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Co-ordinated expression of innate immune molecules during mouse neurulation

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ABSTRACT

The innate immune system is the first line of defence against pathogens and infection. Recently, it has become apparent that many innate immune factors have roles outside of immunity and there is growing evidence that these factors play important functional roles during the development of a range of model organisms. Several studies have documented developmental expression of individual factors of the toll-like receptor and complement systems, and we recently demonstrated a key role for complement C5a receptor (C5aR1) signalling in neural tube closure in mice. Despite these emerging studies, a comprehensive expression analysis of these molecules in embryonic development is lacking. In the current study, we therefore, examined the expression of key innate immune factors in the early development period of neurulation (7.5–10.5 dpc) in mice. We found that complement factor genes were differentially expressed during this period of murine development. Interestingly, the expression patterns we identified preclude activation of the classical and alternative pathways and formation of the membrane attack complex. Additionally, several other classes of innate immune molecules were expressed during the period of neurulation, including toll-like receptors (TLR-2, -3, -4 and -9), receptor for advanced glycation end-products (RAGE), and their signalling adapters (TRAF-4, TRAF-6, TAK-1 and MyD88). Taken together, this study highlights a number of innate immune factors as potential novel players in early embryonic development.

1. Introduction

The first discovery of function of a protein often has the consequence of branding that factor with a label that is difficult to remove. This consequently may impart subtle and unintended bias on subsequent experimental endeavours. This is true for many innate immune factors; for example, the misguided labelling of complement factor C3a as a pro-inflammatory mediator (Coulthard and Woodruff, 2015). However, there are many examples of individual proteins possessing multiple, mutually-exclusive and context-dependent functions. Such bifurcation of function is particularly evident in the developmental context.

There are multiple recent reports of early developmental expression of innate immune factors (Carmona-Fontaine et al., 2011; Denny et al., 2013a; Hawksworth et al., 2014). In the adult, the hepatocytes of the liver are responsible for the production of many of the circulating factors of the innate immune system. In the developing mouse embryo, the liver bud forms around 9.5 dpc (Bort et al., 2006) and contains hepatoblasts, capable of differentiating to cholangiocytes and hepatocytes from 13.5 dpc onwards (Kung et al., 2010). The early hepatoblasts of the liver bud have been demonstrated to produce several circulating serum proteins, such as albumin and alpha-fetoprotein (Germain et al., 1988), however their ability to form the complement factors produced by the adult liver has not, to our knowledge, been assayed. Cells of the myeloid lineage likely to express the receptors of the innate immune system first appear in the yolk sac of the developing embryo, before migration into the aortic region and liver bud (Palis et al., 1999). These progenitor cells reach the primitive circulatory system around 8.5 dpc and the liver around 9.5 dpc (Palis et al., 1999).

Recently, our group demonstrated that the complement C5a receptor (C5aR1) is expressed by neuroepithelial cells during human and mouse neurulation, and is critical for proper mammalian embryonic development under conditions of folate-

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deficiency (Denny et al., 2013a). C5a is the ligand for C5aR1, and is produced as a cleavage product of complement factor C5 (Klos et al., 2013). In the immune system, C5a can be generated through four separate initiation pathways: classical, alternative, lectin and extrinsic protease, each with its own unique propagating zymogens (Manthey et al., 2009; Woodruff et al., 2014). Additionally, numerous regulators modulate the generation of C5a and the action of other cleavage by-products such as C5b, which goes on to form the terminal membrane attack complex (MAC) (Woodruff et al., 2011). Given that C5a may be generated through multiple pathways, and that complement generation and deposition is highly regulated in an immune context, there remains an unanswered question: how C5a may be generated and modulated in the context of mammalian embryonic development, given the importance of its canonical receptor in neurulation (Denny et al., 2013b).

Aside from the complement system, the innate immune system also comprises factors such as toll-like receptors (TLR), formyl peptide receptors (FPR), chemokines, and cytokines. This diverse collection of molecules acts to immediately recognise, opsonise and neutralise pathogens and damaged cells either directly or through the recruitment of leukocytes. In addition, these factors stimulate the activity of the adaptive immune system to respond to the pathogenic insult. Interestingly, whilst many of these factors have well-established roles in innate immunity, many of them were either first discovered, or have recently been discovered, to have non-immune functions (Hashimoto et al., 1988; Hori et al., 1995; Lathia et al., 2008; Okun et al., 2010).

Toll-like receptors were initially discovered for roles in dorsoventral patterning of developing drosophila larva (Hashimoto et al., 1988) and were only later demonstrated to be part of a larger family with roles in pathogen recognition. In mice, this family extends to 13 members, each recognising molecular patterns associated with tissue damage or pathogen invasion (Arumugam et al., 2009). Conversely, the complement system has long been investigated as an important player in innate immunity, and only recently ascribed non-immune roles (Carmona-Fontaine et al., 2011; Denny et al., 2013a; Haynes et al., 2013; Kimura et al., 2003; McLin et al., 2008; Rahpeymai et al., 2006; Rooryck et al., 2011).

McLin and colleagues demonstrated that a number of complement factors are expressed during the early development of xenopus (McLin et al., 2008). This has piqued interest in what early developmental processes complement may be facilitating. Subsequently, complement C3a and its cognate receptor C3aR have been demonstrated to direct cranial neural crest cell chemotaxis (Carmona-Fontaine et al., 2011), and the complement lectin pathway components, collectin 11 (collec11) and MASP1, have also emerged as important participants in craniofacial development (Rooryck et al., 2011).

Given the pleiotropic nature of many innate immune molecules, it is not clear if an early, non-immune developmental role is unique to complement or whether expression of innate immune factors during development is a broader phenomenon. This study, therefore, sought to investigate which innate immune factors were expressed in early stages of embryonic development, specifically during the period of neural tube formation and closure (7.5–10.5 dpc).

2. Methods

2.1. Tissue collection

Animal breeding and tissue collection were performed in accordance with guidelines from the National Health and Medical Research Council of Australia and was approved by the University of Queensland Animal Ethics Committee. Pregnant C57BL/6J dams at 7.5–10.5 days post conception (dpc) were obtained from University of Queensland Biological Resources for use in this study. The day of vaginal plug discovery was designated 0.5 dpc. Mice were sacrificed by cervical dislocation and embryos were removed from the uterus and extra-embryonic membranes and transferred into a 4% formaldehyde/PBS solution freshly prepared from paraformaldehyde, or snap-frozen in liquid nitrogen.

2.2. RT-PCR

Total RNA was obtained from aggregated litters at 7.5, 8.5, 9.5 and 10.5 dpc using RNeasy kit (Qiagen, The Netherlands) according to the manufacturer's instructions. RNA was DNase-treated (TurboDNase, Life Technologies, USA) and 1 μg of total RNA was reverse transcribed (Tetro RT, Bioline, UK). Specific primers for each gene were designed using the NHMC primer design tool. The primers and corresponding annealing temperatures used are detailed in appendix A.

2.3. *Section* in situ *hybridisation*

Section *in situ* hybridisation was performed as per Simmons et al. (Simmons et al., 2007). Briefly, cRNA digoxigenin-labelled probes were generated through ligation of PCR products into a pGEM-Teasy vector (Promega, USA) and transcription with SP6 and T7 RNA polymerase (Promega). Resulting probes were diluted 1:2000 in hybridisation buffer and hybridised overnight on 16 μm 9.5 dpc embryo sections at 65 °C. Following washes with 50% formamide and RNase treatment to remove excess probe, sections were blocked and exposed to anti-digoxigenin-AP antibody (Roche, Switzerland) at 1:2500 dilution overnight at 4 °C. For color development, embryos were incubated in 40 mg/ml NBT and 20 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Roche, Switzerland). Slides were re-fixed in 4% paraformaldehyde, mounted with DEPEX (Sigma–Aldrich, Missouri) and imaged using a digital slide scanner (Aperio, AT2).

2.4. *Whole mount* in situ *hybridisation*

In situ hybridization was performed as per Christiansen et al. (Christiansen et al., 1995). Briefly, embryos at 9.5 dpc were fixed overnight at 4 °C in 4% paraformaldehyde/0.01 M PBS, and taken through a dehydration and rehydration series with methanol. Embryos were permeabilized using 10 mg/ml proteinase K and incubated at 65 °C overnight with 0.5 mg cRNA probe. After removing excess probe, embryos were blocked with 10% goat serum/2% BSA in TBS. Alkaline phosphatase-conjugated anti-digoxigenin IgG (Roche), preadsorbed against embryo antigens, was added in the pre-blocking solution and incubated overnight at 4 °C. For color development, embryos were incubated in 40 mg/ml NBT and 20 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Roche). Embryos were taken through an ethanol dehydration series to remove excess color, re-fixed in 4% paraformaldehyde overnight, and imaged using a stereomicroscope (Leica, M205 FA stereomicroscope).

3. Results and discussion

In the present study, we demonstrate that complement factors normally produced for circulation by adult hepatocytes were also expressed in the murine embryo during the period of neurulation. This period coincides with the early stages of liver development and the movement of myeloid progenitors into the embryo proper. Indeed, in this study, we demonstrate the clear presence of the myeloid cell marker CD11b at 9.5 and 10.5 dpc, whereas it was beyond detection at 7.5 dpc. This coincides with the migration of

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