

ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol
June 2015; 14(3):261-272.

Molecular Diversity of Macrophages in Allergic Reaction: Comparison between the Allergenic Modes; Th1- and -Th2-Derived Immune Conditions

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Received: 30 March 2014; Received in revised form: 24 June 2014; Accepted: 3 August 2014

ABSTRACT

Activated macrophages have been classified into classical (M1) and alternative (M2) macrophages. We aimed to establish a method to yield enough number of macrophages to analyze their molecular, biological and immunological functions. We used drugs; adjuvant albumin from chicken egg whites—Imject Alum (OVA-Alum) and OVA Complete Freund Adjuvant (OVA-CFA), to induce macrophages to M2 and M1 respectively.

We analyzed the phenotype of purified macrophages induced under these immune conditions, using flow cytometry (FACS) to detect cell-surface molecules and the enzyme-linked immunosorbent assay (ELISA) was used to detect cytokines. The cDNA microarray was employed to measure changes in expression level of cell surface protein between M1 and M2 macrophages.

Phenotype analysis of purified macrophages, induced under these immune conditions, showed macrophages induced by OVA-Alum was almost M2 while the proportion of M1 macrophages induced by OVA-CFA was significantly higher. The results also showed higher expression level of macrophage galactose N-acetyl-galactosamine specific lectin-2 protein (MGL1/2-PE), a known M2 macrophage marker, on the surface of Alum-induced macrophages. On the basis of these preliminary data, ELISA results revealed that after macrophage stimulation with lipopolysaccharides (LPS), the level of interleukin (IL)-10 produced by Alum-induced macrophages was higher than the level of IL-10 produced by CFA-induced macrophages. In contrast, the level of tumor necrosis factor-alpha (TNF- α) produced by CFA-induced macrophages was higher than Alum-induced macrophages.

The cDNA microarray confirmed previous results and suggest immunoglobulin-like type 2 receptor alpha (Pilra) as a new marker for M1, macrophage galactose N-acetylgalactosamine-specific lectin 2 (Mgl2) as M2 macrophages marker.

Keywords: Cell Markers; Complete Freund Adjuvant; Cytokines; Hypersensitivity; Inflammatory; Macrophages; Ovalbumin-alum; Th1 cells; Th2 cells

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INTRODUCTION

Helper T (Th) cells release cytokines to regulate the activation of other immune cells in terms of various immune conditions. However, the regulatory mechanisms of Th cells in immune system are still unclear. Functional Th cells have been classified as Th1 and Th2 since the 1980s.¹ Recently, Th cells such as T helper 17 (Th17) cells that release interleukin (IL)-17, and follicular helper T cells that specifically help B cells, have been identified as novel subtypes of Th cells.

Macrophages have been shown to differentiate into two distinct types—M1 and M2—following stimulation by cytokines released from Th1 and Th2 cells, respectively. tumor necrosis factor-alpha (TNF- α) and interferon (IFN)- γ , which are the cytokines mainly produced by Th1, induce M1 differentiation while IL-4, IL-10, and IL-13 which are produced by Th2 or regulatory T cells, promote M2 differentiation.^{2, 3, 4, 5}

Macrophages play pivotal roles in the inflammatory signal pathway in tissues, via release of the pro-inflammatory cytokine; TNF- α that leading to insulin resistance, or the anti-inflammatory cytokine; IL-10 which resulting in down-regulation of insulin resistance⁶. Type I diabetes has been documented as a Th1-dominant disease, in which Kupffer cells exhibit M2 phenotype in response to IL-4, and can be produced by Th2 cells.^{6, 7, 8}

Since the publication of the classification of skin hypersensitive reactions by Gell in 1963², delayed-type hypersensitivity (DTH) has been considered a Th1-dominant immune condition and another DTH-like hypersensitivity has been considered as Th2-dominant immune condition.⁸ In this peritonitis model, DTH or DTH-like hypersensitivity has been induced by complete Freund's adjuvant (CFA) or aqueous solution consisted of aluminum hydroxide and magnesium hydroxide (alum) respectively, in order to generate a large number of macrophages.

We propose a new experimental method for the induction of Th1-dominant hypersensitive peritonitis with the advantage of yielding a large number of M1 macrophages. Moreover, we combined a previous model for Th2-dominant hypersensitive peritonitis with the present model⁹, thereby achieving production of M1 and M2 macrophages via 2 models. Moreover,

we also compared the novel molecular characteristics of these cell types.

MATERIALS AND METHODS

Mice

Male BALB/c mice, 7~8-weeks old, were used for experiments. BALB/c mice were purchased from Kumagai-shigeyasu Co., Ltd, (Japan) and bred under specific pathogen-free conditions at the Animal Research Institute of Tohoku University Graduate School of Medicine, Sendai, Japan. All animal experiments in this study were conducted in accordance with the Tohoku University guidelines for animal experimentation. All the experimental protocols in this study were approved by the Tohoku University Committee for Safety Management of Animals.

Induction of Allergic Inflammation

Allergic inflammation was induced by Albumin made from chicken egg white, OVA, (OVA-alum and OVA-CFA) (THERMO, Rochford, USA and Difco, Michigan, USA) administration to the mouse peritoneal cavity. Total abdominal cells were collected and recovered as previously described.⁹ Production of macrophages in the Th1-immune condition was induced by CFA according to the following steps: first, CFA was lightly sonicated twice (duty cycle, 70%; output control, 3; for approximately 10 seconds once) using a SONIFIER 450 (Branson, Ltd, Japan). One milliliter of the prepared CFA was then mixed with 0.8 ml of PBS and 200 μ L of OVA (2 mg/ml, in PBS). Vigorous mixing (30 min) was necessary in order to emulsify the preparation. Then, 100 μ L of the prepared emulsion was injected subcutaneously (S.C.) into the necks of mice ($n \geq 3$) once on days 0 and then 7. On day 14, the animals were boosted with an intraperitoneal (i. p.) injection of 2 μ g of OVA (in 400 μ L PBS). On day 16, the mice were sacrificed, and peritoneal cells were collected in heparin solution (10 U/ml in PBS containing 0.5% BSA) and peritoneal cells were analyzed using flow cytometry.

Culture and Stimulation of Macrophages

At the indicated time, mice were sacrificed and macrophages were isolated. 10⁶ cells per well were seeded in 12-well plates in RPMI 1640 medium. The

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cells were cultured for 8 hours at 37°C with 5% CO₂ to allow macrophages to adhere to the bottom of the plate. Cell populations were then rinsed 3 times with warm PBS in order to remove suspended cells from adherent macrophages. Adherent macrophages were treated either with 1 ml of 200/400 ng/ml of phorbol 12-myristate 13-acetate (PMA) /ionomycin (PMA/I) (Sigma-Aldrich, Missouri, USA) or 0.1 and 0.01 µg/ml of lipopolysaccharide (LPS) (Sigma-Aldrich) or RPMI-medium and incubated at 37°C with 5% CO₂ for the following 4 and 24 hours, respectively. Four hours following stimulation, half of the cells supernatants were collected prior to centrifugation to remove cells, and then stored at -80°C. Following 4 hours of stimulation, the same volume (half) of fresh reagent was added to the cells and incubated for 24 hours. After 24 hours, the supernatant was collected as described above and stored at -80°C for further experiments.

Flow Cytometry

All antibodies used for flow cytometry were purchased from BioLegend (USA) and staining of macrophages was conducted according to the manufacturer's instructions. Cells were resuspended in propidium iodide solution to separate living cells. Appropriate numbers of cells in suspension (10⁶) were first blocked with anti-mouse blocking solution and stained by macrophage galactose N-acetyl-galactosamine specific lectin-2 (MGL1/2-PE) antibody (1:60 dilution) and then stained with F4/80-FITC (1:100 dilutions). Samples were sorted using a BD FACSCalibur™ flow cytometer (BD Biosciences) for microarray and ELISA analyses. Further data analysis was carried out using BD FACSDiva™ software.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were carried out in accordance with the manufacturer's instructions (R&D Systems). IL-10 and TNF-α levels of Alum and CFA samples were determined by ELISA, and the concentrations were assessed by Berthelot determination methods (ImmunoMini NJ-2300). High-binding 96-well Quantikine ELISA kits (R&D Systems Inc. USA) were used for this assay. Diluent samples (stimulated Alum and CFA-induced macrophages by 0.01 and 0.1 µg/ml LPS), standard, or the positive control of kit was added to each well and incubated for 2 hours at room temperature. Mouse IL-10 conjugate or TNF-α

conjugate were added to each well separately and incubated for 2 hours. Finally, the plates were developed with substrate solution until optimum development occurred at around 30–40 minutes, then the reaction was stopped by stop solution prior to spectrophotometric reading at 450–570 nm.

Gene Expression Level (cDNA Microarray)

Total mRNA was extracted from murine macrophages stimulated by OVA-alum and OVA-CFA and was treated with DNase, using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). The quality and concentration of mRNAs (>1 µg/ml) was confirmed using the spectrophotometer (ND-1000; NanoDrop, USA). Samples were sent to TaKaRa (TAKARA BIO, Inc., Japan) for cDNA microarray analysis. Using the Mouse Genome 430 2.0 Array Chip, approximately 43,000 genes were analyzed by the Affymetrix GeneChip Command Console software (Affymetrix, Santa Clara, USA). Gene expression data were analyzed in terms of global calibration numbers (average signal intensity=500). We conducted 3 independent experiments and selected several genes that had the same character of $|\log_2 [C/A]| \geq 1$.

Statistical Analysis

Values of each group of parametric data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by the Student's *t*-test (two-tailed for independent samples) to determine the differences between each groups. Correlation analysis was conducted using tests of linear regression. $P < 0.05$ was considered to be statistically significant.

RESULTS

Significant Polarization of Macrophages in Mice by Different Adjuvant Injection: CFA and Alum

Macrophages display remarkable plasticity in response to different signals and stimulations. To collect a large numbers of different macrophage types, Alum- and CFA-induced macrophage populations were assessed by flow cytometry (Table 1). Total abdominal cells of BALB/c mice were separated using FSC-A and SSC-A (Figure 1A). The F4/80-FITC positive population was considered as macrophage population (Figure 1A). Finally, the macrophage population coated with F4/80-FITC antibody was collected to assess the specificity of the macrophages induced by Alum or

CFA (Figure 1B). MGL1 and 2 were identified as 2 markers of M2 macrophages.¹⁰ Macrophages induced by Alum showed a peak between 10^3 and 10^4 fluorescence density, whereas macrophages induced by CFA showed a peak around 10^3 fluorescence density. Taken together, the results showed that macrophages induced by Alum were type 2, whereas macrophages induced by CFA were mostly non-M2, maybe M1.

Correlation of Cytokines in Supernatant with Macrophage Type

To understand the diversity functions of macrophages induced by Alum or CFA, various cytokines in cell culture medium were assayed by ELISA following stimulation with LPS. A low dose of LPS (1 ng/ml) has been shown to promote the Th2 immune response.¹¹ The present data shows that Alum-induced macrophages produced higher amounts of IL-10 than CFA-induced macrophages in 0.01

$\mu\text{g/ml}$ LPS (Figure. 2A). In contrast, CFA-induced macrophages produced higher levels of TNF- α than Alum-induced macrophages in 0.1 $\mu\text{g/ml}$ LPS ($p < 0.05$). It must be noted that the numbers of macrophages obtained from individual mice (1×10^6 total abdominal cells per milliliter) induced by Alum or CFA were remarkably different. To resolve this error, this study also investigated the concentration of cytokines produced by macrophages purified from total abdominal cells by BD FACSAriaII (Figure 3).

The result showed macrophages induced by CFA were described as M1, while macrophages induced by Alum mostly were characterized as M2.

DNA Microarray Results and Gene Analysis

This study also investigated the Alum and CFA adjuvant-induced mRNA expression level in total abdominal cells from BALB/c mice by cDNA microarray assay. Genes that were highly expressed or encoded high level of mRNA ($\log_2 (C/A) 2 > 1$) from CFA adjuvant-induced macrophage populations were found to be Th1-immunization inducible genes, such as *Pilra* (Table 2). On the other hand, those ($\log_2 (C/A) 2 < -1$) from Alum adjuvant-induced macrophage populations were found to be Th2-immunization inducible genes (Table 3), like *Mgl2*.

List of New Markers Identified by cDNA Microarray Analysis

For identifying new potential markers for both M1 and M2 macrophages, the following membrane proteins, which may have a receptor activity, have been suggested. Alum-induced macrophages express high level of M2- (Th2)-related genes, such as *Mgl2*, *Slc7a2*, while CFA-induced macrophages express high level of M1-(Th1)-related genes, such as *Pilra* and EGF-like module-containing mucin-like hormone receptor-like sequence 4 (*Emr4*). In summary, *Axl*, component of *Sp100-rs* (*Csprs*), and *Emr4* have been determined to be highly expressed in macrophages induced by CFA, whereas *Gja1*, *Slc7a2*, hyaluronan-mediated motility receptor (*Hmmr*) and G-protein-coupled receptor 183 (*Gpr183*) have been determined to be highly expressed in macrophages produced by induction with alum. The summary of potential biomarkers is shown on table 2.¹¹⁻¹⁴

Table 1. Peritoneal exudate cells in Th₁- or Th₂- immune conditions

Variables		CFA	Alum	P-value
aTotal cell (10^7)	24h	1.88±0.34	0.97±0.36	0.035
	48h	2.47±1.03	1.37±0.42	0.199
	72h	2.02±0.71	1.12±0.00	0.320
bCD16/32*/CCR3*(%)	24h	54.7±4.65	35.6±9.15	0.049
	48h	55.6±12.2	41.0±6.60	0.166
	72h	48.9±1.02	42.3±1.16	0.573
cThe main components				
Eosinophils (%)	24h	54.6±6.11	52.3±2.59	0.590
	48h	63.1±18.6	53.6±4.22	0.474
Neutrophils (%)	24h	24.5±9.08	26.0±4.23	0.813
	48h	14.5±14.9	20.3±4.07	0.573
Lymphocytes (%)	24h	9.17±4.65	6.67±2.31	0.467
	48h	7.00±5.00	9.33±1.53	0.509
Macrophages (%)	24h	10.9±4.68	13.9±4.61	0.464
	48h	15.2±4.80	15.4±9.10	0.950

^{a,b}n=12 ^cCell type was recognized following hematoxylin-eosin staining, n=6

Optimising the experiment under Th1 and Th2 conditions with different time tables. (a) Total peritoneal cell, (b) Immune cell populations by flow cytometry: CD16 (Fc gamma III Receptor) and (Fc gamma II Receptor) are the low affinity receptors for the mouse IgG Fc portion and are expressed by B cells, monocyte/macrophages, NK cells, and neutrophils. C-C chemokine receptor type 3 (CCR3) is detected in Th1, Th2, eosinophils and basophils. (c) Percentage of various cells in immune cell population.

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Table 2. List of potential biomarkers

Gene	Symbol	Log ₂ [C/A] ²	Molecular function	Associated Disease
AXL receptor tyrosine kinase	<i>axl</i>	1.25	Receptor activity	No-small cell lung cancer,
Paired immunoglobulin-like type 2 receptor alpha	<i>pilra</i>	1.17	Receptor activity	Herpes Virus entry receptor
EGF-like module containing, mucin-like, hormone receptor-like sequence 4	<i>emr4</i>	1.04	Transmembrane receptor activity	Alzheimer`s disease and AIDS
Gap junction protein, alpha 1	<i>gjal</i>	-1.16	Transmembrane receptor activity	Gland tumor metastasis
G-protein-coupled receptor	<i>gpr183</i>	-1.27	receptor activity	Tumorigenesis and metastasis of human cancers,
Solute carrier family 7 (cationic amino acid transporter, y ⁺ system), member 2	<i>slc7a2</i>	-1.60	L-ornithine Transmembrane transporter activity	HIV-1 and MLV
183 Hyaluronan mediated motility receptor (RHAMM)	<i>hmmr</i>	-2.33	receptor activity	Aggressive fibromatosis (desmoids tumor)

AIDS: Acquired immune deficiency syndrome; HIV: Human immunodeficiency virus infection; MLV: Murine leukemia virus

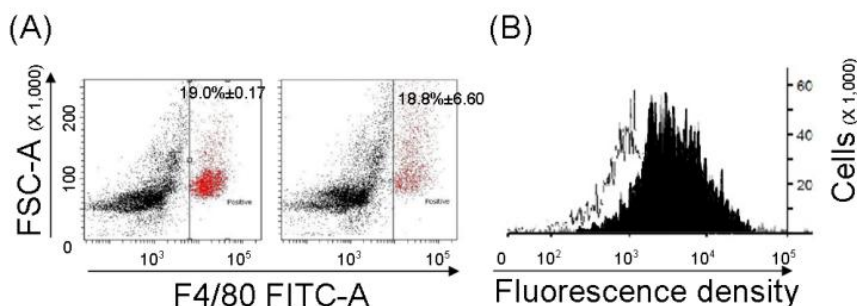


Figure 1. Cell population in Alum and CFA (A) BALB/c mouse peritoneal cells separated using FSC-A and SSC-A. Cells were stained with F4/80-FITC antibody, CFA population showed in (A) left and Alum in (A) right side. (B) F4/80-FITC positive population in Alum and CFA were confirmed by MGL 1/2-PE antibody, CFA peak shown in white and Alum in black color.

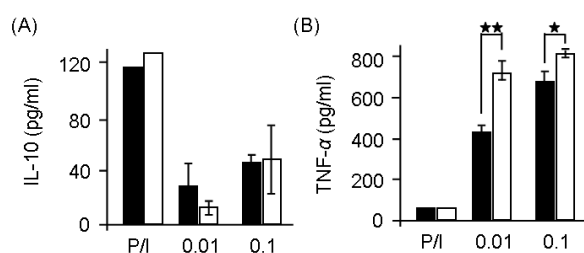


Figure 2. Cytokines of LPS-stimulated CFA or Alum-induced macrophages, LPS (0.01 or 0.1 µg/ml) was added to the medium for 4 or 24 hours. PMA/I was used as a positive control. (A) IL-10 levels in cell culture medium after 24 hours of stimulation, as determined by ELISA. (B) TNF-α level in cell culture medium after stimulation for 4 hours, as determined by ELISA. White column indicates Alum samples and black indicates CFA samples. Bars represent mean ± SD for each mouse and the grouped data (p* < 0.05, ***p* < 0.01, t test (n = 3)).**

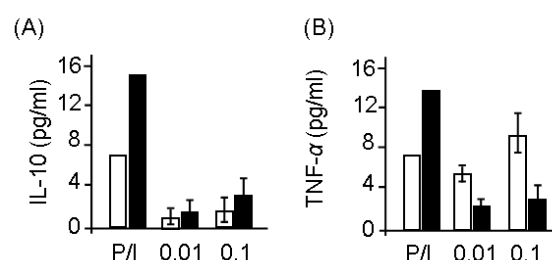


Figure 3. Cytokines of macrophages purified by FACS, Approximately 10⁶ macrophages were purified by F4/80 antibody. (A) Level of IL-10 in cell culture medium after 24 hours of stimulation, as determined by ELISA. (B) TNF-α level in cell culture medium after 4 hours of stimulation, as determined by ELISA. White indicates Alum samples and black indicates CFA samples. Bars represent mean ± SD for each mouse and the grouped data (p* < 0.05, t test (n = 3)).**

Table 3. List of genes showing augmented expression in Th2-immunization

Gene Title	Gene Symbol	log ₂ [C/A]	Chr. Location	Molecular Function	Pathway
sperm associated antigen 5	<i>Spag5</i>	-2.67	chr11 B5 11 44.95 cM	microtubule binding	Cell cycle // Oocyte meiosis // p53 signaling pathway // Progesterone-mediated oocyte maturation
cyclin B1	<i>Ccnb1</i>	-2.45	chr13 D1	protein binding // kinase activity // histone kinase activity	double-stranded DNA binding // single-stranded DNA binding // RNA
RAD51 associated protein 1 (RHAMM)	<i>Rad51ap1</i>	-2.41	chr6 F3 6 61.3 cM	binding	hyaluronan mediated motility receptor
	<i>Hmnr</i>	-2.33	chr11 A5 11 19.0 cM	receptor activity // hyaluronic acid binding	ECM-receptor interaction
denticless homolog (Drosophila)	<i>Dtl</i>	-2.32	chr1 H6	protein binding	Cell cycle
Shc SH2-domain binding protein 1	<i>Shcbp1</i>	-2.32	chr8 A1.2	protein binding // SH2 domain binding	
thymidine kinase 1	<i>Tk1</i>	-2.32	chr11 E1-E2 11 78.0 cM	nucleotide binding	// thymidine kinase activity // ATP binding Pyrimidine metabolism // Drug metabolism - other enzymes // Metabolic pathways
ubiquitin-like, containing PHD and RING finger domains, 1	<i>Uhrf1</i>	-2.18	chr17 D-E1	DNA binding // protein binding /	/ inferred from electronic annotation // ligase activity/budding uninhibited by benzimidazoles 1
homolog, beta (S. cerevisiae)	<i>Bub1b</i>	-2.18	chr2 E5	nucleotide binding // protein serine	/threonine kinase activity // ATP binding
ribonucleotide reductase M2	<i>Rrm2</i>	-2.16	chr12 A1.3 12 7.0 cM	reductase activity // metal ion binding Purine metabolism //	Pyrimidine metabolism // Glutathione metabolism // Metabolic pathways // p53 signaling pathway
kinesin family member 23	<i>Kif23</i>	-2.16	chr9 B	nucleotide binding	// microtubule motor activity // ATP binding
maternal embryonic leucine zipper kinase	<i>Melk</i>	-2.16	chr4 B1 4 26.7 cM	nucleotide binding	// protein serine/threonine kinase activity // ATP binding
baculoviral IAP repeat-containing 5	<i>Birc5</i>	-2.14	chr11 E2	peptidase inhibitor activity Pathways in cancer // Colorectal cancer	cysteine-type endopeptidase inhibitor activity // microtubule binding //
(MIS5 homolog, S. pombe) (S. cerevisiae)	<i>Mcm6</i>	-2.07	chr1 E4 1 66.6 cM	nucleotide binding // DNA helicase activity // single-stranded DNA binding	DNA replication // Cell cycle
Ttk protein kinase	<i>Ttk</i>	-2.01	chr9 E2	nucleotide binding // protein serine/threonine kinase	Cell cycle
peptidylprolyl isomerase (cyclophilin) like 5	<i>Ppil5</i>	-2	chr12 C3	protein binding // isomerase activity	
cyclin A2	<i>Ccna2</i>	-2	chr3 B 3 19.2 cM	protein binding	Cell cycle // Progesterone-mediated oocyte maturation
cyclin B2	<i>Ccnb2</i>	-1.94	chr9 D	0005515 // protein binding // inferred from electronic annotation	Cell cycle // Oocyte meiosis // p53 signaling pathway // Progesterone-mediated oocyte maturation
carbonic anhydrase 4	<i>Car4</i>	-1.94	chr11 C	carbonate dehydratase activity // zinc ion binding // lyase activity	Nitrogen metabolism

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budding uninhibited by benzimidazoles 1homolog (S. cerevisiae)	<i>Bub1</i>	-1.91	chr2 F1 2 73.0 cM	nucleotide binding // protein serine/threonine kinase activity // ATP binding	Cell cycle // Oocyte meiosis // Progesterone-mediated oocyte maturation
ubiquitin-conjugating enzyme E2C	<i>Ube2c</i>	-1.88	chr2 H3 2 93.0 cM	nucleotide binding // ubiquitin-protein ligase activity // ATP binding	Ubiquitin mediated proteolysis
PDZ binding kinase	<i>Pbk</i>	-1.88	chr14 D1 14 28.0 cM	nucleotide binding // protein serine/threonine kinase activity // ATP binding	
DNA segment, Chr 17, human D6S56E5	<i>D17H6S56E-5</i>	-1.83	chr17 B1 17 19.0 cM	structural molecule activity	
chemokine (C-C motif) ligand 7	<i>Ccl7</i>	-1.83	chr11 C 11 46.5 cM	cytokine activity // chemokine activity	Cytokine-cytokine receptor interaction // Chemokine signaling pathway
nucleolar and spindle associated protein 1	<i>Nusap1</i>	-1.8	chr2 E5	DNA binding // microtubule binding	
cystatin F (leukocystatin)	<i>Cst7</i>	-1.8	chr2 G1-G3	cysteine-type endopeptidase inhibitor activity // peptidase inhibitor activity	
RIKEN cDNA 2610021K21 gene	<i>2610021K21Rik</i>	-1.8	chr12 F1	calcium ion binding	
chemokine (C-C motif) ligand 2	<i>Ccl2</i>	-1.79	chr11 C-E1 11 46.5 cM	cytokine activity // chemokine activity // CCR2 chemokine receptor binding	Cytokine-cytokine receptor interaction // Chemokine signaling pathway
antigen identified by monoclonal antibody Ki 67	<i>Mki67</i>	-1.79	chr7 F3-F5	protein C-terminus binding	
polymerase I and transcript release factor	<i>Ptrf</i>	-1.79	chr11 D 11 60.5 cM	protein binding	RNA polymerase I transcription termination factor activity // RNA binding // PPAR signaling pathway
fatty acid binding protein 5, epidermal	<i>Fabp5</i>	-1.76	chr3 A1-3	transporter activity // fatty acid binding // lipid binding	
shugoshin-like 1 (S. pombe)	<i>Sgoll</i>	-1.75	chr17 C	protein binding	Oocyte meiosis
cell division cycle 20 homolog (S.cerevisiae)	<i>Cdc20</i>	-1.75	chr4 D2.1	protein binding // protein C-terminus binding	Cell cycle // Oocyte meiosis // Ubiquitin mediated proteolysis
asp (abnormal spindle)-like, microcephaly associated (Drosophila)	<i>Aspm</i>	-1.7	chr1 F	calmodulin binding	
cyclin-dependent kinase inhibitor 3	<i>Cdkn3</i>	-1.69	chr14 C1	protein serine/threonine phosphatase activity // protein binding	
Zwilch, kinetochore associated, homolog (Drosophila)	<i>Zwilch</i>	-1.68	chr9 D	kinesin family member 20A protein binding	
RAD51 homolog (S. cerevisiae)	<i>Rad51</i>	-1.68	chr2 F1 2 66.8 cM	DNA binding	// damaged DNA binding // DNA-dependent ATPase activity Homologous recombination // Pathways in cancer // Pancreatic cancer
glutaminy-peptide cyclotransferase (glutaminy cyclase)	<i>Qpct</i>	-1.67	chr17 E3	cyclotransferase activity	peptidase activity // acyltransferase activity // glutaminy-peptide

chemokine (C-C motif) ligand 12	<i>Ccl12</i>	-1.66	chr11 C 11 47.0 cM	cytokine activity	// chemokine activity Cytokine-cytokine receptor interaction // Chemokine signaling pathway
TPX2, microtubule-associated protein homolog (Xenopus laevis)	<i>Tpx2</i>	-1.63	chr2 H2	kinase binding // inferred from physical interaction	0019901 // protein kinase binding // not recorded // 0019901 // protein nucleotide binding // protein serine/threonine kinase activity // histone
cyclin-dependent kinase 1	<i>Cdk1</i>	-1.63	chr10 B5.3 10 38.0 cM	kinase activity Cell cycle	// Oocyte meiosis // p53 signaling pathway // Gap junction // Progesterone-mediated oocyte maturation
cell division cycle associated 8	<i>Cdca8</i>	-1.63	chr4 D2.2 4 57.6 cM	protein binding	
SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	<i>Spe25</i>	-1.62	chr2 C3	protein binding	
non-SMC condensin I complex, subunit D2	<i>Ncapd2</i>	-1.61	chr6 F2	histone binding	
kinesin family member 11	<i>Kif11</i>	-1.61	chr19 C2	nucleotide binding // microtubule motor activity // ATP binding	
diaphanous homolog 3 (Drosophila)	<i>Diap3</i>	-1.6	chr14 D3	actin binding // Rho GTPase binding	Regulation of actin cytoskeleton solute carrier family 7 (cationic amino acid L-ornithine transmembrane transporter activity // high affinity arginine
transporter, y+ system), member 2	<i>Slc7a2</i>	-1.6	chr8 A4 8 21.0 cM	transmembrane transporter activity	cysteine-type endopeptidase inhibitor activity // SH3 domain binding // heat
pituitary tumor-tripartite motif-containing 59	<i>Pttg1</i>	-1.6	chr11 A5	Cell cycle // Oocyte meiosis	shock protein binding
protein regulator of cytokinesis 1	<i>Prcl</i>	-1.58	chr7 D3 7 38.0 cM	protein binding	
insulin-like growth factor 1	<i>Igf1</i>	-1.57	chr10 C1 10 48.0 cM	growth factor activity Progesterone-mediated oocyte maturation // Aldosterone-regulated sodium reabsorption // Pathways in cancer //	Oocyte meiosis // p53 signaling pathway // mTOR signaling pathway // Focal adhesion // Long-term depression //
topoisomerase (DNA) II alpha	<i>Top2a</i>	-1.56	chr11 D 11 57.0 cM	DNA binding // chromatin binding // DNA topoisomerase activity	Glioma // Prostate cancer // Melanoma // Hypertrophic cardiomyopathy (HCM) // Dilated cardiomyopathy
stathmin 1	<i>Stmn1</i>	-1.56	chr4 D3 4 65.7 cM	protein binding // tubulin binding	MAPK signaling pathway
kinetochore	<i>Kntc1</i>	-1.54	chr5 F	protein binding	
aurora kinase A	<i>Aurka</i>	-1.54	chr2 H3 2 100.0 cM	nucleotide binding // protein serine/threonine kinase activity // ATP binding	Oocyte meiosis
cell division cycle 20 homolog (S.cerevisiae)	<i>Cdc20</i>	-1.54	chr4 D2.1	protein binding // protein C-terminus binding	Cell cycle // Oocyte meiosis // Ubiquitin mediated proteolysis

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kinesin family member 22	<i>Kif22</i>	-1.53	chr7 F3 7 61.0 cM	DNA binding // microtubule motor activity // ATP binding	nucleotide binding // microtubule motor activity // plus-end-directed
macrophage galactose N-acetyl-galactosamine specific lectin 2	<i>Mgl2</i>	-1.43	Chr11 B3	Sugar binding // carbohydrate binding// purine-nucleotide binding	ribonucleoside-diphosphate reductase activity// G-protein coupled
G protein-coupled receptor 183 essential meiotic endonuclease 1 homolog 1	<i>Gpr183</i>	-1.27	Chr14 E5	G-protein coupled receptor activity// purinergic nucleotide receptor activity	G-protein coupled

^aValues represents an average of three logarithmic values calculated from DNA microarray data. The C and A denote the signal intensity values resulted from CFA (Th1)- and Alum (Th2)-immunization, respectively.

^bThe assignments are based on Mouse Genome Informatics, the Jackson Laboratory (MGI).

^cThe descriptions are cited from the Gene Ontology database of MGI.

Table 4. List of genes showing augmented expression in Th1-immunization

Gene Title	Gene Symbol	log [C/A] ^a	Chr. Location-	Molecular Function-	Pathway
cDNA sequence AF251705	<i>AF251705</i>	2.07	chr11 E2 11 78.0 cM	receptor activity	
hydroxyacyl-Coenzyme A dehydrogenase	<i>Hadh</i>	1.59	chr3 G3	protein binding// coenzyme binding	
component of Sp100-rs	<i>Csprs</i>	1.54	chr1 C5 /// chr8 B1.3	receptor activity	
histocompatibility 2, class II antigen A, alpha	<i>H2-Aa</i>	1.43	chr17 B1 17 18.65 cM	protein binding // peptide antigen binding	
CD8 antigen, alpha chain	<i>Cd8a</i>	1.42	chr6 C 6 30.5 cM	protein binding // protein homodimerization activity	
complement factor B	<i>Cfb</i>	1.34	chr17 B1 17 18.85 cM	serine-type endopeptidase activity // hydrolase activity	
lysophosphatidylcholine acyltransferase 2	<i>Lpcat2</i>	1.32	chr8 C5	calcium ion binding // acyltransferase activity // 1-	
cytochrome P450, family 7, subfamily b, polypeptide 1	<i>Cyp7b1</i>	1.31	chr3 A 3 1.0 cM	monooxygenase activity // oxysterol 7-alpha-hydroxylase activity // heme	
cyclin D2	<i>Ccnd2</i>	1.28	chr6 F3 6 61.1 cM	protein binding // protein kinase binding	
AXL receptor tyrosine kinase	<i>Axl</i>	1.25	chr7 A3-B1 7 6.0 cM	binding	
calponin 3, acidic /// similar to calponin 3, acidic	<i>cnn3</i>	1.22	chr3 G1	acylglycerophosphocholine O-acyltransferase activity	
acidic	<i>Lrg1</i>	1.21	chr17 D 17 10.0 cM		
leucine-rich alpha-2-glycoprotein 1 Nuclear receptor coactivator 7 Calcium/calmodulin-dependent protein kinase ID	<i>Ncoa7</i>	1.19	chr10 A4	actin binding // calmodulin binding // microtubule binding	

Calcium/calmodulin-dependent protein kinase ID	<i>Camk1d</i>	1.19	chr2 A1	nucleotide binding // protein serine/threonine kinase activity // calmodulin-dependent protein kinase activity
paired immunoglobulin-like type 2 receptor alpha	<i>Pilra</i>	1.17	chr5 G2	receptor activity // protein binding
interferon activated gene 205 /// myeloid cell nuclear differentiation antigen	<i>Ifi205</i>	1.14	chr1 H3 /// chr1 H3 1 95.3 cM	protein binding
transforming growth factor, beta 2	<i>Tgfb2</i>	1.12	chr1 H5 1 101.5 cM	receptor binding // transforming growth factor beta receptor binding // growth factor activity
E1A binding protein p400 complement component 4B (Chido blood group)	<i>Ep400</i>	1.1	chr5 F	nucleic acid binding // helicase activity // ATP binding
	<i>C4b</i>	1.06	chr17 B1 17 18.8 cM	endopeptidase inhibitor activity // protein binding
inhibitor of DNA binding 2	<i>Id2</i>	1.05	chr12 B 12 7.0 cM	protein binding // transcription repressor activity // transcription regulator activity
SET domain containing 1A	<i>Setd1a</i>	1.04	chr7 F3	nucleic acid binding // protein binding // methyltransferase activity
EGF-like module containing, mucin-like, hormone receptor-like sequence 4	<i>Emr4</i>	1.04	chr17 C-D 17 34.3 cM	transmembrane receptor activity // G-protein coupled receptor activity // calcium ion binding

^aValues represents an average of three logarithmic values calculated from DNA microarray data. The C and A denote the signal intensity values resulted from CFA (Th1)- and Alum (Th2)-immunization, respectively.

^bThe assignments are based on Mouse Genome Informatics, the Jackson Laboratory (MGI).

^cThe descriptions are cited from the Gene Ontology database of MGI.

DISCUSSION

Although another novel classification of macrophage has been discussed in detail (Classically activated macrophage, regulatory macrophage and wound-healing macrophage) by David M. Mosser and Justin P. Edwards (2008), we still use the classic classification of activated macrophage (M1 and M2 macrophages). We have been interested in understanding the relationship between the phenotype of allergic reaction and activated macrophages. The common understanding about the role of activated macrophages is that M1 macrophages produce inflammatory cytokines, such as TNF, to respond to IFN by Th1 cells while M2 macrophages produce anti-inflammatory cytokines, such as IL-10, to respond to various stimuli, including IL-4 produced by Th2 cells. Recently, a novel model of “phenotypic switch” has been reported by Lumeng et al. During adiposity,

subtypes of adipose tissue macrophages shift to M1 phenotype, while macrophages switch to M2 phenotype in insulin-sensitive state.

The “phenotypic switch” model gave a possible mechanism for explaining accumulation of adaptive type of activated macrophages in tissue according to immune environment. However, the mechanisms of activated macrophage under allergic reaction are still not completely understood. We attempted to demonstrate an accumulation of activated macrophages under DTH or DTH-like allergic conditions, respectively. FACS data suggested that this is a useful way that leads us to separate two distinct macrophages to do more experiments with purified macrophages. An overlap of data implied that macrophages induced by CFA were described as M1, whereas most macrophages induced by Alum were M2 (Figures 1, 2 and 3). In addition, PMA/I-positive control of CFA samples in IFN- γ data and 0.1 μ g/ml LPS-stimulated

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macrophages of CFA samples in IL-12 data were read as positive (data not shown). It is suggested that macrophages from CFA samples have the characteristics of M1 macrophages, at least under 0.1 µg/ml LPS conditions.

Finally, DNA microarray data showed that a large number of genes were transcribed following CFA or Alum stimulation. Among these genes (table 3 and 4), genes encoding proteins with molecular function, receptor activity and membrane-expressed proteins have been identified as novel markers for either M1 or M2 macrophages (Table 2). Mgl2 (table 3) is reported as an M2 macrophage marker, and Mgl2 ($\log_2(C/A)2 = -1.43$), Hmnr, Slc7a, and Gpr183 may be potential markers for M2 macrophages. Axl, Pilra, and Emr4 are potential markers for M1 macrophages.

It is interesting to know the biological and physiological functions of novel markers suggested in this study for M1 and M2 macrophages. Briefly describing the characteristic of markers in immunity provides new view of these macrophage markers.

Axl encodes a protein named "AXL receptor tyrosine kinase" in Homo sapiens, which is a chronic myelogenous leukemia-associated oncogene and also associated with colon cancer and melanoma. PILRa, paired immunoglobulin-like type 2 receptor alpha, is an immune inhibitory receptor. PILRalpha is a herpes simplex virus-1 entry co-receptor that associates with glycoprotein B. Control of cell signaling via SHP-1 is thought to occur through a balance between PILRa-mediated inhibition and PIRLb-mediated activation. EMR4 promotes the exchange of HDP for GTP on the alpha subunit of a heterotrimeric G-protein complex in Alzheimer's disease and AIDS.¹²⁻¹⁷

G-protein-coupled receptor EBI2, GPR183, is one of chemokine receptors expressed in human cancers which controls migration of B cells within lymphoid follicles. In all of Th1-immune or Th2-immune conditions, macrophages require the uptake of exogenous arginine to meet their metabolic demands. Slc7a2, solute carrier family 7 (cationic amino acid transporter, y+ system), member 2, encodes a retroviruses-associated, such as HIV-1 and murine leukemia virus (MLV) transporters (CAT) 2, which could limit the function of macrophages by strong regulation of arginine transport level. The aggressive fibromatosis-associated protein encoded by Hmnr gene, hyaluronan-mediated motility receptor (RHAMM), is involved in cell motility.¹⁸⁻²³

According to our data, a possible mechanism is to consider that activated macrophages may trigger the allergic reaction following accumulation. Moreover, to response to stimuli, Th1- or Th2-immune condition induces macrophage's activation following accumulation by "phenotype switch". M1 macrophages express genes such as Pilra to suppress the immune reaction, which cause pro-inflammation response. Accumulated M2 may highly express genes such as Slc7a2 to develop the inflammatory allergic reaction. However, it is still not clear whether activated macrophage is one of the pathological reasons of allergic reaction, and if these biomarkers were useful in human need to be carefully investigation.

ACKNOWLEDGEMENTS

This study was fully supported by Tohoku University Graduate School of Medicine, Sendai, Japan. We would like to thank Dr. Nosei Inuie and Mr. Zhongbo Li for their assistance.

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