Measuring immune system variation to help understand host-pathogen community dynamics

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SUMMARY

Carefully chosen immunological measurements, informed by recent advances in our understanding of the diversity and control of immune mechanisms, can add great interpretative value to ecological studies of infection. This is especially so for co-infection studies, where interactions between species are often mediated via the host's immune response. Here we consider how immunological measurements can strengthen inference in different types of co-infection analysis. In particular, we identify how measuring immune response variables in field studies can help reveal inter-species interactions otherwise obscured by confounding processes operating on count or prevalence data. Furthermore, we suggest that, due to the difficulty of quantifying microbial pathogen communities in field studies, innate responses against broad pathogen types (mediated by pattern response receptors) may be useful quantitative markers of exposure to bacteria and viruses. An ultimate goal of ecological co-infection studies may also be to understand how dynamics within host-parasite assemblages emerge from trade-offs involving different arms of the immune system. We reflect on the phenotypic measures that might best represent levels of responsiveness and bias in immune function. These include mediators associated with different T-helper cell subsets and innate responses controlled by pattern response receptors, such as the Toll-like receptors (TLRs).

Key words: co-infection, immunology, T-helper cell, Toll-like receptor, cytokine, parasite, pathogen, Treg, Th17, community ecology.

INTRODUCTION

For an invading pathogen, the host's immune response is a key environmental variable determining establishment, survival and fecundity. The immune response against one pathogen may also affect these quantities in co-occurring infections, through cross-species effects. Furthermore, immune responses can influence the survival and fecundity of the host 'resource' itself, through energetic or immunopathological penalties. For these reasons the development of the host immune phenotype is a fundamental consideration in pathogen ecology, including the ecology of interactions between groups of co-occurring pathogen species. However, because vertebrate immunity is multi-faceted and complex, a key challenge for ecological studies of infection is to determine the most meaningful ways in which it can be measured. For instance, the functional compartmentalisation of the immune system and its tendency to operate through multiple counter-regulated effector arms (Reiner, 2007) means that host individuals probably cannot be thought of as displaying a single level of immunocompetence or resistance to all types of pathogen (Long and Nanthakumar, 2004; Viney, Riley and Buchanan, 2005). Resistance to one pathogen, resulting from phenotypic commitment to one arm of the immune system, may produce susceptibility to other pathogens that are controlled by different arms. For some years this was exemplified by the concept that T-helper cell type 1 (Th1) responses are required for controlling microparasites, whereas T-helper cell type 2 (Th2) responses are directed against helminths: and each Th cell type is down-regulated by the other. More recently, however, the idea of a simple Th1 *vs* Th2 axis controlling Th cell responses has been, to some extent, superseded by the discovery of further cross-regulated Th cell subsets (discussed further below). Given this intrinsic complexity, the analysis of immune dynamics in natural populations will arguably be more incisive if it becomes possible to analytically 'decompose' the immune system into measurable elements that reflect its main functional arms (Graham *et al*. 2007). Although the immunological effects of infection in humans and laboratory rodent models are routinely dissected in great detail, the mainstream ecological literature contains little apart from relatively crude measures of 'immune competence' in the context of infection or individual host status (Viney *et al*. 2005; Kennedy and Nager, 2006). Methods are now available that will allow a far more sophisticated exploration of immune phenotypic variation in natural vertebrate populations. In the last 10–15 years great

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steps forward have been taken in understanding the regulation of adaptive immune responses by T-helper cells. At the same time the discovery of new innate immune receptors and responses by antigen presenting cells (APCs) that direct T-helper cell development have led to a greater knowledge of how the innate immune system translates information derived from pathogens into signals that drive adaptive responses (Janeway and Medzhitov, 2002). Levels of mediators involved in this process provide measurable phenotypes that reflect responsiveness and functional bias in the immune system. Indeed, the measurement of detailed immunological phenotypes has often been valuable in describing infection in human populations (e.g. Roberts *et al*. 1993; Grogan *et al*. 1998; Stewart *et al*. 1999; Cooper *et al*. 2001; Elias *et al*. 2001; Turner *et al*. 2003; Booth *et al*. 2004; Jackson *et al*. 2004*a*; Babu *et al*. 2006), albeit that these studies usually focus narrowly on the determinants of resistance/susceptibility or pathology rather than ecosystem function. Given this, the potential now exists to incorporate far more powerful immunological information into parasite community ecology than has previously been attempted. In the following review we outline some of the ways such detailed measurement of immune responses can increase the analytical power of ecological co-infection studies. We will also consider the immunological target variables and measurement techniques that might be most relevant.

Co-infection: the fundamental questions

Most individuals in natural vertebrate populations are infected with parasites and very often these occur as multiple infections, or 'co-infections', with more than one species (Esch *et al*. 1990). Co-infection is of special biological importance if the outcome of multiple infections is different from that predicted if single-species effects were simply superimposed. In this case, the outcome could not be anticipated by a simple additive model, and special knowledge of the consequences of the co-infection state would be required to understand the dynamics of the host-parasite system. This potential for parasite × parasite interaction represents an important biological interest that has implications for the practical management of real-world systems where multiple infections are present. In any co-infection system there are therefore two fundamental initial questions, viewed either from the perspective of the host or parasite. Firstly, from the point of view of the parasite: is the overall mean abundance of any one species altered by the presence of another species or, in other words, does some form of interaction occur? Secondly, from the point of view of the host: do multiple-infection states drive hosts towards phenotypes different from those that would occur in single infection states?

With regard to the first of the two questions posed in the preceding paragraph, there is no doubt that interspecific competitive interactions, including facilitation ('positive competition'), can occur between pathogens in individual hosts (Holmes, 1961; Christensen *et al*. 1987; Behnke, in this special issue). Whilst these interactions can be mediated by a variety of resource or interference competitive mechanisms, they will often be driven indirectly through effects on the host immune system (Behnke, Wakelin and Wilson, 1978; Behnke *et al*. 2001*a*). On the other hand, it remains controversial as to whether competitive processes are generally important in structuring communities of pathogens in nature (Stock and Holmes, 1988; Janovy, Clopton and Percival, 1992; Lotz and Font, 1994; Poulin, 1996; Rohde *et al*. 1998; Poulin and Luque, 2003; Lello *et al*. 2004; Behnke *et al*. 2005; Poulin, 2007), although there are a few instances where strong non-random patterns observed in field data have been followed up by definitive laboratory experiments (e.g. Jackson *et al*. 2006*a*; see also Jackson, Tinsley and Hinkel, 1998; Jackson and Tinsley, 2007).

The lack of widespread evidence of interspecific interaction between parasites in wild populations sits uncomfortably with at least two received facts. Firstly, the vertebrate immune system is known to make strong phenotypic commitments to different functional arms in response to infection (Mosmann *et al*. 1986; Diaz and Allen, 2007; Reiner, 2007). These commitments can affect co-occurring (bystander) parasite species because they influence the fundamental balance of the immune system in terms of its bias towards particular effector mechanisms and also its overall level of responsiveness. There are clear examples in human populations where immune responses relevant to defence against one pathogen are modulated by infection with bystander pathogens. For example, helminth infections in humans have been shown to modulate responsiveness to *Mycobacterium tuberculosis* (see Stewart *et al*. 1999; Elias *et al*. 2001). Secondly, a lack of non-random signals in field data is inconsistent with observations that interactions are often seen in empirical laboratory co-infection models. Understanding these apparent paradoxes is central to the relevance of parasite community ecology to the biology of host-parasite/pathogen interactions.

HOW MEASURING IMMUNE RESPONSES CAN HELP

Exposure vs resistance

Immune responses can be used in ecological infection studies in more than one context. In some cases they may correlate with and be used as a measure of exposure to infection. This might be the case for some specific antibody responses, for example IgG4 responses in human worm infections (Faulkner *et al*.

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2001; Turner *et al*. 2005), where levels may track the amount of antigenic stimulation. The same might apply to innate receptors involved in the recognition of pathogenic organisms, for example pattern response receptors of the types mentioned in sections below may sometimes be up-regulated by the presence of their ligands. In other circumstances, immune variables can be used as a functional measure of resistance or susceptibility. A potential complication may be that the distinction between immune responses as measures of exposure or protection can be blurred. Depending on the kinetics of parasite transmission and the induction and loss of immunity in individuals, there could theoretically be positive or negative associations between infection and mediators that are genuinely involved in protection (Woolhouse, 1992). For example, in young host cohorts with minimal acquired immunity to a pathogen, the induction of an (ultimately protective) immune response may initially depend directly on the force of infection, and therefore produce transient positive associations. On the other hand, in older hosts, some of whom will have acquired effective immunity, the resistant individuals with stronger protective immune responses may generate negative associations. Stratification of field samples by host age and longitudinal study designs with repeat observations for individuals (Woolhouse, 1992) may help detect such situations and identify correlates of resistance. Ultimately, the existence of direct experimental evidence from laboratory models for the role of particular mediators in resistance may add confidence to the interpretation of observational field data. For example, Th2 responses (production of antigen-specific IgE and type 2 cytokines) are associated with resistance in mouse-gastrointestinal nematode infection models (Gause, Urban and Stadecker, 2003), and they also correlate negatively with these infections in age-stratified studies in humans, including re-infection studies, suggesting variation in such responses can be used as a predictor of resistance (Turner *et al*. 2003; Jackson *et al*. 2004*a*).

Cross-sectional association studies

Due to technical and logistical difficulties with capture-recapture studies and interventional experiments in the field, most work on parasite community structure has been based on cross-sectional epidemiological data (e.g. Esch *et al*. 1990; Behnke, in this special issue), usually prevalences and/or counts. One approach with this type of data is simply to assign observed numerical associations between species to the effects of within-host interactions (e.g. Lello *et al*. 2004; Cattadori *et al*. 2007). Such associations can have multiple alternative explanations, requiring caution for their interpretation as evidence for interaction, let alone as estimators of interaction strength (Lello *et al*. 2004). Non-interactive processes that lead to association include correlated age-dependent host exposure. For example, exposure to soil transmitted nematodes may co-vary with age in humans because geophagy reduces as children grow older. Such age-dependence can potentially be adjusted for statistically in some epidemiological data. However, it remains a major problem in field studies of non-human hosts, where age can be difficult to determine beyond a set of very broad categories unless individual sight-resight records are available, or a special technique such as skeletochronology is applied (in suitable lower vertebrates). In addition, differences in the temporal infection trajectories of different parasite species can generate apparent trends (Bottomley, Isham and Basanez, 2005), whilst altered pathogen-induced host mortality in co-infections might disguise some genuine interactions (Brown *et al*. 2006). Perhaps most importantly of all, there are often likely to be correlated age-independent transmission processes due to behavioural and fine-scale spatial variation (Booth and Dunne, 2004; Booth, 2006; Pinot de Moira *et al*. 2007) that cannot always be incorporated into an analysis. For instance, positive associations are frequently seen where parasites share similar transmission strategies, even when easily measured confounder variables are accounted for. A good example of this is the recurring confounder-adjusted positive association (Tchuem-Tchuenté *et al*. 2003; Faulkner *et al*. 2005) between the soil-transmitted nematodes *Ascaris lumbricoides* and *Trichuris trichiura* discussed by Behnke in this special issue and considered further below. Also, it should not be forgotten that correlated underlying host susceptibility may generate non-random interspecific associations between pathogens. Thus there will often be environmentally-determined and intrinsic genetic or ontogenetic variability in pathogen-specific resistance in a host population that might produce correlated susceptibility to different pathogens. Finally, even when interspecific competition is detectable in associations amongst count or prevalence variables, the direction of the underlying interaction may sometimes be obscure. For example, two infections that compete by negative interference involving a common mechanism acting against both species might be positively correlated in count or prevalence data.

Potentially, therefore, correlated exposure or susceptibility, varying temporal infection dynamics, or even parasite-induced host mortality could obscure the numerical signature of competition or facilitation in field data based on individual counts or presence/absence alone (see Fig. 1). Even when such a signature is present, the numerical association may sometimes be of opposite sign to the underlying competitive interaction. Given these problems, the fact that many interactions between parasites and

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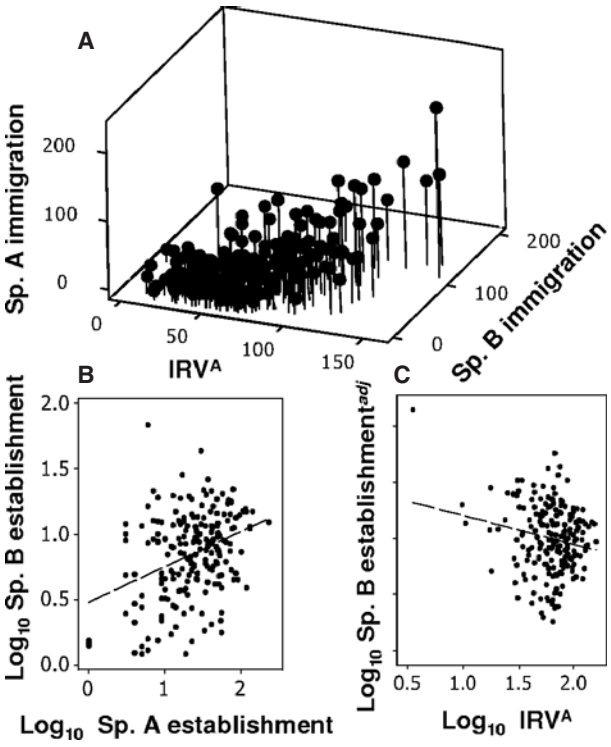


Fig. 1. A simplistic numerical simulation that captures the opposing effects of quantitative covariation respectively generated by transmission processes and cross-immunity. Species A has a negative effect on species B via the immune response it generates (IRV^A). The larval recruitment of both species and IRV^A are highly positively inter-correlated due to non-independent transmission processes (e.g. Booth, 2006). This is simulated (A) by generating random correlated data for each parasite and the immune response in 200 hosts. For the incoming parasite distributions, three random variables (x_1, x_2, x_3) were generated from a negative binomial distribution using the negbin function from the R MASS library. Correlation was introduced by an overlapping sums method, so that numbers of species A, $N^A = x_1 + x_2$ and numbers of species B, $N^B = x_1 + x_3$. To simulate a correlated immune response, a further variable, x_4 was generated from a normal distribution, so that $IRV^A = x_1 + x_4$. This gave data with strong inter-correlation ($R = 0{\cdot}5$–$0{\cdot}6$) amongst the three variables. The parasite variables had means of 34–39 and variances of 1146–1220. The effect of species A on post-larval mortality in species B was simulated by ($1/rsIRV^A$). N^B, where $rsIRV^A$ was IRV^A re-scaled to produce a mean mortality of 80% in the in-coming larval stages of species B. Species A establishment was assumed to be directly dose dependent. Although linear modelling suggested that the numbers of established species A and B were highly significantly positively associated ($P < 0{\cdot}001$) (B), inclusion of IRV^A as an additional variate produced a significant negative association between IRV^A and species B establishment ($P < 0{\cdot}04$). This is shown in (C) by the variation of species B residuals (adjusted for variation in species A) against IRV^A. Although a high immune-mediated mortality in species B was necessary for the effect of IRV^A to become detectable, this was due to the relatively tight inter-correlation of incoming infective stages and IRV^A. However, with greater

pathogens may be mediated through the immune system suggests that measuring functional specific immune response variables (IRVs) in field studies can be of great additional value (Table 1). This is because an IRV, which depends on within-host factors influencing immunogenicity and, in turn, influences parasite mortality processes, is partly independent from other sources of numerical covariation in parasite infection. Also, as IRVs can be key environmental drivers, their covariation with infection may reflect true interaction signs more clearly than covariation in count or prevalence data. In many circumstances, therefore, a specific IRV might be a better 'index' of the potential competitive force exerted by a given pathogen species than the numbers of that pathogen itself. A hypothetical example for two parasite species with strongly positively correlated exposure is illustrated by the quantitative simulation in Fig. 1. Here, one species (A) has an indirect negative influence on another (species B) via the immune response it stimulates. In this case, the amount of immune response (IRV^A) against species A, can be related to the absolute numbers of species B (Fig. 1). As IRV^A will be correlated with, but will partly vary independently from quantitative exposure to species A, it can be adjusted to be independent of the numbers of species A. This would result in removal, to a large degree, of the effects of correlated exposure between species A and B. Any residual association between adjusted IRV^A and species B numbers, if it is of opposite sign to the association in worm numbers, may then be indicative of an interaction through the immune response. In the case of raw associations between IRVs and bystander species numbers that are of opposite sign to the numerical association in individuals, such adjustment is not necessary, as the clear inference is that an immune-mediated interaction has been revealed in the face of a contrasting covariation in numbers. Where both the parasite-parasite and adjusted IRV-parasite associations are of the same sign, interpretation remains problematical because IRVs may to some extent reflect past as well as present infection, and therefore adjustment for current infection may not perfectly account for covariation in exposure. Within certain limitations, therefore, measuring immune responses can provide a valuable extra dimension to the pair-wise analysis of competition amongst parasites and pathogens.

decoupling of immune responses from current infection abundance, which might realistically occur due to long-term and time-lagged build-up of immunity, this association would become more detectable. Furthermore, whilst the present simulation does not take into account functional specific responses against species B, and their possible inter-correlation with responses against species A, such responses could be measured and included in any regression analysis (see example in text).

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Table 1. Potential uses of immune response variables (IRVs) in improving inference in field studies of co-infection

Problem	Analytical use of IRV	Limitations	Examples
Cross-sectional and longitudinal field studies. Parasite interaction obscured in count data by correlated exposure, correlated host susceptibility, temporal infection dynamics or altered host mortality in co-infection.	Using specific IRVs. Improved detection of true interaction sign.	Inferential problem if predicted underlying interaction and 'coincidental' covariation in parasite numbers are of the same sign.	Jackson *et al*. (2004*b*) This study, Fig. 1 (hypothetical) This study, Fig. 2
Community 'pattern' analyses using numerical indices or multi-species null distributional models.	Improved inference of causal mechanisms.		None
Manipulative field experiments.	Improved inference of causal mechanisms.		None
Lack of quantitative measures of microbial infection or exposure in community studies.	Using non-specific IRVs. Global measure of exposure to pathogenic microorganisms.	Non-dose-dependent variation. e.g. confounding by immunomodulatory pathogens or commensal bacteria.	Lauener *et al*. (2002) Ege *et al*. (2006)
Measuring co-infection effects on host characters in cross-sectional studies.	Resolving potential inferential problems. Is covariation in parasite numbers part of a genuine 'co-infection effect' or a confounder?		None

Going beyond simple cross-sectional designs, the addition of a longitudinal component to studies can provide stronger insights into competitive processes by revealing temporally variable or time-lagged patterns of covariation amongst parasite and immune variables. For example (Jackson *et al*. 2004*b*) in an age-stratified study of the recolonization of anthelmintic-treated human hosts by gastrointestinal nematodes found that despite positive covariation in the per infection fecundity (Fig. 2A) of *A. lumbricoides* and *T. trichiura*, each species showed negative fecundity associations with an overall measure of anti-nematode Th2 immune responses at different points within the study design. These associations were restricted to a younger host age class (4–13 years). Whilst only *A. lumbricoides* fecundity was negatively associated with contemporary Th2 responses in the baseline data, at a later sampling point placed 8–9 months after treatment only *T. trichiura* fecundity was negatively associated with baseline Th2 responses. Across all age strata, relatively low re-colonization was also recorded for *T. trichiura* in comparison to *A. lumbricoides* at the later sampling point, and host re-infection probability for *T. trichiura* was negatively associated with baseline anti-nematode Th2 responses. Furthermore, a re-visitation of these data confirms that *T. trichiura* re-infection probability was strongly negatively associated ($P<0{\cdot}01$) with *A. lumbricoides*-specific Th2 responses when *T. trichiura*-specific responses and *A. lumbricoides* abundance were adjusted for (Fig. 2B). For this re-analysis, interleukin (IL)-5 and IL-13 recall responses to species-specific antigens were 'grouped' for each species by taking the sum of the two variables after they had been standardized (zero mean, unit standard deviation). The grouped anti-*A. lumbricoides* Th2 variable was then analysed in a general linear model, with age group, sex and *T. trichiura* re-infection group as factors and anti-*T. trichiura* Th2 response and *A. lumbricoides* infection variables (baseline and 8–9 month) as covariates. The significant cross-species immunity effect confirmed here is the type of correlational 'signature' that would be expected where two species interact negatively via the induction of similar immune responses. Furthermore, the negative associations seen with the immunological variables suggest that at least one of the respective species populations, under the conditions studied, probably *depresses* the other via induced immune responses, despite the existence of the positive correlation in fecundity data.

Community-level comparisons

In addition to within-population studies analysing the pair-wise covariances of parasite species amongst individual hosts, the distributions of multiple species

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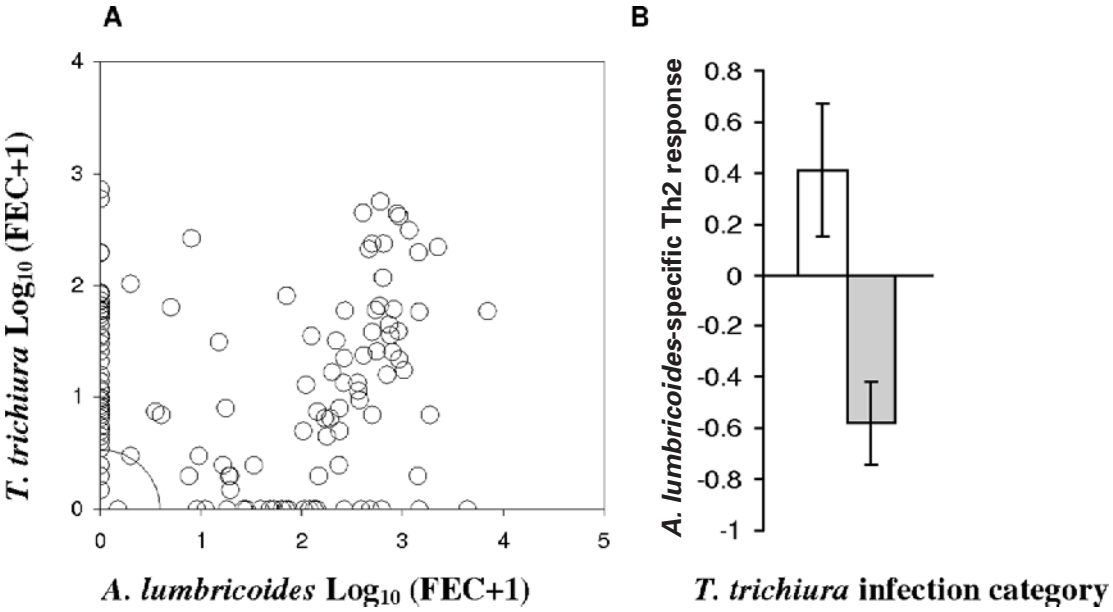


Fig. 2. Using immunological variables to infer negative parasite interactions against a probable background of positive correlation in exposure: an example based on data from Jackson *et al*. (2004*b*). A. Correlated occurrence (based on faecal egg count, FEC, data) of *Ascaris lumbricoides* and *Trichuris trichiura* in humans at Nyomo, Cameroon in baseline data from a treatment/re-infection study. Data point size proportional to number of observations (note large double zero point at origin). Prevalence of the two species was also significantly positively associated before and 8–9 months after treatment. B. Despite positive interspecific covariation in count and prevalence data, an analysis of *A. lumbricoides*-specific Th2 responses indicated that these were negatively associated with *T. trichiura* recolonization at 8–9 months post-treatment, even when *T. trichiura*-specific responses and *A. lumbricoides* FEC were accounted for (see text). Open and shaded bars respectively indicate hosts not re-infected and re-infected by *T. trichuira*; anti-*A. lumbricoides* Th2 responses (y-axis) are represented by residuals from a general linear model (Age class + Sex + anti-*T. trichiura* Th2 responses + *A. lumbricoides* FEC at baseline + *A. lumbricoides* FEC at 8–9 months).

in 'communities' can also be compared amongst individual hosts or groups of hosts (Poulin, 2007). These comparisons can use derived measures of community composition, such as indices of diversity (the species diversity of a community), dominance (the tendency of one or a limited number of species to dominate the community numerically) or similarity indices (measures of compositional similarity between different communities). They might also use distributional metrics, for example nestedness (Poulin, 1996), that reflect the tendency of species to occur with some kind of non-random order. When groups of hosts are compared, these may represent populations at different locations or times (Behnke *et al*. 2001*b*, 2004; Bajer *et al*. 2005), or groupings within populations stratified according to some variable of interest such as age or sex. Community-level patterns, and departures from null distributional models, can give important clues about the processes driving assemblage formation (Poulin, 2007). An interesting possibility is that measures of individual host immune phenotypes could provide a valuable contribution to the interpretations based on the numbers or occurrence of parasites alone (Table 1). If dominance, similarity or diversity indices or particular non-random distributional patterns were associated with immune phenotypic variation, this might provide new insights into immune-mediated processes that could determine community structure. For instance, is it possible to distinguish a 'non-interactive' community structured by positive correlation in exposure from a community with random exposure that is structured by a parasite driving non-specific immunosuppressive effects and hence positive effects on survival in other species? In both cases, one might expect to see positive correlations between species, and perhaps positive associations between some individual species and measures of community diversity. However, if immunological variables were measured, there might be specific predictions to distinguish between the two types of community. In the case of a non-specifically immunosuppressive parasite, for example the heligmosomatid nematodes discussed elsewhere in this special issue (see Behnke), it might be predicted that the 'suppressor' species would be strongly associated with immune hypo-responsiveness or increased activity of regulatory mediators (Elliott *et al*. 2004; Wilson *et al*. 2005; Finney *et al*. 2007). Specific regulatory responses against this species (for example antigen-specific regulatory cytokine production in cellular recall assays) might also be expected to covary positively with community diversity and perhaps negatively with dominance. There might also be negative associations between a suppressor species and

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specific functional responses against other species in the community. Furthermore, similar communities in which the suppressor species is absent might be expected to show increases in the magnitude of specific immunity against remaining community members, and also changes in the relationships of specific IRVs with the abundances of their target species.

Experimental studies

Ultimately, correlational field studies may allow inferences to be made about underlying processes, but usually cannot determine cause and effect. Manipulative field experiments, possibly in conjunction with laboratory studies, will be necessary to fully understand the importance of interaction in parasite communities. A range of field experimental designs could be envisaged, including the addition or removal of some elements of the community (Graham *et al*. 2007; Pedersen and Fenton, 2007) or, the manipulation of host immune responses, such as increasing specific responses to one pathogen by vaccination. If based solely on responses in infection variables (e.g. prevalence or abundance) this type of study could demonstrate the principle that the overall mean population levels of one species can be affected by another. However, unless very simple communities are studied, or study designs become very complex, it is possible that the true magnitude of pair-wise competition would still be obscured by readjustments within multi-species interaction networks. For instance, removal of a non-specifically immunosuppressive species might result in decreases in the abundance of other pathogens. However, these pathogens, in a more immunologically responsive host environment, might then additionally undergo increased immune-mediated negative competition with each other. This, in turn, would mean that any observed population decreases might overestimate the direct effects of the 'immunosuppressor' species in the original community. Indeed, in general, the removal of one species might result in a variety of numerical responses in other community members that could be due, directly, to the absence of interaction with the removed species or, indirectly, to an altered interaction with other species whose behaviour in the host ecosystem has been modified by the removal. Whilst scenarios such as these are likely to form 'knotty' real-world problems, it is arguable that changes in immunological variables in the original and manipulated community might help understand the mechanisms involved and increase the interpretative power of field experiments (Table 1).

Markers of microbial infection pressure

The consideration of immunology may be especially relevant to the study of communities where interactions involving microbes are being considered. Indeed, given the possibility that the host immune system and its associated community of parasites and pathogens represent a complex mutually interdependent system, artificially limiting studies to just a subset of pathogens may ignore important dynamics driving real-world systems. Unlike metazoan parasites, where individuals can be counted, staged and identified with careful work by a competent parasite taxonomist, the infection levels of bacteria and viruses are often less easy to quantify. In fact, it may be impossible even to identify all of the species present. Measuring the broad immunological consequences of infection with certain types of pathogen, for example, stereotypical anti-viral or anti-bacterial pattern responses (see below), might therefore be a powerful way to summarise exposure to particular 'types' of microbial infection in co-infection studies (Table 1). In the context of anti-bacterial responses, it is worth mentioning that the immunological influence of commensal bacteria in the gut may be an important consideration. Interpretational problems might also arise if some pathogens are present that modulate innate immune activity independent of stimulation by co-infecting microbes.

Consequences of co-infection for the host

Leaving aside the existence of competition or facilitation between pathogen species, the consequences of co-infection for the status of the host itself (e.g. Graham *et al*. 2005) are also of great interest and relate to the second fundamental question identified above. Field studies in this area (which are surprisingly rare) might potentially focus on response variables reflecting host condition or fitness. Examples might include specific measures of pathology, the degree of reproductive activity or investment, or markers of stress or adverse physiological states. This type of analysis has sometimes been approached by comparing hosts grouped according to the sets of species they contain, without considering individual infection intensity in the respective pathogens. In the case of correlated exposure rates or correlated underlying susceptibility (see above), this may confound the effects of genuine functional interactions with simple dose-dependent effects in the individual species. Here, to fully dissect 'true' co-infection effects from phenomena linked to coincidentally correlated transmission and establishment rates would require the measurement of interactions on the response variable with respect to intensity of the co-infecting parasites. A further complication arises where the mechanism of interspecies interaction produces immunological facilitation of one species by the other. In this instance, allowing for covariation in parasite numbers that results directly from the within-host interaction

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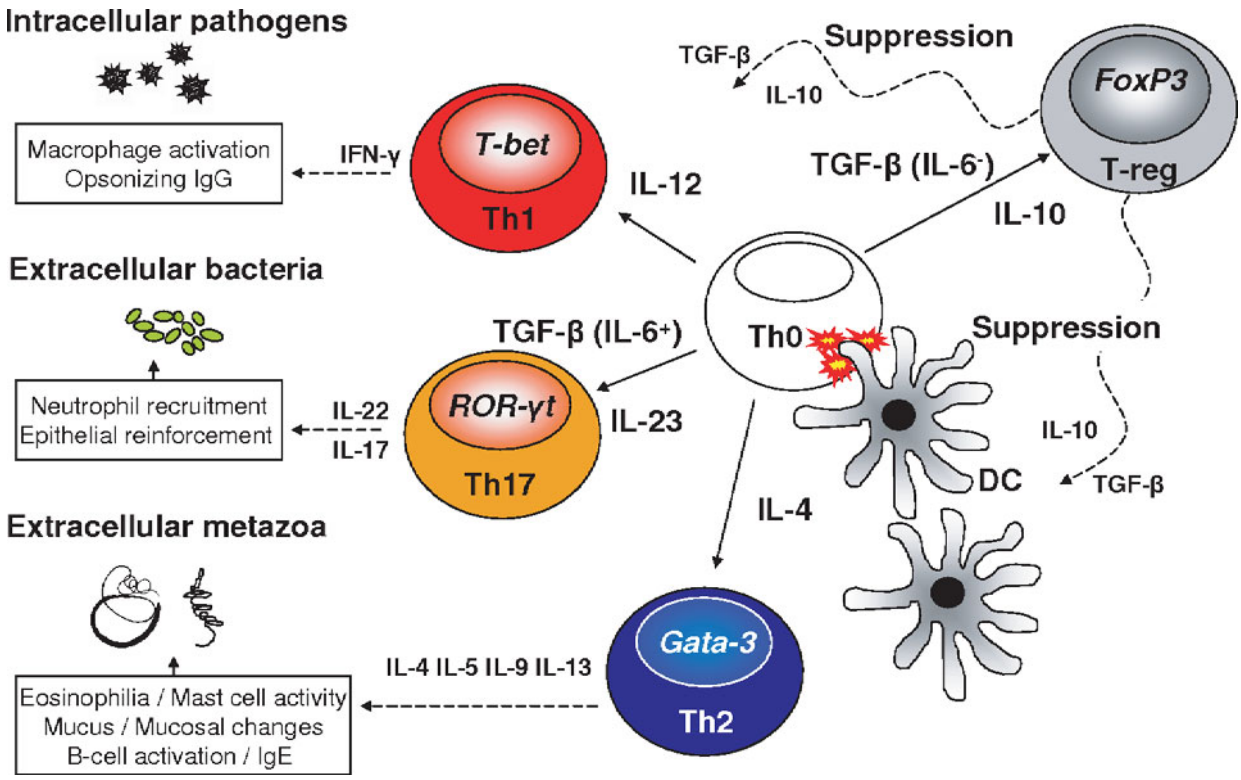


Fig. 3. Division of the immune system into functional arms: diversity in Th cell subsets. Schematic figure to show that under the influence of signals from antigen presenting dendritic cells (DCs), naïve Th cells (Th0) develop either into regulatory cells (Tregs) with over-arching suppressive functions, or into one of three mature phenotypes that drive different terminal effector mechanisms (boxed) appropriate for defence against different pathogen types. Systemically detectable cytokines are associated with the induction (solid lines) and subsequent function (dotted lines) of each effector phenotype.

through the immune system would actually be *removing* some of the special effects of co-infection. This type of inferential problem highlights the importance of understanding underlying immunological mechanisms involved in pathogen × pathogen interactions (Table 1).

The complexity of the immune system

Often, in order to be meaningful, the immunological variables measured in field studies must have some relationship with other biologically significant aspects of the host's phenotype. This could be resistance to particular pathogens or parasites, or some biological marker of condition or fitness. A problem with the analysis of immune responses in ecological studies has been the fact that crude global measures (e.g. blood cell counts, lymphoid organ size, phytohaemagglutinin response, non-specific lymphocyte responsiveness, total circulating antibody levels, levels of complement activity), whilst not to be discounted as useful measures of overall investment, may not always correlate well with biologically significant phenotypic variation (e.g. Goüy de Bellocq *et al*. 2007; Scharsack *et al*. 2007). This is probably because the *type* of immune response elicited (e.g. which subset of lymphocytes) rather than overall immune responsiveness may determine resistance to particular pathogens. In other words, similar amounts of resources devoted to different effector arms may have very different consequences in terms of the resistance – susceptibility profile of the host. Consequently, in a situation where resources are limited, different response types may 'trade-off' against each other (Graham *et al*. 2007) and, in turn, against other non-immunological life-history traits (Martin *et al*. 2004, 2006).

Given the complexity of the immune system, which elements represent the best targets for measurement in immuno-ecological studies? Whilst this is not the place for a full description of how vertebrate immunity works (for this see, for example, Janeway *et al*. 2006), it will be useful to briefly describe components that reflect the responsiveness and functional bias of the immune system and that present easily measurable phenotypes. Of central interest is the T helper cell system, which can be divided into distinct phenotypes that drive different regulatory or effector responses. Naïve Th cells are thought to be multipotent, with phenotypic commitment at maturation being controlled by signalling from APCs. Also of great interest is the responsiveness of PRRs, of which the Toll-like receptor (TLR) system is a particularly important example. These

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innate receptors have wide specificities to molecular patterns that occur on broad categories of pathogen (Pathogen-Associated Molecular Patterns, PAMPs). Typically, one receptor will recognise patterns derived from one or more higher taxonomic grouping of organisms rather than an individual pathogen species or strain. There may also be responses to some endogenous ligands that provide 'danger signals' of pathological damage (Matzinger, 2002). PRRs therefore detect the presence of an invading pathogen, or the damage that it causes. The signalling generated can initiate rapid innate responses. In addition, it can activate APCs and influence their maturation so that they polarize particular Th cell phenotypes (Janeway and Medzhitov, 2002). It is also important to recognise that the innate-adaptive interaction is not a simple 'one-way' control circuit and immuno-regulatory signals generated during adaptive responses also feed back to channel the activities of cells functioning in innate immunity.

T helper cells

$CD4^+$ Th cells are central to the control of adaptive immunity, producing signals that are fundamental to the development of both cellular and humoral responses. As introduced above, mature Th cells show functional division (Fig. 3) and provide cytokine and other signals, including help to B cells, that drive particular effector responses. Secreted cytokines may act locally, but also contribute to a systemic background influencing further innate and adaptive responses. The phenotype of Th cells is reflected in their cytokine production (Fig. 3), and global measurements of these molecules in cell populations can therefore be used to describe the functional balance of the immune system (Graham *et al*. 2007). At present four major phenotypic Th types can be defined, although, in reality, individual cell phenotypes may fall across a complex continuum. In simplified terms, Th1 cells produce responses appropriate for defence against intracellular pathogens. Typically, polarization of Th1 cells is associated with the presence of IL-12, and mature T-cells express the transcription factor *T-bet* and produce interferon (IFN) -γ. Th2 cells drive responses against extracellular parasites, such as parasitic worms. Mature Th2 cells express the transcription factor *Gata-3* and secrete cytokines including IL-4, IL-5, IL-9 and IL-13. Until recently a simple counter-regulation of the Th1 and Th2 arms was thought to be the main control mechanism for Th responses (Mosmann *et al*. 1986). Indeed, this Th1-Th2 paradigm may capture a significant amount of real variation and has been important in understanding resistance-susceptibility variation in a variety of pathogens (Scott *et al*. 1988; Cliffe and Grencis, 2004). It is now recognized that there are also regulatory subsets of $CD4^+$ and $CD8^+$ (Jiang and Chess, 2004) cells that exert an overarching down-regulatory control on effector cell types. The diversity of regulatory Th cell subsets (Treg) is complex and has not been fully defined (Bluestone and Abbas, 2003; Weaver *et al*. 2006), but typical phenotypic characters can include persistent expression of the activation marker CD25 (Sakaguchi *et al*. 1995) and the transcription factor FoxP3 (Ramsdell, 2003). Regulatory cells can be induced in the presence of IL-10 (Wakkach *et al*. 2003) and transforming growth factor (TGF)-β (Chen *et al*. 2003), and also actively secrete one or both of these cytokines. Furthermore, a new functional category of effector T cells with distinctive properties has recently been characterized. These are the Th17 subset (Reiner, 2007; Zhou *et al*. 2007) that typically express the transcription factor *ROR-*γt and secrete IL-17 and IL-22. Th17 cells are thought to be important for pathogens localised at cell membranes, driving neutrophil recruitment and the reinforcement of epithelial defences.

In immuno-ecological studies, phentotypic commitment to each of these arms could be measured by the relative levels of production of their associated cytokines. These could be *in vivo* global measurements (from serum) or *ex vivo* measurements from defined cell subsets, either reflecting constitutive production or production under specific stimulatory conditions (e.g. antigen recall assays). Direct measurements of some circulating antibody isotypes may also, to some extent, reflect Th1/Th2 variation. IgE and, in humans, IgG4 may be a marker for a Th2-biased phenotype. Also, levels of IgG1 and IgG2a respectively reflect levels of Th2 and Th1 activity in mice (Finkelman *et al*. 1988). Transcription factor or cytokine expression could also be measured by quantitative real time reverse transcription PCR (Q-RT-PCR) although, in practice, direct measurements of protein production may be a more sensitive and reliable option. On the other hand, PCR expression analyses may allow rapid processing of samples in the field and provide feasible protocols where cold storage or cell culture facilities are inaccessible, or where non-model organisms are the object of study (see below).

Immuno-ecological studies of the type envisaged would generate large multivariable data-sets. Indeed, it is likely that multiple measurements of different immune mediators would often be desirable to increase the reliability of the phenotypic description (for example, more than one cytokine with overlapping pleiotropic functions may often be measured to reflect a particular Th response type). In such cases, the employment of a multivariate technique, such as principal components analysis (PCA), prior to further analysis may help reduce multiple immune measures into a smaller set of variables, each of which is a linear combination of the original measures. The use of reduced data to carry out

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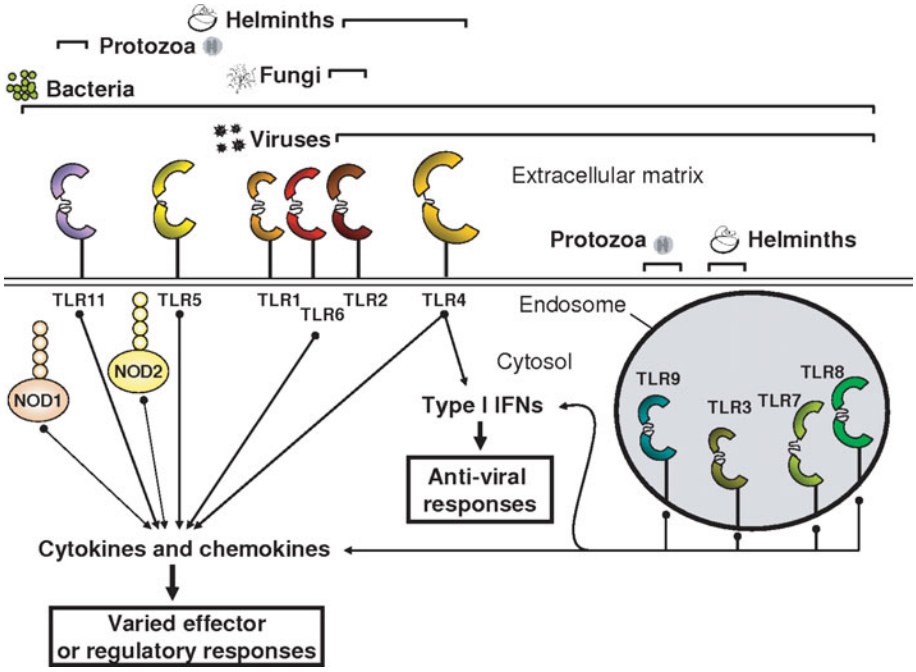


Fig. 4. Schematic representation of the cellular location and function of some important pattern response receptors (PRRs) involved in innate responses and the priming of adaptive immunity. Extracellularly-directed Toll-like receptors (TLRs) and intracellular NOD-like receptors are especially prominent in driving anti-bacterial responses. Endosomal TLRs that detect nucleic acid structures are particularly important in driving responses to viral infection. Brackets indicate known associations between receptor types and PAMPs from different pathogen groups.

central hypothesis tests may be especially helpful to avoid difficulties in interpretation due to multiple testing (e.g. Jackson *et al.* 2004*a*, 2006*b*). Reduced immunological variables might be used to represent different Th phenotypes, or combinations of phenotypes (e.g. Turner *et al.* 2003; Jackson *et al.* 2004*a*, *b*). A similar approach can also, potentially, be adopted to simplify variation in inter-correlated multi-species parasite communities (Legendre and Gallagher, 2001). Host individuals can thus be visualised in a multi-dimensional phenotypic space delimited by a relatively small number of composite immune response or parasite susceptibility variables. Associations between continuous immunological and infection variables can then be analysed by standard univariate statistical modelling techniques. Alternatively, individuals that are ordinated in the phenotypic spaces can be grouped or clustered and then group or cluster membership can be analysed with respect to other variables of interest.

Innate responses through PRRs

A further measure of functional disposition in the immune system is the expression and signalling responses of PRRs on and in cells (Fig. 4). In general, the expression levels and responsiveness of PRRs, such as TLRs, will be altered by exposure to their target ligands (Nomura *et al.* 2000; Guillot *et al.* 2005) and may also respond to the general background of immuno-regulatory signals from both adaptive and innate compartments (Bosisio *et al.* 2002; Tamandl *et al.* 2003; van Aubel *et al.* 2007). In the case of TLRs, many distinct cell populations, including non-immune system cells, differentially express subsets of the overall repertoire of receptor types. Crucially, each TLR recognizes a unique set of ligands associated with particular pathogens. Also, some TLRs are located at the cell surface, to survey extracellular spaces, whilst others are expressed intracellularly. The degree of activity of particular receptor types, responding to particular ligands in particular localities, may therefore carry information about phenotypic commitment or 'immunocompetence' with regard to defence against different types of infection. It might also, perhaps in the same way as Th phenotypes, reflect resource allocation trade-offs between different components of the immune system. Receptor activity would also be expected to mirror the type of pathogenic challenges being experienced (e.g. Jackson *et al.* 2006*b*). Indeed, in the context of the difficulties in quantifying microbial infection mentioned above, levels of TLR expression or responsiveness might be a particularly useful measure of overall exposure to microbial pathogens (e.g. Lauener *et al.* 2002; Ege *et al.* 2006). A further important consideration is that in many TLRs, qualitatively different downstream signalling responses, with different functional consequences, may be generated through stimulation of the same receptor. Thus, individual TLRs may mediate pro-inflammatory responses in some

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circumstances in some cell subsets, or trigger regulatory activity in others (Sutmuller *et al*. 2006; Layland *et al*. 2007). The type of response mediated can depend on the ligand. For a particular ligand it may also be influenced by other responses in the host environment, including responses to infection (e.g. Ince *et al*. 2006). When assaying ligand-specific TLR-mediated responses it may therefore be useful to measure a panel of mediators, such as cytokines, reflecting different possible downstream outcomes. For example, these might be pro-inflammatory (e.g. TNF-α), immunosuppressive (e.g. IL-10), or Type I interferons (IFN-α, IFN-β) important in anti-viral responses (De Maeyer and De Maeyer-Guignard, 1998).

In mammals, the extracellular TLRs (including TLRs 1, 2, 4, 5, 6 and 11) recognize a range of biomolecules from different source pathogens. Their ability to drive pro-inflammatory responses following exposure to bacterial PAMPs makes them a cornerstone of the body's anti-bacterial defences. Intracellular TLRs, expressed within endocytic vesicles (TLRs 3, 7, 8 and 9), detect nucleic acid motifs. Although they may respond to nucleic acids from a range of sources, their intracellular location and ligand specificity make them especially important for responses to viruses, including the production of anti-viral Type I interferon (Seth, Sun and Chen, 2006). Although TLRs are currently seen as the most functionally diverse group of PRRs in vertebrates, with the greatest influence on the development of adaptive responses, other innate receptors are likely to be important. Examples include intracellular NOD-like receptors (Bourhis and Werts, 2007), C-type lectin receptors (Willment and Brown, 2008) and RNA helicase RIG receptors, such as RIG-1 (Yoneyama *et al*. 2004).

In practice, *in vivo* receptor expression levels could be measured directly by fluorescence-activated cell sorting (FACS) analysis, or by Q-RT-PCR analysis of mRNAs. Functional responsiveness could also be quantified in *ex vivo* stimulatory cellular assays by the measurement of cytokine production, or by cell respiratory bursts in study organisms for which specific reagents are lacking (Scharsack *et al*. 2007). Given that a single receptor may stimulate more than one signalling pathway (see above), it would be especially revealing to measure panels of possible down-stream cytokine responses in this type of assay. Data analytical approaches could be similar to those discussed for measures of Th-associated phenotypes in the preceding section. In an example of a study based on cellular assays in humans, Jackson *et al*. (2006*b*) isolated monocytes from a Tanzanian cohort and measured a panel of individual pro- and anti-inflammatory cytokine responses following *in vitro* exposure to two PRR ligands, bacterial lipopolysaccharide (LPS, recognised by TLR-4) and peptidoglycan (now thought to trigger NOD-like receptors). It was found that pro-inflammatory, but not anti-inflammatory, cytokine responses were positively associated with gastrointestinal nematode infection. In particular, there was an increase in TNF-α production in response to LPS in wormier individuals. Whilst this was a simple 'observational' cross-sectional field study and therefore subject to confounding from unmeasured variables, the analysis may have revealed a potential candidate mechanism for interaction between parasitic worms and microbial pathogens that could be investigated further. Increased levels of responsiveness to bacterial molecular patterns might mean changes in resistance/ susceptibility to bacterial pathogens and high TNF-α levels might also have consequences for both resistance and immunopathological damage in other microbial infections such as malaria.

Future directions and application to different systems

It is worth emphasising that, whilst the cross-regulatory nature of many immune pathways and the likelihood of finite resource availability suggest that there should be functional or resource-allocation trade-offs between the different arms of the immune system, this has not been sufficiently investigated in experimental models (Lamb *et al*. 2005; Graham *et al*. 2007). Such trade-offs could occur within the adaptive arm (e.g. Th phenotypes) or innate arm (e.g. different TLR-mediated responses) of the immune system. Likewise, the existence of a trade-off between innate and adaptive immune defences is an intriguing possibility (Martin *et al*. 2007*a*; Wegner, Kalbe and Reusch, 2007) that deserves further experimental investigation. The amount of host investment in innate 'standing' defences that are generated constitutively, such as complement (e.g. Maniero and Carey, 1997), anti-microbial peptides (Woodhams *et al*. 2006) and natural antibodies (Matson *et al*. 2005), should also not be ignored. Furthermore, the 'shape' of functional trade-offs within the immune system needs to be placed in the context of the wider phenotype and possible environmental influences. Thus, anti-pathogen defences are energetically costly and potentially subject to resource allocation trade-offs with other phenotypic traits (Martin *et al*. 2004, 2006). The nutritional status of the host also appears to influence strongly the strength and phenotypic direction of some immune responses (Ing *et al*. 2000; Koski and Scott, 2001; Martin *et al*. 2007*b*). There is therefore now a clear requirement for further experimental studies with laboratory models to elucidate how (and if) different elements of the immune response compete with each other under different conditions of infection and resource provision. There is also an accompanying need for an exploratory phase in field studies of non-human hosts to describe immune phenotypic variation in more detail, investigate its

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important life history correlates (if any), and confirm whether there are associations with pathogen-specific resistance and susceptibility that go beyond simple tracking of exposure/current infection levels. Here, there is a clear advantage to choosing study systems that can be paralleled by existing or new laboratory infection models, given some of the interpretational limits associated with field studies. The extension of immunological measurements to field systems is also, to some extent, limited by the choice of organism and the availability of immunoreagents and/or genome sequence information. Antibody reagents are primarily available for humans and a small number of farmed and model organisms, especially the mouse, *Mus musculus*. This means that direct measurements of protein levels will mainly be possible only where the chosen study organisms are sufficiently closely related to one of these species to allow cross-reactivity. Future studies may well, therefore, focus on rodents, many of which are feasible laboratory and ecological field models and amongst which the level of evolutionary divergence may often allow cross-reactivity with antibodies designed for the laboratory mouse or rat. Studies of wild conspecifics or close relatives of other farmed or domesticated animals, however, may also be possible. The expanding amount of sequence information available in databases may make a wider range of non-model organisms accessible to study through the analysis of expression levels using Q-RT-PCR. Studies in non-mammalian vertebrates, which have often been historically popular, perhaps due to traditional taxon biases or to ease of sampling, are compromised to some degree by the fact that the function of the immune system remains less well understood. For example, in fishes, amphibians and birds, it is unclear whether the Th system shows the same functional division as in humans and mice. Cytokines associated with the Th2 cell subset, although present in birds, have been elusive in ectothermic vertebrates (but see Li *et al*. 2007) and it is possible that some aspects of Th functional differentiation may have evolved along different paths in different vertebrate groups. However, the TLR system (Roach *et al*. 2005) and key pro-inflammatory cytokine mediators (e.g. Novoa *et al*. 2006; Fast, Johnson and Jones, 2007) appear to be relatively structurally and functionally conserved amongst vertebrates and would provide valid candidates for study.

Practical and economic considerations may also affect the complexity of the immunological procedures attempted and the choice between mRNA analysis and the direct measurement of protein. Protein measurement would often be methodologically more robust (e.g. Huggett *et al*. 2005), perhaps making this the method of first choice. A particular advantage of recent cytometric bead array technologies is that they permit multiplexed protein measurements from very small sample volumes at high sensitivities, but these analyses are relatively costly. *Ex vivo* cellular assays or FACS analyses also require rapid access to specialist facilities following sampling, and the labour-intensive nature and/or cost of the procedures may impose limits on the throughput of samples. In the case of mRNA analyses of *in vivo* expression, however, sampling and storage is less problematical (for example, samples of lymphoid tissues may be kept in RNA*later*® at room temperature and analysed after an indefinite period). This may make the processing of large numbers of samples more feasible. A further practical consideration inherent in field studies is non-random sampling due to the vagaries of where and when animals can actually be captured. Fortunately, standard modelling techniques are now available to account for spatially or temporally non-independent data. This includes the application of residual maximum likelihood (REML) mixed models (Paterson and Lello, 2003) which can be implemented in commercially available 'off-the-shelf' software packages or with publicly available routines for free software (R). Crucially for parasitological and immunological data, which often show skewed distributions, non-normal error structures can also be accommodated using both 'fixed' generalized linear models (Wilson and Grenfell, 1997) and 'mixed' generalized linear mixed models (Paterson and Lello, 2003). The availability of these and other advanced methods has removed many of the pitfalls that would previously have compromised the detailed analysis of field parasitological data. Indeed, the key impediment to progress in this field may not be further sophistication in statistical analysis, but technical and logistical issues surrounding the execution of large-scale field studies and the generation of sufficiently high quality parasitological and immunological data. One particular concern should be that the quantification of parasite communities in wild hosts may often be compromised by identification issues, given the accelerating collapse of traditional parasitological expertise in some countries and the limited amount of verified material that has been deposited in databases to aid molecular identifications.

CONCLUSIONS

There is currently a wide gap between our understanding of how the immune system is regulated in laboratory models and the application of this knowledge to ecological studies of infection in natural vertebrate populations. This is especially relevant in the study of co-infection states, where interspecific interactions may often be mediated through the host immune system. Some of the most interesting immunological target variables for future ecological studies are responses associated with different

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T-helper cell phenotypes and mediated through pattern receptor responses, such as the Toll-like receptors. These up-stream mediators trigger and control the plethora of different possible terminal immunological effector mechanisms and, therefore, most parsimoniously reflect the global responsiveness and functional bias of an individual immune system. Field immunological measurements at this level of detail, in a variety of settings, are highly desirable and may help us reveal interspecific interactions that are obscured by confounding processes, and to describe the force of infection by some pathogen groups whose communities are difficult to quantify by direct measurements. Ultimately, an increase in the sophistication of immunological measures used in field and laboratory ecological studies may allow us to understand how trade-offs and functional constraints acting on, and within, the immune system interact with infection and other biological pressures to shape the dynamics of host-parasite communities.

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