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Long-term persistence after acute Q fever of non-infective *Coxiella burnetii* cell components, including antigens*

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Summary

Background: Previous studies of inciting factors for a prolonged post-infection fatigue syndrome after Q fever (variously termed QFS or Q fever associated CFS/ME in the literature) showed that after the acute infection a high proportion of asymptomatic and QFS patients had Q fever antibody and also low levels in PBMC and bone marrow of *Coxiella burnetii* (*C.b.*) DNA with PCR assays directed against three different target sequences in different parts of the coxiella genome. Attempts to isolate a strain of *C.b.* in A/J mice, and cell culture from PCR positive PBMC and bone marrow were consistently negative. The detailed composition of the persisting coxiella residues remains to be defined.

Aim: To retest and provide detailed results on selected PCR positive samples from the Birmingham Q fever outbreak patients tested by a highly sensitive method to detect viable organisms and to determine the nature of the residual coxiella cell components.

Design: Laboratory case study.

Methods: NOD/SCID mice were inoculated with samples from the 1989 Q fever outbreak in Birmingham and followed for evidence of infection and the presence of coxiella DNA and specific antigens in spleen and liver macrophages. A significant, unexpected finding of specific antigen was followed by assessment of its ability to provoke production of inflammatory and non-inflammatory cytokines in mice, in THP-1 human macrophage cell cultures and to induce inflammatory lesions in the skin of guinea pigs hyperimmunized against Q fever vaccine.

Results: Culture of samples from 10 Birmingham Q fever patients in NOD/SCID mice, 12 years from infection did not yield viable *Coxiella burnetii,* as shown earlier. However complexes of material with coxiella antigens were found in mouse spleens in all cases but in significantly greater amounts in samples from those with post Q fever fatigue syndrome. The antigenic complexes [now designated 'immunomodulatory complexes' (IMC)] were shown to stimulate cytokine release in the mice and in the THP-1 macrophages and to provoke an inflammatory reaction on intradermal injection into the skin of Q fever hyperimmunized guinea pigs.

Conclusion: The study identifies a non-infective complex of *C.b.* antigens able to survive in the host and provoke aberrant humoral and cell mediated immunity responses – a possible pathogenic link between initial infection and a subsequent long-term post Q fever fatigue syndrome.

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Introduction

Q fever is a major, clinically debilitating, very costly occupational zoonotic disease affecting the Australian stock raising, meat processing and associated industries. *Coxiella burnetii* (*C.b.*), the causative bacterium of Q fever, is a facultative intracellular pathogen highly evolved to multiply in and transform phagolysosomes of macrophage lineage cells into a sheltered niches [parasitophorous vacuoles (PV)] for the coxiella's survival, nutrition and replication.[1–3]

Although the majority of acute cases of Q fever recover and remain asymptomatic, a minority develop endocarditis, granuloma in various organs or more commonly, a protracted post Q fever fatigue syndrome, variously termed QFS or Q fever associated CFS/ME. Over the last two decades our groups in Adelaide (Australia) and Birmingham (UK) have studied the pathobiology of QFS with particular reference to persistence of the viable coxiella or its cell components and antigens as major inciting factors for the syndrome.[4]

The present report presents detailed laboratory evidence that in QFS viable, infective *C.b.* are rarely, if ever, isolated from Peripheral blood mononuclear cells (PBMCs) or bone marrow (BM), but a complex of antigen and Phase 1 LPS (now designated immunodulatory complex) is regularly present.

Material and methods

Patient groups and samples

All patients came from the cohort of acute Q fever cases in the Q fever outbreak in Solihull, Birmingham, UK in 1989.[4–6] Patients had been classified into various clinical groupings according to asymptomatic recovery or the presence of post Q fever fatigue syndrome (CFS) with or without other co-morbidities.[7,8] Further laboratory investigation concentrated on three main groups: recovered Group 3 (recGr3) patients with laboratory verified acute Q fever, who had made an asymptomatic recovery; QFS Group 5 (QFSGr5) with acute Q fever followed by QFS, but without co-morbidity; QFS Group 6 (QFSGr6) with QFS and other morbidity associated with 'fatigue' e.g. developing endocarditis. Twelve years after the outbreak, subsets recGr3, QFSGr5 and QFSGr6 were sampled for Q fever antibody, and blood leucocytes and BM aspirates were tested by PCR for *C.b.* gene sequences.[6] In the current investigation, samples from 10 patients in these subsets were chosen for inoculation into NOD/SCID mice in an effort to isolate living coxiellas and to ascertain accompanying pathological effects.

Cytosolic fraction from patient PBMCs for IL-10 analysis was separated by differential centrifugation.[9] PBMC homogenates were centrifuged at 1000*g* for 3 min at 4°C. The isolated supernatant was further centrifuged at 14 000*g* for 30 min at 4°C. The resulting supernatant fluid was collected and analysed for IL-10 levels using ELISA (R&D Systems).

Patient samples tested positive for *C.b.* DNA were pooled; lipopolysaccharides (LPS) were extracted using hot phenol–water gradient method.[10] Similarly, control LPS was extracted from Q Vax vaccine (CSL Ltd.).

Inoculation and monitoring of NOD/SCID mice

NOD/SCID 6- to 8-week-old mice (Adelaide University, Australia) were kept in micro isolator cages in specific pathogen-free conditions on sterilized food and water. Groups of 3–6 mice were inoculated intraperitoneally with 0.1 ml of emulsion from BM, or PBMC, or aortic valve specimens and held for the 14–60 days according to the experimental design as described previously.[7,11,12] Control animals received negative blood specimens from PCR and seronegative control persons. Mice were observed daily and weighed weekly. Body weight changes were expressed as body weight index (BWI). The BWI is the relative body weight of an inoculated mouse on a day divided by the relative body weight of sham-infected mice on the same day; the relative body weight is the body weight on each day divided by the body weight at zero time.[12]

Blood was collected from the lateral saphenous vein after 48 h, 7 days and 14 days post-inoculation (DPI) and after euthanasia by cardiac puncture at 60 DPI. Saphenous vein was penetrated with a 25-gauge needle. Blood was collected in a capillary pipette flushed with EDTA; two 20-µl increments were collected. Mouse blood plasma was then analysed for IL-6 and IL-10 levels using ELISA (R&D Systems).

At the designated time points mice were euthanized by inhaled CO_2 overdose. Spleen, liver and BM samples were harvested. Spleens were weighted, and then spleen fragments were taken for histopathology. Other spleen fragments were placed into lysis buffer (Proteinase K 2 mg/ml, SDS 0.5% w/v) and tested by PCR for *C.b.* sequences.

Experiments were performed to a protocol approved by the institutional Animal Ethics Committee and performed in bio-containment

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level 3 laboratories and animal house accommodation (SA Pathology, Site 1 Adelaide, Australia).

Immunocytochemistry and histochemistry

For this purpose NOD/SCID mouse and out bred guinea pig tissues (spleen, liver and/or skin) were collected, formalin fixed (4%, v/v) and paraffin embedded. Sections (5 µm) of paraffin-embedded tissues were cut and mounted on APES coated (frost plus) tissue slides and deparaffinized according to standard protocol. Slides were then counterstained with haematoxylin and eosin (H&E) for microscopic examination.

For *C.b.* antigen detection, sections were pre-treated in citrate buffer (10 mM; pH 6.0) and heated by microwave at 95°C for 10 min, then allowed to cool to room temperature. Antisera and negative sera were diluted in phosphate buffered saline (PBS). Immunostaining was performed on selected slides with primary antibodies for the following markers: (i) *C.b.* Phases I and II antigens (rabbit polyclonal) or mouse Phase I monoclonal (a gift from CSL Ltd., Australia); (ii) macrophages mouse anti-rabbit F4/80; (iii) CD11c rabbit anti-mouse (Sapphire Bioscience); (iv) LAMP-1 (CD107a, BD Biosciences). After washes in PBS/0.05% Tween-20 (Fisher Scientific), sections were incubated with 1:100 dilutions of anti-rabbit or anti-mouse secondary antibodies conjugated to Fluorescein Isothiocyanate (FITC) or Phycoerythrin (PE) (Jackson ImmunoResearch Laboratories Inc.) according to the instructions of the manufacturer. Nuclei were stained with Hoechst 33342 (Invitrogen). The relative concentration of detected fluorescent signals over designated background per microscopic image was estimated semi-quantitatively using Olympus Soft Imaging System.

Image acquisition and analysis

Phase-contrast images were captured using a microscope (IX70; Olympus), UPlanFl 10× NA 0.3 Ph1 and LCPlanFl 20× NA 0.4 Ph1 objectives and a camera (DP12; Olympus). Colour and fluorescent images were captured using a microscope (IX81; Olympus) with 10× UPLSApo NA 0.4, 20× UPLSApo NA 0.75, and 40× UAPO/340 NA 1.15w objectives and CC12 (Soft Imaging System, colour) and Hamamatsu Orca-ER (fluorescent) cameras, respectively. Fluorescence images were acquired using Cell^{R} software (Olympus Soft Imaging System). All other images were acquired and processed using the Analysis software (Olympus Soft Imaging System). For immunofluorescent images where comparisons of staining intensities were made, the images were acquired using the same attenuator and exposure settings.

Guinea pig skin granuloma test

Twelve out bred Hartley guinea pigs (GPs) were immunized by intradermal injection with 80 µg Q fever vaccine (Q Vax, formalin-killed purified whole cell Phase 1 *C.b.*, Henzerling strain, CSL Ltd. Melbourne, VIC Australia). The immunization was repeated after a 2 week period. Two weeks after last Q Vax injection GPs were anesthetized, and both flanks of a guinea pig were shaved prior to intradermal injection of 0.1 ml of different antigens. Up to four sites on each side of the guinea pig were used. Intradermally injected samples consisted of the set of selected patient's PBMC or BM specimens, control PBMC specimens, or Q Vax (60 µg) all in duplicate for each animal. The immunoreactivity of antigens at the injection sites were monitored for 14 days and skin conditions were measured and recorded daily. The extent of the granulomatous reactions was measured with calipers and expressed as the mean of two measurements taken at right angles to each other so as to delineate the reaction area. The results were expressed as the diameter (in millimetres) of the induration reaction as described previously.[13] Similar injections were done to the non-vaccinated GPs used as controls. After 14 days of monitoring, GPs were euthanized. Skin samples were collected from inoculated and control sites, processed and assessed by H&E staining.

Reactogenity of the inocula was scored semi-quantitatively. The inoculated areas were evaluated and compared to the uninoculated area on the back of the same animal. The granulomatous response scoring was as follows: 0, no induration; 1, induration $\leq$4 mm ($S \leq 4$); 2, induration $\leq$10 mm ($4 < S \leq 10$); 3, induration larger than 10 mm ($S > 10$). Statistical analysis of the responses (clinical scores) induced by each of the sample was performed by using paired *t*-test, while a measure of the strength of the responses was represented by the mean ± standard deviation.

Detection of *C.b.* DNA target sequences by PCR

Animals tissue samples were tested by two separate PCR assays, one targeting the 27-kDa outer membrane protein (COM1) and the other detecting the insertion sequence (IS1111a) performed on 5 µl of the DNA extracted from 200 µl of lysed tissue (lysis buffer: proteinase K 2 mg/ml, SDS 0.5% w/v) using the Qiagen (Hilden, Germany) DNA blood kit and

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amplified for 45 cycles using the Roche LightCycler (Basel, Switzerland) as described previously.[14] To avoid false positives, DNA extraction and PCR were performed carefully according to guidelines described previously.[15] The LightCycler software version 4.05 (Roche) was used to determine the C_t values of the PCR products.

Monocyte/macrophage cell culture assay

Monocytoid THP-1 cells were cultured in RPMI 1640 with containing 25 mmol/l HEPES buffer (supplemented with 10% FCS, 1% L-glutamine 200 mmol/l, penicillin 100 U/ml and streptomycin 100 μg/ml) in humidified air, 5% CO_2 at 37°C and maintained at $3–6 \times 10^5$ cells/ml. After washing twice with Ca^{2+}-free Hank's solution (HBSS, Sigma), cells were incubated in 96-well plates (5×10^5/ml) in serum-free RPMI 1640 medium. For cytokine production assay THP-1 monocytes were differentiated into monocyte-derived macrophages (MDMs) by treatment with 0.1 μmol/l phorbol ester (PMA) (Calbiochem) overnight in starvation medium, 0.5% FCS RPMI 1640. Monocyte differentiation to macrophages was confirmed by changes in morphology and then MDMs were incubated with or without ligands [salmonella LPS (100 ng/ml) (Sigma-Aldrich); Q Vax (80 ng/ml) (CSL Ltd.); or extracted coxiella LPS from patient samples confirmed to react with a potent anti-*C.b.* phase 1 antiserum similarly to the LPS extracted from the Q Vax (data not shown]. Cell cultures were stimulated with the ligands for 24–72 h. Supernatants were collected for cytokine measurements using cytometric bead array (BD Biosciences) or ELISA (R&D Systems) according to the manufacturer protocol. To exclude cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase in cell supernatant was performed according to the manufacturer's recommendations (Roche).

BD™ cytometric bead array

Human Inflammation [cytometric bead array (CBA)] system (BD Biosciences) uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. We used CBA to quantify interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α) and interleukin-12p70 (IL-12p70) protein levels in tissue culture supernatant fluids, EDTA plasma or PBMC homogenates. The experiments were done according to the manufacturer protocol. Results were analysed using BD™ CellQuest Software. Briefly, samples were incubated with the combined capture bead cocktail, diluted in capture bead diluent and incubated for 1 h at RT. Following incubation, samples and beads were mixed with the combined cocktail of PE detection antibodies for the five cytokines and incubated for 1 h. Sample was then washed and resuspended in FACS buffer. Cytokine concentrations were measured via quantification of PE fluorescence of samples in reference to a standard curve.

Results

Birmingham cohort patients' specimens inoculated in NOD/SCID mouse failed to induce acute infection, but resulted in *C.b.* antigen accumulation in mouse spleen and other pathological changes *in vivo*

In order to detect residual infectivity of *C.b.* BM/PBMC homogenates from Birmingham patients' were inoculated into NOD/SCID mice in triplicate. The inoculated specimens were positive by PCR with probes for Com 1 (Table 1) and belonged to Birmingham cohort of patients chosen from three clinical groups described in Material and methods section. Control animals were inoculated with patient PBMC homogenates that were PCR and serologically negative for *C.b.*[6]

After inoculation, mice were monitored for sickness behaviour over 60 days and weighed daily according to standard protocol for detection of infection by failure to thrive.[12] Minor, but statistically significant weight loss was noted among mice inoculated with samples from QFSGr5 and 6 during the first week after inoculation. The weight loss recovered by Day 14 afterwards (Figure 1A–C). The subsequent steady gain in weight was consistent with the absence of infection.

The absence of infection was also supported by PCR analyses: all mouse spleen specimens were negative at 1:100 dilutions (Table 1) that strongly indicates a lack of growth and replication of the *C.b.* organisms in the NOD/SCID mice. Overall these data are in agreement with previous results of inoculation of specimens from PCR positive patients into A/J mice, INF-γ KO mice, and human embryo lung fibroblasts cell culture.[6]

However, despite the absence of active infection, a number of changes were observed in the mice. Moderate spleen enlargement ($29 \pm 7\%$ of control spleen size, $P < 0.05$) occurred in mice inoculated with QFS patients' specimens from Groups 5 and 6 (QFSGr5 and 6) when compared to control mice (Figure 2A), not however comparable to massive splenomegaly induced by live coxiellas. Inoculation of formalin-inactivated bacteria

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Table 1 Summary of findings on the 11 samples from 10 patients in the earlier studies compared with those in the present investigation

Patient samples/original tests						Samples inoculated into NOD/SCID mice				
Patient's tissue	Clinical group	Cohort #	Presence of *C.b.* DNA analysed by PCR		Cytokine values	Pathological responses		Presence of *C.b.* DNA by PCR (*COM1*)		Spleen antigen by IFA
			IS 1111a/ COM1	Correct sequences	hIL-10, pg	Enlarged spleen	Increased no. of megakaryocytes	1:1	1:100	
1. PBMC/BM	QFSGr6	57[a]	+/+	+	55.8	+/– –	+	– –	– –	+
2. PBMC/BM		84	+/+	+	185.9	+	+	+/– –	– –	+
3. Heart Valve		84	+/+	n/t		+	+	+	– –	+
4. PBMC/BM	QFSGr5	138[a]	– –/+	+	97.8	+	+	+	– –	+++
5. PBMC/BM		2	+/+	+	26.4	– –	–	– –	– –	+
6. PBMC		44	– –/+	+	45.2	+	++	+	– –	+++
7. PBMC		107	– –/+	+	10.5	+	+	– –	– –	– –
8. PBMC/BM		121	– –/+	+	13.7	+/– –	+/– –	+	– –	+
9. PBMC/BM	recGr3	10	– –/+	+	65.6	– –	+/– –	– –	– –	+++
10. PBMC/BM		78	– –/+	+	14.2	– –	+/– –	– –	– –	+
11. PBMC/BM		117[a]	+/+	+	15.9	– –	– –	+	– –	+
12. PBMC		Neg. Ctrl	– –/– –		18.4	– –	– –	– –	– –	– –
13. PBMC/BM			– –/– –		15.8	– –	– –	– –	– –	– –

Post Q fever fatigue patient specimens (1–11) were analysed for presence of *C.b.* DNA by PCR and then effects of *C.b.* antigens were assessed after inoculation of the samples into NOD/SCID mice (mouse spleen size, haemopoiesis, PCR for bacterial DNA; presence of specific *C.b.* antigens by IFA). Clinical recGr3: no fatigue, no comorbidities; clinical QFSGr5: post Q fever fatigue, no comorbidities; clinical QFSGr6): post Q fever fatigue, with comorbidities as described previously.[5,6]

[a]For Patients #57, 138, 117 PCR results with IS 1111a were at a borderline.

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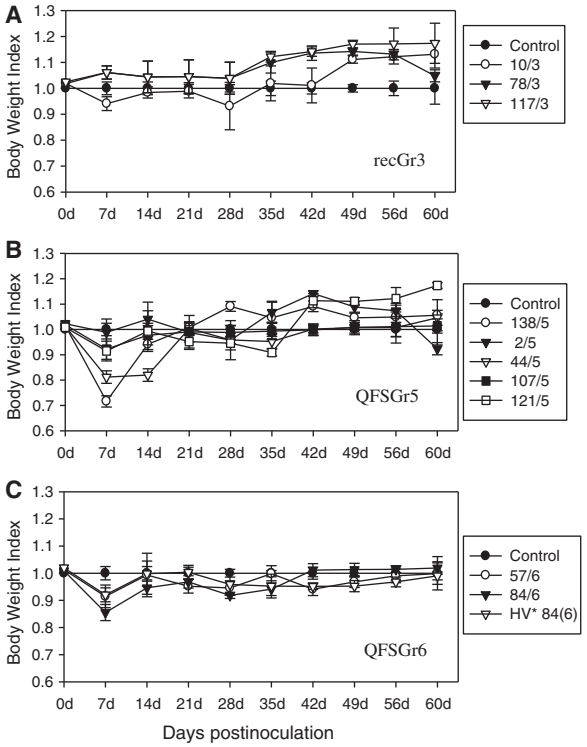

Figure 1. Body weight changes of NOD/SCID mice inoculated with 0.2 ml of PBMC/bone marrow patients' and control specimens from Birmingham Q fever collection. Patient numbers refer to data in Table 1. (**A**) Clinical Group 3, post Q fever patients with no fatigue, no comorbidities (recGr3). (**B**) Clinical Group 5, post Q fever fatigue syndrome, no comorbidities (QFSGr5). (**C**) Clinical Group 6, post Q fever fatigue syndrome (QFS), with comorbidities (QFSGr6). The results (means ± standard deviations) are expressed as the body weight index as described in Materials and methods section.

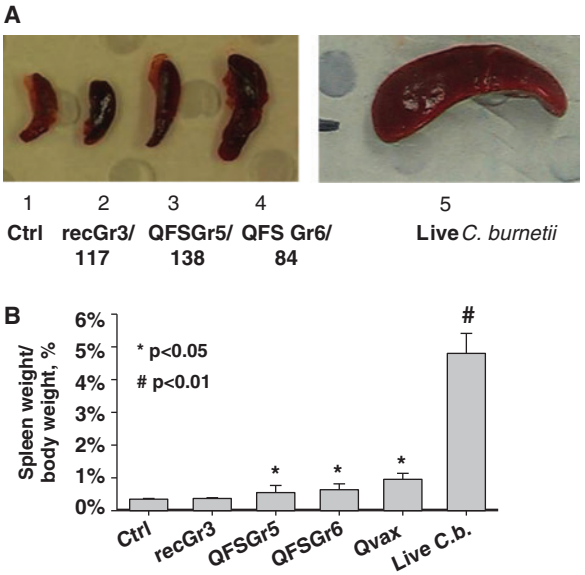

Figure 2. NOD/SCID mouse spleen pathological responses. (**A**) Macroscopic views of NOD/SCID mouse spleen sizes in control mice and mice inoculated with samples from patients in recGr3, QFSGr5 and QFSGr6 compared to splenomegaly with a viable culture of Henzerling strain of *C.b.* Representative photographs are shown. (**B**) Spleen/body weight ratio percent of NOD/SCID mice inoculated with PBMC/BM patients' specimens, Q Vax and Henzerling strain are shown to accompany statistically significant pathological changes in spleen. The results are means ± SE.

(Q Vax, 100 ng) also stimulated moderate spleen enlargement (Figure 2B and Table 2)

Remarkably, in the absence of PCR C_t values indicating presence of coxiella genomes or genomic fragments, sections of mouse spleens showed variable amounts of aggregates stained to detect specific antigen using anti-*C.b.* monoclonal antibody that reacts with Phase 1 coxiella (Table 1 and Figure 3A and B). The antigen was also present in large amounts in sections from the original Birmingham patient heart valve (Patient 84/6)[4] and also at lower levels in the spleens of the NOD/SCID mice inoculated with the valve homogenate (Figure 3A, QFSGr6, Patient 84). Figure 3B demonstrates significant increases in intensities that were found in the QFSGr5 and 6 comparing to control. Coxiella antigens were also found consistently in NOD/SCID mouse BMs and livers inoculated with post-Q fever patient's specimens (data not shown). Overall, although the increases are small there were consistent trend differences in spleen size and weight, and antigen concentration among recGr3, QFSGr5 and QFSGr6. However, the presence of coxiella antigens did not correlate with the low levels of coxiella DNA (Table 1) suggesting that the antigen complexes represent incompletely degraded cell material. The dichotomy between antigen and DNA values suggested that the complex of coxiella proteins and LPS detected by the rabbit polyclonal antiserum with Phases 1 and 2 reactivity, and by the mouse monoclonal antibody to Phase 1 antigen, survives degradation in host cells more successfully than coxiella genomic or transposon DNA.

Consequently, a highly purified whole cell, formalin inactivated vaccine (Q Vax, CSL Pty Ltd.) in which coxiella DNA, proteins and other cell components are intact, was tested in NOD/SCID mice to determine survival patterns of DNA and antigen in mouse splenocytes. We inoculated NOD/SCID mice using Q Vax at different doses ranging from 1 to 100 ng; in triplicate. After 21 days, examination of the mouse spleen sections showed fluorescent aggregates of *C.b.* antigen in amounts proportional to the dose of vaccine inoculated (Figure 4A and Table 2). PCR assay of *C.b* genomic targets in the same samples showed that the coxiella DNA and

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Table 2 Titration of purified formalized whole cell *C.b.* vaccine (Q Vax Henzerling strain) in NOD/SCID mice

NOD/SCID mouse spleen tissue		Pathological responses		
Q Vax dose (ng)	IS 1111a/ COM1 PCR	Enlarged spleen	Increased no. of megakaryocytes	IFA detected antigen
1	+/+	−	−	Present
5	+/+	−	±	
10	+/+	±	+	
50	+/+	+	+	
100	+/+	+	+	

PCR were designed to detect target DNA sequences of COM1 and IS1111a genes. IFA detected Phase 1 *C.b.* antigen with mouse monoclonal antibody. Spleen tissues from Q fever vaccinated NOD/SCID mice were analysed and used as positive controls for data in Table 1 and for discussion of data on Figure 6D and E.

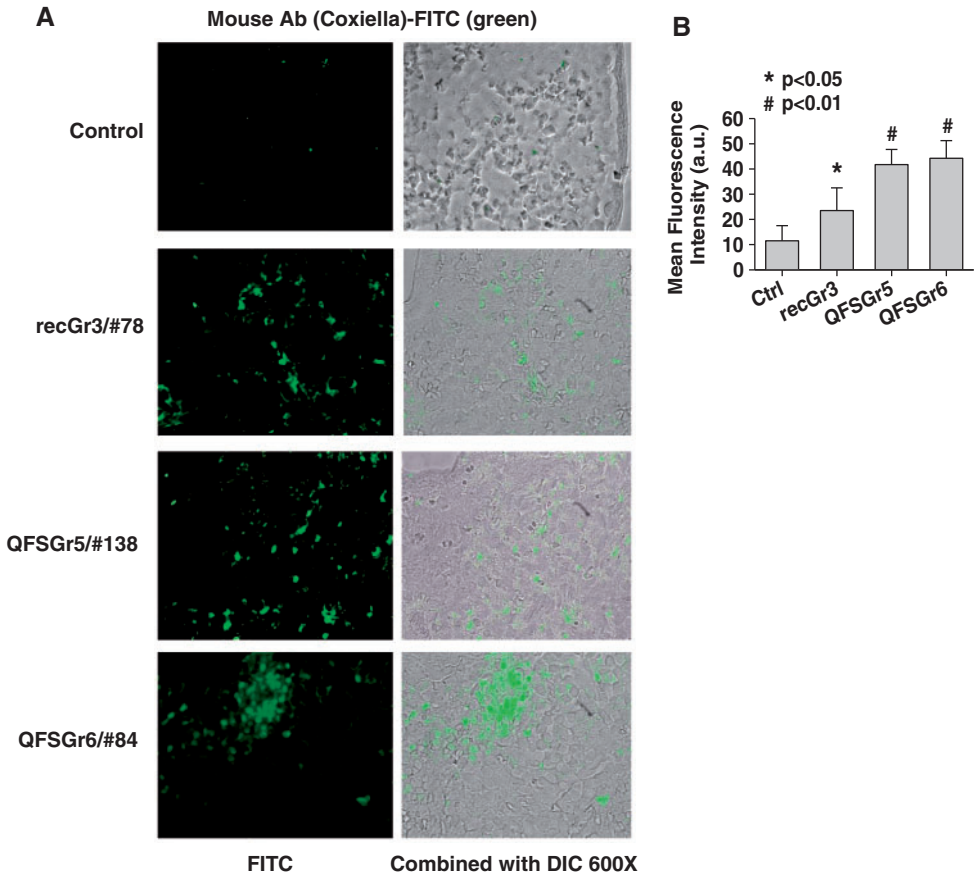

Figure 3. Immunofluorescent detection of *C.b.* antigens in NOD/SCID mouse spleen. (**A**) Illustrative sections of NOD/SCID mouse spleens from group of animals inoculated with PBMC/BM homogenates from seronegative persons (control), and from recGr3, QFSGr5 and QFSGr6 of the Birmingham cohort of Q fever patients. Sections were stained with monoclonal primary antibodies to *C.b.*, visualized with FITC-conjugated secondary antibodies and superimposed with DIC images magnified ×600. (**B**) Estimates of the mean fluorescence intensity analysed for spleen microphotographs taken in quadruplicate for each spleen section of total three mice per patient in patient subgroups. Fluorescence intensity was quantified for all FITC spots over background using Analysis software (Soft-Imaging Systems, Olympus) for each image separately.

antigen concentrations declined in step over the same range of inocula (Figure 4B). Thus, DNA remained intact in NOD/SCID mice after inoculation of Q Vax for at least 21 days. In sharp contrast to these findings, the proportions of DNA and antigens recovered in NOD/SCID mice from BM/PBMC samples taken 12 years after infection in the Birmingham outbreak had only trace amounts of *C.b.* DNA, but

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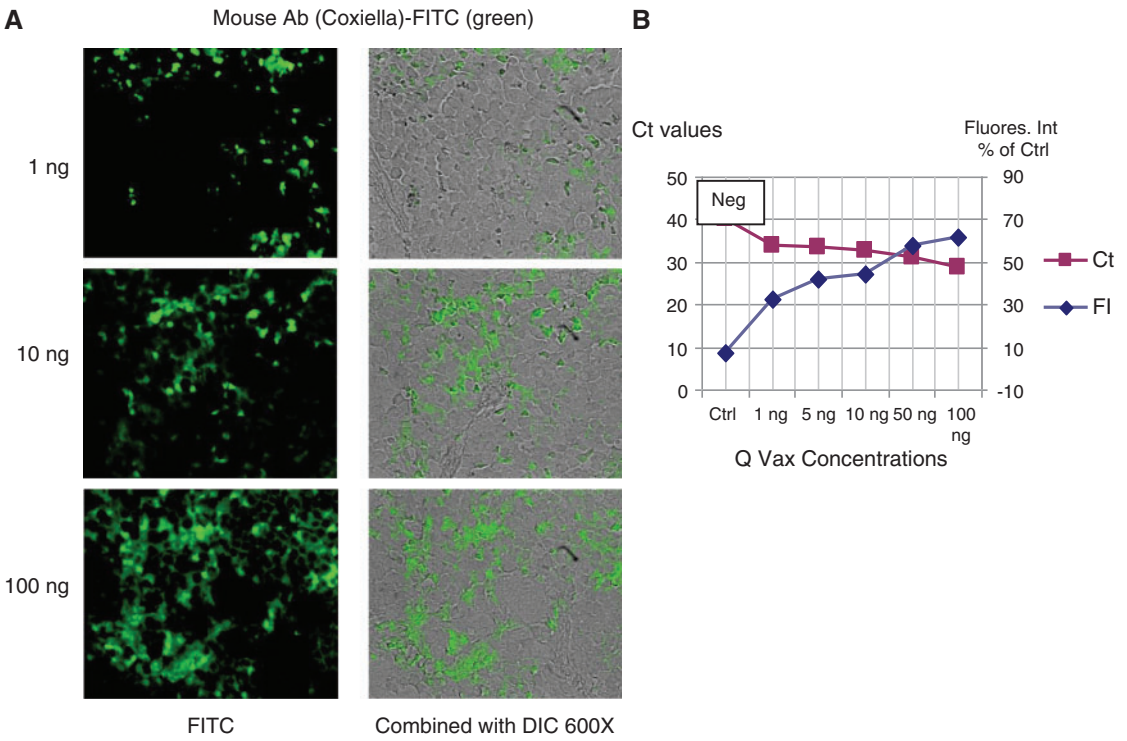

Figure 4. Immunofluorescent detection of *C.b.* antigens in spleen of NOD/SCID mice inoculated with different doses of Q Vax. (**A**) Accumulation of *C.b.* antigens in splenocytes of NOD/SCID mice 21 days after inoculation with 1, 10 and 100 ng of Q fever vaccine. Spleen sections were stained first with mouse monoclonal antibody to *C.b.* Phase 1 antigen, then visualized with FITC-conjugated anti-mouse as a secondary antibody, and combined with DIC images. Magnification is ×600. (**B**) Dual axis curves showing relative *C.b.* target sequence (C_t value) numbers detected by PCR vs mean fluorescence intensity percentage for FITC stained antigen foci calculated per every image in spleens from mice given varying doses of Q Vax.

still significant amount of antigen complexes (Figure 3). These findings help to define the differences between an active viable infection and the effects of antigenic coxiella cell residua: so-called immunomodulatory or immunosuppressive complexes (IMC) as originally suggested by Waag and Williams.[16]

Interestingly, *C.b.* LPS from Henzerling strain purified by hot phenol–water extraction method when inoculated into SCID mice was not detected by IFA and was cleared completely during 14 days after inoculation into NOD/SCID mice suggesting other cell components were linked to LPS in the IMC (data not shown).

Cellular location of *C.b.* antigens in NOD/SCID mouse splenocytes

The fluorescent coxiella IMCs were localized in the marginal zones of the mouse spleen that are occupied by macrophages and dendritic cells (DCs) involved in active phagocytosis of coxiella and subsequent antigen presentation.[17] Double staining technique confirmed that *C.b.* antigens were localized in spleen phagocytes. Sections of spleen from mice inoculated, respectively, with 100 ng of inactivated whole cell Q fever vaccine (control antigen) or with suspension of heart valve from Patient # 84 (QFSGr6, Table 1) both display *C.b.* antigens (green) localized in spleen macrophages and DCs (Figure 5). Red (PE) staining with antiserum to F4/80 (macrophage marker) or CD11c (DCs marker) was used for phagocytes identification (Figure 5A and B). Figure 5A–C shows that *C.b.* antigens are located in spleen phagocytes, particularly in F4/80 labelled macrophages and CD11c expressing DCs. Yellow colour was detected when green and red fluorescence overlapped and served as an indicator for co-localization of the chosen antiserum.

We further detected localization of coxiella IMC in lysosomes of mouse splenocytes. *C.b.* antigens from Patient #138 (QFSGr5, Table 1) and control *C.b.* antigens from Q Vax were detected at lysosomes marked by LAMP-1 (CD107a) (Figure 5D and E). The bright yellow colour indicates that green fluorescence from FITC-labelled *C.b.* coincided with red fluorescence from PE labelled LAMP-1.

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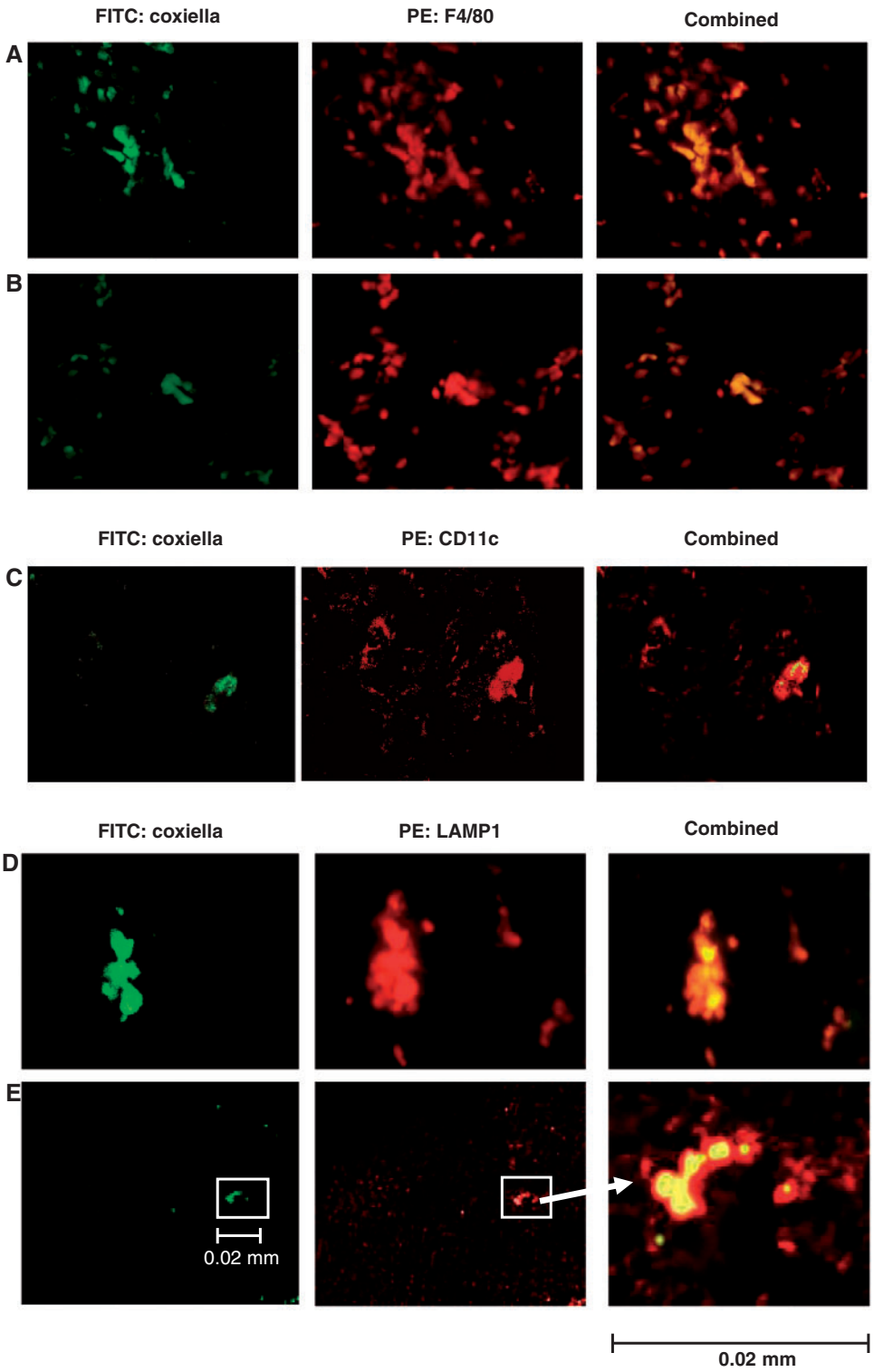

Figure 5. Localization of *C.b.* antigens in NOD/SCID spleen phagocytes. (**A**) NOD/SCID mouse inoculated with 100 ng of Q Vax. (**B**) NOD/SCID mouse inoculated with PBMC from Patient #84/QFSGr6. PE-conjugated F4/80 macrophage marker was used in (A) and (B). Co-localization of *C.b.* antigen in macrophages is indicated by yellow colour, magnification ×1000. (**C**) NOD/SCID mouse inoculated with PBMC from Patient #84/QFSGr6. PE-conjugated CD11c dendritic cells marker was used, magnification ×1000. (**D**) NOD/SCID mouse inoculated with 100 ng of Q Vax. (**E**) NOD/SCID mouse inoculated with PBMC specimen from Patient #138/QFSGr5. For (D and E) PE-conjugated secondary antibodies were used to visualize lysosome marker LAMP-1 in NOD /SCID splenocytes, magnification ×1000. In all experiments, spleen sections were stained with primary mouse monoclonal antibody to *C.b.* Phase 1 antigen and visualized with FITC-conjugated anti-mouse secondary antibodies. Representative microphotographs are shown.

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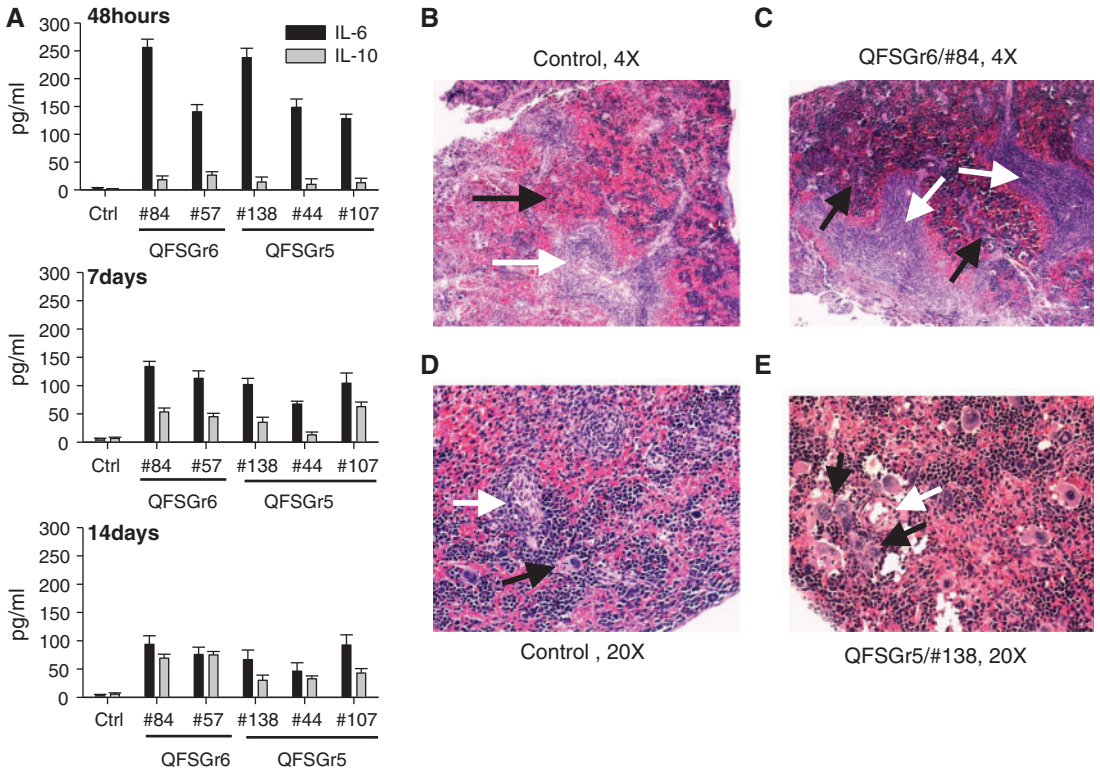

Figure 6. Time-course measurements of IL-6 and IL-10 in mouse plasma and spleen pathology after inoculation of patients' specimens. Each patient's sample was inoculated into three NOD/SCID mice as described in Materials and methods section. (**A**) Mouse blood was collected 48 h, 7 and 14 days after inoculation of patients' specimens and further analysed for the levels of IL-6 and IL-10. Results are means ± SE. (**B**) Control mouse spleen. (**C**) Mouse spleen inoculated with PBMC specimen from Patient #84/QFSGr6. For (B and C) white arrows indicate follicular area; black arrows indicate blue islands of haemopoiesis in the red pulp zone. (**D**) Control mouse spleen. (**E**) Increased megakaryocytes number in mouse spleen after inoculation of PBMC specimen from Patient #138/QFSGr5. For (D and E) white arrows indicate the area of blood vessel; black arrows indicate megakaryocytes. For (**B–E**) H&E staining was used for examination of NOD/SCID mouse spleen sections.

Inoculated patients' specimens stimulated cytokine production and spleen haemopoiesis in NOD/SCID mice

Localization of the *C.b.* antigens at the macrophage lysosome suggested that phagocytosis of the antigens was successful and, thus, production of pro-inflammatory cytokines might be also activated. Additionally, it had been shown previously that live *C.b.* stimulated increases in levels of anti-inflammatory cytokines and, thus, might subvert anti-infectious competence of host, especially its microbicidal activity.[18] Accordingly, we determined the level of release for two cytokines (pro-inflammatory IL-6 and anti-inflammatory IL-10) in mouse blood. Blood was collected by saphenous venipuncture 48 h after inoculation of patient specimens, and on days 7 and 14 afterwards (Figure 6A). Significant increase in level of IL-6 was detected 48 h after inoculation, however substantially decreased in following 2 weeks. On the contrary, the level of IL-10 was gradually increasing. Two weeks after inoculation of patient specimens, the level of IL-10 was still increased compared to the slowly decreasing level of IL-6 suggesting that elevated level of IL-10 might be sufficient to down-regulate macrophage activation and inhibit clearance of the *C.b.* antigens. The level of IL-10 was also measured in patient specimens. We detected significantly increased IL-10 in one sample (QFSGr6/84) and moderate increases of IL-10 in 2 specimens (recGr3/10 and QFSGr5/138) out of the 10 specimens tested (Table 1).

H&E staining of mouse spleen sections did not confirm formation of large replicative vacuoles (LRV) in the enlarged spleen samples indicating the absence of *C.b.* multiplication. However, moderately increased haemopoiesis was detected (Figure 6B–E). We observed moderate expansion of the follicular areas (Figure 6D, white arrows), increased extramedullary haemopoiesis (Figure 6D, black arrows), and increased number of megakaryocytes around blood vessel, as the signs of stimulated thrombocytopoiesis (Figure 6E). These findings

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confirmed activation of host immune responses to the presence of inoculated material.

C.b. antigen complexes stimulated cytokine release by THP-1 cells

NOD/SCID mice do not exhibit wide proliferative T-cell responses to non-specific mitogens (PHA or Con A) stimulation,[19,20] even though specific T- and B-cell responses are present in older SCID mice.[21] Furthermore, NOD/SCID macrophages are immature and do not produce detectable level of IL-1.[21] These multiple deficiencies of NOD/SCID mouse splenocytes make this *in vivo* model hard to interpret, particularly in assessing macrophage responses. Therefore, we used the human monocyte THP-1 cell line to measure the ability of macrophages to respond to putative coxiella antigens/IMC purified from the pooled patients' specimens. Cytokine responses were measured using cytometric beads array (CBA, BD Biosciences) technique detecting five cytokines in culture supernatants. Salmonella LPS was used as positive stimulatory control and confirmed the ability of THP-1 cells to produce high level of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Figure 7). Stimulation with Q Vax (80 ng) and *C.b.* IMC/LPS extracted from pooled patient specimens resulted also in significantly increased levels of cytokines compared with the unstimulated control. However, the degree of responses to Q Vax and *C.b.* LPS is recognized to be less potent compared with the robust responses to salmonella LPS.[22,23] Our findings are in agreement with this statement.

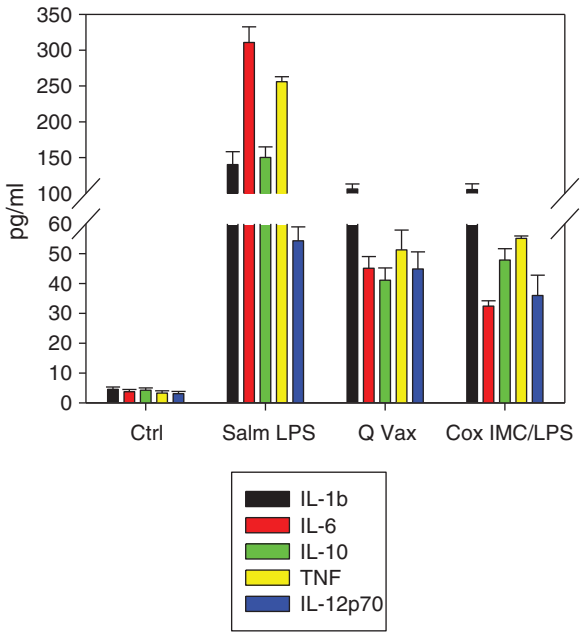

Figure 7. Stimulation of cytokine production in THP-1 cells by salmonella LPS, whole inactivated coxiellas (Q Vax), and pooled LPS extracted from coxiella PCR positive patient's PBMC using hot phenol–water gradient extraction method.[10] Five cytokines in supernatant of cultured THP-1 cells were measured using CBA as described in Material and methods section.

Interestingly, the ratio of IL-6 to IL-10 production had different profile for salmonella and coxiella LPS: for salmonella stimulation IL6/IL-10 ratio was ∼2; for Q Vax stimulation IL6/IL-10 it was ∼1, and for *C.b* IMC stimulation IL6/IL-10 it was <1. However, the level of IL-10 produced by THP-1 cells in response to Q Vax or *C.b.* IMC (putative *C.b.* LPS/protein complexes) stimulation was still high, especially considering the low response level for IL-6. Speculatively, we suggest that IL-6/IL-10 ratio and high level of IL-10 might signal important role in facilitating survival of non-degraded bacterial material.

C.b. IMC produced inflammatory skin lesions in Guinea pigs hyperimmunized with inactivated whole cell Q fever vaccine

So far our investigation of PBMC/BM samples from the Birmingham cohort of Q fever patients have detected presence of *C.b.* proteins (using rabbit polyclonal antiserum to Phase 2 antigen) and complete LPS (using monoclonal antiserum to Phase 1 antigen), which most likely exist as incompletely digested complexes. The complexes were found to locate in lysosomes of cells in the macrophage lineage. This implies that these particles phagocytosed by macrophages should be properly recognized and used for further antigen presentation and activation of cytokine production. We found several indicators, particularly moderate spleen enlargement and activated haemopoiesis, and cytokine production as described above (Figures 2A and 6), that activation of immune system actually happened. However, multiple immune deficiencies of the NOD/SCID mice make it inappropriate to extrapolate the findings from this mouse model to immunologically competent animals and humans.

Consequently, patient's specimens containing *C.b.* antigens were tested for their ability to produce skin lesions after inoculation intradermally into guinea pigs (GPs). GPs were sensitized with multivalent vaccine (Q Vax, CSL) as described in Material and methods section. Following the immunization, patients' specimens, Q Vax and control coxiella PCR-negative specimens were injected intradermally in duplicate into GPs flanks.

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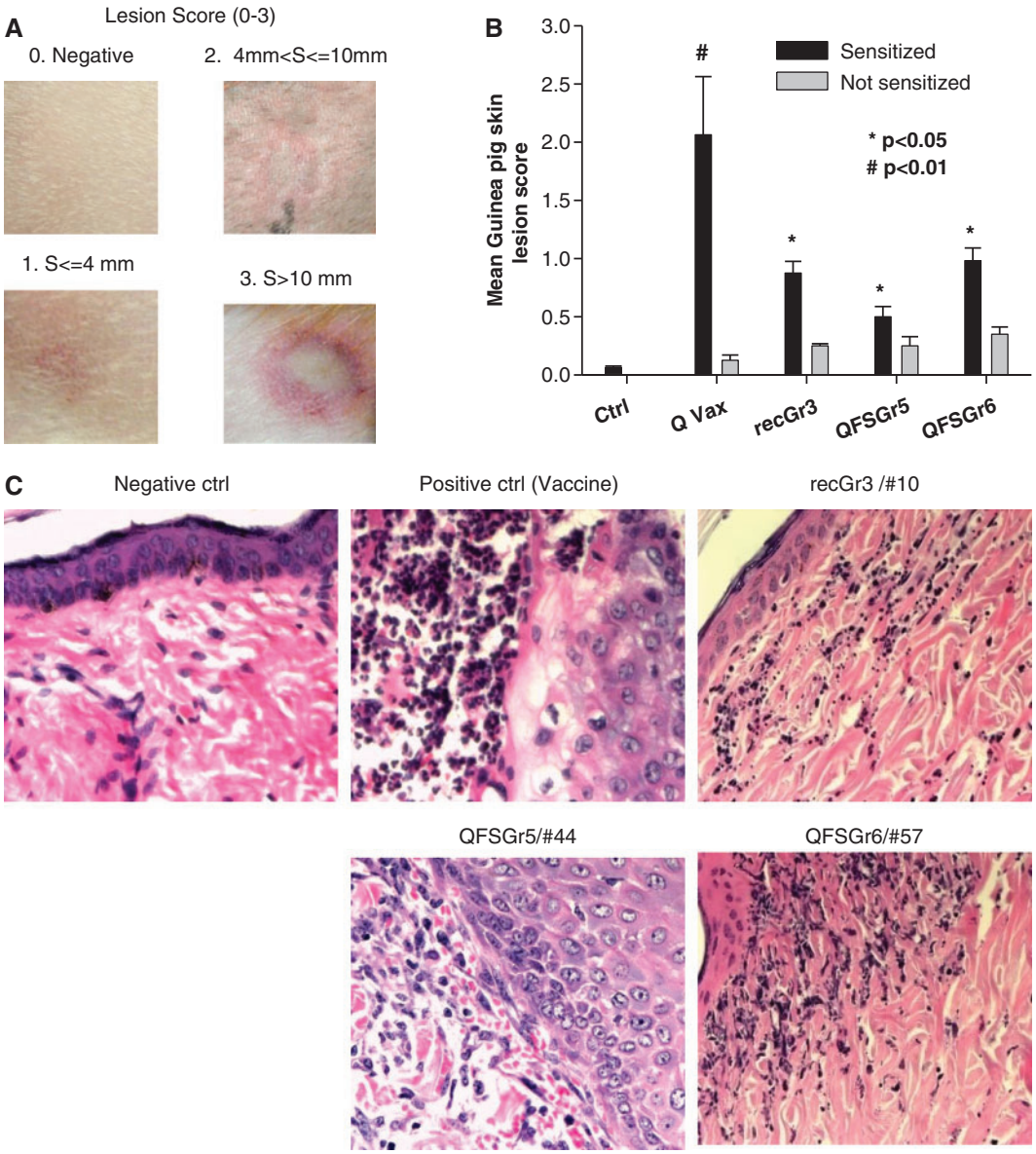

Figure 8. Guinea pig granuloma skin test. (**A**) Lesion scores 0–3: 0—negative (no induration or lesion); 1—induration < or = 4 mm, $S \leq 4$; 2—lesion < or =10 mm, $4 < S \leq 10$; 3—lesion >10 mm, $S > 10$. (**B**) Means of GPs skin score represents sizes of lesion score for sensitized animals with Q Vax vs. not sensitized GPs after challenging of animals with human specimens during 14 days. From recGr3 the specimens were used from Patients #10–44 (QFSGr5)— and patient # 57 (QFSGr6). (**C**) H&E staining was used for examination of GPs skin sections. Representative microphotographs are shown.

The reactions to the inocula at the skin injection sites, particularly such as concomitant inflammatory reactions, were monitored for 14 days. Skin conditions/reactions were measured and recorded daily. GPs inflammatory skin responses were scored 0–3. Granuloma scores are presented on Figure 8A and B. The response to Q Vax was strongly defined and scored above 2. Responses to the patient specimens were significant, but lower than those to Q Vax. After 14 days of monitoring, the animals were euthanized, skin samples were collected and analysed. H&E staining of mouse skin sections confirmed that the injected patients' specimens and Q Vax induced aggregation of mono- and polymorphonuclear neutrophils indicating the hypersensitivity of immunized animals to injected pathogens[13,24] (Figure 8C). Injection of control specimens induced minor erythema during first 24 h after injection that was rapidly cleared afterwards.

Discussion

A post-infection fatigue syndrome (QFS CFS or qCFS) as an incapacitating sequel to acute primary

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Q fever in 10–20% of cases was described in Adelaide SA Australia and in Birmingham (UK) at the end of the 1990s.[4–6,25] Subsequent investigations of its pathobiology proceeded in the Q fever Research Group in Adelaide in collaboration with Jon Ayers and colleagues in Birmingham, and during the last 2 years also in collaboration with the Australian Rickettsial Reference Laboratory (Geelong, VIC Australia).

Our working hypothesis or paradigm for QFS—basically unchanged but refined over the period 1996 to date—is that five main factors are involved. Namely, (i) that after the initial primary infection either the viable coxiella or its antigenic and structural components persist in the body and continue to stimulate the humoral and cell mediated immune (CMI) systems to a variable extent; (ii) that in the majority of patients the immune stimulation is successfully down-regulated and asymptomatic recovery is eventually achieved, albeit slowly, although antibody and a positive intradermal skin test to *C.b.* antigen, signal its low-level persistence; (iii) in the minority of patients who develop QFS, a stable immune homeostasis is not achieved or is easily reversed on re-exposure to the coxiella in their environment; (iv) that the symptom complex of QFS reflects a failure of CMI homeostasis determined by polymorphic immunogenetic background of the individual; (v) that the CMI dysregulation resulting from inappropriate responses of cytokine and other immune mediators alters levels of gene expression in other organ systems thus generating the stereotypic symptom complex of QFS. Some tests of postulates 2, 3 and 4 are detailed elsewhere.[4,6,26–28]

The first hypothesis about survival and nature of the physical state of viable organisms or antigen complexes after acute infection—is a central issue for the paradigm and is the focus of the current investigation.

Prolonged carriage of living *C.b.* in humans after an initial infection is recognized in early stage Q fever endocarditis, in sporadic granulomas of liver, lung, testes or bones, and also in particular by its recrudescence in late pregnancy.[29]

However, we are less certain of the host–pathogen interaction in the longer term after uncomplicated acute primary Q fever. The organism can be isolated from peripheral blood around the very end of the incubation period and for a period of to 8–14 days from the onset of illness up to the appearance of antibody. It may also be present in the urine and tissue samples during the period. But it is apparently absent as a viable entity thereafter.

We reviewed this problem recently to asses whether in uncomplicated patients and in QFS *C.b.* remain as a low-level infection with viable organisms or as subcellular form or as non- or partially degraded cell components retaining antigencity and immunomodulatory ability[4] Previous studies of patients in Australia[6,25] and patients in the 1989 Birmingham (UK) Q fever outbreak in collaboration with Jon Ayers and his colleagues[5,7] showed that as long as 12 years after the outbreak in the Birmingham cohort, without further overt exposure to infection, there were indications of the persistence of coxiella cell material as evidenced by low levels of IgG antibodies and minor levels of coxiella DNA in PCR assays in PBMCs or BM aspirates.[5,7] However attempts to isolate the coxiella by serial passage of PCR positive samples in A/J mice, INFγ KO mice, or by culture in human lung fibroblasts were unsuccessful.[4]

The present report gives technical details of the re-examination of the samples in the highly susceptible NOD/SCID mice, of PBMC/BM from patients 12 years out from the Birminham Q fever outbreak who (i) had made an uncomplicated recovery from acute infection (recGr3); or (ii) had a persisting post-infection fatigue syndrome without other debility (QFSGr5); (iii) or in addition to QFS had a persistent post-infection fatigue syndrome with another debility (QFSGr6), e.g. Patient # 84 eventually shown to have low level, late stage endocarditis requiring valve replacement.[4]

Overall, the NOD/SCID mice inoculated with PCR positive PBMC/BM specimens from 12 patients survived and remained free from severe disease or mortality. Mortality cases would indicate that mice had had an infection. On the contrary, the mice gained weight during the 8 weeks of monitoring. At autopsy PCR analysis showed that all mouse spleens and others specimens were negative at 1:100 dilutions thus excluding replication of viable coxiellas in the NOD/SCID mice (Table 1; Figure 1). These observations confirmed absence of replicable organisms in the PBMC or BM and matched those described previously.[5–7]

Nevertheless some more subtle pathological changes were detected in the mice. Namely, statistically significant moderate weight loss in the first week after inoculation for mice inoculated with samples from clinical groups QFSGr5 and 6 compared with recGr3, and with control mice. The weight loss was recovered afterwards (Figure 1A).

Autopsy also revealed moderate enlargement of spleen in size and weight ($29 \pm 7\%$ of control spleen size, $P < 0.05$) in mice inoculated with specimens from groups QFSGr5 and 6 compared to control mice (Figure 2A and B). The changes were comparable to those induced by the inactivated Q Vax but well short of those present in an active

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infection induced with a small dose of the Henzerling strain of *C.b.* as also shown in Figure 2B.

An unexpected, but highly significant finding was the detection in the mouse spleens of collections of amorphous material that stained specifically with a rabbit polyclonal antiserum to *C.b.* Phases 1 and 2 antigens and also with a mouse monoclonal antiserum specific for the Phase 1 antigen. The mean fluorescence intensity of the antigen increased significantly from the negative control samples through specimens from recGr3 patients and finally to highest values in QFSGr5 and 6 (Figure 3). IFA staining for macrophage cell markers F4/80 and for DCs CD11c and with the FITC coxiella antisera, followed by confocal analysis, located the material in spleen cells of macrophage lineage (Figure 5). Similar confocal analyses located the antigenic material in macrophage phagolysosomes. The material was mostly located in peripheral sinuses of the spleen. The overall pattern of IFA staining was a complete contrast with that of an infection with its collections of stained particles arranged within distended phagocytic vesicles as 'microcolonies' and other antigenic material dispersed through the tissue.

The totality of these observations suggests that unlike the situation in early stage Q fever endocarditis and in the recrudescence *of C.b.* in late pregnancy and at parturition, those Q fever patients who have recovered and are asymptomatic and those with post-infection fatigue syndrome do not have a persistent low-level bacteraemia with viable organisms but rather carry—presumably from the original infection—antigenic coxiella cell components with LPS and proteins and traces of genomic DNA.

Given that the Birmingham cohort of patients under study was 12 years out from their original infection the latter proposition is predictably counter intuitive at first glance.

An alternative, possibly complementary view might suggest survival of the coxiella in an immunlogically 'privileged' site, elsewhere than the PBMC and BM. In this site, there might be a low level of viable organisms able to complete a genomic cycle of replication and death thereby replenishing the traces of DNA and antigen complex detected peripherally. However while such 'privileged' sites may well exist, e.g. reproductive tract, we suggest they are more likely to contain metabolically dormant, *non-replicating* organisms with complete genomes that can be the source of recrudescent infective cells when activated presumably by e.g. specific hormonal changes or alteration of immunological control at parturition.

A pointer to survival of 'dormant' but metabolically and replication-competent coxiellas with complete genomes in the special environment of the reproductive tract in a different state to that in peripheral sites outside the tract may be deduced from the early studies of Lennette *et al*. on pregnant sheep inoculated with *C.b.* in first weeks of pregnancy. Coxiellas were isolated from most organs early on but subsequently became negative. Placenta and foetus were, however, negative. On the verge of parturition placenta and foetus became positive, but not the other sites which only became positive later after parturition as part of the general lateral and vertical dissemination of infection (unpublished studies of Berkley Cal. Viral and Rickettsial Laboratory 1951/3[4,26,27]). Significantly, the products of such reactivation are readily detected as infective coxiella by animal inoculation, eukaryotic cell culture or by PCR in samples of placenta, foetus or milk.

A concept of 'continuous low-level creation' as implied above also does not fit either with the recrudescence model or with the actual findings in our post Q fever fatigue patients. The coxiella genomic DNA we detected although containing sequence-verified target sites for COM1, IS1111a and 16S RNA genes, was fragmented and present in small amounts. So far we have not been able for organizational limitations, to test the material by whole genome amplification to determine whether some coxiella cells with a complete genome are present rather than a composite of different PCR targets on different genomic fragments.

On the other hand as data in Table 1 shows that the antigen aggregates are present in the mouse spleens more consistently than DNA detected by COM1 PCR. Although it may now be deduced from the data with the heart valve from Patient # 84 and BMs from Patients #57, #2 and #117 that the IS1111a gene was in fact present as well as the COM1 gene in the Birmingham outbreak strain, contrary to our earlier conclusion.[6] nevertheless samples from the six remaining patients in the group of 10 were IS1111a negative, but COM1 positive. In contrast, small numbers (~5) of the inactivated whole cell Henzerling strain in Q Vax used as a control were consistently positive for both IS1111a and COM1.[6] Further, as shown in Table 2 and Figure 4, titration of Q Vax at concentrations from 1 to 100 ng showed changes in C_t values and antigen intensity falling in step over the range. If we suppose that a few freshly minted coxiella cells from a process of 'continuous creation' escape from the PV (wherever situated) they might be expected to have the infectivity, DNA and antigen profile of a viable strain and not that of the degraded DNA traces and more resistant antigen complex actually observed in the QFS patient samples.

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It might also be supposed that the IS1111a gene as a transposon element might be more exposed to hydrolytic enzyme degradation than the chromosomal COM1 gene during the 12 years from infection thus explaining our original apparently heterodox PCR findings with the Birmingham samples.

The other major question arising from our study concerns the pathobiological significance of the antigen complexes we now term IMC (see also extended discussion in Marmion *et al*.[4] for analogous evidence its existence in observations from other workers). The IMC clearly carry *C.b.* antigens as detected with the polyclonal and monoclonal antisera. The remaining question was whether they would also stimulate the CMI system to produce cytokines and other immune mediators that have been shown to be dysregulated in the post Q fever fatigue syndrome.[27,28,31,32] There are numbers of pointers from the present work to suggest that they do.

NOD/SCID mice inoculated with chosen Birmingham patients PBMC/BM specimens containing antigen complex (IMC) exhibited a vigorous initial IL-6 response followed by an increasing amounts of IL-10 over the period from 48 h up to post-inoculation 14 days (Figure 6A). Mouse spleen tissue H&E sections showed moderate expansion of follicular mononuclear cell areas (Figure 6D, white arrows). Given the absence of infection-stimulated T and B lymphocytes in SCID mice, the increase was probably in macrophage numbers. In addition there were increased numbers of megakaryocytes (Figure 6D, black arrows). These changes in spleen cellular composition could be a result of elevated IL-6 detected 48 h after inoculation of the specimens. IL-6 has been shown to influence maturation sequence of megakaryocytes.[33]

Stimulation of the THP-1 human monocyte cell line with extracts containing pooled patient coxiella LPS/IMC gave responses of a number of inflammatory cytokines and also, in sequence, IL-10 (Figure 7). However, NOD/SCID mice and the THP-1 cell line are models at some distance from the responses of a healthy immuno-competent animal so the stimulatory effect of the IMC was also tested in guinea pigs—either hyperimmunized (sensitized) with Q Vax or left unimmunized. The guinea pigs were then inoculated intradermally with IMC and extracts of sero-negative human PBMCs used as negative controls, and with Q Vax as a positive control. Figure 8B shows that IMC from patients in recGr3, QFSGr5 and 6 all produced reactions significantly in excess of controls.

In summary, the present work reveals that subjects who have recovered from acute Q fever and are asymptomatic, and those with Q fever post-infection fatigue syndrome (QFS) do not have viable *C.b.* in peripheral blood or BM. They do however circulate for prolonged periods a complex of *C.b* proteins and Phase 1 LPS. This complex—so called IMC—stimulates a raised or modified level of cell mediated immunity with aberrant cytokine responses and cytokine mediated tissue changes in NOD/SCID mice and on intradermal inflammation in hyperimmunized guinea pigs.

There were small but statistically significant differences in the amounts and reactogenicity of IMC in asymptomatic Q fever patients (recGr3) on the one hand compared with post-infection fatigue (QFSGr5 and 6) patients on the other. This mirrors the higher frequency of PCR coxiella genomic reactions observed previously in PBMC of QFSGr5 and 6 compared with recovered/asymptomatic Q fever patients,[6] but at this stage detection of antigen (IMC) or coxiella DNA is not advocated as diagnostic test for QFS.

The central theme of the Q fever-host pathogen relationship is variation of cell-mediated immune sensitivity to Q fever antigens—abnormally low in endocarditis, but abnormally high in QFS. The difference in responses between endocarditis, asymptomatic/recovered and qCFS/QFS patients is considered to rest on immunogenetic differences in handling the IMC and the cytokine responses.[26–28,31]

Our paradigm for persistence of IMC and the maintenance of heightened CMI and cytokine dysregulation points to a possible mechanism linking an initial infection with *C.b.* and the subsequent prolonged dysfunction of various organ systems and the symptom complex of QFS. In this context, a recent collaborative study[34] around Dr Kerr's group is of particular relevance. It measured the level of expression of 88 somatic genes in various subgroups of CFS/ME patients, in patients with endogenous depression and in healthy subjects as controls. This revealed statistically significant different patterns ('gene signatures') between subsets of CFS/ME following infection with EB virus, or parvovirus B19 virus, or with *C.b.* The three subgroups not only differed from each other but also had 'gene signatures' that diverged from the subjects with endogenous depression and those in the group of healthy controls. The aberrant levels of gene expression in the post-infection CFS patients involved somatic genes regulating the immune system and metabolic pathways, among others.

In the study,[34] of the total of 12 subjects in the Birmingham UK area who had IgG antibody to *C.b.* Phase 2 Ag, six patients were from the 1989 Q fever outbreak with laboratory-confirmed acute Q fever and subsequently QFS. Five of the six had an

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unusual gene signature (subtype A) and were drawn from our QFSGr5 and 6 in the Birmingham outbreak who had residual Q fever antibody, traces of coxiella genomic DNA and IMC as described previously.[4–7] The remaining six persons with serological evidence of past *C.b.* infection, but not the part of the outbreak and without QFS, did not have subtype A gene signatures.[34]

The findings on somatic gene modulation, taken in conjunction with our recent review[4] on the role of IMC in modulating macrophage and immune function, IL-2 restriction and IFN-γ formation, permit a hypothetical pathogenetic sequence for QFS: overt clinical Q fever + immunogenetic polymorphism → defective antigen clearance (IMC persistence) → persistent CMI + cytokine dysregulation → cytokine-mediated somatic gene modulation → QFS (i.e. Q fever-associated CFS/ME[34]).

The postulated sequence offers a framework for further tests of concept and a way into possible therapeutic approaches for QFS (e.g. clearance of IMC in laboratory animals or cell culture with IFN-γ). It also suggests a re-evaluation of the form of the continuing antigenic challenge—living intra cellular bacteria or antigenic residua: living viruses or integrated viral genes coding for antigen—in post-infection fatigue syndromes that follow other infective agents.

The importance of disordered cellular immune function in CFS, advanced by the early workers in the field, is re-emphasized by remissions of idiopathic CFS on treatment with methotrexate or by B lymphocyte depletion[35] and offers an indirect support for the paradigm.

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