Intranuclear delivery of the transcription modulation domain of Tbet-improved lupus nephritis in (NZB/NZW) F1 lupus-prone mice

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Excessive expression of Tbet and IFNγ is evidence of systemic lupus erythematosus (SLE) in lupus patients. In this study, the nucleus-transducible form of Transcription Modulation Domain (TMD) of Tbet (ntTbet-TMD), which is a fusion protein between Protein Transduction Domain Hph-1 (Hph-1-PTD) and the TMD of Tbet comprising DNA binding domain and isotype-specific domain, was generated to inhibit Tbet-mediated transcription in the interactomic manner. ntTbet-TMD was effectively delivered into the nucleus of the cells and specifically inhibited Tbet-mediated transcription without influencing the differentiation of other T cell subsets and signaling events for T cell activation. The severity of nephritis was significantly reduced by ntTbet-TMD as effectively as methylprednisolone in lupus-prone mice. The number of Th1, Th2 or Th17 cells and the secretion of their cytokines substantially decreased in the spleen and kidney of lupus-prone mice by ntTbet-TMD treatment. In contrast to methylprednisolone, the marked increase of Treg cells and the secretion of their immunosuppressive cytokine were detected in the spleen of (NZB/NZW) F1 mice treated with ntTbet-TMD. Thus, ntTbet-TMD can improve nephritis in lupus-prone mice by modulating the overall proinflammatory microenvironment and rebalancing T cell subsets, leading to new immune therapeutics for Th1-mediated autoimmune diseases.

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Lupus nephritis (LN) is one of the major manifestations of systemic lupus erythematosus (SLE), which is characterized by proteinuria, heterogeneous histologic alteration, and immune complex deposition in the kidneys.[1,2] SLE has been known to be initiated by dendritic cells and autoreactive B-cell dysregulation, but recent studies suggest that it is mainly affected by several T-cell subsets,[3] among which autoreactive T helper 1 (Th1) cells in particular have been considered to play a major role in the pathogenesis of LN by creating a proinflammatory microenvironment. However, the direct pathogenic role of Th1 cells in LN remains uncertain.[4]

Tbet (encoded by *Tbx21*) is the main transcription factor for Th1 cells, which has a role in controlling the infection of intracellular pathogens. Tbet directly binds to the *Ifng* promoter for the expression of interferon-γ (IFN-γ), which is important for the differentiation of Th1-cell lineage.[5] Although Th1 cells have a pivotal role in clearing intracellular pathogens, the excessive Th1-mediated immune responses can be the reason for their pathogenesis or exacerbating factors for many chronic inflammatory autoimmune diseases[6] such as multiple sclerosis,[7] type 1 diabetes,[8] and Crohn's disease.[9] Other than Th1 cells, Tbet can be expressed in Th17 cells and B cells, which also participates in the pathogenesis of autoimmune diseases. IFN-γ–producing Th17 cells have been identified as a new subpopulation of pathogenic Th17 cells in several autoimmune models, and Tbet expression in B cells mediates IgG2a production, which may, in turn, activate the development of SLE as well as LN.[10,11]

Recently, various monoclonal antibodies blocking the functions of inflammatory cytokines or their receptors are being considered as the main therapeutic modality in many autoimmune diseases. However, many studies have demonstrated that many autoimmune patients showed therapeutic unresponsiveness to these monoclonal antibodies, and their therapeutic efficacy was gradually reduced due to the production of antibodies to these biological agents or functional redundancy of the inflammatory cytokines during disease progression.[12,13] Therefore, the therapeutic efficacy of the current biological agents for LN is not satisfactory to replace cyclophosphamide or mycophenolate mofetil.[14,15] Therefore, novel therapeutic approaches to modulate the functions of the transcription factor responsible for the initiation and maintenance of Tbet-mediated inflammatory microenvironment are critically needed.

In this study, a novel therapeutic strategy was developed to inhibit the function of endogenous Tbet by delivering the transcription modulation domain (TMD) of Tbet into the nucleus of cells *in vitro* and *in vivo* via protein transduction domain Hph-1 (Hph-1-PTD), thereby preventing Tbet-mediated transcription in an interactomically inhibitory manner without using virus-mediated or genetic methods. The functional specificity of nucleus transducible (nt) Tbet-TMD *in vitro* and *in viv*o was investigated, and its *in vivo* therapeutic efficacy in nephritis in lupus-prone (NZB/NZW) F1 mice was analyzed.

RESULTS

Generation of ntTbet-TMD and its intranuclear delivery kinetics

To modulate the functions of Tbet without genetic alteration and the use of virus-mediated methods, ntTbet-TMD was generated in which Hph-1-PTD enables Tbet-TMD to be delivered into the nucleus of the cells *in vitro* and *in vivo* with high efficacy. Tbet-TMD contains the isotype-specific domain and T-box domain, therefore playing a critical role in isotype-specific DNA binding to the Th1-related gene promoter (Figure 1a).[5,16–18] ntTbet-TMD lacking DNA-binding activity (ntTbet-TMD[R164A]) and Tbet-TMD without Hph-1-PTD were generated as the negative controls (Figure 1a). ntTbet-TMD was expressed in the *Escherichia coli* expression system and purified under native condition, and its identity was confirmed by Western blot using anti-FLAG monoclonal antibody and sodium dodecylsulfate-polyacrylamide gel electrophoresis (Figure 1b).

When primary T cells were treated with different concentrations of ntTbet-TMD or for different periods of time, only 0.2 μM of ntTbet-TMD incubated <1 hour was required for the intranuclear delivery (the upper and middle panels of Figure 1c), whereas Tbet-TMD without Hph-1-PTD failed to penetrate the cells at all. Most of the transduced ntTbet-TMD were detected in the nucleus and maintained stability inside the cells for >24 hours (the lower panels of Figure 1c and d). Treatment of the primary T cells with as much as 4 μM of ntTbet-TMD did not show any cytotoxicity (Figure 1e). Therefore, ntTbet-TMD is effectively delivered into the nucleus of the primary T cells in a dose- and time-dependent manner and retains its stability without any cellular cytotoxicity.

Specific inhibition of Th1-cell differentiation by ntTbet-TMD without influencing the differentiation of other T-cell subsets and T-cell activation

To investigate the functional specificity of ntTbet-TMD to Tbet-mediated transcription, HEK 293 cells were cotransfected with wild-type Tbet- or EOMES-expressing vector and *Ifng* promoter-luciferase vector, and then incubated with 4 μM or 1 μM of ntTbet-TMD. Tbet and EOMES are members of Tbox family, which bind to the different regions of *Ifng* promoter.[19] Luciferase activity induced by wild-type Tbet was substantially reduced by ntTbet-TMD in a dose-dependent manner and ntTbet-TMD(R164A) did not affect luciferase activity (Figure 2a). Interestingly, ntTbet-TMD did not influence luciferase expression induced by EOMES, demonstrating that ntTbet-TMD inhibits the transcription of Tbet-inducible genes in an isotype-specific manner.

To examine whether ntTbet-TMD can specifically block the differentiation of naïve T cells into Th1 cells, the differentiation of naïve T cells into Th1, Th2, Th17, or induced regulatory T cell (iTreg) cells was induced by each T-cell subset–polarizing condition in the presence of ntTbet-TMD. The level of IFN-γ secretion by Th1 cells was significantly reduced by ntTbet-TMD and not by ntTbet-TMD(R164A) (Figure 2b). Surprisingly, ntTbet-TMD did not influence the differentiation of naïve T cells into Th2, Th17, or iTreg nor the level of T-cell activation that was evaluated by an analysis of the inducible surface expression of CD69 and IL-2 secretion (Figure 2c–e). Taken together, ntTbet-TMD inhibits Tbet-mediated transcription in a competitively interactomic manner with a high level of isotype specificity and blocks Th1-cell differentiation in a T-cell subset–specific manner without affecting T-cell activation.

In vivo therapeutic efficacy of ntTbet-TMD for nephritis in lupus-prone mice

The therapeutic potential of ntTbet-TMD for nephritis *in vivo* was evaluated by comparing it with that of methylprednisolone in lupus-prone mice. At 23 weeks of age, all the mice exhibited proteinuria higher than grade 2. Two untreated mice died of lupus exacerbation at 28 weeks of age, whereas all other mice treated with Tbet-TMD, ntTbet-TMD(R164A), ntTbet-TMD, or methylprednisolone survived (Figure 3b). At 30 weeks of age, treatment with ntTbet-TMD significantly reduced proteinuria in a dose-dependent manner, and the therapeutic efficacy of 100 μg per mouse of ntTbet-TMD treatment was comparable to that of methylprednisolone, but Tbet-TMD– or ntTbet-TMD(R164A)–treated mice did not alleviate the severity of lupus symptoms (Figure 3c). Also, the level of serum creatinine in 100 μg of ntTbet-TMD– or methylprednisolone-treated mice considerably decreased compared with that in untreated, Tbet-TMD–treated, or ntTbet-TMD(R164A)–treated mice (Figure 3c). These results