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Original Article

TARC and MDC are produced by CD40 activated human B cells and are elevated in the sera of infantile atopic dermatitis patients.

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We report here that human B cells produce thymus and activation-regulated chemokine (TARC/CCL17) and macrophage derived chemokine (MDC/CCL22) if stimulated with anti-CD40 and IL-4. The production was determined by both protein and mRNA level using specific ELISA and semi-quantitative RT-PCR methods. Since the ligand of the TARC and MDC is CCR4, which is specifically expressed on Th2 type T cells, the production of these CC chemokines is likely to play important roles in the T cell and B cell interaction. Consistent with this, ovalbumin (OVA) specific IgE levels, which reflect the T-B cell interaction, are significantly correlated with the amounts of TARC and MDC in sera. Furthermore, we found that TARC and MDC levels are significantly increased in the sera obtained from patients with atopic dermatitis, and that the amounts are correlated with the severity of atopic dermatitis.

Since CD40 ligand and IL-4 are produced by activated T helper cells, these results indicate that TARC and MDC produced by B cells play important roles in the production of antigen specific IgE by the T-B cell interaction and in the pathogenesis of allergic disease.

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Introduction

The human chemokine system currently includes more than 50 chemokines and 18 chemokine receptors. The chemokines are divided into the C, CC, CXC and CX_3C subfamilies according to their NH2-terminal cysteine-motifs[1-3].

Chemokines are small, secreted polypeptides that play important roles in a wide range of inflammatory and immunological processes by recruiting selected subsets of leukocytes[4,5]. CC chemokines TARC has been mapped to human chromosomes 16 and 2, respectively[6,7]. The human macrophage-derived chemokine (MDC)/stimulated T cell chemotactic protein (STCP-1) is encoded by a gene on chromosome 16q13[8-11], and CCR4[12,13] is the receptor for TARC and another macrophage/dendritic cell-derived CC chemokine, macrophage-derived chemokine (MDC)[8]. Both TARC and MDC bind to the same receptor (CCR4), which is selectively expressed by Th2 cells[14]. Memory-effector T cells of the CD4 lineage are now subdivided into Th1 and Th2 types in accordance with their cytokine profiles. Th1 cells produce IL-2, IFN-γ, and TNF-α, for example, and are responsible for cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-13, for example, and are involved in humoral immunity and allergic diseases[15]. Th2-dominant immune responses to environmental allergens in the skin on the basis of undefined genetic predisposi-

tions are the central features of AD[16].

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin that is frequently associated with high serum IgE levels and eosinophilia[17]. Atopic dermatitis is a Th2-type disease.

In this study, the roles of TARC and MDC produced by activated human B cells are discussed. We also showed high amounts of TARC and MDC in the sera of pediatric patients with atopic dermatitis (AD). Thus our data strongly suggest that TARC and MDC are important chemokines in the pathogenesis of AD.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat preparations obtained from healthy volunteers. Separation was achieved by Ficoll (Sigma) density gradient centrifugation.

B Cells Separation

Human B cells were isolated from PBMCs by positive selection with anti-CD19-coated immunomagnetic beads (Dynal, Oslo, Norway) following protocols provided by Dynal. B cells were washed and resuspended in RPMI 1640 culture medium supplemented with 10%(v/v) heat-inactivated (56°C for 30 min) fetal calf serum (FCS) (GIBCO BRL) and collected by centrifugation. Purity of B cells was greater than 99%.

Macrophages Isolation

Macrophages were obtained as adherent cells after a 60 min plating of the PBMCs. Purity was greater than 95%. PBMCs (2×10^7) were suspended in 10 ml of RPMI 1640/10% FCS and used as a source for macrophages. Cell culture flasks (75 cm^2 CORNING 430720), were used routinely for preparation of serum-coated dishes. Two or three milliliters of FCS heat-inactivated at 56°C for 30 min was incorporated into the dish and then incubated at 4°C overnight. PBMCs were added to the dish and incubated for 60 min at 4°C.The adherent macrophages were easily removed by incubation in PBS containing 0.2% ethylenediamine tetraacetate (EDTA) and 5% FCS and recovered as a cell suspension with greater than 95% purity.

Stimulation of Cells

B cells (1×10^5/well) and macrophages (1×10^5/well) were cultured in U-bottom 96-well microtiter plates (SUMILON MS-3096U) and were stimulated with anti-CD40 (1 μg/ml) and IL-4 (1 μg/ml) for the indicated times. The supernatants were assayed by a sandwich type enzyme-linked immunosorbent assay (ELISA). B cells (2.5×10^6/well) were cultured in 24-well flat-bottom culture plates (BECTON DICKINSON) and were stimulated with anti-CD40 (1 μg/ml) and IL-4 (1 μg/ml). At the indicated times, cells were harvested for semi-quantitative RT-PCR analysis.

RNA Isolation and RT-PCR

Total RNA was prepared from B cells stimulated with anti-CD40 and IL-4, with a Total RNA isolation kit (Ambion, ToTally RNA). The RNA was reverse transcribed into cDNA with a First-strand RT-PCR kit (ProSTAR). For PCR, cDNAs were amplified in a 25 μl reaction volume containing a final concentration of 1.5 mM MgCl2, 0.05 units/μl Taq DNA polymerase, 1× PCR buffer, 0.2 mM dNTP Mixture, and the primers (0.5 μM), Primers used for PCR were 5' and 3' TARC (5' -AGCTGGAGGGACCAATGTG-3' and 5' -GACCTCTCAAGGCTTTGCAG-3'), resulting in 212-bp fragment; 5' and 3' MDC (5' -CTGCACTCCTGGTTGTCCTC-3' and 5' -ACGGTCATCAGAGTAGGCTC-3'), producing a 297-bp fragment; 5' and 3' G3PDH (5' -ACCACAGTCCATGCCATCAC-3' and 5' -TCCACCACCCTGTTGCTGTA-3'), amplifying a 451-bp product. The mixture was first incubated for 5 min at 94°C and then cycled 30 times at 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min, (9600 Thermal Cycler, Perkin-Elmer). After amplification, the samples were separated on a 2% agarose gel containing 0.1 μg/ml ethidium bromide, and bands were visualized and photographed using a translucent UV source.

ELISA for MDC

A 96-well flat-bottom plate was coated with 100 μl per well of the capture antibody (R & D Systems), at a concentration of 2 μg/ml in PBS and was incubated at room temperature overnight. The plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 1 hour at room temperature with PBS containing 1% bovine serum albumin (BSA), 5% sucrose and 0.05% NaN3. After washing three times with PBS-T, 100 μl of two-fold dilutions of cell culture supernatants in PBS containing 1% BSA, pH 7.4, was added to the wells. The plates were incubated for 2 hours at room temperature then washed three times with PBS-T. Biotinylated anti-human MDC antibody (R & D Systems) at a concentration of 50 ng/ml in 1% BSAPBS was added and incubation was continued for

2 hours at room temperature. After another washing as described above, 100μl streptavidin HRP at a 1/20,000 dilution (Zymed) was added to each well and incubated for 20 min at room temperature. After washing, the streptavidin-peroxidase substrate (genzyne TECHNE) was added and the plates were incubated at room temperature for 20 min. Finally, 50 μl of stop solution (1 M H_2SO_4) was added to each well, and the results were measured at 450 nm with an automated microplate reader (BIO-RAD, Model 550), OD readings for duplicate wells were averaged. Units of MDC in experimental samples were calculated against a standard curve generated with dilutions of purified recombinant human MDC (R & D Systems).

ELISA for TARC

The harvested supernatants were assayed for TARC with the TARC Quantikine™ high sensitivity kit (R & D Systems). The standard curve for these kits was run according to protocols provided by Quantikine™.

Serum Samples

Serum samples were collected from 16 infantile atopic dermatitis patients (mean age±SD, 3.9±0.11 years), 14 normal subjects(mean age±SD, 3.25±0.57 years) and assayed in our laboratory for TARC and MDC. This study approved by the declaration of Helsinki and informed consent has been obtained from all the subjects prior to the experiment.

Results

TARC and MDC were Produced by Activated Human B Cells.

Purified human peripheral B cells were stimulated by anti-CD40 with or without IL-4, and semiquantitive RT-PCR specific for the TARC and MDC was performed using the RNA obtained from thus cultured B cells. As shown in Fig. 1, anti-CD40 and IL-4 stimulation clearly induced TARC and MDC mRNA expression in human peripheral B cells. The large amounts of TARC and MDC mRNA were induced as indicated by the facts that cDNAs were detectable even if diluted at 1:3125. IL-4 alone induced less amounts of TARC mRNA than anti-CD40 and IL-4 stimulation. Anti-CD40 alone failed to induce detectable amounts of TARC mRNA. As for MDC, IL-4 alone induced small amounts and anti-CD40 alone induced relatively high amounts of MDC mRNA.

To confirm these TARC and MDC production by human B cells, TARC and MDC in the culture super-

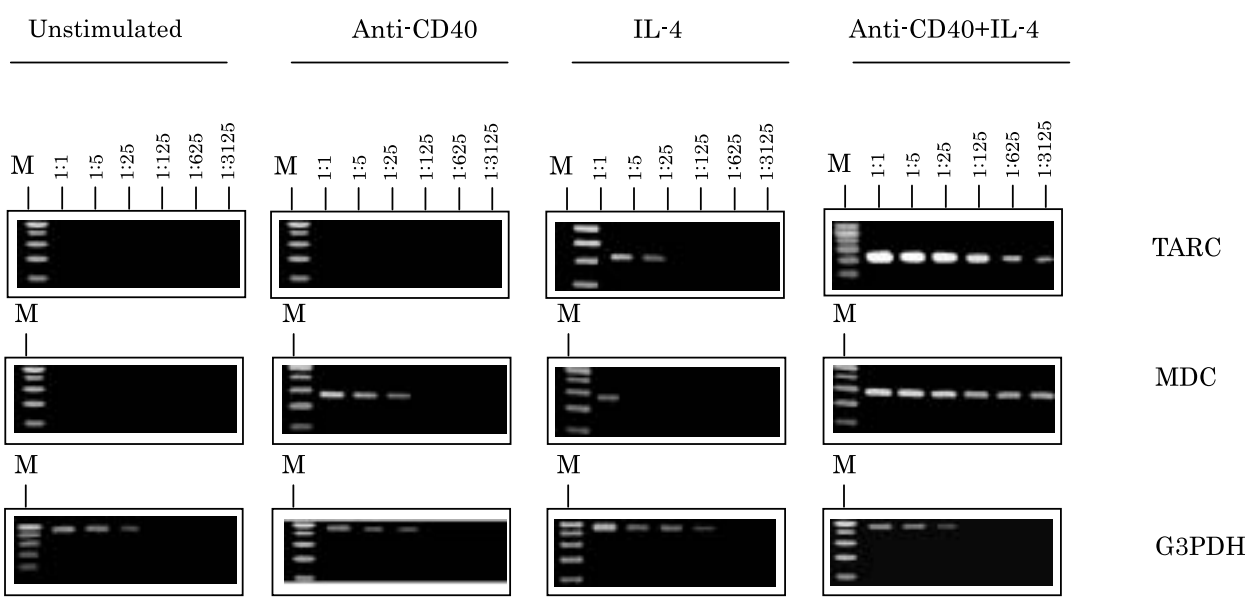

Fig. 1. Semiquantitative RT-PCR analysis of TARC and MDC mRNA expression.
Human B cells (5×10^5/ml) were cultured *in vitro* for 96 hours in the presence of the indicated stimuli. Total RNA was isolated and reverse transcribed and serial 1:5 dilutions of the cDNA prepared from the indicated cells were used as template to PCR-amplify TARC, MDC, and G3PDH. M, Marker.

natants were examined by specific ELISA system. As shown in Fig. 2a, large amounts of TARC were produced by B cells stimulated by anti-CD40 and IL-4. The mean amount of TARC detected at 145 hours after stimulation was 4115.01 ± 1131.97 pg/ml. TARC was detectable in the culture supernatent at 48 hours after stimulation, and it reached maximum levels at 145 hours. IL-4 also induced small amounts of TARC in human B cells. Anti-CD40 stimulation alone failed to induce TARC production.

Similarly, MDC was produced by human peripheral B cells if stimulated with anti-CD40 and IL-4 (Fig. 2b). The production of MDC was relatively rapid if compared with TARC production and reached almost peak levels (6627.32 ± 392.16 pg/ml) at 48 hours after the stimulation. Anti-CD40 alone induced small amounts (2075.10 ± 265.94 pg/ml) of MDC at 120 hours after the stimulation. These results are consistent with the observations obtained by the RT-PCR system, and clearly indicate that activated human B cells produce TARC and MDC.

It has been reported that human macrophages produce MDC and TARC. We compared the amounts of TARC and MDC produced by macrophages and B cells. As shown in Fig. 3a and 3b, both B cells and macrophages produced comparable amounts of TARC and MDC (P=0.65, 0.72, respectively).

Serum TARC and MDC Levels are Correlated with OVA Specific IgE Levels

It is known that anti-CD40 and IL-4 stimulation induces IgE production in human B cells. We compared the serum levels of TARC and MDC and those of OVA specific IgE. We choose OVA specific IgE because this is the most frequently observed antigen specific IgE in infantile AD. As shown in Fig. 4a, there is a significant correlation between serum TARC and OVA specific IgE levels (n = 14, p = 0.005). Similarly, as shown in Fig.4b, a significant correlation between serum MDC and OVA specific IgE was found (p = 0.001). There are significant correlations between serum TARC and MDC levels with OVA specific IgE level expressed in both

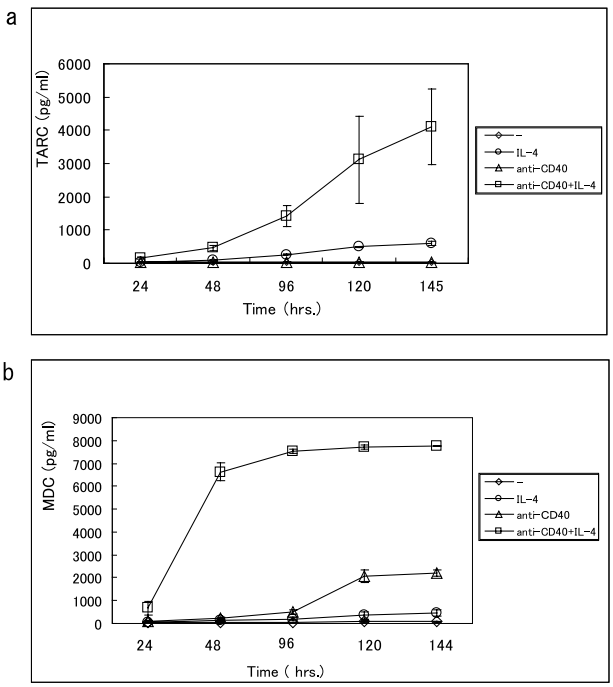

Fig. 2. Inducible expression of the CC chemokine TARC and MDC in B cells.
Human B cells (5×10^5/ml) were plated into 96-well tissue culture plates incubated at 37°C and stimulated with anti-CD40 (1 μg/ml) and IL-4 (1 μg/ml) for the indicated times, and the supernatants were assayed by an ELISA. Each point represents the mean ± SD.
(A) TARC induced by anti-CD40 and IL-4 stimulation.
(B) MDC induced by anti-CD40 and IL-4 stimulation.
In unstimulated (◇), IL-4(○), anti-CD40(△) and anti-CD40 plus IL-4(□).

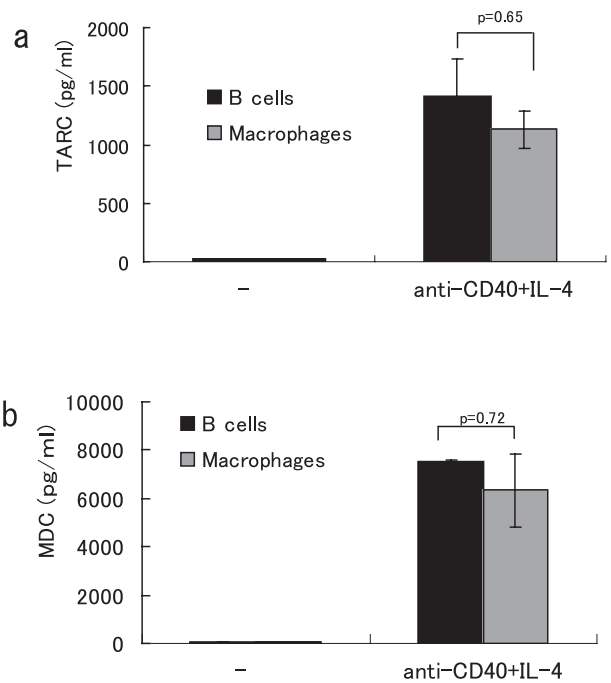

Fig. 3. TARC and MDC production by B cells and macrophages.
Equal numbers of B cells and macrophages (5×10^5/ml) were cultured. Supernatants were collected 3 days after stimulation with anti-CD40 (1 μg/ml) and IL-4 (1 μg/ml). TARC and MDC were measured by ELISA.
(A) TARC production by B cells and macrophages.
(B) MDC production by B cells and macrophages.

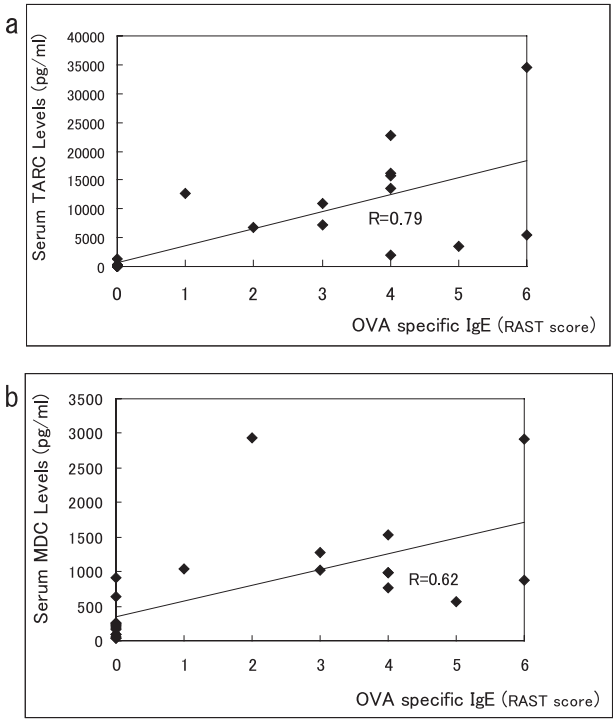

Fig. 4. Correlation of serum TARC and MDC levels with OVA-specific IgE levels.
(A) Comparison between serum TARC levels and OVA-Specific IgE levels patients with AD.
(B) Comparison between serum MDC levels and OVA-Specific IgE levels patients with AD.

RAST scores and specific IgE titers (data not shown).

Serum TARC and MDC Levels are Increased in Patients with AD.

We next examined if serum TARC and MDC would be increased in infantile atopic dermatitis patients. As shown in Fig. 5a and Fig. 5b, significant increases of both TARC and MDC were observed in AD patients (n = 16) and if compared with age matched normal subjects (n = 14) ($P < 0.001$ in TARC, $P < 0.001$ in MDC). The mean ± SD of TARC was 9995.512 ± 6323.255 pg/ml in AD patients and 272 ± 129.61 pg/ml in normal subjects, and the mean ± SD of MDC was 1595.931 ± 1092.55 pg/ml in AD patients and 149.25 ± 95.20 pg/ml in normal subjects. Fig. 5c indicates that there is a significant correlation between TARC and MDC levels in AD patients and normal subjects (P = 0.0003).

The severity of AD and the amounts of TARC and MDC in sera were further analyzed. As shown in Fig. 6a and Fig. 6b, there were significant correlations between serum TARC and MDC levels and severity of

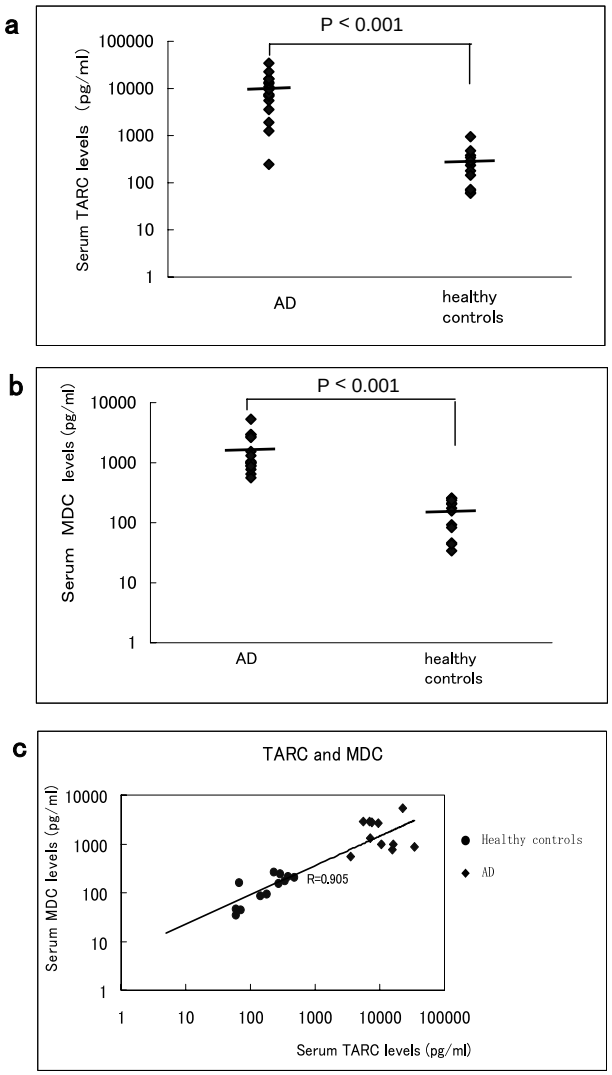

Fig. 5. Sera from atopic dermatitis (n=16) and normal subjects (n=14) were assayed.
(A) Serum TARC levels were measured by ELISA.
(B) Serum MDC levels were measured by ELISA.
(C) Correlation between TARC and MDC levels in AD patients and normal subjects

AD. The severities of AD were based on the internationally approved severity scoring of atopic dermatitis (SCORAD) index. A scoring index (SCORAD) combining extent, severity and subjective symptoms was mathematically derived from the first system and showed a normal distribution of the population studied[18].

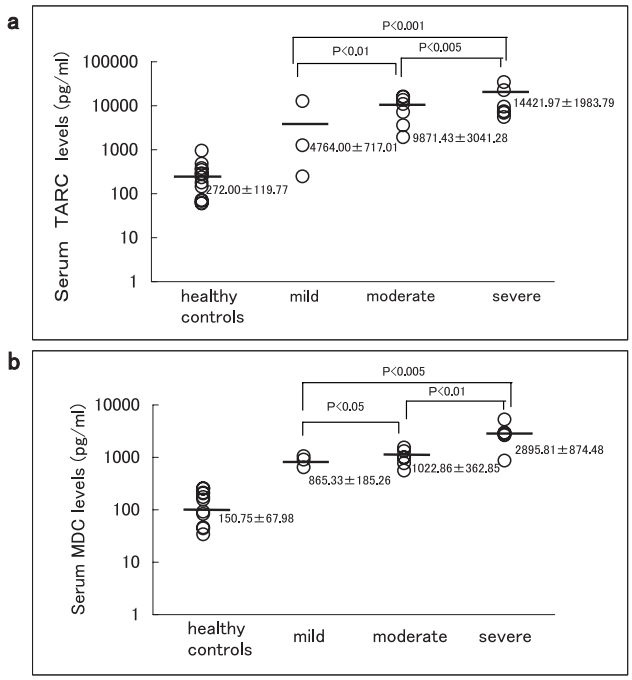

Fig. 6. Comparison of serum TARC and MDC levels among 3 groups of patients with AD.

(A) Serum TARC levels among normal subjects (n=14) and 3 groups of patients with AD: those with mild (n=7) and severe (n=6) disease.

(B) Serum MDC levels among normal subjects (n=14) and 3 groups of patients with AD: those with mild (n=7) and severe (n=6) disease.

Each vertical bar represents mean ± SD of total subjects in individual categories.

Discussion

We found that human B cells produce TARC and MDC if stimulated with anti-CD40 and IL-4. Although macrophages are known to produce TARC and MDC upon the CD40 stimulation[8], it has not been reported previously that human B cells produce these chemokines. The amounts of TARC and MDC produced by B cells were comparable with those produced by macrophages if stimulated with anti-CD40 and IL-4.

The ligand of TARC and MDC is CCR4, which is reportedly expressed on Th2 type T cells. It is also known that CD40 ligand expressed on activated T cells plays essential roles in the B cell activation through the CD40[19,20]. These data suggest that TARC and MDC, produced by CD40 activated B cells, are involved in the T cell and B cell interaction. T cell and B cell interaction is essential for the production of antigen specific IgE. Thus, we next examined if the TARC and MDC production is related with the serum amounts of IgE specific for OVA, one of the most frequently observed antigen which is closely involved in the onset of atopic dermatitis in children. As a result, there was significant correlation between TARC and OVA specific IgE in the sera of infantile atopic dermatitis patients (Fig. 4a). Similarly, there was significant correlation between MDC and OVA specific IgE in the sera of infantile atopic dermatitis patients (Fig. 4b).

From these results, we examined if serum TARC and MDC levels are increased in infantile AD patients. We found that both TARC and MDC were significantly elevated in AD patients if compared with normal age matched subjects (Fig. 5a and b). Furthermore, we also found that the severity of atopic dermatitis is significantly correlated with the amounts of TARC and MDC. The increase of TARC and MDC levels in the sera of AD patients are not simply caused by the inflammation, since it has been reported that TARC and MDC are not increased other inflammatory disorders[21]. Our data do not rule out the possibility that TARC and MDC observed in the sera of AD patients are produced by macrophages. However, significant correlation of OVA specific IgE levels and TARC/MDC indicates that TARC/MDC may be involved in the T-B cell interaction and thus suggest that TARC/MDC in the sera contained, at least in part, those produced by CD40 stimulated B cells.

Taken together, these data suggested that TARC and MDC may play some roles in the onset of infantile atopic dermatitis, probably inducing the T cell and B cell interaction. To confirm this idea we are now doing additional experiments including if B cells obtained from infants produce TARC and MDC as observed in adult B cells and if OVA specific IgE production by peripheral blood mononuclear lymphocytes obtained from AD patients are inhibited by the neutralizing anti-TARC or anti-MDC.

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