Proinflammatory Cytokines and Chemokines at the Skin Interface during Powassan Virus Transmission

TO THE EDITOR

Powassan virus (POWV) is an emerging North American tick-borne flavivirus transmitted to humans by infected tick bites. Ticks transmit pathogens during the complex feeding process of penetrating the skin and stay attached for several days to acquire blood. This process is facilitated by a repertoire of pharmacologically active proteins/ factors in tick saliva (Ribeiro et al., 2006; Kazimirová and Štibrániová, 2013). Thus, skin acts as the interface of the host–pathogen–vector interactions (Wikel, 2013). Skin provides the first line of defense against mechanical and environmental damage and infectious agents (Nestle et al., 2009). In a previous study, which examined cutaneous bite-site lesions from uninfected Ixodes scapularis nymphs, a rapid, proinflammatory progression of the early host response was identified, culminating in the infiltration of innate immune cells by 12 hours after tick infestation (Heinze et al., 2012). Successful transmission of tick-borne POWV has been shown to occur within 15 minutes of I. scapularis attachment (Ebel and Kramer, 2004). In addition, it was demonstrated that during early feeding time points the viral load in the tick salivary glands increases (Alekseev and Chunikhin, 1990). Therefore, the early cutaneous interactions between host immunity and initial tick-mediated immunomodulation are central to successful disease-causing agent transmission. In this study, we sought to characterize tick-induced changes in cutaneous gene expression at the early stages of attachment and feeding by POWV-infected I. scapularis nymphs. This will allow us to demonstrate the effect of a tick-borne virus on immune response at the tick–host interface.

In this study, we generated POWV-infected I. scapularis nymphs by synchronous infection (McNally et al., 2012) and allowed them to feed on 6-week-old female Balb/C mice. Uninfected ticks were used as control. Each treatment group consisted of four mice, each with a capsule containing one tick. At least three out of four mice had successful tick attachment at each time point, providing us with sufficient sample sizes to perform statistical analyses. Three and six hours after tick attachment (hours post infection, h.p.i.), 4 mm mouse skin biopsies were harvested along with the feeding ticks. Ticks and skin were checked for POWV infection, and all the infected ticks/skin biopsies used in this experiment contained POWV RNA. Total RNA was extracted from each skin biopsy and cutaneous immune responses were analyzed by pathway-specific PCR arrays (Supplementary Table S1 online). In total, 456 genes were analyzed with these arrays. Relative fold differences of the immune genes were calculated as previously described (Heinze et al., 2012). These data were then uploaded to ingenuity pathway analysis software for further analysis. Comparative analysis between POWV-infected and uninfected tick attachment sites at 3 and 6 h.p.i. was performed (Supplementary Table S2 online). When all significantly modulated (P<0.05) host genes in the uninfected versus POWV-infected 3 h.p.i. tick-feeding sites were taken into account, there were 40 upregulated genes and 11 downregulated genes (Figure 1a). Of all significantly modulated host genes in the 6 h.p.i. uninfected versus POWV-
infected tick-feeding sites, 13 were upregulated and 46 were downregulated.

The majority of modulated genes in the 3 h.p.i. comparison were significantly upregulated and ‘inflammatory response’ was the top associated reaction at this time point. Several proinflammatory cytokines, such as IL1B and IL6, were significantly upregulated (Figure 2a). IL36A, which is involved in the positive regulation of IL6 production, was also upregulated. IL1B, IL6, and IL36A all influence the quantity of phagocytes and neutrophils during the inflammatory response (Fielding et al., 2008; Rider et al., 2011). TLR4 is another molecule, which was significantly upregulated and associated with the inflammatory response (Figure 2d). Similar to IL1B and IL6, TLR4 is linked to innate immunity. It has been shown that the TLR4 signaling pathways are involved in the innate immune response to viral infections, leading to the induction of additional proinflammatory cytokines (Okumura et al., 2010). In addition, CCR3 was upregulated and it contributes to the chemotaxis of lymphocytes and eosinophils (Fahy et al., 2001). To various extents, IL1B, IL6, IL36A, TLR4, and CCR3 all help establish the proinflammatory environment, which is generated by the chemotaxis of certain immune cells. These relationships are best shown in Figure 1c network, which illustrates their central role in inflammatory response regulation during POWV-infected tick feeding at 3 h.p.i.

In contrast to 3 h.p.i. comparison, the majority of significantly modulated genes at 6 h.p.i. were downregulated, including several proinflammatory cytokines associated with the inflammatory response reaction: IL1B, IL18, IFNg, and tumor necrosis factor (TNF; Figure 2a). In Figure 1d, IFNG, IL1B, and TNF are the most connected to other molecules in this network, indicating that these three cytokines are heavily influenced by molecules that regulate the inflammatory response and cell-to-cell
signaling during POWV-infected tick feeding at 6 h.p.i. Although the majority
of genes modulated at 6 h.p.i. were downregulated, CCL2 was slightly
upregulated (Figure 2a). As CCL2 has chemotactic activity for monocytes
and basophils, this suggests that such immune cells are being recruited to
the bite site after a POWV-infected tick has been feeding for 6 hours.

Ingenuity pathway analysis identified several significantly modulated mole-
cules, which can either be classified under cell death and apoptosis
(Figure 2b) or under signal transduction (Figure 2c). The majority of all mole-
cules in Figure 2b and c were upregulated at 3 h.p.i. and downregulated at 6 h.p.i. Trem1 (triggering receptor expres-
sed on myeloid cells 1) and Traf6 (TNF receptor-associated factor 6) are
two molecules associated with signal transduction. In Figure 1d network, it is
evident that the 3 h.p.i. upregulation of such proinflammatory cytokines such as IL1B and IL6. Specifically, Trem1 stimulates
the release of such proinflammatory cytokines, which in turn amplify the
neutrophil and monocyte-mediated inflammatory response. Traf6 is a pro-
tein that facilitates signaling from the Toll/IL1 family and the TNF receptors. In
response to proinflammatory cytokines, Traf6 transduces signaling in the NF-κB
pathway (Wong et al., 1998).

Traf6 and the NF-κB pathway are also
linked with iNOS (inducible nitric oxide synthase) signaling. NOS2 (inducible
nitric oxide synthase 2) is a reactive free radical, which acts as a biologic
mediator in antimicrobial processes. It is inducible by a combination of lipo-
opolysaccharide and certain cytokines such as IFNγ and TNF (Lau et al., 1995).
IFNγR2 and TNF were both significantly
upregulated (Figure 2a and d), linking these cytokines to the host’s induction of
NOS2 after a POWV-infected tick has been feeding for 3 hours. This NOS2
pattern is consistent with the overall 3 h.p.i. upregulation of other molecules
associated with cell death (Figure 2b).

Finally, our PCR array data demon-
strated that at both 3 and 6 h.p.i., IL2 and IL4 were downregulated (Figure 2a).
Therefore, our data suggest that the host’s induced immune response to POWV-
infected tick feeding does not have a defined Th1 or Th2 profile. This further
supports our conclusion that a complex
proinflammatory environment exists, with
increased granulocyte recruitment, migra-
tion, and accumulation, specifically of
neutrophils, at the POWV-infected tick
feeding loci. Macrophages were also
predicted to undergo apoptosis. Compar-
ning POWV-infected with uninfected tick
feeding loci at 6 h.p.i. predicts decreased
recruitment of neutrophils and phago-
cyes. Our data clearly indicate that
POWV-infected tick feeding recruits
immune cells much earlier than the
uninfected tick feeding (Figures 1 and
2). This could be directly attributed to
POWV infection or changes in tick saliva
secretion, or a synergistic effect of both.
Further research needs to be conducted
to elucidate this phenomenon. To our
knowledge, this is the first report of the
early cutaneous response during POWV
transmission.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Immunological and Statistical Studies of Anti-BP180 Antibodies in Paraneoplastic Pemphigus

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TO THE EDITOR
Paraneoplastic pemphigus (PNP) shows clinically intractable stomatitis and conjunctivitis with polymorphous-cutaneous lesions (Anhalt et al., 1990; Hashimoto, 2001). Histopathology shows intraepidermal-acinolytic bullae and keratinocyte apoptosis (Oursler et al., 1992). Most common features revealed by direct immunofluorescence (IF) are deposition of IgG to keratinocyte cell surfaces and C3 to basement membrane zone (BMZ) (Anhalt et al., 1990; Hashimoto, 2001). In addition, in indirect IF, we encounter occasional reactivity with BMZ of normal skin, and more frequently with epidermal side of 1 M NaCl-split skin.

BP180 is a transmembranous collagenous protein, whose extracellular NC16a and C-terminal domains were identified as immune-dominant regions in bullous pemphigoid (BP) and mucous membrane pemphigoid, respectively (Giudice et al., 1992; Matsumura et al., 1996; Nie and Hashimoto, 1999; Zillikens et al., 1999; Hashimoto et al., 2012). Lamina lucida-type linear IgA bullous dermatosis reacts with LAD-1, truncated-extracellular domain of BP180 (Ishii et al., 2008). Previous mouse model studies revealed that anti-BP180 antibodies can induce blister formation (Zillikens et al., 1999), whereas the pathogenic role of BP230 is currently unclear.

Systemic study for autoantibodies to BP180 in PNP has not been performed, although a few PNP cases showed reactivity with BP180 (Preisz et al., 2004). Although reactivity with BMZ in PNP may be contributed mainly by anti-BP230 antibodies, we suspected a more frequent presence of antibodies to BP180 in PNP sera.

In this study, we investigated IgG anti-BP180 antibodies in 59 PNP patients by various methods. Materials and Methods are described in Supplementary Materials online. All results of IF, immunoblotting (IB), and ELISA studies are summarized in Supplementary Table S1 online. Clinical parameters examined are shown in Supplementary Table S2 online. The results of statistical analyses are

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