomes will be important for genetic selection and vaccine development for helminth infections.

Genes encoding PRRs or their downstream adaptors can be extremely polymorphic and, in human studies, have been associated with enhanced susceptibility to a variety of diseases, including malaria, pneumococcal disease and severe sepsis, as well as with failure to respond to vaccination (reviewed in [4-6]). Polymorphisms have also been found in ruminant PRRs and have been linked with increased susceptibility to mastitis [7], bovine tuberculosis [8] and *Mycobacterium avium subsp. paratuberculosis* infection [9]. A single nucleotide polymorphism (SNP) was recently identified that conferred remarkable natural, genetic resistance to Salmonella and *E. coli* O157:H7 infection in amelanotic (non-black coat) cattle (Steve A Carlson and Tim A Day, PSR Genetics). Interestingly, a mutant β-defensin gene, encoding an innate antimicrobial peptide, is also involved in the melanocortin pathway responsible for black coat color [10], which may explain the strong genetic linkage and would provide one of the clearest examples yet of innate gene polymorphisms and disease resistance in livestock. Polymorphisms in defensin genes have previously been proposed as genetic marker(s) for selecting cattle with increased resistance to mastitis [11].

As shown above, genetic lesions within the innate immune pathways tend to show more subtle phenotypes, with increased susceptibility to specific infections or impaired resistance

to a family of pathogens. This reflects the type of resistance we see within livestock to disease, general variability in modulation of immunity rather than very large shifts in susceptibility. This is not surprising as the innate immune system has many receptors and overlapping pathways which, coupled with the ability to detect multiple PAMPs from pathogens, results in a high level of redundancy within the system. This means that the phenotypes associated with innate immune defects are more subtle and more representative of what is observed in on farm infections. It also means that specific innate stimuli should be used to assess the competency of any innate immune driven pathway within livestock. In contrast genetic lesions within the adaptive immune system tend to result in severe consequences for the immune system with the exception of MHC polymorphisms which operate under a different set of selection criteria (see section 5). Failure to mount a specific adaptive immune response is a severe genetic defect that is likely to be rapidly removed from the gene pool. A complete failure of immunity is extremely abnormal in outbred animals including livestock, however a gradation of responses is what is typically seen. We would argue that the majority of immune variation not due to MHC alleles is driven by subtle alterations within innate immune pathways and as such these should be assayed in any phenotypic driven genetic selection methodologies.

Key points

- The acquired immune response and the immune effector mechanisms they generate, are critical in protecting animals from infectious diseases.
- The variability in the level and type of acquired immunity determines resistance or susceptibility to infection and is heritable.
- Differential stimulation of the innate immune system results in different outcomes of the acquired immune response
- Variability in acquired immunity is in most cases determined by the initial activation of the innate immune system
- Heritability of resistance/susceptibility to many infections/diseases may therefore reside in polymorphism at the level of innate immune genes.

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Chapter 3

Prophylactic and therapeutic modulators to enhance innate immunity.

Table of Contents

Prophylactic and therapeutic modulators to enhance innate immunity.

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Introduction

The innate immune system underpins the whole of immunity, it is essential for the generation of strong effective immunity and responsible for many of the undesirable side effects associated with excessive inflammation. This section of the review will present an analysis of the latest findings on targeting the innate immune system to develop anti and pro-inflammatory drugs, prophylactic immune modulators and improved vaccine formulations. Finally there is a growing appreciation that the immune system is a central mediator and highly integrated in many physiological processes, one very important to farmers is stress which can increase disease susceptibility.

Drugs targeting Innate Immune Pathways.

Large pharmaceutical companies are aggressively developing drugs and therapeutic agents that target the innate immune pathways due to their potent effects on inflammation, infection, autoimmunity, sepsis, immunodeficiency, atherosclerosis, asthma and cancer. The clinical development of molecules that influence innate immunity holds promise for a number of applications relevant to livestock production. This section of the review will focus on the latest developments in manipulation of the innate immune system for therapeutic outcomes. A comprehensive catalogue of innate immune modulators being developed for clinical use will be presented as well as how those therapies are performing in the clinical setting and their potential relevance to livestock.

The discovery of the Toll Like Receptors began the paradigm shift in our understanding of innate immunity from a non-specific phagocytic and complement response to a specific receptor-ligand interaction that initiates defined immune outcomes. Since this discovery new

families of innate immune receptors and signalling pathways have been identified and all of these are being targeted for therapeutic applications [1, 2]. It is an exciting time in the field of innate therapeutics as the discovery of the TLRs was over ten years ago and clinical results from the first generation of innate immune drugs and therapies are only now being published [2-4]. Most of the clinical trials have focussed on using specific TLR agonists to drive increased cancer immunity, hepatitis C clearance, reduction in allergy symptoms and chronic immune diseases. While many of these diseases are not relevant to livestock farmers we will attempt to interpret the clinical findings from these trails to assess the applicability of the general approach or drug to livestock medicine and husbandry.

TLR based clinical therapeutics.

The TLRs are a large family of receptors that monitor the cell surface and endosomal compartments for microbial pathogens and host dysfunction. TLRs activate multiple signalling pathways which increases the diversity of the immune outcome following engagement of a TLR by an agonist. This complexity in cell type expression patterns, cellular location and signal transduction can make therapeutic intervention challenging in terms of delivery, cellular targeting and the specificity of the outcome. The ligands that activate the range of TLRs are extraordinarily diverse and range from proteins to lipids and glycolipids to nucleic acids which add another level of complexity to drug development. Despite these difficulties pharmaceutical manufacturers and scientists have developed small molecule drugs, antibodies and both natural and synthetic ligands that agonise or antagonise nearly all the members of the TLR family. Ideally for livestock use drugs based on TLR interactions would be small synthetic and highly potent. Fortunately it appears that small drug TLR agonists can be developed, Guan *et al*, (2010) screened an extensive small compound library and identified several molecules that stimulated TLR2 with TLR1 and 6 also involved [5].

Many TLR based interventions have gone through the rigorous phase II and III clinical trial process in humans and these have been summarised along with the disease being treated and the outcome of the trial in Table 1. Most of these first generation compounds have been disappointing as anti-cancer agents (see table 1). While TLR based cancer therapies have been very disappointing with the exception of imiquimod and its derivatives [6, 7], it not entirely surprising as cancer is an extremely difficult target for innate therapies due to

localised immunosupression, immune evasion and tolerance[2, 8]. Imiquimod and similar TLR7 agonists have shown efficacy against skin cancer and other skin lesions such as warts, most of which are caused by viral infection raising the possibility that these agents can be used to increase viral immunity [9, 10].

An area that is of keen interest to livestock producers is the use of TLR agonists and antagonists to treat inflammatory diseases and infections. Eritoran a TLR4 antagonist has just finished undergoing trails in humans to reduce severe sepsis symptoms. While phase II trials demonstrated that it was well tolerated by patients and helped reduce mortality rates [11], a larger Phase 3 trial on very ill patients showed no survival benefit [12]. While disappointing that a single TLR antagonist was not sufficient to ameliorate the severe symptoms of systemic bacterial dissemination it is not entirely surprising given that bacteria engage many innate immune receptors. It suggests that treatments aimed at blocking immune driven pathology during systemic bacterial infections will likely require the targeting of a number of innate receptors or shared pathways and intervention before severe sepsis is manifest. Unfortunately systematic pairing of compounds is extremely laborious and slow in a human trail format. This is an area where, if systemic immune pathology driven by over activation of innate immune pathways is known to be a cause of livestock mortality, that more targeted and rapid trials within livestock could be carried out with a range of innate antagonists.

This approach may be applicable to mastitis to reduce production of inflammatory mediators which have systemic side effects, however it would require extremely complex targeting of the therapy to minimise systemic inflammation while not blocking desirable inflammation at the site of infection which is recruiting phagocytic cells to clear the bacteria. The infiltration of phagocytic cells into the mammary glands thought to be important for the clearance of mastitis [13, 14] and neutrophils are rapidly recruited to localised sites of inflammation [15-17]. There is almost no data to indicate that this level of specificity is currently available for controlling the activation of livestock TLRs using drugs.

The nucleic acid sensing TLRs, notably TLR7,8 and 9 are very attractive drug targets as the molecules that are being developed to agonise and antagonise these receptors are typically synthetic, reducing quarantine risks to Australian livestock and are generally quite stable. There is a long history of imiquimod being used as a topical anti-wart therapy before it was identified as a potent activator of TLR7 [10]. It and similar TLR7 based drugs have since shown clinical efficacy against a number of cutaneous viral diseases including genital warts

and superficial basal cell carcinoma [6, 7, 9, 10]. Imiquimod (Aldara™ 3M Pharmaceuticals) is a useful treatment of equine aural plaques and sarcoids [18] and it may be applicable in the treatment of a number of cutaneous lesions where viral aetiologies are suspected. It can be dangerous and toxic if administered in high doses systemically and this has limited its use to topical creams[7].

Interestingly hydroxychloroquine (HCQ) best known as an antimalarial, is often used as part of the antibiotic mix in the treatment of chronic Q-Fever which is highly inflammatory and is transmitted by livestock [19]. It was recently shown that HCQ is an inhibitor of the nucleic acid sensing TLRs, particularly TLR9 [1]. It will be interesting to see if the clinical benefit associated with HCQ treatment of chronic infections with *C. burnetii,* the causative agent of Q-fever, requires binding to the TLRs that recognise nucleic acids. Immunostimulatory RNA and DNA sequences that efficiently activate TLR9 pathways are available for livestock species (M. Elhay, personal communication and [20, 21]) and many inhibitors of these TLRs are currently being developed. Unfortunately there is little evidence of their efficacy as direct therapeutic agents.

Many TLR based therapeutics are being developed as antiviral agents, primarily due to their ability to induce high levels of the type I interferon (IFN) family of antiviral cytokines [22-24]. While some of these agents have been withdrawn from trials many are showing promise for the treatment of hepatitis although the future for further drug development based on innate immune therapeutics is unknown due to the recent success of the antiviral molecules that directly target the virus [25]. It has been shown that TLR7 agonists produce more IFN-α than TLR8 agonists [26] indicating that it may be possible to modulate specific TLRs to generate desired outcomes. High systemic doses of TLR7 and 8 agonists can induce high levels of Type I IFNs which are antiviral however can also lead to side effects such as flu like symptoms, lymphopenia and low blood pressure as seen in the trial of a TLR7 agonist (PF-4878691) in humans [27]. The promise of antiviral TLR agonists will require careful dose estimation and optimisation however they hold great promise for the prevention of viral infections and use as prophylactic and preventative agents. These roles were excellently reviewed by Horscroft *et al*., 2012 which outlines many of the results from human clinical trials using TLR-agonists for antiviral therapies.

Overall there are few examples, apart from imiquimod, where targeting a specific TLR has lead to a clear clinical benefit within either the human or veterinary fields. Many trials are

ongoing and further research is being conducted as there is strong evidence to suggest that TLRs are important in clinical diseases. Unfortunately most of the early clinical trial data using TLR based therapeutics was aimed at developing cancer therapeutics and treatments for advanced sepsis [1, 12, 28] which are not major concerns within the Australian livestock industry. However if conclusions can be drawn from these trails we would suggest that single TLR agonist or antagonist based therapies are not likely to offer significant benefits in complex infectious or inflammatory diseases as most pathogens trigger multiple innate immune pathways. While this can be overcome by targeting the signalling adapters which are common to most TLR pathways such as MyD88, TRIF and Mal, we could find no mention of specific interventions targeting these proteins that are practical for farmers to implement within the short to medium term. Most of the agonists have shown readily detectable immune stimulation in the trials but limited clinical effect [1, 23]. To date the most promising area making use of the diverse array of TLR agonists is their use as vaccine adjuvants and potential prophylactic therapeutics, both of which will be discussed in more detail below.

Inflammasome based therapies.

The inflammasome is a large molecular complex assembled following recognition of Danger Associated Molecular Patterns (DAMPs) and is driven by an association of proteins which contain Caspase Activation and Recruitment Domains (CARDs) leading to the activation of proteolytic caspases that cleave inactive cytokines into their active forms [29]. Recognition of DAMPs by the different inflammasomes typically leads to pyroptosis (inflammatory cell death), IL-1 beta and IL-18 production [29]. Pyroptosis is an effective mechanism to reduce viral and bacterial infection through cell death and enhanced inflammation while IL-18 and IL-1 beta are potent inflammatory cytokines. The inflammasome was discovered after the TLRs and while it is an attractive target for drug discovery projects most are at the early trial stage and untested in clinically relevant models [30].

There are examples of new drugs that target the inflammasome components, one such new entity, acALY18, a novel lipopeptide being developed by Therimunex Pharmaceuticals activates NACHT, LRR and PYD domains containing protein 3 (NALP3). A recent publication claimed that it reduced *Staphylococcus aureus* infection as a prophylactic in mice experiments and “enhanced survival in an experimentally induced Gram negative bacteremia

and overrides the escape mechanism of an obligate intracellular pathogen, *Chlamydia pneumonia"*. It was also more effective than standard-of-care antibiotic therapy in a clinically established multi-factorial bacterial infection [31] and they claim it reduces bacterial numbers in bovine mastitis models (unpublished). Unfortunately we could find no published evidence to support the mastitis statement made in Thacker *et al*. 2012 or any information on how this drug was administered. However, it definitely raises the possibility that inflammasome agonists could be a promising class of antibacterial prophylactic therapies or combination therapies with antibiotics [30, 31]. They may allow farmers to reduce the amount of antibiotic required to treat mastitic cows, which is a desirable outcome as antibiotic resistance is a looming problem in the Veterinary and Human health care sectors [13].

Glyburide is a drug used to treat type2 diabetes, it blocks IL-1 beta release through an unknown mechanism and may be protective against bacterial infection in diabetic patients [32]. Another class of potent inflammasome inhibitors are the P2X7R antagonists which inhibit inflammasome activation induced by extracellular ATP [33]. While it is generally accepted they are anti-inflammatory, specific clinical trial data was not available. Targeting the inflammasome through inhibition of caspase-1 and adenosine type-3 receptors improved the outcome of antibiotic therapy in a murine anthrax model suggesting that the inflammasome is important in infectious disease [34]. Additionally inflammasome dependent pyroptosis and IL-18 helped protect mice from a lung challenge with *Burkholderia pseudomallei*, while IL-1β was deleterious [35]. This suggests that it may be more useful to target individual downstream effectors such as IL-1beta and IL-18 rather than the inflammasome itself in infectious diseases.

Indeed by far the most successful human clinical outcomes related to innate immunity, apart from vaccination, is through the inhibition of cytokines induced following innate immune activation. These include the broad spectrum of anti-TNF-alpha and anti-IL1-beta biopharmaceuticals [29, 36]. The inhibition of TNF-α has revolutionised rheumatoid arthritis treatment and the anti-IL1 beta therapies have been extremely effective as treatments for gout and genetic cryopyrin auto-inflammatory diseases [37, 38]. It will be interesting to follow how effective these treatments will be in infectious diseases which are more relevant to the livestock industry however clinical treatment appears to be much more complex than the inflammatory diseases mentioned above.

The family of biopharmaceutical therapies used to target TNF and IL-1beta and those in development for other cytokines and molecules must overcome a number of hurdles before they can be considered for use within livestock. The cost is currently prohibitive, human based antibody therapies require milligram doses per individual, the costs associated with producing milligram quantities of antibody are currently prohibitive for general use within livestock. Additionally they are biological molecules and as such require cold storage and extensive safety and regulatory assessment before quarantine approval could be granted for use within Australian livestock, further increasing associated costs. Antibody and receptor specificity is often species specific, thus new antibodies specific to ovine and bovine targets would likely need to be developed. This is not beyond the capacity of the animal health industry however there would have to be clear indicators of clinical efficacy in other organisms before companies or organisations would be advised to undertake these tasks; these clear demonstrations are not presently apparent for diseases that cause major economic impacts in Australian livestock farming.

Therapeutics targeting other innate immune receptors and pathways.

Another major category of innate resistance and defence is lectin mediated recognition of glycan moieties on pathogens and particles. Currently, five families of animal lectins are defined in structural terms. i.e. the C-type, I-type and P-type groups, the galectins and the pentraxins. They have roles as defence molecules homing in on foreign or aberrant glycosignatures on pathogens, as glycan transporters and as coordinators of transient or firm cell-cell/cell-matrix contacts [39]. The best characterised lectins with relevance to innate immunity are the scavenger receptor families, the C-type lectins, the mannose binding lectins (MBL) and the collectins. All these receptors have roles in pathogen recognition and resistance from infection, some of these roles involve specific recognition such as the Dectin-1 and 2 mediated recognition of yeast and fungal beta-glucan [40-43] to surface recognition of viruses and bacteria as foreign glycan particles [39]. We could find little evidence for specific therapeutic drugs that target these receptors and pathways, but they remain attractive targets as they are often found at sites where pathogens enter the body and have been genetically associated with resistance to disease [44]. Collectins form part of the innate surfactant barrier within the lung and are associated with immunity from pathogens [45-47].

Polymorphisms in Dectin-1 and its signalling adaptor CARD9 are often found in individuals who are particularly prone to highly virulent fungal infections [44, 48, 49]. Mutations in Mannose Binding Lectin appears to increase susceptibility to mycobacterial disease [50]. Additionally their ability to recognise glycans and particles makes them very attractive self-adjuvanting vaccine delivery vehicles. This use will be discussed further in the review.

General Therapeutic and Prophylactic Strategies Using Innate Immunity

The potential to manipulate innate immunity to generate general resistance to disease is of considerable relevance to livestock owners. We have outlined how animals can be selected for resistance based on immune parameters and the considerations involved. We will now look at whether more direct interventions based on stimulation of the innate immune system can be used to enhance disease resistance. There is considerable evidence to suggest that the innate immune system has a central role in disease resistance through amplified immunity, increased resilience and better wound healing following infection. Activation of innate pathways induces a plethora of anti-infectious downstream effectors, this section will discuss whether this can be harnessed for livestock.

Biodefense.

The activation of innate pathways to prevent infection is being investigated as a Biodefense strategy to minimise infection in the event of a disease outbreak or bioterrorism attack [51]. These are very real threats; with the rise in the global mobility of humans and trade goods it is unlikely even the best quarantine systems will prevent diseases from entering Australia. Also bioterrorism is a real threat with our country's increased role in global affairs and strategic alliances making us a target for extremist groups. Economic disruption by targeting agricultural commodities such as livestock is not beyond the scope of activities some of these groups may engage in. A summary prepared by Amlie-Lefond (2005) on the use of innate immunity for Biodefense identified the following list of questions associated with their use:

1. Which innate immune receptors stimulate effective prophylactic responses to the broadest range of bacterial and viral pathogens?
2. How long does protection last?

3. Could innate immune therapy trigger harmful inflammation?
4. Will innate immune stimulation promote autoimmune reactions or retroviral activation?
5. How important a factor is human genetic polymorphism within the innate immune system for innate immune therapy strategies?

They contacted several companies developing innate therapies and presented their answers. Overall most were optimistic that innate immune modulation could lead to general protective immunity for at least 3 days to 2 weeks depending on the pathogen in question and how effective the innate immune stimulation was [51]. This type of study has immediate applicability to Australian livestock farmers as it may present strategies to initially control and limit the spread of an adventitious agent until authorities can implement more specific control strategies. The obvious threat is foot and mouth disease virus (FMDV) which if released into Australia would require immediate and effective inhibition of transmission to limit its impact. There is a precedent for this type of mobilisation with the recent equine influenza outbreak which was controlled by strict quarantine of equine movement and effective ring vaccination with a new class of equine influenza vaccine that allowed discrimination of infected from vaccinated horses. While this vaccine rapidly induced adaptive immunity it may well have been beneficial to have a stock of defined innate immune modulators to limit the infection while vaccine stocks were being obtained. This may be an area MLA could invest in: we need tools to effectively combat infectious agents immediately and the innate immune system is an obvious candidate to supply at least some level of protection when no vaccine or therapeutic drug is available. The above questions can be used as a guide for further developing future research within this area.

Generalised prophylactic immunity.

There are a number of lines of evidence to suggest that immune stimulation provides generalised immunity within naive individuals and may be a beneficial strategy to pursue to increase livestock resistance and resilience to disease and stress. Mice pretreated with aminoalkyl glucosaminide phosphates (AGPs), which are synthetic TLR4 agonists, had reduced levels of *Listeria moncytogenes* or influenza following challenge [52]. The response of animals and humans to vaccination against a specific disease is often insufficient to explain population wide protection supplied from the vaccine. Aaby argues that we are ignoring many of the off-target effects vaccines have on “general resistance” [53]. There are

two possibilities which may account for general resistance in vaccinates, the first documented is cross reactive T-cell epitopes which help protect against heterologous disease and the second may be increased innate immune activation [53]. Most of the vaccines implicated in general resistance are the live measles vaccine and BCG which induce strong effects in both these areas. A study investigating the BCG vaccine in children showed a small "reduction in neonatal mortality was mainly due to fewer cases of neonatal sepsis, respiratory infection, and fever" [53]. There are cautions with this approach as the high titre measles vaccine when given with diphtheria-tetanus-pertussis (DTP) may have caused increased mortality and was subsequently withdrawn from use within Africa, lending a cautious tale to the role of overstimulation of innate immunity. We have also witnessed this recently in Australia where a batch of swine flu vaccine caused young infants to have febrile seizures due to incorrect estimation of the vaccine dose [54].

This prophylactic use of innate immune agonists may be an effective means of ensuring short term protection of livestock from an adventitious virus, it has been shown that a single intranasal dose of an imidazoquinoline a mixed TLR7/8 agonist significantly reduced nasal influenza viral titres when given 72 h pre-challenge or 6 h post-challenge in a rat model and required induction of type-I IFN [55]. Pre-treatment of mice with CRX-527 was found to enhance both CD4+ and CD8+ T cell responses via induction of significant amounts of IL-12, which offered protection against a challenge by influenza virus and RSV [56]. There have been similar reports of TLR3, TLR9 and TLR4 agonists also offering protection in murine models of influenza ([52, 57, 58] and reviewed in Horscroft *et al*., (2005). Conjugates of the TLR7 agonist SM-360320 with mouse serum albumin was designed to keep the agent within the lung when inhaled, thereby reducing the risk of systemic cytokine induction [59]. Pre-treatment with the conjugate in an infectious model of influenza virus produced significantly delayed mortality while synthetic RNA induced immune responses similar to those observed with a live viral vaccine in humans [60]. Taken together this suggests that nucleic acid based PAMPs may be a viable and cheaper alternative to administration of IFN to prevent or treat viral infections and the rapid spread of pandemics.

Products that activate a broad spectrum of the innate immune system are commercially available and used within Veterinary practice, for example Equimune IV^{TM}, a mixture of mycobacterial cell wall components is sold as a general immune stimulant that is used to treat horses with Equine Respiratory Disease Complex (ERDC) or to prevent infectious disease when transmission is likely. The common causes of ERDC are equine herpesvirus

(rhinopneumonitis), influenza A equine 1 and influenza A equine 2 [61, 62]. Unfortunately there appears to be no peer reviewed published clinical data available on Equimune or its effectiveness against specific pathogens, except when used as an adjuvant [63]. A study by Nichani *et al.*, (2006) used CpG oligonucleotides to induce antigen independent protection of new born lambs from parainfluenza-3 virus. They found that "pre-treatment of newborn lambs with SC but not intratracheal CpG ODN 2 days, but not 6 days prior to the virus challenge was protective". Thus timing and route of administration is critical to success, indeed it is likely that many components will need to be optimised before this approach will be broadly applicable within livestock.

Mastitis in cattle induces distinct "clinical outcomes ranging from acute and life-threatening to chronic and sub-clinical and the differences are underpinned by the nature of the innate immune response activated in the mammary gland by different bacteria" [13]. There is hope that appropriate activation of the innate system can help prevent or clear infection in susceptible animals. This is likely to be more difficult than viruses as most viruses are quite sensitive to systemic IFN-mediated inhibition and we are still not sure whether systemic activation of antibacterial pathways will enhance clearance. While IFN and complement can act systemically, neutrophil and other phagocytic cell mediated clearance of bacterial and mycobacterial infections will likely require potent localised activation of innate immunity making administration difficult. If effective mammary gland delivery vehicles are available then administration of innate stimuli may be an attractive therapeutic or prophylactic prospect. Innate stimuli will likely increase neutrophil and other phagocyte numbers within the blood which may be beneficial for mastitis however their timing will need to be synchronised to times where there is a high likelihood of transmission. A study determining whether inflammasome, TLR or lectin based agonists would be effective therapeutics or prophylactics could be useful in a reliable model of livestock mastitis. As mentioned above the inflammasome activator acALY18 has been proposed to actively reduce mastitis.

Wound healing.

The ability of an organism to tolerate low infectious doses while showing few signs of disease or production loss is thought to result from a combination of innate immunity such as effective immune barriers, innate immune cell recognition, defensins and other immune mediators and resilience mechanisms such as good healing and anti-inflammatory

mechanisms following resolution of infection [64]. While activation of many of the innate immune pathways has been implicated in the first part of the resistance/resilience equation, the inflammasome appears to play an important role in exacerbating fibrosis following wounding. Mice deficient in the ASC protein, an inflammasome adapter molecule, show reduced fibrosis suggesting that chronic IL-1 beta may enhance the fibrotic response and scarring [65, 66]. This has implications for livestock skin and meat quality as fibrotic lesions reduce meat and hide scores, thus any therapy that induced chronic inflammasome activation would need to include analysis of hide and meat scarring within its safety profile.

The role innate receptors play in detection of host molecules following wounding is only just beginning to be worked out but it does offer interesting possibilities for treatments to improve wound healing following procedures such as mulesing, branding and surgery. Interestingly the TLR4 inhibitory antibody 1A6 reduced colitis in mice when administered early in disease, however when administered during the recovery phase slowed healing [67]. This biphasic response supports the notion that innate pathways are active in both the highly inflammatory environment of initial pathogen detection but also regulate wound repair following infection. It is not clear whether this effect is due to recognition of host DAMPs induced via the wound or stimulation of feedback reparative pathways following pathogen recognition which help initiate wound healing and resilience to disease [67]. All innate immune pathways have built in feedback mechanisms that reduce over-activation and chronic stimulation through induction of proteins that inhibit receptor signalling. This probably best described by the observation of endotoxin tolerance, where an organism can be tolerised to lethal doses of LPS by administering it 24 hours following a sub-lethal dose [68]. There is scope to test whether application of defined innate immune stimuli could be used to strengthen the induction of wound healing and resistance to infection. This may have applications in mulesing and wound repair following surgery and for general antibiotic salves.

Gut barriers.

Immune pathology within the gut caused by e.g. *Mycobacterium paratuberculosis*, *Salmonella spp.*, *E. coli* and intestinal parasites impact upon livestock production and profitability. It is well established that gut microbiota influence immune outcomes within the gut in concert with innate immune genes. There is little published work on the effect of modulation of innate immune pathways to either minimise inflammation or enhance immune

barriers within the gut until antibiotic or antiparasitic therapy can remove the pathogenic organism. These are areas that research is required to see if defined innate immune stimulators added within feed can strengthen gut innate immunity by increasing mucosal barriers and general disease resistance and resilience. This could be a viable strategy within very young calves or lambs to aid maturation of their gut immune system and increase mucosal barriers to pathogens. There is evidence that innate stimulation in neonatal lambs leads to maturation of mucosal surfaces, LPS administration to neonatal lamb lungs increased lung maturation and upregulated innate immune receptor expression within the lung [69]. In adults they may also be used to increase defence mechanisms long enough for other therapeutic interventions such as antibiotics or anthelmintics to be effective. Whether defined innate stimuli or modulation of gut immunity with prebiotics is a better approach will require future research (see section 4).

Vaccination and Innate Immunity.

Vaccination will remain a cornerstone of disease and livestock management due to its low cost, high efficacy, duration of effect, safety profile and ease of delivery. These factors have long been recognised by the industry and vaccines are one of modern medicines most spectacular success stories. This success is driving the development of vaccines that treat more than just infectious disease, problems ranging from oestrous control, addiction, cancer and diabetes are all showing promise as vaccine targets. New livestock vaccines also have to meet increasingly stringent safety, biosecurity and regulatory requirements; modern vaccines are not only being asked to do more but they have to do it safer and more effectively than ever before. These demands will require new developments in vaccinology which are likely to come from our increasing understanding of vaccine induced immunity and improved vaccine delivery vehicles. We now have a much better understanding of the immunological parameters that influence vaccination and vaccine delivery agents are becoming more sophisticated. There is great promise that innate stimuli combined with gels, ointments, patches, liposomes, nanoparticles and sublingual capsules as delivery vehicles will compete effectively with or complement the live and killed viral, viral vectors, chemical, bacterial and oil based preparations that dominate the livestock vaccine market.

The need for better vaccines in the Australian Livestock Industry.

Vaccination has been an extraordinarily successful farming intervention, it has allowed livestock farming within areas previously inaccessible due to endemic disease, it has lifted productivity due to cost effective control of livestock pathogens and helped to block zoonotic transfer of diseases to humans. As the planet's population expands there will be an increasing demand for livestock products; however there are constraints on the availability of productive land and serious livestock diseases continue to reduce productivity in an era where increased productivity will be vital to feed a growing and more prosperous population. Heegaard *et al.,* (2011) published an excellent review outlining the use of adjuvants and delivery vehicles within Veterinary species, including livestock [70]. This section will attempt to focus on the innate immune system and how it can be manipulated for vaccine development for diseases that impact Australian farmers. There remain serious issues that continue to plague the Australian livestock industry such as vaccine reactivity, biosecurity, reversion, infectious diseases and dosing regimes that will require a more thorough understanding of how innate immunity can be harnessed to produce more effective interventions.

Currently the vaccines available for livestock mostly combat bacterial and viral disease, many are live attenuated or killed whole organism vaccines which induce long lasting immunity, often life-long, with few or no booster vaccinations required to maintain protective immunity [71]. Although highly effective there are still many problems associated with the use of these vaccines and there are many diseases remaining where vaccines would be of great benefit to the livestock industry. An obvious hole in the livestock vaccination schedule is lack of vaccines for the gastrointestinal nematode parasites and to an extent ticks. Infectious diseases caused by bacteria remain problems within the livestock industry, mastitis develops when the udder is infected with bacteria and can cause sepsis and death of the parent or poor growth in offspring due to milk spoilage [13]. Currently no effective mastitis vaccines are available due to a number of constraints, the infection site is difficult to locally vaccinate and systemic vaccines typically induce poor mucosal responses [72]. Additionally there are many types of bacteria that cause clinical mastitis, including *Streptococcus species*, *E. coli species* and *Staphlococcus species* with each requiring an individual vaccine. Bacteria

also pose a problem due to the transmission of food pathogens from livestock into the human food chain. This is a growing concern; some pathogenic *E.coli* and *Salmonella* strains can contaminate carcasses and persist right through the food chain to the end consumer. A vaccine that targets pathogenic bacteria would help alleviate mastitis, carcass contamination and shedding of pathogenic bacteria.

With an increase in intensive farming practises more likely within Australia to meet the worlds growing demand for protein, bovine respiratory disease (BRD) is likely to become a more common problem. It is commonly observed in feedlot cattle and while the aetiology is not well understood, it appears that viral pathogenesis in the lungs is the main cause of the disease which can be exacerbated by adventitious bacterial infection [73, 74] and is difficult to control [75]. Vaccines against the most common viruses are poorly protective in new born calves which may be due to several factors; however the immaturity of the calf immune system and presence of maternal antibody are common factors [76]. Larson and Step (2012) reviewed the efficacy of vaccines against the bacterial pathogens associated with BRD and found it difficult to assess the clinical benefits from a range of studies [77].

Reactogenicity is a vaccine's propensity to produce severe side effects following administration; these can include localised inflammation, fever to disease-like symptoms. The current Johne's vaccine, Gudair® uses Freund's Complete Adjuvant (FCA) which is highly reactogenic and injection site reactions often persist for years and there are reported cases of accidental injection into humans causing significant discomfort and long term disfiguration [78-80]. A complication with many of the live attenuated viral and bacterial vaccines is that they may recombine with existing virulent viruses or genetically revert to their virulent forms. This was recently very clearly outlined by a study on a herpes virus outbreak in Australian chickens where attenuated vaccine strains recombined to form a dominant virulent virus strain [81]. This study clearly outlined the need for producers and veterinary organisation to investigate methods to reduce the use of attenuated live vaccines and replace them with killed or sub-unit vaccines which pose no threat of recombination or reversion.

Perhaps the biggest threat from infectious diseases to the Australian livestock industry lies with the introduction or evolution of a highly pathogenic organism that has the potential to cause widespread disruption and cost to Australian Producers. Livestock Biosecurity is a real threat, the cost of a foot and mouth disease virus (FMDV) outbreak would be counted in the

millions without immediate containment. The risk could be minimised through the development of successful sub-unit vaccines that rapidly generate immunity and allow fast discrimination between vaccinated and infected animals and would decrease costs associated with an outbreak [82]. Footrot vaccines are available however they suffer from a process known as "antigenic competition" where one antigen is preferentially selected by the immune system over others present within the vaccine. This is particularly relevant to the development of effective footrot vaccines which are based on the fimbrial proteins of the 12 or more pathogenic strains of *Dichelobacter nodosus.* To maintain antibody titres sufficient to protect sheep from all the possible strains/serotypes of *D. nodosus* numerous vaccinations need to be given at least 3 months apart [83], this is not an ideal situation due to increased expenses and animals remain susceptible to disease causing strains during this lengthy process.

Bopriva® a currently approved vaccine available for cattle to control fertility and undesirable oestrous behaviours, suffers from a lack of long term immunity and requires frequent vaccination or "booster shots" for prolonged effect [84]. This restricts the vaccines use to highly intensive cattle farming, such as feedlots where the behavioural controls can recoup the cost of repeated vaccination. Northern cattle free range over large areas and are only mustered annually so treatments in these environments must be fast, single administration, effective and long lasting. Single shot formulations could improve conventional livestock vaccines that require “boosting”, as farmers benefit from reduced labour and diesel costs and it would also help increase compliance and adherence to recommended protocols for vaccination [85]. Duration of effect and dosing schedules of vaccines are important issues across the Australian livestock industry. These are just highlights of unmet needs within the Australian livestock industry that may benefit from the availability of improved safer and more efficacious vaccines.

The research underlying the promise and problems of using innate immunity in vaccine development.

The greatest therapeutic potential of our current understanding of innate immunity comes from the development of more targeted controlled vaccine adjuvants that can be formulated to specifically direct the adaptive immune system to best protect from the infectious

pathogen. This is a promising area for the livestock industry as many pathogens require specific types of immune responses to eradicate. Adjuvants that specifically target innate immune receptors have recently been approved for use in humans suggesting they can be formulated to be safe and effective. The TLR4 agonist monophosphoryl LipidA (MPL) has been formulated into two adjuvants by Glaxo Smith Kline (GSK) AS01 and AS04 which are approved for human use. AS04, a mixture of Alum and MPL forms the basis for the human Cervarix human papiloma virus vaccine and AS01, a mixture of liposomes, MPL and QS21 is the basis of a new malaria vaccine [86]. Murine studies have detailed how important and specific the innate immune system is for sculpting the adaptive immune response and how this interplay affects murine models of disease. However from experience and the literature we know that many adjuvants behave differently within different species, even highly related species. For this reason, this section will attempt to focus on work performed within livestock on the diseases plaguing the industry.

Johne's and mycobacterial diseases.

Clearance and resistance to mycobacterial pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) and *Mycobacterium tuberculosis* (tuberculosis) is most closely associated with IFN-γ produced from CD4+T helper cells and innate type T-cell subsets for clearance of the infection [87] and IL-17 [88]. There is controversy over whether IFN-γ producing $CD4^+$-T-cells are the most effective correlate of protection following mycobacterial infection as innate like T-cells may also be involved [89]. A study by Baldwin et al., 2012 compared a mycobacterial antigen reconstituted in a stable oil-in-water emulsion (SE) or an SE incorporating the TLR4 agonist glucopyranosyl lipid adjuvant (GLA-SE). The ID93/GLA-SE vaccine induced potent polyfunctional T-cells and IFN-γ and showed protection in mice and guinea pigs whereas no protection was observed with ID93/SE [90]. Water in Oil formulations in cattle induced a large effector T-cell and a central memory response, while a cationic-liposome adjuvant induced only a central memory immune response implying that Oil in water formulations may be superior adjuvants for TB immunity [91]. These results highlight the importance of properly formulating subunit vaccines with effective delivery vehicles and innate immune adjuvants for use against tuberculosis. A review by Lang discusses the potential of C-Type Lectin Receptors that activate innate immune cells via the Syk-Card9 pathway as receptors for adjuvants that direct the development of robust Th17 and Th1 responses to subunit vaccines [92]. Interestingly one of

the hallmarks of Freund's Complete Adjuvant, used in the Gudair® vaccine is the induction of IL-17 [93], however its persistence is also linked to vaccine side effects [79]. It is clear that targeting specific innate pathways is likely to contribute substantially to developing effective adjuvants for Johne's disease which requires safer alternatives to Gudair®.

Johne's is very similar to TB [94] and the current BCG vaccine for human and cattle tuberculosis is quite effective however its immunity wanes over time and it is difficult to boost, for reasons which are not entirely clear. Currently the most promising strategies being employed to develop long lasting immunity to TB involve adjuvanted subunit vaccines to boost or prime BCG immunity [95]. These strategies may be applicable for Johne's disease, however induction of the correct protective response is still likely to be the key to effective and safer vaccination for this disease [89, 94]. It is hoped that more targeted innate receptor agonists with better pharmacological safety profiles will be able to induce specific immune responses tailored to drive protective immunity against the difficult to treat mycobacterial diseases. Any candidate adjuvants will need to be combined with appropriate antigens and screened for protection in long-term field experiments within livestock as Johne's disease and tuberculosis have long incubation periods [96].

Sub-Unit vaccines – modulation of immunity with innate adjuvants.

Live vaccines are enormously effective and have been proven to be safe and reliable, however they have an inherent risk of reversion to virulence which occurs with the live human rabies vaccines and was recently reported for chicken anti-herpes virus [81]. Innate immunity offers the promise of improved sub unit vaccines, which typically have a better safety profile and no risk of reversion to virulence than attenuated live vaccines. Adding a defined innate stimulator to existing adjuvants is likely to increase desirable immunity. Our recent results demonstrate that adding a TLR agonist (CpG or pIC) to cationic liposomes or cationic liposomes resuspended in oil dramatically alters the degree and type of immunity elicited within sheep (de Veer personal observations). Extensive work in a number of species by the Vaccine and Infectious Disease Organization, University of Saskatchewan and others has shown that addition of CpG to livestock vaccines generally enhances effectiveness within a number of adjuvant formulations [20, 97-101]. Examples include CpG coupled with host defence peptides and phosphazine induced stronger cell mediated immunity within cattle than Emusigen™ with CpG but not higher IgG titres [102]. A trial looking at the vaccination of lambs with Herpes virus-1 vaccine formulations are improved by the addition of CpG [99]

and close to complete protection against BHV-1 challenge was elicited in the calves immunized with the protein/CpG formulation [103]. CpG improved a killed FMDV vaccine formulation, 80% of the calves immunized with CpG adjuvant, 25% immunized with adjuvant and 50% immunised with inactivated FMDV vaccine were protected from FMDV challenge [104]. The addition of CpG to the BioThrax® anthrax vaccine improved immunological parameters and immunity [105, 106]. CpG is a promising molecular adjuvant that appears to effectively enhance immune responses in livestock and fulfils many of the criteria necessary for effective use within Australia, it is synthetic, stable, easy to store and effective. CpGs and other nucleic acid based immunostimulants are likely to be useful when incorporated into delivery vehicles or vaccine systems where they are retained with the antigen or gradually flow to the lymph node rather than rapidly disseminate throughout the systemic circulation where they may pose safety risks [107].

Shu *et al,* (2000) compared Alum (inflammasome activation and dendritic cell binding), Freund's Complete Adjuvant (FCA) (inflammasome & multiple TLR activation), QuilA (unknown - perhaps inflammasome), dextran sulphate (unknown) combined with mineral oil, or alum in cattle for their effects on *Streptococcus bovis* and *Lactobacillus spp.* antibody titres. Generally all adjuvants increased antibody titres with dextran sulfate being the most potent, however cell mediated immunity was not assessed [108]. There are very few other examples of the incorporation of defined innate immune agonists into livestock vaccine formulations that have presented vaccine trial outcomes. Various lipopeptides including Pam3CysK4 activate bovine dendritic cells (DC) to induce IL-12, MHC molecules and induced T-cells activation and IFN-gamma secretion [109]. Bovine DC and macrophages both produced TNF-alpha, DCs produced more IL-12 and macrophages produced more IL-10 and nitric oxide following stimulation with lipopolysaccharide, poly(I:C)-double-stranded RNA and CpG-DNA [110] which suggests that livestock are capable of sensing a range of common TLR agonists. This is supported by analysis of the bovine genome which contains the relevant TLR genes and indeed a full complement of other innate receptor and adaptor molecules.

Live viral vaccines often require infection and limited replication to induce immunity which is prone to being blocked by maternal antibodies [76]. In contrast a vaccine study using the new generation ISCOM™ adjuvant which combines innate immune stimulation with nanoparticle delivery protected calves following Bovine Respiratory Syncytial Virus (BRSV), a component of vaccines to reduce BRD. Vaccination induced a highly significant

reduction of virus replication in the respiratory tract of calves, associated with higher antibody and T helper cell responses and overcame the inhibiting effect of maternal antibodies [111]. It has to be cautioned that the ISCOM study only looked at a single virus, while the Windeyer study had combined 4 different live virus vaccines however it further highlights the possibilities of combining innate stimulation with innovative delivery vehicles.

Modulation of antibody isotypes using innate immune adjuvants.

Stimulation of the innate immune system with defined adjuvants can modulate T-helper cells to produce a desired set of cytokines to improve vaccine outcomes in disease such as Johne's however most vaccines work by inducing high levels of antibodies. Antibodies directly bind pathogens and block interaction with a target cellular receptor, this is the most accurate correlate of protection for most antiviral vaccines and is typically measured as the neutralisation titre. Antibodies also agglutinate or crosslink pathogens which improves uptake via phagocytic cells types and larger agglutinated pathogens are removed by the spleen. Agglutination is important for bacteria, viruses and small unicellular parasites such as babesia. The third antibody mediated protective mechanism is the activation of the complement cascade which can directly destroy the pathogen or flags it for phagocytosis. Many pathogens have evolved mechanisms to subvert these pathways however high titre antibody production is still the best correlate for protection for almost all the current vaccines sold for livestock and human use. Antibodies are not a homogenous species; each B-cell typically matures to produce a single isotype of antibody, thus the final response to a pathogen consists of a mix of many isotypes. This is important for vaccination as each antibody isotype differs in its ability to induce the three main mechanisms of vaccine efficacy.

The antibody isotype that particular B-cells produce depends on the signals it receives while maturing, these include; location, innate stimuli and the surrounding cytokine milieu. Where B-cells acquire antigen is important, B-cells encountering antigen in areas associated with mucosal surfaces are more likely to switch to produce IgA antibodies which is partially driven by gut production of retinoic acid [112, 113]. Peripheral B-cells are more likely to mature to produce IgG1 or IgG2 and their sub-isotypes. The innate immune system is a powerful driver of B-cell isotype switching as B-cells express many of the receptors and signalling molecules associated with innate immunity and directly take queues from

pathogens or vaccine adjuvants. In addition the innate immune system helps determine the milieu of cytokines produced by T-helper cells and surrounding cells that help direct B-cells class switching. Genetic differences and history of pathogen exposure also contribute the repertoire and proclivity of B-cells to produce certain antibody isotypes. This is illustrated most effectively by the vast literature on the development of allergic diseases which are highly associated with the production of the IgE antibody isotype by B-cells [114]. Indeed innate immune adjuvants are showing promise as components of anti-allergy vaccines that aim at driving B-cell class switching away from the harmful allergenic IgE isotype to less harmful antibodies (reviewed in [115]).

A study displaying how different antibody isotypes and innate immune stimuli influence vaccine efficacy was nicely illustrated in mice by Schmitz *et al.,* 2012. They developed isotype specific monoclonal antibodies to the influenza-A M2 protein and discovered that the IgG2c isotype antibodies were particularly effective at preventing influenza infection while IgG1 antibodies were poor. The addition of a TLR7 agonist to a vaccine using the M2 protein dramatically increased the IgG2c antibody titre and the vaccine's anti-influenza effect [116]. These same methods have broad applicability within livestock vaccination schedules as it is becoming apparent that specific immunity is often required for effective pathogen removal. It is broadly accepted that a type 2 immune response is required to remove parasitic nematodes within livestock, while this is the opposite of the type of immune response that is thought to protect animals from Johne's disease. In type 2 immunity there is little production of IFN-γ and higher production of cytokines such as IL-4, 5 and IL-13. The polarisation of immune responses within outbred animals is rarely as stark as those seen in laboratory mouse strains however infections and vaccine adjuvants do tend to increase the expression of specific sets of cytokines within animals.

Anti-parasitic vaccines

A recent study by Andronicos *et al*., 2010 showed that sheep selectively bred to be resistant to nematode infection showed increased levels of antibody isotypes and cytokine expression patterns associated with type 2 immunity following *T. colubriformis* infection compared with susceptible sheep [117]. Defined PAMPs that promote type 2 immunity have remained elusive, however various extracts from parasitic organisms such as nematodes, trematodes and cestodes tend to polarise immunity to a type 2 response. There is some evidence that

proteases and glycans present within these extracts help to promote type 2 immunity, however the pathways and receptors for these products have remained elusive [118]. A number of commercially available adjuvants such as dextran sulphate and alum promote type 2 immune responses, however how they do this is unknown. Alum can activate the inflammasome in primed monocytes however this is not required for some of its adjuvant effects [119]. Alum increases the association of antigen with dendritic cells which may be responsible for its adjuvant effects [120, 121]. Our laboratory recently tested four adjuvants with a larval antigen purified from *Haemonchus contortus* and found dextran sulphate was the most effective at protecting sheep from subsequent challenge consistent with its ability to induce type 2 immunity [122]. The above studies demonstrate that type 2 immune responses are likely to be beneficial for the protection of Australian livestock from parasitic worms.

Interestingly, *T. colubriformis* feeding and burrowing behaviour caused necrosis in an *in vitro* cultured epithelial cell layer and lead to the release of the IL-33 alarmin [123]. IL-33 has previously been associated with parasitic responses [124] and plays important roles in type 2 immune induction [125], allergy [126] and wound healing [127]. IL-33 forms a family of intracellular alarmins which includes HMGB-1 and IL-1alpha, they are released following cell damage and induce inflammation and direct the immune system [128]. It may be that cell damage and necrosis are intrinsic triggers that drive type 2 immune induction. It will be interesting to see if Alum, dextran sulphate and other type 2 inducing adjuvants induce cellular necrosis and alarmin release. These innate alarmins may be an important source of new more potent adjuvants that skew the immune system to a type 2 response. The currently identified type 2 inducing alarmins are proteins, which usually makes production costs high and more challenging. However if suitable production methodologies can be perfected or synthetic analogues developed these molecules may be highly effective anti-parasitic vaccine adjuvants.

Vaccine Responders and Non-responders.

To successfully vaccinate an animal, a sufficient dose of vaccine must be delivered, the innate immune system must be strongly engaged, the antigen must be suitable presented to the adaptive immune system and a sufficiently broad repertoire of T-cell and B-cell receptors need to be present to recognise the antigen. Failure in any of the immune parameters mentioned above will lead to inefficient vaccine responsiveness in individual animals.

Additionally sickness, stress and maternal antibodies also contribute the variations in vaccine responses observed across a herd and the human population. It is well established that not all vaccinates whether livestock or humans produce sufficient antibodies to be protected, these individuals are known as non or low-responders and can act as reservoirs for disease within livestock. Non-responders or low-responders can become responsive to the vaccine if multiple doses are administered, this is one reason why multiple dosing is common with many vaccines while persistent non-responsiveness to a vaccine is most likely to be due to a genetic defect.

There is a strong genetic basis for variations in vaccine responsiveness, a QTL discovery study in cattle identified QTLs associated with the levels and isotypes of antibodies following administration of an RSV vaccine [129]. There have been many studies within humans looking at the genetic basis for the variation in vaccine responses observed across the community [130]. The Mayo Clinic Vaccines Group have conducted the most comprehensive genetic studies on vaccine immunity looking at the ability to accurately measure and predict vaccine outcomes using genetic testing [131-133]. Their studies of people vaccinated against measels have shown that innate immune pathways are crucial reasons why some individuals fail to respond to vaccination. Genetic lesions within the measles virus receptor CD46, innate pattern-recognition receptors, intracellular signaling intermediates, and cytokines and receptors known to be induced by innate immune pathways were central to control of both humoral and cellular immune responses to measles vaccination [134]. Additionally gene expression studies demonstrated that individuals showing increased stimulation of innate immune pathways were associated with improved vaccine responses [135]. This suggests that improved formulations focussing on potent activation on innate immunity are likely to decrease the requirement for “boosting’ and increase vaccine effectiveness in low-responder populations.

A study on responses to an Anthrax vaccine using the human snp chip with 736,996 snp's passing quality control on 726 individuals identified MHCII alleles and a few candidate genes but had low power to predict outcomes following vaccination [132]. The authors blame missing heritability problems that can confound complex trait studies, with evolutionary selection removing many highly influential genotypes as they are likely to impact fitness. Whole genome based approaches have not found as many genetic associations with disease as experimental calculation would suggest due to a number of shortcomings which are detailed in the following reviews [136, 137]. All single nucleotide (snp) studies on polygenic

traits are confounded by this missing hereditary, however livestock studies can incorporate twin analysis, breeding histories, single sires and more controlled genetic conditions than human studies greatly increasing their power to detect mutations associated with traits. Hindorff *et al.*, (2012) has compiled a database of genetic association studies that is searchable by the trait analysed and is an excellent resource [138].

The innate immune system helps shape the adaptive immune response and as such many of the adaptive immune readouts such as antibody levels may be surrogate markers for an effective innate immune system. An example would be testing antibody levels to clostridial vaccines as a marker for effective activation of innate immune pathways, it is highly likely that the clostridial vaccines contains factors that activate innate immune pathways. Other common vaccines, preferably with defined adjuvant effects could also be used. Due to the high genetic heritability of vaccine responsiveness and the important role of innate immune activation in vaccination it is likely that vaccine responses could inform future calculations of genetic immune fitness. These could reasonably easily be included in estimations of breeding value and an immune component to the extensive list of production traits currently measured.

Measuring vaccine induced immune responses and associating these with genetic markers such as snp's in target livestock breeds may help breed livestock that not only respond more reliably to vaccination but are also more resistance to disease as they have a healthy innate immune system. An even more systematic approach could include testing a defined set of innate immune agonists that activate the different innate immune receptors to generate an innate immune profile which would help eliminate animals that are likely to be susceptible to certain infections and have low responses to certain vaccine adjuvants. While vaccine delivery is beyond the scope of this review, inefficient vaccine delivery and failure to adhere to vaccine schedules by farmers are also likely causes of differential vaccine responses observed in the field.

Using vaccination to track immune competence.

The section on the genetics of immunity nicely outlines the benefits and pitfalls of selecting animals based on immune phenotypes. One of the most accessible immunological phenotypes measurable in livestock is vaccine responsiveness. While human GWAS studies are complicated and generally require very large sample sizes to see specific genetic contributions, studies within livestock benefit from the ability to follow parentage and

actively participate in the selection process through directed breeding. It is highly likely that a high proportion of the underlying variability in the response to vaccination is driven by the initial innate immune response to the vaccine [135]. Measuring adaptive immune responses is a good surrogate for the competency of the underlying innate immune system however determining the relative contribution becomes challenging when the outcome is triggered by compounds that have poorly defined innate immune activatory mechanisms. It becomes even more difficult within a disease context where there are often multiple innate immune stimuli present which will trigger redundant pathways and infective organisms often modulate immunity. This can make determination of genetic input difficult, especially variation within the highly diverse innate immune system. The immune system "see's" non-living vaccines as simplified versions of the infective organism and in the case of sub-unit vaccines often only a single adjuvant or antigen is used, greatly simplifying the system again. This reduced complexity may allow more meaningful links to be made between vaccination phenotypes (IgG levels and isotypes, T-cell number and cell mediated immunity measures) and genetic markers such as snp's and whole genome sequencing.

Genetic selection based on vaccine responsiveness is likely have the added benefit of improving vaccine response rates and allow the measure of whether high vaccine response rates correlate with resistance to other diseases. Along with implicating the pathways targeted by the adjuvant/s in the particular disease state it will also validate whether the approach of selecting for high immune performance enhances resistance to disease in livestock. This strategy is already being employed within cattle with bovine respiratory syncytial virus [129, 139] and this could be used as a blueprint to implement these types of measures more broadly within livestock selection strategies. Incorporation of vaccine responses into genetic screens would allow long term monitoring and inclusion of immune based EBVs into regular genetic improvement programs.

Stress and Innate Immunity.

The detrimental effect of stress on the immune system has been extensively studied in both human and animal models. Stress has been shown to reduce the number and function of a variety of immune cells, decrease antibody production, modulate the production of protective cytokines and can also reactivate previously latent viruses [140]. These effects are mediated primarily by stress hormones, particularly glucocorticoids and catecholamines.

Glucocorticoids have been shown to downregulate the type 1 inflammatory immune response while upregulating the type 2 anti inflammatory response. This involves suppression of pro inflammatory cytokines including IL-12, IL-1 and IL-6 combined with the upregulation of anti inflammatory cytokines IL-4 and IL-10. Catecholamines augment this effect by contributing to the inhibition of IL-12 and increasing IL-10 production [141]. An increase in stress hormones has been shown to increase the growth and virulence of various pathogens [142]. These stress hormones also reduce cellular immunity by diminishing natural killer (NK) cell activity and inhibiting lymphocyte proliferation [140]. It appears that chronic stress skews the immune response away from cellular immunity toward a type 2 anti-inflammatory response, which may influence an individual's susceptibility to infection.

The fact that stress modulates immune function has important implications for animal production practices such as housing, feeding, temperature, handling and transportation. These practices contain potentially stressful periods that may lead to stress induced changes in pathogen susceptibility and disease. Feed withdrawal and handling increases faecal shedding of *E.coli* in cattle [143], sheep [144] and pigs [145]. Additionally, exposure to cold temperature stress increases the resistance of *E.coli* derived from the swine intestinal tract to various antimicrobial agents [146]. The stress associated with transportation induced both an increase in the infection rate and the faecal excretion of *Salmonella* in cattle [147, 148]. [149] found that alleviating heat stress with sprinklers reduced the faecal prevalence of *Salmonella* in dairy cattle before milking. Calving stress was shown to increase the clinical signs of ruminant paratuberculosis, thereby suggesting that stress can also affect mycobacterial infections [141].

The specific biological pathways and immune mechanisms that are affected by stress in an infection state are largely unknown. The studies that investigate the role of stress hormones, including glucocorticoids and catecholamines, are mainly conducted *in vitro* in cells derived from small animal models. Glucocorticoids can increase expression of antiviral inhibitory pathways [150], suppress TLR induced dendritic cell maturation [151] and induce macrophages to induce anti-inflammatory mediators which dampen acute inflammation [152]. Innate immune pathways can also inhibit glucocorticoid induced effects suggesting a strong interaction between the two systems in disease [153]. It is clear that glucocorticoids and other stress mediated compounds can exert multiple inhibitory effects on innate immune pathways (for a review see [154]), however the mechanisms of regulation are poorly characterised and not well understood.

There are a small number of large animal *in vivo* studies that investigate the relationship of stress and immunity in natural or experimental infections, this is a highly complex series of interactions that are only just starting to be elucidated (reviewed in [155]). Weaning stress in cattle induces broad changes in gene expression within bovine leukocytes including many genes involved in both innate immune and adaptive immunity [156] as well as generally increasing the expression and number of innate immune cell types and cytokine in stressed calves [157]. The Bovine respiratory disease complex (BRD) is exacerbated by stress inducing glucocorticoids and stress has been implicated in susceptibility to the disease in cattle [158, 159]. A recent study in calves showed that weaning and maternal separation stress followed by induction of BRD by virus and bacteria did not significantly alter virus shedding but increased innate immune responses in stressed calves compared to stress adapted calves [159]. Interestingly cortisol levels were typically higher in the stress adapted calves when compared to the stressed calves before and after viral challenge suggesting that natural cortisol may reduce innate immune activation following viral and infection and could be protective. The acute stress introduced in this study also included a change in feed in the stressed group which introduced a metabolic perturbation, making interpretation of cortisol and innate immune mechanisms less clear. It is clear that multiple stressors had a detrimental effect on survival of calves following infection and it will be important to understand the mechanisms by which stress alters immunity in livestock and the interventions that can help minimise the impact. Practical measures to reduce stress are more likely to come from better husbandry practises than direct therapies aimed at cortisol or innate immune pathways. Additionally EBVs based on minimising behavioural responses to stressors and immune perturbations following stressors may be beneficial however it will difficult to determine which pathways to target based on our current understanding of the interaction of stress induced effectors and the immune system.

Side effects of innate immune activation.

We have extensively discussed the potential benefits that effective manipulation of the innate immune system may bring to the Australian livestock industry we have not extensively presented problems with the use of innate immune modulators. The anti-TNF-alpha biopharmaceuticals that decrease the activity of cytokines associated with inflammatory conditions such as rheumatoid arthritis slightly elevate the risk of infection, cancer and autoimmune diseases [37]. Additionally the blockade of inflammasome activated IL-1 beta

also showing some increase in mild to moderate infections [160]. These clinical implications are the best indication we have that broad blockade of innate immune pathways may predispose the recipient to infectious agents. This is not surprising as one of the prime roles of innate immunity is to induce and mobilise anti-infection pathways.

The activators or agonists of the innate immune pathways hold great promise for the prophylactic and potentially therapeutic treatment of infections, however they also pose a risk of systemic inflammation that can be harmful. It is well established that LPS a potent TLR4 agonist induces fatal sepsis like symptoms and sheep are particularly susceptible to intravenous LPS mediated toxicity. Imiquimod and other TLR7 agonists have a long history of use in topical skin treatments, however their use as systemic reagents has been hampered by toxicity [7]. This indicates that any systemic use of innate immune stimulators will require careful optimisation of dose, route of administration and testing within the target species. There is no doubt that each innate immune agonist likely has a therapeutic window, however most can also induce severe side effects if this dose is exceeded. It is not just TLR-agonists that can induce severe side effects, systemic administration of a form of bacterial flagellin that could access the cytoplasm resulted in rapid activation of the NAIP5/NLRC4 inflammasome leading to an eicosanoid storm that killed mice within 30 minutes [161]. This drastic outcome is a caution that adjuvants are potent immune modulators and pose serious risks if dose, formulation and route of administration are not intelligently designed and rigorously tested in relevant models. Nanoparticles and particulate delivery systems in general have been implicated in the activation of the inflammasome which has raised safety considerations regarding the future use of particulate therapies [162, 163]. These side effects are a caution that any therapeutic application of innate immunity poses a risk due to the central role of this system in inflammation and immunity. To efficiently employ both activators and inhibitors of innate immunity as livestock prophylactics, therapeutics or vaccine adjuvants will require intelligent design of the compositions of innate immune modulators and systematic dose testing in livestock.

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Chapter 3 - Table 1: Drugs targeting innate immune pathways or components.

Name	Class	Therapeutic use.	Company
TLR			
ANA975	TLR7 agonist	Discontinued due to toxicity	Anadys
Imiquimod	TLR7/8 agonist	Warts, cutaneous cancers.	Various
Resiquimod-R848	TLR7 agonist	Herpes (Trial Discontinued)	Lily and 3M
ANA773	TLR7 agonist	Antiviral	Anadys
Eritoran	Lipid TLR4 antagonist	Sepsis	Eisai
Tolamba	ssDNA allergen conjugate	Discontinued - not efficacious	Dynavax Technologies
VAX-102, VAXFlagellin-HA	TLR5 agonist Flagellin fusion protein	Vaccine Adjuvant - Influenza	VaxInnate
Small molecules	Drugs	Development	Guan et al.,
IPH-3102	TLR3-dsRNA	Cancer & Vaccine adjuvant	Innate Pharma
Ampligen AMP-516	TLR3-dsRNA polyI:U	Chronic fatigue, viral infection.	HemiSpheRx
Monophosphoryl LipidA (MPL)	TLR4 lipid agonist	Vaccine adjuvant	GSK
OMP-174	Glucosamine disaccharide FA	Cancer, vaccine adjuvant	OM Pharma
CBLB502	Flagellin fragment	Stressors	Cleveland biolabs
VTX-1463 and VTX-2337	TLR8 agonist	Antiallergenic, antiviral, cancer	VentiRx Pharma
AZD8848	TLR7 agonist	Asthma and allergy	AstraZeneca
IMO-2055	CpG TLR9 agonist	Cancer	Idera Pharma
MGN-1703 and 1706	dsDNA - TLR9 agonist	Cancer	Mologen AG
ISS1018	CpG TLR9 agonist	Cancer, vaccine adjuvant	Dynavax Technologies
SD-101	P-type CpG TLR9 agonist	Viral infection	Dynavax Technologies
Heplisav	TLR9 agonist	Hepatitis B vaccine	Dynavax Technologies
Agatolimod	CpG TLR9 agonist	Cancer	National Cancer Institute
IMO-2125 and 2134	TLR9 agonist	Viral infection and allergy respectively	Idera Pharma
NuThrax	TLR9 agonist	Anthrax	Emergent Biosolutions
AVE0675	TLR9 agonist	allergy	Sanofi-aventis/Pfizer
DIMS 0150	TLR9	Colitis	InDex Pharma
SMP-105	TLR2	Cancer	Sumitomo Pharma
1A6/NI-0101	TLR4 antagonist antibody	Inflammation - Phase 1 trail Q4 2012	NovImmune
CRX-527	Lipid A mimetic - TLR4 agonist		
SM-360320	TLR7 agonist	Hepatitis C, antiviral agent.	Anadys Pharmaceuticals, Inc.
GS9620	TLR7 agonist	Hepatitis C, antiviral agent.	Gilead Sciences
CpG10101	TLR9 agonist	Hepatitis - discontinued, poor efficacy	Pfizer
PF-4878691 also 852A	TLR7 agonist	Hepatitis C agonist - high dose flu symptoms	Pfizer
poly-ICLC	TLR3 agonist	Antiviral and adjuvant, glioblastoma	Oncovir Inc.
Inflammasome			
Anakinra	IL-1beta inhibitor	Gout, sterile inflammation	
poly[di(sodiumcarboxylatoethylphenoxy)phosphazene (PCEP)			
Vaccinia virus F1L (amino acids 1-44)	Peptide	NLRP1 Inhibitor	Patent Applic # 12/504649
AZD9056	P2X7R antagonist	Rheumatoid arthritis - not effective	AstraZeneca
acALY18	Lipopeptide activates NALP3	Prophylactic anti-infective	Therimunex
Bay 11-7082	Inflammasome and NF-κB inhibitor	Inflammation	Numerous
Parthenolide - Yew fever plant extract	Inflammasome inhibitor	Inflammation	Numerous
Multiple Innate pathways			
Cadi-05	Multiple-Mycobacterial extract	cancer	No clinical benefit

Chapter 4

Impact of microbiota and nutrition on innate immune responses

Table of Contents

Impact of Microbiota and Nutrition on Innate Immune Responses

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Introduction

A major advance in immunology in recent years is the recognition that the gut microbiota plays a major role in both the development of the immune system as well as the strength and quality of the immune response generated. At the basis of this phenomenon, is the interaction of the gut microbiota and its products with the innate immune receptors of the host. In separate developments, nutritional sciences have long recognised the effect of nutritional manipulation and supplements on the composition of bacterial communities within the gastrointestinal tract. The recent mergence of these different areas has dramatically increased our potential to understand disease development and susceptibility, as well as providing new avenues for intervention via nutritional changes to promote health and productivity. This section will give a general overview of these very extensive areas of immunity, gut microbiota and nutrition in as far as they intersect, and will summarise available ways of using this knowledge to manipulate the health of man and livestock.

Immune system of the gastrointestinal (GI) tract

The primary function of the gastrointestinal tract (GI) is the digestion of food and absorption of nutrients needed for the body to function. In order to achieve this, the GI tract is exposed to a constant influx of foreign material and potentially harmful microbial and parasitic organisms. It is therefore not surprising that the GI tissues represent the primary immune organ in the body, containing ~ 60% of total immunoglobulins (Ig) and > 10^6 lymphocytes/gram tissue [1, 2, 3]

Several levels of protection are active at the GI tract to protect it from overwhelming infections [4, 5, 2] (Fig. 1).

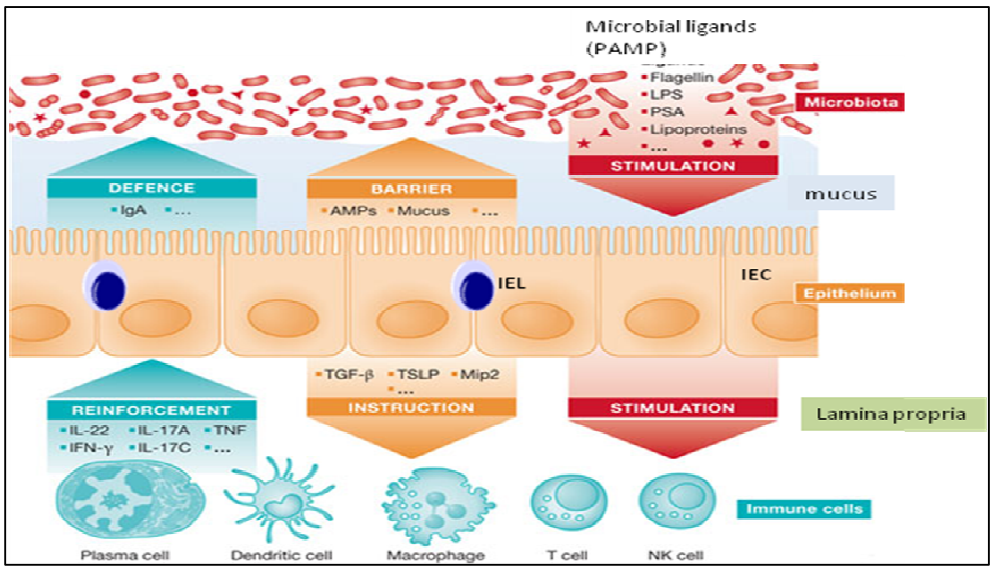

Figure 1: Levels of immune defense against microbiota in the gastrointestinal tract. If microbes cross the mucus barrier, stimulation of PRR on intestinal epithelial cells (IEC) and intraepithelial lymphocytes (IEL) by microbial PAMPs provides the first line of defense through increased production of mucins, inflammatory cytokines and anti-microbial factors (e.g. AMP). Innate immune cells in the underlying tissue provide antigen-specific signals to underlying gut-associated lymphoid tissue (GALT) to reinforce anti-microbial defense with an adaptive immune response, including IgA and various cytokines depending on the initial innate signaling event. (Adapted from [5])

AMP, antimicrobial peptide; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; LPS, lipopolysaccharide; PSA, polysaccharide A; TGF-β, transforming growth factor beta; TNF, tumour necrosis factor; TSLP, thymic stromal lymophopoietin; MIP2, macrophage inflammatory protein 2.

The low pH and the presence of proteolytic enzymes in the stomach provide a hostile environment and are the first barrier for most pathogens entering the GI tract. From stomach to rectum, the gastrointestinal epithelium consists of a single columnar layer of cells which are covered by a layer of mucus. Mucus is a complex and dynamic substance held together by cross linking, highly glycosylated mucin molecules secreted by epithelial cells, in particular the specialized mucus-secreting epithelial cells or goblet cells [6]. There are two types of mucus: an insoluble gel which adheres strongly to the brushborder of epithelial cells and a viscous layer which is soluble in water and covers the gel. Hydrophobicity and mucus

thickness increases from proximal to distal parts of the intestine and colon and mucus viscosity can change during infection or following tissue damage [6].

Mucus also contains antibodies and an array of bioactive molecules secreted by epithelial cells and mucosal glands [6, 7]. Paneth cells are specialised epithelial cells located at the base of intestinal crypts and are particularly active in the constitutive and induced production of lysozymes, antimicrobial factors and proinflammatory cytokines into the lumen. Antimicrobial factors secreted by epithelial cells include the antimicrobial peptide (AMP) families of small, cationic peptides such as defensins. In addition to their microcidal properties, AMPs have additional roles as chemotactic and paracrine signaling molecules that can facilitate the innate inflammatory response and tissue repair during infections [8].

As well as providing a physical barrier, intestinal epithelial cells also display a variety of pattern recognition receptors that are critical for initiation of both the innate and adaptive response [9]. Pattern recognition receptors (PRR) expressed by epithelial cells include members of the Toll-like receptor (TLR) family and intracellular nod-like receptors (NLR) (see Section 1). Under steady state conditions, basal PRR signalling in intestinal epithelial cells is important for intestinal homeostasis and constant renewal of the epithelial barrier, while activation of PRR by pathogens results in an acute inflammatory response and the secretion of an array of cytokines, chemokines and antimicrobial effector molecules. There are also a large number of intraepithelial lymphocytes interspersed in the epithelium layer, comprised primarily of CD8+ T-cells, which are thought to function as suppressor cells and play a role in oral tolerance. $\gamma\delta$-TCR$^+$ intraepithelial cells have also been implicated in the early response to infections by the secretion of antimicrobial peptides [10, 11].

A further line of defense is provided by the sentinel cells present in the subepithelial tissues or lamina propria (Figure 1). Innate receptors have been detected on fibroblasts and many lamina propria leukocytes which act in concert with each other and soluble factors to initiate innate responses and to modulate the adaptive response in the draining lymph nodes.

The adaptive immune response is generated within GALT [1, 12] . GALT includes (jejunal) Peyer's patches and isolated lymphoid follicles variably present in different mucosal tissues and different animal species. GALT are covered by specialised microfold (M)-epithelial cells that have direct contact with the luminal content and can pinocytose soluble antigens present

in the lumen to present to T and B cells resident in the GALT or draining gut lymph nodes for the initiation of an adaptive immune response. This includes the production of secretory IgA (S-IgA) into the lumen, a typical mucosal antibody response. The secretory component of IgA facilitates transport through epithelium and protects the antibody from degradation by intestinal enzymes and toxins. The main function of S-IgA is to prevent attachment of intestinal pathogens (immune exclusion). Most intestinal bacteria are coated with specific IgA and this is probably the reason why most gut bacteria remain on the surface of the mucus without contacting epithelial cells [6].

The constant renewal of the intestinal epithelium is also essential for the maintenance of tissue homeostasis and results in the daily release of 25% of enterocytes into the gut [13].

Ruminant GI tract

The primary difference between a ruminant and non-ruminant mammal is that ruminants have a four-compartment stomach: rumen, reticulum, omasum, and abomasum [14]. Digestion of food in ruminants is primarily carried out by the rumen microflora which contain dense populations of several species of bacteria, protozoa, as well as some yeasts and fungi . Fermentation in a mostly anaerobic environment results in the production of the short-chain fatty acids (SCFA) propionic-, acetic- and butyric acid, carbon dioxide, methane and hydrogen sulphide. Rumen pH typically ranges from 6.5-6.8. Water absorption occurs in the omasum. The abomasum, or fourth stomach, is most similar to the human stomach; it produces hydrochloric acid and digestive enzymes (pepsin) and has a pH ranging from pH 3.5-4.0.

From the abomasum, the digesta enter the small intestine and are mixed with secretions from the pancreas and liver (bile), which elevates the pH to 7-8, suitable for most of the intestinal enzymes (trypsin and chymotrypsin, lipase and amylase). Nutrient absorption mainly occurs throughout the small intestine. Further fermentation of non-digested food particles may occur in the large intestine which is also the place where minerals are absorbed. The colon is the site of most of the water absorption and from where the remaining material is excreted as faeces from the rectum.

Immature ruminants from birth to about 2-3 months of age are functionally non-ruminants. Through the suckling reflex, the reticular groove formed by muscular folds of the reticulum shunts milk directly to the omasum and then abomasum, bypassing the reticulorumen. At

birth, the abomasums of calves is the largest compartment of the stomach. The rumen must be inoculated with microorganisms through licking and environmental contact, and consumption of solid food is required for the rumen to fully develop. A normal, functional rumen does not develop in ruminants raised in sterile conditions.

After weaning, ruminants derive protein from both feed and from microbes that inhabit the rumen.

The immune system of the ruminant gastrointestinal tract and its ability to induce an adaptive immune response has been reasonably well characterised and from the abomasum to the colon resembles in most aspects that of the murine and human GI tract. One major peculiarity of the ruminant GI immune system, shared with some other species, is the presence of ileal Peyer's patches, which are thought to be the primary lymphoid organs for B cell development. Very few studies have characterised the innate immune system of the ruminant GI tract or its composition of innate immune receptors.

Gut microbiota and its effect on immunity

Until recently, intestinal microbiota were mainly studied by microbiologists investigating infections and diseases of the GI tract. In the past decade, it has become clear that the intestinal flora strongly influences maturation and development of the gastrointestinal immunity and that the gut microbial community has co-evolved with the host immune system [12, 15-18]. There is also increasing evidence for a role of intestinal microbiota beyond the local gut immune response with evidence for its involvement in allergies, asthma, diabetes, obesity, cancer and neuropathologies. The interaction between gut microbiota and the immune system is a recent, but fast developing field of research with the potential for discovery of novel means of intervention that can be applied to both human and animal health and livestock production.

Overview of ontogeny and composition of gut microbiota

Most studies on gut microbiota have been performed in mice and humans [3, 19, 20] and will be briefly reviewed here, with reference to ruminants where appropriate. At birth the human alimentary canal is sterile, like that of animals raised in a germ-free environment. After natural birth, human infants are first colonized from maternal flora followed by environmental flora, while infants born through caesarean section obtain most of their

microflora from the environment. Infantile flora evolves towards normal adult flora over the first 24 months of life, depending on diet [3]. Formula-fed babies have different colonization than breast fed. Murine models have shown that mouse pups derive their microbiota from foster mothers, not birth mothers, which can be linked to disease susceptibility [19]. Enteric bacteria colonize the newborn infant in an oral-to-anal direction. About 3 or 4 weeks after birth the flora characteristic for the individual host is fairly well established and, except under unusual circumstances, remains relatively stable throughout life. Equilibrium of normal flora is believed to vary from person to person.

Due to the high acidity, most bacteria do not multiply in the stomach which contains a very low microbial population. Gastric emptying allows many bacteria to survive and pass into the duodenum where neutral pH allows microbial survival and multiplication. Due to the much longer transit time in the large intestine and colon, bacteria are able to reach considerable population levels in these sites and a dramatic change in the enteric flora occurs across the ileocecal valve, with number of microorganisms reaching upward of 10^9 to 10^{12} organisms per gram of colonic content, constituting up to 50% of fecal matter by weight [3]. In intestinal stasis, the bacterial count rises even higher. Forty genera of bacteria, represented by at least 400 species, can be cultured from the feces of a healthy human. The gut microbiota also includes a large number of bacteriophages which contribute to homeostasis of the microbial population.

The microbiota of the mammalian intestine depend largely on dietary polysaccharides as energy sources. Most of these polymers, the non-starch polysaccharides (NSP), are not degradable by the host, but herbivores can derive 70% of their energy intake from microbial breakdown. The herbivorous diet consists largely of plant cell-wall polysaccharides that are not digestible by host enzymes, and 70% of the total gut volume is typically devoted to microbial fermentation [21]. Polysaccharide substrates that are derived from the host rather than the diet are also available, especially in the large intestine and include mucopolysaccharides that are secreted in large quantities from the gut mucosa (mucus and dead cells). The two regions of the vertebrate gastrointestinal tract that have undergone the most specialization for microbial fermentation are the rumen and the large intestine (including the cecum).

Contact between bacteria and food in the colon causes anaerobic fermentation which produces numerous metabolites, varying depending on substrate availability. If the substrate is carbohydrates, short chain fatty acids (SCFA) are produced locally, including acetic, propionic, and butyric acids which are important as a source of energy as well as having important immunomodulatory functions (see further). Production of SCFA increases the luminal pH of the proximal large bowel within an acidic range of pH 5-6. The gut microflora also synthesizes amino acids (from ammonia) and protein, which are utilized by both the microorganisms themselves and the host [22]. Ruminants have a superior ability to convert cellulose into digestible carbohydrates and short-chain fatty acids and to free up the protein confined within cellulose cell wall.

The intestinal microbiota also have the capacity to synthesize a variety of vitamins involved in aspects of microbial and host metabolism, including cyanocobalamin (vitamin B12), pyridoxal phosphate (active form of vitamin B6), pantothenic acid (vitamin B5), niacin (vitamin B3), biotin, tetrahydrofolate (generated from dietary forms of folate) and vitamin K. Other beneficial effects of bacterial activity include lipid hydrolysis and protein breakdown (producing peptides and AA).

In the last decade significant progress has been made in analysing colonic microbiota of mice and humans to understand interaction between human and microbial genomes. Culture-based studies provided the first insights into the complexity of the GI tract microbiota. However, culture methods only allow the detection of cultivable bacteria, are time-consuming, and are biased by the selectivity of the culture medium. Direct molecular approaches using modern genomics techniques, in particular high-throughput sequencing, mostly overcome the limitations of plate culture methods, and are particularly useful for the detection of uncultivable bacteria. Using these culture-independent (metagenomic) methods, it is estimated that at least 500-1,000 different species of 10^{14} microorganisms exist in rodents and humans. While most of the microbiota consist of bacteria, they also contain eukaryotes such as yeast. Metagenomic studies of microbial ecology have also discovered the presence of both a viral and archaebacterial intestinal flora. There are a few studies that indicate that urinary metabolites may be linked to variations in the gut microbiota [23, 24], which may offer a more rapid and cost-effective measure of intestinal microbial activity.

Ruminant gut microbiota

Most microbiota studies in ruminants have concentrated on those present in the rumen, due to their importance in food conversion and methane production. In 2011, a global alliance was set up to accelerate Rumen Microbial Genomics research and generate a reference set of rumen microbial sequences, : 'The Hungate 1000' project (http://www.hungate1000.org.nz/). There are however few studies that relate rumen microbiota to host phenotypic traits. Higher butyrate and valerate is detected in rumens of more efficient cattle compared to less efficient [25].

The pH of ruminal contents is probably the most important ruminal factor affecting the microbial populations and their activities [26]. Protozoa and fungi are more sensitive to pH than bacteria whose pH sensitivity varies depending on the functional groups. Subacute ruminal acidosis (SARA) is a consequence of the general increase in gram positive and decrease in gram negative bacteria, and endotoxin LPS, present on the cell wall of all gram negative bacteria, is thought to contribute to the disease.

Microbial fermentation also takes place in the large intestine of ruminants and the intestinal luminal content constitutes a more favourable environment for bacterial and pathogen growth than the rumen. The microbiota of the rumen and large intestine of cattle has been shown to be very different [27]; however, unlike other species (human, pig, chicken, mice, etc.), studies of the GI microbiota in ruminants are very few and generally limited to faeces [28]. It was recently demonstrated that faecal samples do not necessarily reflect microbial populations in other parts of the GI tract [29] and there is some evidence that regional differences in the microbiome influence mucosal immune system development and maturation in calves [30], making this a promising area of future research.

Interaction of microbiota with the immune system

In addition to providing bioavailability of energy sources and vitamins, gut microbiota are also essential for the maturation of the immune system. In mice and humans, it has been shown that the formation of secondary lymphoid structures (Peyer's patches, mesenteric lymph nodes) occurs before birth, but their size and the development of germinal centers

depends on the postnatal microbial colonization of the gut [20]. Also, isolated lymphoid follicles are seen only postnatally in mice [31].

Commensal bacteria contain PAMPs which are recognised by innate host receptors under normal steady-state conditions and this interaction has a crucial function in the maintenance of intestinal epithelial homeostasis [32]. Mutations in innate receptors have been linked to dysregulation of the intestinal microbiota (dysbiosis) resulting in clinical outcomes such as inflammatory bowel disease (IBD) in humans and increased susceptibility to colitis in mice [33]. Constitutive intestinal inflammation by normal microbiota is avoided through active regulation of recognition. This has been demonstrated in mice deficient in IL-10 which develop massive leukocyte recruitment when reared under normal conditions, but are completely disease free when reared germ-free [34]. Mice deficient in both IL-10 and MyD88 were completely protected from colitis, demonstrating that the critical anti-inflammatory function of IL-10 in intestinal homeostasis is to regulate activation of TLR by the bacterial PAMPs. The innate immune system can also shape the microbiotal profile. For example, the NLRP6 inflammasome complex is highly expressed in intestinal epithelial cells and its activation-induced secretion of IL-18 can prevent gut colonization by colitogenic bacteria in mice, while defective NLRP6 signalling results in pathogen colonization and disease [35]. Conversely, the microbiota can also shape the innate immune response profile. For example, segmented filamentous bacteria are commensal organisms that adhere tightly to the intestinal epithelium, and in mouse models they have been shown to be critical for inducing the development of IL-17 and IL-22-producing cytokines by Th17 cells, which are important for protection against pathogenic bacteria [36]. Feeding of bacterial products to antibiotic-treated mice (peptidoglycan, LPS, flagellin) can stimulate their respective innate receptors (NOD1, TLR4, TLR5) and confer protection against intestinal infections [18]. Peptidoglycans, derived from gut microbiota, have also been shown to have a systemic effect on immunity, by priming the innate immune system after diffusion into the blood stream of mice [37]. Treatment with polysaccharide A (PSA), a product of the human symbiont, *Bacteroides fragilis*, has been shown to protect germfree mice against the induction of inflammatory bowel disease through the induction of IL-10 by regulatory T cells (Treg) [32]. Activation of TLR9 or NOD2 with CpG-DNA or MDP, respectively, resulted in marked reductions in the severity of experimental necrotising enterocolitis (NEC) in mice [38]. Most of these intervention studies have been performed in mouse models and their translational relevance is still to be confirmed. In particular, administration of these potent stimulators of innate

immunity in young animals with will need to be carefully controlled for undesirable side effects.

The gut microbial phenotype is transmissible and co-housing studies in mice have shown that alterations of microbiota composition might by themselves be sufficient to cause mucosal inflammation, independent of genetic background [35]. Different intestinal microbial profiles are associated with higher or lower susceptibilities and this may be of particular importance in the neonatal and infant gut, where the immature microbiota is unstable and susceptible to colonization by pathogenic bacteria [20, 32].

Gut microbiota also plays a role in shaping the adult repertoire of adhesion sites [32]: Neonates contain an 'innate repertoire' but under the action of bacterial glycosidases, an adult repertoire is established and new species are able to bind to the mucins as new sites appear. Resident microflora can also interfere with the expression of host glycosyltransferases, which induce changes in the carbohydrate repertoire of mucins. In this way, even genetically identical individuals can develop their own individual intestinal flora [3].

The expression of PRRs and therefore there interaction with microbial PAMPs can also alter during development. For example, TLR4 expression is high in the human fetus but is downregulated in normal birth. It is thought that the higher expression of TLR4 in children born by Caesarean section may account for their slightly increased risk of developing Crohn's disease [20]. Age-dependent expression of TLR3 on intestinal epithelial cells has also been correlated with susceptibility to infection [39].

Gastrointestinal helminth parasites may be able to interact directly with the intestinal microbiota during their life cycle through secretion of antimicrobial products or changing the nutritional microenvironment or indirectly through immunomodulation. Conversely, the GI microbiota may influence parasite development through metabolic changes as well as modulation of the immune response. Studies to examine these complex interactions have only recently been initiated [40], and promise to provide new avenues for exploration and intervention.

Nutritional influences on Immunity

Like all physiological systems, the immune system requires a balanced nutrition for proper development and functioning and malnutrition is the most common cause of immunodeficiency in humans. In animals, it is well established that nutritional deficiencies lead to depressed immune responses which can result in higher worm burdens [41] [42] and increased susceptibility to bacterial infections such as mastitis [43]. Nutritional requirements vary from species to species and the field of nutrition science is enormous and not within the scope of this review. We will therefore concentrate on nutritional aspects that may have a direct or indirect influence on the innate immune system beyond the provision of a balanced diet.

While many commercial food supplements have been shown to have effects on growth and/or immune responses, their mechanisms of action are in general poorly understood. Considering the new knowledge of gut microbiota and its interaction with the immune system reviewed above, it is likely that many of the observed nutritional effects are based on their ability to influence gut microbiota composition and activity.

Variation in diet can affect the growth and development of different microorganisms leading to different microbiota profiles. In piglets, the composition of the initial weaning diet has been shown to be the primary factor determining the variation in urinary metabolites, a readout of gut microbial activity [24]. The use of organic acids and salts in diet is a long standing concept and common practice to reduce post-weaning diarrhea in piglets [44]. Its primary action is to assist weaning piglets to acidify the digesta in the stomach as they are not able to produce sufficient hydrochloric acid themselves. Lowering the pH in the gut microenvironment favours the growth of lactic acid producing bacteria (LAB) over that of pathogenic organisms that may be more pH sensitive (mostly gram negative). Moreover, unlike inorganic acids, the organic acids are lipophilic and may exert a bactericidal effect as they enter into the bacterial cell by a passive diffusion through the cell membrane of gram negative bacteria, acidifying the cell plasma and disturbing the cell physiology. As mentioned previously, these changes in gut microbiota may also have an influence on the immune response although this has not been examined in most cases of nutrient manipulation.

A clear example of how nutrient processing by microbiota can influence the immune response is provided by the short chain fatty acids (SCFAs) produced by microbial fermentation of non-digestible oligosaccharides derived from the diet [19]. The luminal concentration of intestinal SCFAs can be modified by the amount of fibre in the diet, which in turn affects the composition of the microbiota in favour or LAB [44]. In addition to acting as an energy source for the host, SCFAs exert significant effects on host immune responses. Butyrate can modify the cytokine production profile of helper T cells [19]. and promote intestinal epithelial barrier integrity [45], which in turn can help limit exposure of the mucosal immune system to luminal microbes. Production of another SCFA, acetate, by the microbiota downregulates intestinal inflammation via the G protein-coupled receptor, Gpr43 [46]. SCFA are the only ligand of Gpr43 which is expressed mainly on innate cells such as neutrophils, eosinophils and activated macrophages [46]. SCFA have also been shown to have negative effects on health, in particular in neonates with a developing intestinal tract where they can cause mucosal injury and increased pathology of NEC [47].

Innate immune recognition pathways have also evolved to directly assess the nutrient environment. TLR4 can sense the presence of free fatty acids [48] while ATP is an important activator of the inflammasome [49]. A variety of other immune cell-associated 'sensors' serve to couple information about the local nutrient/metabolite environment to the co-ordination of local immune responses [49] [19].

Food supplementations which have been most commonly used to promote growth with claims to have immunomodulatory effects are summarised below.

Vitamins

Numerous observational studies indicate that deficiencies in vitamins A, C, D, and E can adversely impact immune function.

Stimulation of dendritic cells via TLR2 increases the expression of host genes associated with generation of the immunoactive form of vitamin A (retinoic acid). Vitamin A deficiency has been associated with immunodeficiencies and increased risk of infectious diseases [50]. Thus, vitamin A has the potential to modulate immune responses through direct interactions with immune cells, or indirectly by modulating the composition of the microbiota.

The bioactive form of vitamine D (1,25(OH)2D3 or calcitriol) has been shown to be a potent modulator of the immune response through promotion of a Th2 and Treg response [51] and can directly stimulate the expression of antimicrobial peptides [52].

Vitamins C and E are antioxidants that protect the body against reactive oxygen species (ROS) generated by immune cells to kill pathogens. There are well documented links between vitamin E and neutrophil function: injections or high-level supplementation of vitamin E during the last few months of gestation and during early lactation can both boost the immune systems of the cows by improving neutrophil function and decreasing mammary infections, while ensuring delivery of vitamin E nutrition to the offspring.

Fatty Acids (FA)

Several types of dietary lipids (fatty acids) have been shown to modulate immune function. Polyunsaturated FA (PUFAs) are essential nutrients because they cannot be synthesized by the body and are found in fish, flaxseeds, walnuts, canola oil, nuts etc. Eicosanoids, key mediators of an inflammatory response (e.g. prostaglandins, leukotrienes), have anti-inflammmatory and immunosuppressive effects when derived from omega-3 PUFA, while those derived from omega-6 PUFA derivates, have mostly pro-inflammatory and immunostimulatory effects.

Prostaglandins, a subclass of eicosanoids, are mediators of inflammation, produced by macrophages that act through ligation of a sub-family of cell surface seven-transmembrane receptors, G-protein-coupled receptors (GPCR). Amongst other actions, some prostaglandins are potent neutrophil attractants. Prostaglandins can be derived from linoleic acid and feeding supplemental linoleic acid to dairy cows prepartum has been claimed to improve health [53]. However, the effect of linoleic acid on bovine immune responses has not been proven consistency in limited studies.

Different fat supplementations (palm oil, fish oil, safflower oil) [54] have been assessed for their ability to improve immune response outcomes (antibody responses, PBMC proliferation; measuring adhesion molecules, cytokines before/after LPS in neutrophils and monocytes, acute phase proteins). Thies *et al* [55] compared the effect of different vegetable oil supplements on immune parameters and health outcomes in weaned piglets and found no difference in weight gain and mortality or levels of T cell subpopulations; however, there were significant reductions in phagocytic and NK activities, especially in the fish oil group

confirming the general suppressive effect of omega-3 PUFA. Fish oil supplementation also had a minor beneficial effect in calves [56] and a commercial blend of single and medium-chain saturated FA (NeoTec4™) has been claimed to increase daily weight gain and require fewer medical treatments [57]. In general, supplementation of calf milk replacer is more effective than supplementing calf starter, probably due to bypassing the ruminal fermentation process in the younger calves.

Controlled trials in humans have also demonstrated beneficial effects of fish oil in chronic inflammatory diseases and have retraced the anti-inflammatory effects of omega-3-FA to its binding to a GPCR, predominantly present on macrophages, GPR120 [46]. Macrophages are major producers of the inflammatory cytokines, TNFα and IL6, and these are suppressed after binding of GPR120.

Saturated Medium Chain Fatty Acids (MCFAs) derived from coconut oil or palm kernel oil contain the typical antimicrobial compounds which are naturally present in goat milk (caproic-, caprylic- and capric acids) and are successfully used in animal nutrition to control the microflora, mostly gram negative pathogens, at the intestinal level [44]. The longer MCFAs, lauric- and myristic acids, which are present at high levels in these oils have shown to be very efficient against gram positive pathogens, such as *Clostridium perfringens* and *difficile* and *Streptococcus suis* in poultry and pigs.

A recent study in mice [58] showed that feeding a diet high in saturated (milk-derived) fat, but not polyunsaturated (safflower oil) promoted taurine conjugation of hepatic bile acids resulting in increased availability of organic sulphur and expansion of gut colonization with a sulphite-reducing pathogen, *Bilophila wadsworthia*, making them more susceptible to colitis. Considering the differences in bile constituents in different species, and the general differences in size and metabolism, it remains to be seen how relevant these studies are to other species.

Herbs and Plant extracts

Herb and plant extracts have been used for centuries for their antimicrobial activities and claims to improve immune system, however their mode of action is mostly not understood. They include saponins, tannins, and essential oils particularly prevalent in many tropical

plants (also lignins, flavonoids). It is possible that their antibiotic properties may result in a modified intestinal microbiota and thereby indirectly in an improved immune system. Green tea extracts has been shown to improve the intestinal microbial balance of calves by maintaining high fecal levels of ***Bifidobacterium*** and ***Lactobacillus*** spp. and decreasing those of ***C. perfringens*** [59]. Many *in vitro* studies have also shown direct effects of plant extract on immune cells, however it is not know how this translates to the much more complex *in vivo* situation.

Flavonoids are biologically active polyphenolic compounds ubiquitously found in fruits, vegetables, nuts and plant-derived beverages, such as tea or wine. A large number of publications suggest immunomodulatory and anti-inflammatory properties of these compounds. However, almost all studies are *in vitro* studies with limited research on animal models or human studies. Epidemiological studies in humans have shown no consistent effect of antioxidant (including flavanoid) consumption, although some support supplementation with antioxidants in patients suffering from diseases associated with inflammation and oxidative stress [60].

Sugar cane extract, or polyphenol-rich fractions of Sugar cane extract, has been shown to have some beneficial effects on immune responses and health outcomes in chickens [61] and pigs [62].

In general , it is difficult to draw conclusions of food supplement studies because most are crude and complex preparations and the observed effects can be due to a number of factors provided by the different components e.g. feed palatability, digestibility and nutrient utilisation. In addition, studies purely based on the *in vitro* activity of different nutrients or supplements can be misleading as they do not necessarily reflect the bioavailability after oral administration and absorption; metabolites generated in the GI tract may not have the same biological activity as the original compound.

Probiotics

Antibiotics used at sub-therapeutic levels in feed have a considerable effect on the equilibrium of intestinal flora and the banning of antibiotic growth promoters (AGP) in feed for food producing animals in the EU in 2006 has resulted in a massive increase in the

development of products as alternatives to AGPs. This has dramatically changed animal nutrition, now aiming to create a healthy gastrointestinal microflora, termed ‘ Eubiotic Nutrition’ [44, 63]. This includes the use of probiotics and prebiotics.

Probiotic treatment can be defined as ‘providing living microorganisms to animals that upon ingestion in sufficient numbers exert health benefits beyond basic nutrition’ [3].

Probiotic products must be repeatedly administered to ensure a sufficient population level over time and maintain an effect. Common probiotic organisms are Lactobacilli and Bifidobacteria species, consumed in yogurt and other fermented foods. They are most beneficial in younger individuals where the intestinal flora is not well established and diversified. The mechanisms of action of probiotics within the GI tract may be manyfold, including competing with pathogenic bacteria for nutrients; competing with pathogens for binding sites on the intestinal epithelium; producing compounds that are toxic to pathogens and stimulating the immune system. However, they may also compete for nutrients with the host, they may compromise nutrient absorption by occupying the intestinal lining and, last but not least, they may de-conjugate the bile phospholipids and compromise fat digestion [63]. This is a good strategy in human nutrition where products are typically based on *L. casei* to overcome obesity, but is not desired in nutrition for food producing animals aiming at producing a maximum output of meat, milk or eggs with a minimum of feed input.

Certain probiotics (e.g. *Lactobacillus reuterii*) have been shown to reduce the duration and severity of rota-virus-related diarrhea in children, and antibiotic-associated diarrhea in adults [64]. Oral administration of Gram-positive probiotic bacteria has also been shown to decrease the incidence of necrotizing enterocolitis of the newborn and enhance S-IgA export in the gut lumen [3]. Typically *Bacillus subtilis* is used in animal nutrition to overcome problems with a subacute necrotic enteritis provoked by *Clostridium perfringens*. Their mode of action is however more likely due to their antimicrobial metabolites, the surfactins.

Probiotic supplementation has also been shown to stimulate immunity and reduce overall pre-weaning mortality and post-weaning diarrhea in piglets (reviewed in [65]) and have a significant effect on their mucosal Ig profile [24].

Intestinal microbiota transplantation is another way to control the microbial composition of the gut and in humans is used to manage recurrent infection that is refractory to antibiotic

therapy (12,13). This transplantation approach involves infusing microorganisms from a healthy donor stool into the intestine of a patient to restore the normal microbiota.

The term “probiotic” implies a curative nature of these products which is not always easy to prove. For regulatory purposes US feed Industry in conjunction with FDA and USDA has accepted the more generic term of “direct-fed microbials” (DFM) for microbial-based feed additives which may be sold without approval as long as the microorganism appears on the approved list and no claims of improved health or production are made. Several companies are building up portfolios for animal health that include probiotics e.g Bioniche distributes an equine probiotic ‘designed to balance the intestinal systems in horses and ponies, to boost immunoregulatory responses to illness and inflammation’ particularly under conditions of stress.

Ruminant probiotics

Sudden changes in diet, e.g. after transport to feedlots, may lead to problems when the right bacterial species are not available to digest the new food. It has been suggested that the additions of specific microbial strains could allow feedlot producers to decrease the time it takes to adapt cattle to a high concentrate diet [66].

Fungal DFM have been added to ruminant diets for many years and a number of yeast products are available commercially for use in cattle , mostly live strains of *Saccharomyces cerevisiae* (e.g. Yea-sacc from Alltech, USA) or strains of the fungus *Aspergillus oryzae* (e.g. Amaferm from Biozyme Inc., St. Joseph, MO). Most appear beneficial via changes in ruminal fermentation by oxygen scavenging or providing micronutrients rather than changing the microbiota or immune response [67].

Many bacterial-based DFM are sold for use in calf diets often containing lactobacilli, most commonly *L. Acidophilus* (also *Bifidobacterium*, *Enterococcus* and *Bacillus*). Timmerman et al. [68] showed that a calf-specific probiotic containing six ***Lactobacillus*** species reduced the fecal counts of ***E. coli***. Bacterial DFMs have maximal efficacy in pre-ruminant calves, and most probably act in the lower gut, not the rumen, where they may lower pH to inhibit growth of pathogens. However, there are contradictory reports on their benefit, especially in adult cattle.

Prebiotics

A recent definition of prebiotics is 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the GI microbiota that confers benefits upon host wellbeing and health' [69]. The FAO defines a prebiotic "as a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" [70].

The indigestible portion of plant foods is generally referred to as dietary fibre (or commonly roughage) and includes non-starch polysaccharides, hemi-cellulose, and many other plant components such as resistant starch, resistant dextrins, inulin, lignin, waxes, chitins, pectins, beta-glucans, and oligosaccharides [1]. In general, these complex carbohydrates cannot be digested by the mammalian host as they lack the large repertoire of glycoside hydrolases and polysaccharide lysases needed to cleave the varied glycosidic linkages present in these glycans, and these enzymes are provided by the microbiome. Dietary fibres therefore provide a selective substrate for colonic bacteria and have the ability to alter the microflora of the gut [71] . Prebiotic supplementation of human infant diet has been reported to increase levels of lactic acid bacteria and bifidobacteria, decrease diarrhea, improve allergy symptoms and decrease rates of infection [47, 69, 72, 73].

Prebiotics are usually a more cost-effective way of altering the gut microbiota than probiotics, especially in livestock. Specific carbohydrates are now widely used as prebiotic dietary additives that are designed to manipulate gut metabolism and the bacterial populations in the gut towards a healthy composition [74]. Fermentation of dietary fibres increases production of SCFA, primarily acetate, butyrate and propionate in the gut and a number of studies support direct or indirect immunomodulatory properties of SCFA as mentioned previously.

Oligosaccharides, in particular inulin and oligofructose, have been the primary focus of prebiotic research; they have been shown to increase the growth of 'health promoting bacteria' in the GI of humans and animals. Oligosaccharides are abundant in human milk (5-10g/L) but virtually absent from cow milk (<0.08 g/L) and infant formula, possibly accounting for some of the differences in microbiota between breast-fed and bottle-fed infants [47]. Oligosaccharides in human milk are also more complex (~200 molecular

species) than bovine (~10) or porcine (~29) milk [47]. Oligosaccharides are often only digestible by microbial enzymes and are used in pigs and poultry diets. Oligosaccharides containing mannose or fructose are known to selectively increase the growth of beneficial intestinal bacteria, including lactobacilli and bifidobacteria. Piglets fed lactose had significantly higher levels of lactic acid (but lower butyric acid) in the colon and fewer incidences of experimental necrotizing enterocolitis [75]. It has been advocated that recent advances in glycobiology may allow the generation of oligomers that stimulate lactic acid bacteria at the species rather than the genus level.

Mannan oligosaccharides (MOS), mostly derived from *S. cerevisiae*, are non-digestible oligosaccharides that differ from other prebiotics in that they are not direct nutrients either for intestinal microbiota or for the host but potentially have a positive effect on the health and performance of farm animals [76]. Dietary MOS have been shown to reduce colonization of pathogenic bacteria in poultry trials and when administered in the last 2-3 weeks of gestation and during lactation improved the growth rate of piglets. Weaning is associated with dramatic feed refusal and often result in post-weaning diarrhea and drastic depression in growth rate. In the past, antibiotic growth promoters efficiently prevented the complex post- weaning symptoms. MOS supplementation has shown positive effects on the immune response of weaned pigs, rats, dogs, chickens and can efficiently reduce the number of pathogens post-infection; however it is unable to modify consistently the quantity of harmful species under adequate hygienic conditions [76].

The mode of action of MOS likely involves increasing levels of mannose-binding proteins (collectins) that bind to mannose containing structures present on a number of viruses and bacteria thereby triggering the complement cascade and phagocytosis [77].

β-Glucans are polysaccharides that contain only glucose as structural components and are present in cellulose, cereal bran, the cell wall of yeast, certain fungi, mushrooms and bacteria. Adding either highly purified β-glucan as feed additives to chickens during the first 4 days post-hatch was shown to significantly protects the birds against an enteric infection and enhanced phagocytic capacity [78]. One mode of action is thought to be through the direct interaction of β-glucan with the complement receptor, CR3 [5].

Polydextrose (PDX), an indigestible carbohydrate, has been advocated as a supplement to infant milk formulae to substitute for the abundant oligosaccharides present in human milk [47]. Supplementation of piglets with PDX resulted in a decreased pH in cecal and colon digesta due to increased lactic acid production by lactobacilli. It also decreased levels of inflammatory cytokines, possibly due to less stimulation with pH sensitive intestinal pathogens.

Conclusions

Determining nutritional requirements for optimal performances and formulating feeds that maintain a good immune-status with the addition of appropriate feed additives are key strategies in modern animal nutrition. Optimising dietary fibre and feed energy as well as maximising protein utilization are key strategies in this concept.

The objective must be to avoid various metabolic diseases and to manage enteric disorders by ensuring a healthy gastrointestinal microflora that has been termed “Eubiosis” [44].

There is no doubt that the gut microbiota can influence the immune status of animals and their ability to fight infections. Stimulation of the innate immune system by gut microbes lies at the basis of this interaction. There is also clear evidence that the gut microbiota can be manipulated with appropriate management strategies and nutritional intervention. This can however be best achieved at certain stages when the microbiota is not completely stabilised (birth, weaning, antibiotic treatment) or under stress (transport, feedlot). Much of the livestock research in this area has been performed in chickens and pigs, with ruminant studies just starting to appear. A major challenge is to develop the necessary technologies for rapid identification of resident gut microbiota in ruminants so that comprehensive studies can be performed to correlate microbial ecology with desirable and undesirable production traits. This should also allow more unbiased determination of the effect of different interventions on gut microbiota and the quality of an immune response. The final readout should be the ability of the animal to resist infections that reduce productivity which may require more standardized infection models. Another challenge for scientists working in specialised areas, is to foster collaboration between nutritionists, microbiologists, immunologists and pathologists to arrive at an integrated approach for improving animal health and welfare.

Key points

- Mammals are born with a sterile gut but quickly acquire a resident community of gut microbes (microflora or microbiota) from their mothers and the environment in early life.
- Once the gut microbiota is established, it remains relatively stable in adults and is difficult to change (except through antibiotic treatment).
- There are more microbes in the gut of adults than cells in their bodies.
- A healthy microbiota provides essential nutrients to the host and prevents establishment of pathogenic microbes.
- The composition of the gut microbiota has a strong influence on how the immune system of the host develops, both at the level of the gut as well as systemic.
- Metagenomics is a new, unbiased method to identify and study gut microbiota without culturing.
- We can change the gut microbiota by administering selected populations of health promoting bacteria (probiotics), especially in young animals without a well established microbiota.
- We can also change the gut microbiota by administering food or feed additives that selectively promote the growth of health promoting bacteria within the gut (prebiotics)
- Some nutrients may have additional beneficial effects on the gut immune system, apart from an effect on the gut microbiota, however their mechanism of action is ill defined.

Recommendations

- Establish metagenomic technologies to identify gut microbioata in cattle and sheep.
- Develop experimental models to study the interaction of gut microbiota and innate immunity and the effect of interventions on those (e.g. intestinal loops/cannulae)
- Perform comparative metagenomic studies of the gut microbiota of healthy animals and animals with enteric diseases and correlate these with in vivo metabolic activity (e.g urine metabolites).

- Determine microbiota (microbiome) in animals with different disease resistance phenotypes and relate this to gut innate immune profile (host transcriptomics)
- Study the development of the gut microbiota and innate receptors in neonatal animals and determine effect on disease resistance
- Examine the use of organic acids and salts in diet on the gut microbiota and health status of calves/lambs, bringing in expertise from the pig and poultry industries where this has been well researched.
- Test potential low cost food additives (e.g. sugar cane extract) for their effect on gut micriobiota and immune responses.
- Eubiotic nutrition: Examine the interplay between food and food supplements, the microbiota, the immune system and health, whilst assuring efficient production.

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CHAPTER 5

GENETICS AND HERITABILITY OF INNATE IMMUNITY AND THE GENETIC CONTRIBUTION OF IMMUNITY TO DISEASE RESISTANCE IN LIVESTOCK

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GENETICS AND HERITABILITY OF INNATE IMMUNITY AND THE GENETIC CONTRIBUTION OF IMMUNITY TO DISEASE RESISTANCE IN LIVESTOCK

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DISEASE AND MEASUREMENT OF RESISTANCE TO DISEASE

Although there is considerable exploitable genetic variation in resistance to diseases caused by nutritional deficiency, toxicity, structural abnormality and metabolic dysfunction, and some of this variation might be associated with components of the immune system, this review concentrates on the resistance to infectious disease exclusively.

When we aim to select animals with resistance to infectious disease, we wish to identify those animals that have the capacity to produce offspring that have optimal immune and other mechanisms to perform to their genetic capacity for survival, reproduction and production in an infected environment. Survival and reproductive capacity in a given environment is a measure of adaptation to that environment, or fitness, whereas production is an artificial construct derived from human demands on domestic animals. The tensions between fitness and production are important, well documented, controversial, and will not be discussed in this review except to state that disease can directly impair both, through direct and indirect, interacting and independent mechanisms.

The definitions of disease and resistance to disease are not trivial considerations when it comes to the selection of animals with resistance to disease. Infection and disease are not synonymous. The presence or absence of a specific pathogen can rarely be taken as a proxy

for disease, given that the majority of organisms cause disease in a dose-dependent manner. Most metazoan parasites colonise hosts with aggregated, right-skewed frequency distributions (Rosza et al., 2000), two examples of which are shown in Figure 1. The vast majority of animals have undetectable or light to moderate burdens and show no loss of fitness. Some animals will show varying degrees of loss of production performance and are often referred to by veterinarians as being sub-clinically affected. A smaller proportion will show clear loss of function in one or more body systems, regarded as showing signs of clinical disease.

Measuring a disease resistance phenotype can be logistically challenging and variation in disease resistance in any given study might depend as much on variation in environmental factors (including coinfection), and on genetically determined characteristics intrinsic to the pathogen, as it does on the host. It is rarely possible to identify an intensity of infection at an individual animal or population level that can be taken as a threshold of disease. Studies that aim to quantify genetic variance in disease resistance might use the presence or absence of clinical signs including mortality, presence or absence of infectious agent, level of a biological marker (for example somatic cell count in milk) or serological evidence of prior exposure as measures. In all cases the measure must be carefully selected for the disease in question. The most appropriate measure will depend largely on the prevalence of the pathogen and its pathogenicity. As an example, in areas infested with cattle tick, most or all animals are challenged and most or all animals carry some ticks. Hence, the only meaningful measure of resistance to tick infestation is a quantitative measure of tick burden. Repeated counts of standard-sized ticks over the entire animal or half an animal are the best measure. In contrast, *Mycobacterium avium paratuberculosis* (MAP) is a ubiquitous organism that fails to establish a chronic infection in most cattle and thus occurs with a relatively low frequency in a population, despite sometimes high challenge. Seropositive animals are almost certainly infected and will, given sufficient time, develop clinical signs of disease so it is reasonable to use seropositivity or shedding of MAP as a proxy for disease occurrence. With tick fever caused by *Babesia bovis*, *B. bigemina* or *Anaplasma marginale*, the situation is more complicated. Challenge can be high, with most animals exposed. Most exposed animals become infected and the proportion developing clinical signs of disease varies according to immune status. Infection at low levels persists after clinical signs resolve. Seropositivity is therefore not a good measure of resistance, nor is the presence of the organism in very low densities. Disease can only be inferred if the organism is present at a high intensity and there

is a significant depression of the erythron. The best measure of disease resistance for tick fever is therefore the haematocrit plus demonstration of organism. It could be argued that the production performance of an animal in the face of infection might be an even better measure.

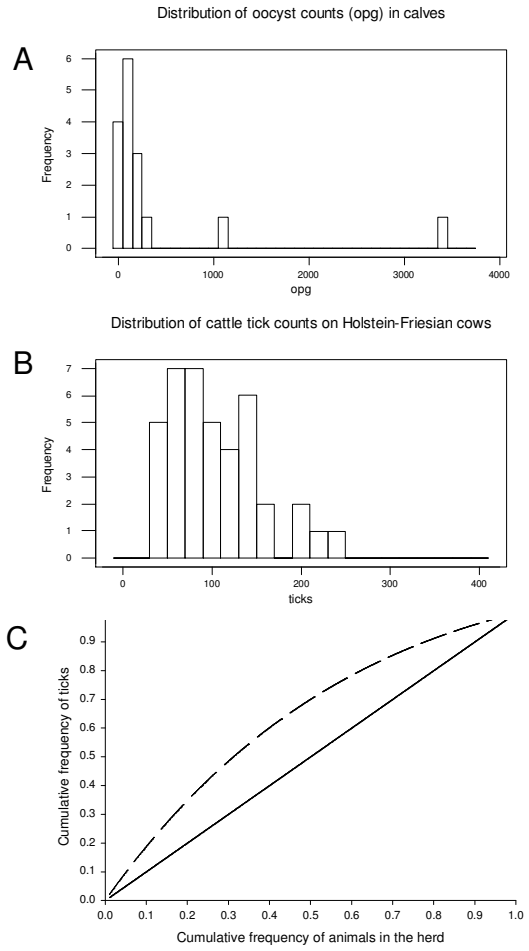

Figure 1. A. Faecal oocyst counts per gram of faeces (opg) in 16 calves, two weeks after being artificially infected by mouth with approximately 40,000 oocysts of *Eimeria bovis* and *E. zuernii*. The majority of the observations are either zero or less than 500 oocysts per gram (opg). A small number of animals excreted very large numbers of oocysts. **B**. The distribution of ticks on 40 Holstein-Friesian cows is less skewed than in A, but still demonstrates the non-normal distribution. **C.** The dotted line represents the cumulative frequency of ticks plotted against cumulative frequency of cows from B and indicates that culling the most heavily infested 10% of the herd would result in a reduction in the current tick population on cattle of close to 20% (Jonsson et al., 2000). The solid line represents the expected relationship if the distribution of ticks on cows approximated a Poisson distribution with equal mean and median.

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Resistance to disease is considered sometimes most appropriately to be a complex or quantitative trait and at other times it might legitimately be considered to be a complex but categorical trait. More rarely disease resistance is a simple, Mendelian trait. A good example of how terminology can be important is provided by enteritis in piglets. If the disease entity is defined as enteritis causing diarrhoea in neonatal piglets, then there are a number of potential aetiological agents, including *E. coli*. Under this definition, there is no doubt that resistance to neonatal diarrhoea is a complex or quantitative trait and will have a relatively low heritability. However, some enterotoxigenic *E. coli* have antigens that bind to F4 (syn. K88) receptors on the enterocytes of piglets, facilitating colonisation and increasing the severity of clinical signs of infection (Chandler et al., 1994). Hence resistance to infection is inherited as an autosomal dominant Mendelian trait. Piglets born without the receptor for the antigen are completely resistant to disease caused by that specific type of *E. coli* but their resistance to other causes of enteritis is not improved. Hence, if the disease is defined as neonatal enteritis, the potential to select for increased resistance might appear to be high in an environment where the dominant pathogen is *E. coli* F4 (syn. K88) but would be low in other environments. Similarly, high heritability of resistance and the probability of antagonistic interactions among disease resistances are more likely to be noted if the diagnosis or definition is made at the level of the pathogen or subtypes of the pathogen rather than if disease is considered at the level of a clinical diagnosis. This distinction between aetiological and clinical diagnosis is important and sometimes overlooked. In general, heritability estimates of traits measured at the molecular level (including innate immune parameters such as isoform and level of expression of acute phase proteins for example) are higher than heritability estimates of traits measured in the field (including resistance to clinical disease).

In summary, when considering the potential for selection of animals with increased resistance to infectious disease it is very important to identify precise selection criteria for the disease in question. These should be the criteria most relevant to the disease problem in hand and should not be simply the criteria that are most readily measured.

GENETIC VARIATION IN RESISTANCE TO DISEASE

Resistance to disease is a fitness trait, so evolutionary theory should predict relatively little variation in resistance, but one of the great surprises in modern genetics is the discovery of substantial genetic variation in resistance to disease. Two of the best examples come from Australian livestock. Variation among cattle in resistance to the cattle tick *Rhipicephalus microplus* is clearly under strong genetic control (Box 1). Similarly resistance to gastrointestinal nematodes in Merino sheep also shows high genetic variation (Box 2). Susceptibility to parasitic diseases in general appears to have a strong genetic component. Other examples include trypanosomes in cattle, flukes in sheep and experimental infections with species of *Leishmania*. The situation is less clear for microbial diseases such as mastitis (Box 3) and respiratory disease (Box 4). Here the heritabilities of clinical disease, although significant, are usually quite low; of the order of 0.1. These low heritability estimates might reflect a genuinely low heritability of the immune response. Alternatively, they may reflect the composite nature of the diseases, a relatively low prevalence, or the difficulty in detecting subclinical disease. Mastitis and respiratory disease can be caused by a variety of diverse pathogens. The genes coding for resistance to different pathogens are not identical and pooling different responses to different pathogens could lower the overall estimate of heritability. In contrast to parasitic diseases where differences among animals in adult tick burdens or nematode eggs in faeces can be clearly counted and essentially all animals can be exposed to infection, exposure to microbial disease within any given sample period is often less than 100%. Consequently it can be difficult to distinguish between susceptible animals which have not been exposed and resistant animals which have been exposed but did not develop disease. Although the heritabilities of many infectious diseases are low, they are not too low to prevent selective breeding. A good example of this is breeding to reduce clinical mastitis in Scandinavian countries (Steine et al 2008). The low heritabilities just mean that the response to selection based on the incidence of clinical disease will be slow.

Sources of genetic variation in disease

About 20 to 30 years ago conventional genetic wisdom held that disease resistance was a fitness trait and would be subject to natural selection which would fix favourable genes and eliminate unfavourable genes. Consequently, there would be little genetic variation in resistance to disease. Technically, non-additive genetic variation could persist but additive variation would be reduced (Box 5 discusses additive and non-additive genetic variation).

Relatively high heritabilities indicate that there is considerable genetic variation but the reasons for this have seldom been considered. Yet the source of genetic variation in resistance to disease has a major bearing on the optimal way to breed for resistance to disease and on the sustainability of selective breeding. There are several possible explanations for the variation in resistance to disease. These include statistical imprecision, genetic load, and some form of balancing selection including trade-offs with maternal immunity, different diseases, nutrition, immunopathology or the effect of parasite evolution. These will be discussed in turn.

Statistical imprecision. Variance components are notoriously difficult to estimate with precision. This is especially true for ratios of variance components such as heritabilities. Therefore in any discussion of heritabilities the possibility that the statistical estimates are imprecise must be considered. However, this appears to be an unlikely explanation for the relatively high heritability of some diseases. For example with nematodes, the heritability of a single faecal worm egg count has been estimated on multiple occasions and is nearly always above 0.2 (Bishop et al. 1996; Bisset et al. 1992; Nguti et al. 2003; Woolaston and Baker 1996). The heritability of pooled egg counts can be even higher but depends upon the repeatability of egg counts and the number of times that separate faecal samples were collected. In addition, faecal egg count is determined by the number of adult worms and their fecundity, which is a consequence of worm length. Nematode size and fecundity is under very strong genetic control (Stear et al. 1997). IgA and IgE play important roles in regulating nematode egg output; both plasma IgA and plasma IgE activity are under strong genetic control (Murphy et al. 2010; Strain et al. 2002). Antibody at the site of infection rather than antibody in the plasma regulates nematode egg production and mucous antibody is likely to be under even stronger genetic control than plasma antibody. Clearly, the evidence for strong genetic control is overwhelming and unlikely to be an artefact of the experimental procedure.

Genetic Load. Another explanation for heritable variation in resistance to disease is genetic load. The immune response involves many different gene products and over 2000 genes are believed to play a role. Purely by chance some of these genes will mutate. Natural selection will take time to eliminate unfavourable genes. Favourable mutations are believed to be a minority but even here many favourable alleles will coexist with less fit alleles before fixation. The persistence of mutations means that, at any given time, many individuals will be polymorphic and consequently there will be genetic variation in the immune response. The

existence of suboptimal alleles is known as the genetic load. While the genetic load is usually considered in the context of defective Mendelian genes it will contribute to genetic variation in the immune response and to variation in resistance to disease. However, the size of the contribution is unclear. Possibly most genetic variation in the immune system consists of genes in a state of flux. Alternatively only a small proportion of polymorphic genes might be in the process of being eliminated. In the latter case most polymorphic genes would then be stable and under some form of balancing selection.

Balancing selection. Balancing selection maintains genes at a stable intermediate frequency. The best known example is haemoglobin where the S allele is maintained in malarial areas. Homozygotes with two copies of the S allele are prone to anaemia because their red cells adopt an abnormal sickle shape. Their life expectancy is reduced but the gene persists because heterozygotes are more resistant to malaria. The gene frequencies are determined by a balance between susceptibility to anaemia and resistance to malaria. A more relevant example for this review is the major histocompatibility complex (MHC; Box 6). The MHC is also under balancing selection and this balancing selection has clearly persisted for a long time. For example, one of the sheep alleles at the *DQA1* locus is identical to a goat allele at the homologous goat *DQA1* locus. As goats and sheep are thought to have diverged over 5 million years ago, this implies that the allele has remained unchanged in both lineages for millions of years. Unfortunately, very few loci have been examined as closely as MHC loci.

Maternal Antibody. Tradeoffs might explain genetic variation under balancing selection. For example, high immune responses in a mother might lead to more passive antibody being transmitted to her offspring. This would increase the protection of the neonate against disease but could interfere with the development of immune responses in the offspring. An example of this is the transmission of antibodies to Coxsackie B virus in humans. The presence or absence of the *E. coli* K88 (syn. F4) receptor in pigs is a useful veterinary example (Edfors-Lilja., et al. 1995). If the dam has the receptor for this strain of *E. coli* and has been infected she will pass protective antibody in colostrum. However, if the offspring do not have the receptor they will be protected even without colostrum, but the K88 (syn. F4) negative females will not offer the advantage of protective passive antibody to their offspring. This trade off is one of the reasons people decide not to select for absence of the K88 (syn. F4) receptor in pigs, despite the availability of genetic tests for this susceptibility.

Resistance and susceptibility to multiple diseases. Another possible trade-off where balancing selection plays a role is between different diseases. Many immunologists believe that the immune system cannot respond effectively to different types of diseases at the same time. Protective responses against many microbial diseases require Th1 helper T cells, particularly virus or facultative intracellular bacteria, while responses to diseases caused by metazoan parasites involve Th2 helper T cells. However, there is very little solid evidence that the immune system cannot respond simultaneously to diverse infectious agents and there is considerable evidence against this view. Several breeds of cattle and sheep are resistant to particular diseases. Examples include the Red Maasai sheep from Kenya which has a high degree of resistance to the nematode *Haemonchus contortus (Baker et al. 2003; Mugambi et al. 1996).* Other breeds of sheep with high resistance to nematodes include the St Croix (Gamble and Zajac 1992), the Gulf Coast Native (Miller et al. 2006), the Barbados Blackbelly (Yazwinski et al. 1981) and in temperate areas the Texel (Sayers et al. 2005). In Africa, several breeds of cattle are relatively tolerant to infection with trypanosomes, most notably the N'Dama (Paling et al. 1991). Indicine breeds are more resistant to the cattle tick *Rhipicephalus microplus* than taurine breeds (Francis 1964). In all these cases there is no evidence that resistance to one disease causes increased susceptibility to other diseases.

In breeding terms, a trade-off would produce a negative genetic correlation between resistance to different diseases but again there is no evidence of negative genetic correlations between different diseases. In fact, there are a number of recent reports showing positive associations between a variety of diseases in dairy cattle (Koeck et al., 2012). In addition to differences between breeds and genetic correlations, a third line of evidence comes from the effect of specific genes. There are now a wide variety of genetic variants that have been associated with resistance to different diseases. Very few of these show that resistance to one disease causes susceptibility to other diseases. Nonetheless, in some livestock studies there is evidence of negative genetic correlation between antibody-mediated immune responses (type 2) and cell- mediated immune responses (type 1), however it was still always possible to identify individuals with robust and balanced antibody and cell-mediated responses (Thompson-Crispi et al., 2012, Mallard et al., 1993). Disease resistance and immune response traits are low to moderately heritable respectively (Koeck et al., 2012), providing evidence that it should be possible to genetically select for improved resistance to a number of economically important diseases simultaneously.

The influence of nutrition on immunity. Ecological immunologists have argued (Read and Allen 2000) that the immune response has an energetic cost, which is undoubtedly true. This could constrain the immune response and lead to suboptimal immune responses; this is more controversial. However, malnourished individuals do mount suboptimal immune responses and this is a particular problem with cattle and sheep because they have evolved to live on grass, which is abundant but a relatively low quality food. Certainly, the blood biochemistry of a sheep is similar to a starved human. Sheep that are not starved do respond to supplementary dietary protein by producing stronger immune responses (Strain and Stear 2001; Wallace et al. 1995; Wallace et al. 1996; Wallace et al. 1998; Wallace et al. 1999). Nematode infection induces a relative protein deficiency and one consequence of this is to reduce the immune response (Wallace et al. 1995; Wallace et al. 1996). This suggests that parasitized animals may show weaker immune responses and be more vulnerable to a multitude of diseases.

One consequence of the cost of immunity could be on production traits. For example, some but not all studies have shown a trade-off between resistance to nematodes and decreased wool production (Eady et al. 1998; McEwan et al. 1992). This probably reflects the requirement of both the immune system and wool production for large quantities of sulphur-containing amino acids. On the other hand, Yorkshire pigs selected for 9 generations for high adaptive immune responses showed significantly increased rate of gain (Wilkie and Mallard 1999). The high immune responder pigs reached market weight 10-12 days sooner than low or average responders and showed a variety of other health attributes. This apparent absence of an impost on production might be an effect of confounding by reduced impact of disease on production given that measurements are made in a disease-endemic environment – in other words there might be a higher basal set point of the immune system of high responders that converts into a more efficient active protective response when the immune system is activated. Alternatively, this might have related to other growth genes being altered during selection. For example, growth hormone concentration was also greater in the high immune responder pigs (Mallard B. A. unpublished data). Additionally, it was also observed that the high immune responders were more aggressive at getting to the feed trough. It is difficult to be definitive about the reasons for this increased growth in the high immune responders but it does demonstrate that it is possible to select livestock for greater immune responsiveness, while at the same time improving growth performance, at least when nutrients are not limiting.

There are also contradictory reports of trade-offs between increased resistance to nematodes and decreased growth rate (Bishop et al. 1996; Bouix et al. 1998; Eady et al. 1998; McEwan et al. 1992). However, this is not the cost of the immune response per se but the immune-mediated pathology induced by immediate IgE-mediated hypersensitivity reactions (Stear et al. 2003). This is considered further below. However, it is clear that nutrition does constrain the immune response under certain situations and impact on disease. This is most marked in malnourished animals but even overtly healthy animals respond positively to additional feeding, especially protein supplementation.

Immunopathology. Immune-mediated pathology could constrain the development of optimal immune responses for disease control and introduce a trade-off that favours balancing selection. An example of immunopathology associated with high immunity was noted in commercial pigs selected for high and low immune responses, in which high responders showed more immune-mediated arthritis following challenge with *Mycoplasma hyorhinis* than low responders (Reddy et al. 2000). However, it is important to note that the high responders had better overall disease scores and survival post-challenge. Nematode infection forms another good example. Here IgA has no effect on growth whereas strong IgE responses are associated with reduced growth rates (Murphy et al. 2010). The mechanism by which IgE affects host growth appears to be the breakdown of tight junctions between epithelial cells leading to relative protein deficiency (Stear et al. 2003). Although immunopathology could conceivably be invoked to explain genetic variation in IgE activity, it does not explain genetic variation in IgA activity. Therefore immunopathology cannot be a general explanation for balancing selection.

Co-evolution. A final explanation for the retention of a high degree of genetic variation in disease resistance is host-parasite co-evolution. Genetic variation in the immune response could exist because genes that improve disease resistance in one generation might decrease disease resistance in subsequent generations as a result of changing environmental conditions. Consequently, natural selection does not fix favourable genes. These genes would then contribute to the genetic load. This scenario is plausible and has been extensively explored by theoreticians and epidemiological modellers. There is little doubt that host-parasite co-evolution does occur but there is very little evidence to support the idea that host-parasite co-evolution contributes to variation in the immune system.

With nematode infections, nematodes predigest food externally and some proteases could digest antibody. Nematodes also produce galectin which binds antibody and presumably prevents antibody attacking the nematode or interfering with digestive enzymes (Chandler 1932; Chandler 1935a; Chandler 1935b; Chandler 1936a; Chandler 1936b). The recognition of parasite molecules by the immune system is often assumed to be an arms race with parasites mutating to evade recognition. However a small-scale comparison of parasite molecules indicated that rates of evolution were very low. For example tropomyosin is a target of a protective IgE response but is almost identical in *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Stear et al. 2011).

This survey of the causes of genetic variation in the immune response suggests that some of the variation in resistance to disease is a consequence of mutation and also supports the idea that the immune response is constrained by nutrition. Both of these causes argue in favour of selective breeding to enhance disease resistance and the immune response. Selective breeding will act to eliminate genes which lead to sub-optimal immune responses. It will also fix genes which improve the efficiency of food conversion. As long as selective breeding involves an index of desirable traits including production, reproduction and disease resistance there is no reason to expect adverse consequences.

STRATEGIES FOR BREEDING TO INCREASE RESISTANCE TO DISEASE

Historically, animal breeders have selected desirable phenotypes and applied statistical analyses to determine which animals had the best breeding values. This has been an enormously valuable undertaking with immense benefits to society including cheap food and wool. The animal breeding world is now changing; advances in genetics, DNA sequencing, statistics and computing are moving animal breeding into an era of genome-based selection. The basic idea is easily understood and compelling. A reference population is phenotyped for all desirable attributes, extensively genotyped and analysed to detect all associations between genetic variants and desirable traits. The genomic breeding value of each animal can then be calculated by adding the value of all genetic variants across the genome. This method is being implemented by chicken and pig breeding companies and by the dairy cattle and industries. Beef and sheep industries are starting to follow; probably sooner in Australia and New Zealand than in Europe and the rest of the world.

A major challenge for breeding for disease resistance is to determine which phenotypes to select. Essentially, there are three strategies, direct selection for resistance to specific diseases, selection for immune responsiveness or selection of animals for fitness or performance traits within a disease-endemic environment.

1. Direct selection for resistance to the most important diseases. The first strategy is to concentrate on the diseases that are believed to be most important to the industry. These might include the most economically relevant diseases, as well as zoonotic pathogens. In Australia there has been some success in exploiting genetic variation among cattle in resistance to ticks by cross breeding of indicine and taurine breeds and in resistance to nematode infection in sheep through selective breeding. The phenotypic characters used for selection are the number of adult female ticks on one side of an animal and the number of nematode eggs in the faeces. This work is likely to continue. However, there is much effort devoted to identifying better markers of resistance to disease, especially resistance to nematodes. Faecal egg counts can be time-consuming to perform and may not be an accurate reflection of resistance to infection or disease.

The main problems with direct selection are heterogeneity in the distribution of disease-causing agents and the fact that the heritability of resistance to most diseases is low, generally below 10 % (Koeck et al., 2012). Nonetheless, there are examples where this has been successful. In Scandinavian countries direct selection for reduced clinical mastitis in dairy cows has been ongoing for two decades (Steine et al., 2008). The results of this selection show that an increase in incidence of mastitis is at least held at bay, whereas previously it was steadily increasing when only production traits were included in the selection index (Steine et al., 2008, Koeck et al., 2012). This strategy is best accomplished in those countries where all disease treatment and recording is tightly regulated as in parts of Scandinavia.

Despite the difficulty of accumulating useful data on disease occurrence, Canada has recently implemented a National Health Recording Program for 8 prevalent diseases so that direct selection can be considered (Koeck et al., 2012). In this case, diseases will be recorded by the dairy producers and the resulting data will need to be carefully reviewed and analysed by the Canadian Dairy Network prior to inclusion in the Canadian lifetime profitability index (LPI) (Koeck et al., 2012). The Canadian data also show positive genetic correlations between most

of these 8 recorded diseases indicating that simultaneous improvement in these health traits is possible. The limiting factors are expected to be consistency and accuracy of disease recording, as well as slow response to selection due to low heritability of these traits.

The identification of genetic markers for specific disease resistance can be augmented by genomic selection. Selection based on genomic estimated breeding values (gEBV) offers increased reliability compared with traditional EBVs and potentially reduces the generation interval (Pszczola et ., 2012). This translates into increased genetic gain at a reduced cost. Realizing these benefits the dairy industry has been particularly quick to implement these genomic values into their selection programs worldwide. In fact, all widely available young sires and many cows in North America and many sires in Europe have gEBVs. The cost of the SNP chip technology is rapidly coming down in price from about $130/50K chip in 2012 to $30/50K in 2013. In addition, it is possible to use smaller chips at even lower price and impute information up to the 800K chip with the current reference information available in Holstein dairy cattle (Segelke et al., 2012).

2. Selection for improved immune responsiveness. The second strategy is to select for improved immune responses as such (Figure 2) (Mallard et al., 1993, Thompson-Crispi et al., 2012a and b, Zhao et al., 2012, Leach et al., 2012). Research in pigs, chickens and cattle has clearly shown that immune responses can be improved by selective breeding. Animals with higher immune responses are generally healthier and more productive. There are a number of points to consider here. First, this approach does not target a particular pathogen but is most often designed to facilitate improvements in broad-based disease resistance (Thompson-Crispi et al., 2012a and b). Since antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) are effective against different pathogens, it is ideal to select for a balanced and robust AMIR and CMIR. This can best be accomplished by estimating breeding values for these two traits and selecting animals that have the highest combined estimated breeding values (EBVs) for both these traits (Mallard et al., 2010). Immune response traits, measured in terms of Ab titres or skin thickness in response to standard antigen preparations are generally moderately heritable, in the range of 25 percent, which is in line with other milk production traits where genetic progress has been consistently achieved (Thompson-Crispi et al ., 2012a, Flori et al., 2011). North American dairy cattle with the highest EBVs for AMIR and CMIR have the lowest occurrence of disease (Thompson-Crispi, 2012b).

Another factor to consider with this approach is that identification of individuals with a more robust adaptive immune response based on EBVs has the ability to capture all of the benefits of the adaptive immune system (i.e. immunological specificity and memory which can generate a quicker and greater response on second and subsequent exposure to the foreign agent), as well as the innate host defense mechanisms required for pathogen recognition and appropriate initiation of adaptive immunity (Figure 3, Wilkie and Mallard 1999). Since breeding values represent the genetic component of the trait, they allow individuals to be compared across herds, regions and even countries. Additionally, these benefits are assured of transmission to future generations of offspring. This method has a sound theoretical basis as it eliminates genes that cause suboptimal immune responses and reduces the genetic load. This process is now being adopted by the Canadian pig and dairy industries.

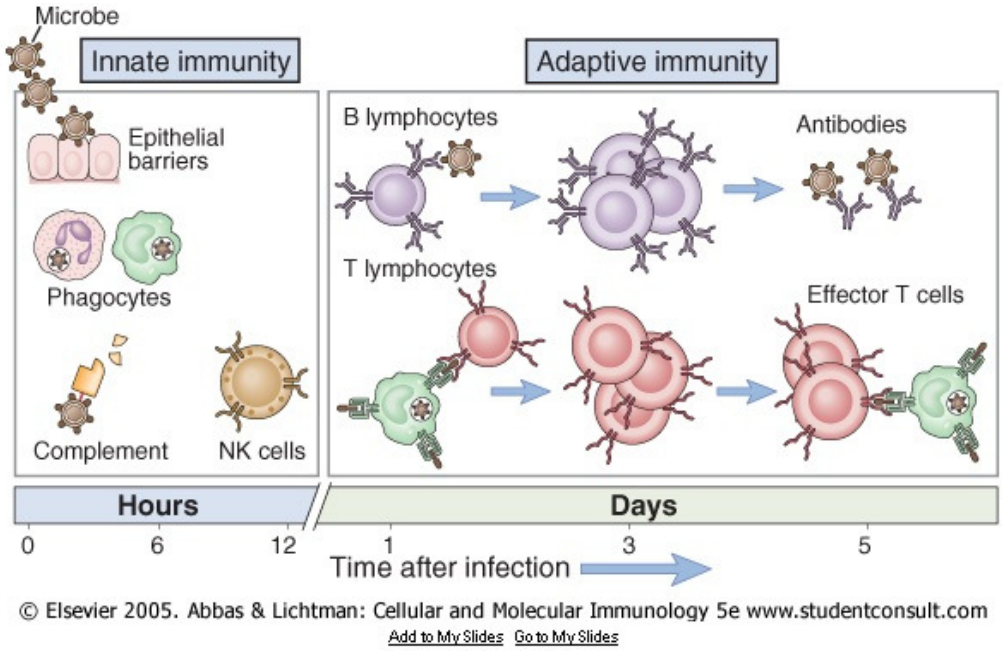

Figure 2. Overview of components of the immune system.

In addition, it is possible to consider selection based on a compilation of heath traits that include direct and indirect selection based on clinical and clinical disease, as well as innate and adaptive immune response traits (Koeck et al., 2012, Thompson-Crispi et al., 2012 a and

b, Bovenhuis et al., 2002). This can work in both conventional and organic farming (Magnusson et al., 2001).

Genomic selection will also be useful for rapidly improving immune response traits. A recent study by the Mallard lab demonstrated that it is possible to identify unique SNPs associated with high and low AMIR using the Illumina 50K SNP panel (Thompson-Crispi et al., 2012 submitted to JDS).

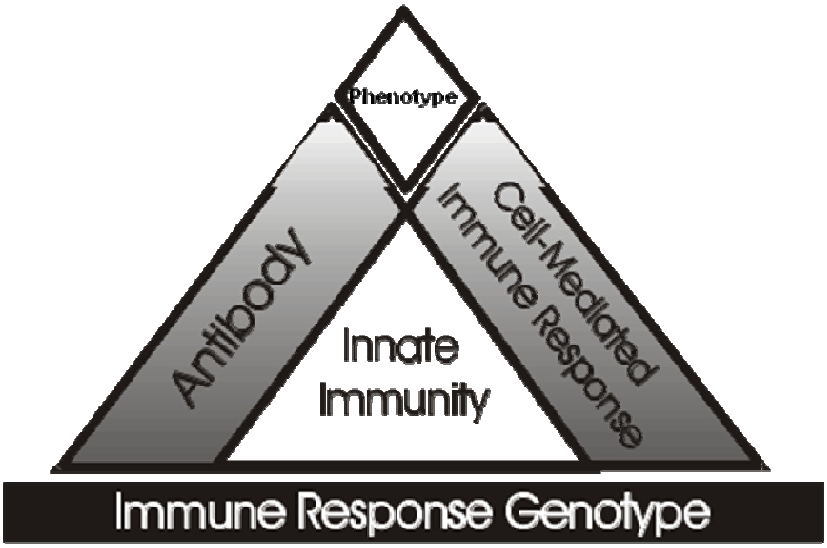

Figure 3. The phenotype of optimal disease resistance should be a function of optimal innate and adaptive host defence mechanisms (from Wilkie and Mallard, 1999).

3. Selection of animals for fitness or performance traits within a disease-endemic environment. It is sometimes forgotten that when we select animals on survival and performance in any environment, we are taking a naturally balanced approach to selecting animals that are adapted to that environment, which is weighted for the prevalence of endemic disease and for the impact of endemic disease on production. The Danish pig industry provides an excellent example of how much progress can be made by the use of a survivability index. Since 2004, the number of live piglets at day-5 of life (LP5) has been included as a selection trait. The associated dramatic reduction in mortality is shown in Figure 4 (Danish Pig Research Centre, 2012). To some extent the benefit of this strategy is complicated by a more or less simultaneous introduction of a programme to increase the frequency of boars with the resistant homozygous F4 ab/ac genotype through Project F4

(Danish Pig Research Centre, 2011). This has resulted in a very high proportion of resistant animals in the population and is consistent with the first strategy described in this section on strategies for increasing resistance to disease.

Longevity and survival are traits that are increasingly included in indices such as the Lifespan and Profitable Life Index (PLI) used by DairyCo, the British dairy research and development agency. For cattle, productive lifespan is a difficult trait to accumulate data for proofs because information on the productive lifespan of daughters will take many years to accumulate. However, this is partly circumvented by the use of traits that are known to be associated closely with survival in the herd, including fertility, susceptibility to mastitis and some specific type characteristics (feet, legs and udder).

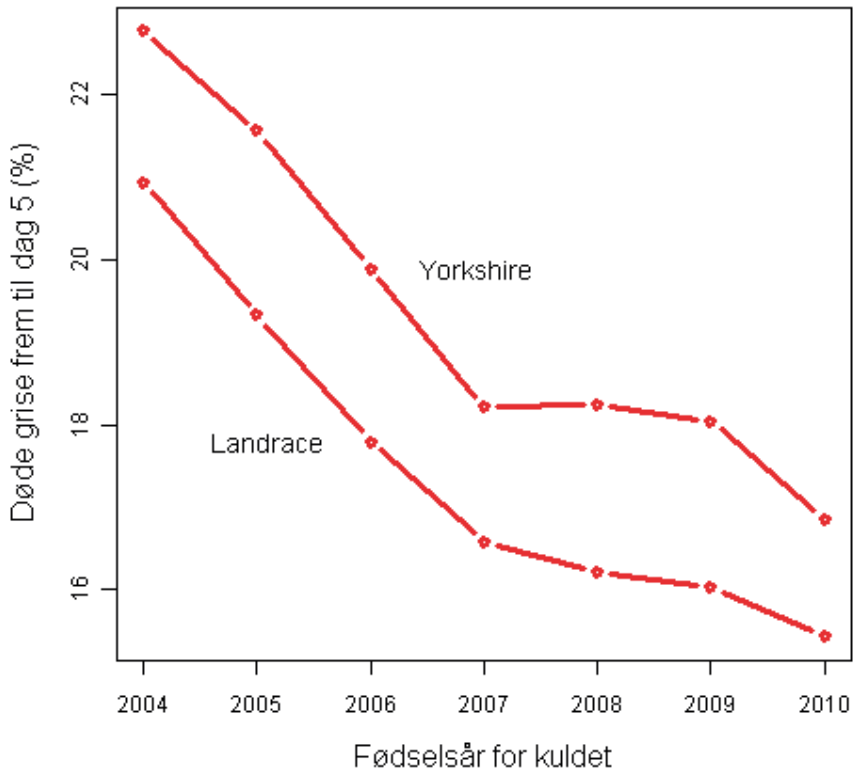

Figure 4. Reduction in piglet mortality in Danish purebred Landrace and Yorkshire performance-recorded herds since the introduction of live piglets at day-5 (LP5) as a selection index in 2004.

CONCLUSIONS

1. There is considerable variation in resistance to disease in cattle and sheep that enables the effective selection of more adapted animals.
2. Accurate definition of the disease entity, the most appropriate measure of resistance and the most appropriate selection goal is essential to ensure effective progress.
3. For most diseases of economic importance in cattle and sheep, when defined at the level of a clinical disease syndrome, resistance can be considered to be a complex or quantitative trait with relatively low heritability but with little evidence of negative correlations with resistance to other disease syndromes. This is because there are frequently several aetiological agents and the mechanisms of resistance are dependent on effectively interacting innate and adaptive arms of the immune response.
4. In cases where disease is more tightly defined in terms of the aetiological agent, sometimes down to bacterial or viral subtype, it might be possible to identify more specific mechanisms that contribute disproportionately to resistance, such as receptor presence or polymorphisms in defensins. These traits will have high heritability estimates (if not rare in the population under consideration) and enable rapid selection. However for these traits, it is also more likely that there will be a trade-off with resistance against other, similar aetiological agents. It remains unlikely that there would be negative genetic correlations with resistance to other clinical disease syndromes.
5. Reduction in the burden of infectious disease can be improved genetically in three distinct strategies.
    a. Specifically by selecting for resistance to important diseases, either directly on the occurrence of disease (eg clinical mastitis) or indirectly on a proxy measure (eg somatic cell score in milk),
    b. Generally by selecting for animals with strong innate and or adaptive immune responses to a standardised antigenic challenge according to a quantifiable scoring system,
    c. Both specifically and generally by selecting for animals that perform well in an environment in which disease is endemic.
6. Heritability estimates for components of the innate and adaptive immune response are high enough to enable relatively rapid improvement.

7. The geographic heterogeneity of disease incidence has important implications for heritability estimates and the extent to which selection should be targeted at specific diseases or specific aetiological agents.
8. High density SNP chips and improved sequencing methodologies are leading to rapid improvement in the technical ability to conduct genome-wide association studies (GWAS), to the extent that the collection of phenotypic data is becoming the chief limitation. GWAS can be used directly in genomic selection and can also identify candidate genes for detailed examination of their role in immunity.
9. In the absence of suggestive information from GWAS, other functional approaches including transcriptomics, proteomics (indeed all of the omics) contribute complementary information that can enable the elucidation of the genetics of disease resistance.
10. Overall, a balanced approach to investigation and implementation is indicated, combining the use of GWAS to identify candidate genes with diverse physiological studies and simultaneously using all three strategies for selection.

RECOMMENDATIONS FOR FUTURE RESEARCH.

1. Develop a tool for the evaluation of antibody-mediated immune response (AMIR) and cell mediated immune response (CMIR) in sheep and the most popular breeds of beef cattle as per the work of Mallard and colleagues in dairy cattle, as this has not been attempted yet. For beef cattle, indicine and taurine cattle would have to be considered independently as evidence is building to suggest that they have differing predispositions to CMIR and AMIR.
2. Consideration should be given to the development of dynamic and flexible health selection indices for cattle and for sheep based on breeding values focused on broad-based disease resistance. This index would include EBV of appropriate immune response variables (AMIR and CMIR, innate components), as well as various other markers of specific disease resistance, according to current economic and zoonotic pathogen prevalence.
3. For tick resistance there is a real need to repeat some of the GWAS done within the Beef CRC, using higher density SNP and proper tick counts rather than tick scores

and using carefully selected populations to enable appropriate comparisons between indicine and taurine cattle and stabilised composite breeds.

4. For mastitis in sheep there is a paucity of information on all aspects of the disease. Studies are required to determine the incidence of mastitis under grazing conditions in Australia and to identify the pathogens present in naturally occurring cases of mastitis. It would be possible to progress with GWAS in the absence of information on pathogens. Perhaps the most useful strategy would be to identify regions of interest found in GWAS in cattle, apply an imputation and fine-mapping approach to enable linkage studies in closely monitored populations of sheep. This could be then validated and applied as a genomic selection tool to improve resistance. While it is certain that various polymorphisms in components of the innate immune response will modulate the resistance of sheep to mastitis, it will be difficult to apply this knowledge confidently without a better understanding of the pathogens involved.
5. The extent to which diversity in the MHC (rather than specific alleles) contributes to the efficiency with which a population responds to challenge with infectious diseases could be readily investigated using the model of bovine respiratory disease (BRD) complex in feedlot cattle. This is one of the fundamental questions of immunogenetics and has important ramifications for selection strategies more broadly. Other aspects of resistance to BRD and responsiveness to vaccines against pathogens contributing to BRD are being investigated using GWAS and functional studies of physiological response to challenge.

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Box 1. Resistance to cattle tick infestation

Differences in the tick burdens of cattle under the same challenge have been well known for many years. Seifert (1971) quotes references in the scientific literature to the resistance of *Bos taurus indicus* cattle and their crosses from as early as 1912. However, host resistance was likely the basis of integrated control of ticks in the thousands of years before the introduction of acaricides or the existence of any scientific literature. The variation in resistance to tick infestation is most marked between *Bos taurus taurus* and *Bos taurus indicus* cattle, taurine cattle given the same exposure carrying between five and ten times as many ticks as indicine cattle.

Resistance to tick infestation in cattle has many effector mechanisms, each of which is likely modulated by many factors. Some of these mechanisms and their modulating factors have been identified and quantified, although much remains to be explained. Tick resistance is mostly manifest against attaching larvae, which attempt to feed often and without success, death occurring mostly within 24 h of host finding (Roberts, 1968). Self grooming is an often neglected but major contributor to variation in tick resistance (Bennett, 1969; Koudstaal et al., 1978). There is some evidence of innate and adaptive immune response to tick infestation and it appears that the relative importance of each differs between indicine and taurine cattle. There is conflicting information regarding the role of humoral immunity in tick resistance, recent studies indicating that IgG is not protective (Piper et al., 2009). Genome wide association studies (GWAS) have suggested several loci as being associated with variation in tick resistance, none being responsible for more than 5% of variance (Porto Neto et al., 2011). There is evidence from gene expression studies and from GWAS to suggest that variation in the extracellular matrix of skin (epidermal growth factors, collagens and other matrix components such as lumican) also contributes to variation in host resistance (Piper et al., 2010). Inflammation alone does not appear to be protective against tick infestation (Constantinoiu et al., 2010). Thus, in the complex tick–host interaction, diverse immune mechanisms and non-immune, structural components contribute to variation in host resistance. The cellular and humoral mechanisms both appear to be relevant, but contribute differently in *B. t. taurus* and *B. t. indicus* cattle.

Resistance can be accurately and repeatably estimated using the method described by Utech *et al*. (1978a). This method uses artificial infestations with approximately 20,000 *R.*

microplus larvae (1 g by weight) and resistance is estimated to be the mean percentage of larval ticks that fail to mature as engorged females, assuming a 1:1 sex ratio among the applied larvae. Cattle with > 98% resistance are considered to be highly resistant, 95 to 98% is considered moderate, 90 to 95% low and < 90% very low. Using this system of measurement, estimates of heritability have been high, ranging from 0.42 to 0.64 (Wharton et al., 1970).

The high genetic contribution to variance in resistance to ticks together with the readily measureable phenotype has enabled the rapid improvement in resistance to tick infestation in taurine breeds by cross breeding with indicine animals and by selection within taurine breeds. Utech and Wharton (1982) selected intensively on sire and dam for tick resistance within the taurine breed Australian Illawarra Shorthorn (similar to Dairy Shorthorn). In ten years they increased mean host resistance from 89.2 to 99%, a level of host resistance about equivalent to that seen in F1 indicine × taurine cattle. There have been no reported attempts to improve the resistance of pure indicine breeds. Although it is possible to select for high levels of resistance from within taurine breeds, in practice few farmers include tick resistance in their selection objectives. One of the main impediments to selection for host resistance to tick infestation is that on a well managed farm, naturally occurring tick burdens are generally quite tightly controlled by applications of acaricide, thereby limiting the expression of resistance (Jonsson et al., 2000; Jonsson and Matschoss, 1998). Artificial infestations on farm are also not seen as a viable practice because of the cost of producing larvae and concerns about biosecurity including the introduction of tick-borne diseases or acaricide resistant ticks.

Genome wide association studies have identified several SNP associated with resistance to tick infestation (Turner et al., 2010; Porto Neto et al., 2011; 2012) however none explain a large amount of the variation. The *RIPK2* gene is a good example of a strong candidate gene for tick resistance, which has been shown to have a role in the innate immune system as well as being implicated in adaptive immunity and in the healing of wounds (Porto Neto et al., 2012). However, despite its significant effect in GWAS and its demonstrated role in recognition of tick Ag, the gene only explains a small percentage of variation in resistance and there is insufficient information to warrant selection on the basis of this gene. At this point in time there appears to be little promise of identifying any single gene that might have alleles that confer a useful level of resistance, nor yet of any panel of genes to do the same.

From a mechanistic perspective, although several studies have demonstrated that there is variation in innate immune responses in resistant and susceptible animals, in most cases an intense innate inflammatory response to ticks is associated with low levels of resistance (eg Piper et al., 2009). The mechanisms of resistance have not been elucidated to the extent that any single component of the immune response, whether innate or adaptive could be clearly identified as being a critical regulator. More information is required regarding the biology of the host's response to infestation.

Box 2. Selection for gastrointestinal nematodes in sheep

Nematodes cause disease and death in livestock and consequently they have been intensively studied. The most important species of nematodes affecting sheep are *Teladorsagia circumcincta* and *Haemonchus contortus.* In cool, temperate areas of the world, such as the North of Britain, Southern Australia, and New Zealand the dominant nematode of sheep is *T. circumcincta*. In hotter areas, such as the southern United States, northern Australia, South Asia and southern and East Africa, *H. contortus* predominates. Interestingly, *T. circumcincta* and *H. contortus* seldom coexist in large numbers; when they occur together one species dominates. A similar situation exists in cattle with the related nematodes *Haemonchus placei* and *Ostertagia ostertagi. Haemonchus* dominates in hotter areas such as subtropical Australia while nematode infections in cooler areas such as the north-eastern United States and southern Australia are predominantly *O. ostertagi.*

Grazing animals are usually infected with several different species of nematode. Other common taxa that infect sheep in the UK include *Trichostrongylus vitrinus*, *Trichostrongylus axei, Nematodirus battus, Nematodirus filicolis, Nematodirus spathiger* and *Cooperia* spp. *Nematodirus* spp. eggs are slightly larger and have a distinctive shape. They are often counted separately but eggs from the other species cannot be easily distinguished from each other and their eggs are counted together.

The life-cycles of the most important nematodes of livestock, such as *T. circumcincta* or *H. contortus* are direct and involve only one host. Eggs are produced in the gastrointestinal tract and are then deposited on pasture in the faeces. The eggs hatch into first-stage larvae and subsequently moult into second-stage larvae. Both first and second stage larvae feed on bacteria in the faeces. The third-stage larvae leave the faeces and some of these larvae are ingested by sheep during grazing. Within the host the third stage larvae undergo two further moults to fourth-stage and fifth-stage larvae. The latter are young adults and once they mature, they breed at species-specific sites within the gastrointestinal tract.

Mature sheep are relatively immune to nematode infection and most breeding ewes produce significant numbers of eggs only during the periparturient period. Along with relatively small numbers of parasites that have survived over winter, these periparturient eggs develop into larvae that initiate infection in naïve lambs.

There is considerable variation among regions and farms in husbandry arrangements. In a typical Scottish upland farm, lambs are born in late March to early April, kept with their mothers until 3-4 months of age then grazed with other lambs until the end of the grazing season when they are 6-7 months of age. At this point transmission of nematodes ceases or falls to low levels. Often, egg counts peak in mid-season, then decline as the immune response begins to control parasite establishment, survival and fecundity. However, this pattern is dependent upon the weather and frequency of anthelmintic treatment and different patterns are observed in different years.

Most parasites are aggregated among their hosts, with an overdispersed distribution. Most hosts have relatively low egg counts while a small proportion have relatively high egg counts. These individuals with high egg output make a disproportionate contribution to parasite transmission between hosts. In contrast, the distribution of the lengths of adult female *Teladorsagia circumcincta* is approximately symmetrical around the mean. This trait has a strong relationship with nematode fecundity; longer females lay more eggs per day.

Aggregation arises because there are differences among hosts in exposure to infection or in the response to infection. Each mouthful of grass ingested by grazing sheep can contain infective larvae. However, faeces and infective larvae are not uniformly distributed on pasture. There are also differences among sheep in grazing behaviour. For example, faster growing sheep may eat more grass. Infected sheep may suffer a temporary anorexia. Possibly, this is an adaptive response to reduce infection. Most sheep will ingest relatively low numbers of infective larvae but some sheep will ingest large numbers.

The second force promoting aggregation of the numbers of adult nematodes in sheep is the immune response. In natural, predominantly *T. circumcincta* infection of lambs, the steadily increasing heritability of egg counts suggests that resistance is largely acquired rather than innate. Resistant sheep have fewer adult nematodes, more inhibited larvae and shorter and less fecund adults. Sheep with more globule leucocytes (discharged mast cells) have fewer adult *T. circumcincta.* Immediate hypersensitivity reactions may prevent the establishment of incoming larvae and possibly expel some adults. Variation in the local concentration of globule leucocytes (discharged mast cells) accounts in a statistical sense for about one-third of the variation in worm numbers.

Sheep with increased mucosal IgA activity against fourth-stage larvae have shorter and less fecund adult worms as well as more inhibited fourth-stage larvae. IgA activity in the abomasal mucosa also varies extensively among animals. IgA activity in the mucosa is correlated with globule leucocyte numbers (r=0.4) consistent with both responses being influenced by Th2 type cytokines.

The distribution of immune responses mirrors the distribution of the associated parasitological variables; globule leucocytes, worm egg counts and adult worm numbers are skewed and overdispersed while both mucosal IgA activity and adult female worm length were consistent with a normal distribution.

There is substantial evidence for genetic variation in resistance to nematodes. The most important component is additive genetic variation. The importance of these components varies over time; for example the maternal component decreases as the lambs mature. In contrast, the additive genetic component is indistinguishable from zero at one and two months of age but increases to one-third of the total variation at 6 and 7 months of age. The delayed development of genetic resistance is consistent with the hypothesis that resistance is due to the development of protective immune responses. A heritability of one-third is similar to the heritability of milk production in dairy cattle or of growth rate in beef cattle. Heritabilities of this magnitude mean that selective breeding for decreased egg output is feasible. Selective breeding for resistance to nematodes has been carried out in Australian and New Zealand sheep and in a small number of farms in Europe.

These values refer to a single egg count at each time-point. In practice, a sensible strategy would be to make replicate counts on each sample and to combine the results from samples taken at different times. A combination of multiple counts and replicate samples would improve the identification of genetically resistant animals and produce faster responses to selection. The more rapid response to selection is a consequence of the fact that mean of several counts is a better estimate of genetic effects and is therefore under stronger genetic control. This observation suggests that resistance to this parasitic disease is strongly influenced by genetic variation in the host; the relatively low estimates of genetic variation in disease resistance may sometimes be a consequence of the difficulty in defining and accurately measuring resistance to disease.

Traditional quantitative genetic analyses successfully predict the response to selection in production traits such as milk yield or growth. In the case of parasitic and infectious diseases traditional approaches underestimate the response to selection, because they take no account of the reduced contamination of the environment. For example, selected animals will produce fewer nematode eggs and mean egg counts of infected lambs will decrease over time. The combination of reduced environmental contamination and increased resistance lead to even greater benefits from selective breeding.

Identifying the genes underlying immune responses can improve our understanding of the host-parasite interaction and allow selective breeding to be enhanced by the use of genetic markers. Two of the major sources of variation in immune responsiveness are variation in antigen recognition, which influences the quality of the immune response and variation in cytokine production, which influences the quantity of the immune response. There are two confirmed genetic markers for *T. circumcincta*. The association between the MHC and nematode infection has been independently confirmed by several groups. The second marker is the interferon gamma locus *Ifng*. This too has been independently confirmed by several groups. These markers are biologically plausible candidate genes: the MHC class II region influences antigen recognition while interferon gamma can influence antigen recognition but its main function is to determine the type of cytokine response. However for both genes, the causative mutations have still to be identified.

The F allele at the *ifng* locus shows dominant susceptibility in male lambs while the polymorphic *DRB1* locus shows heterozygote advantage for some of the traits describing relative resistance to nematode infection. These loci therefore show non-additive gene action. The two loci may be markers for other linked loci in linkage disequilibrium and the non-additive gene action may reflect gene action at the true disease resistance loci. Both loci were more strongly associated with differences in the number of adult *T. circumcincta* than with differences in the number of immature larvae or in worm length or fecundity. Together these two loci accounted for approximately 50% of the additive genetic variation in worm number.

In conclusion, nematode infection in sheep is one of the best understood of all complex diseases. Important genes and the mechanisms that influence variation among animals have been identified but much still remains to be done.

Box 3. Mastitis in cattle and potential for selection in sheep

Mastitis is arguably the most important predominantly bacterial disease in dairy cattle and some progress has been made in the selection of animals that are less susceptible to mastitis. Because of the variety of microorganisms involved and the complex, polygenic nature of resistance to mastitis, it could be argued that resistance to mastitis should be considered as several different traits, dependent on the aetiology (Sørensen et al., 2012). However, the limited availability of data on mastitis aetiology has resulted in most selection being on the basis of milk somatic cell score (SCS), for which data are readily available and on the incidence of clinical mastitis (CM). Heritability estimates are in the order of 0.05 to 0.15 for SCS, depending on the sampling approach used (ie lactation average, single sample, stage of lactation of sample collection) and generally from 0.02 to 0.07 for CM (Rupp & Foucras, 2010). There is a well-established genetic correlation between high milk production and increased risk of CM and high SCS (many authors; Rupp & Foucras, 2010).

Despite these low estimates of heritability of mastitis and SCS, most countries' dairy sectors have introduced selection on the basis of both traits and there has been some evidence of genetic improvement. Since the addition of SCS into the lifetime profit index (LPI) in Canada around 1990 there has been a decline in both SCS and clinical mastitis. Norway, which began selecting cattle on the basis of the incidence of CM has seen a slow reduction in the incidence of CM of 0.15 to 0.27% per year. This slow progress seems likely to continue.

Many QTL have been associated with resistance to mastitis. Rupp & Foucras (2010) summarised the QTL for udder health traits in Holstein and Scandinavian breeds of cattle. Similarly, a number of QTL have been identified for mastitis in dairy sheep, many of which appear to be consistent with those discovered in cattle (Rupp & Foucras, 2010). Almost all bovine chromosomes are represented by significant QTL, but very few of them have been characterised and even fewer functional studies of candidate genes have been conducted. Polymorphism in the MHC, in particular the DRB3 gene, has also been implicated in resistance or susceptibility to mastitis in many studies (Rupp & Foucras, 2010). Some genetic studies have implicated components of the innate immune response to mastitis, which has been recently reviewed from a functional perspective (Wellnitz & Bruckmaier, 2012). The forebrain embryonic zinc finger-like gene (FEZL), which regulates cytokine expression, was associated with SCS (Sugimoto et al., 2006). Clinical mastitis has been associated with

polymorphisms in the *Mucin 7* gene, which codes for an antimicrobial peptide (Nilsen et al., 2009). Several TLR have been examined in relation to mastitis susceptibility and not surprisingly, TLR polymorphisms have been associated with mastitis. In one study on 246 Holstein-Friesian cows from a single herd, 11 SNP were identified and examined within the *boTLR1* gene for their relationship with CM and milk production traits (Russell et al., 2012). Two of the genotypes that were seen were associated with higher incidence of CM and with altered expression of TLR1 and downstream chemokines and cytokines. Increasingly frequently other components of innate immune response are being associated with resistance or susceptibility to mastitis, examples including *lipopolysaccharide binding protein* gene *LBP* (Cheng et al., 2012), *breast cancer 1* (*BRCA1*, Yuan et al., 2012), and *high mobility group box protein* (*HMGB1,* Li et al., 2012), although their real impact on the incidence of disease is far from clear.

Some studies have examined the heritability of individual components of the immune response to intramammary infection. Somatic cell count increase is mainly due to the infiltration of polymorphonuclear leukocytes (PMN) as a response to mammary gland infection. These PMN are a critical component to directly defend against infection and for the initiation of appropriate adaptive immune responses. Sire effects on the function of neutrophils have been demonstrated, with heritabilities of 0.2-0.5 for PMN migration, 0.3-0.7 for PMN phagocytic activity and 0.4-0.5 for serum complement activity (Detilleux et al., 1994).

With respect to mastitis in sheep, almost all published reports relate to dairy ewes, and there is virtually nothing to be found about the aetiology, incidence, impact of mastitis in sheep raised for meat and wool production. One study in the UK has attempted to address this (Conington et al., 2008), but was forced to refer to information on cattle and dairy sheep. In general, estimates of heritability for SCS and incidence of mastitis are similar to those for cattle. The primary challenge has been recording a reliable phenotype in pasture-based ewes during lactation. Given the overlap between sheep and cattle GWAS for mastitis, it would be reasonable to assume that the mechanisms of resistance to infection are similar in sheep and cattle, that many of the pathogens are similar, and consequently that the same challenges will be faced in improving resistance to mastitis in sheep. These challenges are multiplied by the nearly impossible task of recording accurate phenotypes in sheep. Perhaps the most useful strategy would be to identify regions of interest found in GWAS in cattle, apply an

imputation and fine-mapping approach to enable linkage studies in closely monitored populations of sheep. This could be then validated and applied as a genomic selection tool to improve resistance. While it is certain that various polymorphisms in components of the innate immune response will modulate the resistance of sheep to mastitis, it will be difficult to apply this knowledge without a better understanding of the pathogens involved.

Box 4 Bovine respiratory disease complex (BRD)

BRD is a complex of diseases that often progresses from a viral upper respiratory tract infection to a bacterial pneumonia. Because BRD is a clinical and not an aetiological diagnosis, and the pathogenic organisms are many and diverse, it is similar to mastitis from the perspective of selection for disease resistance, particularly via variation in the innate immune response. Viruses commonly associated with BRD include bovine herpesvirus 1 and 3 (BHV-1, BHV-3), bovine parainfluenza 3 virus (PI3); bovine viral diarrhoea virus (BVDV); bovine respiratory syncytial virus (BRSV); bovine adenovirus; bovine rhinovirus; and bovine coronavirus (Griffin, 1998). Of these, BHV-1, PI3, BVD and BRSV are the most common viruses associated with BRD. In one study, 68%, 57%, 27% and 13% of cattle entering the feedlot were serologically positive for BVDV, PI-3; BRSV and BHV1 respectively (Dunn et al., 1991). However, only 10.3% of these cattle had clinical signs of disease. Bacterial pathogens are an important, but not essential, component of BRD. In one study bacteria were cultured from 19% of sick animals, with the most common species being *Mannheimia haemolytica* and *Pasteurella multocida* (7 and 2.7% respectively) (Griffin, 1996). Of the deaths investigated, 53% were attributed to BRD with *P. multocida* more commonly cultured from post-mortem examination than *M. haemolytica* (14% v 9%). Other bacteria isolated from post-mortem examination included *Salmonella* spp. (6%), *Arcanobacterium pyogenes* (10%) and *Histophilus somni* (2%). Bacterial or viral pathogens may cause respiratory disease independently, and the bacterial component may not necessarily follow the viral infection (Griffin, 1998). The important point to note is that BRD is a complex of many infectious agents, so it would be expected that resistance to BRD will require a variety of innate and acquired immune responses.

Vaccines are used to control the incidence and severity of disease. There are commercially available vaccines in Australia to prevent BRD: Rhinogard and Bovilis MH. Rhinogard is directed against the viral infection IBR. Bovilis MH is directed against the bacterial infection *M. haemolytica*. The use of these vaccines in feedlots has resulted in some reduction in BRD occurrence (Colditz et al., 2006) however the economic impact of BRD in the feedlot is still regarded as high.

The genetic contribution to variation in susceptibility to BRD has been established and its heritability is broadly similar to mastitis, between 0.04 and 0.08 (Muggli-Cockett et al., 1992;

Snowder et al., 2006). Nonetheless, there has been relatively less progress in identifying the molecular basis for variation in host resistance to BRD than mastitis, probably for two main reasons. In the first place, beef animals are drawn from a wide variety of breeds and GWAS has been shown to be strongly influenced by breed structure of the base populations. The second reason for relatively less progress with this disease is a lack of clear phenotypic data associated with the difficulty of defining a useful phenotype for GWAS.

Clear differences in breed susceptibility to BRD in the feedlot have been established. Two studies measured the incidence of BRD over six (Muggli-Cockett et al., 1992) and 15 (Snowder et al., 2006) year periods in nine breeds: Hereford, Angus, Red Poll, Pinzgauer, Braunvieh, Limousin, Charolais, Simmental, and Gelbvieh; and three cross breeds. Both studies identified Angus cattle as having the lowest incidence of BRD (11.8%, (Muggli-Cockett et al., 1992); 10.2%, (Snowder et al., 2006)). In contrast, Herefords (19.5%, (Muggli-Cockett et al., 1992); 18.5%, (Snowder et al., 2006)), Pinzgauers (24.6%, (Muggli-Cockett et al., 1992)) and Braunviehs (19.7%, (Muggli-Cockett et al., 1992)) had a higher incidence. Of these susceptible breeds, only Hereford cattle are commonly used in feedlots in Australia. Two studies conducted in Australian feedlots confirmed a high incidence of BRD in Hereford cattle compared with other breeds. One study measured the incidence of BRD in Hereford, Angus, Murray Grey and Santa Gertrudis, and showed Santa Gertrudis animals to be the most resistant to BRD. Treatment for BRD was five times more likely to be needed in Angus, six times more likely in Murray Grey and ten times more likely in Herefords (Cusack et al., 2007). The other study demonstrated an association between the percentage of Herefords introduced into a feedlot and the overall incidence of new cases of BRD and mortality. Herefords were 23% more likely to develop BRD, incurred 29% more in treatment costs and were 25% more likely to die from the disease (Sullivan, 2006). These differences among breeds in susceptibility to BRD strongly suggest that there is a genetic component to disease resistance in cattle.

The major histocompatibility complex (MHC) is discussed at length in Box 6. The interaction of the class I and class II MHC genes with infectious disease has been seen in two different ways:

1) Specific alleles or variants of a gene can be associated with resistance or susceptibility to disease.

2) The overall level of heterozygosity or variability of MHC genes is proposed to be associated with disease resistance. A heterozygote, which shows two alleles or variants of a MHC gene, is expected to recognise a wider variety of pathogens and thus be more resistant to disease than a homozygote, which has only a single allele. This 'heterozygote advantage' predicts an association between MHC variability and disease resistance.

It has been demonstrated, using lymphocytotoxicity testing, that Australian and American Herefords are less polymorphic at the class I *BoLA-A* locus than other breeds (Stear et al., 1988). This low level of variability may be specific to the MHC but, more likely, is due to an overall genome-wide loss of genetic variation. When breeds are formed, a subset of available cattle is chosen and closely bred to achieve desired traits. Such strategies result in the genetic processes of founder effect, inbreeding and genetic drift, all of which lead to a loss in genetic variability. This is supported by data on allele frequency and exclusion probability data from 11 microsatellite loci. It shows clearly that Hereford and Poll Hereford animals are less polymorphic at all loci than 10 other tested breeds, including Angus, Jersey, Brahman, Holstein Friesian, Limousin, Maine Anjou, Saler and Charolais (Vankan et al., 1994; Vankan & Faddy, 1999). The lack of genetic variability in Herefords suggested by these studies together with the multifactorial and multi-agent nature of BRD suggest that lack of variability in the MHC in Hereford cattle might be one cause of the increased susceptibility of that breed.

As with mastitis, a number of components of the innate immune system have been implicated in the resistance of cattle to BRD (see Glass et al., 2012 for a review of this subject), however to date there is no clear evidence that any one of these components is worth pursuing.

Box 5. Additive and non-additive genetic variation

Continuously distributed traits are assumed to be multifactorial i.e. influenced by many different factors. Some of these factors will be different genes; some will be non-genetic factors including chance, climate and nutrition. All these non-genetic factors are called environment. For geneticists, the environment is much more than the wind and the rain.

The performance of an individual for a particular trait is called the phenotypic value (P) and this is made up of a genetic component (genotypic value) and an environmental component E (environmental deviation).

$$P = G + E$$

The environmental deviation is defined to have a mean of zero and consequently, the average performance of a particular genotype is the genotypic value.

The breeding value of an animal is not necessarily equal to the genotypic value. The genotypic value is equal to the breeding value (A - the additive effect) plus a deviation due to dominance (D) and a deviation due to interaction between genes at different loci (I - epistatic deviation)

$$G = A + D + I$$

The additive genetic component is the most important for selective breeding and the dominance deviation and epistatic components are treated as noise. But the dominance and epistatic components are utilised in crossbreeding schemes. The proportion of the variance that is due to genotypic values (VG/VP) is heritability in the broad-sense. In contrast, heritability in the narrow sense is the proportion of the variance that is attributable to variance in breeding values (VA/VP). It is the narrow-sense heritability (h^2) which predicts the response to selection and it is the term of most value to animal breeders. The heritability of a trait and the breeding value of an animal can vary between populations, depending on gene frequencies, type of gene action and environmental effects.

Box 6. The major histocompatibility complex

In mammals, the major histocompatibility complex (MHC) accounts for about 1% of the total genome. It contains over 200 loci, more than half of these are capable of being expressed. The exact number of loci varies among haplotypes. The MHC has been divided into class I, class II and class III regions. In most mammals, the regions form a contiguous block but in the bovidae and cervidae (cattle, sheep, deer and related species) an inversion has moved part of the class II region about 20 million base pairs away from the rest of the region.

The MHC exists in nearly all vertebrates and came into being with the evolution of jawed vertebrates. It does not exist in jawless vertebrates and other more primitive animals. In teleost fish, such as Atlantic salmon, the class I and class II regions occur on separate chromosomes.

CLASS I GENES AND MOLECULES.

Class I molecules are dimeric. They consist of two different proteins. A heavy chain with a relative molecular mass of 45,000 Daltons is non-covalently associated with a light chain of 12,000 Daltons. The dissociated light chain is β-2 microglobulin. Only the heavy chains are encoded by genes within the major histocompatibility complex. The gene for β-2 microglobulin is usually found on a separate chromosome. The C terminus of the heavy chain forms a short cytoplasmic tail joined to a hydrophobic section which spans the plasma membrane of the cell. The extracellular portion forms three globular domains and is glycosylated. The light chain forms a fourth extracellular protein domain.

The two N terminal domains of the heavy chain form an antigen binding site. Two α-helices form the sides while a β-pleated sheet forms the floor. This antigen binding site is usually filled with a short peptide, 8-10 amino acids in length. This peptide is usually derived from proteins produced endogenously within the cell. These endogenously processed peptides can come from host (self) proteins or from intracellular invaders (such as foreign bacteria or viruses).

In most species, such as humans and mice, the class I molecules are encoded by multiple loci. These have been divided into the classical and non-classical loci. The classical loci were the first to be discovered. In humans, they are expressed on nearly all nucleated cells. They are

abundant on myeloid and lymphoid cells, common on kidney, liver and lung but relatively sparse on brain, skeletal muscle and sperm cells. This wide distribution of class I molecules is related to their function which is to signal the presence of foreign molecules to T cells. In humans and mice they are encoded by three loci. They are called HLA-A, B and C in humans and H-2 K, D and L in mice.

The non-classical class I molecules have a restricted distribution, lower levels of polymorphism and appear to differ in function compared to classical class I molecules. In humans, non-classical loci in the class I region include HLA-E, -F and –G.

HLA-E has a low level of polymorphism and is the most divergent class I gene. It has a similar pattern of transcription to classical class I genes. As with classical class I molecules, HLA-E binds to β-2 microglobulin. HLA-E presents a 9 amino acid peptide to the CD94/NKG2 receptor on NK cells and cytotoxic T cells as well as the αβ T cell receptor on some T cells. The 9 AA peptide is derived from the signal sequence of HLA-A, –B, -C and –G molecules. The expression of HLA-E increases when the other HLA class I molecules supply the peptides. This may allow NK cells to check the expression of class I molecules.

HLA-F has a similar pattern of mRNA expression to classical class I genes but does not occur on the cell surface of most cells. It is expressed on the tonsils, spleen and thymus. In many cells, it is localised in the endoplasmic reticulum and Golgi apparatus. It binds a restricted set of peptides. The function of HLA-F is unknown.

The HLA-G locus encodes a molecule that is expressed in extraembryonic tissues (placenta and extravillous membranes) but is absent from most other cells. HLA-G is capable of presenting endogenously-generated peptides to T cells. It may play a role in the maintenance of pregnancy.

MHC class I chain related (MIC) genes are distantly related to class I genes. There are at least 5 sequences at two locations. MICA and MICB are similar in structure to each other. They are expressed on epithelial cells and in fibroblast and epithelial cell lines but, in contrast to classical MHC class I genes, not in B or T lymphocyte lines. They are induced by stress. They are recognised by some intraepithelial gamma-delta T cells ($V\delta1^+$). These cells also

express NKG2D which also recognises MIC. In addition, MIC may promote anti-tumour responses by NK and T cells. Mice do not have a structural homologue of MIC.

The HFE molecule (previously HLA-H) is closely linked to the class I region. It is involved in iron uptake and a mutation (C282Y) is one of the most common causes of haemochromatosis. This disease is characterized by excessive absorption of dietary iron. Humans, like most animals, cannot excrete excess iron. The build up of iron prevents normal organ function, especially of the liver causing cirrhosis, of the heart causing heart failure, of the adrenal glands causing adrenal insufficiency and of the pancreas causing diabetes. Haemochromatosis is one of the most common genetic disorders in people from Northern Europe with an incidence of 0.5%.

Other molecules with similar structures to classical class I molecules are encoded elsewhere in the genome, often on separate chromosomes. These include CD1. The CD1 loci are present on chromosome 1 in humans and chromosome 3 in mice. There are four CD1 molecules in humans. CD1 molecules present lipids and glycolipids to T cells.

In mice. non-classical class I loci include Qa-1, TL, T10 and T22. Qa-1 is the murine homologue of HLA-E. This molecule has a similar function. The TL antigen is abundantly expressed on epithelial cells of the mouse small intestine. It binds to CD8αα on T cells. The TL molecule has a narrow and completely occluded groove and cannot present peptides. The T10 and T22 molecules are recognised by gamma-delta T cells.

CLASS II GENES AND MOLECULES.

Like class I molecules, class II molecules are also dimeric, transmembrane glycoproteins. The α-chain is slightly heavier at just over 30,000 Daltons while the β-chain weighs slightly less than this. The structure of the class II dimer is similar to class I. The N terminal domains on the α- and β-chains form an antigen binding site with the walls formed by two α-helices and floored by a β pleated sheet.

As with class I. the antigen-binding site is usually occupied by a self or a foreign peptide but unlike class I, these peptides are usually generated outside the cell (exogenously). Also, in contrast to class I the bound peptides are usually larger than 10 amino acids, usually 12-24 amino acids in length. In contrast to class I, the peptide extends beyond the groove.

The class II region was originally thought to consist of a single locus called HLA-D in humans. Subsequent studies demonstrated the existence of several loci, called HLA-DP, DQ and DR. Both chains are encoded within the major histocompatibility complex class II region and the loci. These are arranged in matched pairs and are called DRA and DRB for example. There are multiple loci and different individuals can possess different numbers of loci and these are called DRB1, DRB2 and DRB3 for example.

The classical class II molecules have a very restricted tissue distribution compared to class I cells. They are found on dendritic cells, macrophages, B cells and thymic epithelium. Their presence on professional antigen-presenting cells is related to their function which is to prime helper T cells to recognise and respond to extracellular infections. The presence of class II molecules on thymic epithelium is related to the negative selection of maturing T cells in the thymus which recognise self antigens too strongly. Some cells will express class II molecules following activation in response to infection. These cells include endothelial cells of the capillaries, epithelial cells and gamma-delta T cells. Some cells expressing class II on activation, such as Gamma-delta T cells or intestinal epithelial cells, may then act as antigen–presenting cells.

Other non-classical class II loci exist, including HLA-DMA and DMB as well as HLA-DOA and DOB. DMA and DMB are structurally similar to the classical class II genes but are found in a lysosomal-like compartment where peptide loading of class II molecules occurs. The HLA-DM molecule is a heterodimer of the DMA and DMB molecules. It facilitates peptide loading of the classical class II molecules because it helps the class II-associated invariant peptide (CLIP) to dissociate and frees up the antigen-binding site.

HLA-DOA and DOB genes are very similar to the classical class II molecules but have low levels of polymorphism and are expressed at low levels. They are mainly present in intracellular vesicles and they suppress peptide loading by inhibiting HLA-DM.

GENETIC VARIATION.

In most but not all mammals, the class I and class II genes are very polymorphic with more than a hundred alleles at the HLA-A, HLA-B, HLA-C and DRB1 loci. This makes them the most polymorphic loci in the human genome and together with variation in the number of

copies of the class I and class II loci makes the MHC the most variable region of the genome. In some populations over 80% of individuals are heterozygous for some of the most polymorphic loci. Each allele is codominantly expressed and there are a large number of different functional MHC proteins on the cell surface.

These high levels of genetic diversity are too great to be due to neutral evolution and some form of balancing selection seems to maintain MHC diversity. Balancing selection maintains polymorphism and is distinguished from negative or purifying selection which fixes the most advantageous allele and eliminates all other alleles. There are three forms of balancing selection that may act to maintain MHC polymorphism: overdominant selection, frequency-dependent selection and selection that fluctuates in time and space.

Overdominant selection occurs when heterozygotes are fitter than homozygotes. This might occur because heterozygotes can recognise a wider variety of parasite molecules from the same or different species of parasites. Therefore heterozygotes would be more disease resistant than homozygotes. This is plausible and there are several instances where heterozygotes have been shown to be more disease resistant than homozygotes, including humans infected with hepatitis B virus or human immunodeficiency virus-1, Scottish Blackface sheep infected with gastrointestinal nematodes, chickens infected with Rous Sarcoma virus and Chinook salmon infected with infectious haematopoietic necrosis virus. Some authors believe that overdominant selection is not, in itself, sufficient to maintain MHC diversity and suggest that additional forces may act.

Frequency-dependent selection is particular relevant for disease resistance and occurs when the selective forces differ with the frequency of MHC alleles. A rare MHC allele may focus immune responses effectively on a particular bacterial epitope. This allele may then have a selective advantage and will increase in frequency. There is then increased selective pressure on the bacterial species to alter this particular epitope, this could then render the now relatively common MHC allele more susceptible to disease. This process thus increases the frequency of rare alleles and helps them to survive while preventing alleles becoming very common and thus reducing the total number of alleles.

The third form of balancing selection is selection that varies in time or space. This might occur for example, if some species of parasite only occur in certain environments. For

example, malaria is a problem in tropical areas of Africa but not in the cool temperate areas of northern Europe. Alleles that are selected against in malarial areas might survive in cool temperate areas. Alternatively, plagues like the Black Death devastated Europe in the middle Ages, killing approximately one-third of the human population. If individuals with specific MHC genotypes were more susceptible to disease, the frequency of these genotypes would decline and in the plague areas specific alleles could disappear. However, rare alleles could recover in frequency in the absence of the Black Death and uninfected areas of America and Africa would allow susceptible alleles to survive in the population.

Several other lines of evidence suggest that balancing selection acts to maintain MHC polymorphism. Phylogenetic analyses demonstrate that MHC alleles within a species fall into closely related groups that presumably share a common ancestor. These are known as allelic lineages. Remarkably some lineages are more closely related to lineages within closely related species than they are to other lineages of the same species. This suggests that the populations that gave rise to new species contained several MHC alleles and that these alleles have survived for millions of years. This occurrence of identical or very similar alleles in different species is known as trans-species polymorphism. In the absence of balancing selection, alleles are not expected to survive so long.

In the absence of selection (neutral evolution), new alleles will arise by occasional mutations and persist by chance for reasonably short periods of time, in a process known as genetic drift. This creates a characteristic pattern of gene frequencies with one common allele and a smaller number of alleles at a much lower frequencies. The frequency of MHC alleles at the classical class I and class II loci appears more even than this and is likely to be influenced by selection.

Another line of evidence comes from a comparison of point mutation rates in coding regions. Some point mutations create changes in the amino acid structure. They are non-synonymous while synonymous mutations change nucleotides but do not lead to changes in amino acids. A comparison of the number of synonymous and non-synonymous nucleotide substitutions can provide evidence for selection. In most functional genes, the rate of synonymous substitutions exceeds the rate of non-synonymous substitutions; this provides evidence for purifying selection. Most amino acid changes will disrupt protein structure and interfere with function. They are thus eliminated. In contrast, the rate of non-synonymous mutation

exceeds the rate of synonymous mutation in the antigen binding site of most classical MHC molecules in most species. This is evidence for balancing selection.

One feature of the MHC is linkage disequilibrium. Linkage disequilibrium occurs when combinations of alleles at different loci occur more or less frequently than predicted by chance alone. If an allele at locus A has a frequency of 10% and another allele at locus B also has a frequency of 10% then the two alleles should occur together 1% of the time (10% multiplied by 10%). If the alleles occur together more often than this, then this is positive linkage disequilibrium. If they occur more rarely than predicted then this is an example of negative linkage disequilibrium. Linkage disequilibrium does not necessarily involve linked loci and can occur between alleles from loci on different chromosomes. Consequently, some authors prefer to use the term gametic association. Certain combinations of alleles in the human MHC do occur together more commonly than expected. As many of the alleles in the MHC are very old, some authors believe that selection is required to maintain these positive associations.

DISEASE ASSOCIATIONS

One of the many remarkable features of the MHC is the large number of diseases that it has been reported to influence. These disease associations have been thoroughly reviewed by Lechler and Warrens (Anon 2000). The disease associations cover a wide range of mechanisms. They include the production of specific antibodies or cytotoxic T cell responses as well as linked loci, such as HFE in haemochromatosis (mentioned above). We shall discuss a selected set of these disease associations to illustrate general points. The diseases include Insulin-dependent diabetes mellitus, Goodpasture's disease, narcolepsy and systemic lupus erythematosus..

Insulin-dependent Diabetes Mellitus is also known as juvenile diabetes or type I diabetes. It is lethal unless treated by administration of insulin. It is an autoimmune disease that results from the immune-mediated destruction of the insulin producing beta cells of the pancreas. Many different genes contribute to the development of type I diabetes and non-genetic factors also play a role as shown by concordance rates in identical twins of 50% or less. The most important genetic influence is due to the major histocompatibility complex. The DQB1*0302-DQA1*0301-DRB1*04 haplotype is associated with diabetes in most populations studied and has a relative risk of approximately 20. The loci outside the MHC

have relative risks less than 2. The most highly associated HLA molecule is HLA-DQ3.2 which is encoded by the DQB1*0302 and DQA1*0301 alleles. The disease associated properties of the DQ3.2 molecule are largely influenced by the binding pockets for the 4^{th} and 9^{th} residues of the antigenic peptide. However, the critical peptides for the development of diabetes have still to be identified.

Goodpasture's disease is an inflammation of the kidneys (glomerulonephritis) that is often associated with bleeding into the lungs. The disease is rapidly progressive and the kidneys can be destroyed within days of the initial diagnosis. The disease is rare with an incidence of 1 case a year per million Caucasians. Other races have an even lower incidence. The disease is characterized by the presence of antibodies that react with the glomerular basement membrane. These antibodies bind to the 230 amino acid carboxy-terminal NC1 domain of the α3-chain of type IV collagen. Almost all patients with Goodpasture's disease inherit a haplotype with the DRB1*1501, DRB5*0101 and DQB1*0602 alleles. The primary association appears to be with DR molecules. Other DR alleles also appear to confer increased susceptibility (DRB1*04) or resistance to disease (DRB1*0701). The susceptibility alleles DRB1*1501 and DR4 share a common motif (amino acids RFLDRYF) in the DR β chain. This motif forms part of the antigen-binding site and influences peptide binding to DR molecules. In particular disease-associated alleles have positively charged amino acids at position 13 (R or H) and a positively charged glutamine residue at β70 while protective alleles encode hydrophobic amino acids at β13 (Y or F) and a negatively charged amino acid at β70. The strong HLA associations with Goodpasture's disease probably involves differential presentation of the α3-chain of type IV collagen by resistant and susceptibility DR molecules. For example, two peptides from the NC1 domain bind better to Dr15 than DR7. Alternatively, abnormal processing of the NC1 domain may expose cryptic epitopes that bind more efficiently to susceptibility alleles.

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness in conjunction with disturbed night time sleep and cataplexy (sudden loss of muscle tone). There is a near total association with HLA-DQB1*0602. This allele is in strong linkage disequilibrium with HLA-DRB1*1501 and it can be difficult to disentangle the effects of these loci. One hypothesis is that the HLA susceptibility alleles allow immune responses against neurons in the brain that produce the orexin protein that regulates sleep patterns. In principle, class II HLA molecules could influence the specificity of the immune response in several ways.

They could directly bind peptides (determinant selection); if peptides from self molecules are recognised by some but not all MHC molecules this could lead to autoimmune responses and associations between HLA alleles and autoimmune disease. Alternatively, MHC molecules could produce holes in the T cell repertoire. Here there is no T cell with a receptor that can recognise a given antigen. T cells with certain T cell receptors might be coincidentally self-reactive and deleted during T cell development. A third explanation is that MHC molecules might induce immunosuppression.

Systemic lupus erythematosus is a chronic autoimmune disease that is occasionally fatal. Diagnosis can be elusive with symptoms varying among individuals and even among the same individual at different times. Typical symptoms include fever, joint pain and fatigue. Some 30-50% of affected individuals have a butterfly rash. The disease is multifactorial with both genetics and environment influencing disease development. The most important genes lie within the HLA system. Three classes of genes have been implicated: Class II, complement and other class III genes. The most common haplotype in western European Caucasoid lupus patients is HLA-A1-B8-Cw-DR3-C4AQ*0-C4*B1-C2*C-B*fS. The class II alleles appear to influence disease susceptibility through the production of specific autoantibodies. Independently, the null allele (C4AQ*0) at the C4A complement locus influences disease susceptibility, possibly through less efficient clearing of immune complexes, with increased deposition and pathology in tissues. Other loci such as TNFβ and Fas (CD95/APO-1) have also been implicated in disease susceptibility.

INFECTIOUS DISEASES

Most of the disease associations have involved autoimmune diseases or diseases of unknown aetiology. However, the fundamental role of the immune system is protection against microbial and parasitic diseases. Fewer associations with infectious diseases have been reported but this may reflect the greater difficulty in obtaining suitable samples. However associations have been reported for malaria, leismaniasis, filariasis, mycobacterial infections, AIDS and hepatitis. Among domestic animals, associations with resistance to the cattle ticks, nematodes and enzootic bovine leucosis have been reported by at least two separate laboratories for each disease.

A case-control study of over 2000 individuals from the Gambia reported that those with the class I allele HLA-B*5301 had a reduced risk of severe malaria in childhood (defined as

cerebral malaria and anaemia). Those with the class II haplotype HLA-DRB1*1302-DQB1*0501 were also but independently protected. The protective effect of the of the HLA-B5301 molecule might be mediated by class I restricted cytotoxic T cells. HLA-B53-restricted cytotoxic T cells recognise a nonapeptide from a liver stage specific molecule.

A similar study in Kenya did not confirm these associations but found a protective association with HLA-DRB1*0101. The differences between the studies may be due to genetic differences between the host or parasite populations or differences in other factors such as parasite intensity. Alternatively, the failure to confirm the original results may mean that the associations are statistical artefacts.

The HLA system has also been implicated in susceptibility to infection with human immunodeficiency virus 1 (HIV-1) and the progression to Acquired Immune Deficiency Syndrome (AIDS). HLA class I haplotypes have been associated with the rate of disease progression but class II associations are less convincing. In particular, individuals with the extended haplotype HLA-A1-B8-DR3 develop AIDS more rapidly than individuals lacking this haplotype. This association is with the class I rather than class II region. In addition, patients with increased levels of heterozygosity in the classical class I loci (HLA-A, -B and –C) developed AIDS more slowly than their more homozygous counterparts.

TRANSPLANTATION

The survival of kidney and heart but not liver grafts is enhanced when donor and recipient share HLA haplotypes. Grafts from HLA identical siblings have an exceptionally good outcome, which may be related to the fact that siblings share complete haplotypes or possibly to the increased accuracy of typing within families.

Chapter 6

Summary of recommendations

Table of Contents

General recommendations

- With the increasing use of high throughput technologies and systems biology (e.g.proteomics, massive parallel sequencing) a large amount of data is created that cannot always be interpreted or used at the time or by one laboratory. The high level of public investment in these studies means that efforts should be made to ensure that the information is stored in open access databases, that include appropriate search and analysis tools, so it can be interrogated by a broad range of researchers and practitionars. In order for this to be preserved as a future resource, regular curation is also critical.
- Ruminants are valuable experimental models and have the advantage of large tissue volume; efficient animal usage should therefore be maximised. This could be done by making animals and/or tissues from all MLA-supported projects and resource flocks available to all researchers and/or the establishment of a tissue bank as is done for human diseases (e.g. cancer) consortia.
- There is a great need for more defined immunological readouts to better select relevant SNPs in livestock.

Innate immunity toolkit

- Basic research to discover the innate immune pathways (including non-coding RNAs) involved in disease susceptibility and immune responsiveness. Once identified, these can then be targeted for therapeutic intervention, biomarker diagnostics, vaccine improvement or selective breeding.
- Develop practical *in vitro* and *in vivo* assays/read-outs to measure innate immune status in sheep and cattle. These can include adaptive immune read-outs (e.g. antibody- and cell-mediated immunity) and testing a defined set of innate immune agonists that activate the different innate immune receptors to generate an innate immune profile. This may necessitate generation of sheep- and cattle-specific reagents. These assays can then be used for selective breeding and determining EBVs for immune competence.
- Construct an innate immunity genomic and transcriptomic database and annotated website for sheep and cattle.

- Identify SNPs in innate immune genes and construct an innate gene array, coupled with defined innate stimuli, to assess EBV for innate immune competence of an animal – cooperate with existing projects where possible (e.g. IAEA/FAO)

Recommendations for specific diseases

Mastitis (sheep)

- Caused by infection of the mammary gland.
- Pathogens can vary with livestock species and environment; *Mannheimia* species are a common cause of mastitis in sheep but not cattle (Omaleki *et al*, Vet Microbiol 153 (2011) 67–72).
- Mostly occurs within the first 2 weeks of lactation.
- Periparturient immune suppression may predispose to mastitis by microbes that are normally commensals.
- Severity depends on the animal response and bacterial virulence factors.
- Immune defence mainly effected by neutrophils (free radicals but also cause tissue damage) and macrophages.

Recommendations

- Epidemiological studies to determine incidence of disease and contribute data for GWAS or linkage studies potentially based on imputed genotypes from bovine studies.
- Metagenomic analysis to identify pathogens in different commercial flocks in Australia.
- As *M. haemolytica* causes both mastitis, pneumonia and septicaemia in sheep, developing a vaccine against this organism may be cost-effective.
- Analyse innate immune response of mammary gland pre- and post-partum and after infection
- Develop reliable model of mastitis
- Assess protective effect of local/systemic administration of different innate stimulators on disease outcome
- Include mastitis diagnosis in GWAS-based gEBV selection studies/screens

Bovine Respiratory Disease

- Major cause of morbidity and mortality in feedlot cattle; result from pathogen exposure and stress e.g. new arrivals in feedlot and post-weaning stress in calves
- Both viral and bacterial pathogens; typically bacterial follows viral infections.

Recommendations

- Investigate general innate boosting at times of high susceptibility (feedlot/weaning/transport) with different innate agonists. Need to target specific innate pathways to determine optimal prophylactic effect.
- Investigate appropriate adjuvants for development of subunit vaccines for the most prevalent viral pathogens.
- Selective breeding for high immune responders against pathogenic viral strains associated with BRD
- Investigate the role of MHC diversity rather than presence of specific alleles

Johne's disease (or Paratuberculosis)

- A contagious, chronic and sometimes fatal infection that primarily affects the small intestine of ruminants.
- Caused by the bacterium *Mycobacterium avium paratuberculosis* (MAP).
- Infection occurs in the first few months of an animal's life but clinical signs of disease may not show up for many months to years later.
- Infects macrophages which fuse together to form multinucleated giant cells
- Antibody production late in infection indicative of clinical signs and imminent death.
- In an endemic herd, only a minority of the animals develop clinical signs;
- Infection is contagious, i.e. it can spread from one animal to another.
- Most animals either eliminate the infection or become asymptomatic carriers.
- Has been compared to human Crohn's disease, a type of chronic inflammatory bowel disease, probably caused by impaired innate immunity and gut microbiota
- Innate immune activation of intestinal epithelial cells is thought to contribute to pathology (Pott et al. Cell Microbiol 2009:1802-15).

Recommendations

- Examine changes in microbiota and innate stimulation of gut in normal and diseased animals.
- Examine effect of probiotic and prebiotic supplements in calves on gut microbiota and disease development.
- A safer and effective vaccine than currently used (Gudair®) is required. New vaccine adjuvants based on specific innate stimuli should be rationally designed to elicit an appropriate cell mediated response (*i.e.* high innate stimulation, induction of IFN and IL-17 producing T cells) with reduced side-effects.
- Perform long-term vaccine trials in sheep to establish efficacy with defined read outs of IFN-g and IL-17 producing cells throughout the study.
- Investigate depot or longlasting vaccine formulations, as the maintenance of effector cells appears to be required for immunity or suppression of clinical disease.
- GWAS on sheep using the new HD SNP chip to identify resistance markers.

Ecto-parasites:

- No protective immune response against blowfly (sheep) and buffalo fly (cattle).
- Differences in immune response and resistance against tick in some cattle breeds
- Some success with tick vaccines using 'hidden' antigen but the need for boosting to maintain protective antibody levels limits its practical use.

Recommendations

- Develop and/or assess efficacy of single shot vaccines/delivery devices in well controlled trials to improve response to existing tick vaccine (Bm86).
- Look at innate differences between breeds before and after infection using appropriate experimental models (*in vivo* cannulation/*in vitro* skin explants/biopsies)
- Comparative transcriptomic expression and mutation analysis of innate pathways stimulated in susceptible and resistant cattle before/after infection to identify potential mechanisms and genetic markers of resistance.
- Although GWAS has been undertaken for resistance to tick infestation, further studies using high density SNP and well-standardised and validated tick counts to compare indicine and taurine cattle, supplemented with meta-analysis of transcriptomic data, should result in improved ability to identify mechanisms of resistance.
- Proteomic analysis to identify immunosuppressive and immunomodulatory molecules
- This is an area where a strong systems biology approach is likely to be beneficial.

Endoparasites

- Gastrointestinal nematodes are the major parasitic diseases in sheep and cattle
- There is good evidence for development of natural immunity after repeated infections.
- There are currently no commercial vaccines against these parasites.
- Immunity against worms is different from that of bacteria and viruses and likely requires the use or development of different adjuvants

Recommendations

- Examine the effect of parasitism on microbiota and vice versa – correlate with immunity and responder/non-responder phenotype
- Develop and test specific adjuvants for anti-helminth vaccines.
- Immune phenotyping using industry resource flocks to find innate correlates of immunity
- Using information from biological read-outs above, generate a defined expression array to measure innate immune competence and correlates with resistance/susceptibility

Breeding for disease resistance

- Selective breeding is moving towards genomic selection
- To optimise selection for disease resistance we need to identify disease resistant animals phenotypically before genome wide testing
- The optimal weighting between disease resistance and production traits needs further research

Recommendations

- Better genomic, immunological and statistical tools to identify resistant animals
- A comparison of selection for enhanced immune responsiveness and resistance to specific diseases

Vaccines; Responder and non-responders/ single shot vaccine

- Vaccines are a highly effective disease control tools.
- Vaccination can be harnessed as a resource for selection of desirable traits.
- The current explosion in vaccine formulation research should allow more targeted approaches to vaccine development and inclusion in immune phenotype selection strategies.
- Single shot vaccines would be desirable for Northern Cattle farmers.
- Development of effective sub-unit vaccines to replace live vaccines would be desirable but may require development of pathogen-specific adjuvant systems

Recommendations

- Measure innate immune competence and correlates with responder/non-responder phenotype to vaccination.
- Adjuvants are essential innate stimulators in most vaccines but their action can be very species specific. Different adjuvants/immune pathways will be required for different pathogens. Selective stimulation of the innate immune system with different adjuvant systems should be performed in sheep & cattle and related to specific adaptive outcomes and protection against disease targets.
- Candidate innate stimuli that are being validated as adjuvants in other species could be screened in appropriate sheep and cattle models.
- Due to the high genetic heritability of vaccine responsiveness, this trait could be included in estimations of breeding value and provide an immune component to the list of production traits currently measured.
- Measuring vaccine induced immune responses and associating these with genetic markers such as SNPs in target livestock breeds; this may help breed livestock that not only respond more reliably to vaccination but are also more resistant to disease.

Biodefence, new viral epidemic and FMDV

- New viral diseases can arise unexpectantly (e.g. Hendra)
- Agricultural Bioterrorism is a potential threat within Australia.

- An FMDV outbreak would be extremely costly if not rapidly contained.
- Vaccination is the most effective long-term strategy to containing and controlling a viral outbreak.
- Innate immunity may provide a relatively inexpensive mechanism to help early containment of infectious viral agents until vaccine induced immunity has time to manifest.

Recommendations.

- Test a panel of innate stimuli for an ability to induce safe but effective levels of systemic type I interferon.
- Focus on synthetic innate stimulators to minimise quarantine problems and test for persistence within the animal.
- Test these molecules in suitable viral challenge models for an ability to induce resistance and the duration and timing of resistance to viral infection following challenge.
- Test different delivery vehicles to maximise the persistence of systemic interferon and better control the levels produced within a cohort of livestock.
- If resistance to challenge can be demonstrated, incorporate economic impact into existing models on the impact of an FMDV outbreak in Australia.

Combined Glossary

A Disintegrin and metalloproteinase domain-containing protein (ADAM): A protease that cleaves TNFα from its membrane anchor.

Acaricides: Drugs that are used to treat or prevent tick infestations.

Additive genetic variation: The part of the genetic variance within a trait that can be attributed to the average effects of substituting one allele for another at a given locus or at multiple loci (gene areas) governing a polygenic trait.

Aetiological agents: Causal agents, the direct agent that causes disease or symptoms.

Agglutinate: The result of antibodies binding to and clustering many molecules, pathogens or cells together into a large complex. Often used as an assay for the presence of antibody.

Agonist: A molecule or substance that activates a receptor or pathway.

Alarmin: A family of often intracellular proteins that bind to receptors on innate immune cells and causes inflammation following necrotic cell death.

Alum: A common vaccine adjuvant based on Aluminium salts.

Aminoalkyl glucosaminide phosphates (AGPs): A family of synthetic chemicals that activate TLR4.

Amyloid plaques: Amyloid protein bundles found in the brain of people with Alzheimers disease. They can activate innate immune pathways through the inflammasome.

Antagonist: A molecule or substance that blocks or inhibits a receptor or pathway.

Anthelmintics: A family of drugs that are used to treat infections with various helminth (worm) parasites.

Antibiotic growth promoters (AGP): Antibacterial substances administered in sub-therapeutic doses to promote growth;

Antibiotics: Anti microbial drugs, typically used to treat bacterial infections.

Antibody-mediated immune responses (AMIR): Immune responses driven by antibodies produced by B cells; these can include complement mechanisms, direct neutralisation of viruses and antibody-mediated phagocytosis. Also known as humoral immunity as the B cells do not need to be at the site to mediate an effect.

Antigen–presenting cells (APCs): specialised white blood cells that can present antigen through MHC class II molecules on their surface. They most typically include; dendritic cells, macrophages and B-cells..

Antimicrobial peptides (AMP): Peptides 12 -50 amino acids long that can kill microbial pathogens and may also play a role in influencing/modulating the immune response

AP-1: A transcription factor.

Apoptosis: A non-inflammatory cell death mechanism.

Apoptosis-associated speck-like protein containing a CARD (ASC): A family of intracellular adaptor molecules that link inflammasome receptors to caspases.

Atherosclerosis: A disease caused by a build up of fatty plaques on the wall of arteries, eventually blocking them. Thought to involve inflammatory mechanisms.

Autosomal dominant: A dominant trait or allele present on a non sex chromosome.

Babesia bovis*, *B. bigemina* or *Anaplasma marginale: unicellular blood pathogens that are transmitted by tick's.

***Bacillus Calmette–Guérin* (BCG):** A vaccine against tuberculosis that is prepared from a strain of attenuated (weakened) live bovine tuberculosis bacillus, *Mycobacterium bovis*.

Bacterial type III Secretion Systems (T3SS): A specialised apparatus present on pathogenic bacteria that can inject toxins, proteins and PAMPs into the cytoplasm of mammalian cells.

Bacteriophages: viruses that infect bacteria

Balancing selection: A genetic mechanism or complex set of mechanisms that maintain the presence of gene variants (alleles) at an intermediate frequency.

Biomarkers: Molecules derived from the organism that on their own or combined with other measurements accurately determine the presence of disease.

Bovine Respiratory Disease (BRD): A complex of diseases that often progresses from a viral upper respiratory tract infection to a bacterial pneumonia and can be fatal. This can be a serious problem in feedlot type housing.

C –type Lectins: A large family of glycan binding proteins that activate innate immune pathways via the syk/card adaptors.

Caspase Recruitment Domain (CARD): A protein domain that is involved in protein-protein recognition. It typically binds other CARD domain contain proteins and is very common in proteins that transducer signals that induce apoptosis and activation of caspases.

Caspase: A family of proteases (proteins that cut other proteins) involved in immunity and cell death. Inflammasome activation causes the activation of caspases.

Catecholamines: A family of small hormones that are involved in the stress response, members include adrenaline and dopamine.

Cathepsins: A family of proteases with many diverse functions.

CCL: A chemokine family defined by the presence of a cysteine-cysteine-leucine amino acid motif within the protein.

CD14: See LPS binding protein.

CD4+ T-cell: A type of T-cell primarily implicated in supplying T-cell help to B-cells and producing cytokines. Also called helper T-cell.

CD8+ T-cell: A type of T-cell primarily implicated in killing cells infected with intracellular pathogens and producing cytokines. Also called cytotoxic T-cell.

Cell-mediated immune responses (CMIR): This is driven by T-cells, it involves many mechanisms where T-cells directly interact with infected target cells through antigen presented on cellular MHC molecules and either kill them or release immune mediators at the site.

Cestodes: A family or clade of flatworms that often cause disease in livestock and other species. Examples include tapeworms.

Chemokines: A large family of small proteins that primarily coordinate the movement of immune cells including the recruitment of different cell types to inflammatory sites.

Codominantly: Where two alleles equally influence a trait, they are both expressed.

Co-evolution: Where two or more species or genes within a single species reciprocally affect each others' frequency or evolution.

Colitis: Inflammation of the colon

Collectins: A diverse family of soluble proteins that bind different glycan (sugar) structures on the surface of pathogens triggering a diverse array of innate immune pathways.

Colony stimulating factors (CSF): A family of cytokines involved in the production and recruitment of monocytes and macrophages.

Colostrum: First milk feed that contains an extremely high amount of maternal antibodies that can transfer protection against diseases to newborn animals.

Commensal: A bacteria or fungi that livews within or on another organism that is typically non pathogenic. Normally associated with the standard gut flora.

Co-stimulatory molecules: Molecules present on antigen presenting cells that are required for activation of naive T-cells. Examples include CD80 and CD86.

Coxiella burnetii: A small Gram-negative bacterium that can cause Q-fever.

CpG or CpG-DNA: A type of immunostimulatory DNA sequence abundant in the genome of microbes.

Crohn's disease: A type of inflammatory bowel disease characterised by chronic inflammation of the gastrointestinal tracts, possibly associated with the gut microbiota.

Cryopyrin: Another name for the NALP3 protein.

C-type lectin domain family 9 (CLEC9): A C-type lectin family member involved in the recognition of dying cells.

CXCL: A chemokine family defined by the cysteine-X-cysteine-leucine amino acid motif within the protein.

Cytokines: A very large family of small proteins that regulate many aspects of immunity including inflammation and resolution following inflammation.

Danger Associated Molecular Patterns (DAMPs): The products produced by host cells following tissue damage and stress that bind to recognition receptors and activate innate immune pathways.

DEAD/H-box helicases: A family of RNA helicases involved in various aspects of RNA homeostasis including, translation, transcription and RNA splicing. Some members also activate innate immune pathways apon recognition of dsRNA.

Dectins: A sub family of C-type lections that detect the presence of beta glucans.

Defensins: A family of small peptide based antimicrobial molecules.

Delayed type hypersensitivity: A delayed (typically 12-24h) reaction that involves activation and recruitment of T-cells and subsequent production of inflammatory mediators.

Dendritic cells (DC): Potent antigen presenting cells, they express very high levels of MHC class II and readily activate T-cells.

Dextran sulphate: A large polymer of dextran sugars with a sulphate side group on each sugar. Highly negatively charged. Often used as a vaccine adjuvant that primarily induces Type 2 or humoral immune responses.

Diphtheria-tetanus-pertussis (DTP): A mixed vaccine that protects from infection from the bacteria that cause Diphtheria, tetanus and whooping cough.

Direct-fed microbials (DFM): Microbial-based feed additives without proven efficacy data that can be sold as long as health or production claims are not made.

DNA sensors: A family of proteins that detect DNA within the cytoplasm and activate innate immune pathways, typically antiviral defence pathways. Members include; STING, AIM proteins, DAI, Ku70 and IFI16.

dsRNA: double stranded RNA.

Effector Triggered Immunity (ETI): A specialised system of activation of immunity following detection of cellular perturbations induced by pathogens.

Endoplasmic reticulum (ER): An intracellular vesicle or structure where many aspects of intracellular protein transport and translation are facilitated.

Endosomal compartments: Endosomes are vesicles within cells that transport extracellular fluid and molecules collected by phagocytosis. They are also involved in homeostatic transport of cell components.

Endothelial: Present on the outside surface of vessels within the body, including the veins and arteries. Eg: Endothelial cells are the cells that line veins and arteries and are exposed to the blood.

Enterocytes: Intestinal epithelial cells

Epistatic: A gene that interacts with another gene to mask or influence the effects of that gene on a trait.

Epithelial: Present on the outside surface of the body, including the skin, lungs and gut lumen. Eg: Epithelial cells are the barrier cell type exposed to the outside environment.

Equine Respiratory Disease Complex (ERDC): A complex of diseases in horses that often progresses from a viral upper respiratory tract infection to a bacterial pneumonia and can be fatal.

Erythron: The total number of all red blood cells and their precursors.

Estimated Breeding Values (EBVs): A value assigned to how well the offspring of an individual are likely to perform within a given set of parameters.

F4 ab/ac genotype: A set of alleles or gene variants within pigs that encode the F4 fimbrial adhesion molecule which Enterotoxic *E. coli* strains bind to and utilise to anchor to and invade the gut wall.

Freund's Complete Adjuvant (FCA): A very potent adjuvant formulation consisting of an emulsified oil and water mix containing mycobacterial components.

Fungal beta-glucan: A glycan (sugar) structure found in the cell wall of fungi and yeast.

G protein coupled receptors: 7 transmembrane proteins which transduce signals via the intracellular G-proteins that activate the lipid based secondary messengers inositol phosphate and diacylglycerol.

Gastrointestinal tract (GI tract): Combined stomach and intestines.

Genetic drift: The changes in an allele or gene that occur through random chance and mutation.

Genetic gain: A measure of the improvement in the genes over time due to the imposition of selective regimes.

Genetic load: The amount of sub-optimal alleles present within a population.

Genome-wide association studies (GWAS): Where the whole genome or representative areas spanning the whole genome are screened for selective markers. Most commonly performed with SNPs, however direct sequencing of the genome and analysis is increasingly being used.

Genomic Estimated Breeding Values (gEBV): A value assigned to how well an animal's genetic quotient is likely to perform within a given set of parameters. It is a measure of how many "good" genes or alleles the individual is likely to pass onto its offspring.

Globule leukocyte: A type of leukocyte (white blood cell) that is present in the epithelium of mucosal tissues, particularly after repeated helminth infections; thought to be derived from mast cells.

Glucocorticoids: A family of small steroidal hormones that mediate multiple effects, including immune suppression.

Glycosylated: The protein or entity has glycan (sugar) residues attached to it.

GTPase: A family of enzymes that can bind and hydrolyze guanosine triphosphate (GTP) producing GDP which is a potent secondary messenger within cells.

Gut-associated lymphoid tissue (GALT): Organized structures of leukocytes present within the gastrointestinal tissue that can directly respond to antigenic challenge in the GI tract.

Haematocrit: A measure of the density of red blood cells within the blood.

Haplotypes: The mix of MHC molecules that an individual has.

Helicase domain: A protein domain that recognises dsRNA and unwinds it into single stranded RNA.

Heritability (h^2): A prediction of the response of a trait or disease to the imposition of selection. It is the term of most value to animal breeders.

Heritability estimates: A measure of the genetic contribution to the trait or disease being measured. What proportion of the trait is due to genes and genetic differences within a population and not due to environmental influences.

Heterozygotes: An individual that has two different alleles or variants of a gene. Usually one from the paternal and one from the maternal parent.

High mobility group box protein (HMGB1)*:* A protein that is produced within cells and activates innate immune pathways upon cell death. An alarmin.

High throughput DNA and RNA sequencing: The use of next generation sequencing technologies to identify the sequence and quantity of large amounts of DNA and RNA.

Homozygotes: An individual that has two identical alleles or variants of a gene. Usually one from the paternal and one from the maternal parent.

Humoral immunity: B-cell driven immunity, antibody mediated immunity.

IL-10, IL-18 etc: Types of cytokines (interleukins)

Immediate hypersensitivity: A very early inflammatory reaction that can be caused by activation of either the innate or acquired immune system or pre-existing antibody.

Immunoglobulin-A (IgA): A type of antibody typically associated with mucosal sites and release into the gut.

Immunoglobulin-E (IgE): A type of antibody that potently activates mast cells and basophils triggering allergic like symptoms.

Immunoglobulin-G (IgG): A family of antibody molecules that contains many sub-types such as IgG1, IgG2 and IgG4 and is predominantly produced in a secondary/acquired immune response.

Immunoglobulin-M (IgM): A highly multivalent antibody isotype that is generally produced early after infection.

Immunopathology: Disease or damage and symptoms caused by the immune system.

Immunostimulatory RNA and DNA: Cells contain pattern recognition receptors that detect RNA and DNA causing activation of innate pathways. These receptors are only expressed within compartments where cellular RNA and DNA do not occur naturally. Additionally, specific nucleotide sequences and modifications can increase the innate stimulatory capacity of RNA and DNA.

Indicine and Taurine: Two strains of cattle. Indicine cattle *(Bos. indicus)* typically have a hump over the shoulders and Brahman cattle are a good example of an indicine breed. Taurine cattle *(Bos, taurus)* are “humpless” cattle and a good example is the angus breed.

Inflammasome: A family of proteins that detect and transmit many different types of stimuli that activate immunity, primarily involved in the production of IL-1beta and IL-18.

Inflammatory bowel disease (IBD): Chronic inflammation of the colon and small intestine.

Interferon (IFN): A family of cytokines with potent antiviral and immunomodulatory functions.

Interferon Regulated Genes (IRGs): The genes that are induced or repressed following activation of the IFN-signalling pathways.

Interleukin-1 (IL1): A large family of inflammatory cytokines induced by inflammasome activation and activated by caspases.

Interleukin-33 (IL-33): An intracellular alarmin cytokine.

ISCOM™ adjuvant: A self assembling nanoparticle which combines Quil A saponins with cholesterol and phospholipid and effectively activates both T and B-cell pathways.

JAK/STAT signaling pathway: The combination of Janus kinases and signal transducers and activators of transcription (STATS) that transducer the signals following the activation of many cytokine receptors.

Janus kinases: A family of kinases that are involved in the signal transduction of many cytokines.

Kinases: A very large family of proteins that add phosphate groups to proteins which often dramatically alters their functions.

Lactic acid producing bacteria (LAB): Gram positive bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation; they are generally tolerant of low pH

Lectins: A diverse family of proteins that bind different glycan (sugar) structures.

Leishmania: A unicellular (protozoa) parasite that causes leismaniasis and is transmitted by sand fleas.

Leucine Rich Repeats (LRRs): A flexible protein domain that contains many leucine residues. Repeats of this domain are found on many recognition receptors in innate immunity.

Linkage disequilibrium: Linkage disequilibrium occurs when combinations of alleles at different loci occur more or less frequently than predicted by chance alone.

Lipopolysaccharide (LPS): The major component of the cell wall of gram negative bacteria; potent stimulator of innate immunity also known as endotoxin.

Lipopolysaccharide binding protein: A serum protein (CD14) that binds lipopolysaccharide (major component of cell wall of gram negative bacteria and facilitates interaction with the TLR4 receptor and activation of innate immune pathways.

Lymph node: The structure where naive T- and B-cells reside and are activated. It has a highly coordinated structure that faciliatates activation of adaptive immunity.

Lymphocytotoxicity: Many T-lymphocytes can directly kill cells infected with pathogens through MHC recognition; they can also release potent inflammatory mediators that can cause toxicity and side effects.

Lymphopenia: A lower than normal number of white blood cells within circulating blood.

Lysosome: An intracellular organelle where proteins and other cellular components are broken down and recycled. It is also the organelle where phagocytosed material is broken down and many antigen fragments are produced to "load" onto MHC II molecules. Endosomes can mature into lysosomes.

Major Histocompatibility Complex (MHC): A genomic region that contains a large family of genes (alleles) that encode cell surface molecules involved in presentation of antigen to T-cells.

Memory: As an immunological term it refers to the process where the adaptive immune system once activated can "remember" antigens by producing a pool of long lived T- and B-cells specific for the antigen.

Mendelian trait: A selective trait that follows standard Mendelian inheritance ratios.

Metagenomic: The direct study of genetic material present in a sample; for example by direct sequencing of gut microbial DNA without prior bacterial cultures.

Microbiota, also referred to as **microflora:** The terms encompass all microorganisms living in a particular environment.

Micro-RNAs (miRs): Small RNA molecules that are produced within cells to specifically inhibit the translation of target mRNA molecules.

Mitogen-activated protein kinases (MAP kinases): A family of kinases that are involved in many intracellular signalling pathways including inflammation.

Monocytes: A cell type that circulates within the blood that is rapidly recruited to sites of inflammation and can mature to become a potent antigen presenting cell.

Mucosal IgA: A special type of antibody complex that is secreted into mucosal tissues.

Muramyl dipeptide (MDP): The bioactive peptidoglycan motif common to all bacteria.

***Mycobacterium avium paratuberculosis* (MAP):** A mycobacterium that causes Johne's disease in sheep.

Myeloid differentiation primary response gene (88) (MYD88): A universal adapter protein that links innate immune receptors (TLRs) with other intracellular signalling molecules to activate innate immune pathways

NACHT, LRR and PYD domains containing proteins (NALPs): A family of inflammasome pattern recognition receptors.

NAIP/NLRC: A family of inflammasome pattern recognition receptors.

Natural killer (NK) cell: A type of cell that is at the interface of innate and adaptive immunity. They often produce high amounts of cytokines and can kill cells similar to cytotoxic T-cells.

Necrotising enterocolitis (NEC): A disease of the young with unknown cause, characterised by inflammation and death of intestinal tissue, predominantly in the colon.

Negative genetic correlation: Selection for one trait adversely affects another desirable trait.

Nematodes: A family of roundworms that often cause disease in livestock and other species. Examples include *Haemonchus spp.* and *Trichostrongulus spp*.

NFκB: A transcription factor that is critical for in the induction of many cytokines and chemokines following activation of innate immune pathways. It exists as a cytoplasmic protein complex that apon activation translocates to the nucleus to induce or suppress gene expression.

Nod-Like Receptors (NLRs): A family of proteins that are localised to the cytoplasm and recognise intracellular activators of innate immune pathways including, peptidoglycans and compaonents of bacterial secretion systems.

Non coding RNA (ncRNA): RNA molecules produced within cells that do not code for proteins. They include miRNA's, ribosomal RNA and other regulatory RNA types.

Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). A member of a family of cellular proteins that function to inhibit the NF-κB transcription factor by masking the nuclear localization signals (NLS) of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm.

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2): An intracellular pattern recognition receptor that recognizes bacterial peptidoglycans.

Nucleotide-binding oligomerization-domain protein-like receptor protein 6 (NLRP6): Part of an inflammasome complex involved in microbiota regulation and inflammation of the colon.

Oxidative stress: An imbalance in the redox state within a cell that can trigger protein damage through free radical production and activation of innate immune pathways, primarily the inflammasome.

Pam3CysK4: A lipopeptide that specifically activates TLR2.

Parenchymal cells: Cells that are structural and involved in standard tissue functions.

Pathogen Associated Molecular Patterns (PAMPs): The products produced by pathogens that bind to pathogen recognition receptors and activate innate immune pathways. Molecular structures of microbes recognised by host pattern recognition receptors.

Pathogen Recognition Receptors (PRRs): A broad class of proteins that detect the presence of pathogen products outside and within cells, they include TLRs, NODs and inflammasome proteins.

Pentraxins: A family of inflammatory proteins that include Serum Amyloid P component (SAP) and C reactive protein (CRP) which are involved in pathogen recognition and inflammation.

Peptidoglycan: A small molecule that is often found within the surface of bacteria composed of a glycosylated peptide chain (sugar and amino acids). They are potent bacterial PAMPs.

Periparturient period: A short period of time around birth. Typically associated with a rise in maternal nematode egg production.

Peripheral blood mononuclear cells (PBMC): White blood cells without granulocytes (neutrophils & eosinophils).

Peyer's patches (PP): Organised lymphocyte aggregates in the gut tissue. In ruminants, there are two types: jejunal PP can initiate immune responses, while ileal PP are thought to be sites of B cell development.

Phagocytosed, phagocytosis: The process that cells use to engulf or "eat" large structures outside the cell. A phagocytic cell is one that is capable of performing this function.

Phenotypic data: A measurement of both the genetic and environmental components of a trait.

Phosphatases: Proteins that cleave phosphate groups off other proteins. Theya re the opposite of kinases.

Plasmacytoid DCs (pDCs): A type of DC that is involved in the recognition of viral infections and the production of IFN.

Poly I:C: A long synthetic polymer of cytidine and inosine nucleotides that is an effective dsRNA mimetic.

Polyfunctional T-cells: T-cells that produce more than one cytokine.

Polygenic: Multiple genes influence the trait.

Polymorphic: More than one type of individual within a species or population. A trait or many traits have many forms within a population.

Polymorphonuclear leukocytes (PMN): Usually refers to neutrophils, a highly phagocytic cell type that specialises in removing and killing bacteria and other small pathogens. Potent producers of innate inflammatory cytokines.

Polysaccharides: Long chains of sugar molecules (mono/disaccharides).

Pro-IL: The inactive form of an interleukin before it is cleaved into its active form by a caspase.

PYHIN family: A family of proteins that contain a pyrin and HIN domain. Commonly refers to NOD family of PRRs.

Pyrin domain: A protein domain involved in protein-protein interaction commonly found in proteins involved in inflammation and apoptosis.

Pyrogen: A factor that induces fever.

Pyroptosis: An inflammatory cell death mechanism.

Quartile Trait Loci (QTL): A statistical probability that an area of the genome is involved in inheritance of a trait.

Quil A: A family of saponin (detergent) based adjuvants derived from the Quillaja saponaria plant.

Reactogenicity: A vaccine's propensity to produce severe side effects following administration; these can include localised inflammation, fever and disease-like symptoms.

Receptor-interacting protein (RIP): A family of proteins that are involved in the signal transduction of NOD receptors to activation of the NFκB and MAP kinase pathways.

Regulatory T cells (Tregs): T-cells that downregulate or suppress immune responses and induce immune tolerance; usually through the production of the immunosuppressive cytokines TGF-beta and Interleukin 10 (IL-10).

Respiratory syncytial virus (RSV): A common virus that leads to mild, cold-like symptoms. It can be more serious in young animals.

Rhipicephalus microplus: Cattle tick.

RIG-I-Like Helicases (RLHs): A family of RNA helicases that detect dsRNA within the cytoplasm and activate innate immune pathways, typically antiviral defence pathways.

***RIPK2* gene:** A gene that encodes a serine/threonine kinase which is an important component of many signaling complexes in both the innate and adaptive immune pathways.

Secretory IgA (S-IgA):A complex of immunoglobulin (of IgA isotype) and peptides that allows its secretion into the luminal mucus for protection against pathogens.

Septic shock: Widely disseminated inflammation that is typically caused by bacteria within the blood stream and release of many bacterial PAMPs. Can be fatal.

Serologically positive/negative: The animals blood does or does not contain antibodies specific for the infecting agent respectively.

Short-chain fatty acids (SCFA): Fatty acids composed of less than 6 carbons, produced by bacterial fermentation in the gut, including acetate, butyrate and propionate.

Signal Transducers and Activators of Transcription (STATs): A family of transcription factors that are involved in the signal transduction and activation of gene expression of many cytokines.

Signal transduction: The process and components that transfer the information derived from activation or repression of a receptor to the nucleus or compartment where the ultimate function is determined. It typically involves coordinated protein pathways such as kinases and adaptors, however lipids and nucleotide analogs are also potent signal transducers.

Signalling adapters (innate pathways): Intracellular proteins or molecules that transmit the ligand binding signal from the receptor to the nucleus. Most have defined protein domains that interact with the receptor and each other.

Single Nucleotide Polymorphisms (SNPs): A single DNA nucleotide position that is variable between individuals within a population. These are common within outbred species and can be measured and tracked through generations for selection of specific areas of DNA.

Supressors of cytokine signaling (SOCS): A family of proteins that bind to cytokine receptors and inhibit subsequent signal transduction.

Syk-Card9: A family of signalling adaptor proteins that transmit activation of lectin and other pattern recognition receptors.

Systemic Lupus Erythematosus (SLE): An autoimmune disease that may be caused by aberrant activation of innate immune pathways.

Th17 cells: T-cells characterised by the production of IL-17 and commonly associated with mycobacterial and fungal infections and autoimmunity.

Th17 immunity: Typically associated with the production of IL-17 by T-cells and is commonly associated with mycobacterial and fungal infections and autoimmunity.

Tolerise and tolerance: An immunological term where antigens are ignored by the immune system as they are not perceived as foreign but rather as “self”.

Toll IL1 Receptor (TIR) domain: A protein domain found in the cytoplamic side of many innate immune receptors that is involved in the recruitment of adaptor proteins to the receptor.

Toll-like Receptors (TLRs): A large family of pathogen recognition receptors that detect a very broad array of both pathogen and host molecules and activate innate immune pathways via adaptor proteins.

Transcriptomics: The use of high throughput technologies to study the expression patterns of RNA molecules within cells or tissues.

Trematodes: A family of flatworms that often cause disease in livestock and other species. Examples include liver flukes.

Tubules: A bundle of protein fibres that form the framework within a cell that maintains its architecture and shape. Tubules are typically composed of actin, tubulin and other associated proteins.

Tumour necrosis factor (TNF): A potent inflammatory cytokine that requires protease activity to release it from the cell surface. Inhibition of TNFα is a very good therapy for rheumatoid arthritis.

Type-1 immunity: Typically associated with the production of IFN-gamma by T-cells and an increase in the number of CD8 T-cells.

Type-2 immunity: Typically associated with the production of IL-4, IL-5 by T-cells and and good activation of B-cells to produce antibody. Limited involvement of CD8 T-cells.

Vesicles: Intracellular lipid membrane "bags" that are used to traffic many molecules within the cell.

Viral vectors: Modified viruses that can be used to deliver RNA or DNA to cells.

Zoonotic pathogens: Pathogenic agents that can be transmitted between different species.

α-helices and β-pleated sheet: Different protein folds.

Experts consulted

Name and affiliation	Field of expertise
Dr Stuart Barber, School of Veterinary Sciences, The University of Melbourne	Mastitis
Dr Rob Bischof, Dept Physiology, Monash University	Immune system of the mammary gland
Prof Charles Mackay, Dept Immunology, Monash University	Immune responses and gut microbiota
Profs Julian Rood and Ben Adler, ARC-Centre of Structural and Microbial Genomics, Monash University	Microbial genomics
Ir Andre Meeusen, Senior Nutritionist, Kemira ChemSolutions, APPLIED NUTRITIONAL SERVICES CommV.	Livestock nutrition
Assoc Prof David Piedrafita, School of Applied Sciences & Engineering, Monash University	Gastrointestinal nematode and liverfluke infections
Professor B.A. Mallard, Ontario Veterinary College, University of Guelph, Canada	Breeding for disease resistance and immune responsiveness
Professor B. Wilkie, Ontario Veterinary College, University of Guelph, Canada	Breeding for disease resistance and immune responsiveness
Professor A. Peregrine, Ontario Veterinary College, University of Guelph, Canada	Epidemiology of livestock disease
Dr Seyemehdi Eman, Ontario Veterinary College, University of Guelph, Canada	Immune responses in cattle
Mathur Pramod, TOPIGS Research Centre, IPG	Pig Breeding
Professor A,. Forbes, Merial, UK	Parasites of Livestock
Dr Louise Matthews, University of Glasgow	Modelling the response to selection for disease resistance
Dr Jacques Cabaret, INRA, France	Host-parasite interactions
Dr Carole Moreno INRA, France	Genetic variation in resistance to disease

Dr Beatriz Guttierez Gil, University of Leon, France	Genetic variation in resistance of sheep to disease
Dr Johannes Buitkamp, LfL, Bavaria, Germany	Genetic resistance to disease in livestock
Dr T. Leinster, University of Edinburgh	Biodiversity
Dr C. Cobbold, University of Glasgow	Biodiversity
Dr R. Reeve, University of Glasgow	Biodiversity
Dr Marc Drillich, VetMedUni Vienna	Herd health and genetics
Mr Anton Wagner, ZAR (Rinderzucht Austria – Austrian Cattle Breeding), Vienna	Bovine genetic selection policy
Dr Emily Piper, Science Leader, Animal Genetics Laboratory, University of Queensland, Australia	Bovine genetics
Ms Alison Glasgow, Signet Genetics, UK	Bovine genetic selection policy
Dr Ashley Mansell, CiiiD, Monash Institute of Medical Research, Melbourne Australia	Innate immunity signaling
Dr Richard Ferrero, CiiiD, Monash Institute of medical Research	Helicobacter pylori, bacterial pathogenesis, host-pathogen interactions
Prof Doug Gollenboch, University of Massachusetts, Worchester, MA USA	Innate immunity and host-pathogen interactions
Dr Kate Fitzgerald, University of Massechusetts	Innate immunity signaling
Prof Eicke Latz, University of Bonn, Germany	Inflammasomes and TLR signaling
Dr Anthony Coyle, Pfizer, Boston MA USA	Drug development in infection and inflammatory diseases
Prof Luke O'Neill, Trinity College, Dublin	Innate immunity signaling and drug development
Dr Andrew Bowie, Trinity College Dublin	DNA sensors and viral inhibition of innate immunity
Prof. Nicholas Gay, Dept Biochemistry, Cambridge, UK	Structure-function of innate immune signaling
Dr Brian Dalrymple, CSIRO Brisbane.	Genetic selection, bioinformatics.
Dr Richard Whittington, University Sydney, Cambden	Johne's disease - models and vaccines.

Dr Andrew Bean, CSIRO Australian Animal Health Laboratory, Geelong.	Innate immune antiviral strategies.
Dr Sam Gill, MLA	gEBVs
Dr Jill Maddox, Melbourne University, Veterinary School	Genetic selection and mapping in livestock
Dr John Mc Ewan, NZ Ag Research.	SNP array
Hans Daetwyler, DPI, Attwood Victoria	SNP and livestock genetics
Dr Paul Wood, Private Consultant.	Livestock Vaccines
Dr Julius van der Werf, University of New England	Genetic selection practicalities

Relevant conferences/meetings attended

Conference title	Participants
Lorne Infection & Immunity, Lorne Australia February 2012	Paul Hertzog Michael de Veer
Innate Immunity: Sensing the Microbes and Damage Signals. Keystone, Colorado, US. March 2012	Michael de Veer
International Society for Animal Genetics, 33rd conference of, Cairns, Australia. July 2012	Nick Jonsson Mike Stear Bonnie Mallard
International Veterinary Vaccines and Diagnostic Conference, August 2012,Cairns, Australia	Els Meeusen Michael de Veer
TLROZ, Melbourne Australia. August 2012	Paul Hertzog Michael de Veer
International Workshop on Genetic Resistance to Parasites in Small Ruminants, Gran Canaria, Spain. September 2012	Mike Stear Els Meeusen
International Symposium: Alternatives to Antibiotics, Challenges and Solutions in Animal Production. September 2012, Paris, France	Els Meeusen
Exploratory Conference on the Mathematics of Biodiversity, Barcelona	Mike Stear
International Cytokine Society and International Society of Interferon and Cytokine Research, Geneva, Switzerland, Sept 2012	Paul Hertzog