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Functions of Metallothionein Generating Interleukin-10-Producing Regulatory CD4$^+$ T Cells Potentiate Suppression of Collagen-Induced Arthritis

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Abstract Metallothionein, a cysteine-rich stress response protein that is naturally induced by a variety of immunologic stressors, has been shown to suppress autoimmune disorders through mechanisms not yet fully defined. In the present study, we examined the underlying mechanisms by which metallothionein might mediate such regulation of autoimmunity. Naïve CD4$^+$ T cells from metallothionein-deficient mice differentiated to produce significantly less IL-10, TGF-γ, and repressor of GATA, but more IFN-γ and T-bet, when compared with those from wild-type mice. The levels of IL-4 and GATA-3 production were not different between the two groups of mice. Conversely, treatment with exogenous metallothionein during the priming phase drove naïve wild-type CD4$^+$ T cells to differentiate into cells producing more IL-10 and TGF-β, but less IFN-γ than untreated cells. Metallothionein-primed cells were hyporesponsive to restimulation, and suppressive to T cell proliferation in an IL-10-dependent manner. Lymphocytes from metallothionein-deficient mice displayed significantly elevated levels of AP-1 and JNK activities in response to stimulation compared with those from wild-type controls. Importantly, transgenic mice overexpressing metallothionein exhibited significantly reduced susceptibility to collagen-induced arthritis and enhanced IL-10 level in the serum, relative to their nontransgenic littermates. Taken together, these data suggest that metallothionein is able to promote the generation of IL-10- and TGF-β-producing type 1 regulatory T-like cells by downregulating JNK-dependent AP-1 activity. Thus, metallothionein may play an important role in the regulation of Th1-dependent autoimmune arthritis, and may represent both a potential target for therapeutic manipulation and a critical element in the diagnostic assessment of disease potential.

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Induction and maintenance of self-tolerance are essential to maintain immune homeostasis. In addition to the thymic deletion of autoantigen-reactive T cells, active suppression mediated by T cells and T cell factors have emerged as crucial mechanisms that maintain peripheral tolerance. Under certain circumstances, the mechanism of active suppression seems to be naturally induced as a consequence of homeostatic regulation, although the physiological conditions underlying this induction remain to be further studied.

Factors that play a role in the maintenance of normal physiology include metallothionein (MT). MT is a low molecular weight (about 7 kDa), cysteine-rich, metal-binding protein that is readily inducible in most tissues upon exposure to a variety of cellular stressors such as heavy metal cations, reactive oxygen species (ROS), bacterial toxins, and acute phase cytokines [10, 15, 26, 49]. Although the action of MT at the cellular level is not yet fully understood, MT has been proposed as an intracellular regulator of some transcription factors, since antiapoptotic and mitogenic activities of MT in neoplastic cells are dependent on its interaction with the p50 subunit of NF-κB [1]. Reports of MT in various extracellular compartments at significant levels and the expression of MT-specific-receptor on astrocytes [16] suggest that another way in which extracellular MT may evoke physiologic changes is by ligand-like interactions with target cells. This role for extracellular MT is underscored by the recent report of MT's action as a chemokine [53].

Functions of MT have been implicated in the maintenance of physiologic homeostasis *via* several ways. MT can

protect essential cellular compartments from toxicants, including heavy metals and free radicals, by binding and sequestering them [4, 34]. MT also serves as a reservoir of essential metals such as zinc and copper [21]. In addition, several lines of evidence suggest that MT is intimately involved in the maintenance of immune homeostasis by counteracting autoimmune-mediated damage. The serum concentrations of MT have been reported to be depleted as rheumatoid arthritis progresses, and could be significantly enhanced by cortisone treatment, which subsequently results in significant clinical improvement [36]. Targeted disruptions of the *Mt1* and *Mt2* genes have been found to elicit much more severe autoimmune diseases in viable motheaten *Hcph*^{mev} mice and experimental autoimmune encephalomyelitis (EAE)-established mice [32, 45]. Conversely, administration or induction of MT resulted in significantly reduced clinical manifestations of collagen-induced arthritis (CIA), EAE, and streptozotocin-induced diabetes [8, 42, 44, 54]. Interestingly, this suppressive effect correlated with the elevated frequency of TGF-β-producing cells [54], suggesting that MT may mediate the induction of regulatory cells that play a crucial role in the mechanism of active suppression.

Several subsets of regulatory T cells with distinct phenotypes and mechanisms of action have been identified. These include type 1 regulatory T (Tr1) cells, which secrete high levels of IL-10 and low to moderate levels of TGF-β with little or no production of IL-2 and IL-4 [20]. Initially, Tr1 clones were generated by chronic antigenic stimulation of $CD4^+$ T cells in the presence of IL-10 [20]. These clones could suppress the proliferation of $CD4^+$ T cells in response to antigen (Ag), and prevent colitis induced in SCID mice by pathogenic $CD4^+CD45RB^{hi}$ splenic T cells. The suppressive effects of Tr1 clones were reversed by neutralizing IL-10 with antibody (Ab), suggesting that, regardless of their Ag specificities, Tr1 cell suppression is a bystander effect mediated through the production of IL-10. More recently, different routes other than IL-10 costimulation have been proposed to induce the differentiation of Tr1 cells, despite the sole dependence of its suppressive activity on IL-10. For instance, *in vitro* manipulation of naïve $CD4^+$ T cells with immunosuppressive drugs, vitamin D3 plus dexamethasone, and neutralizing Abs to IL-4 and IL-12 has facilitated the expansion of Tr1-like cells that exclusively secrete IL-10 and can prevent EAE [3]. These investigations suggest that Tr1 cells can be differentiated *in vivo* under diverse conditions.

The present study was designed to test the possibility that MT, naturally induced under physiological stress, can provoke the differentiation of regulatory T cells bearing Tr1-like phenotypes. We found that MT-deficient naïve $CD4^+$ T cells differentiated into effector cells without IL-10- and TGF-β-producing capacity. This defect could be reversed by addition of exogenous MT. These *in vitro* functions of MT appear to be recapitulated *in vivo*, since MT transgenic mice were not susceptible to arthritis development induced under type 1 helper T (Th1) predominant conditions. Thus, our study proposes MT as a novel physiological inducer of Tr1-like phenotypes and provides a link between environmental stress and adaptive immunity.

MATERIALS AND METHODS

Mice

MT-deficient mice (129S7/SvEvBrd-Mt1^{tm1Bri}Mt2^{tm1Bri}/J) [34] and their controls (129S1/SvImJ) were purchased from The Jackson Laboratories. C57BL/6 mice were obtained from The Orient Corporation (Seoul, Korea). The MT transgenic B6/129Sv mice [43] purchased from The Jackson Laboratories were backcrossed with DBA/1J mice for more than 7 generations to obtain the DBA/1 MT transgenic mice used in the present study. All mice were bred and maintained under specific pathogen-free conditions at the animal facilities of Hanyang University according to the animal protocol guidelines of Hanyang University (Seoul, Korea).

Cell Culture Reagents

Cells were cultured in RPMI 1640 (JBI, Deagu, Korea) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol, and 2 mM L-glutamine (all from Gibco). Recombinant murine IFN-γ and IL-10 were purchased from BD Pharmingen and recombinant murine IL-4 from Peprotech (London, U.K.). The following monoclonal Abs used for the cell culture were purchased from BD Pharmingen: anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), anti-IL-12p70 (C17.8), anti-IL-10 (JES5-16E3), anti-CD3ε (145-2C11), and anti-CD28 (37.51).

In Vitro T Cell Differentiation and Cytokine Assays by ELISA

Splenic naïve $CD4^+$ T cells were purified by negative selection using the naïve T cell $CD4^+CD62L^+CD44^{lo}$ isolation columns (R&D Systems) according to the manufacturer's instructions. The purified fraction contained more than 95% of $CD4^+CD44^{lo}$ cells. Naïve $CD4^+$ T cells were stimulated with 10 μg/ml immobilized anti-CD3ε and 2 μg/ml anti-CD28 Abs (neutral conditions). In some experiments, the cells were stimulated in the presence of murine IL-12 (10 ng/ml) and anti-IL-4 monoclonal Ab (5 μg/ml) for Th1 differentiation, or murine IL-4 (10 ng/ml), anti-IL-12 (5 μg/ml), and anti-IFN-γ (5 μg/ml) monoclonal Ab for Th2 differentiation. After 5–6 days, cells were washed and re-stimulated with anti-CD3ε (10 μg/ml) and anti-CD28 (2 μg/ml) Abs for 24 or 48 h. To test the effects of exogenous MT or IL-10 on T cell differentiation, 10 μM purified rabbit MT-I+II (Sigma), 10 ng/ml recombinant

murine IL-10, or 10 μg/ml anti-IL-10 monoclonal Ab was added during the priming period. The culture supernatants were collected at 24 h for IFN-γ and IL-4 measurement and 48 h for TGF-β and IL-10 measurement [27]. The concentrations of these cytokines were determined using the R&D DuoSet ELISA Development Systems (R&D Systems) according to the manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Splenocytes from MT knockout (KO) and wild-type mice were stimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) Abs for 24 or 48 h in the presence of 10 ng/ml IFN-γ or 10 ng/ml IL-4 and assayed by RT-PCR as described [54]. To determine the level of repressor of GATA (ROG) transcripts in Tr1 cells, splenocytes were stimulated with anti-CD3ε, anti-CD28 and 10 ng/ml IL-10 for 7 days, washed, and restimulated with anti-CD3ε and anti-CD28 for 8 h. The primer sequences used were as follows: ROG sense, 5' CTC TCT GGA GTC AGA ATC AGC TAA 3'; ROG antisense, 5' AGC GCT GAG GAC AGA GGC TAC AGG 3'; T-bet sense, 5' TTC CCA TTC CTG TCC TTC ACC G 3'; T-bet antisense, 5' GGA AGG TCG GGG TAG AAA C 3'; GATA3 sense, 5' TCT GGA GGA GGA ACG CTA ATG G 3'; GATA3 antisense, 5' GAA CTC TTC GCA CAC TTG GAG ACT C 3'; β2 microglobulin (β2m) sense, 5' TGA CCG GCT TGT ATG CTA TC 3', β2m antisense, 5' CAG TGT GAG CCA GGA TAT AG 3'.

Proliferation Assays and Suppression Assays

Lymph node cells (2×10^5 cells/well) primed in the presence of MT or IL-10 were restimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.2 μg/ml) for 3 days in the presence or absence of 10 μg/ml neutralizing Ab to IL-10. ^{3}H-thymidine (1 μCi/well) was added to the culture for the last 16 h, and the incorporated radioactivity was measured using a scintillation counter.

To determine the suppressive capacity of exogenous MT- or IL-10-primed T cells on responder cell proliferation, MT- or IL-10-primed T cells were cocultured with lymph node cells from C57BL/6 mice under the stimulation with immobilized anti-CD3 and soluble anti-CD28 Abs in the presence or absence of 10 μg/ml anti-IL-10 Ab for 3 days. T cell proliferation was assayed as described above.

EMSA and Western Blot Assays

Splenocytes were stimulated with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) for 1 h and nuclear extracts were prepared as previously described [37]. DNA-binding reactions were performed using 5 μg of nuclear extract in 12-μl reactions containing 2 μg poly dI-dC competitor and ^{32}P-labeled double-stranded oligonucleotide (5' CGC TTG ATG AGT CAG CCG GAA 3'). DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer as previously described [37]. To determine the levels of c-Jun and c-Fos protein expression, splenocytes were stimulated as above overnight, followed by standard Western blot methods using anti-c-Jun (SC44X) and anti-c-Fos (SC-253X) polyclonal Abs obtained from Santa Cruz Biotechnologies.

JNK Kinase Assays

Splenocytes from MT KO and wild-type mice were starved in medium without serum for 2 h and stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 15 min. The cells were lysed with a solution containing 0.1% SDS, 0.5% deoxycholate sodium, 1% NP-40, 0.1 mM sodium orthovanadate, and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml PMSF). Cell lysates were incubated with 2 μg of anti-JNK Ab (Santa Cruz Biotechnologies) and 20 μl Protein A-agarose beads overnight at 4°C. Anti-JNK immunoprecipitates were resuspended in 10 μl kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT) containing 1 μg c-Jun-GST (Cell Signaling Technology). Reactions were initiated by adding 10 μCi γ-^{32}P-ATP and incubated for 30 min at room temperature. The c-Jun-GST phosphorylation was assessed by autoradiography of the SDS-PAGE. The amount of JNK protein in each sample was determined by Western blot analyses.

Induction and Evaluation of CIA

MT transgenic and nontransgenic DBA/1 mice (7–8 weeks old) were immunized with 100 μg of bovine type II collagen (CII, Chondrex) emulsified in complete Freund's adjuvant (Chondrex) by intradermal infection at the base of the tail. Two weeks later, mice were subjected to booster immunization with 50 μg of bovine CII emulsified in incomplete Freund's adjuvant (Chondrex). Starting on day 25 after primary immunization, mice were inspected for disease progression. The severity of arthritis was graded for each paw using the following scoring system: 0= normal, 1=slight swelling on 1 or 2 joints, 2=modest swelling on more than 2 joints, and 3=extreme swelling of the entire paw and/or ankylosis [38]. Each limb was graded, resulting in a maximal clinical score of 12 per animal and expressed as the mean arthritic index on a given day.

For histopathologic examinations, hindpaws were removed on day 38 after primary immunization, fixed in 10% phosphate-buffered formalin, and decalcified in 5.5% EDTA in phosphate-buffered formalin. Decalcified paws were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Arthritic changes in the ankle and foot were scored as previously described [30], where 0=normal, 1= weak leukocyte infiltration but no erosion, 2=modest infiltration and weak erosion, 3=severe infiltration and invasion of bones, and 4=loss of bone integrity.

Sera were collected from CIA-established mice on day 38 post primary immunization to measure levels of CII-specific IgG by ELISA, as described previously [28]. Mouse serum at a 1:10,000 dilution was tested, and serum collected from CIA mice marking maximal symptoms was serially diluted and used as standard for the CII-specific IgG. IL-10 concentration in the sera was measured by standard sandwich ELISA using the R&D DuoSet ELISA Development Systems (R&D Systems). The detection limit for IL-10 in the ELISA was <30 pg/ml.

RESULTS

Development of Effector CD4$^+$ T Cells from MT-Deficient Mice

In MT-deficient mice, subsets of CD4$^+$, CD8$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells in the spleen and lymph nodes were found in normal numbers (data not shown). Thus, T cell development appeared normal in naïve mice despite the MT deficiency.

In light of the investigations that intracellular redox regulators and some MT inducers are able to modulate proliferation and differentiation of T lymphocytes [40, 41, 46, 47], we tested whether MT deficiency could influence the development of CD4$^+$ T effector cells during *in vitro* stimulation. Cells from MT KO mice produced approximately 7-fold less IL-10 and 2-fold less TGF-β than those from their wild-type littermates when naïve CD4$^+$CD44^{hi}CD62L^{hi} T cells were stimulated through the TCR and costimulatory CD28 molecules without cytokines and neutralizing Abs to cytokines (neutral conditions) for 5–6 days, washed, and restimulated (Fig. 1A). The levels of IFN-γ and IL-4 production were not significantly different between MT KO and wild-type mice under these conditions. However, cells from MT KO mice produced over 3.4-fold more IFN-γ than those from their wild-type counterparts, when effector cells were developed under the Th1 conditions, whereas there was no difference in the level of IL-4 production between mouse strains under the Th2 conditions (Figs. 1B and 1C). These results demonstrated that naïve CD4$^+$ T cells primed through only TCR/CD28 costimulatory pathways in MT-deficiency condition did not efficiently develop into IL-10- and TGF-β-producing cells. Moreover, MT deficiency facilitated the process of Th1 polarization, but had no influence on the process of Th2 polarization.

MT Deficiency Influences the Expression Levels of T-bet and ROG

T-bet and GATA3 are master transcription factors that direct naïve CD4$^+$ T cells to differentiate toward Th1 and Th2 cells, respectively [17, 39]. ROG, a new member of the poxviruses and zinc finger family of transcriptional

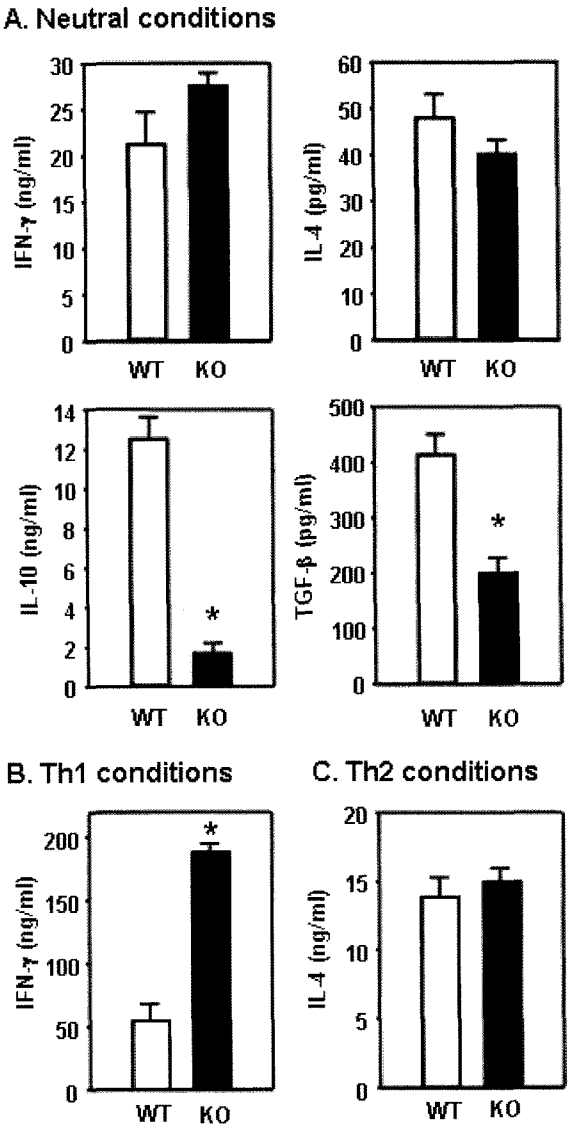

Fig. 1. Defective IL-10 and TGF-β production but enhanced IFN-γ production in MT-deficient CD4$^+$ T cells.
Untouched naïve CD4$^+$CD44$^+$CD62L^{hi} T cells from MT knockout (KO) and wild-type (WT) mice were cultured with anti-CD3ε and anti-CD28 Abs. In some experiments, IL-12 and anti-IL-4 Ab were added for Th1 conditions (**B**), and IL-4, anti-IL-12, and anti-IFN-γ Abs were added for Th2 conditions (**C**). After 5–6 days, cells were washed and restimulated with anti-CD3ε and anti-CD28 Abs for 1 or 2 days. Culture supernatants were subjected to ELISA. Representative results of more than five experiments are shown. *$P < 0.05$ (Student's *t*-test).

repressor that mediates negative regulation of T cell activation, has been recently proposed as a new marker of Tr1 cells [9]. Our data show that lymph node cells from normal mice express ROG transcripts upon restimulation after priming in the presence of IL-10 (Fig. 2B). In response to stimulation with anti-CD3 and anti-CD28 Abs, ROG expression was detected in lymph node cells from normal mice (Fig. 2A, lane 3). However, cells from MT KO mice did not express ROG under any tested conditions

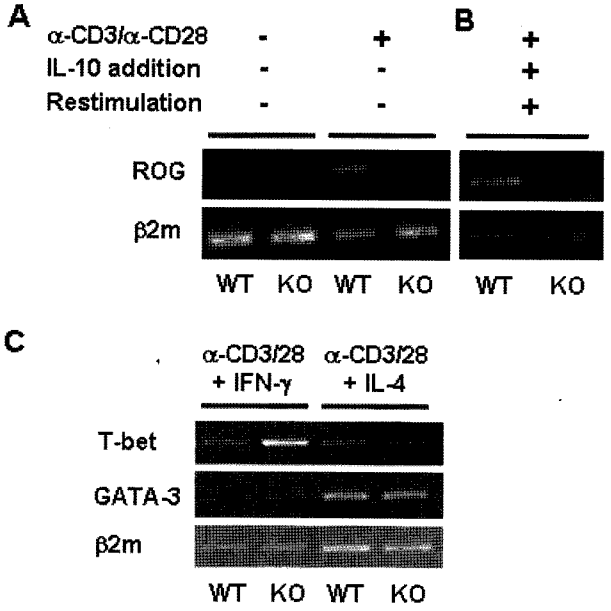

Fig. 2. Downregulation of ROG but upregulation of T-bet expression in MT-deficient T cells.
A and **C**. Splenocytes from MT knockout (KO) and wild-type (WT) mice were stimulated with anti-CD3ε and anti-CD28 Abs without (**A**) or with IFN-γ (**C**, lanes 1 and 2) or IL-4 (**C**, lanes 3 and 4) for 24 h. **B**. Cells were stimulated with anti-CD3/anti-CD28 Abs plus IL-10 for 5–7 days, washed, and restimulated without IL-10 for 8 h. Cells were assayed by RT-PCR. Representative results of more than three experiments are shown.

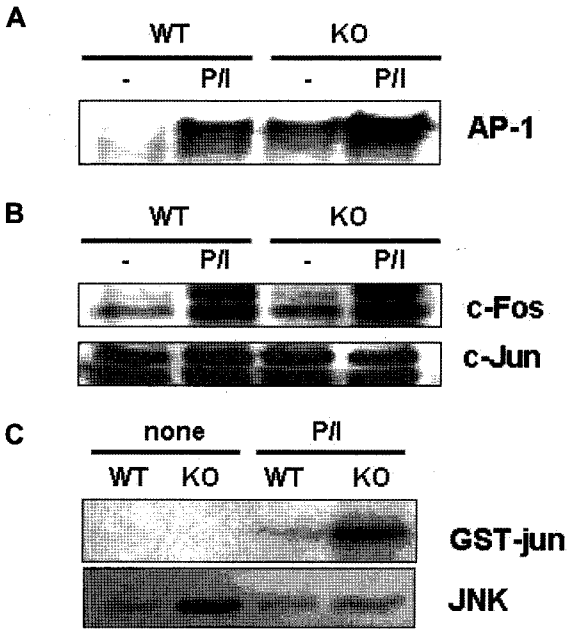

Fig. 3. Enhanced activity of AP-1 in MT-deficient mice.
A. Spleen cells from MT knockout (KO) and wild-type (WT) mice were stimulated with PMA plus ionomycin (P/I) for 1 h. Nuclear fractions were extracted and the DNA-binding activity of AP-1 was assessed by EMSA. **B**. After overnight stimulation, cells were lysed. Whole cell lysates were resolved by SDS-PAGE and assayed by Western blot methods using anti-c-Fos and anti-c-Jun Abs. **C**. Spleen cells were stimulated with PMA plus ionomycin for 15 min. Cell lysates were subjected to immunoprecipitation with anti-JNK antibody, followed by kinase assays (upper panel) and immunoblotting with anti-JNK Ab (lower panel). Representative results of more than three experiments are shown.

(Figs. 2A and 2B). In contrast, the level of T-bet expression was much higher in MT KO cells than in normal cells, when cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IFN-γ for 24 h (Fig. 2C, lanes 1 and 2). Upon stimulation with TCR/CD28 plus IL-4 stimulation, GATA-3 expression was induced in both MT KO and wild-type cells at a comparable level (Fig. 2C, lanes 3 and 4). Thus, these data support the results in Fig. 1, demonstrating that MT deficiency led to a defect in Tr1 cell development but facilitated Th1 cell development.

Enhanced Activation of AP-1 in MT-Deficient Mice

It has previously been shown that AP-1 activity is essential for the signal transduction of Ag-stimulated T cells and that inhibition of AP-1 activity results in the functional unresponsiveness of T cells to antigenic stimulation [23, 50]. To determine whether MT-mediated regulation of T cell differentiation is associated with the level of AP-1 activity, splenocytes obtained from MT-deficient and wild-type mice were stimulated with PMA plus ionomycin and assayed by EMSA. The DNA-binding activity of AP-1 from MT KO cells was remarkably higher than that from wild-type controls in both unstimulated and stimulated conditions (Fig. 3A).

AP-1 is regulated at the levels of both *jun* and *fos* gene transcription and posttranslational modifications of their gene products. To test whether the increase in AP-1 activity of MT-deficient cells is attributed to the enhanced levels of c-Fos and c-Jun proteins, the expression level of these proteins were assessed by Western blot analyses. Whereas c-Fos expression was induced following stimulation, c-Jun did not vary after stimulation. These protein levels were not significantly different between two strains of mice (Fig. 3B), indicating that the difference of AP-1 activity between mice was not caused by regulation of protein synthesis.

Mechanisms by which AP-1 activity is regulated at the posttranscriptional level include phosphorylation of c-Jun by MAPK such as JNK [13]. To assess whether MT KO-associated upregulation of AP-1 activity results from enhanced JNK activity, its kinase activity from MT KO cells was compared with that from wild-type cells. JNK activity was induced in PMA/ionomycin-treated cells from both mice, but the induction level of MT KO cells was remarkably higher than that of wild-type cells (Fig. 3C). Taken together, these results demonstrate that MT deficiency resulted in upregulation of AP-1 activity through a JNK-dependent pathway rather than regulation of *de novo* synthesis of the proteins composed of AP-1.

Exogenous MT Enhances the Development of IL-10-Producing Regulatory T Cells

The finding that MT-deficient $CD4^+$ T cells were defective in their differentiation to IL-10- and ROG-expressing cells suggests that MT has the potential to promote the development of Tr1 cells. To address this possibility, $CD4^+$ T cells from wild-type mice were stimulated with anti-CD3 and anti-CD28 Abs in the presence of exogenous MT for 5–6 days, washed, and restimulated in the absence of MT. Cells primed in the presence of MT produced dramatically more IL-10 and TGF-β than those in the absence of MT, whereas no significant difference was evident in the production of IFN-γ and IL-4 regardless of

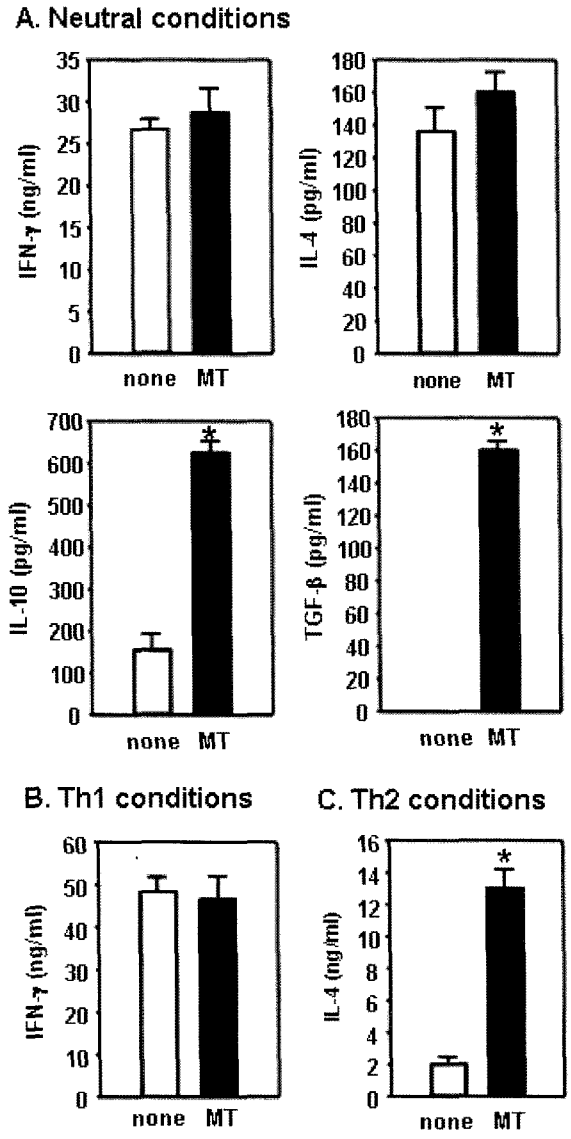

Fig. 4. Generation of Tr1-like cells by priming with MT. **A.** Lymph node cells from C57BL/6 mice were primed with anti-CD3e and anti-CD28 Abs in the presence or absence of 10 μM MT. In some experiments, cells were primed under the Th1 (**B**) or Th2 (**C**) conditions. After 7 days, cells were washed and restimulated for 1 or 2 days. Culture supernatants were subjected to ELISA. Representative results of more than three experiments are shown. $*P < 0.05$ (Student's *t*-test).

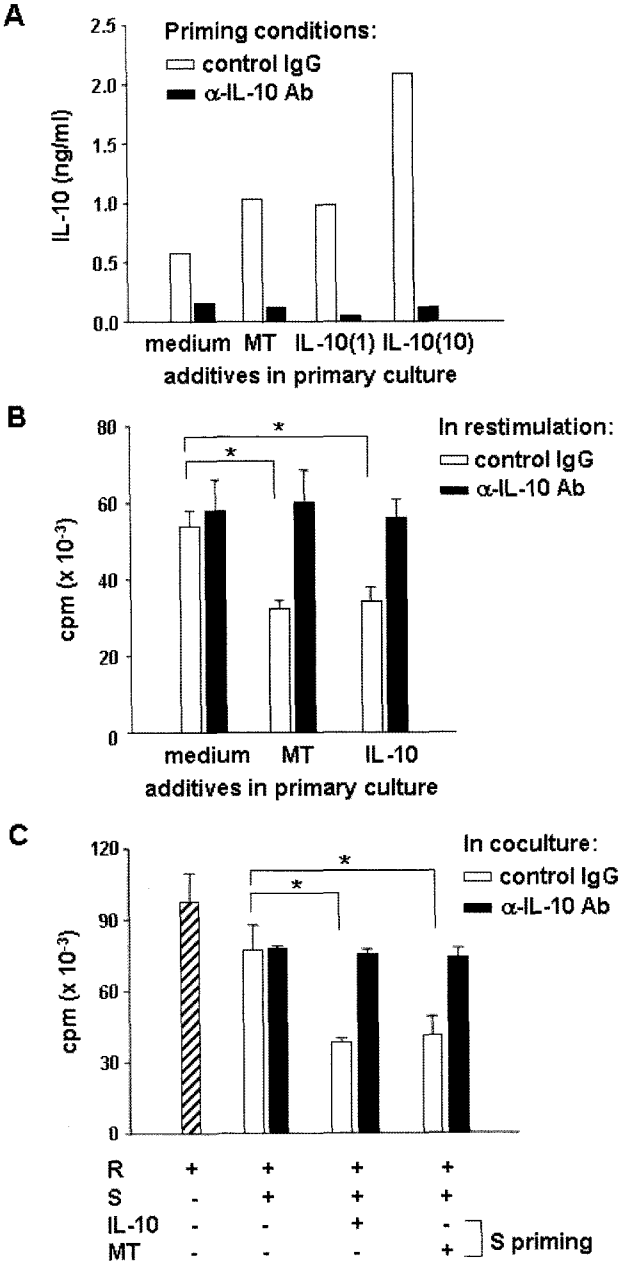

Fig. 5. Activity of MT-primed T cells in an IL-10-dependent manner. Lymph node cells from C57BL/6 mice were primed with anti-CD3ε and anti-CD28 Abs in the presence or absence of 10 μM MT or IL-10 (1 or 10 ng/ml). To neutralize IL-10, anti-IL-10 Ab or control IgG was added to some wells (**A**). After 5–7 days of culture, cells were washed and re-stimulated in the absence (**A**) or presence of anti-IL-10 or control IgG (**B** and **C**). **A.** After 2 days, the culture supernatants were analyzed to measure the level of IL-10 using cytokine ELISA. **B.** After 3 days, proliferation of the cells was assessed by ^{3}H-thymidine incorporation assays. **C.** MT- or IL-10-primed cells (S) were cocultured with naïve syngenic lymphocytes (R) in the presence of stimulation with anti-CD3ε and anti-CD28 Abs for 3 days, followed by ^{3}H-thymidine incorporation assays. All these results are representative of five independent experiments. $*P < 0.05$ (Student's *t*-test).

treatment with MT (Fig. 4A). The IL-10 produced by wild-type $CD4^+$ T cells following MT priming was comparable to that resulting from priming with 1 ng/ml recombinant murine IL-10 (Fig. 5A). When the cells were primed under the Th2 conditions in the presence of MT, they produced more IL-4 than those in the absence of MT (Fig. 4C). Under the Th1 conditions, cells produced huge amounts of IFN-γ irrespective of MT treatment during the priming period (Fig. 4B). To examine whether such activity of MT was dependent on IL-10 production, neutralizing Ab to IL-10 was added during the time of primary stimulation. Neutralization of IL-10 during priming dramatically reduced the IL-10 producing capacity of MT-primed cells as well as that of IL-10 primed cells (Fig. 5A). Like IL-10-primed cells, MT-primed cells were significantly less proliferative in response to TCR/CD28 costimulation and suppressive to responder cells, and anti-IL-10 Ab added during proliferation and suppression assays fully reversed these phenomena (Figs. 5B and 5C). Thus, these results demonstrate that naïve $CD4^+$ T cells exposed to MT during priming favor differentiation to Tr1-like cells retaining IL-10-producing capacity, which is dependent on IL-10 production during the

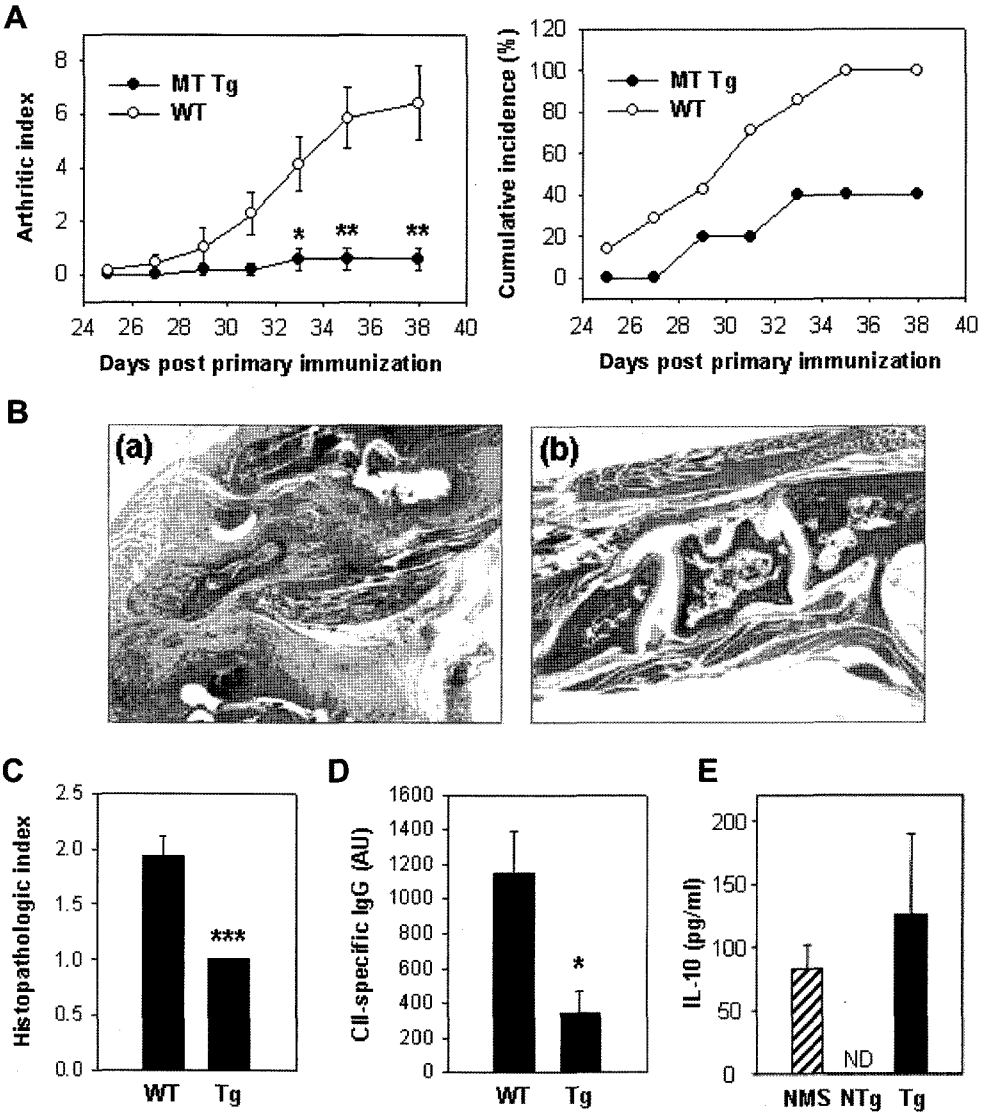

Fig. 6. Suppression of CIA in MT transgenic mice.
MT transgenic (Tg) and nontransgenic (WT) DBA/1 mice were immunized with CII/CFA emulsion at day 0, followed by booster immunization with CII/IFA at day 14. **A.** Joint swelling was graded from days 25 to 38. **B.** On day 38 post-primary immunization, the severity of synovitis from hindpaws of sacrificed mice were examined by histopathological studies. Figures are representative of each group of mice; a, WT; b, MT Tg. **C.** The mean histopathologic scores of each group are plotted as a histopathologic index, as described in Materials and Methods. **D** and **E.** Sera collected at day 38 were assayed to measure the levels of CII-specific IgG and IL-10 concentrations by ELISA. AU, arbitrary unit; NMS, pre-immune serum obtained from MT transgenic and nontransgenic mice; ND, not detectable. These results were reproducible in two independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Student's *t*-test.

primary stimulation. The hypoproliferative and suppressive activities of MT-primed cells were also IL-10-dependent, similar to functional characteristics of Tr1 cells.

MT Overexpression Suppresses CIA

CIA is a widely used model for studying human rheumatoid arthritis, since it resembles rheumatoid arthritis in that both cellular and humoral mechanisms are involved in the pathogenic process [52, 54]. The initiation of disease in CIA is largely dependent on the development of Th1 cells, and adoptive transfer of $CD25^+$ natural regulatory T cells could ameliorate the onset and progression of the disease [38]. However, whether Tr1 cells can suppress CIA has not yet been defined. We tested whether MT overexpression, which may promote *in vivo* Tr1 cell development, can overcome the progression of CIA. Whereas 100% of normal DBA/1 mice were severely arthritic, only 40% of MT transgenic DBA/1 mice exhibited weak symptoms of arthritis (Fig. 6A). This aspect was consistent with the significantly reduced histopathological manifestations of synovitis and serum level of CII-specific IgG in MT transgenic mice (Figs. 6B–6D). Importantly, the serum IL-10 level from CIA-established MT transgenic mice was elevated compared with that from pre-immune mice (Fig. 6E). In contrast, IL-10 was not detectable in the serum from the CIA-established nontransgenic mice, indicating downregulation of IL-10 production following Th1-dominated disease progression. Thus, these results implicate that MT plays a suppressive role *in vivo* in Th1-dominated autoimmune disease, presumably by enhancing IL-10-producing regulatory T cells.

DISCUSSION

In the present study, we demonstrated that MT production can act as a physiological stimulus for differentiation of IL-10- and TGF-β-producing $CD4^+$ T cells using *in vitro* polarization systems. MT-deficient naïve $CD4^+$ T cells have defects in the development process of these cells, which could be offset by addition of exogenous MT during priming, suggesting that the pool of MT doing the regulation is extracellular. These cells share many phenotypic and functional features with Tr1 cells, which are characterized by robust secretion of IL-10 and TGF-β with negligible IL-4 secretion, high levels of ROG expression, hypoproliferation to TCR/CD28 stimulation, and IL-10 dependency. Most importantly, MT transgenic mice, which are remarkably less susceptible to CIA than their nontransgenic littermates, exhibit enhanced serum levels of IL-10, suggesting that overexpression of MT can drive the induction of Tr1-like cells *in vivo* and this mechanism may play a critical role in the suppression of Th1-dominated autoimmune arthritis.

It has been previously reported that factors other than IL-10 can drive naïve $CD4^+$ T cells to differentiate into Tr1 or Tr1-like cells. For example, a combination of immunosuppressive drugs, vitamin D3 and dexamethasone, induced Tr1-like cells that produce IL-10 but not IL-5 and IFN-γ [3]. Co-engagement of CD3 and the complement regulator CD46 in the presence of IL-2 induced a Tr1-specific cytokine phenotype in human $CD4^+$ T cells, linking CD46 as a physiologic stimulus for Tr1 cell differentiation [24]. Studies have shown that $CD4^-CD8^-$ dendritic cells can prime Tr1 cells and that dendritic cells exposed to myeloma cell lysates or cyclooxygenase-2-overexpressing glioma favor the induction of Tr1-like cells. These results suggest an important role of the microenvironment provided by dendritic cells in the Tr1 cell differentiation [2, 19, 55]. Our results regarding MT-mediated Tr1-like cell induction provide a novel physiological process of Tr1-like cell development through a $CD4^+$ T cell-autonomous pathway, since MT is a naturally occurring protein that is produced at elevated levels when cells are exposed to a variety of cellular stress, and our system did not employ any other cells including dendritic cells but naïve $CD4^+$ T cells.

How extracellular MT is functionally complementary to endogenous MT is not clear. Following induction, MT can be released to the extracellular environment via unknown mechanisms, as metallothionein has been found at significant levels in extracellular compartments, such as blood, urine, liver sinusoids, renal tubule lumina, bronchoalveolar spaces, and pancreatic ducts [5, 12, 14, 22]. These observations, along with the identification of a MT-specific receptor on astrocytes [16] and multiple immunomodulatory effects of extracellular MT [32, 54], suggest that extracellular MT may transduce its signal into the cell by engagement of a specific cell surface receptor or by interactions of a more nonspecific nature. One outcome of the signals triggered by extracellular MT is induction of endogenous MT expression, leading to an enhanced level of intracellular MT. Indeed, addition of exogenous MT to a culture of splenocytes has been shown to increase the level of MT in the culture supernatants [35], supporting our hypothesis regarding a positive feedback link between extracellular MT and intracellular MT.

The function of MT to regulate the fate of naïve $CD4^+$ T cells might be related to the level of AP-1 activity, since MT-deficient $CD4^+$ T cells displayed an increase in DNA-binding activity of AP-1. Similarly, it has been reported by other investigators that NF-κB activity was enhanced in MT KO splenocytes [6, 11]. Apart from regulation of cellular proliferation, transformation, and death, AP-1 and NF-κB have been known to regulate T cell activation and differentiation. For instance, increased AP-1 activation confers resistance against anergy induction on Ag-specific T cells [23] and *in vivo* anergized T cells have defects in the AP-1 pathway due to both reduced protein expression and perturbed posttranslational modifications [50]. It has

also been shown that, in the absence of AP-1, NF-AT imposes a genetic program of lymphocyte anergy that counters the program of productive activation mediated by the cooperative NF-AT:AP-1 complex [33]. Furthermore, NF-κB and AP-1 activities were inhibited in the Tr1-like cells whose generation was induced by dexamethasone and vitamin D3 [3]. With respect to these investigations, regulation of AP-1 and NF-κB may be related to functional unresponsiveness to antigenic stimulation and generation of Tr1-like phenotypes. MT-mediated induction of anergic and regulatory T cells may relate to potential regulation of AP-1 and NF-κB. In addition, it should be noted that, without *in vitro* stimulation, MT KO T cells exhibit a remarkably enhanced activity of AP-1 relative to wild-type cells, suggesting that MT might play a role in the inhibition of activation to maintain the quiescent state by regulating AP-1 activity. Given that maintenance of quiescence is essential for the maintenance of self-tolerance, MT may serve as an important regulator of the spontaneous development of autoimmune responses.

Proof of how MT regulates AP-1 may be data showing that MT-deficient cells have enhanced JNK kinase activity, a critical regulator of AP-1 transactivation. JNK can be activated by exposure of cells to many forms of environmental stress such as radiation and redox stress [13]. The formation of ROS can be increased in T cells fully activated by TCR/CD28 costimulation, and ROS or lipid peroxidation products may activate stress kinases, such as JNK, ERK, and p38, leading to activation of redox-sensitive transcription factors including NF-κB and AP-1 [48]. On the other hand, studies in both cell-free systems and *in vivo* pancreatic islet transplantation systems have demonstrated that MT is able to scavenge a wide range of ROS including superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide at higher efficiency than other antioxidants such as glutathione [29, 31]. In this respect, MT may serve to regulate the overall levels of oxidants that are produced in activated T cells. MT deficiency may give rise to an abundance of ROS that activate oxidant-sensitive molecules, such as JNK, AP-1, and NF-κB. Moreover, other antioxidants like rosmarinic acid and chlorogenic acid can inhibit ROS generation and result in inhibition of JNK activation [18, 25], supporting this hypothesis. Alternatively, MT may exert its activity by directly interacting with transcription factors, since MT has been shown to distribute into the nucleus [51] and can bind directly to the p50/RelA form of NF-κB [1].

We found that abnormality of the MT gene quantity also perturbed the balance between Th1 and Th2 cell development. Under the Th1-polarizing conditions, the development of Th1 cells was significantly enhanced in MT-deficient $CD4^+$ T cells compared with wild-type cells, whereas there was no difference between these strains in Th2 development under the Th2-polarizing conditions. These results were further confirmed by the expression levels of T-bet and GATA-3. This aspect was reversed by addition of exogenous MT during the priming period. The presence of exogenous MT during priming facilitated Th2 cell development, when Th2-polarizing conditions were provided. However, IFN-γ production, an indicator of Th1 cell development, was not influenced by the presence of exogenous MT. This result is likely in line with the report showing that autoAg-specific Th2 cells and Tr1 cells were concomitantly induced by the same stimulation [7]. Our results indicate that MT can skew development of Th1/Th2 cells toward Th2 dominance. This mechanism, in concert with Tr1-like cell functions, may contribute to the amelioration of Th1-dominated autoimmune responses.

In summary, signaling events mediated by MT induced the development of $CD4^+$ T cells to a Tr1-like phenotype. This observation establishes a link between cellular stress and adaptive immunity. Not only are Tr1 cells essential for maintaining peripheral tolerance and preventing autoimmunity, but they also presumably modulate the host's immune response to many environmental stressors. This putative role of MT in regulating cellular immune responses might also account for how physiologic stress modulates the adaptive immunity. Our identification of MT as a physiological stimulus for Tr1-like cell differentiation should facilitate further characterization of regulatory cell populations and might provide a therapeutic strategy for generating such cells to ameliorate T-cell-mediated pathology.

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