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Innate immunity in an *in vitro* murine blastocyst model using embryonic and trophoblast stem cells

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The immune system has two broad components—innate and adaptive immunity. Adaptive immunity becomes established only after the onset of hematopoiesis, whereas the innate immune system may be actively protecting organisms from microbial invasion much earlier in development. Here, we address the question of whether the innate immune system functions in the early-stage embryo, i.e., the blastocyst. The innate immune system was studied by using *in vitro* blastocyst models, e.g., embryonic stem (ES) and trophoblast stem (TS) cell cultures. The expression of Toll-like receptors (TLR)-2, -3, and -5 could be detected in both ES and TS cells. The expression of interferon (IFN)-β was induced by the addition of polyinosinic:polycytidylic acid [poly(I:C)] in TS cells, but not ES cells, although TLR-3 was expressed at the same level in both cell types. In turn, ES cells responded to IFN-β exposure by expressing IFN-induced anti-viral genes, e.g., RNA-dependent protein kinase and 2′, 5′-oligoadenylate synthetase (OAS). Neither a reduction in ES cell proliferation nor cell death in these cultures was observed after IFN-β stimulation. Furthermore, OAS1a expression was induced in ES/TS co-cultures after poly(I:C) stimulation, but was not induced when either cell type was cultured alone. In conclusion, TS cells react to poly(I:C) stimulation by producing IFN-β, which induces IFN-inducible genes in ES cells. This observation suggests that the trophectoderm, the outer layer of the blastocyst, may respond to viral infection, and then induce anti-viral gene expression via IFN-β signaling to the blastocyst inner cell mass.

[**Key words:** Innate immunity; Blastocyst; Trophoblast stem cell; Embryonic stem cell; Toll-like receptors; Type-I interferon]

The immune system largely consists of two broad components—innate and adaptive immunity (1). In turn, adaptive immunity comprises humoral immunity, largely governed by B cells that produce high-affinity antibodies, and cellular immunity, largely governed by T cells that recognize processed peptide antigens presented by antigen presenting cells in the context of the major histocompatibility complex proteins. During development, the adaptive immune system is established after the initiation of hematopoiesis, which gives rise to B and T cells, among others (2). By contrast, innate immunity is an evolutionarily conserved system that provides a first line of protection against invading microbial pathogens and is activated by Type-I and III interferons (IFNs) (3,4). Most types of mammalian cells can produce IFNs upon viral infection by sensing cytoplasmic non-self DNA or RNA, or by recognizing pathogenic molecular signatures via 'pattern recognition receptors', e.g., Toll-like receptors (TLRs), Sting, and RIG-I–like receptors. TLRs are evolutionarily conserved membrane proteins that are critical for the activation of mammalian innate immunity (5). TLR signaling leads to the production of IFNs, which, in turn, induce the expression of anti-viral proteins, such as RNA-dependent protein kinase (PKR) and 2′, 5′-oligoadenylate synthetase (OAS) (3).

The innate immune system is functional in insects (6–8), which have no lymphocytes (T or B cells). Because the mammalian embryo, prior to the onset of hematopoiesis, has no adaptive immune system, another defense system is necessary. The innate immune system is functional in mice by embryonic day 12–13 post-coitum (E12–13) as demonstrated by the fact that mouse embryonic fibroblasts (MEFs), which are generally prepared from E12–13 embryos, have a strong innate immune response, i.e., IFN-β expression is strongly induced after TLR stimulation (9–11). The innate immune system likely plays a major role in protecting against microbial invasion before hematopoiesis is established. Hematopoiesis begins in the yolk sac at E7.5 (12). The purpose of this study was to investigate whether the innate immune system has been established in the early-stage embryo, i.e., in the blastocyst before implantation into the uterus. The blastocyst consists of the inner cell mass (ICM), which gives rise to the body of the organism, including the germ cells, and the trophectoderm, which gives rise to the placenta. The blastocyst is surrounded by the zona pellucida (ZP). Early-stage embryos, from the non-fertilized egg to the pre-hatched blastocyst, are surrounded by the zona pellucida,

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which may serve as a barrier from viruses and bacteria that exist in the oviduct and uterus. After the blastocyst hatches from and releases the zona pellucida, the bare blastocyst is exposed to infectious hazards, such as viruses and bacteria in the uterus. Because embryonic stem (ES) cells are derived from the ICM (13), ES cells are thought to be an *in vitro* model of ICM, and their differentiated cells might mimic the developing embryo. The ICM is surrounded in the blastocyst by a trophectoderm layer. Some foreign substances could pass through the ZP and come into contact with the trophectoderm. Because the trophoblast stem (TS) cell line is derived from blastocyst trophectoderm, it might be useful to investigate the behavior of TS cells as an *in vitro* model of the immunological roles of trophectoderm in blastocyst development.

Due to the comparatively easier access to ES cells as compared to TS cells, there are a few reports about the expression of TLR*s* in ES cells derived from the 129 mouse strain (14–16), but no reports as yet in TS cells. In this study, we began by establishing a mouse TS cell line from the BALB/cA mouse strain, which is of the same origin as the mouse ES cell line, ST1 (17). Then, we investigated the TLR expression patterns and innate immune responses in ES and TS cells, and their co-culture as an *in vitro* blastocyst model, to test whether the blastocyst may have the components for a functioning innate immune system.

MATERIALS AND METHODS

Preparation and culture of early-stage mouse embryos 2.5-day embryos were collected from superovulated BALB/cAJcl female mice (CLEA Japan, Tokyo, Japan) crossed with BALB/cAJcl male mice (CLEA Japan). 2.5-day embryos were cultured for one day in Brinster's BMOC-3 medium (Life Technologies Japan, Tokyo, Japan) to develop into blastocysts. The animal protocol was approved by the Animal Experimentation Committee of the Tokyo Institute of Technology.

Cell culture All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. The mouse ES cell line, ST1, was originally established from the BALB/c strain (17). ST1 cells were grown on feeder layers of mitomycin C–treated MEFs in order to maintain stem cells in an undifferentiated state, as previously described (18). In this study, to remove MEF cells, ST1 cells were cultured on gelatin-coated plates without the MEF feeder layer in ESGRO Complete PLUS Clonal Grade medium (Chemicon, Temecula, CA, USA). TS cell line, CAT1, was originally established on MEF feeder layer in TS medium supplemented with 25 ng/mL human recombinant FGF4 (R&D Systems, MN, USA) and 1 μg/mL heparin (Sigma–Aldrich Japan, Tokyo, Japan). TS medium is RPMI 1640 medium (Life Technologies Japan) with 20% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 1 mM sodium pyruvate (Life Technologies Japan), 100 μM 2-mercaptoethanol (Sigma–Aldrich Japan), 2 mM L-glutamine, penicillin and streptomycin (Life Technologies Japan) (19). MEF conditioned medium was collected after three days culture on MEF feeder cells in TS medium. To remove MEF cells for this study, CAT1 cells were cultured on gelatin-coated dishes in 70% MEF conditioned medium/TS medium with FGF4 and heparin. The mouse monocyte–macrophage cell line, RAW264 (RIKEN Cell Bank, Ibaraki, Japan), was cultured in MEM supplemented with 10% FBS and penicillin and streptomycin (Life Technologies Japan). MEF was cultured in DMEM (Life Technologies Japan) with 10% FBS, penicillin and streptomycin (Life Technologies Japan). For co-cultures stimulated by polyinosinic:polycytidylic acid [poly(I:C)], we used GFP-expressing ST1 and DsRed-expressing CAT1.

Establishment of the cell lines carrying DsRed2 or AcGFP expression vector The expression vectors of DsRed2 and AcGFP fluorescence protein genes, pCAGDsRed2-neo and pCAGAcGFP-neo, respectively, were controlled by the CAG promoter (20–23). These expression vectors were introduced in the CAT1 or ST1 cells by the electroporation method as described previously (23). Briefly, 1×10^7 cells/0.9 mL in PBS of the cells was mixed with 25 μg of linearized pCAGDsRed2-neo/Eco RI or pCAGAcGFP-neo/Eco RI, respectively, and were transfected by electroporation method. After electroporation, the cells were seeded onto 100-mm diameter gelatin-coated plastic dishes. The culture medium containing 400 μg/mL or 1 mg/mL G418 was changed at a day after the electroporation. G418-resistant colonies were picked up. The clones were maintained as well as the parental cells were. The expression of those clones was confirmed by the flow cytometer (Beckman Coulter, CA, USA), and then finally each clone with the strongest expression of the DsRed2 or AcGFP gene was established as $CAT1^{Red}$ or $ST1^{Green}$ cell line, respectively.

RT-PCR and real-time PCR Total RNA was isolated using a kit from Kurabo (Tokyo, Japan). After genomic DNA was removed by treatment with DNase I (Promega, Tokyo, Japan), the RNA samples (5 μg) were reverse transcribed using a Superscript II First-Strand Synthesis System Kit (Life Technologies Japan) with an oligo(dT) primer (Life Technologies Japan). PCR was performed using Ex Taq DNA polymerase (Life Technologies Japan) with the primer sets shown in Table 1. For the quantitative analysis of IFN-β, OAS1a, and PKR expression, real-time PCR was carried out using the StepOnePlus Sequence Detection System (Applied Biosystems, Tokyo, Japan). Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) was used as an internal housekeeping reference gene. Gene expression was quantified using the $\Delta\Delta C_T$ method. Primer sequences are listed in Table 1.

TABLE 1. Primer information for RT-, real-time, and genomic PCR.

primer	Forward Sequence	Reverse Sequence
Toll-like receptor 1	GAGTGTTTGTGAATGCAGTTGG	TAGCTCATTGTGGGACAAATCC
Toll-like receptor 2	TAGGGGCTTCACTTCTCTGC	GAGACTCCTGAGCAGAACAGC
Toll-like receptor 3	ACTGGATGGCCATTTTTACC	AGAGAACAGGTGCGTCAACC
Toll-like receptor 4	TTCTTCTCCTGCCTGACACC	TTCCTGGGGAAAAACTCTGG
Toll-like receptor 5	CCAGACACATCTGTGAGACACC	GCATCTCGATTCAAGACTTCG
Toll-like receptor 6	AAGAAAATGGTACCGTCAGTG CTGG	AAGGCCAGGGCGCAAACAAAGT
Toll-like receptor 7	CCTTCAAGAAAGATGTCCTTGG	GGAGAGATGCTTGGTATGTGG
Toll-like receptor 8	GAGAAACAAACGTTTTACCTTCC	TTTCAAAGACTCAGGCAACC
Toll-like receptor 9	TCTGAGAGACCCTGGTGTGG	AAACGGGGTACAGACTTCAGG
RIG-I	TGCCACAGTCAGAGACAACG	TGCCATCTGAAACACTGAGC
MDA5	GCGGGAATGAGTCAGGTGTAA	GGCTCGGGGGATACTCTTTTT
IPS-1/MAVS	TGCTGTGTTGACGTTCTGG	AAACTCGAGTCACTTGATCAGC
Sting	CCCGAGTCTCGAAATAACTGC	TGAGGAGTCTTGGCTCTTGG
Oct3/4	TTGGAGAAGGTGGAACCAAC	AGATGGTGGTCTGGCTGAAC
Cdx2	GAAACCTGTGCGAGTGGATG	GCAGCCAGCTCACTTTTCCT
IFNAR1	ATGGGACTACATTGCGTCTGC	TTCTTGAGGGTGAACTCTGG
Hprt	GTAATGATCAGTCAACGGGG	AGCTTTACTAGGCAGATGGC
IFN-β (real-time)	CAGGCAACCTTTAAGCATCAG	CTTTGACCTTTCAAATGCAG
PKR (real-time)	GGAAAATCCCGAACAAGGAG	CCCAAAGCAAAGATGTCCAC
OAS1a (real-time)	TGTCCTGGGTCATGTTAATAC	CCGTGAAGCAGGTAGAGA
Hprt (real-time)	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
Sry (genomic)	GCCCTTTTTCCAGGAGGCA	CAGTGGGGATATCAACAGGCT
Hprt (genomic)	TCTCGAAGTGTTGGATACAGGC	AGCTTTACTAGGCAGATGGC

Genomic PCR Genomic DNA was prepared from mouse cells or tissue using a kit from Kurabo. Genomic PCR was performed using Ex Taq DNA polymerase (Life Technologies Japan) with the primer sets listed in Table 1.

Immunostaining ES and TS cells were cultured on gelatin-coated glass coverslips in a six-well plate. Colonies of ES or TS cells were fixed with 4% paraformaldehyde/PBS for 10 min and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. The fixed samples were incubated in Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 30 min, and then incubated with the primary antibody for 2 h, followed by the secondary antibody for 1 h at room temperature. The following antibodies were used: rabbit immunoglobulin (IgG) anti Oct3/4 (1:400; Santa Cruz Biotech), rabbit IgG anti Cdx2 (1:50; Cell Signaling Technology). DAPI was used for nuclear staining.

Cell proliferation assay Cells were seeded on a gelatin-coated 96-well plate at 5×10^3 cells/well, in the appropriate medium for ES or TS cell culture ± different concentrations of TLR ligands. The proliferative rates of these cells were evaluated 36–60 h after poly(I:C) treatment using a WST-8 cell viability test kit (Nacalai Tesque).

RESULTS

Establishment of a TS cell line Morulae were collected from BALB/cA female mice 2.5 day post-coitum (dpc), and were cultured to blastocysts. Three blastocysts were cultured on the feeder layer in the TS medium. During the culture, the blastocysts hatched and attached to the feeder layer within 2 days. On day 3–4, the blastocyst outgrowths emerged and grew out. The growing outgrowths were trypsinized into single cells, which were sown on the feeder layer again. When TS-like colonies became visible on culture day 7–16, seven independent colonies were picked, and were passaged