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Trade-offs within the immune systems of female White-footed Mice, *Peromyscus leucopus*

L. B. MARTIN II,*† Z. M. WEIL,* J. R. KUHLMAN* and R. J. NELSON*†‡

*Departments of *Psychology and †Neuroscience, and ‡Evolution, Ecology and Organismal Biology, Ohio State University, Columbus, OH 43210, USA*

Summary

1. In many vertebrates, immune activity is compromised when other expensive activities are concurrent. One explanation for such patterns includes trade-offs between immune activity and other expensive physiological processes. Trade-offs among different immune responses themselves may also occur, but thus far few data exist to substantiate them.

2. We predicted that immune activity in female White-footed Mice, *Peromyscus leucopus*, would be weak (relative to sham-treated controls) if another immune response was already ongoing. To test this hypothesis, we examined (i) the effects of inflicting a cutaneous wound on cell-mediated immune activity one day after wounding, and (ii) the effects of inducing cell-mediated immune activity on the cutaneous wound-healing process when wounds were inflicted one day after the immune challenge.

3. Prior wounding dampened cell-mediated immune responses and induction of cell-mediated immune activity altered progression of wound healing. Immune challenges did not affect reproductive tissue masses, however, as has been detected in males of this species. Also, concentrations of circulating glucocorticoids, which are known modulators of immune activity, were not dramatically different between treatment and sham groups.

4. In sum, our results provide evidence that some immune responses can negatively influence other recent immunological activity. Further study is warranted, however, to pinpoint the molecular mechanisms underlying these apparent trade-offs and determine whether induction of immune activity may sometimes prime instead of hinder subsequent immune responses.

Key-words: Cell mediated, delayed type hypersensitivity, immunocompetence, life history

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Introduction

Animals are constantly accosted by disease-causing agents. Variability in traits promoting disease-resistance therefore, including immune activity, is counterintuitive because animals could benefit from maintaining high levels year-round. Variability in immune activity over time is believed to be a consequence of the counterbalance organisms must make among competing costly process in different environmental contexts (Nelson & Demas 1996). Indeed, abundant evidence has indicated that the immune system is expensive. Artificial increases in reproductive responsibilities (especially nestling provisioning) decrease immune activity in multiple species of passerine birds (Moreno *et al*. 1999; Ardia 2005); likewise, pregnancy and lactation depress immune activity in Siberian hamsters (*Phodopus sungorus*; Drazen *et al*. 2003). Conversely, induction of immune activity increases energy expenditure (Demas *et al*. 1997; Martin *et al*. 2003), depresses reproductive behaviour (Aubert *et al*. 1997; Bonneaud *et al*. 2003; Weil *et al*. 2006) and decreases tissue growth (Prendergast *et al*. 2004; Martin 2005) in both rodents and passerines. Subsequently, up-regulation of one physiological process mandates the down-regulation of others.

Such trade-offs between the immune and reproductive systems have been well studied. However, one potential trade-off that has received little attention includes trade-offs within the immune system itself. Activation of one immune response may negatively

†Author to whom correspondence should be addressed. E-mail: lmartin@mail.psy.ohio-state.edu

influence a second immune response if both take place in close temporal proximity. To date however, this hypothesis has been only indirectly addressed in vertebrates, and thus evidence is inconclusive. In Red Jungle Fowl (*Gallus gallus*) for example, two measures of immune activity were positively related to comb length, a sexually selected trait, prior to the breeding season. During the breeding season however, one was positively related but another was negatively related to comb length (Zuk & Johnsen 1998). In invertebrates, evidence for trade-offs within the immune system is stronger, but based solely on genetic correlations (Walters & Pawlik 2005). Antibacterial defence in Egyptian Cotton Leafworms (*Spodoptera littoralis*) exhibited a negative genetic correlation with haemocyte density, but haemocyte density was positively genetically correlated with two other immune indexes (Cotter *et al*. 2004). Altogether, trade-offs within the immune system appear to occur, but strong evidence indicating that one immune response can directly impair another remains lacking in vertebrates.

Here, we tested whether infliction of a small cutaneous wound would suppress a cutaneous T-cell-mediated immune response (delayed-type hypersensitivity; DTH) induced 24 h later in White-footed Mice (*Peromyscus leucopus*; Pyter *et al*. 2005). We also tested the reverse possibility, namely whether induction of a cutaneous DTH response would affect progression of cutaneous wound healing when wounding occurred 24 h after a DTH challenge. These two particular immune responses were chosen for several reasons. First, both take place in the skin, thus differences among challenge sites in terms of vascularisation and/or cytokine receptor density and distribution should minimally influence results. Second, both involve inflammatory immune responses mediated by both adaptive and innate cells (e.g. T cells (adaptive) and macrophages and granulocytes (innate)), which are integral to rapid and effective healing of wounds and DTH responses (Turk 1967; Elgert 1996; Viswanathan & Dhabhar 2005). These characteristics should provide an ideal opportunity for detecting trade-offs between two related but distinct integrative measures of immune activity. Although we expected negative interactions between immune responses, it was possible that a first immune response could prime a second. Our experimental design allowed us to determine not only whether interactions between immune responses occurred, but also the direction of this interaction (e.g., antagonism *vs* synergism).

In addition to searching for interactions within the immune system, we asked whether induction of two types of immune activity would impact the reproductive tissues of female animals. A single immune challenge to male *Peromyscus leucopus* decreased testes mass by 22% (Derting & Compton 2003); we predicted that multiple immune challenges would have similar if not more dramatic effects in females of the same species. We also addressed whether effects of prior immune responses on subsequent immune responses were related to the activity of the hypothalamic–pituitary–adrenal axis by comparing blood corticosterone concentrations between control and treatment groups at the end of the experiment (Dhabhar & McEwen 1999; Sapolsky *et al*. 2000). Hormone concentrations were not compared over the course of immune responses to prevent the process of blood sampling from affecting the immune responses measured.

Materials and methods

ANIMALS AND GENERAL PROCEDURES

Female White-footed Mice (*P. leucopus*) were bred in our colony at The Ohio State University initiated from stock purchased from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC). Mice were weaned at 18–21 days of age and housed singly in polypropylene cages in a 16:8 h light : dark photoperiod (lights off 15.00 hours EST) before and throughout experiments. Ambient temperature and relative humidity were maintained at 22·5 ± 1 °C and 50 ± 5%, and mice were provided with *ad libitum* access to food (Harlan TekLad 8640 Indianapolis, IN) and filtered tap water before and throughout experiments. Once mice reached adulthood (~9 weeks old), each mouse was housed singly and randomly assigned to a group: DTH–wound healing ($n = 8$), wound healing–DTH ($n = 8$), sham–DTH ($n = 8$) or sham–wound healing ($n = 8$). For the wound healing–DTH group, mice were sensitised to DNFB (2,4-dinitro-1-fluorobenzene) 1 week prior to the experiment and wounded 6 days later; DTH was induced the following day. Sham–DTH mice were sensitized and challenged at the same time as wound–DTH mice, but were not wounded 6 days later. For the DTH–wound healing group, mice were sensitized to DNFB, but wounding occurred 24 h after DTH challenge. Sham-wound mice were sensitised and wounded at the same time as DTH–wound animals, but were not challenged with DNFB. All mice in all groups were sensitised to DNFB. Sensitization involved anaesthesia (isoflurane in O_2 enriched-air) followed by administration of 50 µl of DNFB [0·5% (w/v) in 4 : 1 acetone/olive oil vehicle] to a shaved area on the rump for two consecutive days (beginning 6 days prior to either wounding or DTH challenge; Pyter *et al*. 2005).

At the conclusion of the study, body mass (to the nearest 0·1 g) was measured, mice were killed humanely (decapitated under deep isoflurane anaesthesia), and blood was collected for radioimmunoassay of corticosterone. Reproductive tissues (uterus, ovaries and ovarian fat pads) were then collected from all mice, cleaned of connective tissue and weighed to the nearest 0·1 mg within 3 min of collection (to minimize desiccation). Blood samples were not taken during or prior to immune measures to prevent the bleeding process from confounding immune responses. Owing to animal availability, the study was conducted in two blocks

with similar numbers of mice in each treatment group spread across the two blocks. One mouse in the sham–wounded group and two mice in the sham–DTH group died prior to the end of the experiment. During all procedures, mice were handled and anaesthetized equally. All procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee and complied with the US-NIH regulations.

WOUND HEALING

Mice were anaesthetised (as above) and a patch of fur (~90 mm^2) was shaved between the scapulae (Marucha *et al*. 2000; Rojas *et al*. 2002; Glasper & Devries 2005; Martin *et al*. 2006c). This shaved region was sterilized (70% ethanol), and two wounds (3·5 mm diameter) were made in the skin using a sterile, disposable biopsy punch (Miltex Instrument, Bethpage, NY). Between 10.00 and 14.00 h immediately following wounding and for 5 days thereafter, wounds and a reference standard (a 3·5 mm diameter circle) were photographed using a digital camera (Nikon Coolpix 775, Tokyo, Japan). In each photo, entrance wounds and reference standards were traced at 800× magnification, and relative wound areas were calculated using graphic design software (Canvas 6, Deneba Systems, Miami, FL). Sham-treatment for wounding involved shaving the dorsum, sterilising skin and touching a biopsy punch to the shaved area.

DELAYED-TYPE HYPERSENSITIVITY (DTH)

Delayed-type hypersensitivity to DNFB is a common measure of T-cell mediated immune activity in rodents. In order for a local inflammatory response to DNFB to be generated, mice must first be exposed to the antigen to allow receptive T cells (those possessing receptors complementary of components of the DNFB molecule) to proliferate in circulation (Elgert 1996). Subsequent exposure of a mouse to DNFB elicits a cascade of immunological activity orchestrated by educated T cells at the site of exposure. This immune activity consists of inflammation and oedema induced by degranulation of local and circulating innate immune cells recruited to the site of DNFB challenge by (predominantly) T cells receptive to DNFB (Turk 1967). Unlike phytohaemagglutinin (PHA) and other mitogens commonly used in ecological immunology, DNFB does not non-specifically activate resident T cells (i.e. cross link ~40% of T-cell receptor types, inducing proliferation of those clonotypes without costimulation from antigen-presenting cells; Martin *et al*. 2006b). For this reason, in this study and many others from our lab and elsewhere, we sensitized mice following a standard protocol prior to DTH measurements. As with all other studies, we interpret a larger swelling response post-DNFB challenge to indicate a stronger immune response.

Mice were anaesthetised, and thickness of both pinnae was measured using a constant-loading micrometer (Mitutoyo America, Aurora, IL; Pyter *et al*. 2005). Immediately afterwards, 20 µl of DNFB [0·7% (w/v) in 4:1 acetone/olive oil] was applied to right pinnae, and left pinnae were treated with 20 µl of vehicle. Every 24 h for the following 5 days, thickness of both pinnae were measured; swelling was expressed as the ratio of right pinna each day relative to its pre-challenge thickness. All DNFB applications and pinnae measurements were performed between 10.00 and 14.00 h, and all were made on the same region of the pinnae as prior measurements. Sham-treatment for DTH involved application of vehicle to both ears followed by measurement of pinnae.

CORTICOSTERONE ASSAY

Serum corticosterone was assayed using a double-antibody ^{125}I kit (MP Biomedicals, Costa Mesa, CA; cross-reactivity with other steroids <1%). The assay was conducted following the guidelines set by the manufacturer, except all samples were diluted 1:1000 because *Peromyscus* have much higher corticosterone than domestic mice (Glasper & Devries 2005). Intra-assay variation was <10% and lower detection limit was 5 ng ml^{-1}.

DATA ANALYSIS

All percentage data were square-root transformed; 1-sample Kolmogrov–Smirnov tests and Levene's tests indicated that parametric statistical analyses were appropriate. DTH responses and wound healing were compared between treatment and sham groups using repeated-measures GLM with treatment (sham *vs* immune challenge) and block (run one *vs* run two of the experiment) as factors. Corticosterone concentrations and reproductive organ masses (uteri, ovaries and ovarian fat pads) were compared between sham and treatment mice (sham-wound *vs* DTH-wound and sham-DTH *vs* wound-DTH) using independent *t*-tests (after $\log_{10}$-transformation due to non-normal data distribution). All statistics were conducted using SPSS v. 12 with $\alpha \leq 0{\cdot}05$.

Results

EFFECT OF WOUND HEALING ON DTH RESPONSES

DNFB treatment induced significant cell-mediated immune responses (i.e. swelling) in all mice ($F_{5,50}$ = 58·5, $P < 0{\cdot}001$). DTH responses were dampened, however, by prior wounding (treatment × time: $F_{5,50}$ = 2·63, $P = 0{\cdot}03$; Fig. 1a), but this effect was influenced by experimental block (group × time: $F_{5,50}$ = 2·96, $P = 0{\cdot}02$). However, the block effect was in the same direction between blocks (as indicated by a

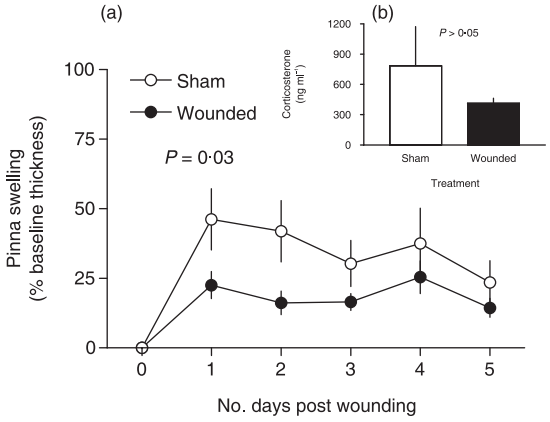

Fig. 1. (a) Delayed-type hypersensitivity responses to DNFB and (b) baseline corticosterone concentrations ($\log_{10}$-transformed) in wounded and sham-treated female *P. leucopus*. Bars are means ± 1 standard error of the mean (SEM).

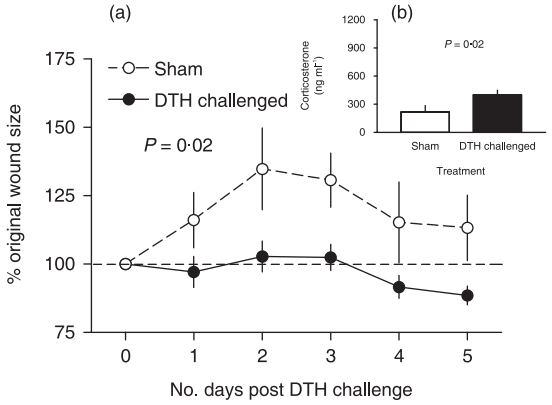

Fig. 2. (a) Wound healing and (b) baseline corticosterone concentrations ($\log_{10}$-transformed) in female *P. leucopus* challenged with DNFB or vehicle prior to wounding. Dashed horizontal line represents initial wound size (standardized). Bars are means ± 1 SEM.

non-significant three-way interaction; treatment × group × swelling: $F_{5,50} = 0{\cdot}98$, $P > 0{\cdot}05$), indicating a consistent but differentially effective influence of wounding on DTH responses in *P. leucopus*. Neither blood corticosterone concentrations ($t_{12} = 0{\cdot}98$, $P > 0{\cdot}05$; Fig. 1b) nor body mass or any reproductive tissue was influenced by immune challenges or any other factor at the end of the experiment (all $P > 0{\cdot}05$, raw and body-mass adjusted; Table 1).

EFFECTS OF DTH INDUCTION ON WOUND HEALING

Wounds healed over time in all mice ($F_{5,55} = 5{\cdot}84$, $P < 0{\cdot}001$). Prior induction of a DTH response (treatment × time: $F_{5,55} = 2{\cdot}94$, $P = 0{\cdot}02$; Fig. 2a), however, affected progression of healing. Sham-treated mice showed physical inflammation at the wound site (wound size >100% original size) whereas DTH-challenged mice on average did not (Fig. 2a). In this part of the experiment, experimental block did not influence healing (group × time: $F_{5,55} = 1{\cdot}07$, $P > 0{\cdot}05$) as in the above group. Also, corticosterone concentrations were significantly elevated at the end of the experiment in DTH-challenged *vs* sham-challenged controls ($t_{13} = -2{\cdot}66$, $P = 0{\cdot}02$; Fig. 2b). However treatment and sham mice did not differ in body mass or reproductive organ mass (all $P > 0{\cdot}05$, raw or body-mass adjusted; Table 1) post-immune challenges.

Discussion

To survive and reproduce, an animal must adaptively allocate resources among competing physiological systems in a fashion complementary of current or impending environmental conditions (Nelson & Demas 1996; Ricklefs & Wikelski 2002). Such adjustments occur among the immune system and reproduction and somatic growth (Bonneaud *et al.* 2003; Prendergast *et al.* 2004; Martin 2005). Based on data from this study, it is apparent that trade-offs also occur within the immune system itself. Wounding and induction of a DTH response altered subsequent DTH activity and wound healing progression, respectively, in female White-footed Mice. One of the best supported explanations of these phenomena involves the high costs of mounting immune responses. Indeed, immune function is expensive in terms of energy, resources and time (Lochmiller & Deerenberg 2000; Demas 2004; Klasing 2004). In passerine birds, DTH challenges are as expensive as other fitness-related processes (Martin *et al.* 2003). Although estimates of the energetic costs of wound healing are lacking, production and movement of immune cells to a distant site may be sufficiently

Table 1. Somatic and reproductive organ mass among groups of female *Peromyscus leucopus*

		Sham–wound (7)		DTH–wound (8)		Sham–DTH (6)		Wound–DTH (8)	
Group*	Units	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body	g	19·2	0·6	20·3	0·6	19·3	0·8	21·0	0·9
Uterus	mg	713·8	136·6	858·7	130·9	696·7	195·4	681·6	83·3
Paired ovaries	mg	154·3	22·3	156·9	10·8	123·0	24·4	191·3	27·7
Paired ovarian fat pads	mg	99·1	15·2	122·5	17·0	115·0	29·0	258·0	130·7

*Number in parentheses indicates sample size.

expensive to hinder inflammation if another inflammatory process is ongoing already.

One other explanation of the results of this experiment involves the activity of cytokines either at the site of immunological insult or in systemic circulation. Pro-inflammatory cytokines, in particular tumour necrosis factor α (TNFα), are integrally involved in the inflammatory components of both DTH responses and wound healing (Hubner *et al*. 1996; Viswanathan & Dhabhar 2005). In this study, multiply immune-challenged mice may have been unable or reluctant to elevate pro-inflammatory cytokines to levels where both DTH responses and wound healing could be maximized. Such regulation may represent an effort to prevent anaphylaxis, septic shock or activation of unnecessary (and costly) febrile responses (Råberg *et al*. 1998). Inflammation is indeed a double-edged sword; it provides a broadly effective type of defence but, if unchecked, it can promote autoimmunity (Klasing 2004). One factor that must be reconciled if this hypothesis is to be supported is how cytokines produced locally could act systemically. Indeed, for cytokines generated post-wounding to affect DTH responses, cytokines created at the wound site would have to reach the general circulation or evoke broad changes in the immune system that could alter the state of the immune system elsewhere. Such local effects on systemic processes are not unprecedented. One of the best recognized phenomena of the early stages of parasite infections is a Th-1/Th-2 shift (Menger & Vollmar 2004). Early postinfection, a global bias in the cytokines that predominate in circulation (pro *vs* anti-inflammatory) develops as parasites activate different populations of T-helper lymphocytes (Graham 2002), hence the Th-1/Th-2 nomenclature (Janeway 2004). In our study, activation of DTH responses may have inhibited some pro-inflammatory pathways leading to reduced inflammation at the wound site.

The absence of physical inflammation at the wound site (Fig. 2a) was the major difference between sham and DTH-challenged mice. Although one may assume that a more rapidly healed wound is adaptive, which is in part valid (Glaser & Kiecolt-Glaser 2005), the absence of inflammation at the wound site in DTH-challenged mice indicates at least some compromising of the immune systems of DTH-challenged mice. Indeed, multiple studies have found that an absence of inflammation at the wound site promotes bacterial infection (Marucha *et al*. 2000; Rojas *et al*. 2002). Subsequently, if T-cell mediated immune activity is engaged at another body site, wounds may heal at comparable rates, but in the first several days of the healing process, bacterial resistance may be compromised. Similarly, if animals are wounded during territorial defence or in competition over resources, then T-cell mediated inflammation, which is involved in defence against viral infections, may be weakened.

The existence of the Th-1/Th-2 biases described above highlights a possible, but unrealized, outcome of our experiments: synergy between immune responses induced in close temporal proximity. Indeed, although immune activity is expensive, it is possible that in some cases prior immune activity may prime the immune system for subsequent immune responses. For example, helminth infections typically induce a Th2 bias in their hosts (Graham 2002). Thus, resistance of a second helminth infection (or another extracellular parasite) may be achieved via a pre-existing bias in the immune system driven by an ongoing infection with a related parasite. Even this synergy may sometimes be influenced by resource-based trade-offs. However, primary and secondary infections with multiple parasite types would have to happen close in time for this to occur. Otherwise, temporal incongruence may allow hosts to compensate for increased immunological demands (by taking in more food or decreasing investments in other expensive behaviours) prior to the second immune challenge.

In the future, research on potential synergy within the immune system would be useful, as would research regarding the manifestation of trade-offs in captive animals. Antagonistic interactions among components of the immune system were initially predicted to occur because of the costliness of immune activity in general (Demas *et al*. 1997; Lochmiller & Deerenberg 2000; Martin *et al*. 2003). However, the lack of demanding conditions in the lab could potentially have eliminated or at least minimized trade-offs. Perhaps trade-offs continue to manifest in captivity because the signalling mechanisms that were selected to prioritize physiological processes in natural settings (e.g. cytokines) still operate in captive ones (Martin *et al*. 2006a; Ricklefs & Wikelski 2002). A similar explanation may hold for an unexpected outcome of this study, particularly the absence of an effect of immune challenge on reproductive tissues. Alternatively, the potentially higher maintenance costs of male *vs* female reproductive tissues (Martin *et al*. 2006a) and the presence of immuno-enhancive oestrogens in females *vs* males (Kovacs *et al*. 2004) may explain inconsistencies with previous work.

Altogether, the trade-offs between immune defences detected in this study probably represent prior selection for adaptive counterbalancing among physiological systems that animals have been forced to make over evolutionary time. Corticosterone does not appear to be integrally involved in regulating these trade-offs because this hormone was elevated only following a DTH response, not postwounding. On the other hand, avoidance of repeated blood sampling may have prevented detection of important changes in this hormone involved in mediating trade-offs. As such, we cannot rule out corticosterone as an important influence on the inter-immune trade-offs detected here. Evidence from other studies, however, suggests that cytokines probably are important mediators of the trade-offs detected in these experiments, and thus warrant further study. Finally, studies concerning

interactivity among different components of the immune system would be informative (Zuk & Johnsen 1998), as it remains unsettled whether antagonism within the immune system is a general phenomenon or if synergism between immune components often occurs.

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