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Abbreviations: PCR, polymerase chain reaction; cMCT, canine mast cell tumor.

Abstract

Screening for expression of the high affinity receptor for IgE by reverse transcriptase PCR, revealed that almost all canine mast cell tumors expressed FcεRIα mRNA, supporting the rationale for developing anti-neoplastic treatments based on molecules that could target this receptor. Use of cytotoxic cytokines to trigger an apoptotic signal is one strategy for inducing cell death in malignant mast cells. The coding sequences for canine IgE and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were identified through genome analyses. Selected regions of the coding sequences for these genes were cloned and compared to the predicted genome sequences. The Fc region of canine IgE, death domain of canine TRAIL and an IgE Fc : TRAIL fusion construct were generated and epitope-tagged proteins expressed, using a eukaryotic expression system. Specific binding of recombinant canine IgE Fc-containing proteins to recombinant human FcεRIα and to a canine mast cell tumor line expressing FcεRIα (C2), but not one failing to express FcεRIα (MCLA), was demonstrated. Specific binding of the IgE:TRAIL fusion protein was not abrogated by the TRAIL moiety. These results are proof of principle that canine IgE targeting to FcεRIα can be used as a platform for selective delivery of therapies to FcεRIα-expressing cells, potentially enhancing their therapeutic index and efficacy.

1. Introduction

Canine mast cell tumors (cMCTs) are the most common skin malignancy in dogs (Brodey 1970; Finnie and Bostock, 1979; Bostock 1986; Dobson et al., 2002), likely arising from neoplastic transformation of resident tissue mast cells or their progenitors. Breed, location, stage, gross appearance, size, presence of paraneoplastic syndromes and several proliferation markers have been variably associated with prognosis, however the most consistent predictor of cMCT recurrence, metastasis and survival is histopathological grade (Bostock, 1973; Patnaik et al., 1984; O' Keefe, 1990; Gerritsen et al., 1998; Mullins et al., 2006). Although dogs with a localised cMCT are often cured by local therapy (surgery and/or radiation therapy), those with an inoperable primary mass or confirmed disseminated disease usually die of their disease (reviewed by Welle et al., 2008; Blackwood et al., 2012). The use of both local (e.g. surgery and radiation therapy) and systemic therapies (e.g. chemotherapy, receptor tyrosine kinase inhibitors) is limited by the potential for adverse effects, some of which can be idiosyncratic, cumulative and/or permanent in nature. Thus, there is a need to develop novel therapies for cMCTs with improved efficacy and higher therapeutic indices.

Mast cells avidly and specifically bind to IgE via the high-affinity IgE receptor, FcεRI. Cross-linkage of surface IgE by antigen leads to mast cell degranulation and inflammation, an important defence against parasite infestation. In addition to disease caused by malignant transformation, mast cells are also involved in type I hypersensitivity reactions in allergic disease. Additional beneficial and pathophysiological roles for mast cells are emerging, although many of these can be undertaken by other, more numerous cells (reviewed by Rao & Brown, 2008), and mast cells are not absolutely required for survival, as demonstrated by genetically-modified mouse strains lacking these cells (C57BL/6-KITW^{sh}-/W^{sh-}, Grimbaldeston et al., 2005). However, other genetic mutations associated with mast cell deficiency can lead to significant developmental problems in cells of other lineages. WBB6F_1-W/W^{v} mice have reduced erythrocyte, granulocyte, platelet and mast cell

numbers (Kitamura et al., 1978), and although such mice are more likely than wild type mice to die in a model of acute septic peritonitis involving cecal ligation and puncture, their survival can be enhanced solely by reconstitution with cultured wild type mast cells (Echtenacher et al., 1996).

FcεRI is constitutively expressed by mast cells and basophils during early differentiation, but lower level, inducible expression has also been found on mammalian eosinophils, monocytes, platelets and dendritic cells (Thompson et al., 1990; Rottem et al; 1992; Joseph et al., 1997; Kinet 1999; Kita et al., 1999; Seminario et al., 1999). The affinity and avidity of IgE for FcεRI exceeds that of other immunoglobulin/Fc receptor interactions by several orders of magnitude (Ravetch and Kinet, 1991; Maenaka et al., 2001; McDonnell *et al.,* 2001; Wan et al., 2002). Previous work, evaluating the role of each IgE domain in binding to its high affinity receptor, has shown that $C_H\varepsilon3$ is critical for binding to FcεRI, but $C_H\varepsilon2$ and $C_H\varepsilon4$ are also required for high affinity and avidity binding (Keown et al., 1997; Garman et al., 2000 & 2001; McDonnell et al., 2001; Wurzburg and Jardetzky, 2002; Hunter et al., 2008). In a previous study, a partially dimerized $C_H\varepsilon2$-4 recombinant canine protein was used to raise an anti-canine IgE antibody which detected native canine IgE, suggesting that the quaternary structure of the recombinant $C_H\varepsilon2$-4 protein was similar to native canine IgE, despite the lack of a $C_H\varepsilon1$ domain (Ledin et al., 2006). Hunter et al. (2008) reported an alternative cell-based binding assay using RBL-2H3 cells stably transfected with rcFcεRIα and elegantly evaluated the role of each IgE heavy chain domain in binding to the receptor.

IgE has been used as a delivery platform for targeting murine and human mast cells, when linked to Pseudomonas toxin or pro-apoptotic Bcl-2 protein family members. These fusion proteins were capable of binding to and inducing apoptosis in mast cells *in vitro* and *in vivo*. (Fishman and Lorberboum-Galski, 1997; Fishman et al., 2000; Belostotsky and Lorberboum-Galski, 2001) A $rmIgE_{301\text{-}473}$-Pseudomonas exotoxin A chimeric protein was highly cytotoxic to both malignant murine mast cell lines and bone marrow-derived mast cells, while having no effect on other cell lines of

haematopoietic lineage (Fishman and Lorberboum-Galski, 1997), demonstrating its specificity for FcεRI-expressing cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, TNFSF10) is a type II membrane protein, an extracellular fragment of which can be cleaved to generate soluble TRAIL (sTRAIL; amino acids 37-281) (Wiley et al., 1995; Pitti et al., 1996; Mariani and Krammer, 1998). In humans and rodents, healthy cells display a repertoire of death and decoy TRAIL receptors, whereas neoplastic/transformed cells preferentially express death receptors over decoy receptors (Sheridan et al., 1997; Simonet et al., 1997; Pan et al., 1998; LeBlanc and Ashkenazi, 2003). Therefore, neoplastic cells are generally more susceptible than healthy cells to TRAIL-mediated apoptosis, making this a tempting therapeutic strategy (LeBlanc and Ashkenazi, 2003). Although the canine TRAIL receptor repertoire is poorly characterized and there are no TRAIL receptor genes identifiable in the syntenic region of the canine genome, compared to human and mouse, we and others have demonstrated that canine neoplastic mast cells are susceptible to the apoptotic effects of TRAIL (Rong et al., 2008; Elders et al., 2009).

The aim of the current study was to generate a recombinant canine IgE Fc protein that was capable of binding to the FcεRI receptor expressed on canine mast cells. The intention was that this recombinant canine IgE Fc molecule could then be modified to allow specific targeting approaches to be evaluated for treatment of canine mast cell tumors. Since we had previously shown that a canine MCT line (C2) was susceptible to TRAIL-mediated apoptosis, a canine IgE-TRAIL fusion protein was planned in the first instance to test the concept that this targeting strategy was feasible.

2. Materials and methods

2.1 Case recruitment and sample collection

Biopsy samples were obtained from dogs undergoing surgical resection of a mass, suspected or cytologically confirmed as cMCT. Tissue was stored in RNA*later*® (Qiagen, Crawley, UK) at -20 °C prior to molecular analysis. All tumors had histopathology or cytology performed and, where available, representative slides were reviewed by a single pathologist (K.C.S.) using a published grading scheme (Patnaik et al., 1984). In one patient, tumor tissue was disaggregated and cultured to generate a novel cMCT line, designated MCLA, which demonstrated metachromatic granule staining with Toluidine blue and was positive for expression of chymase and tryptase (Elders, 2009). Residual canine EDTA blood, following completion of processing of a diagnostic sample, was used to provide canine genomic DNA for IgE cloning. Similarly, residual lymph node tissue, following diagnostic processing of a lymphoma sample, was used to provide cDNA for TRAIL cloning.

2.2 Cells and cell culture

Chinese Hamster ovary (CHO) cells (obtained from ECACC), C2 canine mastocytoma cells (a generous gift from Dr. Birgit Helm, University of Sheffield; permitted by the originator, Prof. W. Gold, University of California) (Lazarus et al., 1986) or MCLA cells were propagated at 37 °C, 5% CO_2, in 75 cm^2 flasks in Eagle's minimal essential medium, supplemented with 5% foetal bovine serum (FBS), 1% non-essential amino acids, 50 µg/ml gentamicin (all Sigma) and 1% L-glutamine (Invitrogen, Paisley, UK) (culture medium). For experiments, cells were dissociated using Accutase™ (PAA Laboratories, Hampshire, UK) and cultured in phenol red-free minimal essential medium (Invitrogen), supplemented with 10% FBS, 1% non-essential amino acids, 1% L-glutamine and 50 µg/ml gentamicin.

2.3 Nucleic acid extraction and polymerase chain reaction

Tumor tissue or cultured cells were homogenized in Lysis Solution for total RNA (Sigma, Poole, UK) and RNA extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). On-column (RNase-free DNase set, Qiagen) and in-solution (Turbo DNase™, Ambion, Austin, USA) digestion

steps were performed to remove contaminant genomic DNA. Reverse transcription of mRNA into complementary DNA (cDNA) was performed using oligo$(dT)_{15}$ primer and ImProm-II reverse transcriptase (Promega, Southampton, UK).

Polymerase chain reaction was used to amplify genes of interest using specific primers (Table 1). Each 25 µl reaction contained 1 µl both sense and anti-sense primers (final concentration 10 pmol/µl) and cDNA or gDNA (1 µl) as template. Reactions also contained 1× Hi-Spec Additive, 1× NH_4 buffer, $MgCl_2$ (final concentration 2.0-3.0 mM), dNTP (final concentration 10 mM) and 0.5 IU Immolase™ DNA polymerase per reaction (all from Bioline, London, UK), made up to 25 µl with water. Reactions were heated to 95 °C for 10 mins, followed by 20 - 35 cycles at 94 °C for 40 s, 55 – 62.5 °C for 30 s, and 72 °C for 1 min; with a final extension step at 72 °C for 7 mins using a G Storm thermocycler (Gene Technologies Ltd, Essex, UK). For PCR products that were to be cloned, a proof-reading DNA polymerase (Easy-A® High-Fidelity PCR Cloning Kit, Stratagene, Amsterdam, The Netherlands) was used in place of Immolase.

Horizontal gel electrophoresis was used to separate PCR products, using 1-2% agarose (Bioline) gels containing 1× SafeView Nucleic acid stain™ (NBS Biologicals Huntingdon, UK). Amplicons were visualized under 590 nm ultra-violet light, using the ImageMaster® VDS Gel Documentation System (Pharmacia Biotech, Uppsala, Sweden).

2.4 Cloning of inserts into plasmid DNA vectors

PCR amplicons were extracted (GenElute™ gel extraction kit; Sigma), cloned into the pSC-A® vector (Stratagene) and transformed into *E. coli* (Solopack™, Stratagene). Plasmid DNA was then isolated using the GenElute™ Plasmid Miniprep Kit (Sigma) and sequencing to confirm the integrity of the inserts (Geneservice, Cambridge UK). Donor (recombinant pSC-A) and recipient (pSecTagA®; Invitrogen) plasmid DNA were digested using the indicated restriction enzymes to allow directional subcloning. Following purification, insert DNA was ligated into digested recipient vector (Quick-Stick™ DNA Ligase, Bioline) and *E. coli*. transformed. Endotoxin-free plasmid DNA was prepared from

E. coli cultures using GenElute™ Endotoxin-free Plasmid Midiprep Kit (Sigma), according to the manufacturer's instructions.

2.5 Transfection of Chinese hamster ovary cells

Cells for transfection were seeded at 1×10^5 cells per well into 24-well plates in a 500 µl volume of culture medium containing 5% FBS. When 90-95% confluent, the medium was replaced with FBS and antibiotic-free medium. Cells were transfected with 800 ng endotoxin-free recombinant plasmid DNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Plates were incubated for 6 h then the plasmid-containing medium was replaced with medium containing 10% FBS but lacking antibiotics, and after a further 18 h this medium was replaced with culture medium. After 72 h incubation, the supernatant, containing recombinant protein, was recovered, centrifuged (13.8 ×*g*, 5 mins), filtered (0.22 µm filters, Millex® GV syringe filter unit, Sigma), and used for experiments.

2.6 Detection of recombinant proteins

Pre-blocked, nickel coated ELISA strips (Ni-NTA HiSorb®, Qiagen) were used to capture polyhistidine-tagged recombinant proteins from transfected CHO supernatants (100 µl/well). After 2 h incubation, wells were washed with PBS supplemented with 0.1% Tween 20 (Sigma). A caprine anti-c-myc:HRP conjugate (A190-104P; Universal Biologicals, Cambridge, UK), diluted 1:5,000 in phenol red-free MEM/10% FBS was added for 2 h, followed by six washes. One hundred microliters of substrate (Supersensitive TMB Liquid Substrate for ELISA; Sigma) was added per well and incubated for 20 min. Twenty five microliters of 0.5 M sulphuric acid (SLS, Nottingham, UK) was added to stop the reaction, and the optical density (O.D.) at 540 nm subtracted from that at 450 nm to generate absorbance values per well. The ELISA was modified by substituting the anti-c-myc antibody with either a polyclonal caprine anti-canine IgE:HRP conjugate (1:10,000 dilution of AHP946P, Abd Serotec Ltd., Oxford, UK), or a polyclonal caprine anti-human TRAIL antibody (1:100

dilution of AF375, AbD Serotec) which was in turn detected using a polyclonal anti-caprine IgG:HRP conjugate (1:10,000 dilution of OBT1500P, Abd Serotec). Samples of transfected CHO supernatants, containing recombinant protein, were also submitted to HESKA (Fribourg, Switzerland) for analysis using their validated Allercept® canine IgE ELISA (Foster et al., 2003).

2.7 Western blotting

Polyhistidine-tagged proteins were purified from CHO supernatants using the MagneHis™ Protein Purification System (Promega). Purified recombinant proteins (32.5 µl) were added to 5 µl of 500 mM dithiothreitol (DTT) reducing agent and 12.5 µl NuPAGE® LDS sample buffer (both Invitrogen) and incubated at 70 °C for 10 mins. Proteins were separated by PAGE using 4 - 20% gels and sodium dodecyl sulphate (SDS) running buffer (both PAGEgel.com, California, USA), in the X-cell *SureLock*™ Mini-cell (Invitrogen) at 200 V constant for 35 mins. Proteins were then transferred to nitrocellulose membranes under reducing conditions in the X-Cell II™ Blot Module (Invitrogen) and transfer buffer (PAGEgel.com) at 30 V constant for 1 h. Membranes were rinsed, blocked overnight at 4 °C in 50 ml PBS/5% Marvel, then incubated with murine anti-His:HRP conjugate (MCA1396P, Abd Serotec, diluted 1:2500 in 25 ml PBS/5% Marvel™/0.1% Tween 20) at room temperature for 1 h. After washing, immunoreactivity was detected by chemiluminescence using the ECL™ Western blotting analysis system (GE Healthcare, Chalfont St Giles, UK) and film (Kodak X-Omat AR™, Kodak, Harrow, UK).

2.8 Assessment of FcεRIα receptor-binding activity of recombinant proteins by flow cytometry

C2 (FcεRIα positive mastocytoma line) and MCLA (FcεRIα negative mastocytoma line) cells (2×10^5 cells in a 100 µl volume of culture medium) were incubated at 4 °C for 30 mins. Cells were then centrifuged at 1200 ×*g* for 10 mins, resuspended and incubated at 4 °C for 2 h in 200 µl of each sterile-filtered CHO cell supernatant (transfected with either $IgE_{100\text{-}427}$, $TRAIL_{114\text{-}282}$, or $IgE_{100\text{-}427}$:$TRAIL_{114\text{-}282}$ fusion construct). Cells resuspended in supernatant from mock transfected CHO cells

acted as negative controls. Cells resuspended in PBS/0.5% canine IgE heterohybridoma supernatant (Bethyl Laboratories) were used as positive controls for IgE binding.

Cells were centrifuged and supernatants aspirated. Cell pellets were resuspended in 200 µl PBS alone or containing caprine anti-canine IgE:FITC (1:200 of AHP946F, AbD Serotec) or murine anti-c-myc:FITC (1:200 of MCA2200F, Abd Serotec) and incubated for 20 mins. Following repeat centrifugation, cell pellets were resuspended in 200 µl PBS for flow cytometric analysis, (FACSAria, BD Biosciences, Erembodegem, Belgium) counting up to 20,000 events. Data were analysed using FlowJo (Tree Star Inc., Oregon, USA)

3. Results

3.1 Analysis of FcεRIα mRNA expression in canine mast cell tumors and selected mastocytoma cell lines

The canine FcεRIα coding sequence (762 bp), consisting of 5 exons, is located on chromosome 38 (GenBank Accession# NM_00110766.1, Goitsuka et al., 1999). Screening primers, designed to amplify a 273bp fragment, were used to demonstrate that 23 of the 25 canine MCT biopsy samples were positive for FcεRIα mRNA expression (Fig. 1A), suggesting that this represents a suitable target for potential immunotherapeutic strategies. The C2 mastocytoma cell line but not the newly established MCLA mast cell tumor line, expressed FcεRIα mRNA (Fig. 1B).

3.2 Generation of recombinant canine IgE Fc and IgE Fc-TRAIL fusion constructs

The canine IgE heavy chain gene, encoding $V_H\varepsilon$ and $C_H\varepsilon1$-4, is located on chromosome 8 and consists of 5 exons (GenBank Accession# XM_548007.1). Using genomic DNA as template, primers were designed at the start of exon 3 (encoding $C_H\varepsilon2$) and the end of exon 5 (encoding $C_H\varepsilon4$), to generate a PCR amplicon spanning the $C_H\varepsilon2$-4 coding region, but which contained additional intronic sequence. This was cloned into the pSecTagA vector and following sequence verification, plasmid

DNA was transfected into Chinese hamster ovary (CHO) cells. Isolation of cDNA from the transfected cells and further PCR analysis revealed three amplicons (Fig. 2), the smallest of which was consistent with splicing out of both introns. This was confirmed by sequencing, and this canine $C_H\varepsilon 2\text{-}4$ coding region was subsequently cloned into the pSecTagA vector between the *HindIII* and *EcoRI* sites (pSecTagA/rcIgE$_{100\text{-}427}$).

The canine TRAIL gene, consisting of 5 exons is located on chromosome 34 (GenBank Accession# NM_001130836.1). Using cDNA prepared from a canine lymphoma biopsy as template, canine TRAIL-specific primers were used to generate an amplicon encoding the predicted C-terminal TNF-like death domain (amino acids 114-282), which was cloned into pSecTagA (pSecTagA/rcTRAIL$_{114\text{-}282}$). In addition, a fusion construct was generated by subcloning the TRAIL sequence, between *EcoRI* and *XhoI* sites, downstream of and in-frame with the IgE Fc fragment to generate a fusion construct (pSecTagA/rcIgE$_{100\text{-}427}$: rcTRAIL$_{114\text{-}282}$).

3.3 Expression of recombinant canine IgE Fc and IgE Fc-TRAIL fusion proteins

Chinese hamster ovary cells were transfected with pSecTagA containing, rcIgE$_{100\text{-}427}$, rcTRAIL$_{114\text{-}282}$, or rcIgE$_{100\text{-}427}$: rcTRAIL$_{114\text{-}282}$ fusion constructs. Polyhistidine and c-myc epitope-tagged recombinant proteins were detected by ELISA in the supernatants of CHO cells transfected with the three constructs (Fig. 3A), while none was found in cell lysates (data not shown). This ELISA was modified by substitution of the anti-c-myc detection antibody with either an anti-canine IgE conjugate (Figure 3B), or an anti-human TRAIL antibody (Figure 3C). This confirmed expression of the relevant epitope-tagged recombinant proteins containing canine IgE Fc and/or TRAIL protein domains.

Western blotting of recombinant proteins, purified from supernatant, revealed polyhistidine-tagged proteins, somewhat heavier than the predicted weights of the translated

sequences (rcIgE$_{100-427}$: 42.3kDa; rcIgE$_{100-427}$:TRAIL$_{114-282}$: 61.6kDa; rcTRAIL$_{114-282}$: 26kDa), possibly consistent with glycosylation and/or inefficient cleavage of the signal peptide (Fig. 4).

3.4 Binding of recombinant canine IgE Fc and IgE Fc-TRAIL fusion proteins to FcεRIα

Using the Allercept system, it was demonstrated that rcIgE$_{100-427}$ and rcIgE$_{100-427}$:TRAIL$_{114-282}$ were able bind to recombinant FcεRIα, whereas rcTRAIL$_{114-282}$ was unable to do so (Fig. 5). By flow cytometric analysis, C2 cells (FcεRIα positive) bound monoclonal canine IgE as well as rcIgE$_{100-427}$ (Figure 6A), whereas MCLA cells (FcεRIα negative) failed to demonstrate any binding to monoclonal IgE or rcIgE$_{100-427}$ (Figure 6B). Competitive inhibition of binding of rcIgE$_{100-427}$ to C2 cells (detected with anti-myc antibody) was demonstrated by co-incubation with monoclonal IgE, with MFI values falling from 64.4 in the absence of the monoclonal antibody to 27.5 in the presence of the monoclonal antibody. In addition to binding to rcIgE$_{100-427}$, C2 cells were able to bind to the rcIgE$_{100-427}$:TRAIL$_{114-282}$ fusion protein but not rcTRAIL$_{114-282}$ (Figure 6C).

4. Discussion

The features of the canine patients and cMCT recruited for this study are similar to previous reports (Sfiligoi et al., 2005; Mullins et al., 2006; Newman et al., 2007; Thamm and Vail, 2007). Although a retrospective study of archived tumor tissue would have produced a much larger cohort, we have found RNA extracted from formalin-fixed material to be of relatively poor quality, compared to tissue specifically preserved for molecular analysis (Stell, 2008). FcεRIα mRNA expression was consistent throughout the biopsy samples assessed in the study, which contained both well-differentiated and poorly-differentiated tumors. Although mRNA expression does not necessarily indicate expression of functional protein, consistent FcεRIα mRNA expression suggests that development of IgE-based therapies might be worthwhile. The logical next step would be to

285 analyse a sample cohort composed primarily of high grade, poorly-differentiated or metastatic MCTs
286 where IgE-based immunotherapeutics could be particularly valuable, given that the majority of high
287 grade and disseminated tumors are refractory to current anti-cancer treatments.

288 Established from a cross-breed dog with tumor recurrence, C2 mastocytoma cells are highly
289 differentiated, expressing surface FcεRI capable of binding canine IgE (Lazarus et al., 1986, Brazis et
290 al., 2002; Hunter et al., 2009). The MCLA mastocytoma cell line was established from a Labrador
291 retriever with a cytologically diagnosed, metastatic MCT. Cells demonstrated typical morphology,
292 although they failed to express FcεRIα. Expression of FcεRIα might have been absent at the time of
293 biopsy, consistent with a neoplasm derived from an early mast cell precursor cell (Thompson et al.,
294 1990; Rottem et al., 1992), de-differentiation and loss of FcεRIα expression in a tumor arising from a
295 later precursor or a mature mast cell, or might have been downregulated during establishment of
296 the cell line, in the absence of IgE which stimulates expression of the receptor (Furuichi et al., 1985;
297 Hsu and MacGlashan, 1996; Yamaguchi et al., 1997; Kubo et al., 2001). There were no detectable KIT
298 exon 11 mutations in this cell line, which also lacked KIT mRNA expression (data not shown). This
299 might also be evidence of an early mast cell precursor lineage, although canine mast cell lines lacking
300 KIT expression and KIT mutation have been reported which are also independent of stem cell factor
301 for their propagation (Ohmori et al., 2008). However, it is difficult to reconcile the lack of FcεRI and
302 KIT mRNA expression with the characteristic morphological appearance of this cell line and the
303 expression of chymase and tryptase mRNA (data not shown), which would suggest more advanced
304 differentiation.

305 Native IgE (with dimerized heavy chains) binds to the FcεRIα chain on the cell surface with a
306 prolonged half-life, compared to other types of Fc receptor, whereas IgE heavy chain monomers are
307 rapidly internalized (Menon et al., 1986). In the current study, constructs were designed to allow
308 dimerisation of the heavy chain fragments of $rcIgE_{100\text{-}427}$ as this might facilitate long-term cell surface
309 binding for detection and in the case of $rcIgE_{100\text{-}427}$:$TRAIL_{114\text{-}282}$ would also increase the local

concentration of TRAIL death domain moieties on the cell surface for signalling through relevant TRAIL receptors. However, formation of inappropriate intra-molecular disulphide bonds or intermolecular disulphide bonds between $rcIgE_{100-427}$ proteins, might have resulted in an altered tertiary and/or quaternary structure. There are two unpaired cysteine residues within the fusion protein (IgE_{100CYS} and $TRAIL_{230CYS}$) that could potentially lead to misfolding. $Cysteine_{230}$ in human TRAIL is important for trimerisation, with mutation of this amino acid abrogating its apoptosis-inducing effect (Seol and Billiar, 2000; Trabzuni et al., 2000; Kelley et al., 2001). An IgE construct lacking the N-terminal cysteine was generated (pSecTagA/IgE_{99-427}) although this failed to express recombinant protein when transfected into CHO cells (data not shown).

An ELISA exploiting the polyhistidine epitope tag for capture and c-myc tag for detection consistently demonstrated recombinant proteins secreted into the supernatant of transfected CHO cells, although the relative immunoreactivity varied between experiments, likely reflecting differences in transfection efficiencies in different wells. Substituting the c-myc antibody with anti-canine IgE or anti-TRAIL antibodies revealed the presence of IgE and/or TRAIL epitopes in the recombinant proteins, suggesting adequate levels of protein folding. Furthermore, a modified Allercept system demonstrated binding of epitope-tagged $rcIgE_{100-427}$ to a recombinant FcεRIα conjugate, usually employed to detect serum IgE in dogs suffering from allergic disease. Similar binding of the $rcIgE_{100-427}$:$TRAIL_{114-282}$ fusion protein was also demonstrated using this assay, confirming that addition of the TRAIL component did not interfere with the ability of the IgE Fc to bind to its cognate receptor.

Binding of recombinant IgE Fc constructs to canine FcεRI was further investigated by flow cytometry, taking advantage of the availability of the novel FcεRI negative MCLA mast cell line as a control for the FcεRI positive C2 mastocytoma line. Specific binding of recombinant $IgE_{100-427}$ to C2 cells was demonstrated, which could be inhibited by monoclonal canine IgE. These results provide strong supportive evidence for specific binding of $rcIgE_{100-427}$ to canine FcεRI. The C2 cellular binding

335 experiments were conducted at 4 °C and it might be the case that at 37 °C rcIgE$_{100-427}$ proteins could
336 possibly be endocytosed and internalized. This could have been detected through incubating cells
337 for varying periods at 4 °C or 37 °C and comparing the binding of recombinant proteins on the cell
338 surface by anti-c-myc/anti-cIgE staining. Alternatives could have included assessing binding at 37 °C
339 in the presence or absence of a metabolic inhibitor (e.g. sodium azide) to prevent internalisation, or
340 the use of confocal microscopy.

341 The authors have previously demonstrated the preferential efficacy of rhTRAIL$_{114-281}$ in
342 inducing apoptosis in C2 cells compared to non-neoplastic MDCK cells (Elders et al., 2009). Herein,
343 rcTRAIL$_{114-282}$ was selected as a prototype cytotoxic agent that was added to the IgE delivery system,
344 as this would potentially combine selective targeting and selective killing of malignant cells of this
345 lineage. This particular TRAIL fragment is similar to the one shown to induce apoptosis in several
346 canine neoplastic cell lines (Rong et al., 2008), although the rcTRAIL protein used by Rong et al.,
347 (2008) is slightly bigger than the current protein (by 3 amino acids at the N-terminus) and the
348 current construct expressed C-terminal c-myc and polyhistidine tags.

349 Although receptor-binding studies demonstrated that rcIgE$_{100-427}$:TRAIL$_{114-282}$ fusion protein
350 was able to bind to C2 cells, subsequent cell viability assays failed to demonstrate any increased
351 levels of apoptosis when C2 cells were cultured in the presence of the recombinant fusion protein
352 (data not shown). Reasons for the lack of biological activity of the fusion protein include inadequate
353 concentration, interference from other components in the cell supernatant or mis-folding of the
354 recombinant protein. Furthermore, whereas IgE monomers form dimers, TRAIL functions exclusively
355 as a trimer (Trabzuni et al., 2000). Thus, the fusion protein as designed would not have an intrinsic
356 apoptosis-inducing effect. Further experiments combining IgE Fc-TRAIL fusion protein with TRAIL
357 monomers, attempting to enhance C-terminal trimerisation also failed to demonstrate any biological
358 effect (data not shown).

359

5. Conclusions

The specific binding of recombinant canine IgE Fc and a TRAIL fusion protein to canine mast cells expressing FcεRI is proof-of-principle that an IgE-targeted approach is a feasible strategy for mast cell-directed immunotherapeutics in dogs. TRAIL targeted to mast cells via IgE offered the possibility of induction of apoptosis specifically in neoplastic mast cells; however, this proved inefficacious *in vitro*. Future work aims to generate alternative IgE-Fc fusion proteins that can be utilized for antibody-directed enzyme prodrug therapy (ADEPT). As other cells seem capable of performing the majority of the beneficial functions of mast cells *in vivo*, the therapeutic index for such IgE-based immunotherapeutics is likely to be high for cMCT-bearing patients, provided that adequate endo-and ecto-parasite control is implemented during therapy, with likely repopulation of the tissues with mast cells from bone-marrow derived precursors after treatment is complete.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Fig. 1. Screening of canine MCT biopsies and mastocytoma cell lines for expression of FcεRIα mRNA. PCR was used to assess FcεRIα mRNA expression (273 bp amplicon) in (A) cMCT biopsies (B-Z) and (B) mastocytoma cell lines C2 and MCLA (MC). PCR products were separated by agarose gel electrophoresis and visualized under UV illumination. La: 100 bp molecular weight ladder. H2O: water negative control.

Fig. 2. PCR screening for canine IgE mRNA in control and transfected CHO cells. PCR was used to amplify the C_Hε2-4 region of canine IgE from cDNA prepared from CHO cells transfected with the pSecTagA vector containing this region of the genomic sequence. PCR products were separated by agarose gel electrophoresis and visualized under UV illumination. L = 100 bp molecular weight ladder. H = water negative control; G = genomic DNA positive control; UC = untransfected CHO cDNA; UN = untransfected CHO NRT; TN = transfected CHO NRT; TC = transfected CHO cDNA, which produced amplicons with neither (largest fragment), one or both (smallest fragment) introns excised.

Fig. 3. Detection of recombinant proteins in transfected CHO supernatant. Untransfected (Mock) and pSecTagA/rcIgE$_{100-427}$ (IgE), pSecTagA/rcIgE$_{100-427}$: rcTRAIL$_{114-282}$ (IgE/TRAIL), pSecTagA/rcTRAIL$_{114-282}$ (TRAIL) transfected CHO supernatants were harvested 90 h post transfection. Supernatants were applied to wells of Ni-NTA HisSorb plates and recombinant epitope-tagged proteins detected using by ELISA using anti-c-myc (A) anti-canine IgE (B) or anti-TRAIL (C) antibody conjugates. A polyhistidine-tagged recombinant human TRAIL protein was used as a positive control in (C). Results are show as the mean of triplicate wells ± SEM, corrected by subtracting the mean value from wells

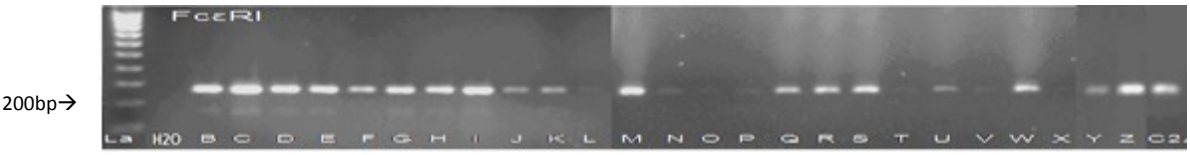
where medium only were applied. Experiments were repeated, with demonstration of recombinant protein expressed from each construct, although the relative levels of recombinant protein immunoreactivity varied between transfections. O.D. = optical density.

Fig. 4. Detection of recombinant proteins in transfected CHO supernatants by Western blotting. Recombinant protein, purified from CHO supernatant using MagneHis beads, were separated under reducing conditions by SDS PAGE and immunoblotted. Recombinant protein was detected using anti-polyhistidine:HRP conjugate and enhanced chemiluminescence with 5 and 30 min exposure of autoradiography film. The arrow indicates histidine-tagged recombinant canine TRAIL after 5 minutes exposure, which is more apparent after 30 min exposure. Lad = Histidine-tagged molecular weight ladder; U = unpurified, untransfected CHO supernatant; I = purified $IgE_{100-427}$; F = purified $IgE_{100-427}$:$TRAIL_{114-282}$; T = purified $TRAIL_{114-282}$.

Fig. 5. Binding of $rcIgE_{100-427}$ and $rcIgE_{100-427}$:$TRAIL_{114-282}$ in the Allercept assay. Recombinant proteins within transfected CHO supernatants were captured using HisSorb strips (A) or Maxisorb ELISA plates coated with anti-canine IgE antibody (B) and their ability to bind to the FcεRIα chain was assessed using the Allercept system. IgE = pSecTagA/$IgE_{100-427}$-transfected CHO supernatant; IgE:TRAIL = pSecTagA/IgE_{100-}427 : $TRAIL_{114-282}$-transfected CHO supernatant; TRAIL = pSecTagA/ $TRAIL_{114-282}$-transfected CHO supernatant.

Fig. 6. Binding of recombinant proteins to C2 and MCLA mastocytoma cells. C2 (A and C) or MCLA cells (B) were incubated with mock-transfected CHO supernatant (medium) or supernatant containing $rcIgE_{100-427}$ (rcIgE), $rcIgE_{100-427}$:$TRAIL_{114-282}$ (rcIgE/TRAIL) or $rcTRAIL_{114-282}$ (rcTRAIL), followed by labelling with polyclonal anti-canine IgE:FITC (panel B) or polyclonal anti-c-myc:FITC (panel C) or in the absence of a secondary antibody (panel A) and analysed by flow cytometry. The graphs show histogram overlays of cells incubated with the various recombinant proteins. The tables show mean fluorescence intensity (MFI) data for each incubation condition.

596 **Figure 1A**



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599 **Figure 1B**

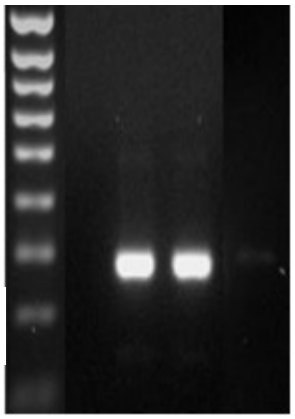

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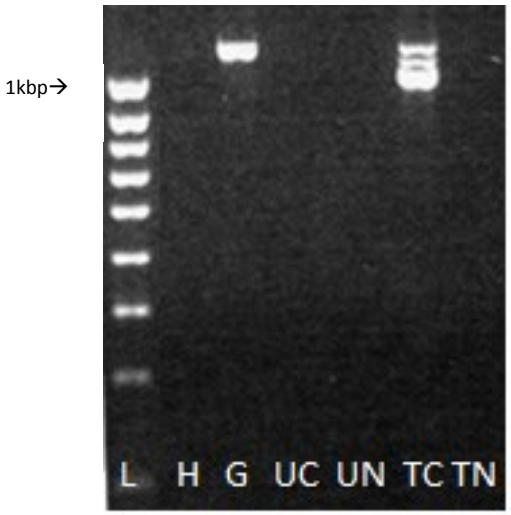
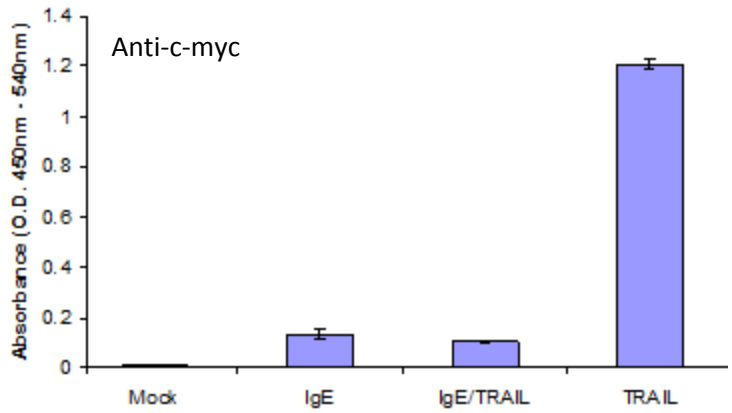
Figure 2



606 **Figure 3A**



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610 **Figure 3B**

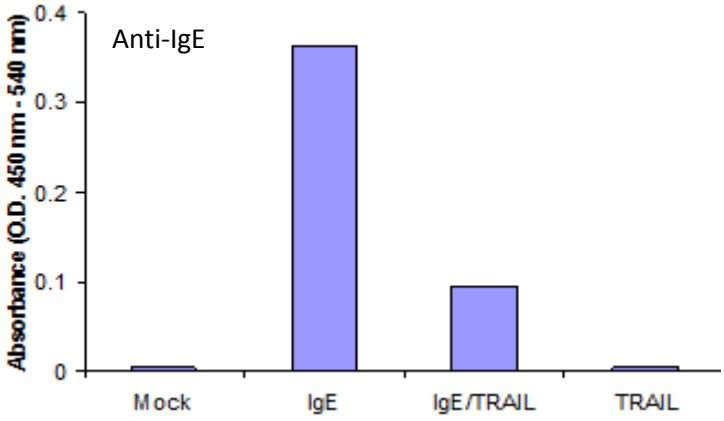

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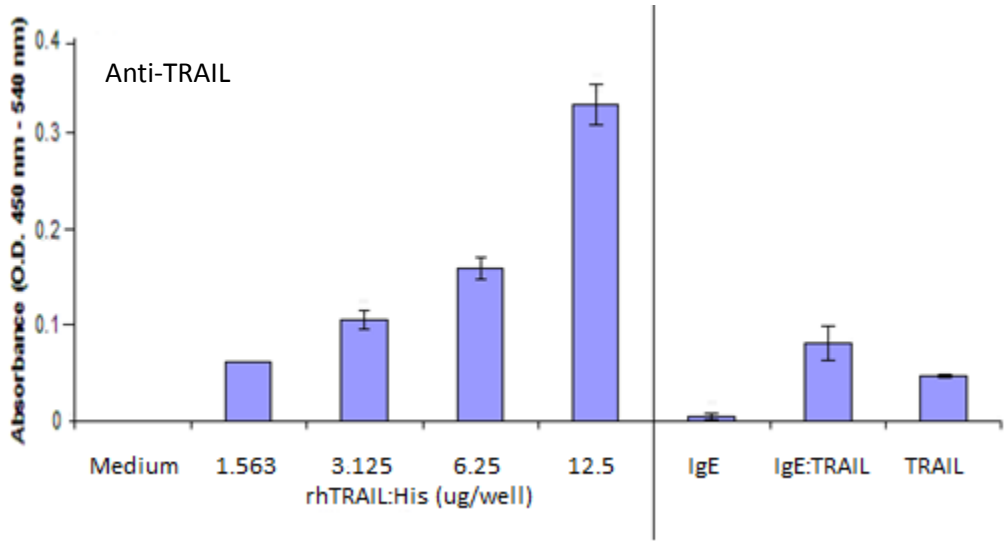
Figure 3C


Anti-TRAIL
Absorbance (O.D. 450 nm - 540 nm)
0.4
0.3
0.2
0.1
0
Medium
1.563
3.125
6.25
12.5
IgE
IgE:TRAIL
TRAIL
rhTRAIL:His (ug/well)

618 **Figure 4**

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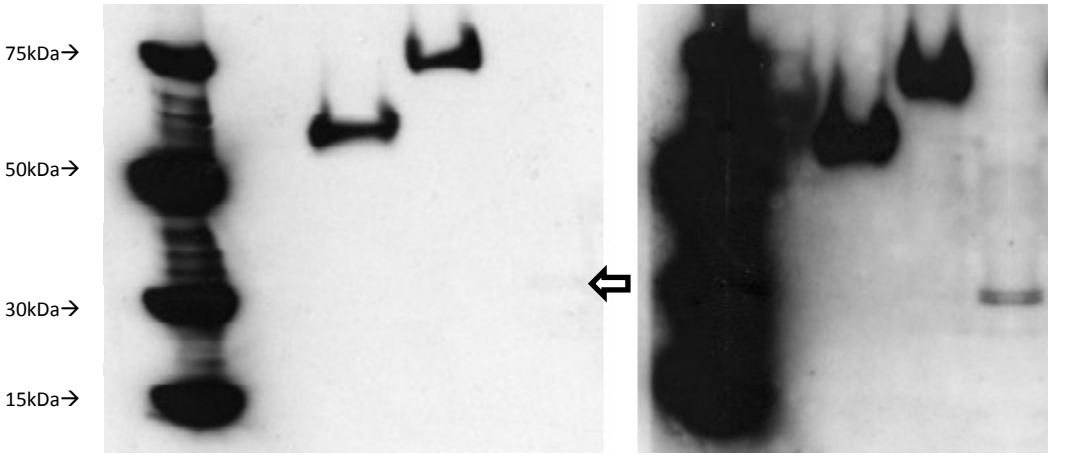

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622 **Figure 5**

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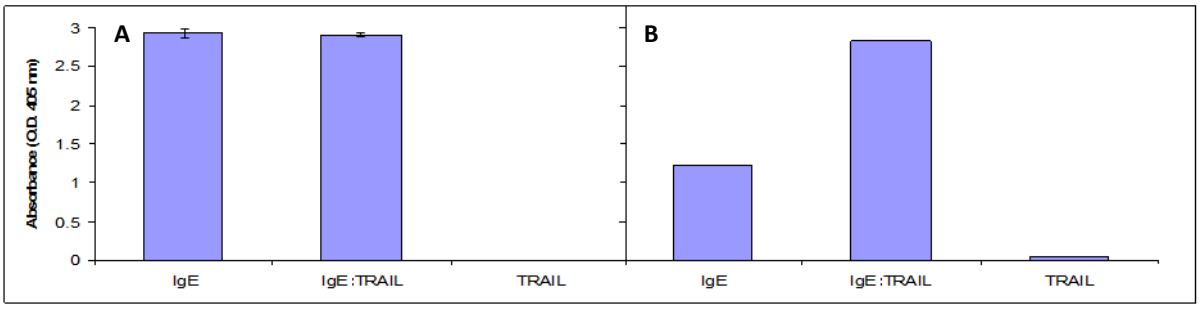

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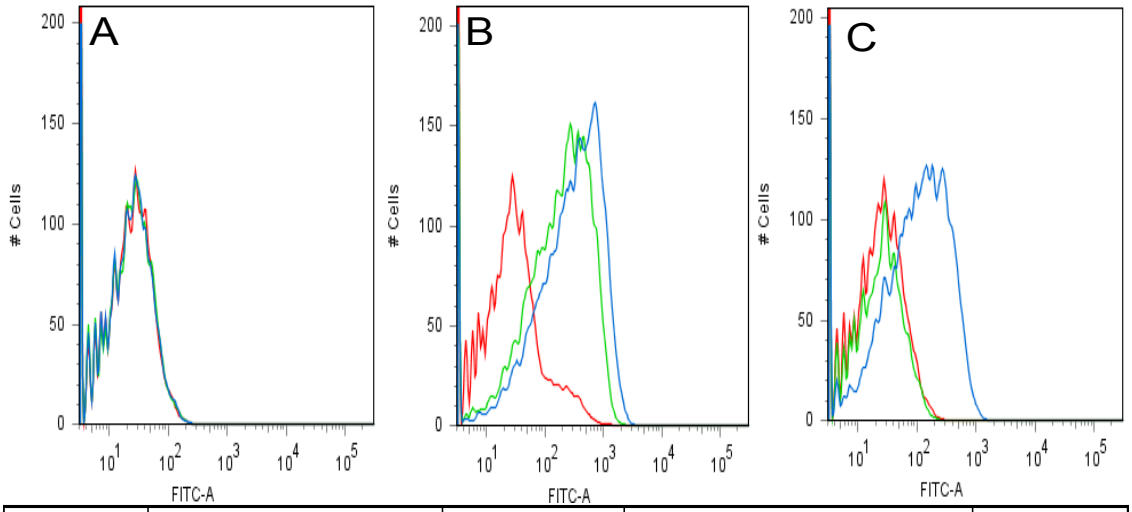
Figure 6A

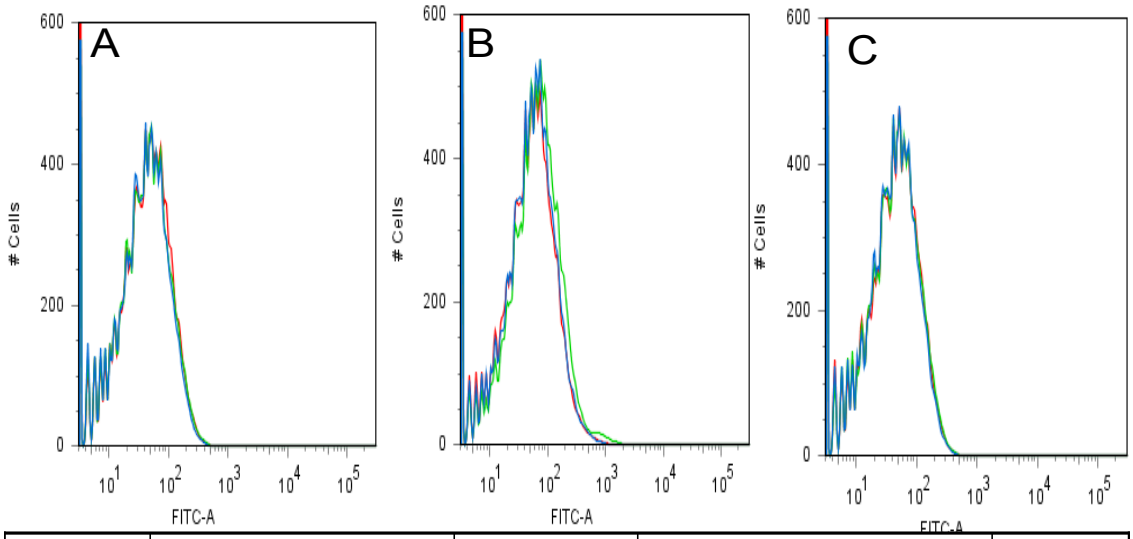
C2 cells



	Antibody	Medium	Monoclonal cIgE	rcIgE
A	None	8.53	8.61	8.59
B	cIgE:FITC	11.39	133.9	219.89
C	c-myc:FITC	8.97	8.77	64.6

Figure 6B

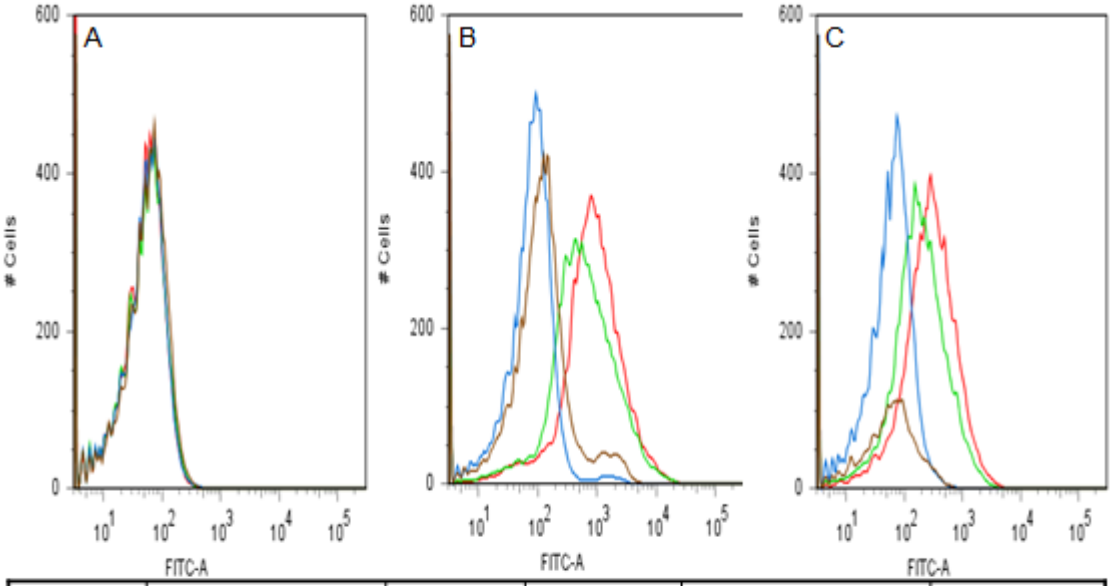
MCLA cells



		Medium	Monoclonal cIgE	rcIgE
A	None	20.04	29.74	19.54
B	cIgE:FITC	19.26	36.63	20.42
C	c-myc:FITC	18.23	27.55	20.12

Figure 6C

C2 cells



		Medium	rcIgE	rcIgE/TRAIL	rcTRAIL
A	None	34.14	29.85	29.57	29.69
B	cIgE:FITC	85.59	670.5	474.23	55.02
C	c-myc:FITC	29.22	227.84	145.75	37.22

Table 1. Primers used in study

Target gene	Primer sequence (5'-3')	Size (bp)	Genbank #
FCERIA	F: AGTGGGGAGTACAGGTGTCG R: GCCTGAGCAGGAATAGTTGC	273	NM_00110766.1
IGHE	F: AAGCTTATGTGCCTTAAACTTCATTCCG R: TTTACCGGGGGTTTTGGACAC	988(cDNA) 1152(gDNA)	XM_548007.1
TRAIL	F: CGAGGTTCTCAGAGAGTAGCT R: CTCGAGGCAGCGTATTTTGCCGATTA	522	NM_001130836.1

F = forward/sense primer; R = reverse/antisense primer. Restriction enzyme sites (*Hind*III: AAGCTT and *Xho*I: CTCGAG) are shown underlined.

Table 2. Details of MCT-bearing patients.

Identifier	Age	Breed	Sex	Tumor site	Differentiation review	Location review
B	10y11m	Crossbred (Lurcher)	FN	Tail	Intermediate	Skin/SQ
C	7y6m	German shepherd dog	FN	Visceral	Poor	Skin
D	9y5m	Crossbred	FN	Thigh	Intermediate	SQ
E	8y9m	Staffordshire bull terrier	ME	Scrotum	Intermediate	Skin
F	9y1m	Rhodesian ridgeback	ME	Stifle	Well	Skin
G	8y11m	Labrador retriever	FN	Interdigital	Well	Skin
H	9y1m	Staffordshire bull terrier	MN	Muzzle	(Cytology only)	
I	11y	Crossbred (collie-type)	FN	Ventral abdomen	Poor	Skin
J	8y11m	Staffordshire bull terrier	MN	Thigh	Intermediate	SQ
K	6y4m	Labrador retriever	MN	Thigh	Intermediate	Skin
L	11y3m	Crossbred	FN	Ventral abdomen	(Cytology only)	
M	7y	Golden retriever	FE	Flank	Intermediate	Skin
N	8y1m	Labrador retriever	FN	Thigh	Intermediate	Skin/SQ
O	11y10m	Shih Tzu	MN	Prepuce	Intermediate	SQ
P	8y2m	Labrador retriever	FE	Thigh	Intermediate	Skin
Q	11y1m	Labrador retriever	MN	Scrotum	(Cytology only)	
R	9y1m	Staffordshire bull terrier	ME	Thigh	Intermediate	SQ
S	12y7m	Labrador retriever	ME	Ventral abdomen	(Cytology only)	
T	11y	Labrador retriever	MN	Prepuce	Intermediate	SQ
U	9y10m	Staffordshire bull terrier	FN	Thorax	Intermediate	SQ
V	9y5m	Labrador retriever	MN	Pinna	Intermediate	Skin
W	10y	Labrador retriever	FN	Flank	Intermediate	SQ
X	6y	Jack Russell terrier	MN	Thigh	Intermediate	Skin
Y	5y	Boxer	FN	Antebrachium	Intermediate	SQ
Z	8y4m	Staffordshire bull terrier	FN	Calf	Poor	Connective Tissue

FE: female entire; FN: female neutered; LTF: histopathology slides lost to follow-up; ME: male entire; MN: male neutered; SQ: subcutaneous.

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