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Administration of an Antigen at a High Dose Generates Regulatory CD4⁺ T Cells Expressing CD95 Ligand and Secreting IL-4 in the Liver¹

Tomohiro Watanabe,* † Masaru Yoshida,* † Yasuhiko Shirai,* Masashi Yamori,* Hideo Yagita, ‡ Toshiyuki Itoh,* † Tsutomu Chiba, † Toru Kita,* and Yoshio Wakatsuki² *

Ags administered orally at a high dose are absorbed in immunogenic forms and perfuse the liver, which raises a question regarding the relevance of hepatic lymphocyte activation to the systemic hyporesponsiveness against the ingested Ag. Oral administration of 100 mg of OVA to the mice led to massive cell death of OVA-specific (KJ1-26⁺) CD4⁺ T cells by Fas-Fas ligand (FasL)-mediated apoptosis in the liver, which was associated with the emergence of hepatic KJ1-26⁺CD4⁺ T cells expressing FasL. Hepatic CD4⁺ T cells in OVA-fed mice secreted large amounts of IL-4, IL-10, and TGF-β₁, upon restimulation in vitro and inhibited T cell proliferation. Adoptive transfer of these hepatic CD4⁺ T cells to naïve mice and subsequent antigenic challenge led to suppression of T cell proliferation as well as IgG Ab responses to OVA; this effect was mostly abrogated by a blocking Ab to FasL. i.p. administration of an Ag at a high dose also generated hepatic CD4⁺ FasL⁺ T cells with similar cytokine profile as T cells activated by oral administration of Ags at a high dose. Finally, we did not see an increase in FasL⁺ cells in the hepatic CD4⁺Vβ8⁺ T cell subset of MRL/lpr/lpr mice given staphylococcal enterotoxin B, indicating the requirement for Fas-mediated signals. These hepatic CD4⁺FasL⁺ regulatory cells may explain the tolerogenic property of the liver and play roles in systemic hyporesponsiveness induced by an Ag administered at a high dose. *The Journal of Immunology, 2002, 168: 2188–2199.

The liver is perfused by the blood from the gastrointestinal tract and the systemic circulation draining into hepatic sinusoids via the portal vein and the hepatic artery, respectively. Therefore, antigenic materials absorbed in the intestine may potentially activate hepatic lymphocytes by being delivered to the liver by portal blood flow. The physiological relevance of this lymphocyte activation in the liver to the systemic hyporesponsiveness against the Ags absorbed in the intestine is not well understood. In the liver, sinusoids contain the fenestrated endothelium with large cytoplasmic gaps, which may facilitate maximal contact by T cells with Ag-presenting dendritic cells and endothelial cells (1–4). These anatomical features suggest that the liver may serve as a lymphoid organ wherein lymphocytes are activated by Ags circulating in the portal and arterial blood flow. In fact, administration of donor cells via the portal vein primes the recipient by the donor-Ags and leads to donor-Ag specific hyporesponsiveness (5–8). Furthermore, the liver itself is such a tolerogenic organ that transplantation of an allogeneic liver sometimes requires little or no immunosuppressive therapy. Recent clinical trials in patients with insulin-dependent diabetes mellitus have revealed that islet transplantation via the portal vein can result in good metabolic control due to the long survival of transplanted cells in the liver (9). Thus, the liver may be closely associated with peripheral immune tolerance against cellular and noncellular Ags that circulate throughout the liver via the portal vein.

Oral administration of Ags has been recognized as a method to prevent or delay the onset of disease associated with immune responses to self and non-self Ags (10). In fact, oral administration of surrogate autoantigens has been applied to the treatment of rheumatoid arthritis and multiple sclerosis (11, 12). The induction mechanism involved in the oral tolerances depends on the dose of Ags administered. Thus, administration of Ags at a low dose leads to the emergence of T cells producing suppressive cytokines, whereas a high dose leads to clonal anergy and deletion of T cells (10, 13–15). However, in the latter case where a part of the Ag is absorbed in an immunogenic form, it is still largely unknown whether generation of suppressor cells associates with clonal deletion or how T cells residing outside of gut-associated lymphoid tissue (GALT) are involved in the induction of tolerance. If other organs besides GALT are involved in the induction and maintenance of high dose tolerance, one such candidate could be the liver for the anatomical and immunological reasons stated above. In support of this idea, animals with a porta-caval shunt show enhanced delayed-type hypersensitivity (DTH) responses to ingested Ags (16). In a similar context, Ab titers to intestinal flora are usually elevated in patients with chronic liver disease or porta-caval shunts (17). Furthermore, administration of donor cells via the portal vein leads to cytokine production in the liver and induction of tolerance (18, 19). Moreover, liver sinusoidal endothelial cells can take up circulating Ags and mediate Ag-specific tolerance (20). Based on these findings, we asked whether Ags administered orally at a high dose would activate Ag-specific T cells in the liver.

¹ Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; DTH, delayed-type hypersensitivity; FasL, Fas ligand; IHL, intrahepatic lymphocyte; KO, knockout; PI, propidium iodide; PP, Peyer’s patch; SEB, staphylococcal enterotoxin B; Tg, transgenic.

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and how this cell activation leads to events that may regulate systemic immune responses. In the present study we report that oral administration of Ags at a high dose leads to the emergence of CD4+ T cells (in the presence of IAα) (DO11.10) were administered 100 mg of OVA (Sigma-Aldrich, Germany). In the liver, spleen, and PP, double-positive (CD4+ CD8+) T cells were <1% (confirmed by flow cytometric analysis). Purified CD4+ T cells (5 × 10⁵/animal) were cultured for 3 days with 1 μg/ml OVA 323-339 peptide in the presence of irradiated splenic APC (2.5 × 10⁵/well) from BALB/c mice in U-bottom 96-well culture plates (Sumitomo Bakelite, Tokyo, Japan). For cytokine assays, the culture supernatants were collected at 48 h for IFN-γ and IL-2 and at 72 h for IL-4, IL-10, and TGF-β1. Proliferation and cytokine production were evaluated in triplicate assay.

**Cytokine analysis**

The concentrations of IL-2 and IL-4 were determined using the IL-2-dependent CTLL-2 and IL-4-dependent CT.4S cell lines, respectively (29, 30). Standards for these assays consisted of human IL-2 (Takeda Pharmaceutical, Osaka, Japan) and murine IL-4 (PeproTech, London, U.K.), respectively. The concentrations of IL-10 and IFN-γ were measured by sandwich ELISA as described previously (31). Pared Abs (capture and secondary biotin-conjugated) and recombinant cytokines were purchased from BD Pharmingen and PeproTech, respectively. Color was developed by adding a substrate solution (Sigma 104; Sigma-Aldrich) after incubating with alkaline phosphatase-labeled anti-biotin Ab (Zymed Laboratories, San Francisco, CA). The OD was measured at 405 nm with a microplate reader (SOFT max; Molecular Devices, Sunnyvale, CA). TGF-β1 was measured using an ELISA kit (Promega, Madison, WI).

**IL-4 assay of hepatic KJ1-26 Fasl−/− cells and KJ1-26 Fasl+ cells**

Hepatic KJ1-26 Fasl−/− cells and KJ1-26 Fasl+ cells were purified by anti-FITC Multisort kit (Miltenyi Biotec). Briefly, IHL from DO11.10 mice fed OVA were stained with FITC-conjugated KJ1-26 mAb and biotin-conjugated anti-Fasl mAb, then incubated with anti-FITC microbeads, followed by positive selection. After cleaving off the beads by release and stop solution, purified KJ1-26−/− cells were incubated with streptavidin microbeads for separation of KJ1-26−/− Fasl−/− cells and KJ1-26−/− Fasl+ cells. The purity of each population was >90% (confirmed by flow cytometric analysis). Purified KJ1-26−/− Fasl−/− cells and KJ1-26 Fasl+ cells (2.5 × 10⁵/well) were stimulated in vitro by plate-bound anti-CD3 mAb (10 μg/ml; 2C11; BD Pharmingen) for IL-4 analysis.

**Detection of suppressor activity in vitro**

Suppressor activity of CD4+ T cells was determined by a coculture assay as described below. The responder cells were splenic CD4+ T cells (5 × 10⁵/well) from 3-wk-old naive DO11.10 mice and were stimulated by 1 μg/ml OVA peptide 323–339 presented by irradiated splenic APC from BALB/c mice (2.5 × 10⁵/well) in U-bottom 96-well plates. To this culture were added the graded dose of test cells (modulator cells), which consisted of CD4+ T cells purified from DO11.10 mice immunized orally five times and then irradiated before the culture. The cultures were incubated for 72 h, and 1 μCi of [3H]thymidine was added for the final 16 h. Cells were harvested using a multiple cell harvester, and incorporated thymidine was determined using a Topcount microplate scintillation counter (Packard Instrument, Meriden, CT). Cultures were also prepared using soluble anti-CD3 mAb (1 μg/ml) instead of antigenic peptide to examine the suppressor activity of hepatic CD4+ T cells against Ag-nonspecific T cell proliferation. We used neutralizing Abs to IL-4 (11B11; BD Pharmingen), IL-10 (JES5-2A5; BD Pharmingen), TGF-β1 (1D11; R&D Systems, Minneapolis, MN), and Fasl (MFL4) for T cell proliferation in this coculture assay. As controls, rat IgG1 (BD Pharmingen), mouse IgG1 (BD Pharmingen), and hamster IgG (ICN) were used.

**Measurement of cytotoxicity of hepatic CD4+ T cells**

A previously described protocol was used to measure the cytotoxicity of hepatic CD4+ T cells (32). Jurkat T cells were labeled with 10 μCi/ml [3H]thymidine and served as target cells. Effector T cells were hepatic
FIGURE 1. Clonal deletion of hepatic Ag-specific CD4+ T cells by apoptosis in DO11.10 mice after administration of OVA. A, The proportion of CD4+ KJ1-26+ T cells. B, The percentage of annexin V+ cells in KJ1-26+ T cells. DO11.10 mice were fed 100 mg of OVA or PBS every other day for a total of five times, and then mononuclear cells were prepared from the liver, spleen, and PP 3 days after the fifth feeding. A, Cells were stained with FITC-conjugated KJ1-26 mAb and PE-conjugated anti-CD4 mAb. Dead cells were excluded by PI staining. The number in each panel shows the percentage of CD4+ KJ1-26+ T cells. B, Cells were stained with PE-conjugated KJ1-26 mAb, and annexin V binding assay was performed using an annexin V apoptosis detection kit. Percentages of annexin V+ cells in the KJ1-26+ gate were expressed as the mean ± SD. *, p < 0.05 vs PBS control in each organ. The results shown are representative of three independent experiments.

CD4+ T cells purified from DO11.10 mice fed OVA or PBS and were combined with labeled target cells (5 × 10^3/well) at different target:effector ratios. Eight hours later, labeled unfragmented DNA with high m.w. was harvested onto glass-fiber filters and radioactivity was measured. Results are expressed as the percentage of specific cytotoxicity and calculated as ((cpm without effector cells − cpm with effector cells)/cpm without effector cells) × 100. We used neutralizing Ab to FasL (MFL4) in this cytotoxicity assay. As a control, hamster IgG (ICN) was used.

Gated on KJ 1-26+ cells

Feeding frequency

FIGURE 2. CD25 and FasL expression after oral administration of OVA. DO11.10 mice were fed 100 mg of OVA every other day for a total of five times. At the indicated time points, mice were killed, and then mononuclear cells were prepared from liver, spleen, and PP. An analysis gate was set on the KJ1-26+ and PI-negative cells. Cells were stained with PE-conjugated KJ1-26 mAb, FITC-conjugated anti-CD25 mAb, and biotinylated anti-FasL mAb followed by streptavidin-RED670. The number in each panel shows the percentage of CD25+FasL+ T cells. The results shown are representative of two independent experiments.
Evaluation of tolerogenicity by adoptive transfer of CD4+ T cells to naive BALB/c mice and antigenic challenge

To determine in vivo functions, hepatic CD4+ T cells were transferred to BALB/c recipient mice that were subsequently immunized with OVA. Six-week-old naive male BALB/c mice were injected i.p. with 1 × 10^6 CD4+ T cells purified from the PP, spleen, and liver of DO11.10 mice orally immunized with 100 mg of OVA or PBS five times and killed 3 days after the fifth feeding. The next day after the transfer, the recipient mice were immunized s.c. with 100 μg of OVA in complete CFA (Life Technologies), followed by boost-immunization with 100 μg of OVA in IFA s.c. 1 wk later. DTH responses, T cell proliferation, cytokine production, and OVA-specific Ab responses were measured 7 days after the booster immunization.

Proliferative responses and cytokine production of splenic CD4+ T cells in recipient mice were measured by stimulation with OVA (0.1 mg/ml) or soluble anti-CD3 mAb (1 μg/ml) in the presence of splenic APC. OVA-specific Ab was measured by ELISA as described previously (33). Pooled immune sera obtained from BALB/c mice were used as a control, and the Ab titer equivalent to a 2^10 dilution of immune sera was defined as 1 arbitrary unit. DTH responses were performed as previously described (34). Footpad swelling was measured 48 h later with a dial thickness gauge (Ozaki MFG, Tokyo, Japan).

Administration of anti-mouse FasL mAb to recipient BALB/c mice

BALB/c mice, which received hepatic CD4+ T cells from DO11.10 mice fed OVA, were injected i.p. with 0.5 mg of anti-mouse FasL mAb (MFL4) or control hamster IgG (ICN) every 3 days from the first day of s.c. immunization. These animals were immunized s.c. by OVA, and then proliferative responses of splenic CD4+ T cells and anti-OVA Ab responses were evaluated as described above.

Treatment of MRL wild-type and MRL/lpr/lpr mice with SEB

MRL+/- and MRL/lpr/lpr mice, 6–8 wk of age, were treated i.p. with 100 μg of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL) dissolved in PBS or with PBS alone. Mice were killed 3 days after treatment, and then IHL and spleen cells were analyzed by flow cytometry.

Statistical analysis

Student’s t test was used to evaluate the significance of the differences. Statistical analysis was performed with the StatView v.4.5 program (Abacus Concepts, Berkeley, CA). A value of p < 0.05 was regarded as statistically significant.

FIGURE 3. Fas/FasL expression (A) and percentage of annexin V+ cells (B) in KJ1-26+ T cells after oral administration of OVA accompanied by treatment with anti-FasL mAb. DO11.10 mice were fed 100 mg of OVA or PBS every other day for a total of five times. Mice were also injected i.p. with 0.5 mg of anti-FasL mAb (MFL4) every 3 days from the first day of oral immunization. Mice were killed 3 days after the fifth feeding, and then mononuclear cells were prepared from liver, spleen, and PP. A, Cells were stained with PE-conjugated KJ1-26 mAb, FITC-conjugated anti-Fas mAb, and biotinylated anti-FasL mAb, followed by streptavidin-RED670. An analysis gate was set on the KJ1-26 and PI-negative cells. B, Percentages of annexin V+ cells in the KJ1-26+ gate were determined as described in Fig. 1B and expressed as the mean ± SD. *, p < 0.05; **, p < 0.01 (vs PBS control in each organ). The results shown are representative of three independent experiments.
**Results**

**Cell death in the liver of mice administered OVA**

Administration of OVA (100 mg) for five times decreased the percentage of CD4⁺KJ1-26⁺ T cells in the liver and the PP of DO11.10 mice, which effect was not seen in the spleen (Fig. 1A). The number of total lymphocytes was slightly decreased in liver and PP after feeding of OVA (liver, 1.41 ± 0.49 × 10⁶/mouse to 1.20 ± 0.25 × 10⁶; PP, 2.85 ± 0.52 × 10⁶ to 2.23 ± 0.45 × 10⁶), whereas such a decrease was not seen in the spleen (78.8 ± 8.5 × 10⁶ to 78.9 ± 21.0 × 10⁶). The decrease in the percentage of CD4⁺KJ1-26⁺ T cells was associated with an increase in KJ1-26⁻ annexin V⁺ T cells in liver and PP (Fig. 1B). No significant changes in the percentage of annexin V-positive KJ1-26-negative cells were seen in either organ after oral administration of OVA (liver, 15.6 ± 6.4 vs 18.4 ± 2.1; spleen, 7.6 ± 1.9 vs 9.0 ± 2.3; PP, 8.8 ± 3.3 vs 12.1 ± 6.9). Taken together, these data indicated that the absolute number of CD4⁺KJ1-26⁺ T cells in the liver and PP had decreased by apoptotic cell death upon feeding of OVA. This decrease in the number of T cells was Ag specific, since there were no remarkable changes in the number of CD4⁺KJ1-26⁺ T cells in the mice fed keyhole limpet hemocyanin. Histological examination as well as a serum transaminase assay revealed no evidence of liver dysfunction in mice given OVA (data not shown).

**Emergence of Ag-specific T cells expressing CD25 and FasL in the liver**

To assess antigenic cell activation in the liver, we analyzed the expressions of CD44, CD25, and CD69 on CD4⁺KJ1-26⁺ T cells. Upon feeding of OVA, expression of CD44 and CD69 on CD4⁺KJ1-26⁺ T cells increased in all tissues examined (data not shown). Interestingly, after the fifth feeding of OVA, a large population of CD4⁺KJ1-26⁺CD25⁺ T cells appeared in the liver. Because annexin V-positive CD4⁺KJ1-26⁺ cells appears upon OVA feeding in the liver, we studied the time course of FasL and CD25 expression in KJ1-26⁺ cells. As shown in Fig. 2, a large population of KJ1-26⁺CD25⁺FasL⁺ cells appeared only in the liver 3 days after the fifth feeding of OVA. The emergence of KJ1-26⁺CD25⁺FasL⁺ cells in the liver was dependent on the frequency of OVA feeding. Thus, a significant increase in KJ1-26⁺CD25⁺FasL⁺ cells was first observed in the liver after the third feeding, and the proportion of these T cells further increased to 38.0% of KJ1-26⁺ cells after the fifth feeding. Interestingly, a considerable population of KJ1-26⁺CD25⁺FasL⁺ cells still persisted in the liver 14 days after the last feeding. This may indicate that the phenotype of CD25⁺FasL⁺ cells in hepatic KJ1-26⁺ T cells is stable for a considerable period, and maintenance of the population requires stimulation by OVA. In contrast, a significant

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**FIGURE 4.** Enhanced expression of FasL on Ag-specific CD4⁺ T cells associates with clonal deletion in the adoptive transfer model. Splenic CD4⁺KJ1-26⁺ T cells (2.5 × 10⁶/mouse) were adoptively transferred into BALB/c mice via the i.v. route. After 3 days, mice were fed PBS or 100 mg of OVA every other day for a total of five times. At the indicated time points, IHL and splenocytes were collected and stained with FITC-conjugated KJ1-26 mAb, PE-conjugated anti-CD4 mAb, and biotinylated anti-FasL mAb, followed by streptavidin-RED670. Dead cells were excluded by PI staining. The number in each panel shows the proportion of CD4⁺KJ1-26⁺ T cells or FasL⁺ cells gated on CD4⁺KJ1-26⁺ T cells. The results shown are representative of three independent experiments.
increase in KJ1-26<sup>CD25<sup>+</sup>FasL<sup>+</sup></sup> in spleen or PP was not detected at any of the time points studied despite repetitive stimulation by OVA.

The results indicative of cell death and FasL expression raised the question of Fas expression and Fas-FasL interaction in KJ1-26<sup>CD25<sup>+</sup></sup>/H11001 T cells in the liver. As shown in Fig. 3A, only in the liver did we see a large population of Fas<sup>+</sup> cells in KJ1-26<sup>+</sup> T cells. Feeding of OVA for five times led to the emergence of Fas<sup>+</sup>FasL<sup>high</sup> double-positive cells and FasL<sup>high</sup> single-positive cells in KJ1-26<sup>+</sup> T cells in the liver. The emergence of FasL<sup>high</sup> cells in the liver was abolished by treatment with a blocking Ab to FasL, and only Fas<sup>+</sup>FasL<sup>low</sup> T cells were increased. In contrast, the emergence of Fas<sup>+</sup>FasL<sup>high</sup> cells in the KJ1-26<sup>+</sup> T cell subset was almost negligible in spleen and PP, so that the effect by the Ab was undetectable in these organs. As shown in Fig. 3B, treatment with the blocking Ab reduced the proportion of annexin V-positive KJ1-26<sup>+</sup> T cells in liver. Thus, Fas and FasL expressed in KJ1-26<sup>+</sup> T cells were functional. Taken together, OVA feeding led to the emergence of KJ1-26<sup>+</sup>FasL<sup>+</sup> cells in the liver as a result of apoptosis of KJ1-26<sup>+</sup>Fas<sup>+</sup> cells, which was mediated by Fas-FasL interaction. Requirement of Fas-mediated signal is further studied using Fas-mutant mice as described below. As to the expression of CTLA-4 in KJ1-26<sup>CD25<sup>+</sup></sup> T cells, no remarkable changes were seen between mice fed OVA and mice fed PBS (data not shown).

Clonal deletion associates with emergence of CD4<sup>+</sup>KJ1-26<sup>+</sup>FasL<sup>+</sup> T cells in the liver of the mice adoptively transferred with KJ1-26<sup>+</sup> cells

To assess the possibility that high precursor T cell frequency in mice with a Tg background might have affected the antigenic cell activation and subsequent cell death seen in our previous studies, we repeated experiments using the adoptive cell transfer model described by Jenkins et al. (22). As shown in Fig. 4, clonal expansion of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells followed by deletion were observed in the liver of the recipient mice administered OVA, which was associated with an increase in FasL<sup>+</sup> cells in the hepatic CD4<sup>+</sup>KJ1-26<sup>+</sup> T cell subset. In contrast, the proportion of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells and FasL expression remained unchanged in the spleen of mice fed OVA. Thus, even under the condition of lower precursor frequency in the recipient mice, OVA feeding led to cell death of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells, which is coupled with emergence of FasL<sup>+</sup>KJ1-26<sup>+</sup> T cells in the liver.

FIGURE 5. Cytokine production by CD4<sup>+</sup> T cells in DO11.10 mice. A–E, Ag-specific cytokine production by CD4<sup>+</sup> T cells in DO11.10 mice administered orally OVA (■) or PBS (□) every other day for a total of five times. Mice were killed 3 days after the fifth administration. CD4<sup>+</sup> T cells (5 × 10<sup>4</sup>/well) were stimulated for 3 days in vitro with OVA peptide (1 μg/ml) presented by irradiated syngeneic spleen cells of non-Tg mice (2.5 × 10<sup>5</sup>/well). Culture supernatants were collected at 48 h for IL-2 (A) and IFN-γ (B) and at 72 h for IL-4 (C), IL-10 (D), and TGF-β<sub>1</sub> (E). *<i>p < 0.05;</i> **<i>p < 0.01</i> (vs PBS control in each organ). F, IL-4 secretion by hepatic T cell subset in mice fed OVA or PBS. The purified T cell subpopulation in the liver (2.5 × 10<sup>4</sup>/well) was stimulated for 3 days in vitro with plate-bound anti-CD3 mAb (10 μg/ml), and culture supernatants were analyzed for IL-4 production. *<i>p < 0.05;</i> **<i>p < 0.01</i> (vs PBS KJ-26<sup>+</sup> T cells). Results are expressed as the mean ± SD. The results shown are representative of three independent experiments.
Cytokine production by hepatic CD4⁺ T cells

We next assessed the functional alteration of OVA-specific CD4⁺ T cells that survived apoptosis in the liver. Hepatic CD4⁺ T cells displayed a marked decrease in IL-2 secretion in response to stimulation by OVA peptide (Fig. 5A). No significant change in IFN-γ secretion was seen with hepatic CD4⁺ T cells after administration of OVA (Fig. 5B). To the contrary, IL-4 secretion by hepatic CD4⁺ T cells was remarkably augmented (Fig. 5C). In contrast to hepatic CD4⁺ T cells, IL-4 secretion by PP CD4⁺ T cells was unchanged, and that by splenic CD4⁺ T cells was even decreased. The secretion of IL-10 and TGF-β₁ by CD4⁺ T cells was increased by feeding of OVA in all tissues studied (Fig. 5, D and E). Thus, the most striking finding in the cytokine study was that IL-4 secretion by hepatic CD4⁺ T cells was enhanced in mice fed OVA.

To exclude the possibility that T cells expressing an endogenous TCR might be involved in the functional alteration, we repeated the same experiment using Rag2 KO/DO11.10 mice. We confirmed that feeding of OVA suppressed IL-2 secretion (56.9 ± 7.9 vs 18.5 ± 11.9 Japan reference unit/ml; p < 0.05) and enhanced the secretion of IL-4 (159 ± 9 vs 696 ± 157 pg/ml; p < 0.01), IL-10 (350 ± 170 vs 1560 ± 110 pg/ml; p < 0.01), and TGF-β₁ (75.4 ± 12.2 vs 194 ± 46.8 pg/ml; p < 0.05) by hepatic CD4⁺ T cells stimulated with OVA peptide. Moreover, by sorting cells, we identified the IL-4 high producer cells with the hepatic KJ1-26⁺ FasL⁺ T cell subset in mice fed OVA (Fig. 5F). Taken together, OVA feeding led to the development of OVA-specific T cells secreting immunosuppressive lymphokines in the liver.

In vitro suppressor function of hepatic CD4⁺ T cells

In the next series of experiments we tested the regulatory function of CD4⁺ T cells that developed in liver after OVA feeding. As shown in Fig. 6A, hepatic CD4⁺ T cells obtained after OVA feeding suppressed OVA-specific proliferation by naive CD4⁺ T cells in the coculture. In contrast, PP or splenic CD4⁺ T cells did not show significant suppression (data not shown). Hepatic CD4⁺ T cells also inhibited the proliferation by anti-CD3-stimulated splenic CD4⁺ T cells (Fig. 6B). Thus, feeding of an Ag generated CD4⁺ T cells with Ag-nonspecific suppressor activity in the liver.

We next determined whether this suppressor activity of hepatic CD4⁺ T cells is mediated by a Fas/FasL interaction or by secretion of suppressive cytokines such as IL-4, IL-10, and TGF-β₁. Blockade of the Fas/FasL interaction by an Ab abrogated most of the suppressor activity of hepatic CD4⁺ T cells in the coculture (Fig. 6). In contrast, restoration of the proliferative response by treatment with anti-IL-4, anti-IL-10, and anti-TGF-β₁ was far less effective than that by anti-FasL Ab at any dose examined (1, 10, and 100 μg/ml). Thus, FasL appeared to play a predominant role in mediating the suppressor activity of hepatic CD4⁺ T cells developed in the mice fed OVA.

Killing activity of hepatic CD4⁺ T cells

As shown in Fig. 7, hepatic CD4⁺ T cells purified from mice fed OVA demonstrated a dose-dependent cytotoxicity against Fas⁺ Jurkat T cells, which was not seen with the hepatic CD4⁺ T cells purified from control mice fed PBS. Blockade of the Fas/FasL

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FIGURE 6. Immunosuppressive activity of hepatic CD4⁺ T cells is mediated by FasL. Splenic CD4⁺ T cells from nonimmunized DO11.10 mice (responder cells; 5 × 10⁴/well) were stimulated in vitro with 1 μg/ml OVA peptide (A) or soluble anti-CD3 mAb (B) in the presence of irradiated syngeneic spleen cells from non-Tg mice (2.5 × 10⁵/well). To these cultures we added hepatic CD4⁺ T cells from DO11.10 mice fed PBS or OVA (modulator cells; 1.25 × 10⁵/well). Modulator cells were irradiated before use. Neutralizing Abs to FasL, IL-4, IL-10, or TGF-β₁ (1, 10, or 100 μg/ml) were added to the culture of CD4⁺ T cells stimulated by OVA peptide or anti-CD3. The cultures were incubated for 72 h, and 1 μCi of [³H]thymidine was added for the final 16 h. The results are expressed as the mean ± SD, and proliferation was evaluated in triplicate. **, p < 0.01; *** p < 0.001 (compared with proliferation of 100 mg of OVA-treated liver/control Ab). Baseline proliferation in control medium was always <500 cpm. The results shown are representative of three independent experiments.
interaction by anti-FasL mAb inhibited the cytotoxicity of hepatic CD4+ T cells. Thus, FasL expressed in the hepatic CD4+ T cells is functional and mediates killing activity.

In vivo suppressor activity by hepatic CD4+ T cells

In the next series of experiments we tested in vivo regulatory activity of hepatic CD4+ T cells developed in mice fed OVA. For this purpose, donor DO11.10 mice were administered 100 mg of OVA or PBS every other day for a total of five times. We then transferred the same dose of CD4+ T cells taken from the PP, spleen, or liver to naive BALB/c recipients, which were subsequently immunized s.c. with OVA/CFA. As shown in Fig. 8A, the recipient mice showed significant reductions in anti-OVA IgG response only when they received hepatic CD4+ T cells of mice fed OVA. Moreover, anti-CD3-stimulated proliferation by splenic CD4+ T cells was significantly suppressed in the mice transferred with hepatic CD4+ T cells of mice fed OVA (Fig. 8B). DTH responses and splenic T cell proliferation specific to OVA were also significantly reduced in the recipient mice transferred with hepatic CD4+ T cells from mice fed OVA (data not shown), which was associated with a decrease in IFN-γ (Fig. 8C). In contrast, neither PP nor splenic CD4+ T cells transferred suppressor activity to recipient mice. As shown in Fig. 8, D and E, anti-FasL treatment abrogated most of the suppressor activity of hepatic CD4+ T cells in both Ab responses to OVA and proliferative responses to anti-CD3 (Fig. 8E) and OVA (data not shown), which was not seen with a control Ab. Taken together, hepatic CD4+ T cells from mice fed Ag at a high dose have suppressor activity in vivo, which was mostly mediated by FasL.

Generation of suppressor cells in the liver does not depend on GALT

To determine whether the development of KJ1-26+CD25+FasL+ T cells in the liver requires exposure to an Ag in the intestine, DO11.10 mice were injected i.p. with 1 mg of OVA every other day for a total of five times. As shown in Fig. 9A, i.p. administration of OVA also generated KJ1-26+CD25+FasL+ T cells in liver, which was not seen in PP or spleen. Upon antigenic stimulation in vitro, the secretion of IL-2 and IFN-γ by hepatic CD4+ T cells was remarkably suppressed in the mice administered i.p. of OVA (Fig. 9, B and C). In contrast, i.p. administration of OVA enhanced the secretion of IL-4, IL-10, and TGF-β1 by hepatic CD4+ T cells (Fig. 9, D–F). Thus, generation of CD4+CD25+FasL+ T cells producing Th2-type cytokines in the liver does not require exposure to Ags in the intestine.

Generation of hepatic CD4+ FasL+ T cells requires Fas expression

We showed that CD4+FasL+ T cells were selectively generated upon administration of OVA in the liver of DO11.10 mice and in the mice adoptively transferred with KJ1-26+ T cells. In the final series of experiments, we determined whether Fas-mediated stimulation is required for the generation of CD4+FasL+ T cells in the liver of mice with a non-Tg background. For this purpose, MRL wild-type (+/+ ) and MRL/lpr/lpr mice were treated i.p. with SEB. Three days after SEB treatment, the proportion of PI-positive CD4+Vβ8+ T cells had increased in the liver and spleen of MRL+/+ mice, indicating the activation-induced cell death of CD4+Vβ8+ cells. In contrast, the proportion of PI-positive CD4+Vβ8+ T cells remained unchanged in the liver and spleen of MRL/lpr/lpr mice treated with SEB (data not shown). This cell death in the CD4+Vβ8+ population was associated with emergence of cells coexpressing Fas and FasL in the Vβ8+ subset in the liver of MRL+/+ mice (Fig. 10A). Furthermore, SEB treatment increased the percentages of cells expressing FasL and CD25 in the CD4+Vβ8+ subset in both the liver and spleen of MRL+/+ mice (Fig. 10B). Interestingly, a significant increase in FasL- and CD25-positive cells in the CD4+Vβ8+ subset was seen in the liver of MRL+/+ mice treated with SEB, whereas such an increase was not seen in the liver of MRL/lpr/lpr mice treated with SEB. Thus, administration of SEB increases FasL+ cells in the CD4+ T cell subset reactive to SEB in the liver of non-Tg mice as a result of Fas-mediated cell death in the liver.

Discussion

In this study we demonstrated that the feeding of an Ag at a high dose can activate Ag-specific T cells in the liver and concomitantly induce two pathways known to be involved in the induction of peripheral tolerance, i.e., clonal deletion of Ag-specific T cells and generation of Ag-nonspecific regulatory CD4+ T cells expressing FasLhigh and secreting IL-4, IL-10, and TGF-β1. We showed that the Fas/FasL interaction is required for the generation of CD4+FasLhigh cells in the liver and for the effector mechanism of the suppression in vivo.

As for the origin of the regulatory T cells described in this study, we postulate that two pathways are possible. First, these regulatory T cells may be generated in situ in the liver by antigenic activation.
FIGURE 8. FasL mediates suppressor activity of hepatic CD4+ T cells in vivo. A–C, Donor DO11.10 mice were fed 100 mg of OVA or PBS every other day for a total of five times and were killed 3 days after the fifth feeding. CD4+ T cells (1 x 10^6/mouse) in liver, PP, and spleen of donor mice were purified and transferred to recipient BALB/c mice. Recipient mice were immunized and boosted s.c. with 100 μg of OVA. Ab responses (A), proliferative responses (B), and IFN-γ secretion (C) by splenic CD4+ T cells of the recipient mice were measured 2 wk after the first immunization. Splenic CD4+ T cells (1 x 10^5/well) of the recipient mice were stimulated by soluble anti-CD3 mAb (1 μg/ml) in the presence of irradiated syngeneic spleen cells (5 x 10^5/well) from non-Tg mice. A, Pooled immune sera obtained from BALB/c mice were used as a control, and the Ab titer equivalent to a 2^{10} dilution of immune sera was defined as 1 arbitrary unit. B, For proliferative responses, the cultures were incubated for 72 h, and 1 μCi of [3H]thymidine was added for the final 16 h. For analysis of IFN-γ production, culture supernatants were collected at 48 h (C). *, p < 0.05; **, p < 0.01 vs PBS control in each organ. D and E, Hepatic CD4+ T cells (1 x 10^6/mouse) from donor DO11.10 mice were purified and transferred to recipient BALB/c mice, followed by immunization as described above. Mice transferred with hepatic CD4+ T cells were injected i.p. with 0.5 mg of anti-mouse FasL mAb (MFL4) or control hamster IgG every 3 days from the first day of immunization. Ab responses and proliferative responses of splenic CD4+ T cells in the recipient mice were measured as described above. **, p < 0.01. The results are expressed as the mean ± SD.

by hepatic APC. In fact, hepatic sinusoidal endothelial cells and dendritic cells can present Ags (3, 4, 35). Alternatively, these regulatory T cells can develop in GALT (14) and then migrate to the liver. However, i.p. injection of OVA led to the emergence KJ1-26"FasL+" T cells secreting IL-4/IL-10/TGF-β1, and i.p. injection of SEB led to the emergence of CD4"Vα8" cells expressing FasL in MRL+/+ mice. Moreover, CD4"KJ1-26"CD25"FasL^{high} T cells were not seen in PP or spleen during the feeding of OVA from the first through the third time, during which period a considerable population of CD4"KJ1-26"CD25"FasL^{high} T cells appeared in the liver. Thus, CD4"KJ1-26"CD25"FasL^{high} T cells in the liver are not likely to migrate from PP or spleen to liver.

Using MHC class I-restricted TCR Tg mice, Crispe et al. (36, 37) reported that systemic administration of an antigenic peptide resulted in the deletion of peripheral T cells specific to the Ag and that activated CD8+ T cells are selectively trapped in the liver and eliminated by apoptosis. In the present study the proportion of annexin V-positive OVA-specific T cells increased after OVA feeding, suggesting that the liver is also the site for cell death of class II-restricted T cells. Thus, antigenic activation can lead to the death of Ag-specific T cells in the liver for both class I and class II-restricted T cells regardless of the route of Ag administration. However, in the case of class II-restricted CD4+ T cells, the liver can have a 2-fold role in immune regulation, since activation by the Ag at a high dose not only eliminated Ag-specific T cells but also produced regulatory CD4+ T cells expressing FasL^{high} in the liver.

Peripheral hyporesponsiveness can be achieved by generation of CD4+ T cells secreting anti-inflammatory cytokines. In this regard, the enhanced secretion of anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β1 by hepatic CD4+ T cells should be noted. The profile of the lymphokines produced by hepatic CD4+ T cells was similar to that of T cells in GALT after administration of an Ag at a low dose (13, 14). Thus, although we do not exclude the possibility that suppressor function by hepatic CD4+ FasL^{high} T cells in the recipient mice was mediated by cytokines, FasL appears to play a major role in the effector phase. In fact, DTH was suppressed in the recipient mice that received hepatic CD4+ T
cells obtained from IL-4 KO/DO11.10 mice administered OVA (T.
Watanabe and Y. Wakatsuki, unpublished observations). How-
ever, enhanced secretion of IL-4 by hepatic CD4⁺ T cells might
have still contributed to the suppressor effect, because it is reported
that IL-4 increases cell surface expression of FasL on CD4⁺ T
cells and enhances FasL-mediated cytotoxicity in vivo (38). In
addition, it has been reported that Th1-type, but not Th2-type, cells
preponderantly undergo Fas/FasL-mediated activation-induced
cell death upon antigenic activation or by anti-CD3 stimulation
(39, 40). Compatible to these, Vella et al. (41) reported that the
survival of T cells is promoted by IL-4 via the expression of Bcl-2
and Bcl-xL. Furthermore, KJ1-26⁺FasL⁺ T cells produce more
IL-4 than KJ1-26⁺FasL⁻ cells in the liver of mice administered
OVA. Collectively, we postulate that antigenic activation of CD4⁺
T cells in the liver may preferentially delete T cells that express
Fas and lack IL-4 production by a death signal provided by FasL
expressed on IL-4-producing cells. This fratricidal mechanism
cells secreting IL-4, IL-10, and TGF-β₁. A, CD25 and FasL expression
in KJ1-26⁺ T cells after i.p. administration of OVA. DO11.10 mice were injected i.p. with 1 mg of OVA or PBS every other day for a total of five times, and then mononuclear cells were prepared from the liver, spleen, and PP 3 days after the fifth injection. Cells were stained as described in Fig. 2. Dead cells were excluded by PI staining. The number in each panel shows the percentage of FasL⁺ CD25⁺ T cells. B–F, Ag-specific cytokine production by CD4⁺ T cells in DO11.10 mice administered i.p. OVA or PBS. CD4⁺ T cells (5 × 10⁴/well) were stimulated as described in Fig. 5. Culture supernatants were collected at 48 h for IL-2 (B) and IFN-γ (C) and at 72 h for IL-4 (D), IL-10 (E), and TGF-β₁ (F). Results are expressed as the mean ± SD. The results shown are representative of two (A) or three (B–F) independent experiments. *, p < 0.05; **, p < 0.01 (vs PBS control in each organ).

FIGURE 9. Intraperitoneal administration of OVA at a high dose also generated CD4⁺CD25⁺FasL⁺ T cells secreting IL-4, IL-10, and TGF-β₁. A, CD25 and FasL expression in KJ1-26⁺ T cells after i.p. administration of OVA. DO11.10 mice were injected i.p. with 1 mg of OVA or PBS every other day for a total of five times, and then mononuclear cells were prepared from the liver, spleen, and PP 3 days after the fifth injection. Cells were stained as described in Fig. 2. Dead cells were excluded by PI staining. The number in each panel shows the percentage of FasL⁺ CD25⁺ T cells. B–F, Ag-specific cytokine production by CD4⁺ T cells in DO11.10 mice administered i.p. OVA or PBS. CD4⁺ T cells (5 × 10⁴/well) were stimulated as described in Fig. 5. Culture supernatants were collected at 48 h for IL-2 (B) and IFN-γ (C) and at 72 h for IL-4 (D), IL-10 (E), and TGF-β₁ (F). Results are expressed as the mean ± SD. The results shown are representative of two (A) or three (B–F) independent experiments. *, p < 0.05; **, p < 0.01 (vs PBS control in each organ).
cells and survival of Th2 cells in the liver; both CD4+ T cells share the same clonotype as KJ1-26.

Although a massive reduction in the number of Ag-specific T cells was a common feature of CD4+ T cells in PP and liver, both in vitro and in vivo studies have indicated that suppressor activity was much stronger with hepatic CD4+ T cells than with PP CD4+ T cells, probably due to the high expression of FasL in the former. The reason why we did not see a significant increase in FasL+ cells in PP CD4+ T cells could be explained by the fact that FasL+ CD4+ T cells appear at a much earlier time point after antigenic exposure and express lower levels of FasL in PP CD4+ T cells than hepatic CD4+ T cells, probably due to the high expression of FasL in the former. The reason why we did not see a significant increase in FasL+ T cells in PP CD4+ KJ1-26+ cells could be explained by the fact that FasL+ CD4+ T cells appear at a much earlier time point after antigenic exposure and express lower levels of FasL in PP CD4+ T cells than hepatic CD4+ T cells. As shown in Fig. 2, FasL expression in KJ1-26+ T cells in PP showed a peak expression 3 days after the first feeding. Similar to the results reported by Chen et al. (15), massive cell death of KJ1-26+ PP T cells was observed after the first feeding of OVA in our study (T. Watanabe and Y. Wakatsuki, unpublished observations). Thus, the generation mechanism of CD4+ FasL+ T cells appears to be different between PP and the liver.

In this study we found that administration of Ag at a high dose produced Ag-specific CD4+ FasL+ cells in the liver of mice with various backgrounds: DO11.10 mice, BALB/c mice adoptively transferred with KJ1-26+ T cells, and MRL+/- mice. Especially the result obtained in the MRL+/- mice has a 3-fold meaning. First, Fas-mediated stimulation is required to generate CD4+ FasL+ cells in the liver. This was corroborated by the experiment using anti-FasL Ab in DO11.10 mice and the one performed in Fas mutant MRL mice. Second, generation of CD4+ FasL+ cells in liver does not require oral administration of Ags. This idea is also compatible to the results of i.p. injection of OVA in DO11.10 mice. Third, this phenomenon can occur in mice with T cells not bearing Tg TCR.

Bonfoco et al. (42) reported that i.v. administration of SEB induced a marked up-regulation of FasL in IHL, which, in turn, killed activated lymphocytes, leading to peripheral T cell deletion. We speculate that Ag-specific CD4+ FasL+ cells generated in the liver could deliver a death signal at two stages: one in the effector phase to Fas-positive activated T cells by an Ag-nonspecific manner, and the other in the induction phase to Fas-positive APC interacting with Ag-specific T cells by cognate interaction. Thereby, the overall effects by CD4+ FasL+ cells may contribute to the maintenance as well as the induction of peripheral tolerance.

NKT cells exist in a high percentage in the liver of C57BL/6 mice (43), which deserves our attention here. In fact, Ilan et al. (44) reported that hepatic NKT cells play a substantial role in the induction of oral tolerance in experimental colitis model using C57BL/6 mice. NKT cells are characterized by the invariant Ag receptor coded by Vα14Jα281 gene segments (45). However, hepatic NKT cells are not likely to play a crucial role in our system.
since the proportions of KJ1-26 +/Vα14 + T cells and KJ1-26+/CD122 + T cells were unchanged in the liver between mice fed OVA and mice fed PBS (KJ1-26+/Vα14 +, 1.4 vs 1.1%; KJ1-26+/CD122 +, 2.6 vs 2.9%). Finally, we consider that studies on the emigration of CD4 +/Fas(high) + T cells from the liver to the periphery would consolidate the role played by the liver in the induction and maintenance of orally induced systemic hyporesponsiveness.

In conclusion, our study provides a new concept that deletion of Ag-specific T cells and induction of regulatory T cells are concomitant events in the liver after encountering an Ag at a high dose. A hepatic CD4 + T cell subset expressing Fas(high) and secreting IL-4/IL-10/TGF-β1, the development of which is driven by Ag exposure and mediated by Fas/FasL interaction, may play a regulatory role and confer a tolerogenic property on the liver.

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