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Research paper

MHC class II transcription is associated with inflammatory responses in a wild marine mammal

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ABSTRACT

Inflammation is one of the most important non-specific and rapid responses that a vertebrate can elicit in response to damage or a foreign insult. To date, despite increasing evidence that the innate and adaptive branches of immunity are more intricately related than previously thought, few have examined interactions between the Major Histocompatibility Complex (MHC, a polymorphic region of the vertebrate genome that is involved with antigen presentation) and inflammation, and even less is known about these interactions in an eco-immunological context. Here, we examined the effect of MHC class II DRB gene multiplicity and transcription on phytohemagglutinin (PHA)-induced inflammation during the early stages of development of California sea lions. Neither constitutive nor expressed *ZacaDRB* diversity was found to be associated with pup responses to PHA at any of the stages of pup development. However, for two-month-old pups, those with a specific MHC-DRB locus (*ZacaDRB-A*) tended to have less efficient responsive inflammation. Transcription of distinct MHC-DRB loci was also linked to PHA-induced inflammation, with patterns that varied markedly between ages, and that suggested that ongoing infectious processes could limit the capacity to respond to a secondary challenge. Life history constraints and physiological processes associated with development of California sea lions, in conjunction with their changing pathogenic environment could explain the observed effects of MHC class II transcription on PHA-induced inflammation. To our knowledge, ours is the first study to examine the importance of expressed *vs.* constitutive MHC loci on inflammation in a natural population.

1. Introduction

The phytohemagglutinin (PHA) skin-swelling challenge is commonly used in ecoimmunology studies (Kennedy and Nager, 2006; Brock et al., 2014). Intradermal injection of a PHA and subsequent measurement of responsive swelling are commonly used as indicators of inflammatory potential of vertebrates (Martin et al., 2006; Brown et al., 2011; Brock et al., 2012). PHA is recognized as a pathogen-associated molecular pattern (PAMP) that induces non-specific inflammation, one of the main innate responses of the immune system (Hoebe et al., 2004; Medzhitov, 2008; Unitt and Hornigold, 2011). Given that PHA-induced inflammation involves proliferation and infiltration of both humoral and cellular immune effectors in the challenged tissue, it has been suggested that the process requires a considerable investment of resources (Kennedy and Nager, 2006; Martin et al., 2006; Brown et al., 2011; Brock et al., 2012).

To date, despite the growing number of publications on the evolutionary ecology of the Major Histocompatibility Complex (MHC), an extremely polymorphic region of the vertebrate genome that is involved with antigen presentation (Bernatchez and Landry, 2003; Sommer, 2005; Piertney and Oliver, 2006), few studies have examined the relationship between MHC polymorphism and the inflammatory response (Bonneaud et al., 2005, 2009). Furthermore, examination of local inflammatory responses in the context of systemic MHC class II transcription patterns remains unknown for free-ranging vertebrates. Once activated by inflammatory cytokines, professional antigen-presenting cells such as macrophages and dendritic cells engulf the 'offending' molecules or cells, and process and present antigenic peptides to specialized lymphocytes (*e.g.* T and B cells), in a process that relies on MHC class II transcription (Hoebe et al., 2004; Bernatchez and Landry, 2003).

PHA-induced inflammation was found to be context-dependent during early development of the California sea lion, *Zalophus californianus* (hereafter, CSL), relying on high body condition of young pups and later depending on the absence of active infections (Vera-Massieu et al., 2015). The CSL MHC class II DRB (hereafter, *Zaca-DRB*) comprises a family formed by at least ten loci (DRB-A to DRB-J) that have limited variability but are present as diverse genotypic configurations in each individual (Bowen et al., 2004). Particular *ZacaDRB* loci have been found to be associated with specific pathologies (Bowen et al.,

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2005; Barragán-Vargas et al., 2016), and it has been proposed that *ZacaDRB* diversity could reflect local adaptation to pathogens and environmental conditions from geographically disparate populations (Bowen et al., 2006a).

Because deployment and maintenance of immune responses are energetically-costly processes (Lochmiller and Deerenberg, 2000; Norris and Evans, 2000), and transcription of MHC class II genes can indicate active infections (Schrum et al., 2003), we hypothesized that transcription of *ZacaDRB* genes by circulating antigen-presenting cells will be inversely related to the magnitude of PHA-induced inflammation of CSL pups, regardless of their constitutive *ZacaDRB* polymorphism.

2. Materials and methods

2.1. Field work and collection of samples

Fifty-one CSL pups from the breeding colony of Granito Island in the Gulf of California (29°33′43″ N, 113°32′04″ W) were sampled at two weeks (n = 23; hereafter neonates; born in 2013), two months (n = 11; born in 2012), and six months (n = 17; born in 2012) of age. Each cohort contained only pups that had not been captured previously. Pups were challenged with PHA (see details in Vera-Massieu et al., 2015). For each pup, skin and whole blood samples were obtained. Skin samples were collected from the right hind flipper and preserved in 96% ethanol. Blood (7–10 ml) was collected from the caudal gluteal vein using Vacutainer® tubes with sodium heparin (BD Biosciences, USA), and immediately centrifuged at 2500 rpm. Buffy coats (blood leukocytes) were separated, preserved in RNAlater stabilizing solution (Qiagen, USA), and stored in liquid nitrogen until analysis.

2.2. MHC genotyping and transcription

Genomic DNA was extracted from the skin samples following a proteinase K and phenol–chloroform protocol (Sambrook and Russell, 2001). Total RNA was extracted from each buffy coat using Trizol (Sigma, USA) according to the manufacturer's protocol, and 15 μg of RNA was reverse-transcribed with a Quantitect Reverse Transcription Kit (Qiagen, USA). Genomic DNA and the buffy coat cDNA were analyzed using ten sequence-specific forward primer pairs (ZCDRB194A to ZCDRB194J), each paired separately with a reverse primer DRBlociR (primer sequences can be seen in Bowen et al., 2006a, 2006b). The region amplified spans the peptide binding region of the CSL MHC class II DRB molecules (Bowen et al., 2004, 2006a, 2006b). The analysis of DNA and cDNA allowed us to determine diversity as well as transcription (presence/absence) of each *ZacaDRB* gene. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal PCR control for the *ZacaDRB* transcription assays. Sequence-specific amplification (SSP-PCR; Bowen et al., 2006a) was conducted in an ABI 3100 PCR system (Applied Biosystems, USA), and products were electrophoresed in ethidium bromide-stained 2.0% agarose gels and then visualized with a UV transilluminator. Transcription of each *ZacaDRB* gene was observed as amplification of a band of ~250 bp and an additional ~100 bp band for GAPDH (internal control). Absence of *ZacaDRB* gene transcription was observed as a single band for GAPDH. Each SSP-PCR was run in duplicate.

2.3. Data analysis

ZacaDRB diversity was calculated as the number of MHC class II DRB genes present in genomic DNA (hereafter, constitutive *ZacaDRB* diversity) and in the buffy coat cDNA (hereafter, expressed *ZacaDRB* diversity). For each age class, we built generalized linear models (GLMs) to examine the effect of constitutive and expressed *ZacaDRB* diversity, and of the presence or absence of specific *ZacaDRB* loci on the magnitude of the PHA-induced swelling. We included body condition, number of circulating antigen-presenting cells (*i.e.* lymphocytes, monocytes, basophils and eosinophils), and neutrophil to lymphocyte ratio (NLR) as explanatory variables that have previously been found to vary significantly for those age classes (see details in Vera-Massieu et al., 2015). Pup body condition was estimated by calculating a scaled mass index which takes into account the scaling between body components and body size (SM*i*; Peig and Green, 2010). This index is useful as a proxy for assessing energetic resources in sexually dimorphic species, and has shown trade-offs in CSL pups (Vera-Massieu et al., 2015). Before building the GLMs, we examined differences in MHC transcription patterns between both sampling years. As we found no evidence that expression levels varied, we did not include sampling year as an explanatory variable in the models. Considering the moderate sample sizes of our study, we used a jackknife resampling approach to systematically rerun each GLM leaving out one observation at a time for each model (Sokal and Rohlf, 1995). Only variables that maintained significance after jackknife resampling were kept in the models. The main results were invariant under this resampling suggesting that our results are robust in spite of the sample sizes. All statistical analyses were performed in R version 3.0.2 (R Development Core Team, 2008). The R package bootstrap was used for jackknife resampling.

3. Results

Neither constitutive nor expressed *ZacaDRB* diversity was associated with pup responses to PHA at any of the age classes examined. However, when examining associations between particular *ZacaDRB* loci and inflammation, we found significant patterns that varied between age classes. For neonate pups, responses to PHA were related to *ZacaDRB-A*, where individuals that expressed this locus (14 out of 20) mounted a more pronounced inflammatory response (GLM; $F_{1,19} = 6.69$, $p = 0.0185$; Fig. 1). Pups that expressed *ZacaDRB-A* also had a more evident response to PHA when the numbers of circulating eosinophils were low (see Table S1 in Supplementary material). Eosinophil counts were a significant term in the model, and the overall fit of the model improved when including this variable (Fig. 1 in Supplementary material). None of the other expressed *ZacaDRB* loci helped explain variations in the

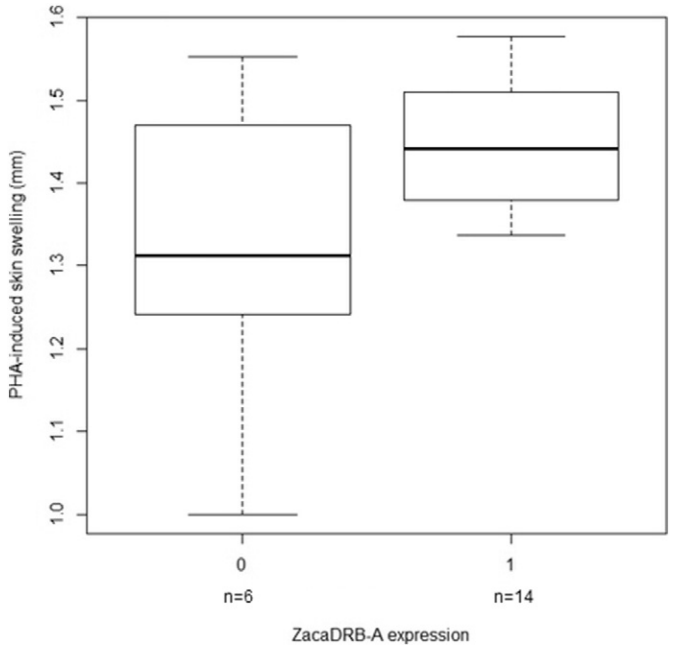

Fig. 1. PHA-induced swelling in neonate pups in terms of *ZacaDRB-A* transcription. Individuals whose peripheral antigen-presenting cells expressed *ZacaDRB-A* elicited more pronounced responses to the PHA challenge (0 = no transcription; 1 = transcription of that specific locus). The bold lines show the median responses for both categories, while the boxes encompass the quartiles and the whiskers indicate the endpoint data. The number of pups that expressed or not expressed the *ZacaDRB-A* locus is indicated beneath each column.