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Research paper

Relative quantitative kinetics of interferon-gamma and interleukin-10 mRNA and protein production by activated ovine peripheral blood mononuclear cells

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ABSTRACT

Interferon-gamma (IFN-γ) and interleukin (IL)-10 are cross-regulatory cytokines capable of driving and controlling the adaptive host immune response. The inter-relationship between IFN-γ and IL-10 expression has not been defined in sheep despite biological evidence suggesting that they perform similar functions to their orthologues described in other species. To address this, we have developed a quantitative (q)PCR method to assess relative levels of IFN-γ and IL-10 mRNA expression in activated ovine peripheral blood mononuclear cells (PBMC) and compared the kinetics of mRNA expression with amounts of cytokine secreted by the cells over a 96 h period. PBMC were collected from sheep immunised with the nominal antigen ovalbumin (Ova) and re-stimulated *in vitro* with antigen and the T cell mitogen concanavalin A (ConA). The recall response to antigen was characterised by a single peak in IFN-γ mRNA expression at 48 h of culture (13-fold increase over unstimulated cells) and relatively lower expression of IL-10 mRNA (average 2–3-fold increase over the 96 h culture period). Antigen-driven IFN-γ protein concentration was greatest at the end of the culture period (96 h) whereas IL-10 protein level was not elevated above that observed in unstimulated cells. The typical response to ConA was greater for both cytokines, with IFN-γ mRNA expression peaking at 6 h of culture (133-fold increase) then declining rapidly whereas IL-10 mRNA expression peaked at 24 h (16-fold increase) and declined more gradually. Despite these differences in the relative kinetics of mRNA expression in mitogen-activated PBMC, the typical pattern of protein expression of the two cytokines was similar. Both showed a gradual rise in protein concentration starting from 12 h of culture which was still rising at the end of the culture period (96 h). These data demonstrate that the kinetics of mRNA expression for IFN-γ and IL-10 in activated ovine PBMC do not necessarily correlate with detectable protein in culture.

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1. Introduction

Cytokine biology is central to T cell function and the development of adaptive immune responses 'tailored' for controlling different types of pathogens. The first description of two distinct types of CD4+ve T helper(h) clones (Th1 and Th2) in mice was based on their mutually exclusive cytokine profiles, with Th1 cells producing interferon-gamma (IFN-γ) and Th2 cells producing interleukin (IL)-4 (Mosmann et al., 1986). These findings led to the establishment of one of the most important paradigms of modern immunology, namely that Th1/Th2 subsets cross-regulate through mutual inhibition. It was studies on the mechanistic nature of this inhibition that led to the discovery of 'cytokine synthesis inhibitory factor' (CSIF), now known as IL-10 (Bashyam, 2007; Fiorentino et al.,

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1989). IL-10 was identified for its capacity to block IFN-γ synthesis and therefore regulate inflammatory immune responses. The importance of IL-10-mediated immune regulation is exemplified by the lethal, uncontrolled immune responses in IL-10 knock-out mice infected with the protozoan pathogen *Toxoplasma gondii*, a strong driver of the inflammatory immune response (Gazzinelli et al., 1996). CD4+ve T cell subset differentiation and cross-regulation are now known to be much more complex than the original Th1/Th2 paradigm. Regulatory T cells (Treg), Th17 and the recently described Th9 subset, all of which express defined cytokine profiles, have been described in mice (Veldhoen et al., 2008; Zhu and Paul, 2008).

The capability to measure cytokine production and define function is essential for investigating the development of host immune responses and understanding disease pathogenesis. It is well recognised that the capability to study Th subsets and define immunoregulatory pathways in veterinary species lags behind that of mouse and human to the extent that we do not know if the subsets even exist in other species. Such a knowledge gap clearly impacts on vaccine design and disease control, particularly in light of that fact that there is growing evidence to suggest that mechanisms of immune activation and immunological paradigms established in rodents do not necessarily translate to other mammalian species (Kirman and Seder, 2003; Wattegedera et al., 2008).

The cloning and expression of cytokines, and other immunoregulatory molecules, from several veterinary species have accelerated in recent years (Entrican et al., 2009). However, invariably, molecular probes to study mRNA expression are developed and exploited long before antibodies are produced (and properly characterised) to measure protein production. Consequently, the measurement of gene transcription is more commonly measured than protein production. The potential shortcoming of the molecular approach alone is the difficulty of inferring levels of protein production from mRNA expression. Ideally, it is desirable to have the capability to quantify both mRNA expression and protein production for any given molecule of interest to reliably define the kinetics of cytokine induction. With a view to expanding this capability in sheep, we report here on the development of quantitative (q)PCR to measure relative expression of mRNA encoding IFN-γ and IL-10 in mitogen-driven and antigen-specific recall responses of peripheral blood mononuclear cells (PBMC) and compare this expression with protein production over time.

2. Materials and methods

2.1. Animals

Eight, three-and-a-half-year-old, female Scottish Blackface ewes derived from the breeding stock at Moredun Research Institute were maintained on pasture. The ewes were immunised with chicken egg ovalbumin (Ova) two years prior to this study. Briefly, the immunisation protocol consisted of a sub-cutaneous primary injection of 1 ml containing 1 mg of the nominal antigen (Ova; Sigma, Dorset, UK) emulsified in complete Freund's adjuvant (Sigma) followed by a 1 ml booster immunisation four weeks later consisting of 0.5 mg Ova emulsified in incomplete Freund's adjuvant (Sigma). These ewes had demonstrable antigen-specific cellular recall responses up to eighteen months after immunisation (Wattegedera et al., 2008).

2.2. Lymphocyte stimulation assay (LSA)

PBMC were prepared from 30 ml of blood collected from the jugular vein according to a previously described protocol (Wattegedera et al., 2004). The cells were adjusted to a final concentration of 2×10^6 cells/ml in 500 μl of Iscove's Modified Dulbecco's medium (Gibco, Paisley, UK) supplemented with 10% FBS (PAA, Hanninger, Austria), 50 μg/ml gentamycin (Aventis Pharma Ltd., Kent, UK) and 50 μM β-mercaptoethanol (Sigma) and seeded in 24-well flat-bottom tissue culture plates (Costar, Corning, NY, USA). 500 μl of Ova at 20 μg/ml, 500 μl of ConA (extract from *Concanavalia ensiformis*, ICN Biochemicals, Cleveland, OH, USA) at 10 μg/ml (positive control), or 500 μl of culture medium (unstimulated negative control) were added to the cells to give a final volume of 1 ml per well and the cells were cultured at 37 °C/5% CO_2 in a humidified incubator. Cells and culture supernatant were harvested at 6, 12, 24, 48, 72 and 96 h. At each of these time-points, the cells and culture supernatant were removed to conical centrifuge tubes and centrifuged at $290 \times g$, 4 °C for 5 min. The cell-free supernatant was removed and stored at −20 °C until analysis for cytokines. The cell pellet was re-suspended mechanically using cell lysis buffer (Qiagen, West Sussex, UK) containing β-mercaptoethanol (Sigma) to produce cell lysates which were then stored at −20 °C. Samples representing a time zero sample point of unstimulated cells in culture medium alone were harvested as baseline controls.

2.3. Analysis of culture supernatant by ELISAs

Culture supernatants were tested for IFN-γ using the BOVIGAM™ commercial ELISA (Prionics, Schlieren-Zurich, Switzerland), according to the manufacturer's instructions. Quantification was performed using a standard curve generated using known concentrations of recombinant bovine IFN-γ (kindly supplied by Dr. Steven Jones, Pfizer Animal Health, Parkville, Australia). IL-10 was detected by ELISA using the monoclonal antibodies (mAbs) CC318 and CC320 (Kwong et al., 2002). Quantification was performed using recombinant bovine IL-10 expressed in Chinese Hamster Ovary (CHO) cells using the pEE14® expression vector according to a previously published protocol (Wattegedera et al., 2004). The IL-10 mAbs and recombinant protein were generated and provided within the BBSRC/RERAD Immunological Toolbox Consortium (http://www.immunologicaltoolbox.com/).

2.4. Preparation of cDNA

Total RNA was prepared from cell lysates using an RNeasy® Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions, including the additional on-column DNaseI digestion step to remove contaminating

genomic DNA. The integrity of the RNA was confirmed by the visualisation under UV light of the 28S and 18S bands from each RNA sample with 1 μl run on an agarose gel. 15 μl of RNA from each sample was reverse-transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, Cheshire, UK) according to the manufacturer's instructions and then stored at −20 °C.

2.5. Primer and probe design and optimisation for qPCR

Details of the primers and probes for ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ovine IFN-γ and ovine IL-10 are shown in Table 1. Sense and antisense primers were predicted to be located in two consecutive exons of the respective genes. The IFN-γ TaqMan® probe, containing the VIC® reporter dye and 6-carboxytetramethylrhodamine (TAMRA™) was designed to span the junction of two exons to ensure discrimination between cDNA and possible contaminating genomic DNA. Since no exon/intron information is available for ovine IFN-γ, the primers and probe were designed by aligning the ovine and bovine IFN-γ sequences, accession numbers X52640 and M29867 respectively (Genbank), and then modifying the previously described primers and probe for bovine IFN-γ (Leutenegger et al., 2000). The IL-10 TaqMan® probe, containing the VIC® reporter dye and 6-carboxytetramethylrhodamine (TAMRA™) was designed from the ovine cDNA sequence (Dutia et al., 1994). TaqMan® qPCR was carried out using the ABI PRISM 7000 Sequence Detection System (SDS). Primer and probe concentrations were optimised for all genes following the Applied Biosystems protocol using complementary (c)DNA. For the cytokine targets, the cDNA was derived from transfected CHO cells expressing ovine IFN-γ or ovine IL-10. For GAPDH, the cDNA was derived from the ovine adenocarcinoma ST-6 cell line (Norval et al., 1981).

2.6. qPCR protocol

Three separate qPCR reactions were conducted using 5 μl of cDNA from each sample and each of the IFN-γ, IL-10 or GAPDH primers and probes. TaqMan® Universal PCR Master Mix (Applied Biosystems) was used at the recommended concentration. Each sample was assayed in triplicate using thermal cycling conditions of 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Experimental samples from each time-point were analysed on the same 96-well plate to reduce within sample-point variation. Analysis was performed using the ABI Prism 7000 SDS software 1.2.3.

2.7. Validation of qPCR and application of relative quantification method

Data were quantified using relative quantification by the comparative cycle threshold (C_T) method (Cikos et al., 2007). To validate this quantification method, the amplification efficiencies of the experimental targets (IFN-γ and IL-10) need to be approximately equal to the amplification efficiency of the reference target (GAPDH). A serial dilution of an experimental sample was used for this validation. The $\log_2$ input was plotted versus ΔC_T (target–reference) for each cytokine giving differences in the slope values of less than 0.2 (data shown in Table 1). The comparative C_T method was then used to determine the relative difference in cytokine expression between stimulated and unstimulated cells, using the cells cultured in medium alone as the calibrator.

GAPDH has been shown to serve as an appropriate calibrator housekeeping gene for quantitative analyses (Montagne et al., 2001; Tudor et al., 2009). The amplification of the GAPDH gene for each test sample was used to standardise the amount of template DNA present in each reaction and optimised for use in this experimental system. The arithmetic mean of three C_T values for each of the eight samples (each ewe) for treatment against time was calculated and transformed using the formula 40-C_T. The transformed value (40-C_T) value was the plotted for treatment over time and the higher values represented greater amounts of cytokine mRNA (Fig. 1). This process demonstrated that GAPDH gene expression varied with different treatments as shown by the divergence of the lines extending over time for the experimental samples. It was not possible to attribute mRNA expression on a per cell basis from the cell harvesting protocol and therefore it was necessary to remove the influence of proliferating cells on the gene expression values. To achieve this, the cytokine gene expression C_T values were normalised against the most appropriate baseline (GAPDH C_T values for each treatment at each time-point), giving ΔC_T values. The $\Delta\Delta C_T$

Table 1
Quantitative PCR primer/probe sequences for amplification and detection of target and endogenous genes.

Target	Primer/probe	Sequence (5′–3′)	Slope of $\log_2$ versus ΔC_T (cytokine-GAPDH)	Genbank accession number/reference
GAPDH[a]	Fwd	GCATCGTGGAGGGACTTATGA	n/a	AF035421
	Rev	GCCATCACGCCACAGCTT		
	Probe	(6-FAM)-CACTGTCCACGCCATCACTGCCA-(TAMRA)		
	Fwd	TGGATATCATCAAGCAAGACATGTT		
IFN-γ	Rev	GGTCATTCATCACCTTGATGAGTTC	−0.0418	X52640/M29867
	Probe	(VIC)-CAGATCATCCACCGGAATTTGAATCAGCA-(TAMRA)		
	Fwd	GAGCTGCCTTCGGCAAAGT		
IL-10	Rev	CCCTTAAAGTCATCCAGCAGAGA	−0.1704	Dutia et al. (1994)
	Probe	(VIC)-AAGACTTTCTTTCAAATGAAGGACCAACTGAACA-(TAMRA)		

Key: Fwd, forward; Rev, reverse; 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.
[a] Primers and probe designed by Dr. Christina Cousens (Moredun Research Institute).

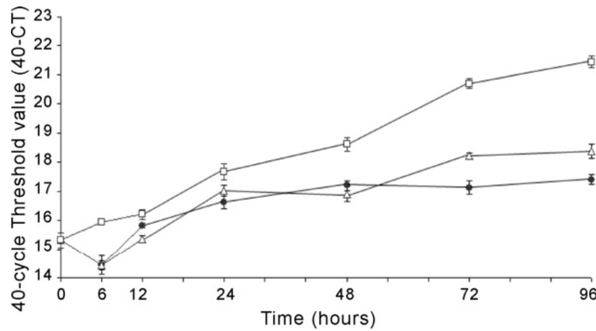

Fig. 1. GAPDH mRNA expression in activated ovine PBMC. PBMC collected from eight sheep were re-stimulated *in vitro* with antigen (Ova, △) or mitogen (ConA, □). Unstimulated PBMC (medium alone, ●) served as a negative control. Total RNA was prepared from cell lysates as described in Section 2 and GAPDH mRNA expression (represented by 40-cycle threshold; 40-C_T) measured by real-time TaqMan® PCR. Error bars represent the standard error of the arithmetic mean of PBMC from eight sheep at each time-point.

was calculated by subtracting the calibrator ΔC_T from each sample ΔC_T, and the fold-change determined by the equation $2^{-\Delta\Delta C_T}$ (Cikos et al., 2007).

2.8. Statistical analyses

The qPCR data were transformed using the $\Delta\Delta C_T$ method and responses to Ova and ConA for each animal were compared using a repeated measures model. The lack of independence between successive measurements on the same animal was modeled using an AR(1) power model. Data for IFN-γ and IL-10 expression were analysed separately.

The ELISA data showed increasing variability with higher values and also contained many zero readings that were unsuitable for inclusion in a simple repeated measures model. The data were analysed using the area under the response curve (AUC) method with respect to time for both Ova and ConA and the log of the AUC values used in the subsequent analyses. The difference between the mean values for Ova and ConA were investigated using a paired *t*-test separately for IFN-γ and IL-10.

All data were analysed using the one-sample *t*-test or REML directive in Genstat 10th Edition.

3. Results

3.1. Comparative expression of mRNA encoding IFN-γ and IL-10 by activated PBMC

The levels of IFN-γ and IL-10 mRNA expression in PBMC prior to normalisation with GAPDH are shown in Fig. 2. The mean C_T values have been transformed by deducting the number from 40. The transformed values (40-C_T) have been used to generate the box and whisker plots. The baseline values for cytokine mRNA expression were derived from unstimulated PBMC and higher 40-C_T correspond to greater mRNA expression. It can be seen that there is inter-animal variation in both IFN-γ and IL-10 mRNA expression in unstimulated cells over time, with greater inter-animal variability in IL-10 expression at time zero, as shown by the inter-quartile range of the box and whisker plots (Fig. 2a and d).

The patterns of IFN-γ mRNA expression over the time-course of the experiment differ between unstimulated cells (Fig. 2a), the Ova-stimulated cells (Fig. 2b), representing the specific antigen recall response and the mitogen (ConA)-stimulated cells (Fig. 2c), representing a polyclonal response, with highly significant changes in mean IFN-γ mRNA expression in response to Ova and ConA ($p < 0.001$) over time. The Ova-specific recall response induces IFN-γ mRNA expression that increases at a steady rate over time to a plateau at 72–96 h (Fig. 2b). In contrast, ConA rapidly induces a high level of IFN-γ mRNA expression by 6 h, with little deviation over the course of the experiment (Fig. 2c).

The levels of expression of mRNA encoding IL-10 are generally lower than those for IFN-γ, irrespective of time-point or treatment (Fig. 2a–f). Interestingly, the level of IL-10 mRNA expression in the Ova-stimulated cells is almost non-existent and indistinguishable from unstimulated cells (Fig. 2d and e), which is different from the pattern observed for IFN-γ. However, as for IFN-γ, IL-10 mRNA is rapidly expressed in response to ConA (within 6 h) and remains at the same level for the course of the experiment (Fig. 2f).

3.2. Comparative production of IFN-γ and IL-10 protein by activated PBMC

The levels of IFN-γ and IL-10 in the culture supernatants of unstimulated cells were very low and close to the detection limits of the respective ELISAs, as described in the legend of Fig. 3. As for the mRNA expression, differences in protein production between the two cytokines were observed following antigen re-stimulation or mitogen activation of PBMC (Fig. 3). Both antigen and mitogen stimulation of PBMC induced highly significant changes in mean IFN-γ production over time ($p < 0.001$). Ova-specific IFN-γ was first detected in the 48 h culture supernatants and mean levels continued to increase over the course of the experiment, with intra-animal variability most evident at 72 and 96 h (Fig. 3a). In contrast to the antigen-specific recall response, IFN-γ was detected earlier in the culture supernatants of the ConA-activated cells (12 h), continuing to accumulate in the culture supernatants at a steady rate to the 96 h sample point. Appreciable intra-animal variability was evident from 48 h onwards (Fig. 3b).

There was very little antigen-specific production of IL-10. Although there was a trend towards increasing amounts as the experiment progressed, the levels of IL-10 in the supernatants of the Ova-stimulated PBMC were close to the detection limit of the ELISA and indistinguishable from those derived from unstimulated PBMC at all time-points (Fig. 3c). As for IFN-γ, IL-10 began to appear in the culture supernatants of mitogen-activated PBMC at 12 h and levels continued to increase over the course of the experiment (Fig. 3d). Unlike Ova stimulation, ConA stimulation induced highly significant changes in mean IL-10 production over time ($p < 0.001$). There was clear intra-animal variation in the mitogen-induced IL-10, with

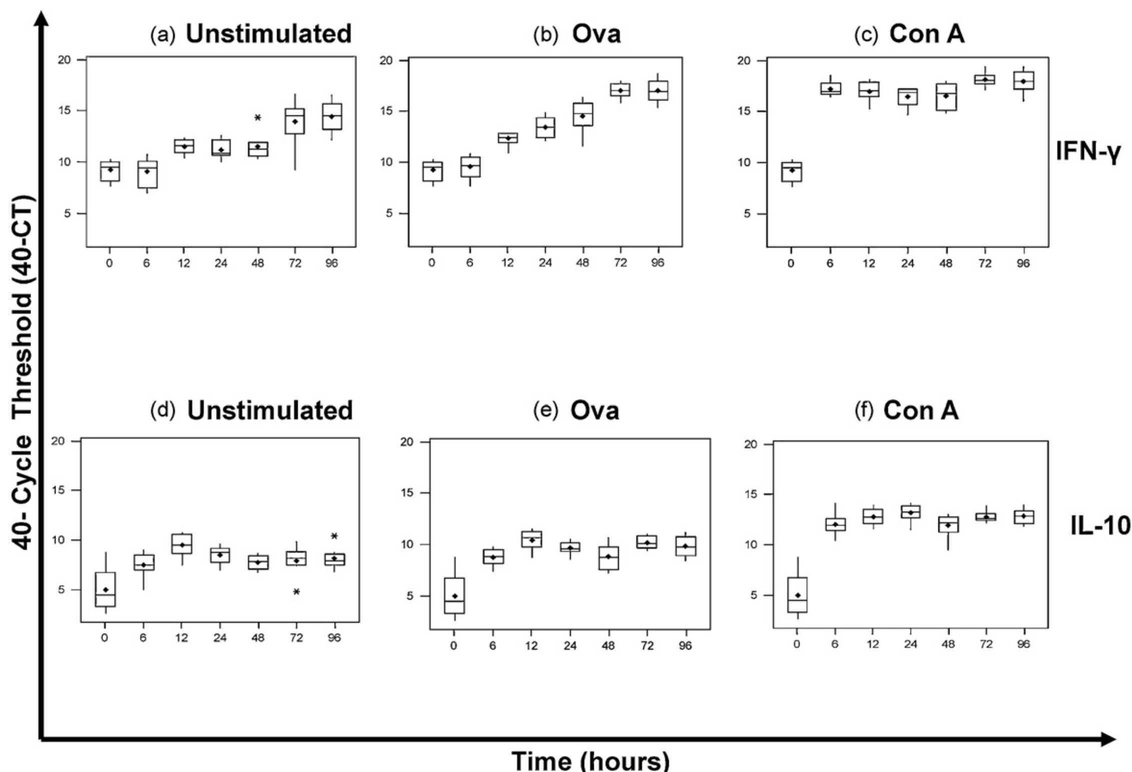

Fig. 2. IFN-γ and IL-10 mRNA expression in activated ovine PBMC. PBMC collected from eight sheep were re-stimulated *in vitro* with antigen (Ova; b and e) or mitogen (ConA; c and f). Unstimulated PBMC (medium alone; a and d) served as negative controls. Total RNA was prepared from cell lysates as described in Section 2 and IFN-γ and IL-10 mRNA expression (represented by 40-cycle threshold; 40-C_T) measured by real-time TaqMan® PCR. Boxplots represent the inter-quartile ranges of the individual data points from the group of eight animals. The box bottom represents the 1st quartile (Q1) and the box top the 3rd quartile (Q3) value. The horizontal line through each box represents the median value and ◆ denotes the arithmetic mean. Whiskers extend to the highest/lowest value within the outlier limits, set at the points Q1–1.5(Q3–Q1) and Q3 + 1.5(Q3–Q1). Outliers (*) are those values falling outside the outlier limits.

outlier animals being identified by the statistical algorithm (Fig. 3d).

3.3. Correlation of cytokine mRNA expression with protein production using the relative quantification method

The C_T values shown in Fig. 2 for cytokine mRNA expression in PBMC over time were all relative to the unstimulated cells harvested at time zero. To ensure that the intra- and inter-sample-point differences were not simply due to variations in cell numbers, C_T values for unstimulated PBMC were deducted from C_T values for the corresponding antigen- or mitogen-activated PBMC at each time-point and normalised to the equivalent GAPDH C_T value to give fold-changes in cytokine mRNA expression relative to GAPDH (Fig. 4).

Using this adjustment to allow the direct comparisons to be made, it can be seen that there was a marked difference between the pattern and magnitude of IFN-γ mRNA expression and protein production by PBMC stimulated with Ova and ConA (Fig. 4a). In ConA-activated PBMC, the peak mean mRNA expression occurred at 6 h (133-fold increase compared to unstimulated cells), dropped rapidly by 12 h (39-fold increase) then continued to decline steadily to a 2.3-fold increase by 96 h. In contrast, the mean amount of IFN-γ protein in the supernatants of the same cultures was greatest at 96 h, the point of lowest mRNA expression. In the antigen-stimulated PBMC, IFN-γ mean mRNA expression peaked later at 48 h (13-fold increase), decreasing to a 6.0-fold mean increase by 96 h (Fig. 4a). As with the ConA-stimulated cells, the greatest mean amount of IFN-γ was found in the 96 h culture supernatant.

The expression profile for IL-10 following ConA activation differed from that observed for IFN-γ. At the 6 h time-point (the peak for IFN-γ) there was a mean 9.4-fold increase in the level of IL-10 mRNA expression. However, this continued to rise to a peak at 24 h (16-fold increase) which was both relatively later and lower than that observed for IFN-γ (Fig. 4b). The mean levels of IL-10 mRNA declined less rapidly than those of IFN-γ but had decreased to a similar level by 96 h (2.7-fold increase). Despite the different kinetics of IFN-γ and IL-10 mRNA expression in ConA-activated cells, the relative patterns of protein expression for both cytokines were almost identical (Fig. 4a and b). For Ova-stimulated PBMC, a modest but significant ($p < 0.001$) increase in mean IL-10 mRNA expression was observed for each sample point over the time-course of the experiment, with the lowest being a 1.8-fold mean increase (24 h) and the highest a 3.3-fold mean increase (6 h). The amounts of IL-10 protein in the Ova-stimulated PBMC culture supernatants were close to

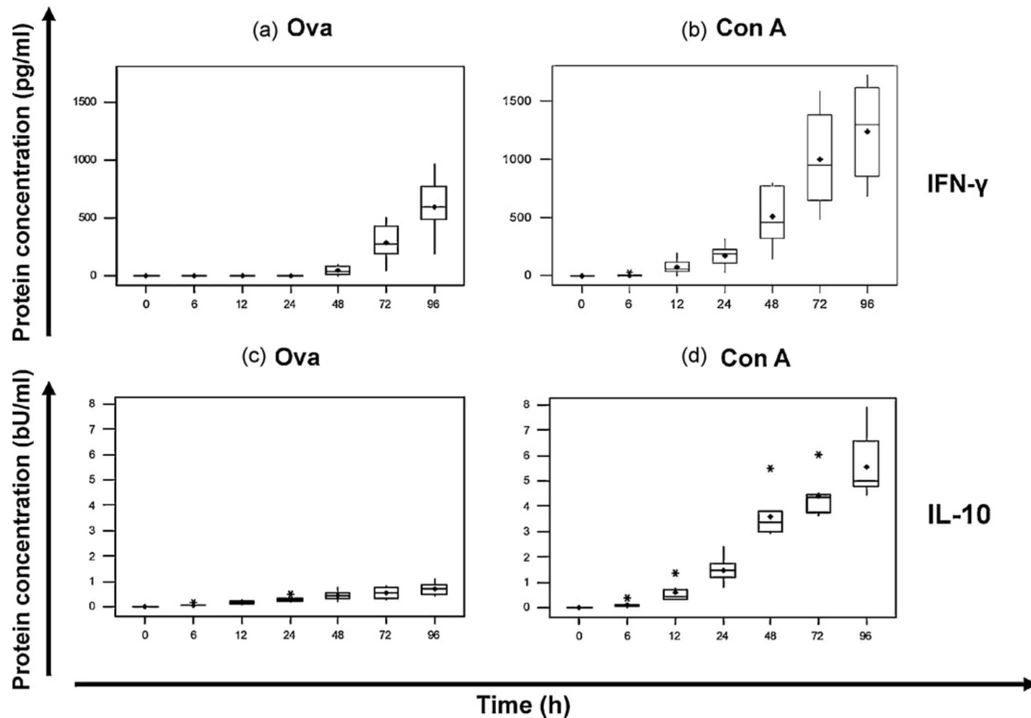

Fig. 3. IFN-γ and IL-10 protein expression by activated ovine PBMC. PBMC collected from eight sheep were re-stimulated *in vitro* with antigen (Ova; a and c) or mitogen (ConA; b and d). Culture supernatants were harvested as described in Section 2 and IFN-γ and IL-10 protein expression measured by ELISA. IFN-γ was quantified against a recombinant bovine IFN-γ standard and expressed as pg/ml. IL-10 was quantified against a recombinant bovine IL-10 standard and expressed as biological units per ml (bU/ml). Boxplots represent the inter-quartile ranges of individual data points from the group of eight animals. The box bottom represents the 1st quartile (Q1) and the box top the 3rd quartile (Q3) value. The horizontal line through each box represents the median value and ◆ denotes the arithmetic mean. Whiskers extend to the highest/lowest value within the outlier limits, set at the points Q1–1.5(Q3–Q1) and Q3 + 1.5(Q3–Q1). Outliers (*) are those values falling outside the outlier limits. Plots for unstimulated cells are not shown since values were very low. Neither cytokine was detectable at 6 h. IFN-γ was also undetectable in the 12, 24 and 48 h culture supernatants. At 72 and 96 h, the average values for IFN-γ were 24 and 83 pg/ml respectively. The concentrations of IL-10 at 12, 24, 48, 72 and 96 h were 0.07, 0.13, 0.20, 0.24 and 0.23 bU/ml respectively.

the detection limit of the ELISA and similar to those in unstimulated cells.

4. Discussion

Hypothesis-driven research into host–pathogen interactions and identification of immunological correlates of protection are dependent on having the appropriate tools and reagents to answer specific research questions. The range of tools to conduct such studies in veterinary species has expanded greatly in recent years. However, many gaps in our capabilities still remain, and although we can infer immune function from comparative data across species, it is not reliable to make broad assumptions (Entrican et al., 2009). To address one of the gaps in our understanding of cytokine inter-relationships in sheep, we report here on the kinetics of ovine IFN-γ and IL-10 mRNA and protein expression during antigen-specific recall responses and mitogenic activation of PBMC *in vitro*. Furthermore, we describe a novel combined quantitative PCR method for these two cytokines that allows relative analysis of their gene expression during immune cell activation.

The value of developing a relative quantitative PCR method based on a housekeeping gene is that it allows comparisons to be drawn between different targets. We selected GAPDH as the housekeeping gene to normalise the template cDNA since this has been shown previously to be a suitable reference for normalising ovine cytokine gene transcription (Montagne et al., 2001). In the Montagne et al. (2001) study, the GAPDH signal was found to remain constant between animals, between different organs (spleen and lymph nodes) and irrespective of infection status (*Salmonella abortusovis*) and was therefore considered suitable to be used as a reference to quantify ovine IL-1β, IL-4, IL-12, TNF-α and IFN-γ expression using a competitive PCR method. However, a more recent study (Budhia et al., 2006) compared GAPDH and ATPase (among others) as housekeeping genes for quantifying the expression of the same cytokines by quantitative PCR. When using ConA-activated ovine PBMC to validate their method, they found differential expression of these housekeeping genes between unstimulated and ConA-activated ovine PBMC over time when normalised against total RNA and input cell number. In keeping with those findings, we also found that mean GAPDH expression differed between ConA-stimulated and unstimulated PBMC and also changed over time. The most likely explanation is that increasing GAPDH expression is reflective of increasing numbers of cells due to proliferation in response to mitogenic activation. Normalising

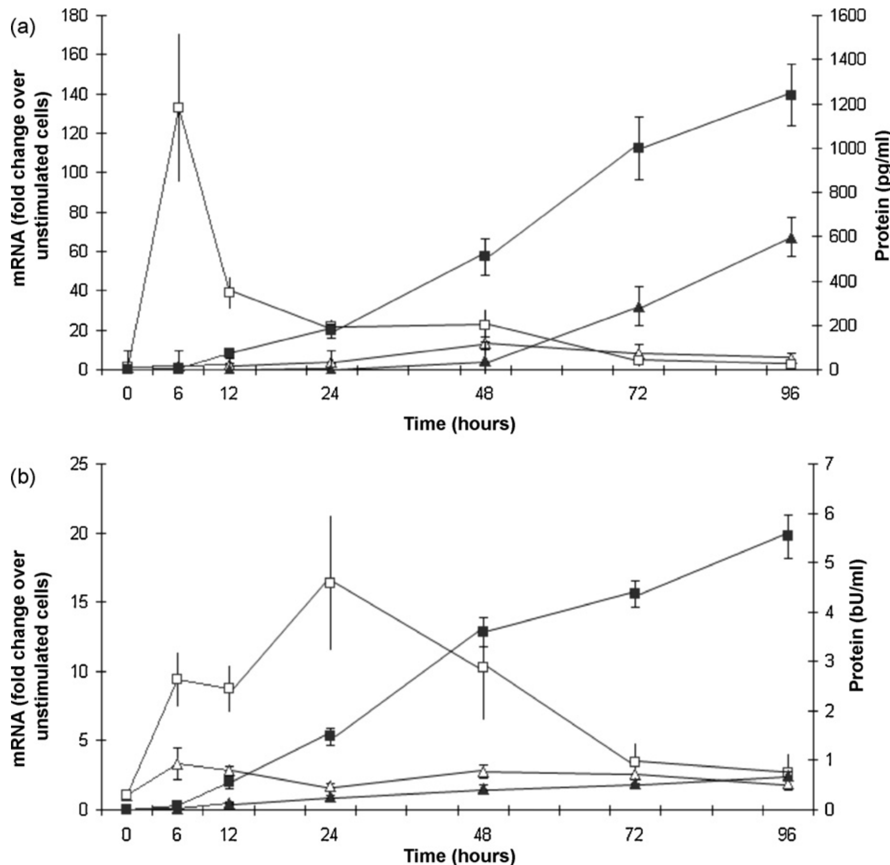

Fig. 4. Quantitative comparison of IFN-γ and IL-10 mRNA and protein expression by activated ovine PBMC. PBMC collected from eight sheep were re-stimulated *in vitro* with antigen (Ova, △/▲) or mitogen (ConA, □/■). Total RNA was prepared from cell lysates as described in Section 2. IFN-γ, IL-10 and GAPDH mRNA expression were measured by real-time TaqMan® PCR. IFN-γ (a) and IL-10 (b) were quantified by normalisation against GAPDH within each treatment for each time-point and values shown as fold-change relative to unstimulated PBMC (medium alone). IFN-γ (a) and IL-10 (b) protein expression were measured by ELISA. IFN-γ was quantified against a recombinant bovine IFN-γ standard and expressed as pg/ml. IL-10 was quantified against a recombinant bovine IL-10 standard and expressed as biological units per ml (bU/ml). mRNA is represented by open symbols (□ and △), protein is represented by closed symbols (■ and ▲). Error bars represent the standard error of the arithmetic mean.

against total RNA is one method of standardisation. Since the principle aim of our study was to develop a capability to examine the relative kinetics of IFN-γ and IL-10 mRNA expression (and protein) over time, we normalised against GAPDH within each treatment at each time-point and not simply against total RNA. This allowed reliable comparisons to be drawn between the cytokines as the cells proliferated.

Taking this approach, it can be seen from Fig. 4 that IFN-γ mRNA expression peaks earlier and at a higher mean relative level than mRNA encoding IL-10 in both antigen- and mitogen-activated ovine PBMC. Despite the different kinetics of mRNA expression, protein measurements in the culture supernatants followed an almost identical pattern, indicating that quantitative mRNA measurements for these cytokines do not necessarily predict protein production (Fig. 4). This is not unique to the cytokines in this study. Tagaya et al. (1996) found that PMBC stimulated with LPS and IFN-γ strongly up-regulated IL-15 mRNA, but only low amounts of protein were detectable by ELISA or in a CTLL-2 proliferation assay.

The patterns of mRNA expression also demonstrate the impact of sample collection time on the relative expression of IFN-γ and IL-10. For example, in this experimental system, relative quantification of IFN-γ versus IL-10 mRNA 6 h after ConA activation gives a mean ratio of 14.1:1, whereas at 12 h the mean ratio is 4.5:1 and at 24 h drops to 1.3:1. Thus the early dominance of the IFN-γ response over IL-10 would not be observed in ConA-activated PBMC harvested at 24 h. In contrast, for antigen-stimulated cells the greatest mean ratio of IFN-γ:IL-10 mRNA was observed at 48 h (4.8:1). The greater magnitude and speed of the mitogen-driven response relative to the antigen-driven response is to be expected, based on the relative numbers of PBMC responding in each case and the requirement for antigen processing to occur in the Ova-stimulated cultures (Figs. 2–4). The low/absent production of IL-10 by the Ova-stimulated PBMC is in keeping with a previous study looking at longitudinal cytokine expression in Ova-immunised ewes where IFN-γ predominated (Wattegedera et al., 2008). This is likely to be reflective of the immunisation regime used to elicit the Ova-specific

response, namely antigen delivery using Freund's Complete Adjuvant, a strong promoter of the inflammatory host immune response (Wattegedera et al., 2008).

Other groups have shown that cytokine mRNA expression tends to peak between 3 and 24 h of mitogenic stimulation (Budhia et al., 2006; Sullivan et al., 2000) depending on the host species, cell type and culture conditions. Whilst measurement of mRNA by qPCR is very useful in providing relative quantification of different cytokines in an immune response, other factors potentially play a more significant role in the development of immunity such as mRNA turnover or stability as determined by post-transcriptional control mechanisms. Post-transcriptional control of cytokine production is very complex and dependant on multiple factors. IL-10 and IFN-γ are known to be regulated by the adenine and uridine-rich elements binding proteins (ARE-BP) tristetraprolin (TTP) and Hu antigen R (HuR) respectively. These proteins positively or negatively regulate mRNA stability and/or translation to protein depending on other factors in the microenvironment (reviewed by Anderson, 2008).

Direct comparisons of relative protein expression for IFN-γ and IL-10 were not possible in this study due the nature of the ELISA standards. The IL-10 ELISA was calibrated using a recombinant standard that was quantified in biological units/ml whereas the IFN-γ ELISA was calibrated using a recombinant standard quantified in pg/ml. Efforts to address this for bovine and ovine IL-10 are underway. However, even if using relative quantification as pg/ml was possible, there is a further factor that needs to be taken into account when drawing comparisons of cytokine expression at the protein level as opposed to the mRNA level. ELISAs will only detect excess cytokine in the culture supernatants and do not therefore account for cytokine that has been produced but has not been exported by the cell or indeed cytokine that has been exported but then utilised by the cells in culture. Since cytokine receptor expression is not uniform across different phenotypes within PBMC, different cytokines will therefore be utilised at different rates, and thus ELISA measurements will not therefore necessarily reflect the amount of protein being produced.

IFN-γ is a marker of innate immune activation and of adaptive immune responses used in several species as an indicator of polarisation of CD4+ve T cells to a Th1 phenotype (Zhu and Paul, 2008). Although the Th1/Th2 paradigm has never been definitively proven in sheep, IFN-γ performs a similar function as it does in other species, inducing cellular pathways that mediate host immune control of intracellular pathogens (Rocchi et al., 2009). In contrast, IL-10 is widely regarded as an immunoregulatory cytokine. Although IL-10 can interfere with clearance of intracellular pathogens and therefore be counter-active to IFN-γ in terms of host defence, it also performs a very important function in reducing or preventing immunopathology resulting from uncontrolled immune activation (Couper et al., 2008). We have previously shown that IL-10 is capable of regulating mitogen-induced cell proliferation and IFN-γ production by ovine PBMC, suggesting that it performs a similar cross-regulatory function in sheep as described for other species (Wattegedera et al., 2004).

In conclusion, this is the first report of the relative kinetics of IFN-γ and IL-10 mRNA expression in antigen- and mitogen-activated ovine PBMC and the relationship to protein in the culture supernatants. The technologies described here provide a means to define inflammatory and regulatory immune responses in sheep for two inter-related cytokines thereby underpinning disease pathogenesis studies.

Conflict of interest

None of the authors have any conflicts of interest relating to this publication.

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