From Editorial Office

Dear Prof. Dr. Contributor,

Your manuscript entitled, ‘Fuelling an immune response: An ultrastructural study of immune-stimulated lymph nodes’ has been published in journal, Applied Cell Biology.

Complementary 5 Printed Reprints & Journal’s Issue

Complementary 5 Printed reprints and manuscript containing Journal’s issue in hardcopy will be supplied to corresponding author. Rs.500/- is charged towards packaging, handling and postal charges.

Shipping Address Form

1. Name in Full: ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________

2. Mailing Address: ____________________________________________________________
   ____________________________________________________________
   ____________________________________________________________
   ____________________________________________________________

3. Email Address: ____________________________________________________________

Payment Mode for Postal Charges

Demand Draft of Rs. 500/- should be made payable in favor of ‘Trade Science Inc’, payable at Rajkot. Send DD at following address

Trade Science Inc.
126 Prasheel Park, SanjayRaj Farm House, Nr. Saurashtra University, Rajkot 360 005, Gujarat - INDIA.
Fuelling an immune response: An ultrastructural study of immune-stimulated lymph nodes

**Tips for checking Galley Proof**

1. Print Guidelines for Publication available at [http://www.tsjournals.com](http://www.tsjournals.com)
2. Carefully check, (i) Title, (ii) Authors and Addresses, (iii) Corresponding Author’s E-mail Address, (iv) Abstract, (v) Keywords, (vi) Introduction, (vii) Experimental, (viii) Results and Discussion, (ix) Conclusions, (x) Acknowledgment, (xi) References and Footnotes.
3. Make sure all symbols, subscripts, superscripts, equations, reaction schemes, chemical structures, legend of figures, captions of tables are correct.
4. It is corresponding author’s responsibility to check sent galley proof. If once correction(s) will be receive, we are not taking any responsibility regarding remained/not corrected information.
5. 2nd galley proof will not be sent in any case.
6. No claim will be considered after ms is published.
7. Corrected galley proof must be returned within 3 days after receiving via e-mail at galleyproofs@tsijournals.com with subject line galley proof and manuscript number.
8. If we aren’t receiving galley proof within 4 days we will publish manuscript in as appeared as in galley proof.

**Galley Proof Department**
Trade Science Inc.
Fuelling an immune response: An ultrastructural study of immune-stimulated lymph nodes

S.Daya, H.A.Davies, F.M.Colyer, J.Tuffnell, A.J.Loughlin, H.A.MacQueen*
Department of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, (UK)
E-mail: h.a.macqueen@open.ac.uk
Received: 12th November, 2012 ; Accepted: 18th February, 2013

ABSTRACT

A growing body of evidence suggests that adipocytes, and the triacylglycerol fatty acids within them, play an important role in supporting the inflammatory immune response. Inflammatory cytokines produced by an activated lymph node cause the release of fatty acids from neighbouring adipocytes, and the fatty acids are taken up into lymph node lymphoid cells, where they can be used as precursors for eicosanoid and plasma membrane synthesis, or as a fuel source. The mechanism of transport of fatty acids from adipocytes across the lymph node capsule and into lymphoid cells remains to be elucidated. Here we present evidence from light and electron microscopy that small fat-filled cells are found within the lymph node, and that 24 hours after an immune challenge they are associated with dendritic cells. The small adipocytes express S100 protein and insulin receptor, characteristics of mature adipocytes. Our observations suggest a mechanism for delivering triacylglycerol fatty acids directly to their site of use in supplying components for an immune response.

KEYWORDS

Lymph node; Adipocytes; Dendritic cells; Immune challenge.

INTRODUCTION

The immune system accounts for about 15% of the resting metabolic rate and thus represents a major energy consumer among human organ systems[1,2]. Adipose tissue is well equipped to provide the high level of energy required by the immune system and, according to Calder and co-workers, the metabolic activity of adipose tissue and the energy requirements of the immune system have led to the evolutionary development of a feedback relationship between the two systems. When the immune system is challenged locally or systemically, an important and constant flow of energy is required; fatty acids and glutamine are essential lipogenic precursors and also the preferred fuel for the proliferation of lymphocytes in response to an immune stimulus[3].

Circulating fatty acids play an essential role in supplying lymphoid tissue with energy and metabolic precursors for membrane components, and substrates for the synthesis of complex lipids such as leukotrienes, prostacyclins, thromboxanes and prostaglandins[4] and there is growing evidence that adipocytes, and the triacylglycerol fatty acids stored within them, also play an important role in supporting the inflammatory immune response at a local level. It has long been known that some cells in the lymph node contain lipid droplets,
but the polyunsaturated fatty acids transferred following an immune challenge have been shown to derive from perinodal adipocytes[5] and the role of the ‘resident’ lipid droplets in this process is unclear. Inflammatory cytokines produced by an activated lymph node result in the release of fatty acids from neighbouring perinodal adipocytes, and the fatty acids are taken up directly by lymph node lymphoid cells[5,6]. These authors have demonstrated that this effect appears first in the immediately perinodal adipocytes, and spreads to the surrounding adipocytes. This effect is likely to be cytokine-mediated: the expression of tumour necrosis factor receptor I is weak on perinodal adipocytes in sites that have not been exposed to an immune challenge, but is upregulated on adipocytes surrounding immune-stimulated lymph nodes[7]. This upregulation is seen on all perinodal adipocytes, not just those in close proximity to blood or lymphatic vessels.

It is of interest to establish the mechanism by which the triacylglycerols (TAG) contained within perinodal adipocytes can be made available, rapidly and specifically, to lymphoid cells responding to an immune challenge. The involvement of all the adipocytes surrounding the lymph node, and the speed and specificity of the process[7,8] suggest that direct transfer of TAG across the node capsule might be taking place. The lymph node capsule is made of collagen and has a subcapsular sinus. The capsule maintains tissue architecture and organisation in the node and is thought to prevent migration of cells from the lymph node into peripheral tissues. However, several studies have suggested that the cellular and extracellular density of the capsule increases during lymph node development[9-11] and it is known that the lymph node increases in size to almost twenty times its original size after an immune challenge. Tanegashima and co-workers reported a lymph node embedded in the centre of a human thymus and showed that adipocytes infiltrated the capsule and subcapsular area surrounding the thymus, indicating that in some circumstances the capsule is not impermeable to cells[12].

Dendritic cells are well known as migratory and scavenger cells. They are found in significant numbers among the adipocytes surrounding immune-challenged lymph nodes, and their numbers are modulated by dietary fatty acids[13]. They are involved in the adipocyte-lymphoid cell transactions in Crohn’s disease, where they modulate lipolysis[14], and it has been reported that developing dendritic cells accumulate lipid[15]. Thus dendritic cells are possible candidates for involvement in the transference of TAG into the lymph node. It is unclear whether this would involve the transfer of extracellular TAG, or whether whole adipocytes might be introduced into the lymph node.

In this study we have investigated the structure and cell populations present in lymph nodes that have, or have not, been subjected to a localised immune challenge. We present evidence of a population of small adipocyte-like cells within lymph nodes in a rat model system, and show that following a localised immune challenge to the lymph node, the adipocytes are observed in close association with dendritic cells. Our hypothesis is that small adipocytes from the perinodal adipose tissue can enter lymph nodes, where they act as reservoirs of TAG fatty acids. Following an immune stimulus to the node, the TAG reservoirs are ‘tapped’ by dendritic cells which then transfer the TAG to other cells where the immune responses are taking place.

**MATERIALS AND METHODS**

**Animals**

Sprague-Dawley rats were bred in-house and maintained in accordance with the Animals (Scientific Procedures) Act, 1986, and the project was approved by the Open University Animal Ethics Advisory Group. At the age of 7-9 months, when the animals’ mass was between 200-350g, the left popliteal lymph node was stimulated by injecting bacterial lipopolysaccharide (LPS; Sigma-Aldrich Company Ltd, Gillingham, UK) subcutaneously at 10 µg per kg body weight in phosphate-buffered saline (PBS) into the lower left hind limb. The right popliteal lymph node in the same animal was injected with vehicle only and used as the unstimulated control. The animals were killed 24 hours later by Schedule 1 methods. Fifteen rats were used in this study.

**Isolation of lymph nodes**

The left and right popliteal adipose depots were dissected out and placed in cold Hanks balanced salt solution (Invitrogen Ltd, Paisley, UK). The lymph nodes were extracted by removing the surrounding adipose tissue with 2 pairs of fine forceps on an ice block. For experiments involving visualisation of the isolated lymph node, great care was taken to remove all the adipose
tissue, and this was verified microscopically. For some experiments a dissection microscope was used to aid the removal of the lymph node capsule. For experiments involving visualisation of the lymph node capsule, a layer of perinodal adipose tissue was retained around the intact node to avoid accidental disruption of the capsule.

**Lymph node sectioning**

For immunohistochemical analysis by light microscopy, the lymph nodes were flash-frozen in isopropanol on dry ice, embedded in OCT medium (Raymond A Lamb Limited, Eastbourne, UK) and stored at -80°C for long term tissue preservation. They were sectioned at 10 μm intervals in a standardised orientation (with the end of the ovoid lymph node that was closest to the knee joint being the first end to be sectioned) on a Leica CM1900 cryostat (Leica Microsystems Ltd, Milton Keynes, UK) and the sections were collected serially onto gelatine-chromium coated microscope slides. They were fixed in 4% paraformaldehyde in PBS for 1h at 4-8°C and rinsed three times in cold phosphate buffered saline (PBS). The sections were termed either early (first 20 sections collected), mid (the second 20) or late.

For transmission electron microscopy, the lymph nodes were immersion-fixed in 3.75% acrolein in 2% paraformaldehyde in 0.1 mol l⁻¹ phosphate buffer, PB) for 24 hours followed by a further 24 hours in 4% paraformaldehyde at 4°C. They were enrobed in 15% agar in PBS and 50 μm sections cut on a Leica cryostat.

**Oil red O staining**

Lymph node sections on slides were rinsed in 60% isopropanol, then 0.5 ml of the Oil red O working solution was added for 15 minutes at room temperature (RT). The slides were rinsed in 60% isopropanol and after washing in PBS were counterstained with 0.5 ml of Gill’s haematoxylin for 5 minutes. After further PBS washes, the slides were mounted in 50% glycerol and viewed using a Nikon Microphot-FX (Nikon UK Ltd, Kingston upon Thames, UK).

**Immunostaining for light microscopy**

Primary antibodies were either rabbit anti-S100 protein αβ (used at 1 in 200; Abcam Ltd, Cambridge, UK) or mouse anti-insulin receptor β subunit (used at 1 in 100; Chemicon Europe Ltd, Chandlers Ford, UK). Slides were incubated overnight at 4°C. Appropriate fluorescent secondary antibodies [goat anti-mouse IgG Cy3 conjugated (1:200), goat anti-rabbit IgG Cy3 conjugated (1:200) and goat anti-rabbit IgG FITC conjugated (1:400) (Chemicon Europe Ltd, Chandlers Ford, UK), or goat anti-mouse IgG FITC conjugated (1:200) (Abcam Ltd, Cambridge, UK)] were allowed to react for 1 hour at RT. Nuclei were counterstained with 5 mg ml⁻¹ 4,6-diamidino-2-phenyl-indole-dihydrochloride (Invitrogen Ltd, Paisley, UK). The sections were mounted in an anti-fade mountant (DakoCytomation Ltd, Ely, UK) and viewed using an Olympus BX-UCB fluorescence microscope (Olympus UK Ltd, Southall, UK).

**Scanning electron microscopy**

Lymph nodes were dissected out and immersion fixed in 4% paraformaldehyde in PB. They were then immersed in 2.3M sucrose for 3h at RT as a cryoprotectant, then immersed in liquid nitrogen. Once frozen they were fractured by gentle pressure from a scalpel blade. Following freeze-fracture, samples were osmicated in 1% osmium tetroxide in PB for 2h, washed in PB, then dehydrated in a graded acetone series. The samples were critical point dried in CO₂, mounted onto stubs, sputter-coated with gold in a Polaron SC7640 sputter coater, and viewed with a Zeiss Supra 55VP FEG scanning electron microscope (Carl Zeiss Ltd., Cambridge, UK).

**Pre-embedding immunolabelling for transmission electron microscopy**

Free-floating 50 μm thick lymph node tissue sections were incubated in 1% sodium borohydride in PB for 30 minutes at RT to block excess aldehyde groups and then rinsed copiously with PB. Endogenous peroxidase activity was blocked for 5 minutes with 3% hydrogen peroxide in 10% methanol in PB and sections placed into a cryoprotectant solution (25% sucrose and 3% glycerol in 0.05 mol l⁻¹ phosphate buffer) for 15 minutes, then permeabilised by freeze-thawing in liquid nitrogen. The sections were rinsed in 0.1 mol l⁻¹ tris buffered saline (TBS) and the non-specific binding sites blocked in 0.5% bovine serum albumin in TBS for 30 minutes. The primary antibodies (against S100 protein and insulin receptor, as used for light microscopy) were appropriately diluted in 0.1% bovine serum albu-
min in TBS and the sections incubated with shaking for 24h at room temperature, followed by 24h at 4°C.

The sections were rinsed in TBS and the secondary antibodies added (biotinylated goat anti-mouse IgG (1:200) and biotinylated goat anti-rabbit IgG (1:200), Vector Laboratories Ltd, Peterborough, UK), and the sections incubated at room temperature for 1h. After washing, the sections were incubated with Avidin Biotin Complex (ABC; Vector laboratories Ltd, Peterborough, UK) and incubated for 30 minutes at room temperature. Finally, the sections were rinsed in TBS and reacted with 0.02% diaminobenzidine (DAB; Sigma-Aldrich Company Ltd, Gillingham, UK) solution in PB in the presence of 0.01% hydrogen peroxide. The DAB reaction was checked using a Nikon 120 light microscope (Nikon UK Ltd, Kingston upon Thames, UK). The sections were washed and placed in 2% osmium tetroxide in PB for 1 hr at room temperature. Following washing, the tissue was dehydrated through a graded acetone series and infiltrated in an Epon and acetone mix overnight at RT. The sections were flat-embedded in Epon between sheets of aclarfluorhalocarbon film, and polymerised at 60°C overnight. The film was carefully removed and the sections glued onto prepared Epon blocks with cyanoacrylate.

Ultrathin sections of 70nm were cut on a Leica UCT ultramicrotome (Leica Microsystems Ltd, Milton Keynes, UK) and collected on carbon/formvar films on copper slot grids. Sections were counter-stained with uranyl acetate and Reynolds’ lead citrate and viewed using a JEOL JEM 1010 transmission electron microscope operating at 80kV (JEOL Ltd, Welwyn Garden City, UK). Micrographs were taken at various magnifications.

Post-embedding EM immunogold labelling

One Epon block from a stimulated and one from an unstimulated lymph node was used for post-embedding immunogold labelling. Ultrathin sections, 90nm thick, were collected on filmed nickel slot grids and all treatments performed by floating the grids on 30µl drops of reagents. The sections were initially etched with 1% periodic acid for 10 minutes, followed by 1% sodium metaperiodate for 10 minutes. After blocking non-specific binding, the primary antibody, mouse anti-OX62 (ABD Serotec), was used at 1 in 5 in PBS containing 0.8% BSA, 0.1% cold water fish gelatin and 1% normal goat serum and sections incubated overnight at RT. After washing, the sections were incubated for 2h at RT in a 1 in 50 dilution of the secondary antibody, goat anti-mouse IgG coupled to 15nm gold particles (British BioCell International, UK). The reaction was fixed by floating the grids on 2% glutaraldehyde in PBS for 5 minutes before final washing in deionised water.

The sections were counter-stained with uranyl acetate and Reynolds’ lead citrate before examination in a JEOL JEM 1400 transmission electron microscope. Digital images were captured using an AMT XR60 camera.

RESULTS

Oil red O staining in the unstimulated lymph node

Serial sectioning of entire lymph nodes revealed a small number of Oil red O positive cells throughout the node (Figure 1). The Oil red O-stained cells were observed together in patches. They were noticeably smaller in size than adipocytes from adipose tissue, ranging from 10-15 µm in diameter, compared to 50-70 µm diameter for mature adipose tissue adipocytes, and were at the top of the size range found for lymph node cells.

S100 protein and insulin receptor expression in lymph nodes

In unstimulated lymph nodes, a number of cells were positive for the S100 protein and for the insulin receptor, and the number and distribution of these cells did not notably change following an immune challenge (Figure 2). Such cells were located throughout the lymph node, in early, mid and late sections (Figure 3). These cells expressed adipocyte-specific markers, but were similar in size to other lymph node cells.

Scanning electron microscopy

SEM analysis revealed that adipocyte-like cells are readily distinguishable deep within both stimulated and unstimulated lymph nodes (Figure 4A and B). These adipocytes are readily distinguishable from adipocytes contaminating the external surface (Figure 4C), and some, seen in stimulated nodes only, appear to be closely apposed to other cell types (Figure 4D).

Transmission electron microscopy

We used transmission electron microscopy to fur-
Figure 1: Oil Red O staining (arrowheads) in serial sections (A, early section; B, mid section; C, late section) through an unstimulated lymph node; counter-staining with haematoxylin. Bars = 10 μm.

Figure 2: Expression of insulin receptor (A-C) and S100 αβ (D-F) in lymph nodes. A, D = isotype controls; B, E = unstimulated lymph node; C, F = immune-stimulated lymph node. All sections x 200.

Figure 3: Expression of S100 αβ (A-C) and insulin receptor (D-F) in serial sections through unstimulated lymph nodes. A, D = early sections; B, E = mid sections; C, F = late sections. A-C, stained with FITC-anti S100αβ; D-F, stained with Cy3-anti-insulin receptor. All sections are counterstained with DAPI. Bars = 100 μm.
An ultrastructural study of immune-stimulated lymph nodes

Full Paper

ther characterise the associations seen between the putative adipocytes and other cells in the lymph node. We observed cells in very close contact in immune-stimulated lymph nodes, as shown in Figure 5, but were unable to detect these close relationships in unstimulated nodes (8 rats examined by TEM). In order to confirm the identity of the cells involved in these associations, we undertook immunogold labelling. Putative adipocytes were labelled with anti-S100αβ, as shown by arrows in Figure 5. Dendritic cells were identified by labelling with anti-OX62, shown in Figure 6. In this case, labelling was sparse and mostly cytoplasmic but appeared to be limited specifically to the lymphoid cells in question, suggesting that the association observed in immune-challenged lymph nodes is indeed between adipocytes and dendritic cells.

Structure of the lymph node capsule

In order to assess the feasibility of our hypothesis that cell trafficking occurs directly across the lymph node capsule, we examined the structure of the capsule by both scanning and transmission electron microscopy. Figure 7 reveals that in both stimulated (Figure 7A, B, C, D) and unstimulated (E, F) lymph nodes, the capsule has a very open structure, with loosely-spaced bundles of collagen fibres running in several directions and cells between the bundles. The micrographs show (A, C, E, F) that adipocytes are regularly found intruding into the capsule.

DISCUSSION

In this study we have examined serial sections of rat popliteal lymph nodes isolated 24 hours after an immune challenge to one of the nodes, using the unchallenged popliteal node as a control. Lipid-containing cells, as assessed by Oil red O staining were found throughout the lymph node (Figure 1), regardless of immune status. Using the expression of S100αβ and insulin receptor as markers, we observed that cells staining positive for these molecules could also be found throughout both immune-challenged and unchallenged lymph nodes (Figures 2 and 3). These putative adipocytes were at the top end of the size range of
lymph node lymphoid cells but smaller than most of the adipocytes seen in surrounding adipose tissue. Scanning electron microscopy of freeze-fractured lymph nodes showed adipocyte-like cells deep within the nodes, that could be readily distinguished from adipocytes contaminating the node surface (Figure 4C). In particular, in stimulated nodes (Figure 4B, D, E) the adipocytes appeared to be in contact with highly ruffled processes from other cells within the node. Transmission electron microscopy confirmed that the adipocyte-like cells expressing S100αβ and insulin receptor were found closely associated with lymphoid cells (Figure 5C, D), but so far we have observed these intimate associations only in immune-stimulated lymph nodes. The lymphoid cells most often closely associated with the adipocytes appeared, morphologically (Figure 5C, D).

Figure 5: Transmission electron micrographs of immune-stimulated lymph nodes immunolabelled with anti-S100αβ (arrowheads). C and D show close associations between adipocyte-like cells and lymphoid cells. Bars: A, C = 2 μm; B = 5 μm; D = 1 μm.
D) and by OX62 labelling (Figure 6), to be dendritic cells. We further examined the ultrastructure of the lymph node capsule, and determined that it is unlikely to form an impenetrable barrier to cells, and indeed both dendritic cells and adipocyte-like cells are regularly seen in sections of lymph node capsule (e.g. Figure 7E). All these results support our hypothesis that small adipocytes are deployed from perinodal adipose tissue into lymph nodes, where they serve as reservoirs to fuel an immune response, and that this process is mediated by dendritic cells.

The presence of lipid-containing cells in lymph nodes is well known. As expected from many previous observations, Oil red O stained cells were seen throughout the lymph node. However, from preliminary immunoperoxidase staining studies (not shown) most of these cells did not appear to express the adipocyte markers, and are therefore likely to represent the cells containing Gall bodies.

Our hypothesis is supported by other previous work. S100αβ protein has been shown to be expressed in immunoreactive giant cells in lymphoid tissue[17]. These cells had the morphology dendritic cells, with irregularly-shaped, euchromatic nuclei, phagosomes of various diameters, numerous mitochondria and microfilaments in the perikaryon, and processes free of cell organelles. Furthermore, it has been shown that some follicular dendritic cells stain for the S100 protein and are present mainly in primary follicles with a few cells distributed in the secondary follicles[18]. This is consistent with the localisation that we have found (Figure 3). A number of studies published in the 1970s and 1980s have reported expression of the insulin receptor by lymph node lymphoid cells, and have variously linked expression to lymph node activation[19-23]. More recently, Helderman[24] showed that insulin exerts a regulatory role for T helper cells to provide help for insulin receptor synthesis by B cells, while also serving a number of intermediary metabolic functions in the activated lymphocytes. However, the role of insulin receptors in specific lymphoid cell types remains unclear. Interestingly, Fossum & Vaaland[25] observed aggregates of lipid-laden cells in lymph nodes from mice. They described these cells as having granules containing osmophilic material, probably lipid, and resembling dendritic cells in morphology. It is possible that these authors were observing the pairs of cells that we report here, or that they were observing dendritic cells which were filled with TAG received from adipocytes.

Our electron microscopical analysis of LPS-stimulated lymph nodes showed that adipocyte-like cells were present in the nodes. Immunolabelling confirmed that...
the putative adipocytes expressed S100\(\alpha\beta\) protein; they shared morphological and receptor characteristics with mature adipocytes in adipose tissue but were much smaller in size. They were frequently observed in close proximity to dendritic cells (Figures 4 and 5), but only following an immune challenge. We suggest that these cell pairings represent a functional association and form part of the mechanism that conveys triacylglycerol fatty acids to the lymph node cells.

The mechanism and timing of formation of these cell pairings is not clear. One possibility is that small adipocytes can migrate directly across the capsule into the lymph node, and that following immune challenge they form associations with dendritic cells within the node. Another possibility is that upon immune challenge dendritic cells migrate out into the perinodal adipose tissue and capture whole or partial adipocytes, returning to the lymph node with a supply of TAG. Finally it is possible that dendritic cells already exist in perinodal adipose tissue in a pairing with adipocytes (or fragments derived from them), and immune challenge merely activates their migration into the lymph node. However, these last two models do not easily explain the presence of adipocyte-like cells in unstimulated lymph nodes. Note that, in order to facilitate any of these mechanisms the lymph node capsule must be leaky, presenting no physical barrier to cell passage, and indeed the evidence presented here does indicate that the structure of the capsule is very likely to allow this (Figure 6).
**CONCLUSION**

We have for the first time shown that small adipocytes are present deep inside lymph nodes. These adipocytes express S100αβ protein and the insulin receptor, their cytoplasm is occupied by a single lipid droplet and they show the morphological characteristics of mature adipocytes found in adipose tissue. Following an immune challenge to the lymph node, the adipocyte-like cells are observed paired with dendritic cells, and we propose that this is a functional association that facilitates a rapid immune response. We propose that direct migration of small adipocytes across the lymph node capsule is possible, and show that the ultrastructure of the capsule is sufficiently loose to permit this migration.

**ACKNOWLEDGEMENTS**

This work was supported by a Biotechnology and Biological Sciences Research Council studentship to SD, and by The Open University. We gratefully acknowledge the support of Steve Walters, Karen Evans and Agata Stramek in this study. SD contributed to the design of the experiments and carried out the light microscopy. HAD, FMC and JT carried out the electron microscopy and HAD also critically reviewed the manuscript. AJL contributed to the concept and design, and the critical review process. HAM contributed to the concept and design, and drafted the manuscript.

**REFERENCES**