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Anterior Pituitary Progenitor Cells Express Costimulatory Molecule 4Ig-B7-H3[1]

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Stem/Progenitor cells in the postnatal pituitary gland are embedded in a marginal cell layer around Rathke's pouch. However, the nature and behavior of anterior pituitary progenitor cells remain unclear. We established bovine anterior pituitary progenitor cell line (BAPC)-1 from the anterior pituitary gland, which expressed stem/progenitor cell-related genes and several inflammatory cytokines. To characterize and localize these pituitary progenitor cells, we produced a mAb (12B mAb) against BAPC-1. The 12B mAb recognized the 4Ig-B7-H3 molecule, which is a costimulatory molecule and negative regulator in T cell activation. WC1$^+$ $\gamma\delta$ T cells in young bovine PBMC express the 4Ig-B7-H3 molecule, but few or no 4Ig-B7-H3-immunoreactive cells are expressed in PBMC in adult cattle. The 12B-immunoreactive cells in the bovine anterior pituitary gland were localized around Rathke's pouch and expressed IL-18 and MHC class II. However, the number of 12B-immunoreactive cells was lower in adult than in young cattle. BAPC-1 expressed IL-18 and MHC class II, and demonstrated phagocytotic activity. BAPC-1 also had the ability to promote CD25 expression in PBMC after 5 days of coculture, and blocking 4Ig-B7-H3 × 12B mAb enhanced their expression of CD25. In addition, the 12B-immunoreactive cells were observed around the pars tuberalis closely bordering the median eminence and in the blood vessels of the primary portal plexus in the anterior pituitary gland. These results suggest that an established BAPC-1 may originate from these progenitor cells, and that the progenitor cells with 4Ig-B7-H3 may play a critical role in the immunoendocrine network. *The Journal of Immunology,* 2008, 181: 6073–6081.

I t is well known that pituitary function is regulated by the hypothalamic-pituitary-adrenal axis. Recent studies show that several cytokines, such as IL-1, IL-6, IL-11, IL-18, macrophage-migration inhibitory factor, and leukemia inhibitory factor, and their specific receptors are expressed in the anterior pituitary gland and play an important role in the neuroendocrine system by autocrine or paracrine pathways as immune-endocrine mediators (1–5). It has also been reported that neural multipotent precursor cells act as immunomodulators during inflammation of the CNS, which promotes long-lasting neuroprotection (6), and that the marginal cell layer around Rathke's pouch has been proposed to contain a stem/progenitor cell compartment in the postnatal pituitary (7, 8).

Cells in the marginal cell layer of Rathke's pouch in the anterior pituitary gland express stem/progenitor molecules. One of them is Nestin, a class VI intermediate filament protein, whereas the others are Notch, Wnt, and the sonic hedgehog signaling factors (7, 9–11). We have reported that constituent cells of the marginal cell layer of Rathke's pouch in the bovine and porcine pituitary expressed IL-18 (12, 13). IL-18 is a pleiotropic cytokine, which can

trigger the cytokine signaling pathway and initiate a cytokine cascade immune responses (14). Our previous studies show that IL-18 and its receptor (IL-18R) are expressed in specific cell types of the bovine and porcine anterior pituitary, and that there are substantial differences in the distribution of IL-18 and IL-18R between cattle and pigs (3, 4). These data suggest that IL-18 may be an immunoendocrine mediator both through autocrine and paracrine pathways. Recently, it has been revealed that the corticotrophs of the rat anterior pituitary gland express IL-18 (1), and that IL-18 induces the expression of IL-1α and pro-IL-1β, and also the release of IL-6 from mixed glia in vitro (15). In addition, IL-18 has a role in the homeostasis of energy intake as well as insulin sensitivity (16). These results indicate that inflammatory mediators regulate anterior pituitary cell function through the immune-endocrine axis, and that stem/progenitor cells of the anterior pituitary gland also have a role as immune-endocrine mediators. However, little is known about the population and localization of immune-endocrine mediator cells in the anterior pituitary gland.

We have established bovine anterior pituitary progenitor cell line (BAPC)[3]-1 (13). BAPC-1 express stem/progenitor cell-related genes, such as Nestin, Nanog, Oct-4, Ptch1, Notch1, Hes1, Lrp, Fzd4, Lhx3, Pitx1, and pit-1, whereas they also express several cytokine mRNAs, including IL-1, IL-6, IL-7, IL-12, IL-15, and IL-18. In addition, stimulation with IL-18 causes the induction of IL-1β and IL-8 mRNA expression in BAPC-1. Although IL-18 is expressed in somatotrophs as it is in the cells of Rathke's pouch in the bovine anterior pituitary gland (4), BAPC-1 is not immunoreactive for growth hormone. These findings suggest that BAPC-1

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[3] Abbreviations used in this paper: BAPC, bovine anterior pituitary progenitor cell line; LC-MS/MS, liquid chromatography/tandem mass spectrometry.

Table I. *Primer sequences used in this study*

Targets	Forward Primer	Reverse Primer	Annealing Temperature	Product Size (bp)
4Ig-B7-H3 1	CTGCCTCTCGAGAAGATGCT	AACCTGGACTTCCACTGCAC	55°C	765
4Ig-B7-H3 2	GTGCAGTGGAAGTCCAGGTT	TGATCTTTCTCCAGCACACG	55°C	744
4Ig-B7-H3 3	GGGCGCTAATGGTACCTACA	CCTCGCGGTCAGACTATTTC	55°C	324
Nanog	ACCCGGAGATCTTCACCTTT	TTGATGTCCTGGGACTCCTC	55°C	527
Oct-4	GGTTCTCTTTGGAAAGGTGTTC	ACACTCGGACCACGTCTTTC	55°C	314
Fzd4	TCACTGTGCTCACCTTCCTG	CTTCGTCCCATCCTTTTGAA	55°C	572
B7-1	GAACCGCACCATCACTGACA	TAATGGTCCAGGTCAGGTGC	56°C	485
B7-2	GACCTTGAGACTCCACAACG	GTAGAGCTGCAATCCAGAGG	59°C	490
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	55°C	452

may be a pituitary progenitor cell and act as an immune-endocrine mediator through cytokine expression.

To localize the BAPC-1 cell type in the pituitary gland, and thereby possibly identify pituitary progenitor cells, we immunized BALB/c mice with BAPC-1 and produced mAbs. After selection on the basis of reactivity against the membrane protein of BAPC-1, we established a mAb that we named 12B. mAb 12B recognizes the 4Ig-B7-H3 molecule, which is a costimulatory molecule and a negative regulator in T cell activation (17). This mAb also reacted to cells in the marginal cell layer around Rathke's pouch. In addition, we observed that BAPC-1 expressed MHC class II and had a phagocytic activity. These data indicate that pituitary progenitor cells may be constituent cells of the marginal cell layer, suggesting new aspects of the immunoendocrine network in the anterior pituitary gland.

Materials and Methods

Animals

Thirteen Holstein cattle (males and females, up to 36 mo of age) were used. The cattle were housed in pens under natural lighting conditions. Additionally, 12 BALB/c mice (7 wk old, male) were purchased from Japan SLC. These animals were handled in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care of Tohoku University.

Development of anti-BAPC-1 mAb

BAPC-1 were maintained in DMEM (Life Technologies) supplemented with 10% FBS (10% FBS-DMEM). The 12B mAb (mAb, IgG2b) was obtained by immunizing three mice with BAPC-1. Briefly, mice were immunized i.p. with 1×10^7 BAPC-1 cells. After 14 days, mice were i.v. immunized again and sacrificed 5 days later. The splenocytes were fused with SP2/0-ag14-K13 mouse myeloma cells in the presence of 50% (w/v) polyethylene glycol (4000). Fused spleen-myeloma cells were selected by culturing in HAT medium (RPMI 1640 medium containing 2 mM glutamine, 0.2% glucose, 1 mM pyruvic acid, 10% FBS, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). The hybridoma cells were screened for anti-BAPC-1 Ab production by flow cytometry, as described below, and cloned by limiting dilution. mAb was produced in ascetic fluid of BALB/c mice primed with pristine, and the mAb was purified by a MABTrap kit (Amersham Biosciences). The subclass and type of mAb were determined by a mouse mAb isotyping kit (Dainippon Sumitomo Pharma).

Flow cytometry

BAPC-1 cells were treated with a sucrose/EDTA buffer (pH 7.5; 0.45 M sucrose, 0.36% EDTA, 0.1% BSA in PBS) for 4 min, detached by 0.04% trypsin in PBS, and resuspended in PBS. Approximately 1×10^6 BAPC-1 cells were incubated with 10 μg/ml 12B mAb and anti-CD45 (×50; VMRD) for 30 min at 4°C, washed three times, and then resuspended in PBS. The cells were incubated with FITC-labeled goat anti-mouse IgG (×25; Southern Biotechnology Associates), washed three times, and resuspended in FACS Flow diluent (BD Immunocytometry Systems) for flow cytometric analysis.

Bovine PBMC were obtained from the jugular veins of three 7- to 13-wk-old calves and three 20- to 60-mo-old Holstein cows and adjusted to 1×10^7 cells/ml, as previously described (18). PBMC were incubated with 12B mAb and MM1A (CD3, IgG1), CACT138A (CD4, IgG1), BAQ111A (CD8, IgM), CACT61A (TCR-N12, IgM), or B7A1 (WC1-N1, IgM) mAb for 30 min at 4°C. These Abs were purchased from VMRD and used at a 1/50 dilution. Subsequently, PBMC were incubated with FITC-labeled goat anti-mouse IgG2b Ab (×25; Southern Biotechnology Associates) and PerCP-labeled rat anti-mouse IgG1 (×5; BD Biosciences) or PE-labeled goat anti-mouse IgM (×25; Southern Biotechnology Associates) Ab. All data that were acquired by FACSCalibur (BD Biosciences) were analyzed by CellQuest software (BD Biosciences).

Immunohistochemistry

The anterior pituitary glands of four 13-wk-old calves and three 20- to 36-mo-old steers were immersed in Zamboni's fixative overnight at 4°C. The tissues were then paraffinized and cut to 2 μm thickness. The deparaffinized sections were washed three times with PBS for 5 min and incubated in 1.5% normal goat serum for blocking. The sections were incubated with 12B (10 μg/ml), TH14B (MHC class II, VMRD; ×100), or mouse anti-human IL-18 (5 μg/ml) mAb overnight at 4°C. For the identification of MHC class II-immunoreactive cells, sections were incubated with DakoCytomation Target Retrieval Solution High pH (DakoCytomation) for 5 min at 121°C before blocking as an Ag retrieval technique. After several washings, the sections were then incubated with Simplestain MAX-PO (M) (Nichirei) as a secondary Ab for 1 h. After washing with PBS, the sections were visualized with 0.025% 3.3'-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris buffer (pH 7.6) and counterstained with Mayer's hematoxylin. To determine the colocalization of the 12B and MHC class II, or 12B and IL-18 immunoreactive cells, mirror section technique was performed, as previously described (4).

Immunocytochemistry

BAPC-1 were seeded into Sonicseal slides (Nunc) and cultured to ~80% confluence. Cells were fixed with 4% paraformaldehyde for 10 min at 4°C, permeabilized with acetone for 10 min at −20°C, and then washed five times with PBS. The chamber slides were incubated in PBS containing 1.5% normal goat serum for 20 min and then treated with primary Abs in PBS overnight at 4°C. After washing, the chamber slides were incubated with FITC-conjugated goat anti-mouse IgG Ab (×400; Sigma-Aldrich) for 1 h and then counterstained with ToPro3 (Molecular Probes) for 10 min. These slides were observed using a confocal laser microscope (MRC-1024; Bio-Rad).

Identification of 12B Ag molecule

BAPC-1 were detached and resuspended in PBS. Approximately 5×10^6 BAPC-1 cells were incubated with 3 μg/ml 12B mAb in PBS for 30 min at 4°C. After several washes with PBS, the cells were lysed in 0.5% Triton X-100 buffer and subsequently centrifuged at 9200 × g for 10 min at 4°C. The supernatant was immunoprecipitated using μMACS protein G (Miltenyi Biotec), according to the manufacturer's instructions. The sample was separated by SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen Life Technologies). The 12B-reactive protein at 75 kDa was cut and then identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis (APRO Life Science Institute).

Western blotting

The 12B-reactive protein was prepared by immunoprecipitation, as described above, separated by SDS-PAGE, and electrotransferred onto Immobilon-P membrane (Millipore). After the electrotransfer, the membrane was blocked with 0.05% Tween 20/PBS (PBS-T) containing 1% BSA and 1.5% normal horse serum overnight at 4°C. After three washings with PBS-T for 10 min, the membrane was treated with 1 μg/ml 12B mAb or goat anti-human B7-H3 Ab (R&D Systems). The membrane was washed
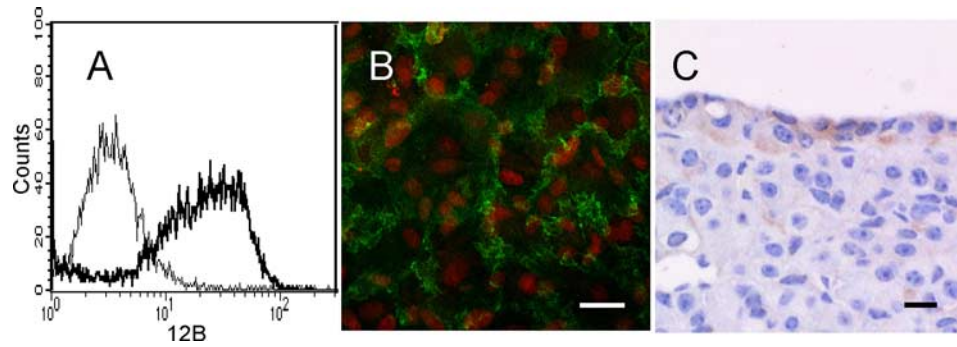
FIGURE 1. Reactivity of mAb 12B in BAPC-1 and anterior pituitary tissue. BAPC-1 were screened by flow cytometry for reactivity to the 12B Ab (*A*). The *y*-axis and *x*-axis show cell counts and staining intensity, respectively. The gray line was the negative control stained only with the secondary Ab. BAPC-1 were cultured to ~80% confluence and stained with 12B (*B*). The immunohistochemical expression of the 12B Ab in a paraffin-embedded bovine anterior pituitary gland (*C*). Scale bars = 50 μm (*B*) and 10 μm (*C*).

and incubated with a biotinylated goat anti-mouse IgG Ab or rabbit anti-goat IgG Ab (Vector Laboratories) containing 1% FBS for 2 h. After five washes, the membrane was treated with ABC-PO kit (Vector Laboratories) for 1 h. Immunoreactive proteins were visualized using a diaminobenzidine detection kit (Vector Laboratories).

Partial DNA sequence of bovine 4Ig-B7-H3 and gene expression analysis by RT-PCR

Total RNA from BAPC-1 was isolated using TRIzol (Invitrogen Life Technologies) and reverse transcribed for 60 min at 50°C using Superscript III (Invitrogen Life Technologies). Reverse-transcription reaction products were amplified with specific primer pairs (Table I) designed from the predicted sequence (accession XM 864408). The amplification was performed in 1 cycle for 5 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 59°C, and extension for 1 min at 72°C. Each single band was purified from the gel and sequenced (accession AB271019).

To determine the expression of 4Ig-B7-H3, total RNA from the anterior pituitary gland, hypothalamus, and PBMC from a 13-wk-old calf were extracted and subjected to RT-PCR, as described above. Reverse-transcription reaction products were amplified with the primer pairs for 4Ig-B7-H3. As the housekeeping gene, PCR was also performed with G3PDH primers. The amplification was performed in 1 cycle for 5 min at 94°C, 20 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. Each of the specific primer pairs is summarized in Table I.

Phagocytosis assay

BAPC-1 were seeded into 4-well chamber slides (Nunc) and cultured to ~80% confluence. Yellow-green fluorescent microspheres (1 $\times$ 10^7 beads, diameter 1 μm; Molecular Probes) were added to the cells and incubated for 1 or 12 h. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. The cells were counterstained with ToPro3 for 10 min and were observed by the confocal laser microscope.

Separation and phenotypic analysis of 12B-positive cells from PBMC

Freshly isolated PBMC were firstly incubated with 12B mAb for 30 min at 4°C, and washed three times with PBS containing 0.5% BSA and 2 mM EDTA. These cells were then incubated with 20 ml of rat anti-mouse IgG (2a + 2b) Ab conjugated with magnetic beads (Miltenyi Biotec) for 30 min at 4°C. The magnetic bead-labeled PBMC were positively separated using Auto MACS magnetic columns (Miltenyi Biotec). The cells passing through the magnetic column were collected as 12B-negative cells. The purity and phenotypes of the collected cells were analyzed by FACS analysis using mAbs, as described above, and RT-PCR. Each of the specific primer pairs is summarized in Table I (19, 20). Nanog and Fzd4 primer pairs were designed from predicted sequences or from other species, and the PCR products were sequenced (AB257753).

Effect of BAPC-1 and 12B mAb on CD25 expression in PBMC

BAPC-1 cells (1 $\times$ 10^4 cells/cm^2) were seeded into 12-well plates (Nunc) and cultured to ~80% confluence. Bovine PBMC were added to 12-well plates (1 $\times$ 10^4 cells/well) and cultured with or without the BAPC-1. The

4Ig-B7-H3 was neutralized by adding 12B mAb (10 μg/ml) to the wells. After 5 days, PBMC were harvested and analyzed for the expression of CD25 (CACT116A, $\times$50; VMRD) by FACS analysis as the activation marker (21, 22).

Results

Isolation of mAb 12B

To identify cell surface markers expressed in BAPC-1 and the localization of this cell type in the anterior pituitary gland, mice were immunized twice with BAPC-1. Four hybridoma clones were identified as producing mAbs reacting to BAPC-1. One clone termed 12B (IgG2b) reacted strongly with BAPC-1 and was used for further studies (Fig. 1*A*). The 12B mAb was also used for the immunocytochemistry and immunohistochemistry. The cell surface of BAPC-1 was stained with 12B mAb (Fig. 1*B*). In the bovine anterior pituitary gland, 12B-immunoreactive cells were localized around Rathke's pouch (Fig. 1*C*). No staining was observed in tissue sections treated with an unrelated mouse IgG primary Ab (negative control; data not shown).

Identification and LC-MS/MS analysis of 12B Ag

To identify the 12B Ag, immunoprecipitation was performed using BAPC-1 incubated with 12B mAb (Fig. 2). A band at ~75 kDa
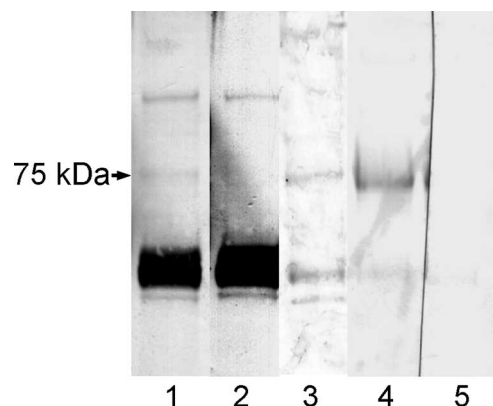
FIGURE 2. Identification of 12B-reactive molecule. BAPC-1 cell lysate was separated by SDS-PAGE and subjected to Western blot analysis (*lanes 1* and *2*). The 12B mAb-immunoprecipitated BAPC-1 sample was separated by SDS-PAGE (*lane 3*) and transferred to a polyvinylidene difluoride membrane (*lanes 4* and *5*). Western blotting analysis showed that 12B mAb recognizes a protein of 75 kDa. *Lane 1*, 12B mAb. *Lane 2*, Mouse control IgG. *Lane 3*, CBB staining. *Lane 4*, Goat anti-human B7-H3. *Lane 5*, Normal goat serum.

Table II. *Predicted peptides by LC-MS/MS*

Peptides	Organisms	Accession No.	Total Score
CD276 (4Ig-B7-H3)	Bovine	869501	247
Lysozyme	Bovine	841217	52
Secretoglobin, 2A	Human	4505171	52

was detected by Western blotting, extracted from the staining gel, and subjected to LC-MS/MS analysis (*lanes 1* and *3* in Fig. 2). The LC-MS/MS results from the database search are summarized in Table II. The database search identified three peptides, as follows: bovine CD276 Ag (4Ig-B7-H3), bovine lysozyme, and human secretoglobin 2A. Four different peptides allowed the identification of the 12B Ag molecule. Each peptide corresponded to a transmembrane protein termed 4Ig-B7-H3 (23) (gray boxes in Fig. 3). When Western blotting was performed using an anti-human B7-H3 Ab, the Ab also detected a specific band at ~75 kDa as for the 12B mAb (*lane 4* in Fig. 2). Previous reports have shown that B7-H3 has two isoforms generated by alternative splicing. These are 2Ig- and 4Ig-B7-H3. The 4Ig-B7-H3 is expressed in the hu-

man, but 2Ig-B7-H3 is not (23, 24). We were not able to identify any band corresponding with 2Ig-B7-H3 in the 12B-immunoprecipitated sample, and nothing was identified in cells treated with normal mouse IgG as the primary Ab (negative control) (*lanes 2* and *5* in Fig. 2).

Identification of bovine 4Ig-B7-H3 partial mRNA and its expression

Although there are no reports of the sequence of bovine 4Ig-B7-H3 mRNA, the sequences of the peptide immunoprecipitated by 12B mAb were matched to a predicted sequence derived from bovine annotated genomic sequence (Fig. 3). The mRNA expressed in BAPC-1 was sequenced using specific primer pairs designed from the predicted sequence. PCR products were determined (accession AB271019) and predicted for a 528 aa sequence (Fig. 3). There were three differences in the nucleotide sequence between the predicted sequence and PCR-determined sequence. The determined sequence demonstrated extensive homology (93.9%) with human 4Ig-B7-H3. The peptide sequences identified by LC-MS/MS analysis were also matched with the determined sequence (gray boxes in Fig. 3).

FIGURE 3. Position of identified peptides in the bovine 4Ig-B7-H3 amino acid sequence. The 75-kDa band immunoprecipitated by the 12B mAb was cut from the CBB-stained gel. Four peptide sequences were identified by LC-MS/MS analysis and matched to human 4Ig-B7-H3 amino acid sequence (gray boxes). The amino acid sequence of bovine 4Ig-B7-H3 was determined by specific primer pairs designed from the predicted sequence, and was the same as the four portions of the immunoprecipitated peptide sequences (gray boxes). Bovine predicted: predicted 4Ig-B7-H3 sequence that is derived from an annotated genomic sequence (XP869501). Bovine sequenced: the determined sequence from BAPC-1 cDNA (accession AB271019). Human: the registered sequence of human mRNA (NP 001019907).
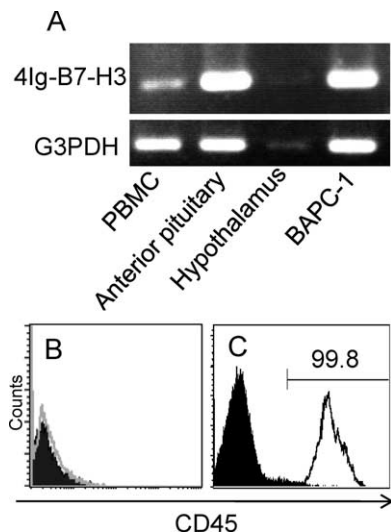
FIGURE 4. Expression of 4Ig-B7-H3 and CD45 in BAPC-1 and bovine cells. Total RNA was isolated from bovine PBMC, the anterior pituitary gland, the hypothalamus, and BAPC-1, and subjected to RT-PCR (*A*) using the specific primer pair for 4Ig-B7-H3 3 in Table I. The products marked with an arrow result from the RT-PCR. The housekeeping gene, G3PDH, served as the internal standard. FACS analysis was used to determine the expression of CD45 in BAPC-1 (*B*) and PBMC (*C*). The gray-filled histograms are the negative controls that were stained with a secondary Ab only.

To investigate 4Ig-B7-H3 expression in a 13-wk-old calf, RT-PCR was performed using total RNA extracted from PBMC, the hypothalamus, and the anterior pituitary gland (Fig. 4*A*). The 4Ig-B7-H3 mRNA was expressed in PBMC and in the anterior pituitary, but only to a very small extent in the hypothalamus. BAPC-1 expressed 4Ig-B7-H3 mRNA, but not a hematopoietic lineage marker CD45 (Fig. 4, *B* and *C*). A specific band was also detected using other primer pairs used for the sequencing (data not shown).

Identification of 12B-reactive cells in PBMC

Previous studies have shown that immature and mature dendritic cells are brightly stained by anti-4Ig-B7-H3 Abs in humans, and that normal lymphocytes and the cultured NK, B, and T cell lines are not reactive with this Ab (23, 25). Fig. 4 shows that the expression of 4Ig-B7-H3 mRNA was detected in bovine PBMC. Therefore, flow cytometric analysis was performed to identify 12B-reactive cells. The 12B-reactive cells accounted for ∼16.4% of PBMC from the 13-wk-old calves. However, PBMC from the 20-mo-old cows did not react with the 12B mAb (Fig. 5, *A* and *B*). Following this, we assessed the surface distribution of the 12B-reactive molecules on PBMC from the 13-wk-old calves. Thirty percent of CD3^{+} cells (T cells) reacted with the 12B mAb, which accounted for 14.2% of the PBMC (Fig. 5*C*). However, the 12B mAb did not react with CD4^{+} and CD8^{+} cells (Fig. 5, *D* and *E*). Fifty percent of TCR1^{+} (γδ T) cells were positive for 12B mAb (Fig. 5*F*). In the 12B-reactive γδ T cells, almost all WC1^{+} cells were stained with 12B mAb (Fig. 5*G*). These results indicate that 12B-reactive cells in PBMC are WC1^{+} γδ T cells.

Localization of 12B Ag in the pituitary gland

The BAPC-1 cells were established from the anterior pituitary gland of a 7-wk-old calf (13). Because we were not clear of the origins of BAPC-1, we performed immunohistochemistry using the 12B mAb on the anterior pituitary glands of 13-wk-old calves and 20-mo-old adult steers (Fig. 6). In the calves, 12B-reactive
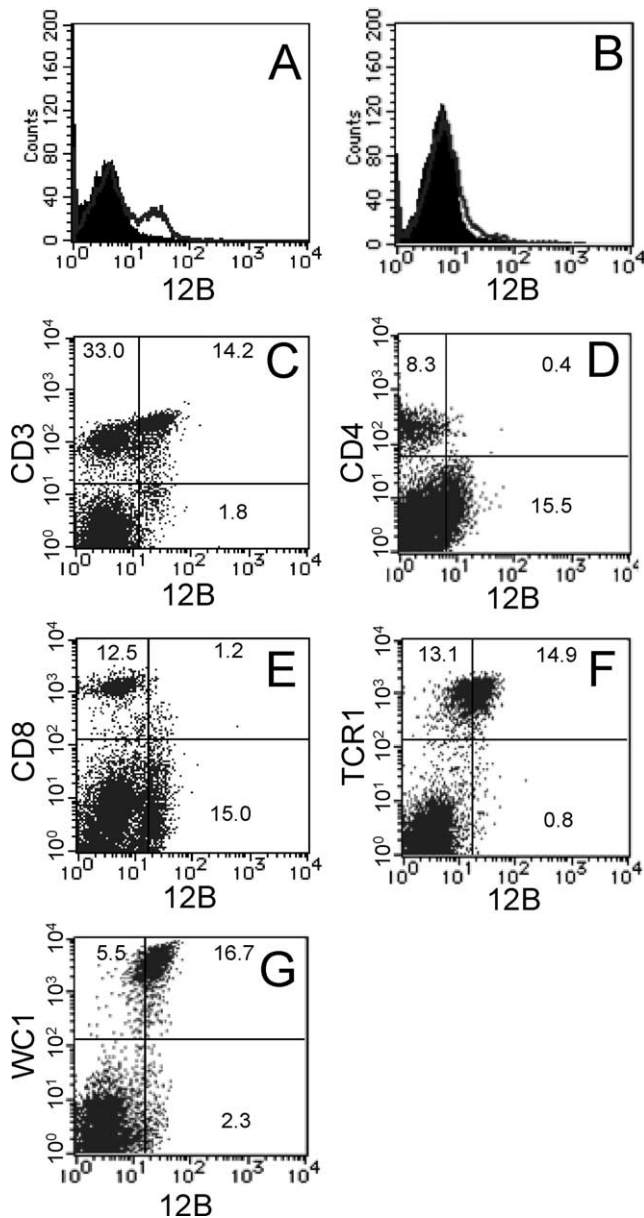
FIGURE 5. Flow cytometric analysis of 4Ig-B7-H3-positive cells in bovine PBMC. PBMC from a 13-wk-old calf (*A*) and a 5-year-old cow (*B*) were analyzed using the 12B mAb. The gray-filled histograms are negative controls that were stained with only a secondary Ab. The PBMC from the 13-wk-old calf were also analyzed with 12B and CD3 (*C*), CD4 (*D*), CD8 (*E*), TCR1 (*F*), or WC-1 (*G*) Abs and detected by dual-color flow cytometric analysis. The percentage of cells staining positively is indicated in each box.

cells were localized around Rathke's pouch in the anterior lobe (Fig. 6*A*). Stained cells were also localized to the same area in the adult steers; however, the number of positive cells in adult steers was lower than that of the calves (Fig. 6*E*). The 12B-reactive cells were observed around the pars tuberalis in the anterior lobe of both the young and adult animals, which adhered closely to median eminence and had blood supply via the primary portal plexus (Fig. 6, *B* and *F*). Some 12B-reactive cells were observed in the intermediate lobe and existed around Rathke's pouch sparsely in both the young and adult animals (Fig. 6, *C* and *G*). In the neural lobe of both samples, some pituicytes were immunoreactive for the 12B mAb, and the reactivity of 12B mAb in the intermediate and neural lobe of the adult steers was stronger than in calves (Fig. 6, *D* and

FIGURE 6. Localization of 4Ig-B7-H3-expressing cells in bovine pituitary gland. Immunostaining was performed with 12B mAb on pituitary glands from 13-wk-old (*A–D*) and 19-mo-old (*E–H*) cattle. RP: anterior lobe section around Rathke's pouch. PT: anterior lobe section around the pars tuberalis. IL: intermediate lobe section around Rathke's pouch. NL: Neural lobe. Scale bars = 10 μm.
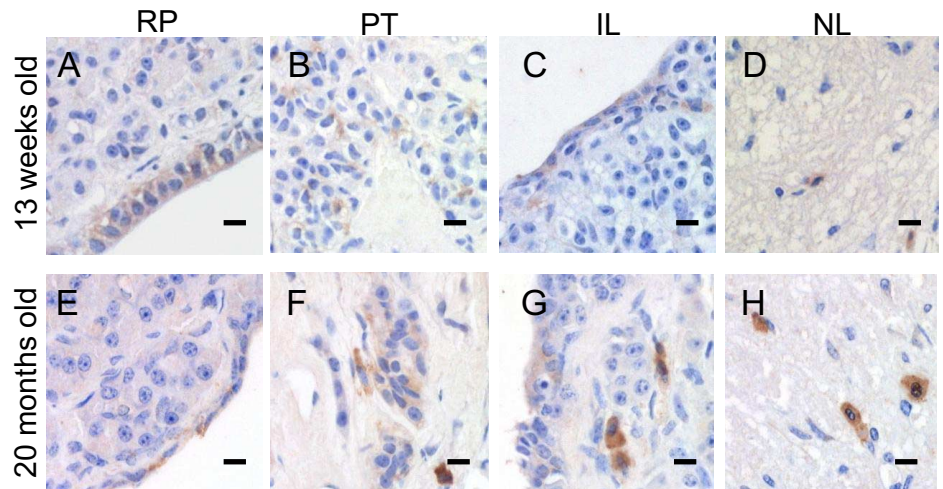
H). In addition, the cells in the pouch and pars tuberalis showed the same reactivity in both the calves and the adult steers.

Colocalization of 12B and MHC class II Ags in the anterior pituitary gland

B7-H3 is known to be a costimulatory molecule in APCs, which also express MHC class II Ag (26). Immunocytochemistry shows that BAPC-1 also expressed MHC class II (Fig. 7*A*). In the anterior pituitary gland, some 12B-positive cells weakly expressed MHC class II (arrows in Fig. 7, *B* and *C*). Cells that strongly expressed MHC class II were negative for 12B mAb (arrowheads in Fig. 7, *B* and *C*). IL-18-reactive cells in the layer of Rathke's pouch also

reacted for 12B mAb; however, IL-18-reactive cells in the hormonal cells were not reactive for 12B mAb in the other regions (arrows in Fig. 7, *D* and *E*).
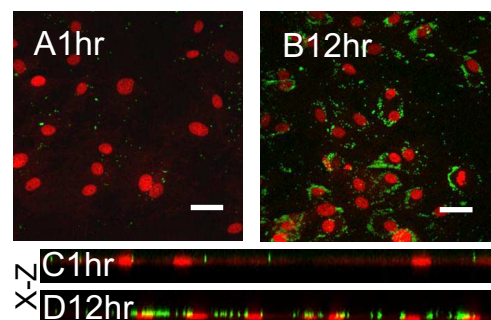
Phagocytosis assay of BAPC-1

To analyze phagocytic activity of BAPC-1, the cells were incubated with yellow-green fluorescent microspheres for 1 and 12 h. BAPC-1 were able to incorporate some microspheres into the cytosol after 1 h, and numerous microspheres were phagocytosed by BAPC-1 after 12 h (Fig. 8, *A* and *B*). BAPC-1 also phagocytosed FITC-labeled *Escherichia coli* after a 12-h incubation (data not shown). The X-Z section revealed that microspheres were incorporated into the cytoplasm of BAPC-1 and detected around their nuclei (Fig. 8, *C* and *D*).

Expression of B7 family and pluripotency-related genes in BAPC-1

The 12B-positive and 12B-negative cells were collected from PBMC using MACS magnetic columns and subjected to FACS analysis to evaluate the phenotype of the 12B-positive cells. The purity of the 12B-positive cells was 93.2%, and their persistence in 12B-negative cells was 19.6% (Fig. 9, *A* and *G*). The 12B-positive cells were largely composed of CD3$^+$ T cells (84.5%), and WC1$^+$ γδ T cells accounted for ~50% of these. However, the populations of CD4$^+$ and CD8$^+$ cells in these were quite low (Fig. 9, *B–F*). In contrast, WC1$^+$ cells and γδ T cells accounted for ~2.8 and 13.5% of the 12B-negative cells, respectively (Fig. 9, *K* and *L*).
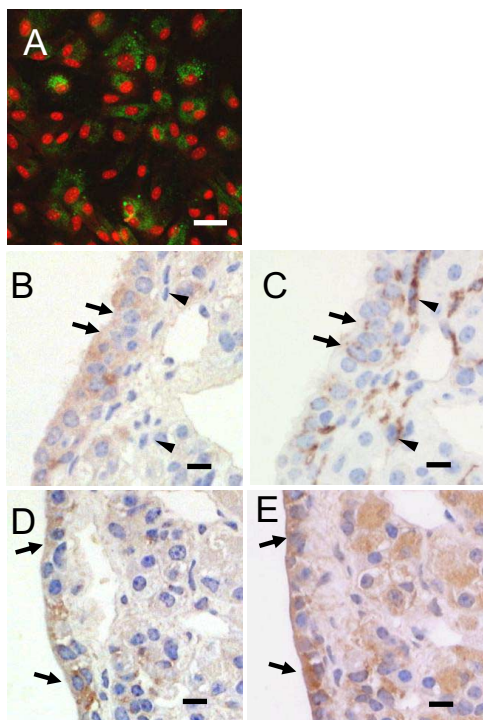
FIGURE 7. Localization of 12B and MHC class II-expressing cells in the anterior pituitary gland. BAPC-1 were stained with MHC class II Ab (*A*). A couple of mirror sections were stained with Abs against 12B (*B*) or MHC class II (*C*), and 12B (*D*) or IL-18 (*E*) in the anterior pituitary gland of a 13-wk-old calf. Arrows show the colocalized cells with 12B and MHC class II, or 12B and IL-18. Arrowheads show MHC class II-positive cells without 12B reactivity. Scale bars = 50 μm (*A*) and 10 μm (*B–E*).

FIGURE 8. Phagocytosis activity of BAPC-1. BAPC-1 were incubated with microspheres (green) for 1 (*A* and *C*) or 12 h (*B* and *D*). The cells were counterstained with ToPro3 (red). X-Z: X-Z plane pictures from consecutive *z*-sections and three-dimensional reconstruction. Scale bars = 10 μm.
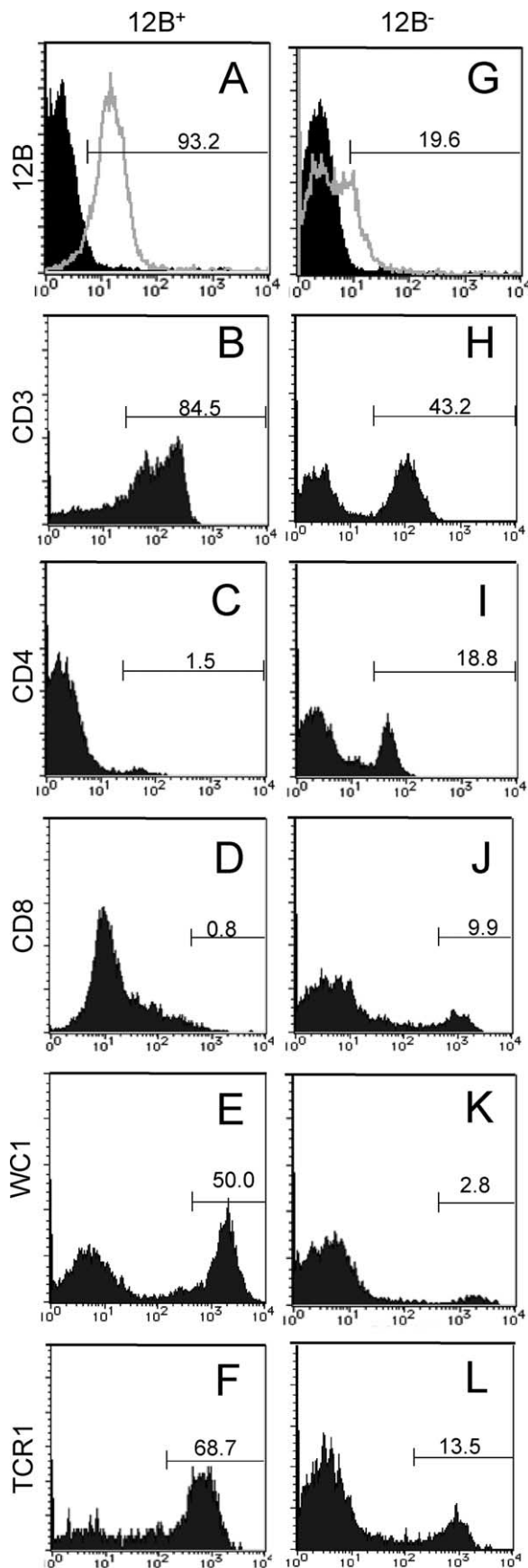
FIGURE 9. Identification of 12B-positive cells in PBMC. PBMC were treated with 12B mAb and collected by the positive or negative selection using Auto MACS magnetic columns. The purity was confirmed using anti-mouse IgG2b (*A* and *B*, solid lines). The 12B-positive (12B$^+$) and
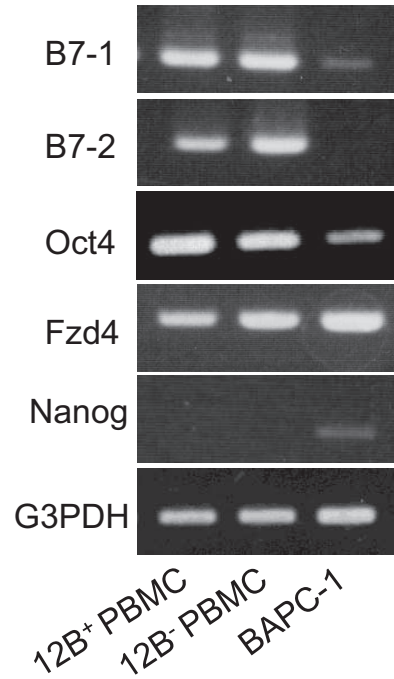
FIGURE 10. Expressions of the B7 family and pluripotency-related genes in BAPC-1. Cells were separated from PBMC by 12B-positive or 12B-negative selection using Auto MACS magnetic columns. Total RNA was isolated from PBMC and BAPC-1. The cDNA was subjected to PCR using the specific primer pair of the indicated genes in Table I. G3PDH was used as the control.

Next, we investigated the expression of the B7 family and pluripotency-related genes in BAPC-1 and 12B-positive and 12B-negative PBMC (Fig. 10). The 12B-positive and 12B-negative PBMC expressed the B7 family of genes, a pluripotent cell marker Oct-4 (20), and a canonical Wnt signal receptor Fzd4, but did not express Nonag, which is also known as a key marker of stem cells (27). In contrast, BAPC-1 marginally expressed B7-1, but not B7-2. Of the pluripotency-related genes, BAPC-1 expressed Oct-4 to a lower extent than the PBMC, but the mRNA expression of Nanog was detected in BAPC-1. Although BAPC-1 expressed the 12B Ag (4Ig-B7-H3), there were quite large differences in the expression of phenotypic markers between BAPC-1 and 12B-positive PBMC.

Effects of BAPC-1 and 12B mAb on expression of CD25 in PBMC

The 4Ig-B7-H3 molecule recognized by 12B mAb is known to be a costimulatory molecule and a negative regulator in T cell activation (20, 21). However, it is not clear how the interaction between PBMC, cells like BAPC-1, and the 4Ig-B7-H3 molecule affects cell function in the marginal cell layer of Rathke's pouch in the anterior pituitary gland. We cocultured PBMC and BAPC-1 with or without 12B mAb and measured the expression of activated T cell marker CD25 by FACS analysis (Fig. 11). After 5days of culture, CD25-positive cells were not observed in the single culture of PBMC with or without 12B mAb. In contrast, CD25-positive cells accounted for ~34.8% of the cells in the coculture.

12B-negative (12B$^-$) cells were subjected to FACS analysis using anti-WC1 (*C* and *D*), CD3 (*E* and *F*), CD4 (*G* and *H*), CD8 (*I* and *J*), and TCR1 (*K* and *L*) Abs. The gray-filled histograms are the negative controls that were stained with the secondary Abs only.
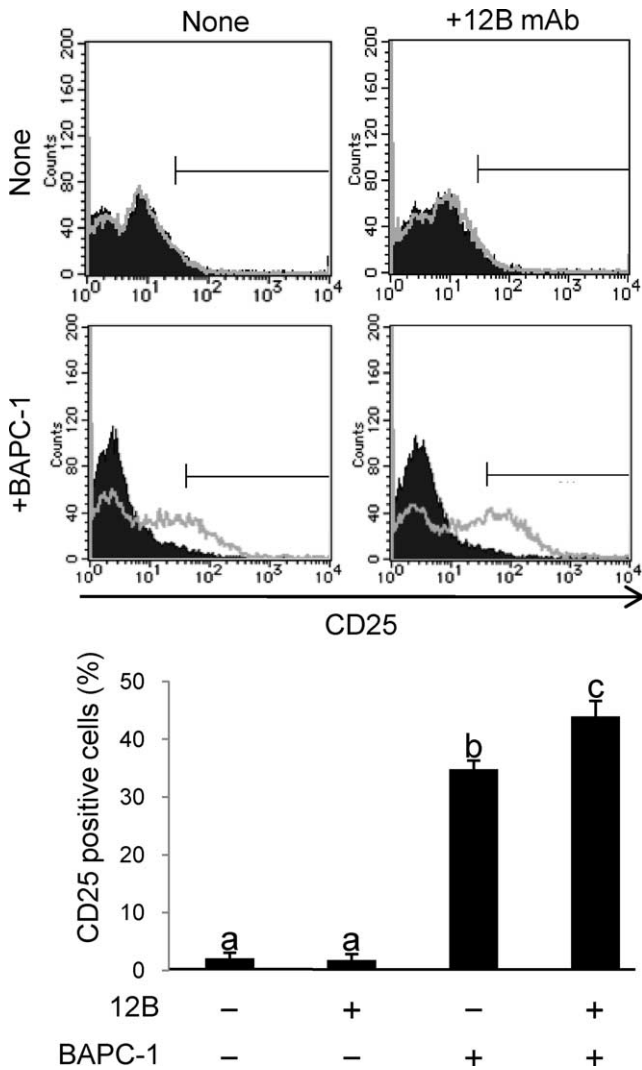
FIGURE 11. Effects of BAPC-1 and 12B mAb on the expression of CD25 in PBMC. BAPC-1 were cultured to ~80% confluence in 12-well plates. PBMC were added to the wells and cocultured with or without BAPC-1 and/or 12BmAb. After 5 days, the expression of CD25 in PBMC was measured by FACS analysis. The gray-filled histograms are negative controls that were stained with the secondary Ab only. The data are expressed as means ± SEM ($n = 3$). Values with different letters are statistically different ($p < 0.01$). Results are representative of three identical experiments.

In addition, supplementation with 12B mAb significantly increased the population of CD25-positive cells to 44.0% in the coculture. No morphological changes were observed in BAPC-1 after 5 days of coculture (data not shown). These data suggest that BAPC-1-like cells and the 4Ig-B7-H3 molecule may regulate the cell function of PBMC in the layer of Rathke's pouch in the pituitary gland.

Discussion

In this study, we revealed that the 12B mAb, raised against BAPC-1, recognized the 4Ig-B7-H3 molecule, and that 12B-reactive cells were localized in the layer of Rathke's pouch in the pituitary gland. Rathke's pouch has been proposed to embody a stem/progenitor cell compartment. BAPC-1 expresses stem/progenitor cell-associated molecules and factors (13); however, it is not yet clear from what kind of cell BAPC-1 originates. Considering the immunohistochemical data on the expressions of MHC

class II and IL-18, BAPC-1 may originate from a stem/progenitor cell in the layer of Rathke's pouch in the anterior pituitary gland. The 4Ig-B7-H3 is a member of the B7 family of cell surface markers (23, 24). In both the human and mouse, B7-H3 binds to an unidentified receptor expressed on activated T cells and NK cells, and exerts an inhibitory function on their cell-mediated responses (17, 25, 28). It is known that B7-H3 mRNA is expressed in many tissues and organs, such as skeletal muscle, kidney, pancreas, heart, and brain, suggesting that it may also play a number of roles outside of the immune system (24, 26).

Cattle have large numbers of circulating $\gamma\delta$ T cells, of which the majority express a WC1 molecule (29). The WC1 molecule is expressed in ruminants and pigs, but not in humans and mice (30, 31). WC1$^+$ $\gamma\delta$ T cells are decreased in number in older cattle (32). WC1$^+$ $\gamma\delta$ T cells can form up to 35% of the PBLs of yearlings and 5–10% in adults. WC1$^+$ $\gamma\delta$ T cells in the young bovine PBMC expressed a 4Ig-B7-H3 molecule, but little or no 4Ig-B7-H3-immunoreactive cells could be found in PBMC of adults (Fig. 5). It is also known that cultured WC1$^+$ $\gamma\delta$ T cells can act as APCs for $\alpha\beta$ T cells (33). In contrast, WC1$^+$ $\gamma\delta$ T cells inhibit the proliferation of CD4$^+$ T cells in response to *Mycobacterium avium subsp. paratuberculosis* Ags (34). WC1$^+$ $\gamma\delta$ T cells represented the bulk of the population of 12B-positive cells in the PBMC, and 12B-positive cells clearly expressed the Oct-4 mRNA (Fig. 10). Because Oct-4 plays an essential role to prevent embryonic stem cells from differentiating (27), WC1$^+$ $\gamma\delta$ T cells in PBMC may have primitive phenotypes.

Although BAPC-1 marginally expressed B7-1, but not B7-2, the expressions of these mRNA were observed in 12B-positive cells (WC1$^+$ $\gamma\delta$ T cells) of PBMC (Fig. 10). A recent study has shown that B7-1 and B7-2 have significant implications for the manipulation of immune responses and tolerance related with regulatory T cells (35). In addition, TLR4 ligation enhances the expression of B7-1 and decreases B7-2 on human oral mucosal Langerhans cells with tolerogenic properties, and contributes to the tolerogenic state (36). These reports suggest that bovine WC1$^+$ $\gamma\delta$ T cells of PBMC may have some tolerogenic functions.

Given that 4Ig-B7-H3-positive cells in the layer of Rathke's pouch were fewer in adult cattle than in young animals in the present experiments (Fig. 6), there may be some differences in cellular immunomodulatory functions in the layer of Rathke's pouch between young and adult animals. In the rat anterior pituitary gland, Nestin-immunoreactive cells in the layer of Rathke's pouch are also fewer in the adult than in the young rat (11). These results indicate that there may be fewer progenitor cells in the layer in adult animals than in young animals.

The 4Ig-B7-H3 molecule is a member of the B7 family, which is critical for the regulation of cell-mediated immune responses (37). B7-1 and B7-2 are other molecules of the B7 family and can activate T cells by cross-presentation (38). BAPC-1 also express MHC class II and have phagocytic activity, but do not express the mRNA of B7-2 (Fig. 10). Human mesenchymal stem cells also express MHC class II, but do not express B7-1 and B7-2, and display the immunomodulatory function (39, 40). BAPC-1 had the ability to promote CD25 expression in PBMC after 5 days of coculture, and blocking 4Ig-B7-H3 × 12B mAb enhances their expression of CD25 (Fig. 11). These results suggest that BAPC-1 may regulate the function of T cells in a similar manner to human mesenchymal stem cells. Our immunohistochemical data also show the colocalization of IL-18 and the 4Ig-B7-H3 molecule in the layer of Rathke's pouch. In contrast, the immunoreactivity of MHC class II in the cells of Rathke's pouch seemed weak and demonstrated an intracellular localization in the cytoplasm. These results also suggest that the stem/progenitor cells in the layer of

Rathke's pouch may have an immunomodulatory activity, but not the typical ability of Ag presentation.

In conclusion, we have succeeded in identifying the costimulatory molecule 4Ig-B7-H3 in bovine anterior pituitary progenitor cells in the layer of Rathke's pouch. We have established BAPC-1, which is indicated to originate from these progenitor cells, and expresses the same molecule. We believe the progenitor cells expressing the 4Ig-B7-H3 molecule may play a critical role in anterior pituitary functions, such as in the immunoendocrine network.

Disclosures

The authors have no financial conflict of interest.

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