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The class II major histocompatibility complex molecule BoLA-DR is expressed by endothelial cells of the bovine corpus luteum

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Abstract

Cells expressing class II major histocompatibility complex (MHC) molecules are found within the corpus luteum (CL) of several species. Expression and localization of class II MHC molecules in the bovine CL were examined in the present study. Immunohistochemical evaluation revealed class II MHC molecules on single cells in early CL (days 4 and 5 post-estrus). Two class II MHC-expressing cell types were observed in midcycle CL (days 10–12 post-estrus), single cells similar to those observed in the early CL, and endothelial cells. Not all endothelial cells expressed class II MHC, and further investigation revealed expression of only one type of class II MHC molecule, DR, on endothelial cells. Class II MHC was also localized to endothelial cells in late CL (day 18 post-estrus). Steroidogenic luteal cells were negative for class II MHC throughout the estrous cycle. Quantitative RT-PCR revealed higher ($P<0.05$) concentrations of mRNA encoding the α-subunit of DR (DRA) in late CL when compared with those in the early CL. *DRA* mRNA abundance was also measured in cultures of mixed luteal and luteal endothelial (CLENDO) cells, in the presence or absence of tumor necrosis factor-α (TNF). No differences were found in the *DRA* mRNA concentration between mixed luteal and CLENDO cell cultures, and TNF had no effect on *DRA* mRNA concentration in both cell types. Expression of DR by endothelial cells of the midcycle CL may induce anergy of T lymphocytes, or stimulate them to secrete products that enhance normal luteal function.

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Introduction

Evidence supporting the role of immune cells in the regulation of luteal function is accumulating. Macrophages and T lymphocytes have been detected in the corpus luteum (CL) of several species in numbers that vary with stage or functional state of the CL (Bagavandoss *et al.* 1990, Bränström *et al.* 1994, Lawler *et al.* 1999, Penny *et al.* 1999, Krusche *et al.* 2002, Nagaosa *et al.* 2002, Townson *et al.* 2002, Komatsu *et al.* 2003, Neuvians *et al.* 2004). The presence of mRNAs encoding various cytokines and the corresponding proteins has also been demonstrated in luteal tissue (Telleria *et al.* 1998, Penny *et al.* 1999, Petroff *et al.* 1999, Sakumoto *et al.* 2000, 2006, Krusche *et al.* 2002, Townson *et al.* 2002, Komatsu *et al.* 2003, Neuvians *et al.* 2004, Nishimura *et al.* 2004) T-cell cytokines such as interleukin-1β (IL1B), IL6, tumor necrosis factor-α (TNF), and interferon-γ (IFNG) modulate the synthesis of progesterones and prostaglandins by granulosal, thecal, and luteal cells *in vitro* (Nothnick & Pate 1990, Fairchild & Pate 1991, Benyo & Pate 1992, Gorospe *et al.* 1992, Alpizar & Spicer 1994, Townson & Pate 1994, 1996, Del Vecchio & Sutherland 1997, Young *et al.* 1997, Breard *et al.* 1998). Collectively, these observations suggest the potential for infiltrating immune cells to be activated within luteal tissue, and the cytokines they produce to modulate luteal function.

The activation of T lymphocytes occurs via a receptor–ligand type interaction between the T-cell receptor for antigen and major histocompatibility complex (MHC) molecules on the surface of cells stimulating T-cell activation (Altman *et al.* 1990). Two distinct types of

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MHC molecules, class I and class II MHC molecules, are involved in the activation of T lymphocytes. Class I MHC molecules are found on all nucleated cell types, with several notable exceptions. These molecules bind with intracellularly generated peptide fragments, and the presence of complexes of peptide–class I MHC at the cell surface allows cells to interact with $CD8^+$ (cytotoxic) T lymphocytes (Groothuis *et al.* 2005). This interaction forms the basis for immune system surveillance of somatic cells for the presence of viral proteins or aberrantly synthesized endogenous proteins (Fruh *et al.* 1997, Cresswell *et al.* 2005, Rock & Shen 2005). Class II MHC molecules also bind with peptide fragments, but expression of class II MHC molecules is more restricted. Class II MHC molecules allow interaction of cells with $CD4^+$ (helper) T cells, and, classically, class II MHC expression by the so-called professional antigen-presenting cells of the immune system (macrophages, dendritic cells, and B lymphocytes) has been regarded to be of greatest significance, due to the necessity of these molecules for proper immune system function (Scholl & Geha 1994, Grusby & Glimcher 1995, Rohn *et al.* 1996). However, class II MHC expression is not limited to these cells, and it has become apparent that expression under normal physiological as well as pathological conditions significantly impacts the function of various tissues (Knolle & Limmer 2001, Pober *et al.* 2001, Kelly *et al.* 2003). Finally, three types of class II MHC molecules, referred to as DP, DQ, and DR, are expressed on professional antigen-presenting cells.

Expression of class II MHC molecules by the cells of the CL has been demonstrated in several studies (Khoury & Marshall 1990, Benyo *et al.* 1991, Kenny *et al.* 1991, Bukovský *et al.* 1995, Bowen & Keyes 1999, 2000, Lawler *et al.* 1999, Penny *et al.* 1999, Lehman *et al.* 2000, Hoffmann *et al.* 2004). Macrophages would certainly account for a percentage of the class II-positive cells in the CL, but expression of class II MHC by cells other than macrophages has been convincingly demonstrated (Khoury & Marshall 1990, Benyo *et al.* 1991, Kenny *et al.* 1991, Bukovský *et al.* 1995, Lehman *et al.* 2000, Hoffmann *et al.* 2004). In the bovine CL, expression of class II MHC has been demonstrated in several studies (Benyo *et al.* 1991, Penny *et al.* 1999, Lehman *et al.* 2000), but data are lacking on the convincing demonstration of the identity of class II MHC-expressing cells in the bovine CL. The present study was undertaken to identify the cell type(s) in the bovine CL that expresses class II MHC, since identification of these cells is crucial to understanding the role of the immune system in the regulation of luteal function. The overall hypothesis is that cells in addition to macrophages express class II MHC in the bovine CL, and that expression of class II MHC on these cells changes with the functional status of the CL.

Materials and Methods

Reagents

Powdered Hams F-12 culture medium, gentamicin, fetal bovine serum, SuperScript II Reverse Transcriptase, and TRIzol reagent were purchased from Invitrogen. Recombinant RNasin and dNTPs were purchased from Promega. Random hexamer primers were acquired from Amersham Pharmacia Biotech. Oligonucleotide primers were obtained from Operon (Huntsville, AL, USA). DyNAmo HS SYBR Green qPCR kits were purchased from MJ Research (Waltham, MA, USA). Fluorescein-labeled Griffonia (Bandeiraea) simplicifolia lectin-1 (FITC-BS-1), non-specific mouse IgG, normal horse serum, and histological grade BSA were purchased from Vector Laboratories (Burlingame, CA, USA). Non-immune rabbit serum, 4′,6-diamidino-2-phenylindole (DAPI), BSA (fraction V), and HEPES were acquired from Sigma Chemical Company. Type I collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Bovine luteinizing hormone (LH; AFP II 743B) was provided by the National Hormone and Peptide Program and AF Parlow (Torrence, CA, MD, USA). Insulin–transferrin–selenium (ITS) premix was obtained from Collaborative Research Products. Anti-bovine class II MHC monoclonal antibodies (mAbs) and anti-bovine CD172a mAb were purchased from VMRD, Inc. (Pullman, WA, USA). Rabbit anti-rat P450 side-chain cleavage enzyme (CYP11A1) polyclonal antiserum was obtained from Research Diagnostics, Inc. (Concord, MA, USA). Mouse anti-human CD68 mAb was purchased from Dako (Carpinteria, CA, USA). Alexa Fluor 546-conjugated goat anti-mouse $F(ab')_2$ fragment and Alexa Fluor 488-conjugated goat anti-rabbit $F(ab')_2$ fragments were purchased from Invitrogen. Agarose was acquired from Amresco (Solon, OH, USA). Tissue culture flasks were from Corning (Corning, NY, USA). Unless otherwise specified, all other chemicals, reagents, and supplies were purchased from Sigma Chemical Co. or VWR Scientific Products (West Chester, PA, USA).

Animals and tissue collection

For all experiments except those involving CL endothelial (CLENDO) cells, corpora lutea were collected from normally cycling, multiparous, lactating dairy cows between 3 and 6 years of age. Corpora lutea were removed by transvaginal lutectomy on days 4, 5, 10–12, or 18 post-estrus (day 0, day of estrus), and were cut into four equal pieces. Two pieces were snap-frozen in liquid nitrogen and stored at −80 °C until RNA was extracted. The remaining pieces were embedded in Tissue-Tek optimal cutting temperature (OCT) medium, frozen in liquid isopentane chilled in liquid nitrogen, and stored at −80 °C until frozen sections for immunohistochemistry were prepared. Handling of animals and surgical procedures were carried out in accordance with

procedures approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Immunohistochemistry

Luteal tissues embedded in OCT were sectioned at a thickness of 7 μm on a Leica CM 1850 cryostat (Leica Microsystems, Bannockburn, IL, USA). Tissue sections were mounted on Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ, USA) and stored at −20 °C until use. Prior to use in immunohistochemical procedures, unfixed tissue sections were air dried for 10 min at 27 °C, then fixed in ice-cold 95% ethanol for 10 min. After fixation, sections were washed (3×5 min in ice-cold PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 0.5 mM $MgCL_2{\cdot}6H_2O$, pH 7.4)) and used in immunohistochemical procedures. Immunohistochemical procedures were performed on sections from a minimum of four CL removed at each time point listed above.

Primary antibodies, their specificity, commercial source, and the working dilutions used in immunohistochemical procedures are listed in Table 1. In the first procedure, steroidogenic luteal cells were identified using rabbit anti-CYP11A1 antiserum in conjunction with Alexa Fluor 488-conjugated goat anti-rabbit $F(ab')_2$ fragment as a 2° Ab (steroidogenic cells display green fluorescence), and cells expressing class II MHC cell surface antigens were identified using a cocktail of three monoclonal anti-bovine class II MHC antibodies in conjunction with Alexa Fluor 546-conjugated goat anti-mouse $F(ab')_2$ fragment as a 2° Ab (labeled cells display red fluorescence). Following fixation and wash steps, sections were incubated for 30 min at 27 °C in PBS containing 1% histological grade BSA (PBS/BSA) with 10% normal horse serum. Sections were rinsed (1× 3 min with ice-cold PBS) and incubated at 27 °C for 2 h with anti-CYP11A1 antiserum diluted in PBS/BSA containing 10% normal horse serum and 2% normal bovine serum (1° Ab diluent). Slides were washed and sections were incubated at 27 °C for 2 h with mouse anti-bovine class II MHC mAbs. In parallel, luteal tissue sections were incubated with a cocktail of mouse anti-bovine CD172a and mouse anti-human CD68 mAbs to identify monocytes and macrophages, as described previously (Townson *et al.* 2002). Slides were washed again and incubated at 27 °C for 3 min with 2° Abs diluted 1:200 each in PBS/BSA containing 2% normal bovine serum (2° Ab diluent). Slides were washed again and counterstained with 3 μg/ml DAPI in PBS (nuclei display blue fluorescence). Non-immune rabbit serum and non-specific mouse IgG were substituted for the anti-CYP11A1 antiserum (not shown) and mouse anti-bovine class II MHC mAbs respectively as negative controls to confirm primary antibody specificity.

In the second procedure, endothelial cells were identified using FITC-BS-1 (labeled endothelial cells display green fluorescence), as previously described (Clark *et al.* 2004), and cells expressing class II MHC cell surface antigens were identified using the same antibodies and dilutions as in the first experiment. Following fixation and wash steps, sections were incubated for 30 min at 27 °C with FITC-BS-1 diluted 1:500 in PBS. Slides were then washed, and sections were incubated for 30 min at 27 °C in PBS/BSA with 10% normal horse serum. Slides were then rinsed and sections were incubated at 27 °C for 2 h with mouse anti-bovine class II MHC antibodies. Slides were washed again and incubated at 27 °C for 30 min with 2° Ab diluted 1:200 in 2° Ab diluent. Slides were then washed and counterstained with 3 μg/ml DAPI in PBS. Non-specific mouse IgG was used instead of mouse anti-bovine class II MHC mAbs as a negative control to demonstrate specificity of antibody binding.

Tissue sections were examined using an Olympus BX51 microscope equipped with an Olympus reflected fluorescence system (Olympus America, Melville, NY, USA). Images were collected using an Olympus MagnaFire digital camera. Fluorescence micrographs were qualitatively assessed to determine the identity of cells expressing class II MHC molecules.

Isolation and culture of mixed luteal cells and luteal endothelial cells

Cultures of mixed luteal cells were prepared from bovine CL using procedures described previously (Pate

Table 1 Primary antibodies used in immunohistochemistry.

Antibody	Specificity	Source	Working dilution
H42A	Bovine class II MHC DP	VMRD, Pullman, WA, USA	1:2000
TH81A5	Bovine class II MHC DQ	VMRD, Pullman, WA, USA	1:2000
TH14B	Bovine class II MHC DRa	VMRD, Pullman, WA, USA	1:2000
RDI-P450 SCCabr	Bovine CYP11A1	Research Diagnostics, Concord, MA, USA	1:1000
DH59B	CD172a (bovine monocytes/granulocytes)	VMRD, Pullman, WA, USA	1:200
EBM11	CD68 (bovine activated macrophages)	Dako, Carpinteria, CA, USA	1:150

& Condon 1982). Cell culture was performed in a humidified atmosphere of 5% CO_2 in air at 37 °C. Dispersed luteal cells (4×10^6 cells/flask) were cultured in serum-coated 25 cm^2 flasks in a total of 4 ml Hams F-12 containing insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), gentamicin (20 μg/ml), and LH (1 ng/ml). The cells were allowed to adhere overnight, medium was replaced, and the cultures were exposed to either 0 or 50 ng/ml TNF for 48 h, the latter concentration having been shown to affect function, viability, and gene expression in cultures of mixed bovine luteal cells (Townson & Pate 1994, Petroff *et al.* 2001, Cannon & Pate 2003). Total RNA was extracted from the cells after 48 h of culture. Culture experiments were repeated thrice.

Purified endothelial cells from bovine CL (CLENDO cells) were purchased from Cambrex Bioscience (Bio-Whittaker, Inc., Walkersville, MD, USA), as described previously (Cavicchio *et al.* 2002, Pru *et al.* 2003, Liptak *et al.* 2005). In the present study, endothelial cells from frozen aliquots (passages 3–5; 5000 cells/cm^2) were cultured in a growth factor-containing medium (microvascular endothelial cell medium-2 (EGM-2MV)), as recommended by the supplier with 3% fetal bovine serum in 60 mm dishes. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture medium was replaced every 48 h until cells were 80–90% confluent, at which point medium was changed and cultures were maintained in a basal (serum- and growth factor-free) endothelial cell basal medium (EBM-2) medium for 24 h, as described (Pru *et al.* 2003). Prior to treatment, the medium was removed and replaced with fresh EBM-2 for an equilibration period of 3 h before the administration of treatments. Cultured cells were then treated with 0 or 50 ng/ml TNF for 48 h, the latter concentration having been shown to induce signal transduction pathways in bovine CLENDO cells (Pru *et al.* 2003). Total RNA was extracted from cells after 48 h of culture. Culture experiments were repeated thrice.

RNA extraction and RT-quantitative PCR (RT-qPCR)

RNA was extracted from luteal tissue collected during the estrous cycle using TRIzol reagent. Frozen luteal tissue was homogenized in TRIzol using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY, USA), and total cellular RNA was isolated according to manufacturer's specifications. For extraction of total RNA from mixed luteal cell or CLENDO cultures, cells were collected in RLT lysis buffer and RNA was isolated using the RNeasy kit (Qiagen) according to the procedures specified by the manufacturer.

Sequences, annealing conditions, GenBank accession numbers for corresponding targets, and references (Aida *et al.* 1994, Hartung *et al.* 1995, Stewart *et al.* 1996) for primer sequences used in RT-PCR are listed in Table 2. Steady-state concentrations of *DRA* and *GAPDH* mRNA were determined in total RNA extracted from bovine luteal tissues ($n=5$ CL per time point). Concentrations of *DRA* and *GAPDH* mRNA were also determined in total RNA isolated from cultures of mixed luteal cells ($n=3$) and CLENDO cells ($n=3$). In addition, the presence and relative abundance of *STAR* and *PECAM1* (a cell adhesion molecule used as an endothelial cell marker; Albelda *et al.* 1990, Levy *et al.* 2001) mRNAs were assessed in mixed luteal and CLENDO cell culture samples as a means of determining purity and composition of cultures.

PCR procedures were performed using an MJ Research Opticon 2 real-time PCR thermal cycler. Prior to PCR, RT using random hexamer primers was performed on 2 μg total RNA. PCR was then performed on 200 ng reverse-transcribed cDNA, using the DyNAmo HS SYBR Green qPCR kit according to manufacturer's instructions. Thermal cycling was carried out using the following conditions: denaturation at 94 °C for 30 s; annealing (see Table 2 for temperatures) for 30 s; and extension at 72 °C for 60 s, for a total of 32 cycles. Fluorescence values in each tube were measured at the end of each cycle using single acquisition mode. Melting curve analysis was performed after the end of the last cycle. Melting curve analysis in conjunction with gel electrophoresis of amplified products was used to verify amplification of a single product in each sample, and identification of amplified products was confirmed by sequencing.

Table 2 Primer sequences used in real-time RT-PCR assays.

Target	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)	GenBank accession no.	Reference
DRA	Forward: 5′-GGAAGAAGGAGACGGTGT-3′ Reverse: 5′-CAGGAAGACCGTCTGTGA-3′	305	54	X78308	Aida *et al.* (1994)
PECAM1	Forward: 5′-GTTCAGCGAAGTTCTGCGAG-3′ Reverse: 5′-CTTGCTGGCTGTGGTCTTGT-3′	229	58	U35433	Stewart *et al.* (1996)
GAPDH	Forward: 5′-AAGATTGTCAGCAATGCC-3′ Reverse: 5′-ACAGACACGTTGGGAG-3′	293	56	BC102589	–
STAR	Forward: 5′-CCTCTCTACAGCGACCAA-3′ Reverse: 5′-TCGTGAGTGATGACCGTG-3′	311	58	Y17259	Hartung *et al.* (1995)

For RNA samples collected from luteal tissue during the estrous cycle, all samples were assayed in duplicate using *DRA* and *GAPDH* primers. Fluorescence values of *DRA* from duplicate wells were standardized to the corresponding *GAPDH* values. Standardized values were then used to calculate steady-state concentrations of *DRA* message in each sample, using a homologous standard curve prepared from a purified *DRA* PCR product. Similar procedures were performed on RNA samples from mixed luteal and CLENDO cell cultures. In addition, the presence and abundance of *STAR* and *PECAM1* mRNAs in samples isolated from these cultures were assessed. Fluorescence values for *STAR* and *PECAM1* amplifications were standardized to corresponding *GAPDH* values as described previously. Since no standard curve was used to quantify the concentrations of these messages, comparisons were made using mean fluorescence, as previously described (Cannon *et al.* 2006).

Statistical analysis

One-way ANOVA was performed to determine whether differences ($P<0.05$) existed between mean steady-state *DRA* mRNA concentrations in luteal tissue samples collected on different days of the estrous cycle. The Student–Newman–Keuls procedure was used to determine differences between specific means. Within each culture type, the effect of TNF on amounts of *DRA*, *STAR,* and *PECAM1* mRNAs was determined using Student's *t*-test. Since TNF had no effect on the concentrations of any of these messages, data from untreated and TNF-treated cultures were pooled according to the cell type (mixed luteal or CLENDO cells), and differences in the amounts of *DRA*, *STAR*, and *PECAM1* mRNAs between cell types were determined using Student's *t*-test.

Results

In order to determine whether steroidogenic cells of the bovine CL express class II MHC molecules, polyclonal anti-rat CYP11A1 was used to identify the steroidogenic cells, while a cocktail of three anti-bovine class II MHC mAbs was used to identify cells expressing class II MHC. Steroidogenic luteal cells were negative for class II MHC at all stages of the estrous cycle examined (Fig. 1). In days 4 and 5 CL, class II MHC mAbs labeled single cells dispersed throughout the tissue, which was similar to the staining pattern observed when luteal tissues were stained with anti-monocyte and -macrophage mAbs (Fig. 1a and d versus b, c, e and f). In days 10–12 CL, class II MHC mAbs labeled single cells with an appearance similar to cells labeled by anti-monocyte and -macrophage mAbs (Fig. 1g versus h and i), and also cells with a distribution and arrangement within the tissue reminiscent of capillary endothelial cells (Fig. 1i, arrows). In day 18 CL, anti-class II MHC staining was similar to that observed in midcycle CL (Fig. 1g–i versus j–l), although the luteal microvasculature was less distinct at this time.

To determine whether endothelial cells expressed class II MHC, FITC-BS-1 was used to identify endothelial cells, while cells expressing class II MHC were identified as in the previous experiment. Similar to results in days 4 and 5 CL from the previous experiment, class II MHC expression was localized to single cells dispersed throughout the tissue. Endothelial cells expressing class II MHC were not present in early CL, as indicated by a lack of colocalization of class II MHC and FITC-BS-1 staining (Fig. 2a, b, d and e). In days 10–12 CL, in addition to single class II MHC-expressing cells similar to those observed in the early CL, colocalization of FITC-BS-1 and class II MHC staining revealed the presence of class II MHC-expressing endothelial cells (Fig. 2g and h, arrows), while other endothelial cells were negative for class II MHC. In day 18 CL, class II MHC expression was less distinct in endothelial cells, although some colocalization of FITC-BS-1 and class II MHC staining was apparent (Fig. 2j and k). Tissues in which primary antibodies were replaced with non-immune rabbit serum or non-specific mouse IgG were devoid of staining throughout (Fig. 2c, f, i and l; data for non-immune rabbit serum not shown). To determine whether luteal endothelial cells express each of the three types of class II MHC molecules (DP, DQ, and DR), the mAbs used as a cocktail in the first and second immunohistochemical studies were applied individually to stain midcycle luteal tissue in a third study. Endothelial cells in the midcycle CL expressed only DR (Fig. 3).

Steady-state concentrations of *DRA* mRNA were lower in days 4 and 5 CL when compared with those in day 18 CL (Fig. 4; $P<0.05$; $n=5$ CL per time point). The variation in *DRA* mRNA concentration was highest in the midcycle CL when compared with the other times examined; therefore, although the concentration of *DRA* mRNA appears higher in the midcycle CL when compared with that in days 4 or 5 CL, this difference was not significant ($P>0.05$).

To further confirm the expression of DRA by luteal endothelial cells, the presence and steady-state concentrations of *DRA* mRNA in cultures of mixed luteal and CLENDO cell cultures were evaluated. The purity of culture types was assessed using RT-qPCR to measure amounts of *STAR* and *PECAM1* mRNAs in samples from each culture, and the PCR was allowed to continue for a maximal number of cycles to amplify even the slightest amount of these two mRNAs. After 45 cycles, electrophoretic analysis revealed a single 311 bp amplicon of *STAR* mRNA in some samples from CLENDO cultures, while large amounts of *PECAM1* mRNA were present in all CLENDO cell

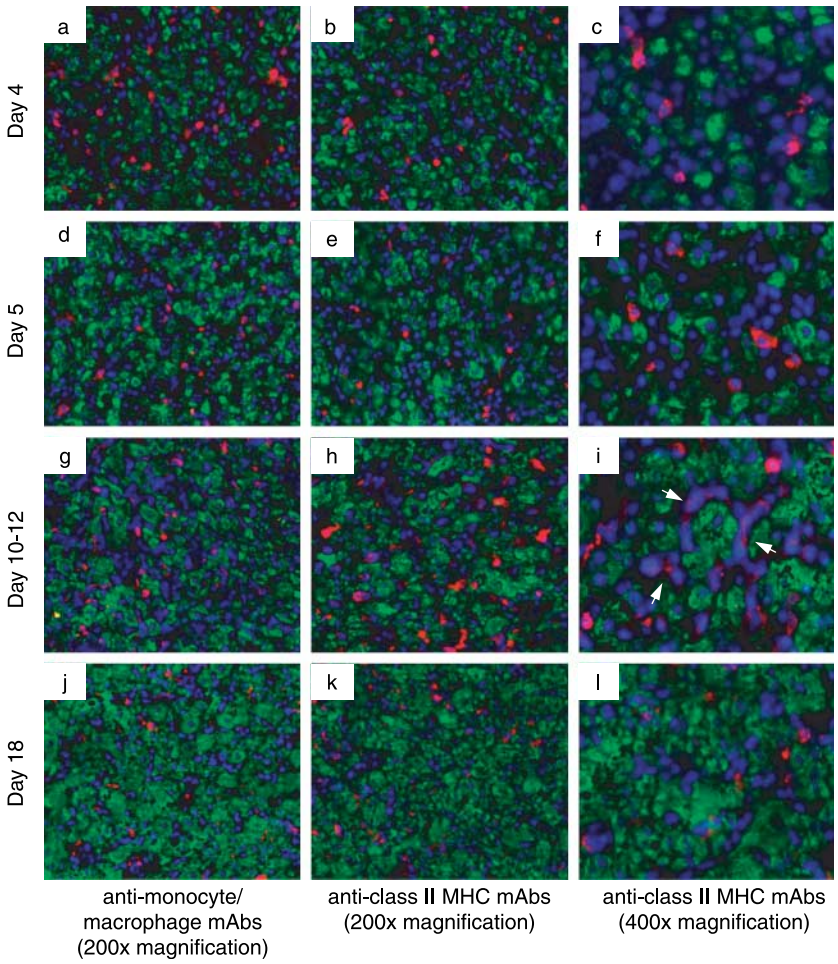

Figure 1 Fluorescent micrographs of bovine luteal tissue sections (7 μm) collected on days 4, 5, 10–12, and 18 of the estrous cycle. Steroidogenic cells were identified with anti-CYP11A1 antiserum (green fluorescence) and cells expressing class II MHC were identified using anti-class II MHC mAbs (red fluorescence in b, c, e, f, h, i, k, and l). Sections were also labeled with anti-monocyte and -macrophage mAbs (red fluorescence in a, d, g, and j). Cell nuclei were visualized with DAPI (blue fluorescence). White arrows (i) indicate putative endothelial cells labeled with class II MHC mAbs. Magnifications are listed below each column.

cultures (Fig. 5a, upper panels). TNF had no effect on the abundance of *STAR* or *PECAM1* mRNA in CLENDO cells ($P>0.05$, $n=3$; data not shown). In samples from mixed luteal cell cultures, *STAR* mRNA was abundant, and a single 229 bp *PECAM1* amplification product was also clearly visible (Fig. 5a, lower panels). TNF did not affect amounts of *STAR* or *PECAM1* mRNA in mixed luteal cell cultures ($P>0.05$, $n=3$; data not shown). The concentration of *STAR* mRNA was much higher ($P<0.05$) in mixed luteal cell cultures when compared with that in CLENDO cultures in which *STAR* mRNA was detectable, while the concentration of *PECAM1* mRNA was approximately tenfold higher ($P<0.05$, $n=3$) in CLENDO cells when compared with that in mixed luteal cells (Fig. 5b). A single 305 bp amplification product corresponding to *DRA* mRNA was present in cultures of CLENDO cells as well as in mixed luteal cells (Fig. 6, upper panel). TNF had no effect on *DRA* mRNA concentrations in both cultured cell types ($P>0.05$; Fig. 6, graphs), and concentrations of *DRA* mRNA were not different between cultures of mixed luteal and CLENDO cells ($P>0.05$).

Discussion

In the present study, we have demonstrated the expression of the class II MHC molecule DR by endothelial cells of the bovine CL. In the early CL, we observed class II MHC expression on single cells with a tissue distribution similar to monocytes and macrophages (Penny *et al.* 1999, Townson *et al.* 2002; and present study). Expression of class II MHC on macrophages is not surprising and would be expected. However, in midcycle (days 10–12) CL, staining was also observed on cells, whose distribution and architecture relative to the surrounding tissue were reminiscent of endothelial cells (Farin *et al.* 1986, O'Shea *et al.* 1989). In the second experiment, class II MHC was colocalized with a subpopulation of endothelial cells in the midcycle CL. Further investigation revealed that these cells express only DR, while putative macrophages express all the three types of class II MHC molecules, DP, DQ, and DR. However, not all endothelial cells were labeled with class II MHC mAbs, and expression of class II MHC by endothelial cells was less distinct in late CL, possibly due to the loss of a well-defined microvasculature relative to the midcycle CL.

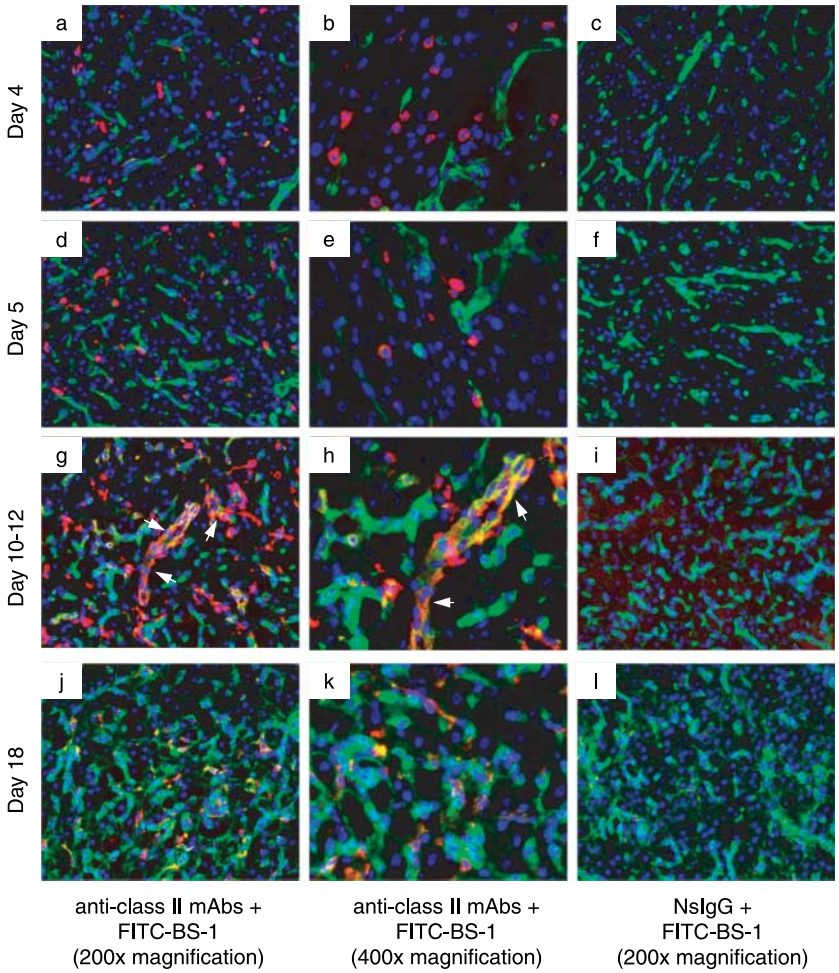

Figure 2 Fluorescent micrographs of bovine luteal tissue sections (7 μm) collected on days 4, 5, 10–12, and 18 of the estrous cycle. Endothelial cells were identified with FITC-labeled BS-1 lectin (green fluorescence) and cells expressing class II MHC were identified using anti-class II MHC mAbs (red fluorescence). Cell nuclei were visualized with DAPI (blue fluorescence). Yellow staining indicates colocalization of FITC-BS-1 lectin and class II MHC mAbs. White arrows (g and h) indicate endothelial cells expressing class II MHC. Sections in which anti-class II MHC 1° mAbs were substituted with non-specific IgG (c, f, i, and l) are shown to demonstrate antibody specificity. Magnifications are listed below each column.

Steady-state concentrations of *DRA* mRNA were higher in the late CL relative to the early CL, but due to a large amount of variation among animals, concentrations of *DRA* mRNA in the midcycle CL were not significantly different from the early or late CL. This increase in *DRA* expression is consistent with the expression of DRA protein by both putative macrophages and a subpopulation of endothelial cells in the midcycle and late CL, whereas only putative macrophages in the early CL express class II MHC. The elevation in steady-state *DRA* mRNA concentrations as the CL ages also agrees with the flow cytometric data of Benyo *et al.* (1991), who observed an increase in the number of class II MHC-expressing cells in the midcycle CL when compared with that in the early CL using flow cytometry.

Expression of class II MHC in the bovine CL has been examined in previous studies, with varying results. Fairchild & Pate (1989) initially demonstrated that IFNG induces expression of class II MHC on cultured bovine luteal cells, and the aforementioned flow cytometric study (Benyo *et al.* 1991) subsequently evaluated the expression of class II MHC on freshly isolated cells from dispersed bovine CL. Flow cytometric evaluation of dispersed bovine luteal cells in that study revealed three cell populations in midcycle and late luteal tissue: small cells (which presumably consist of small steroidogenic cells and macrophages, since cell <10 μm were excluded from analysis), large dense cells, and large less dense cells. The small and large less dense cells expressed class II MHC in the midcycle and late CL. The existence of two populations of large luteal cells, differing in amount of secretory granules and intracellular lipid droplets, had been noted previously (Hansel *et al.* 1987). However, when considering the data from the present study, it seems plausible that the large less dense cells expressing class II MHC in the study by Benyo *et al.* (1991) may have been small clusters of class II MHC-expressing endothelial cells, rather than single large cells. In preliminary immunofluorescent studies, we observed small clusters of endothelial cells labeled with class II MHC mAbs in dispersed luteal cell suspensions. These clusters were composed typically of four to eight endothelial cells, and were of similar size to large steroidogenic luteal cells (Cannon & Pate unpublished observations).

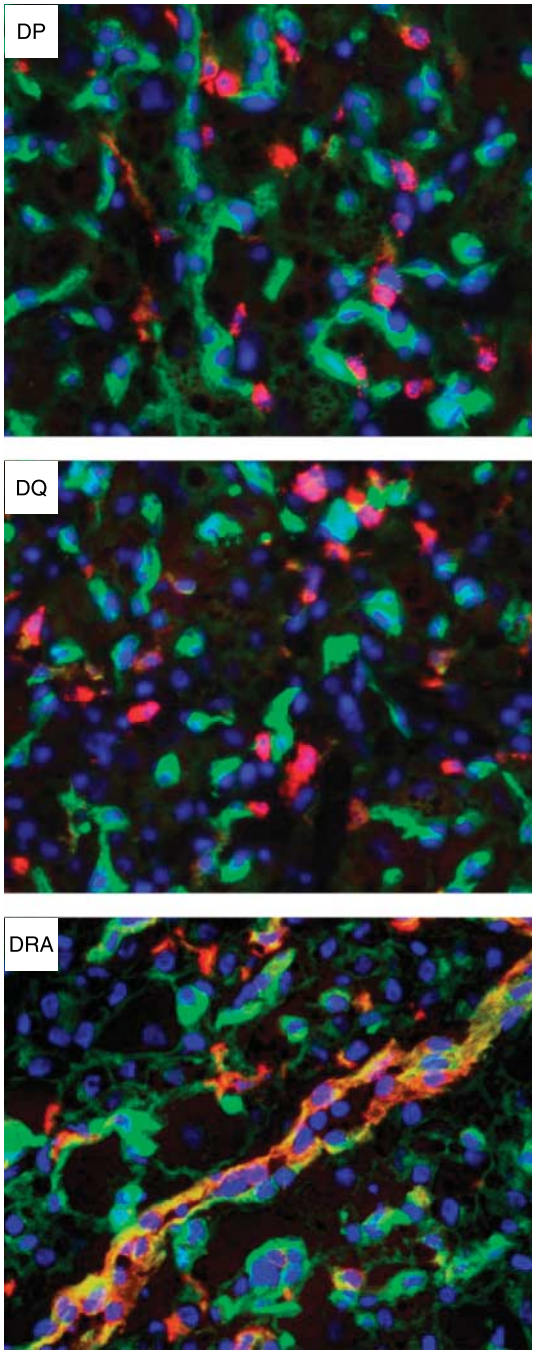

Figure 3 Fluorescent micrographs of midcycle (day 10) bovine luteal tissue sections (7 μm) stained with mAbs H42A (DP), TH81A5 (DQ), or TH14B (DRA) individually (red fluorescence). Endothelial cells were identified with FITC-labeled BS-1 lectin (green fluorescence). Cell nuclei were visualized with DAPI (blue fluorescence).Yellow staining indicates colocalization of FITC-BS-1 and class II MHC mAbs. Magnification, 200×.

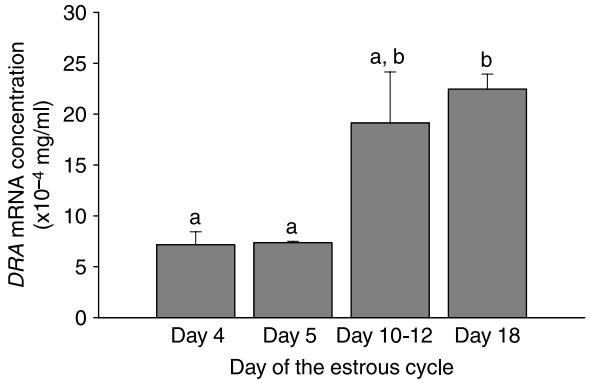

Figure 4 Steady-state concentrations of *DRA* mRNA in bovine CL collected during the estrous cycle. Bars represent mean concentrations of *DRA* mRNA in total RNA samples extracted from luteal tissue collected on days 4, 5, 10–12, or 18 of the estrous cycle ($n=5$ CL per time point). Different letters denote significant differences ($P<0.05$).

Therefore, it is possible that the class II MHC-positive large less dense cells originally observed (Benyo *et al.* 1991) consisted, at least in part, of small clusters of DR-expressing luteal endothelial cells. The results of that study agree with those of the present study, in that large dense cells in that study (presumably steroidogenic luteal cells) did not express class II MHC at any time during the estrous cycle.

The presence of cells expressing DRA was observed in a more recent immunohistochemical study (Penny *et al.* 1999), but these authors did not identify the individual cell type(s) expressing DRA. The methods employed differ somewhat from those of the present study, in that we have used immunofluorescent techniques to localize antibody binding, whereas Penny *et al.* (1999) used enzymatically based colorimetric detection. Additionally, acetone was used as a fixative for frozen sections in that study, but we have found that, at least in our hands, ethanol is superior to acetone for maintaining cell morphology and tissue architecture in frozen luteal tissue sections, thus allowing for better resolution of cell types.

Our results appear to agree with those of Lehman *et al.* (2000), who observed class II MHC expression on a subpopulation of cultured endothelial cells derived from bovine CL. Flow cytometry was used in that study to evaluate class II MHC expression on various cultured bovine luteal endothelial cell subtypes (Lehman *et al.* 2000), and therefore no information on expression of class II MHC by endothelial cells *in situ* could be derived. Ours is the first study, to the best of our knowledge, in which the expression *in situ* of class II MHC by bovine luteal endothelial cells has been demonstrated.

Cultures of mixed luteal and CLENDO cells were used to further confirm the observation that the class II MHC molecule DR is expressed by luteal endothelial cells. Analysis of *STAR* and *PECAM1* mRNAs indicated that the CLENDO cell cultures used in the present study were highly enriched cultures of luteal endothelial cells, whereas the mixed luteal cell cultures were composed largely of steroidogenic cells, but were also likely to contain some endothelial cells. *DRA* mRNA was found in both mixed luteal and CLENDO cell cultures, with no

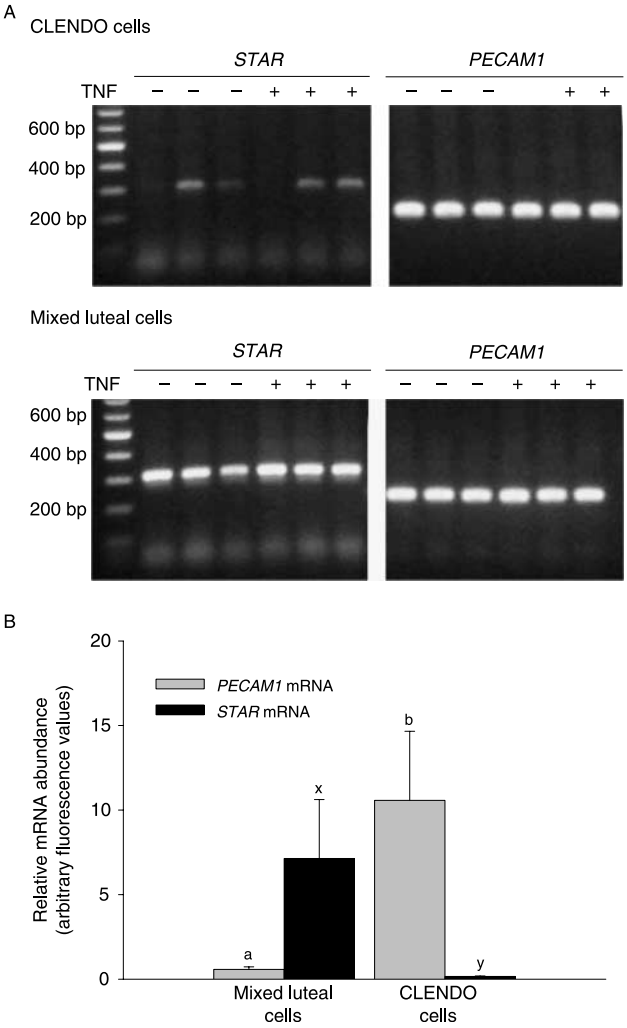

Figure 5 (A) Agarose gels showing PCR amplification products using *STAR*- and *PECAM1*-specific primers in samples extracted from cultures of CLENDO cells (top panel) or mixed luteal cells (bottoms panel). Cultures were treated with 0 or 50 ng/ml TNF ($n=3$ cultures per treatment) for 48 h. (B) Relative abundance of *STAR* and *PECAM1* mRNA in cultures of CLENDO and mixed luteal cells. Bars represent mean amounts of PECAM1 (gray bars) or STAR (black bars) mRNAs (expressed in arbitrary fluorescence units) in samples extracted from CLENDO or mixed luteal cell cultures. Different letters indicate a difference in relative abundance of mRNA between CLENDO (a and b) and mixed luteal cell (*x* and *y*) cultures ($P<0.05$, $n=3$).

differences in steady-state concentrations of *DRA* mRNA between cultures. The presence of *DRA* mRNA in CLENDO cultures confirms the immunohistochemical results demonstrating the presence of DRA in endothelial cells of the CL. The presence of relatively large amounts of *DRA* mRNA in mixed luteal cell cultures seems enigmatic, given the lack of expression of class II MHC by steroidogenic cells observed throughout the first part of this study. Macrophages, along with the minor endothelial cell contaminant suspected to be present in mixed luteal cell cultures, could account for the presence of *DRA* mRNA in these cultures. Alternatively, steroidogenic cells may contain *DRA* mRNA, but express an aberrant form of the protein that is not recognized by the antibody using immunohistochemical methods. Finally, the possibility exists that a distinct cell type, which is localized adjacent to endothelial cells in tissue sections, but is not isolated with CLENDOS, is responsible for DRA expression. Such non-professional antigen-presenting cells (ITO cells) have been identified in the liver (Winau *et al.* 2007), but no such cell has yet been described in the CL.

It was of interest to note that TNF had no effect on *STAR*, *PECAM1*, or *DRA* mRNA concentrations in both mixed luteal and CLENDO cells. TNF has been shown to reduce steady-state *PECAM1* mRNA concentrations in bovine endothelial cells (Stewart *et al.* 1996). However, in the flow cytometric study by Lehman *et al.* (2000), TNF had no effect on PECAM1 and class II MHC expression in cultured bovine luteal endothelial cells, which supports the present findings.

Endothelial cells make up an estimated 50% of the cells in the CL (Farin *et al.* 1986, O'Shea *et al.* 1989), and a great deal of attention has been focused on the role of endothelial cells in luteal function. Prostaglandin $F_{2\alpha}$ enhanced the production of endothelin-1 (EDN1) by luteal endothelial cells *in vitro* (Girsh *et al.* 1996*a*), and EDN1 inhibited progesterone production by cultured luteal-like cells (Girsh *et al.* 1996*b*), presumably via a protein kinase C-dependent pathway (Sen *et al.* 2006). TNF stimulated the secretion of $PGF_{2\alpha}$, EDN1, and monocyte chemoattractant protein 1 (CCL2) by luteal endothelial cells (Okuda *et al.* 1999, Cavicchio *et al.* 2002), and induced apoptosis of luteal endothelial cells, possibly via a ceramide-dependent mechanism that involved the production of reactive oxygen species (Pru *et al.* 2003). In addition, activated lymphocytes stimulated CCL2 secretion by cultured luteal endothelial cells, and this stimulation was contact dependent (Liptak *et al.* 2005). It is unknown whether this contact-dependent stimulation was MHC dependent. However, it is possible that within the midcycle CL, endothelial cells interact with lymphocytes in a class II MHC-dependent manner, resulting in the production of CCL2 by endothelial cells. Class II MHC-dependent activation of T lymphocytes by bovine luteal cells has been demonstrated *in vitro* (Petroff *et al.* 1997).

Microvascular endothelial cells play an integral role in the regulation of the immune system, since they regulate recruitment of T cells into the tissue via chemokine and cell surface adhesion molecule expression (Pober 1999). Expression of class II MHC molecules by endothelial cells is most often associated with either pathogenic autoimmune disease or graft rejection (Denton *et al.* 1999, Turesson 2004), although some types of endothelial cells apparently express class II MHC molecules in the absence of a pathological inflammatory condition (Pober 1999). The common belief that class II MHC expression by endothelial cells results in stimulation of T-cell activation by endothelial cells is supported by numerous studies

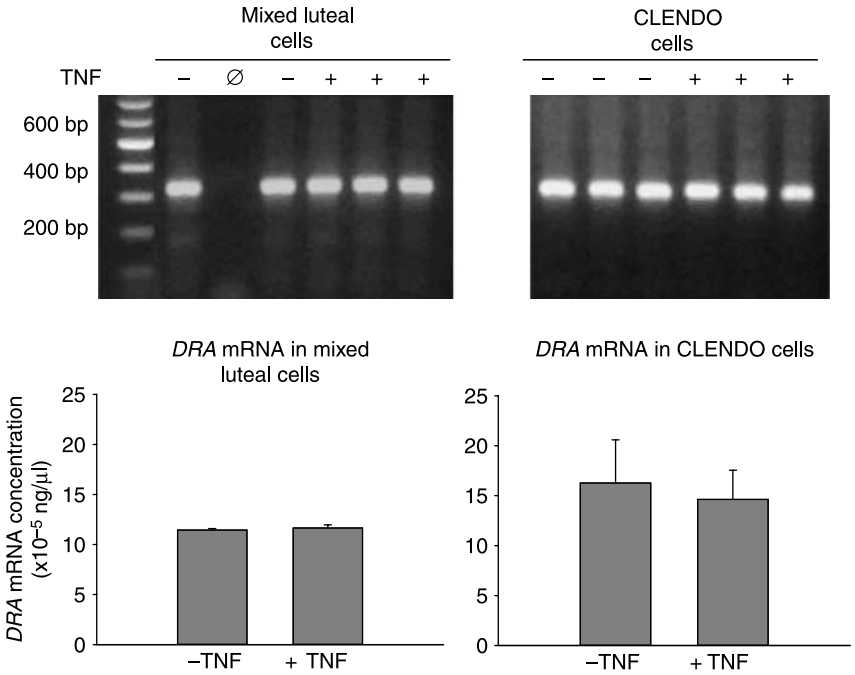

Figure 6 Expression of *DRA* in CLENDO and mixed luteal cell cultures. Upper panels display agarose gels showing PCR amplification products using *DRA*-specific primers in samples extracted from CLENDO or mixed luteal cell cultures. Ø indicates a lost sample (empty gel lane). Graphs show steady-state concentrations of *DRA* mRNA in cells cultured in the absence or presence of 50 ng/ml TNF ($n=3$ cultures per treatment). Bars represent mean concentrations of *DRA* mRNA in total RNA extracted from CLENDO or mixed luteal cell cultures.

(Choi *et al.* 2004). However, T-cell activation by class II MHC-expressing endothelial cells should not be considered the rule, since there are notable exceptions in which endothelial cells do not induce T-cell activation (Marelli-Berg *et al.* 1996, 1999, Katz *et al.* 2004) and even induce T-cell anergy, an induced state of inactivation (Denton *et al.* 1999, Kawai *et al.* 2000, Khayyamian *et al.* 2002, Appleman & Boussiotis 2003, Tokita *et al.* 2006). In addition to induction of anergy, microvascular endothelial cells from other tissues can also promote production of pro-inflammatory as well as anti-inflammatory cytokines by T lymphocytes, and these observations demonstrate the ability of endothelial cells to regulate the activity of migrating T lymphocytes.

With regard to the CL, microvascular endothelial cells are the first cells that will be encountered by T lymphocytes circulating through and infiltrating the CL. It is therefore likely that luteal endothelial cells, by necessity, interact with infiltrating T lymphocytes, and in doing so may regulate their effector functions. It is noteworthy that several recent studies have observed increases in immune cells, cytokines, and expression of genes significant to immune function in the midcycle CL (Townson *et al.* 2002, Cannon & Pate 2003, Cannon *et al.* 2006), while several reports demonstrate supportive effects of secreted products of macrophages and T lymphocytes on luteal cell function (Hughes *et al.* 1991, Chen *et al.* 1992, Ness & Kasson 1995, Sugino *et al.* 1998, Pate & Keyes 2001). Expression of the class II MHC molecule DR by a subpopulation of luteal endothelial cells may be a means by which regulation of infiltrating T lymphocytes takes place. Thus, although it has been suggested for some time that the immune system facilitates the process of luteal regression (Pate 1995, Davis & Rueda 2002), it may be possible that the immune system also participates in maintenance of normal luteal function. In this scenario, class II MHC-expressing luteal endothelial cells could induce a state of anergy in T cells infiltrating the CL during the luteal phase, thus preventing potentially detrimental activation of T cells prior to the time of luteal regression. In light of the present findings, the role of the immune system in regulation of luteal function may need to be reconsidered, in that the immune system may not only facilitate luteal regression, but also support development and normal function of the CL prior to initiation of luteolysis.

In conclusion, we have presented the evidence that a subpopulation of endothelial cells in the bovine CL expresses the class II MHC molecule DR, with expression absent in the early CL but present and readily detectable at midcycle, and also in late CL. CLENDO cells were shown to contain *DRA* mRNA, confirming the immunohistochemical observations from the first part of the study. The precise role of DR expression on bovine luteal endothelial cells remains to be determined, but it is possible that DR expression on luteal endothelial cells during midcycle modulates the response of T lymphocytes present in the luteal microvasculature in a way that supports normal luteal function.

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