

## $\gamma\delta$ -TCR<sup>+</sup> CD2<sup>-</sup> lymphocytes are recruited into bovine mammary gland after stimulation

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**ABSTRACT:**  $\gamma\delta$ -T-Cell Receptor (TCR) lymphocytes were detected in mammary gland lavages collected from 10 clinically healthy virgin heifers before and after intramammary stimulation with synthetic muramyl dipeptide analogue. Using two-colour flow cytometry, CD2<sup>+</sup> and CD2<sup>-</sup> subsets of  $\gamma\delta$ -TCR lymphocytes were analyzed. CD2<sup>+</sup>  $\gamma\delta$ -TCR lymphocytes markedly prevailed over CD2<sup>-</sup> cells in intact mammary gland: 88.9 ± 4.9% of  $\gamma\delta$ -TCR lymphocytes were CD2<sup>+</sup>. After stimulation, neutrophils and  $\gamma\delta$ -TCR lymphocytes were recruited into the mammary gland. Among  $\gamma\delta$ -TCR lymphocytes, CD2<sup>-</sup> cells were mainly responsible for their expansion. After stimulation, 60.8 ± 13.4% of  $\gamma\delta$ -TCR lymphocytes were CD2<sup>+</sup> ( $P < 0.01$  when compared with mammary gland lavages before stimulation). It follows from the present study that the cells seem to be involved in the first phase of a response to an infection affecting mammary gland.

**Keywords:** cattle; udder; mastitis

Despite intensive udder health care, mastitis is one of the most significant diseases causing serious losses to the economy in the dairy industry. Apart from soluble factors, resident and incoming leucocytes play an important role in the mammary gland defence system against invading pathogens. Macrophages and lymphocytes are the predominant cell populations of healthy mammary glands in the middle lactation. An injury to mammary glands induces the recruitment of leucocytes (mostly neutrophils) from the blood into the mammary gland (Concha et al., 1986; Miller et al., 1990, 1991). The role of recruited neutrophils – to phagocyte invading bacteria – seems to be clear. Contrary to that, the exact role of lymphocyte subsets in mediating mammary gland defence has not been elucidated yet.

A lot of studies described changes of lymphocyte subset distribution in mammary gland secretion either during lactation (Park et al., 1992; Asai et al., 1998, 2000; Yamaguchi et al., 1999; Van Kampen et al., 1999; Leitner et al., 2000a) or after mam-

mary gland stimulation (Soltys and Quinn, 1999; Inchaisri et al., 2000; Leitner et al., 2000b; Ebling et al., 2001; Riollet et al., 2001; Rivas et al., 2001). Only two of the reports mentioned counts of  $\gamma\delta$ -TCR lymphocytes. Soltys and Quinn (1999) detected an increased number of these cells in mammary gland during acute mastitis caused by staphylococcal or streptococcal infections. On the contrary, no changes in the numbers of  $\gamma\delta$ -TCR lymphocytes were found during chronic *Staphylococcus aureus* infection (Riollet et al., 2001).

It is known that  $\gamma\delta$ -TCR lymphocytes represent a large population of lymphocytes in cattle (Clevers et al., 1990; Hein and Mackay, 1991). There are two basic subpopulations of  $\gamma\delta$ -TCR lymphocytes based on the expression of a unique molecule known as WC1. The first subpopulation is WC1<sup>+</sup> and CD2<sup>-</sup>, and the second is WC1<sup>-</sup>, but CD2<sup>+</sup>. A subset of WC1<sup>-</sup>CD2<sup>+</sup> cells also express the antigen CD8 as  $\alpha$ -homodimer (Hein and Mackay, 1991; Morrison and Davis, 1991; Wijngaard et al., 1992, 1994; Crocker et al., 1993). The situation concerning

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$\gamma\delta$ -TCR lymphocyte subsets is complicated by the fact that there are many monoclonal antibodies that recognize different subpopulations (MacHugh et al., 1993; Parsons et al., 1993; Davis et al., 1996).

It has been described that respective subsets of  $\gamma\delta$ -TCR lymphocytes have different tissue distribution. WC1<sup>+</sup> CD2<sup>-</sup> cells are mostly localized in peripheral blood and WC1<sup>-</sup> CD2<sup>+</sup> cells are mostly localized in peripheral lymphoid compartments, including spleen and intestinal mucosa (Mackay and Hein, 1989; Wyatt et al., 1994, 1996; Waters et al., 1995; MacHugh et al., 1997; Wilson et al., 1998; Rebelatto et al., 2000). The biological relevance of CD2 expression on  $\gamma\delta$ -TCR lymphocytes is not clear. Since the CD2 molecule is involved in antigen presentation via interaction with molecule CD58, one can say that upregulation of CD2 expression is associated with the activation of the cells.

In this context, the aim of our study presented here was to extend knowledge of  $\gamma\delta$ -TCR lymphocytes in mammary gland secretions by the detection of CD2 on these cells. We used a model of intact glands of virgin heifers that we successfully applied in many previous studies (Rysanek et al., 2001, 2005; Sladek and Rysanek, 2000, 2001a, 2005; Sladek et al., 2002, 2005). Stimulation was done with a synthetic muramyl dipeptide analogue. Muramyl dipeptide is part of the cell membrane of Gram-positive bacteria. Therefore, stimulation with this substance imitates an infection of the mammary gland by *Staphylococci* and/or *Streptococci*.

## MATERIAL AND METHODS

**Animals and sample collection.** The study was carried out using peripheral blood samples and mammary gland lavages collected from 10 clinically healthy Bohemian Red Pied  $\times$  Holstein virgin heifers (i.e. cows that have not been used in breeding), aged 16–18 months.

Samples of peripheral blood were collected from the jugular vein into heparin-containing test tubes (15 i.u./ml) from all animals immediately before mammary gland stimulation.

Mammary gland lavages (MGL) were obtained by irrigation of respective udder quarters, with 20 ml of sterile buffered 0.01M saline (phosphate-buffered saline, PBS), pH 7.2, 37°C. Samples (0.1 ml) of the lavage fluids were inoculated onto blood agar and incubated aerobically at 37°C for 24 h for the detection of bacterial contamination. The remaining

fluids were used for the detection of lymphocyte subsets in MGL before stimulation.

Stimulation was performed, irrespective of bacteriological evaluation results, immediately after the first irrigation by intramammary administration of 10 ml of synthetic muramyl dipeptide analogue (MurNAc-L-Abu-D-IsoGln, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, 50  $\mu$ g in 1 ml of PBS). After 24 h, the mammary glands were lavaged with 20 ml of PBS. Fluids were used for the detection of lymphocyte subsets in MGL after stimulation.

Mammary gland quarters with aberrant secretions or with bacterial contamination were excluded (Sladek and Rysanek, 2001b). Consequently, twenty-two quarters were included in the study.

**Differential leucocyte counts.** Differential leucocyte counts were calculated from blood/cell suspension smears stained with May-Grünwald and Giemsa-Romanowski.

**Lymphocyte subsets enumeration.** Lymphocyte subsets were enumerated by flow cytometry, using the indirect staining technique. The whole blood lysis technique was used for the analysis of peripheral blood samples. Fifty microlitres of blood were incubated in tubes (12  $\times$  75 mm) with monoclonal antibodies at room temperature for 15 min. Erythrocytes were lysed by adding 3 ml of a hemolytic solution (8.26 g of NH<sub>4</sub>Cl, 1 g of KHCO<sub>3</sub>, 0.037 g of Na<sub>4</sub> EDTA per 1 litre of distilled water). The suspensions were centrifuged, supernatants were discarded, secondary antibodies were added, and the tubes were incubated at 4°C for 20 min. Thereafter, 3 ml of washing solution (1 g of NaNO<sub>3</sub> and 1.84 g of Na<sub>4</sub> EDTA per 1 litre of PBS) were added into the tubes. The suspensions were centrifuged, supernatants were discarded and the obtained pellets were resuspended in washing solution.

For MGL analysis, the cells were adjusted to 1  $\times$  10<sup>6</sup>/ml with washing solution supplemented with 10% of heat-inactivated goat serum (blocking of non-specific Fc-fragment mediated staining). After 20 min, 100  $\mu$ l of the cell suspension was incubated with primary monoclonal antibodies at 4°C for 15 min. The cells were washed with washing solution, centrifuged and the supernatants were discarded. Secondary antibodies were added, and the tubes were incubated at 4°C for 20 min. After a further washing, the cells were resuspended in the washing solution.

The following monoclonal antibodies (mAb) were used in the study as follows: Mouse mAb anti-bovine  $\gamma\delta$ -TCR (GB21A, IgG2b, Davis et al., 1996) was counterstained with mouse mAb against a bovine homologue of CD2 (CC42, IgG1, Davis and Splitter, 1991). Mouse mAb against a monocyte/macrophage specific antigen CD14 (VPM65, IgG1, Jungi et al., 1997) and against pan-leucocytary CD45 (CC1, IgG1, Bembridge et al., 1995) were used for the detection of gating purity. All mAbs were from Serotec Ltd., Oxford. FITC-conjugated and R-PE-conjugated goat anti-mouse IgG2b and IgG1, respectively, (Southern Biotechnology Associated, Inc.) were used as secondary antibodies. Negative control samples were stained with the secondary antibody only.

Lymphocyte subsets were enumerated using the FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and the CELLQuest™ software. At least 20 000 events were read in each run. A gate was set to the lymphoid compartment using light scatter characteristics (forward versus diagonal scatter values) and the results were verified by tests with monoclonal antibodies detecting CD45 and CD14 antigens. Only cells that showed expression of CD45 but were negative for CD14 were considered to be lymphocytes.

Propidium iodide was used to stain DNA in dead and damaged cells in order to exclude these events from analysis. Approximately 15% of events were propidium iodide positive. They were mostly included in the region of debris and did not contaminate the region containing lymphocytes.

The percentage of positive cells was determined in log-scale dot-plots. The cursor was set so that only 1% (in the peripheral blood) or 2% (in mammary lavages) of the events were positive in negative control. The WinMDI™ software was used for off-line data processing.

**Statistical analysis.** The results are presented as means  $\pm$  SD. Since the data were paired, paired non-parametric Wilcoxon test was used for the comparison of appropriate data. The *P* values lower than 0.05 were considered statistically significant. All calculations were performed with Prizma® (Graph Pad Software, Inc.).

## RESULTS

### Gating of samples

More than 98% of cells in the gate set for the lymphoid compartment using light scatter characteristics were positive for pan-leucocytary antigen CD45. Less than 2% of the cells were positive for CD14 antigen. It means that gating purity was high and almost all cells in the gate were lymphocytes.

### Leucocyte subset distribution

In peripheral blood, lymphocytes represented the major population ( $57.3 \pm 12.5\%$ ), and was followed by neutrophils ( $31.2 \pm 11.3\%$ ) and monocytes ( $11.5 \pm 2.9$ ). MGL before stimulation was practically

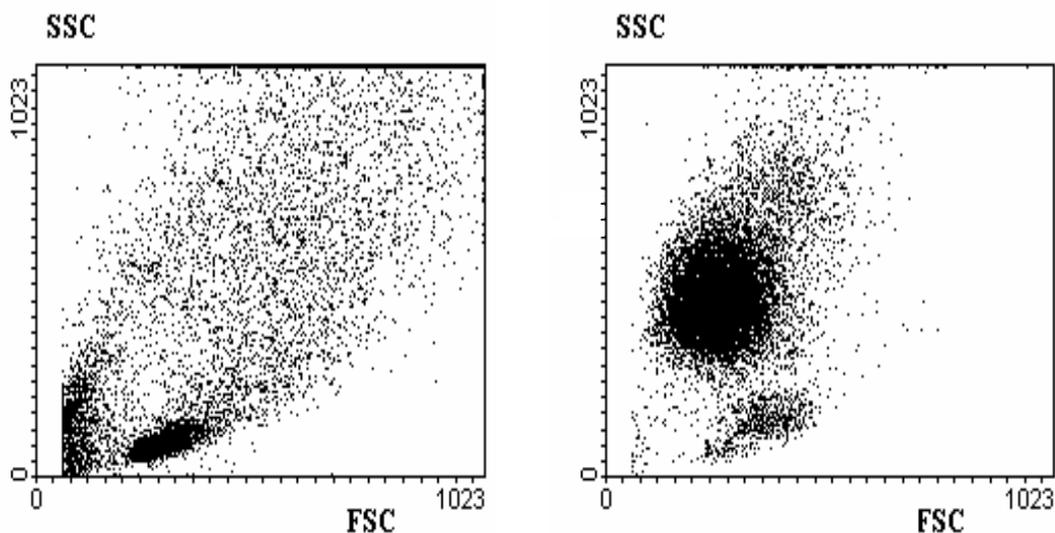


Figure 1. Light scatter characteristics of the cells from a mammary gland lavage before (left) and after (right) stimulation. FSC (forward scatter) represents cell size; SSC (side scatter) represents cell granularity

free of granulocytes ( $1.8 \pm 1.2\%$ ). The proportion of lymphocytes was  $45.2 \pm 11.3\%$ . Udder stimulation with muramyl dipeptide analogue led to a massive migration of granulocytes into the MGL ( $82.1 \pm 15.2\%$ ,  $P < 0.01$ ). The proportion of lymphocytes decreased to  $7.4 \pm 7.7\%$  ( $P < 0.01$ ). Representative dot-plots are shown in Figure 1.

### Lymphocyte subset distribution

$\gamma\delta$ -TCR lymphocytes in peripheral blood represented  $27.6 \pm 6.8$  percent of all lymphocytes. In MGL before stimulation,  $9.4 \pm 4.7$  percent of lymphocytes were of this subpopulation ( $P < 0.01$  when compared with peripheral blood). The stimulation resulted in an increase in the proportion of  $\gamma\delta$ -TCR lymphocytes; up to  $16.4 \pm 7.2$  percent ( $P < 0.01$  when compared with MGL before stimulation).

We investigated both subsets of  $\gamma\delta$ -TCR (i.e.  $CD2^+$  and  $CD2^-$ ), and they were found in all tested samples. In peripheral blood,  $CD2^-$  cells were recorded more frequently than  $CD2^+$  cells (93:7 on average). Contrary to that,  $88.9 \pm 4.9$  percent of  $\gamma\delta$ -TCR in MGL before stimulation expressed the antigen CD2. The stimulation led to a decrease in the proportion of CD2 positive  $\gamma\delta$ -TCR lymphocytes. After stimulation, only  $60.8 \pm 13.4$  percent of  $\gamma\delta$ -TCR lymphocytes expressed the CD2 ( $P < 0.01$  when compared with MGL before stimulation). Representative dot-plots are shown in Figure 2.

### DISCUSSION

The aim of our study was to extend the knowledge of  $\gamma\delta$ -TCR lymphocytes in bovine mammary gland secretion on a model of intact glands of virgin heifers.  $\gamma\delta$ -TCR lymphocytes comprise a large population of all lymphocytes in cattle (Clevers et al., 1990; Hein and Mackay, 1991).

The exact functions of  $\gamma\delta$ -TCR lymphocytes have not been clearly recognized as of yet, but there is a general agreement that the cells participate in connecting innate and adaptive immune mechanisms (Boismenu and Havran, 1997; Hayday, 2000,) and play an important role in many physiological and pathological processes. For example, they are involved in antiinfectious immunity and local regulation of inflammation, including the mammary gland (Park et al., 1994). It was also shown that  $\gamma\delta$ -TCR lymphocytes are antigen-presenting cells with the capability to produce co-stimulatory molecules and cytokines (Collins et al., 1998).

In our study, we described that  $CD2^+$   $\gamma\delta$ -TCR lymphocytes markedly prevails over  $CD2^-$  cells in intact mammary gland. It is consistent with the situation in other lymphoid compartments described so far. After stimulation, neutrophils and  $\gamma\delta$ -TCR lymphocytes are recruited into the mammary gland. Among the  $\gamma\delta$ -TCR lymphocytes,  $CD2^-$  cells were mostly responsible for their expansion. Although the reason for the different tissue distribution of distinct  $\gamma\delta$ -TCR subsets has not been recognized

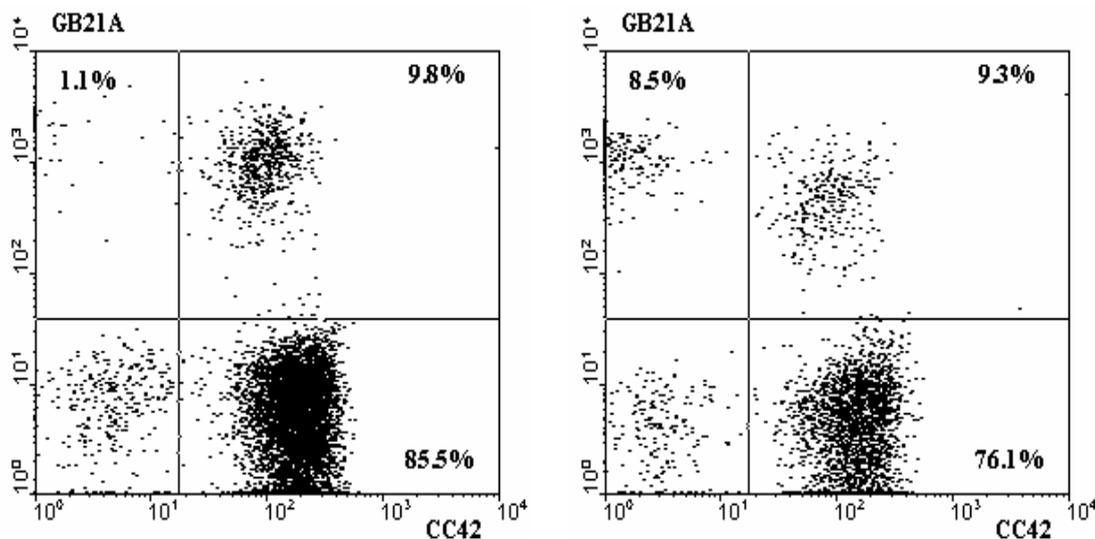


Figure 2. Two-colour flow cytometric analysis of cells from a mammary gland lavage before (left) and after (right) stimulation. Cells were stained as described in Material and Methods for  $\gamma\delta$ -TCR (GB21A) and CD2 (CC42) antigens. Percentages represent relative proportions of the cells positive for respective antigens

yet, the mechanism involved in this phenomena has partly been described (Wilson et al., 2002). The authors showed that  $\gamma\delta$ -TCR lymphocytes, which accumulated in tissues, expressed higher levels of  $\alpha 4\beta 7$  integrins. It should also be admitted that the relationship between  $WC1^+CD2^-$  and  $WC1^-CD2^+$  is not known. Downregulation of  $WC1$  and expression of  $CD2$  may be associated with the stage of maturation.

Although there is no information regarding the exact role of  $\gamma\delta$ -TCR lymphocytes in mammary gland – such as production of cytokines, the cells seem to be involved in the first phase of a response to an infection affecting mammary gland. Their importance in mucosal immunity is also supported by the fact that TCR $\delta$  knock-out mice are deficient in IgA (but not IgM or IgG) production in response to oral administration of tetanus toxoid (Fujihashi et al., 1996).

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