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Research paper

Isolation, characterization, and functional analysis of ferret lymphatic endothelial cells

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ABSTRACT

The lymphatic endothelium (LE) serves as a conduit for transport of immune cells and soluble antigens from peripheral tissues to draining lymph nodes (LNs), contributing to development of host immune responses and possibly dissemination of microbes. Lymphatic endothelial cells (LECs) are major constituents of the lymphatic endothelium. These specialized cells could play important roles in initiation of host innate immune responses through sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including toll-like receptors (TLRs). LECs secrete pro-inflammatory cytokines and chemokines to create local inflammatory conditions for recruitment of naïve antigen presenting cells (APCs) such as dendritic cells (DCs) to sites of infection and/or vaccine administration. In this study, we examined the innate immune potential of primary LEC populations derived from multiple tissues of an animal model for human infectious diseases – the ferret. We generated a total of six primary LEC populations from lung, tracheal, and mesenteric LN tissues from three different ferrets. Standard RT-PCR characterization of these primary LECs showed that they varied in their expression of LEC markers. The ferret LECs were examined for their ability to respond to poly I:C (TLR3 and RIG-I ligand) and other known TLR ligands as measured by production of proinflammatory cytokine (IFNα, IL6, IL10, Mx1, and TNFα) and chemokine (CCL5, CCL20, and CXCL10) mRNAs using real time RT-PCR. Poly I:C exposure induced robust proinflammatory responses by all of the primary ferret LECs. Chemotaxis was performed to determine the functional activity of CCL20 produced by the primary lung LECs and showed that the LEC-derived CCL20 was abundant and functional. Taken together, our results continue to reveal the innate immune potential of primary LECs during pathogen-host interactions and expand our understanding of the roles LECs might play in health and disease in animal models.

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1. Introduction

The lymphatic vasculature (LV) is often described as a network of unidirectional, blind-ended capillaries and larger collecting vessels made up of a single layer of loosely overlapping cells – lymphatic endothelial cells

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(LECs) (Jurisic and Detmar, 2009; Wang and Oliver, 2010). Since the LE is often located within micrometers beneath mucosal surfaces, they are likely to be among the first cells to participate in early host innate immune responses upon contact with microbes, host inflammatory signals, and vaccine antigens. LECs secrete chemoattractant cytokines (chemokines), such as CCL20, which recruit immature DCs to sites of inflammation, and CCL21, which draws antigen-loaded mature DCs into the collecting lymphatic vessels and then downstream into the LN paracortices, wherein a unique environment is created to optimize activation of adaptive immune responses (Luther et al., 2000; Saeki et al., 1999).

Model human LECs express functional toll-like receptors (TLRs) that recognize multiple pathogen-associated molecular patterns (PAMPs) on microbes (Garrafa et al., 2011; Pegu et al., 2008). TLR activation results in signaling that triggers the production of pro-inflammatory cytokines and chemokines, including type I interferons (IFNs), that are crucial not only for pathogen clearance during innate responses, but also enhance the induction of antigen-specific responses during subsequent adaptive immunity (Akira and Takeda, 2004; Beutler, 2009). Thus, the use of TLR ligands as vaccine adjuvants to increase vaccine efficacy in inducing host immune responses is an attractive strategy for development of next generation vaccines. Recent studies on the use of TLR ligand-conjugated vaccines have been promising in non-human primates (Kwissa et al., 2012) as well as small animal models such as mice (Zhang and Matlashewski, 2008; Zhu et al., 2010) and ferrets (Fang et al., 2010). However, different TLR ligand adjuvants mediate distinct cellular and molecular profiles of early innate responses in the periphery and the lymphatic organs of non-human primates (Kwissa et al., 2012). Despite their potential, there is still limited understanding of the local and systemic immune responses and potential toxicities associated with their use in vivo.

Ferrets are becoming an increasingly examined small animal model for the study of human diseases, including neurobiology (Atkinson et al., 1989; Bock et al., 2010; Medina et al., 2005), cancer (Kim et al., 2006), and infectious diseases of viral and bacterial origin (Chu et al., 2008; Geisbert et al., 2010; Martina et al., 2003; Svitek and von Messling, 2007; Woods et al., 2002). The use of the ferret as an animal model for studying human respiratory diseases has offered a number of advantages. First, ferret airways resemble and share many anatomical and physiological similarities to humans making them useful for study of human respiratory infections (Bruder et al., 2010). In addition, ferrets are highly susceptible to a number of human respiratory pathogens that often require no laboratory adaptation prior to infection (Belser et al., 2011; O'Donnell and Subbarao, 2011). Furthermore, ferrets are considered an accurate small animal model to study both human and avian influenza (Bouvier and Lowen, 2010). In this regard, the ferret model is used to study not only seasonal and highly pathogenic avian influenza virus pathogenicity, but also viral transmission (Herfst et al., 2012; Russell et al., 2012) and the development of vaccines and antiviral therapeutics (Banner and Kelvin, 2012).

Despite the utility and increasing use of this animal model there is still a major lack of ferret-specific reagents for use in research, despite efforts that have been invested to obtain reagents to enable development of ferret-specific assays at the cellular and molecular levels (Bruder et al., 2010; Camp et al., 2012). Molecular cloning and phylogenetic analysis of ferret immune-related genes provides tools to assess the inflammatory cytokine and chemokine profiles in infected animals and determine their importance in disease progression and/or clearance of infection (Nakata et al., 2008; Qin et al., 2013). The expression of functional TLRs by human LECs (Pegu et al., 2008) has highlighted that the LE could be a target for new vaccine adjuvancy strategies, alongside monocytes and DCs. In this light, we isolated, cultured, and characterized primary LECs from multiple ferret tissues, and determined their responsiveness to known TLR ligands by measuring the production of proinflammatory cytokine and chemokine mRNAs using real time RT-PCR. In addition, we also cloned and sequenced ferret LEC marker partial cDNAs for in situ hybridization (ISH) analysis to probe the lymphatic vasculature in ferret tissues. Altogether, these findings provide insight into the function and microanatomy of ferret lymphatics and establish a foundation for examination of the roles of LECs during infection and immunization.

2. Methods

2.1. Ferrets and tissue processing

The ferrets from which tissues for histological analysis and isolation of LECs were obtained were available from other non-infectious studies, and were 6–7 month old females that ranged in weight from 695 to 825 g. These ferrets were vaccinated for Canine Distemper virus, descented, and single housed at the University of Pittsburgh. All animal work was approved by the University of Pittsburgh Institutional Animal Care and Use Committee, although the tissues contributing to these studies were excess tissues available at necropsy.

2.2. RT-PCR, cloning, and phylogenetic analysis of ferret LEC markers

Due to the lack of complete ferret genomic sequence information, design of ferret-specific primers for amplification of ferret cDNAs, including LEC markers (Table 1), was based on published canine sequences available in GenBank, National Center for Biotechnology Information (NCBI). Previous analyses of ferret cytokine cDNAs reported that ferret sequences were closely related to canine sequences (Nakata et al., 2008). Total cellular RNAs were obtained from ferret lung, spleen, and LN tissues, both untreated and stimulated overnight with unmethylated CpG oligonucleotides (ODN), poly I:C, or lipopolysaccharide (LPS). RNA extractions were performed using Trizol (Life Technologies, Rockville, MA, USA) according to the manufacturer's recommendation. Total RNA (2 μg) was reverse transcribed using oligo-dT primer (Promega, Madison, MI, USA) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, MI, USA). The resulting cDNAs for each tissue

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