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Original article

Purification and characterisation of bovine WC1$^+$ γδ T lymphocytes from peripheral blood

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Abstract – In order to isolate and characterise resting WC1$^+$ γδ T cells from cattle, we developed a protocol for purifying these cells by negative selection from peripheral blood. The purification method included five steps: separation of mononuclear cells on lymphoprep, depletion of monocytes by adherence to plasma-coated gelatin, enriching T cells on a nylon wool column, depleting CD2$^+$ T cells by sheep red blood cells (SRBC), and finally depleting CD4$^+$ and CD8$^+$ T cells by the magnetic cell sorting technique (MACS). This procedure proved efficient and reproducible, and the purity of the isolated WC1$^+$ γδ T cells was more than 97% as analysed by flow cytometry (FACS). Cytokines and costimulatory molecules mRNA expression was assessed by the reverse transcriptase polymerase chain reaction (RT-PCR) technique in freshly isolated resting WC1$^+$ T cells. We found that purified uncultured WC1$^+$ T cells express TNF-α, CD28, CTLA-4 and IL-2Rα mRNA transcripts but do not express those for IL-2, IL-4, IL-6, IL-10 and IFN-γ. The expression of CD28 and CTLA-4 transcripts on bovine WC1$^+$ T cells indicates that these genes are evolutionarily conserved.

antigen / cytokine mRNA expression / γδ T lymphocyte

Résumé – Purification et caractérisation des cellules bovines T γδ WC1$^+$ du sang périphérique. Afin de purifier et de caractériser les cellules T γδ du type WC1$^+$ d'origine bovine, nous avons développé un protocole de purification par sélection négative de ces cellules contenues dans le sang périphérique. La méthode de purification comporte cinq étapes, à savoir : la séparation des cellules mononucléés sur lymphoprep, la déplétion des monocytes par adhérence sur de la gélatine couverte de plasma, l'enrichissement des cellules T sur colonne de nylon, la déplétion des cellules T du type CD2$^+$ par formation des rosettes avec les globules rouge du mouton, et enfin la déplétion des cellules T CD4$^+$ et CD8$^+$ par la technique de « Magnetic cell sorting » (MACS). Le procédé s'est révélé

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efficace et reproductible, tandis que la pureté des cellules isolées analysées par cytométrie de flux, s'élevait à plus de 97%. Sur les cellules fraîchement isolées, nous avons étudié au moyen de la technique de "reverse transcriptase polymerase chain reaction" (RT-PCR) l'expression de l'ARN messager des cytokines et des molécules de costimulation. Nous avons ainsi mis en évidence que les cellules T γδ WC1$^+$ fraîchement isolées, non cultivées, expriment les ARNm du TNF-α, du CD28, du CTLA-4 et de IL-2Rα tandis que les ARNm de l'IL-2, l'IL-4, l'IL-6, l'IL-10 et de l'IFN-γ n'étaient pas exprimés. L'expression du CD28 et du CTLA-4 sur les cellules T γδ WC1$^+$ indiquerait que ces gènes ont été conservés durant l'évolution.

antigène / expression ARNm cytokine / lymphocyte T γδ

1. INTRODUCTION

Several domestic animal species, including ruminants and pigs, possess a large number of circulating γδ T cells [20, 29]. This contrasts with the very small number of these cells in humans [26] and mice [2]. In bovine, γδ T cells constitute a prominent population of peripheral blood mononuclear cells (PBMC), reaching up to 20-30% in young calves. These cells express a unique family of high molecular weight surface molecules, termed Workshop Cluster 1 (WC1) [9, 31]. The cells are further characterised by the CD2$^-$ CD4$^-$ CD8$^-$ CD3$^+$ TCR γδ $^+$-phenotype [9, 22]. WC1 has been identified on sheep, bovine and pig γδ T cells by mAb reacting with common epitopes [7, 13]. In addition, there is a small population of WC1$^-$ γδ T cells, many of which express CD2 and/or CD8 [35].

Several methods have been developed for enriching γδ T cells. Positive purification of WC1$^+$ T cells from PBMC (stained with mAb CC15 (anti-WC1) and successively sorted on Mini Magnetic Cell Sorting (MiniMACS) and Fluorescence Activated Cell Sorter (FACS)) has been described [11]. The binding of anti-WC1 mAb to γδ T cells has been reported either to induce reversible growth arrest in proliferating IL-2- dependent γδ T lymphocytes [25] or to augment the proliferation of these cells in an autologous mixed leukocyte reaction as well as augmenting proliferation induced by anti-CD3 or anti-CD5 mAbs [17]. Negative purification of WC1$^+$ T cells has been reported by other authors [17], using monocyte depleted-PBMC stained with a cocktail of monoclonal antibodies on mouse T-cell immunocolumn and culture overnight with human recombinant interleukin-2 (IL-2). Addition of IL-2 to purified WC1$^+$ T cells resulted in a significant increase in proliferation [11]. The purification of WC1$^+$ T cells in order to obtain resting cells for the in vitro study thus remains a challenge.

In this paper we describe a procedure for the purification of resting WC1$^+$ T cells. Purity was assessed by the FACS method using mAbs that differentiate the (CD2$^-$, CD4$^-$, CD8$^-$, CD3$^+$, and WC1$^+$) γδ T cells from the (CD4$^+$, CD8$^-$) and (CD8$^+$, CD4$^-$) αβ T cells. We further studied the expression of costimulatory molecules and of cytokines mRNA in uncultured, non-activated purified WC1$^+$ T cells. WC1$^+$ γδ T lymphocytes purified under these conditions provide excellent starting material for the examination of their functional characteristics in vitro.

2. MATERIALS AND METHODS

2.1. Materials

Phosphate-buffered saline (PBS) without calcium and magnesium, culture medium containing RPMI 1640, 2 mM L-glutamine, 25 mM hepes buffer, 100 IU·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 0.5 μg·mL^{-1} fungizone and 10% foetal calf serum (FCS) were supplied from

Gibco BRL (Gibco BRL, Grand Island, NY, USA). Lymphoprep was purchased from Nycomed (Nycomed Pharma, Oslo, Norway), and 2-aminoethylisothiouronium bromide (AET) and concanavalin A (ConA) from Sigma (Sigma Immunochemicals, St Louis, MO, USA). Goat anti-mouse IgG-labelled paramagnetic beads (GAMIg-MACS beads), MACS columns and MACS separator were supplied from Miltenyi (Miltenyi Biotech Gmbh Bergisch Gladbach, Germany). Fluorescein isothiocyanate (FITC)-conjugated F (ab') 2 fragment of rabbit anti-mouse immunoglobulin (FITC-F (ab') 2 fragment) was purchased from Dako (Dako A/S, Glostup, Denmark). The following murine mAbs reported within the International Ruminant Leukocyte Antigen Workshop [21] were used: CC42, MM1A, CC8, CC63, and CC15 specific for CD2, CD3, CD4, CD8, and WC1 respectively. Flow cytometry analysis was performed on FACScan (Becton Dickinson, Mountain View, California, USA).

2.2. Preparation of bovine WC1$^+$ T cells

WC1$^+$ T cells were negatively isolated from peripheral blood as depicted in Figure 1. Using lymphoprep, PBMC were iso-

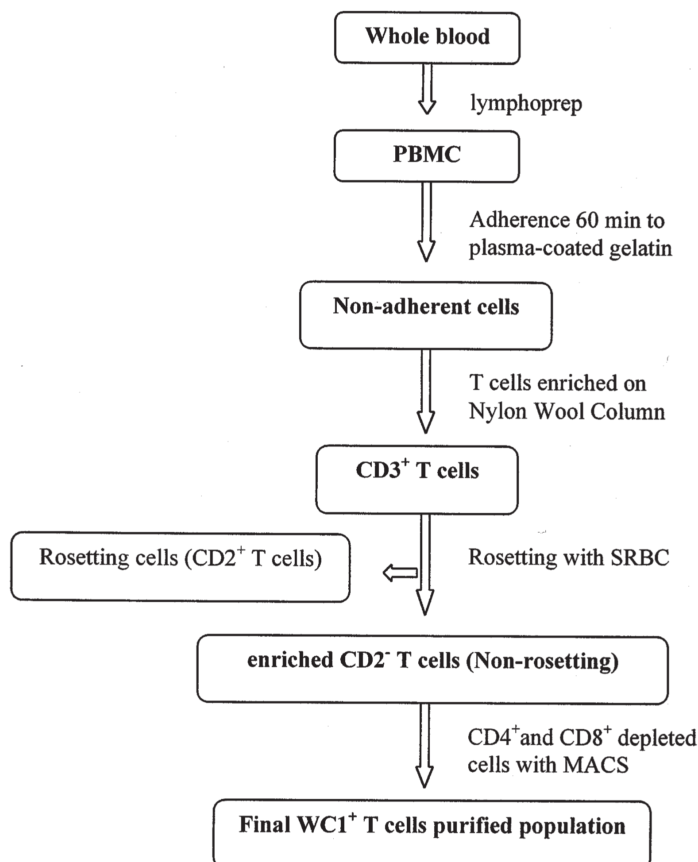

Figure 1. Flow diagram of the negative purification protocol for WC1$^+$ $\gamma\delta$ T lymphocytes.

lated from heparinised peripheral venous blood from 6-12-month-old conventionally raised cattle. PBMC at 5×10^6 cells·mL^{-1} were resuspended in culture medium and allowed to adhere for 60 min to plasma-coated gelatin as described by Goddeeris et al. [15]. Non adherent cells were enriched in T cells by passing the cells through a nylon wool column to remove B cells and remaining monocytes as previously described [5]. $CD2^+$ T cells were depleted by rosetting T cells with AET-treated sheep red blood cells (SRBC). Briefly, enriched T cells suspended at 2.5×10^6 cells·mL^{-1} were added to an equal volume of 2% SRBC in culture medium, incubated for 15 min at 37 °C and centrifuged for 10 min at $190 \times g$. The supernatant medium was discarded and the cell pellet allowed to incubate for 1 h on ice. The cells resuspended in 15 mL of culture medium were layered onto lymphoprep and spun for 30 min at $635 \times g$. Enriched $CD2^-$ T cells at the interface were harvested and washed twice in PBS. $WC1^+$ T cells were further separated from $CD4^+$ and $CD8^+$ T cells using the MACS technique [30] whereby the non-rosetting population, coated with anti-CD4 and anti-CD8 mAbs, was incubated with GAMIg-MACS beads at 4 °C for 15 min and passed through the MACS column. Labelled cells were retained on the column in a magnetic field and the negative fraction was collected.

2.3. Flow cytometry

Single color FACS staining was performed using mAbs to CD2, CD3, CD4, CD8 or WC1. Cells were washed twice with ice-cold PBS containing 1% BSA. For 30 min, 10^6 cells per test were incubated on ice with an appropriate dilution of mAb, washed with PBS and further incubated for another 30 min on ice with the FITC- F (ab´) 2 fragment. After washing, phenotypic analysis was performed on a FACScan flow cytometer.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from purified uncultured $WC1^+$ T cells (10^7) and from PBMC activated with ConA [23] by lysis in the ultraspec^{TM} RNA-reagent (AMS Biotechnology Ltd, Witney, Oxon, UK). RNA concentrations were determined by spectrophotometer readings. The products used for the cDNA synthesis were from Gibco BRL except where otherwise stated. The cDNA was synthesised for 1 h at 37 °C in a 40 µL cocktail containing 1 µg total RNA in diethyl pyrocarbonate (DEPC)-dH_2O, 2 µL oligo-dT primer, at 500 µg·mL^{-1}, 4 µL of a 10 mM dNTP mix, 1 µL RNasin at 40 U·mL^{-1} (Promega, Madison, WI, USA), 1 µL Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) at 200 U·mL^{-1}, 8 µL of $5 \times$ concentrated RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), and 4 µL of 1 mM DTT. This was further incubated at 65 °C for 5 min. cDNA (3 µL per primer pair) was added to 5 µL of $10 \times$ concentrated PCR buffer (300 mM Tris-HCl pH 9.0, 50 mM $MgCl_2$, 300 mM KCl, 0.5% (W/W) W-1 buffer), 1.25 µL of each 10 mM dNTP, 2.5 µL of each 10 µM sense/antisense primers (Tab. I), 0.5 µL Taq polymerase at 5000 U·mL^{-1}, and dH_2O to a final volume of 50 µL. The PCR reactions were performed in a PCR thermocycler under the following conditions: for β-actin, IL-2 and IL-4: one cycle (1 min at 94 °C), followed by 35 cycles (94 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min), and for IL-6, IL-10, IFN-γ, TNF-α, IL-2Rα, CD28, and CTLA-4: one cycle (1 min at 94 °C), followed by 35 cycles (94 °C, 30 sec; 60 °C, 45 sec; 72 °C, 1 min), and for the final extension cycle: 10 min at 72 °C. Reaction products (10 µL) were visualised after electrophoresis on 2% agarose containing ethidium bromide.

2.5. Determination of primer specificity

Primers were designed based upon bovine specific GenBank sequence infor-

Table I. Bovine cytokine and antigen primers.

Gene	Fragment (bp)	Primer 5'→3'	Reference
IL-2R	358	AGCACTTTCTCTTCCCAGC[a] CCCGAAGAACATAGGGAGAAA	This publication
CD28	571	TCCTGGCTCTCAACTTCTTCC GCGACTATATGAACATGACCCC	This publication
CTLA-4	435	GACTTGGTGGACATCTAGG CGTCATTGATCCAGAACCATGC	This publication
IL-2	548	ACGGGGAACACAATGAAAGAAGT CGTAGGGCTTACAAAAAGAATCT	[8]
IL-4	423	GTCTTTCAGCGTACTTGT TGCATTGTTAGCGTCTCCT	[3]
IL-6	534	CCTTCACTCCATTCGCTGTC TGCGTTCTTTACCCACTCG	[14]
IL-10	518	ACAGCTCAGCACTGCTCTGTT CG TTGTCATGTAGGATTCTATG	[11]
IFN-γ	426	CTGTGGGCTTTTGGGTTTTTCTG CTCTTCCGCTTTCTGAGGTTAGA	[8]
TNF-α	549	CTGCACTTCGGGGTAATCGG CAGGGCGATGATCCCAAAGTA	[10]
β-Actin	400	ACGTAGCAGAGCTTCTCCTTGATG CCTTTTACAACGTGCGTGTG	[3]

[a] Sequences are listed as sense followed by antisense.
bp: base pairs.

mation M20818, X93304 and X93305 for Bovine IL-2Rα, CD28 and CTLA-4 respectively. Primer specificity was demonstrated through sequencing of PCR products, where all amplified sequences were found to be identical to the published sequences.

3. RESULTS

3.1. Purification of WC1+ T cells from bovine blood

From six independent experiments the range in cell number and in percentage of CD2$^+$, CD3$^+$, CD4$^+$, CD8$^+$ and WC1$^+$ T cells recovered after each step are presented in Table II. Upon PBMC separation on Lymphoprep, $0.8 \times 10^9 - 1.0 \times 10^9$ cells were obtained from 300 mL of blood. The adherence step on plasma-coated gelatin removed 80% to 90% of monocytes as analysed by flow cytometry (not shown).The non-adherent cells containing 80-85% CD3$^+$ T cells were allowed to run into the nylon wool column in order to deplete the non T-lymphocytes and the remaining monocytes. This selection step yielded a population of more than 97% CD3$^+$ T lymphocytes containing an average of 44% (range: 40-49)

Table II. Analysis of range cell number and range percentage of specific T cells in six independent experiments.

	n[a]	Range and mean % T cells[b]				
		$CD2^+$	$CD4^+$	$CD8^+$	$CD3^+$	$WC1^+$
PBMC	$0.8 – 1.0 \times 10^9$	ND[c]	17-22 [18.5]	11-14 [13]	65-72 [68.5]	32-48 [39.8]
Non-adherent cells	$8.4 – 9.0 \times 10^8$	ND	20-24 [22.5]	15-18 [16.5]	80-85 [82.3]	46-52 [48.3]
$CD3^+$ T cells	$1.9 – 2.8 \times 10^8$	40-49 [44.5]	25-28 [26]	16-20 [19]	> 97[d]	51-58 [54]
Enriched $CD2^-$ T cells	$7.6 – 9.0 \times 10^7$	17-22 [19.5]	11-14 [12]	5-8 [6.5]	> 97	79-82 [80.5]
Selected $WC1^+$ T cells	$4.2 – 7.5 \times 10^7$	< 1[d]	< 1	< 1	> 97	> 97

[a] n represents the range cell number of six independent experiments.
[b] The columns give the range percentage of T cells and the mean [percent] as evaluated by flow cytometry.
[c] ND: not done.
[d] Straight value in percent, no range, no mean.

of $CD2^+$, 26% (range: 25-28) of $CD4^+$, 19% (range: 16-20) of $CD8^+$ and 54% (range: 51-58) of $WC1^+$ T cells. The next step of rosetting T lymphocytes with SRBC, undertaken in order to remove $CD2^+$ T cells, yielded a $CD2^-$ T cell population enriched to about 80% (range: 79-82) of $WC1^+$ T cells with almost 19% (range: 17-22) of $CD2^+$ T cells in excess. Enriched $CD2^-$ T cells were incubated with a mixture of anti-CD4 and anti-CD8 mAbs, and applied to the MACS column in order to remove the $CD4^+$ and $CD8^+$ T cells. The final fraction of cells passing through the MACS column represented consistently >97% $WC1^+$ T cells and less than 1% of the other T cells. The cell viability assessed by the trypan blue exclusion test was always greater than 98%.

3.2. Phenotypic analysis

To evaluate the efficiency of the procedure, purified cells and PBMC were stained with mAbs specific for bovine CD2, CD3, CD4, CD8, and WC1 leukocyte molecules and the percentage of the populations evaluated by FACS analysis. As depicted in Figure 2, up to 97% of the purified cells expressed WC1 and CD3 antigens and less than 1% CD4, CD8 or CD2 T antigens. In PBMC, $WC1^+$, $CD4^+$ and $CD8^+$ T cells represented 42%, 21% and 14% respectively.

3.3. Expression of costimulatory molecules on freshly isolated $WC1^+$ T cells

The expression of the primary T-cell costimulatory receptors CD28, CTLA-4 and IL-2Rα (CD25) was assessed by RT-PCR. In $WC1^+$ T cells RT-PCR was conducted on RNA extracted from freshly isolated uncultured cells using primers listed in Table 1 as shown in Figure 3A. Gel electrophoresis of RT-PCR band products are the size of 571 bp, 435 bp and 358 bp cor-

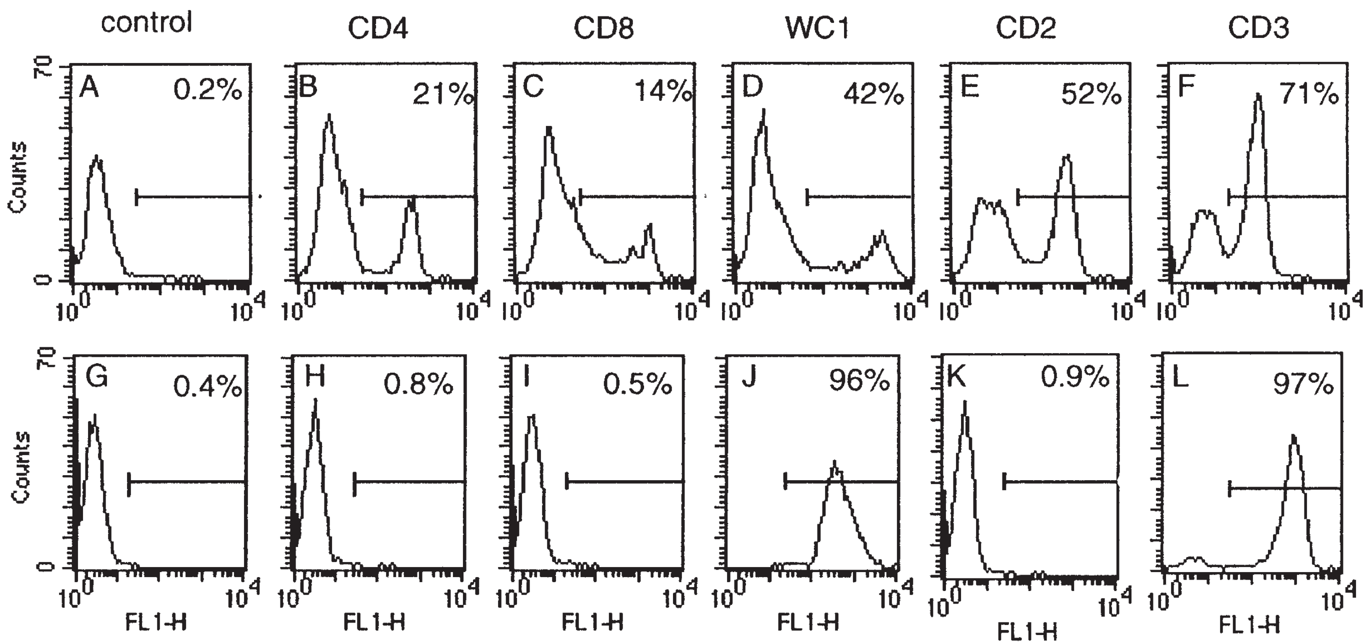

Figure 2. FACS analysis of PBMC and purified WC1$^+$ γδ T cells. PBMC (A-F) and purified WC1$^+$ T cells (G-L) were analysed by indirect immunofluorescence for the five major surface determinants of T cells including CD4 (B and H), CD8 (C and I), WC1 (D and J), CD2 (E and K) and CD3 (F and L) using the mAbs CC8 (anti-CD4), CC63 (anti-CD8), CC15 (anti-WC1), CC42 (anti-CD2) and MMA1 (anti-CD3) and FITC-F (ab') 2 fragment as second reagent. For controls, the FITC-F (ab') 2 fragment was used alone (A and G).

responding to CD28, CTLA-4 and IL-2Rα respectively. DNA sequencing of these bands confirmed that all amplified mRNA transcripts were identical to the published sequences.

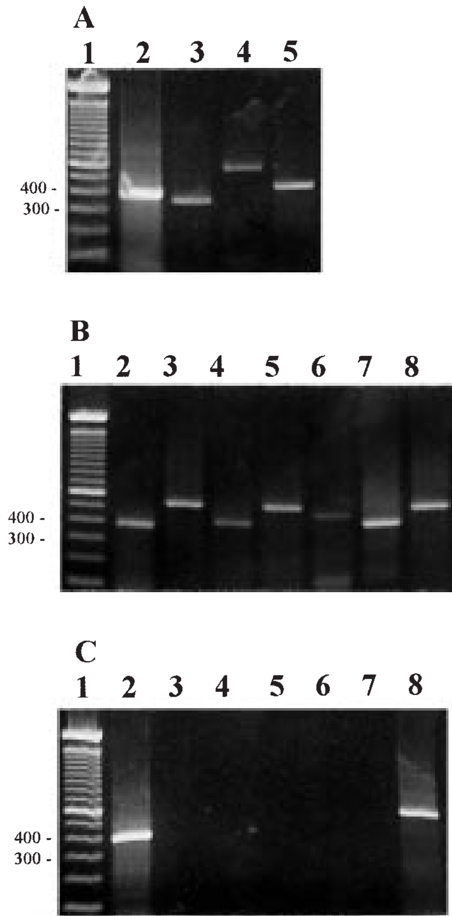

Figure 3. Detection of costimulatory molecules and cytokine mRNA expression on freshly isolated resting WC1$^+$ T cells. (A) β-actin (lane 2), IL-2Rα (lane 3), CD28 (lane 4) and CTLA-4 (lane 5) mRNA expression on uncultured WC1$^-$ T cells. Cytokine mRNA expression for β-actin (lane 2), IL-2 (lane 3), IL-4 (lane 4), IL-6 (lane 5), IL-10 (lane 6), IFN-γ (lane 7) and TNF-α (lane 8) assayed following early PBMC ConA activation (B) for positive control and uncultured WC1$^-$T cells (C). For reference, a 100 pb DNA ladder marker is shown on lane 1.

3.4. Cytokine expression by freshly purified resting WC1$^+$ T cells

Using RT-PCR, we examined mRNA expression from a range of cytokines in freshly purified uncultured WC1$^+$ T cells. While TNF-α mRNA seemed to be constitutively expressed, other cytokines including IL-2, IL-4, IL-6, IL-10 and IFN-γ were undetectable in resting uncultured WC1$^+$ T cells but were detected in PBMC activated with ConA (Fig. 3B).

4. DISCUSSION

For the comprehensive study of the physio-pathologic role of the γδ T cells, it seems essential to characterise the non-activated cells in order to establish a basic comparison to the functional activation. The hereto used methods of purification, being positive purification [11] or negative purification of γδ T cells cultured in IL-2 [17] have both generated activation of these cells. Using a combination of cell separation methods, we tentatively developed a negative procedure for purifying bovine resting WC1$^+$ γδ T cells from peripheral blood. We first removed the majority of the monocytes from PBMC by the method of adherence to plasma-coated gelatin as described by Goddeeris et al. [15]. Second, the non-T lymphocytes and the remaining monocytes in the non-adherent cells were depleted on a nylon wool column. These two preliminary manipulations permitted to enrich the CD3$^+$ T lymphocytes from 70% in PBMC to more than 97% from which 50% were CD2$^+$ T cells. Knowing that WC1$^+$ T cells do not express CD2 antigens [9, 22], the next step was aimed at removing the CD2$^+$ T cell population. The rosetting technique with SRBC allowed a reduction of 50% of them, meanwhile increasing the percentage of WC1$^+$ cells from 55% to 80% without decreasing the CD3$^+$ T cells. Furthermore, the percentage of the CD4$^+$ and CD8$^+$ T cells decreased from 25-28% and 16-20% in the

CD3+ pool to 11-14% and 5-8% respectively (Tab. II). Ultimately, removing CD4+ and CD8+ T cells by the magnetic cell sorting technique yielded highly purified T cells presenting with the phenotype CD2- CD4- CD8- CD3+ WC1+ defining the majority of bovine γδ T cells in peripheral blood [9, 22]. Our method dealing with easy and improved techniques was reproducible in at least six standard experiments and has proved so far quite efficient in obtaining pure WC1+ γδ T cells.

However, the most important consideration lies in the activation state of the cells. Activated γδ T cells express cytokine mRNA and produce cytokines as reported by many authors [4, 11, 12, 34]. The analysis of the purified bovine WC1+ T cells shows constitutive expression of TNF-α mRNA. The production of TNF-α transcripts by γδ T cells has been reported in several species including humans and mice [1, 16]. The biosynthesis of this cytokine is largely controlled at the translational level, therefore TNF-α mRNA may be present without synthesis of the corresponding protein [33]. The other cytokines examined, including IL-2, IL-4, IL-6, IL-10 and IFN-γ, were undetectable (Fig. 3C). The detection of these transcripts in Con A stimulated PBMC demonstrated that the primers were effective and that the purification procedure did not activate the resting cells.

We further addressed the question of costimulatory molecules expressed on the purified cells. Indeed, full activation of T cells in response to foreign antigens depends on the engagement of the T-cell receptor (TCR) by the peptide-major histocompatibility complex (MHC) followed by costimulatory signals which are generated by other receptor-ligand interactions between the APCs and the T cells [19, 32]. CD28 and CTLA-4 are the primary T-cell costimulatory receptors. Upon interaction with their ligands, CD80 and CD86, T-cell proliferation and IL-2 synthesis are enhanced [24, 27]. CD28 is constitutively expressed on all murine T cells as well as on 80% of human T cells [24] and once activated, the cells express the CTLA-4 molecule [28]. The expression of transcripts of these molecules has been recently reported for ovine γδ T cells isolated from efferent lymph cells [18]. In this study, we report on the results of the expression of costimulatory molecules on the isolated γδ T cells. The WC1+ T cells showed the expression of CD28 and CTLA-4 mRNA , suggesting that these genes are evolutionarily conserved on γδ T cells altogether. Studies are in progress to determine whether the proliferation of WC1+ T cells requires the ligands of CD28/CTLA-4 on the surface of APCs.

IL-2Rα mRNA expression was also demonstrated. The production of IL-2Rα transcripts on uncultured WC1+ T cells from sheep has been reported [18, 36]. This finding is in striking contrast to the situation in αβ T cells where the α-chain of IL-2Rα is not expressed on most freshly isolated cells and is only transiently expressed following antigenic or mitogenic stimulation [6, 18]. The biosynthesis of IL-2Rα seems to be largely controlled at the transcriptional and translational levels [36]. Thus, detection of IL-2Rα mRNA does not necessarily represent the synthesis of the corresponding protein. The analysis of PBMC from gnobiotic calves by Collins et al. [11] also indicated that the CD25 molecule was constitutively expressed on resting WC1+ T cells and was further up-regulated following stimulation of the cells.

In conclusion, the described WC1+ T cell purification protocol is a reliable and easy method for the negative selection of resting cells with high purity. This method circumvents the possible inopportune activation occurring through mAbs by positive purification and through IL-2 culture supplement in negative purification methods as described in earlier reports. We recommend this protocol whenever enriched γδ T cells are to be used for functional studies. The method may also be useful in other species with special reference to sheep and pigs,

where WC1+ γδ T cells possess the same phenotype as in bovine.

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