

## Activated macrophages for treating skin ulceration: gene expression in human monocytes after hypo-osmotic shock

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### SUMMARY

Macrophages play a major role in almost all stages of the complex process of wound healing. It has been previously shown that the incorporation of a hypo-osmotic shock step, in the process of monocyte-concentrate preparation from a blood unit, induces monocyte/macrophage activation. As the macrophages are produced using a unique, closed and sterile system, they are suitable for local application on ulcers in elderly and paraplegic patients. Enhanced phagocytosis by the activated cells, as well as increased secretion of cytokines such as IL-1, IL-6, were detected in a recent study which are in accord with the very encouraging clinical results. In the present study, we used DNA microarrays to analyse the differential gene expressions of the hypo-osmotic shock-activated monocytes/macrophages and compare them to non-treated cells. Of the genes that exhibited differences of expression in the activated cell population, 94% (68/72) displayed increased activity. The mRNA levels of 43/68 of these genes (63%) were found to be 1.5-fold or higher (1.5–7.98) in the activated macrophages cell population as compared to the non-treated cells. Only four genes were found to have lower mRNA levels in the activated cells, with ratios of expression of 0.62–0.8, which may suggest that the changes are insignificant. A significant number of the genes that showed increased levels of expression is known to be directly involved in macrophage function and wound healing. This may correlate with the increased secretion of different cytokines by the activated macrophages depicted previously. Other groups of genes expressed are known to be involved in important pathways such as neuronal growth and function, developmental defects and cancer. The hypo-osmotic shock induces a gene expression profile of cytokines and receptors in the activated cells. These may evoke potential abilities to produce a variety of protein products needed in the wound healing process and may bring to light possibilities for other therapeutic applications of these cells.

**Keywords** cytokines gene expression hypo-osmotic shock macrophages

### INTRODUCTION

The monocytes, which constitute 1–6% of the nucleated peripheral blood cells, egress the blood stream into tissues and differentiate into a variety of macrophages, depending on the nature of the signals in the local microenvironment [1,2].

Their phagocytic abilities and diverse secretory potential place the monocytes/macrophages in the centre of the wound healing process [1,3–5]. This biologically complex sequence of events involves cellular and molecular processes such as cell migration, inflammation, angiogenesis, collagen synthesis and deposition and re-epithelialization [5,6]. The monocytes/

macrophages have key functions in almost every stage of the process. Upon initiation of the inflammation stage, the macrophages secrete IL-1 that induces the rapid recruitment of inflammation cells from the circulation into the wound [7–9]. As phagocytes, they help in the digestion of bacteria and debridement [10] and, in the later stages of the wound-healing process, they secrete IL-6, which is believed to influence endothelial cell proliferation and the initiation of angiogenesis [11–13]. The macrophage serves as the coordinator of this proliferative process, by producing growth factors such as PDGF BB, TGF- $\alpha$ , TGF- $\beta$ 1, VEGF, FGF, EGF and IGF-1, necessary in the wound-healing process [5,8,14].

It seems that the age of the macrophages may play a role in the process. Application of rabbit-antimouse macrophage serum to wounds of young mice delayed wound healing, reducing it to the rate observed in old mice [15]. Moreover, wound repair

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was enhanced in old mice by local injection of monocytes/macrophages derived from young mice [16].

We have developed a method for preparation of human activated monocytes/macrophages from randomly collected blood units in a closed, sterile system, which are used successfully, within 24 h, in the treatment of human chronic decubital ulcers [17,18]. The macrophage preparation process includes a step of hypo-osmotic shock which induces monocyte/macrophage activation, without having to introduce additional substances (LPS, Inf- $\gamma$ , etc.) to the cell culture [19–23]. The hypo-osmotic shock was found to be an effective trigger for monocyte/macrophage activation, as depicted using phagocytosis, and secretion of IL-1 and IL-6 as test parameters [24]. To further investigate the possibility that other products of the activated monocyte/macrophage may have been triggered but not yet secreted into the surrounding medium, the changes in mRNA levels of the cells submitted to hypo-osmotic shock were studied using DNA microarrays. The present report summarizes our observation of increased expression of mRNA of interleukins, growth factors and receptors in the hypo-osmotic shock-treated cells as compared to non-treated controls.

## MATERIALS AND METHODS

### *Preparation of monocytes/macrophages*

Monocytes/macrophages were prepared in a closed sterile system as previously described [17] (USA patent no. 6,146,890, D. Danon) with some modifications. Briefly, a whole blood unit collected into a triple blood bag system was separated into packed red blood cells, white blood cells (WBC) and plasma. The bags containing the plasma and WBC layer were connected to the macrophage preparation system using a sterile connecting device (Terumo Sterile Tubing Welder SC-201 A, Tokyo, Japan). Transfer of 60 ml CaCl<sub>2</sub> (80 mM) to the plasma bag induced a coagulation that was completed after 2 h of incubation at 37°C. The obtained autologous serum served as the growth medium for the monocytes/macrophages.

A control sample was prepared from each blood unit by attaching an additional culture bag to the preparation system. The WBC layer was divided equally into the two culture bags, of which only one was submitted to hypo-osmotic shock by the addition of 100 ml distilled water. After 45 s, 10 ml of a 10-fold concentrated saline solution was added to re-establish isotonicity. In parallel, 110 ml of isotonic saline was transferred into the control culture bag. The set was centrifuged at 600 g for 10 min and the supernatant of both bags was decanted. Equal volumes of autologous serum (30 ml) were then transferred into each bag and incubated for 1 h at 37°C, after which the supernatant of the culture bags was decanted. The bags were rinsed with fresh serum and transferred into the 'sink bag', rinsing away all the non-adherent cells. At this stage, a layer of monocytes/macrophages adhered to the inner surface of both culture bags. A new volume (30 ml) of serum was placed into each bag, which was then incubated for 24 h. Following incubation, the cells were harvested by rubbing the inner surfaces of the plastic bags against each other. The cell suspension was transferred into 50 ml sterile polyethylene tubes and centrifuged at 600 g for 10 min. The supernatant was discarded and the cells were resuspended in 1 ml of autologous serum. Cells were counted in a Newbauer haemocytometer after suspension in trypan blue (1:1 in 0.4% trypan blue solution) for evaluation of cell count and percentage of vitality. They were then centrifuged

at 600 g for 6 min and the cell pellet was frozen at -70°C for further studies. Pools of monocytes/macrophages from 12 blood units was collected and frozen. Pools of the control saline-washed cells contained  $5 \times 10^7$  cells and those from the hypo-osmotic shock-treated cells contained  $7.5 \times 10^7$  cells.

### *RNA extraction*

Total RNA was isolated using TRIZOL reagent (GIBCO BRL, Life Technologies, NY, USA). Homogenization of the cells was performed by the addition of 1 ml of reagent per  $1 \times 10^7$  cells. The cell suspension was divided into 1-ml tubes. RNA was extracted by adding 0.2 ml chloroform to each tube, mixing and storing the samples for 2–3 min at RT, followed by a 15-min centrifugation at 12 000 g in 4°C. RNA precipitation was achieved by transferring the aqueous phase into a new tube, adding 0.5 ml isopropanol, mixing and storing the samples for 5–10 min at RT. Centrifugation at 12 000 g in 4°C was performed for 10 min. RNA was washed by mixing the pellet with 1 ml 75% ethanol and centrifuging at 7500 g 4°C for 5 min. The RNA pellet was air-dried for 5–10 min and then dissolved in 50  $\mu$ l by pipetting and incubating at 55–60°C for 10 min. After solubilization, all samples were frozen at -70°C.

### *DNA microarrays*

*cDNA array hybridization.* We used the cytokine/receptor (268 genes) array of human cDNAs spotted on a nylon membrane (Atlas Filter Arrays, Clontech, Palo Alto, CA, USA). The filters also included housekeeping control cDNAs and negative controls spotted in duplicate dots. A complete list of the cDNAs and controls as well as their accession numbers is available at <http://www.clontech.com/atlas/genelists/index.html>. Total RNAs were treated with DNaseI according to the manufacturer's instructions and used for cDNA synthesis. A 3- $\mu$ l mix containing 5  $\mu$ g of total RNA and 1  $\mu$ l of  $\times 10$  CDS primer mix (specific for each filter array, provided by the manufacturer Clontech, USA) was incubated at 70°C for 2 min followed by additional incubation at 48°C for 2 min. To this mix, 8  $\mu$ l of master mix (containing 2  $\mu$ l  $\times 5$  reaction buffer, 1  $\mu$ l  $\times 10$  dNTP mix, 3.5  $\mu$ l [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol, 10 mCi/ml, Amersham/Pharmacia Biotech, Amersham, UK), 0.5  $\mu$ l 100 mM DTT and 1  $\mu$ l MMLV Reverse Transcriptase (50 U/ $\mu$ l) was added, mixed and incubated for 25 min at 48°C. The reaction was terminated by adding 1  $\mu$ l of  $\times 10$  termination mix at room temperature. The radioactive-labelled cDNA mix was fractionated on a Chroma Spin-200 column (Clontech Laboratories Inc., USA) and fractions that comprise the first peak of radioactivity were pooled for each cDNA synthesis reaction. In each set of hybridization, equal counts were taken for control and experimental labelled cDNA probes. The labelled cDNA probe was then mixed with 1/10th vol. of  $\times 10$  denaturing solution (1 M NaOH, 10 mM EDTA) and incubated at 68°C for 20 min. Following the incubation, 5  $\mu$ l (1  $\mu$ g/ $\mu$ l) of cot-1 DNA and equal volume of  $\times 2$  neutralizing solution (1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0) were added and incubated at 68°C for 10 min. Denatured, labelled cDNA was then added to 5 ml of ExpressHyb solution (Clontech) with 1 mg of sheared salmon sperm DNA (Sigma, St Louis, MO, USA) and mixed. This hybridization solution was added to the Atlas cDNA expression array membrane, which was prehybridized in 10 ml of expressHyb hybridization solution at 68°C for 1 h. Hybridization proceeded overnight at 68°C in a roller bottle. Membranes were washed once with prewarmed 2XSSC/1%SDS for 30 min and once or twice with

0.5XSSC/0.5%SDS for 30 min at 68°C with constant agitation. The membranes were exposed to Fuji X-ray films at -70°C with intensifying screens.

**Analysis of hybridization signals.** The cDNA microarray autoradiograms were scanned and the images were analysed using AtlasImage 1.01 software (Clontech, USA). The background was calculated using default external background calculation that takes into consideration the background signals at blank space between the different panels of the arrays. Signal threshold was set as background-based signal threshold. The signal intensities were normalized globally using the sum method (AtlasImage, Clontech, USA). A report of differentially expressed genes was generated based on ratio and intensity differences.

#### Interleukins

Samples of supernatants were collected and stored at -60°C for further interleukins titration by ELISA kits. In order to refer the interleukins production to the number of monocytes, they were marked with CD-14 fluorescent antibody (CD-14 is a common marker for both monocytes and macrophages, thus including all the relevant cell population).

#### Sample collection

The culture bags containing the adherent cells were incubated with 30 ml of autologous serum for 24 h. Aliquots of 1.5 ml supernatant were sampled from each bag after 24-h incubation. The level of interleukins in the serum before incubation served as baseline. All samples were centrifuged at 12 000 *g* for 5 min and the supernatant was transferred into clean Eppendorf tubes and stored at -60°C.

#### CD-14 marking

After 24 h incubation, the cells were harvested by rubbing the inner surfaces of the plastic bags against each other; the cell suspension was transferred into 50-ml sterile polyethylene tubes and centrifuged at 600 *g* for 10 min. The supernatant was discarded and the cells were resuspended in 1 ml of serum. Cells were counted in a Newbauer haemocytometer after suspension in trypan blue (1:1 in 0.4% trypan blue solution) for evaluation of cell count and percentage of vital cells. The cells were concentrated to  $10 \times 10^6$ /ml by centrifugation (600 *g* for 10 min) and serum was added to the sediment, as required. The 50  $\mu$ l suspension (containing  $5 \times 10^5$  cells) was then transferred into a new conic tube. Ten microlitres of CD-14 was added (CD-14 FITC mouse antihuman, monoclonal, Ancell, Italy) and incubated at 4°C for 25 min in the dark. At the end of the incubation, the cells were washed twice with 1 ml cold PBS (600 *g* for 10 min) and resuspended in 1 ml cold PBS. The samples were measured for fluorescence by FACS for evaluation of macrophage percentage in the collected cell population.

#### Interleukins quantification

IL-1 $\beta$  and IL-6 were titrated using Edogen ELISA kits, Cambridge, MA, USA.

Samples were measured in duplicate and the mean result of the interleukin concentration in the supernatant was corrected to  $10^6$  monocytes. The level of the interleukins in pure serum of each blood unit was subtracted from each sample.

## RESULTS

Table 1 summarizes 72 genes whose expression level ratios differed from the hypo-osmotic shock-induced cells (shown in Fig. 1) and the controls (Fig. 2), and a graphic comparison of the data is presented in Fig. 3.

Among the genes that exhibited differences of expression in the activated cell population, 94% (68/72) displayed increased activity and only four genes were found to have lower mRNA levels in the activated cells, with ratios of expression of 0.62–0.8, which may suggest that the changes are insignificant.

The mRNA levels of 43/68 (63%) of genes that expressed increased activity were found to be 1.5-fold or higher (1.5–7.98) in the activated macrophages cell population compared to the non-treated cells. To correlate between the gene profiles and the relevant cytokines production, the secretion of IL-1, IL-6, TGF- $\beta$  and GM-CSF by both the hypo-osmotic shock-treated macrophages and the controls were measured. The levels of IL-1 measured in the supernatant of the hypo-osmotic shock-treated monocytes/macrophages after 24 h of incubation, ranged between 1.7 and 123 times higher than those of the controls ( $P = 0.004$ ) in all blood samples. (Table 2). The augmentation in the levels of IL-6 was even more pronounced, ranging from 6.6 to 175-fold increase in the hypo-osmotic shock-treated macrophages, compared to their matched controls ( $P = 0.001$ ) (Table 2).

Interestingly enough, no increase was found in the levels of TGF- $\beta$ , as measured after the 24-h incubation, in both cell preparations from seven different blood units tested (Table 3). However, the levels of TGF- $\beta$  in the cell growth medium (serum) were relatively high. Levels of GM-CSF were not detected in the osmotic-shock or the control cell preparations after incubation of the cells for 24 h, nor were they found in the serum medium before incubation (data not shown).

## DISCUSSION

Activated human monocytes/macrophages, produced by hypo-osmotic shock, have been used successfully for the treatment of decubital chronic ulcers for few years [17,18]. This procedure is of special interest since no toxic additives or chemical reagents are used, making the clinical application safe. In addition, increased secretion of cytokines, such as IL-1, IL-6 as well as enhanced phagocytosis, were detected by the activated cells, all bearing relevance to the wound-healing process [24].

In this study, we used DNA microarrays to detect differential gene expressions of activated monocytes/macrophages and compared it to that of non-treated cells. Of the genes that exhibited differences of expression in the activated cell population, 94% (68/72) displayed increased activity, and only four genes were found to have lower mRNA levels in the activated cells, with ratios of expression of 0.62–0.8, which may suggest that the changes are insignificant. The mRNA levels of 68 genes were found to be increased in the activated macrophage cell population when compared with the non-treated cells. The expression ratios of 43 of these genes (63%) were found to be 1.5-fold or higher (1.5–7.98).

The genes, whose level of expression was significantly increased, can be divided into four groups:

- Genes known to be directly involved in macrophage function and wound healing. These include TGF- $\beta$ , MIF, FGF-8, TNF receptors, IGF BP, BMP1, IGF-I receptor, monocyte IL-1 $\beta$ ,

**Table 1.** Ratios of 72 genes whose expression levels were found to differ between the hypo-osmotic shock-activated cells and the controls. Bold characters indicate factors known to be related to wound healing

Accession no.	Coordinate	Gene/protein name	Ratio
L38734	10 I	Ephrin-B2 precursor*	0.62
M10051; X02160	12 E	Insulin receptor	0.72
M26880	1 A	Ubiquitin <sup>1</sup>	0.76
X97057	20 J	WNT-10B precursor; Wnt-12	0.8
M65199	18 L	Endothelin ET2	1.25
X01057; X01058; X01402	4 I	IL-2 receptor $\alpha$ subunit precursor (IL-2 receptor $\alpha$ subunit; IL2RA); TAC antigen; CD25	1.25
M73980	21 G	Neurogenic locus notch protein homolog 1 precursor (NOTCH 1); translocation-associated notch protein (TAN 1)	1.26
U04806; U03858	19 C	SL cytokine precursor, FLT3 ligand (FLT3LG)	1.29
M31159; M35878	11 O	Insulin-like growth factor-binding protein 3 precursor (IGFBP3)	1.33
U28811; U64791	17 G	Cysteine-rich fibroblast growth factor receptor; Golgi membrane sialoglycoprotein MG160 (GLGI)	1.34
U14188	10 K	Ephrin A3 precursor (EFNA3); EPH-related receptor tyrosine kinase ligand 3; LERK-3; EHK1L; EFL2	1.34
X74764	23 K	Neurotrophic tyrosine kinase receptor-related 3 TKT precursor	1.35
X53799	7 B	Macrophage inflammatory protein 2 $\alpha$ -(MIP -2 $\alpha$ ); growth-regulated protein beta (GRO-beta)	1.36
L20861	20 H	Wnt -5a	1.36
M24545	7 D	Monocyte chemotactic protein 1 precursor (MCP1); monocyte chemotactic and activating factor - MCAF monocyte secretory protein JE; monocyte chemotactic protein 1; HC11; small inducible cytokine A2 (SCYA2)	1.37
Y00787	3 J	Monocyte-derived neutrophil chemotactic factor - (MDNCF); IL-8 precursor; T-cell chemotactic factor; neutrophil activating protein 1; lymphocyte-derived neutrophil activating protein; protein3-10C	1.38
M74178	6 L	Macrophage stimulating protein (MSP); hepatocyte growth factor-like protein	1.38
X78686	7 E	Granulocyte chemotactic protein 2 (GCP2); neutrophil activating peptide ENA-78	1.38
L20471	22 F	Basigin precursor (BSG);leucocyte activation antigen M6; collagenase stimulatory factor; extracellular matrix metalloproteinase inducer (EMMPRIN); 5F7; CD147 antigen	1.38
X03438	6 J	Granulocyte colony -stimulating factor precursor (G-CSF); plutiprotein; CSF3	1.38
J03634	16 F	Erythroid differentiation protein	1.39
X01394	14 I	Tumour necrosis factor	1.4
M21121	7 F	RANTES proinflammatory cytokine	1.4
U32659	4 E	IL-17 precursor; cytotoxic T-lymphocyte- associated antigen 8 (CTLA8)	1.42
X04602; M14584		3 HIL-6 precursor; B-cell stimulatory factor 2 (BSF2); interferon beta-2 (IFNB2); hybridoma growth factor	1.43
X54936	18 N	Placenta growth factor precursor 1 and 2	1.46
U06863	22 H	Follistatin-related protein precursor	1.46
K00558	1 E	Brain-specific tubulin $\alpha$ subunit	1.46
X14454	6 E	Interferon regulatory factor 1	1.47
X06233	6 I	MRP-14 (calcium binding protein in macrophages MIF-related)	1.48
U90875	12 L	Cytotoxic ligand TRAIL receptor	1.49
X72304	22 E	$\beta$ thromboglobulin-like protein	1.5
M11220	6 K	Granulocyte-macrophage colony stimulating factor (GM-CSF)	1.54
D10924	22 M	Stromal cell derived factor 1 receptor (SDF1 receptor); fusin; CXCR4; leucocyte-derived seven transmembrane domain receptor (LESTR); LCR1	1.58
M95667 + M11730	18 G	ERBB2 receptor protein-tyrosine kinase; neu proteo-oncogene; c-erbB2 + HER2 receptor	1.59
M15530	6 G	B-cell growth factor (BCGF1)	1.59
D16431	22 K	Hepatoma-derived growth factor	1.59
M22489	15 J	Bone morphogenic protein 2a	1.63
L06801	4 B	IL-13 precursor; NC30	1.67
X06234	6 H	Migration inhibitory facto-related protein 8 (MRP-8) calcium-binding protein in macrophages MIF-related) calgranulina; leucocyte L1 complex light subunit; S100 calcium-binding protein A8; cystic fibrosis antigen (CFAG)	1.68
M32977; M27281	19 F	Vascular endothelial growth factor precursor (VEGF); vascular permeability factor (VPF)	1.69
D10923	22 L	G-protein-coupled receptor HM74	1.7
L15344	4 C	IL-14 precursor; high molecular weight B-cell growth factor	1.71
X56932	1 H	23-kD highly basic protein; 60S ribosomal protein LI3A	1.76
L08187	9 I	Cytokine receptor EB13	1.77
X53655; M37763	9 D	Neurotrophin-3 precursor (NT-3); neurotrophic factor (HDNF); nerve growth factor (NGF-2)	1.81
M60316	16 C	Bone morphogenic protein 7 precursor (BMP7); osteogenic protein 1(OP1)	1.82
M62402	12 D	Insulin-like growth factor binding protein 6 precursor (IGF-binding protein 6; IGFBP6;IBP6)	1.9



Table 1. (Continued)

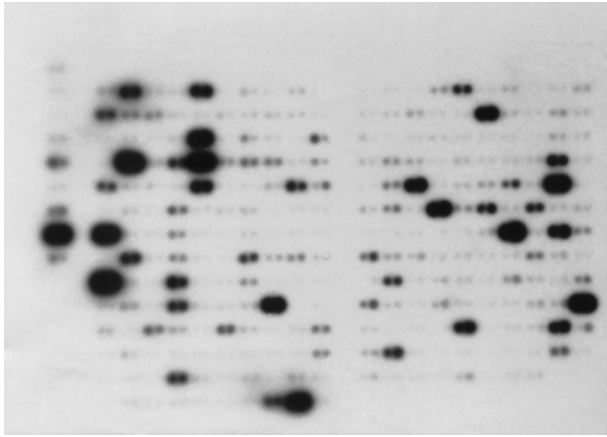
Accession no.	Coordinate	Gene/protein name	Ratio
M19154; M22045; M22046; Y00083	15 G	TGF $\beta$ -2 precursor; glioblastoma-derived T-cell suppressor factor; bsc-1 cell growth inhibitor; polyergin; cetermin	1.9
M61176	8 L	Brain-derived neurotrophic factor (BDNF)	1.93
M96955 + M96956	18 B	TDGF1 + TDGF3 + CRGF; CRG	1.99
U84401	20 F	Smoothened; GX	2.18
X04434; M24599	12 F	IGF I receptor	2.21
M57627	3 L	IL-10 precursor; CSIF	2.25
M25667	10 E	Neuromodulin; axonal membrane protein GAP-43; PP46; protein F1; calmodulin-binding protein p57	2.33
M86528 + S41522 + S41540 + S41541	11 I	NT-4 NT-5 + NT6- $\alpha$ + NT6- $\beta$ + NT6- $\gamma$	2.36
L00587	22 C	Calcitonin receptor	2.44
K02770	3 C	IL-1 $\beta$ precursor; catabolin	2.44
M22488 + U50330	15 I	Bone morphogenetic protein 1 BMP1 + procollagen C-proteinase (pCP-2)	2.49
M35410	11 N	IGF BP-2	2.5
M32315 + M55994	14 K	TNF-receptor + TNF-receptor 2; TBP2	2.51
X03541	9 J	High affinity nerve growth factor receptor precursor; trk-1 transforming tyrosine kinase protein; p140-TRKA; p68-trk-T3 oncoprotein	2.61
X02851	3 B	IL-1 $\alpha$ precursor; haematopoietin-1	2.66
U36223	17 B	FGF-8; AIGF; HBGf8	2.67
M23452	6 N	MIP1- $\alpha$ ; tonsillar lymphocyte LD78 $\alpha$ protein; GOS19-1 protein; PAT 464.2; SIS - $\beta$ ; small inducible cytokine A3 (SCYA3)	2.7
D11086	4 K	IL-2 receptor $\gamma$ ; cytokine receptor common $\gamma$ chain precursor; p64	2.78
U95299	21 I	Notch 4	3.15
X74979	23 J	Epithelial discoidin domain receptor 1 precursor (EDDR1; DDR1); cell adhesion kinase (CAK); TRKE; RTK6	3.25
M84747	5 E	IL-9 receptor precursor	3.36
X02812; J05114	15 F	TGF $\beta$	3.4
X00351	1 G	Cytoplasmic $\beta$ actin	4.04
U02687	19 G	Stem cell tyrosine kinase 1 (STK1); FL cytokine receptor precursor; tyrosine-protein kinase receptor flt3; CD135 antigen	4.51
M13982	3 F	IL-4 precursor; B-cell stimulatory factor 1 (BSF-1); lymphocyte stimulatory factor 1	7.98

bone morphogenic protein 7, VEGF, MRP-8 and GM-CSF. The methodology and interpretation used in this study, which enabled detection of differentially expressed genes that are known to be involved in monocyte/macrophage activation, metabolism and effective functions, supports and validate the observations of their importance in the wound-healing process.

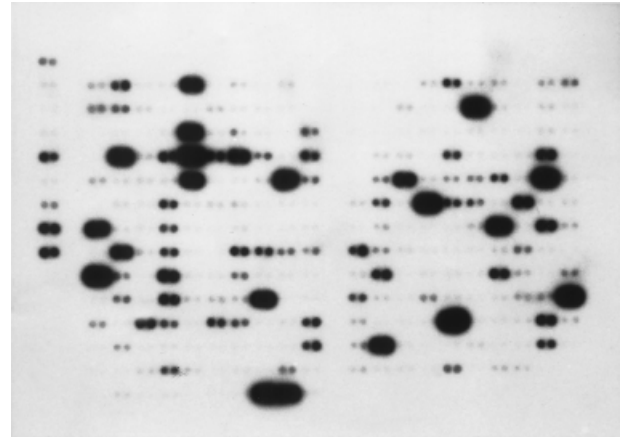
- Genes characterized in this study that were not associated previously with macrophage function and wound healing should be studied further in depth in order to elucidate their specific role and potential clinical applications in the process. Several attractive genes were shown to be expressed preferentially in the activated cell population. One example is the expression of the FLT3 growth factor receptor tyrosine kinase (FLT3R, STK-1), which was shown to be significantly induced (4-51-fold) after activation. Interestingly, FLT3 ligand was recently shown to induce macrophage activation in mice [25]. In bovine bone marrow culture, the combination of FLT 3 ligand with GM-CSF and IL-4 (both found to be induced in our study) resulted in the generation of dendritic cells [26]. Calcitonin receptor mRNA was also induced in the hypo-osmotic shock-treated cells. However, the calcitonin receptor protein is usually expressed on osteoclasts, rather than monocytes/macrophages [27]. Moreover, IL-4, as well as other cytokines such as IL-1 $\alpha$ , which were also found to be induced in a very significant way

in our study, were associated with osteoclast formation in a monoblastic cell line [28,29]. It will be interesting, therefore, to study whether osteoclast activation can also be induced by the hypo-osmotic shock protocol.

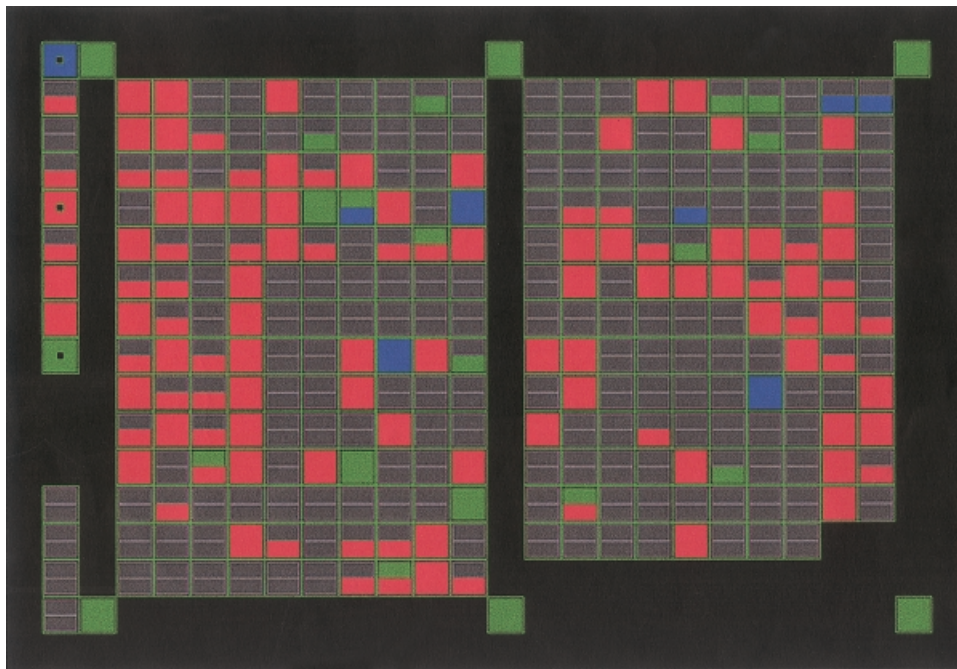
- The third group includes genes associated with neuronal development and function. These include nerve growth factor receptor (TRK), high affinity nerve growth factor receptor precursor, neuronal growth protein GAP-43, NT-4, NT-6 and nerve growth factor (NGF-2). The increased expression of mRNAs encoding 'neuronal' genes can be explained by their parallel involvement in both the nervous system and the monocyte/macrophage lineage of the haematopoietic system. Alternatively, a functional cross-talk between monocytes/macrophages and neuronal cells can be proposed. Indeed, a great body of evidence has accumulated in recent years concerning the involvement of monocytes/macrophages and macrophage-secreted factors in the nerve regeneration processes [30]. The identification of the various proteins up-regulated in monocyte/macrophage activation by differential expression might lead to the characterization of molecules with therapeutic potential in neuroprotection and neuroregeneration.
- The fourth group of genes found to be expressed preferentially in the activated monocytes/macrophages includes genes that encode components of developmental controlling pathways



**Fig. 1.** Differential hybridization of monocytes/macrophages activated by hypo-osmotic shock, to the cytokine/receptor filters.



**Fig. 2.** Differential hybridization of control monocytes/macrophages to cytokine/receptor filters.



**Fig. 3.** Comparative hybridization of control and hypo-osmotic shock activated monocytes/macrophages, using the AtlasImage™ software. The blue boxes indicate genes expressed at lower levels in the activated cell population, compared with the controls. The red boxes indicate genes expressed at higher levels in the activated cell population. The green boxes indicate genes with no significant difference in expression levels in both groups and the grey boxes indicate genes expressed at a background level.

such as Notch, Wnt and smoothened. These key pathways are central to the development and function of various tissues and genetic aberrations in several genes encoding different components of these pathways were associated in developmental defects and cancer. Interestingly, glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a key player in the Wnt/Wingless signalling pathway, was shown recently to be involved in the signalling pathway induced by the tumour-necrosis factor  $\alpha$  (TNF- $\alpha$ ) proinflammatory cytokine [31].

Several reservations should be borne in mind when the results of DNA microarray experiments are considered. The analysis of

mRNA levels does not necessarily reflect the level and activity of the respective protein product, as translational and post-translational processes can have a major effect on the product. The comparison performed in this study between gene expression ratio and the measurement of cytokine levels illustrates the discrepancy between mRNA and protein levels in some examples. For some cytokines, such as IL-1 and IL-6, there was a good correlation between the increased expression at both the mRNA and the protein levels. For other cytokines such as GM-CSF and TGF- $\beta$  no such correlation was found. The results obtained from mRNA studies should therefore be complemented by analyses of protein levels and function.

**Table 2.** Levels of IL-1 and IL-6 secreted by monocytes/macrophages activated by hypo-osmotic shock and their respective controls, measured in the supernatant of eight individual blood samples, after 24 h of incubation time

Blood sample no.	IL-1 (pg/ml)		IL-6 (pg/ml)	
	Control	Osmotic shock	Control	Osmotic shock
1	1	20	35	348
2	1	123	33	1 523
3	4	204	114	20 000
4	7	393	383	20 000
5	55	697	935	20 000
6	30	53	5.9	500
7	39	72	26.8	289
8	12	29	134	883

A statistically significant difference was found in both interleukins between the stimulated cells and their controls,  $P = 0.004$  and  $P = 0.001$ , respectively.

**Table 3.** Levels of TGF  $\beta$  levels in hypo-osmotic shock stimulated monocytes/macrophages and their controls, measured in the supernatant of the individual blood samples, after 24 h of incubation time

Blood sample no.	TGF- $\beta$ (pg/ml)		
	Serum	Control	Osmotic shock
1	250	279	294
2	240	260	264
3	433	481	567
4	260	250	221
5	683	733	650
6	375	423	481
7	433	365	375

There was no statistically significant effect of the hypo-osmotic shock stimulation on secretion of TGF- $\beta$  to the supernatant.

Note: high levels of TGF- $\beta$  are detected in the serum that serves as growth medium to the cells (controls and hypo-osmotic shock stimulated).

We restricted our conclusions to those genes where the expression ratio was higher than 1.5, having in mind the technical limitations inherent in the methodology and the potential sample to sample variability. However, many genes which demonstrated only borderline increased expression are known to be involved meaningfully in macrophage activities, thus one should not always expect to find unequivocal quantitative correlation. For instance, when the interleukins IL-6 and IL-1 were measured by both the DNA microarray and ELISA methods, no quantitative correlation was found. While there was a 6.6–175-fold increase in the levels of IL-6 secreted by the activated cells, at the protein level, the mRNA precursor was only 1.4-fold higher than that of the controls. Conversely, the IL-1 mRNA was expressed 2.4 times higher in the activated cells, with a 1.7–123-fold increase at the protein level. We decided to include those uncertain candidates in the data presented to enable other researchers access

to this information. One should bear in mind that the cells activated by hypo-osmotic shock in our system are not composed of a pure macrophage population, but contain certain numbers of other cells such as lymphocytes and granulocytes. The gene profile reported in this study may therefore represent the expression profile of that mixture, enriched for activated monocytes/macrophages. As the clinical utilization of these cell preparations has already been demonstrated, the characterization of the various genes contributing to the therapeutic effects is important, irrespective of the exact cellular origin. As an example, monocytes and macrophages were shown to express both IL-4 and IL-13 receptors [32]. While IL-4 mRNA was significantly increased in the hypo-osmotic treated cells (7.98-fold), IL-13 mRNA was elevated only moderately. Whether the source of IL-4 or IL-13 in the activated cells is the monocytes/macrophages or some other 'contaminating' cells remains to be tested. However, a more important issue, in our opinion, is to study the correlation and contribution of these cytokines to the therapeutic utility of the activated cell mixture.

The system described was designed to create activated monocytes/macrophages for clinical use from random blood units donated by healthy volunteers. Further studies are necessary where activation of the macrophage population is performed using agents such as LPS and interferon- $\gamma$  and compared to the gene expression profiles described in the present results. This will strengthen our understanding and shed more light on the basic macrophage biology and their potential therapeutic applications in other fields.

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