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## Brief communication: Condensed tannins for priming innate immunity

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### INTRODUCTION

The immune system of sheep, cattle and other ruminants contains unusually large numbers of gamma-delta ( $\gamma\delta$ ) T lymphocytes. In lambs and calves, these cells may comprise more than 50% of the T cells circulating in blood while in rodents and primates, they typically account for less than 5% of blood-borne T cells. The  $\gamma\delta$  T cells localise within mucosal surfaces of all species, particularly in the gastrointestinal tract. Compared to their rodent and human counterparts, ruminant  $\gamma\delta$  T cells have a greatly expanded repertoire of antigen receptors (Hein & Dudler, 1993, 1997). They also express unique accessory molecules in their cell membrane which may reflect a differential capacity of the ruminant  $\gamma\delta$  T cells to contribute to immune defence (Hein & Mackay, 1991).

The precise contribution of  $\gamma\delta$  T cells to immune protection requires more research. A dominating theory is that  $\gamma\delta$  T cells contribute to early innate immune defence at mucosal surfaces through the local proliferation and release of cytokines and other mediators (Casetti & Martino, 2008; Jutila *et al.*, 2008). Using an *in vitro* culture system, it was discovered that tannins from unripe apple peel functioned as direct, antigen-independent, non-specific agonists for bovine  $\gamma\delta$  T cells as evidenced by augmented entry into cell cycle and up-regulation of the interleukin-2 alpha receptor (Holderness *et al.*, 2007). In an *in vivo* experiment to test the extent to which condensed tannins (CT) exert resistance to nematodes in sheep, a CT-containing diet of willow (*Salix* spp.) produced higher numbers of  $\gamma\delta$  T cells in their blood during early stages of the experiment (Ramirez-Restrepo *et al.*, 2010). This provided the suggestion that CT may directly affect some aspects of immunity to nematodes but highlighted the need to further investigate the use of CT from forages species to activate  $\gamma\delta$  T cells and up-regulate immune capacity in grazing ruminants in a more controlled manner.

The  $\gamma\delta$  T cells are the only bovine cell type that has been found to be affected by plant tannins (Jutila *et al.*, 2008). It needs to be established if

ovine  $\gamma\delta$  T cells also have this response. The aim of this study was to determine if CT from forage plants was able to prime  $\gamma\delta$  T cells from both calves and lambs in a similar manner to that reported for apple peel CT with bovine cells in overseas research (Holderness *et al.*, 2007). The plant species were selected on their potential to be used in grazing systems by lambs and calves. A positive outcome would suggest that natural dietary components may directly prime innate immune cells residing in the intestinal wall, independent of antigens, and provide further evidence for the use of CT to control gastrointestinal parasites in grazing systems.

### MATERIALS AND METHODS

Fresh vegetative growth of broadleaf dock (*Rumex obtusifolius*), canary clover (*Dorycnium rectum*), sulla (*Hedysarum coronarium*), bird's foot trefoil (*Lotus corniculatus*) and big trefoil (*Lotus pedunculatus*) was collected from experimental plots located at AgResearch Grasslands, Palmerston North. Willow (*Salix* spp.) leaves were collected from cuttings obtained from the Greater Wellington Regional Council's Nursery near Masterton. The six plant samples were stored at -20°C until condensed tannins were extracted from them using the method of Sivakumaran *et al.* (2004).

Blood was collected from six lambs and six calves by jugular venipuncture into 10 mL Vacutainers<sup>®</sup> with ethyldiaminetetraacetic acid (EDTA) anti-coagulant. Both the calves and lambs were from systems which implemented rearing practices that were typical of New Zealand farms. That is, lambs at foot on pasture and calves separated from their mothers and housed indoors. The calves and lambs were approximately 10 weeks old and unweaned at the time of blood collection. Blood was held at ambient temperature for transport to the laboratory. Peripheral blood lymphocytes (PBL) were immediately isolated from the blood samples by density gradient centrifugation. The PBL from each animal were then cultured for 48 h in RPMI (Rosswell Park Memorial Institute) culture medium containing 5% bovine fetal serum and supplemented with extracted CT at a concentration

of 0, 5, 10 and 20 µg/mL. Evidence of priming was detected by measuring the expression of CD25 (interleukin-2 receptor) on the γδ T cells by flow cytometry using monoclonal antibodies (mAbs) in a direct fluorochrome staining procedure. Antibodies were conjugated to either Alexa 488 or Alexa 647 as recommended by the producer (Invitrogen). Bovine γδ T cells were stained with clone CACTB14A conjugated to Alexa 488, ovine γδ T cells with clone 127.5/86D directly conjugated to Alexa 488, and CD25 (clone CACT116A which reacts with bovine and ovine CD25) directly conjugated to Alexa 647. For analysis, gates were set around the mononuclear cell population. Dead cells were excluded by staining with 7AAD.

Exploratory investigation of the data indicated that background expression was different between individual calves and lambs (P <0.001). Therefore,

for the final analysis, the percentage of stimulated γδ T cells from each calf or lamb was adjusted for background stimulation. Within calf or lamb mean values from assays without CT added were subtracted from the values obtained for assays with CT. Adjusted values were subjected to an analysis of variance based on a 6 (CT sources) x 3 (concentration) factorial design (PROC MIXED, SAS, 2003). Plant species, CT concentration and their interaction were included in the model. The calf or lamb from which the cells were obtained was incorporated into the analysis as a random effect to control variation occurring as a result of repeating the analyses across six animals. The treatment with no CT added was not included in the analysis of variance but the mean percentage of CD25 expression without CT addition has been included in Table 1 for comparison.

## RESULTS AND DISCUSSION

The expression of CD25 on the γδ T cells isolated from calves was stimulated by the presence of CT although the response was not as great as that seen with apple peel extract in the study of Holderness *et al.* (2007). The priming capacity of the CT was dependent on the plant species from which the CT was extracted (P <0.001; Table 1). Sulla and broadleaf dock gave the greatest up-regulation of CD25 while willow gave the poorest response (Table 1). The response to CT was dependant on the concentration of the CT used in the cell culture (P <0.05). A greater CT concentration gave a greater expression of CD25 when using the CT extracts from broadleaf dock, canary clover, sulla and big trefoil (Table 1).

In comparison to the calf results, the expression of CD25 on the γδ T cells isolated from lambs in response to added CT was minimal (Table 1). Although this study was not designed to provide a between species comparison, the results are indicative of species specific differences in the γδ T cell receptor repertoire. Other factors that may have contributed to the difference in the results between the lambs and calves

**TABLE 1:** Change in the percentage expression of CD25 (as an indicator of priming for γδ T cells) from calves and lambs when the γδ T cells were cultured in the presence of condensed tannin (CT) extracted from six plant species at three concentrations relative to the expression without CT. The without CT data was not subjected to analysis of variance but included in the table for comparison purposes.

Plant species	CT concentration (µg/mL)	Percentage expression of CD25 (%)	
		Calves	Lambs
Without CT <sup>1</sup>	0	13.7	5.9
Broadleaf dock ( <i>Rumex obtusifolius</i> )	5	19.3 <sup>a</sup>	0.0 <sup>a</sup>
	10	35.0 <sup>b</sup>	1.1 <sup>b</sup>
	20	38.7 <sup>b</sup>	2.6 <sup>b</sup>
Canary clover ( <i>Dorycnium rectum</i> )	5	6.5 <sup>a</sup>	0.4
	10	22.2 <sup>b</sup>	0.6
	20	33.2 <sup>b</sup>	0.7
Sulla ( <i>Hedysarum coronarium</i> )	5	25.6 <sup>a</sup>	0.0
	10	36.0 <sup>ab</sup>	0.0
	20	40.5 <sup>b</sup>	0.0
Bird's foot trefoil ( <i>Lotus corniculatus</i> )	5	26.2	0.6
	10	26.0	1.1
	20	32.7	1.6
Big trefoil ( <i>Lotus pedunculatus</i> )	5	22.2 <sup>a</sup>	0.0
	10	24.2 <sup>ab</sup>	0.0
	20	37.4 <sup>b</sup>	0.0
Willow ( <i>Salix</i> spp.)	5	18.6	0.0
	10	24.0	0.1
	20	28.9	0.1
Pooled SEM		12.0	0.6
Significance:			
Plant species		***	NS
CT concentration		*	*
Plant species x CT concentration		NS	NS

Within plant species, mean values with different superscripts are different at P <0.05.

include: differences in diet, differences in antigen exposure and differences in the maturity levels of the two animal species at the time of blood sampling. These factors need to be investigated further or controlled in further experiments in order to establish the most appropriate approach for utilising CT-containing forages for the priming of innate immunity in grazing ruminants. Ramirez-Restrepo *et al.* (2010) reported increased numbers of circulating  $\gamma\delta$  T cells by feeding willow to sheep but the willow-fed sheep had elevated circulating  $\gamma\delta$  T cells at Day 0 of the trial and greater parasite burdens which may have prompted the greater  $\gamma\delta$  T cell numbers rather than feeding willow itself. In this current *in vitro* study, there was no evidence of increased CD25 expression on lamb  $\gamma\delta$  T cells for the willow CT. This suggests that willow is not capable of eliciting a response on the immune response via  $\gamma\delta$  T cells or that non CT components of the willow are more likely to have a role. With the  $\gamma\delta$  T cells from lambs, the up-regulation of the interleukin-2 receptor with broadleaf dock CT was dependant on the CT concentration used in the cell cultures ( $P < 0.05$ ). It is possible that a CT concentration greater than 20  $\mu\text{g}/\text{mL}$  is required to elicit a substantial response in sheep.

With  $\gamma\delta$  T cells from bovine animals, plant tannins are able to induce interleukin-2 receptor up-regulation and also augment cell division (Holderness *et al.*, 2007). The current results suggest that the agonist activity is also evident with CT from broadleaf dock, canary clover, sulla and big trefoil but the response is poor for  $\gamma\delta$  T cells from sheep. It is possible that natural compounds other than CT, present within plants, could also be agonists for  $\gamma\delta$  T cell activity. The dependency of priming capacity on the concentration of CT indicates that in the grazing system the greater the exposure of the gastro-intestinal mucosa to CT, the greater the response on  $\gamma\delta$  T cell priming. Further research is needed to establish if the priming activity of CT on  $\gamma\delta$  T cells that is seen *in vitro* is able to translate to a priming ability *in vivo* with a consequent measurable improvement in the resilience of the animal to gastro-intestinal nematodes.

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