

11-1-2010

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Del Galdo, Francesco; Wermuth, Peter J; Addya, Sankar; Fortina, Paolo; and Jimenez, Sergio A,  
"NF $\kappa$ B activation and stimulation of chemokine production in normal human macrophages by the gadolinium-based magnetic resonance contrast agent Omniscan: possible role in the pathogenesis of nephrogenic systemic fibrosis." (2010). *Jefferson Institute of Molecular Medicine Papers and Presentations*. Paper 2.  
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*Ann Rheum Dis* 2010 69: 2024-2033

doi: 10.1136/ard.2010.134858

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# NF $\kappa$ B activation and stimulation of chemokine production in normal human macrophages by the gadolinium-based magnetic resonance contrast agent Omniscan: possible role in the pathogenesis of nephrogenic systemic fibrosis

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## See Editorial, p 1895

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Accepted 12 September 2010

## ABSTRACT

**Objective** Nephrogenic systemic fibrosis (NSF) is a generalised fibrotic disorder occurring in certain individuals with renal insufficiency exposed to gadolinium-based contrast agents (GdBCA) for MRI. Histopathological examination of affected tissues shows increased numbers of activated macrophages. To elucidate the mechanisms responsible for macrophage activation, the effects of the GdBCA Omniscan on normal human macrophage global gene expression, chemokine production and nuclear factor  $\kappa$ B (NF $\kappa$ B) activation was examined.

**Methods** Normal human monocyte-derived macrophages were incubated with Omniscan (50 mM) and their gene expression analysed by microarrays and real-time PCR. Macrophage chemokine production was assayed by multiplex ELISA. NF $\kappa$ B activation was assessed by NF $\kappa$ B nuclear localisation and quantitation of intracellular levels of inducible nitric oxide synthase (iNOS) protein. A specific cell-permeable NF $\kappa$ B peptide inhibitor was used to abrogate NF $\kappa$ B stimulation of chemokine and iNOS protein levels. CCL8/MCP-2 in affected skin of patients with NSF was examined by indirect immunofluorescence.

**Results** Omniscan caused a profound change in the transcriptome of differentiated human normal macrophages *in vitro*, including a large increase in the expression of genes encoding CC and CXC chemokines. It induced rapid nuclear localisation of NF $\kappa$ B and stimulation of iNOS protein levels and chemokine production which were blocked by an NF $\kappa$ B inhibitory peptide. CCL8/MCP-2, the most upregulated chemokine following *in vitro* macrophage exposure to Omniscan, was strongly increased in NSF-affected skin.

**Conclusion** The GdBCA Omniscan induces potent stimulation of macrophage gene expression, NF $\kappa$ B activation and increased NF $\kappa$ B-mediated production of CC and CXC chemokines and iNOS. These alterations may play a crucial role in the pathogenesis of NSF.

## INTRODUCTION

Nephrogenic systemic fibrosis (NSF), previously known as nephrogenic fibrosing dermopathy, is a generalised fibrotic disorder occurring in individuals with renal insufficiency following exposure to gadolinium-based contrast agents (GdBCA) used to enhance MRI.<sup>1–5</sup> Clinically, NSF displays many similarities to the clinical manifestations of

systemic sclerosis including severe and usually progressive skin induration, progressive and eventually incapacitating joint flexion contractures and fibrotic involvement of numerous internal organs including lungs, heart, diaphragm and striated muscles.<sup>1–8</sup> The association of NSF with GdBCA administration has been supported by extensive clinical and epidemiological studies describing the onset of NSF in close temporal relationship with GdBCA use<sup>9–14</sup> and the presence of Gd in affected tissues from patients with NSF.<sup>15–18</sup> Furthermore, potent functional effects of GdBCA have recently been described on human peripheral blood monocytes,<sup>19</sup> human skin organ cultures<sup>20</sup> and cultured human dermal fibroblasts.<sup>21,22</sup>

Following intravenous administration, GdBCA distribute rapidly through the intravascular and extracellular space compartments and Gd chelates are excreted by glomerular filtration, with >95% excreted by 1 day, a biological elimination half-life of approximately 1.5 h and no detectable biotransformation, decomposition or serum protein binding.<sup>23,24</sup> Because of the renal excretion of these compounds and the demonstrated deposition of Gd in affected tissues of patients with NSF, the accumulation of Gd in tissues in patients with renal insufficiency is believed to be a crucial event in the pathogenesis of this condition. Histopathological studies of tissues from affected individuals with NSF suggest that, during the early stages of the disease, the cellular elements involved in the pathogenesis of NSF are activated macrophages<sup>7</sup> and bone marrow-derived collagen-producing fibrocytes that infiltrate the affected tissues.<sup>25,26</sup> We examined whether GdBCA could exert a direct effect on human macrophage function and found that Omniscan, the GdBCA studied, causes a remarkable change in the transcriptome of normal human macrophages with a potent stimulation of macrophage chemokine gene expression and production and activation of the nuclear factor  $\kappa$ B (NF $\kappa$ B) pathway. We further demonstrated that CCL8/MCP-2 was the most upregulated chemokine in Omniscan-treated macrophages and was also markedly increased in NSF affected skin. The multifunctional cytokines and chemokines released from normal macrophages upon exposure to Omniscan may be able to recruit circulating mononuclear cells and fibrocytes and

activate tissue resident fibroblasts to initiate a fibrotic process as discussed recently.<sup>19,22,27</sup> Thus, the data presented here indicate that the stimulation of chemokine production and activation of the NF $\kappa$ B pathway in macrophages may play a crucial role in the pathogenesis of NSF.

## MATERIALS AND METHODS

### Macrophage isolation and differentiation

Normal human peripheral blood buffy coat preparations or leuko-reduction filters were obtained from the Thomas Jefferson University Hospital Blood Bank following Institutional Review Board approval. Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat or the leukoreduction filters by Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and enriched for monocytes by adherence to plastic culture dishes for 2 h as described.<sup>28</sup> To obtain terminally differentiated macrophages the monocytes were cultured with 60 ng/ml M-CSF (BioVision, Mountain View, California, USA) and 25 ng/ml interleukin 10 (IL-10) (BioVision) for 7 days as described.<sup>28</sup> Macrophages were activated by incubation in the same medium for an additional 24 h with 50 nM phorbol myristate acetate (PMA; Acros Organics, Morris Plains, New Jersey, USA). Activated macrophages ( $5 \times 10^5$  cells/ml) were exposed for 24 h to 50 mM Omniscan (GE Healthcare, Lawrence, Massachusetts, USA). No significant effects on cell numbers or increased cytotoxicity were observed as examined by the WST-1 assay (Roche Diagnostics, Indianapolis, Indiana, USA). Certain experiments were performed with differentiated macrophages prepared exactly as described above except that they were not activated by culture with PMA. Macrophage samples cultured with an equal volume of phosphate buffered saline (PBS) served as negative controls. Macrophage culture supernatants were isolated and filtered and maintained frozen for subsequent studies. Isolation of total RNA from macrophages was performed as previously described for human monocytes.<sup>19</sup> The Omniscan employed for all the studies was tested and verified by the manufacturer to be free from endotoxin contamination. The endotoxin-free status of Omniscan was further confirmed utilising the Etodate Assay (Sigma-Aldrich, St Louis, Missouri, USA) based on *Limulus Ameobocyte Lysate* (LAL) gel formation, according to the manufacturer's instructions.

### Analysis of global gene expression patterns

Global gene expression analysis was performed employing microarrays. For this purpose macrophages from two different donors prepared and activated as described above were incubated for 24 h with either PBS or 50 mM Omniscan. Total RNA was extracted using the RNeasy protocol from Qiagen (Valencia, California, USA). RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, California, USA). Total RNA (2  $\mu$ g) from control and Omniscan-treated samples (two replicates in each group) were used for Affymetrix one cycle target labelling as described by the manufacturer (Affymetrix, Santa Clara, California, USA). Each of four Affymetrix HG U133 plus 2.0 arrays were hybridised for 16 h with biotin-labelled fragmented cRNA (10  $\mu$ g) in 200  $\mu$ l hybridisation mixture according to the manufacturer's protocol. Arrays were washed and stained using GeneChip Fluidic Station 450 and hybridisation signals were amplified using antibody amplification with goat IgG (Sigma-Aldrich) and anti-streptavidin biotinylated antibody. Chips were scanned

on an Affymetrix GeneChip Scanner 3000 using GeneChip Operating Software version 3.0. Raw data were normalised using Robust Multichip Average and normalised to control samples with GeneSpring GX v7.3.1 and GX 10.0 software (Agilent). A volcano plot was used to identify differentially expressed genes using parametric testing assuming equal variances and no multiple testing correction. Pathway analysis of differentially expressed genes was performed employing Ingenuity software (Ingenuity Systems, Redwood City, California, USA).

### Real-time PCR validation

Expression levels of CCL2 (MCP-1), CCL8 (MCP-2), CXCL10 (IP10) and CXCL11 (ITAC) were assayed by real-time quantitative PCR using SYBR Green chemistry (Applied Biosystems, Foster City, California, USA) following a standard amplification protocol on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The following primers were employed:

$\beta$ -Actin: forward 5'-TTGCCGACAGGATGCAGAA-3', reverse 5'-GCCGATCCACACGGAGTACTT-3'; CCL2 – chemokine, CC motif, ligand 2: forward 5'-ACCAGCAGCAAGTGTCCCAA-3', reverse 5'-TTTGCTTGTCCAGGTGGTCCAT-3'; CCL8 – chemokine, CC motif, ligand 8: forward 5'-TCATGCTGAAGCTCACACCTT-3', reverse 5'-AGAATTGCCATTGCACAACCTT-3'; CXCL10 – chemokine, CXC motif, ligand 10: forward 5'-ACTGCCATTCTGATTGCTGCC-3', reverse 5'-TGATGCAGGTACAGGTACAGT-3'; CXCL11 – chemokine, CXC motif, ligand 11: forward 5'-ACTCCTTCC AAGAAGAGCAGCA-3', reverse 5'-CCATGCCCTTCACACTCATGTT-3'.

Relative quantification was performed by arbitrarily setting the expression level of the PBS negative control at 100 and by expressing changes in transcript levels of other samples relative to this control sample. Relative differences in each PCR sample were corrected using human  $\beta$ -actin mRNA as an endogenous control. Some experiments were performed employing terminally differentiated macrophages without prior activation (not exposed to PMA) to confirm that the observed effects were not dependent on PMA activation. Other experiments were performed with macrophages incubated with 500  $\mu$ M caldiumide to exclude any potential effects that the chelator present in the Omniscan preparations might have caused on the macrophage chemokine gene expression.

### Multiplex ELISA

SearchLight proteome array analyses (Pierce Biotechnology, Woburn, Massachusetts, USA) were conducted to measure the levels of CCL8, CXCL10, CCL2 and CXCL11 in culture supernatants from terminally differentiated and activated macrophages following incubation with 50 mM Omniscan for 24 h.<sup>29</sup> Briefly, culture supernatant samples were diluted 1:2, 1:50 or 1:1000 and then incubated for 1 h on the array plates which had been prespotted with capture antibodies specific for each protein. The plates were decanted and washed three times with PBS before addition of a mixture of biotinylated detection antibodies to each well. Following incubation with detection antibodies for 30 min, the plates were washed three times and incubated for 30 min with streptavidin horseradish peroxidase. The plates were again washed and SuperSignal Femto chemiluminescent substrate (Pierce Biotechnology) was added. The plates were immediately imaged using the SearchLight imaging system and data were analysed using ArrayVision software (GE Healthcare, Chalfont St Giles, UK).

## Extended report

**Quantitative assessment of iNOS protein levels**

Terminally differentiated macrophages were exposed to various concentrations of Omniscan (5–50 mM) for 90 min and the intracellular levels of inducible nitric oxide synthase (iNOS) protein were examined in cell lysates of control and Omniscan-treated cells. The amounts of iNOS present in the macrophage samples were measured with the Quantising human iNOS ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. The values of iNOS protein present in the samples were expressed as U/ml of cellular lysates by comparison with a standard curve prepared employing recombinant iNOS protein provided by the manufacturer. The calculations were performed within the linear range of the standard curve for all samples.

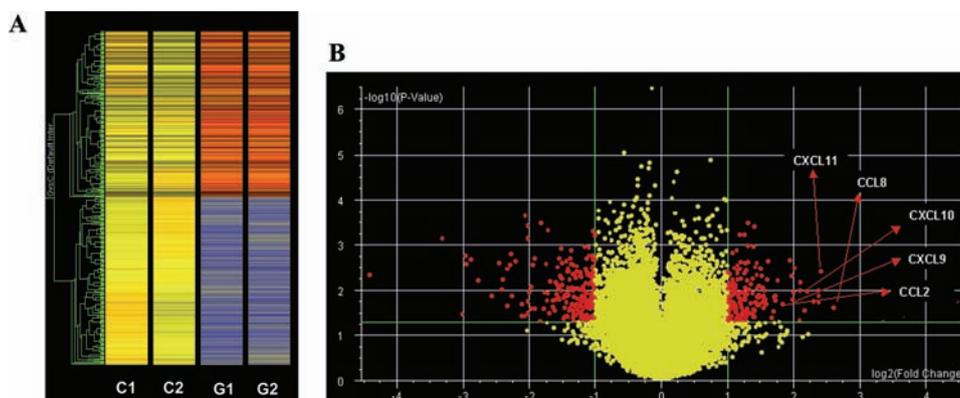
**Indirect immunofluorescence studies**

CCL-8 protein was analysed in a sample of normal skin obtained from a healthy individual and in affected skin from three different patients with NSF by immunofluorescence using a specific anti-CCL8 rabbit polyclonal antibody (ABCAM, Cambridge, Massachusetts, USA). Isotype control staining was performed using rabbit IgG (Sigma, St Louis, Missouri, USA). For NF $\kappa$ B localisation studies, macrophages were exposed to Omniscan or saline for 30 min and then fixed and permeabilised. Anti-p65-NF $\kappa$ B antibodies (Cell Signaling, Danvers, Massachusetts, USA) were used at a 1:100 dilution according to the manufacturer's instructions. Secondary antibodies were affinity purified sheep FAB' anti-rabbit IgG, Cy3 conjugated (Sigma, St Louis, Missouri, USA). Paraffin-embedded sections from skin were deparaffinated with two changes of xylene for 10 min each and then two changes of ethanol for 5 min each. For antigen retrieval the tissue sections were boiled in 10 mM citrate buffer pH 6.0 for 20 min. The sections were then rinsed in PBS for 2 min and incubated with 5% (w/v) normal sheep serum for 20 min at room temperature to block non-specific binding sites. The primary antibody incubation step was performed in a dark humidified chamber overnight at 4°C for CCL8. Tissue sections were then incubated with the sheep polyclonal antibodies (1:200). The unbound antibodies were removed from the section following

each incubation with three changes of PBS for 2 min each. Tissue sections or fixed cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) and analysed using a Zeiss LSM 510 META Confocal Laser Scanning Microscope System. Zeiss META confocal microscopy software was used in balancing signal strength. The breakthrough of the DAPI signal into the red and green channels was recorded separately and subtracted from the DAPI blue channel. Each image was scanned eight times to accurately separate signal from noise. Panels were assembled using Adobe Photoshop software without any RGB modification. Quantitative analysis of fluorescence was performed using ImageJ software (NIH National Institute of Mental Health, Bethesda, Maryland, USA). The integrated density of fluorescence was calculated in three biopsies and expressed as mean $\pm$ SD. An unpaired two-tailed t test was used to calculate p values.

**NF $\kappa$ B inhibition studies**

To demonstrate the participation of NF $\kappa$ B activation in the observed results, macrophages exposed to Omniscan were pretreated by incubation with a cell-permeable NF $\kappa$ B inhibitory peptide.<sup>30,31</sup> The inhibitory peptide was the I $\kappa$ B-NBD peptide, which consists of an antennapedia (AP) homeodomain that confers cell permeability, and the T735 to E745 region of I $\kappa$ B $\beta$  which is the NEMO binding domain. This peptide blocks the interaction of NEMO, an I $\kappa$ B complex regulatory protein, with the I $\kappa$ B complex. The control peptide consists of the AP homeodomain sequence alone. The I $\kappa$ B-NBD and control peptides (Marligen Biosciences, Ijamsville, Maryland, USA) were dissolved in dimethyl sulphoxide to a concentration of 6.7 mM. Cells were washed with PBS and exposed to 200  $\mu$ M of either I $\kappa$ B-NBD or the control peptide for 2 h. This incubation was followed by addition of either PBS or PBS with 5, 10, 25 or 50 mM Omniscan for 30 min. Cells were washed twice in PBS and lysed in 1 ml iNOS buffer. iNOS protein levels were determined by ELISA as described above. In other experiments the effects of the cell-permeable NF $\kappa$ B inhibitory peptide on chemokine expression by macrophages incubated either in PBS alone or exposed to 50 mM Omniscan were examined.



**Figure 1** (A) Dendrogram showing gene expression differences induced by 50 mM Omniscan in normal human differentiated macrophages. RNA was isolated from replicate samples of normal human differentiated macrophages incubated under control conditions (C1 and C2) or cultured with 50 mM Omniscan (G1 and G2), labelled and applied to Affymetrix human U133 2.0 Plus microarrays. Dendrograms are reflective of the genes with a differential expression of more than twofold in the two experimental conditions. In the dendrogram shown, a shorter arm indicates higher similarity whereas a longer arm indicates lower similarity. (B) Volcano plot of differentially expressed transcripts in the presence of Omniscan. There are four positive and four negative relative expression values (VG) and log<sub>2</sub> (fold change) shown on the x-axis of the figure. The y-axis is negative base 10 logarithm of the p value for the gene F test (F1). Genes that are significant at the 0.05 multiple test adjusted level are shown in red. Several transcripts of interest exhibiting twofold or greater increases of expression with p values <0.05 in Omniscan-exposed cells are indicated by arrows.

## RESULTS

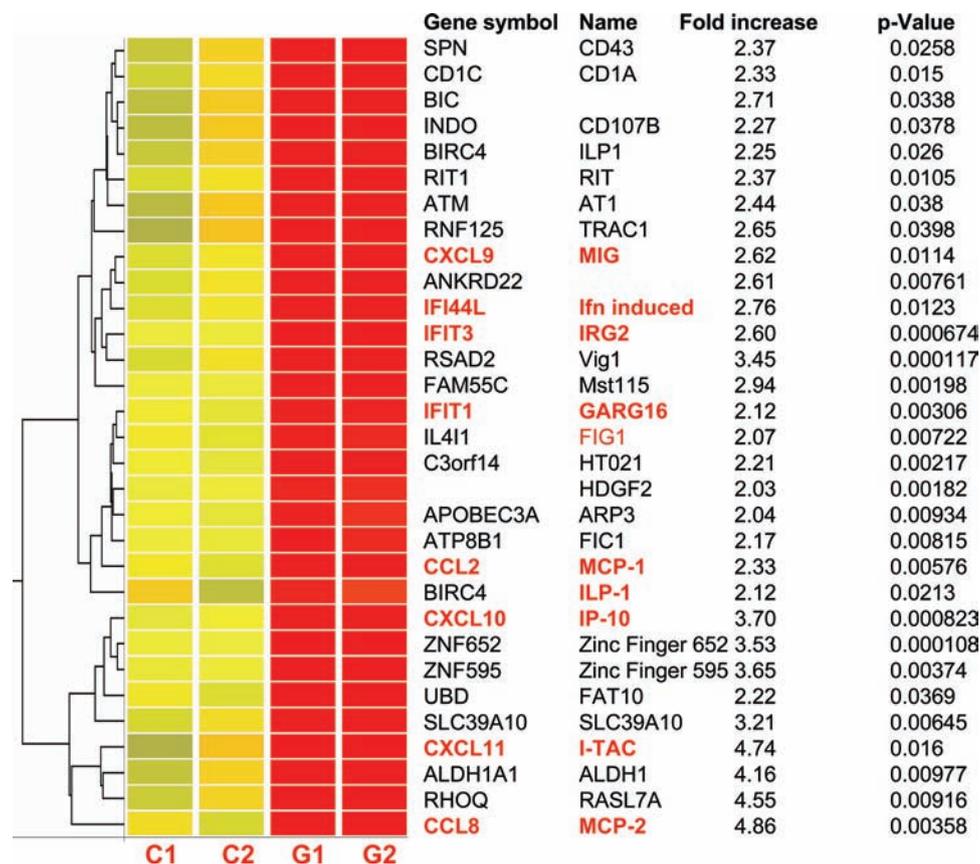
## Global gene expression of control and Omniscan-exposed normal human macrophages

Comparison between the average gene expression signals for the replicates between Omniscan-exposed and control macrophages revealed 551 differentially expressed genes (more than twofold) (figure 1A). Table 1 in the online supplement shows a list of all the differentially expressed genes. In many instances the differential expression was of a large magnitude (up to 19-fold). A volcano plot analysis of the differentially expressed transcripts (figure 1B) revealed that 240 of them were upregulated and 311 were downregulated by more than twofold with high statistical significance ( $p < 0.05$ ). Of these 240 upregulated transcripts, 31 had a signal intensity  $> 100$  and corresponded to genes with a known function on the NCBI database. We defined the transcripts for these 31 genes as representing the 'macrophage Gd signature'. Five of these 31 transcripts corresponded to chemokines and three to interferon-inducible genes. The complete list is shown in figure 2. CEL files for all the samples analysed will be made publicly available through the NCBI following publication.

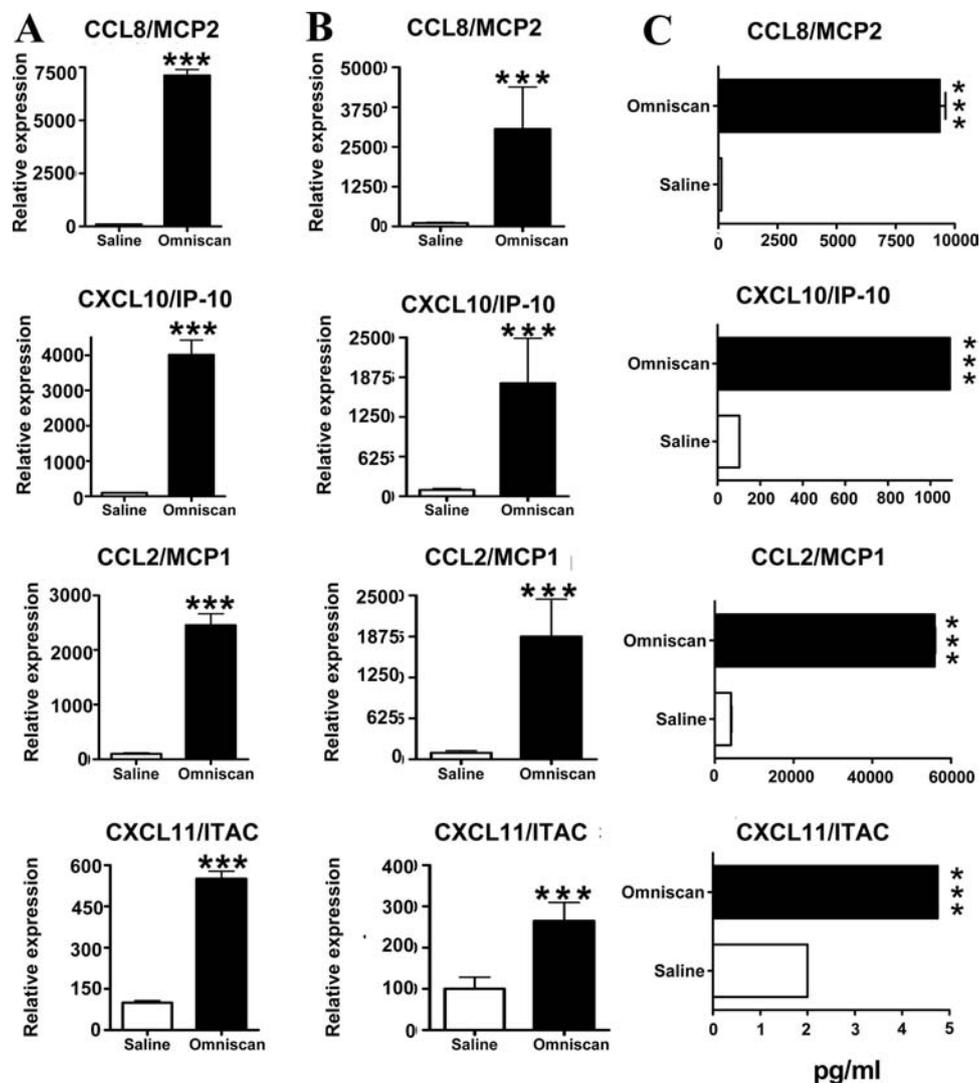
## Real-time PCR validation

To validate the microarray results and to further characterise the chemokine expression levels in macrophages following incubation with Omniscan, we performed real-time quantitative PCR analysis of mRNA levels for CCL2 (MCP-1), CCL8

(MCP-2), CXCL10 (IP10), CXCL11 (ITAC) and CXCL12 (SDF1) on the same samples employed for the microarray studies. The results confirmed and quantified the microarray results, indicating that expression of these genes was increased in normal differentiated and activated human macrophages in response to exposure to 50 mM Omniscan (figure 3A). Omniscan caused a potent stimulation of the expression of CCL8 (669 $\pm$ 108-fold increase), CXCL10 (401 $\pm$ 72-fold increase), CCL2 (245 $\pm$ 36-fold increase) and CXCL11 (551 $\pm$ 48-fold increase) compared with the PBS control levels. To examine whether previous PMA activation of macrophages was required for Omniscan stimulation of chemokine expression, four similar experiments were performed with macrophages without PMA stimulation. The results in figure 3B show that PMA activation is not required to elicit the potent stimulation of chemokine expression in macrophages following exposure to Omniscan, and that the pattern of chemokine stimulation was qualitatively similar between samples obtained from Omniscan-exposed macrophages with or without previous PMA activation. Studies with macrophages cultured with caldiameter showed that the chelator alone had no detectable effects on macrophage chemokine gene expression (data not shown). No toxic effects were observed in the macrophages exposed to 50 mM Omniscan, as indicated by the absence of cytotoxicity in WST-1 soluble tetrazolium cytotoxicity assays and the observation of similar levels of house-keeping gene transcripts in quantitative PCR arrays (data not shown).



**Figure 2** The macrophage Gd signature genes. Comparisons were performed between transcript expression levels of cells exposed to 50 mM Omniscan and phosphate buffered saline (PBS) control macrophages on the Affymetrix U133 2.0 Plus microarray, which yielded a group of 31 transcripts excluding orphan genes and transcript redundancies. Genes were considered significant if the p value of the differences was  $< 0.05$ , the signal intensity of their expression was  $> 100$  and the fold change average signal intensity was  $> 2$ . Highlighted in red are transcripts for chemokines of the interferon pathway.



**Figure 3** Effects of Omniscan on the expression and production of chemokines in normal human differentiated macrophages. (A) mRNA expression levels of four chemokines following treatment of phorbol myristate acetate (PMA)-activated macrophages with 50 mM Omniscan for 24 h. Results are expressed as mean  $\pm$  SD percentage difference of three replicate samples analysed by quantitative RT-PCR. The phosphate buffered saline (PBS) control was arbitrarily set to 100% expression. (B) mRNA levels of four chemokines expressed by non-PMA-activated macrophages following incubation with Omniscan. (C) Levels of secreted chemokines assessed by multiplex ELISA in the supernatants from PMA-activated macrophages cultured under the same conditions as in (A). The results are expressed as pg/ml of samples analysed in duplicate, each at three different dilution; \*\*\* $p < 0.01$ .

#### Multiplex ELISA of tissue culture supernatants from microarray studies

Culture media isolated from normal human differentiated and activated macrophages exposed to 50 mM Omniscan were analysed by the Searchlight Proteome Array Assay. Consistent with the mRNA expression results, we observed a marked increase in the production and secretion of various chemokines compared with the levels from control macrophages incubated with PBS. The concentrations of selected cytokines found in the culture medium of Omniscan-stimulated macrophages are shown in figure 3C.

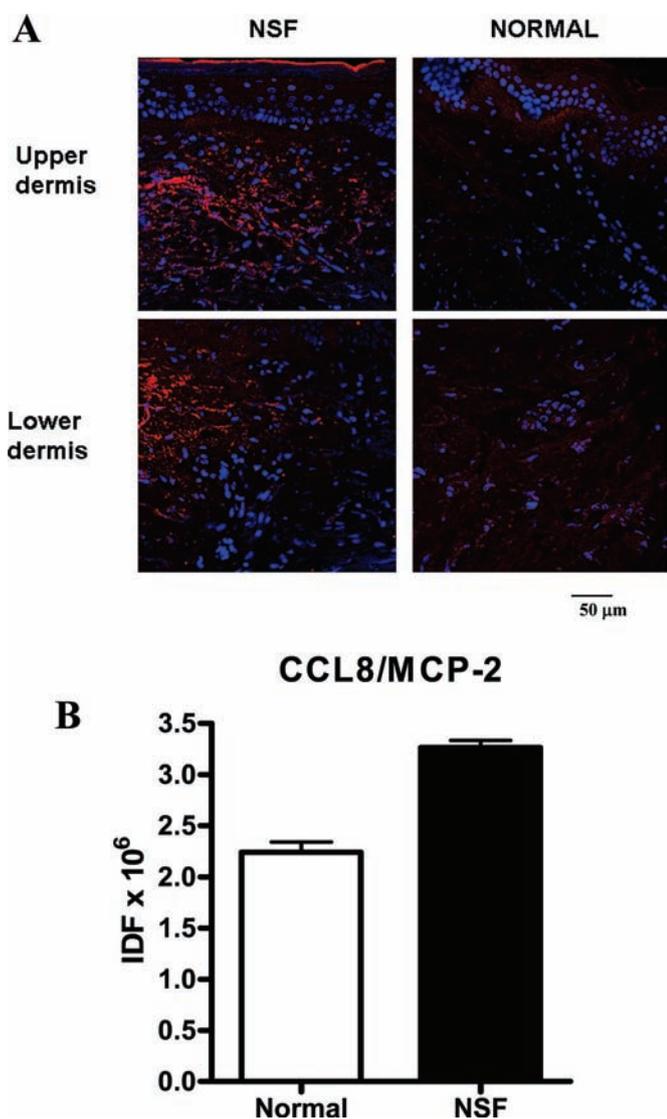
#### MCP-2/CCL8 expression is increased in NSF skin

To further validate the *in vitro* results and to determine whether the observed increase in chemokine production was pertinent to the pathogenesis of NSF, we examined the expression of MCP-2/CCL8, the most upregulated chemokine in the microarray, in paraffin-embedded skin biopsies from affected

skin from three patients with NSF. Immunofluorescence studies followed by quantitative confocal microscopy analysis showed that, in contrast to normal skin from a healthy individual, affected skin from three patients with NSF displayed a marked increase in the level of fluorescence for MCP-2/CCL8 (figure 4A); the increase was apparent in both the upper and lower dermis and was consistent in the three biopsies examined ( $p < 0.0001$ , figure 4B).

#### Pathway analysis of microarray data revealed strong NF $\kappa$ B activation

Pathway analysis of all the significantly (more than twofold,  $p < 0.05$ ) differentially increased genes in the transcriptome of Omniscan-treated macrophages indicated a strong activation of the NF $\kappa$ B pathway (figure 5). All the macrophage chemokines found to be upregulated in response to Omniscan exposure are downstream targets of NF $\kappa$ B activation. Given the strong NF $\kappa$ B pathway activation suggested by these results, all reagents were



**Figure 4** Confocal microscopy imaging of MCP-2/CCL8 expression in the skin of patients with nephrogenic systemic fibrosis (NSF). (A) Affected skin samples from three different individuals with NSF were examined for MCP-2/CCL8 tissue expression with similar results. The results of one illustrative patient are shown. MCP-2/CCL8 antibodies (ABCAM) are stained in red. Nuclei are counterstained with DAPI in blue. Note the intense staining in both the upper and lower dermis of the NSF biopsy whereas normal skin displays only a dim fluorescence for MCP-2, mostly around a vessel in the lower dermis. Original magnification  $200\times$ . (B) Quantitative analysis of MCP-2/CCL8 antibody fluorescence in skin samples from a normal control and from three patients with NSF. The integrated density of fluorescence (IDF) of the normal skin (open bar) is compared with the average IDF of the three NSF skin biopsies (closed bar) and the values are expressed as arbitrary IDF units ( $p < 0.0001$ ).

confirmed to be endotoxin-free using a LAL gel formation assay (data not shown).

#### Omniscan induced NF $\kappa$ B nuclear localisation in terminally differentiated macrophages

To confirm functionally the NF $\kappa$ B activation suggested by the pathway analysis, we examined the effects of Omniscan on NF $\kappa$ B nuclear localisation in normal macrophages. As shown in figure 6A, in macrophages incubated under control conditions the localisation of NF $\kappa$ B is mostly cytoplasmic. In contrast,

as early as 5 min after macrophage incubation with 50 mM Omniscan, a striking NF $\kappa$ B nuclear localisation was observed, indicating a strong activation of this pathway. The observed nuclear localisation was completely abrogated by pretreatment of the macrophages with a cell-permeable NF $\kappa$ B inhibitory (I $\kappa$ B-NBD) peptide (figure 6B) but was not changed by pretreatment with the AP control peptide.

#### Omniscan stimulated iNOS protein levels in differentiated normal human macrophages through NF $\kappa$ B activation

To further confirm the activation of NF $\kappa$ B, we measured the levels of iNOS protein in cell lysates from differentiated macrophages without prior PMA activation following incubation with various concentrations (5–50 mM) of Omniscan (figure 6C,D). The basal level of iNOS in control macrophages was  $\sim 0.015$  U/ml of cell lysate. Following 30 min incubation with all concentrations of Omniscan there was a potent increase in iNOS levels (10- to 25-fold) with a dose response that reached a plateau at 25 mM (figure 6C). To examine the role of NF $\kappa$ B in the stimulation of iNOS protein levels, the effects of NF $\kappa$ B inhibition by pretreatment with the inhibitory I $\kappa$ B-NBD peptide were analysed. The results are shown in figure 6D. Exposure to Omniscan caused a concentration-dependent increase in iNOS protein levels, with 5 mM inducing a 36-fold increase and 50 mM a 157-fold increase. This stimulation was essentially completely abolished by incubation with the I $\kappa$ B-NBD inhibitory peptide but was not affected by the AP control peptide.

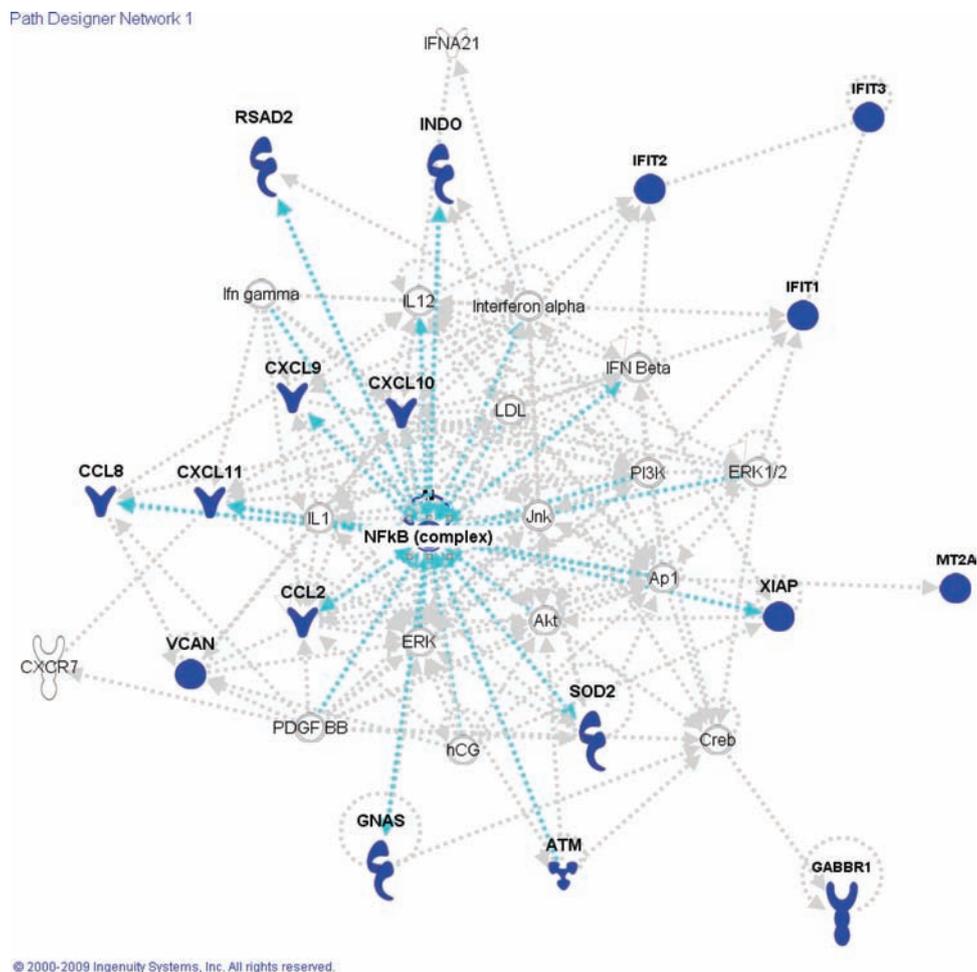
#### Omniscan stimulation of chemokine gene expression is dependent on NF $\kappa$ B activation

To determine whether NF $\kappa$ B activation was required for Omniscan stimulation of macrophage chemokine production, terminally differentiated macrophages without prior PMA stimulation were preincubated with the NF $\kappa$ B cell-permeable inhibitory peptide and then exposed to Omniscan (50 mM) for 24 h. Quantitative assessment of transcript levels showed that pretreatment with the I $\kappa$ B-NBD peptide completely abrogated the Omniscan-induced upregulation of CCL8, CXCL10 and CXCL11 and reduced by  $\sim 85\%$  the upregulation of CCL2 (figure 6E). These results demonstrated that exposure to Omniscan elicits potent chemokine gene expression stimulation in macrophages that is dependent on NF $\kappa$ B activation but independent of prior PMA activation.

#### DISCUSSION

The activation of tissue macrophages is known to play a critical role during the wound healing process, orchestrating both the onset and the resolution of the fibrotic phase.<sup>32–34</sup> The study of the pathological processes occurring in systemic fibrotic diseases such as systemic sclerosis has revealed the presence of macrophages in the affected tissues, particularly during the early stages of tissue involvement.<sup>35–36</sup> Macrophage infiltration has also been described in the affected skin of patients with NSF,<sup>7</sup> and electron scanning microscopy and energy dispersive x-ray spectroscopy revealed intracellular Gd deposits in macrophages in affected NSF skin.<sup>15</sup> These elegant studies, however, did not provide any functional evidence that the Gd deposits caused functional effects or alterations in the macrophage cell populations.

The studies described here examined directly the role of an interaction between GdBCA and macrophages by exposing normal differentiated human macrophages to the GdBCA Omniscan.



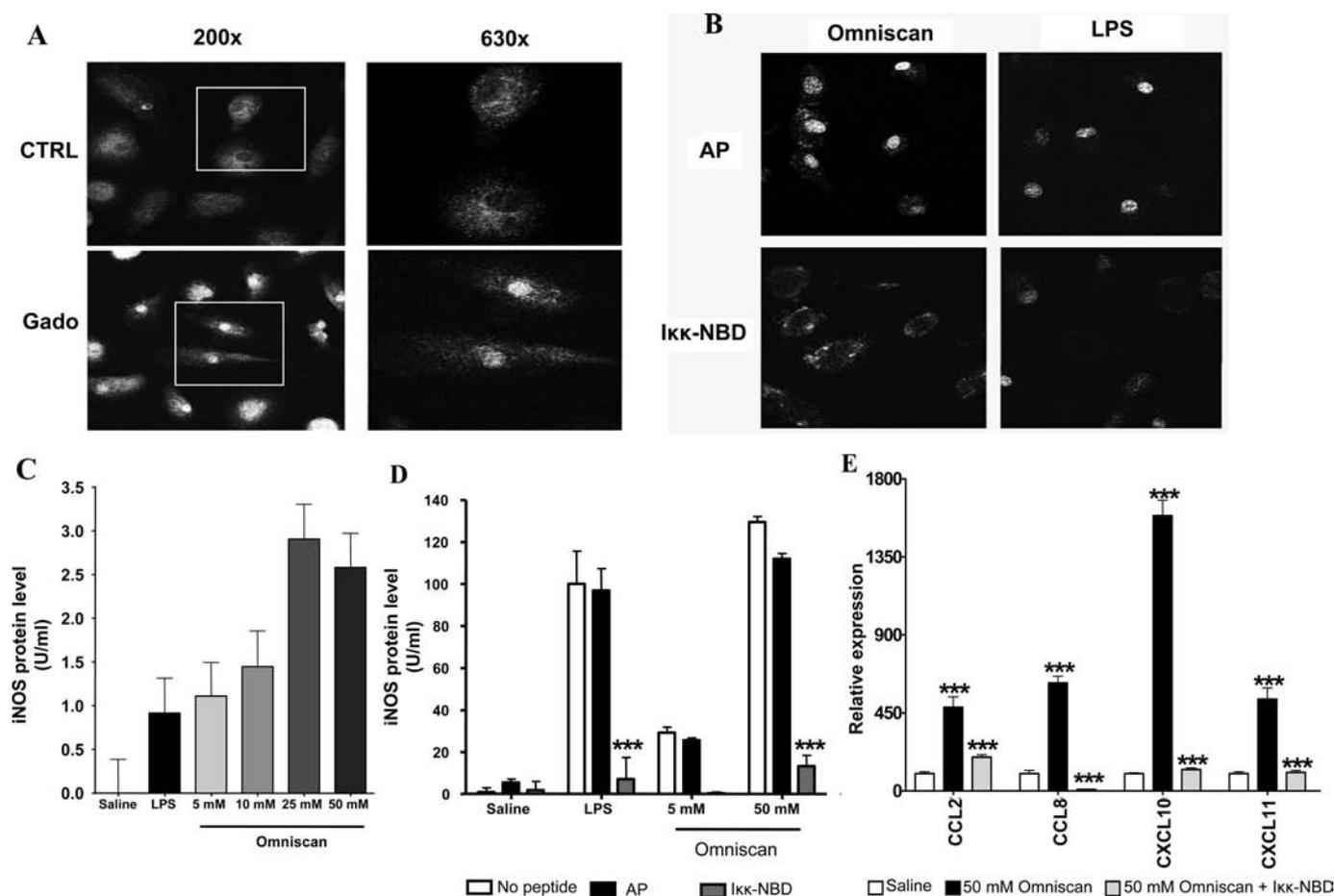
**Figure 5** Pathway analysis of Omniscan upregulated transcripts. Ingenuity software pathway analysis of the genes most upregulated by Omniscan indicated that the most represented pathway was the nuclear factor  $\kappa$ B (NF $\kappa$ B) signalling pathway. The upregulated genes represented in the pathway are shown in blue symbols according to Ingenuity classification.

This compound was chosen because it is the most common GdBCA to which patients who subsequently developed NSF had been exposed. Another reason for our focus on this GdBCA was that, in a previous study, we performed a detailed comparison of several GdBCA on PBMC function<sup>19</sup> and found that, despite numerous quantitative and qualitative differences in the levels of cytokine/chemokine stimulation between the different GdBCA, the most potent effects were observed with Omniscan. However, we also performed a microarray analysis on terminally differentiated normal human macrophages incubated with another GdBCA, Gd-DTPA. The effects of Omniscan were substantially stronger in inducing macrophage activation than those induced by Gd-DTPA.

The microarray analysis of transcriptome changes occurring in normal human macrophages as a result of exposure to Omniscan demonstrated a remarkable change in their transcriptome that results in the differential expression of 551 genes, as depicted in figure 1 and listed in table 1 in the online supplement. Although the Gd-DTPA data are not described, a table of the differentially expressed genes in human macrophages following Gd-DTPA stimulation is included in table 2 in the online supplement. A volcano plot analysis of the most differentially expressed genes following macrophage exposure to Omniscan revealed the increased expression of genes encoding numerous chemokines known to be potent chemoattractants for bone marrow-derived cells. Although the concentrations of GdBCA

used here are substantially higher than those found in the circulation of individuals with normal renal function following their administration for imaging studies, the actual concentrations of these compounds in the tissues of patients with renal insufficiency are very likely to be much greater than those calculated from pharmacokinetic studies. For example, in a study of patients with chronic renal failure on haemodialysis receiving 0.1 mmol/kg administrations of Gd-DTPA (Magnevist) before haemodialysis, serum concentrations as high as 50  $\mu$ mol Gd/ml were observed.<sup>37</sup> Furthermore, recent studies have demonstrated the selective accumulation of Gd in macrophages and fibrocytes in affected skin from patients with NSF,<sup>15</sup> suggesting that the distribution of GdBCA in affected tissues is not homogeneous and that GdBCA can be concentrated in tissue macrophages resulting in cellular exposure to much higher concentrations of these compounds.

The reasons why only certain individuals with renal insufficiency exposed to GdBCA develop NSF are not known. However, it is very likely that there is great variability in the cellular response to GdBCA among individuals. Indeed, in our previous study<sup>19</sup> we observed that PBMCs from different donors display different patterns of activation following incubation with similar concentrations of the same contrast agent. We have suggested that this variation must be related to a permissive genetic background that may influence the threshold and the pattern of cellular activation.



**Figure 6** Cellular localisation of nuclear factor  $\kappa$ B (NF $\kappa$ B) in Omniscan-treated differentiated macrophages and Omniscan stimulation of macrophage inducible nitric oxide synthase (iNOS) protein levels and chemokine production through NF $\kappa$ B activation. (A) Omniscan induction of NF $\kappa$ B nuclear localisation. Confocal microscopy analysis of terminally differentiated macrophages exposed to 50 mM Omniscan (Gado) for 5 min or maintained in phosphate buffered saline (PBS). Staining for NF $\kappa$ B p65 was shifted into the nuclei in the Omniscan-treated cells within 5 min of exposure. (B) Inhibition of nuclear localisation of NF $\kappa$ B p65 by pretreatment with the inhibitory I $\kappa$ κ-NBD peptide. Panels are representative of three different experiments on normal human terminally differentiated macrophages (magnification 200 $\times$ ). Antennapedia homeodomain (AP) peptide alone was used as a control peptide. (C) Dose-dependent stimulation of iNOS protein levels by Omniscan. iNOS protein levels following lipopolysaccharide (LPS) and Omniscan at concentrations of 5–50 mM were measured. Cells were incubated for 30 min and iNOS protein levels measured in cell lysates using an ELISA and calculated by comparison with a standard curve obtained with human iNOS recombinant protein. The assays were performed in triplicate. Values are expressed in U/ml according to the instructions from the manufacturer (R&D Systems). (D) Omniscan induction of iNOS protein levels is dependent on NF $\kappa$ B activation. iNOS protein levels in terminally differentiated macrophages exposed to either LPS as positive control or Omniscan in the presence of a control peptide or of the inhibitory I $\kappa$ κ-NBD peptide. AP peptide alone was used as control. iNOS protein levels were normalised to the values obtained with macrophages treated with LPS in the absence of any peptide. Values are presented as average and SE of triplicates. (E) Omniscan stimulation of chemokine expression is dependent on NF $\kappa$ B activation. Chemokine expression in terminally differentiated macrophages exposed to either LPS as positive control or Omniscan in the presence of a control peptide or of the inhibitory I $\kappa$ κ-NBD peptide. AP peptide alone was used as control. Percentage expression of chemokines was normalised on macrophages treated with PBS in the absence of any peptide. The other values were calculated as multiples thereof. Values are presented as mean and SE of triplicates: \*\*\* $p$ <0.01).

The results shown in figure 3A confirmed and quantified the results of the microarray experiments indicating that the expression of numerous chemokine genes was increased in normal differentiated human macrophages. Furthermore, the results shown in figure 3B show that these effects were independent of PMA macrophage activation. Assessment of chemokine protein levels in media of GdBCA-exposed macrophages confirmed the increase in production of CCL8, CCL2, CXCL9 and CXCL11 (figure 3C). Most importantly, immunofluorescence studies with confocal microscopy of affected skin from three patients with NSF indicated that CCL8, the most upregulated chemokine, was markedly increased in comparison with normal skin (figure 4A,B).

To gain an insight into the mechanisms responsible for the remarkable increase in the macrophage production of these

chemokines in response to Omniscan exposure, we analysed the cluster of upregulated genes by pathway analysis. This analysis indicated that most of the chemokines found to be increased following GdBCA exposure are known to be induced as a result of the activation of NF $\kappa$ B (figure 5). To determine whether the observed macrophage activation was caused by stimulation of the NF $\kappa$ B pathway we analysed the nuclear localisation of NF $\kappa$ B and the levels of iNOS in differentiated macrophages following incubation with Omniscan (figure 6). We observed that 5 min incubation was sufficient to induce essentially complete nuclear localisation of NF $\kappa$ B, indicating a strong activation of this pathway. Furthermore, Omniscan induced a potent dose-dependent stimulation of iNOS protein levels and chemokine production in the same cells. Inhibition of intracellular NF $\kappa$ B with a cell-permeable inhibitory peptide showed that NF $\kappa$ B activation was

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necessary for the observed increase in iNOS levels and stimulation of chemokine expression in normal macrophages following Omniscan exposure.

The data reported here show that GdBCA can induce a potent activation of normal macrophages and suggest that a similar mechanism may be responsible for the macrophage activation observed in NSF-affected tissues. Although these observations strongly implicate macrophage participation in this unique fibrotic process, the role of any cell type in NSF is presumptive since no single cell type has been definitively demonstrated to be responsible for the spectrum of histological and pathological changes observed in this condition. Indeed, we consider it highly unlikely that a single cell type is responsible for mediating all the effects of GdBCA in the induction of NSF. However, the contention that macrophages may play an important role in this process is supported by strong experimental evidence indicating that macrophages play a role in wound healing<sup>32–34</sup> and in the pathogenesis of systemic sclerosis.<sup>35–36</sup> The involvement of monocytes and macrophages suggested here does not preclude effects of GdBCA on other cells present in lesions such as fibrocytes and fibroblasts. Furthermore, it is widely accepted that there is an increased number of fibrocytes (CD34) in the skin of patients with NSF. These cells are of bone marrow origin and home to the skin following a specific chemokine gradient. In this sense, a specific activation of macrophages causing increased production and a gradient of chemokines is very likely to occur and to be crucial in the process.

Although the studies reported here were conducted *in vitro*, the kinetics of response we observed, including a potent NFκB nuclear localisation as early as 5 min following exposure to Omniscan, suggest that normal human macrophages are capable of reacting directly to the Gd-chelate complex. The kinetics of response and the activation of NFκB are similar to those observed following lipopolysaccharide stimulation, suggesting a possible involvement of Toll-like receptor (TLR) signalling in this process. Additional studies are in progress to further examine the role of TLR signalling in GdBCA-induced macrophage activation in the initiation and progression of tissue fibrosis.

The results described here show conclusively that Omniscan is able to activate macrophages and induce their production of numerous chemokines that are known to be involved in the pathogenesis of tissue fibrosis. Indeed, we found potent stimulation of chemokines such as CXCL10 which have been shown to be raised in the serum of some patients with systemic sclerosis<sup>38</sup> and to participate in wound healing.<sup>39</sup> Furthermore, we clearly demonstrate that the activation responsible for their production is NFκB-mediated. Thus, the principal conclusion of the studies described here is that the GdBCA Omniscan, when in contact with terminally differentiated macrophages, is able to activate them towards a profibrotic phenotype and therefore this could trigger a sequence of events that is responsible for the development of the clinical picture of NSF in a genetically permissive background. Most importantly, this paradigm could be used to investigate the very early events in the pathogenesis of idiopathic tissue fibrotic disorders such as systemic sclerosis or idiopathic pulmonary fibrosis, conditions in which the triggering events are not known, thus precluding a detailed analysis of the early steps in their pathogenesis. In this regard, environmentally-induced diseases such as GdBCA-induced NSF may offer a very valuable model to study the cascade of early events that lead to a given pathological fibrotic disease phenotype.

**Acknowledgements** The authors acknowledge the excellent assistance of Susan V Castro and Carol Kelly in the preparation of this manuscript.

**Funding** Supported by an Investigator Initiated Grant (IIG) from GE Healthcare to SAJ, by grants from NIH (R01 AR-019616) to SAJ, the Scleroderma Research Foundation and the Dermatology Foundation to FDG and the PA Department of Health (SAP4100026302) to PF. PJW was supported by NIH Training Grant (T32 AR-007583) to SAJ.

**Ethics approval** This study was conducted with the approval of the Thomas Jefferson University Internal Review Board.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Competing Interests** None.

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