Membrane-Bound Metallothionein 1 of Murine Dendritic Cells Promotes the Expansion of Regulatory T Cells In Vitro

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Exposure to environmental toxicants can alter a range of cellular functions involved in the immune response. Increased expression of the stress protein metallothionein 1 (MT1) is one example thereof. Previously, it has been reported that MT1 has several immunosuppressive properties. Furthermore, we earlier showed that functionally tolerogenic dendritic cells (DCs) expressed increased mRNA levels of MT1. Here, we demonstrate that dexamethasone-treated murine DCs are functionally tolerogenic and produce MT1. However, these DCs do not actively transport MT1 to the cell membrane and their regulatory function does not depend on MT1. Alternatively, ZnCl₂-treated murine DCs transport MT1 to the cell surface are tolerogenic and promote the expansion of T cells with a regulatory phenotype. Moreover, the membrane-bound MT1 was shown to be essential for ZnCl₂-treated DCs to exert their regulatory function. On the basis of this, MT1 can be used as a new marker for functionally tolerogenic DCs. Additionally, we have found a new mechanism for tolerogenic DCs to exert their immune regulatory function.

Key Words: metallothionein 1; stress protein; dendritic cells; immune regulation.

Mammalian metallothionein 1 (MT1) is a low weight, cysteine-rich stress protein especially induced after exposure to environmental stressors like heavy metals (Goering and Klaassen, 1983; Hunziker and Kagi, 1985). The main function of MT1 is to scavenge heavy metals and either serve as a reservoir for essential heavy metals or to redistribute toxic metals from the nucleus into the cytosol to reduce genotoxic damage (De Lisle et al., 1996; Goering and Klaassen, 1983). In addition, MT1 is a potent antioxidant as it scavenges reactive oxygen species to provide protection to other cellular constituents (Thornalley and Vasak, 1985). Following exposure to stress, MT1 is released into the extracellular environment and is therefore found in physiological fluids like serum, urine, and milk (Bremner et al., 1987; Milnerowicz and Chmerek, 2005; Nordberg et al., 1982). The exact mechanism of secretion into the extracellular environment is not yet understood because MT1 does not possess hydrophobic signal sequences that are necessary for normal excretion of proteins. Therefore, MT1 is considered to be secreted by a nonclassical mechanism similar to that of the cytokines interleukin (IL)-1α and IL-1β (Muesch et al., 1990; Stevenson et al., 1992).

MT1 is mainly synthesized in the liver but other tissues, like the thymus and some immune cells can also produce MT1 (Coto et al., 1992; Mesna et al., 1995). Furthermore, acute-phase cytokines like IL-1, IL-6, and tumor necrosis factor (TNF)-α were found to induce the expression of MT1 (Cousins and Leinart, 1988; Sato et al., 1994). These combined findings indicate a role for MT1 in the inflammatory response. Indeed, earlier reports showed that extracellular MT altered cytotoxic T-lymphocyte activity (Youn and Lynes, 1999), suppressed humoral immunity in vitro and in vivo (Canpolat and Lynes, 2001; Lynes et al., 1993), induced a respiratory burst in macrophages coinciding with a weaker antigen-specific T-cell response (Youn et al., 1995), functioned as a chemoattractant (Yin et al., 2005), and induced differentiation of naïve CD4+ T cells into T cells with a regulatory phenotype (Huh et al., 2007). Several studies showed that MT1 has a protective role in experimental autoimmune models like collagen-induced arthritis and experimental autoimmune encephalomyelitis (Huh et al., 2007; Penkowa et al., 2003; Youn et al., 2002).

Earlier, we found that tolerogenic DCs treated with the phytonutrient carvacrol in combination with heat stress featured a highly increased mRNA expression of MT1. These DCs were functionally tolerogenic as they were suppressive in a mouse model of rheumatoid arthritis (Spiering et al., 2012). In the present study, we examined whether MT1 produced by tolerogenic DCs was able to induce a regulatory phenotype in T cells.
We found that MT1 present on the cell surface of ZnCl₂-treated DCs could promote the expansion of FoxP3⁺ regulatory T cells and this result was MT1 dependent. These observations indicate that manipulation of MT1 expression on tolerogenic DCs can be used as therapeutic intervention in T-cell-dependent autoimmune diseases.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Charles River Laboratories. DO11.10 mice (ovalbumin (OVA)-TCR Tg) and TCR-5/4E8-Tg mice (proteoglycan (PG)-TCR Tg) (Berlo et al., 2006) were bred under specified pathogen-free conditions. Experiments were approved by the Utrecht University Animal Experimental Committee.

Treatment of bone marrow–derived dendritic cells. Bone marrow–derived dendritic cells (BMDCs) were cultured as described before (Spiering et al., 2012). On day 8, BMDCs were seeded in 12-well plates at 1 × 10⁶ cells/ml. The next day, 0.4 µg/ml dexamethasone (DEX; Sigma), 12nM vitamin D₃ (vitD₃; Sigma), 10 or 40 µg/ml ZnCl₂ (Merck), 50ng/ml TNF-α (Tebu-Bio), 0.1, 1, 10, or 100ng/ml lipopolysaccharide (LPS; Escherichia coli 0127:B8; Sigma) were added to the culture, and cells were harvested at indicated time points. As a control, cells were left untreated.

Analysis of mRNA expression by quantitative reverse transcriptase-PCR. BMDCs were treated as described above. Cells were harvested at indicated time points and mRNA isolation, cDNA synthesis, and quantitative reverse transcriptase-PCRs (RT-PCRs) were performed as described elsewhere (Spiering et al., 2012). Primers used for quantitative RT-PCR: hypoxanthine-guanine phosphoribosyltransferase (HPRT) (forward 5'-CTG-GTT-AAA-AGG-ACC-TCT-CC-G-3', reverse 5'-TGA-AGT-ACT-CT-A-TAT-AGT-CA-GAG-CA-3'), MT1 (forward 5'-AAG-AGT-GAG-TTG-GGA-CAC-CTT-3', reverse 5'-GAG-ACA-ATA-CAA-TGG-CCT-CC-3'), IL-10 (forward 5'-GGT-TGG-CAA-GCC-TTA-TGG-3', reverse 5'-ACC-TGC-TCC-ACT-GGC-TTG-CT-3'), glucocorticoid-induced leucine zipper (GILZ) (forward 5'-AAC-ACC-GAA-ATG-TAT-CAG-ACC-C-3', reverse 5'-GTT-TAA-CCG-AAA-CCA-AAT-CCC-CT-3'), IL-12p40 (forward 5'-CGA-AGC-ACT-GCA-AGC-CAG-GA-3', reverse 5'-AAC-TTG-AGG-GAG-AAG-TAG-GGA-TT-3'), and TNF-α (forward 5'-GCC-TCA-TCA-GAT-CAT-CTT-CT-3', reverse 5'-GCT-AGG-ACG-TGG-GCT-ACA-3'). The relative amount of target mRNA normalized to HPRT mRNA was calculated according to the method described by Pfaff (2001).

Preparation of cell lysates and Western blot analysis. BMDCs were treated as described above. After 24h of recovery, 1 × 10⁶ treated and untreated washed BMDC pellets were resuspended in 40 µL Laemmli sample buffer (60mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue). Ten microliters of the supernatant of 10-min boiled lysates were loaded to a 15% SDS-polyacrylamide gel electrophoresis gel. Following electrophoresis, proteins were transferred to a PVDF membrane (immobilon, pore size 0.2 µm; Millipore) using a semidy transfer (Trans-Blot SD, Bio-Rad). After overnight blocking, the membrane was incubated with monoclonal mouse anti-MT1 (UC1MT, 3.3 µg/ml, Enzo Life Sciences). Monoclonal mouse anti-γ-tubulin (5.4 µg/ml, Sigma) was used as a loading control. Blots were subsequently incubated with rabbit anti-mouse-horseradish peroxidase (HRP) (0.26µg/ml; Dako). All antibodies were diluted in PBS containing 0.5% blocking reagent (Roche) and 0.05% Tween-20. Protein levels were detected using Luminata Crescendo Western HRP substrate (Millipore) and Amersham Hyperfilm ECL (GE Healthcare).

In vitro effect of BMDC secreted MT1 on antigen-specific T cells. BMDCs were treated as described above. After 6h of recovery, treated and untreated BMDCs were plated in a 96-well plate at a concentration of 2 × 10⁶ cells/well. Antigen was added at a final concentration of 100 µg/ml. CD4⁺CD25⁺ T cells were isolated from spleens of DO11.10 or PG-TCR transgenic mice as described before (Broere et al., 2008) with the addition of a monoclonal antibody to CD25 (PC-61) to remove CD25⁺ cells and labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE). Antigen-specific CFSE-labeled CD4⁺CD25⁺ T cells (2 × 10⁶) were added to DC-antigen mixtures and 72h later, cells were analyzed. For blocking studies, 4 µg anti-MT1 (UC1MT) was added to the wells (Canpolat and Lynes, 2001; Yin et al., 2005).

In vitro effect of exogenous MT1 on CD4⁺ T cells. CFSE-labeled wild-type splenic CD4⁺CD25⁺ T cells were stimulated with magnetic M-450 Tosylactivated Dynabeads (Dynal Biotech) coated with anti-CD3 (145-2C11) and anti-CD28 (PV1-17-10) in a 1:1 ratio. Anti-CD3/anti-CD28-coated beads (2 × 10⁶) were added to 1 × 10⁶ CFSE-labeled T cells in a 96-well plate. Rabbit liver MT1 (Enzo Life Sciences) was added at 10µM or cells were left untreated. After 72h, cells were measured for proliferation and FoxP3 expression.

Flow cytometric analysis of surface markers, MT1, and FoxP3. BMDCs were treated as described above. After 6h of recovery, cells were stained with allopurinol (APC)-anti-CD11c (HL3) plus one of the following antibodies: phycoerythrin (PE)-anti-CD4 (3/23), fluorescein isothiocyanate (FITC)-anti-CD86 (GL1), or PE-anti-1-Adi/Ed (M5/114) (BD Biosciences). For analysis of MT1 expression, cells were allowed to recover for 24h. For extra-cellular staining, cells were stained with APC-anti-CD11c plus biotinylated anti-MT1 (UC1MT) in combination with streptavidin-FITC (BD Biosciences). For total MT1 staining, cells were stained with APC-anti-CD11c (HL3), then fixed and permeabilized for 20min with Cytofix/Cytoperm solution (BD Biosciences), washed, and incubated with biotinylated anti-MT1 and streptavidin-HRP (BD Biosciences). Antigen-specific T cells were stained with V450-anti-CD4 (RM4-5) (BD Biosciences) after 72h of coculture, and FoxP3 staining was carried out with a FoxP3 (FJK-16S; PE labeled) staining kit as instructed (eBioscience). Flow cytometry was performed on a FACS Canto (BD Biosciences).

Statistical analysis. Statistical analysis was carried out using Prism software (version 4.0, Graphpad Software Inc.). Significance level was set at p ≤ .05 and paired (Figs. 1F, 2F, and 3A) or unpaired (Fig. 3B) 2-tailed Student’s t tests were applied.

Cytokine analysis by Luminex. BMDCs were treated as described above. After indicated times of recovery, supernatants were collected and analyzed for cytokine secretion. Fluoresceinlabeled microbeads coated with capture antibodies for simultaneous detection of IL-10 (JES5-2A5), TNF-α (G281-262), and IL-12p70 (9A5) (BD Biosciences) were added to 50 µl of culture supernatant. Cytokines were detected by biotinylated antibodies IL-10 (SXC-1), TNF-α (MP6-3X3), IL-12p70 (C17.8), and PE-labeled streptavidin (BD Biosciences) and analyzed on a Luminex model 100 XYP (Luminex).

Collection of blood samples. Buffy coat of 500ml whole blood from 1 healthy donor was obtained from Sanquin Blood Bank Utrecht. Donors gave written informed consent.

Isolation and treatment of monocyte-derived DCs. Human peripheral blood mononuclear cells were prepared from buffy coats by Ficoll density gradient centrifugation. CD14⁺ monocytes were positively isolated using CD14 microbeads (MACS; Miltenyi) according to the manufacturer’s protocol. CD14⁺ cells were cultured in X-VIVO 15 medium in the presence of human granulocyte-macrophage colony-stimulating factor (0.1 µg/ml) and IL-4 (25 ng/ml) for 6 days. After 6 days, monocyte-derived DCs (moDCs) were treated with several concentrations (mentioned in Supplementary Figure 2) DEX, ZnCl₂, or VitD₃. As a control, cells were left untreated.

RESULTS

DCs With Membrane-Bound MT1 Are Functionally Tolerogenic

MT1 has been demonstrated to exhibit various immunosuppressive functions (Canpolat and Lynes, 2001; Huh et al., 2007;
In addition, carvacrol-heat stress–treated DCs had a tolerogenic phenotype and produced high levels of MT1 (Spiering et al., 2012). To study whether MT1 producing DCs could induce a regulatory phenotype in T cells, we treated DCs with 2 nontoxic concentrations of ZnCl₂, an inducer of MT1 (Tandon et al., 2001). Total MT1 expression was measured after ZnCl₂ treatment to confirm the production of MT1 (Figs. 1A and 1B). In addition, as MT1 needs to be on the cell membrane of the DC to exert a potential regulatory function on T cells, MT1 was also measured on the cell surface of untreated and ZnCl₂-treated DCs. As shown in Figure 1C, MT1 was present on the cell surface of ZnCl₂-treated DCs but not of controls.

Furthermore, the phenotype of ZnCl₂-treated DCs was investigated. ZnCl₂-treated DCs exhibited a tolerogenic phenotype with reduced levels of the costimulatory molecule CD86 and major histocompatibility class II (MHCII) (Fig. 1D) and increased mRNA expression levels of the anti-inflammatory cytokine IL-10 and the tolerogenic DC marker IL-T3 (Chang et al., 2002). In addition, IL-12 mRNA levels were reduced, but TNF-α mRNA levels were increased in ZnCl₂-treated DCs (Fig. 1E). On protein level, small changes in these cytokines could be observed as well (Supplementary Figure 1).

Finally, the potential capacity of DCs with membrane-bound MT1 to induce a FoxP3+ regulatory phenotype in T cells was studied. T cells cocultured with ZnCl₂-treated DCs had higher percentages of FoxP3+ T cells compared with T cells cocultured with untreated controls, indicating that ZnCl₂-treated DCs can induce a regulatory phenotype in T cells or promote the proliferation of existing FoxP3+ T cells (Fig. 1F).

In sum, treatment of DCs with the MT1 inducer ZnCl₂ induced a tolerogenic DC phenotype. Moreover, ZnCl₂-treated DCs displaying membrane-bound MT1 were able to induce a FoxP3+ phenotype in T cells or preferentially induce the proliferation of existing FoxP3+ T cells compared with FoxP3+ T cells.

### Functionally Tolerogenic DCs Produce MT1

To investigate whether other previously described tolerogenic DC inducers also increased MT1 expression, DCs were treated with the following compounds: DEX, vitD₃, TNF-α, or LPS (Hamdi et al., 2007; Healy et al., 2008; Penna and Adorini, 2000; Salazar et al., 2008). All these compounds have also been reported to induce the expression of MT1 in different cell types (De et al., 1990; Hanada et al., 1995; Iijima et al., 1990; Karin et al., 1980; Mesna et al., 1995; Sato et al., 1994). Interestingly, in DCs, DEX treatment but not any of the other treatments induced elevated mRNA levels of MT1 (Fig. 2A). In addition, total MT1 protein levels were increased after DEX treatment; however, MT1 could not be measured on the cell membrane of DEX-treated DCs (Figs. 2B and 2C), indicating that MT1 production is increased after DEX treatment but not transported to the cell surface. DEX-treated DCs had a tolerogenic phenotype with increased mRNA levels of IL-10 and the tolerogenic DC...
MT1 Is an Essential Mediator in Tolerogenic Function of ZnCl$_2$-Treated DCs

Although both ZnCl$_2$ and DEX treatment induced a tolerogenic phenotype and function in DCs, only ZnCl$_2$-treated DCs showed cell surface expression of MT1. It has been described that exogenous administration of MT induced the differentiation of naive CD4$^+$ T cells into T cells with a regulatory Tr1-like phenotype (Huh et al., 2007). Furthermore, activation of T cells in the presence of exogenous MT1 increased the percentage of FoxP3$^+$ T cells (Fig. 3A). In addition, to investigate if DC membrane-bound MT1 was responsible for the increased percentage of antigen-specific FoxP3$^+$ T cells, T cells were cultured with their cognate antigen and ZnCl$_2$-treated DCs in the absence or presence of an MT antibody earlier described as blocking

These data indicate that functionally tolerogenic DEX-treated DCs produced MT1 but did not transport MT1 to the cell surface.
agent (Canpolat and Lynes, 2001; Yin et al., 2005). Untreated
and DEX-treated DCs were used as controls. As shown in
Figure 3B, the tolerogenic function of ZnCl₂-treated DCs was
MT1 dependent because MT1 blocking inhibited the increased
percentage of FoxP3⁺ T cells induced by ZnCl₂-treated DCs.
In contrast, MT1 blocking had no effect on the percentage of
FoxP3⁺ T cells when DEX-treated DCs were used.

In conclusion, both DEX and ZnCl₂ treatments increased
the production of MT1 and induced a tolerogenic DC that was
able to increase the number of FoxP3⁺ T cells. However, only
in ZnCl₂-treated DCs, membrane-bound MT1 was essential to
exert their tolerogenic function.

DISCUSSION

The stress protein MT has been described earlier as an
immunosuppressive agent that affects several different cells of
the innate and adaptive immune system (Canpolat and Lynes,
2001; Huh et al., 2007; Lynes et al., 1993; Youn et al., 1995;
Youn and Lynes, 1999). Furthermore, carvacrol plus thermal
stress–treated DCs with a tolerogenic phenotype and function
produced high mRNA levels of MT1 (Spiering et al., 2012).
Tolerogenic DCs can achieve immune suppression via the
induction of regulatory T cells and are hereby a promising
immunotherapeutic target in T-cell-driven autoimmune dis-
ases (Steinman, 2003). In the present study, we demonstrated
that membrane-bound MT1 present on ZnCl₂-treated tolero-
genic DCs induced a regulatory FoxP3⁺ phenotype in antigen-
specific CD4⁺ T cells. Via in vitro or in vivo modification,
tolerogenic DC properties can be induced and numerous studies
have shown beneficial effects in experimental animal models
(Phillips et al., 2008; Stoop et al., 2011; Thomson and Robbins,
2008). Several immunosuppressive molecules, like vitD3 and
DEX, have been identified that induce a tolerogenic DC pheno-
type. In addition, vitD3 and DEX-treated DCs are function-
ally tolerogenic as they are able to induce antigen-specific
regulatory T cells. VitD3 has been described to block DC matu-
ration, prevent the release of proinflammatory cytokines (Penna
and Adorini, 2000), and induce expression of immunoglobulin-
like transcript 3 with which they can anergize CD4⁺ T cells
(Chang et al., 2002). DEX can induce the expression of GILZ
implicated as important factor in the induction of a FoxP3⁺
regulatory phenotype in antigen-specific T cells (Hamdi et al.,
2007). Our findings demonstrated that, although DEX-treated
DCs produce MT1, the stress protein was not exported to the
cell surface or secreted into the medium (Fig. 2C and data not
shown). In addition, blocking of MT1 did not significantly
reduce the percentage of FoxP3⁺ T cells. Therefore, for DEX
DCs, MT1 can be used as a tolerogenic DC marker but is not
responsible for a tolerogenic DC function. The glucocorticoid
GILZ is more likely to be essential for the tolerogenic func-
tion of DEX DCs (Hamdi et al., 2007). In contrast, the toxicant
ZnCl₂ was identified as a novel tolerogenic DC inducing mole-
ecule. DCs treated with ZnCl₂ exhibited a tolerogenic phenotype
and were functionally tolerogenic, as demonstrated by their
ability to preferentially increase the number of antigen-specific
FoxP3⁺ T cells. Moreover, we showed that the production and
membrane-bound presence of MT1 on the DC was essential for
the tolerogenic function of ZnCl₂-treated DCs. In addition,
human moDCs treated with ZnCl₂ also produced and translo-
cated MT1 to the cell surface, indicating a potential role for
MT1 in the human immune system (Supplementary Figure 2).

Exactly how MT1 could induce a regulatory phenotype in T
cells is not known. For astrocytes in the brain and renal prox-
imal tubule cells in the kidney, the megalin receptor was shown
to bind MT1 (El Refaey et al., 1997; Fitzgerald et al., 2007;
Klassen et al., 2004). However, for immune cells such as T
cells, no such receptor has been described yet. Nevertheless,
MT1 was found to interact directly with the plasma membrane
of T cells and macrophages (Borghesi et al., 1996; Youn et al.,
1995). Furthermore, binding of exogenous MT to splenocyte
cell membranes was shown to induce intracellular produc-
tion of MT (Borghesi et al., 1996). It has been suggested that

**FIG. 3.** Tolerogenic function of ZnCl₂-treated DC is MT1 dependent. A, CFSE-labeled CD4⁺CD25⁻ T cells were stimulated with anti-CD3/anti-CD28-
coated beads in the absence or presence of MT1. After 3 days, FoxP3 expression was measured by flow cytometry. B, DCs were treated with DEX, ZnCl₂,
were left untreated. After 6h of stimulation, DCs were used as APC to present OVA or PG to antigen-specific CD4⁺CD25⁻ T cells in the absence or presence
of MT1 antibody. Results were depicted relative to untreated DCs in the absence (black) or presence (white) of MT1 antibody. Results in (B) are representative of
4 independent experiments. * P < 0.05. Abbreviations: APC, antigen presenting cell; CFSE, 5,6-carboxy-succinimidyl-fluorescein-ester; DC, dendritic cell; DEX,
dexamethasone; MT1, protein metallothionein 1; OVA, ovalbumin; PG, proteoglycan.
NF-κB activity is negatively regulated by MT (Sakurai et al., 1999) and that the p50 subunit of NF-κB acts as a negative regulator of FoxP3 (Jana et al., 2009). In this way, MT could have a positive effect on the expression of FoxP3 in T cells.

In conclusion, we have discovered a new marker for tolerogenic DCs and a new mechanism for tolerogenic DCs to exert their tolerogenic function in the induction of a regulatory phenotype in T cells. Herewith, inducers of the stress protein MT1 seem to offer novel opportunities for the development of functional tolerogenic DCs for the treatment of autoimmune diseases and transplantation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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