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Detection of Membranous Protein Alterations on BCG-activated Macrophages Involved in Contact-dependent Tumoricidal Processes

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Abstract

Activated macrophages possess tumoricidal ability associated with a cell-cell contact mechanism. However, the nature of the cell surface proteins involved in this tumoricidal process is not yet known. In this research, macrophages activated by bacterium *Mycobacterium bovis bacillus Calmette-Guerin* (BCG) and by thioglycolate (TGC) were chosen as two comparative models for the large-scale detection of cell surface protein alterations involved in this process. A proteomics approach involving SDS-PAGE followed by LC-MS/MS was used to characterize membrane proteins of the two models. A total of 421 uniquely expressed proteins were identified on the BCG-activated macrophage membrane. Functional groups indicating signal transduction, transport, and cell adhesion, among others, were significantly enriched in this group of proteins. In addition, 42 proteins associated with the plasma membrane were detected. These membrane proteins may contact tumor cells directly and play important roles in the tumoricidal process. In general, this study provides an initial database of candidate proteins that can now be screened as potential regulators of the adherence-dependent tumoricidal properties associated with macrophages.

Keywords: Macrophage activation; Tumor cell killing; Membranous protein; Proteomics

Abbreviations

BCG: Bacterium *Mycobacterium bovis bacillus Calmette-Guerin*; TGC: Thioglycolate ; LPS: Lipopolysaccharide; GM-CSF: Granulocyte-Microphage Colony-Stimulating Factor; M-CSF: Macrophage Colony-Stimulating Factor; TNF: Tumor Necrosis Factor; NO: Nitric Oxide; PBS: Phosphate Buffered Saline; MTT, 3-(4,5-dimethylthia zol-2-yl)-2-5-diphenylterazolium bromide; MGI: Mouse Genome Information; iNOS: inducible NOS; LFA-1: Lymphocyte Function-associated Antigen-1; ICAM: Intercellular Adhesion Molecules; Prdx: Peroxiredoxin; PC: Phosphatidylcholine; PA: Phosphatidic Acid; EZR: Ezrin

Introduction

The functions of macrophages as the first line of defense in the innate immune system have been well documented. Based on their differences in capacities and functions, macrophages can be characterized as unstimulated, primed, or activated (MacKay and Russell, 1986). For activated macrophages, there are two major categories of activating agents: 1) components associated with microorganisms, such as lipopolysaccharide (LPS) and the bacterium *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (Rietschel and Brade, 1992); and 2) macrophage activating cytokines, such as granulocyte-microphage colony-stimulating factor (GM-

CSF) and macrophage colony-stimulating factor (M-CSF) (Ulich et al., 1990).

Over the past decades, activated macrophages have been identified as having a key role in the defense against tumor cells. Accumulating evidence indicates that activated macrophages are able to effectively recognize, bind, and subsequently lyse tumor cells (Klostergaard et al., 1991; Klimp et al., 2002). This tumoricidal process occurs by both direct and indirect mechanisms. In the former condition, products harmful to tumor cells, such as oxygen radicals and tumor necrosis factors (TNFs), can be directly released to the tumor site (Feinman et al., 1987). Alternatively, macrophages can play an indirect role by secreting cytokines or by presenting antigens, thereby regulating immune responses against tumors. One of the major mechanisms by which macrophages mediate tumor cytotoxicity appears to be a process that relies on cell-to-cell contact (Cleveland et al., 1974; Tsung et al., 2002). However, the proteins on the cell surface that contribute to specific tumor cell recognition have not yet been identified.

In previous studies (Werb and Gordon, 1975), *in vivo* administration of inflammatory agents such as thioglycolate (TGC) were found to significantly enhance the subsequent *in vitro* stimulation of peritoneal macrophages. And TGC-elicited and resident peritoneal macrophages showed very few changes in the pattern of proteins synthesized after LPS treatment (Largen and Tannenbaum, 1986). In our earlier study (Zang et al., 2007), we found that both *in vivo* BCG-activated and *in vitro* LPS-induced macrophages were recruited as effector cells in cytotoxicity assays. And the LPS-induced macrophages were pre-elicited by TGC *in vivo*. Although LPS is one of the most potent *in vitro* activators of macrophages, we observed that BCG-activated macrophages exhibited much higher tumoricidal activities than did LPS-induced macrophages. Furthermore, since the activated macrophages had been fixed in 1% paraformaldehyde, metabolic release of classical tumor killing mediators such as TNF and nitric oxide (NO) was unlikely. The molecules responsible for the tumoricidal responses were unclear.

Changes in gene expression profile of macrophages in response to BCG have been analyzed (Begum et al., 2004). However, the membrane protein alternations involved are still undiscovered. In the current study, we have further analyzed activated macrophage membrane proteins. We report on 421 unique proteins seen in response to BCG activation but not to TGC activation. According to their functional features, we have categorized these unique proteins into groups including transport, cell organization and biogenesis, signal transduction, cell adhesion, and apoptosis. By providing an initial database of likely candidates for cytotoxicity responses, we set the stage for future detailed gene expression and functional studies that will clarify the tumoricidal mechanism used by activated macrophages.

Materials and Methods

Reagents

LPS from *Escherichia coli* serotype 055:B5 was purchased from Sigma (Saint Louis, MO). 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. All cell culture media and supplements were obtained from Invitrogen Corporation. SDS gels were homemade following the guidelines of Molecular Cloning (Sambrook, 2000).

Mice and cell lines

Female C57BL/6 mice aged 8-12 weeks were purchased from the Animal Division of Jilin University and were maintained under pathogen-free conditions. The MCA207 cell line was obtained from Dr. Kangla Tsung (Stanford University, CA). MCA207 is a methylcholanthrene-induced tumor cell series, which is transplantable into C57BL/6 mice. The cells were maintained in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FCS, 100μg/ml streptomycin, and 100 IU/ml penicillin.

Preparation of mouse peritoneal macrophages activated by BCG and TGC *in vivo*

Mice weighing 20g were primed with 2mg BCG by *i.p.* injection. Another two *i.p.* injections of 1 mg BCG were given on the 10th and the 20th days. Peritoneal macrophages were collected and transferred to RPMI-1640 tissue culture medium three days after the last *i.p.* injection (McCarron et al., 1984). Cells were respectively centrifuged at 300g for 5 min at 4 °C and resuspended in RPMI-1640 medium supplemented with 5% fetal calf serum. The cells were then plated in 35×15mm cell culture dishes. After 2 hours of incubation at 37 °C in 5% CO_2, non-adherent cells were removed with 5 washes with 2 ml warm PBS. Adherent cells were collected using a sterile cell scraper. Cells were pooled and centrifuged at 300g at 4 °C for 10 min. After carefully removing the supernatant, the cells were washed twice with PBS for the subsequent experiments.

To obtain TGC-elicited macrophages, peritoneal macrophages were harvested from mice 3 days after they had been

given 2 ml 3% thioglycolate medium by *i.p.* injection (Gordon et al., 1974). Cells were collected following the protocol mentioned above. For TGC-elicited macrophages, RPMI-1640 medium containing 5μg/ml LPS was added. After 24 hours of incubation at 37 °C in 5% CO_2, LPS-induced macrophages were prepared as control effector cells for the subsequent cytotoxicity assay.

By respectively fixing BCG-activated and LPS-induced macrophages in 1% paraformaldehyde in PBS at room temperature for 20 min, the fixed effector cells were washed twice with culture medium and suspended in medium at a concentration of 5×10^6 cells/ml. For the assay, a suspension of 1×10^4 MCA207 target cells was added to three out of four wells of a 96-well plate (blank wells served as controls) and incubated for 10-30 min. A 0.1 ml aliquot of effector cell suspension was then added to each well and the plates were incubated at 37°C for 48-60 hours. Non-adherent effector cells and dead tumor cells were removed by gently washing the wells 3 times with medium. The number of viable tumor cells was then determined by MTT assay. After dissolving the formazan, the absorbance was recorded directly using a microplate reader (Model 550, Bio-RAD) at a wavelength of 570 nm. The cytotoxicity was calculated with the following formula:

cytotoxicity= [1-(absorbance at 570 nm of target + effector cells)/(absorbance at 570 nm of target cells only)]×100%.

Preparation of macrophage membrane proteins, mass spectrometry, and data analysis

BCG-activated and TGC-elicited macrophages were harvested as previously described. Membrane proteins from activated macrophages were extracted following the kit manufacturer's protocol (ProteoExtract Native Membrane Protein Extraction Kit; Calbiochem, USA). Protein concentration was determined with a BCA Assay Kit (BIOS, Beijing) and 150μg protein was applied to a 12% bis-Tris SDS-PAGE gel. After visualization by Coomassie blue staining, entire gel lanes were cut into eight pieces of equal size and subjected to in-gel tryptic digestion (Shevchenko et al., 1996). The extracted peptides were analyzed using a LCQ deca plus system equipped with a capillary HPLC system (Thermo Finnigan, San Jose, CA, USA). First, microcore RP column (RP-C18, 0.15mm×120mm, ThermoHypersil, San Jose, CA, USA) was used to separated the protein digests The mobile phases used for reverse phase were 0.1% formic acid in water, pH 3.0 (A), and 0.1% formic acid in 100% v/v ACN (B). Peptides were eluted using a 0 to 80% linear gradient of solvent B over 60 min. The flow rate of the pump was 200 μl/min and reduced to about 2 μl/min after the split. An ESI IT mass 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 was automatically set by the LCQ Deca XP system. After acquisition of a full scan, three MS/MS scans were acquired for the next three most intense ions using dynamic exclusion for 6 hours.

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 the IPI mouse protein database (V3.15.1). The following search parameters were used (Zhang et al., 2007): 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 were based on Delta CN (≥ 0.1) and Xcorr (one charge ≥ 1.9, two charges ≥ 2.2, and three charges ≥ 3.75). Protein identification results were extracted from a SEQUEST output file with the in-house software BuildSummary. Sub-cellular classifications were performed with online GO analysis (www.informatics.jax.org/gotools/MGI_Term_Finder.html) according to the accession number of proteins in the Mouse Genome Information (MGI) database.

Results

Cytotoxicity assays were carried out to compare the tumoricidal abilities of BCG-activated and LPS-induced macrophages (Zhang et al., 2007). After activation by BCG *in vivo*, the tumoricidal activity against MCA207 cells was significantly promoted from 14.59% to 89.06%. In contrast, the tumor cell killing capacity of macrophages induced by LPS *in vitro* exhibited less change (from 8.91% to 12.61%).

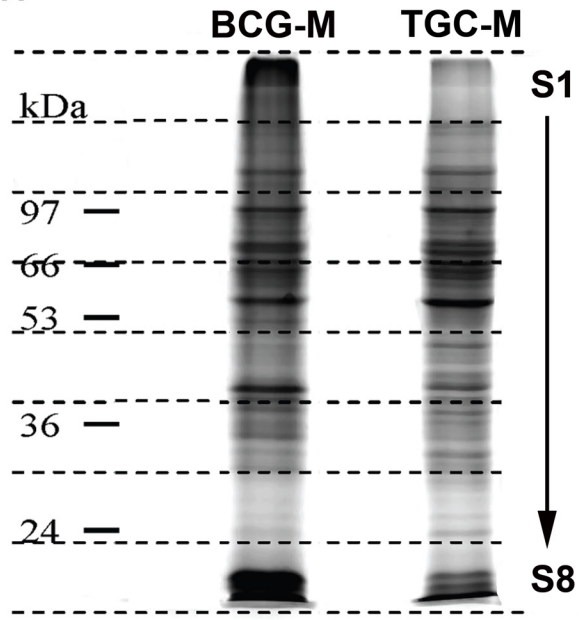
Identification of activated-macrophage membranous proteins

Representative SDS-PAGE gel lanes are shown in Figure 1A and the numbers of proteins identified in each gel strip are indicated in Figure 1B. The peptide mass fingerprints of the in-gel tryptic digest fraction are shown in Supplementary File 1. In total, 1762 proteins were recognized in BCG-activated macrophages samples and 1518 proteins in the samples of TGC-induced macrophages (Supplementary File 2 and 3).

Unique membrane proteins of BCG-activated macrophages

Comparing the two lists of identified proteins, we filtered

A



B

	S1	S2	S3	S4	S5	S6	S7	S8
TGC-M	119	145	183	212	195	191	182	291
BCG-M	238	168	189	237	225	214	198	293

Figure 1: A, SDS-PAGE separation of macrophage membrane proteins. Proteins were separated on 12% Tris-Bis gel and stained to enable protein identification (Zhang et al., 2007). Eight equally spaced sections were excised and subsequently used for MS/MS analysis. B, protein numbers of each slice (S1-S8) identified by LC-MS/MS.

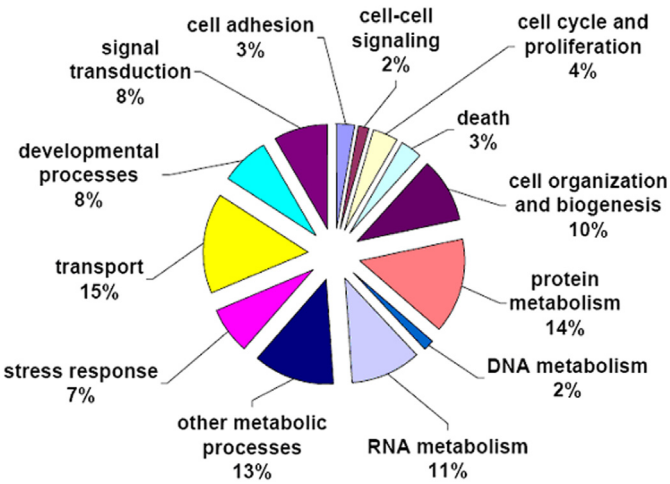


Figure 2: Functional groups of uniquely expressed proteins identified from the BCG-activated macrophages membrane.

out redundant data and consequently confirmed that there were 421 unique proteins on BCG-activated macrophage membranes. The details of the identified proteins are listed in Supplementary File 4.

To elucidate potential functions of these proteins, we categorized them by GO analysis. The functional categories included the following groups(Figure 2): transport (15%), protein metabolism (14%), other metabolic processes (13%), RNA metabolism (11%), cell organization and biogenesis (10%), signal transduction (8%), developmental processes (8%), stress responses (7%), cell cycle and proliferation (4%), cell adhesion (3%), death (3%), DNA metabolism (2%) and cell-cell signaling (2%). The unique proteins were also assigned subcellular locations by GO classification. They were found to distribute throughout the cell, in subcellular organs such as Golgi apparatus, ribosomes etc. Of the initial 421 unique proteins, 42 were determined to be plasma membrane proteins (Table 1).

Discussion

Previous studies have shown that activated macrophages play an important role in tumor killing processes. However, the mechanisms used for tumoricidal functions are not yet clearly understood. During macrophage activation, 25% of all observed genes exhibit alterations in expression (Ehrt et al., 2001). This creates a logistics problem for determining which of the several hundred genes that are involved in the activation are true and reliable mediators of tumoricidal processes.

In an earlier study (Zhang et al., 2007), we also observed that the tumoricidal capabilities of activated macrophages were retained even after they had been fixed by paraformaldehyde. This suggested that tumoricidal activity was contact-dependent and not mediated by metabolically released factors. Moreover, BCG-activated macrophages exhibited much stronger tumor cells killing abilities than did LPS-induced macrophages, which induced us to analyze certain molecular alterations involved in the tumor killing process.

The combined SDS-PAGE and LC-MS/MS approach taken in the current study has allowed us to discriminate membrane proteins that were unique to activated macrophage and which would therefore be likely candidates for involvement in a contact-dependent tumor killing process. Our results also allowed the other unique proteins differentially expressed in BCG-activated macrophages to be sorted into a variety of functional groups including transport, cell organization and biogenesis, signal transduction, stress responses, cell cycle and proliferation, cell adhesion, and death.

IPI	MGI	Protein name	Peptides identified	Sequence coverage	Mw/PI
IPI00323897	MGI:1858202	RAB11a, member RAS oncogene family	8	47.44%	24262.32/6.14
IPI00115892	MGI:96534	CD74 antigen	4	14.70%	31557.38/8.61
IPI00122973	MGI:96392	Icam1, intercellular adhesion molecule 1	4	10.24%	58844.26/5.79
IPI00132286	MGI:96606	integrin alpha L	4	6.53%	128344.12/5.75
IPI00130118	MGI:105066	RAB10, member RAS oncogene family	3	20.50%	22540.85/8.58
IPI00271059	MGI:105071	RAB4B, member RAS oncogene family	3	16.43%	23628.83/5.8
IPI00135655	MGI:1928744	vesicle-associated membrane protein	3	16.12%	26815.01/7.84
IPI00330862	MGI:98931	ezrin	3	5.81%	69275.84/5.83
IPI00229277	MGI:2429943	Gbp5, guanylate binding protein 5	2	6.10%	66970.12/5.91
IPI00323251	MGI:102772	Gbp2, guanylate binding protein 2	2	6.28%	66739.5/5.56
IPI00126083	MGI:1341878	Ehd1, EH-domain containing 1	2	4.49%	60602.88/6.35
IPI00622270	MGI:109356	Snap23, synaptosomal-associated protein 23	2	16.19%	23260.81/4.88
IPI00111359	MGI:95804	Gsr, glutathione reductase	2	4.60%	53662.53/8.19
IPI00457611	MGI:1858222	Sept9, septin 9	2	4.46%	65575.04/9.01
IPI00132279	MGI:105370	Bst1, bone marrow stromal cell antigen 1	1	4.18%	34616.48/5.49
IPI00118420	MGI:107742	Stra6, stimulated by retinoic acid gene 6	1	3.58%	73775.23/8.91
IPI00109254	MGI:1926446	Cd274, CD274 antigen	1	3.79%	32780.49/5.85
IPI00469307	MGI:96829	Lrpap1, low density lipoprotein receptor-related protein	1	3.61%	42215.14/7.35
IPI00463402	MGI:1339977	type I receptor-associated protein	1	24.84%	17497.23/5.4
IPI00265452	MGI:2149209	otoancorin	1	1.40%	126615.48/5.44
IPI00227838	MGI:1336171	guanine nucleotide binding protein	1	21.62%	8155.39/9.14
IPI00112252	MGI:2158329	parvin, gamma	1	3.93%	37576.33/5.42
IPI00355808	MGI:2685438	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2	1	0.84%	106804.91/6.22
IPI00272690	MGI:87874	angiotensin I converting enzyme	1	1.22%	150918.55/6.1
IPI00130095	MGI:1351465	Ras-GTPase-activating protein SH3-domain binding protein 1	1	3.66%	51828.97/5.41
IPI00130872	MGI:1277979	integrin beta 2-like	1	2.44%	81605.73/6.68
IPI00329927	MGI:104753	neurofascin	1	1.69%	138315.67/5.94
IPI00223023	MGI:2388361	gliomedin	1	4.19%	59136.82/8.71
IPI00268377	MGI:2153063	parvin, beta	1	3.83%	41532.89/5.82
IPI00330923	MGI:2180140	tumor necrosis factor (ligand) superfamily, member 15	1	9.13%	27725.06/8.43
IPI00108870	MGI:99611	Eph receptor B2	1	1.01%	110760.19/5.4
IPI00284505	MGI:1858236	cadherin, EGF LAG seven-pass G-type receptor 3	1	0.45%	358462.87/6.15
IPI00420735	MGI:2653880	G protein-coupled receptor 156	1	2.88%	87040.73/7.97
IPI00411158	MGI:2441950	latrophilin 3	1	2.41%	110733.18/7.14
IPI00420315	MGI:3042776	inositol 1,4,5-triphosphate receptor interacting protein	1	3.06%	63298.11/5.44
IPI00118475	MGI:97612	plasminogen activator	1	4.89%	35428.28/6.44
IPI00132868	MGI:1913105	solute carrier family 28	1	3.64%	72970.6/7.96
IPI00553454	MGI:1339710	myosin, heavy polypeptide 2	1	0.95%	181438.54/5.59
IPI00126346	MGI:95895	histocompatibility 2, class II antigen A	5	31.25%	28093.02/4.64
IPI00408489	MGI:2150309	Down syndrome cell adhesion molecule-like 1	1	3.29%	40482.42/9.39
IPI00331044	MGI:99400	ATPase, Cu++ transporting	1	1.34%	161909.38/6.22
IPI00268475	MGI:99668	sialic acid binding Ig-like lectin 1	2	2.65%	182979.05/6.2

Table 1: Forty-two members of uniquely expressed proteins on BCG-activated macrophages plasma membrane.

Classic molecules involved in macrophage-activation

Classic tumor killing mediators such as inducible NOS (iNOS) and TNF (MGI:2180140) were well represented in previous results (Feinman et al., 1987; MacMicking et al., 1997; Weinberg, 1998). For example, iNOS expression in mouse macrophages resulted in high-level NO production. When macrophages contacted tumor cells, NO generated by iNOS can infiltrate tumor cells and initiate tumor killing functions (Fehr et al., 1997; Tsung et al., 2002; Bosca et al., 2005). The mechanism by which TNF is able to kill tumor cells involves the binding of TNF to TNF-receptors expressed on target cells (Loetscher et al., 1990; Schall et al., 1990).

In the current study, macrophages used as effector cells were fixed by paraformaldehyde and would not be expected to perform metabolic functions such as iNOS and TNF release. However, these compounds were useful for characterization as membrane markers of activated macrophages.

Another marker protein detected to be uniquely expressed in BCG-activated macrophages was the chitinase 3-like protein (MGI: 1330860). The chitinase family includes Ym1 and Ym2 isoforms (Zhao et al., 2005). However, Ym1 is also a membrane marker of cytokine-associated myeloid (M2) macrophages, which are not the major effector cells that kill tumor cells (Ghassabeh et al., 2006). We identified membrane markers of both M1 and M2 activated macrophages following BCG infection. The existence of macrophage intrapopulation heterogeneity probably represents responses against both infection and tumorigenesis.

Proteins related to cell adhesion

Cell-cell adhesion is an important process during morphogenesis. It ensures the tight contact between neighboring cells necessary for cell segregation and the morphological and functional differentiation of different tissues. During tumor progression, disruption of cell-cell contact plays a pivotal role in metastasis as well as in the dedifferentiation process that accompanies the malignant phenotype. In terms of biological processes, in the current study, about 3% of the identified unique membranous proteins were classified into this group.

One of the interesting molecules was an ITGAL encoding protein (MGI: 96606), a known positive regulator of cell-cell adhesion that encodes the integrin alpha L chain. Integrins are characterized as heterodimeric integral membrane proteins, which are composed of an alpha chain and a beta chain (Akiyama, 1996). The integrin lymphocyte function-associated antigen-1 (LFA-1) expressed on all leukocytes is composed of alpha integrin and the beta 2 chain (ITGB2). LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligands, ICAMs 1-3 (intercellular adhesion molecules 1 through 3), and also functions in lymphocyte co-stimulatory signaling (Sumimoto et al., 1994; Akiyama, 1996; Sumimoto et al., 1996).

We also recognized a member of the flamingo subfamily (MGI: 1858236), which is part of the cadherin superfamily (Pettitt, 2005). The flamingo subfamily consists of nonclassic-type cadherins; a subpopulation that does not interact with catenins. The flamingo cadherins are located at the plasma membrane and have nine cadherin domains, seven epidermal growth factor-like repeats, and two laminin A G-type repeats in their ectodomain (Usui et al., 1999). Seven transmembrane domains have been characterized as being unique to this subfamily. It is hypothesized that these proteins are receptors involved in contact-mediated communication, with cadherin domains acting as homophilic binding regions and the EGF-like domains being involved in cell adhesion and receptor-ligand interactions (Vincent et al., 2000). The specific function of this particular member has not been determined.

Proteins related to signal transduction and cell-cell conjunction

Eight percent of our unique proteins were categorized into this signal transduction and cell-cell conjunction group. For example, a Stat1 encoding protein (MGI: 103063) was detected only in samples from BCG-activated macrophages. The protein encoded by Stat1 was a member of the STAT protein family, which was first discovered as a key group of proteins involved in cytokine signaling. In response to cytokines and growth factors, STAT family members can be phosphorylated by receptor-associated kinases, after which they form homo- or heterodimers. These dimers then translocate to the cell nucleus where they act as transcription activators (Lim and Cao, 2006). The proteins of this family can be activated by various ligands including interferon-alpha (IFN-α), EGF, interferon-gamma (IFN-γ), PDGF and IL6 (Cao et al., 1996; Linnekin et al., 1997; Shen et al., 2001; Colomiere et al., 2009). This protein mediates the expression of a variety of genes that are thought to be important for cell viability in response to different cell stimuli and pathogens.

Enzymes

A group of enzymes detected only in the sample from BCG-activated macrophages included proteins such as peroxiredoxin 2 (Prdx2) (MGI: 109486) and PLD4 (MGI:

2144765).

Prdx2 is a member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. Prx2 is the third most abundant protein in erythrocytes, and competes effectively with catalase and glutathione peroxidase to scavenge low levels of hydrogen peroxide, including that derived from hemoglobin autooxidation (Low et al., 2008). The peroxiredoxin family enzymes may play a protective role against oxidative stress in cells. Another member of the family, peroxiredoxin 5 (Prdx5), may contribute to the antiviral activity of CD8 (+) T-cells (Sensi et al., 2005). These findings implied that the peroxiredoxin family might have a proliferative effect and may play a role in cancer development or progression.

Phosphatidylcholine (PC)-specific PLDs catalyze the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline (Corrotte et al., 2006). A range of agonists acting through G protein-coupled receptors and receptor tyrosine kinases stimulate this hydrolysis. PLDs are also involved in the EGFR1 Signaling Pathway. PLD isoforms have been suggested to coordinately regulate macrophage adherence dependent phagocytosis (Foster and Xu, 2003; Iyer et al., 2004; Corrotte et al., 2006). Therefore, PLDs have emerged as potential regulators of several critical aspects of cell physiology, including vesicle transport, endocytosis, exocytosis, cell migration, and mitosis (Hitomi et al., 2004; Iyer et al., 2004; Iyer et al., 2006; Kim et al., 2006).

Proteins with unknown identity or function

In addition to some unidentified proteins, we obtained matches to a number of metabolic proteins in the database

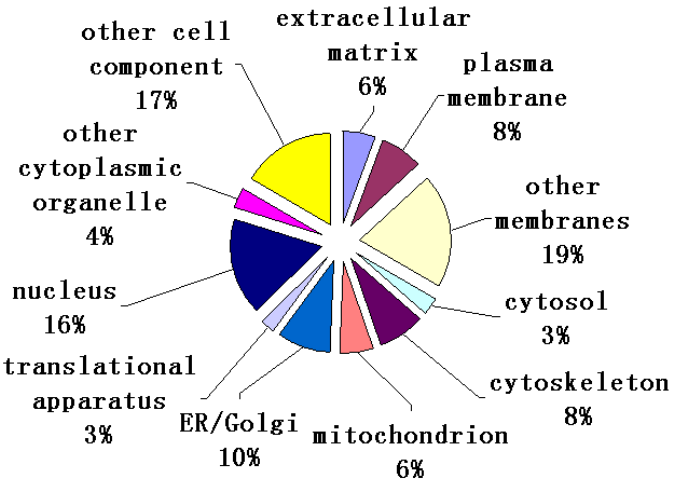


Figure 3: Subcellular localization of uniquely expressed proteins identified from the BCG-activated macrophages membrane

whose function was uncertain with respect to the tumoricidal process. These included proteins involved in cell cycle and proliferation, protein metabolism, RNA metabolism, DNA metabolism, and other metabolic processes. Most of these proteins were involved in cell cycling and metabolic activities, suggesting that activation alters the cell structure and the metabolic activities of macrophages and therefore may have indirect effects on the tumoricidal functions of activated macrophages.

Proteins located on the plasma membrane

Through GO analysis, 42 proteins were localized to the plasma membrane proteins, making them likely candidates for cell contact. Since the activated macrophages mediate tumor cell killing in a contact-dependent manner, any of these 42 plasma membrane proteins may be involved in mediating the tumoricidal process. The top three functional categories of these 42 members were signal transduction (31%), transport (24%) and cell adhesion (21%). All of these functions are extensively involved in either the physiological processes or the pathology of cells.

An attempt was made to predict the interaction relationships among the proteins, based on the protein interaction information databases such as DIP (Xenarios et al., 2002), BIND (Bader et al., 2003) and IntAct (Hermjakob et al., 2004) [Figure 4]. Querying the KEGG pathway database (http://www.genome.jp/kegg/), these members appeared to be proteins involved in regulating cell communication, including adherents such as integrin and ezrin (EZR). Both integrin and EZR can interact with more than one protein. Ezrin has been reported as a potential regulator of cell-cell

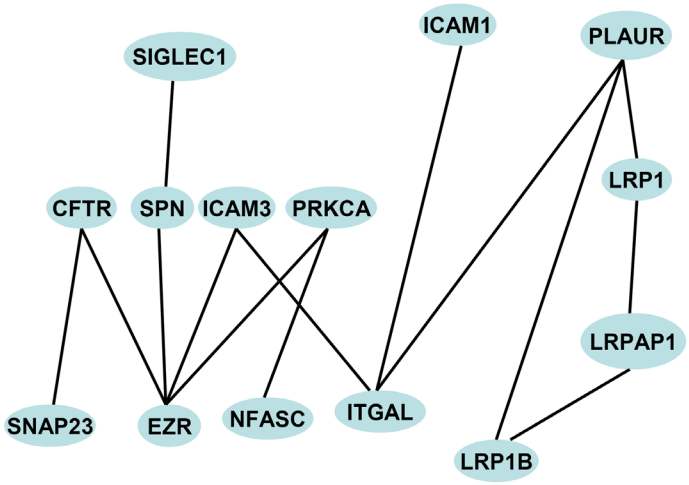


Figure 4: The interactions among the proteins located on plasma membrane. Each node represented one protein; lines indicated the interactions. This figure was generated by the online server Genes2Network (http://actin.pharm.mssm.edu/genes2networks/).

and cell-matrix adhesion and plays an important role in the control of adhesion and invasiveness of cancer cells (Hiscox and Jiang, 1999).

Among these proteins, ICAM-1 was also distinguished. Intercellular adhesion molecules are structurally related members of the immunoglobulin supergene family and are ligands for the β2 integrin molecules present on leukocytes. ICAM-1 is expressed constitutively at low levels on endothelial cells and on some lymphocytes and monocytes, and its expression can be significantly increased in the presence of cytokines (TNFα, IL-1, IFNγ) and reactive oxygen species. ICAM-1 participates in trafficking of inflammatory cells, in cell-cell interactions during antigen presentation, in microbial pathogenesis, and in signal transduction through outside-in signaling events. Depending on the cell type examined, ICAM-1 engagement has been documented to activate specific kinases through phosphorylation, resulting in transcription factor activation and increased cytokine production, increased cell membrane protein expression, reactive oxygen species production, and cell proliferation.

In conclusion, using a proteomics approach, we have reported a number of protein expression changes that occurred in macrophages activated by BCG and induced by TGC. These proteins were involved in cell adhesion, cytoskeleton interactions, immune responses and apoptosis. The results supported all of the known function of activated macrophages in killing tumor cells. Furthermore, 42 proteins located on plasma membrane were detected, any of which could be potential regulators of the cell adhesion dependent tumoricidal process. Further characterization of these proteins should provide clues to their roles in the tumoricidal process mediated by BCG-activated macrophages.

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