Veterinary Immunology and Immunopathology 146 (2012) 237–244

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Condensed tannins from Botswanan forage plants are effective priming agents of γδ T cells in ruminants

O. Tibe[a,b,*], A. Pernthaner[c], I. Sutherland[c], L. Lesperance[d], D.R.K. Harding[b]

[a] Botswana College of Agriculture, Private Bag 0027, Gaborone, Botswana
[b] Institute of Fundamental Sciences, Massey University, Turitea Campus, Palmerston North 4442, New Zealand
[c] AgResearch, The Hopkirk Research Institute, Private Bag 11008, Palmerston North 4442, New Zealand
[d] 25 St Hill Lane, Havelock North, Hawkes Bay, New Zealand

ARTICLE INFO

Article history:
Received 19 January 2012
Received in revised form 1 March 2012
Accepted 5 March 2012

Keywords:
γδ T cells
Innate
Priming
Condensed tannins

ABSTRACT

The potential impact of extracts from forage plants on γδ T cell activity in ruminants was evaluated using an *in vitro* immunoassay. This study investigated whether plant extracts could prime γδ T cells *via* up-regulation of CD25 (interleukin-2 receptor alpha). Purified Sephadex LH-20 fractions, isolated from *Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius* and *Grewia flava*, were screened against γδ T cells on kid, lamb and calf peripheral blood lymphocytes. Condensed tannins (CT) from *G. flava* significantly primed γδ T cells in kids up to 64.75% at 10 μg/mL, which was statistically significant relative to the negative control at 22.66% ($p = 0.004$). CT from *T. oleifolius* also induced priming of γδ T cells in kids, while fractions from *V. rotundifolium* and *V. verrucosum* induced minimal priming of γδ T cells. In contrast, there was no significant priming of γδ T cells from lambs and calves for any of the tested fractions ($p > 0.05$). These findings suggest that CT from a selected range of Botswanan forage plants can stimulate the immune system *in vivo* in selected ruminant species and may participate in enhancing host innate immune responses.

1. Introduction

Young cattle, sheep and goats possess relatively high proportions of gamma-delta (γδ) T lymphocytes, a subset of T lymphocytes, which play a major role in the innate immune response. These γδ T cells express γδ T cell receptors (TCR) which recognise an array of antigens and ligands (Hedges et al., 2005).

While γδ T cells represent a small fraction of T-lymphocytes in the human peripheral blood (0.5–5%), they can be present in much higher proportions (30–70%) in the blood stream of young ruminants, as well as in chickens (Gertner et al., 2007; Hedges et al., 2005; Nath, 2008). A significant difference in the γδ T cell proportions between animal species is observed only in the peripheral blood. γδ T cells represent the major lymphocyte subset in the intestinal mucosal linings, reproductive and the respiratory tracts of all species, where they could be strategically placed to have a role for innate (*i.e.* first line of defence) immune responses to pathogens (Williams, 2001), quick clearance of infected cells (Hayday, 2000), and/or the prevention of re-infection (Komori et al., 2006). They also guard the extracellular environment by ensuring tissue restoration and by participating in wound healing of damaged epithelial surfaces (Chen et al., 2002).

In contrast to αβ T cells which recognise processed antigens presented on the major histocompatibility complex (MHC), γδ T cells recognise antigens independent of MHC presentation (O'Brien et al., 2007). As such, γδ T cells can directly recognise ligands such as the phosphoantigens, the alkyl amines, the aminobisphosphanates, and

* Corresponding author at: Institute of Fundamental Sciences, Massey University, Turitea Campus, Palmerston North 4442, New Zealand. Tel.: +64 6 356 9099; fax: +64 6 350 5682.
E-mail addresses: Olekile_Tibe@hotmail.com, o.tibe@massey.ac.nz (O. Tibe).

0165-2427/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2012.03.003

other non-peptide antigens, which are not presented on the MHC (Casetti and Martino, 2008). In addition, low and high molecular weight compounds such as phenolics and condensed tannins (CT) are recognised (Gertner et al., 2007). Holderness et al. (2007) reported that CT from unripe apple peel significantly primed bovine γδ T cells *in vitro* as measured *via* up-regulation of CD25. Similarly, CT from forage plant extracts primed lamb and calf γδ T cells *in vitro*, although the activity was not as significant as in CT from apple peel (Schreurs et al., 2010). Ramírez-Restrepo et al. (2010) reported that lambs fed temperate willow blocks containing CT had a higher proportion of γδ T cells in their blood than those fed lucerne. Conversely, in a consecutive *in vivo* study, young sheep fed willow did not exhibit different levels of primed γδ T lymphocytes, although their worm burdens were lower than in the relevant control animals (Mupeyo et al., 2010).

The priming capacity of CT from forage plants on goat γδ T cells *in vitro* has not been investigated. Hence, in addition to priming of bovine and ovine γδ T cells, the effects of CT on the expression of CD25 on γδ T cells in goats and their contribution to the innate immune responses warrant further investigation.

Smallholding farmers in Botswana have reported feeding a range of parasitic and browse plants to small ruminants temporarily over a short period of time (about 1–2 months) in the dry season to meet their nutritional requirements and to improve their health (Madibela et al., 2000). However, there is limited information on the effects of CT from these forage plants on the ruminant immune system. Therefore, the main aim of this study was to investigate the potential impact of purified CT from Botswanan forage plants on the innate immunity, particularly on the priming of γδ T cells in kids, calves and lambs *via* up-regulation of CD25.

2. Materials and methods

2.1. Plant material

Fresh samples of four different plants (*Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius* and *Grewia flava*) were collected at Botswana College of Agriculture (BCA) farm in Gaborone, Botswana in February 2009 (summer). These samples, consisting of the leaves and small stems, were freeze-dried and couriered to New Zealand under Ministry of Agriculture and Forestry (MAF) approval.

2.2. Extraction

Freeze-dried and ground leaves and small stems (100 g) were extracted with aqueous acetone (3:7, v/v, 3L) containing ascorbic acid (1 g/L) and strained through cheesecloth to remove plant debris. The filtered extract was concentrated *in vacuo* using a rotary evaporator at 40 °C to remove acetone and the aqueous solution was subsequently defatted with dichloromethane. The aqueous layer was then concentrated *in vacuo* to remove residual dichloromethane and freeze-dried to yield a brown CT crude extract.

2.3. Fractionation

The fractionation on Sephadex LH-20 of the CT crude extract was carried out according to the method of Sivakumaran et al. (2006). Briefly, the CT extract (5 g) was dissolved in aqueous methanol (1:1, 30 mL), loaded onto an SR 25 × 300 Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) and equilibrated with aqueous methanol (1:1). Four fractions (F1-F4; 150 mL each) were obtained after elution with aqueous methanol (1:1). Elution with aqueous acetone (3:7) yielded two fractions (F5; 150 mL) and (F6; 350 mL), which were concentrated *in vacuo* and freeze-dried. In summary, fractionation yielded purified CT containing fractions (represented by F6) from each plant: *V. verrucosum* (aqueous acetone; 3:7), *T. oleifolius* (aqueous acetone; 3:7), and *G. flava* (aqueous acetone; 3:7) and these fractions were used in the immunoassays. F5 could not be used for the assays due to insufficient yield. For *V. rotundifolium*, CT were present in the first fraction (F1, aqueous methanol; 1:1).

2.4. Condensed tannin content

The extractable, protein-bound and fibre-bound CT concentrations were determined by the butanol-HCl colorimetric assay according to the method of Jackson et al. (1996). All CT concentrations were determined using CT extracted from *Lotus pedunculatus* as a standard reference (Sivakumaran et al., 2006).

2.5. Animals and experimental design

All animal experiments were approved by the Massey University Animal Ethics Committee (AEC). Two replicates were run for each concentration of each CT (5 and 10 μg/mL) with cells of the blood collected from four kids, four calves and four lambs into sodium heparin tubes ($n = 8$). Blood samples from different animals were used in all the experiments. For pilot studies, Vacutainers (10 mL) with EDTA as anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) were used for collection of whole blood from the animals.

2.6. Isolation of peripheral mononuclear blood lymphocytes (PBMCs)

Peripheral blood lymphocytes were isolated from the blood samples as previously (Ramírez-Restrepo et al., 2010). Blood samples were centrifuged to collect the buffy coat. Cells were then overlaid over Histopaque and PBL separated by density gradient centrifugation. The cells were washed twice and re-suspended to the desired concentration (1×10^6 cells/mL) in RPMI culture media containing antibiotics, L-glutamine, HEPES, and 5% foetal calf serum.

2.7. Cell culture

Two purified CT extracts (5 mg) from each of *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava* were dissolved in phosphate buffered saline (PBS, 1 mL), sterile filtered and kept at −20 °C until used. Stock solutions were

further diluted to give a 500 μg/mL concentration. PBMCs from each animal were cultured with each Sephadex LH-20 CT containing fractions at 5 and 10 μg/mL or Concanavalin A (positive control) or PBS (negative control) at 5 μg/mL at 37 °C and 5% CO_2 for 48 h. The optimal concentrations of 5 and 10 μg/mL were established prior to tests at higher concentrations of 20 and 50 μg/mL, which were found to be toxic to the cells (data not shown).

2.8. Cell analysis

Cell analysis to measure the priming of γδ T cells was performed according to the modified method of Ramírez-Restrepo et al. (2010). Briefly, cells were stained with a pool of monoclonal antibodies (mAbs) specific for surface antigens for γδ T cells (anti-γδ TCR; clone H1-176) and CD25 (anti-interleukin-2 receptor alpha IL-2Rα, clone CACT116A). MAb (clone H1-176) was originally produced and characterised by the sheep immunology group at the Basel Institute for Immunology, while MAb (clone CACT116A) was obtained from the Veterinary Medical Research and Development (VMRD, Pullman, WA, USA). These mAbs were conjugated to appropriate fluorochromes Alexa 488 or Alexa 647 (Molecular Probes, Invitrogen, Eugene, USA). The cells were incubated at room temperature for 15 min, washed twice and re-suspended in PBS containing 1% (v/v) of 7-amino-actinomycin D (7AAD). 7AAD was included in all staining procedures to exclude dead cells from the analysis. Cells were analysed on a flow cytometer (Beckton Dickson, FACSCalibur, NJ, USA). Data were analysed and figures produced by using FlowJo software (Tree Star, Inc. Ashland, USA). Thirty thousand events were routinely acquired for analysis. Gating strategy was employed to differentiate lymphocytes on light scatter (side scatter-SSC and forward scatter-FSC) profiles from monocytes, granulocytes, and debris followed by exclusion of dead cells with 7AAD (Fig. 1A and B). Finally, the proportion of γδ T cells which are CD25 positive (γδ $TCR^+/CD25^+$) relative to the total γδ T cell population was calculated and the mean fluorescent intensity (MFI) of CD25 on γδ T cells was identified (Fig. 1C).

2.9. Statistical analysis

Data on the priming of γδ T cells and their mean fluorescent intensity of kids, calves and lambs by the plant extracts were analysed using Minitab 15 statistical software (v15.1). Differences between treatments for each animal were analysed using one-way ANOVA procedures. The means from two pooled experiments were considered significantly different when $p < 0.05$. The expression of CD25 on γδ T cells induced by the tested fractions was compared to that induced by PBS and Con A. In addition, data were subjected to an analysis of variance based on a 4 (CT sources) × 2 (concentration) × 3 (lamb, kid and calf) factorial design (General Linear Model, Minitab v15.1) to investigate for interaction effects. The model included plant–animal, plant–concentration, concentration–animal and plant–animal–concentration interaction effects. The model included the fixed effects of concentration. The plant and animal species (calf, lamb and kid) were incorporated in the model as random variables to control variation because the analysis was repeated across the four animals. The least square difference (LSD) test was used to separate least square means and were considered significantly different when $p < 0.05$. The results for the factorial design are reported as the

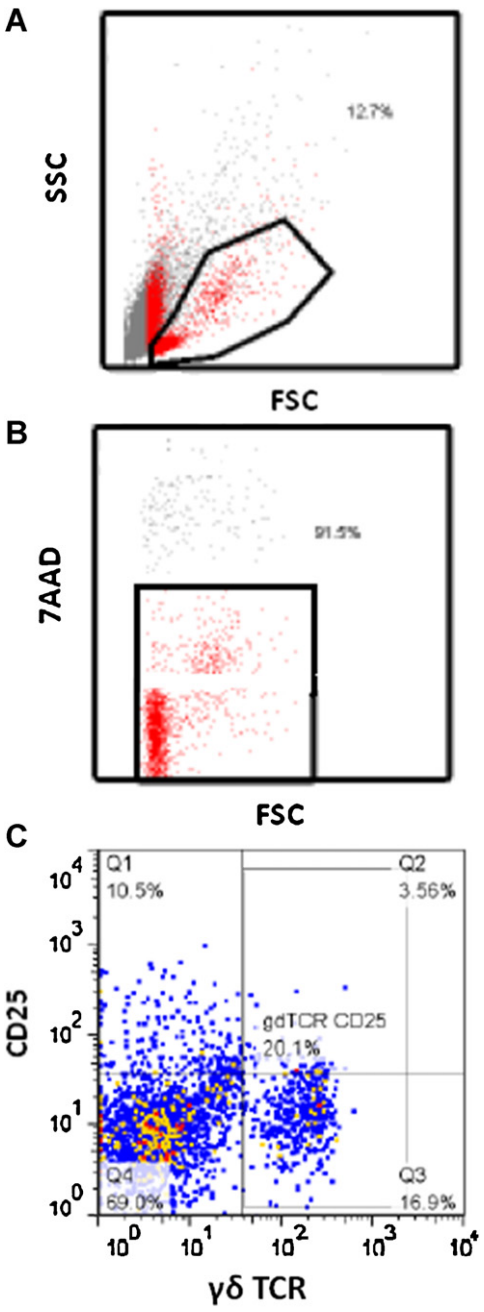

Fig. 1. Gating strategy was employed in: (A) differentiation of lymphocytes by their characteristic FSC (forward scatter)/SSC (side scatter) profiles from monocytes, granulocytes, and debris; (B) identification of viable cells by exclusion of 7AAD positive cells; (C) identification of MFI (mean fluorescent intensity) of CD25 on γδ T cells and proportion of γδ T cells expressing CD25 on γδ T cells.

Table 1
Condensed tannin concentration (g/100 g DM) of forage plants collected in February 2009 (summer) by the butanol-HCl colorimetric assay.

Plant name	Free	Protein-bound	Fibre-bound	Total
Viscum rotundifolium	0.1	0.4	<0.1	0.5
Viscum verrucosum	3.9	2.0	0.1	6.0
Tapinanthus oleifolius	2.0	2.9	0.2	5.1
Grewia flava	6.9	5.0	0.7	12.7

least square means from the two final pooled experiments.

3. Results

3.1. Condensed tannin concentration

CT content results from four forage plants are shown in Table 1. The total CT concentrations ranged from 0.5 to 12.7% (g/100 g DM). *G. flava* leaves had the highest CT concentration (12.7%) followed by *V. verrucosum* (6.0%) and *T. oleifolius* (5.1%). *V. rotundifolium* contained trace CT content of 0.5%.

3.2. Effects of plant extracts on priming of γδ T cells from kids

The response of γδ T cells derived from the blood of kids was dependant on the plant species from which the CT were purified (Table 2). CT from *G. flava* gave the highest proportion of primed γδ T cells from kids up to 64.75% at 10 μg/mL relative to primed γδ T cells at 22.66% by the negative control ($p = 0.004$). *G. flava* had the highest CT content (12.7%). These results are in agreement with their MFI of CD25 expression on γδ T cells with 69.65 ($p = 0.002$). CT from *T. oleifolius* showed increase in the proportion of primed γδ T cells up to 45.70% at 10 μg/mL, although not statistically significant ($p = 0.102$). Although there was no significant difference in priming of the cells from kids at 5 μg/mL, there was an indication of priming by CT from *T. oleifolius* (45.30%) ($p = 0.110$). CT from *V. rotundifolium* and *V. verrucosum* induced a minimal increase in the proportion of primed γδ T cells, although not statistically significant. The results of priming of γδ T cells from kids by CT from *G. flava* and *T. oleifolius* are also represented in Fig. 2A. A sub-fraction of γδ T cells (32.2%) in kids was primed by CT from *G. flava* at 10 μg/mL, while non-γδ T cells were not primed by CT from *G. flava*. CT from *T. oleifolius* increased expression of CD25 on γδ T cells in kids at 10 μg/mL. CT from *T. oleifolius* and *G. flava* exclusively primed γδ T cells in the blood of kids in contrast to polyclonal activation by the mitogen Con A.

3.3. Effects of plant extracts on priming of γδ T cells in calves

While significant priming of γδ T cells in the blood of kids by CT from *G. flava* and *T. oleifolius* was obtained, the plant extracts did not prime γδ T cells in the blood from calves (Table 3). The proportion of primed γδ T cells by the plant extracts was not statistically significantly relative to the negative control ($p = 0.308$ at 5 μg/mL and $p = 0.475$ at 10 μg/mL) ($p < 0.05$). These results are consistent with their MFI of CD25 on γδ T cells. However, there was an indication of an increase in the proportion of primed γδ T cells by CT from *G. flava* at 18.62% relative to the negative control with primed cells at 9.94%. In all the tested fractions, unprimed γδ T cells were predominant (Fig. 2B). In addition, the tested extracts did not prime the non-γδ T cells. In the positive control nearly all γδ T cells and non-γδ T cells were primed, while for the negative control all γδ T cells were not primed.

3.4. Effects of plant extracts on priming of γδ T cells in lambs

The priming of γδ T cells from lambs was minimal in comparison to response from goats (Table 4). There were no significant differences in the proportion of primed γδ T cells by CT from all the tested extracts ($p = 0.966$ at 5 μg/mL and $p = 0.844$ at 10 μg/mL). However, there was an indication of elevation of primed γδ T cells (43.45%) by CT from *G. flava* relative to the negative control (32.49%). The mean percentage expression of CD25 on γδ T cells of lambs induced by the plant extracts was in agreement with MFI of CD25 on γδ T cells and was not statistically significant ($p = 0.922$ at 5 μg/mL and $p = 0.659$ at 10 μg/mL). The results in Fig. 2C show that CT from *T. oleifolius* and *G. flava* induced the expression of CD25 on γδ T cells in lambs at 10 μg/mL, although the priming of γδ T cells by the positive control was lower. Con A did not prime extensively non-γδ T cells in lambs in contrast to polyclonal activation by Con A in kids.

3.5. Effects of animal species, concentration and their interaction on priming γδ T cells

In addition to the effects of plant species on priming of γδ T cells, the cellular response was also dependent on the animal species. The mean proportion of primed γδ T cells in the blood of kids (35.95%) was higher than those in lambs (28.80%) and calves (12.14%) ($p = 0.015$) at both concentrations. The increase in the concentration of the CT extracts in the cell culture did not induce significant impact on the priming of γδ T cells ($p = 0.357$). However, the priming of γδ T cells in kids was induced at higher concentration of 10 μg/mL, suggesting concentration-dependence and cell proliferation induction. No plant × animal ($p = 0.072$), plant × concentration ($p = 0.107$), concentration × animal ($p = 0.173$), and plant × animal × concentration ($p = 0.833$) interactions on priming γδ T cells occurred. In addition, no plant × concentration ($p = 0.275$), plant × animal ($p = 0.317$), concentration × animal ($p = 0.453$), no plant × concentration × animal ($p = 0.064$) interactions occurred in the MFI of CD25 on γδ T cells (data not shown).

4. Discussion

The results obtained demonstrate that CT containing extracts from Botswanan forage plants, particularly *G. flava* and *T. oleifolius*, prime γδ T cells derived from the circulating blood of young goats. This is in agreement with reports in the literature that CT from broadleaf dock (*Rumex*

Table 2
Mean percentage of primed γδ T cells in kids relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments ($n = 8$) and comparison of γδ T cell responses was made to the non-stimuli.

Plant species extract and controls	Concentration (μg/mL)	Mean % of primed γδ T cells ($CD25^+$γδ TCR^+) ± SEM	Mean MFI of CD25 on γδ TCR^+ ± SEM
Controls			
Non-stimuli		22.66 ± 6.32	18.77 ± 1.38
Con A	5	95.60 ± 1.02 ($p = 0.000$)	461.50 ± 28.5 ($p = 0.000$)
Plant species and extraction solvent			
V. rotundifolium (Fr-1, 50% methanol)	5	28.40 ± 11.0	24.60 ± 6.60
	10	29.90 ± 10.2	23.50 ± 5.12
V. verrucosum (Fr-6, 70% acetone)	5	25.57 ± 9.65	26.92 ± 3.85
	10	31.32 ± 6.57	20.93 ± 2.89
T. oleifolius (Fr-6, 70% acetone)	5	36.50 ± 15.1 ($p = 0.286$)	40.20 ± 18.7 ($p = 0.302$)
	10	45.70 ± 10.1 ($p = 0.102$)	49.20 ± 12.5 ($p = 0.340$)
G. flava (Fr-6, 70% acetone)	5	45.30 ± 10.3 ($p = 0.110$)	43.10 ± 13.8 ($p = 0.310$)
	10	64.75 ± 6.61 ($p = 0.001$)	69.65 ± 9.24 ($p = 0.002$)
Significance (overall)	5	0.591	0.481
	10	0.018	0.001

Abbreviations and significance: MFI: mean fluorescent intensity, Fr: fraction, No significance in some of the tested extracts with p values not shown: $p > 0.05$.

Table 3
Mean percentage of primed γδ T cells in calves relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments ($n = 8$) and comparison of γδ T cell responses was made to the negative stimuli.

Plant species extract and controls	Concentration (μg/mL)	Mean % of primed γδ T cells ($CD25^+$γδ TCR^+) ± SEM	Mean MFI of CD25 on γδ TCR^+ ± SEM
Controls			
Non-stimuli (PBS)		9.94 ± 2.36	22.82 ± 4.96
Con A	5	75.95 ± 18.3 ($p = 0.012$)	656.00 ± 270 ($p = 0.058$)
Plant species and extraction solvent			
V. rotundifolium (Fr-1, 50% methanol)	5	15.81 ± 2.37	27.45 ± 4.96
	10	13.43 ± 1.08	26.20 ± 3.41
V. verrucosum (Fr-6, 70% acetone)	5	10.21 ± 2.17	22.90 ± 5.20
	10	14.42 ± 1.48	27.70 ± 3.59
T. oleifolius (Fr-6, 70% acetone)	5	10.58 ± 2.34	23.70 ± 4.42
	10	11.06 ± 3.15	23.45 ± 5.50
G. flava (Fr-6, 70% acetone)	5	8.54 ± 2.34	26.48 ± 5.21
	10	18.62 ± 6.54	32.27 ± 5.58
Significance (overall)	5	0.308	0.960
	10	0.475	0.634

Abbreviations and significance: MFI: mean fluorescent intensity, Fr: fraction, NS: not significant, No significance in all the tested extracts ($p > 0.05$), except Con A ($p < 0.05$).

Table 4
Mean percentage of primed γδ T cells in lambs relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments ($n = 8$) and comparison of γδ T cell responses was made to the non-stimuli.

Plant species extract and controls	Concentration (μg/mL)	Mean % of primed γδ T cells ($CD25^+$ γδ TCR^+) ± SEM	Mean MFI of CD25 on γδ TCR^+ ± SEM
Controls			
Non-stimuli (PBS)		32.49 ± 13.2	27.40 ± 11.6
Con A	5	34.11 ± 18.2 ($p = 0.945$)	179.40 ± 67.5 ($p = 0.068$)
Plant species and extraction solvent			
V. rotundifolium (Fr-1, 50% methanol)	5	37.52 ± 13.3	28.20 ± 11.6
	10	35.02 ± 11.7	23.83 ± 9.24
V. verrucosum (Fr-6, 70% acetone)	5	24.08 ± 13.0	22.90 ± 11.3
	10	20.15 ± 12.5	20.70 ± 11.9
T. oleifolius (Fr-6, 70% acetone)	5	34.57 ± 16.8	31.50 ± 13.8
	10	32.75 ± 14.3	29.40 ± 12.8
G. flava (Fr-6, 70% acetone)	5	37.47 ± 17.8	40.40 ± 19.1
	10	43.45 ± 18.4	47.90 ± 19.8
Significance (overall)	5	0.966	0.922
	10	0.844	0.659

Abbreviations and significance: MF: mean fluorescent intensity, Fr: fraction, NS: not significant, No significance in all the tested extracts ($p > 0.05$), except Con A.

obtusifolius) and sulla (*Hedysarum coronarium*) significantly primed γδ T cells from the circulating blood of calves *in vitro* (Schreurs et al., 2010). In the same study (Schreurs et al., 2010), there was minimal stimulation of γδ T cells from lambs following the addition of the plant extracts. According to Ramírez-Restrepo et al. (2010), the numbers of γδ T cells in the peripheral blood was elevated in sheep fed temperate willow blocks. In addition, CT from unripe apple peel significantly primed γδ T cells and augmented cell division (Holderness et al., 2007). This activity has been observed in humans and mice (Jutila et al., 2008). It has been proposed that the variability in the response of γδ T cells to stimulation with the plant extracts could be due to differences in the chemical structure and concentrations of CT (Hummer and Schreier, 2008; Spencer et al., 2007).

In the present study, CT from *G. flava* and *T. oleifolius* significantly primed γδ T cells derived from the blood of kids, but not those derived from lambs or calves. This indicates that the γδ T cell responses of related animal species such as goat, sheep and cattle may vary considerably (Hein and Mackay, 1991). It is proposed, therefore, that goat γδ T cells may have been evolved to recognise selected range of CT from forage plants. It is noted that goats are browsers in contrast to lambs and cattle, which are grazers (Alonso-Díaz et al., 2010; Hoste et al., 2005). Browsers may ingest a wide range of plants containing high CT, which may stimulate innate immune cells in the gut mucosa. Therefore, further studies should be conducted to establish whether γδ T cells in other browsing species such as deer could be stimulated by CT from these forage species. Other factors that could have contributed to observed species differences in the priming of cells are the history of antigen exposure, diet and age, and the physiological state of the animal.

In addition to the observed differences in plant and animal species effects on the priming of γδ T cells, the *in vitro* cellular response in the kids was induced at low CT concentrations, apoptosis (programmed cell-death) occurring at high concentrations. Normally, *in vivo*, high concentrations of CT are regulated and toxic levels are inhibited from reaching the bloodstream (Jutila et al., 2008). A small proportion of CT is absorbed in the upper gastrointestinal tract, although the mechanisms of absorption are still unclear (Stevenson and Hurst, 2007). Therefore, the observed priming of γδ T cells in the kids at these low concentrations suggests that the results may have physiological significance.

The stimulation of γδ T cells by CT is receptor-mediated, and is subtle, direct and antigen-independent (Holderness et al., 2008). CT have been shown to be ligands for γδ T cell receptors and this receptor binding results in functional changes of the cell such as CD25 expression and synthesis

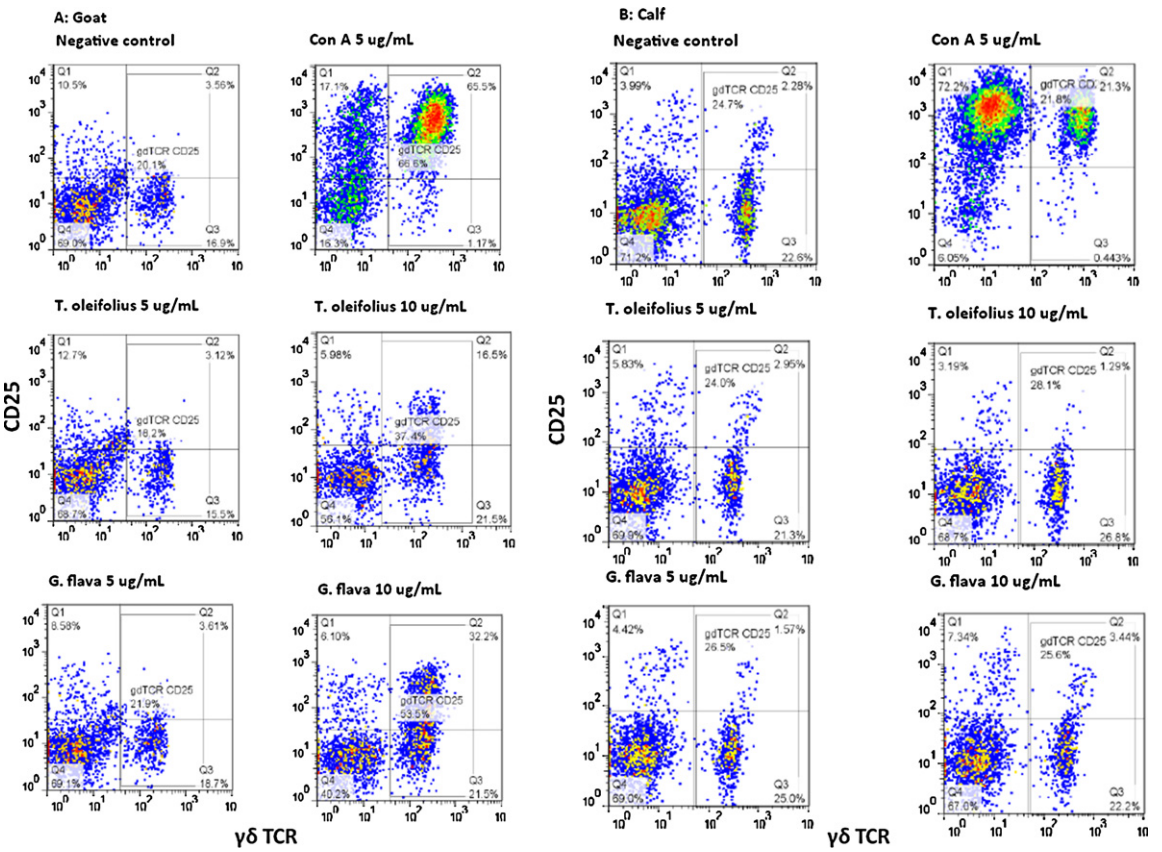

Fig. 2. Results are representative of plant CT extracts on priming γδ T cells in kids, calves and lambs. (A) Priming in kids by *T. oleifolius* and *G. flava* at 10 μg/mL and by Con A at 5 μg/mL. No priming in kids by *T. oleifolius* and *G. flava* at 5 μg/mL. (B) No priming in calves. (C) Priming in lambs by *T. oleifolius* and *G. flava* at 10 μg/mL. No priming of cells of lambs by the negative control, *T. oleifolius* and *G. flava* at 5 μg/mL.

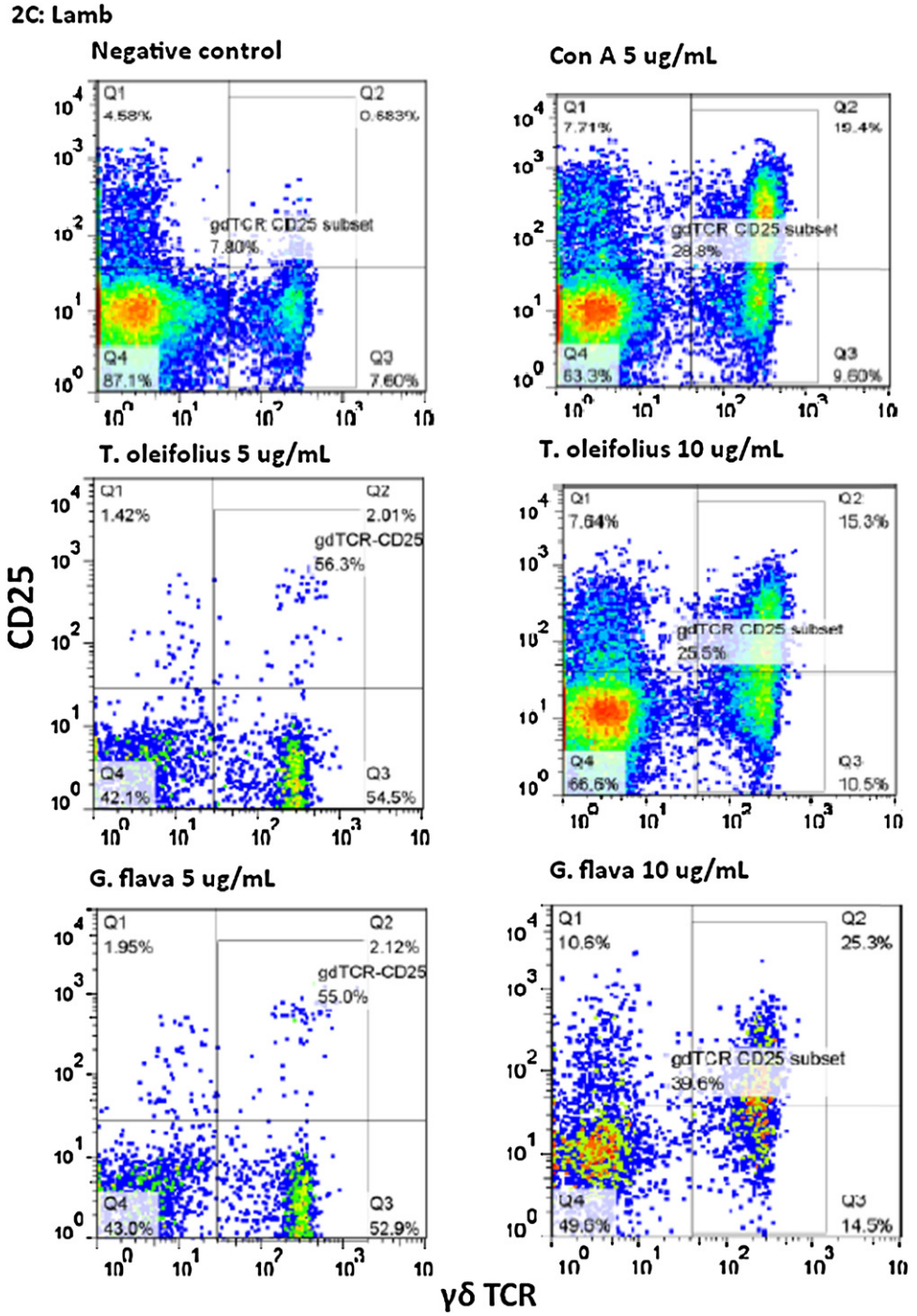

Fig. 2. (*Continued*).

of interleukin IL-2 (Ferrero et al., 2007). Primed γδ T cells may be able to respond more quickly and robustly in downstream responses to pathogens. The priming event induced by CT enables the interaction of IL-2 with IL-2R, which in turn initiates cell proliferation (Parkin and Cohen, 2001). In addition, the activated γδ T cell can migrate, and home into sites of infection, where they produce pro-inflammatory effector cytokines such as interferons (IFNγ) and tumor necrosis factor (TNFα) (Jutila et al., 2008).

The γδ T cells are the predominant lymphocytes in the mucosal linings of all animal species and play a significant role in innate immunity (Holderness et al., 2008). The priming of γδ T cells *in vitro* implies a similar process may occur in the gut mucosa of goats, which is commonly exposed

to pathogens such as bacteria, viruses and gastrointestinal nematodes (Hein and Mackay, 1991). However, the potential impact of the extracts from *G. flava* and *T. oleifolius* on the ruminant immune system and on gastrointestinal nematodes *in vivo* has not been investigated.

In conclusion, the current study presents new information on the priming of γδ T cells from young goats following exposure to CT from *G. flava* and *T. oleifolius*. Should this priming also occur *in vivo*, the feeding of these plants may have a positive impact on the ability of the animals to respond to pathogens, such as gastrointestinal nematodes. It remains to be seen if priming is restricted to specific subsets of γδ T cells such as those represented in workshop cluster 1 (WC1), and if the stimulated cells could reduce the effects of parasitism.

Acknowledgements

The authors would like to thank Dr. Dave Leathwick (AgResearch Grasslands, Palmerston North, New Zealand) and Mr. Christian Sauermann (Massey University, Institute of Veterinary and Animal and Biomedical Sciences, Palmerston North, New Zealand) for providing animal (kids, lambs and calves) blood samples for this study. Thanks also to Mr. Francis Kweku Amagloh at Massey, Institute of Food Nutrition and Human Health for advice on statistical analysis. Dr. Bryce Buddle and Dr. Axel Heiser are also thanked for critiquing the manuscript. Finally, thanks to the Botswana College of Agriculture (BCA) for providing sponsorship for the study.

References

Alonso-Díaz, M., Torres-Acosta, J., Sandoval-Castro, C., Hoste, H., 2010. Tannins in tropical tree fodders fed to small ruminants: a friendly foe? Small Ruminant Res. 89, 164–173.

Casetti, R., Martino, A., 2008. The plasticity of γδ T cells: innate immunity, antigen presentation and new immunotherapy. Cell. Mol. Immunol. 5, 161–170.

Chen, Y., Chou, K., Fuchs, E., Havran, W.L., Boismenu, R., 2002. Protection of the intestinal mucosa by intraepithelial γδ T cells. Proc. Natl. Acad. Sci. 99, 14338–14343.

Ferrero, I., Michelin, O., Lescher, I., 2007. Antigen recognition by T lymphocytes. In: Encylopaedia of Life Sciences. John Wiley and Sons, pp. 1–10.

Gertner, J., Scotet, E., Poupot, M., Bonneville, M., Fournie, J., 2007. Lymphocytes: gamma delta. In: Encylopaedia of Life Sciences. John Wiley and Sons, pp. 1–10.

Hayday, A., 2000. γδ Cells: a right time and a right place for a conserved third way of protection. Annu. Rev. Immunol. 18, 975–1026.

Hedges, J., Lubick, K., Jutila, M., 2005. γδ T cells respond directly to pathogen-associated molecular patterns. J. Immunol. 174, 6045–6053.

Hein, W.R., Mackay, C.R., 1991. Prominence of γδ T cells in the ruminant immune system. Immunol. Today 12, 30–34.

Holderness, J., Hedges, J., Daughenbaugh, K., Kimmel, E., Graff, J., Freedman, B., Jutila, M., 2008. Response of T cells to plant-derived tannins. Crit. Rev. Immunol. 28, 377–402.

Holderness, J., Jackiw, L., Kimmel, E., Kerns, H., Radke, M., Hedges, J., Petrie, C., McCurley, P., Glee, P., Palecanda, A., 2007. Select plant tannins induce IL-2Rα up-regulation and augment cell division in γδ T cells. J. Immunol. 179, 6468–6478.

Hoste, H., Torres-Acosta, J.F., Paolini, V., Aguilar-Caballero, A., Etter, E., Lefrileux, Y., Chartier, C., Broqua, C., 2005. Interactions between nutrition and gastrointestinal infections with parasitic nematodes in goats. Small Ruminant Res. 60, 141–151.

Hummer, W., Schreier, P., 2008. Analysis of proanthocyanidins. Mol. Nutr. Food Res. 52, 1381–1398.

Jackson, F.S., Barry, T.N., Lascano, C., Palmer, B., 1996. The extractable and bound condensed tannin content of leaves from tropical trees, shrubs and forage legumes. J. Sci. Food Agric. 71, 103–116.

Jutila, M., Holderness, J., Graff, J., Hedges, J., 2008. Antigen-independent priming: a transitional response of bovine T-cells to infection. Anim. Health Res. Rev. 9, 47–57.

Komori, H., Meehan, T., Havran, W., 2006. Epithelial and mucosal γδ T cells. Curr. Opin. Immunol. 18, 534–538.

Madibela, O.R., Boitumelo, W.S., Letso, M., 2000. Chemical composition and *in vitro* dry matter digestibility of four parasitic plants (*Tapinanthus lugardii*, *Erianthenum ngamicum*, *Viscum rotundifolium* and *Viscum verrucosum*) in Botswana. Anim. Feed Sci. Technol. 84, 97–106.

Mupeyo, B., Barry, T., Pomroy, W., Ramírez-Restrepo, C., López-Villalobos, N., Pernthaner, A., 2010. Effects of feeding willow (*Salix* spp.) upon death of established parasites and parasite fecundity. Anim. Feed Sci. Technol. 164, 8–20.

Nath, I., 2008. Immune mechanisms against intracellular pathogens. In: Encylopaedia of Life Sciences. John Wiley and Sons, Chichester, pp. 1–14.

O'Brien, R., Roark, C., Jin, N., Aydintug, M., French, J., Chain, J., Wands, J., Johnston, M., Born, W., 2007. T-cell receptors: functional correlations. Immunol. Rev. 215, 77–88.

Parkin, J., Cohen, B., 2001. An overview of the immune system. The Lancet 357, 1777–1789.

Ramírez-Restrepo, C., Pernthaner, A., Barry, T., López-Villalobos, N., Shaw, R., Pomroy, W., Hein, W., 2010. Characterization of immune responses against gastrointestinal nematodes in weaned lambs grazing willow fodder blocks. Anim. Feed Sci. Technol. 155, 99–110.

Schreurs, N., Pernthaner, A., Hein, W., Barry, T., 2010. Condensed tannins for priming innate immunity. Proc. New Zeal. Soc. An., 288–290.

Sivakumaran, S., Rumball, W., Lane, G.A., Fraser, K., Foo, L.Y., Yu, M., Meagher, L.P., 2006. Variation of proanthocyanidins in *Lotus* species. J. Chem. Ecol. 32, 1797–1816.

Spencer, P., Sivakumaran, S., Fraser, K., Foo, L., Lane, G., Edwards, P., Meagher, L., 2007. Isolation and characterisation of procyanidins from *Rumex obtusifolius*. Phytochem. Anal. 18, 193–203.

Stevenson, D., Hurst, R., 2007. Polyphenolic phytochemicals – Just antioxidants or much more? Cell. Mol. Life Sci. 64, 2900–2916.

Williams, D.A., 2001. Inflammatory cytokines and mucosal injury. J. Natl. Cancer Inst. 29, 26–30.