T Cell Proliferation by Direct Cross-Talk between OX40 Ligand on Human Mast Cells and OX40 on Human T Cells: Comparison of Gene Expression Profiles between Human Tonsillar and Lung-Cultured Mast Cells

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T Cell Proliferation by Direct Cross-Talk between OX40 Ligand on Human Mast Cells and OX40 on Human T Cells: Comparison of Gene Expression Profiles between Human Tonsillar and Lung-Cultured Mast Cells

Jun-ichi Kashiwakura,* Hidenori Yokoi,† Hirohisa Saito,*‡ and Yoshimichi Okayama2*†

Mast cells (MCs) are the primary effector cells in allergic reactions and have also been found to activate T cells and to reside in close physical proximity to T cells. However, the molecular mechanisms involved in the MC-T cell interaction remain unclear. We hypothesized that human tonsillar MCs, which locate in the interfollicular areas, might interact with T cells. Thus, we first established a culture system of human tonsillar MCs and then compared gene expression profiles of tonsillar MCs with that of lung MCs before and after aggregation of FcεRI by using high-density oligonucleotide probe arrays. Here we show that resting tonsillar MCs, when compared with lung MCs, revealed significantly higher expression levels for CC chemokines (CCL3 and 4), which recruit T cells, and for TNFR superfamilies (OX40 ligand and 4-1BB ligand), which induce proliferation of T cells. After aggregation of FcεRI, not only tonsillar MCs but also lung MCs up-regulated the expression of these molecules. We confirmed that T cell proliferation is induced in direct cross-talk by the MC surface molecule OX40 ligand. These results suggest that human MCs may play important roles in adaptive immunity through the T cell responses. The Journal of Immunology, 2004, 173: 5247–5257.

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production (17–21). Moreover, it has been reported that OX40L has the ability to induce the differentiation of naive CD4⁺ T cells into Th2 cells producing IL-4 in vivo and in vitro (22–26).

To explore what molecules produced by MCs can activate T cells, we compared the gene expression profile of tonsillar MCs with that of lung MCs by using high-density oligonucleotide probe arrays (GeneChip; Affymetrix, Santa Clara, CA). We found that expression of some chemokines and costimulatory molecules was specifically up-regulated in tonsillar MCs. Among them, the expression of OX40L on tonsillar MCs was significantly high compared with that on lung MCs. We confirmed that T cell proliferation is induced in direct cross-talk by OX40L on MCs.

**Materials and Methods**

**Cytokines and Abs**

The human rIL-3, rIL-6, and recombinant stem cell factor (rSCF) were purchased from Intergen (Purchase, NY). The following mouse anti-human mAbs were purchased: anti-tryptase (clone G3, Chemicon International, Temecula, CA; clone AA1, DakoCytomation, Carpinteria, CA), antichymase (clone B7; Chemicon International), anti-kir (clone YB5.88; BD Pharmingen, San Diego, CA), anti-FceRI (clone CRA-1; Kyokuto, Tokyo, Japan), anti-CD3 (clone UCHT-1, eBioscience, San Diego, CA; clone OKT3, Ortho Diagnostics, Westwood, MA), anti-CD14 (clone M 2-ME (Invitrogen Life Technologies, Grand Island, NY) containing 3 μg/ml fungizone (Invitrogen Life Technologies) and 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies). Cells were then separated by density-gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). The interface containing mononuclear cells was collected. Lineage-negative (Lin⁻) tonsillar cells were negatively selected from the mononuclear cells by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) using a mixture of magnetic microbead-conjugated Abs against CD4, CD8, CD11b, CD14, CD16, CD19, and CD22 (Miltenyi Biotec) according to the manufacturer’s instructions. Then, Lin⁻ tonsillar cells were incubated with anti-κir mAb for 30 min, and the Lin⁺ tonsillar cells were positively selected from Lin⁻ tonsillar cells by MACS using a magnetic microbead-conjugated Ab against mouse IgG1 (Miltenyi Biotec). Lin⁺ tonsillar cells were suspended in IMDM supplemented with 1% insulin-transferrin-selenium (Life Technologies), 50 μM 2-ME (Invitrogen Life Technologies), 100 U/ml penicillin-streptomycin, and 0.1% BSA (complete IMDM). For methylcellulose culture, the Lin⁺ Kir⁺ tonsillar cells were suspended in 0.3 ml of complete IMDM. The cells were mixed by shaking the tubes for >1 min with 2.7 ml of serum-free Iscove’s methylcellulose medium supplemented with 200 ng/ml rSCF, 50 ng/ml rIL-6, and 1 ng/ml rIL-3. IL-3 was added until 4 wk. The cell suspension was incubated at 0.3 ml per well in the 24-well plate at 37°C in 5% CO₂. Every 2 wk, 0.3 ml of fresh methylcellulose medium containing 200 ng/ml rSCF and 50 ng/ml rIL-6 was layered over the methylcellulose cultures.

**Purification and culture of human tonsillar MCs**

Fresh samples of tonsils were obtained after tonsillectomy at the Juntendo University School of Medicine (Tokyo, Japan) with informed consent. Tonsillar MCs were enzymatically dispersed as described previously (27–29). The dispersed cells were resuspended by IMDM (Invitrogen Life Technologies, Grand Island, NY) containing 3 μg/ml fungizone (Invitrogen Life Technologies) and 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies). Cells were then separated by density-gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). The interface containing mononuclear cells was collected. Lineage-negative (Lin⁻) tonsillar cells were negatively selected from the mononuclear cells by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) using a mixture of magnetic microbead-conjugated Abs against CD4, CD8, CD11b, CD14, CD16, CD19, and CD22 (Miltenyi Biotec) according to the manufacturer’s instructions. Then, Lin⁻ tonsillar cells were incubated with anti-κir mAb for 30 min, and the Lin⁺ tonsillar cells were positively selected from Lin⁻ tonsillar cells by MACS using a magnetic microbead-conjugated Ab against mouse IgG1 (Miltenyi Biotec). Lin⁺ tonsillar cells were suspended in IMDM supplemented with 1% insulin-transferrin-selenium (Life Technologies), 50 μM 2-ME (Invitrogen Life Technologies), 100 U/ml penicillin-streptomycin, and 0.1% BSA (complete IMDM). For methylcellulose culture, the Lin⁺ Kir⁺ tonsillar cells were suspended in 0.3 ml of complete IMDM. The cells were mixed by shaking the tubes for >1 min with 2.7 ml of serum-free Iscove’s methylcellulose medium supplemented with 200 ng/ml rSCF, 50 ng/ml rIL-6, and 1 ng/ml rIL-3. IL-3 was added until 4 wk. The cell suspension was incubated at 0.3 ml per well in the 24-well plate at 37°C in 5% CO₂. Every 2 wk, 0.3 ml of fresh methylcellulose medium containing 200 ng/ml rSCF and 50 ng/ml rIL-6 was layered over the methylcellulose cultures.

**Purification and culture of human lung MCs**

Macroscopically normal human lung resected during surgery was obtained at Teikyo University School of Medicine (Tokyo, Japan) and processed, after obtaining informed consent. Lung MCs were isolated as described previously (30). The final purity of MCs was 95–99%.

**Generation of adult peripheral blood-derived MCs**

Adult peripheral blood-derived MCs were generated as described previously (30). The final purity of MCs was 95–99%.

**Further purification of MCs**

To obtain high purity of MCs, cultured MC preparation was incubated with anti-κir mAb for 30 min, and then MCs were positively selected from cultured MC preparation by MACS using a magnetic microbead-conjugated Ab against mouse IgG1.

**Purification of CD4⁺ T cells**

CD4⁺ T cells were negatively selected from the mononuclear cells by MACS using a mixture of magnetic microbead-conjugated Abs against CD8, CD11b, CD14, CD16, and CD19 according to the manufacturer’s instructions. Then, CD4⁺ T cells were positively selected by MACS using magnetic microbead-conjugated Ab against CD4. Purity of CD4⁺ cells was >98%.

**Purification of human peripheral blood monocytes**

Human monocytes were separated as described previously (30). The final purity of monocytes was >95%.

**Activation of MCs**

For aggregation of FcεRI, MCs were sensitized with 1 μg/ml human myeloma IgE (CosmoBio, Tokyo, Japan) at 37°C for 48–72 h. After washing, the cells were challenged with either 15 μg/ml rabbit anti-human IgE Ab (DakoCytomation) or the culture medium alone at 37°C for 30 min for histamine assay and for the indicated time period for other assay. For aggregation of FcγRI, MCs were preincubated with 30 ng/ml IFN-γ (R&D Systems) for 48 h and biotinylated IgG1 (Calbiochem, Darmstadt, Germany) for the last 16 h. Biotinylated IgG1-sensitized MCs were challenged with 1 μg/ml streptavidin-alkaline phosphatase (BD Pharmingen) for the indicated time period. For stimulation of MCs via C5aR, MCs were challenged with 3 × 10⁻⁷ M C5a (Sigma-Aldrich, St. Louis, MO) for 30 min for histamine assay. For histamine release experiments, the cells were suspended in Tyrode solution as described previously (31). In other experiments, the cells were suspended in complete IMDM containing rSCF and rIL-6.

**Isolation of RNA and real-time quantitative RT-PCR**

Isolation of total RNA and real-time quantitative RT-PCR for OX40L, 4-1BBL, and GAPDH were performed as described previously (30). Relative expression levels were determined using cycle threshold values and the Compared Ct method to adjust for coamplification of housekeeper gene levels, 2-fold amplification/cycle rates, and the expression reference level of control samples (32).

**Immunocytochemistry of cultured MCs**

MCs were fixed in 4% acetone for 10 min after cytocentrifugation onto glass slides and then were washed in TBS (2 × 5 min) and treated for 20 min with TBS containing 1% BSA, 1% nonfat milk, and 10 mg/ml human IgG (ICN Biomedicals, Aurora, OH). Anti-tryptase mAb (clone AA1; 1/100 dilution) or isotype control mouse IgG1 was applied for 16 h at 4°C. After washing (3 × 5 min), bound Abs were visualized by the alkaline-phosphate-anti-alkaline-phosphatase techniques (DakoCytomation). The cell preparations were counterstained with Mayer’s hematoxylin.

**Flow cytometric analyses**

Flow cytometric analysis of MCs was performed as described previously (30). The mean fluorescence intensities of MCs stained with specific Abs and those stained with control Abs were obtained as described previously (30).

**Electron microscopic analysis**

For electron microscopic analysis, section of a tonsil and cultured tonsillar MCs were fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for 2 h. After rinsing in sodium phosphate buffer overnight, the samples were postfixed in 2% osmium tetroxide in 0.1 M sodium phosphate buffer at 4°C for 2 h and dehydrated with ethanol and propylene oxide. The samples were then embedded in Epok 812 (Okenshoji, Tokyo, Japan). At least 20 randomly selected MCs were examined and photographed with an electron microscope (JOEL, Tokyo, Japan) with an acceleration potential of 80 kV.

**GeneChip expression analysis**

Human genome-wide gene expression was examined by using the Human Genome U133A probe array (GeneChip; Affymetrix), which contains the oligonucleotide probe set for ~23,000 full-length genes and expressed sequence tags, according to the manufacturer’s protocol (Affymetrix) and previous reports (33–35). Further data analysis was performed using GeneSpring software version 5.1 (Silicon Genetics, San Carlos, CA). To normalize the staining intensity variations among chips, the average difference
(AD) values for all genes on a given chip were divided by the median of all measurements on that chip. To eliminate changes within the range of background noise and to select the most differentially expressed genes, data were used only if raw data values were <100 AD and the gene expression was judged to be present by Affymetrix data analysis. Hierarchical clustering analysis with standard correlation was used to identify gene clusters. The separation ratio was set at 0.5. Normalization values below 0 were set to 0. Data were considered significant when 1) expression changed by at least 1.5-fold (activation program) and 2) increased gene expression included at least one present call (Affymetrix algorithm). Furthermore, we used two donors’ average after normalization. The restriction to classify genes as up-regulated or down-regulated was applied to the normalized value. The expression levels of genes of the same cells analyzed twice showed significant correlation (coefficient of correlation = 0.997). Under the criteria above, the reproducibility of the expression level differences that were seen among different cells under different conditions was confirmed.

**Histamine release experiments**

Histamine in the supernatants and cell pellets was measured using an ELISA kit (MBL). The net percentage of histamine release was calculated from the ratio of each sample with spontaneous release subtracted against total histamine. MCs (1 × 10⁶) were used for each assay.

**ELISA for CCL3 and CCL4**

CCL3 and CCL4 were measured by ELISA kits purchased from R&D Systems. The sensitivities of the assays of CCL3 and CCL4 were 10 and 4 pg/ml, respectively.

**Confocal laser scanning microscopy**

Confocal laser scanning microscopy of MCs was performed as described previously (30).

**T cell proliferation assay**

Polyclonal proliferation of purified naïve CD4⁺ T cells was determined using cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) (36). Resting CD4⁺ T cells as responder cells were placed in 10 µg/ml anti-CD3 mAb (clone OKT3) or isotype control mouse IgG1 coating plate at 5 × 10⁵ cells per well in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS with only IgE-sensitized MCs at ratios of 1:1, 2:1, 5:1, and 10:1 of effector to stimulator cells in a total volume of 0.2 ml in each well. MCs or a positive control cell line, MT-2 cells, were used after pretreating the cells with 50 µg/ml mitomycin C. The cells were incubated for 5 days. At the end of the experiments, cells were incubated with 20 µl of the cell proliferation reagent WST-1 for 4 h. The absorbance of the treated samples against a blank control was measured using a GE-Nios (Tecan Japan, Tokyo, Japan). As a positive control, peripheral blood mononuclear cells were used after pretreatment of cells with 50 µg/ml mitomycin C (37). In some experiments, CD4⁺ T cells were cultured with MT-2 cells in an anti-CD3 mAb coating plate with neutralizing anti-OX40L mAb (0.1–30 µg/ml, clone 159403) for 5 days. In the peripheral blood-derived MC and CD4⁺ T cell coculture experiment, the IgE-sensitized MCs were activated with anti-IgE for 24 h. After first confirming up-regulation of OX40L expression by FACS, the MCs were treated with mitomycin C and cultured with or without CD4⁺ T cells. To investigate the contribution of mediators released by activated MCs, we harvested the MC supernatants in expressed as the mean ± SEM.

**Statistical analysis**

Differences between the two groups were analyzed by paired or unpaired Student’s t test and were considered significant at p < 0.05. Values are expressed as the mean ± SEM.

**Results**

**Establishment of in vitro culture system of tonsillar MCs**

In tonsils, MCs exist in the interfollicular area and they are surrounded by T cells (Fig. 1A) (38). We confirmed that these T cells were CD4⁺CD8⁻ cells by immunohistochemistry (data not shown). The MCs in the area contain scroll, particle, and mixed-type granules (Fig. 1B), but they do not express any granules with regular crystalline arrays resembling those frequently present in MCs from skin (39). We hypothesized that human tonsillar MCs might interact with T cells in the tissue. Because it was difficult to obtain highly purified tonsillar MCs with any method (27–29), we first tried to culture Lin⁻ kir⁺ cells from dispersed tonsillar cells in methylcellulose culture medium and we found MC colonies (60 MC colonies from 1 g of tissue; Fig. 1C). After 4-wk culture in methylcellulose culture medium, we transferred the cells into IMDM liquid medium following the standard method for culture of adult peripheral blood-derived MCs (40). However, the number of cells decreased in this system. Thus, we continued the culture of dispersed tonsillar MCs in methylcellulose medium after 4 wk and without any decrease in the number of cells. Examination of the cells after 8-wk culture showed them to be microscopically normal. An example of 8-wk cultured MCs cyto centrifuged onto a microscope slide and stained with anti-tryptase mAb is shown in Fig. 1D. Using this technique, we determined the number and percentage of MCs in the culture population. The number of tryptase-positive (tryptase⁺) cells after enzymatic dispersion was 2.1 ± 0.1 × 10⁵/g (Fig. 1E).
FIGURE 2. Characterization of human cultured tonsillar MCs. A–G, The cell surface expression of FceRI, kit, CD3, CD14, CD19, CD54, and CD88 on human cultured tonsillar MCs. Cultured tryptase^-gated tonsillar cells were stained by anti-FceRI (A), anti-kit (B), anti-CD3 (C), anti-CD14 (D), anti-CD19 (E), anti-CD54 (F), and anti-CD88 (G). H and I, Intracellular staining of tryptase^-gated freshly isolated (H) and cultured tonsillar cells (I) with anti-chymase mAb. In A–I, bold lines indicate Abs against FceRI, kit, CD3, CD14, CD19, CD54, and chymase. Dotted line showed isotype control Abs. J, Histamine release from cultured tonsillar MCs after FceRI aggregation or C5a stimulation. Each result is the mean ± SEM of two experiments. Spontaneous release from tonsillar MCs was 1.1 ± 0.02%.
The number of tryptase$^+$ cells gradually increased during the culture and reached 1.1 $\pm$ 0.3 $\times$ 10$^3$/g at 8 wk ($p < 0.05$; Fig. 1E). The percentage of freshly isolated tryptase$^+$ cells in Lin$^-$ kit$^+$ cells of dispersed tonsillar cells was 13.3 $\pm$ 3.5% (Fig. 1F). After 8 wk, the percentage of tryptase$^+$ cells increased to 95.8 $\pm$ 1.6% ($p < 0.01$; Fig. 1F). We examined the structure of 8-wk cultured tonsillar MCs by using electron microscopy. As can be seen in Fig. 1G, cultured tonsillar MCs contained immature granules with incomplete deposition and condensation of dense granule materials. This is one characteristic of human MCs cultured from umbilical cord blood cells (39) and from PBMC CD34$^+$ cells (41). The tonsillar MCs contained scroll-type granules, particle-type granules, and mixed-type granules (Fig. 1, G and H), but no crystalline granules, indicating that although the granules were immature, their types were similar to those of MCs in the interfollicular area of tonsils (Fig. 1B).

Characterization of cultured tonsillar MCs

It has been reported that enzymatically dispersed tonsillar MCs express FcεRI, kit, and CD54, but not CD3, CD14, CD19, and CD88 (42). As described above, >95% of 8-wk cultured tonsillar cells expressed tryptase. To investigate whether cultured tonsillar MCs express the same cell surface Ags as freshly isolated tonsillar MCs, such as FcεRI and kit, we performed flow cytometry analysis using PE-conjugated anti-FcεRI, kit, CD3, CD14, CD19, CD54, or CD88. As expected, ~100% of tryptase$^+$-gated cells expressed FcεRI and kit (Fig. 2, A and B). In agreement with a previous report on freshly isolated tonsillar MCs (42), the cultured MCs also expressed CD54, but not CD3, CD14, CD19, and CD88 (Fig. 2, C-G). Next, we compared the expression of chymase in freshly isolated MCs to that of 8-wk cultured MCs by flow cytometry. Using intracellular staining, both MCs were almost 100% chymase positive, but cultured MCs showed higher intensity of chymase expression than did freshly isolated MCs (Fig. 2, H and I). Thus, cultured tonsillar MCs showed an immunological pattern similar to that of freshly isolated tonsillar MCs. Because dispersed tonsillar MCs were reported to degranulate by aggregation of FcεRI, but not by C5a-mediated stimulation (43), we examined the histamine release from cultured tonsillar MCs after cross-linking of FcεRI and stimulation of C5a. As can be seen in Fig. 2J, IgE-dependent stimulation induced ~20% histamine release, but C5a caused minimal degranulation.

Gene expression profile of cultured tonsillar MCs

As described above, tonsillar MCs are closely associated with T cells in the interfollicular zones of the tonsils, but MCs are not closely associated with T cells (44, 45). Therefore, we hypothesized that resting tonsillar MCs express molecules that interact with T cells and that resting lung MCs do not. To clarify the specific gene expression profile in human tonsillar MCs, we evaluated ~23,000 genes by comparing expression levels with that in cultured lung MCs. To avoid the contribution of gene expression from contaminating cells in the culture population to GeneChip results, we positively selected resting tonsillar and lung MCs. To avoid the contribution of gene expression from contaminating cells in the culture population to GeneChip results, we positively selected resting tonsillar and lung MCs. Expression levels are shown as AD values obtained by GeneSpring software and selected if the mean of expression levels was >200 and flag was present. Numbers in the overlapping region of the Venn diagram represent shared MC-specific genes. Numbers of cultured tonsillar or lung MC-specific genes are shown inside the circles. B. Comparison of mRNA expression levels of CCL3 and CCL4 in human tonsillar and lung MCs. Expression levels are shown as AD values obtained by GeneChip analysis (see Materials and Methods). C. Comparison of CCL3 and CCL4 production from resting culture tonsillar and lung MCs. Resting tonsillar and lung MCs were cultured in serum-free culture medium for 6 h. Supernatants were collected and the concentrations of CCL3 and CCL4 were measured by ELISA. The production of CCL3 and CCL4 are the mean $\pm$ SEM of two and three independent experiments using two and three different donors, respectively. ND. Not detected.

Figure 3. Gene expression profiles in resting human cultured tonsillar and lung MCs. A. The comparison of resting human cultured tonsillar and lung MCs. The expression levels of cultured tonsillar and lung MCs were normalized by GeneSpring software and selected if the mean of expression level was >200 and flag was present. Numbers in the overlapping region of the Venn diagram represent shared MC-specific genes. Numbers of cultured tonsillar or lung MC-specific genes are shown inside the circles. B. Comparison of mRNA expression levels of CCL3 and CCL4 in human tonsillar and lung MCs. Expression levels are shown as AD values obtained by GeneChip analysis (see Materials and Methods). C. Comparison of CCL3 and CCL4 production from resting culture tonsillar and lung MCs. Resting tonsillar and lung MCs were cultured in serum-free culture medium for 6 h. Supernatants were collected and the concentrations of CCL3 and CCL4 were measured by ELISA. The production of CCL3 and CCL4 are the mean $\pm$ SEM of two and three independent experiments using two and three different donors, respectively. ND. Not detected.

4 The on-line version of this article contains supplemental material.

5 Supplemental Table 1 contains gene array data submitted to GEO. The accession number for "resting lung mast cells" is GSM29629; for "resting tonsillar mast cells1" is GSM29630; and for "resting tonsillar mast cells2" is GSM29631.
lungs as Fig. 3C), suggesting that only the resting tonsillar MCs have the ability to recruit T cells.

The 613 genes were specifically expressed in cultured lung MCs (Fig. 3A, I). These included CCL1, CXCL1, CXCL12, IL-13RIIa, and fibronectin receptor (data are shown in supplemental Table I).

Expression of OX40L and 4-1BBL on cultured tonsillar MCs

Interactions between costimulatory molecules and their receptors are crucial for the activation of T cells, the prevention of tolerance, and the development of T cell-dependent immunity (46–48). We found that certain costimulatory molecules such as OX40L (TNFSF4) and 4-1BBL (TNFSF9) were up-regulated in tonsillar MCs compared with lung MCs (Table I). Because the expression levels of OX40L and 4-1BBL in tonsillar MCs were more than three times higher than those in lung MCs (Table I), we focused on OX40L and 4-1BBL, which have been reported to induce T cell proliferation and cytokine production through OX40 and 4-1BB on T cells (16-18). To confirm whether tonsillar MCs express OX40L and 4-1BBL on their cell surface, we used FACS analysis using anti-OX40L and anti-4-1BBL mAb. In agreement with the results of the GeneChip analysis, the tonsillar MCs expressed the OX40L and 4-1BBL on their surface. The mean fluorescence intensity ratios of OX40L-to-control and 4-1BBL-to-control were 4.3 and 1.6, respectively (Fig. 4, A and B). In contrast, human lung MCs showed only minimal expression of these molecules (Fig. 4, Ba and Bb). To examine primary MCs isolated from tonsils for the presence and localization of OX40L, we next performed confocal laser scanning microscopy of MCs using anti-OX40L mAb. As can be seen in Fig. 4Cb, OX40L was expressed both on their surface and in their cytoplasm. However, 4-1BBL expression in the MCs was below the detection level (data not shown). In agreement with the results for cultured lung MCs, primary isolated lung MCs showed minimal expression of OX40L and 4-1BBL (Fig. 4, Da and Db).

Up-regulation of OX40L, 4-1BBL, CCL3, and CCL4 in MCs after aggregation of FcεRI

To examine whether the specifically up-regulated genes in resting cultured tonsillar MCs, which include CC chemokines, costimulatory molecules, and TNFSFs/TNFRSFs, are further up-regulated after aggregation of FcεRI, we comprehensively examined the expression levels in anti-IgE-activated tonsillar MCs by using GeneChip. Because the transcriptomes for cytokines related to inflammation were reported to be up-regulated at early time points in peripheral blood-derived MCs after FcεRI-mediated activation (30), we chose 6 h as the anti-IgE stimulation period in our experiments. Genes with expression levels that changed in response to stimulation were selected on the basis of repeated differences in the expression levels of the treated and untreated samples.

We found that some genes were further up-regulated after aggregation of FcεRI in the specifically up-regulated genes in the resting tonsillar MCs (1646 genes). These contained CC chemokines such as CCL3 and CCL4, TNFSFs such as OX40L and Fas ligand, and TNFRSFs such as CD30 (Table II). Next, we confirmed the up-regulation of OX40L and 4-1BBL expression in anti-IgE-stimulated tonsillar MCs by real-time RT-PCR and flow cytometric analysis. As can be seen in Fig. 5A, OX40L and 4-1BBL mRNA were maximal at 3 h after FcεRI aggregation. After aggregation of FcεRI, OX40L and 4-1BBL expression on tonsillar MC surfaces increased 1.3- and 1.1-fold, respectively (Fig. 5B). This might be attributable to the high spontaneous expression of OX40L and 4-1BBL on tonsillar MCs. We further confirmed the up-regulation of CCL3 and CCL4 production from tonsillar MCs after aggregation of FcεRI using ELISA (Fig. 5C).

To investigate whether these CC chemokines and TNFSFs/TNFRSFs were up-regulated in human lung MCs after aggregation of FcεRI, we performed GeneChip analysis using anti-IgE-activated human lung MCs. The results revealed that not only tonsillar MCs but also lung MCs up-regulate the expression of these CC chemokines and TNFSFs/TNFRSFs (Fig. 5C and Table II). We confirmed that human lung MCs up-regulated OX40L after FcεRI aggregation (Fig. 5D), suggesting that human lung MCs may interact with T cells at inflammation sites. Furthermore, we used peripheral blood-derived MCs to confirm the up-regulation of OX40L expression after aggregation of FcεRI or FcγRI. As can be seen in Fig. 5E, peripheral blood-derived MCs up-regulated OX40L after the aggregation of FcεRI or FcγRI.

### Table I. Comparison of gene expression levels of costimulatory molecules, TNFSFs, and TNFRSFs between resting tonsillar and lung MCs

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<td>ID0G_T</td>
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<td>2107.1</td>
<td>1.5</td>
</tr>
<tr>
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<td>NP_003811</td>
<td>1086.1</td>
<td>407.9</td>
<td>2.7</td>
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</table>

*Gene expression levels are shown as AD values obtained from GeneChip expression analysis (see Materials and Methods). These genes were selected if the gene expression in tonsillar MCs was judged to be present by Affymetrix data analysis. “Expression ratio of tonsillar to lung MCs” means the value of the ratio of each AD values of tonsillar to lung MCs.

*APRIL, a proliferation-inducing ligand.*
the effect of mediators released from activated MCs on T cell proliferation, we used resting tonsillar MCs. Resting CD4+ T cells were cultured with the MCs at ratios of 1:1, 2:1, 5:1, and 10:1 of effector cells to stimulator cells in the presence of anti-CD3 mAb. Because it is known that T cells are also capable of promoting MC activation (49, 50), MCs were treated with mitomycin C. As can be seen in the upper panel of Fig. 6A, when the ratio of effector cells to stimulator cells was in the range of 2:1 to 1:1, MCs could induce significant proliferation of CD4+ T cells (p < 0.05 compared with MCs alone). The OD_{450} values of T cells alone (5 × 10^4/well) on the plates coated with anti-CD3 mAb and isotype control mouse IgG1 were 0.008 ± 0.006 and 0.048 ± 0.010, respectively (n = 4 and 3, respectively). OD_{450} value of MCs alone increased according to increase in the number of MCs (2.5 × 10^4 to 5 × 10^4 MCs/well). The cell proliferation reagent WST-1 is cleaved to formazan dye by mitochondrial dehydrogenases in viable cells (51, 52). In this experiment, the increase in the number of MCs in a well results in the augmentation in formazan dye produced by viable cells (51, 53). The lower panel of Fig. 6A shows the effect of peripheral blood monocytes as a positive control, on polyclonal proliferation of CD4+ T cells using the same assay method. The OD_{450} value of monocytes alone was also detectable. When the ratio of CD4+ T cells to monocytes was 1:1, monocytes induced marked proliferation of CD4+ T cells. To determine the contribution of OX40L on T cell proliferation, CD4+ T cells were cocultured with MCs in anti-CD3 mAb coating plate with neutralizing anti-OX40L mAb. To determine the optimal concentrations of anti-OX40L mAb, CD4+ T cells were cocultured with mitomycin C-treated MT-2 cells, which express high levels of OX40L, with various concentrations of anti-OX40L mAb (0.1~30 μg/ml). The results showed that the neutralizing anti-OX40L mAb inhibited the proliferation of CD4+ T cells in a concentration-dependent manner and that 10 μg/ml and 30 μg/ml concentrations of anti-OX40L mAb significantly inhibited T cell proliferation (p < 0.01 or p < 0.05, data not shown). Because of the limited availability of human tonsillar MCs, we used 10 μg/ml anti-OX40L mAb in this study. The proliferation was significantly inhibited by the addition of the neutralizing anti-OX40L mAb (p < 0.01; Fig. 6B). Finally, we investigated whether T cell proliferation would be induced when anti-IgE-activated peripheral blood-derived MCs, which up-regulate OX40L expression, were substituted for tonsillar MCs and whether such an effect would be inhibited by anti-OX40L mAb. Anti-IgE-activated peripheral blood-derived MCs were treated with mitomycin C and then were cultured with or without CD4+ T cells. As can be seen in Fig. 6C, when the ratio of effector cells to stimulator cells was 1:1, the MCs induced significant proliferation of CD4+ T cells (p < 0.01), but

Table II. Comparison of gene expression levels of costimulatory molecules, TNFSFs, and TNFRSFs between IgE-dependent activated tonsillar and lung MCs.

<table>
<thead>
<tr>
<th>Costimulatory molecules</th>
<th>Accession</th>
<th>Tonsillar MCs</th>
<th>Lung MCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD54</td>
<td>P05362</td>
<td>1.3</td>
<td>3.4</td>
</tr>
<tr>
<td>TNFSFs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX40L (TNFSF4)</td>
<td>A39680</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>FasL (TNFSF6)</td>
<td>P48023</td>
<td>3.4</td>
<td>1.8</td>
</tr>
<tr>
<td>TNFRSFs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD30 (TNFRSF8)</td>
<td>P28908</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>TRAIL-R2 (TNFRSF10B)</td>
<td>1D0G_T</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Death receptor 6 (TNFRSF21)</td>
<td>NP_055267</td>
<td>5.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* The values are shown as the ratio of AD values of activated MCs to those of control resting MCs. These genes were selected if the gene expression in activated tonsillar MCs was judged to be present by Affymetrix data analysis.

FIGURE 4. FACS analysis of OX40L and 4-1BBL expression on tonsillar and lung MCs. Cultured tonsillar (A) and lung (B) tryptase+ gated cells were stained using anti-OX40L mAb (Aa or Ba) or anti-4-1BBL mAb (Ab or Bb). C, Localization and expression of OX40L in human primary isolated tonsillar MCs. Primary isolated tonsillar MCs were first incubated with anti-trypwise (green) and anti-OX40L (red) and then were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (blue). OX40L is shown alone (Ca) or merged with DAPI (Cc), whereas tryptase is shown merged with DAPI (Ca). A differential interference contrast image of primary isolated tonsillar MCs is also shown (Cd). Original magnification, ×400. D, Primary isolated lung MCs were stained with anti-OX40L mAb (Da) or anti-4-1BBL mAb (Db) and were analyzed by FACS. In A, B, and D, bold lines show OX40L- or 4-1BBL-stained cultured tryptase+ cells. Dotted lines show isotype control Abs. The results of one representative experiment of two are shown.
FIGURE 5. Up-regulation of OX40L, CCL3, and CCL4 in MCs after aggregation of FceRI on FcγRI. A. IgE-sensitized tonsillar MCs were incubated with (●) or without (○) anti-IgE for indicated time points, and OX40 or 4-1BBL mRNA expression was examined by real-time RT-PCR. Data are shown as the fold induction of OX40L or 4-1BBL expression level against 0-h-stimulated tonsillar MCs. The results of one representative experiment of three using three different donors are shown.

B, IgE-sensitized tonsillar MCs were incubated with (Bb or Bd) or without (Ba or Bc) anti-IgE for 2 h, and OX40L (upper panels) and 4-1BBL (lower panels) expression was examined by FACS. The bold lines show OX40L- or 4-1BBL-stained cultured MCs, and the dotted lines show isotype control Abs.

C, Production of CCL3 and CCL4 from tonsillar and lung MCs after aggregation of FceRI. (Figure legend continues)
neither activated MCs alone nor resting MCs alone induced an increase in OD450 values (Fig. 6C). Supernatants from mitomycin C-treated anti-IgE-activated MCs from the same donor failed to exert a stimulatory effect. Similarly, to determine the contribution of OX40L to T cell proliferation, CD4+ T cells were cocultured with activated MCs on an anti-CD3 mAb-coated plate with neutralizing anti-OX40L mAb (3, 10, and 30 μg/ml), and proliferation was found to be significantly inhibited by the addition of the anti-OX40L mAb in a concentration-dependent manner (p < 0.05; Fig. 6D). The OD450 value of the activated MCs plus T cells on the plate coated with isotype control mouse IgG1 was 0.089 ± 0.013 (n = 3). Mitomycin C-treated resting peripheral blood-derived MCs, which were used as a negative control, did not induce T cell proliferation, and the neutralizing anti-OX40L mAb did not affect the T cell proliferation.

**Discussion**

MCs are known to activate T cells in autoimmune diseases and infection (6, 8). However, it has not been clear what molecular mechanisms may be involved in MC-T cell interaction. Here we have shown that MCs express OX40L on their surface (Figs. 4 and 5) and that MCs induce T cell proliferation in an OX40L-dependent manner (Fig. 6). We established cell cultures of human tonsillar MCs (Fig. 1), which showed characterization similar to that of freshly dispersed tonsillar MCs in all aspects examined (Fig. 2). To find out candidate molecules that have the ability to induce T cell proliferation, we compared the gene expression profiles between tonsillar and lung MCs (Fig. 3) and found that some CC chemokines, costimulatory molecules, and TNFSFs/TNFRSFs were up-regulated in tonsillar MCs (Figs. 3 and 4). Among them, OX40L is highly up-regulated in resting tonsillar MCs, and the coculture of resting tonsillar MCs and T cells induced T cell proliferation (Fig. 6). Upon activation by cross-linking of FcεRI or FcγRI, human MCs up-regulated OX40L (Fig. 5), and anti-IgE-activated MCs induced T cell proliferation via OX40L/OX40-mediated cross-talk (Fig. 6), suggesting that MCs interact with T cells not only in lymph nodes but at sites of inflammation as well.

The first question is whether cultured tonsillar MCs are really the same as in vivo tonsillar MCs. Although the microenvironment may change the characterization of MCs (54), we cultured tonsillar and lung MCs under the same preparation method. A comparison of gene expression profiles revealed significant differences (Fig. 3). In agreement with the results of chymase expression of tissue MCs (55), cultured tonsillar MCs showed higher intensity of the expression than did cultured lung MCs (data not shown). These findings suggest that lung or tonsillar Lin− kir+ cells might be committed to lung or tonsillar MCs, respectively. As we noticed the colony formation and proliferation of tonsillar and lung MCs during our culture system (Fig. 1), dispersed tonsillar or lung cells would contain MC progenitors and immature MCs.

A large fraction of the gene expression profiles of cultured tonsillar MCs were comparable with other MCs, including cultured lung MCs. However, CCL3 and CCL4 were specifically expressed in resting cultured tonsillar MCs, but not resting cultured lung MCs at the mRNA and protein levels (Fig. 3, B and C). These results suggest that tonsillar MCs may recruit the T cells in tonsils without activation. In agreement with our data, it has been reported that CCL3 and CCL4 have the ability to recruit T cells into lymph nodes and that MCs are one of the major sources of CCL4 (56). It has been reported that both TNF-α concentration and the recruitment of circulating T cells were increased within draining lymph nodes after peripheral MC activation (8). In our results, TNF-α expression was up-regulated in tonsillar MCs after FcεRI aggregation, but not in resting tonsillar MCs (data not shown). After aggregation of FcεRI, CCL3 and CCL4 production in tonsillar MCs was further increased (Fig. 5C). These results suggest that T cells may be recruited by tonsillar MCs in the resting state and that T cells may be further accumulated by MCs after activation.

We have shown that unstimulated tonsillar MCs expressed OX40L and 4-1BBL on their surface (Fig. 4). It has been reported that cord blood-derived cultured human MCs express CD80 and that human mast cell lines, HMC-1, express CD40L on their surface (13). However, we found only minimal expression of CD80, CD86, and CD40L on the surface of human MCs by FACS, and FcεRI activation did not up-regulate the expression of these molecules (data not shown). Both OX40L and 4-1BBL can induce proliferation of T cells and differentiation from naive T cells to Th2 cells (18, 20, 26). Thus, we hypothesized that tonsillar MCs might induce the activation of T cells by interaction with OX40L/OX40L-mediated cross-talk (Fig. 6), suggesting that MCs interact with T cells not only in lymph nodes but at sites of inflammation as well.

(Figure legend continued) IgE-sensitized MCs were cultured with or without anti-IgE for 6 h. Supernatants were collected and the concentrations of CCL3 and CCL4 were measured by ELISA. The results are presented as the mean ± SEM of four independent experiments (tonsillar MCs) and of one experiment (lung MCs). ND, Not detected. D, FACS analysis of OX40L on IgE-dependent activated cultured lung MCs. For aggregation of FcεRI, MCs were preincubated with IgE for 48 h. IgE-sensitized MCs were incubated with (Db) or without (Dc) anti-IgE for 24 h. The bold lines show OX40L-stained cultured MCs, and the dotted lines show isotype control Abs. E, FACS analysis of OX40L on IgE-(Ea and Eb) or IgG1-dependent (Ec and Ed) activated peripheral blood-derived MCs. For aggregation of FcεRI, MCs were preincubated with IgE for 48 h. For aggregation of FcγRI, MCs were preincubated with IFN-γ and biotinylated IgG1 for 48 h. IgE or biotinylated IgG1-sensitized MCs were incubated with (Eb or Ed) or without (Ea or Ec) anti-IgE or streptavidin-allophycocyanin for 24 h. The bold lines show OX40L-stained cultured MCs, and the dotted lines show isotype control Abs. The results shown are representative of two independent experiments using two different donors.
MCs could present immunogenic peptides, from soluble Ags endocytosed through fluid to T cells mediated by OX40L and MHC class II molecules.

After activation of MCs, we also noticed that apoptosis-inducible genes such as Fas ligand were induced (Table II). However, co-culture of MCs with T cells induced T cell proliferation under our experimental conditions. Thus, further fine tuning of molecular mechanisms may exist in the interaction between MCs and T cells to determine whether proliferation or apoptosis of T cells is induced.

In this report, we demonstrated for the first time that tonsillar MCs activate T cells through direct cross-talk by OX40L/OX40. We identified a subset of genes that was specifically expressed in cultured tonsillar MCs but not lung MCs by using GeneChip. This finding provides further data to support the assumption of functional heterogeneity among different tissue-derived human MCs (58, 59). Specifically expressed genes in cultured tonsillar MCs include CC chemokines such as CCL3 and CCL4 and TNFSFs such as OX40L and 4-IBBL. Here, we focused on OX40L and 4-IBBL and found that OX40L was up-regulated in lung MCs after FcεRI aggregation. Also, OX40L was up-regulated in peripheral blood-derived MCs after aggregation of FcγRI. Taken together, human MCs might be involved in the induction of adaptive immune responses via recruitment and activation of T cells in allergic responses or autoimmune diseases.

**Acknowledgments**

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**References**


**FIGURE 6.** Induction of T cell proliferation by coculture with tonsillar MCs. A, Proliferation of T cells by coculture with a various number of tonsillar MCs. CD4+ T cells were cultured with resting tonsillar MCs (upper panel) or mitomycin C-treated monocytes (lower panel) in anti-CD3 mAb coating plate for 5 days. T cell proliferation was determined by WST-1 assay. OD450 values of MCs alone and T cells plus MCs are shown as open and filled circles, respectively (upper panel). OD450 value of CD4+ T cells alone in anti-CD3 mAb coating plate was 0.008 ± 0.006 (n = 4). Data are expressed as the mean ± SEM of triplicate wells. The results of one representative experiment of two are shown. OD450 values of human monocytes alone and CD4+ T cells plus monocytes are shown as open and filled squares, respectively (lower panel). *, p < 0.05; **, p < 0.01; when OD450 value of T cells plus MCs is compared with OD450 value of MCs alone. B, CD4+ T cells were cultured with tonsillar MCs in anti-CD3 mAb coating plate with or without neutralizing anti-OX40L mAb. After 5 days, proliferation of T cells was detected. All assays were performed in triplicate. The results are shown as the mean ± SEM of three independent experiments using three different donors. ***, p < 0.01, when OD450 value of cells with neutralizing anti-OX40L mAb is compared with OD450 values of cells without neutralizing anti-OX40L mAb or with control mIgG1. C, Proliferation of T cells by coculture with various numbers of anti-IgE-activated peripheral blood-derived MCs. Anti-IgE-activated MCs were treated with mitomycin C and then were cultured with (●) or without (□) CD4+ T cells in anti-CD3 mAb coating plate. Mitomycin C-treated resting MCs were cultured with (●) or without (□) CD4+ T cells in anti-CD3 mAb coating plate. The OD450 values of the supernatants from activated MCs (see Materials and Methods) plus T cells and from resting MCs plus T cells are shown as filled and open triangles, respectively. T cell proliferation was determined by WST-1 assay. OD450 value of T cells plus MCs in mouse IgG1 coating plate was 0.049 ± 0.008 (n = 3). Data are expressed as the mean of triplicate wells. ***, p < 0.01, when OD450 value of T cells plus activated MCs is compared with OD450 value of activated MCs alone. D, Effect of resting peripheral blood-derived MCs on CD4+ T cell proliferation. CD4+ T cells were cultured with resting (□) or activated (●) peripheral blood-derived MCs in anti-CD3 mAb or mouse IgG1 coated plates with or without neutralizing anti-OX40L mAb (3–30 μg/ml), and T cell proliferation was determined by WST-1 assay. ***, p < 0.05, when OD450 value of cells with anti-OX40L mAb is compared with OD450 value of cells without anti-OX40L mAb.


