Presence and interaction of inflammatory cells in the spleen of Atlantic cod infected with *Francisella noatunensis*

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Abstract

Serious infectious diseases accompanied by macrophage-dominated chronic inflammation, are common in farmed Atlantic cod. To increase knowledge relating to morphological aspects of such inflammatory responses, cod were challenged with *Francisella noatunensis,* an important bacterial pathogen of this fish species. Tissue and cell dynamics in the spleen were examined sequentially over a period of 60 days. Small clusters of mainly macrophage-like cells staining for non-specific esterase and acid phosphatase, developed with time. These foci were transiently infiltrated by pleomorphic proliferating cells of unknown nature and by granulocyte-like cells staining for peroxidase and lysozyme. The latter cell-type, which appeared to be resident in the red pulp of control fish, migrated into the inflammatory foci of infected fish. Cells expressing genes encoding IFN-γ and IL-8 increased in number during the study period. Bacteria were detected only in the macrophage-like cells and their number increased despite the extensive inflammation. Our results demonstrate an intimate spatial relationship in inflammatory foci between at least three cell types. The presence of granulocyte-like cells, together with macrophage-like cells, suggests pyogranulomatous inflammation as a more appropriate descriptive term than granulomatous inflammation.

Introduction

Inflammation in fish, including cod, includes cell responses of the monocyte, granulocyte and lymphocyte lineages. As macrophages in many cases appear to be the most common cell type, these inflammations are frequently described as granulomatous (Gudmundsdottir B.K., Bjørnsdottir, Gudmundsdottir S. & Bambiret 2006; Roberts & Rodger 2001). The kidney and spleen are important components of the immune system, and inflammation and tissue destruction may develop if infectious agents are not promptly eliminated from these organs.

In cod, highly phagocytic cells of typical macrophage morphology and function, including bean-shaped nuclei (Sørensen, Sveinbjørnsson, Dalmo, Smedsrød & Bertheussen 1997) and phagocytic cells (Øverland, Pettersen, Rønneseth & Wergeland 2010) described as neutrophils with irregular shaped nuclei (Rønneseth, Wergeland & Pettersen 2006) have been described. Hence, differentiation of macrophages and granulocytes in cod by routine histology, may be difficult.

Unfortunately, there are few specific cellular markers for leukocytes in cod and characterisation of cellular responses in histological sections is therefore limited to description of cell morphology, detection of enzymes typical for different cell types (Rønneseth *et al*. 2006; Sørensen *et al*. 1997) and demonstration of known response markers. The properties of cod macrophages cannot therefore be as fully evaluated as those of e.g. mammalian macrophages (Leenen & Champel 1993). Non-specific esterase (NSE) and acid phosphatase (ACP), but not peroxidase (PO) have been detected in head kidney macrophages of cod (Sørensen *et al*. 1997). Although NSE has been described in lymphocytes in fish (Afonso, Ellis & Silva 1997), it is more consistently detected in fish monocytes and macrophages (Afonso *et al*. 1997; Grove, Johansen, Reitan & Press 2006; Press, Dannevig & Landsverk 1994; Tavares-Dias 2006) and is also regarded as a reliable marker for macrophages in mammals (Kaplow 1981). ACP is present in macrophages of many fish species although it also has been detected in other leukocytes (Afonso *et al*.1997). In the spleen of normal cod, cells described as neutrophils have been shown to

produce PO (Rønneseth *et al*. 2006). This enzyme has been used as a neutrophil marker in cod (Rønneseth *et al*. 2006) and mammals (Faurschou & Borregaard 2003). Although it is absent in the granulocytes of some fish species examined (Zinkl, Cox & Kono 1991) it is present in activated macrophages of fish (Afonso, Lousada, Silva J., Ellis & Silva M.T. 1998) and mammals (Rodrigues, Rodriguez, Russo & Campa 2002). Lysozyme acts against bacterial invaders in fish (Saurabh & Sahoo 2008) and goose-type lysozyme has been shown to be highly expressed in normal cod spleens (Larsen, Solstad, Svineng, Seppola & Jørgensen 2009). Rabbit antiserum (Inami, Solem, Jørgensen & Larsen 2010) raised against a recombinant cod g-type lysozyme (Larsen *et al*. 2009) has been shown, immunohistochemically, to stain granulocyte-like cells (GCLC) in the spleen of cod (Inami *et al*. 2010). Thus, although no general statement can be made relating to enzymes as markers for leukocytes in cod, they may nevertheless serve as tools for characterising cells involved in inflammatory processes.

The cytokine IFN-γ has been cloned from cod (Furnes, Seppola & Robertsen 2009) and other fish species (Milev-Milanovic, Long, Wilson, Bengten, Miller & Chinchar 2006; Zou, Carrington, Collet, Dijkstra, Yoshiura, Bols & Secombes 2005) and its functional properties appear to be as described in mammals (Zou *et al*. 2005), i.e. macrophage activation (Boehm, Klamp, Groot & Howard 1997). In channel catfish it was concluded, as is established in mammals (Schroder, Hertzog, Ravasi & Hume 2004), that IFN-γ is produced by immune cells, i.e. natural killer cells and T cells (Milev-Milovanovic *et al*. 2006). To the best of our knowledge, these cell types have not as yet been identified in cod. IL-8 (CXCL8) is a small cytokine produced by many cell types, and attracts leukocytes including neutrophils in mammals (Mukaida, Harada & Matsushima 1998). While most teleost species examined lack the motif responsible for the neutrophil-attracting abilities of IL-8 (Chen, He, Baoprasertkul, Xu, Li, Serpion, Waldbeiser, Wolters & Liu 2005; Laing, Zou, Wang, Bols, Hirono, Aoki & Secombes 2002), it is present in gadoids including haddock (*Melanogrammus aeglefinus*) (Corripio-Miyar, Bird, Tsamopoulos & Secombes 2007) and Atlantic cod (Seppola, Larsen, Steiro, Robertsen & Jensen 2008).

In the present study we have used the term macrophage-like cells (MLCs) and GCLCs instead of macrophages and granulocytes, respectively. This is based on similarities in cell morphology and descriptions of macrophages and granulocytes from other vertebrates combined with the presence of different enzymes.

The bacterium *Francisella noatunensis* (Mikalsen, Olsen, Tengs & Colquhoun 2007) was utilised to provoke an experimental chronic inflammation. Disease caused by this Gram-negative, facultative intracellular bacterium is associated with systemic infection and multi-organ granuloma (Olsen, Mikalsen, Rode, Alfjorden, Hoel, Straum-Lie, Haldorsen & Colquohoun 2006), and is currently a serious threat to the Norwegian cod farming industry (Nylund, Ottem, Watanebe, Karlsbakk & Krossøy 2006). The aim of this study was to describe morphologically the sequential manifestation of a chronic inflammation in cod and to study and discuss related immunological features from a qualitative perspective.

Materials and methods

Fish, feed and environment

Unvaccinated Atlantic cod of approximately 160g were obtained from a commercial hatchery, transported to Tromsø Aquaculture Research Station (Norway) and kept in 24h light in 300 l in-door tanks supplied with filtered sea water of 12°C and fed once a day with dry feed (Dana feed, Horsnes, Denmark). After an acclimatation period of 10 days, the fish were randomly distributed into two tanks in two different rooms and acclimatised for an additional 10 days.

Cultivation of bacteria for infectivity trial

F. noatunensis (type strain NCIMB 14265) was grown on cystein heart agar (Difco, US) with 5% sheep blood (Olsen et al., 2006) (CHAB) at 22˚C for 7 days. A single colony was transferred to 10ml liquid medium for *F. noatunensis* (Pharmaq, Norway) and incubated on a shaker at 16˚C overnight. The culture was diluted with 0.9% NaCl to

optical density 0.2, which by plate counting was shown to correspond to approximately 10^9 colony-forming units (cfu) ml^{-1}. Infectious doses were generated by diluting the bacteria suspension to 5×10^6 cfu ml^{-1}.

Anesthezia and injection, sampling of fish and processing of samples

Fish were anesthetized in water containing 60 mg $liter^{-1}$ metacain and injected intraperitoneally with 100µl 0.9% NaCl (control fish) or 100µl medium with *F.noatunensis.* Two 5 mm-thick slices of spleen from 10 non-injected fish sampled before the start of the experiment, and 4 control fish and 6 infected sampled 7, 15, 30 and 60 days post-infection (d.p.i.) were fixed for 10-16 h in 4% paraformaldehyde at 4C° and thereafter transferred to 70% ethanol or phosphate-buffered saline (PBS, 0.01M, pH 7.3) with 15% sucrose, respectively. For enzyme staining on cryosections, the tissue stored in PBS with sucrose was embedded in OCT-Tissue tek® (Chemi-teknik AS, Norway) within 5 days, snap frozen in liquid nitrogen and prepared as described below.

Routine histology, immunohistochemistry, immunofluorescences and enzyme staining

Tissues stored in ethanol were embedded in paraffin, and 3 µm-thick sections cut and stained with haematoxylin and eosin (H&E) (Stevens 1990). Single sections of spleen sampled 30 and 60 d.p.i. were stained with Martius Scarlet Blue (MSB) (Bradbury & Gordon 1990). Immunohistochemical staining and *in-situ* hybridization were performed on paraffin-embedded tissues and enzyme staining on cryosections. Sections for the staining methods described below were selected to include representative pathological changes

Immunohistochemistry

All immunohistochemical (IHC) staining techniques were performed on de-waxed and rehydrated paraffin sections following antigen retrieval by microwave heating in citrate buffer essentially as described by Shi *et al*. (Shi, Chaiwun, Young, Cote & Taylor 1993).

The different staining procedures were performed on sections from the same tissue blocks.

Immuno-histochemical staining for *F. noatunensis* was performed on one control fish and two infected fish from each sampling using a polyclonal antibody as described by Zerihun et al. (in press). Amplification of the signal intensity on spleens sampled 7 and 15 d.p.i., was achieved using En Vision (Dako, Denmark) kit with an alkaline and fast red visualisation system. For spleens sampled 30 and 60 d.p.i., a biotinylated anti-rabbit immunoglobulin secondary antibody (Dako)**,** a streptavidin-alkaline-phosphatase complex (GE Healthcare, US) and fast red were used. Sections from cod with confirmed francisellosis were immuno-histochemically stained with polyclonal antibody and normal rabbit serum as positive and negative controls, respectively.

Proliferating cell nuclear antigen (PCNA) was detected according to a procedure modified from Gjessing et al (Gjessing, Kvellestad, Ottesen & Falk 2009) using mono-clonal anti-PCNA clone PC10 (Dako). Staining was performed on one control fish and two infected fish from each sampling using En Vision (Dako) kit with a peroxidase and diaminobenzidine visualisation system. Sections of pyloric ceca, containing crypts with many mitotic cells, were included for positive and negative control and stained with- and without- primary antibody, respectively.

For demonstration of g-type lysozyme, sections were incubated with polyclonal rabbit anti-cod g-type lysozyme (Inami *et al.* 2010). Staining for lysozyme was performed on one control fish and on two infected fish sampled 7, 15 and 30 d.p.i. The secondary antibody was a biotinylated anti-rabbit immunoglobulin (Dako) and streptavidin-alkaline-phosphatase complex (GE Healthcare) and fast red were used for visualization. Sections of spleen from a control fish were stained with normal rabbit serum as negative control.

Double immunofluorescences for lysozyme and peroxidase (PO)

Demonstration of lysozyme and PO co-localisation was performed on a 5µm thawed, air-dried cryosection from a control fish. Firstly, PO was replaced by depositions of biotin-

labeled tyramide by incubation of the section with biotinyl tyramide, a PO substrate (provided by TSA™ Biotin system-kit, PerkinElmer, USA) for 30 min. After washing in Tris-buffered saline (TBS 0.05M, pH 7.6) the sections were incubated with rabbit anti-cod g-type lysozyme. Biotin-labeled tyramide and lysozyme were visualized using Streptavidin texas red (PerkinElmer) and Alexa 488 goat anti-rabbit (Invitrogen, USA), respectively.

Enzyme histochemistry

Five µm thick cryosections from one control fish and one infected fish from each sampling were thawed and air dried for 1h and incubated in a humid chamber with solutions as described below for detection of PO, ACP and NSE, respectively. The various staining procedures were performed on sections from the same tissue blocks. Unless otherwise stated incubations were performed at room temperature, sections washed in tap water and finally mounted in Aquatex® (Chemi-teknik).

PO was demonstrated according to the method of Ganasin (Ganassin, Shirmer & Bols 2000). Briefly, sections were fixed, washed and incubated for 10min in the dark at 37ºC with Fast diaminobenzidine tablets (Sigma, USA) staining solution. To inhibit PO, a serial section was incubated with 10mM phenylhydrazine added to the incubation solution. The sections were counterstained with hematoxylin.

Demonstration of ACP was done as described by Lodja (Lodja, Gossrau & Scheibler 1976) and as previously performed on Atlantic salmon tissues (Press, Reitan & Landsverk 1995). Briefly, sections were incubated for 1h at 37ºC in a solution containing napthtol AR-TR phosphate and hexazonium-p-rosaniline at pH 5.5. To inhibit ACP, a serial section was incubated with 10mM NaF added to the incubation solution. Sections were washed in tap water and left for 2h in 10% neutral phosphate buffered formalin.

Detection of NSE was performed according to the method of Pearse (1972). Briefly, sections were incubated for 15min in a solution containing a-naphtylacetate and

hexazonium-p-rosaniline. To inactivate NSE, a serial section was preheated for 5min at 80ºC in distilled water.

Construction of probes for IFN-γ and IL-8

Isolation of RNA

RNA was isolated from head kidney tissues of Atlantic cod with Trizol™ reagent (Life Technologies Burlington, USA) according to the manufacturer's instructions. The RNA precipitate was air dried for 5–10 min., resuspended in diethylpyrocarbonate (DEPC)-treated water and quantified using the NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, USA).

To amplify Atlantic cod IFN-γ and IL-8 genes, two sets of primers were designed based on genomic DNA sequences of the cod interferon gamma- (gene bank reference gi 224381711; Furnes et al., 2009) and IL-8- genes (gene bank reference gi 157652603; Seppola et al., 2008). The primers for IFN-γ were L-3322 5′GGGAAGGTGTTGGAGGTTTA 3′and R-3323 5′ATCTGTATGTTGCCCAGTTGC 3′, producing an amplicon of 200bp. The primers for IL-8 precursor were L-3301 5′AGATGACAAGCGGCAAAATC 3′ and R-3302 5′GCAACACCAAACAAGAAGCA 3′, producing an amplicon of 460 bp. One step RT-PCR amplifications were performed with Superscript-2™ III One-Step RT-PCR system with Platinum® Taq High Fidelity (Invitrogen) according to the manufacturer instructions.

The prepared PCR mixtures were subjected first to 30min. reverse transcription at 60°C and 3 min. denaturation at 94°C or 95°C for IFN-γ and IL-8 respectively. This was followed by 40 amplification cycles (IFN-γ : 94°C for 25 sec, 60°C for 30 sec and 72°C for 30 sec or IL-8: 95°C for 25 sec, 55°C for 30 sec and 72°C for 30 sec) and a 7 or 6 min elongation step at 72°C for IFN-γ and IL-8, respectively.

Probe preparation

The amplified PCR products of IFN-γ and IL-8 were purified using Nucleospin extract II (Macherey-Nagel, GE) and inserted into pGEM®–T Vector Systems (Promega, USA) according to the manufacturer's recommendation and transformed into *E. coli* competent cells. Plasmid DNA from recovered recombinants was purified using Nucleospin® Plasmid (Macherey-Nagel).

Digoxigenin-labeling of RNA probes

Purified plasmid DNA was digested with restriction enzymes HindIII and PvuII (Promega) overnight at 37°C. For verification of templates, plasmid DNA with insert was sequenced with primers for IFN-γ, IL-8 and M13 primers. *In vitro* transcription was performed using template and the DIG RNA Labelling Kit (Roche Diagnostics, Germany) with the appropriate T7 and SP6 polymerases to generate DIG-labelled RNA anti- and same-sense probes, respectively. Dig-labelled RNA probes were purified using illustra™ probe Quant™ G-50 micro columns probe purification kit (GE Healthcare) according to the manufacturers' instructions.

***In situ* detection of cells expressing IFN-γ and IL-8**

***In situ* hybridization**

In situ hybridizations were performed on de-waxed and re-hydrated paraffin sections from one control fish sampled 60 d.p.i. and one infected fish from each sampling. The different staining procedures were performed on sections from the same tissue blocks.

Incubations were performed at room temperature unless otherwise stated. Serial sections were incubated with anti-sense and positive-sense probes as positive and negative controls, respectively. To avoid ribonuclease contamination, all specimen handling and subsequent procedures were carried out using gloves, chloroform-treated glassware and instruments and diethylpyrocarbonate-treated (DEPC, Sigma) solutions.

In situ hybridization was performed according to Solstad *et al*. (Solstad, Stenvik & Jørgensen 2007). Briefly, after incubation in PBS II (0.02 M, pH 7.3), treatment with

Nonidet P40 (Roche Diagnostics), washing in PBS II, treatment with proteinase K (Finnzymes, Finland) at 37ºC, incubation with 4% phosphate-buffered paraformaldehyde, washing in PBS II , acetylation and a final washing in PBS II, the sections were prehybridised in hybridization buffer [1× Denhards solution, 2× SSC (sodium chloride/sodium citrate, 1× SSC: 0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.5], 0.25 µg μl^{-1} calf thymus DNA (Sigma), 10% dextran sulphate and 40%(v/v) formamide) at 55ºC (IL-8) or 60ºC (IFN-γ) for one hour. The buffer was then removed and 6 ng μl^{-1} (IL-8) and 10 ng μl^{-1} (IFN-γ) of heat-treated probe dissolved in hybridization buffer were added to the sections and incubated at 55ºC (IL-8) or 60ºC (IFN-γ) overnight.

Washing and immunodetection

The slides were subjected to two 10min. washes in SSC solutions of increasing stringency [2×, 1×, and 0.5×, 0.1× SSC preheated to 55°C (IL-8) or 60°C (IFN-γ)].

Immunodetection of IFN-γ was performed using peroxidase conjugated anti-digoxigenin fab fragments (Roche Diagnostics). Signal amplification was accomplished using a TSA™ Biotin system-kit (PerkinElmer) according to the instructions, and visualization was performed using ACE as a substrate. For IL-8, immunodetection was performed using alkaline phosphatase-conjugated anti- digoxigenin Fab-fragments (Roche Diagnostics) and visualization performed with fast red.

Results

Pathology, immunohistochemical staining for *Francisella* and PCNA

All fish had normal appetite and showed no clinical signs of disease throughout the experimental period of 60 days.

Inflammatory foci developed in the internal organs of infected fish throughout the experimental period. In the spleens of three of six fish examined 15 d.p.i. a few nodules less than 1mm in diameter were macroscopically visible on the organ surface or in organ sections . At 30 d.p.i. many small, white nodules were observed in all examined fish, and

at 60 d.p.i these were large and protruding. As the kidney is also hematopoetically active, we chose to focus on histopathological changes in the spleen as a secondary lymphoid organ. The foci were dominated by MCLs containing an increased number of intracellular bacteria and other more transiently present cell types. There was some variation among fish sampled simultaneously, as new foci apparently emerged at different times during the first weeks. We therefore present the main findings according to the sequence in which they were initially observed.

A few small aggregations containing some MLCs, i.e. large cells of varying size with an abundant eosinophilic cytoplasm and a few cells of unknown nature were seen in the red pulp at 7 d.p.i (Fig. 1A-C). A number of the unidentified cells showed blastic nuclei, some of which stained for PCNA, demonstrating proliferation. Staining for *F. noatunensis* revealed low numbers of intracellular bacteria within vacuole-like structures in a few MLCs. Neither these aggregates nor larger foci observed after 15-60 days could be related to the ellipsoids.

At 15 d.p.i. many small foci were identified within the red pulp (Fig. 1D). A few of the MLCs within the foci posessed intracellular, vacuole-like structures containing low numbers of bacterial cells staining for *F. noatunensis* (Fig. 1E-F). The foci were encircled and infiltrated by cells we denote as pleomorphic basophilic cells (Fig. 1E).

At 30 d.p.i. some of the MLCs in the foci (Fig. 2A) contained intracellular, vacuole-like structures containing bacterial cells staining positively for *F.noatunensis.* Bundles of mainly elongated pleomorphic basophilic cells (Fig. 2B), many of which stained for PCNA (Fig. 2C), were observed in the periphery, and penetrated inwards to create a conglomerate-like appearance for the foci (Fig. 2 A-C). MLCs did not stain for PCNA (Fig. 2C). In a few fish a small proportion of the larger foci studied, displayed cores of dead cells.

The size of the foci, the number of infected MLCs and the size of intracellular colonies increased during the study period. At 60 d.p.i. many of the MLCs contained intracellular

vacuole-like structures with a substantial number of bacteria, all staining for *F.noatunensis* (Fig. 2 D-F). Some foci were rounded and expanded within a pseudocapsule while others were more irregular and apparently infiltrating the surrounding parenchyma (Fig. 2 D-F). The pseudocapsule did not stain for collagen with the MSB method. No pleomorphic basophilic cells or cells staining for PCNA were identified in any foci at this stage. Some larger foci displayed centers with dead cells.

The histological appearance of spleens from control fish was in accordance with that of other teleosts (Fange & Nilsson 1985). Ellipsoids and red pulp were seen, but melanomacrophage centres or organised lymphoid tissues were not. Small numbers of cells staining for PCNA, indicating proliferation, were scattered through the red pulp of both control and infected fish throughout the experimental period.

Staining for PO and lysozyme

Double staining revealed co-localisation of lysozyme and PO within medium-sized cells with an elongated to lobulated nucleus, i.e. GCLCs (Fig. 3 A-B). In control fish, GCLCs staining for PO and lysozyme were scattered through the red pulp (Fig. 3 C-E), whereas in infected fish these lysozyme and PO-positive cells appeared to be partially concentrated within foci (Fig. 3 C,F, I). At 7 d.p.i.aggregations corresponding to those seen in HE-stained sections were shown to contain some slightly pleomorphic cells that stained for lysozyme and PO. At 15 and 30 d.p.i. cells morphologically similar to pleomorphic basophilic cells (Fig. 3 F-I) and staining for lysozyme and PO were observed within and along the outer margins of foci. In addition, in the center of one focus examined, in a fish sampled 30 d.p.i., several apparently dead cells staining very intensely for PO were observed. Few cells staining for PO were seen in the foci at 60 d.p.i.

Staining for ACP and NSE

In control and infected fish sampled at 7 d.p.i., low numbers of cells in the red pulp stained for ACP and NSE. At 15 and 30 d.p.i., some cells in the red pulp external to the foci and several MLCs in the centre of many foci stained for both enzymes (Fig. 4 A-C).

In addition, many apparently dead cells staining intensely for both enzymes (Fig. 4 E-F), were observed in the centre of few foci in a single fish sampled thirty d.p.i.. After 60 days very few MLCs in the centre of the foci stained for NSE; although the number staining for ACP increased (Fig. 4D).

Staining for inflammatory cytokines

IFN-γ

Putative lymphocyte-like cells expressing IFN-γ were detected in the red pulp of all fish; low numbers of cells in control fish (Fig. 5A-B), a moderate number of cells at 7-30 d.p.i. and in large numbers of cells at 60 d.p.i. (Fig. 5C-D).

IL-8

In the control fish (Fig. 5E) and the infected fish sampled at 7 d.p.i., only a few cells in the red pulp stained for IL-8. At 15, 30 and 60 d.p.i., there was an apparent slight increase in the number of cells staining for IL-8 in the red pulp and after thirty days a few cells of unknown nature stained within the foci (Fig. 5G-H).

Discussion

Intraperitoneal injection of cod with *F. noatunensis* produced a chronic systemic infection. Multiple expanding and infiltrating inflammatory foci, developed in the spleen. Pleomorphic proliferating cells infiltrated the foci transiently and a movement of resident GCLC from the red pulp towards the foci was also observed. Despite increasing numbers of IFN-γ expressing cells in the red pulp and an extensive cellular reaction, the number of intracellular bacteria in MLCs increased during the experimental period of 60 days, indicating defeat of host defenses.

Increasing numbers of MLCs staining for ACP and NSE, many with cytoplasmic vacuole-like structures containing *F. noatunensis,* were seen within the foci. The

increased staining intensity of ACP in MLCs is in accordance with results reported for lipopolysaccharide-stimulated cod macrophages *in vitro* (Sørensen *et al*.1997) and salmon macrophages in vaccine-induced inflammations (Press *et al*. 1995). The observation of many infected MLCs and the continuously increasing number of bacteria per cell agrees with studies performed on fish (Soto, Fernandez, Thune & Hawke 2010), including cod (Furevik, Pettersen, Colquhoun & Wergeland 2011) and mammals (Clemens & Horwitz 2007) infected with *Francisella* spp. *In vitro* studies have indicated that cod granulocytes, putatively identified as neutrophils, challenged with *F. noatunensis* contain few bacteria (Furevik, Pettersen, Colquhoun & Wergeland 2011). This is in agreement with the current study in which bacteria were not observed in this cell type. *F. tularensis* has the ability to inhibit leukocyte defense mechanisms and replicate in the host cell cytoplasm (Clemens *et al*. 2007). This, and the pathology and immune response to *Francisella* infection described in zebrafish (Vojtech, Saunders, Conway, Osland & Hansen 2009) as well as the present study, suggests that similar virulence factors may exist in the fish pathogenic species.

Expression of IFN-γ in the control fish is in accordance with results indicating that this cytokine is constitutively expressed in cells of the gadoid spleen (Furnes *et al*. 2009) and mammalian lymphocytes (Schroder *et al*. 2004). The increased expression of IFN-γ in infected fish was confirmed by real time quantitative PCR of the same individual fish as the present study (Ellingsen unpublished). This is in agreement with current views on the role of this key cytokine in host resistance to intracellular agents and its ability to activate macrophages (Boehm *et al*. 1997). The increasing number of bacteria despite increased number of IFN-γ producing cells is in accordance with the situation in francisellosis in mammals, in which the bacterium suppresses downstream events initiated by IFN-γ (Parsa, Butchar, Rajaram, Vremer, Gunn, Schleisinger & Tridandapani 2008), rendering the macrophage less able to fight the infection. While the results from the present study suggest a predominantly macrophage response, the continued development of pathological changes and increasing numbers of *F. noatunensis* strongly indicates unsuccessful elimination of the bacteria.

Highly pleomorphic and frequently elongated basophilic cells could be detected between 15 –and 30 d.p.i. within the processes of infected fish only. Cells staining for PCNA, with a similar morphology and localization were detected simultaneously. The cytoplasmic basophilia and presence of possible mitotic figures indicate cell proliferation and that the PCNA-positive cells represent the basophilic cells in the mitotic phase. Cells staining for lysozyme and PO displayed similar morphology and were detected simultaneously in the same areas. This raises the question as to whether these pleomorphic basophilic cells actually contained these enzymes or not. As lysozyme and PO are usually present in differentiated cells only we conclude that there is most probably one population of pleomporhic, proliferating cells, many of which stain for PCNA, and another population of pleomorphic cells (GCLCs discussed below) staining for the enzymes.

The transient presence of the basophilic cells within the foci indicates that they are an important part of the cellular defense mechanism in cod suffering francisellosis. The precise nature of these cells is unknown and they have not, to the best of our knowledge, previously been reported in fish. On the one hand, their increasing number and proximity to MLCs could indicate that they are proliferating cells of the monocyte lineage. In mammals there is great morphological heterogeneity in the monocyte phagocyte system and local proliferation of resident tissue macrophages is important for the renewal and maintenance of many macrophage types (Gordon & Taylor 2005). On the other hand, we did not detect transition forms between these and the MLCs, leaving the identity of the basophilic cells unknown. The apparently transient role in the development of foci seems to be in accordance with results from diagnostic investigations of farmed cod with chronic francisellosis, in which these cells are not a prominent feature (unpublished results). This is probably due to submission of fish for diagnostic purposes late in the disease course.

Rather homogenous staining for lysozyme was observed in paraffin sections. However, both lysozyme and PO staining was observed in granules following double-staining of cryosections. The homogenous staining pattern of lysozyme was therefore related to the tissue processing, and is in agreement with Inami (Inami *et al.* 2010). Common cell

staining for lysozyme is also in accordance with the high lysozyme activity reported in the spleen of cod (Larsen *et al*. 2009). On the one hand, the granular staining for lysozyme and PO in cells with a bean-shaped to lobulated nucleus is in accordance with studies of mammalian neutrophils (Faurschou et al., 2003). On the other hand, even if these enzyme-containing cells are not proliferating (as suggested above), the presence of the enzymes in highly pleomorphic cells is unexpected, as neutrophils in mammals are rounded and not elongated. The apparently slightly increased number of IL-8-expressing cells may also indicate a granulocyte lineage, as studies have demonstrated that IL-8 *in vitro* has multiple influences on neutrophils, including neutrophil attraction (Mukaida *et al*.1998) and changes in morphology (Thelen, Peveri, Kernen, Vontscharner, Walz & Baggiolini 1988). The apparent migration of lysozyme and PO positive cells from the red pulp to foci indicates that they play a significant role in the inflammatory response.

It was difficult to relate the presence of lysozyme and PO staining cells in the red pulp and the partial redistribution of such cells to foci of infected fish with the situation observed in H&E stained sections. This was further complicated by the presence of the basophilic pleomorphic cells. The results indicate a resident granulocyte population in the red pulp and migration of these cells to foci of infectious inflammation, where they are transiently present during the formation of the foci. This is similar to the situation described for bacterial infections in fish and mammals.

In conclusion, the inflammatory response in cod infected with *F. noatunensis* is characterized by the formation of chronic progrediating inflammatory foci. The dominating cell types in the inflammatory foci were MLCs, pleomorphic cells with lysozyme, and PO and pleomorphic basophilic proliferating cells, apparently representing a novel cell type. The concurrent presence of GCLC indicates that this inflammation in cod should be described more as pyogranulomatous than granulomatous. These extensive pathological changes were detected in apparently healthy cod. Despite an extensive cellular response, the disease continued to develop, which is in accordance with results from spontaneous cases of francisellosis in farmed cod (unpublished results).

Investigation of pathological changes in cod kept at higher water temperatures should be included in further studies. The route of infection and the high number of bacteria inoculated in this study does not reflect a typical field situation and experiments including cohabitation and including more quantitative approaches should be performed in order to draw a more complete picture of pathogenesis. As the MCLs plays a crucial role in host defense against infection with *F. noatunensis*, electron-microscopical studies of the bacteria within the MLC should be performed to determine their precise location and fate. Also the role of granulocytes and IL-8 as well as the relationship between the various differing pleomorphic cells should be further investigated.

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Figure legends

Figure 1 Atlantic cod challenged intraperitoneally with the bacterium *Francisella noatunensis*. Histological sections of spleen stained with hematoxylin and eosin (A-B, D-E), immunohistochemistry for proliferating cell nuclear antigen (PCNA) (C) and the bacterium (F). Fish sampled after 7 (A-C) and 15 (D-F) days. Scale bar for A, D shown in A, scale bar for B-C, E-F shown in B. (A) In the red pulp, few aggregates of macrophage-like (MLC) and other cells, as detailed in B. (B) Large MLCs with abundant eosinophilic cytoplasm and moderately blastic nuclei (right arrow), cells of unknown nature with hyperchromatic nuclei and slight to moderate amounts of basophilic cytoplasm (arrowhead) and some cells of unknown nature with extensive blastic nuclei (left arrow). (C) $PCNA^+$ cells (brown) of unknown nature and with blastic nuclei in an area of aggregated cells. (D) Inflammatory foci in the red pulp, in the lower left corner with a smaller (arrows) partly detailed in E. (E) Many MLCs with moderately or extensively blastic nuclei and cytoplasmic vacuole-like structure (lower arrow). Also, many clustered cells of unknown nature, denoted as pleomorphic basophilic cells due to their elongated or rounded forms (arrowheads), basophilic cytoplasm and hyperchromatic nuclei. (F) Area of similar changes as seen in (D-E) showing staining (red) for the bacterium in what appears to be a cytoplasmic vacuole within a MLC.

Figure 2 Atlantic cod challenged intraperitoneally with the bacterium *Francisella noatunensis*. Histological sections of a spleen stained with hematoxylin and eosin (A-B, D-E), immunohistochemistry for proliferating cell nuclear antigen (PCNA) (c) and the bacterium (F). Fish sampled after thirty (A-C) and sixty (D-F) days. Scale bar for A,D shown in A, scale bar for B-C shown in B, scale bar for E-F, shown in E. (A) A poorly-confined and irregular inflammatory focus that contained many macrophage-like cells (MLCs,) with abundant eosinophilic cytoplasm (arrow, detailed in B) and infiltrating bundles of pleomorphic basophilic cells (arrowhead, as described in 1E). (C) Same focus as in a-b, with $PCNA^+$ nuclei (dark brown, arrow) of many cells with morphology and location as pleomorphic basophilic cells and no staining of MLCs. (D) Many rounded foci with peripheral concentrically arranged cells indicating expanding growth within a pseudocapsule formed from compressed stromal tissue. Further, the arrow depicts a

larger poorly confined focus indicating infiltrative growth into surrounding tissue. (E) Microcolonies of bacteria within what appears to be cytoplasmic vacuoles in MLCs in the foci. (F) Demonstration of intracytoplasmatic staining pattern (red) for bacteria in cells within the processes and pseudocapsule with flattened cells (arrow).

Figure 3 Histological sections of spleen of Atlantic cod. A cryosection double-stained using immunofluorescense for lysozyme (A) and peroxidase (PO, B). Paraffin sections stained immunohistochemically for lysozyme (C-D, F-G, I) and cryosections stained by enzyme histochemistry to demonstrate PO (E, H). From control fish (A-E) and from fish sampled fifteen (F-H) and thirty days (I) after intraperitoneal challenge with the bacterium *Francisella noatunensis*. Scale bar for A-B, D-E, G-H shown in B, scale bar for F, I shown in F. (A-B) One granulocyte-like cell (GCLC) with positive staining for lysozyme (A) and PO (B). Note the moderately elongated nucleus. (C) Many cells in the red pulp, but apparently no cells in the ellipsoids (arrow), as detailed in b, stained for lysozyme (red). (D) GCLCs with bean-shaped or lobulated nuclei displayed homogenous staining of the entire cytoplasm for lysozyme (arrow). Note possible non-stained MLCs (arrowhead). (E) Cells of similar morphology to those positive in (D) showed granular cytoplasmic staining for PO (arrowhead). A slight, diffuse and apparently unspecific staining was seen in erythrocytes (arrow). (F) Compared to uninfected fish in C, the cells staining for lysozyme were fewer in the red pulp and partly clustered in groups corresponding to the inflammatory foci. (G) Close up of part of clustered cells in F, to show that cells staining for lysozyme were only partly similar to those in D as they had a morphology similar to basophilic pleomorphic cells (Fig. 1 E and 2B-C). (H) Cells staining for PO with a similar morphology as lysozyme staining cells in G. (I) Many cells staining for lysozyme in foci infiltrated by elongated cells, similar to those seen in G.

Figure 4 Atlantic cod challenged intraperitoneally with the bacterium *Francisella noatunensis*. Histological sections of spleen stained by enzyme histochemistry for acid phosphatase (ACP) (A, D-E) and non specific esterase (NSE) (B-C, F). Fish sampled after thirty (A-C, E-F) and sixty (D) days. Scale bar for a-b, e-f shown in a, scalebar for c-d, shown in c. (A) Staining for ACP in parts of cytoplasm of macrophage like cells

(arrow) in the red pulp. (B) In red pulp, cells staining for NSE with similar morphology as cells staining for ACP. (C) An inflammatory focus with many cells staining for NSE. (D) Note the diffuse staining pattern of many cells in the central part (arrows) of a focus. (E-F) In the centre of few foci there was a very intense staining of ACP and NSE in cells in the central part of the focus.

Figure 5 Histological sections of spleen of Atlantic cod. Cells expressing IFN-γ (A-D) and IL-8 (E-H) detected by *in situ* hybridisation. Control-fish (A-B, E-F), fish sampled after sixty-(C-D) and thirty days (G-H) after intraperitoneal challenge with the bacterium *Francisella noatunensis*. Scale bar for A, C, E, G shown in A. Scale bar for D, H shown in D. Scale bar for b, f shown in B. (A) Many putative lymphocyte-like cells expressing IFN-γ (brown) were seen in the red pulp, some in the adventitia of an artery (arrow) and a vein (arrowhead), and in blood vessels as detailed in B. (C) Area between inflammatory foci in red pulp with an increased number of cells staining for IFN-γ as detailed in D; These cells, including some cells with a narrow rim of cytoplasm (i.e. putative lymphocyte-like cell, arrow) showed different amounts of cytoplasm. (E) Cells expressing IL-8 (red) in the red pulp. (F) Staining of cells also in the ellipsoidal wall. (G) Increased number of positive cells in the red pulp and some positive cells infiltrating the focus as detailed in (H).

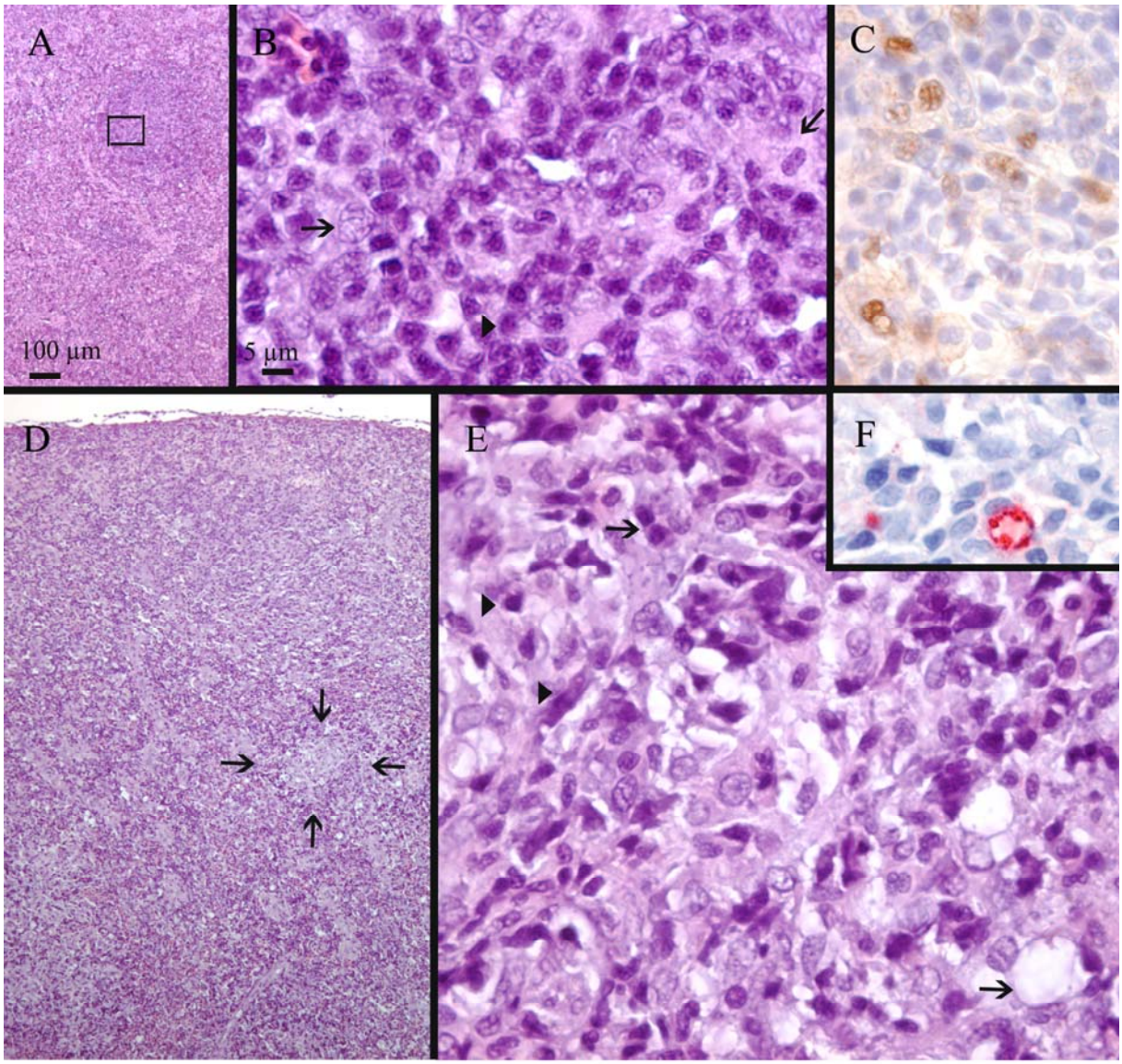

Figure 1

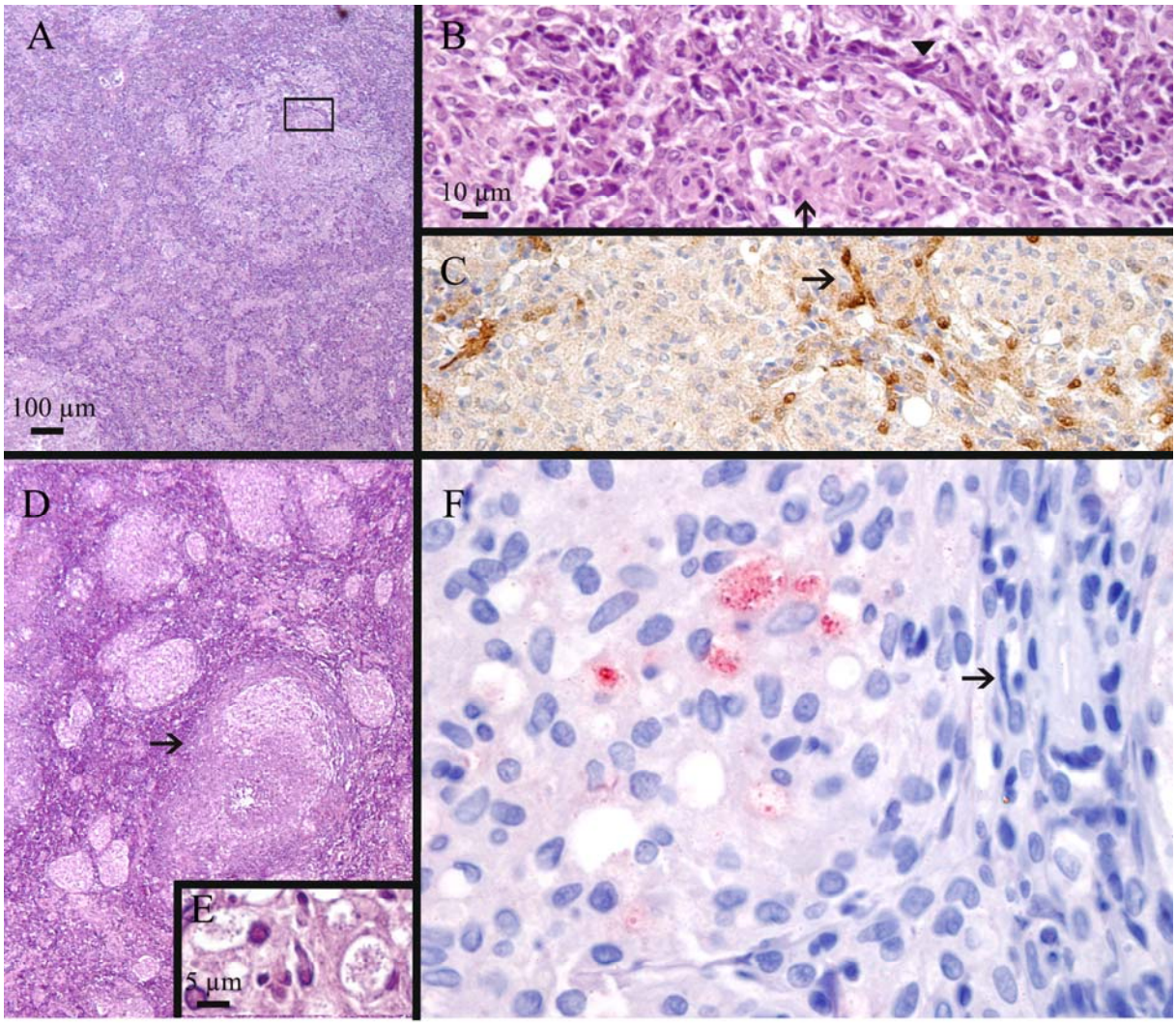

Figure 2

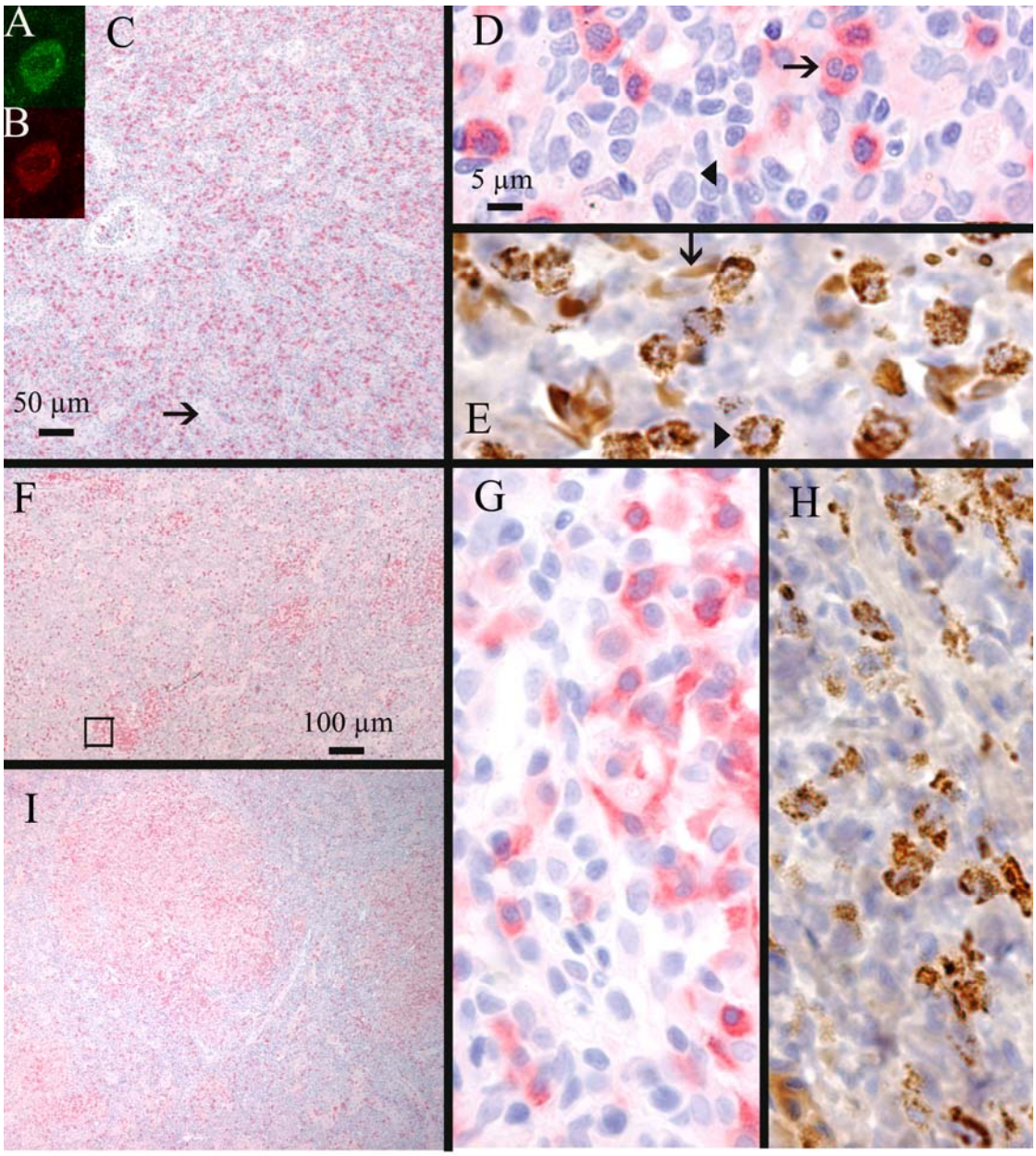

Figure 3

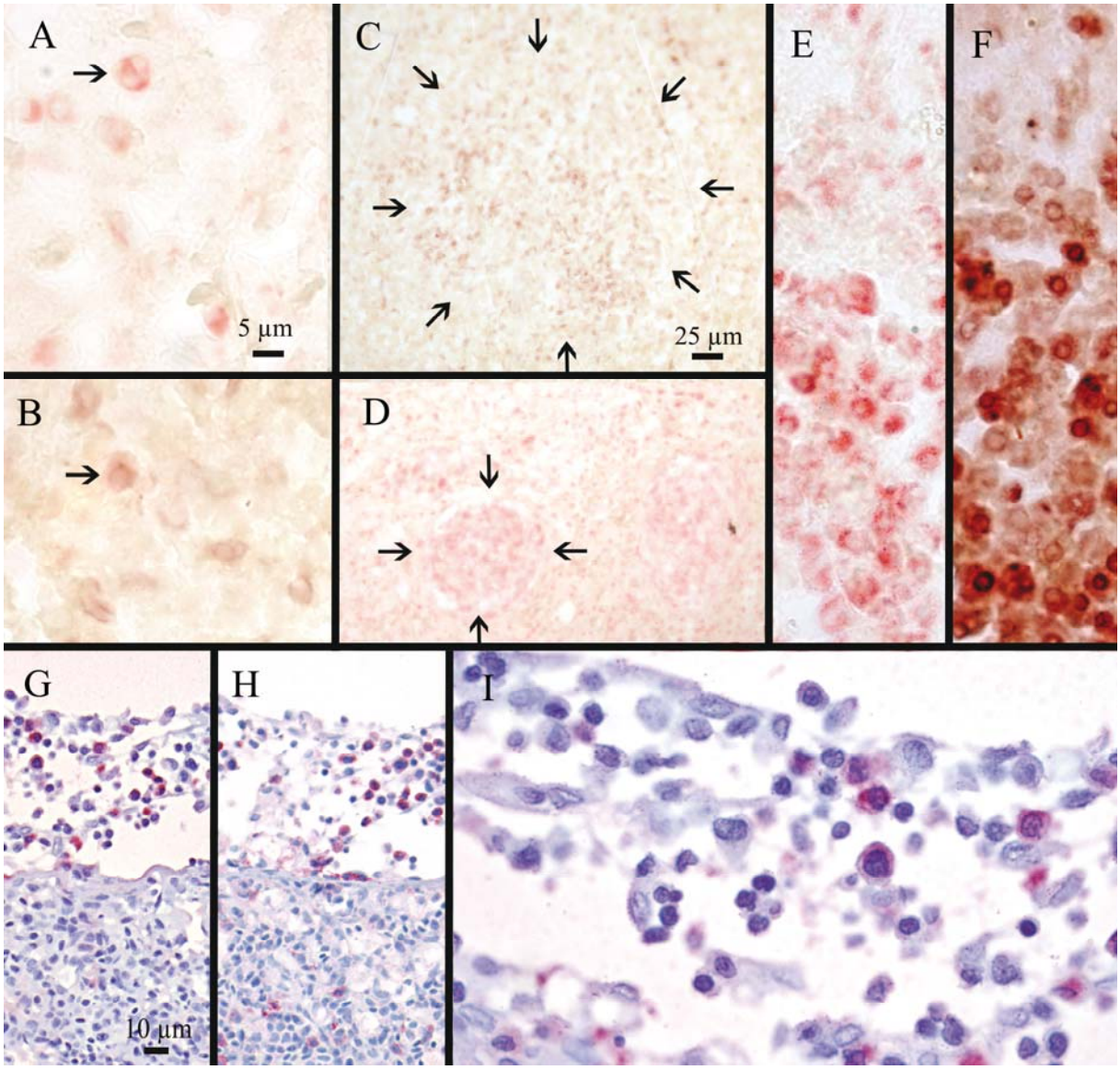

Figure 4

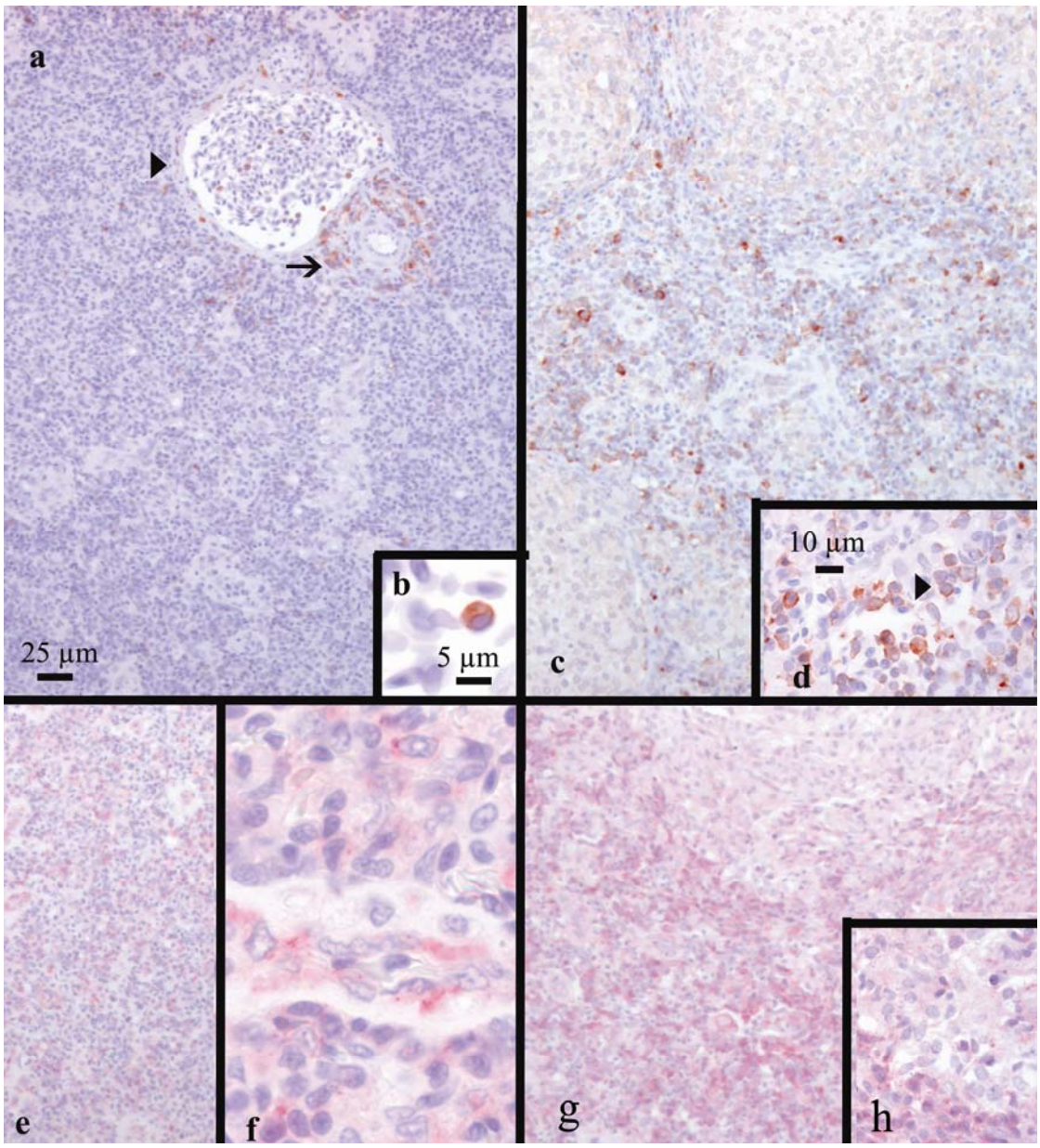

Figure 5