

# Expression of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes

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## Abstract

Interferon-tau (IFN $\tau$ ) acts locally on the endometrium to suppress estrogen and oxytocin receptor expression and block luteolysis in ruminants. Systemic administration of conceptus homogenates or recombinant ovine IFN $\tau$  does not block luteolysis or enhance pregnancy rates in sheep or cattle, respectively. However, IFN $\tau$  up-regulates expression of the antiviral protein Mx throughout the entire uterine wall during early pregnancy. These studies determined if conceptus-derived IFN $\tau$  also up-regulates Mx expression in components of the circulating immune system that migrate through the endometrial wall. In experiment one, peripheral blood mononuclear cells (PBMC) were isolated from ewes at D26 post-artificial insemination (AI) and Mx mRNA levels examined by

Northern and slot-blot hybridization. Pregnancy resulted in a two-fold increase in Mx mRNA levels compared to bred, non-pregnant ewes at D26. In experiment two, PBMC were isolated from ewes at AI, and every three days from D9 to D30. Results showed a four-fold increase in Mx mRNA levels in PBMC from pregnant versus bred, non-pregnant ewes at D15. Increased Mx mRNA, which remained elevated through D30, was accompanied by increased levels of Mx protein. These results show that pregnancy recognition signaling rapidly induces Mx gene expression in PBMC, and are the first to suggest that IFN $\tau$  activates gene expression in components of the circulating immune system.

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## Introduction

Pregnancy recognition in domestic ungulates involves local regulation of endometrial gene expression by the conceptus that results in reduced or altered production of the luteolytic signal, prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ; Bazer *et al.* 1995). This is in contrast to pregnancy recognition in primates, which involves a direct luteotrophic effect on the corpus luteum (CL) by conceptus-produced chorionic gonadotropin (Bazer *et al.* 1995). Ruminant conceptuses secrete IFN $\tau$ , the signal for pregnancy recognition, during the second and third week of pregnancy (Bazer *et al.* 1995; Godkin *et al.* 1982). IFN $\tau$  prevents increases in endometrial estrogen and oxytocin receptors, to abrogate oxytocin-induced luteolytic pulses of PGF<sub>2 $\alpha$</sub> , and maintains CL function (Spencer *et al.* 1995).

IFN $\tau$  is a member of the Type I IFN family, which also includes IFN  $\alpha$ ,  $\beta$ , and  $\omega$  (Samuel 1991). IFN $\tau$  signaling through the Type I IFN receptor and Janus Kinase (JAK)–signal transducer and activator of transcription (STAT) signal transduction pathway (Stewart *et al.* 2001) induces a number of genes in the ovine uterus including

2',5' oligoadenylate synthetase (Johnson *et al.* 2001),  $\beta$ 2-microglobulin (Vallet *et al.* 1991), IFN regulatory factor 1 (Spencer *et al.* 1998), ubiquitin cross-reactive protein (Johnson *et al.* 2000), and Mx protein (Charleston & Stewart 1993; Ott *et al.* 1998). While the functions of many of these proteins in the antiviral response are well characterized, their roles during early pregnancy are not.

Mx proteins are monomeric GTPases, which, depending on the species of animal and type of virus are potent inhibitors of viral replication (Samuel 1991). Although the antiviral effects of Mx are generally directed against negative-stranded RNA viruses (e.g. orthomyxovirus), their expression is induced in all cells that possess Type I IFN receptors and is generally predictive of viral infection (Haller *et al.* 1998). Recently Mx mRNA and protein were shown to be elevated from epithelium (by day 13) to myometrium (by day 15) within the uterine wall in pregnant ewes and levels remained elevated through day 25 (Ott *et al.* 1998). In addition, Mx mRNA levels were elevated in the corpus luteum in response to injections of roIFN $\tau$  into the uterine lumen (Spencer *et al.* 1999).

These results indicated that IFN $\tau$  was either: 1) acting directly on all uterine cell types (i.e., epithelial, stromal and myometrial) and on the CL; or 2) inducing substances (cytokines) that have paracrine/endocrine effects on uterine cells and other organs including the ovaries; or 3) affecting components of the uterine mucosal and circulating immune systems which then affect the various uterine cells and CL. The present studies tested the hypothesis that conceptus-derived IFN $\tau$ , expressed during pregnancy recognition signaling, increases Mx gene expression in PBMC.

## Materials and Methods

### Animal models

For experiment one, 60 mature, white-faced, ewes from the U.S. Sheep Experiment Station (USSES, Dubois ID) were synchronized and bred either by transcervical or laparoscopic AI (AI=D0; Stellflug *et al.* 2001). At D26, blood (10 ml) was collected by jugular venipuncture into EDTA-containing vacutainer tubes (Sherwood Medical, St. Louis MO). PBMC were isolated as described below. Pregnancy was determined by assaying serum for pregnancy-specific protein B (PSPB; Biotracking Inc, Moscow ID) and lambing dates and number of lambs born were recorded. In experiment two, 34 mature Suffolk ewes were synchronized and bred by laparoscopic AI. Blood (20 ml) was collected by jugular venipuncture at D0, and every three days from D9 to D30, and PBMC were isolated. Pregnancy was confirmed by real-time ultrasonography and PSPB assay at D30.

### PBMC isolation

Blood was kept on ice until processed. Samples were centrifuged at 300 *g* for 20 min at 4 °C. The buffy coat was removed and resuspended in 0.87%Tris-NH<sub>4</sub>CL lysis buffer at a 1 to 5 ratio. Samples were incubated for 5 min at 37 °C and centrifuged at 300 *g* for 10 min. The supernatant was removed and pellets were washed with 10 ml 1X PBS and centrifuged for 10 min at 300 *g*. After removal of supernatant, cell pellets were either frozen at -80 °C for protein extraction, or lysed with 2 ml TRIZOL (Life Technologies, Grand Island NY) and stored at -80 °C for RNA extraction.

### RNA extraction, Northern and slot-blot analysis

Total cellular RNA was extracted using TRIZOL according to manufacturer's instructions. RNA was quantified by absorbance at 260 nm. To establish size and number of Mx transcripts in PBMC, RNA (5  $\mu$ g) was electrophoresed in a 1% agarose/0.615 M formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell, Keene

NH) by capillary blotting. For quantification of Mx mRNA levels in PBMC, RNA (5  $\mu$ g) was transferred to a nylon membrane by vacuum filtration (Minifold II, Schleicher & Schuell, Keene NH). Blots were probed with a biotin-labeled ovine Mx anti-sense cRNA probe (Ott *et al.* 1998) using the North2South Hybridization kit (Pierce, Rockford IL) and chemiluminescent signal was quantified using a Bio-Rad Fluor-S MultiImager system and Quantity One software (Bio-Rad, Hercules CA). Slot-blots were stripped and re-probed with an ovine 18s rRNA cRNA probe to correct for variations in RNA loading.

### Protein isolation and Western blot analysis

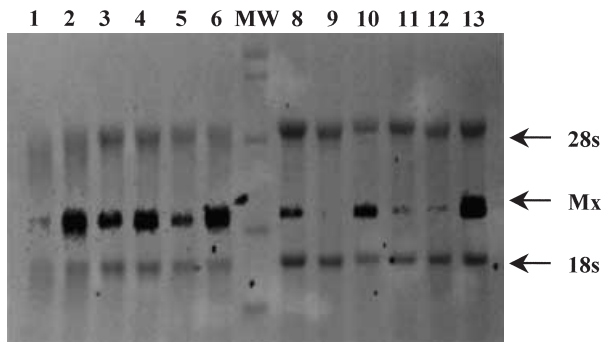
Total cellular protein was extracted using M-PER reagent (Pierce, Rockford IL), according to manufacturers instructions. Protein concentration of samples was quantified by BCA assay (Pierce, Rockford IL) with bovine serum albumen as the standard. Proteins (8  $\mu$ g/sample) from PBMC isolated from pregnant and bred, non-pregnant ewes at D15 and D18 were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (BA83, Schleicher & Schuell, Keene NH). Following blocking of non-specific binding sites in 5% non-fat dry milk in Tris-buffered saline and Tween 20 (TBST) for 2 h at 25 °C, membranes were incubated with a 1:1000 dilution of a polyclonal rabbit ovine Mx peptide antiserum (#90618-2; 0.7  $\mu$ g/ml) at 4 °C overnight. Goat anti-rabbit IgG (0.8  $\mu$ g/ml) labeled with horseradish peroxidase was used at a 1:200 000 dilution as secondary antibody. Chemiluminescent signal was developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford IL) and quantified using the Fluor-S Multi-Imager system and Quantity One software.

### Analysis

Chemiluminescent signal was analyzed using GLM procedures of SAS (Version 8.1, SAS Inc, Gary NC). The model included, where appropriate, status (pregnant versus bred, non-pregnant), ewe nested within status, day (0, 9, 12, 15, 18, 21, 24, 27, and 30) and appropriate interactions. Error terms in the F test were according to the expectation of mean squares for error. Signal for 18s rRNA was run as a covariate in the model to correct for variations in loading. Results are reported as adjusted Least Squares Means (LSM) and pooled standard errors.

## Results

Northern blot analysis (Fig. 1) detected a single, approximately 2.5 kb, band in PBMC isolated from pregnant and

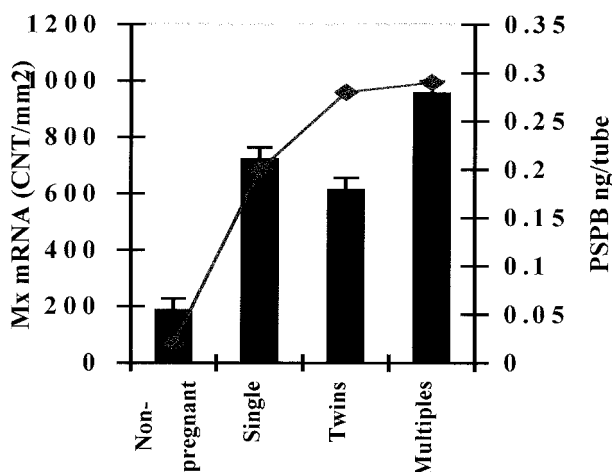


**Figure 1** Northern Blot analysis of Mx mRNA from PBMC at D26 post-AI. Lanes 1–6 represent pregnant ewes and lanes 8–13 represent non-pregnant ewes. Mx mRNA migrated at  $\sim 2.5$  kb.

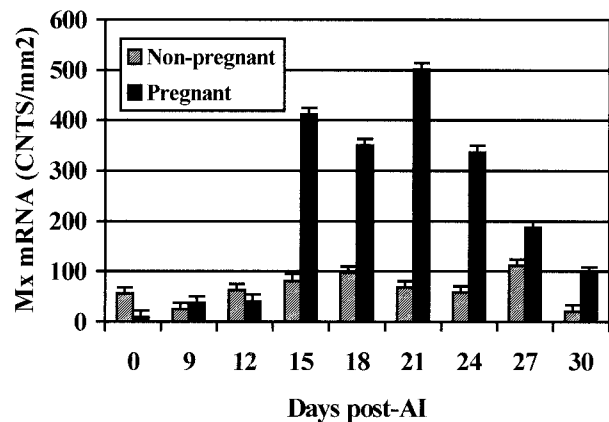
bred, non-pregnant ewes, which agrees with the known size of the ovine uterine Mx cDNA (Charleston and Stewart 1993; Ott *et al.* 1998).

Slot blot analysis (Fig. 2) of total cellular RNA isolated from PBMC collected at D26 post-AI showed a four-fold increase in Mx mRNA levels in pregnant versus bred, non-pregnant ( $n=26$ ) ewes ( $P<0.01$ ). In addition, ewes carrying multiples (triplets or quads;  $n=10$ ) had higher Mx mRNA levels than those carrying singles ( $n=10$ ) or twins ( $n=9$ ;  $P<0.05$ ). Results from the PSPB assay confirmed pregnancy status and, as reported previously, levels of PSPB were correlated with number of lambs born (Willard *et al.* 1995).

Experiment 2 examined the temporal expression of Mx mRNA during early pregnancy in sheep (Fig. 3). Results in Fig. 2 are a representative subset of all ewes and depict results from four pregnant and four bred, non-pregnant



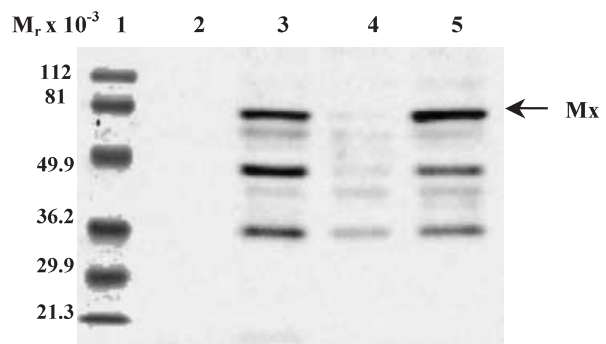
**Figure 2** Mx mRNA abundance in PBMC from D26 post-AI. Mx mRNA levels were  $\sim 4$  fold greater in pregnant versus bred, non-pregnant ewes at D 26 ( $P<0.01$ ). PSPB levels (line) confirmed pregnancy status and were correlated with number of lambs born.



**Figure 3** Temporal expression of Mx mRNA in PBMC from D0 to D30 post-AI. Mx mRNA levels were increased in pregnant ( $n=4$ ) compared with bred, non-pregnant ( $n=4$ ) ewes beginning at D15 and remained elevated through D30 ( $P<0.01$ ).

ewes during the first 30 days following insemination. This allowed analyzing all replicates on a single blot to eliminate problems associated with signal intensity between blots. Results showed Mx mRNA levels increased in pregnant ewes beginning at D15 ( $P<0.01$ ). Levels peaked at D21 and gradually declined thereafter. At D30, Mx levels in pregnant ewes remained elevated two-fold compared to bred, non-pregnant ewes ( $P<0.01$ ).

Mx protein expression in PBMC from representative D15 and D18 pregnant and bred, non-pregnant ewes was examined by Western blotting. Mx protein ( $\sim 75$  kDa) was not detected in either D15 or D18 open ewes, but was strongly up-regulated in PBMC from pregnant ewes on both days (Fig. 4). Two additional bands ( $\sim 48$  and  $36$  kDa) were detected in PBMC from pregnant ewes.



**Figure 4** Western blot analysis of Mx protein expression from PBMC at D15 and D18 post-AI. Lanes 2 and 4 represent a bred, non-pregnant ewe at D15 and D18 respectively. Lanes 3 and 5 represent a pregnant ewe at D15 and D18 respectively. Mx protein was up-regulated in PBMC at D15 and D18 of pregnancy.

## Discussion

Results demonstrate a rapid and sustained activation of Mx gene expression in PBMC in response to pregnancy recognition signaling, and indicate that, in addition to local effects of IFN $\tau$ , there is rapid systemic response in sheep. In addition, Mx expression did not increase in PBMC when pregnancy was not established (bred, non-pregnant ewes). These findings are novel because pregnancy recognition signaling by IFN $\tau$  was heretofore considered to result solely from local regulation of endometrial gene expression (Stewart *et al.* 2001; Johnson *et al.* 2001; Vallet *et al.* 1991; Spencer *et al.* 1998; Johnson *et al.* 2000; Charleston & Stewart 1993; Ott *et al.* 1998; Spencer *et al.* 1999) and suppression of estrogen and oxytocin receptor expression to abrogate luteolytic pulses of PGF $_{2\alpha}$  (Spencer *et al.* 1995). Sheep conceptuses begin to produce high levels of IFN $\tau$  between D12–14 post-mating (Bazer *et al.* 1995). Results from the present study indicate that Mx gene expression increases 4–5 fold in PBMC within 24–48 h of initial IFN $\tau$  signaling (Bazer *et al.* 1995). This systemic response to IFN $\tau$  continued through D30 post-AI; 8–10 days after conceptus production of IFN $\tau$  is detectable (Farin *et al.* 1989). Immunoblotting with Mx antiserum detected three distinct bands, one of which co-migrated with rhuMxA (supplied by M. Horisberger, Novartis, Basel, Switzerland; not shown). The two lower molecular weight bands may represent multiple Mx genes or partially degraded protein. Although Mx levels were not measured on days 13 or 14, the magnitude of the increase observed on day 15 suggests that Mx could be detectable in PBMC earlier. Although these studies do not establish if the effect is direct or indirect, we propose that pregnancy recognition signaling involves both local and systemic activation of IFN $\tau$ -induced genes.

Recently, Dixit & Parvizi (2001) demonstrated that bovine pregnancy was accompanied by increased production of adrenocorticotropin and nitric oxide by cultured bovine PBMC. This increase occurred as early as day 7 of pregnancy and levels remained elevated through the first half of gestation. The authors, however, ruled out involvement of IFN $\tau$  in this response. Although the authors did not suggest a mechanism for this increase, they postulated that it occurred as a result of interactions between the immune and endocrine systems at the fetal/maternal interface. Emond *et al.* (2000) demonstrated *in vitro* that IFN $\tau$  stimulated granulocyte-macrophage colony-stimulating factor gene expression in bovine leukocytes and endometrial stromal cells, independently of its effects on prostaglandin E (PGE) production. This activation remains to be confirmed *in vivo*. A role for PGE as a luteotropin delivered locally from the uterus to the ovary has long been postulated (Ellinwood *et al.* 1979), and IFN $\tau$  increased PGE production by bovine endometrial epithelial and stromal cells *in vitro* (Asselin *et al.* 1997).

However, a role for PGE in the peripheral circulation during early pregnancy has not been established.

At present, the only known stimuli for Mx expression are Type I IFNs and virus, and its only defined function is in the antiviral response where Mx plays a primary role in blocking replication of negative-stranded RNA viruses (Haller *et al.* 1998). The simplest explanation for the present results is that Mx expression is up-regulated in the PBMC of early pregnancy to block potential viral infections; perhaps offsetting the immuno-modulatory actions of progesterone (Gill 1985). Recent results clearly indicate that progesterone interacts with IFN $\tau$  to regulate uterine gene expression (Johnson *et al.* 2001, Johnson *et al.* 2000), including the Mx gene (Ott *et al.* 1999). Whether this is the case for Mx expression in PBMC remains to be determined. However, the temporal up-regulation of Mx in the uteri of cyclic ewes, and spatial distribution of Mx within the uteri of pregnant ewes (Ott *et al.* 1998), combined with the present results suggest a potential role for Mx outside the response to viral infection.

In summary, these results highlight the rapid communication that occurs between the peri-implantation conceptus and the maternal circulating immune system during pregnancy recognition signaling. Although the function of Mx in PBMC during early pregnancy remains to be determined, differential Mx expression between PBMC of pregnant and bred, non-pregnant ewes offers an opportunity for early diagnosis of pregnancy loss, which has broad potential application for enhancing reproductive management of domestic ruminants.

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