PRODUCTION AND UTILISATION OF INTERLEUKIN-15 IN MALIGNANT CATARRHAL FEVER

Ian E. Anderson[1], David Deane[1], Sandi Swa[2], Jackie Thomson[1], Iris Campbell[1], David Buxton[1], Xiao-Qing Wei[3], James Stewart[4], George Russell[1] and Haig, D.M[1]*.

[1]The Moredun Research Institute, Edinburgh, Scotland, U.K. [2] c/o the Moredun Research Institute, Edinburgh, Scotland, U.K. [3]The University of Cardiff, Wales, U.K. [4]The University of Liverpool, U.K.

*Corresponding author: Dr D. M. Haig, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Scotland.

TEL: 0131 445 5111

FAX: 0131 445 6111

E-mail: david.haig@moredun.ac.uk

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SUMMARY

Malignant catarrhal fever is an often fatal lymphoproliferative disease of ungulates caused by either Alcelaphine herpesvirus-1 (AlHV-1) or Ovine herpesvirus-2 (OvHV-2). The pathogenesis is poorly understood, but appears to involve an auto-destructive pathology where active cytotoxic cells destroy areas of a variety of tissues. Interleukin-15 is a cytokine that develops and maintains cytotoxic cells and may therefore have a role in the pathogenesis of MCF. We show herein that the virus-infected large granular lymphocytes (LGL) obtained from MCF-affected rabbit tissues exhibited a similar proliferative response to both IL-15 and to IL-2 in culture, but their activated cytotoxic enzyme (BLT-esterase) content was maintained at higher levels in the presence of IL-15 compared to IL-2. The LGLs did not produce IL-15 (either mRNA or protein). Abundant expression of IL-15 mRNA and protein was detected in tissues rich in MCF lesions. Interestingly, IL-15 production was associated with the MLN and appendix of OvHV-2-infected rabbits where necrotic lesions were frequent, but not in the same tissues of AlHV-1-infected rabbits that lacked necrotic lesions. The cellular source of the IL-15 was predominantly lymphocyte-like cells that lacked B cell or monocyte- macrophage markers. Only a few IL-15+ cells (<10%) co-localised with pan T cells or CD8+ T cells. The results show a marked abundance of IL-15 in MCF, associated with tissue lesions that confers on the cytokine a potential role in MCF pathogenesis.

INTRODUCTION

Malignant Catarrhal Fever (MCF) is an acute, often fatal lymphoproliferative disease of cattle, deer and other susceptible ungulates (Reid, 2000; Coulter *et al.,* 2001). The known causative agents are either of two related γ-herpesviruses – Alcelaphine herpesvirus-1 (AlHV-1) and Ovine herpesvirus-2 (OvHV-2), the genome sequences of which have been obtained (Ensser *et al*., 1997; Hart *et al*., 2007). The natural reservoir host for AlHV-1 is the wildebeest (*Connochaetes spp*), and for OvHV-2 is the sheep (*Ovis aries)*. In these animals there is no apparent disease and the virus persists as a benign infection. However, a proportion of cattle, deer or other susceptible species that are in the vicinity of infected lambs or wildebeest calves can become infected and develop MCF which is normally fatal. MCF is currently a serious problem in cattle in Indonesia and Africa and bison in the USA. Rabbits develop MCF after experimental infection with AlHV-1 or OvHV-2 that is characteristic of the disease in cattle (Buxton and Reid 1980; Buxton *et al*., 1984).

MCF is characterised by hyperplasia of lymphoid organs and areas of necrosis in these and in non-lymphoid tissues associated with the infiltration of large numbers of T lymphocytes and lymphoblasts. Vasculitis is common (Liggitt and DeMartini 1980a,b; Anderson *et al*., 2007). A feature of MCF is that there is severe pathology in susceptible hosts in spite of the presence of only a few virus-infected cells (as detected by virus DNA). The virus-infected cells in both AlHV-1 and OvHV-2 MCF are lymphocytes at lesion sites in the tissues of MCF-affected animals (Bridgen *et al*., 1992; Simon *et al*., 2003) leading to our current hypothesis that MCF is caused by the auto-destruction of tissues by indiscriminately-cytotoxic lymphocytes, produced as a consequence of MCF virus-infection (Swa *et al*, 2001).

In contrast to the situation *in vivo*, virtually all large granular lymphocyte T cells (LGL) in culture contain viral gene transcripts and antigen (Swa *et al*., 2001). These are derived from the lymph

nodes, spleen, corneal and other tissues of MCF-affected animals (Reid *et al*., 1983, 1989). The LGL transmit MCF when injected into rabbits or other susceptible species. The phenotype of the cultured LGL from OHV-2-infected cattle or AlHV-1 or OvHV-2-infected rabbits is generally characteristic of T cells (Schock *et al*., 1998; Swa *et al*., 2001). Other characteristics of LGL in culture are: constitutive, indiscriminate (i.e. non-MHC-associated) cytotoxicity; lack of mitogen-stimulated proliferation in culture; and in the case of OvHV-2 cattle LGLs, lack of IL-2 mRNA and protein expression, but expression of inflammatory cytokines (e.g. TNF-α and IL-1β) and IFN-γ (Schock *et al*., 1998; Swa *et al*., 2001). We have previously shown that the activated phenotype of the LGLs, associated with MCF virus infection and in the absence of exogenous IL-2, involves constitutively-activated Lck and Fyn src kinases (Swa *et al*., 2001). The LGL represent a population of virus-infected T cells found *in vivo* and are a valuable resource for studying virus-cell interaction in the pathogenesis of MCF.

Interleukin-15 is a pleiotropic cytokine involved in both innate and adaptive immune responses to infection (Ohteki 2002; Waldeman, 2006) that utilises a specific α receptor along with the common β and γ receptor components of the four alpha helix bundle cytokine family that includes IL-2. It is produced by a variety of tissue cells as part of an inflammatory response and stimulates the survival and proliferation of cytotoxic T cells, NK cells and NKT cells. Its production is normally highly-regulated. However, dysregulated expression of IL-15 is associated with a spectrum of autoimmune diseases that involve cytotoxic T cells and/or NK cells (Ruchatz *et al*., 1998; Villasden *et al*., 2003), and over-expression in transgenic mice leads to fatal T-NK cell leukaemia (Fehninger and Caligiuri, 2001). This spectrum of activity could indicate a possible contribution of IL-15 to the pathogenesis of MCF. We are testing the hypothesis that IL-15, dysregulated as a consequence of infection, is involved in a pathway to recruit, activate and maintain the cytotoxic activity of T cells in the tissues of MCF-affected animals. To test this

hypothesis, in the first instance we need to demonstrate that IL-15 is produced and utilised in animals with MCF. Thus, the objectives of the present study were: first, to determine whether LGLs from MCF-affected animals express IL-15 and/or respond to IL-15. Secondly, to determine whether IL-15 was expressed in the lesion-containing tissues of MCF-affected animals.

METHODS

Animals. Nineteen New Zealand White rabbits, 2-3 months of age were used in this study. A group of eight rabbits were inoculated intravenously (IV) with 1-2 x 10^6 AlHV-1-infected rabbit lymphoid cells and another group of eight rabbits with 1-2 x 10^6 OvHV-2-infected rabbit lymphoid cells (see below). A control group consisted of three uninfected rabbits. Rectal temperatures were monitored and on the second day of a febrile response (defined as $\geq 40^oC$) rabbits were euthanased. The animal experiments were carried out with the approval of the Moredun Research Institute's experiments and ethics committee and complied fully with the Home Office of Great Britain and Northern Ireland "Animals (Scientific Procedures) Act 1986".

Viruses and the development of LGL cell lines. AlHV-1 strain C500 (Handley *et al*., 1995; Ensser *et al*., 1997; Swa *et al*., 2001) was maintained by passage in rabbits using infected lymphoid tissue as described above. OvHV-2 strain BJ1035 was originally from a cow with MCF in the UK (Schock *et al.*, 1998; Swa *et al*., 2001; Thonur *et al*., 2006; Hart *et al*., 2006) and was maintained in rabbits by IV inoculation of infected LGL (described below) first, then subsequently with rabbit lymphoid cells.

The AlHV-1-infected LGL cell lines (BJ1859, BJ1860, BJ1263) were generated by injecting AlHV-1 C500-infected rabbit lymphoid cells (a pool of ~10^6 viable MLN and spleen cells from frozen stock) intravenously into rabbits. Two days after a rise in rectal temperature >40^oC, the rabbits were euthanased. MLN and spleen cells were harvested, single cell suspensions prepared

and cultured at 5-10 x 10^6 cells/ml Iscove's modified Dulbecco's medium containing 10% FCS (IMDM) and 10U/ml interleukin-2 (IL-2, (Eurocetus, U.K.) in 25 cm^2 flasks.

The BJ1035, BJ1104, BJ1044 and BJ1196 LGL lines were derived from cattle naturally-infected with OvHV-2 (Schock *et al*., 1998; Swa et al., 2001; and unpublished data). The BJ1857 LGL line was derived from the lymphoid tissue of a rabbit with MCF induced by intravenous IV injection of ~ 10^6 OvHV-2-infected rabbit lymphoid tissue cells (the OvHV-2 originally from a clinical case of MCF and passaged in rabbits). The LGL were routinely passaged in IMDM containing IL-2. AlHV-1 + and OvHV-2+ LGLs developed after 2-3 weeks of splitting and refeeding the cells with medium containing IL-2 on an approximately weekly basis. After 8 weeks the phenotype of the cells had stabilised (Table 1). AlHV-1 genes and proteins were detected in the LGL using PCR for AlHV-1 DNA and indirect immuno-fluorescence using serum antibody to AlHV-1 proteins from an infected rabbit using techniques described previously (Swa et al., 2001). The presence of OvHV-2 DNA in the cells was detected by PCR as described previously (Swa *et al.,* 2001). OvHV-2 virus proteins were detected within the cells using antibodies from the serum of a convalescent cow and an indirect immunofluorescence technique (Swa *et al*., 2001). LGL phenotype analysis was by indirect immunofluorescence test using the antibodies identified in Table 1.

Control rabbit or bovine cells were T cells derived from peripheral blood mononuclear cells (PBMC) that were obtained by centrifugation of blood over lymphoprep (Nyegard, Oslo, Norway). 2 x 10^6/ml PBMC were stimulated with 5μg/ml Con-A for three days. 2 x 10^5 lymphoblasts/ml medium were expanded for 1-2 weeks in 10U/ml IL-2. The cells were split and refed with the IL-2 and fresh medium every 3-5 days. The control cells were a mixture of CD8+ and CD4+ T cells (rabbit) and CD4+, CD8+ and γδ TCR+ T cells (bovine) and were negative for AlHV-1 or OvHV-2 viral DNA or antigens.

BLT-esterase detection in LGLs. Cytotoxic cell BLT-esterase activity was detected in the LGL and control cells using the tryptase-specific substrate N-α-benzyloxy-carbonyl-L-lysine thiobenzyl ester (BLT, Sigma, Poole, U.K.) as described previously (Haig *et al*., 1996). Cell lysates and cell-free supernates from culture (50µl) were added to 96-well flat-bottomed plates and 200µl of a mixture of 0.3mM BLT and 0.33mM dithio-bis-(2-nitrobenzoic acid) in PBS pH 7.2 was added. Incubation was for 1 hour at 37^{o}C. The reaction was terminated by placing the plates on ice and adding 5mM Pefabloc® protease inhibitor (Boehringer-Mannheim)/ well. Plates were read on an ELISA plate reader at an absorbance of 412nm. Enzyme activity was expressed as Abs_{412}/hour at 37^{o}C/ ml sample (Abs).

Experimental procedure for cytokine stimulation of the LGLs

LGL and control T cells were stimulated for 6 days with various doses of IL-2 or IL-15 (Peprotech Ltd). Viable cell numbers were counted, and the BLT-esterase assay performed on culture supernates (not shown) and cell lysates.

Tissue processing and sampling

Rabbits were infected with either rabbit adapted (by *in vivo* passage) BJ1035 (the OvHV-2 virus that has recently been sequenced (Hart *et al*., 2007) or rabbit-adapted C500 (AlHV-1) as described above. Uninfected rabbit tissues were collected as controls. At autopsy, various tissues were examined for gross signs of MCF. Samples of appendix, kidney, liver, lung, mesenteric lymph node (MLN) and spleen were collected and divided into aliquots for: (1) Formalin fixation for histological analysis of H&E stained sections. (2) 4% paraformaldehyde fixation for IL-15mRNA detection by the *in situ* hybridisation technique and IL-15 antigen by immunohistochemistry (IHC)(see below); (3) Zn salts fixative for IHC using mabs to rabbit alloantigens for cell phenotype analysis. In addition, aliquots of lymphoid cell samples (appendix,

spleen, MLN) were taken fresh to prepare single cell suspensions to obtain cell extracts for western blot analysis of tissue IL-15. All fixation and tissue preparation procedures have been described previously (Gonzales *et al*., 2001; Anderson *et al.*, 2007). Standard 5μm-thick serial sections were cut and mounted on Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany) and dried overnight at 37^{o}C. One section from each paraformaldehyde-fixed tissue was stained with haematoxylin and eosin (H&E). Remaining sections were stored in slide boxes at 4^{o}C until required.

Histopathology

In all infected rabbits (AlHV-1 or OvHV-2), MCF was confirmed by clinical and histological criteria as described previously (Anderson *et al*., 2007). The histopathology of MCF lesions in the different tissues was defined in H&E sections as (a) areas of hyperplasia/ lymphoid cell accumulation (including cells exhibiting mitotic indices) and (b) necrosis (focal tissue destruction). In lymphoid tissues where hyperplasia and/or necrosis were present a score of + was given. In tissues where no changes were present a score of - was assigned. In non-lymphoid tissues, large interstitial lymphoid cell accumulations (affecting >25% of the area of tissue) were scored as ++ and smaller less frequent interstitial accumulations (affecting less than 25% of the area of the tissue) as +. Tissues with no interstitial lymphoid cell accumulations were given a score of - .

Immunohistochemistry

Sections were dewaxed and hydrated using standard procedures. Immunohistochemical labelling was performed manually using the Sequenza rack and coverplate technology (Shandon, Runcorn, UK). The EnVision Plus HRP system (Dako, Ely, UK) was used to detect antibody binding as described previously (Gonzales *et al*., 2001; Anderson *et al.*, 2007). Labelled sections were examined microscopically. Anti-rabbit alloantigen mabs used were: RTH1A (rabbit CD4-

specific), ISC29E (rabbit CD8-specific), RTH 26A (rabbit pan-T-specific), and MRB 29-A (rabbit B cell-specific). These mabs have been described previously (Anderson *et al.,* 2007). For IL-15 detection, mab 247 (R&D systems) was used on paraformaldehyde-fixed tissue treated (after dewaxing) for antigen retrieval with sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 minutes at 95^{o}C. The 247 mab has previously been shown to be specific for rabbit IL-15 (Xiong *et al.*, 2005) as well as for human and murine IL-15.

In situ hybridisation

In situ hybridisation was used to detect and locate IL-15 mRNA in the tissues of MCF-affected and uninfected control rabbits. Rabbit IL-15 cDNA was prepared from mRNA obtained from the MLN of a MCF-affected rabbit and the DNA sequenced. The predicted amino acid sequence was the same as that of rabbit IL-15 described previously (Xiong *et al*., 2005). For riboprobe preparation, restriction enzyme fragments (between 200bp and 500bp) of the IL-15 cDNA were cloned into the transcription vector pSPT 18/19 (Roche diagnostics, Lewes, England). Both sense and antisense RNA probes were prepared by *in vitro* transcription from the T7 and SP6 promoter incorporating digoxigenin UTP (Roche). The concentration of each labelled probe was determined by dot blot. *In situ* hybridisation was as described previously (Anderson *et al.,* 2001), using an Omnislide thermal cycler and wash module (Hybaid, Ashford, England). Briefly, sections were de-waxed and rehydrated and then treated with 7.5µg/ml proteinase-K in 200mM HCL for 15 minutes at 37^{o}C. After post-fixation with 4% paraformaldehyde, sections were acetylated for 10 minutes with 100mM tri-ethanolamine pH 7.0, 0.25% acetic anhydride and then pre-hybridised for 2h at 52^{o}C in 6 x SSC, 45% de-ionised formamide, 5 x Denharts solution and 256µg per ml bovine liver RNA (Sigma). Hybridisation solution was 48% deionised formamide, 5 x Denharts solution, 1000U /ml heparin (Sigma), 0.1% Triton-X 100, 24mM EDTA, 24mM Pipes (Sigma), 720 mM NaCl, 7.2% dextran sulphate (Sigma) and 512µg/ml bovine liver RNA containing digoxigenin-labelled probes (sense or antisense) at concentrations between 0.1 to 0.4

ng/µl. Hybridisation was overnight at 52^{o}C. Sections were washed and treated with RNase to remove unbound probe. Bound probe was detected using anti-digoxigenin antibody Fab fragments conjugated to alkaline phosphatase as described (Anderson *et al*., 2001). Sections were counterstained with haematoxylin.

Western blots

Both Mab 247 and the murine soluble IL-15 receptor protein (sIL15R, his-tagged; Ruchatz et al., 1998; Smith et al., 2000; Wei et al., 2001) were used to detect rabbit IL-15 by western blot. A single cell suspension of each tissue was prepared and the cells lysed in lysis buffer (0.8% CHAPS in Tris-buffered saline pH8.3 with proteinase inhibitors ('Complete',Boehringer Mannheim, UK). Lysates were adjusted to1mg/ml and 10µl samples run on 15% SDS-PAGE gel lanes under reducing conditions. After electro-transfer of proteins to nitrocellulose, the sheets were treated with 1% casein in PBS for 1 hour. After several washes (PBS containing 0.5M NaCl and 0.5% Tween 80), blots were incubated for 1 hour with either 2µg/ml sIL-15R or 1µg/ml biotinylated mab 247 in wash buffer. After washing, bound receptor or antibody was detected by either 2µg/ml anti-his-HRP conjugate (Roche) for the sIL-15R or 2µg/ml strepatavidin-HRP conjugate for mab 247 detection. Visualisation was by ECL chemiluminesence.

Statistical analysis of data.

Student's *t*-test was performed on data normalised by $\log_{10}$ transformation.

RESULTS

IL-15 supported large granular lymphocyte proliferation and BLT-esterase content.

Table 1 shows the phenotype of the LGL cells used in this study. This was similar to virus-infected LGL lines derived from MCF-affected animals and described previously (Schock et al., 1998; Swa et al., 2001). The LGLs showed characteristics of T cells (CD2+ or CD5+, and with a proportion of each line except BJ1035 expressing CD8). A constitutively-activated LGL phenotype was indicated by the presence of BLT-esterase. LGLs were viral DNA positive by PCR and >90% of the cells in each line were positive for virus antigen (either AlHV-1 or OvHV-2 - not shown).

Figure 1 (rabbit LGLs and control T cells) and figure 2 (cattle BJ1035 LGL line and control T cells) show that both the AlHV-1-infected LGL and OvHV-2-infected LGL lines exhibited IL-15 dose-dependent proliferation over 6 days in culture that was similar (in terms of growth curves) both to the response of the cells to IL-2 and also to control non-infected T cell lines stimulated with IL-15 or IL-2 (Fig. 1A, C, E, G; Fig. 2 A,C). The addition of a half maximal dose of IL-2 (1U/ml) with a range of IL-15 concentrations to the rabbit LGL or control T cell lines did not reveal any synergistic effect of the combination over either cytokine alone, although an increased proliferation compared to either cytokine alone was observed (Fig. 1A,C,E,G).

The content of BLT-esterase per 10^6 LGL cells in culture in general showed an inverse relationship to cell proliferation in the presence of both IL-2 and IL-15 (Fig. 1D,F,H compared to Fig. 1C, E,G and Fig. 2D compared to Fig. 2C) in that enzyme content per cell declined as cell numbers increased. However, cells grown in IL-15 maintained higher levels of enzyme /10^6 cells over the dose range 10-100ng/ml than those in IL-2 (Fig. 1D,F,H and Fig. 2D, $P<0.01$ for IL-15 at 10ng/ml, 50ng/ml or 100ng/ml compared to IL-2 at 1, 5 or 10U/ml). In contrast, in control uninfected cells cultured in the higher doses of IL-15 (10ng/ml, 50ng/ml and 100ng/ml) there was

a dose-dependent increase in the content /10^6 cells of BLT-esterase compared to cells in IL-2 (Fig. 1B, $P<0.001$ for 50ng/ml or 100ng/ml IL-15 compared to 5U/ml and 10U/ml IL-2 and Fig 2B, $P<0.007$ for 10ng/ml or 100ng/ml IL-15 compared to 5U/ml or 10U/ml IL-2). Thus, IL-15 at higher doses maintained (LGLs) or induced BLT-esterase (control T cells) in the cells whereas IL-2 did not. In the rabbit cells, the combination of 1U/ml IL-2 with different doses of IL-15 showed decreased cell content of BLT-esterase compared to cells stimulated with IL-15 alone.

LGLs did not express IL-15

The LGL lines (Table 1) were analysed for the expression of IL-15 mRNA by RT-PCR and for IL-15 protein in cell lysates and supernates by western blot. In addition pelleted LGL cell pellets (after centrifugation) were embedded in paraffin wax and taken through the *in situ* hybridisation technique along with other tissues for the detection of IL-15mRNA. Neither IL-15 mRNA nor IL-15 protein was detected in any of the LGL preparations. Figure 3A shows the absence of IL-15 mRNA in the BJ 1859 AlHV-1+ LGL line by *in situ* hybridisation. Fig 3B and C show a positive control and sense negative control respectively for the assay run at the same time.

Tissue cells from MCF-affected animals expressed IL-15.

IL-15 expression in cells or tissues was measured by *in situ* hybridisation (for IL-15 mRNA), IHC and western blot (for IL-15 antigen). Table 2 shows a semi-quantitative score for the frequency of IL-15 labelled cells measured by mab 247 in the various lymphoid and non lymphoid tissues of AlHV-1-infected or OvHV-2-infected animals and uninfected controls and comparing this with a lesion score for the tissues (Anderson *et al.,* 2007*).*

The results of the analysis revealed high frequencies of IL-15-expressing cells in both lymphoid (appendix, MLN, spleen) and non-lymphoid tissues (particularly liver and lung) from rabbits infected with OvHV-2 (Table 2; Fig 3B, Fig 4B,C,E). In general, IL-15 expression was associated with MCF lesions (any or combinations of: areas of necrosis, lymphocyte

accumulations or lymphoid hyperplasia). In contrast to OvHV-2-infected rabbits, there was a relative lack of IL-15-expressing cells in the MLN and appendix of AlHV-1-infected animals (Table 2; Fig. 4B or C compared to Fig. 4A). There was an association between the presence of necrotic lesions and IL-15-expressing cells in the MLN and appendix of OvHV-2+ MCF, and a lack of necrotic lesions and a relatively low level of IL-15-expressing cells in the MLN and appendix of AlHV-1+ MCF that was similar to that seen in uninfected control animals (Table 2). In the spleen, necrotic lesions were not observed but hyperplasia was present in both AlHV-1 MCF and OvHV-2 MCF. There were less IL-15-expressing cells in the AlHV-1 group compared to the OvHV-2 group, but this was not marked (Table 2). In non-lymphoid tissues, lymphoid cell accumulations were more abundant in AlHV-1-infected compared to OvHV-2-infected animals, but there was no obvious difference between the groups in the frequency of IL-15+ cells (Table 2; Fig. 3D,E,F for IL-15mRNA+ cells and controls in AlHV-1-infected rabbit liver).

The distribution of IL-15mRNA-expressing cells in the tissues was the same as for IL-15 antigen-expressing cells. In all tissues in either infection, the IL-15-expressing cells were predominantly mononuclear cells within areas of mainly lymphocyte accumulation and particularly located to interfollicular (T cell) areas in lymphoid tissue (e.g. Fig. 4B,C,D). IL-15 mRNA and protein expression was also associated with some epithelial and endothelial cells within or adjacent to lesions (e.g. Fig 4E,F). However, in serial sections comparing IL-15-expressing cells and CD4+ cells or B cells (by digital overlay), there was no co-localisation. In spite of T cells labelled with the pan T cell mab or CD8-specific mab and the majority of IL-15-expressing cells locating to interstitial regions of lymphoid tissues and within mononuclear cell accumulations in non-lymphoid tissues, only a minority of these (estimated at <10%) co-localised in the serial sections (e.g. Fig. 4C compared to Fig. 4D).

The validity of the semi-quantitative scoring method was confirmed for lymphoid tissue (appendix and MLN) from infected and control animals by the intensity of IL-15 protein bands by western blot (Fig. 5A,B shows MLN samples). IL-15 was found in cell lysates but not in cell

culture concentrated supernates. Fig. 5A,B, shows in addition that the mab 247 and smIL-15R respectively bind to rabbit IL-15, defined by its M.Wt (16kDa) and the fact that it binds to a sIL-15 receptor.

DISCUSSION

In this study, we have shown that the virus-infected LGLs of cattle and a rabbit infected with OvHV-2 or rabbits infected with AlHV-1 responded to IL-15 but did not produce it. In addition we have shown that there was an abundance of IL-15 (mRNA+ cells, IL-15 antigen+ cells and IL-15 western blot) expressed in lesion-rich areas of tissues of both AlHV-1 and OvHV-2 MCF-affected rabbits. In disease-affected MLN and appendix there was a marked difference between OvHV-2 MCF and AlHV-1 MCF as there was a high frequency of IL-15 expressing cells associated with necrotic lesions in OvHV-2 MCF and a lack of IL-15 expressing cells and necrotic lesions in these tissues in AlHV-1 MCF.

LGLs are virus-infected cells obtained from the tissues of MCF-affected animals and are thought to represent the key cells involved in the pathogenesis of MCF (Reid *et al*., 1983; Schock *et al*., 1998; Swa *et al*., 2001). They are indiscriminately cytotoxic for a variety of target cells in culture, and exhibit a constitutively active *lck* and *fyn* kinase signalling pathway (Swa *et al*., 2001). The LGLs did not produce IL-15, which is therefore not involved in an autocrine loop maintaining the phenotype of the LGLs. This is in contrast to HTLV-1-infected T cells where IL-15 and IL-15R are induced, probably to expand the number of target T cells for HTLV-1 infection (Azimi *et al*., 2000; Mariner *et al*., 2001).

However, LGLs responded to IL-15 demonstrating that LGLs express a functional receptor for IL-15 as well as for IL-2. This was shown for LGLs from rabbits infected with either OvHV-2 or AlHV-1 and for a cattle LGL line infected with OvHV-2. IL-15 maintained the cell content of the

cytotoxicity-associated enzyme BLT-esterase in proliferating LGLs at higher levels than IL-2, even though both cytokines stimulated similar rates of cell growth. IL-2 exhibited a dominant negative effect on IL-15-mediated BLT-esterase maintenance per cell when both cytokines were used together. Thus, IL-15 could be an important cytokine for maintaining active cytotoxic cells in MCF. In control uninfected T cells, IL-15 but not IL-2 stimulated the accumulation of BLT-esterase in a dose-dependent fashion, indicating its capacity to develop cytotoxic cells. The BLT-esterase assay detects a tryptase that is part of the perforin-granzyme cytolytic pathway in activated cytotoxic cells and a tryptase of mast cells (Garcia-Sanz *et al.,* 1987; Griffiths and Mueller, 1991; Haig *et al*., 1996). The results are consistent with the function of IL-15 in mouse and man where it recruits, stimulates the development of and maintains the cytotoxic phenotype of cytotoxic cells that include CD8 CTLs, CD4 CTLs, NK cells and NK T cells (Waldemann, 2006). IL-15 is also anti-apoptotic (Marks-Konczalik *et al*., 2000) and dysregulation leading to over-expression of IL-15 can lead to expansion of cytotoxic cells and autoimmunity (Waldeman, 2006). This may be occurring in MCF. Although IL-2 will stimulate proliferation and activation of cytotoxic cells, it does not maintain active cytotoxic cell proliferation as it induces activation-induced cell death (AICD) and the maintenance of regulatory T cells (T_{regs}) that limit T cell responses (Marks-Konczalik *et al.,*2000; Fontenot *et al*., 2005).

We have shown that the IL-15 in MCF-affected animal tissues was produced mainly by mononuclear cells and some epithelial and endothelial cells in areas containing MCF lesions (lymphocyte accumulations in non-lymphoid tissue; hyperplasia and areas of necrosis in lymphoid tissue). As mab 247 is the only antibody we have found that reacts with rabbit IL-15, a quantitative assay (ELISA) of IL-15 production was not possible.

To determine the source of the IL-15, we looked for co-localisation (by IHC in serial sections) of IL-15+ mononuclear cells with lymphocyte subsets. This was seen only with a few pan-T+ cells

and CD8+ T cells in interstitial areas of lymphoid tissue and in lymphocyte accumulations in non-lymphoid tissue. There was no co-localisation with CD4+ T cells or B cells. CD14+ monocyte/macrophage cells are rare in the rabbit tissues studied (Anderson et al., 2007) and in this study there was no co-localisation detected of IL-15+ cells with these (not shown). Thus, the identity of the majority of IL-15+ cells in the mononuclear cell population is unknown. This needs to be revisited using confocal techniques when more rabbit mononuclear cell-specific antibodies and IL-15-specific antibodies become available. In mouse and man, the production of IL-15 has been described mainly from monocytes, macrophages, dendritic cells, epithelial cells and endothelial cells (Oppenheimer-Marks *et al*., 1998; Dubois *et al*., 2002; Ohteki *et al.,*2001). Although T cell expression of IL-15 has been recorded, there is little information on these cells. Recently however, human T cells have been shown to constitutively produce low levels of IL-15 that acts through autocrine or juxtacrine loops to promote *ex vivo* homeostatic T cell proliferation (that *in vivo* would maintain the T cell pool)(Miranda-Carus et al., 2005). Our study in rabbits is the first we are aware of to show IL-15 production in abundance predominantly from lymphocyte-like cells *in vivo* during a virus infection.

In other species, several isoforms of IL-15 have been described, the most common of which is a membrane bound form (Dubois *et al*., 2002; Sato *et al*., 2007). This allows IL-15 -IL-15R interactions to occur mainly by cell contact, involving low levels of cytokine maintained over a long period of time. In this study, the nature of the IL-15 was not fully investigated, although cytokine was found in cell lysates but not in concentrated supernates by western blot, suggesting a predominance of cell-associated (non-secreted) IL-15.

In previous studies, we showed that there are differences in tissue tropism in the MCF caused by AlHV-1 versus OvHV-2 (Buxton et al., 1984; Schock and Reid, 1996; Anderson et al., 2007). In general, there was less tissue necrosis and more lymphoid cell accumulations in AlHV-1

compared to OvHV-2. In particular, there was marked tissue necrosis in the appendix and MLN of OvHV-2-infected rabbits that was absent in AlHV-1-infected animals. In AlHV-1, MCF lesions were more apparent in peripheral lymphoid tissues (popliteal lymph node, prescapular lymph node and prefemoral lymph node) than in the central lymphoid tissues (appendix and MLN)(Schock and Reid, 1996; Anderson *et al.,* 2007). This distribution was the converse for OvHV-2 MCF. In this study, we confirm this observation with respect to MLN and appendix and in addition demonstrate that IL-15 producing cells and the quantity of IL-15 were greatly increased in the tissues from OvHV-2 –infected animals when compared to those of AlHV-1 infected rabbits. Importantly, it is known that lymphoid cell hyperplasia and lymphocyte accumulation lesions *per se* are not associated with the onset of clinical MCF whereas necrotic lesions are (Buxton *et al*., 1984). The association of IL-15 with necrotic lesions is circumstantial evidence of a role for IL-15 in the pathogenesis of MCF.

In other species, IL-15 is an inflammatory cytokine produced early in infections that can initiate cytokine cascades (that include TNF-α and IFN-γ) and develop and expand activated cytotoxic cells (McInnes *et al.,* 1997,1998). In this role, it can be involved in either host protective responses to viruses or, if dysregulated, in autoimmune pathogenesis. In HSV infection for example, IL-15 is required as part of a host inflammatory and immune response to recruit and activate cytotoxic T cells and NK cells that are involved in virus clearance (Tsunobuchi *et al*., 2000; Ashkar *et al.,* 2003). In addition to a protective role, IL-15 has also been implicated in several autoimmune diseases. For example, in several rodent models of autoimmunity, blocking IL-15 with antibodies or soluble receptors abrogated the severity of the disease (Ruchatz *et al.,* 1998; Smith *et al*., 2000; Villadsen *et al.,* 2003). MCF-affected animals die in spite of a marked IL-15 response in the tissues. This supports the contention that IL-15 is involved in MCF pathogenesis and not virus clearance. The results of the present study are consistent with the view

that IL-15 is an active participant in MCF by supporting indiscriminate auto-destruction of host tissues by cytotoxic T or NK cells induced as a consequence of infection.

We conclude that the abundance of IL-15 in MCF - affected tissues, associated with areas rich in MCF lesions confers on the cytokine a possible role in the pathogenesis of MCF. This is further supported by the fact that IL-15 stimulates the proliferation and maintenance of cytotoxic activity in LGLs in culture. These cells, obtained from the tissues of MCF-affected animals are thought to play a key role in MCF pathogenesis.

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Table 1

Phenotype analysis of the LGLs and control T cells used in the study (% positive cells).

Cells*	CD2	CD5	CD4	CD8	CD21	IgM	γδT	BLT-esterase
Control bovine T cells	50	99	43	18	<1	nd	32	48
Control rabbit T cells	nd	97	19	76	nd	<2	nd	34
BJ1035 OvHV-2+ bovLGL	98	98	<1	<1	<1	nd	<1	98
BJ1104 OvHV-2+ bovLGL	97	98	<2	93	<2	nd	5	94
BJ1044 OvHV-2+ bovLGL	96	96	32	56	<1	nd	11	nd
BJ1196 OvHV-2+ bovLGL	98	nd	<1	52	nd	nd	<1	nd
BJ1857 OvHV-2+rabLGL	nd	98	<1	76	nd	<1	nd	95
BJ1859 AlHV-1+ rabLGL	nd	50	<1	50	nd	<1	nd	83
BJ1860 AlHV-1+ rabLGL	nd	<1	<1	45	nd	<1	nd	88
BJ 1263 AlHV-1+ rabLGL	nd	99	2	50	nd	<1	nd	89

*Bov = bovine, rab = rabbit, nd = not done. All LGL exhibited the morphology of large granular lymphocytes and >91% of all LGL line cells were positive for either OvHV-2 or AlHV-1 antigen by immuno-fluorescence. The LGL lines were positive for OvHV-2 or AlHV-1 DNA by PCR, whereas the control uninfected cells did not contain virus DNA.

FACScan analysis of cells labelled by indirect immunofluorescence technique. Murine monoclonal antibodies used: clone CC42 anti-bovine CD2; clone CC8 anti-bovine CD4; clone CC17 anti-bovine CD5; clone CC63 anti-bovine CD8; clone CC15 anti-bovine γδ T cell receptor (γδTCR); clone CC21 anti-bovine CD21; clone KEN-4 anti rabbit CD4; clone KEN-5 anti-rabbit CD5; clone 12C7 anti-rabbit CD8; clone NRBM anti-rabbit IgM. All from Serotec, U.K. or the Institute for Animal Health, Compton. BLT-esterase cytotoxic enzyme detection *in situ* by cytochemistry as described in Haig *et al*., 1996.

Table 2: Expression of IL-15 in AlHV-1 MCF compared to OvHV-2 MCF.

		Lymphoid tissues									Non-lymphoid tissues					
		Appendix			MLN			Spleen			Kidney		Liver		Lung	
Rab	virus	IL15*	N†	H‡	IL15	N	H	IL15	N	H	IL15	L§	IL15	L	IL15	L
O1	OvHV2	3	+	+	3	+	+	3	-	+	nd	nd	3	+	nd	nd
O2	OvHV2	nd	nd	nd	3	+	+	2	-	+	1	-	1	-	1	-
O3	OvHV2	nd	nd	nd	3	+	+	3	-	+	2	+	1	-	1	-
O4	OvHV2	3	+	+	3	+	+	3	-	+	0	-	2	+	2	+
O5	OvHV2	3	+	+	3	+	+	3	-	+	1	+	3	+	1	-
O6	OvHV2	3	+	+	nd	nd	nd	3	-	+	0	+	2	+	2	+
O7	OvHV2	3	+	+	3	+	+	1	-	+	1	-	2	+	3	+
O8	OvHV2	3	+	+	3	+	+	2	-	+	0	-	1	+	3	+
A1	AlHV1	1	-	+	1	-	+	1	-	+	1	++	1	++	1	+
A2	AlHV1	1	-	+	1	-	+	1	-	+	1	++	-	+	1	++
A3	AlHV1	0	-	+	0	-	+	nd	nd	nd	0	++	2	++	2	++
A4	AlHV1	1	-	+	1	-	+	1	-	+	0	++	1	++	2	+
A5	AlHV1	0	-	+	0	-	+	3	-	+	1	+	3	++	3	++
A6	AlHV1	1	-	+	1	-	+	2	-	+	1	++	3	++	2	++
A7	AlHV1	1	-	+	1	-	+	3	-	+	0	+	3	++	3	++
A8	AlHV1	0	-	+	0	-	+	1	-	+	0	-	2	++	2	++
C1	control	0	-	-	1			1	-	-	0	-	-	-	-	-
C2	control	1	-	-	0			1	-	-	0	-	-	-	-	-
C3	control	1	-	-	1			2	-	-	0	-	-	-	-	-

*Total IL-15+ cells (>90% with lymphocyte morphology) scored as follows: 0 = <10/mm^2; 1 = 10-50/mm^2; 2 = 50-200/mm^2; 3 = >200/mm^2. †Areas of necrosis (N): - = none; + = areas of necrosis seen. ‡Hyperplasia (H): - = no change compared to uninfected control tissue; + = increase with respect to control uninfected tissue. §In non-lymphoid tissues, lymphoid cell accumulations (L) were scored as follows: - = none; + = small infrequent accumulations; ++ = larger, more frequent accumulations. Rab = rabbit, nd = not done.

Figure Legends

Figure 1. Rabbit LGL and control T cell proliferation and BLT-esterase expression in culture with interleukin-15 and interleukin-2. 5 x 10^5 LGL or control T cells/ml medium seeded on day 0 and cultured with the cytokines for 6 days. In the absence of IL-15 or IL-2, LGLs declined in number over the 6 day period.

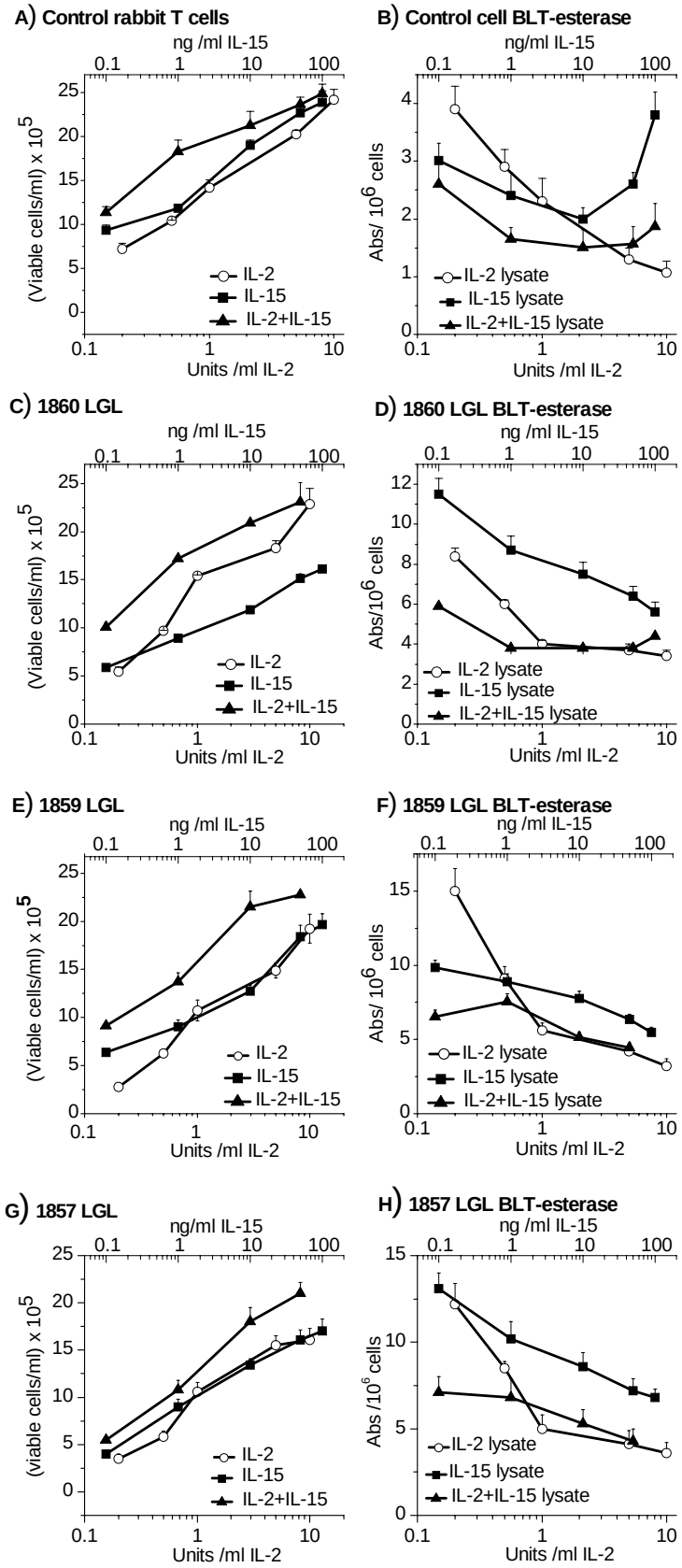
Figure 2. Bovine LGL and control T cell proliferation and BLT-esterase expression in culture with interleukin-15 and interleukin-2. 2.5 x 10^5 LGL or control T cells/ml medium seeded on day 0 and cultured for 6 days. In the absence of IL-15 or IL-2, LGLs declined in number over the 6 day period.

Figure 3. IL-15 mRNA detected by *in situ* hybridisation in MCF-affected tissues, but not in an LGL line. (A) AlHV-1+ LGL line 1859. (B) A positive control for (A) processed at the same time, which shows IL-15mRNA+ mononuclear cells in the interstitial area of a MLN from an OvHV-2-infected rabbit (O4 in Table 2). (C) Sense RNA probe, control for (B) at a lower magnification. (D) H&E stained section of an AlHV-1-infected rabbit liver (A6 in Table 2) showing a lymphocyte accumulation lesion. (E) serial section of (D), sense RNA probe negative control for (F) which is a serial section of (D) showing antisense RNA probe detection of IL-15mRNA+ mononuclear cells in the lymphocyte accumulation lesion.

Figure 4. Immuno-histochemistry detection of IL-15 antigen-expressing cells in MCF-affected tissues using the 247mab. (A) Detail of an area of an AlHV-1-infected rabbit appendix (A2 in Table 2), showing a cluster of three IL-15+ mononuclear cells (arrowhead). (B) IL-15+ mononuclear cells in the interstitial region of an appendix of an OvHV-2-infected rabbit (O4 in Table 2). (C) Lower power magnification of (B) for a comparison with (D), a serial section of (B/C) that shows T cells labelled with the pan-T mab. There is <10% overlap of +ve cells in this

comparison. (E) IL-15 antigen associated with appendix dome epithelium (arrowheads) from an OvHV-2-infected rabbit (O8 in Table 2). (F) Control for (E) at a lower magnification using the IHC labelling technique in the absence of primary 247 mab. N=area of necrosis.

Figure 5. Western blot for IL-15 detection in MLN lysates from uninfected control and virus-infected rabbits. (A) 247 mab western blot showing IL-15 (M.Wt. 16kDa) in MLN cell lysates from a control uninfected rabbit (C1 in Table 2), two AlHV-1-infected rabbits (A4 and A7 in Table 2) and two OvHV-2-infected rabbits (O2 and O8 in Table 2). (B) The same samples as in (G) probed with the murine sIL-15 receptor. The presence of a higher M.Wt. band shown here is sometimes but not always seen in the IL-15 western blots with either sIL-15R or the 247 mab. All lanes received 25ug protein.


A) Control rabbit T cells
ng /ml IL-15
(Viable cells/ml) x 10^5
Units /ml IL-2
IL-2
IL-15
IL-2+IL-15
B) Control cell BLT-esterase
ng/ml IL-15
Abs/ 10^6 cells
Units /ml IL-2
IL-2 lysate
IL-15 lysate
IL-2+IL-15 lysate
C) 1860 LGL
D) 1860 LGL BLT-esterase
E) 1859 LGL
F) 1859 LGL BLT-esterase
G) 1857 LGL
H) 1857 LGL BLT-esterase

1 Figure 1.

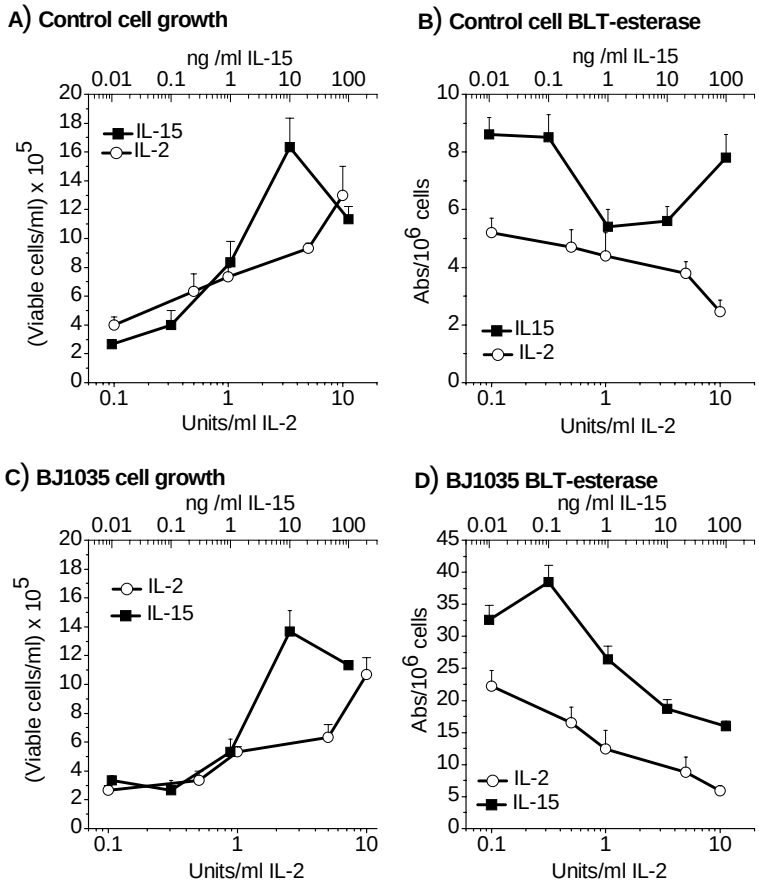
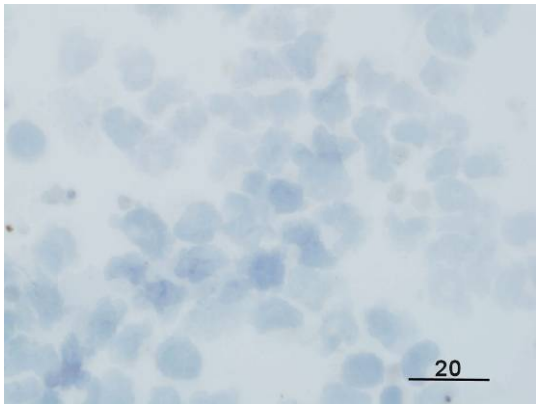

A) Control cell growth
ng /ml IL-15
0.01 0.1 1 10 100
(Viable cells/ml) x 10^5
IL-15
IL-2
Units/ml IL-2
B) Control cell BLT-esterase
Abs/10^6 cells
IL15
IL-2
C) BJ1035 cell growth
D) BJ1035 BLT-esterase

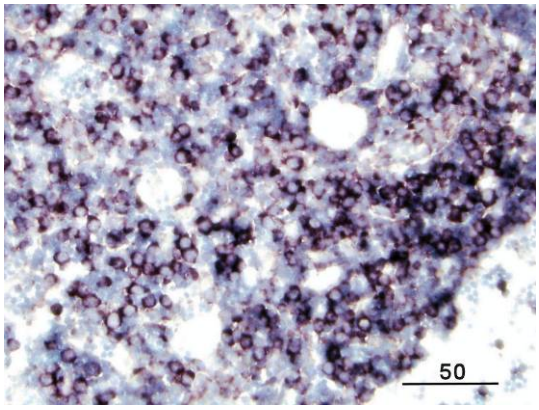
Figure 2.

Figure 3

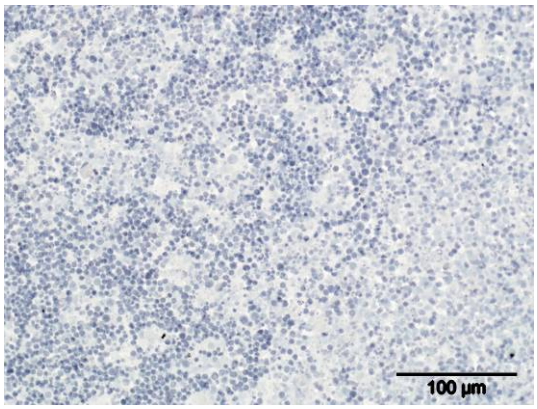
A)



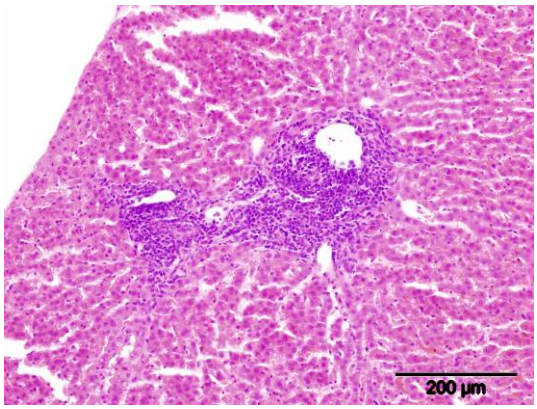
B)



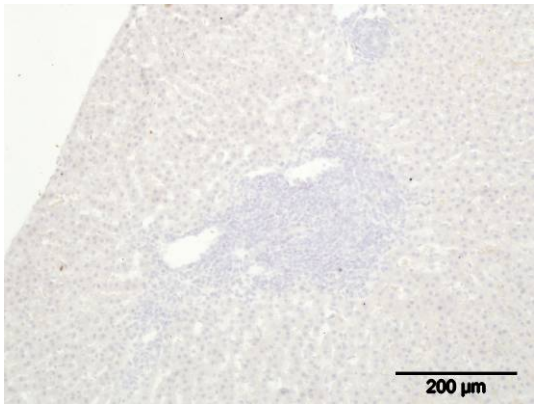
C)



D)



E)



F)

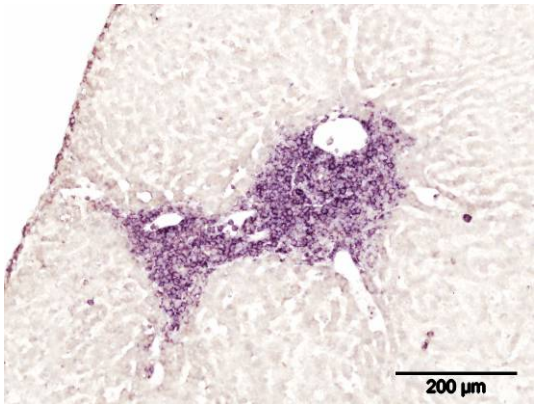
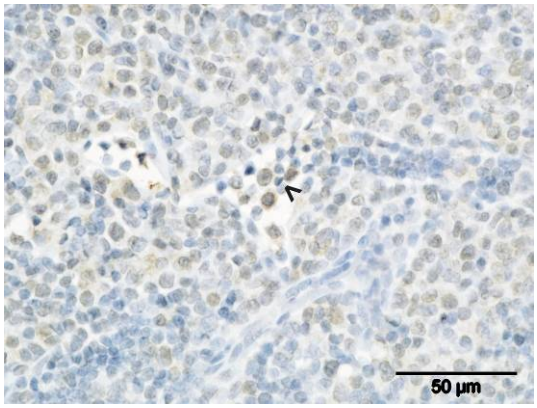
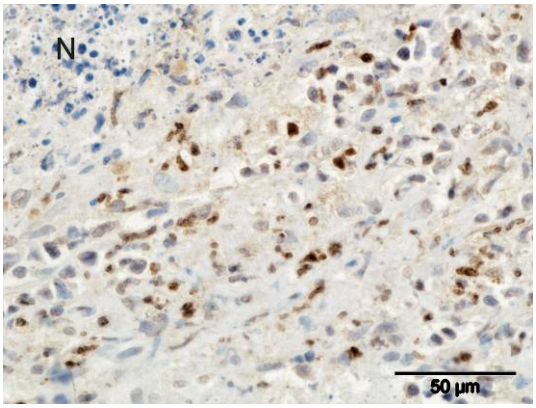


Figure 4

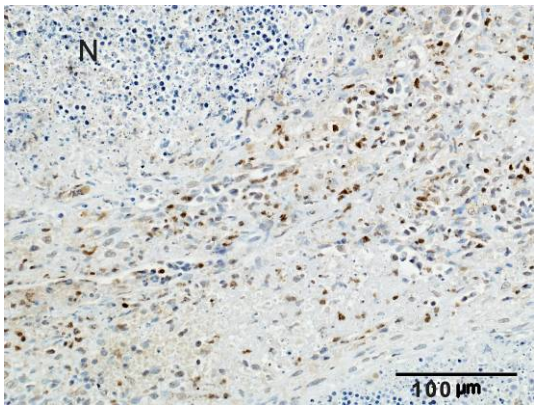
A)



B)



C)



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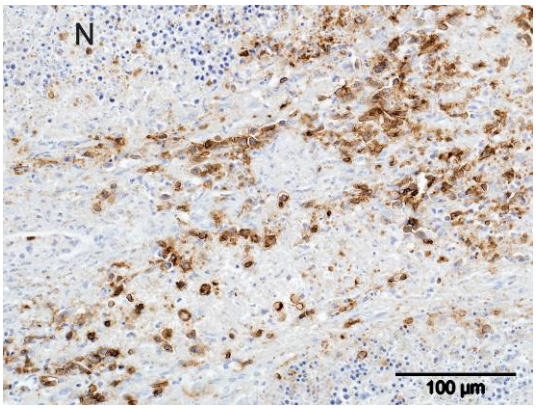

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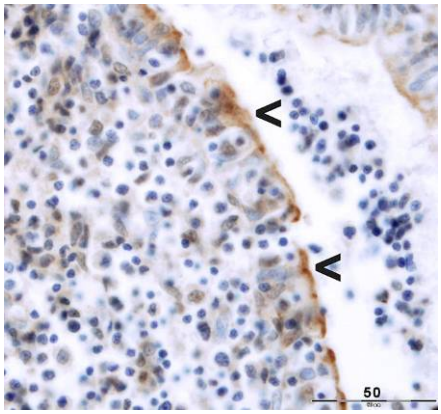

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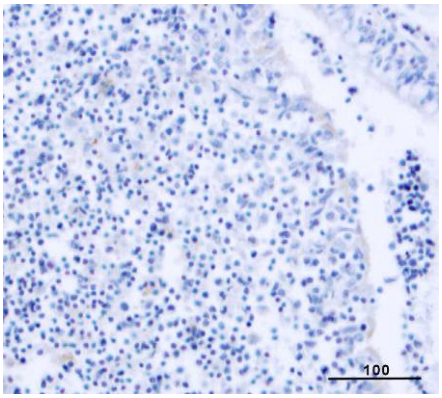
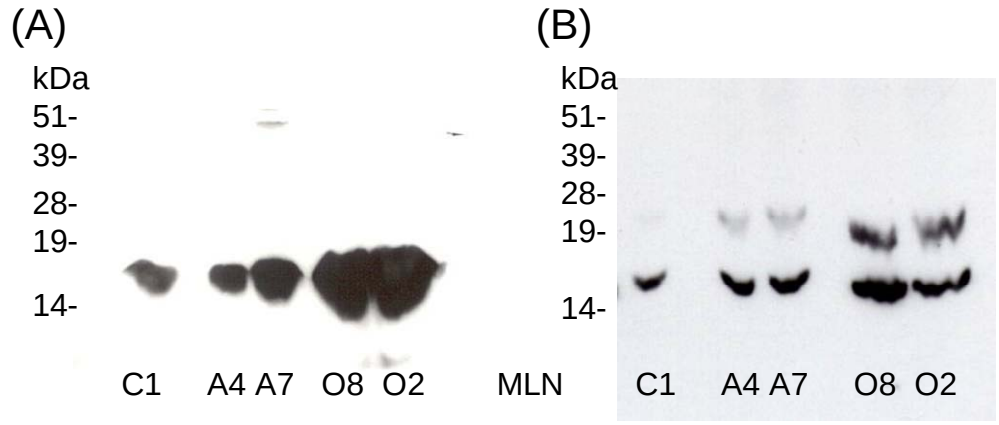


1 Figure 5


(A)
kDa
51-
39-
28-
19-
14-
C1
A4
A7
O8
O2
MLN
(B)
kDa
51-
39-
28-
19-
14-
C1
A4
A7
O8
O2