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Abstract

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Metallothioneins negatively regulate IL-27–induced type 1 regulatory T-cell differentiation

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IL-27–induced type 1 regulatory T (Tr1) cells suppress autoimmunity by producing IL-10. Signal transducer and activator of transcription (STAT) 1 and STAT3 have been described as key transcription factors that promote IL-10 secretion from Tr1 cells induced by IL-27. However, the molecular pathways for negatively regulating Tr1 cell differentiation remain elusive. Here, we show that IL-27 induces metallothioneins (MTs) that in turn prevent Tr1 cell development. MT expression leads to the reduction of STAT1 and STAT3 phosphorylation under Tr1 differentiation condition, resulting in impaired IL-10 production. Accordingly, Tr1 cells derived from MT-deficient mice showed an increased ability to produce IL-10 and potently suppress experimental autoimmune encephalomyelitis upon adoptive transfer. Moreover, activation of STAT1 and/or STAT3 can overcome the suppression of IL-10 by MTs, indicating a dynamic balance between STATs and MTs in regulating IL-10 during Tr1 cell differentiation.

Results
Late Expression of MTs in IL-27–Induced Tr1 Cells. To gain insight into the differentiation of IL-27–induced Tr1 cells, we performed a comparative microarray analysis of developing Tr1 cells at 72 h after stimulation with IL-27. We found that MT1 and -2 were highly expressed in IL-27–induced Tr1 cells generated from naïve CD4+CD25−CD62L−CD44low T cells compared with T cells similarly activated without the presence of differentiating cytokines (Th0) (Fig. S1A). We then analyzed kinetics of MT1 and MT2 expression during the differentiation of Tr1 cells with IL-27. In contrast to Th0 cells, which express only marginal levels of MT1 and -2, both MT isoforms were highly expressed in IL-27–induced Tr1 cells (Fig. S2). Although MT expression was dramatically enhanced in Tr1 cells after 72 h, we failed to detect any significant MT expression before 48 h. Interestingly, this delayed induction of MTs coincided with the induction of IL-10 in developing Tr1 cells (Fig. S2). We also examined the expression levels of MT1 and MT2 in different subsets of CD4+ T cells. We found that both MTs were highly expressed in Tr1 and Th helper 17 cells (Th17), whereas Th1 or Th2 cells exhibited modest expression of MT1 and MT2 (Fig. S1B).

MTs Impair IL-10 Expression in IL-27–Induced Tr1 Cells. We then investigated the role of MTs on IL-27–induced Tr1 cells by differentiating naïve CD4+ T cells from WT or Mtmice. Although IFN-γ production from Tr1 cells was unaffected in the absence of MTs, the frequency of IL-10–producing cells and the secretion of IL-10 were notably enhanced in Tr1 cells derived from Mtmice (Fig. 1A and B). We and others have previously shown that TGF-β1 and IL-6–derived Th17 cells also express IL-10 (11, 12). MTs are expressed at high levels in Th17 cells differentiated with TGF-β1 and IL-6, whereas MT-deficient Th17 cells did not show a difference in IL-10 or other Th17 cytokines (Fig. S3A). Addition of recombinant MT1 or MT2 during differentiation of naïve CD4+ T cells in vitro and in vivo. At the mechanistic level, we found that, in the absence of MTs, IL-27 induces increased phosphorylation of STAT1 and STAT3 but not STAT4, resulting in enhanced IL-10 production. Furthermore, compared with WT Tr1 cells, Mtmice were more efficient in their ability to suppress effector T cell proliferation and inhibit the development of experimental autoimmune encephalomyelitis (EAE). Taken together, our data suggest that MTs act as negative regulators for IL-27–induced Tr1 cells.


The authors declare no conflict of interest.

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cells with IL-27 severely impaired IL-10 secretion, but it did not affect IFN-γ production. IL-17 secretion from differentiating Th17 cells was not impaired, whereas IL-10 secretion was modestly decreased in cells treated with MT1 or MT2 (Fig. S3B). Analysis of the signature Tr1 cytokines from WT and Mt−/− Tr1 cell cultures showed increased IL-10 and unchanged IFN-γ production at mRNA level as well. Additionally, we found that both Il21 and Il27r, which are critical for Tr1 cell development (1, 2), were also up-regulated in the absence of MTs (Fig. 1C). To assess whether MTs play a role in Tr1 cells generated with other stimuli than IL-27, we differentiated naïve sorted CD4+ T cells using vitamin D3 and dexamethasone (13). We observed that the Mt−/− Tr1 cells exhibited elevated IL-10 production under both vitamin D3 and dexamethasone stimulation after 72 h. The enhancement of IL-10 became more profound when these two were combined (Fig. S3C). Altogether, these data indicate that MTs impede IL-10 secretion from Tr1 cells.

We then determined whether endogenous overexpression of MTs can reverse the phenotype we observed in the Mt−/− Tr1 cells. Retroviral overexpression of GFP-tagged MT1 or MT2 in WT T cells under IL-27 stimulation resulted in reduced expression of IL-10 and had no effect on IFN-γ, as detected by intracellular staining and ELISA (Fig. 1D and E). Furthermore, we sorted out the GFP+ cells from the MT1 or MT2 retroviral transduced Tr1 cells and examined the expression of other key genes expressed in Tr1 cells. Consistent with MT-deficient T cells, the expression of Il10, Il27r, and Il21 genes was down-regulated, whereas Ahr and IFN-γ were unchanged by MT1 or MT2 over-expression (Fig. 1F). Thus, our data demonstrate that MTs negatively regulate Tr1 differentiation by inhibiting IL-10 production and impairing the IL-27 signaling pathway by repressing Il27r and Il21 expression.

To test whether MTs are also relevant for human Tr1 cell biology, we differentiated human Tr1 cell in vitro to analyze MT expression and the function of MTs in these cells. More than 10 isoforms of MT have been identified in the human genome, compared with only 3 isoforms in the mouse genome. According to the National Center for Biotechnology Information HomoloGene database, human MT1E and MT1H are the closest homologs to murine MT1 and MT2, respectively. Therefore, we decided to test the mRNA expression level of these two isoforms in human Tr1 cells. We observed that Mtle and Mt1h were highly expressed in human Tr1 cells compared with Th0 cells (Fig. S4A). To understand the relevance of MTs in human Tr1 cells, we differentiated human Tr1 cells in the presence of recombinant MT1 and MT2 proteins. We observed that both MT1 and MT2 significantly suppressed the IL-10 production of human Tr1 cells (Fig. S4B and C). Moreover, we observed that the supernatants from cultured Tr1 cells inhibited proliferation of bystander CD4+ T cells, whereas the supernatants from the Tr1 cells cultured with additional MT1 or MT2 exhibited reduced ability to suppress proliferation, which was abrogated by anti-IL-10 antibody (Fig. S4D). These data suggest that the inhibitory functions of human Tr1 cells are also negatively regulated by MTs.

**MTs Negatively Regulate STAT1 and STAT3 Activation.** Previous studies have shown that STAT1 and STAT3 are both critical for the induction of IL-10 production in Tr1 cells (6, 14). To assess whether MTs regulate Tr1 differentiation by influencing the activation of STATs, we examined the activation level of STAT1 and STAT3 after IL-27 stimulation. To this end, naïve CD4+ T cells from WT and Mt−/− mice were activated with IL-27 and phosphorylation of bystander CD4+ T cells, whereas the supernatants from the Tr1 cells cultured with additional MT1 or MT2 exhibited reduced ability to suppress proliferation, which was abrogated by anti-IL-10 antibody (Fig. S4D). These data suggest that the inhibitory functions of human Tr1 cells are also negatively regulated by MTs.

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STAT3 [MSCV-ires-GFP (MIG) vector, GFP reporter] and stimulated the cells with IL-27. Overexpression of either STAT1 or STAT3 could reverse the suppressive effect of MT1 or MT2 on IL-10 production (Fig. 2 E–G). These results imply that MTs inhibit Tr1 cell differentiation and potentially compete with positive regulators of IL-10 such as STATs, subsequently defining the STAT-driven threshold for the induction of IL-10. However, our results also suggest that both STAT1 and STAT3 are dominant over MTs in the regulation of IL-10 during Tr1 cell polarization. Thus, expression levels and activation status of STAT1/3 and MTs may form a kinetic balance to control IL-10 production in Tr1 cells.

MTs Control the Induction of Tr1 Cells in Vivo. To study the relevance of MTs in expanding Tr1 cells in vivo, we examined IL-10-producing Tr1 cells generated from CD4^+CD25^-CD44^-CD62L^- memory T cells from WT versus Mt^-/- mice. We found that IL-10 secretion from memory T cells from Mt^-/- mice was increased by over 50% compared with WT mice (Fig. 3 A). It has previously been shown that repeated in vivo treatment with anti-CD3 antibody induces Tr1 cells, which are dependent on IL-27 for their generation (1, 16). Because MTs are expressed in other tissues, such as liver, it is not clear whether the in vivo increase in IL-10 is due to a direct effect on T cells. To exclude any effects from the nonhematopoietic cell-derived MTs in our system, we generated bone marrow (BM) chimeras in which the WT host were reconstituted with either WT or Mt^-/- BM. Eight weeks after reconstitution, we repeatedly administered anti-CD3 or an isotype control antibody to the WT and Mt^-/- BM chimera mice. After treatment, we analyzed the Tr1 cell frequency in peyer's patches (PP) and lamina propria (LP). To rule out the confounding effects by other IL-10-producing T-cell subsets and specifically examine Tr1 cells, we analyzed IL-10 production by gating on CD4^+IL-17 Foxp3^- cells. We found a significant increase in Tr1 cells in Mt^-/- BM chimera's PP and LP compared with WT chimeras (Fig. 3 B and C). These results further emphasize that MTs regulate the generation of IL-10^+ Tr1 cells by specifically acting on the hematopoietic compartment in vivo.

Enhanced Suppressive Capacity of IL-10 Producing T Cells in the Absence of MT in Vivo. To further understand the role of MTs and their relevance to the function of Tr1 cells in vivo, we studied the impact of MT-deficient Tr1 cells in an adoptive transfer model of EAE. We first immunized WT or Mt^-/- mice with myelin oligodendrocyte glycoprotein (MOG)35-55 peptide with Freund's Complete Adjuvant (CFA). Ten days following immunization we isolated lymphocytes from immunized WT or Mt^-/- mice and reactivated them under various conditions. The response was antigen specific as depicted by the proliferation with MOG but not with OVA peptide (Fig. 4 A–D). When these cells were restimulated with MOG35-55 and
Discussion

As one of the suppressive T cell subsets, Tr1 cells have been described to regulate inflammation, graft-versus-host disease, and autoimmunity by producing IL-10 (3). The results presented in this study show that the nonenzymatic proteins MT1 and MT2 can negatively regulate the production of IL-10 but not IFN-γ during Tr1 cells' development and thus regulate the anti-inflammatory properties of those cells. Loss of MT1 and MT2 enhanced IL-10 production within Tr1 cells upon IL-27 or vitamin D3/dexamethasone stimulation without affecting IFN-γ. Furthermore, the enhancement of IL-10 increases the capacity of MT-deficient Tr1 cells to suppress effector T cells, in turn leading to the abatement of autoimmunity. Our results reveal that MTs blunt IL-10 secretion from Tr1 cells by preventing the activation of the transcription factor STAT1 and STAT3. Consistent with these data, anti-CD3-induced generation of Tr1 cells was increased in the absence of MTs in vivo, underscoring the key role of MTs in regulating IL-10 production in Tr1 cells.

Although the key roles of MTs in mediating heavy metal detoxification have been abundantly documented (20), their implication in the control of gene transcription is still unclear. It has been proposed that MT could bind to the p50 subunit of the NF-kB complex, thereby increasing its ability to act as a transcriptional activator (21). So far, there is no evidence showing that MTs have any DNA-binding sites, making it unlikely that they directly regulate gene expression. However, it has been shown that MTs can control the binding of the estrogen receptor to its DNA-binding site by modulating zinc levels (22). Our data suggest that MTs regulate induction of pSTAT1 and pSTAT3, both of which are required for induction of many important cytokine-driven functions in T cells. Whether MTs directly bind to STAT1 or STAT3 and interfere with their phosphorylation or indirectly by interfering with Jak-mediated phosphorylation is not clear at this stage. However, because it was reported that Zinc binding disrupts the association of STAT3 with Jak2 kinase (23), we can speculate that zinc might be involved in the MT-driven inhibition of phosphorylation of STAT1/3. Nonetheless loss of MTs resulted in enhanced phosphorylation and transcription of STAT1 and STAT3, suggesting STAT1/3 but not STAT4 as one of the targets of MTs. Although MTs are also expressed in Th17 cells, MT expression does not regulate the expression of proinflammatory proteins such as IL-17. We cannot rule out the effect of MTs in IL-10 production by Th17 cells, because IL-6 is one of the cytokines that induces IL-10 and activation of STAT3 in Th17 cells (24).

Proinflammatory cytokines such as IL-6 induce MTs by activating the transcription factor STAT1 and STAT3 (10). Endotoxin (LPS) produced during bacterial infection has also been shown to elevate the MT expression level (10). Both LPS and IL-6 can initiate STAT1 and STAT3 expression and activation. pSTAT1 and pSTAT3 in turn can directly bind to the promoter of MTs and potentiate its transcription (10), forming a feedback inhibitory loop whereby STAT1/3 induction of MTs results in decreased induction and/or activation of STAT1/3. This mechanism of action would be reminiscent of the action of the suppressors of cytokine signaling 3 (SOCS3), which is induced by STAT3 and limits STAT3 phosphorylation, thereby dampening the secretion of proinflammatory cytokines like IL-17 (25). Here, the induction of MTs by IL-27 would limit the induction of Tr1 cells to prevent excessive immune regulation that might favor the emergence of viral infections or cancers.

Both STAT1 and STAT3 are phosphorylated upon IL-27 signaling (6), leading to transactivation of IL-10. The absence of MTs results in hyperphosphorylation of both STAT1 and STAT3 under the stimulation of IL-27. On the other hand, either STAT1- or STAT3-deficient Tr1 cells exhibit reduced MT1 and MT2 expression. We thus hypothesize that MTs and STATs compete with each other during Tr1 cell development to control IL-10 production. Although MT1/2 overexpression can lead to IL-10 suppression from Tr1 cells, coregulation of STAT1/3 results in restoration of IL-10. This result indicates that STAT1 or STAT3 can override MT-dependent suppression of IL-10 during Tr1

IL-23, which has been reported to induce and reactivate Th17 cells (17), IL-17 production from CD4+ T cells was not altered (Fig. 4 A and B). Although we did not see a significant change in IL-10 production with specific antigen MOG55–55 by flow cytometry, we indeed observed a higher production of IL-10 in CD4+ T cells from Mt−/− mice than WT mice when T cells were reactivated in the presence of MOG55–55, in the cultured supernatant, which was further amplified in the presence IL-27 (Fig. 4 C and D). Moreover, IFN-γ production by cells from WT and Mt−/− mice was comparable following either stimulation condition (Fig. 4 A–D). Consistent with our in vitro data, these results confirm the specific role MTs play in the regulation of IL-10 in IL-27-stimulated Tr1 cells.

Next, we tested whether Mt−/− Tr1 cells display enhanced ability to suppress autoimmunity because IL-10 production has been used as a criterion for Tr1 cell anti-inflammatory activity. We first generated pathogenic effector T cells from MOG-immunized WT mice. Simultaneously, WT or Mt−/− T cells were cultured in the presence of MOG and IL-27 to generate antigen specific Tr1 cells. Because Mt−/− mice are on the SV129 background and this strain is not optimal for EAE induction by MOG55–55 (18), we used an alternative recipient strain (SV129 × B6) F1, which were genetically compatible with SV129 and were susceptible to EAE following adoptive transfer (19). We then adoptively transferred WT effector T cells with or without differentiated Tr1 cells from either WT or Mt−/− mice at a ratio of 3:1. As expected, WT Tr1 cells significantly suppressed EAE development. However, Mt−/− Tr1 cells suppressed disease more efficiently than WT Tr1 cells both in terms of disease severity and incidence (Fig. 4E). We further tested the ability of Tr1 cells to suppress EAE by titrating the Tr1 cells. Mt−/− Tr1 cells exhibited the suppressive capacity on the disease process, whereas the suppressive effect of WT Tr1 cells was no longer dominant when the ratio of the effector T cells (Tcell)-to-Tr1 was 5:1 (Fig. S5). Using the same adoptive transfer system, we labeled WT effector cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) before transfer. On day 4 after transfer, lymphocytes were isolated from lymph nodes of the recipient mice and CFSE− effector cell proliferation was analyzed. We found significant reduction of effector cell proliferation if they had been transferred together with Tr1 cells. Moreover, Mt−/− Tr1 cells displayed a superior suppressive capacity, inhibiting effector T cell proliferation more profoundly compared with WT Tr1 cells (Fig. 4F).
MTs, which are found in the gut and can be induced by the repetitive administration of anti-CD3, have been shown to induce Tr1 cells in the gut (1, 16). Repetitive administration of anti-CD3 can induce Tr1 cells in the gut more efficiently when used with Zn-MT2 treatment resulting in neuroprotective effects within the CNS (28). Moreover, in the Zn-MT2 treatment within the CNS, MT proteins were found to be elevated significantly when we used MT−/− mice (129/Sv B6 F1) recipients adoptively transferred with WT MOG +CD4+ T cells (35–55) or OVA +CD4+ T cells (35–55) were restimulated with plate-bound anti-CD3 (145-2C11, 1 μg/mL) and/or dexamethasone (29, 30). In this study, we established the correlation between MTs and IL-10 production within Tr1 cells and their proinflammatory role in the EAE model. Passive transfer of EAE mice allowed us to dissect the function of MTs specifically in T cells, excluding any effects due to the neuroprotective role of MTs. We and others have shown that the repetitive administration of anti-CD3 antibody can induce Tr1 cells in the gut (1, 16). Repetitive administration of anti-CD3-induced Tr1 cells in the gut more efficiently when we used MT−/− BM chimera compared with WT mice, further emphasizing the importance of the cellular environment in the control of MT responses. These data further suggest that MTs have divergent functions in the immune response, depending on tissue and cells. It is also important to evaluate distinct effects of MTs in specific circumstances to identify all of their various and sometimes contradicting functions. Altogether, we have identified MT1 and MT2 as negative regulators of Tr1 cell differentiation and IL-10 production. We also illustrate the balance between MT and STAT signaling in controlling Tr1 cell development. Our work emphasizes that MTs, beyond their essential role in the regulation of metal homeostasis, also shape the quality of immune responses in vivo. By identifying a specific function for MTs in Tr1 cells, our study also provides a target for development of selective therapeutic strategies for regulating Tr1 cell expansion and autoimmunity.

Materials and Methods

Animals. MT−/− mice (129Sv-MT1MT2−−/−), 129Sv (control), 129Sv B6 F1, 2D2, Star1−/− and C57BL/6 mice were purchased from Jackson Laboratory. Star3−/− mice were a kind gift from John O'Shea (National Institutes of Health, Bethesda). Mice were housed in conventional, pathogen-free facilities at the Harvard Institute of Medicine. All experiments were undertaken in accordance with guidelines from the Committee on Animals at Harvard Medical School.

In Vitro T-Cell Differentiation. Naive (CD4+CD62L−CD25−) or memory (CD4+CD62L+CD25+) CD4+ T cells from spleens and lymph nodes of WT or MT−/− mice were purified by fluorescence-activated cell sorting (FACS). The purity of isolated T-cell populations routinely exceeded 98%. Naive CD4+ T cells were stimulated with plate-bound anti-CD3 (145-2C11, 1 μg/mL) and anti-CD28 (PV-1, 1 μg/mL) in the presence of the following reagents: mouse IL-27 (50 ng/mL) or vitamin D3 (50 pg/mL; Sigma), and/or dexamethasone (0.05 and **P < 0.01 (Student t test, error bars show SD)).

Fig. 4. MTs regulate suppressive capacity of Tr1 cells in vivo. Lymphocytes were isolated from lymph nodes of WT and MT−/− mice 10 d after immunization with MOG +OVA and were restimulated with MOG +OVA or OVA +IL-23. (A and B) EAE development in WT (129/Sv B6 F1) recipients adoptively transferred with WT MOG−/− specific effector T cells with (3:1) or without WT or MT−/− Tr1 cells. (Right) Data are expressed as a linear-regression curve of the disease score over time. (F) Experimental setup as E, except for labeling WT effector T cells with CFSE before adoptive transfer. Four days after transfer, the percentage of proliferating CFSE-labeled CD4+ T cells in the lymph nodes was determined by flow cytometry. The data are representative of three independent experiments. *P < 0.05 and **P < 0.01 (Student t test, error bars show SD).
(30 &mu;g/mL, Sigma) for Tr1 cells; human TGF-β1 (2 ng/mL) and mouse IL-6 (20 ng/mL) for Th17 differentiation; mouse IL-12 (5 ng/mL) and anti-mouse IL-4 (10 &mu;g/mL; 11B11) for Th1 differentiation; or mouse IL-4 (10 ng/mL) and anti-mouse IL-12 (10 &mu;g/mL; C17.8) for Th2 differentiation; or human TGF-β1 (2 ng/mL) for iTregs. All recombinant cytokines were purchased from R&D Systems.

**Statistical Analysis.** Statistical analysis was performed using Prism software (GraphPad). P values < 0.05 were considered significant.


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