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γδTCR+ cells of the pregnant ovine uterus express variable T cell receptors and contain granulysin

Annette Fox[a], Jill F. Maddox[b], Mike J. de Veer[c], Els N. Meeusen[c,*]

[a] Oxford University Clinical Research Unit, Hanoi, Viet Nam
[b] Faculty of Veterinary Science, The University of Melbourne, Victoria 3010, Australia
[c] School of Biomedical Sciences, Monash University, Victoria 3800, Australia

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ABSTRACT

γδ T cells are a prominent granulated cell population in the ruminant pregnant uterus and both their number and granule size increase dramatically during pregnancy. Anchor-RT-PCR was used to assess TCRδ gene usage by γδ T cells from the uterine epithelium of pregnant sheep. The TCRδ genes obtained exhibited distinct combinatorial and junctional diversity and only two out of nine V–D–J rearrangements sequenced were identical. Furthermore, two of the Vδ elements used are also expressed in peripheral blood, indicating that γδTCR use in sheep epithelia is neither restricted nor site-specific, similar to humans but in contrast to findings in mice. Protein analysis of purified, granulated uterine γδ T cells revealed the presence of large amounts of the antimicrobial peptide, granulysin. The results of the present study indicate that ovine uterine γδTCR+ intraepithelial lymphocytes have the potential to recognise diverse antigens and may have a role in protecting the uterus from infection during pregnancy and parturition. A similar protective role for γδ T cells may exist in the human decidua parietalis.

1. Introduction

Although γδ T cells represent a substantial or dominant proportion of intraepithelial lymphocytes (IEL) within various surface epithelia in humans, ruminants and mice (Kronenberg and Havran, 2007; Meeusen et al., 2001), their exact function and antigen recognition properties have not been fully determined. γδ T cells in murine skin and reproductive tract epithelium express homogeneous TCR and have therefore a limited capacity to recognise diverse antigens; it has been proposed that they respond to a conserved antigen expressed by epithelial cells after exposure to stressful stimuli (Asarnow et al., 1988). In support of this proposal, murine γδ T cells can respond to conserved heat shock proteins and to stressed keratinocytes *in vitro* (Chien and Konigshofer, 2007; Kronenberg and Havran, 2007). These results in mice contrast to humans, where extensive TCR diversity has been found in γδ T cell clones from female reproductive tissues (Christmas et al., 1993, 1995). γδTCR diversity is generated by rearrangement of pools of V, J, (D) and C elements during T cell development. Diversity is increased by junctional modifications including addition of non-germline encoded (N) nucleotides and P nucleotides, exonucleolytic deletion of nucleotides from the 3′ ends of rearranging elements and simultaneous use of more than one D element in TCRδ genes (Chien and Konigshofer, 2007; Kronenberg and Havran, 2007).

γδ T cells represent up to 60% of lymphocytes in the peripheral blood of sheep and express extremely diverse TCR using a particularly large number of Vδ elements (Hein and Dudler, 1993). γδ T cells are also a prominent population in the uterine epithelium of sheep (Meeusen et

* Corresponding author at: Biotechnology Research Laboratory, School of Biomedical Sciences, Bld 13f, Monash University, Victoria 3800, Australia. Tel.: +61 3 9905 2513; fax: +61 3 9905 2547.
E-mail address: els.meeusen@med.monash.edu.au (E.N. Meeusen).

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al., 1993). In contrast to the majority of blood γδ T cells, ovine uterine γδTCR$^+$ IEL express a unique, WC1$^-$, CD45R$^+$, CD8^{+low} surface phenotype and contain distinct cytoplasmic granules (Fox et al., 1998; Meeusen et al., 1993). From mid to late pregnancy, uterine γδTCR$^+$ IEL disappear from the placentomes but their number increases dramatically in the non-invaded inter-placentomal areas (from ~25% to 70% of IEL) (Lee et al., 1992; Majewski et al., 2001; Meeusen et al., 1993). In addition, both the number and size of their cytoplasmic granules increase significantly during pregnancy (Meeusen et al., 1993). At birth, there is a sudden and dramatic decline in the number of uterine granulated γδ T cells and they show clear signs of degranulation, suggesting a distinct role for granule proteins during parturition (Lee et al., 1992; Nasar et al., 2002). In the present study, the diversity of TCR usage by the uterine γδTCR$^+$ IEL was assessed, by characterizing TCRδ gene usage, and the identity of a major granule constituent was revealed by N-terminal peptide sequencing.

2. Materials and methods

2.1. Animals and tissue collection

Pregnant Merino cross ewes were purchased from a commercial farm and housed indoors at the School of Veterinary Science, The University of Melbourne, under close veterinary supervision. Ewes were killed at late pregnancy (110–140 days of gestation) by intravenous injection of an overdose of sodium pentobarbital. All experimental sheep carried a healthy foetus and showed no signs of infection or inflammation after gross and histological examination by a veterinary pathologist. Tissues were collected from the inter-placentomal (i.e. non-invasive) endometrial areas only for further studies.

2.2. Purification of uterine intraepithelial γδ T cells

Cells were prepared from the inter-placentomal uterine epithelium of a pregnant ewe at 110–140 days of gestation as described previously (Meeusen et al., 1993). For RNA preparation, γδ T cells were purified by indirect positive selection with sheep anti-mouse IgG(Fc) conjugated magnetic beads after reaction of the cells with anti-γδ TCR mAb, 86D, according to the manufacturer's protocol (Immunotech S.A. Marseille, France). For gel fractionation, cells were incubated with CD45R mAb and conjugated magnetic beads in PBS with 0.1% azide and 5 mM EDTA before separating with a magnet. Bound and unbound fractions were collected, washed in PBS and stored at −70 °C until use.

2.3. Anchor PCR

RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method and converted to cDNA with M-MLV reverse transcriptase (Unites States Biochemical, USA) and oligo dT. cDNA was filtered through a S300 Microspin^{TM} column (Pharmacia) and RNA hydrolysed as described previously (Troutt et al., 1992). The anchor oligonucleotide sequence and preparation by 5′ phosphorylation and 3′ blocking was as described by Troutt et al. (1992). The anchor was ligated to the cDNA using T4RNA ligase (New England Biolabs) according to previously described methods (Tessier et al., 1986). Primers complimentary to the anchor (Troutt et al., 1992) and to a stretch of the TCRδ chain constant region (Hein and Dudler, 1993) were used for PCR. Amplifications were carried out using a hot start followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. PCR products were cloned into a T vector prepared from pBluescript® SK(+/−) phagemid as described previously (Holton and Graham, 1991). Cloned products were fully sequenced in both directions and alignments carried out using FastA and Gap software (Wisconsin Sequence Analysis) with default settings.

2.4. SDS-PAGE and amino acid sequencing

CD45R bound and unbound cells were solubilised in non-reducing SDS buffer and run on a 3 tier tricine SDS-PAGE gel (16% lower gel, 10% spacer gel and 4% stacking gel) and stained with Coomassie blue. Preparations containing large amounts of the selected band were blotted onto Problott membranes (Applied Biosystems, Inc), proteins visualized by staining with Coomassie blue and the selected band excised from the blot and subjected to Edman degradation sequencing as described previously (Tkalcevic et al., 1995).

3. Results

3.1. γδ T cells in sheep uterine epithelium express a diverse TCR repertoire

TCRδ genes expressed by γδ T cells in sheep uterine epithelium were assessed by cloning and sequencing the products of anchor-PCR. Nine clones were fully sequenced in both directions and found to encode productive TCRδ gene rearrangements (EMBL Accession No's AJ005903–910). Since genomic sequences for sheep TCRδ elements are not available, the V–D and J–D boundaries were predicted by comparing sequences to each other and to genomic sequences for human, mouse and sheep TCRδ elements in GenBank. The predicted V elements were also translated into amino acid sequences for comparison. The nine clones derived from uterine IEL encoded seven clearly different V elements (Table 1). Clones 3 and 9 used the same V element and were the only two completely identical clones. Clones 4 and 8 also appeared to use the same V element with only a single base difference in the C terminal amino acid which probably reflects exonuclease activity and N nucleotide addition rather than a genomic difference. The seven V elements differed from each other by at least 9 amino acids. Differences were mainly clustered around position 30 and 60, equivalent to the complimentarity determining regions 1 and 2. All V elements were 342 bp in length except for that of clone 2 which was 348 bp long.

In addition to using multiple V elements, the nine sheep uterine IEL TCRδ genes sequenced used two different J elements identified previously in sheep peripheral blood