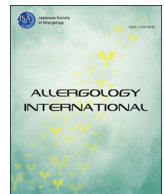




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Letter to the Editor

Metallothioneins are required for human basophil interleukin-4 gene induction via FcεRI stimulation

Dear Editor,

Metallothioneins (MTs) are low-molecular-weight (Mr, 7 kDa) cysteine-rich proteins that exhibit high affinities for divalent heavy

metal ions.¹ MTs assist in the detoxification of trace metals, such as cadmium, and in maintaining homeostasis with respect to essential metals, such as copper and zinc (Zn), under physiological conditions.¹

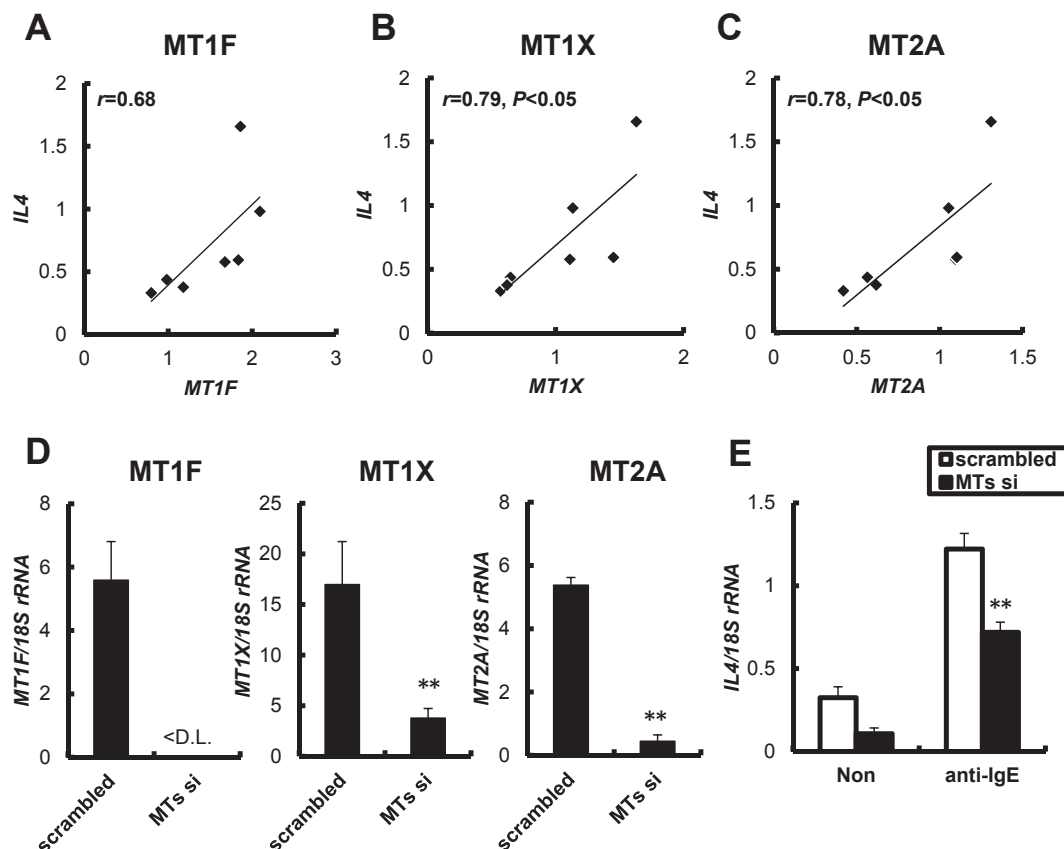


Fig. 1. Human basophils require MTs to induce IL-4 gene transcription. (A–C) Levels of IL4, MT1F, MT1X, and MT2A transcripts in blood basophils were measured relative to 18S rRNA using real-time RT-PCR. Correlations between MT1F and IL4 (A), MT1X and IL4 (B), and MT2A and IL4 (C) expression are shown. Basophil purity was >95%. Student's *t*-tests were used for statistical analyses and *P*-values are indicated. *R*-values were determined using Pearson's correlation tests. (D–E) Human basophils isolated from 200 ml of venous blood collected from each healthy donor were transfected with siRNA (Sigma–Aldrich) using a two-step electroporator. The viability of the transfected cells ranged from 41% to 46%. (D) Real-time RT-PCR analysis of MT1F, MT1X, and MT2A transcripts in MT-knock down and control basophils relative to 18S rRNA. (E) MT-knock down (closed bars) and control (open bars) basophils were stimulated with anti-human IgE for 2 h. IL4 transcript levels (normalised to 18S rRNA) were measured using real-time RT-PCR. Data represent means $\pm$ SD; ***P* < 0.01 by Student's *t*-tests compared with control basophils. <D.L., below detection limit. Data representative of two experiments are shown. The primers were used for PCR reactions as listed in Supplementary Table 1. The primers, except for 18S rRNA, were designed and synthesized by TAKARA.

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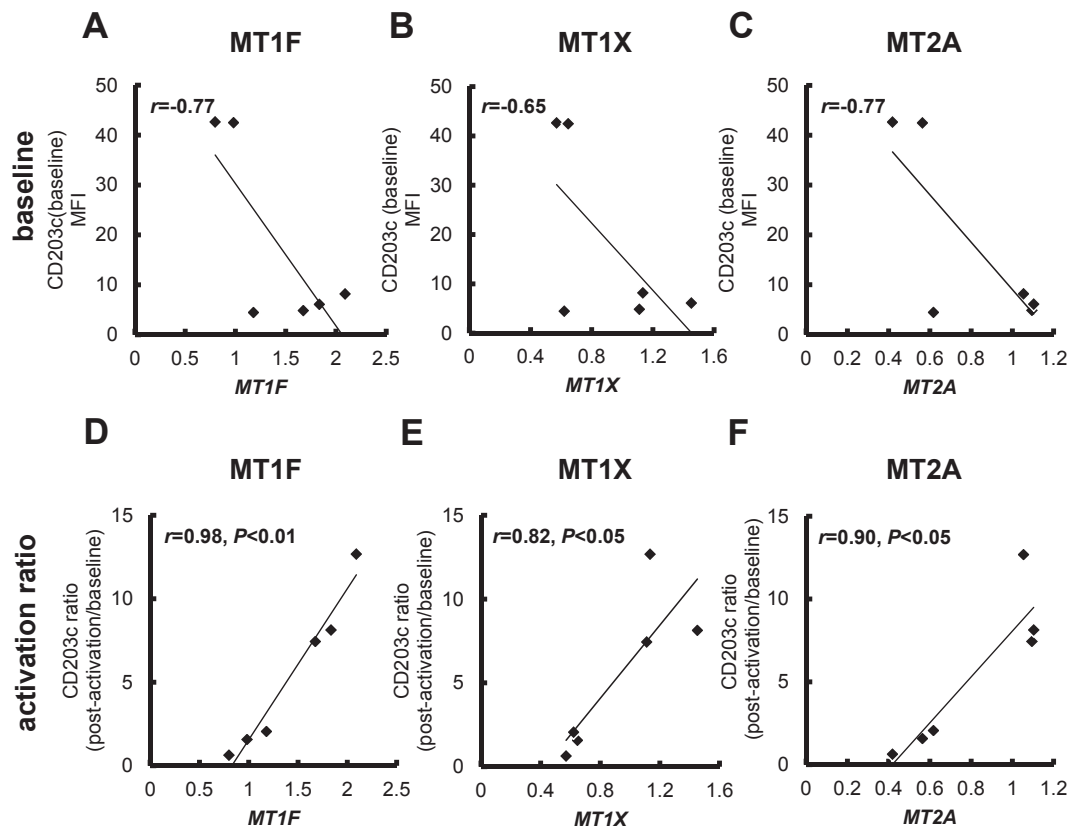


Fig. 2. MTs are involved in FcεRI-induced CD203c up-regulation. Venous whole blood isolated from six of the donors in Figure 1 were treated with EDTA and stained with fluorescein isothiocyanate (FITC)-conjugated CRTH2 Ab, PC7-conjugated CD3 Ab, and phycoerythrin (PE)-conjugated CD203c Ab in the presence or absence of anti-IgE Ab. The mean fluorescence intensity (MFI) of CD203c on basophils (CRTH2⁺CD203c⁺CD3⁺ cells) was measured by flow cytometry. The correlation between MFIs of CD203c (baseline) and MT1F (A), MT1X (B), or MT2A (C) and relative induction ratios (post-activation/baseline) of CD203c and MT1F (D), MT1X (E), or MT2A (F) are shown. Student's *t*-tests were used for statistical analyses; *P*-values are as indicated. *R*-values were determined using Pearson's correlation tests.

Basophils are a major source of interleukin (IL)-4.² In addition, several recent murine studies revealed that basophils are variously involved in immune regulation and allergic inflammation via IL-4. Basophil-derived IL-4 contributes to the development of antigen-specific Th2 cells.³ Basophil-derived IL-4 also initiates eosinophilic inflammation in lungs,⁴ and plays a regulatory role in IgE-mediated allergic skin inflammation.⁵ However, the molecular mechanism of basophil IL-4 production, especially in humans, remains unclear. We previously demonstrated that MT-dependent Zn regulation is critical for FcεRI-mediated T cells (NFAT) signalling and IL-4 gene transcription in murine basophils.⁶ Here, we explored the role of MTs in human basophil IL-4 gene induction.

MTs are grouped into four classes: MT1, MT2, MT3, and MT4.¹ In humans, MTs are encoded by a family of genes consisting of 10 functional isoforms: seven MT1 genes (*MT1A*, *MT1B*, *MT1E*, *MT1F*, *MT1G*, *MT1H*, and *MT1X*), *MT2A*, *MT3*, and *MT4*.¹ To determine which isoforms are involved in regulating basophil function in humans, quantitative real-time RT-PCR was used to examine the profile of MT gene expression in blood basophils. Whole blood was collected from seven healthy volunteers after obtaining informed consent. Blood basophils were separated from EDTA-treated whole blood by Ficoll gradient centrifugation and then negatively selected using a Human Basophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Among the 10 functional MT-family genes, *MT1F*, *MT1X*, and *MT2A* were detected in basophils isolated from all donors (Fig. 1A–C and data not shown). These

data indicate that MT1 and MT2 are involved in regulating basophil function.

To clarify whether MTs are involved in IL-4 gene induction in human basophils, we measured the level of *IL4* transcripts in blood basophils using quantitative real-time RT-PCR, and assessed the correlation between *IL4* transcript levels and levels of the *MT1F*, *MT1X*, and *MT2A* genes. The expression levels of *MT1F* and *IL4* were slightly correlated (Fig. 1A), although the correlation was not statistically significant. However, the levels of *MT1X* and *MT2A* were highly correlated with *IL4* levels (Fig. 1B–C). These results suggest that MT1 and MT2 are involved in IL-4 gene transcription in human basophils.

To verify this finding, we transfected blood basophils with siRNAs targeting *MT1F*, *MT1X*, and *MT2A* to knock down the majority of MT1 and MT2 expression. We first confirmed that specific siRNA treatment reduced the expression of *MT1F*, *MT1X*, and *MT2A* mRNAs (Fig. 1D). The induction of *IL4* mRNA expression by FcεRI was significantly inhibited in the MT-knock down human basophils compared to control basophils (Fig. 1E), indicating that MTs are required for FcεRI-mediated IL-4 gene induction.

The surface antigen CD203c is an ecto-nucleotide pyrophosphatase phosphodiesterase expressed on human basophils and is extensively used as a basophil activation marker.⁷ To further examine the relationship of MTs and basophil activation status, we assessed the expression level of CD203c on basophils isolated from the blood of six of the seven donors from Figure 1 before and after IgE-stimulation, in order to evaluate basophil activation

status at baseline and post-activation, respectively. We analysed the correlation between the expression levels of CD203c and *MT* transcript levels in each donor. There was no correlation between the baseline levels of CD203c and *MT* levels (Fig. 2A–C). However, the activation ratios (post-activation/baseline) of CD203c expression had a strong correlation with *MT* levels (Fig. 2D–F), indicating that *MT*s are involved in FcεRI-induced CD203c up-regulation. Furthermore, we assessed the correlation between serum IgE levels and *MT* levels in each donor and found that there was no correlation between them (Supplementary Fig. 1).

In this study, we found that the *MT* and *IL4* transcript levels in human basophils were highly correlated (Fig. 1B–C). Knock down experiments further confirmed that *MT*s were required for FcεRI-induced *IL-4* gene transcription in human basophils (Fig. 1D–E). Thus, the requirement of *MT*s for FcεRI-induced *IL-4* gene transcription is conserved. However, the mechanism responsible for the contribution of *MT*s to FcεRI-induced *IL-4* gene transcription in human basophils remains unknown. Our prior study in mice showed that *MT*s are necessary for maintaining the intracellular Zn level, which regulates the CaN/NFAT-mediated basophil *IL-4* induction.⁶ In human basophils, FcεRI signals induce the nuclear translocation of NFAT and its binding to the *IL-4* promoter.⁸ In this study, we examined the effect of the CaN inhibitor cyclosporine A (CSA) on FcεRI-induced *IL-4* gene transcription in human basophils. CSA pretreatment significantly diminished the FcεRI-stimulated increase in *IL4* mRNA expression (Supplementary Fig. 2), indicating that the CaN/NFAT pathway is responsible for FcεRI-mediated *IL-4* gene induction, even in human basophils. Taken together, these findings strongly suggest that *MT*s are involved in FcεRI-induced *IL-4* gene transcription through NFAT activation in human and murine basophils.

Recent murine studies showed that *IL-4* enables basophils to play opposing inflammatory and regulatory roles in allergic inflammation.^{4,5} In humans, basophils reportedly infiltrate the peripheral tissues during allergic inflammation, such as in the case of bronchial asthma or atopic dermatitis.^{9,10} However, it remains unclear whether basophil-derived *IL-4* is involved in the pathogenesis of these human allergic diseases. In this study, we demonstrated that *IL4* transcription in human basophils rises as the transcription of several *MT* genes increases (Fig. 1B–C). We further revealed that FcεRI stimulation induced a higher degree of CD203c up-regulation in basophils from donors with high *MT* levels than in basophils from donors with low *MT* levels (Fig. 2D–F). These findings suggested that *MT* levels influence basophil *IL-4* secretion and activity potential in each individual. Therefore, further investigation, including analysis of *MT* expression levels in basophils from allergic subjects, may shed light on the involvement of basophil-derived *IL-4* in human allergic diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2016.03.005>.

Conflict of interest

The authors have no conflict of interest to declare.

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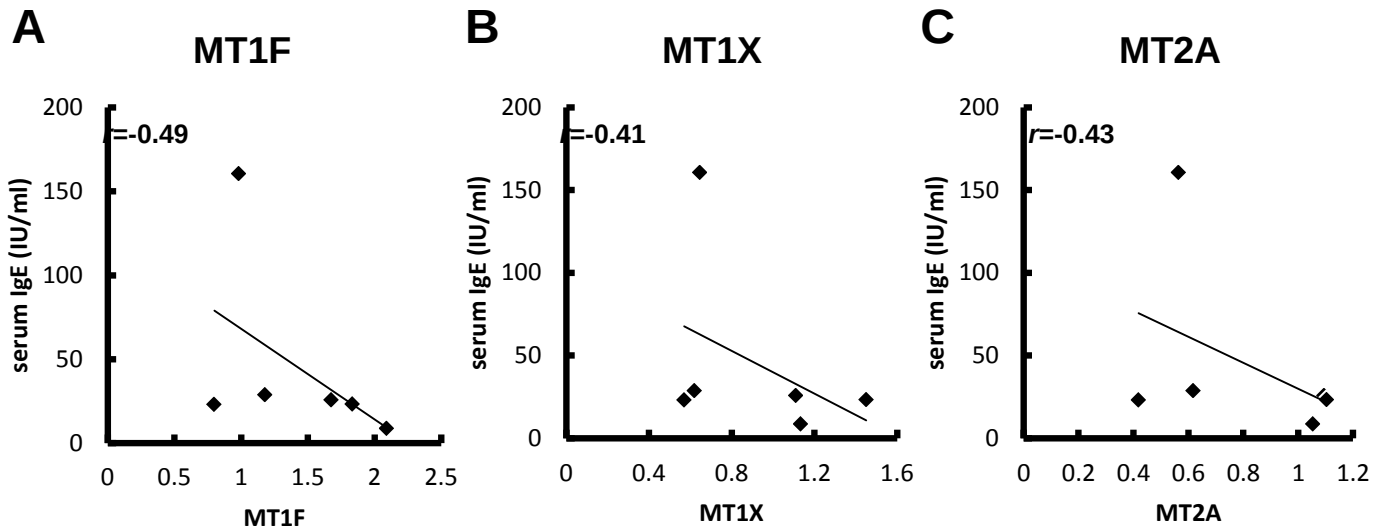
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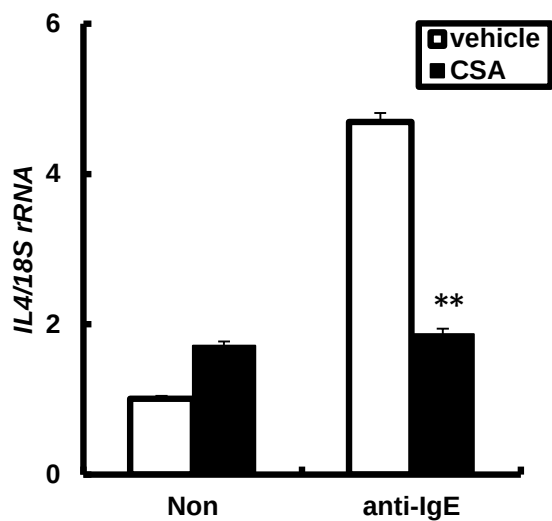
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Supplementary Fig. 1. The levels of serum IgE and MTs are not correlated in human basophils.

Serum was separated from venous blood isolated from the same donors as in Figure 2. The concentration of IgE in serum was measured with an enzyme-linked immunosorbent assay kit (Abcam). The correlations between serum levels of IgE and *MT1F* (A), *MT1X* (B), or *MT2A* (C) are shown. Student's *t*-tests were used for statistical analyses; *R*-values were determined using Pearson's correlation tests.



Supplementary Fig. 2. FcεRI-induced IL-4 production is dependent on a calcineurin-mediated pathway.

Human basophils isolated from venous blood collected from healthy donors were treated with 2 μM cyclosporin A (CSA, Calbiochem) (closed bars) or vehicle (open bars) for 20 min, then stimulated with anti-human IgE for 2 h. The level of *IL4* transcription (normalised to *18SrRNA*) was then measured using SYBR Green dye and real-time PCR. Data show the mean+SD. ***p*<0.01, compared with vehicle-treated basophils, Student's t-test. The data shown are representative of three independent experiments.

Supplementary Table 1. Primer sets used for Real-Time PCR Analyses

Gene product	Direction	PCR primers
MT1F	Forward	5'-CGCTTCTCTCTTGGAAAGTCCAGT-3'
	Reverse	5'-CTTCTTGCAGGAGGTGCATTTG-3'
MT1X	Forward	5'-CGGGAACTCCTGCTTCTCCTT-3'
	Reverse	5'-CTTCTTGCAGGAGGTGCATTTG-3'
MT2A	Forward	5'-CAGGGCTGCATCTGCAAAG-3'
	Reverse	5'-GAATATAGCAAACGGTCACGGTCA-3'
IL-4	Forward	5'-AGCAGCTGATCCGATTCCTGA-3'
	Reverse	5'-TCCAACGTACTCTGGTTGGCTTC-3'
18SrRNA	Forward	5'-AGTCCCTGCCCTTTGTACACA-3'
	Reverse	5'-GATCCGAGGGCCTCACTAAAC-3'