Article

Proteomic Analysis of Macrophages: A Potential Way to Identify Novel Proteins Associated with Activation of Macrophages for Tumor Cell Killing

Lingbing Zhang[1], Haoxuan Zhu[1, 3], Yanni Lun[1], Dongmei Yan[1], Leyang Yu[1], Bairong Du[1] and Xun Zhu[1, 2, 4]

One major mechanism through which macrophages effectively kill tumor cells requires cell to cell contact, indicating that certain molecules expressed on cell surface of activated macrophages may mediate the tumoricidal capability. Tumor necrosis factor (TNF) and nitric oxide (NO) are the two classical mediators of tumor cell death. However, evidence of discrepancy is accumulating indicating these known mediators do not appear to account for the broad and potent tumoricidal activity of macrophages. To obtain a full repertoire of tumoricidal activation-associated membrane proteins, we combined one-dimensional SDS-PAGE with capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using this technique, we identified 454 activated macrophage specifically expressed proteins with extremely high confidence, including most known activation markers of macrophages, such as NO synthase (iNOS), Ym1, cyclooxygenase, etc. Membrane bound TNF-α was also identified on activated macrophages. However, it was also detected on thioglycolate elicited macrophages, indicating this molecule may not play a key role in conjugation-dependent tumor cell killing. In contrast, although NO has not been assigned as an effector molecule of conjugation-dependent tumoricidal pathway, iNOS was identified from membrane fraction of activated macrophages, suggesting NO may be involved in conjugation-dependent tumoricidal mechanism, because iNOS association with plasma membrane is ideally suited to deliver NO directly into the contacted tumor cells. This research provides not only new insights into macrophage conjugation-dependent tumoricidal mechanisms, but also a valuable data set of macrophage activation associated membrane proteins, thus providing better understanding of the functional mechanisms of macrophages in anti-tumor and other biological processes. ***Cellular & Molecular Immunology*****. 2007;4(5):359-367.**

Key Words: macrophage, membrane protein, mass spectrometry, macrophage activation

Introduction

The role of macrophages in the defense against tumors has been investigated extensively over the last decades. Compelling evidence has suggested that activated macrophages have the capacity to recognize and destroy tumor cells (1, 2). Also, the mechanisms by which macrophages kill tumor cells have been studied in a large number of *in vitro* experiments (3-6). One major mechanism through which macrophages effectively kill tumor cells requires cell to cell contact (3, 6), indicating that certain molecules expressed on cell surface of activated macrophages may mediate the tumoricidal capability. Tumor necrosis factor (TNF) and nitric oxide (NO) produced by activated macrophages are the two classical mediators of tumor cell death (7-9). However, evidence of discrepancy is accumulating indicating these two mediators do not appear to account for the broad and potent tumoricidal activity of activated macrophages (6-11). These conflicting observations may be reasoned by the pleiotropic nature and ill-defined tumoricidal mechanisms of macrophages. Specific macrophage tumor cell killing pathway can be modulated depending on the type of activation stimuli (5, 6, 10-12).

Intraperitoneal infection of mice with the intracellular bacterium *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) stimulates a strong classic Th1 response which has

[1]Department of Immunology, School of Basic Medical Sciences, Jilin University, Changchun 130021, China;

[2]Changchun Botai Medicine and Biological Technology Company Limited, Changchun 130012, China;

[3]College of Life Science, Jilin University, Changchun 130021, China;

[4]Corresponding to: Dr. Xun Zhu, Department of Immunology, School of Basic Medicine, Jilin University, Changchun 130021, China. Tel: +86-431-8508-5050, Fax: +86-431-8508-5050, E-mail: zxunzhux@vip.sohu.com

Received Sep 16, 2007. Accepted Oct 22, 2007.

been a model for study of macrophage-mediated tumoricidal mechanisms (3, 12-14). Previous studies of tumoricidal mechanism of BCG-activated macrophages suggested that membrane TNF and NO are the two predominant effector pathways (12, 15). However, our recent study demonstrated that BCG-activated macrophages must possess effector molecules other than the above mentioned two. This finding prompted us to identify novel molecules responsible for the contact-dependent tumoricidal activity of BCG-activated macrophages. Recently the studies of macrophage contact-dependent tumoricidal mechanisms have been carried out mainly with antibody blocking approach. Obviously, it is impossible to find molecules that have not yet been identified by previous studies with this approach. Therefore, new approach to identify overall pattern of membrane proteins expressed on activated macrophages is needed.

Previous studies have tried to identify new molecular markers of macrophage activation for tumor cell killing through identification of changes in the total cellular proteins by two-dimensional gel electrophoresis (2-DE) (16, 17). However, the identified molecules have not been associated with macrophage killing function in a cause-and-effect way (18). In addition, recent proteomics studies have demonstrated that membrane proteins are extremely under-represented on 2-D-gels and the analysis of very hydrophobic proteins such as integral membrane proteins remains a challenge for current proteomics approaches (19-23). Two factors contribute to the difficulty in analyzing membrane proteins. First, many integral membrane proteins are highly hydrophobic, which results in low solubility and a tendency to aggregate and precipitate in aqueous media. Second, many membrane proteins possess basic pIs and are expressed in low copy numbers (24, 25). Although some progresses such as the introduction of thiourea and zwitterionic detergent have been made towards improving the solubility and separation of membrane proteins (26, 27), the best strategy for membrane protein analysis is still the combination of one-dimensional SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (28, 29).

In this report, we analyzed the membrane proteomes of BCG infection-activated and thioglycolate-elicited macrophages respectively with the combination of SDS-PAGE and LC-MS/MS. Among the two lists of over 1,000 identified proteins, 454 proteins were uniquely detected in the membrane fraction of BCG infection-activated macrophages, including most of the previously identified macrophage activation markers. Furthermore, a significant number of the proteins identified are of unknown function and identity. Results of this research not only evoke new scientific insights into macrophage activation and tumoricidal properties, but also provide a valuable repertoire of macrophage membrane proteins.

Materials and Methods

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was obtained from Sigma (Saint Louis, MI). Recombinant murine interferon-γ (IFN-γ) was purchased from Peprotech Inc. (Rocky Hill, NJ). BCG vaccines were obtained from Changchun Institute of Biological Products. 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenylterazolium bromide (MTT) was purchased from Sigma. FITC-conjugated rat anti-mouse CD11b monoclonal antibody was obtained from BD PharMingen (San Diego, CA). Biotinylated anti-mouse TNF-α antibody was obtained from R&D Systems, Inc. Av-FITC was obtained from BD PharMingen. All cell culture and supplements were obtained from Invitrogen Corporation, unless stated otherwise.

Mice and cell line

Female C57BL/6 mice at 8-12 weeks of age were obtained from Animal Division of Jilin University and maintained under a pathogen-free condition. All experimental procedures were approved by the university committee on the use of live animals in research. MCA207 cell line was obtained from Dr. Kangla Tsung (Stanford University, CA). MCA207 is a methylcholanthrene-induced transplantable tumor in C57BL/6 mice. The cell was maintained in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin and 100 IU/ml penicillin.

Cell preparation

Mice weighting more than 20 g were primed by *i.p.* injection with 2 mg BCG. At days 10 and day 12 after the first infection, mice were boosted with another twice *i.p.* injection of 1 mg BCG. Three days after the last *i.p.* injection, mice were killed and peritoneal cells were collected by *i.p.* injection 10 ml RPMI1640 medium. Peritoneal fluid was withdrawn through a small incision in the anterior abdominal wall. Peritoneal exudate cells were enriched for macrophage using the method of Kumagai et al. (30). Briefly, peritoneal fluid from each mouse was centrifuged at 300× g for 5 min at 4°C and resuspended in RPMI1640 medium supplemented with 5% FCS. The cells were plated on 35 mm cell culture dish (Corning, New York, NY) that had been previously blocked with FCS. Nonadherent cells were removed by washing 5 times with 5 ml warm phosphate buffered saline (PBS) after incubation for 2 h at 37°C in 5% CO_2. Adherent cells were scrapped using a cell scrapper (Corning, New York, NY). Cells from 8 dishes (mice) were pooled. The purity of the isolated cells was analyzed by flow cytometry. The cells were washed once with PBS and used for subsequent experiments.

Cytotoxicity assay

BCG infection-activated macrophages were obtained as described above. Thioglycolate induced peritoneal macrophages were harvested from mice 3 days after the mice had been given 2 ml 3% thioglycolate medium *i.p.* Purified macrophages were fixed using 1% paraformaldehyde in PBS at room temperature for 20 min. For cytotoxicity assay, the fixed effector cells were harvested and washed twice with culture medium and suspended in medium at a concentration of 5×10^6 cells/ml. Effector cells (0.1 ml) were added to

triplicating wells in 96-well, flat-bottom plates containing 1 × 10^4 MCA207 target cells added 10-30 min before. Control wells contained effector cells only. Plates were incubated at 37°C for 48-60 hours. Next, the nonadherent effector cells and dead tumor cells were removed through gentle washing of the wells with medium for 3 times. The number of viable tumor cells was determined by a method proposed by Mosman (31). Briefly, 10 μl of PBS containing dissolved MTT (5 mg/ml) were added into each well. Following incubation of the tumor cells with MTT for 4 h, the plate was centrifuged, medium was carefully removed and the blue-dark formazan was dissolved with dimethylsulphoxide. The absorbance was recorded directly after dissolving the formazan using a microplate reader (Model 550, Bio-RAD) at a wavelength of 570 nm. The cytotoxicity was calculated with formula as follows: % cytotoxicity = [1 - (absorbance at 570 nm of target + effector cells) / (absorbance at 570 nm of target cells only)] × 100

Flow cytometry analysis

Macrophages were collected as described above. For each analysis, 5 × 10^5 cells were stained with FITC-conjugated antibody specific for CD11b and TNF-α. Isotype-matched irrelevant antibodies were used as control. The specific antibodies used were rat anti-mouse CD11b (clone M1/70) and rat anti-mouse TNF-α. Antibody-stained cells were analyzed by flow cytometry (FACStar; Becton Dickinson).

Macrophage membrane protein preparation

Membrane proteins from 50 mg frozen macrophage pellet were extracted using the ProteoExtract™ Native Membrane Protein Extraction Kit (Calbiochem, USA), following the instruction protocol of the manufacturer. Protein concentration was determined by BCA Assay kit (BIOS, Beijing), and 150 μg of protein was applied on a 12% bis-Tris SDS gel. After visualization by Coomassie staining, each gel strip was cut into 8 pieces of equal size and subjected to in-gel tryptic digestion essentially as previously described (32).

Liquid chromatography/tandem mass spectrometry and data analysis

The extracted peptides from each gel piece were analyzed using an LCQ Deca XP plus system (Thermo Finnigan, San Jose, CA, USA). HPLC separation was performed with a capillary LC pump. The flow rate of the pump was 200 ml/min and was about 2 ml/min after split. The mobile phases used for reverse phase were 0.1% formic acid in water, pH 3.0 (A), and 0.1% formic acid in ACN (B). Peptides were eluted using 0 to 80% linear gradient of solvent B in 60 min. An ESI ITmass spectrometer (LCQ Deca XP, Thermo Finnigan) was used for peptide detection. The positive ion mode was employed and the spray voltage was set at 3.2 kV. The spray temperature was set at 170°C for peptides. Collision energy is automatically set by the LCQ Deca XP system. After acquisition of a full scan mass spectrum, three MS/MS scans were acquired for the next three most intense ions using dynamic exclusion. Peptides and proteins were identified using Bioworks Browser3.1 SR1 ALPHA7 software (Thermo Finnigan), which uses the MS and MS/MS spectra of peptide ions to search against IPI mouse protein database (V3.15.1) with the following search parameters: cleavage enzyme, trypsin (KR); fixed modification, carbamidomethyl (Cysteine); variable modifications, oxidation (Methionine); mass tolerance for precursor ions, 3.0000; mass tolerance for fragment ions, 0.0000. The protein identification criteria that we used was based on Delta CN (≥ 0.1) and Xcorr (one charge ≥ 1.9, two charges ≥ 2.2, three charges ≥ 3.75). Protein identification results were extracted from SEQUEST out file with the in-house software BuildSummary. Sub-cellular classifications were performed with GOA (http://www.ebi.ac.uk/GOA/) according to the accession number of proteins in Uniprot.

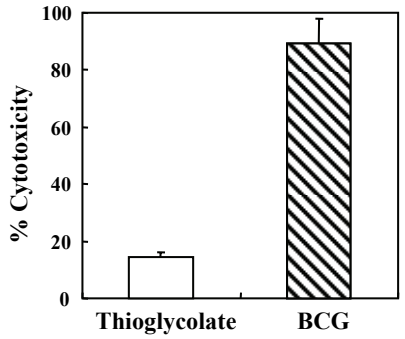

Figure 1. *In vitro* cytotoxicity of paraformaldehyde-fixed macrophages activated with BCG.

Results

Membrane bound TNF-α independent tumoricidal capacity of BCG activated macrophages

In order to focus on the contact-dependent tumoricidal function and exclude any secretion of cytotoxic factors, the cytotoxicity assays were carried out using paraformaldehyde fixed macrophages as effectors. As the experiment in Figure 1 indicates macrophages activated by BCG *in vivo* possess potent MCA207 cells killing ability at E:T ratio of 50:1. In contrast, inflammatory macrophages induced by thioglycolate did not show cytotoxicity in these assays. Previous studies

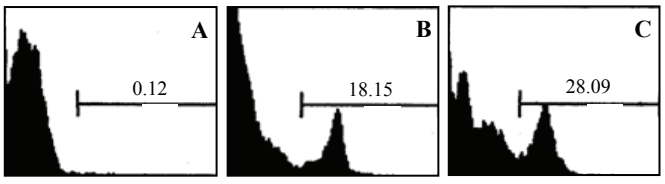

Figure 2. TNF-α expression level on the surface of BCG *in vivo* activated macrophages analyzed by flow cytometry. (A) Isotype stained control; (B) BCG activated macrophages; (C) thioglycolate elicited macrophages. The number indicated the positive percentage.

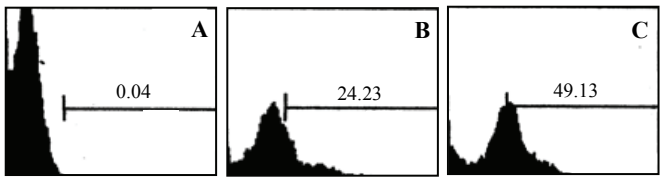

Figure 3. TNF-α expression level on the surface of LPS *in vitro* treated macrophages analyzed by flow cytometry. (A) Isotype stained control; (B) untreated macrophages; (C) LPS treated macrophages. The number indicated the positive percentage.

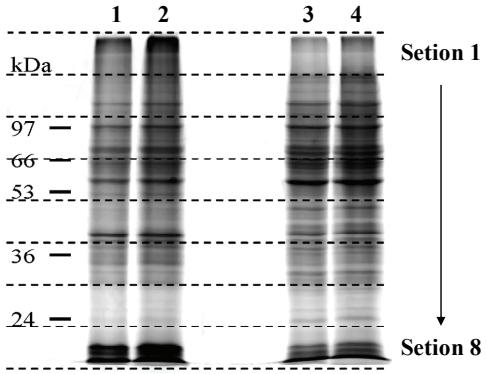

Figure 4. One-dimensional SDS-PAGE separation of macrophage membrane proteins. Proteins were separated on 12% Tris-Bis gel and stained to enable protein identification. Eight equally spaced sections were excised and subsequently used for downstream analysis. Lanes 1 and 2, membrane proteins from BCG activated macrophages; Lanes 3 and 4, membrane proteins isolated from thioglycolate elicited macrophages.

demonstrated the expression of membrane form of TNF-α may explain why chemically fixed macrophages remain cytotoxic to some types of tumor cells (12). Therefore, we analyzed the expression level of TNF-α on macrophage. To our surprise, BCG activation did not increase the expression level of TNF-α compared with thioglycolate elicited macrophages (Figure 2). As a positive control, LPS stimulation *in vitro* can strongly enhance the expression of membrane bound TNF-α (Figure 3).

High-throughput proteomic analysis of macrophage membrane proteomes

We applied cutting edge proteomics techniques to obtain valuable information about membrane protein expression patterns in macrophages under different conditions. Purified membrane proteins were isolated from BCG-activated and thioglycolate elicited mouse peritoneal macrophages and

Table 1. List of known membrane proteins identified by this study

IPI	Name	PepCount	Cover Percent	Sequence	Charge	Xcorr
IPI00120674.2	CD11b	54	22.00%			
IPI00320605.3	CD18	53	39.82%			
IPI00125726	NOS	33	19.49%			
IPI00157508	Ym1	22	22.36%			
IPI00343568.2	CD180	17	25.72%			
IPI00469218.1	CD107a	13	15.48%			
IPI00331214.4	CD36	11	23.35%			
IPI00126186.1	CD206	9	5.43%			
IPI00133082.2	CD177	8	8.32%			
IPI00222968.1	CD107b	6	10.12%			
IPI00132286.1	CD11a	6	6.53%			
IPI00114641.2	CD98	5	10.90%			
IPI00308990.3	CD14	4	13.93%			
IPI00132474.3	CD29	4	4.14%			
IPI00323053.4	CD45	3	2.79%			
IPI00266149.2	CD44	3	4.05%			
IPI00342691.2	TLR	3	5.55%			
IPI00123957.4	CD97	2	2.35%			
IPI00128152.1	CD243		1.02%	KAGAVAEEVLAAIR.T	2	2.6168
IPI00125199.1	CD191		3.38%	K.WLPFLSVDQLER.T	2	2.4599
IPI00129253.2	CD205		0.87%	K.FVSRLMRENYNITMR.V	2	2.2163
IPI00649263.1	CD300		7.27%	K.AAGPPSEQAQSLEGDLCYADLSLK.Q	2	3.7648
IPI00122411.1	F4/80		3.01%	K.SAPVSLQSAATSVSLVLEQATTWFELSK. E	2	3.4332

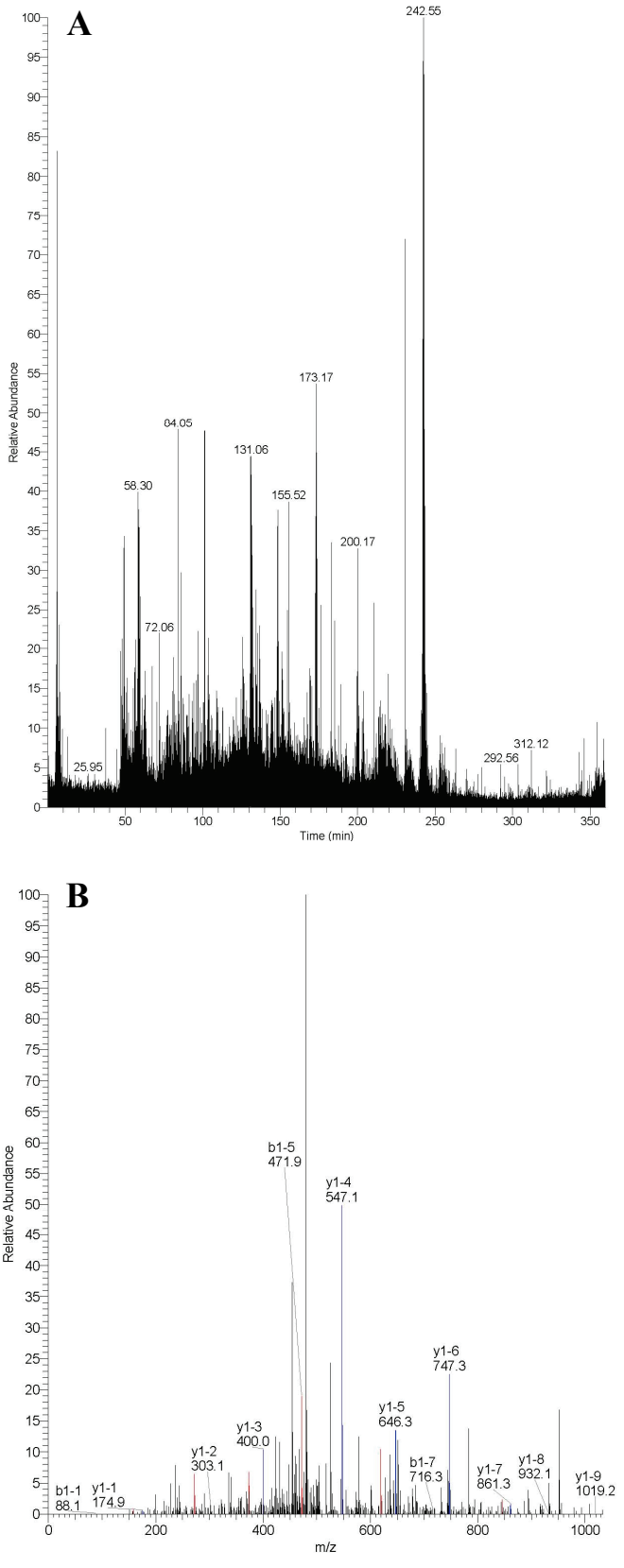

Figure 5. Typical HPLC/MS/MS analysis. (A) Total ion current chromatogram of capillary-HPLC/MS/MS of the tryptic peptides from gel slice 2 in SDS-PAGE; (B) MS/MS spectrum of peptide SAITVFPQR, unique to iNOS.

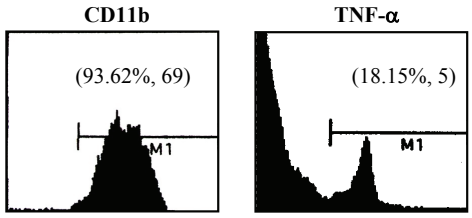

Figure 6. Expression of surface proteins on macrophages. Flow cytometric analysis of macrophages for expression of CD11b and TNF-α. Numbers in upper right corner indicated the expression level of the molecules and the number of detected peptides derived from the molecule.

separated by one-dimensional SDS-PAGE (Figure 4). The proteins present in each gel slice were subjected to trypsinolysis, and the resulting peptide fragments were detected using the LCQ Deca XP system (Figure 5). Using the criteria mentioned above, we obtained a list of over 1,000 proteins identified in each of the two populations of macrophages respectively. To analyze the available information about the identified membrane proteins manually, many of the identified proteins were found to be known markers of peritoneal macrophages. These included CD11b, F4/80, CD14, CD86, CD18, CD16, CD44 and Toll-like receptor-1. Other CD antigens detected were CD243, CD98, CD107a, CD107b, CD191, CD36, CD97, CD205, CD206, CD177, CD180, CD300, CD45, and CD29 (Table 1). Several members of the integrins, integrin αM, integrin β-2, integrin α5 and integrin β-1 were also detected. In addition, a significant number of the identified proteins were "hypothetical proteins" that have not yet been reported to be translated into proteins (33). Further analyses of these proteins using bioinformatics tools, gene expression and specific antibodies will likely yield new scientific insights into macrophage biology.

To assess the sensitivity of the method, we selected CD11b and TNF-α, two classical proteins expressed on macrophages, for analysis by flow cytometry. The expression

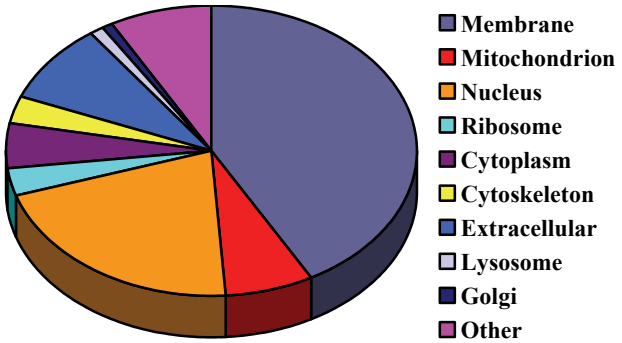

Figure 7. The subcellular distribution of identified macrophage activation associated proteins.

Table 2. Partial list of proteins with at least 8 peptides identified from activated macrophages

IPI	PepCount	Cover Percent	MW	PI	Identified Name
IPI00125726.5	33	19.49%	130575.3	7.76	Nitric oxide synthase, inducible
IPI00126585.1	30	35.09%	30128.28	6.76	H-2 class II histocompatibility antigen, A beta chain precursor
IPI00126346.1	25	31.25%	28093.02	4.64	H-2 class II histocompatibility antigen, A-B alpha chain precursor
IPI00323897.2	24	47.44%	24262.32	6.14	Ras-related protein Rab-11A
IPI00157508.2	22	22.36%	44458.39	5.42	Chitinase 3-like protein 3 precursor
IPI00111285.3	19	30.64%	58480.92	7.37	PREDICTED: immunoresponsive gene 1
IPI00553538.2	17	40.74%	15272.90	11.13	Histone H3.1
IPI00130118.1	17	20.50%	22540.85	8.58	Ras-related protein Rab-10
IPI00115892.1	16	14.70%	31557.38	8.61	Splice Isoform Long of H-2 class II histocompatibility antigen, gamma chain
IPI00654O26.1	14	62.22%	9416.94	9.65	clone: l920073D12 product: H2A histone family, member Z, full insert sequence
IPI00462008.2	14	21.98%	29064.35	8.79	29 kDa protein
IPI00554929.1	14	8.58%	83194.28	4.97	Heat shock protein HSP 90-beta
IPI00115977.1	14	22.07%	65799.16	7.53	NAD-dependent malic enzyme, mitochondrial precursor
IPI00271059.2	14	16.43%	23628.83	5.8	Ras-related protein Rab-4B
IPI00308162.3	13	17.87%	74569.74	8.43	Calcium-binding mitochondrial carrier protein Aralarl
IPI00323496.5	13	20.58%	47571.76	6	Interferon-inducible GTPase
IPI00127280.4	13	12.57%	19331.74	5.21	Myeloid bactenecin
IPI00116688.1	13	13.70%	24416.43	4.76	Ras-related protein Rab-3D
IPI00130015.3	12	13.20%	52376.44	6.41	Dipeptidyl-peptidase 1 precursor
IPI00312980.3	12	19.16%	37924.03	5.88	H-2 class I histocompatibility antigen, Q7 alpha chain precursor
IPI00129803.1	11	5.25%	79005.27	9.01	Antigen peptide transporter 1
IPI00121788.1	11	33.67%	22176.50	8.26	Peroxiredoxin-1
IPI00138892.2	10	36.72%	14728.26	9.87	Uba52 protein
IPI00317340.1	9	11.17%	77837.73	8.86	clone: 9830118D19 product: lactotransferrin. full insert sequence
IPI00461407.1	9	30.05%	23331.66	10.18	PREDICTED: similar to ATP synthase. H+ transporting, mitochondrial F1 complex. O subunit
IPI00121842.2	9	9.25%	77604.21	8.04	TAP2
IPI00133218.2	8	27.96%	21466.93	8.9	clone: l830086G02 product: ADP-ribosylation factor-like 10C, full insert sequence
IPI00133082.2	8	8.32%	87090.54	5.43	CD177 antigen
IPI00122048.1	8	6.74%	115968.7	5.44	Na+/K+-ATPase alpha 3 subunit
IPI00624138.1	8	13.35%	38521.85	5.66	PREDICTED: similar to RT1 class la. locus A2 isoform 1
IPI00114368.2	8	15.42%	24609.44	8.68	Vesicle trafficking protein SEC22b

levels of the two molecules were 93.62% and 18.15%, respectively. Both molecules were identified using the method described here and the peptide numbers identified were in accordance with their abundance (Figure 6). Thus, all membrane proteins from high to moderate abundance can be identified by the method.

Proteins uniquely expressed by BCG-activated tumoricidal macrophages

Proteins identified from each gel piece at the same size position were compared manually between the two samples. Only the proteins detected in BCG-activated macrophages were selected. Complication of the proteins from all the eight comparisons resulted in a list of 454 proteins which were identified from activated microphages only. Among these 454 proteins, 168 were identified with at least two peptides (Table 2). This set of proteins represents an extremely high confidence profile of membrane protein specifically expressed in activated macrophages. To assess the efficacy of the method for the enrichment of membrane proteins, we classified the 454 identified proteins according to the gene ontology annotation (GOA). Among these 454 proteins, 285 proteins have a gene ontology annotation for cellular component. Among the annotated proteins, 117 (42%) were assigned as membrane proteins. The remained annotated proteins were assigned as other organelles proteins, although there must be some membrane associated proteins among them (Figure 7). Investigating the available information about the differentially expressed proteins manually, the classical marker of macrophage tumoricidal activation, iNOS, was detected with 33 peptides, indicating a very high expression level of this protein. Another mouse macrophage

activation-associated marker protein, cyclooxygenase (18), was also detected. To our surprise, the recently discovered macrophage alternative activation marker (34), Ym1, was detected too. In addition to those known macrophage activation-associated proteins, several novel proteins were identified specifically on activated macrophages which are potential markers for macrophage tumoricidal activation.

Discussion

This study is the first to investigate the conjugation-dependent tumoricidal mechanisms of activated macrophages utilizing the recently developed mass spectrometry-based proteomics techniques. Although protein changes associated with macrophage tumoricidal activation have been extensively described (16, 17), it has been difficult to recognize a consistent correlation between macrophage tumoricidal activity and cell surface protein phenotype. A number of parameters have contributed to the complexity of these analyses, including short-live of the tumor cell killing activity, the low expression level of plasma membrane proteins, multiple changes during the process of activation, and continuously adapting their functional pattern in response to the changing environments (35). As a result, the molecular basis of macrophage activation for tumor cell killing is not yet completely understood.

Our approach to the investigation of macrophage conjugation-dependent tumoricidal activity has been through a comparison between BCG infection-activated macrophages and control macrophages that are elicited by thioglycolate. Previous work had suggested that this macrophage population was representative of macrophages with cell contact-dependent tumor cell killing ability (14). Since the membrane-bound TNF-α has been ascribed as the predominant effector molecule in conjugation-mediated cytotoxicity of BCG infection-activated macrophage, we determined the expression level of TNF-α with flow cytometry. To our surprise, the membrane bound TNF-α has not been increased compared with thioglycolate-elicited macrophages, which is conflicted with previous studies (14). The following possible explanations for this conflicting result may be proposed. First, the membrane bound TNF-α may be created at the point of cell-to-cell contact, which has not been determined by previous studies. Second, macrophages can induce tumor cell death through more than one effecor mechanisms. This can also be demonstrated by the cytotoxicity assay, because MCA207 tumor cell is resistant to TNF-α mediated cytotoxicity (Dr. K. Tsung, personal communication).

Our study represents the first proteomics analysis of the membrane proteins of a Th1 response-activated macrophage using a combination of SDS-PAGE with LC-MS/MS. This proteomics approach provides us with a valuable opportunity to acquire intriguing observations about macrophage tumoricidal ability. More than 1,000 proteins were identified in this research, of which 454 were uniquely detected in BCG infection-activated macrophages. Whereas the majority of identified proteins are differential quantitatively, our attention was focused on the BCG infection-activated macrophage uniquely expressed proteins. In addition, the strong difference of tumoricidal activity between activated macrophages and control macrophages suggested the protein expression difference seems more likely qualitative rather than quantitative.

Among the 454 specifically identified proteins, several macrophage activation associated proteins have been identified, including inducible NO synthase (iNOS), Ym1, cyclooxygenase, leucine-rich repeat-containing protein 25, metalloendopeptidase, myeloid bactenecin, interferon induced transmembrane protein 1. It is very interesting to detect iNOS in macrophage membrane fraction, because NO produced from iNOS is an important effector molecule of macrophage conjugation-independent tumoricidal capability. This finding can explain the discrepancy on the role of NO in macrophage tumoricidal activity (5), because iNOS association with plasma membrane is ideally suited to deliver NO directly into the contacted tumor cells. It is also interesting to identify Ym1 in BCG infection-activated macrophages, because Ym1 has been ascribed as a signature of type II cytokine-associated myeloid (M2) (36). Available information suggests that the classically activated M1 macrophages are the only effector cells killing tumor cells. The only explanation for this discrepancy is the existence of macrophage intrapopulation heterogeneity; despite they are 97.5% CD11b positive in this study.

We found that a significant proportion of the specifically identified proteins were not assigned as membrane proteins according to GO annotation. This is mainly due to the membrane isolation kit used in the experiment. In contrast to the two-phase partitioning technique, where detergents are used to separate membrane proteins based primarily on their intrinsic hydrophobicity, the kit's scalable differential extraction procedure selectively extracts integral membrane and membrane-associated proteins based on their actual association with cellular membrane. This has lead to the isolation of many novel membrane-associated proteins that have not been isolated by the two-phase partitioning technique. Because one of the primary long-term goals of this study is to identify protein(s) in cell contact-dependent tumoricidal activity, more detailed studies are needed to determine the role of these proteins in macrophage activation.

Applying the proteomics strategy to characterize macrophage membrane proteins provides us not only with a feasible strategy to investigate the conjugation-dependent tumoricidal mechanisms of macrophage, but also a valuable opportunity to acquire exciting findings about macrophage biology. With 2 lists of over 1,000 proteins identified from activated and inflammatory macrophages respectively with high confidence. Although many results of this study need further validation, the data themselves represent a valuable source of knowledge about activated macrophage membrane proteomes and provide directions for further studies. Many pioneering further studies are currently carried out in our laboratory and have been bringing some promising results. However, we analyzed membrane proteins of macrophages from C57BL/6 inbred mice, one of the most widely used

laboratory mouse strains. It should be noted that their genetic background exerts a strong influence on many functions of macrophages (37). Thus the proteomics analysis of macrophages from mice of different strains may give some different results.

In conclusion, we have accomplished a pilot study of macrophage conjugation-dependent tumoricidal mechanisms utilizing the cutting edge proteomics technologies. This study not only yields some new scientific insights into macrophage tumoricidal mechanisms, but also provides us with a valuable list of macrophage membrane proteins, which has great potential for identification of some novel membrane proteins from macrophages.

Acknowledgements

We are grateful to Dr. Kangla Tsung (Stanford, CA) for helpful advice on the research and the manuscript. We thank RCPA (Research Centre for Proteome Analysis, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) for providing the proteomics technology.

References

1. Klimp AH, De Vries EG, Scherphof GL, Daemen T. A potential role of macrophage activation in the treatment of cancer. Crit Rev Oncol Hematol. 2002;44:143-161.
2. Bingle L, Brown NJ, Lewis CE. The role of tumor-associated macrophages in tumor progression: implications for new anticancer therapies. J Pathol. 2002;19:254-265.
3. Cleveland RP, Meltzer MS, Zbar B. Tumor cytotoxicity *in vitro* by macrophages from mice infected with mycobacterium bovis strain BCG. J Natl Cancer Inst. 1974;52:1887-1895.
4. Klostergaard J, Leroux ME, Hung MC. Cellular models of macrophage tumoricidal effector mechanisms *in vitro*: Characterization of cytolytic responses to tumor necrosis factor and nitric oxide pathways *in vitro*. J Immunol. 1991;147:2802-2808.
5. Cui S, Reihner JS, Mateo RB, Albina JE. Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or independent mechanisms. Cancer Res. 1994; 54:2462-2467.
6. Tsung K, Dolan JP, Tsung YL, Norton JA. Macrophages as effector cells in interleukin12-induced T cell-dependent tumor rejection. Cancer Res. 2002;62:5069-5075.
7. Weinberg JB. Nitric oxide production and nitric oxide synthase type 2 expressions by human mononuclear phagocytes: a review. Mol Med. 1998;4:557-591.
8. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15:323-350.
9. Feinman RD, Henriksen-DeStefano D, Tsujimoto M, Vilcek J. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. J Immunol. 1987;38:635-640.
10. Hicks AM, Willingham MC, Du W, Pang CS, Old LJ, Cui Z. Effector mechanisms of the anti-cancer immune responses of macrophages in SR/CR mice. Cancer Immun. 2006;6:11-19.
11. Chauhan P, Sodhi A, Tarang S. Cisplatin-treated murine peritoneal macrophages induce apoptosis in L929 cells: role of Fas-Fas ligand and tumor necrosis factor-tumor necrosis factor receptor 1. Anticancer Drugs. 2007;18:187-196.
12. Klostergaard J, Stoltje PA, Kull FC Jr. Tumoricidal effector mechanisms of murine BCG-activated macrophages: role of TNF in conjugation-dependent and conjugation-independent pathways. J Leukoc Biol. 1990;48:220-228.
13. Meltzer MS, Tucker RW, Breuer AC. Interaction of BCG-activated macrophages with neoplastic and nonneoplastic cell lines *in vitro*: Cinemicrographic analysis. Cell Immunol. 1975; 17:30-42.
14. Klostergaard J, Leroux ME, Ezell SM, Kull FC Jr. Tumoricidal effector mechanisms of murine BCG-activated macrophages: Mediation of cytolysis, mitochondrial respiration inhibition, and release of intracellular iron by distinct mechanisms. Cancer Res. 1987;47:2014-2025.
15. Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity *in vivo*. Proc Natl Acad Sci U S A. 1994;91:9407-9411.
16. MacKay RJ, Russell SW. Protein changes associated with stages of activation of mouse the macrophages for tumor cell killing. J Immunol. 1986;137:1392-1398.
17. MacKay RJ, Russell SW. Protein phenotypes of mouse macrophages activated *in vivo* for tumor cell killing. J Leukoc Biol. 1987;42:213-221.
18. Phillips TA, Kujubu DA, MacKay RJ, Herschman HR, Russell SW, Pace JL. The mouse macrophage activation-associated marker protein, p71/73, is an inducibel prostaglandin endoperoxide synthase (cyclooxygenase). J Leukoc Biol. 1993; 53:411-419.
19. Nyman TA, Rosengren A, Syyrakki S, Pellinen TP, Rautajoki K, Lahesmaa R. A proteome database of human primary T helper cells. Electrophoresis. 2001;22:4375-4382.
20. Nebl T, Pestonjamasp KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ. Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. J Biol Chem. 2002; 277:43399-43409.
21. Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc Natl Acad Sci U S A. 2003;100:5813-5818.
22. Li N, Mak A, Richards DP, et al. Monocyte lipid rafts contain proteins implicated in vesicular trafficking and phagosome formation. Proteomics 2003;3:536-548.
23. Von Haller PD, Donohoe S, Goodlett DR, Aebersold R, Watts JD. Mass spectrometric characterization of proteins extracted from Jurkat T cell detergent-resistant membrane domains. Proteomics 2001;1:1010-1021.
24. Corthals GL, Washinger VC, Hochstrasser DF, Sanchez JC. The dynamic range of protein expression: a challenge for proteomic research. Electroohoresis. 2000;21:1104-1115.
25. Wilkins MR, Gasteiger E, Sanchez JC, Bairoch A, Hochstrasser DF. Two-dimensional gel electrophoresis for proteome projects: the effects of protein hydrophobicity and copy number. Electrophoresis. 1998;19:1501-1505.
26. Rabilloud T. Use of thiourea to increase the solubility of membrane proteins in two-dimensional. Electrophoresis. 1998; 19:758-760.
27. Perdew GH, Schaup HW, Selivonchick DP. The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. Anal Biochem. 1983;135:453-455.
28. Adam PJ, Boyd R, Tyson KL, et al. Comprehensive proteomic analysis of breast cancer cell membranes reveals unique proteins with potential roles in clinical cancer. J Biol Chem. 2003;278: 6482-6489.
29. Zhao YX, Zhang W, KhoYJ, ZhaoYM. Proteomic analysis of integral plasma membrane proteins. Anal Chem. 2004;76:1871-

1823.
30. Kumagai K, Itoh K, Hinuma S, Tada M. Pretreatment of plastic petri dishes with fetal calf serum: A simple method for macrophage isolation. J Immunol Method. 1979;29:17-25.
31. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Method. 1983;65:55-63.
32. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. 1996;68:850-858.
33. Zhang LB, Lun YN, Yan DM, et al. Proteomic analysis of macrophages: A new way to identify novel cell-surface antigens. J Immunol Method. 2007;321:80-85.
34. Raes G, De Baetselier P, Noël W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. J Leukoc Biol. 2002;71:597-602.
35. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenviroments. J Leukoc Biol. 2004;76:509-513.
36. Ghassabeh GH, De Baetselier P, Brys L, et al. Identification of a common gene signature for type II cytokine-associated myeloid cells elicited *in vivo* in different pathologic conditions. Blood. 2006;108:575-583.
37. van Erp K, Dach K, Koch I, Heesemann J, Hoffmann R. Role of strain differences on host resistance and the transcriptional response of macrophages to infection with Yersinia enterocolitica. Physiol Genomics. 2006;25:75-84.