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Regulatory Functioning T Cell Population Contributing to the Mediation of Endotoxin Tolerance

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Summary. Endotoxin tolerance (ET) has been considered to develop mainly due to the hyporesponsiveness of monocytes and macrophages. We examined the role of T cells in ET mice. T cells derived from ET mice showed a decreased level of interferon-γ (IFN-γ) production in comparison to those from the control mice when T cells were co-cultured with peritoneal exudate cells (PEC) adherent cells from the controls or lipopolysaccharide (LPS)-injected ET mice. After anti-CD3 stimulation, IFN-γ induction by T cells derived from ET mice was significantly decreased in comparison to those from the control mice. On the other hand, interleukin-10 (IL-10) secretion by T cells derived from ET mice was higher than in those of the control mice. When T cells with a high CD62L expression (CD62L^{high} T cells) were co-cultured with PEC adherent cells, the secretion of IFN-γ tended to decrease more than when these were co-cultured with T cells with a low CD62L expression (CD62L^{low} T cells). Our data suggest that T cells expressing CD62L^{high} T cells in ET mice can play a suppressive role in antigen-presenting cells through their cytokine production.

Key words— endotoxin tolerance, interferon-γ, interleukin-10, CD62L^{high} T cell, natural regulatory T cell.

INTRODUCTION

Endotoxin tolerance (ET) was initially described in animal models with repetitive exposure to bacterial endotoxin or lipopolysaccharide (LPS).[1)] The mechanism of ET has been understood to consist of two phases: an early phase associated with altered cellular activation, and a late phase associated with the development of specific antibodies against the polysaccharide chain of gram-negative organisms.[2)] Macrophages and monocytes are generally rendered tolerant and thereafter demonstrate an altered response when rechallenged with either bacterial endotoxin or LPS. The phenotype of tolerant macrophages and monocytes is characterized by the decrease of tumor necrosis factor alpha (TNF-α) production,[3)] changed interleukin-1 and interleukin-6 release,[4,5)] enhanced cyclooxygenase-2 activation, the inhibition of mitogen activated protein kinase activation,[6)] and impaired nuclear factor-κ B translocation.[7)] There is increasing evidence for ET in clinical systemic inflammatory response syndrome and sepsis;[8)] however, very few reports have previously described the interaction between antigen-presenting cells (APC) and CD4$^+$ T cells and their cytokine production that may induce a patient to develop a chronic phase of infection. ET may limit the magnitude of the inflammatory cell responses, thus resulting in a failure to adequately control infection; it may also help to limit the collateral tissue damage caused by vigorous antimicrobial immune responses. Recently, CD4$^+$ T cells which express CD25 have been reported to play a critical role in maintaining peripheral tolerance during infection.[9,10)] CD4$^+$CD25$^+$ T cells, which play a regulatory role, have been reported to express CD62L at high levels (CD62L^{high} T cells).[11,12)] In this study, we focused on CD62L^{high} T cells in the LPS tolerant mouse spleen, then we aim to clarify whether the cells play a privotal role in regulating interferon-γ (IFN-γ) production.

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Abbreviations – ET, endotoxin tolerance; CD62L^{high} T cells, high CD62L expression; CD62L^{low}T cells, low CD62L expression; IFN-γ, interferon-γ; IL-10, interleukin-10.

MATERIALS AND METHODS

Animals

Male C57BL/6N mice were purchased from Charles River Laboratories (Kanagawa). They were maintained in a specific pathogen-free environment. The mice were 8-12 weeks of age at the time of use.

Low-dose endotoxin models

Groups of mice were injected intraperitoneally (i.p.) on days -7, -5, -3 and -1 with either 2 μg (100 μg/kg) of lipopolysaccharide (LPS) (Klebsiella pneunoniae derived LPS dissolved in phosphate buffered saline (PBS), purchased from SIGMA) as an ET group or an equivalent volume of PBS as a control group. On day zero, both groups received i.p. 10 mg (500 mg/kg) of D-GalN (SIGMA) and 2 μg of LPS. Their sera were harvested for a TNF-α assay at 90 min after D-GalN and LPS injection. Thereafter, the mice were observed for 84 h and their survival rates were recorded.

Culture medium

RPMI 1640 medium was purchased from GIBCO and supplemented with 100 U/ml penicillin G and 100 μg/ml streptomysin sulfate (GIBCO), fetal bovine serum inactivated by heating (GIBCO), 0.1 mM nonessential amino acids (GIBCO), 1 μM sodium pyruvate (GIBCO), and 5 x 10^{-5} M2-ME (Sigma-Aldrich, St.Louis, MO, USA).

Measurement of TNF-α, IFN-γ, interleukin-10 (IL-10)

Cytokine levels were determined by an enzyme linked-immuno-sorbent assay (ELISA). Antibodies were coated onto the wells of the microtiter strips. Samples which is well known the contents of these cytokines, were pippeted into these wells by the addition of a biotinylated monoclonal second antibody. After the removal of any excess second antibody, an enzyme was added. After incubation and washing to remove all the unbound enzyme, the substrate solution was added. Cytokine levels were determined by measuring the optical density at 450 nm using a visible plate reader (Biotrak).

Preparation of peritoneal exudate cells (PEC) adherent cells

The PEC were harvested from mice that had received an i.p. injection of 2 ml of a 3% thioglycollate mediua (BECTON DICKINSON) three days previously. The PEC were washed three times by centrifugation and then resuspended in an ice-cold culture media. After removing any contained red blood cells, these cells were then plated in 6-well tissue culture clusters. The cells were incubated at 37C° in a 5% CO_2/95% air atmosphere for two h. Adherent cells were thereafter collected.

Fractionation of T cells and CD62L^{high} T cells or a low CD62L expression (CD62L^{low} T cells)

T cells in the splenocyte were concentrated by passing them through a nylon wool column (WAKO). After a 45 min incubation at 37C°, the first 15 ml pass through the nylon wool column was collected. B cells were removed using magnetic beads, these cells containing about 90-95% T cells. The purified T cells were further isolated by a panning technique using T-25 and T-75 flasks precoated with goat anti-rat immunoglobulin antibody/anti-CD62L antibody (MEL14). CD62L^{high} T cells were obtained as cells attached to flasks to the precoated goat anti-rat immunoglobulin antibody. The cells not attached to these flasks were obtained as T cells with a CD62L^{low} T cells. Analyses of the cell surface phenotypes were conducted by direct immunofluorescence staining of 0.5-1 x 10^6 cells with conjugated anti-CD3 in order to identify T cells, or with conjugated anti-CD62L in order to identify L-selectin expressed T cells. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (BD Biosciences, Sunnyvale, CA, USA).

Anti-CD3 activation

T cells were activated *in vitro* by incubating 4 x 10^6 cells in a 24-well plate precoated with an anti-CD3 monoclonal antibody containing a two ml culture media. After fourty-eight h incubation, the supernatant fluid was obtained. Following this, the cytokine levels of IFN-γ and IL-10 were determined.

Statistical analysis

Data were expressed as the mean ± SD. The Mann-Whitney *U*-test and Student's *t*-test were used to examine differences between paired data. A value of $P < 0.05$ was considered to be a significant difference.

RESULTS

Survival of mice and TNF-α secretion

To ascertain whether ET had been induced or not, we checked the survival of LPS-injected mice and TNF-α secretion in serum in comparison with control mice (Fig.1). The ET group (n = 7) showed a significantly

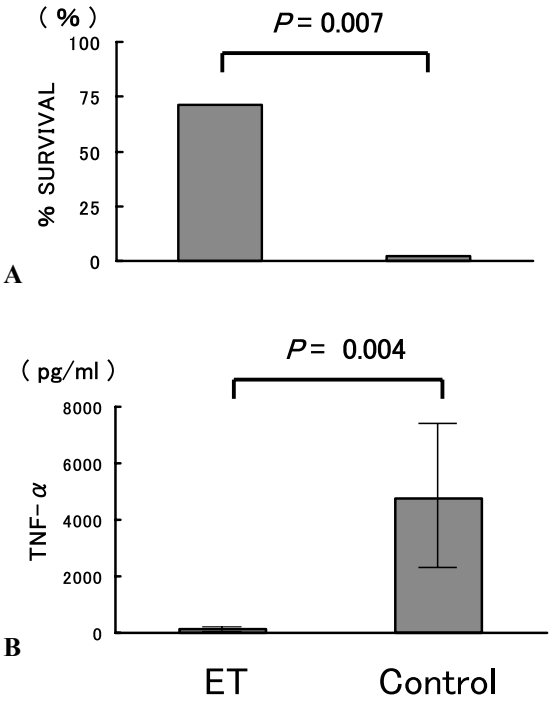

Fig. 1. Survival of mice and TNF-α secretion. **A.** Endotoxin tolerance (ET) group (n = 7) showed a significantly decreased lethality in comparison with the control group (n = 7). The ET group showed a 71% survival at 24 h after injection, and the rest survived for 84 h. The control group showed 0% survival at 24 h after injection (P = 0.007). **B.** Sera from both groups that were injected both lipopolysaccharide (LPS) and D-GalN 90 min later were tested for tumor necrosis factor alpha (TNF-α) secretion. TNF-α could not be detected in the sera of either group prior to receiving LPS and D-GalN. Ninety minutes later, TNF-α was markedly lower in the ET group than in the control group (mean, 118 pg/ml vs 4758 pg/ml; P = 0.004).

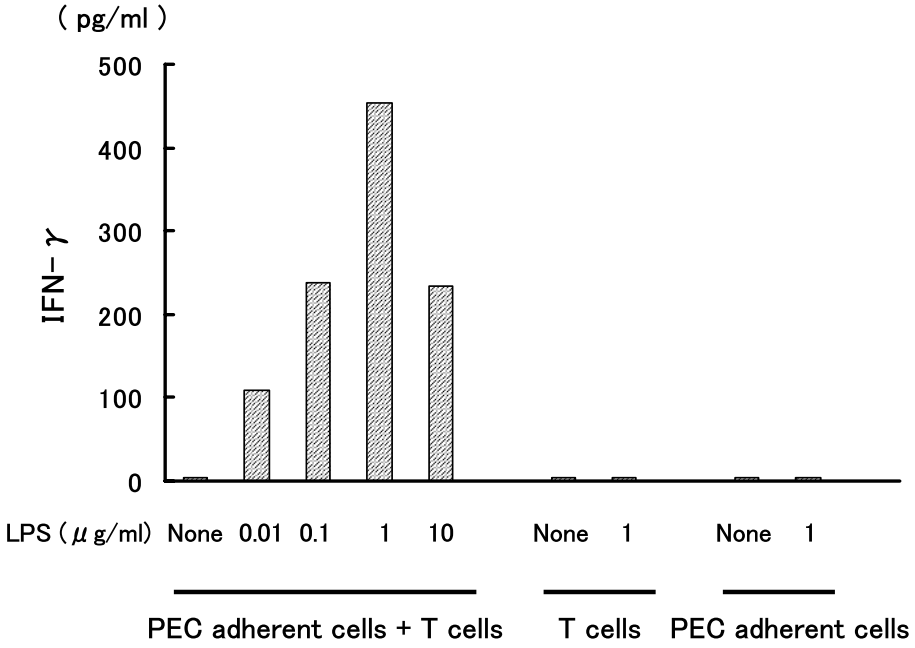

Fig. 2. Induction of interferon-γ (IFN-γ) secretion by stimulation with LPS. To examine whether IFN-γ secretion by T cells could be changed in various concentration of LPS (0.01 to 10 μg/ml), normal PEC adherent cells (5 x 10^5/well) and T cells (1 x 10^6/well) were co-cultured with various concentrations of LPS in a 24-well plate. After 16 h of incubation, the supernatants were obtained and assayed for IFN-γ. IFN-γ secretion increased most when the concentration of LPS was 1 μg/ml. Moreover, IFN-γ was not detected when each T cells or PEC adherent cells were cultured, even when only they were stimulated with 1 μg/ml of LPS.

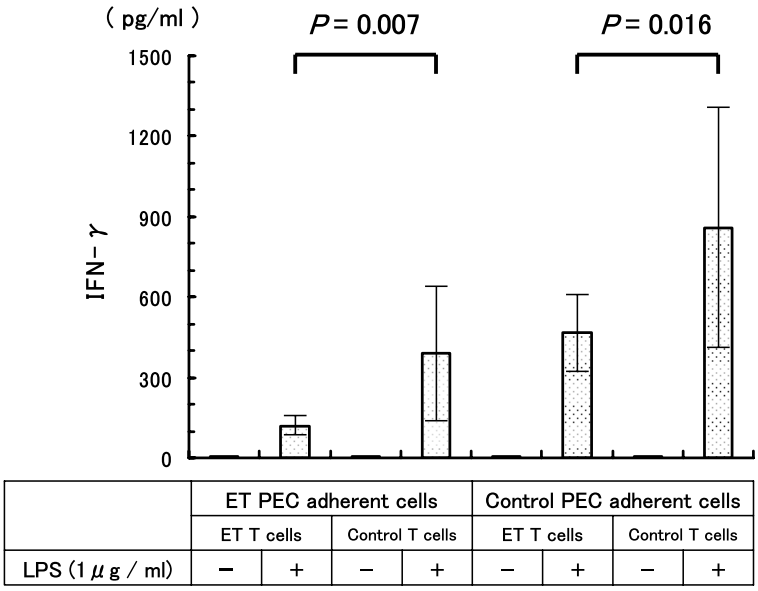

Fig. 3. IFN-γ secretion co-cultured with PEC adherent cells and T cells, with or without LPS. T cells were co-cultured with PEC adherent cells derived from either ET or control mice, with or without LPS (1 μg/ml) *in vitro*. IFN-γ was not detected in any of the groups co-cultured without LPS. In the presence of ET PEC adherent cells, a significant decrease in IFN-γ secretion was observed when they were co-cultured with T cells derived from ET mice in comparison with T cells derived from control mice (mean, 120 pg/ml vs 388 pg/ml; $P = 0.007$). When T cells derived from ET or control mice were co-cultured with control PEC adherent cells, IFN-γ was markedly higher than PEC adherent cells derived from ET mice. A significant decrease in IFN-γ was also observed when control PEC adherent cells were co-cultured with T cells derived from ET mice in comparison with T cells derived from control mice (mean, 468 pg/ml vs 857 pg/ml; $P = 0.016$).

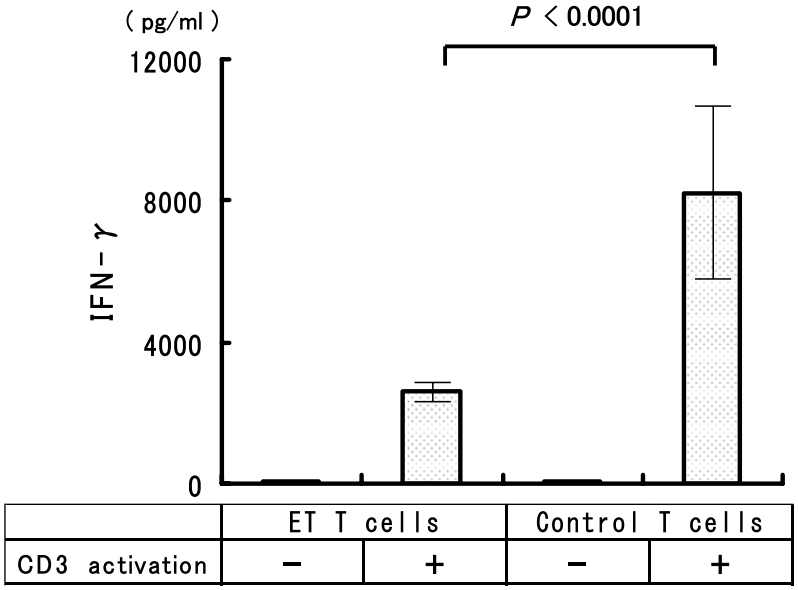

Fig. 4. IFN-γ secretion by T cells after anti-CD3 stimulation. Both T cell were activated with anti-CD3. The supernatant fluid was obtained and IFN-γ was measured. IFN-γ was found to decrease significantly when T cells derived from ET mice were plated in comparison with control mice (mean 2591 pg/ml vs 8190 pg/ml; $P < 0.0001$).

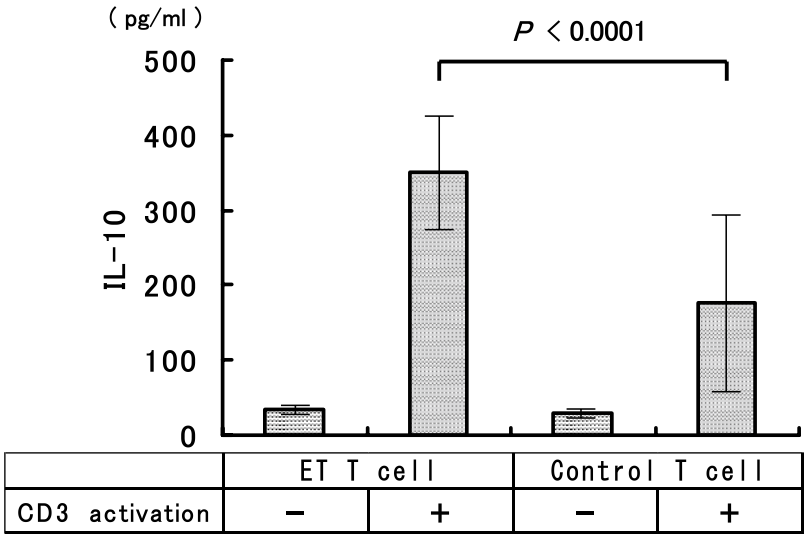

Fig. 5. Interleukin-10 (IL-10) secretion by T cells after anti-CD3 stimulation. IL-10 secretion was found to be significantly higher when they were cultured with the T cells derived from the ET mice than the control mice (mean, 350 pg/ml vs 177 pg/ml; $P < 0.0001$).

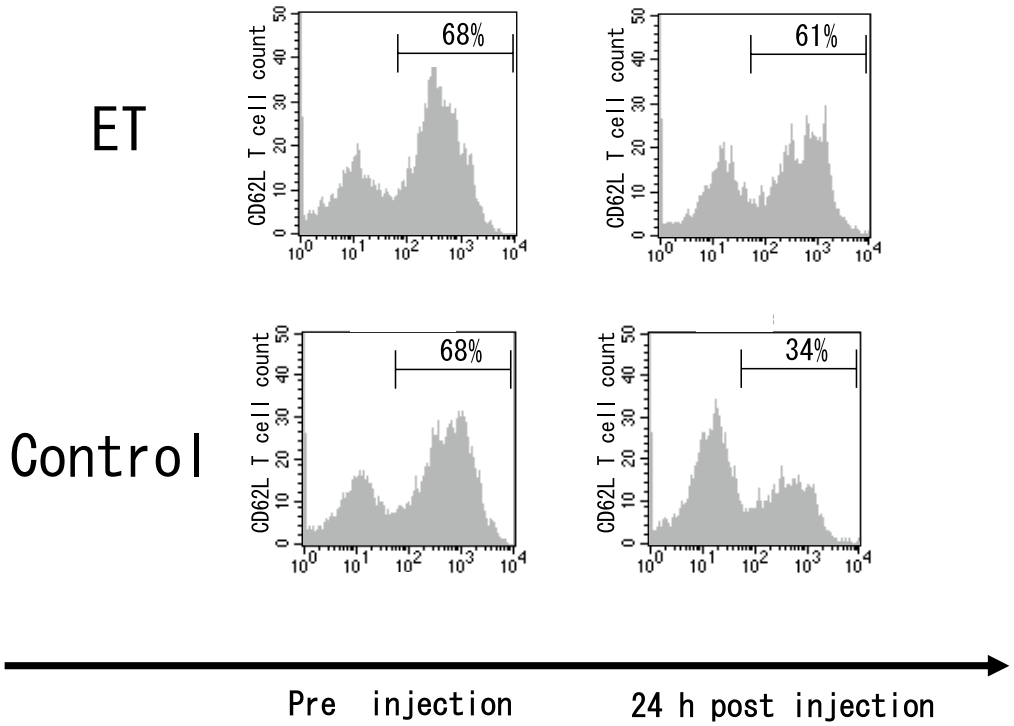

Fig. 6. Kinetics of a high CD62L expression ($CD62L^{high}$T cells). We ascertained the kinetics of $CD62L^{high}$ T cells from mouse spleens after the injection of D-GalN and LPS with a FACScan. The ratio of $CD62L^{high}$ T cells were equivalent in both groups prior to receiving LPS and D-GalN. Twenty-four hours later, the ratio of $CD62L^{high}$T cells decreased in the control group. However, in the ET group, the ratio of $CD62L^{high}$ T cells did not change in comparison to that before the injection.

Table 1. Functions of $CD62L^{high}$ T cells and $CD62L^{low}$ T cells

		LPS (1 μg/ml)	IFN-γ (pg/ml)
ET PEC adherent cells	ET $CD62L^{high}$	–	ND
		+	49.01
	ET $CD62L^{low}$	–	ND
		+	54.88
	Cont $CD62L^{high}$	–	ND
		+	100
	Cont $CD62L^{low}$	–	ND
		+	164
Cont PEC adherent cells	ET $CD62L^{high}$	–	ND
		+	577.8
	ET $CD62L^{low}$	–	ND
		+	728.8
	Cont $CD62L^{high}$	–	ND
		+	1120
	Cont $CD62L^{low}$	–	ND
		+	1117

$CD62L^{high}$ T cells or $CD62L^{low}$ T cells were co-cultured with PEC adherent cells either with or without LPS (1 μg/ml) *in vitro* and IFN-γ was measured. IFN-γ was not detected in any of the groups co-cultured without LPS. In the presence of ET PEC adherent cells, a decrease in IFN-γ secretion was observed when they were co-cultured with $CD62L^{high}$ T cells in comparison with $CD62L^{low}$ T cells derived from both groups. On the other hand, when $CD62L^{high}$ T cells or $CD62L^{low}$ T cells from both groups were co-cultured with control PEC adherent cells, IFN-γ was higher than when co-cultured with ET PEC adherent cells with those. A decrease in IFN-γ was also observed when they were co-cultured with $CD62L^{high}$ T cells in comparison with $CD62L^{low}$ T cells in the ET group. However, when the $CD62L^{high}$ T cells and $CD62L^{low}$ T cells of control were co-cultured, IFN-γ secretion was equal. When $CD62L^{high}$ T cells derived from both groups were co-cultured, IFN-γ tended to decrease more than when they were co-cultured with $CD62L^{low}$ T cells. ND, not detectable.

decreased lethality in comparison to the control group (n = 7), with a 71% survival at 24 h after injection, and the rest surviving for 84 h. The control group showed 0% survival at 24 h after injection ($P = 0.007$). Sera from both groups that were injected with both LPS and D-GalN 90 min later were tested for TNF-α secretion. TNF-α could not be detected in sera of either group prior to receiving LPS and D-GalN. Ninety min later, TNF-α was markedly lower in the ET group than in the control group (mean, 118 pg/ml vs 4758 pg/ml; $P = 0.004$).

Induction of IFN-γ secretion by stimulation with LPS

To examine whether IFN-γ secretion by T cells could be changed in various concentrations of LPS (0.01 to 10 μg/ml), normal PEC adherent cells (5 x 10^5 /well) and T cells (1 x 10^6 /well) were co-cultured with various concentrations of LPS in a 24-well plate. After sixteen h of incubation, the supernatants were obtained and assayed for IFN-γ (Fig.2). IFN-γ secretion increased most when the concentration of LPS was 1 μg/ml. Moreover, IFN-γ was not detected when each T cells or PEC adherent cells were cultured only even if they were stimulated with 1 μg/ml of LPS.

IFN-γ secretion co-cultured with PEC adherent cells and T cells, with or without LPS

To clarify whether the T cell functions of ET mice changed in comparison with those of control mice,

IFN-γ from T cells was measured. T cells were co-cultured with PEC adherent cells derived from either ET or control mice, with or without LPS (1 μg/ml) (Fig.3). IFN-γ was not detected in any of the groups co-cultured without LPS. In the presence of ET PEC adherent cells, a significant decrease in IFN-γ secretion was observed when they were co-cultured with T cells derived from ET mice in comparison to T cells derived from control mice (mean, 120 pg/ml vs 388 pg/ml; $P = 0.007$). When T cells derived from ET or control mice were co-cultured with control PEC adherent cells, IFN-γ was markedly higher than PEC adherent cells derived from ET mice. A significant decrease in IFN-γ was also observed when control PEC adherent cells were co-cultured with T cells derived from ET mice in comparison with T cells derived from control mice (mean, 468 pg/ml vs 857 pg/ml; $P = 0.016$). This showed that even when the same PEC adherent cells were co-cultured, a decrease in IFN-γ production could be observed when they were co-cultured with T cells derived from ET mice.

IFN-γ and IL-10 secretion by T cells after anti-CD3 stimulation

To examine the T cell function further, both were activated with anti-CD3. The supernatant fluid was obtained and IFN-γ was measured (Fig.4). As a result, IFN-γ was found to decrease significantly when T cells derived from ET mice were plated in comparison to control mice (mean, 2591 pg/ml vs 8190 pg/ml; $P < 0.0001$). After anti-CD3 stimulation, we examined the IL-10 secretion which was observed to play a role in hyporesponsiveness to LPS (Fig.5). The IL-10 secretion was thus found to be significantly higher when they were cultured with T cells derived from ET than control T cells (mean, 350 pg/ml vs 177 pg/ml; $P < 0.0001$).

Kinetics of CD62L^{high} T Cells

We ascertained the kinetics of CD62L^{high} T cells from mouse spleens after the injection of D-GalN and LPS with a FACScan (Fig.6). The ratio of CD62L^{high} T cells were equivalent in both groups prior to receiving LPS and D-GalN. Twenty-four hours later, the ratio of CD62L^{high} T cells decreased in the control group. However, in the ET group, the ratio of CD62L^{high} T cells did not change in comparison to that before the injection.

Functions of CD62L^{high} T cells and CD62L^{low} T cells

To clarify whether the functions of CD62L^{high} T cells and CD62L^{low} T cells changed or not, CD62L^{high} T cells or CD62L^{low} T cells were co-cultured with PEC adherent cells either with or without LPS (1 μg/ml) *in vitro* and IFN-γ was measured (Table 1). IFN-γ was not detected in any of the groups co-cultured without LPS. In the presence of ET PEC adherent cells, a decrease in IFN-γ secretion was observed when they were co-cultured with CD62L^{high} T cells in comparison with CD62L^{low} T cells derived from both groups. On the other hand, when CD62L^{high} T cells or CD62L^{low} T cells from both groups were co-cultured with control PEC adherent cells, IFN-γ was higher than when co-cultured with ET PEC adherent cells. A decrease in IFN-γ was also observed when they were co-cultured with CD62L^{high} T cells in comparison with CD62L^{low} T cells in the ET group. However, when CD62L^{high} T cells and CD62L^{low} T cells of the control group were co-cultured, IFN-γ secretion was equal. When CD62L^{high} T cells derived from both groups were co-cultured, IFN-γ tended to decrease more than when they were co-cultured with CD62L^{low} T cells.

DISCUSSION

We speculate the suppressive T cells contribute to the mechanisms of the down regulation as well as IL-10 production by macrophages. Exposure to a wide variety of stimuli has been shown to confer a state of ET. ET is not specific to the action of LPS, and cross reactivity with various exogeneous stimuli occurs.[13] Macrophages and monocytes that are exposed to LPS are rendered tolerant and manifest a profoundly altered response when these are rechallenged with LPS.[13] In this study, our experiments were performed according to a previously established method by Barton BE et al..[14] Their experimental findings showed that the treatment with D-GalN affected the UDP ribosylation in the liver; however, the other organs were not affected by either this treatment or with D-GalN treatment in the absence at a LPS lethal dose.[14] Our experimental system induced a tolerant state for LPS stimulation through TNF-α production and survived after an additional injection of LPS and D-GalN, which were lethal doses for the control mice.

A co-culture with PEC adherent cells and 1 μg/ml of LPS induced IFN-γ production of splenic T cells in both groups, while IFN-γ was not detected in either T cells or PEC adherent cells alone. The IFN-γ production was significantly decreased in the case of PEC adherent cells from the ET mice in comparison to those from control mice. PEC adherent cells from the ET group showed a reduced IFN-γ inducing ability to splenic T cells (both ET T cells and control T cells) in comparison with those from the control group. Moreover, those down regulations were augmented by an additional co-culturing of the ET T cells compared with control T cells in both groups. These results suggest that the T cell function is therefore also down-regulated in ET mice. IL-10 production co-cultured with ET T cells was higher than

when co-cultured with control T cells, thus indicating that IL-10 also plays a role in the regulation of effector cells which mediate ET partly through IFN-γ. These data are somewhat controversial when compared with the findings by Karp et al..[15] They described how the priming of human peripheral blood monocytes with LPS ablated the interleukin-12 (IL-12) productive capacity via the suppression of IL-12 gene transcription.[15] This IL-12 suppression during ET was not dependent on either the presence of IL-10 or transforming growth factor-β, and IL-12 production was not maintained by IFN-γ or granulocyte-macrophage colony-stimulating factor.[15] However, they did not allow T cells to be added to their experimental system, and their methodology thus differed from ours.

Copious amounts of data have shown that ET mediates an improved innate immune effector function through the alteration of cytokine production. Multiple noteworthy reports have suggested that ET changes the way that LPS interacts with either cell surface receptors or membrane components, such as the membrane G protein, Gi3 alpha.[16] Moreover, toll-like receptor (TLR) four expression was down-regulated in LPS tolerant macrophages, and this finding correlated with decreased inflammatory cytokines. TLR4-MyD88-dependent signaling is supposed to play an important role in tolerant cells.[17,18,19] IRAK and MARK are also key proximal signal transductions which independently affect ET.[20,21]

It remains unclear whether T cells regulate the effector function and are related to the mediation of ET. Hiura et al. reported that the $CD62L^{high}$ T cell had a regulatory function in the draining lymph nodes during tumor progression.[22] $CD62L^{high}$ $CD4^+CD25^+$ regulatory T cells suppressed either $CD4^+$ or $CD8^+$ effector T cell functions, including cytokine production and cell proliferation. Accordingly, we checked IFN-γ secretion from $CD62L^{high}$ T cells and $CD62L^{low}$ T cells.

The exposure of natural regulatory T (Treg) cells to LPS induces the upregulation of activation markers on their cell surfaces, thereby enhancing the natural Treg cell survival and proliferation.[23] Whether bacterial pathogens can trigger the production of cytokines favoring the recruitment of natural Treg cells therefore remains to be determined. Our data suggested that IFN-γ production by $CD62L^{high}$ T cells population tended to be lower than that by $CD62L^{low}$ T cell population, which seemed to indicate that Treg cells thus play a role in the induction of ET.

Our findings would seem to indicate several limitations which merit consideration. $CD8^+$T cells or $CD25^-$T cells were included in our $CD62L^{high}$ T cells, which may thus offset the regulatory functions. However, these cells could at least be separated into naïve or regulatory functioning T cells, suggesting that Treg cells induced by endotoxin are primed in the spleen during ET and that the balance between anti-bacterial effector cells and $CD62L^{high}CD4^+CD25^+$ Treg cells may therefore determine the outcome of inflammation. It might thus be possible to orchestrate an adaptive immune reaction by manipulating the balance of effector and regulatory T cells priming against gram-negative bacteria as well as with tolerant macrophages and monocytes. Further experiments are required to clarify the contribution of T cells to the induction of ET.

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