TLR4 Hyperresponsiveness via Cell Surface Expression of Heat Shock Protein gp96 Potentiates Suppressive Function of Regulatory T Cells

Jie Dai, Bei Liu, Soo Mun Ngoi, Shaoli Sun, Anthony T. Vella and Zihai Li

*J Immunol* 2007; 178:3219-3225; doi: 10.4049/jimmunol.178.5.3219

http://www.jimmunol.org/content/178/5/3219

References

This article cites 47 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/178/5/3219.full#ref-list-1

Why *The JI?* Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
TLR4 Hyperresponsiveness via Cell Surface Expression of Heat Shock Protein gp96 Potentiates Suppressive Function of Regulatory T Cells

Jie Dai,* Bei Liu,* Soo Mun Ngoi,* Shaoli Sun,† Anthony T. Vella,* and Zihai Li²**

As one of the main mediators of the endoplasmic reticulum unfolded protein response, heat shock protein gp96 is also an obligate chaperone for multiple TLRs including TLR4. We demonstrated recently that enforced cell surface expression of gp96 in a transgenic (Tg) mouse (96tm-Tg) conferred hyperresponsiveness to LPS and induced TLR4-dependent lupus-like autoimmunity. In this study, we investigated the function of CD4⁺CD25⁺ Foxp3⁻ regulatory T cells (Treg) in these mice in light of the important roles of Treg in the maintenance of peripheral tolerance against self-Ag as well as the increasing appreciation of TLR signaling on the regulation of Treg. We found that the development of Treg was not impaired in 96tm-Tg mice. Contrary to the prediction of dampened Treg activity, we discovered that the suppressive functions of Treg were increased in 96tm-Tg mice. Inactivation of Treg during the neonatal stage of life exacerbated not only organ-specific diseases but also systemic autoimmunity. By crossing 96tm-Tg mice into the TLR4 null background, we demonstrated the critical roles of TLR4 in the amplification of Treg suppressive function. These findings illustrate that gp96 plays dual roles in regulating immune responses by augmenting proinflammatory responses and inducing Treg function, both of which are dependent on its ability to chaperone TLR4.

Our study provides strong support to the notion of compensatory Treg activation by TLR ligation to dampen inflammation and autoimmune diseases. The Journal of Immunology, 2007, 178: 3219–3225.

The mechanism by which Treg is functionally regulated remains unclear. Treg express variable levels of CTLA-4, glucocorticoid-induced TNFR-related gene product (GITR), TGF-β, IL-10, and other molecules (1). In vitro, the suppressive functions of Treg are dependent on cell-cell contact between Treg and CD4⁺CD25⁻ effector T cells (Teff) and independent of soluble factors, such as IL-10 and TGF-β (1). However, TGF-β and IL-10 are required for the inhibitory function of Treg in vivo (7). The roles of CTLA-4 and GITR have also been implicated as mediators of Treg function (7, 8). Recently, it was found that TGF-β induces the generation of Foxp3⁺ Treg, which were completely inhibited by a proinflammatory cytokine IL-6 (9).

TLRs are important pattern recognition receptors for host to recognize and respond to pathogen-associated molecular patterns such as LPS (10). The folding and surface expression of TLR1, TLR2, and TLR4 are dependent on an endoplasmic reticulum chaperone gp96 (11). TLR ligation is generally thought to suppress Treg function (12). Two categories of TLR activity that downregulate the function of Treg have been discovered: suppression of Treg by direct ligation of TLR8 (13) and TLR2 (14, 15) on Treg; and blunting Treg function in a TLR4-dependent fashion by rendering Treg less responsive to TLR8 (16). In this regard, suppression of Treg was believed to be essential for the initiation of the adaptive immune response.

Paradoxically, ligation of TLR4 (17, 18) and TLR5 (19) on Treg was recently reported to have an opposite enhancing effect on the function of Treg. In addition, antagonizing MD1 was also found to decrease the threshold of LPS activation of dendritic cells (DC) and to enhance the induction of Treg (20). The Treg-amplifying effect of TLR4 ligation in this situation could potentially play critical roles in curtailing a late-phase immune response when an infection is contained and thus provide a feedback inhibition mechanism against TLR-dependent inflammatory process. However, direct effect of TLR4 ligation on Treg has been challenged (14, 19, 21–23). More importantly, it is unclear whether TLR4 amplification in vivo could lead to enhanced Treg function.

We previously reported that enforced cell surface expression of gp96 (96tm) in an otherwise non-autoimmune prone C57BL/6 mice induces systemic lupus-like autoimmune diseases (24). Furthermore, we found that 96tm expression confers hyperresponsiveness to LPS and the spectrum of autoimmunity in 96tm-transgenic (Tg) mice is...
completely abrogated in the absence of TLR4 (25). In this study, we addressed two interrelated questions. First, what is the impact of TLR4 hyperfunction on the function of $T_{reg}$ in vivo? Second, to what extent does $T_{reg}$ functional alteration, if any, contribute to the development of systemic autoimmune diseases in 96tm-Tg mice? We provide in vivo and in vitro evidence that surface expression of gp96 enhanced suppressive $T_{reg}$ function, which attenuated autoimmunity vide in vivo and in vitro evidence that surface expression of gp96 enhanced suppressive $T_{reg}$ function, which attenuated autoimmunity in 96tm-Tg mice. More importantly, we discovered that gp96-mediated enhancement of $T_{reg}$ function was dependent on TLR4. Our study is the first example of curtailing TLR4 hyperactivation via $T_{reg}$ on the organismal level to prevent systemic autoimmune diseases, thus providing strong support to the notion of compensatory $T_{reg}$ activation by TLR ligation to dampen inflammation.

Materials and Methods

**Mice**

Membrane-bound gp96 Tg (96tm-Tg) mice were generated as previously described (24). C57BL/6 mice were purchased from The Jackson Laboratory. TLR4 $^{-/-}$ mice were provided by Dr. R. Medzhitov (Yale University, New Haven, CT). Mice were maintained by the Center for Laboratory Animal Care of the University of Connecticut Health Center (Farmington, CT) according to the established guidelines (Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences).

**Abs and other reagents**

All Abs used for flow cytometry were from BD Pharmingen or eBioscience, unless otherwise stated. Anti-CD25 Ab (clone PC61) was obtained from American Type Culture Collection. FITC-labeled anti-CD25 Ab (clone 7D4) was used to confirm the inactivation efficiency of CD4 $^{+}$CD25 $^{+}$ T cells.

**Flow cytometry**

Briefly, cells were washed with staining buffer (cold PBS plus 2% heat-inactivated FCS), blocked on ice with a purified anti-FcyRII/III Ab (clone 93; eBioscience) for 10 min, followed by staining with various fluorochrome-conjugated Ab against interested surface markers. After staining, cells were then washed and analyzed on FACSCalibur (BD Biosciences). Dead cells were gated out by propidium iodide exclusion. For intracellular staining, cell surface markers were first stained as described, followed by fixation in 2% formaldehyde buffered with PBS, washing and permeabilization with 0.25% saponin in the staining buffer. Further staining was done using fluorochrome-conjugated Ab against respective intracellular proteins. In some experiments, cells were activated in vitro with 50 ng/ml PMA and 1 µg/ml ionomycin for 5 h in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich).

**In vitro suppression assay**

CD4 $^{+}$CD25 $^{+}$ $T_{reg}$ and CD4 $^{+}$CD25 $^{-}$ $T_{eff}$ were purified using the Regulatory T Cell Purification kit (Miltenyi Biotec) according to the manufacturer’s protocol. CD4-depleted splenocytes were irradiated (3000 rad) and used as APCs. In 96-well round-bottom plates, each well contained $5 \times 10^{5} T_{reg}$, $5 \times 10^{4}$ APCs, titrated number of $T_{reg}$ in the presence or absence of soluble anti-CD3e Ab (2.5 µg/ml). Cells were cultured at 37°C for 72 h. During the last 6 h, cells were pulsed with 1 µCi of $[^{3}H]$thy midine, harvested by an automated 96-well plate harvester, and the cpm were determined by an automated liquid scintillation counter (Microplate Scintillation and Luminescence Counter; Packard Instrument). The suppression index was calculated as $\frac{[CPM_{(Teff without Treg)} - CPM_{(Teff with Treg)}]}{CPM_{(Teff without Treg)}} \times 100%$.

**Determination of staphylococcal enterotoxin A (SEA)-specific CD4 $^{+}$ T cell response**

Mice were treated with either control IgG or PC61 (anti-CD25) 3 days before immunization. Inactivation efficacy was verified by flow cytometry using anti-CD25 (7D4) Ab recognizing a different epitope from PC61. One day after i.p. injection of 1 µg of SEA, 25 µg of LPS (from Salmonella typhimurium; Sigma-Aldrich) was administered at the same route. Mice were bled for the kinetic analysis of SEA-specific V$\beta$3 $^{+}$ CD4 $^{+}$ T cells as well as control V$\beta$14 $^{+}$ CD4 $^{+}$ T cells in peripheral blood. At day 16, mice were sacrificed, and the percentage and the absolute number of SEA-selective and nonselective CD4 $^{+}$ T cells was determined from various organs.

**Inactivation of $T_{reg}$ during the neonatal stage of life**

Newborn mice were treated with PC61 Abs (500 µg) or control rat IgG by i.p. injection every 4 days for four doses, starting before 9 days of age until day 21. Antinuclear Ab (ANA) was tested using sera obtained at weeks 8, 10, and 12. Mice were sacrificed 5 mo later, for evaluation of disease and serum level of cytokines.

**ELISA**

ELISA was used for the quantification of serum cytokines (BD Pharmingen) according to the manufacturer’s protocol.

**Histology**

Various organs were fixed in 4% formaldehyde buffered with PBS. The 5-µm sections were cut using Shandon Cryotome E (Thermo Electron), stained with H&E by standard methods, and examined by a light microscopy. ELISA was performed by single blind testing, and the severity of the gastritis was scored as follows: 0 (no inflammation), 1 (mild lymphocytic infiltration), 2 (moderate lymphocytic infiltration), or 3 (severe lymphocytic and neutrophil infiltration).
Immunofluorescence detection of glomerulonephritis and clinical evaluation of the severity of kidney disease

Samples of 5-µm cryosections of kidneys were incubated with blocking reagent (Vector Laboratories), stained with biotinylated anti-mouse IgG followed by FITC-conjugated streptavidin. Each sample was examined under a fluorescent microscope. The fluorescence staining intensity was graded as 0 (negative), 1 (slight staining), 2 (moderate staining), or 3 (bright staining). Based on an average of 83.5 ± 20.3 glomeruli evaluated per mouse (mean ± SD, obtained from a total of 40 mice), the mean fluorescence intensity was calculated as the index for the disease severity.

Detection of ANA

HEp-2-coated slides (INOVA Diagnostics) were incubated with diluted sera, followed by FITC-conjugated goat anti-mouse Ig, and examined by a fluorescence microscope. The fluorescence staining intensity was graded as 0 (negative), 1 (slight staining), 2 (moderate staining), or 3 (bright staining). Based on an average of 83.5 ± 20.3 glomeruli evaluated per mouse (mean ± SD, obtained from a total of 40 mice), the mean fluorescence intensity was calculated.

RT-PCR

Total RNA was extracted from purified wild-type (WT) or 96tm-Tg Treg cells using a NucleoSpin RNA II kit (with DNase I on-column digestion), according to the manufacturer’s protocol (Clontech Laboratories). Total RNA was reverse transcribed using Superscript II and oligo(dT)12–18 primer (Invitrogen Life Technologies) per the manufacturer’s protocol. The resulting cDNA obtained from 200 ng of total RNA was amplified for genes of interest for 35 cycles, using the following primers: gp96 (forward) 5'-ggattcctctctctgacctggc-3', (reverse) 5'-tcagaaggactcctatgtgg-3', 96tm (forward) 5'-acaccaagggcttaggagatt-3', (reverse) 5'-gatgatggtagcaccaccc-3', and β-actin (forward) 5'-catgactctagttgg-3', (reverse) 5'-ttcttcctgtacagcc-3'.

Statistical analysis

Student’s t test or Mann-Whitney U test was used for the statistical analysis using Prism software (GraphPad software). General linear model univariate and multivariate analysis were performed using SPSS 12.0 software. Values of p < 0.05 were considered statistically significantly different.

Results

Normal development of 96tm-Tg Foxp3+ Treg

Both male and female 96tm-Tg mice developed the serological/histological evidence of autoimmunity around 4 mo. To understand the possible involvement of Treg in the pathogenesis of autoimmune disease, we first analyzed Treg in 5-mo-old 96tm-Tg mice and age- and gender-matched WT mice to determine whether there was any developmental alteration of Treg in 96tm-Tg mice. By intracellular staining for Foxp3 protein, the best marker for CD4+CD25+ natural Treg, we determined the percentage and absolute number of Treg in the spleen and thymus of both WT and 96tm-Tg mice (3). We found that within the CD4+CD8+ single-positive population, ~7% cells expressed surface CD25 (Fig. 1A) and 2–3% were CD4+Foxp3+ T cells in both Tg and WT mice (Fig. 1B). No Foxp3 positivity was found in CD4+ populations. Similar to thymocyte Treg distribution, splenic CD4+CD25+ populations were comparable between WT and Tg mice, either in percentage (Fig. 1C) or in absolute number (data not shown). Most of these cells were phenotypically Treg as over 80% of them were also CD4+CD25+.

FIGURE 2. Cytokine profile of WT and 96tm-Tg splenocytes after polyclonal activation in vitro. Splenocytes from 5-mo-old WT and 96tm-Tg mice were incubated with PMA/ionomycin for 5 h in the presence of brefeldin A. Intracellular staining of individual cytokine and respective isotype controls were performed after surface staining of CD4 and CD25. Data of one representative mouse from three mice is shown for all, except that the staining for IL-10, and its isotype control represents analysis of 12 mice.

FIGURE 3. Increased suppressive function of CD4+CD25+ splenic Treg in 96tm-Tg mice in vitro. CD4+CD25+ Treg were purified from WT or 96tm-Tg splenocytes, respectively. A total of 5 × 10^5 CD4+CD25+ Teff were cocultured in the presence of soluble anti-CD3e mAb for 72 h with medium, 0.5 × 10^5 (1:1) or 5 × 10^5 (1:1) Treg. [3H]Thymidine was added during the last 6 h. A, A total of 5 × 10^5 CD4-depleted splenocytes from WT mice were used as APCs. B, A total of 5 × 10^5 CD4-depleted splenocytes from 96tm-Tg mice served as APCs. C, Comparisons of suppressive function between WT and 96tm-Tg Treg from young and old mice. Proliferation obtained in the absence of anti-CD3e mAb was generally under 200 cpm (data not shown). Symbols represent results from one individual experiment with Treg purified from the pooled splenocytes of two to three mice. The bars represent the mean values.
positive for Foxp3 (data not shown). Fewer than ~5% of CD4+CD25+ splenocytes were Foxp3+, Tg CD4+CD25+ T cells expressed similar levels of GITR on their cell surface (Fig. 1C), although no surface CTLA-4 or TGF-β was detectable on unstimulated splenic CD4+CD25+ T cells from either WT or 96tm-Tg mice (data not shown). The total number of CD4+Foxp3+ cells in the spleen was the same between WT and Tg mice, although the percentage of CD4+Foxp3+ T cells in the spleen of 96tm-Tg mice was slightly reduced (Fig. 1D).

To gauge possible functional differences between Tg and WT Treg, we next activated splenocytes with polyclonal activators PMA and ionomycin for 5 h, followed by intracellular staining for a variety of cytokines including IL-10, IL-2, IFN-γ, IL-4, and TNF-α. Under this condition, neither WT nor Tg CD4+CD25+ Treg produced a substantial level of cytokines, although both WT and 96tm CD4+CD25+ cells made an appreciable amount of TNF-α and IL-2. The cytokine profile of CD4+CD25+ population from Tg and WT mice appeared to be similar (Fig. 2). Taken together, we conclude that there is no significant impact either positively or negatively by 96tm on the development of Treg.

Augmented regulatory function of 96tm-Tg Treg

Next, we performed functional analysis of Treg from WT and Tg mice using a standard in vitro suppression assay to determine whether 96tm Treg retains suppressive function. CD4+CD25+ Treg were purified from the WT and Tg splenocytes by sequential negative (CD4− lineage mixture) and positive (CD25+) selections, which were then cocultivated with CD4+CD25− T eff in the presence of splenic APCs (CD4+ T cell-depleted populations) and soluble anti-CD3e Ab for 3 days. T cell proliferation was indexed by [3H]thymidine incorporation during the last 6 h of culture. In the absence of Treg, both WT and Tg T eff proliferated robustly (Fig. 3, A and B). In contrast, neither WT nor Tg Treg proliferation divided, which is in agreement with the anergic property of Treg in vitro (1). The proliferation of Treg was greatly reduced in the presence of either WT or Tg Treg at 1:10 or 1:1 ratio (Fig. 3, A and B). The suppressive function of 96tm-Tg Treg was significantly more pronounced than that of WT Treg on the per cell basis, regardless whether T eff and APCs were of WT or Tg origin in these assays (Fig. 3).

The experiments were performed using mice that were at least 4-mo-old when autoimmune disease was already detectable serologically. The functional alteration of Treg in 96tm-Tg mice might be affected by the pathological state of these mice. Furthermore, it was reported that aging had a significant negative effect on the inhibitory functions of mouse and human Treg (8, 26, 27). To determine the impact of age and disease on the functional differences between WT and Tg Treg, we examined the suppressive functions of Treg at 6–8 wk of age when none of the WT and Tg mice had signs of autoimmunity. We found that there was already a trend of increased Treg function from 96tm-Tg mice at the young and predisease stage (Fig. 3C). The fact that there was no age-dependent decline of Tg Treg function further reinforced the notion that the expression of cell surface gp96 potentiates the function of Treg.

Regulatory effects of 96tm-Tg Treg on T eff in vivo

If Treg is hyperfunctional in 96tm-Tg mice in vivo, inactivation of Treg might lead to more robust immune responses. To test this possibility, we kinetically analyzed the expansion of endogenous SEA-specific CD4+ T cells after immunization with SEA, using low-dose LPS (25 μg) as an adjuvant (28, 29), in the presence or absence of functional Treg. We injected mice with the mAb PC61, which recognizes the α-chain of the IL-2R, CD25; this Ab is known to specifically deplete Treg (30–33). The report of inactivation rather than depletion of the CD4+CD25+ Treg in vivo by
anti-CD25 Ab (34) was most likely related to distinct Ag specificity of anti-CD25 Ab (32, 33). PC61 was i.p. administered 3 days before SEA immunization. SEA-specific Vβ3+ CD4+ T cells in the peripheral blood were analyzed by flow cytometry (Fig. 4A). The kinetics of the expansion of WT and Tg SEA-specific Vβ3+ CD4+ T cells mirrored each other well when Treg was unmanipulated. In the absence of functional Treg, the Ag-specific Vβ3+ CD4+ T cells expanded much more in both WT and Tg mice, but the magnitude of expansion of Vβ3+ CD4+ T cells from 96tm-Tg mice were substantially greater than WT Vβ3+ CD4+ T cells (p = 0.049 by univariate regression analysis) (Fig. 4A). Furthermore, the increased percentages of Ag-specific Vβ3+ CD4+ T cells were also found in other lymphoid compartments, such as spleen (p = 0.005 by multivariate analysis) or inguinal, axillary, and cervical lymph nodes (p = 0.006) and mesenteric lymph nodes (p = 0.008) (Fig. 4C). In contrast, non-SEA-specific Vβ14+ CD4+ T cells in peripheral blood as well as lymph nodes remained constant in either WT or Tg mice (Fig. 4, B and D). Our data demonstrated that Tg Treg were not only hyperfunctional in vitro, but also exerted significantly more suppressive regulatory functions on the expansion of CD4+ T cells in vivo.

Exacerbation of autoimmunity in 96tm-Tg mice by inactivation of Treg

Having observed the increased suppressive function of Tg Treg in vitro and their regulatory roles in vivo, we hypothesized that Treg in 96tm-Tg mice represented a significant brake on the development of autoimmune diseases. If so, removal of such a brake might lead to exacerbation of systemic autoimmunity. We tested this hypothesis by injecting PC61 into 96tm-Tg mice beginning at the neonatal stage (within 9 days after birth) every 4 days for four injections. Down-regulation of CD25 was confirmed by flow cytometric analysis of peripheral blood 4 days after the last PC61 injection (data not shown). Inactivation of Treg by this method was transient when PC61 was given neonatally. All mice fully recovered the number of Treg in the peripheral blood as early as 1 wk after the last PC61 injection. PC61-treated 96tm-Tg mice were sacrificed at 5 mo for analysis of autoimmune disease, at which point the distribution of CD4+ T cells, CD8+ T cells, and B cells were comparable between mice treated with PC61 and mice treated with control Ig (data not shown). The percentage and cellularity of Treg also returned normal (data not shown).

However, PC61-treated 96tm-Tg mice manifested a significant exacerbation of autoimmunity by the appearance of ANA at least 2 wk earlier (age 10 wk) than control Ig-treated mice (age 12 wk or older) (Fig. 5A) and the worsening of their immune complex-mediated glomerulonephritis (Fig. 5B). Furthermore, we found that the serum level of IL-12/IL-23 p40 subunit was significantly increased in PC61-treated mice (Fig. 5C), whereas serum IL-1β, TNF-α, and IL-6 remained undetectable in mice treated with PC61 or control Ig (data not shown). Given our recent finding that IL-12 was pathological in the lupus-like autoimmune diseases in 96tm-Tg mice (35), we conclude that Treg inactivation in 96tm-Tg mice accelerates systemic autoimmune diseases.

To determine whether Treg inactivation also leads to worsening of organ-specific autoimmunity, we performed extensive histological analysis of 96tm-Tg mice including lacrimal glands, sublingual glands, stomachs, thyroids, lungs, and sex organs (testicles, coagulating glands, or ovaries). We found significant gastritis in PC61-treated 96tm-Tg mice but not in PC61-treated WT mice nor in the control Ig-treated 96tm-Tg/WT mice, supporting once again that autoimmunity initiated by expression of cell surface gp96 can be potentiated by inactivating Treg (Fig. 6). Our finding is striking as it was reported that effective Treg deletion via day 3 thymectomy (d3Tx) led to accelerated dsDNA autoantibody response, but not the exacerbation of lupus glomerulonephritis in a lupus-prone mouse model (36, 37).
TLR4-dependent hypersuppressiveness of 96tm-Tg T_{reg}

We have shown recently that 96tm is a chaperone for TLR4, resulting in hyperresponsiveness of 96tm-expressing cells to LPS (25). We further demonstrated that TLR4 is critical for the onset of autoimmunein 96tm-Tg mice. Because direct TLR4 engagement on T_{reg} has been reported to enhance regulatory function of T_{reg} (17), we hypothesized that the enhanced T_{reg} activity in 96tm-Tg mice was the direct effect of increased chronic triggering of TLR4 due to 96tm expression on T_{reg}. This hypothesis necessitates the expression of 96tm by T_{reg}. As expected, we found that Tg T_{reg} but not WT T_{reg} expressed transcripts for 96tm (Fig. 7A). More importantly, we found that T_{reg} from TLR4 null 96tm-Tg mice lost their superior ability to inhibit the proliferation of Teff (Fig. 7B), demonstrating that the increased suppression by 96tm-Tg T_{reg} in comparison to WT T_{reg} was mediated by TLR4. Our study is the first example of TLR4 hyperfunction in vivo in inducing increased T_{reg} function to curtail TLR4-dependent autoimmunein.

Discussion

Enforced cell surface expression of a TLR-chaperone gp96, in the 96tm-Tg model, leads to breakdown of the immunological tolerance and the development of autoimmune diseases (24, 25). This process is dependent on TLR4, which is hyperfunctional as a result of the chaperone function of 96tm, but not the direct stimulation of TLR4 by 96tm (25). TLR4 activation has been implicated in regulating the function of T_{reg} in vitro (17), although this conclusion remains controversial (14, 19, 21–23). The availability of 96tm-Tg mice allowed us to examine the impact of TLR4 hyperfunction on T_{reg} in vivo. We found that the suppressive functions of T_{reg} were increased in 96tm-Tg mice (Fig. 3); inactivation of T_{reg} during the neonatal stage of life exacerbated both systemic and organ-specific autoimmune diseases (Figs. 5 and 6). By crossing 96tm-Tg mice into the TLR4 null background, we further discovered that the increased suppressive function of T_{reg} was TLR4-dependent (Fig. 7). Our study has thus demonstrated that TLR4 hyperfunction can indeed lead to intensified T_{reg} function without altering the development of T_{reg}.

Thymectomy on day 3 has been widely used to investigate the roles of T_{reg} in vivo. One enigma is that day 3 thymectomy leads to organ-specific rather than the systemic autoimmunein such as lupus. Depending upon mouse strain and gender, thymectomy on day 3 may induce inflammation in the stomach, thyroid, ovary, testicle, prostate, or other organs (38, 39). For example, C57BL/6 mice are resistant to the development of autoimmunein after thymectomy on day 3, including gastritis (38). Even in BALB/c mice that are more susceptible to day 3 thymectomy-induced gastritis (38, 40), inactivation of T_{reg} is necessary but not sufficient to induce autoimmunein gastritis (31). Yet, inactivation of T_{reg} in our 96tm-Tg mice, which were in C57BL/6 background was sufficient to induce gastritis (Fig. 6), and exacerbate the systemic autoimmunein as indexed by the worsening of glomerulonephritis and earlier emergence of ANA (Fig. 5). Our finding demonstrated that T_{reg} exerts a significant brake in the development of autoimmunein diseases in 96tm-Tg mice. It offers a striking example that T_{reg} can suppress both organ-specific and systemic autoimmunein in vivo.

The increased suppressive functions of T_{reg} from 96tm-Tg mice were directly demonstrated in vitro (Fig. 3). T_{reg} from Tg mice were able to exert much more suppression on the proliferation of either WT or Tg Teff regardless whether 96tm was expressed on APCs. Moreover, unlike WT mice that have age-dependent reduction of T_{reg} activity (8, 26, 27), we found no decline of Tg T_{reg} function with age (Fig. 3C). The increased Tg T_{reg} function was also indirectly demonstrated in vivo, as we observed much more robust and systemic expansion and survival of SEA-specific Tg CD4+ T cells after the inactivation of T_{reg} (Fig. 4). T_{reg} need to be activated before they can function. This requires IL-2 (5, 41) and costimulation, such as B7-CD28/CTLA-4 interaction (6, 42). Additional signals capable of regulating the T_{reg} activity remain elusive. TLR are ancient molecules that detect microbial products and differentiate “non-self” from “self” (43). Engagement of TLRs with their ligands induces the activation of innate immune. As a consequence, the TLR-induced proinflammatory environment can further promote the activation of the adaptive immunein (44). Recently, the roles of TLRs in regulating the functions of T_{reg} have gained increasing attention (18). In response to LPS, mouse DCs produces IL-6, which is partially responsible for releasing T_{reg} from T_{reg} inhibition (16). In contrast, reversal of T_{reg} function could be DC-independent (13). For example, TLR8 activation dampens the functions of T_{reg} directly, which is dependent on MyD88-IRAK4 signaling pathway in the T_{reg} autonomous fashion. Moreover, it has been shown that T_{reg} selectively express TLR4 (17) and that interaction of T_{reg} with LPS induces their suppressive function. In another study, TLR2 signaling was shown to promote the survival of CD4+CD25+ T_{reg} as these cells were significantly decreased in TLR2−/− mice (45).

More recently, heat shock protein 60 was reported to be able to enhance the function of human T_{reg} in vitro via TLR2 in a manner that is dependent on both cell-cell contact and immunosuppressive cytokines IL-10 and TGF-β (46). Our present work is consistent with the roles of TLR4 activation in directly enhancing T_{reg} function (17, 18), providing a strong piece of in vivo evidence that excessive TLR activation could be counter-balanced by amplified T_{reg} function.

Several unanswered questions remain. For example, what are the differences if any in the mechanisms of suppression between WT and Tg T_{reg}? Our analyses so far have found no discernible distinctions between the two, including their ability to proliferate in vitro, the activation status measured by the level of CD3ζ chain phosphorylation, the cell surface marker of CD103 (47) (data not shown), and their cytokine profile in response to polyclonal activation (Fig. 2). Secondly, the number of T_{reg} in WT and Tg is similar. The coordinated actions of Foxp3 and NFAT are critical for the development of T_{reg} (4), yet it is unclear whether expression of gp96 on cell surface might affect this process qualitatively. Thirdly, there are many flavors of T_{reg} in vivo (48). In particular, natural Foxp3+ T_{reg} can be converted into inducible T_{reg} in the presence of appropriate environment such as the presence of TGF-β and IL-10. It is unclear whether these processes were in someway altered in 96tm-Tg mice.

In summary, we have shown that cell surface expression of a TLR-chaperone, gp96, induced lupus-like diseases and elevated T_{reg} function without altering the development of CD4+CD25+ Foxp3+ T_{reg}. Our study represents the first example of curtailing TLR4 hyperactivation via T_{reg} on the organinal level to prevent systemic autoimmunein diseases. Further studies into the mechanisms of this process in the context of infection, tissue injury, inflammation, and autoimmunein pathology should be critical and fruitful in guiding the development of new immunotherapeutics against these conditions.

Acknowledgments

We thank Drs. Leo Lefrançois, Mark Manula, and Robert Clark for helpful discussions; Yi Yang and Matthew Staron (both from Dr. Zihai Li’s laboratory) for stimulating interactions during the course of the study as well as critical analysis of the data; and Dr. Mark Goldstein for reading and editing of the manuscript.