



Contents lists available at ScienceDirect

Veterinary Parasitology

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



Trichostrongylus colubriformis induces IgE-independent CD13, CD164 and CD203c mediated activation of basophils in an in vitro intestinal epithelial cell co-culture model

Sinead M. Corvan^{a,b,*}, Linda Agnew^b, Nicholas M. Andronicos^{a,b}

^a CSIRO Animal Health and Food Science, F.D McMaster Laboratory, Armidale NSW 2350, Australia

^b School of Science and Technology, University of New England, Armidale NSW 2350, Australia

ARTICLE INFO

Article history:

Received 7 January 2014

Received in revised form

30 September 2014

Accepted 7 October 2014

Keywords:

T. colubriformis

Helminth parasite

IL33

Epithelial

Basophil

ABSTRACT

Gastrointestinal nematodes pose a major risk to the farming of small ruminants worldwide. Infections are typically controlled by anthelmintics, however as resistance to anthelmintics increases, it is necessary that the mechanism of host responses are understood in order to develop alternative control options. It is hypothesised that basophils are involved in the initiation of an anti-parasite immune response, independent of IgE. In this study, the in vitro activation states of CD203c⁺ basophil-like KU812 cells were determined in the presence of *Trichostrongylus colubriformis* parasitised HT29 epithelial cells with or without mucin. Cell surface expression of CD164, CD107a and CD13 antigens on gated CD203c⁺ cells were determined and qRT-PCR was used to examine gene expression changes of IL33 (a Th2 cytokine) and the high affinity IgE receptor (FcεR1α) within the co-culture. When KU812 basophils encountered *T. colubriformis* and/or mucin in a parasitised epithelium, the basophils increased cell surface expression of CD13 and CD164 antigens, independent of IgE. *T. colubriformis* also increased the number of CD203c⁺ KU812 cells that expressed CD13 and CD164 antigens. These data support the in vivo observations of *T. colubriformis* primary infections in guinea pigs and sheep.

Crown Copyright © 2014 Published by Elsevier B.V. All rights reserved.

1. Introduction

Gastrointestinal nematode (GIN) parasites pose a major health and productivity problem to the livestock sector globally (Miller and Horohov, 2006; Sackett et al., 2006). Typically, GIN parasite infections are controlled by grazing management and anthelmintic treatment, however widespread emergence of anthelmintic resistance in nematode parasites (Kaplan, 2004) has increased the need for

the development of protective anti-GIN vaccines (Lejambre et al., 2008). By deliberately mimicking the host–parasite interactions that initiate an immune response to GIN, such as damage to intestinal epithelial cells, a vaccine might be designed which is effective against GIN (Andronicos et al., 2012). Knowledge of the activation states and types of innate immune cells that initially respond to the danger signals (Matzinger, 1994) induced by exposure to GIN is expected to be critical for the development of such an effective anti-GIN vaccine.

To successfully combat helminth parasites such as *Trichostrongylus colubriformis* (*T. colubriformis*), a predominantly Th2 response is required. Although research supports the idea that there are several mechanisms for a protective anti-parasite response may exist for example

* Corresponding author at: CSIRO Animal Health and Food Science, F.D McMaster Laboratory New England Hwy, Armidale NSW 2350, Australia. Tel.: +61 2 6776 1361; fax: +61 2 6776 1333.

E-mail address: Sinead.Corvan@csiro.au (S.M. Corvan).

passive immunization of anti-CarLA antibodies (Harrison et al., 2008). However, a Th2 type activity predominantly facilitates a protective host response in *T. colubriformis* immunized sheep. Th2 responses induced by GIN are generally characterized by increases in inflammatory cells such as mucosal mast cells/globular leukocytes (Cardia et al., 2011), basophil infiltration and activation (Rothwell and Love, 1975); (Huxtable and Rothwell, 1975), eosinophilia (Amarante et al., 2007), production of type 2 (IgG1, IgE and IgA) anti-parasite antibodies (Andronicos et al., 2010) and the release of bioactive substances such as histamine and leukotrienes (Bendixsen et al., 1995; Jones et al., 1990) at the site of infection. Although immune responses to helminth parasites follow this general trend, the response to different nematodes varies considerably (Gause et al., 2003) and responses from related host species towards the same parasite is also variable (Harris and Gause, 2011). Recently, it has been postulated that the cytokines IL25, IL33 and TSLP may provide a link between the innate and adaptive immune responses to GIN (Coffman, 2010). In sheep genetically resistant to GINs it appears that initiation of Th2 type cytokine production responses are mediated, at least in part, by the release of IL33 from parasite damaged cells (Andronicos et al., 2012).

New insights have recently emerged supporting a role for basophils in the initiation stages of the immune response (Gibbs, 2005). Several studies in mice have demonstrated that basophils are a source of Th2 cytokines, driving the production of Th2 cells (Sullivan and Locksley, 2009). Basophils can also act as antigen presenting cells (APCs) driving Th2 and IgE responses against protease, protein allergens and parasites (Nakanishi, 2010). Both mucosal mast cells and basophils can activate B cells directly to produce IgE, IL-4 or IL-13 (Henz et al., 2001). Indeed during a primary *T. colubriformis* infection in guinea pigs the number of basophil/mast cells increases in the jejunum (Rothwell, 1975). Moreover, impaired basophil activity in juvenile mice is associated with a delayed Th2 immune response (i.e. decreased Th2 cytokine production, IgE responses, mouse mast cell protease 1 levels, and goblet cell proliferation) during primary infection with *Nippostrongylus brasiliensis* (Nel et al., 2011). Basophils have been shown to secrete IL4 and IL13 early in the immune response when stimulated with IL18 or IL33, promoting Th2 differentiation independently of IgE (Kroeger et al., 2009).

The aim of this study was to determine if basophils are activated in an IgE-independent manner by *T. colubriformis* larvae in a damaged mucosal epithelial environment. The parasite-human epithelial cell co-culture system established by Andronicos et al. (2012) was modified and used to examine the activation of a human basophil-like cell line by *T. colubriformis* larvae. These parasites have been shown to establish patent infections in humans (Sato et al., 2011).

2. Materials and methods

2.1. Cell lines

All cell culture reagents were obtained from Invitrogen, USA and all fluorochrome-conjugated antibodies

were from Biolegend, USA unless otherwise stated. The human colonic epithelial cell line HT29 and the human basophil cell line, KU812 were routinely passaged according to EDAAC guidelines using McCoy's 5a media supplemented with 10% heat-inactivated FCS and 1× penicillin-streptomycin or in RPMI supplemented with 10% FCS and 1× penicillin-streptomycin respectively. Cells were incubated at 37 °C in a 5% CO₂ humidified atmosphere.

2.2. Protein preparations

Post exsheathment antigen (PEA) from infective L3 McMaster *T. colubriformis* larvae and whole cell lysate (WCL) from HT29 cells were produced as described previously (Andronicos et al., 2012). Preparations were aliquoted and stored at –80 °C until required. Porcine mucin (40 mg/ml; Sigma, USA) was dissolved in sterile complete RPMI medium (20 ml) overnight at 4 °C. The solution was then aliquoted and frozen at –20 °C until required. All protein concentrations were determined using BCA protein assay using BSA as a standard.

2.3. Air/liquid interface intestinal epithelial cell-parasite co-culture system

The epithelial cell-parasite co-culture model described by Andronicos et al. (2012) was used with the exception that KU812 cells were co-cultured with the HT29 cells for testing (where indicated). Briefly, after culturing HT29 cells in six-well plates using an air-liquid interface architecture for 24 h in a humidified incubator, plates were initially prepared by adding 1 × 10⁶ KU812 cells to the well inserts and the plate incubated for a further 1–2 h. To establish the remainder of the time course experiments, 1 × 10⁶ KU812 cells in 250 µl of complete RPMI media were added to each of the inserts. Complete RPMI media (250 µl) was added to control inserts and previously exsheathed *T. colubriformis* L3 larvae in 250 µl complete RPMI media with or without 2 µM ivermectin (Sigma, USA) were added to the insert. In some treatments exsheathed larvae were also resuspended with or without 2.5 mg/ml porcine mucin in 250 µl complete RPMI media. When PEA was included as a treatment it was added at 1 mM in 250 µl of complete media to the insert containing cells. All plates were incubated at 37 °C in a 5% CO₂ humidified atmosphere for up to 7 days.

2.4. Parasite motion assays

Exsheathed *T. colubriformis* L3 larvae were co-cultured with either HT29 cells or HT29 and KU812 cells with or without 2 µM ivermectin or 2.5 mg/ml mucin and their motion recorded using ProgRes C10 camera (Jenoptik, Germany) attached to a TE300 inverted microscope (Nikon, Germany). To assess the motility changes in the parasites under the different conditions, the video microscopy was recorded using the live image option of the ProgRes CapturePro 2.1 software (Jenoptik, Germany) and captured from the screen using ZD Soft Screen Recorder 5.4. Parasite motion under different co-culture conditions was

Table 1

Forward and reverse primer sequences used in the study.

Primer	Sequence (5'–3')
IL33 (forward)	TGACTTTCAGGAACCCATGA
IL33 (reverse)	TTGGCATGAGAGATCAGGTG
FceR1 α (forward)	ATGAAGAAGATGGCTCCTGC
FceR1 α (reverse)	ATTGTGGAACCATTTGGTGG

quantified using the Image J wrmtrck plugin (based on MTrack2 by Nico Stuurman).

2.5. Flow cytometric analysis

HT29 and KU812 cells in the co-culture system were incubated for 5 min with TrypLE to dissociate them, then washed twice with sterile PBS and centrifuged for 5 min at $200 \times g$. Each cell pellet was resuspended in 1 ml ice-cold binding buffer (Hanks buffered saline solution without Ca^{2+} or Mg^{2+} (HBSS) supplemented with 0.1% BSA) and 200 μl of each was added to wells in a 96 well V-bottom plate for flow cytometric analysis. The remaining 800 μl of each sample was reserved for total RNA isolation by centrifuging the cells for 5 min at $200 \times g$. The supernatant was discarded and the cell pellets snap-frozen in liquid nitrogen and stored at -80°C until required. Samples containing parasites were processed by flow cytometry as described by Andronicos et al. (2012). In samples containing mucin, the cells were diluted with 10 ml of sterile PBS before centrifugation at $200 \times g$ for 5 min at 4°C . The cells that were processed for flow cytometry were incubated with 200 μl of ice-cold binding buffer containing either a cocktail of CD13-APC, CD107a-Pacific Blue, CD164-FITC and CD203c-PE CD-specific antibodies or relevant isotype control fluorochrome conjugated antibodies (Biolegend, USA) for 30 min in the dark at 4°C . The cells were washed three times with PBS by centrifuging the 96 well plates containing cells at $200 \times g$ for 3 min at 4°C . Cell pellets were resuspended in 200 μl ice-cold binding buffer and cell surface marker data acquired using a LSRII flow cytometer (BD Biosciences, USA) or a FlowSight flow cytometer (Amnis, USA). Flow cytometric 4 colour compensation was performed using the AbC anti-Mouse Bead Kit conjugated to fluorophore antibodies (Invitrogen, USA). Flow cytometric data were analysed using FCS Express version 3.0 (De Novo, USA) or IDEAS application software version 6.0 (Amnis, USA).

2.6. Quantitative RT-PCR

Expression of IL33 and FceR1 α transcripts in the models were determined using quantitative reverse transcriptase PCR (qRT-PCR) according to the method described in Andronicos et al. (2010). Primers used in qRT-PCR are described in Table 1.

2.7. Statistical analysis

Statistics for the parasite motion assays were calculated using one way ANOVA repeated measures followed by a Dunnett's Multiple Comparison Test analysis in Prism 5

(GraphPad, USA). Statistics for the flow cytometry and RT-qPCR experiments were calculated using two-way ANOVA repeated measures analysis in Prism 5 (GraphPad, USA). Genex (BioRad, USA) was used to analyse qRT-PCR data relative to Genorm reference genes.

3. Results

3.1. Motility inhibition of exsheathed *T. colubriformis* L3 larvae

It was previously demonstrated that the motility of *T. colubriformis* larvae induces the release of IL33 from necrotic HT29 cells in an air-liquid interface culture model (Andronicos et al., 2012). To examine the effect of motility on the activation of basophils in our co-culture model, it was important to quantify the motility of the parasite in the presence of motility inhibition agents such as mucin and ivermectin. The average speed of *T. colubriformis* in culture with or without motility inhibitors remained constant within each condition from days 1 to 7 (Fig. 1A). However, as expected ivermectin ($P < 0.001$) and mucin ($P < 0.01$) significantly inhibited the average speed of *T. colubriformis* larvae in culture.

There were significant decreases for the average body bend rates for ivermectin (0.8236 ± 0.7074 ; $P = 0.0029$) and mucin (2.274 ± 0.4485 ; $P = 0.0316$) treated parasites compared to untreated (4.417 ± 2.005) *T. colubriformis* (Fig. 1B); however, there were no significant differences across time for each treatment. The low body bends per minute average of ivermectin treated parasites confirmed the visual observation that most of the parasites were restricted to a spasmodic twitching motion indicating paralysis (Andronicos et al., 2012). Some ivermectin-treated *T. colubriformis* retained sinusoidal motility in the co-culture system, which contributed to the significantly reduced, but non-zero average speed parameter (Fig. 1A), whereas mucin treated *T. colubriformis* had a slower average speed and reduced bending motion and frequency in co-culture.

To estimate if the motility inhibitors affected the circular or sinusoidal motion of the *T. colubriformis* (Andronicos et al., 2012) the ratio of the distance travelled:number of tracks travelled by the parasites in co-culture was calculated (Fig. 1C). There were no significant differences in the number of tracks for mucin and ivermectin treated *T. colubriformis* compared to untreated control parasites in co-culture. However, ivermectin treated parasites travelled significantly ($P = 0.0370$) less distance than untreated or mucin treated *T. colubriformis*. This is consistent with ivermectin and mucin acting through different mechanisms to reduce parasite movement, and is consistent with our expectations.

The parasite average speed in co-culture with or without motility inhibitors were correlated with body bend, number of body bends per minute and distance travelled to determine motility differences associated with the various motility inhibitors (Table 2). There were significant positive correlations between average parasite speed and both number of body bends per minute and distance traversed when *T. colubriformis* larvae were co-cultured without motility inhibitors. In contrast, when the parasites were

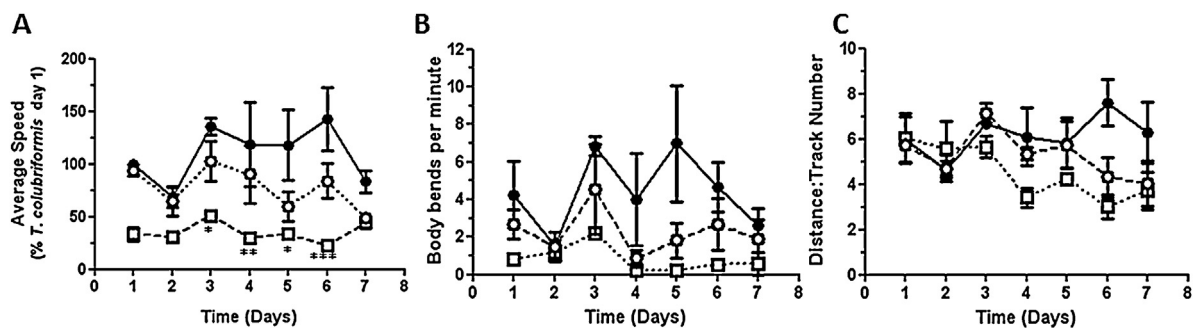


Fig. 1. Quantification of the motility of exsheathed *T. colubriformis* L3 larvae KU812-HT29-parasite co-culture system in RPMI complete media (●), 1 μ M ivermectin (□) or mucin (○). (A) Average velocity, (B) body bends per minute and (C) distance:track number ratio. The motility of exsheathed parasites in the respective co-culture formats were captured using video microscopy for 2 min on each day and quantified using the Fiji wrmtrck plugin ($n=3$; means $\pm$ SEM; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

co-cultured with mucin or ivermectin, significant correlations to average speed were only observed for distance travelled or number of body bends per minute respectively. Thus, the paralysed *T. colubriformis* larvae accounted for the minimal distances travelled and the reduction of the average speed observed in the co-culture system. The mucin treated parasites were significantly slower than the control treatment parasites but still traversed the cell lawn using reduced sinusoidal motion possibly due to the increased viscosity of the mucin in the co-culture environment.

3.2. CD203c expression in cell co-culture experiments

Human basophil-like cells were added to the parasite-epithelial cell co-culture system (Andronicos et al., 2012) to assess changes in the expression of basophil CD activation markers during the early phase of parasitism. To assess changes in CD activation marker status in basophil-like cells, we needed a method to first distinguish the KU812 cell population from the HT29 cell population during flow cytometric analysis. To determine appropriate cell markers to achieve this, the expressions of CD203c, CD164, CD107a and CD13 on individual monocultures of either KU812 basophil-like cells or intestinal HT29 cells under basal conditions were performed. Results indicated that KU812 cells express significant ($P=0.0081$) amounts of CD203c compared to the isotype control cells (Fig. 2A). In contrast, CD203c was not expressed by HT29 cells when grown under basal monoculture conditions (Fig. 2B). Comparison of the CD203c protein expression signatures from basal monocultures of KU812 and HT29 cells indicated that the

KU812 cell line expressed significantly ($P<0.0001$) more CD203c than HT29 cells (Fig. 2C). In contrast, the CD164, CD107a and CD13 expression differentials between KU812 cells and HT29 cells did not adequately discriminate these two populations during flow cytometric analysis (data not shown). Overlaid flow cytometric scatter plots of KU812 and HT29 monocultures were used to establish CD203c⁺ and CD203c⁻ gates (Fig. 2D) to identify KU812, from HT29 cells during the co-culture experiments. The CD203c⁺ gate was set such that the majority of monoculture HT29 CD203c⁻ cells were excluded (95 ± 0.62 CD203c⁻ and 1.4 ± 0.29 CD203c⁺) from the gate. In contrast, approximately 58 ± 10.8 of monoculture KU812 cells were gated in CD203c⁺ gate whereas approximately 75 ± 16 of isotype control KU812 cells were present in the CD203c⁻. Four colour flow cytometric analysis demonstrated that CD203c⁺ cells co-expressed CD13 (Fig. 2E) and CD164 (Fig. 2F) activation antigens at day 4 of the co-culture system. Moreover, 3 colour image flow cytometric analysis (FlowSight flow cytometer, Amnis) indicated that on day 4 of co-culture a subset of the CD203c⁺ cells expressed both CD164 and CD13 cell surface antigens simultaneously (Fig. 2J).

The percentage of cells expressing CD203c significantly decreased during the time course of the experiment when KU812 and HT29 cells were co-cultured with ivermectin ($P<0.0001$) and mucin ($P<0.0001$; Fig. 2G). In contrast, the percentage of cells expressing CD203c did not significantly change over time compared to day 0 when only HT29 and KU812 cells were co-cultured together. This suggested that mucin and ivermectin reduce the amount of CD203c⁺ cells in the co-culture system independent of the parasite over the 7 day culture period. However, enough CD203c⁺ remained in the co-culture system after 7 days for quantification. Thus the expression of CD203c was used to discriminate KU812 cells from HT29 cells during the flow cytometric analysis of the co-culture system. Hence CD203c⁺-gated populations from the co-culture experiments have been designated as KU812 basophil-like cells for the remainder of this study.

The addition of motile *T. colubriformis* larvae to the KU812, HT29 co-culture induced a significant ($P=0.0001$) time-dependent decrease in expression of KU812 cell surface CD203c at days 5 and 7 compared to uninfected or

Table 2

Pearson correlation coefficients of the average speed of *T. colubriformis* in co-culture for 7 days with the motility inhibitors ivermectin and mucin.

Inhibitor	Pearson correlation of average <i>T. colubriformis</i> speed to		
	Body bend	Bend number per min	Distance
None	0.1931	0.8407 ^a	0.8571 ^a
Ivermectin	0.2612	0.5087 ^b	0.3374
Mucin	0.3128	0.3758	0.6103 ^c

^a $P<0.0001$.

^b $P=0.0185$.

^c $P=0.0033$.

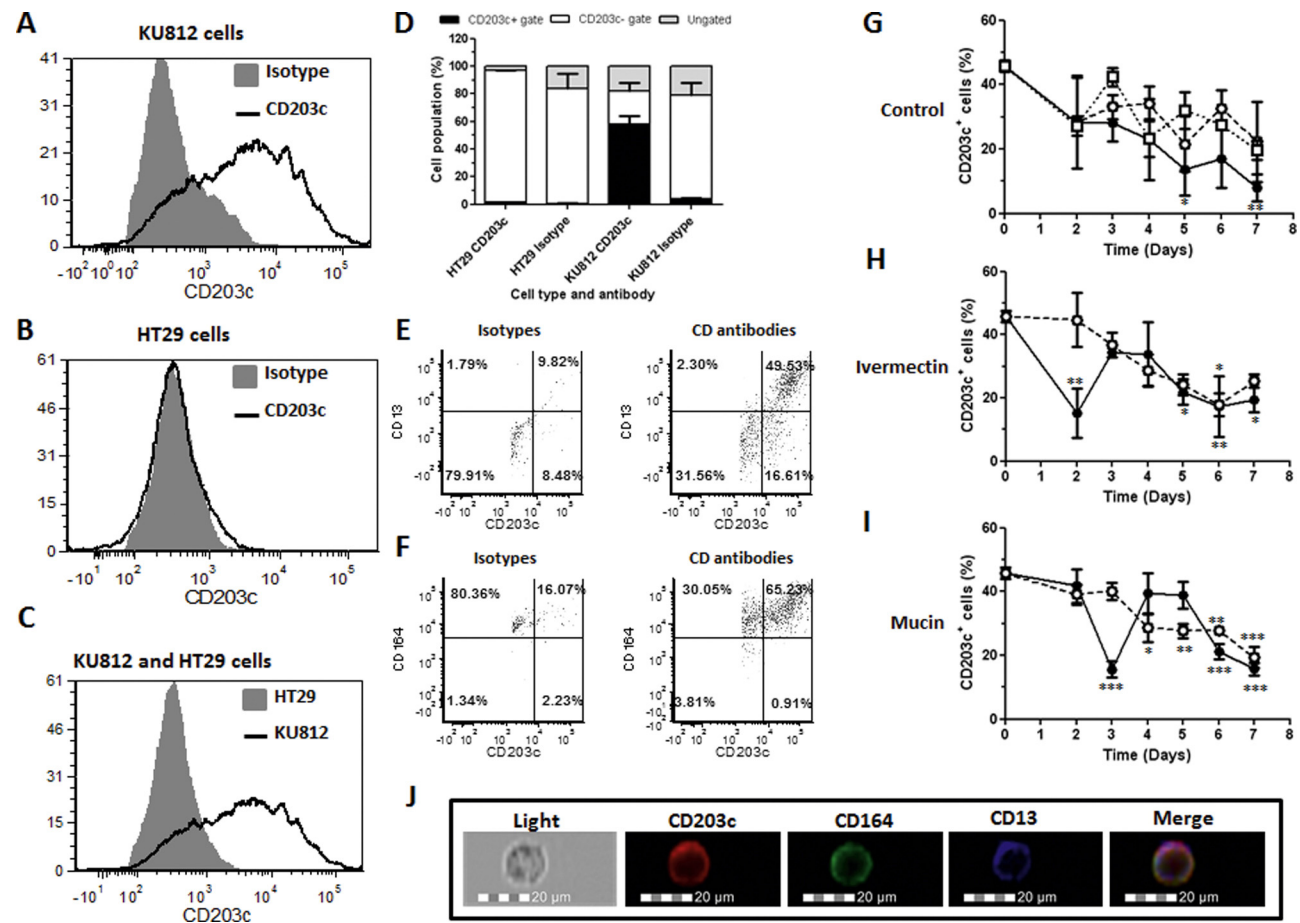


Fig. 2. Expression of CD203c on KU812 basophil-like cells and HT29 intestinal epithelial cells. (A) Flow cytometric histogram plots of CD203c on the surface of KU812 basophil-like cell grown in monoculture probed with CD203c-phycoerythrin antibody (-) or isotype control- phycoerythrin antibody (■). (B) Flow cytometric histogram plots of CD203c on the surface of HT29 intestinal cell grown in monoculture probed with CD203c-phycoerythrin antibody (-) or isotype control-phycoerythrin antibody (■). (C) An overlay histogram comparison of CD203c expression on HT29 cells (■) and 004BU812 cells (-). (D) The percentage of HT29 and KU812 cells grown in monoculture and probed with either CD203c-phycoerythrin antibody within the defined CD203c⁺ gate (■), CD203c⁻ gate (□) and un-gated events (■). (E) Scatter plots of cells from KU812-HT29-*T. colubriformis* co-cultures treated with mucin for 4 days and probed with either an isotype control antibody cocktail or CD203c⁺ and CD13⁺ specific antibody cocktails. (F) Scatter plots of cells from KU812-HT29-*T. colubriformis* co-cultures treated with mucin for 4 days and probed with either an isotype control antibody cocktail or CD203c⁺ and CD164⁺ specific antibody cocktails. (G) Changes in the percentage of CD203c⁺ cells co-culture population when cultured with HT29 cells and *T. colubriformis* (●); HT29 cells (○); or PEA (□); (H) HT29 cells and ivermectin (○) or HT29 cells, *T. colubriformis* and ivermectin (●); (I) HT29 cells and mucin (○) or HT29 cells, *T. colubriformis* and mucin (●) ($n = 3$; means $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (J) Images of a CD164⁺/CD203c⁺/CD13⁺ cell from the HT29-KU812 cells of the co-culture system. Individual channels are shown for CD164 (green) CD203c (red), CD13 (blue) and a merged image.

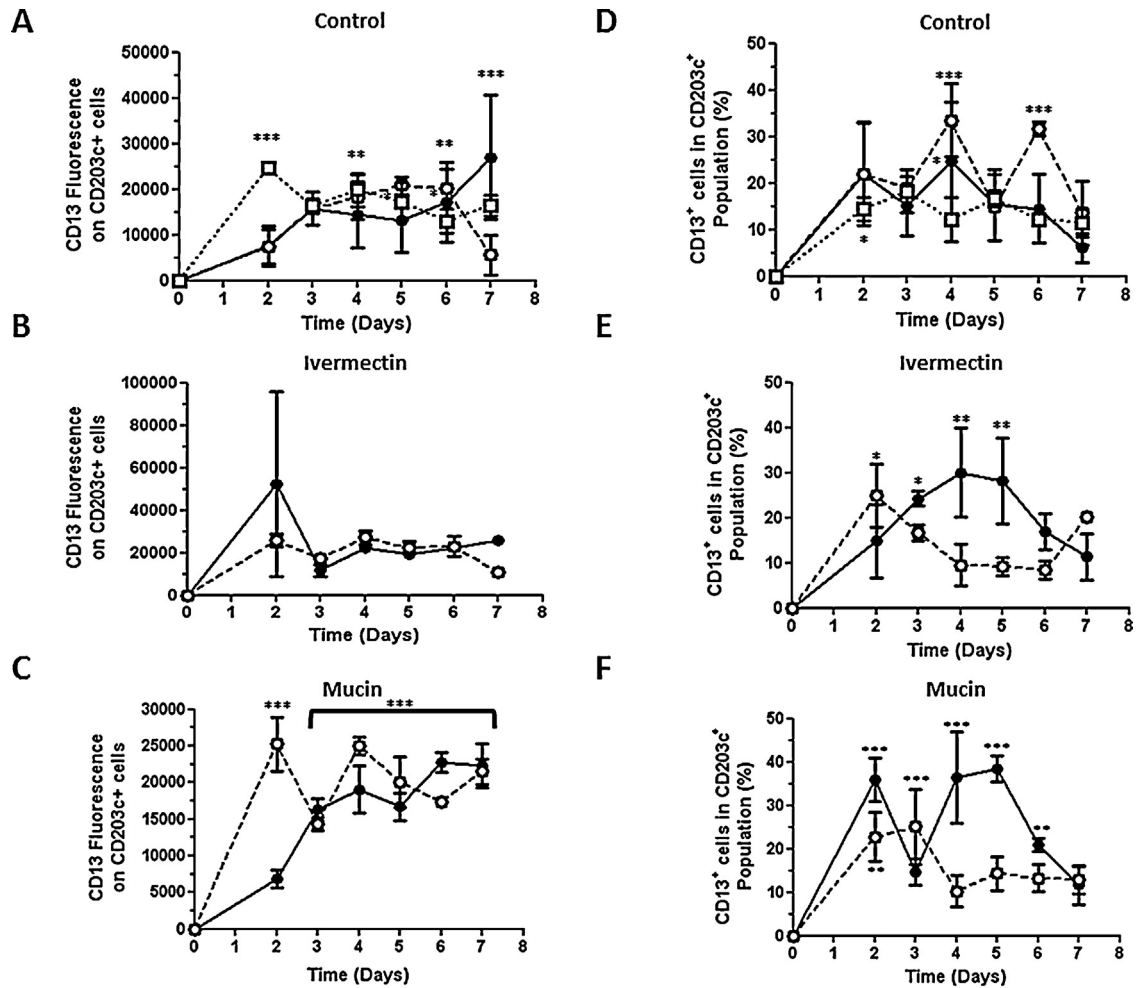


Fig. 3. Expression of CD13 on CD203c⁺ KU812 basophil cells co-cultured with HT29 cells and *T. colubriformis*. (A–C) CD13 expression mean fluorescence intensity on CD203c⁺ cells co-cultured with: (A) HT29 cells and *T. colubriformis* (●); HT29 cells (○); or PEA (□); (B) HT29 cells and ivermectin (○) or HT29, *T. colubriformis* and ivermectin (●); (C) HT29 cells and mucin (○) or HT29 cells, *T. colubriformis* and mucin (●). (D–F) Percentage of CD13⁺ cells in the CD203c⁺ cell population co-cultured with: (D) HT29 cells and *T. colubriformis* (●); HT29 cells (○); or PEA (□); (E) HT29 cells and ivermectin (○) or HT29 cells, *T. colubriformis* and ivermectin (●); (F) HT29 cells and mucin (○) or HT29 cells, *T. colubriformis* and mucin (●) ($n = 3$; means $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

PEA treated co-cultures (Fig. 2G). At day 2 there was a significant ($P < 0.001$) reduction in the number of KU812 cells in the co-culture system when ivermectin treated *T. colubriformis* were present compared to the ivermectin only treated co-cultures (Fig. 2H). Similarly, there was a significant ($P < 0.001$) decrease in KU812 cells on day 3 when *T. colubriformis* was co-cultured with HT29 and KU812 cells in the presence of mucin compared to cells cultured without mucin. However there were no significant differences in the number of CD203c⁺ KU812 cells in parasite infected co-cultures treated with motility inhibitors compared to parasite infected control co-cultures. Collectively these data suggest that CD203c could be used to determine the IgE-independent expression of CD13, CD107a and CD164 activation markers on CD203c⁺ KU812 basophils in the presence of *T. colubriformis* and parasite motility inhibitors.

3.3. CD13 expression in cell co-culture experiments

There was a significant ($P < 0.0001$) time-dependent increase in the intensity of CD13 expression on CD203c⁺ KU812 basophil cells in the control co-culture with PEA and motile *T. colubriformis* larvae. However, there were no significant differences in the CD13 expression levels on CD203c⁺ KU812 cells between PEA, *T. colubriformis* and cells only treatments, despite observation of a significant ($P = 0.0057$) interaction (Fig. 3A). There was significant ($P = 0.0335$) increase in the percentage CD13⁺ CD203c⁺ KU812 cells co-cultured with PEA, motile *T. colubriformis* or cells alone. There was a small, but significant ($P = 0.0019$) increase in CD13 expression in CD203c⁺ cells in the ivermectin treated co-cultures (Fig. 3B). In contrast, mucin induced a highly significant ($P < 0.0001$), time-dependent increase in the expression of CD13 on CD203c⁺ basophil

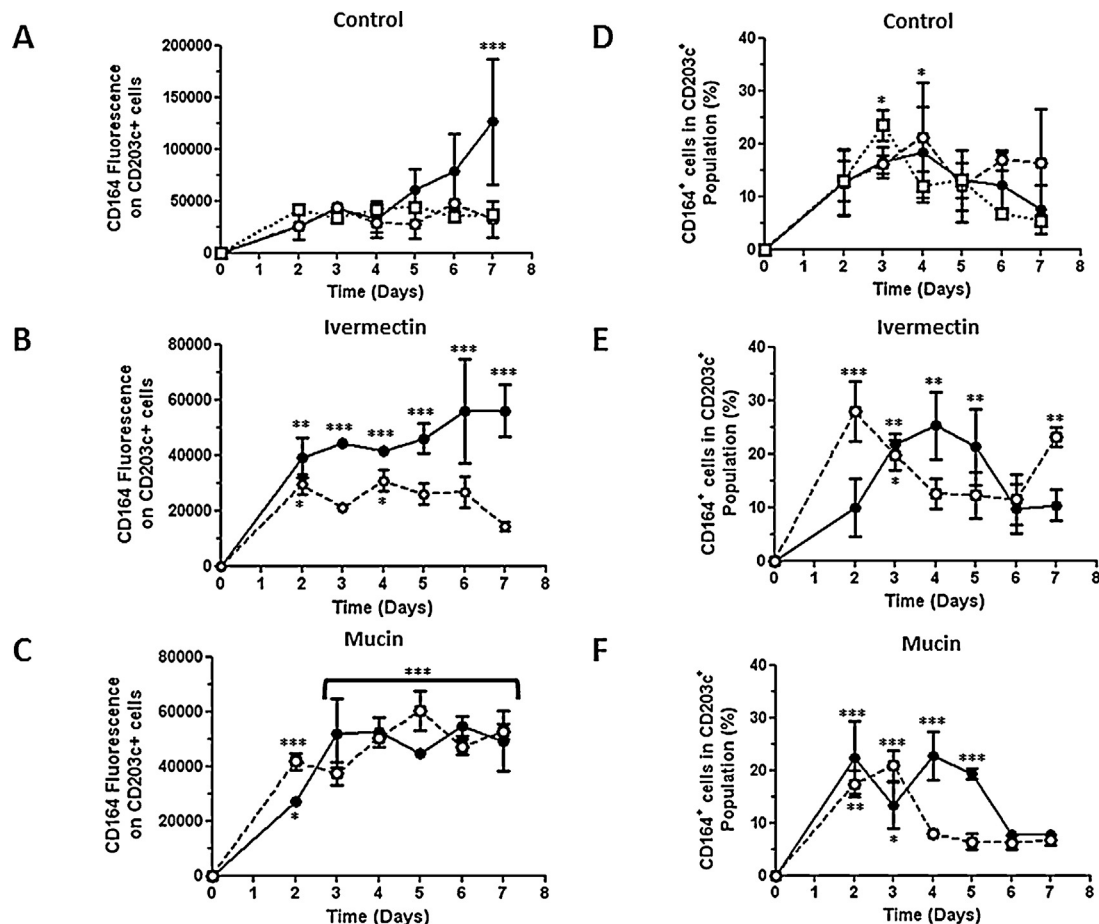


Fig. 4. Expression of CD164 on CD203c⁺ KU812 basophil cells co-cultured with HT29 cells and *T. colubriformis*. (A–C) CD13 expression mean fluorescence intensity on CD203c⁺ cells co-cultured with: (A) HT29 cell and *T. colubriformis* (●); HT29 cells (○); or PEA (□); (B) HT29 cells and ivermectin (○) or HT29, *T. colubriformis* and ivermectin (●); (C) HT29 cells and mucin (○) or HT29, *T. colubriformis* and mucin (●). (D, E, F) Percentage of CD164⁺ cells in the CD203c⁺ cell population co-cultured with: (D) HT29 cell and *T. colubriformis* (●); HT29 cells (○); or PEA (□); (E) HT29 cells and ivermectin (○) or HT29, *T. colubriformis* and ivermectin (●); (F) HT29 cells and mucin (○) or HT29, *T. colubriformis* and mucin (●) ($n=3$; means $\pm$ SEM; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

cells on days 3–7 compared to day 0 cells (Fig. 3C). This increase in CD13 expression was independent of the presence of *T. colubriformis* larvae except at day 2 for the mucin treatment where the presence of *T. colubriformis* significantly ($P<0.0001$) reduced the expression of CD13 3.7 fold.

The percentage of CD13⁺ cells in the CD203c⁺ population significantly ($P<0.0001$) increased over time in the presence of ivermectin and mucin (Fig. 3E and F). The presence of *T. colubriformis* in the mucin and ivermectin co-culture systems significantly ($P<0.05$) increased the number of CD13⁺ basophils on days 4 and 5. These data suggest that mucin increases CD13 expression on basophil cells independent of *T. colubriformis*, however, addition of the parasite further increased the number of basophil cells expressing CD13.

3.4. CD164 expression in cell co-culture experiments

The intensity of CD164 expression levels and the percentage of CD164⁺ cells in the CD203c⁺ basophil population showed a similar profile to that observed for CD13. For example, CD164 expression on CD203c⁺ KU812

basophil cells significantly ($P=0.0028$) increased in a time-dependent manner (Fig. 4A). In a similar fashion to CD13, a significant increase in CD164 expression on CD203c⁺ KU812 cells, compared to day 0 was not observed until day 7 of co-culture when motile *T. colubriformis* larvae were co-cultured without a motility inhibitor (Fig. 4A). Addition of ivermectin and mucin to the co-culture system significantly increased the intensity of CD164 expression on CD203c⁺ KU812 cells after 2 days ($P<0.0001$, Fig. 4B and C). The presence of ivermectin-inhibited parasites induced a further increase in the intensity of CD164 expression on KU812 cells on days 3, 5 and 7 compared to cell only control co-cultures treated with ivermectin (Fig. 4B).

In the same manner as CD13 expression, the percentage of CD203c⁺ cells expressing CD164 in the presence of *T. colubriformis* and ivermectin or mucin there was a significant ($P<0.01$) increase in the percentage of CD164⁺ cells compared to the ivermectin and mucin control co-cultures on days 4 post inoculation (Fig. 4E and F).

Since the abundance of basophils circulating in the blood is approximately 0.5% of the total systemic blood cell population, small numbers of basophils are predicted to

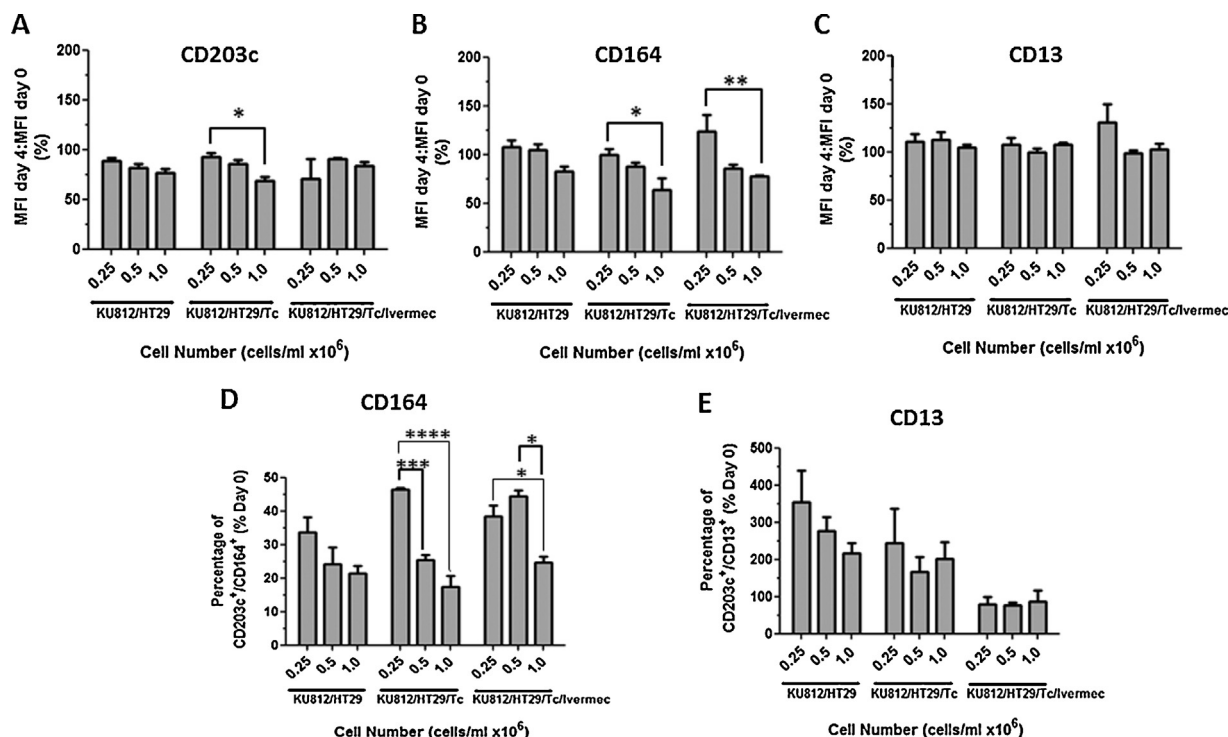


Fig. 5. Number of KU812 basophil cell dose responses in the parasite-cell co-culture system. The percent median fluorescence intensity (MFI) expression of (A) CD203c, (B) CD164 and (C) CD13 on CD203c⁺ KU812 basophil cells after four days of co-culture with HT29 cells, HT29 cell plus *T. colubriformis* or HT29 cells plus *T. colubriformis* plus ivermectin divided by the MFI of the corresponding population of cells at day 0 under the same co-culture conditions. (D) The percentage of CD164⁺ cells within the CD203c⁺ cell population on day 4 of the different culture conditions divided by the corresponding population of cells at day 0 under the same co-culture conditions. (E) The percentage of CD13⁺ cells within the CD203c⁺ cell population on day 4 of the different culture conditions divided by the corresponding population of cells at day 0 under the same co-culture conditions ($n = 3$; means $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

enter the intestines at the site of a *T. colubriformis* infection. Therefore a dose response of KU812 basophil-like cells from 2.5×10^5 to 1×10^6 cells per well examined under the HT29, HT29/*T. colubriformis* or HT29/*T. colubriformis*/Ivermectin co-culture conditions after 0 days (co-cultures were established for 1 hour) and four days. The cells were stained with either the CD-specific cocktail of antibodies or a cocktail of isotype control antibodies and examined flow cytometrically using a FlowSight flow cytometer (Amnis, USA). The ratio as a percentage of the MFI of day 4 CD203c⁺ cells divided by MFI of day 0 cells were plotted. Fig. 5B and C demonstrates that there was either a significant increase or no significant change in the MFI CD164 or CD13 on CD203c⁺ cells when 2.5×10^5 cells per well were included in the various co-culture systems compared to 5×10^5 and 1×10^6 cells per well. Similarly, 2.5×10^5 cells per well in the co-cultures resulted in unchanged cell population numbers or significantly greater populations of CD203c⁺ cells expressing cell surface CD164⁺ or CD13⁺ under the different co-culture conditions. These data suggest that the same types of basophil responses were apparent when low (2.5×10^5 cells) or high (1×10^6 cells) basophil cell numbers were included in the various parasite-cell co-cultures. In fact some basophil responses were more pronounced when lower 2.5×10^5 KU812 cells were used in the co-culture experiments.

In contrast to CD13 and CD164, CD107a expression was observed on less than 5% of CD203c⁺ cells under the conditions examined in the co-culture system (data not shown). Collectively, these data suggest that the IgE-independent expression of the ecto-enzymes CD203c, CD13 and CD164 was increased on KU812 basophil-like cells in the presence of the motility inhibitors mucin and ivermectin independent of *T. colubriformis*; however, the parasite induced further expression of these activation markers on a greater number of basophil cells in an epithelium environment (Fig. 6).

3.5. IL33 and FcεR1α mRNA expression

After culture of KU812 and HT29 cells under the aforementioned conditions for 7 days, IL-33 and FcεR1α expression profiles were determined. In contrast to the flow cytometry data it was technically beyond the feasibility of this study to separate the KU812 cells from the HT29 cells. However, qRT-PCR analysis of HT29 monocultures inoculated with motile *T. colubriformis* demonstrated that there was a significant ($P = 0.0007$), 15 fold increase in IL33 gene expression in HT29 cells two to six days after *T. colubriformis* challenge compared to day 0 cells and accounted for approximately 30% of the total variation (Fig. 5A). These data confirm the data presented in Andronicos et al. (2012) that suggest that *T. colubriformis* induces IL33 gene

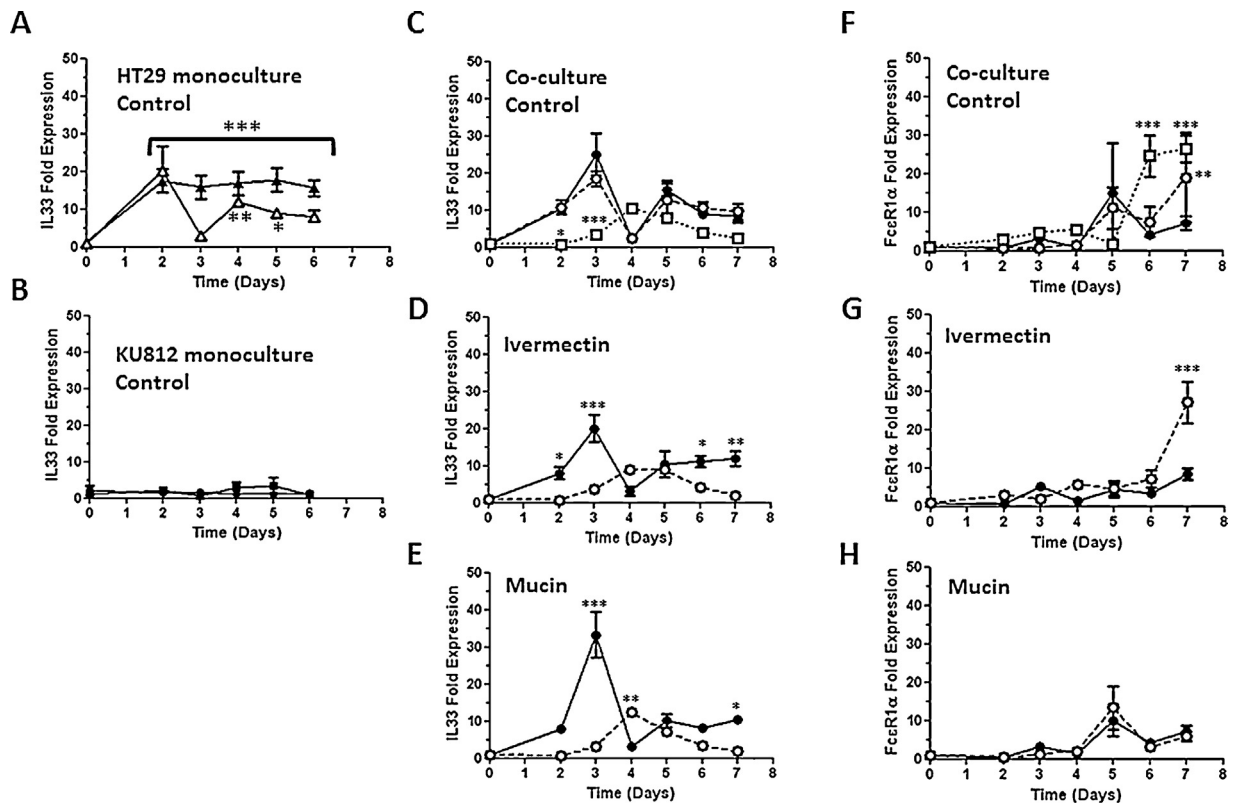


Fig. 6. IL33 and FcεR1α gene expression analysis of the parasite-cell co-culture system. (A) IL33 gene expression in HT29 cells in the presence (▲) and absence (△) of motile *T. colubriformis* parasites. (B) IL33 gene expression in KU812 cells in the presence (▲) and absence (△) of motile *T. colubriformis* parasites. (C) IL33 expression in the co-culture system in the presence of *T. colubriformis* (●); HT29 cells only (○); or PEA (□). (D) IL33 expression in the parasite-cells co-culture with ivermectin in the presence (●) or absence (○) of *T. colubriformis* (E) IL33 expression in the parasite-cells co-culture with mucin in the presence (●) or absence (○) of *T. colubriformis*. (F) FcεR1α expression in the co-culture system in the presence of *T. colubriformis* (●); HT29 cells only (○); or PEA (□). (G) FcεR1α expression in the parasite-cells co-culture with ivermectin in the presence (●) or absence (○) of *T. colubriformis* (H) FcεR1α expression in the parasite-cells co-culture with mucin in the presence (●) or absence (○) of *T. colubriformis* (●) ($n = 3$; means $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

expression in HT29 cells via a direct interaction. However, IL33 expression was not significantly expressed in KU812 cell monocultures (Fig. 5B). IL33 qRT-PCR was performed on a mixed population of HT29 and KU812 cells to determine if the parasites or their components induce the expression of IL33 mRNA within the culture system. Co-culture of KU812 cells with HT29 cells caused a significant ($P < 0.0001$) biphasic (days 2–3 and 5–7) increase in IL33 mRNA expression compared to day 0 (Fig. 5C–E). These increases in IL33 mRNA expression accounted for between 30% and 40% of the total variation observed. Similarly the presence of *T. colubriformis* larvae in the co-culture significantly ($P < 0.005$) increased IL33 gene expression accounting for 14–20% of the total variation in the system. Three days after co-culturing HT29 and KU812 cells with *T. colubriformis* larvae in the presence of the motility inhibitors ivermectin and mucin a significant ($P < 0.0001$) pulse of increased IL33 gene expression of 16 and 30 fold, respectively, was observed in cells when compared to inhibitor only treated cells (Fig. 5D and E). Collectively, this suggests that the major source of IL33 gene expression in the co-culture model was from the HT29 epithelial cells.

These samples were also analysed by qRT-PCR for FcεR1α gene expression (Fig. 5F–H). However, only treatment of the co-culture system with PEA induced a significant ($P < 0.0001$) increase (24 fold) in FcεR1α gene expression as compared to day 0. Collectively, these data suggest that only PEA was able to induce a delayed increase in FcεR1α gene expression in KU812 cells whereas increased IL33 gene expression from HT29 epithelial cells were coincident with increased CD203, CD13 and CD164 expression on the basophil-like KU812 cells in the co-culture model.

4. Discussion

The major site of *T. colubriformis* colonisation in sheep is within the first three metres of the jejunum proximal to the pancreas (Barker, 1975; Wagland et al., 1996). During a *T. colubriformis* infection the parasite kills the epithelium (McClure et al., 1992); goblet cells secrete mucus (Harrison et al., 1999) and immune cell such as mast cells (Harrison et al., 1999), basophils (Rothwell and Love, 1975) and eosinophils (Douch, 1989) infiltrate the site of infection.

Parasite-naïve innate immune cells therefore come into contact with the larvae in the presence of a damaged epithelium and mucus.

Previously we developed a parasite-intestinal epithelial cell model using the HT29 human colonic cell line (Andronicos et al., 2012). The human HT29 cell line was used in the *T. colubriformis* co-culture model because an ovine jejunum epithelial cell line had not been established. Secondly, *T. colubriformis* is zoonotic (i.e. infects humans) and studies have reported a high incidence of *T. colubriformis* in certain populations, specifically, in Laos, Japan and Iran. In 2003, a nationwide survey found that of all the school children surveyed, 19.1% were positive for hookworm infections, and it is believed that *T. colubriformis* is the main zoonotic species causing these infections. In these areas with high levels of *T. colubriformis* infections, human-to-human, animal-to-animal, animal-to-human, and human-to-animal transmission is possible, increasing the risk of infection (Sato et al., 2011). Finally, in response to a primary bolus challenge of *T. colubriformis* in genetically resistant sheep there was altered gene expression of oxidant, antioxidant and mucous genes down the longitudinal axis of the sheep's small intestine, distal to the site of primary infection (Menzies et al., 2010). Thus we suggest that the use of a human colonic cell line in this model is valid. Therefore, whilst not ideal, we have used HT29 cells in the modified parasite-epithelial cell co-culture model previously developed by Andronicos et al. (2012). Similarly, the human basophilic cell line KU812 was included in the modified parasite-epithelial cell model to investigate the activation of KU812 basophil-like cells by GIN in the absence of IgE. Basophils comprise less than 1% of circulating leukocytes. Thus, their use in research is limited by this low frequency, as well as by their short half-life of only a few days (Karasuyama et al., 2009). One strategy to examine the function of basophils using a reductionist in vitro model is to employ basophil-like cell lines such as KU812 cells in an in vitro cell culture model to elucidate in vivo biological mechanisms (Hosoda et al., 2002; Shim et al., 2002; Tare et al., 2010). Finally, porcine mucin was also included in this model since pigs can host *T. colubriformis* parasites intestinal infections (Nganga et al., 2008). As expected the inclusion of porcine mucin decreased the motility of *T. colubriformis* in the modified co-culture model. However, a caveat of the modified co-culture model may be due to the interspecies differences of using porcine mucin with human cell lines which may have contributed to some of the observed gene expression changes. Thus, we modified the parasite-HT29 epithelial cell model by incorporating the KU812 human basophil cell line and porcine mucin to examine the activation of basophil cells in a parasitised epithelium.

The expression of the basophil activation antigens CD13, CD164 and CD107a were specifically examined on CD203c⁺ KU812 cells to determine the IgE-independent activation of basophils under *in vitro* conditions designed to simulate the initial colonisation of *T. colubriformis* into an epithelial mucosa: (1) with or without mucin and (2) in the presence of an anthelmintic such as ivermectin. Data presented here suggests that in the absence of IgE, KU812 basophil-like cells increase cell surface expression of the activation

ecto-enzymes CD203c, CD13 and CD164 when the motility inhibitors mucin and ivermectin were present, independently of *T. colubriformis*. However, *T. colubriformis* induced a greater number of these CD203c⁺ basophils to express increased levels of CD13 and CD164 in the presence of both mucin and ivermectin in a damaged epithelial environment. *T. colubriformis* also modified IL33 gene expression in the co-culture system concomitant with increased basophil cell surface ecto-enzyme expression signatures. Thus, in the presence of *T. colubriformis*, mucin and epithelial cells, KU812 basophil-like cells were activated independently of IgE *in vitro*.

In vivo, gastrointestinal nematodes traverse the site of infection tissues and release excretory/secretory (E/S) products. *T. colubriformis* E/S antigen preparations have demonstrated biologically activity that includes stimulating epithelial cell proliferation (Huby et al., 1999; Hoste et al., 1995), up regulation of TNF α receptor (Riffkin et al., 2000) and aspartyl protease inhibitor activity (Shaw et al., 2003). In an attempt to define if a *T. colubriformis* E/S preparation had biological activity towards basophil-like cells in an intestinal epithelial cell environment, the co-culture model was treated with a *T. colubriformis* post-exsheathment antigen (PEA) preparation. PEA induced an increase in basophil cell surface CD13, on about 10% of cells but no increase in CD164 expression was observed. Hence, PEA in the absence of the motility of viable *T. colubriformis* was able to partially activate the KU812 basophil-like cell line in the presence of an intact epithelial cell monolayer in vitro. This suggested that both the motion of the parasite as well as the E/S of *T. colubriformis* may contribute to the activation of basophil-like cells. In addition, *T. colubriformis* PEA was the only treatment examined that induced a significant increase in the IgE receptor Fc ϵ RI α gene expression. This late phase (days 6 and 7) increase in Fc ϵ RI α expression suggested that a component of basophil IgE-independent activation involved the up regulation of the cell surface receptor for IgE by parasite E/S. Collectively these data suggested that a *T. colubriformis* E/S preparation was able to modify gene expression of basophil-like cells in an in vitro co-culture model.

During *Nippostrongylus brasiliensis* infections in mice, basophils at the site of infection were found to be essential to the establishment of immune responses that facilitated the expulsion of the parasite from adult animals (Nel et al., 2011). Since there is essentially no parasite-specific IgE present during a primary *T. colubriformis* challenge of helminth naïve sheep (Andronicos et al., 2010) and that histaminogenic mast cells, eosinophils and basophils increase in number at the site of *T. colubriformis* infections, it is speculated that these cells are activated in an IgE-independent manner by *T. colubriformis* colonisation of the intestinal epithelium. The activated cells release histamine and other bioactive substances thereby participating in the initial establishment the host immune response. Two types of IgE-dependent activation events have been demonstrated in basophil-like KU812; early IgE-mediated activation involving up regulation of cell surface CD203c, CD13 and CD164 and later CD63 and CD107a IgE-mediated activation (Hennersdorf et al., 2005). Data presented in this study has demonstrated that mucin and *T. colubriformis* induced the

up-regulation of the CD203c cluster of basophil activation markers on KU812 cell in the absence of IgE. In contrast, CD107a, a member of the CD63 cluster late IgE activation markers, was not up regulated by mucin or *T. colubriformis*. These data suggest that in the absence of parasite-specific IgE basophils can be partially activated by mucin and *T. colubriformis* in the presence of damaged epithelium.

Mice infected with *Tricuris muris* had an increased capacity to reject the parasite when inoculated intraperitoneally with IL33 (Humphreys et al., 2008). IL33 is postulated to be involved in the establishment of the Th2-dominant anti-parasite inflammatory response. *T. colubriformis*-mediated intestinal epithelium damage induces the release of IL33 from these cells (Andronicos et al., 2012). However, IL33 is also a mechanically responsive cytokine which is secreted by living fibroblasts in response to mechanical stress (Kakkar et al., 2012). Thus, IL33 from parasite-damaged or mechanically stressed cells may contribute to the activation of KU812 basophil cells in the co-culture system. Hence, IL33 is released from epithelial cells (Andronicos et al., 2012) due to the cytotoxic activity of the parasite may contribute to the Th2 response mounted in the intestine during parasite challenge. For example, IL33 through binding to its receptor (ST2) activates isolated basophils by inducing IL4 and IL13 gene expression and secretion of these cytokines (Suzukawa et al., 2008). Furthermore, KU812 cells, which express the IL33 receptor, functionally respond to IL33 binding by increasing the expression of numerous cytokines including IL5, IL13 and eotaxin (Tare et al., 2010). Similarly, murine bone marrow mast cells (BMMC) also express ST2 and in vitro treatment of these cells with IL33 induces the expression of IL13 and IL6 (Tung et al., 2014). IL33 does not directly induce degranulation or migration of basophils but enhances IgE-dependent and independent activities of basophils and mast cells (Silver et al., 2010; Tare et al., 2010). Interestingly, there was a significant parasite-dependent increase in IL33 gene expression in the co-culture system one day prior to an increase in the number of basophils that expressed the CD203c cluster of early activation antigens.

In conclusion, these *in vitro* data, derived from our parasite-cell co-culture model, suggested that when basophils encounter *T. colubriformis* in a compromised epithelium they are activated in an IgE-independent manner. The activation is mediated by increasing CD203c, CD13 and CD164 cell surface ecto-enzyme expression. These responses are predicted to enhance the contribution of basophils to the establishment of an anti-parasite immune response. These data support the *in vivo* observations made during *T. colubriformis* primary infections in guinea pigs and sheep. Thus the addition of a basophil cell line and mucin to the epithelial cell-parasite co-culture has proved a valuable modification to the initial base model allowing the initial immune responses that facilitate the establishment of protective acquired anti-parasite immunity in mammals to be examined. We are currently developing a second generation ovine-specific parasite gut epithelial cell co-culture model using immortalised sheep intestinal epithelial cells. We hope to examine the initial immune responses to parasites in the intestine of sheep with the

intention of identifying synthetic agents that mimic these responses.

References

- Amarante, A.F.T., Rocha, R.A., Bricarello, P.A., 2007. Relationship of intestinal histology with the resistance to *Trichostrongylus colubriformis* infection in three breeds of sheep. *Pesquisa Vet. Brasil* 27, 43–48.
- Andronicos, N., Hunt, P., Windon, R., 2010. Expression of genes in gastrointestinal and lymphatic tissues during parasite infection in sheep genetically resistant or susceptible to *Trichostrongylus colubriformis* and *Haemonchus contortus*. *Int. J. Parasitol.* 40, 417–429.
- Andronicos, N.M., McNally, J., Kotze, A.C., Hunt, P.W., Ingham, A., 2012. *Trichostrongylus colubriformis* larvae induce necrosis and release of IL33 from intestinal epithelial cells in vitro: implications for gastrointestinal nematode vaccine design. *Int. J. Parasitol.* 42, 295–304.
- Bendixsen, T., Emery, D.L., Jones, W.O., 1995. The sensitization of mucosal mast-cells during infections with *Trichostrongylus-Colubriformis* or *Haemonchus-Contortus* in sheep. *Int. J. Parasitol.* 25, 741–748.
- Cardia, D.F.F., Rocha-Oliveira, R.A., Tsunemi, M.H., Amarante, A.F.T., 2011. Immune response and performance of growing Santa Ines lambs to artificial *Trichostrongylus colubriformis* infections. *Vet. Parasitol.* 182, 248–258.
- Coffman, R.L., 2010. Immunology. The origin of TH2 responses. *Science* 328, 1116–1117.
- Gause, W.C., Urban, J.F., Stadecker, M.J., 2003. The immune response to parasitic helminths: insights from murine models. *Trends Immunol.* 24, 269–277.
- Gibbs, B.F., 2005. Human basophils as effectors and immunomodulators of allergic inflammation and innate immunity. *Clin. Exp. Med.* 5, 43–49.
- Harris, N., Gause, W.C., 2011. To B or not to B: B cells and the Th2-type immune response to helminths. *Trends Immunol.* 32, 80–88.
- Harrison, G.B., Pulford, H.D., Gatehouse, T.K., Shaw, R.J., Pfeffer, A., Shoemaker, C.B., 1999. Studies on the role of mucus and mucosal hypersensitivity reactions during rejection of *Trichostrongylus colubriformis* from the intestine of immune sheep using an experimental challenge model. *Int. J. Parasitol.* 29, 459–468.
- Harrison, G.B., Pulford, H.D., Doolin, E.E., Pernthaner, A., Shoemaker, C.B., Hein, W.R., 2008. Antibodies to surface epitopes of the carbohydrate larval antigen CarLA are associated with passive protection in strongylid nematode challenge infections. *Parasite Immunol.* 30, 577–584.
- Henz, B.M., Maurer, M., Lippert, U., Worm, M., Babina, M., 2001. Mast cells as initiators of immunity and host defense. *Exp. Dermatol.* 10, 1–10.
- Hosoda, M.I., Yamaya, M., Suzuki, T., Yamada, N., Kamanaka, M., Sekizawa, K., Butterfield, J.H., Watanabe, T., Nishimura, H., Sasaki, H., 2002. Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J. Immunol.* 169, 1482–1491.
- Hoste, H., Nano, J.L., Mallet, S., Huby, F., Fournel, S., Rampal, P., 1995. Stimulation of HT29-D4 cell growth by excretory/secretory products of the parasite nematode *Trichostrongylus colubriformis*. *Epithelial Cell Biol.* 4, 87–92.
- Huby, F., Nano, J.L., Mallet, S., Hoste, H., 1999. Effects of the excretory/secretory products of *Trichostrongylus colubriformis* on the growth of different cell lines. *Int. J. Parasitol.* 29, 697–702.
- Huxtable, C.R., Rothwell, T.L.W., 1975. Studies of responses of basophil and eosinophil leukocytes and mast-cells to nematode *Trichostrongylus-Colubriformis*—ultrastructural changes in basophils and eosinophils at site of infection. *Aust. J. Exp. Biol. Med.* 53, 437–445.
- Jones, W.O., Windon, R.G., Steel, J.W., Otteridge, P.M., 1990. Histamine and leukotriene concentrations in duodenal tissue and mucus of lambs selected for high and low responsiveness to vaccination and challenge with *Trichostrongylus-Colubriformis*. *Int. J. Parasitol.* 20, 1075–1079.
- Kakkar, R., Hei, H., Dobner, S., Lee, R.T., 2012. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *J. Biol. Chem.* 287, 6941–6948.
- Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol.* 20, 477–481.
- Karasuyama, H., Mukai, K., Tsujimura, Y., Obata, K., 2009. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9, 9–13.
- Kroeger, K.M., Sullivan, B.M., Locksley, R.M., 2009. IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38alpha-dependent pathway. *J. Leukoc. Biol.* 86, 769–778.

- LeJambre, L.F., Windon, R.G., Smith, W.D., 2008. Vaccination against *Haemonchus contortus*: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture. *Vet. Parasitol.* 153, 302–312.
- Matzinger, P., 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12, 991–1045.
- Menzies, M., Reverter, A., Andronicos, N., Hunt, P., Windon, R., Ingham, A., 2010. Nematode challenge induces differential expression of oxidant, antioxidant and mucous genes down the longitudinal axis of the sheep gut. *Parasite Immunol.* 32, 36–46.
- Miller, J.E., Horohov, D.W., 2006. Immunological aspects of nematode parasite control in sheep. *J. Anim. Sci.* 84 Suppl., E124–E132.
- Nakanishi, K., 2010. Basophils are potent antigen-presenting cells that selectively induce Th2 cells. *Eur. J. Immunol.* 40, 1836–1842.
- Nganga, C.J., Karanja, D.N., Mutune, M.N., 2008. The prevalence of gastrointestinal helminth infections in pigs in Kenya. *Trop. Anim. Health Prod.* 40, 331–334.
- Nel, H.J., Hams, E., Saunders, S.P., Mangan, N.E., Smith, P., Atzberger, A., Flavell, R.A., Akira, S., McKenzie, A.N., Fallon, P.G., 2011. Impaired basophil induction leads to an age-dependent innate defect in type 2 immunity during helminth infection in mice. *J. Immunol.* 186, 4631–4639.
- Riffkin, M.C., Seow, H.F., Wood, P.R., Brown, L.E., Jackson, D.C., Scheerlinck, J.P., 2000. *Trichostrongylus colubriformis* extract upregulates TNF-alpha receptor expression and enhances TNF-alpha sensitivity of L929 cells. *Immunol. Cell Biol.* 78, 575–579.
- Rothwell, T.L.W., 1975. Studies of responses of basophil and eosinophil leukocytes and mast-cells to nematode *Trichostrongylus Colubriformis*. 1. Observations during expulsion of 1st and 2nd infections by guinea-pigs. *J. Pathol.* 11 (6), 51–60.
- Rothwell, T.L.W., Love, R.J., 1975. Studies of responses of basophil and eosinophil leukocytes and mast-cells to nematode *Trichostrongylus Colubriformis*. 2. Changes in cell numbers following infection of thymectomized and adoptively or passively immunized guinea-pigs. *J. Pathol.* 116, 183–194.
- Sacket, D., Holmes, P., Abbott, K., Jephcott, S., Barber, M., 2006. Assessing the economic cost of endemic disease on the profitability of the Australian beef cattle and sheep producers. Sydney: Meat & Livestock Australia Report No.: Final Report.
- Sato, M., Yoonuan, T., Sanguankiat, S., Nuamtanong, S., Pongvongsa, T., Phimmayoi, I., Phanhanan, V., Boupha, B., Moji, K., Waikagul, J., 2011. Short report: human *Trichostrongylus colubriformis* infection in a rural village in Laos. *Am. J. Trop. Med. Hyg.* 84, 52–54.
- Shaw, R.J., McNeill, M.M., Maass, D.R., Hein, W.R., Barber, T.K., Wheeler, M., Morris, C.A., Shoemaker, C.B., 2003. Identification and characterisation of an aspartyl protease inhibitor homologue as a major allergen of *Trichostrongylus colubriformis*. *Int. J. Parasitol.* 33, 1233–1243.
- Shim, S.Y., Ichikawa, A., Yamashita, M., Katakura, Y., Teruya, K., Mochizuki, Y., Tobinaga, E., Shirahata, S., 2002. Construction of an in vitro allergy reaction evaluation system using human leukemia cell lines. *Cytotechnology* 40, 75–83.
- Silver, M.R., Margulis, A., Wood, N., Goldman, S.J., Kasaian, M., Chaudhary, D., 2010. IL-33 synergizes with IgE-dependent and IgE-independent agents to promote mast cell and basophil activation. *Inflamm. Res.* 59, 207–218.
- Sullivan, B.M., Locksley, R.M., 2009. Basophils: a nonredundant contributor to host immunity. *Immunity* 30, 12–20.
- Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S., Matsushima, K., Ohta, K., Yamamoto, K., Yamaguchi, M., 2008. An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J. Immunol.* 181, 5981–5989.
- Tare, N., Li, H., Morschauser, A., Cote-Sierra, J., Ju, G., Renzetti, L., Lin, T.A., 2010. KU812 cells provide a novel in vitro model of the human IL-33/ST2L axis: functional responses and identification of signaling pathways. *Exp. Cell Res.* 316, 2527–2537.
- Tung, H.Y., Plunkett, B., Huang, S.K., Zhou, Y., 2014. Murine mast cells secrete and respond to interleukin-33. *J. Interferon Cytokine Res.* 34, 141–147.