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ORIGINAL ARTICLE

Mite-antigen Stimulates *MAL* Expression in Peripheral Blood T Cells of Mite-sensitive Subjects

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ABSTRACT

Background: Differential gene expression in CD3+ T cells from allergic patients stimulated with allergen will provide important information on the responses of T cells.

Methods: After stimulation of peripheral blood mononuclear cells (PBMCs) with mite extracts, levels of gene transcription were examined in CD3+ T cells from allergic patients.

Results: Stimulation of PBMCs from mite specific IgE positive subjects resulted in specific upregulation of *MAL* transcription levels that was mediated by IL-4 secretion. The MAL protein in IL-4 stimulated primary T cells preferentially localized in glycolipid-enriched membrane (GEM) microdomains. When MAL was exogenously expressed in primary T cells, CD3ζ was concomitantly enriched, along with the expression of MAL, in GEM microdomains.

Conclusions: GEMs are important for the formation and stabilization of TCR signaling complexes. Therefore, MAL may play a role in the formation of GEMs in activated T cells and the high expression of MAL may contribute to Th2 immune response.

KEY WORDS

atopic dermatitis, glycolipid-enriched membrane microdomains, lipid raft, MAL, T cell

INTRODUCTION

MAL, a proteolipid present in the glycolipid-enriched membrane (GEM) microdomain, has been reported to be expressed in T cells of the immune system, the central nervous system, gray mater of the cerebral cortex, and the thyroid gland.[1,2] *MAL*, cloned from T cells, is expressed in the intermediate and late stages of T cell differentiation and is thought to play a specific role in the differentiation process.[3] In polarized epithelial cells, MAL is a component of the raft machinery for apical sorting of both membrane and secretary proteins.[4,5] As shown by co-immunoprecipitation studies, MAL associates with GPI anchored proteins (CD59, CD55, and CD48) as well as Src-like tyrosine kinases (Lck and Fyn).[6] Such associations point to a role for MAL as a linker protein for GPI-anchored protein and Src-like kinase mediated events that occur in GEM microdomains. However little is known about MAL expression during immune or allergic reactions.

After cellular presentation of antigen, the T cells produce cytokines to regulate the immune response. Th2 cells producing IL-4, IL-5 and IL-13 are involved in the development of allergic diseases.[7,8] Any gene showing altered expression levels in T cells during the allergic response could be assumed to have a key role. Mite antigen is a typical allergen that precipitates diseases such as atopic dermatitis, allergic asthma and perennial allergic rhinitis.

One technique for studying the allergic reaction *in vitro* is to stimulate peripheral blood mononuclear cells (PBMCs) with the mite extracts and measure the transcriptional levels of genes in T cells. Using transcription analysis by oligonucleotide microarray technique, we found that mRNA levels of *MAL* increased in CD3+ cells in an asthmatic patient after stimulation of PBMCs with mite extracts. To study

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the possible role of *MAL* expression in allergic diseases, we confirmed the upregulation of *MAL* mRNA in a number of atopic subjects. We further examined the mechanism of upregulation of *MAL*, localization of MAL protein in GEM microdomains, and expression levels of *MAL* in Th2-polarized cells. We will discuss the relevance of high expression of MAL mRNA in atopic individuals.

METHODS

STUDY POPULATION AND SAMPLE COLLECTION

Blood samples were collected from both healthy subjects and patients with atopic dermatitis (AD) and/or asthma (AS). Patients with AD were diagnosed according to the criteria of Hanifin.[9] Diagnosis of AS was based on the asthma prevention and management guidelines.[10] Serum IgE levels specific for house dust mite *Dermatophagoides pteronyssinus* (Dp IgE) were determined using enzyme-linked immunosorbent assay kits (CAP IgE FEIA, Pharmacia Upjohn Inc.). For real time PCR quantification of mRNA in mite-extract stimulated PBMCs, a total of 37 samples were obtained from 8 Dp IgE (–) healthy donors, 10 Dp IgE (+) healthy donors, 2 Dp IgE (–) patients and 17 Dp IgE (+) patients. The 2 Dp IgE (–) subjects included both an AS and AD subject. The 17 Dp IgE (+) cohort consisted of 7 AS, 5 AD, and 5 subjects that suffered from both AS and AD. The AD patients were treated with topical glucocorticoid ointments. A milder steroid (mainly hydrocortisone acetate) was used for treating the face and a stronger steroid (mainly dexamethasone valerate) for the body. None of the AD patients had been treated with systemic glucocorticoids. The moderate and severe asthmatics were treated with inhalational glucocorticoids (beclomethasone propionate). During periods of exacerbation, inhaled β2 agonists were added to the regimen. Glucocorticoids were not used for the mild asthmatics, but inhaled β2 agonists were used as needed. None of the asthmatics had been routinely treated with systemic glucocorticoids. This study was approved by the research ethics committee of the National Research Institute for Child Health and Development. Written informed consent to participate in the study was obtained from volunteers after providing them with detailed information about the study and subjects' rights.

LEUKOCYTE SUBSET PREPARTION

PBMCs were prepared as described previously.[11] CD3+ cells were isolated from PBMCs using a magnetic cell sorter (CD3 Micro Beads and MS+ columns, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The percentage of CD3+ cells in column yields was > 97%. CD16- cells (eosinophils), obtained from the sedimentary granulocyte fraction of the Ficoll-Hypaque gradients, were isolated by negative selection using a magnetic cell sorter. The percentage yield of eosinophils was > 97%. CD14+ cells (monocytes) were prepared from cells remaining after separation of CD3+ cells. The cells remaining after separation of CD14+ cells were used as a source of B cells. CD16+ granulocytes remaining after separation of CD16- cells were the source for neutrophils.

CD8+ cells were prepared from PBMCs of a healthy volunteer by magnetic sorting. CD4+ cells were isolated from cells remaining after separation of CD8+ cells using CD4 multisort microbeads (Miltenyi Biotec). After removing the magnetic particles from the CD4+ cells, CD45RA+ cells were isolated. CD45RO+ cells were prepared from the cells remaining after separation of CD45RA+ cells.

REAL TIME QUANTITATIVE RT-PCR

Real time quantitative RT-PCR for quantification of gene expression was performed using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, USA) as described previously.[11] DNase-treated total RNAs were mixed with random primer (Invitrogen Co., Carlsbad, USA), and first strand cDNAs were synthesized. The primer sequences for *MAL* (accession No. X76223) were AAAAGCCCTGCCCTGTTGCT (forward), CCCCGAACAAGAAGGTCCCC (reverse), and the probe sequence was TGCTGTGTTTACTCTCCCGTGTGCC. The primer sequences for *IL-4 receptor* α (*IL-4Rα*, accession No.X52425) were CGACTTGTGAACGAGTTGTTGG (forward), TTCAGTGAGACAGAGGCAGGTG (reverse), and the probe sequence was TGTTGTAACTGCCCAAGGCATGTTTTGC. Levels of β-actin mRNA were used as an internal standard and quantified in each sample.

MICROARRAY ANALYSIS OF MITE ANTIGEN STIMULATED LEUKOCYTE

Approximately 100 ml of peripheral blood was collected from a single patient with allergic chronic bronchial asthma and a single normal healthy subject for PBMC preparation. PBMCs were cultured in RPMI1640 supplemented with 10% fetal calf serum, penicillin (100 unit/ml) and streptomycin (100 μg/ml), using humidified conditions and 5% CO_2. Cultures were stimulated with mite extracts (5 μg/ml each mite extract from *D. pteronyssinus* and *D. farinae*, COSMO BIO, Tokyo, Japan) both with and without dexamethasone (100 nM). At 12 h and 40 h after stimulation, CD3+ cells were isolated and total RNA was extracted. Gene expression was analyzed using an oligonucleotide array (GeneChip HuGene FL Array, Affymetrix, Santa Clara, CA, USA). It is well known that the majority of individuals who are positive for specific IgE to *D. pteronyssinus* (Dp) are also positive for specific IgE to *D. farinae* (Df). Therefore subjects were not tested for Df IgE levels, but a mix-

ture of both Dp and Df extracts was used to assure optimal responses. In a preliminary experiment, PBMCs from allergic patients were stimulated with various concentrations of mite extract mixtures and IL-5 levels in the supernatants were determined. Stimulation with the mixture containing 5 μg/ml of each extract resulted in the maximum IL-5 secretion and this concentration was used for all experiments. Because glucocorticoids are known to suppress transcriptional upregulation of various genes in stimulated leukocytes,[12] dexamethasone was used to evaluate glucocorticoid susceptibility of gene expression.

QUANTITATIVE PCR OF MITE ANTIGEN STIMULATED LEUKOCYTE

PBMCs were prepared from 10 ml blood samples. Approximately 2×10^6 PBMCs were stimulated in 1 ml of RPMI1640 supplemented with 10% fetal calf serum in the presence of mite extracts both with and without dexamethasone. After 24 h, the cells were collected, washed with PBS, and RNA was extracted. IL-4 in the culture supernatants was quantified by ELISA (Quantikine HS Human IL-4 ELISA Kit, R & D Systems, Inc., Minneapolis, USA). For some experiments neutralizing IL-4 antibody (200 ng/ml, purified rat anti-human IL-4, PharMingen, San Diego, USA) was used. After RNA extraction, gene expression was quantified using real time PCR.

CYTOKINE STIMULATION OF PRIMARY T CELLS

CD3+ cells were stimulated by culture on plates coated with CD3 monoclonal antibody (10 μg/ml, Orthoclone OKT3, Ortho Biotech, South Raritan, USA) to induce cell growth. The cells were diluted to a density of 5×10^5 cells/ml and cultured for 5 days in RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml) and IL-2 (200 U/ml, Imunace, Shionogi Pharmaceutical, Osaka, Japan). The expanded T cells were then diluted in the same medium to a density of 5×10^5 cells/ml for an additional 3 days of culture on a non-surface treated plate. The final culture of cells was washed and cultured at a density of 1×10^6 cells/ml in IL-2 free medium supplemented with different cytokines. Cytokines were used at the following concentration ; 2 ng/ml of IL-4 (Pharmingen), 2 ng/ml of IL-12 (Intergen), 200 ng/ml of IFN-γ (Sigma, Saint Louis, USA), and 100 IU/ml of IFN-α (Sigma).

CLONING AND SUBCELLULAR LOCALIZATION OF GFP-MAL FUSION PROTEIN

MAL gene was amplified using a UniGene clone plasmid (AW25038, NCBI) as a template. The amplified fragment was cloned into a multiple cloning site of pEGFP-C3 (Clontech, Palo Alto, USA) to construct a pEGFPMAL1 plasmid. The plasmid DNA was transfected to Jurkat cells, and a stable transformed cell line, GFPMAL1, was obtained. For the induction of GFP-MAL fusion protein, GFPMAL1 cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 25 ng/ml) and ionomycin (1 μg/ml).

POLARIZATION OF CORD BLOOD MONONUCLEAR CELLS

Umbilical cord blood was collected from healthy full-term neonates immediately after delivery. Mononuclear cells were isolated by Ficoll density gradient centrifugation[13] and stimulated with 2 ug/ml PHA (Difco, Detroit, USA) in the presence of 2 ng/ml IL-12 and 200 ng/ml neutralizing anti-IL-4 for Th1 polarization or 2 ng/ml IL-4 and 5 ng/ml neutralizing anti-IL-12 (Genzyme, Cambridge, USA) for Th2 polarization. Cells were washed once on day 3 and expanded until day 10 in RPMI1640 medium supplemented with 10% FCS containing 4 ng/ml IL-2 for Th1 polarization or 4 ng/ml IL-2 and 2 ng/ml IL-4 for Th2 polarizing condition. During expansion, cell densities were maintained at a concentration that did not exceed 2×10^6 cells/ml by diluting the culture with the respective medium. When stimulated with PMA and ionomycin, the Th2 polarized cells, but not Th1 polarized cells, secreted IL-4 and IL-5. The Th1 polarized cells produced higher levels of IFN-γ than Th2 cells (data not shown).

RETROVIRAL MEDIATED TRANSDUCTION OF MAL IN T CELLS

For the expression of exogenous MAL in human primary T cells, the retroviral transduction system was modified.[14-16] Briefly, *MAL* cDNA was amplified by PCR and cloned into the EcoRI cloning site of pBabe-CLXIP to construct a MAL expression pBabeMALs plasmid. Viral supernatant was prepared by transfecting 293-EBNA packaging cells with plasmid DNAs of PCLA-ampho, pCLAG and pBabeMALs using two kits (RetroMax System, Imgenex, San Diego, USA and TransIT-LTI). CD3+ cells prepared from PBMC were cultured and maintained for 3 days in a CD3 monoclonal antibody coated plate with the medium containing 200 u/ml of IL-2. On day 3, the cells were transduced with retrovirus for 8 hours on a retronectin (Takara Shuzo, Kyoto, Japan) coated plate. The cells were harvested and cultured for expansion in fresh medium. On day 6, the cells were transferred to the medium containing 0.2 μg/ml of puromycin to select transduced cells. On day 21, cells were harvested and used for GEM protein analysis.

PREPARATION OF GEM MICRODOMAINS AND WESTERN BLOT ANALYSIS

GEM microdomains were isolated by standard procedures described by Fernando Martin-Belmonte *et al.*[4] with slight modifications. Approximately 1×10^8 harvested T cells were washed with PBS and suspended

in 108 solution (150 mM of NaCl, 10 mM of Tris HCl pH 7.4, 5 mM of EDTA, 0.2% Triton X100) containing protease inhibitors (1 mM of PMSF, 18 μg/ml of aprotinin, 50 μg/ml of leupeptin, 1 mM of benzamidine and 0.7 μg/ml of pepstatin). The cell suspension was incubated for 30 min at 4℃ with periodic agitation and then homogenized by passing the sample through a 22-gauge needle. The extract was adjusted to contain 42.5% sucrose in a final volume of 3 ml, placed at the bottom of a centrifuge tube and overlaid with 6 ml of 35% sucrose and 4 ml of 5% sucrose. The stepwise gradients were centrifuged for 16 h at 38,000 rpm at 4℃ in a RPS40T rotor (Hitachi Koki Co., Tokyo, Japan). Fractions of 1 ml were harvested from the top of the tube and analyzed by immunoblots. Twenty μl per lane of each fraction was used for SDS polyacrylamide gel electrophoresis. Western blot analysis was performed as described previously.[17] Various antibodies were used as probes. Based on the aa sequence of human MAL,[3] rabbit polyclonal antibody was raised against MAL N-terminal peptide (MAPAAATGGSTLP) and then affinity purified. The antibody recognized recombinant MAL protein as well as endogenous MAL protein in raft fractions (data not shown). Rabbit LAT polyclonal antibody (Upstate Biotechnology, Lake Placid, USA), mouse CD3ζ monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and cholera toxin B subunit peroxidase conjugate (Sigma) were used.

STATISTICAL ANALYSIS

Data were analyzed for significance by a repeated measure ANOVA and Spearman's correlation coefficients by rank were obtained using StatView J-5.0 computer software (SAS Institute Inc., USA).

RESULTS

CORRELATION OF *MAL* EXPRESSION LEVELS WITH ALLERGIC DISEASE AND IL-4 RECEPTOR EXPRESSION LEVELS

To identify allergic disease-associated genes, PBMCs from both an atopic patient and a healthy subject were stimulated with the mite extracts and the expression profiles in the isolated CD3+ T cells were analyzed using an oligonucleotide array. The expression of *MAL* gene exon 4 (GeneBank accession No. X76223) was higher in the atopic patient than in the healthy subject at both 12 and 40 h after the antigen stimulation. The increase in *MAL* expression was suppressed in the presence of dexamethasone (data not shown).

To confirm the results of the microarray experiments, PBMCs from patients and healthy donors were stimulated with the mite extracts and the *MAL* expression levels were quantified by PCR. We also analyzed mRNA levels of *IL-4 Rα* as an allergic disease-related gene. The primers and probe for *MAL* were designed based on the *MAL* sequence registered on the chip and those for *IL-4Rα* were designed based on the coding sequence of the gene. Changes in the *MAL* expression levels related to the presence or absence of the mite antigen stimulation are shown in Figure 1A. A majority of patients were positive for the Dp IgE antibody, indicating allergy to the mite antigen. The *MAL* levels without stimulation were similar (approximately 1,000 copy/ngRNA) in the 4 groups (Dp IgE (−) healthy donors, Dp IgE (+) healthy donors, Dp IgE (−) patients and Dp IgE (+) patients). With mite extract stimulation the levels were increased an average of 2.4 times in the Dp IgE (+) healthy donors and 3.3 times in the Dp IgE (+) patients. In contrast, the increase was less in the Dp IgE (−) healthy donors (1.4) and Dp IgE (−) patients (1.0). Repeated ANOVA analyses showed a significant difference ($p < 0.01$) in *MAL* level elevation among the 4 groups. Post hoc tests showed that the *MAL* levels tended to be more elevated ($p = 0.075$) in the Dp IgE (+) healthy donors than in the Dp IgE (−) healthy donors and that the *MAL* levels were significantly ($p < 0.05$) elevated in the Dp IgE (+) patients when compared to the Dp IgE (−) healthy donors. When all samples were divided into two groups (the Dp IgE (+) group and the Dp IgE (−) group) the *MAL* levels were significantly elevated in the Dp IgE (+) group ($p < 0.05$). The expression profile of *IL-4 Rα* was quite similar to that of *MAL*. *IL-4Rα* levels were significantly ($p < 0.05$) elevated in the Dp IgE (+) healthy donors when compared to the Dp IgE (−) healthy donors and significantly ($p < 0.01$) elevated in the Dp IgE (+) patients when compared to the Dp IgE (−) healthy donors. The *IL-4Rα* levels were also significantly elevated in the Dp IgE (+) group ($p < 0.01$) compared to the Dp IgE (−) group. The change in expression levels of *MAL* between the unstimulated and stimulated samples was tightly correlated with that of *IL-4* Rα ($p = 0.94$, $p < 0.0001$, Fig. 1C). Upregulation of both *MAL* and *IL-4Rα* was completely inhibited in the presence of dexamethasone (data not shown). When the expression levels in various subsets of peripheral blood leukocytes were quantified, *MAL* was expressed at the highest levels in CD3+ cells (Fig. 2A). In the T cell subsets, *MAL* was expressed at a higher level in CD4+ cells than in CD8+ cells (Fig. 2B) and at similar levels in CD4+CD45RA+ and CD4+CD45RO+ subsets.

RELATIONSHIP OF IL-4 TO CONTROL OF INDUCTION OF *MAL*

Next, we examined the mechanism of *MAL* upregulation in CD3+. In most mite specific IgE positive subjects, IL-4 was detected after stimulation of cells with mite extracts (Fig. 1B). At the same time, the *MAL* expression was also increased. To examine the involvement of IL-4 in *MAL* upregulation, IL-4 secreted

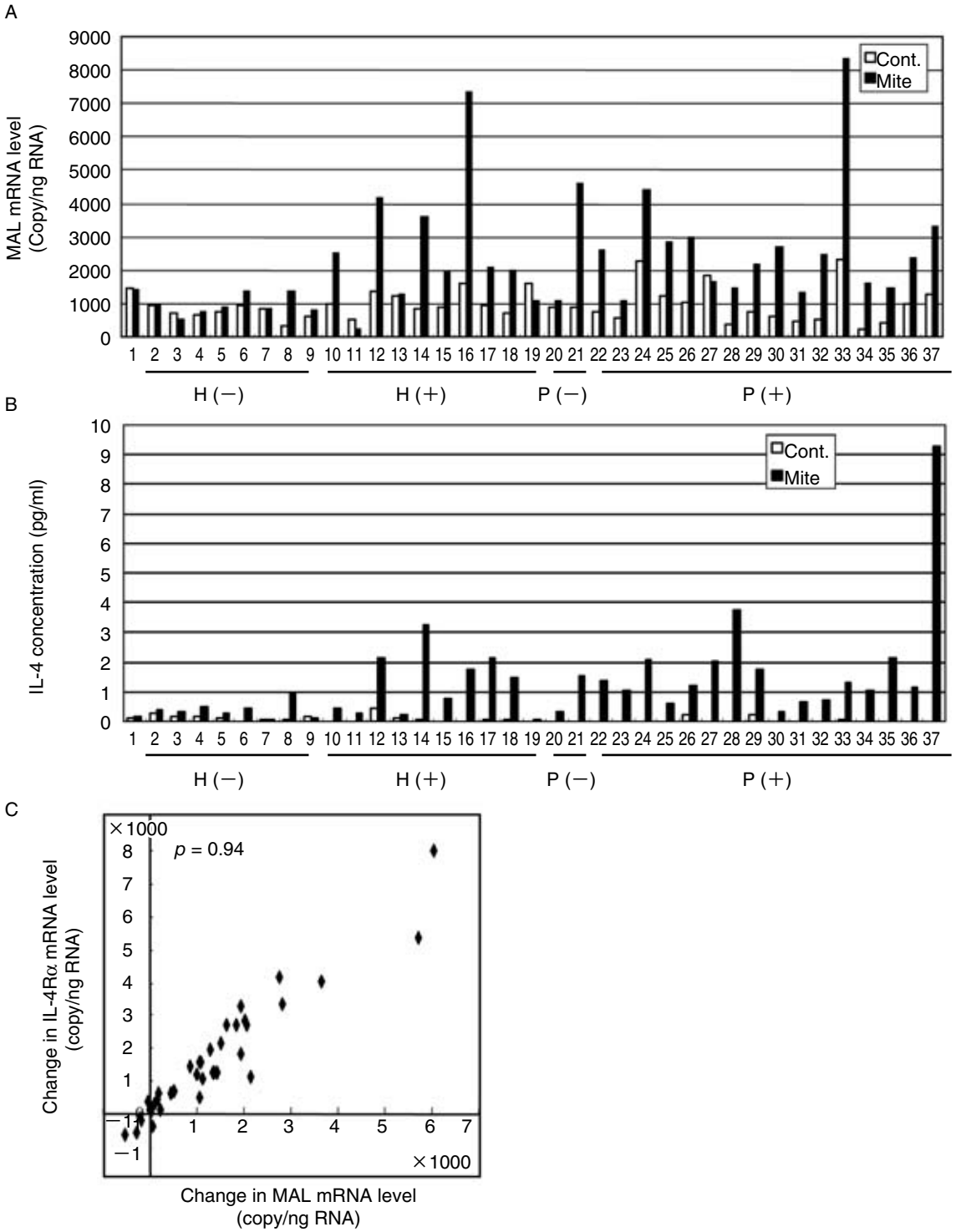

Fig. 1 Upregulation pf *MAL* mRNA and IL-4 levels in PBMCs stimulated with mite extracts. PBMCs from healthy (H) and patient (P) samples were cultured in the presence (Mite) or absence (Control) of mite extracts. Specific IgE positive (+) and negative samples (−) are indicated. After 24 h, total RNAs were extracted from the cells and mRNA levels of *MAL* were examined by quantitative PCR (**A**). Concentrations of IL-4 secreted into the culture supernatant were determined by ELISA (**B**). Correlation between the changes in expression of both *MAL* and *IL-4 receptor* α after stimulation with mite extracts (**C**).

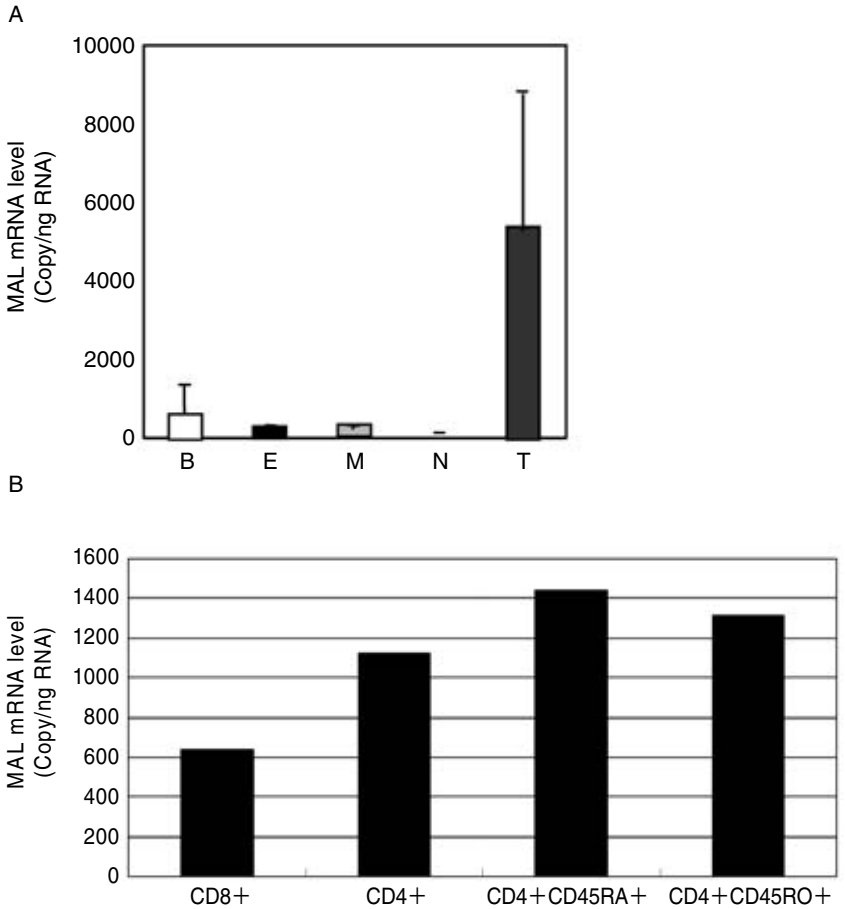

Fig. 2 High transcription levels of *MAL* in peripheral blood T cells. Peripheral blood leukocyte subsets were prepared from 5 healthy subjects and mRNA levels of *MAL* were examined by quantitative PCR. B: B cells, E: eosinophils, M: monocytes, N: neutrophils and T: T cells (**A**). T cell subsets were prepared from a healthy subject and mRNA levels of *MAL* were examined (**B**).

in the culture was neutralized by adding IL-4 mAb. The upregulation of *MAL* mRNA by mite extracts was totally attenuated in the presence of IL-4 mAb (Fig. 3). Next, primary T cells were cultured in the presence of various cytokines. During cultivation for 0–48 h, mRNA for *MAL* was induced after stimulation with IL-4, but not with IL-12, IFN-γ, IFN-α (Fig. 4), or IL-13 (data not shown). These results strongly suggested that IL-4 is necessary for induction of *MAL* mRNA.

EXPRESSION OF MAL PROTEIN IN GEM MICRODOMAINS

Since MAL preferentially localizes in the GEM microdomains, we explored the possibility that the IL-4 induced MAL plays a role in T cell GEM microdomains. GFP-MAL protein was expressed in Jurkat cells and the localization was examined. Accumulation of GFP-MAL protein on the cell surface membrane was observed (Fig. 5). We further examined the localization of MAL in detergent insoluble GEM microdomains. MAL protein levels in GEM fractions were examined by Western blotting and detected with polyclonal antibody to a MAL peptide (Fig. 6). The MAL protein preferentially localized in GEM fractions, and the protein level of MAL of IL-4 stimulated CD3+ T cells was higher than that of non-treated cells. In contrast, the level of LAT, a protein marker for lipid rafts of T cells,[18] was unchanged even after IL-4 stimulation.

EXPRESSION OF MAL IN T CELLS CULTURED UNDER TH2 POLARINZING CONDITION

Since IL-4 occupies a critical role in the development of Th2 cells, we compared the MAL levels between Th1 and Th2 polarized cells that were derived from cord blood mononuclear cells. No differences between Th1 and Th2 polarized cells were detected for either mRNA levels (data not shown) or protein levels in GEM microdomains (Fig. 7).

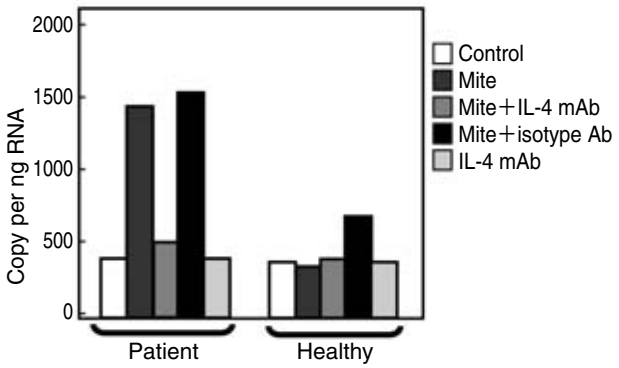

Fig. 3 Involvement of IL-4 in upregulation of *MAL* by stimulation with mite extracts. PBMCs from both allergic and healthy subjects were stimulated with mite extracts in either the presence or absence of IL-4 monoclonal antibody (mAb). Total RNAs were extracted and mRNA levels of *MAL* were examined by quantitative PCR. Data is shown from cultures stimulated with mite extracts (Mite), mite extracts in the presence of IL-4 mAb (Mite + IL-4 mAb), isotype antibody (Ab) (Mite + isotype Ab), unstimulated (Control), and IL-4 mAb alone (IL-4 mAb).

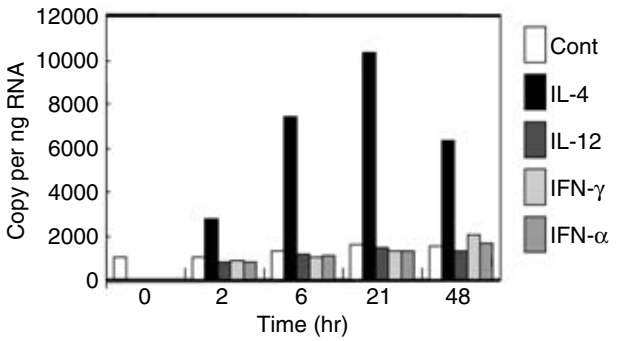

Fig. 4 Upregulation of *MAL* mRNA by IL-4 in primary T cells. Primary CD3+ cells were stimulated with IL-4, IL-12, IFN-γ or IFN-α for various time periods. The cells were harvested and *MAL* mRNA was measured by quantitative PCR.

RECRUITMENT OF CD3ζ PROTEIN IN GEM AFTER FORCED EXPRESSION OF MAL

CD3ζ chain is crucial for T cell receptor mediated signaling. Tyrosine phosphorylated CD3ζ translocates to GEM microdomains upon T cell activation.[19] To examine the effect of MAL on CD3ζ localization, MAL was exogenously expressed in human primary T cells by retroviral transduction and the protein levels of CD3ζ in GEM fractions were examined. The protein levels of CD3ζ in GEM microdomains were markedly increased coincidentally with MAL (Fig. 8). There were no significant changes in the levels of two marker molecules, LAT and GM1 ganglioside. These

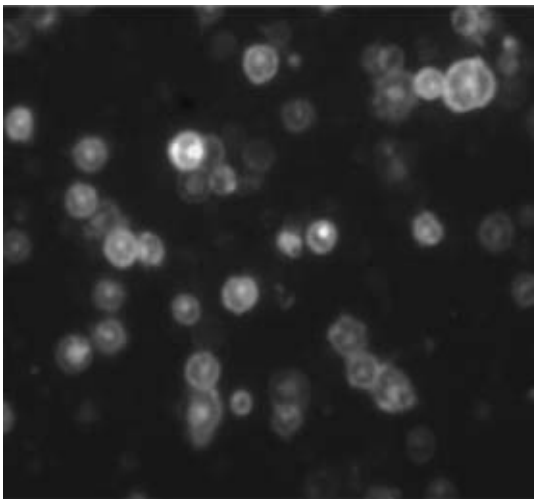

Fig. 5 Subcellular localization of GFP-MAL fusion protein at the cell surface membrane in Jurkat cells. A stable transformant expressing GFP-MAL fusion protein was derived from Jurkat cells that were transfected with pEGFPMAL1. Protein expression was induced with PMA and ionomycin for 24 h and the cells were examined by fluorescent microscopy.

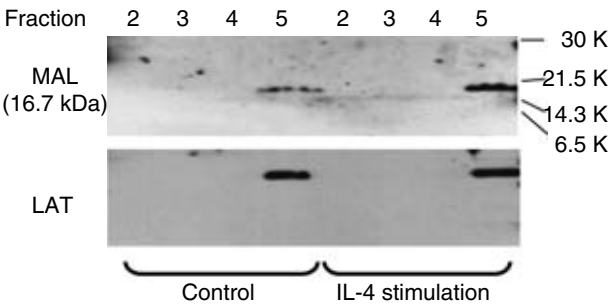

Fig. 6 Induction of MAL protein in GEM microdomains of primary T cells after stimulation with IL-4. CD3+ cells (1×10^8) that were expanded with anti-CD3 stimulation were incubated with 2 μg/ml of IL-4 for 24 h. The cells were harvested and GEM microdomains were prepared by sucrose density gradient centrifugation. MAL and LAT proteins were detected in the GEM fractions (No. 2, 3, 4 and 5) by Western blotting.

results suggest that the MAL protein plays a role in recruiting CD3ζ into the GEM microdomains.

DISCUSSION

Our data showed that 1) *MAL* transcription levels were upregulated specifically in CD3+ T cells when PBMCs from Dp IgE (+) subjects were stimulated with mite extracts 2) IL-4 secretion as a result of mite antigen stimulation was critical for the upregulation of *MAL* and 3) IL-4 stimulation of primary T cells re-

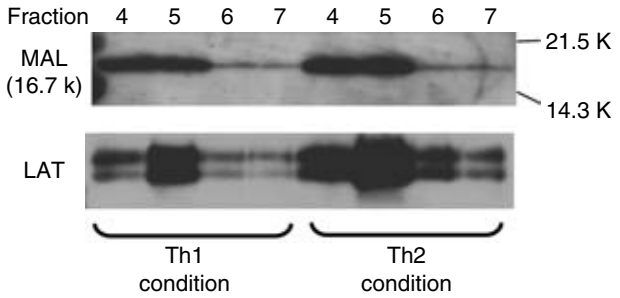

Fig. 7 MAL protein levels in GEM microdomains of Th1 polarized and Th2 polarized T cells. Cord blood mononuclear cells were cultured in Th1 or Th2 polarizing condition. The cells were harvested and GEM microdomains were prepared by sucrose density gradient centrifugation. MAL and LAT proteins were detected in the GEM fractions (No. 4, 5, 6 and 7) by Western blotting.

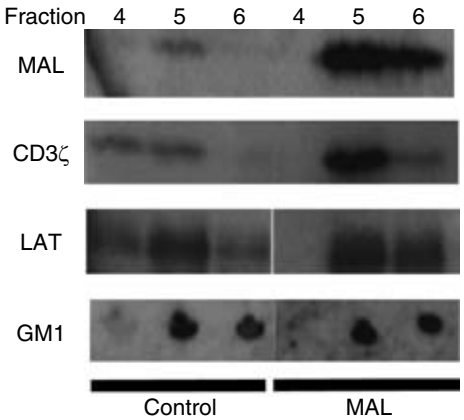

Fig. 8 Enrichment of CD3ζ protein in GEM microdomains after forced expression of MAL in primary T cells. MAL was exogenously expressed in human primary T cells by retroviral transduction. The transduced cells (1×10^8) were harvested and the GEM microdomains were prepared. The protein levels of MAL, CD3ζ and LAT in GEM fractions (No. 4, 5 and 6) were examined by Western blotting. The levels of GM1 were detected using cholera toxin B subunit-peroxidase conjugate.

sulted in enrichment of MAL protein in GEM microdomains.

The IL-4 levels that were secreted in supernatant after stimulation of PBMCs with mite extract were as low as sub- picograms per ml to several picograms per ml. Such low levels probably reflected the limited presence of a T cell subpopulation that recognized mite antigen as well as the IL-4 consumption by IL-4R bearing cells in PBMCs.[20]

A high positive correlation of the mRNA expression profiles between *MAL* and *IL-4Rα* was observed in our study. In addition the upregulation with mite-extract stimulation was suppressed in the presence of dexamethasone for both *MAL* and *IL-4 Rα*. These findings suggest that the regulatory mechanisms for transcription may be similar for these two genes. IL-4 exerts its biological effects by binding to the IL-4R complex and upregulates the expression of the human, as well as mouse, IL-4Rα chain in an autocrine manner[21] through STAT6 dependent signaling.[22-24] From our data-base search of the *MAL* genome sequence, a number of consensus sequences for STAT6 (TTCNNNNGAA) were found in the introns 1, 2 and 3 and the −4–10 kb upstream region of transcriptional start site (data not shown). The identification of these sites support our interpretation that IL-4 mediated STAT6 signaling may regulate the transcription of *MAL*.

In this study, we clearly demonstrated that IL-4 mediates MAL expression in human CD3+ T cells, leading to the hypothesis that MAL might be involved in Th2 differentiation. Studies of *MAL* mRNA expression in various leukemic T-cell lines, as well as in mature T cell clones, support its role in the intermediate and late stages of differentiation.[3] Since a previous study showed that committed Th1 and Th2 cells have distinct patterns of membrane compartmentalization into lipid rafts,[25] it is reasonable to speculate that the role of MAL may be to regulate the differential raft conformation. In our study of Th2 polarized cells, marked upregulation of MAL was not observed when compared to Th1 polarized cells, even though the polarized Th2 cells had been cultured in the presence of IL-4. These data suggested that MAL was not involved in the last stage of Th2 polarization of cord blood cells under our experimental conditions. Further studies of different conditions, such as the use of T cell clones, are needed to clarify the role of MAL in Th2 differentiation.

The TCR has a multi-subunit structure composed of the polymorphic αβ heterodimer and the invariant CD3 γ, δ, ε and ζ chains. All components of CD3 complex possess the immune receptor tyrosine-based activation motif (ITAM). Phosphorylation of tyrosine residues within the ITAM motif is critical for TCR signal transduction. Recent studies point to the importance of lipid rafts for the formation and stabilization of the TCR signaling complexes. Specifically, T cell activation leads to the recruitment of activated TCR and associated signal transducing molecules in the lipid rafts.[26] The integrity of lipid rafts is a prerequisite for efficient T cell receptor signal transduction.[27] Most of the phosphorylated CD3ζ accumulates into the GEM microdomains after T cell activation.[19] The compartmentalization of MAL into GEM microdomains suggests that MAL may also be an important component for T cell signal transduction. The concomitant upregulation of CD3ζ and MAL that occurs during exogenous expression of MAL in primary T cells further suggests the possible involvement of

MAL in modulating T cell receptor signaling.

Considering that IL-4 mediates MAL expression, we can speculate that other allergens such as Japanese cider pollen, may also upregulate MAL expression in antigen specific IgE (+) subjects. Further studies of the expression levels of MAL after stimulation with other antigens should confirm this hypothesis. Taken together, MAL may be involved in the pathophysiology of allergic diseases by modifying the Th2 immune response in antigen specific IgE (+) individuals.

Differential expression profiling of genes as it relates to patients with atopic diseases has been reported[11] and is expected to become a diagnostic tool in the near future. Our studies show that 1) *MAL* mRNA is upregulated in cells after addition of mite extracts and 2) that the upregulation of *MAL* correlates closely with anti-mite specific IgE and reflects increased secretion of IL-4. These observations support the usefulness of examining *MAL* expression levels in both *in vitro* analysis of allergic responses, as well as in diagnosis and detailed characterization of allergic diseases.

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