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Identification of Genes Involved in the Suppression of Antibody Production from Human Peripheral Blood Lymphocytes

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Pretreatment with L-leucyl-L-leucine methyl ester (LLME) is a prerequisite for peripheral blood mononuclear cells (PBMCs) to produce antigen-specific antibodies when sensitized with an antigen. Little information, however, is available regarding the mechanisms involved in LLME-induced augmentation of antibody production from PBMCs that are antigen sensitized. In the present study, we attempted to identify the genes involved in the suppression of antibody production from PBMCs that was not treated with LLME, but sensitized with an antigen. Using subtractive screening, we obtained 63 independent genes, including 17 EST genes, that are specific for LLME-nontreated PBMC. Among these genes, the expression of heavy chain ferritin (H-ferritin), CC chemokine ligand 18 (CCL18), and matrix metalloproteinase 12 (MMP12) were augmented in LLME-nontreated PBMCs, suggesting that inflammatory factors might be involved in the suppression of antibody production in LLME-nontreated PBMCs.

Key words: L-leucyl-L-leucine methyl ester; *in vitro* immunization; antibody production

Monoclonal antibodies (mAb) bind to target molecules with high specificity and affinity. Hence, they are widely used in the diagnosis of and therapies for various diseases such as cancer, rheumatoid arthritis, and allergies. But, it is important to use human mAb to avoid undesirable side effects in patients. To generate human mAb *in vitro*, we developed an *in vitro* immunization protocol using human peripheral blood mononuclear cells (PBMCs). The *in vitro* immunization protocol used to induce antigen-specific antibody production from PBMCs consisted of two steps, pretreatment of PBMCs with L-leucyl-L-leucine methyl ester (LLME), and sensitization of PBMCs with the antigen in the presence of muramyl dipeptide (MDP), interleukin (IL)-2, and IL-4.[1)]

LLME is a lysosomotropic reagent that selectively eliminates monocytes, cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and $CD8^+$ T cells but not B cells and $CD4^+$ T cells from human PBMCs.[2–5)] Although LLME is known to show toxic effects against these cells by inducing lysis and apoptosis, little information is available regarding the causes for induction of antibody production from PBMCs immunized *in vitro* after LLME treatment.[6)] In the present study, we attempted to identify the genes involved in the suppression of antibody production from PBMCs immunized *in vitro*, and to present the possible mechanisms for this suppression.

Materials and Methods

Cell preparation. PBMCs were isolated from peripheral blood of healthy volunteers by Ficoll density gradient centrifugation using a lymphocyte separation medium (LSM; Organon Teknika, Durham, NC). The PBMC were treated for 20 min with 0.25 mM LLME (Bachem, Torrance, CA) in ERDF medium (Trace Scientific, Melbourne, Australia) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

In vitro immunization. LLME-treated PBMCs and nontreated PBMCs (5×10^6 cells each) cultured in ERDF medium supplemented with 10% FBS were sensitized with mite extract (ME; $10\,\mu g \cdot ml^{-1}$; LSL, Tokyo) along with $10\,U \cdot ml^{-1}$ IL-2 (Genzyme, Cambridge, MA), $1\,\mu g \cdot ml^{-1}$ IL-4 (PeproTech, London, UK), 50 mM 2-mercaptoethanol (Life Technologies, Grand Island, NY), and $10\,\mu g \cdot ml^{-1}$ MDP (Bachem, Bubendorf, Switzerland).

Enzyme-linked immunosorbent assay (ELISA). The concentration of antibodies secreted into the culture supernatant was determined by ELISA. In brief, 96-well

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microtiter plates (Nunc, Naperville, IL) were coated with anti-human IgM or IgG antibodies (Tago, Burlingame, CA) for 2 h at 37 °C. After blocking the plates with 1% ovoalbumin (Wako) in 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl (PBS) for 2 h at 37 °C, culture supernatants were added to the plates and incubated overnight at 4 °C. The captured IgM and IgG were detected by adding horseradish peroxidase-conjugated goat anti-human IgM or IgG antibodies (Tago) and analyzing the subsequent reaction using 0.3 mg·ml^{-1} 2.2′-azinobisdiammonium salt substrate solution (Wako) in 0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2. The absorbance at 405 nm was measured using an ELISA reader.

RT-PCR. Total RNA was prepared from the PBMCs using a GenElute mammalian total RNA kit (Sigma, St. Louis, MO). Total RNA (500 ng) was used as a template for cDNA synthesis reaction in a total volume of 25 µl using MMLV reverse transcriptase (Promega, Madison, WI). Subsequently, PCR was performed using 1 µl of the cDNA synthesis reaction mixture and specific primers. The PCR primers were as follows: H-ferritin-sense, 5′-ACTACTTTGACCGCGATGATG-3′; H-ferritin-antisense, 5′-AAACCCCAACATGCATGCACT-3′; MHC class II DR α-sense, 5′-CATAGCTGTGCTGATGAGCG-3′; MHC class II DR α-antisense, 5′-GGAGATAGTGGGAACTTGAGG-3′; β-actin-sense, 5′-ACCCACACTGTGCCCATCTA-3′; β-actin-antisense, 5′-CGGAACCGCTCATTGCC-3′; CCL18-sense, 5′-ATGAAGGGCCTTGCAGCTGCCCT-3′; CCL18-antisense, 5′-TCAGGCATTCAGCTTCAGGTCGC-3′; KBF2-sense, 5′-TCAGGCATTCAGCTTCAGGTCGC-3′; KBF2-antisense, 5′-TCAGGCATTCAGCTTCAGGTCGC-3′; MMP12-sense, 5′-ACTGCTTCTGGAGCTCTTCC-3′; MMP12-antisense, 5′-CGTAGTCAACATCCTCACGG-3′. Amplification was performed through 25–30 PCR cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min). Reaction products were separated by polyacrylamide gel electrophoresis on a 4% gel and stained using SYBR Gold (Molecular Probes, Eugene, OR).

Subtraction. Poly $(A)^+$ RNA was prepared from LLME-treated and nontreated PBMCs with a Quick Prep Micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ). cDNA libraries were constructed with a SMART cDNA synthesis kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol, and tester and driver cDNA were synthesized using specific primers included in the PCR-Select cDNA subtraction kit (Clontech). We then subtracted driver cDNA from tester cDNA and specifically amplified the subtracted cDNA fragments with the primers used for preparation of the tester and driver cDNA. The detailed schematic protocols were provided in the instruction manual supplied with the PCR-Select cDNA subtraction kit. The amplified fragments were purified and ligated to pGEM-T Easy vector (Promega). The sequence of fragments was determined using a DNA sequencer (3100 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA).

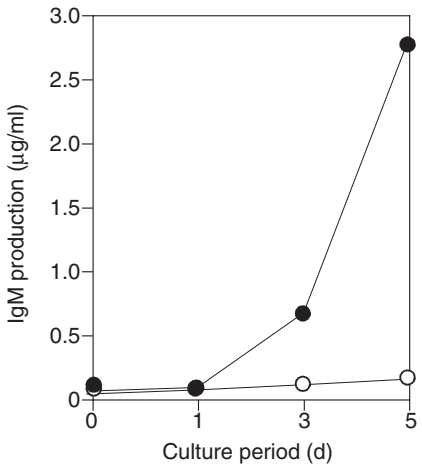

Fig. 1. Treatment with LLME Induces Antibody Production from PBMCs Immunized *in Vitro*.

LLME-treated (●) and nontreated (○) PBMCs were sensitized with mite extract in the presence of IL-2 (10 U·ml^{-1}), IL-4 (10 ng·ml^{-1}), and MDP (10 µg·ml^{-1}). After 1, 3, and 5 d of culture, the amount of IgM antibody secreted into the supernatant was determined by sandwich ELISA.

Results and Discussion

LLME treatment induces antibody production in PBMCs sensitized with antigen

Previously we reported that LLME-treated (LLME(+)) PBMCs, but not nontreated (LLME(−)) PBMCs, induce antibody production when sensitized with an antigen (Fig. 1). In this study, we attempted to identify the suppressive factors involved in the suppression of antibody production in LLME(−)-PBMCs sensitized with an antigen.

LLME(+)- and LLME(−)-PBMCs were collected at days 0, 1, and 3 after *in vitro* immunization and subjected to subtractive screening. The cDNA libraries prepared from LLME(+)- and LLME(−)-PBMCs were used as the driver cDNA and tester cDNA respectively, and independent cDNA clones were cloned from these. Clones obtained by subtracting a cDNA library prepared from LLME(+)-PBMCs from one from LLME(−)-PBMCs should be by derived from the cells removed by the LLME treatment. As a result of sequence determination and homology search analysis against Genbank using the BLAST program, we identified 49 cDNA clones and classified them to 36 independent cDNA clones, including 13 EST clones (Table 1). These clones were obtained by subtracting that prepared from LLME(+)-PBMCs from the cDNA prepared from LLME(−)-PBMCs; this suggests that these genes are involved in the suppression of antibody production from LLME(−)-PBMCs. Next, the cDNAs prepared from LLME(−)-PBMC immunized *in vitro* and subsequently

Table 1. Identification of the Subtracted cDNA Clones

Tester	Driver	Clone identified	Accession no.
LLME(−)	LLME(+)		
0d	0d	Ferritin, heavy polypeptide1, mRNA	NM_002032
		Major histocompatibility complex, class II, DR alpha, mRNA, complete cds	BC032350
		Heat shock protein HSP70 (HSPA7) gene, complete sequence	AF093759
		Poly (rC) binding protein2 (PCBP2), transcript variant 1, mRNA	NM_005016
		Ribosomal protein L7a (RPL7A), mRNA	NM_000972
		DC48 mRNA, complete cds	AF271776
		Solute carrier family 7, member7, mRNA, complete cds	BC010107
		Thrombospondin-1 gene, partial cds	U12471
		EST sequence	AC022692
		EST sequence	AC026696
		EST sequence	AC129913
1d	1d	A disintegrin and metalloproteinase domain 19 (ADAM19), trascript variant 2, mRNA	NM_033274
		Exportin5 (XPO5), mRNA	NM_020750
		Chemokine (C–C motif) ligand 18 (pulmonary andactivation-regulated) (CCL18), mRNA	NM_002988
		Zinc finger protein 44 (KOX 7), mRNA, complete cds	BC032246
		Membrane spanning 4-domains, subfamily A, memaber 6A (MS4A6A), transcript variant 1, mRNA	NM_152852
		Ribonuclease RNase A family, 1 (pancreatic), transcript variant 4, mRNA, complete cds	BC005324
		Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (p105), mRNA, complete cds	BC051765
		Utophin (homologous to dystrophin) (UTRN), mRNA	NM_007124
		Etoposide induced 2.4 mRNA (EI24), transcript variant 1, mRNA	NM_004879
		SEC3-like 1 (S. cerevisiae) (SEC3L1), transcript variant 1, mRNA	BC02050
		EST sequence	AC008039
		EST sequence	BC000116
		EST sequence	BX640634
		EST sequence	BC032220
3d	3d	Inositol polyphosphate-4-phosphatase, type II, 105 kDa (INPP4B), mRNA	NM_003866
		Matrix metallopeptidase 12 (macrophage elastase) (MMP12), mRNA	NM_002426
		Actin, beta, mRNA, complete cds	BC001301
		H2K binding factor 2 (KBF2) mRNA, complete cds	L08904
		SH2 domin containing molecule 1B, mRNA, complete cds	BC022407
		AT rich interactive domain 4B (RBP1-like) (ARID4B), transcript variant 1, mRNA	NM_016374
		Basic transcription factor 3a (BTF3a) gene, complete cds	M90357
		Adenylate cyclase 7 (ADCY7), mRNA	NM_001114
		ADP-ribosylation-like factor 6 interacting protein 5, mRNA, complete cds	BC020797
		Ferritin, heavy polypeptide1, mRNA	NM_002032
		EST sequence	AL137792
		EST sequence	NM_032196
LLME(−)	LLME(+)		
0d	1d	Phosphoglycerate kinase (PGK1) mRNA, partial cds	L00160
		Ribosomal protein L14, mRNA	BC016379
		Ferritin, heavy polypeptide1, mRNA, complete cds	BC063514
		EST sequence	BC008322
		EST sequence	AC019206
0d	3d	IL-1 receptor-associated-kinase-M mRNA, complete cds	AF113136
		HSF protein (EPB41L3) mRNA, partial cds	AF515797
		Chemokine (C–C motif) ligand2 (CCL2) mRNA	NM_002982
		mRNA for lectin-like oxidized LDL receptor, complete cds	AB010710
		Thrombondin1 (THBS1), mRNA	NM_003246.2
		EST sequence	AL832212
		EST sequence	BC015134

Continued on next page.

cultured for different periods were used for subtractive screening to identify the genes involved in the maintenance of suppressed antibody production in the LLME(−)-PBMCs sensitized with an antigen. We identified 11 independent cDNA clones, including 3 EST clones (Table 1).

Inflammation factors were involved in the suppression of antibody production in nontreated PBMCs

We selected five genes, *viz.*, MHC class II DRα, H2K binding factor 2 (KBF2), chemokine ligand 18 (CCL18), matrix metalloproteinase 12 (MMP12), and ferritin heavy chain (H-ferritin), cloned by the subtractive

Continued.

LLME(−) 1d	LLME(−) 0d	Major histocompatibility complex, class II, DR alpha (HLA-DRA), mRNA	NM_019111
		Heat shock 70 kDa protein 8 (HSPA8), transcript variant 2, mRNA	NM_153201
3d	0d	mRNA for pleckstrin (p47)	X07743
		BSCv mRNA, partial cds	AB033767
		CD69 antigen (p60, early T-cell activation antigen), mRNA, complete cds	BC007037
		Chondroitin sulfate proteoglycan 2 (versican) (CSPG2), mRNA	NM_004385
		Nuclear receptor subfamily 3 group C, member1 (glucocorticoid receptor) (NR3C1) gene, complete cds	AY436590
		mRNA for the chondroitin sulphate proteoglycan versican, V1 splice variant; precuresor peptide	X15998
		EST sequence	AC026743
		EST sequence	AL365272
		EST sequence	AF312913
LLME(+) 1d	LLME(−) 0d	Cytokine inducible SH2-containing protein, transcript variant 2, mRNA, complete cds	BC064354
		Lymphoyte activation-associated protein mRNA, complete cds	AF123320
		EST sequence	BC062451

screening, and assessed their expression levels in the LLME(−)-PBMCs. MHC class II DRα and KBF2 are known to be involved in antigen recognition and presentation at the start of an immune reaction. MHC class II DRα is a component of HLA-DR on antigen presenting cells (APC), and it plays a key role in antigen presentation to T cells.[7] Although KBF2 is known to bind to the H2K molecule, its function is not completely understood.[8] As shown in Fig. 2, no significant changes in the expression levels of these genes in LLME(−)- or LLME(+)-PBMCs during the culture period were detected. This indicates that these genes are not involved in the downregulation of antibody production observed in LLME(−)-PBMCs.

Next we focused on CCL18, MMP12, and H-ferritin, which are known to be involved in inflammation. CCL18 is a member protein of the chemokine superfamily that plays an important role in inflammatory responses.[9] MMP12 acts on proinflammatory factors, such as cytokines, chemokines, and other proteins, to regulate varied aspects of inflammation and immunity.[10–12] Recently, it has been reported that MMP12 regulates an inflammatory response by controlling the activity and mobilization of chemokines such as CCL18.[13] Further, H-ferritin is known to be augmented by the inflammatory environment, and it elicits a suppressive function of dendritic cells (DC), resulting in attenuation of immune responses.[14–16] As Fig. 2, shows the expressions of these genes were significantly augmented in LLME(−)-PBMCs, indicating that the inflammatory responses were strongly elicited in LLME(−)-PBMCs. Further, this suggests that an inflammatory reaction might lead to suppression of antibody production after *in vitro* immunization, probably through induction of the suppressive function of DC, although the exact roles of these proteins in antibody production must be investigated in future study.

In previous study, we found that IL-10, which is produced in the supernatant of LLME(−)-PBMCs,

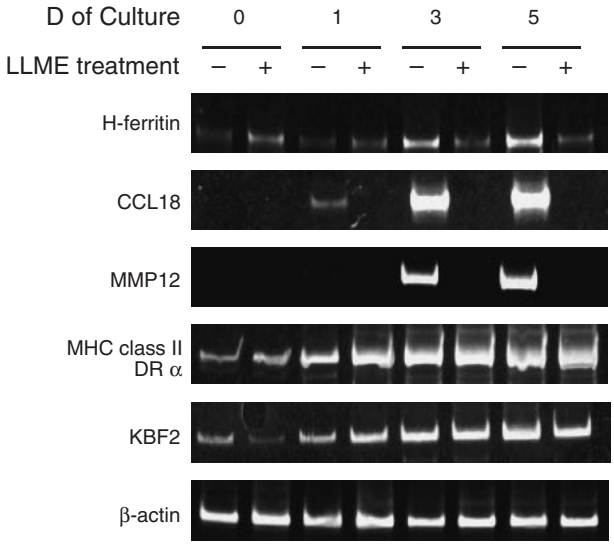

Fig. 2. Expression Profiles of Genes Obtained by Subtractive Screening.

LLME-treated and nontreated PBMC were sensitized with an antigen, as previously described. After 1, 3, and 5 d of culture, total RNA was isolated from the PBMCs and subjected to RT-PCR analyses to assess the gene expression profiles of H-ferritin, CCL18, MMP12, MHC class II DRα, KBF2, and β-actin. PCR conditions and primers are described in "Materials and Methods."

suppresses antibody production after *in vitro* immunization the functional alteration of DC (which occurs during preparation). Based on this finding along with the fact that IL-10 induces secretion of CCL18 from DC, IL-10 is believed to elicit suppression of antibody production after *in vitro* immunization by inducing functional alteration of DC mediated by inflammatory factors. In addition, it has been reported that inflammatory factors are secreted by monocytes, macrophages, and several types of dendritic cells, suggesting that these cells might be involved in the suppression of antibody production from LLME(−)-PBMCs immunized *in vitro*.[17,18]

In conclusion, we suggest that various inflammatory factors such as H-ferritin, CCL18, and MMP12 produced by cells in LLME(−)-PBMCs might suppress antibody production from PBMC immunized *in vitro* by inducing the functional alteration of DC.

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