Brief Definitive Report

Production of Nitric Oxide in the Synovial Membrane of Rheumatoid and Osteoarthritis Patients

By Iain B. McInnes,*‡ Bernard P. Leung,* Max Field,‡ Xiao Q. Wei,* Fang-Ping Huang,* Roger D. Sturrock,‡ Andrew Kinninmonth,§ Jeffrey Weidner,‖ Richard Mumford,‖ and Foo Y. Liew*

*From the *Department of Immunology, University of Glasgow, Glasgow, GII GNT United Kingdom; ‡Centre for Rheumatic Diseases, University Department of Medicine, and §Department of Orthopaedics, Glasgow G31 2ER Royal Infirmary, Glasgow, United Kingdom; and ‖Division of Analytical Biochemistry, Department of Immunology and Inflammation, Merck Research Laboratories, Rahway, New Jersey*

Summary

We have demonstrated spontaneous nitric oxide (NO) production by primary synovial cultures from rheumatoid (RA) and osteoarthritis patients. Increased NO production followed addition of staphylococcal enterotoxin B. Immunochemical double staining with specific anti–human inducible NO synthase (iNOS) and nonspecific esterase (NSE), or anti-CD68 (markers for tissue macrophages) showed that although many lining layer cells in RA synovium expressed iNOS, most (~90%) were NSE^- and $CD68^-$, with only a minor population (~10%) which were $iNOS^+$, $CD68^+/NSE^+$. These data demonstrate the capacity for high output of NO by human synovial tissue and show that, although human macrophages can express high levels of iNOS, the majority of cells expressing iNOS are fibroblasts. We also report that synoviocytes, and macrophage cell lines, cultured with the NO donor, *S*-nitroso-acetyl penicillamine, produced high concentrations of tumor necrosis factor (TNF)-α. These results suggest that NO may mediate pathology in RA through the induction of TNF-α production.

Rheumatoid arthritis (RA) is characterized by chronic inflammatory infiltration of the synovium, with destruction of cartilage and underlying bone, mediated by cytokines, metalloproteinases and superoxide radicals (1). Nitric Oxide is produced constitutively by endothelial (eNOS) or neuronal NO synthases, or in higher concentrations by inducible NO synthase (iNOS) after stimulation by bacterial products and cytokines, including IFN-γ, TNF-α, and IL-1 (2, 3). As such, it has emerged as an important regulatory and effector mediator in several models of inflammation (3), including streptococcal cell wall and adjuvant arthritis in the rat (4, 5) and renal and articular pathology in MRL-*lpr/lpr* mice (6, 7). iNOS activity has been demonstrated in rodent synoviocytes and macrophages, and in rodent and human neutrophils, chondrocytes and mast cells (2, 8–10). Evidence for NO production in human synovium is provided by elevation of nitrite levels in synovial fluid relative to serum in RA patients (11) and by demonstration of elevated urinary nitrate/creatinine ratios in RA (12). Furthermore, a recent report has suggested the presence of iNOS in human synovium and implied that macrophages are the major source of synovial NO (13).

We now provide direct evidence for spontaneous NO production by human synovial tissue from RA and osteoarthritis (OA) patients, which may be further upregulated by bacterial superantigen. We have shown that the predominant cellular source of NO is the synovial fibroblast. Furthermore, NO activated synovial cells produced TNF-α, a critical proinflammatory cytokine in RA synovitis (1).

Materials and Methods

Patients/Cell Culture. Synovial tissue was obtained at arthroplasty from 25 patients satisfying American College of Rheumatology criteria for RA (14), and 7 with clinical and radiological features of OA (mean age 62, range 28–83 yr). Single-cell suspensions were prepared from 13 RA and 7 OA tissues as previously described (15). Cell subsets assessed by FACS® analysis fell within ranges previously reported (15). 10^6 cells/ml were cultured in triplicate in complete Dulbecco's MEM, 10% FCS (GIBCO BRL, Paisley, UK) for 72 h unless indicated. LPS, stephylococcal enterotoxin B (SEB) (Sigma Chemical Co., Poole, UK), N^G-monomethyl-L-arginine (L-NMMA) (provided by Dr. H. Hodson, Wellcome, Beckenham, UK). L-NMMA did not reduce cell viability or proliferation by synovial cultures to SEB (data not shown).

NO Measurement. NO production was measured as its oxidative product, nitrite, using the Griess reaction as described (7). Sensitivity was 2 μM.

Immunohistochemistry. Acetone fixed frozen sections (3–6 μm) from 10 RA synovia, blocked with goat/human serum, were incubated with rabbit antiserum against a human iNOS peptide (NO53), (16) then with biotin goat anti–rabbit Ig (DAKO, High Wycombe, UK), streptavidin-alkaline phosphatase (DAKO), fast red (Vector, Peterborough, UK) and hemotoxylin for light mi-

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Table 1. *Production of NO by Primary RA and OA Synovial Cultures*

Patients	Duration	Drug Therapy	Unstimulated		SEB*	
RA	*yr*			*Nitrite μM*		
RA1	14	H	9.3	(3.0)	33	(7)*
RA2	20	M	13	(1.7)	34	(0.5)
RA3	5	M + H + P	51	(2.7)	131	(6)
RA4	13	G	<2		6	(2.2)
RA5	9	M	10.5	(1.3)	16.2	(0.5)
RA6	19	A + P	<2		11.7	(3.2)‡
RA7	22	S + P	10.1	(0.3)	32	(5.9)
RA8	20	—	<2		<2	
RA9	15	D	10.4	(2)	8.8	(1.0)‡
RA10	20	—	5.2	(1.2)	6.7	(2.8)‡
RA11	10	—	3.2	(0.5)	44	(8)
RA12	10	M	4.3	(0.5)	41	(3.9)
RA13	15	—	<2		19	(2.5)
OA						
OA1	10		15	(0.5)	32	(3.6)
OA2	7		<2		3.3	(1.0)‡
OA3	5		<2		3.2	(1.0)‡
OA4	10		22	(2.5)	57	(8)
OA5	3		128	(26)	98	(8)
OA6	5		10	(3.3)	24	(3)
OA7	8		76	(9)	112	(8)

SEB-stimulated RA synovial tissues produce enhanced nitrite levels (mean ± SD, $P < 0.01$).
*Optimal response to SEB (range 1–10 μg/ml) at 72 h for each patient. ‡1 μg/ml LPS present. G, IM gold; S, sulphasalazine; M, methotrexate; H, hydroxychloroquine; D, penicillamine; A, azathioprine; and P, prednisolone.

croscopy. For double immunofluorescence, murine anti-CD3 or anti-CD68 was followed by FITC Fab_2 goat anti-mouse Ig (DAKO), and anti-iNOS antiserum by biotin goat anti-rabbit Ig and PE-streptavidin (DAKO). Positive staining was acquired by confocal microscopy (Nikon Optiphot-2; Bio-Rad, Hertfordshire, UK). Normal rabbit serum (NRS) or murine IgG1 (DAKO) of irrelevant specificity served as controls and were negative even after coincubation with anti-CD68 or NRS, respectively (data not shown). For neutralization experiments, the immunizing iNOS peptide YRASLEMSAL, rat iNOS peptide YEEPKATRL (COOH terminus rat iNOS), or recombinant human eNOS (gift of Dr. I. Charles, The Cruciform Project, University College London, UK) at 50 μg/ml were incubated overnight at 4°C with anti-iNOS before staining. Nonspecific esterase (NSE) was detected as described (17) on sections stained with anti-iNOS developed with fast blue (Vector) to contrast with NSE. Staining was assessed by two histologists counting >500 cells in more than three high power fields per section (×400). The percentage of iNOS positive cells is expressed as (positive cells)/(total number cells in field) ×100.

RT-PCR. RT-PCR was performed as previously described (8). Primers for human iNOS were a gift from Dr. I. Charles: -5′-GCCTCGCTCTGGAAAG-3′ and 5′-TCCATGCAGCAACCTT-3′. Human chondrocyte iNOS cDNA and β-actin primers 5′-CCACACTGTGCCCATCTACGAGGGGT-3′ and 5′-AGGGCAGTGATCTCCTTCTGCATCCT-3′ (Genosys, Cambridge, UK) served as internal controls. The PCR product was sequenced (Amersham Life Science, Buckinghamshire, UK) and was identical to that predicted from human chondrocyte iNOS (8).

Induction of TNF-α Synthesis by NO. Synoviocyte/macrophage cultures were prepared using adherent cells after 16 h culture of primary synovial tissue digests, or synovial fluid mononuclear cells obtained using lymphoprep (Nycomed Pharma, Oslo, Norway). U937 cells were matured for 24 h with 10 nM PMA (Sigma). Cells at 2×10^6/ml were stimulated with *S*-nitroso-acetyl penicillamine (SNAP) or *N*-acetyl penicillamine (NAP, gifts from Dr. I. Charles) for 48 h and TNF-α concentrations determined by ELISA using paired antibodies (MAb1, MAb11, sensitivity <10 pg/ml; PharMingen, San Diego, CA). Cell viability was >90% by trypan blue exclusion. U937 cells cultured with 1 mM SNAP for 48 h, then pulsed with 1 μCi [^{3}H]thymidine for 6 h, show similar uptake to controls (data not shown).

Statistical Analysis. Comparison between groups was by Mann-Whitney test. Paired samples were compared using a *t* test or Wilcoxon matched pairs signed rank sum test.

Results and Discussion

Nitrite production by primary synovial cultures from 9/13 RA patients and 5/7 OA patients was detected in the absence of exogenous stimulation (Table 1). Together with the demonstration of iNOS mRNA expression in synovial tissue (Fig. 1), these observations show that NO generation has been initiated in vivo in both RA and OA synovium. No difference was observed between nitrite produced by synovial cultures from RA and OA tissues, nor was serum acute phase response correlated with nitrite levels (data not shown). The superantigen SEB induces T cell proliferation, cytokine secretion, and upregulates mononuclear cell cytokine expression after MHC class II binding (18, 19). Addition of SEB to synovial cultures from RA and OA patients clearly induced increased NO synthesis in a dose-

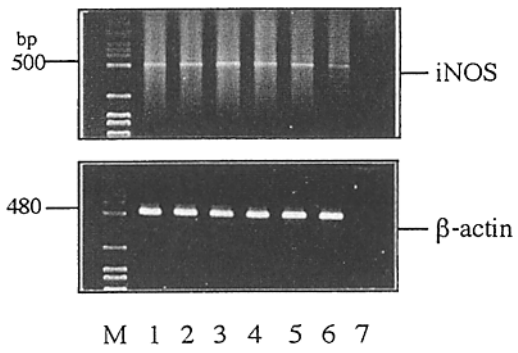

Figure 1. RT-PCR for human iNOS mRNA in snap-frozen synovium. Lanes *1–4*, representative RA patients; lane *5*, representative OA patient; lane *6*, human iNOS cDNA; lane 7, negative control.

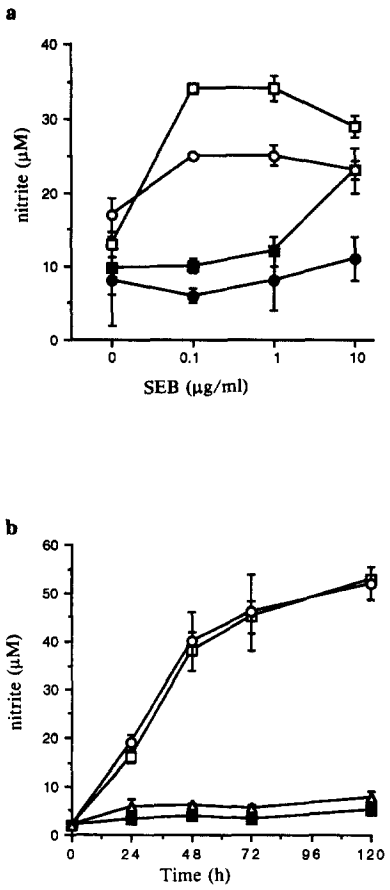

Figure 2. (*a*) SEB-induced nitrite production by synovial cultures from representative patients RA12 (*open*) and OA06 (*closed*). □, SEB 1 μg/ml; ○, SEB (1 μg/ml) + LPS (1 μg/ml). (*b*) Nitrite production by RA synovial tissue over time (representing four similar experiments). Supernatants were harvested at times indicated and kept for simultaneous assay. □, SEB (1 μg/ml); ○, SEB (1 μg/ml) + LPS (1 μg/ml); staphylococcal exterotoxin, SEB (1 μg/ml) + L-NMMA (1 mM); △, LPS (1 μg/ml).

and time-dependent manner (Fig. 2, Table 1). When LPS (0.1–10 μg/ml) was added with SEB, no significant increase in nitrite concentration was observed. Production of nitrite was inhibited by addition of L-NMMA (1 mM), a competitive iNOS inhibitor (Fig. 2 *b*). Thus, in addition to cartilage (20), human synovial membrane itself has the potential for significant NO generation.

Previous efforts to localize iNOS in human synovium used cross-reacting antibodies to rodent iNOS (13). We used rabbit antiserum raised specifically against a peptide from human iNOS (17), which recognized neither human eNOS nor rodent iNOS. Intracytoplasmic staining (Fig. 3 *a*) was observed in 10 RA synovia in the following distribution (mean ± SD [range]): lining layer 19 ± 13.1 [2–56], interstitium 5 ± 5.6 [0–20], aggregates 7 ± 7.8 [0–37] ($P < 0.001$ LL vs Is or Agg). Variable iNOS staining between patients reflected the heterogeneity of spontaneous nitrite production in primary culture. $iNOS^+$ smooth muscle and endothelial cells were identified around blood vessels in 8/10 RA tissues. Staining was abolished by preincubation with human iNOS peptide (Fig. 3 *b*), but not with either rodent iNOS peptide or recombinant human eNOS (Fig. 3 *c*), demonstrating specificity for human iNOS.

The RA synovial lining layer consists primarily of activated macrophages and fibroblasts (1). Double staining with NSE and anti-iNOS (Fig. 3 *d*) revealed that the majority of $iNOS^+$ cells (89 ± 5%) in the lining layer, or interstitium, were NSE^- and therefore unlikely to be macrophages (Ta-

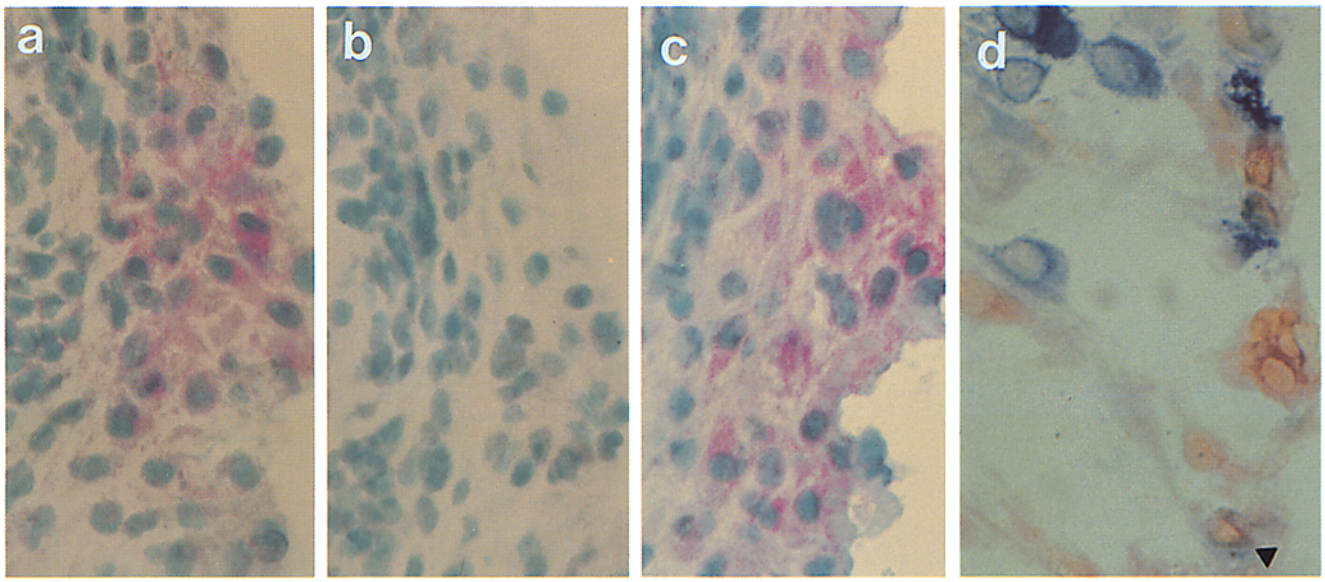

Figure 3. RA synovial lining layer stained with (*a*) anti–human iNOS antiserum (fast red), preincubated with (*b*) human iNOS peptide, or (*c*) anti–rodent iNOS peptide. Preincubation with human eNOS was similar to (*c*). (*d*) Double stain with anti-human iNOS (fast blue) and NSE (red-brown): arrow shows a double-labeled cell (×300).

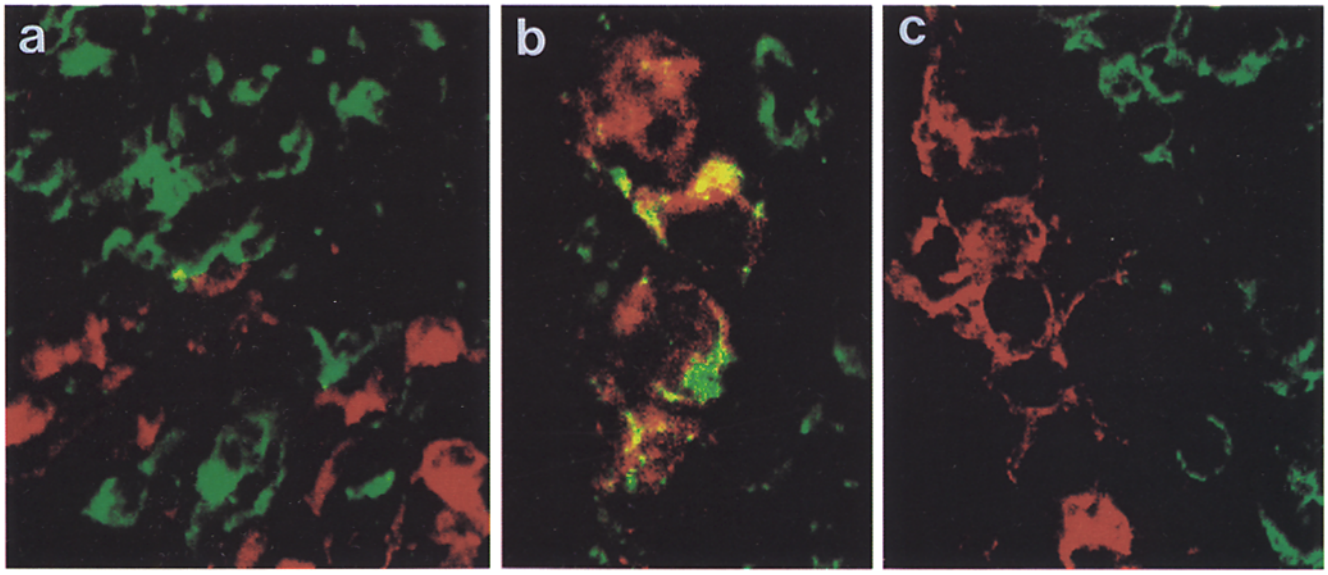

Figure 4. Confocal images of RA synovial sections double-stained with anti–human iNOS (red) and (*a*) anti-CD68 (green), showing that most iNOS-positive cells are $CD68^-$ (×250); (*b*) anti-CD68 at higher magnification, showing that $CD68^+/iNOS^+$ cells are present (double stain appears yellow [×500]); or (*c*) anti-CD3 (green), showing that T cells are $iNOS^-$ (×400).

ble 2). Confocal microscopy also demonstrated that most $iNOS^+$ cells were $CD68^-$ (Fig. 4 *a*), although $iNOS^+/CD68^+$ cells were occasionally observed (Fig. 4 *b*). Thus, although synovial macrophages possess the capability of NO generation, fibroblastlike synoviocytes are the predominant source of NO in vivo. Moreover, a majority of NSE^+ and $CD68^+$ cells in the lining layer were $iNOS^-$, indicating that most macrophages are not directly involved in NO production (Table 2). $CD3^+$ synovial T cells were all $iNOS^-$ (Fig. 4 *c*). The shared species and isotype of anti-CD3 with anti-CD68 antibody provided an additional specificity control for the double staining observed with anti-iNOS and anti-CD68.

There is currently controversy as to the capacity of human macrophages to produce NO (21, 22). Optimal conditions for iNOS expression in murine macrophages are apparently distinct from those required in the human, perhaps reflecting the tissue of origin, since blood monocytes are mainly used in human experiments. Where NO production by human macrophages was detectable (22), levels

Table 2. *Colocalization of iNOS with NSE in RA Synovium*

$NSE^+/iNOS^-$	$NSE^-/iNOS^+$	$NSE^+/iNOS^+$
	Percent positive cells	
49 ± 24	45 ± 23	5 ± 3.1
(0–80)	(16–93)	(0–12.5)

RA sections (n = 4) were double stained with anti–human iNOS and NSE. Greater than 250 lining layer and interstitial area cells positive for NSE and/or iNOS were counted per section and the number of single or double positive cells expressed as a percentage of the total number of stained cells counted (mean ± SD [range]).

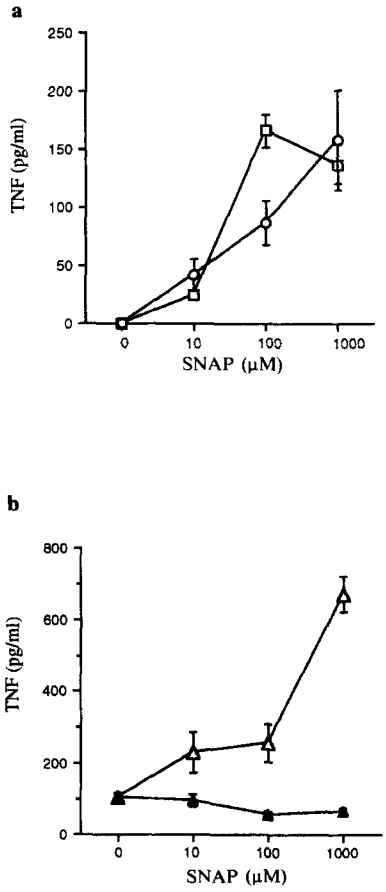

Figure 5. TNF-α production (mean ± SEM) in response to SNAP by (*a*) adherent cells from RA synovial fluid (SFAC) (○, n = 4), or RA synovial tissue (□, n = 2); or (*b*) PMA-matured U937 cells (△, SNAP; ▲, NAP: representative of three similar experiments). NAP did not induce TNF-α production from SFAC (n = 3, data not shown). 2 mM SNAP contained <0.0015 IU LPS by limulus amebocyte lysate assay (E-toxate; Sigma).

were an order of magnitude lower than those from murine macrophages. Our study demonstrates clearly that some synovial macrophages in RA can express high levels of iNOS. The reason why only a limited number of macrophages is activated to produce NO is at present unclear.

We next investigated a potential role for NO in the synovial membrane. U937 cells or synovial mononuclear cells cultured with the NO donor, SNAP, produced TNFα in a dose-dependent manner (Fig. 5). RA synovial macrophages are activated, producing proinflammatory cytokines such as IL-1, TNF-α (1), and IL-15 (23) and clinical trials using monoclonal anti-TNF-α therapy indicate that such cytokine generation is critical in ongoing synovitis (24). However, factors responsible for TNF-α upregulation remain unclear. Our data clearly show that synovial macrophages may express iNOS, but are unlikely to be the principle producers of NO. A reciprocal pathway may exist whereby NO from synovial fibroblasts enhances proinflammatory cytokine production by macrophages, which in turn may upregulate iNOS expression (2, 3), thereby generating a positive feedback loop. NO also upregulates MMP production (25) and is implicated in IL-1β mediated inhibition of proteoglycan synthesis (26), suggesting a proinflammatory role for NO. However, NO levels in our synovial cultures are sufficient to suppress T cell proliferation and may contribute to the hyporesponsiveness of synovial T lymphocytes (27). In addition, an acute chondroprotective role for endogenous NO in bovine cartilage has recently been proposed (28). Thus, in contrast to the situation in animal models in which NO is usually detrimental (4, 5), the net effect of NO production in human arthritis remains unclear.

Both RA and OA synovia produced NO. Although some OA synovial tissue contains an inflammatory infiltrate, the etiology of OA is not considered to be inflammatory. NO production is therefore unlikely to be a unique feature of primary inflammatory arthritides, but may reflect a nonspecific synovial response to injury or inflammation with potential protective or pathologic consequences.

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Address correspondence to Foo Y. Liew, Department of Immunology, University of Glasgow, UK.

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