Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2005.02985.x

Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection

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Accepted for publication 19 October 2005
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Summary

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced human monocyte-derived macrophage (GM-Mϕ) or macrophage CSF (M-CSF)-induced human monocyte-derived Mϕ (M-Mϕ) are distinct in terms of the resistance to *Mycobacterium tuberculosis*. To elucidate the role of molecules involved in the functional differences between these Mϕs, we investigated the gene expression profiles using microarray. After culture of $CD14^+$ monocytes with CSFs, Mϕs were cultured with or without bacillus Calmette–Guérin (BCG) (GM-Mϕ-BCG and M-Mϕ-BCG). The gene expression profiles from these cells were compared. Chemokines highly expressed in M-Mϕs were selected and evaluated for anti-mycobacterial activity and superoxide production. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕ and M-Mϕ, respectively. After stimulation with BCG, three chemokine genes (*Osteopontin* (*SPP1*), *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were highly expressed in M-Mϕ-BCG when compared to those in GM-Mϕ-BCG. A significantly increased resistance to *M. tuberculosis* H37Ra was observed after the stimulation of GM-Mϕ with SPP1 or CXCL7. Superoxide production levels of SPP1- or CXCL7-stimulated GM-Mϕs were higher than those of GM-Mϕs without stimulation. These results indicate that both SPP1 and CXCL7 might have a role in the resistance against mycobacteria, at least in part, through augmenting reactive oxygen intermediate production in Mϕs.

Keywords: GM-CSF, M-CSF, macrophage, microarray

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most important burdens on human health [1]. Both environmental and genetic factors contribute to the development of the disease, which approximates 10% of the infected subjects [2]. Twin studies provided the evidence that human genetic factors could influence the development of tuberculosis [3]. The genetic basis of susceptibility to mycobacteria has been clarified partly by the recent identification of defects in the molecules of the interferon (IFN)-γ-mediated immune pathway, such as IFN-γ receptors 1 and 2 [4,5], interleukin (IL)-12 receptor-β1 [6], IL-12p40 [7] and STAT1 [8]. In addition, linkage and/or association studies have demonstrated many susceptibility genes, such as *HLA* [9], *NRAMP1* [10], *IFN-G* [11], *TNF-A* [12], *IL-10* [12], *IL-1RA* [13], *MBL* [14], *VDR* [15] and *TLR2* [16].

The immune response against mycobacteria is mounted in a complex process. In the host, mycobacteria dwell chiefly within macrophages (Mϕs). Following activation, Mϕs produce a wide range of cytokines/chemokines and activate T cells. IFN-γ secreted by activated T cells and natural killer (NK) cells is one of the principal Mϕ activating factors, and acts as the central cytokine in the control of mycobacterial infection. Activated T cells stimulate anti-mycobacterial machinery in Mϕs, which includes reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [17].

Mϕs that play a pivotal role in the mycobacterial infections are heterogeneous in nature, with different phenotypes and functions. They are derived predominantly from peripheral blood monocytes, and differentiate to specific cells in target tissues. Peripheral blood monocytes need colony-stimulating factors (CSFs) such as granulocyte-macrophage (GM)-CSF or macrophage (M)-CSF for their survival and

differentiation *in vitro*. GM-CSF-induced monocyte-derived macrophage (GM-Mϕ) and M-CSF-induced monocyte-derived macrophage (M-Mϕ) are distinct in their morphology, cell surface antigen expression and function. GM-Mϕ and M-Mϕ show susceptibility and resistance to mycobacteria, respectively [18,19].

To determine novel host resistance or susceptibility genes in mycobacteria infection, we investigated the differences in the gene expression profiles between GM-Mϕ and M-Mϕ with a high-density oligonucleotide microarray containing approximately 30 000 human genes. The expression profiles of each Mϕ subset were analysed with and without the stimulation of bacillus Calmette–Guérin (BCG) (GM- and M-Mϕ-BCG). Our results enlarged the views in the immunological mechanisms against mycobacteria, especially in the roles of several chemokines.

Materials and methods

Mϕ culture

Peripheral blood mononuclear cells (PBMC) were prepared from blood buffy coats of eight different healthy donors separately by density gradient centrifugation using lymphocyte separation medium (Cappel, Aurora, OH, USA). $CD14^+$ monocytes were purified (> 95%) from PBMC using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), with anti-CD14 monoclonal antibody (mAb)-coated microbeads and an FcR blocking reagent (Miltenyi Biotec). $CD14^+$ monocytes were cultured at a concentration of 5×10^4 cells/100 μl in 96-well tissue culture plates or at a concentration of 5×10^5 cells/2 ml in 6-well tissue culture plates with RPMI-1640 (Invitrogen Japan KK, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD, USA), and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml; Sigma-Aldrich, St Louis, MO, USA) in an incubator containing 5% CO_2 at 37°C. Cells were stimulated with GM-CSF (100 ng/ml, PeproTech, London, UK) (GM-Mϕ) or M-CSF (75 ng/ml, PeproTech) (M-Mϕ). Optimal conditions were maintained by refreshing the medium and cytokines every 3 days. After 7 days of culture, a fraction of the cells were stimulated with BCG (10 mg/ml, BCG Tokyo 172; Japan BCG Laboratory, Japan) for 3 h (GM- and M-Mϕ-BCG). During BCG stimulation, a culture medium without antibiotics was used. For the analysis of anti-mycobacterial function and superoxide production, GM-Mϕs were stimulated with or without different concentrations of a chemokine: osteopontin (SPP1) (Biogenesis, Poole, UK: 0·02, 0·25 and 2·5 μg/ml), CXCL7 (Sigma-Aldrich: 0·05, 0·15 and 0·5 μg/ml) or CCL11 (Wako, Osaka, Japan: 0·5, 5 and 50 ng/ml) for another 6 days.

Bacterial preparation and infection to Mϕs

M. tuberculosis H37Ra was grown for 1 week in Middlebrook 7H9 liquid medium (Difco, Detroit, USA) at 37°C and aliquots were frozen at −80°C. In each experiment, an aliquot was thawed and grown in 7H9 medium to mid-exponential growth phase. The culture was sonicated (time: 10 s, output: 1, duty: 80%) (Branson Sonifier 250, CT, USA) to disperse bacilli before the infection. Both types of Mϕ layers were exposed to H37Ra for 3 h in a multiplicity of infection ratio of 1 : 1 in triplicate, washed three times and reincubated in the culture medium (RPMI-1640 plus 10% FBS) with antibiotics. After culture, the medium was removed and sterile phosphate-buffered saline was added to each well. The cells in the bottoms of the wells were scraped with a sterile scraper (Techno Plastic Products AG, Transadingen, Switzerland) and then sonicated as mentioned previously. Serial dilutions of the bacterial suspensions were plated on Middlebrook 7H10 agar plates (Difco). Colonies on the agar plates were counted 3 weeks after inoculation.

RNA isolation

Mϕs were harvested at 7 days after culture with CSF, and after further 24 h with BCG stimulation. Total RNA was extracted using RNA Extraction Kit, Isogen (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. All experiments were performed according to the guidelines of the ethics committee of Kyushu University.

Microarray processing

mRNA was amplified linearly using an Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX, USA). In brief, mRNA (1·5 μg) was reverse transcribed to synthesize complementary DNA (cDNA) using an oligo(dT) primer bearing a T7 RNA polymerase promoter. Second-strand synthesis was carried out to make a transcription template. *In vitro* transcription of the cDNA with incorporation of amino allyl UTP was performed to produce multiple copies of amino allyl-labelled anti-sense RNA (aRNA). After purification, amino allyl-labelled aRNA was reacted with N-hydroxy succinimide esters of Cy3 and Cy5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the Mϕ samples and a standard control, respectively. Uncoupled dye molecules were removed using Micro Bio-Spin P-30 Tris chromatography columns (Bio-rad, Hercales, CA, USA). Cy3- and Cy5-labelled products were mixed together in the same amounts. After the aRNA was fragmented in a buffer containing 40 mM Tris-acetate, 100 mM CH_3COOK and 30 mM $(CH_3COO)_2Mg.4H_2O$ at 94°C for 15 min, the hybridization buffer (5 × SSC, 0·5% SDS, 4 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 10% formamide) was added. The hybridization was performed by incubating 60 μl of the product into three Acegene Human oligo chip 30K slides (Hitachi Software Engineering, Yokohama, Japan). Each slide was rinsed with a solution provided by the manufacturer (Hitachi Software Engineering). Two microarray experiments for each Mϕ subset were conducted, using two

RNA mixtures, each one equally combined from four independent cell cultures.

Signal detection and data analysis

Fluorescence signals for approximately 30 000 spots in slides were detected separately by fluorescent image analyser FLA-8000 (Fuji Film, Tokyo, Japan) for Cy3 and Cy5. Hybridization intensities were processed using Arrayvision software version 6·0 (Imaging Research, Ontario, Canada). Signal and background intensities were determined by the median pixel values. Local background values were determined as the average of four background spots around each gene spot. All spots in the image (for both Cy3 and Cy5 signals) were evaluated for a possibility of dusts, to lower the probability of false data in all experiments. GeneSpring version 6·2. (Silicon genetics, Redwood City, CA, USA) was used for data analysis. According to the GeneSpring instruction, normalization of the data was performed using the 'Lowess method' [20]. Spots with dust, or with signal values of which the Cy5 or Cy3 channels were less than three times of background, were excluded.

*Taq*Man real-time quantitative reverse transcriptase-PCR (qRT-PCR)

The same RNA used in the microarray analysis was employed for qRT-PCR. The cDNA was synthesized from the RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously [21]. PCR primers and the target probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from ABI (Applied Biosystems, Foster City, CA, USA) as a *Taq*Man GAPDH control reagent kit. PCR primers and *Taq*Man probes for *FN1* and *FCGR2B* genes were purchased from ABI as assay reagents (Assays-on-Demand™, Gene Expression Products) with the following numbers: Hs00415006_m1 for *FN1* and Hs00414000_m1 for *FCGR2B*, and used according to the instructions of the manufacturer. The qRT-PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) [22]. To calculate the relative amount of gene expression, the value of each gene expression was divided by that of the internal control, GAPDH. The analysis was carried out in duplicate samples.

Flow cytometry

Flow cytometric analysis was performed using an EPICS XL (Beckman Coulter, Miami, FL, USA). Multi-colour staining was performed by fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-cyanin 5·1 (PC5)-conjugated mAbs against the following markers: HLA-DR, CD14, CD71, CD44, CXCR2 and appropriate controls (Immunotech, Marseille, France).

Superoxide production assay

Superoxide production by Mϕs was determined as described previously [23]. GM-Mϕs were cultured with or without a chemokine for 7 days. After treatment with trypsin (Invitrogen), cells were harvested, washed and resuspended in Hanks's balance salt solution (HBSS) (Invitrogen) (5×10^4/0·5 ml). They were stimulated with antibody-opsonized zymosan (1 mg/ml, Sigma-Aldrich) at 37°C, and the reaction was terminated by the addition of SOD (50 μg/ml, Sigma-Aldrich). The chemiluminescence was counted for 35 min with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) using a luminometer (Auto Lumat LB953; EG & G Berthold).

Statistical analysis

Data in qRT-PCR, colony forming unit (CFU) counting and superoxide production assays were assessed by Student's *t*-test using SPSS software version 11.

Online supplemental material

The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession number GSE3408.

Results

Characterization of GM- and M-Mϕ

After culture with GM- and M-CSF for 1 week, peripheral blood monocytes differentiated into GM-Mϕ and M-Mϕ, respectively. These two types of Mϕs showed distinct features in their phenotypes and functions. Although both Mϕs expressed HLA-DR, GM-Mϕs strongly expressed CD71 and M-Mϕs were strongly positive for CD14 (Fig. 1a). M-Mϕs showed a higher resistance to *M. tuberculosis* H37Ra and a higher superoxide production than GM-Mϕs (Figs 2 and 3B), as reported by Akagawa [18].

Comparison of the constitutive gene expression levels between GM- and M-Mϕ, by microarray and quantitative PCR

To identify the molecules involved in the functional differences between GM- and M-Mϕ, we compared the constitutive gene expression profiles in each Mϕ using microarray (Fig. 1b). The 10 most up-regulated genes, which are a result of comparison between these Mϕ, are listed in Table 1. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕs and M-Mϕs, respectively, both of which encode proteins that potentially interact with *M. tuberculosis*. These microarray data were confirmed by qRT-PCR. As shown in Fig. 1c, the

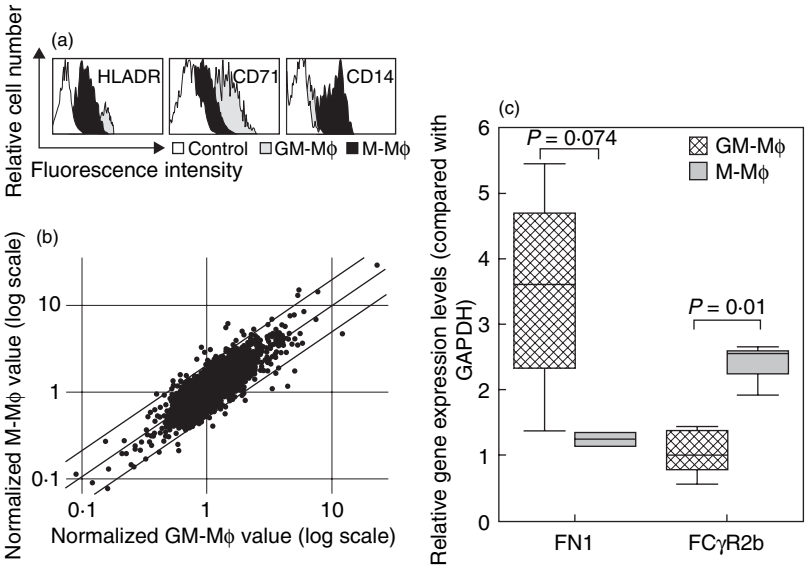

Fig. 1. (a) Phenotypic characteristics of GM-Mϕ and M-Mϕ, generated from human CD14$^+$ monocyte. (b) The scatter-plot between two types of Mϕs in their constitutive states. Each spot is the representative of normalized data in logarithmal scale from the average of two values from each cell type. (c) The qRT-PCR analysis for *FN1* and *FCGR2b* gene expression levels, which were the most up-regulated genes in each Mϕ (Table 1).

expression levels of *FN1* and *FCGR2B* genes were increased in GM-Mϕs and M-Mϕs, respectively, although the difference of *FN1* expression levels did not reach the statistical significance.

Comparison of the gene expression levels between GM- and M-Mϕ with and without BCG exposure by microarray

When we compared the gene expression profiles between GM- and M-Mϕ with and without BCG, *IL-1B* showed the highest expression among BCG-stimulated genes in both Mϕs (Table 2). Also, *SOD2* gene was listed among highly expressed genes in both Mϕs after BCG stimulation (Table 2). Then, we compared the gene expression profiles between GM-Mϕ-BCG and M-Mϕ-BCG (Table 3). *Osteopontin* (*SPP1*) was the most up-regulated gene in M-Mϕ-BCG compared with GM-Mϕ-BCG, suggesting the protective role of *SPP1* in M-Mϕ against mycobacteria. Analysis of genes according to the gene ontology (GeneSpring software) revealed that four HLA-related genes were included in the 10 most up-regulated genes in GM-Mϕ-BCG compared with M-Mϕ-BCG, while three chemokine genes (*SPP1*, *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG.

Effects of 3 chemokines on the growth of *M. tuberculosis* H37Ra in GM-Mϕ

We selected three chemokine genes (*SPP1*, *CXCL7* and *CCL11*) as the candidate genes that potentially contribute to the protective function of M-Mϕs. To clarify the possible effects of these chemokines on the resistance of M-Mϕs against *M. tuberculosis*, GM-Mϕs were cultured in the presence of different concentrations of one of these chemokines for 6 days, and their protective abilities against *M. tuberculosis* H37Ra were evaluated (Fig. 2). SPP1 or CXCL7-stimulated GM-Mϕs significantly inhibited the growth of H37Ra 6 days after the infection with the organism, while CCL11 stimulation had no effects on it (Fig. 2).

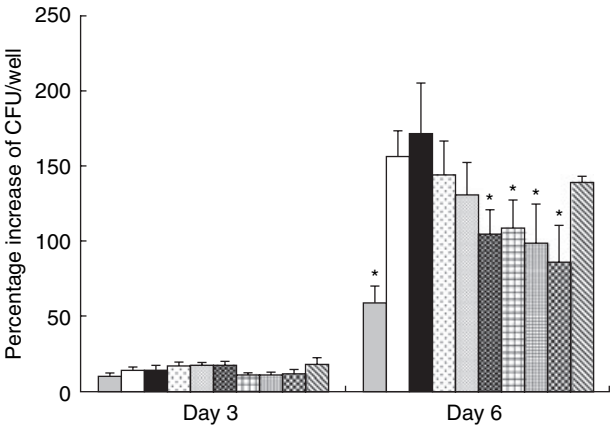

Fig. 2. Inhibition of *M. tuberculosis* H37Ra growth in Mϕs by SPP1 and CXCL7. Mtb colony-forming unit (CFU) assay was performed on days 3 and 6 after H37Ra-Mϕ exposure. M-Mϕ ▩, GM-Mϕ with granulocyte macrophage-colony stimulating factor (GM-CSF) only □ and GM-Mϕ without any cytokines ■ were cultured as controls. GM-Mϕs were stimulated with three different chemokines: for SPP1, 0.02 ▩, 0.25 ▩ and 2.5 ▩ μg/ml; for CXCL7, 0.05 ▩, 0.15 ▩ and 0.5 ▩ μg/ml. Data for CCL11 ▩ are shown only for the results using the highest concentration in the experiments (see Methods for details). Mean values and standard deviations of triplicates are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

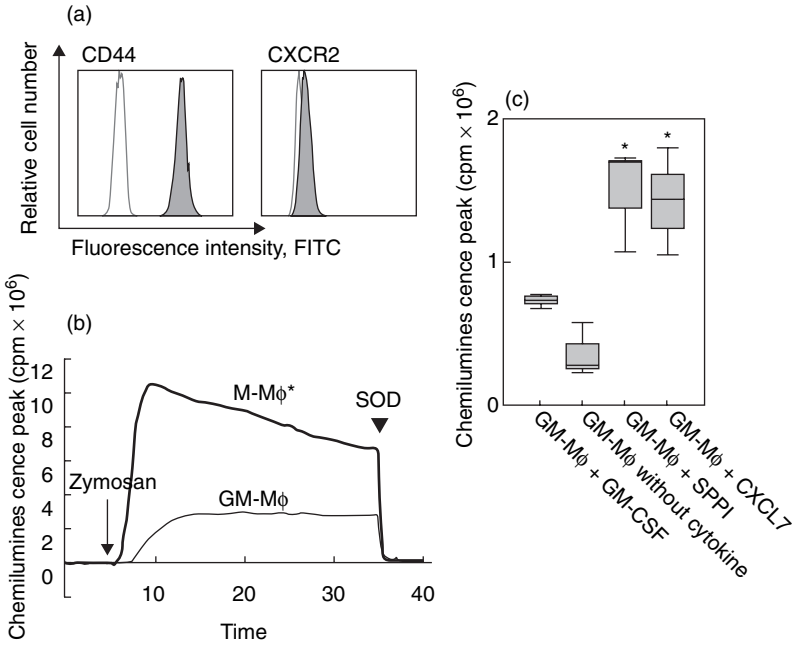

Fig. 3. Increased superoxide production from GM-Mϕs after the stimulation with SPP1 and CXCL7. (a) Receptor expression for SPP1 (CD44) and CXCL7 (CXCR2) on the surface of GM-Mϕ, determined by flow cytometry. (b) Superoxide production by the Mϕs measured by a change of chemiluminescence. The Mϕs (5.0×10^4 cells) were stimulated with antibody-opsonized zymosan (arrow), and the chemiluminescence change was monitored continuously for 40 min with DIOGENES. SOD was added to terminate the reaction (arrowhead). (c) Superoxide production from GM-Mϕs with or without chemokine stimulation. Representatives of three independent experiments are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

Table 1. Gene expression profiles of GM-Mϕ and M-Mϕ. (a) Genes which up-regulated in GM-Mϕ (top 10) compared to those in M-Mϕ; (b) genes which up-regulated in M-Mϕ (top 10) compared to those in GM-Mϕ.

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	FN1	ENSG00000115414	Fibronectin 1	3·81
2	Unknown	ENSG00000079310	ensembl prediction	3·12
3	Unknown	ENSG00000085063	ensembl prediction	2·93
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor; scya7	2·86
5	AD 158	AL136919–1	Hypothetical protein; dkfzp586j1119	2·83
6	ARPC2	AF116702–1	pro2446	2·56
7	KIAA1838	XM_035688–1	Hypothetical protein xp_035688; loc94580	2·53
8	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	2·53
9	HBD	NM_000519–1	Haemoglobin, delta	2·35
10	ABCC3	AF085692–1	Multidrug resistance-associated protein 3b; mrp3	2·17
(b)				
1	FCGR2B	NM_004001–1	fc fragment of igg, low affinity iib, receptor for (cd32)	4·14
2	Unknown	ENSG00000024862	ensembl prediction	3·90
3	MHC Ag	L34093–1	MHC class ii hla-dq-alpha chain	3·64
4	Unknown	ENSG00000126461	ensembl prediction	3·01
5	C15orf12	AK001830–1	cDNA flj10968 fis clone place1000863 moderately similar to putative mitochondrial 40 s ribosomal protein yhr148w	2·90
6	Unknown	AC069384·3.87217·105230·1	ensembl genscan prediction	2·85
7	Unknown	AP002767·1.52387·73825·2	ensembl genscan prediction	2·84
8	MMP9	NM_004994–1	Matrix metalloproteinase 9 preproprotein	2·82
9	TM7SF1	NM_003272–1	Transmembrane 7 superfamily member 1 (up-regulated in kidney)	2·70
10	Unknown	AC003958·1.1·127834·1	ensembl genscan prediction	2·50

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 2. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in GM-Mϕ (a), and in M-Mϕ-BCG compared to those in M-Mϕ (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	IL1B	NM_000576–1	Interleukin 1, beta	46·66
2	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	13·62
3	MT1G	XM_048213–1	Metallothionein 1 g	10·71
4	CLECSF9	AB024718–1	Macrophage c-type lectin mincle; mincle	6·28
5	CCL1	M57502–1	Secreted protein i-309; scya1	5·96
6	BCL2A1	NM_004049–1	bcl2-related protein a1	5·85
7	AKR1C3	L43839–1	3-alpha-hydroxysteroid dehydrogenase; 3alpha-hsd	5·72
8	Unknown	AC005027·2.1·157073·2	ensembl genscan prediction	5·62
9	GRO1	NM_001511–1	Gro1 oncogene (melanoma growth stimulating activity, alpha)	5·61
10	MT1H	NM_005951–1	Metallothionein 1 h	5·38
(b)				
1	IL1B	NM_000576–1	Interleukin 1, beta	74·62
2	CCL20	NM_004591–1	Small inducible cytokine subfamily a (cys-cys), member 20	59·71
3	ARHGEF1	NM_004706–1	Rho guanine nucleotide exchange factor 1	56·29
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor	27·49
5	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	25·94
6	IL8	NM_000584–1	Interleukin 8	17·98
7	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	16·33
8	SERPINB2	NM_002575–1	Serine (or cysteine) proteinase inhibitor, clade b (ovalbumin), member 2	15·81
9	TNFAIP6	NM_007115–1	Tumour necrosis factor, alpha-induced protein 6	15·29
10	H1F2	NM_005319–1	h1 histone family, member 2	14·06

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 3. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in M-Mϕ-BCG (a) and in M-Mϕ-BCG compared to those in GM-Mϕ-BCG (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	HLA-DRA	NM_019111–1	Major histocompatibility complex, class ii	6·86
2	HLA-DMA	NM_006120	Major histocompatibility complex	6·79
3	ID2	NM_002166–1	Inhibitor of dna binding 2, dominant negativ ehelix-loop-helix protein	6·06
4	HLA-DP	S66883–1	Major histocompatibility complex class ii antigen beta chain	5·81
5	HLA-DQA	L34093–1	MHC class II hla-dq-alpha chain	5·30
6	PRG1	NM_002727–1	Proteoglycan 1, secretory granule	4·88
7	RGC32	NM_014059–1	rgc32 protein	4·72
8	TNFSF13B	NM_006573–1	Tumour necrosis factor (ligand) superfamily, member 13b	4·44
9	Unknown	AC026785·3.13728·33112·2	ensembl genscan prediction	4·31
10	Unknown	XM_016170–1	Hypothetical protein xp_016170; loc88021	4·17
(b)				
1	SPP1	NM_000582–1	Secreted phosphoprotein 1 (osteopontin)	22·37
2	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	19·20
3	CXCL7	NM_002704–1	Pro-platelet basic protein (NAP2, SCYB7, CTAP3, PPBP)	15·08
4	FLJ20033	NM_017629–1	Hypothetical protein flj20033	9·43
5	LOC64182	NM_022359–1	Similar to rat myomegalin	8·82
6	Unknown	BC000845–1	Unknown (protein for image:3457769)	7·01
7	C8B	NM_000066–1	Complement component 8, beta polypeptide	6·78
8	Unknown	BC006174–1	Unknown (protein for image:4053618)	6·73
9	STK4	NM_006282–1	Serine/threonine kinase 4	6·62
10	CCL11	NM_002986–1	Small inducible cytokine subfamily a (cys-cys), member 11 (eotaxin)	6·60

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Effects of SPP1 and CXCL7 on GM-Mϕs

The expression of cell surface receptors for SPP1 (CD44) and CXCL7 (CXCR2) on GM-Mϕ were confirmed (Fig. 3a). To investigate further the mechanism of increased resistance of SPP1- or CXCL7-stimulated GM-Mϕs against *M. tuberculosis*, superoxide production by Mϕs was investigated. After the stimulation with antibody-opsonized zymosan, M-Mϕs produced a higher amount of superoxide than GM-Mϕ (Fig. 3b). Superoxide production by GM-Mϕs was significantly enhanced after the stimulation with SPP1 or CXCL7 (Fig. 3c). The reaction was terminated by SOD, which inhibits cytochrome *c* reduction (Fig. 3b). These results suggested that increased superoxide production was one of the mechanisms of increased resistance of SPP1- or CXCL7-stimulated Mϕs against *M. tuberculosis*.

Discussion

GM-Mϕs and M-Mϕs show distinct features, although both Mϕs come from the same origin ($CD14^+$ PMNC). It has been reported that GM-Mϕs show a susceptibility to *M. tuberculosis*, while M-Mϕs have a resistance to *M. tuberculosis* with a greater FcγR-mediated phagocytic capacity and a higher capability of ROI production [18].

In our experiment, *FN1* that encode fibronectin (FN) was expressed predominantly in GM-Mϕs compared with M-Mϕs (Table 1a). FN is expressed constitutively in the lung [24]. *M. tuberculosis* binds to the FN by FN attachment proteins on the surface of *M. tuberculosis*. After fibronectin opsonization, *M. tuberculosis* can be phagocytosed easily via complement receptors and integrin receptors [25,26]. Therefore, it is possible that increased FN production led to the enhanced *M. tuberculosis* load into the cells. On the other hand, *FCGR2B* was highly expressed in M-Mϕs compared with GM-Mϕs (Table 1b). It was reported that *FCGR2B* expression levels were increased in peripheral blood monocytes in patients with tuberculosis compared with healthy controls by microarray analysis [27]. In contrast to FcγR1, FcγR2a and FcγR3, FcγR2b is an inhibitory receptor that does not contain immunoreceptor tyrosine-based activation motifs (ITAM) [28]. Therefore, FcγR2b seems to modulate inflammatory responses and inhibits phagocytosis of Mϕs [25]. Further analysis for the role of FcγR2b in *M. tuberculosis* infection would be necessary.

IL-1B and *SOD2* expression levels were up-regulated in both types of Mϕs after the stimulation with BCG (Table 2), which was consistent with the previous report [29]. IL-1β is produced by activated Mϕs following *M. tuberculosis* infections, and is an important mediator of cellular anti-mycobacterial activities [30]. The importance of IL-1 for the generation of protective immunity against mycobacterial infection was clarified using *IL-1*-knock-out mice [31]. Cell wall components of *M. tuberculosis* are known to induce *IL-1B* expression in human monocytes and macrophages [32]. In addition, increased *IL-1B* gene expression was observed in bronchoalveolar lavage cells from tuberculosis patients compared with those from healthy individuals [33]. Mϕ stimulation triggers an oxidative burst and the generation of superoxide anions (O_2^-) and other ROI in Mϕs [34]. SOD2 may play a role in protection of Mϕs against ROI in *M. tuberculosis*-infected Mϕs. None the less, the protective function of M-Mϕs against *M. tuberculosis* in contrast to GM-Mϕs do not seem to be obtained solely by the increased expression of these molecules, because these molecules were also highly expressed in GM-Mϕs.

Three chemokines were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG (Table 3b). In *M. tuberculosis* infections, chemokines contribute to the recruitment of other immune cells, especially of T cells, and the formation and maintenance of granuloma [35]. We also found that GM-Mϕs, which were stimulated with SPP1 and CXCL7, were more bacteriostatic to *M. tuberculosis* than unstimulated GM-Mϕs (Fig. 2). This is the first description that these two chemokines played protective roles against *M. tuberculosis* in humans. After BCG stimulation, the ratio of *SPP1* expression was highest in M-Mϕ compared with GM-Mϕ (Table 3b). SPP1 is a multi-functional protein that is expressed in both alveolar and peritoneal Mϕs [36]. *SPP1* knock-out mice were more susceptible to *M. tuberculosis* with small and immature granuloma formation in their lungs [37]. *M. tuberculosis* infection of primary human alveolar macrophages causes a substantial increase in *SPP1* gene expression [38]. Many investigators recognize SPP1 as a proinflammatory cytokine, which causes cellular adhesion of inflammatory leucocytes. Furthermore, SPP1 promotes chemotaxis and adhesion of human peripheral blood T cells [39] and enhances their IFN-γ production [40]. It is worthy of notice that its expression can be used as a prognostic marker in patients with *M. tuberculosis* infection [41].

CXCL7 is a cleavage product of platelet basic protein with a length of 70 amino acids [42]. In neutrophils, CXCL7 induces an increase of cytosolic calcium concentration, chemotaxis, exocytosis, production of ROI, degranulation and elastase release [42,43]. Although there is a recent report showing that CXCL7 can modulate the synthesis of IL-12 in Mϕs [44], the role of CXCL7 in Mϕs has not been well determined.

In addition, we demonstrated that SPP1 and CXCL7 facilitated the production of superoxide in GM-Mϕs after the stimulation with antibody-opsonized zymosan particles. The high production of ROI in Mϕs following *M. tuberculosis* infections may be compatible with the high expression of SOD2 in microarray results from BCG-stimulated Mϕs, which may play an important role in preventing Mϕ damages induced by ROI. On the other hand, RNI production in GM-Mϕs was not increased after the stimulation with SPP1 or CXCL7 (data not shown). Immunologically activated Mϕs can generate superoxide anion and other ROI [34]. Mice

deficient in the NADPH oxidase complex have a susceptibility to *M. tuberculosis* infection [45]. In humans, patients with chronic granulomatous disease are more susceptible to *M. tuberculosis* [46]. Although our data showed that SPP1 and CXCL7 may play an important role against *M. tuberculosis*, possibly through the up-regulation of superoxide production in Mϕs, it is possible that their anti-mycobacterial effect could be based on other mechanisms.

In summary, our data showed that Mϕs can secret a large array of molecules that induce host defence after the exposure to BCG. Among them, we found new roles of SPP1 and CXCL7 against *M. tuberculosis* in Mϕs. Further analysis of these molecules using iRNA will confirm the role of these chemokines in *M. tuberculosis* infection more clearly.

Acknowledgements

This work was supported by the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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