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Characterization of immune modulating functions of γδ T cell subsets in a gnotobiotic pig model of human rotavirus infection

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ABSTRACT

We characterized immune modulating functions of porcine γδ T cell subsets in rotavirus infection using a gnotobiotic pig model of human rotavirus infection and sort-purified lymphocyte autologous co-cultures. We demonstrated that CD2+CD8− and CD2−CD8− γδ T cells have mainly pro-inflammatory function as evident by directly secreting IFN-γ or promoting CD4+ αβ T cell proliferation and IFN-γ production, whereas CD2+CD8+ γδ T cells mainly exert regulatory T cell function by expressing FoxP3, secreting IL-10 and TGF-β or increasing IL-10 and TGF-β production by CD4+ αβ T cells. γδ T cells responded to rotavirus infection by increasing TLR2, TLR3, TLR9 expression and IFN-γ and/or TGF-β production. The CD8− subsets likely differentiate into CD8+ subset by acquiring CD8 expression, explaining in part the apparently dual functions of CD2+CD8+ and CD2+CD8− subsets. Thus, both CD8+ and CD8− γδ T cell subsets can contribute to anti-rotavirus immunity and to the maintenance and restoration of intestinal and systemic homeostasis.

1. Introduction

Studies of γδ T cells in humans, mice and cattle have revealed the diverse immune functions of different γδ T cell subsets; however the immune functions of porcine γδ T cell subsets have not been clearly identified. Pigs are the most important large animal models for human biomedical research and are also used as donors for xenotransplantation, thus characterization of the immune modulating functions of porcine γδ T cell subsets is vitally important. Because the lack of monoclonal antibodies distinguishing specific variable (V) and diversity (D) segments of porcine γδ T cell TCRs (*i.e.*, the Vγ and Vδ antibodies used for studying γδ T cell subsets in humans and mice), the equivalent phenotypes between human and porcine γδ T cell subsets have not been determined. However, all mammalian species studied share two principal subsets of γδ T cells in terms of their distributions. One subset is predominant in tissues including mucosal and skin surfaces (Vδ1 in humans and mice; WC1−CD8+ in cattle, and CD2+CD8+ in pigs), and the other subset is predominant in the blood (Vδ2 in humans; Vγ1 in mice, WC1+ in cattle, and CD2−CD8− in pigs) [1–5]. Functionally, γδ T cells in humans, mice and cattle have also been divided into two principal subsets: the pro-inflammatory and anti-inflammatory subsets [6]. Circulatory γδ T cells are mainly pro-inflammatory and act as primary responders for invading pathogens, including viruses, bacteria and parasites; whereas tissue-specific γδ T cells have pro-inflammatory and anti-inflammatory dual functions depending on the anatomic locations and the microenvironment. γδ T cells located in mucosal and skin surfaces are largely anti-inflammatory and contribute to the maintenance of the epithelial integrity.

Phenotypically, porcine γδ T cells are defined by the monoclonal anti-porcine antibody Tcr1-N4 (clone PGBL22A) that recognizes a determinant on a constant region of porcine T cell receptor (TCR) δ chain [7]. The total γδ T cells are divided into three subsets based on the surface

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expression of CD2 and CD8αα (CD2+CD8+, CD2+CD8− and CD2−CD8−) [8]. Our previous study focusing on porcine γδ T cell subset distribution and kinetics in response to enteric virus infection showed that rotavirus infection significantly increased frequencies of CD2+CD8+ γδ T cell subset and decreased the CD8− subsets in ileum, spleen and blood of gnotobiotic (Gn) pigs at post-inoculation days (PID) 3–5 [4]. However, the exact immune functions of each porcine γδ T cell subset are yet to be identified.

γδ T cells can respond directly to pathogen-associated molecular patterns (PAMPs) without any help from antigen presenting cells (APCs) by increasing PAMP receptor expressions, such as Toll-like receptors (TLRs) [9]. Freshly isolated human γδ T cells have been stimulated *via* the TCR ligand in the presence of poly(I:C) without the presence of APCs to enhance IFN-γ production [10], which indicated that human γδ T cells express the poly(I:C) receptor TLR3. Also, human Vγ2Vδ2 T cells expressed TLR2 mRNA and produced IFN-γ under the stimulation of a TLR2 agonist (Pam3Cys) [11]. Pietschmann et al. [12] compared the different TLR expression patterns among human Vδ1 and Vδ2 T cells and found that TLR2, TLR3 and TLR6 proteins were detected in both Vδ1 and Vδ2 T cells. Mouse γδ T cells were also found to express TLR2 and TLR4 [13]. However, it is unknown if porcine γδ T cells express TLRs and whether TLR expressing γδ T cells are involved in the immune responses to rotavirus infection.

The objective of the present study is to characterize the immunological functions of the three porcine γδ T cell subsets in the face of enteric virus infections. The frequencies of TLR2, TLR3, TLR9 and FoxP3 expression, and IFN-γ and TGF-β production among the three γδ T cell subsets in Gn pigs were determined after human rotavirus (HRV) inoculation. The cytokine production profiles of the three γδ T cell subsets after phosphoantigen isopentenyl pyrophosphate (IPP) stimulation and the influence of each γδ T cell subset on CD4+ αβ T cell cytokine production and proliferation in sort-purified autologous co-cultures were examined by using enzyme-linked immunosorbent (ELISA) and multi-color flow cytometry assays. Our findings indicate that, similar to other mammalian species studied previously (humans, mice and cattle), porcine γδ T cells express TLRs and can be divided into two major functional subsets, the pro-inflammatory (CD2+/−CD8−) and the anti-inflammatory (CD2+CD8+) subsets. CD2+/−CD8− γδ T cells can exert anti-viral effector cell functions by producing IFN-γ and promoting the proliferation and IFN-γ production by CD4+ αβ T cells. CD2+CD8+ γδ T cells from the intestine and spleen have characteristics of regulatory cell functions (express FoxP3 and produce TGF-β and IL-10). However, CD2+CD8+ γδ T cells also have anti-viral functions (produce IFN-γ) in early stage of rotavirus infection.

2. Materials and methods

2.1. Viruses and inoculums

The virulent Wa strain HRV (G1P1A [8]) (from Dr. Linda Saif, The Ohio State University) was passaged through Gn pigs and the pooled intestinal contents from the 27th passage were used for inoculation or challenge of Gn pigs at a dose of 1×10^5 fluorescent focus-forming units (FFU). The median infectious dose (ID_{50}) of the HRV in Gn pigs was approximately 1 FFU [14]. The HRV infection in Gn pigs was confirmed by fecal virus shedding using ELISA and cell culture immunofluorescence (CCIF) assays as previously described [14].

The cell-culture adapted attenuated Wa strain HRV (from Dr. Linda Saif, The Ohio State University), derived from the 34th passage in African green monkey kidney cells (MA104) [15] was used for inoculation of a subgroup of Gn pigs at 5×10^7 FFU/dose and was used to prepare semi-purified Wa HRV antigen by centrifugation through a 40% sucrose cushion as described [16]. The semi-purified Wa HRV antigen was used in the CD4+ T cell proliferation assay in the co-culture studies.

2.2. Inoculation of gnotobiotic pigs

Near-term pigs of Landrace and Large White cross breed were derived from pregnant sows by surgery and maintained in germ-free isolator units as described [17]. Pigs were fed with commercial ultra-high temperature (UHT)-treated sterile milk. All pigs were confirmed germ-free prior to rotavirus or norovirus exposure. Pigs were given 8 ml of 100 mM sodium bicarbonate to reduce gastric acidity 10 min before virus inoculation. All animal experimental procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committees of Virginia Polytechnic Institute and State University.

For the study of TLR expressing γδ T cell responses [PID 0 ($n = 7$), 3 ($n = 7$) and 5 ($n = 4$)] and IFN-γ [PID 0 ($n = 7$), 3 ($n = 4$) and 5 ($n = 4$)] or TGF-β [PID 0 ($n = 3$) and 5 ($n = 4$)] producing γδ T cell responses, Gn pigs (both males and females) were randomly assigned to the HRV and mock control groups. At 5 days of age (PID 0), Gn pigs in HRV groups were orally inoculated with 1×10^5 FFU virulent Wa HRV in 5 ml of Dulbecco's modified Eagle's medium (DMEM). Control pigs were given an equal volume of the diluent. The pigs were euthanized on PID 0, 3 and 5 to isolate mononuclear cells (MNCs) from ileum, spleen and peripheral blood as described [15].

In the following studies, Gn pigs were inoculated with rotavirus or norovirus and colonized with or without a probiotic lactobacilli strain, as specified in each experiment. These Gn pigs were shared with other research projects. MNCs from these Gn pigs were used in the present study because the inoculations and ages do not affect the interpretation of the data regarding the function of each γδ T cell subset and the use of animal resources was maximized. In the studies of FoxP3 expression among γδ T cell subsets and antigen presenting cell (APC) function of γδ T cells, Gn pigs were mono-associated with (FoxP3 expression study, $n = 3$) or without (APC function study, $n = 3$) the *Lactobacilli acidophilus* NCFM strain as we previously described [18]. The pigs were inoculated with two oral doses of attenuated Wa HRV at 5×10^7 FFU/dose in 5 ml of DMEM at 5 (PID 0) and 15 (PID 10) days of age, challenged with 1×10^5 FFU of virulent Wa HRV on PID 28, and euthanized on PID 35 [post-challenge days (PCD) 7]. MNCs from ileum, spleen and peripheral blood were isolated as previously described