Oral ingestion of *Lentinula edodes* mycelia extract can restore the antitumor T cell response of mice inoculated with colon-26 cells into the subserosal space of the cecum

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Abstract. We previously reported that oral ingestion of *Lentinula edodes* mycelia (L.E.M.) extract can inhibit the growth of a subcutaneously established melanoma in a T cell-dependent manner via mitigation of regulatory T cell (Treg)-mediated immunosuppression. In this study, we tested the antitumor effect and mechanism of oral ingestion of L.E.M. extract following inoculation of murine colon carcinoma colon-26 (C26) cells into the subserosal space of the cecum (i.c.) of syngeneic mice. In this model, the primary site of the immune response was gut-associated lymphoid tissue (GALT), which is known to be an immunological tolerance-inducing site for numerous dietary antigens. Oral ingestion of the L.E.M. extract suppressed the growth of i.c.-inoculated C26 cells in a T cell-dependent manner and restored the T cell response of the mesenteric lymph nodes and the spleen, not only to a tumor antigen-derived peptide, presented on H-2L^d molecules, but also to C26 cells. I.c. inoculation of C26 cells increased the potential of CD4$^+$ T cells of the mesenteric lymph nodes to produce transforming growth factor (TGF)-β, but ingestion of the L.E.M. extract decreased the ability of both CD4$^+$ and CD8$^+$ T cells in the mesenteric lymph nodes to produce this immunosuppressive cytokine. Although ingestion of L.E.M. showed only a marginal effect on Tregs in this model, this treatment significantly reduced the plasma levels of TGF-β and IL-6, both of which were increased in the i.c. C26-inoculated mice. In summary, our results indicate that oral ingestion of L.E.M. extract can restore antitumor T cell responses of mice even when the primary antitumor immune response is elicited in GALT, and provide important implications for anticancer immunotherapy of human colon cancer.

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Introduction

Studies on tumor-reactive cytotoxic T lymphocytes (CTLs) and tumor-related antigens have enabled the design of specific anticancer immunotherapies (1,2). Anticancer vaccines and adoptive immunotherapy have been applied clinically, although their efficacy has been unsatisfactory (3). Although several explanations of their unsatisfactory performance could be proposed, the major reason that these therapies fail is believed to be due to the emergence of immunosuppressive cells, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (4,5). In addition, the tumor-bearing state is accompanied by chronic inflammation, and it is thought that the inflammatory state of cancer patients inhibits the efficacy of anticancer immunotherapy (6,7).

Lentinula edodes mycelia extract (L.E.M.) is a dried powder of a hot water extract of the mycelia of *L. edodes* before germination, which were cultured in a medium composed of bagasse and rice bran (8). This L.E.M. extract has been reported to exhibit antitumor activity and immunomodulatory effects both *in vitro* and *in vivo* (9,10). L.E.M. can mitigate inflammation in the liver of mice (11), suggesting that it also has an anti-inflammatory effect. In addition, we recently reported that oral ingestion of L.E.M. extract can inhibit the growth of a subcutaneously established B16 melanoma in a T cell-dependent manner via mitigation of Treg-mediated immunosuppression (12). However, the question remains as to how an orally ingested L.E.M. extract affects the immune system in tumor-bearing hosts. The first effect of an orally ingested L.E.M. extract is likely to be on gut-associated lymphoid tissue (GALT). Immune responses are usually suppressed in GALT by Treg in order to maintain oral tolerance (13,14). In addition, the antitumor T cell response has been reported to be impaired in GALT compared to non-GALT (15). Despite these reports, the antitumor effects of immunomodulators such as L.E.M. and the T cell response in GALT following their ingestion have not yet been fully elucidated.

In this study, we tested the antitumor effect of an oral ingestion of L.E.M. extract, and the mechanism of its effect, in a mouse model in which murine colon carcinoma colon-26 (C26) cells were inoculated into the subserosal space of the cecum (i.c.) of syngeneic mice. Ingestion of L.E.M. extract suppressed the growth of i.c.-inoculated C26 cells in a T cell-dependent manner, and restored tumor-specific T cell responses of the mesenteric lymph nodes (LNs) and the spleen. In addition, it appeared that restoration of the antitumor T cell response by ingestion of L.E.M. extract was due to decreases in the plasma levels of TGF-β and IL-6, both of which increased after i.c. inoculation of the C26 cells. Our results indicate that oral ingestion of L.E.M. extract can restore antitumor T cell responses in mice even when the primary antitumor immune response is elicited in GALT. These results have important implications for anticancer immunotherapy of human colon cancer.

Materials and methods

Mice. BALB/c (H-2d: 6 weeks old) and BALB/c nu/nu (H-2d: 6 weeks old) female mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) and were kept under a specific pathogen-free condition. Mice were used at 7 weeks of age for experiments. Experiments were performed according to the ethical guidelines for animal experiments of the Kobayashi Pharmaceutical Co., Ltd.

Tumor cell lines and reagents. C26 and RENCA are colon and renal cell carcinoma cell lines, respectively, of BALB/c mice origin. Both cell lines were maintained *in vitro* in RPMI-1640 medium (Sigma-Aldrich Japan, Tokyo, Japan), supplemented with 10% heat-inactivated FBS (Thermo Trace, Melbourne, Australia) and 1% penicillin-streptomycin (Wako Japan, Osaka, Japan). A dried powder of L.E.M. extract was prepared by extraction with hot water of cultured *L. edodes* mycelia before germination in medium composed of bagasse and rice bran, as previously reported (16).

Assay of in vivo antitumor effect. The antitumor effect of L.E.M. *in vivo* was assayed as previously reported (15). Briefly, the abdominal wall of each mouse was opened under anesthesia and C26 cells ($1x10^6$), in a volume of 50 μl, were inoculated into the i.c., followed by closure of the abdominal wall. Mesenteric LNs were used as the tumor-draining LNs. Sham-operated controls were mice that were inoculated with complete medium into the i.c. following the same operational procedure. After 3 days, the mice were freely fed food containing L.E.M. extract at a final concentration of 1 or 2% (w/w). On day 14, all mice were sacrificed and tumor weights were determined.

Assay of peptide-specific and C26-specific T cells. To test T cell responses against a C26-derived antigen, an H-$2L^d$-binding AH1 peptide (SPSYVYHQF) derived from an endogenous retroviral gene product in a murine colon tumor (17) was used. The measles virus hemagglutinin (M.V.H.) peptide (SPGRSFSYF) (18) was used as an H-$2L^d$-binding control peptide. Both peptides were purchased from PH Japan Co. Ltd. (Hiroshima, Japan), and were >90% pure. Spleen and mesenteric LN cells were harvested, pooled and stimulated *in vitro* with each of the indicated peptides in the presence of 20 U/ml IL-2 for 3 days at a cell dose of $5x10^5$ cells/well in 96-well flat plates. To test reactivity against cancer cells, spleen and tumor-draining LN cells were harvested, pooled and stimulated *in vitro* with C26 or RENCA in the presence of 20 U/ml IL-2 for 3 days at a cell dose of $5x10^5$ cells/well in 96-well flat plates. Cancer cells were inactivated by treatment with 100 μg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) for 90 min. The level of IFN-γ in the culture supernatants was determined using an ELISA kit (Invitrogen Japan, Tokyo, Japan).

Flow cytometric analysis. Spleen cells and tumor-draining LN cells were stained with a FITC-conjugated anti-mouse CD4 mAb (eBioscience, Kobe, Japan), and a PE-Cy5-conjugated anti-mouse/rat Foxp3 mAb (eBioscience), and were analyzed by FACS analysis using an EPICS flow cytometer (Beckman Coulter Japan, Tokyo, Japan).

Measurement of plasma TGF-β1 and IL-6. Peripheral blood was sampled using heparin to avoid contamination with platelet-derived TGF-β1. The plasma levels of TGF-β1 and IL-6 were determined using ELISAs.

Preparation of $CD4^+$ T cells and $CD8^+$ T cells of the mesenteric LNs. To examine the effect of L.E.M. ingestion on immunosuppression in GALT, mesenteric LN cells were collected from C26-bearing mice. Purified $CD4^+$ and $CD8^+$ T cells were negatively isolated using CD4 and CD8 T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. The whole LN cell population, $CD4^+$ T cells or $CD8^+$ T cells ($1x10^5$ cells) were cultured for 3 days without stimulation, and the level of TGF-β1 in the culture supernatants was determined using an ELISA.

Statistical analysis. Data were statistically evaluated using an unpaired two-tailed Student's t test. $P<0.05$ was considered statistically significant.

Results

Antitumor effect of oral ingestion of L.E.M. extract on i.c.-inoculated C-26 cells. In order to establish a mouse model in which GALT was the site of antitumor immune responses, BALB/c mice were inoculated into the i.c. with C26 cells. The effect of ingestion of L.E.M. extract was then analyzed by freely feeding the mice with food containing the L.E.M. extract at a final concentration of 1 or 2% (w/w). On day 14, all of the mice were sacrificed and the tumor weights were determined by weighing the ceca of the mice. As shown in Fig. 1A, the growth of the i.c.-inoculated C-26 tumor cells was significantly suppressed in mice that were fed with a 2% L.E.M. extract diet, compared to the growth of the untreated group. Representative photographs of the ceca of tumor-bearing mice are shown in Fig. 1B. Based on this result, subsequent experiments were performed using a 2% L.E.M. extract diet. We next determined whether the L.E.M.-induced antitumor effect was T cell-dependent by performing a similar experiment using T cell-deficient nude mice. No difference in tumor growth was observed between the untreated control mice and the 2% L.E.M.-fed mice (Fig. 1C). These results indicate that

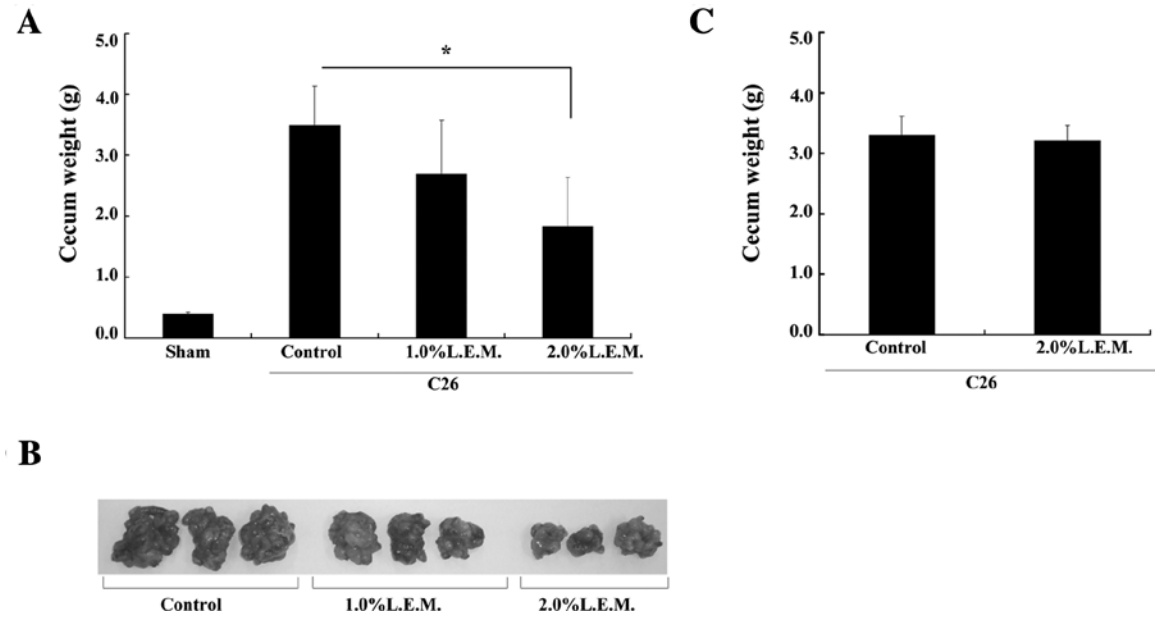

Figure 1. Antitumor effect of oral ingestion of L.E.M. extract on i.c.-inoculated C26 tumor cells. (a) BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. L.E.M. feeding began 3 days after tumor inoculation. Fourteen days after tumor inoculation, the mice were sacrificed and the ceca were weighed. The data represent means ± SD of eight mice. For the sham-operated control, the mice were inoculated in the i.c. with complete medium. *Statistical significance ($p<0.05$). (b) Representative photographs of the ceca are shown. (c) BALB/c nu/nu mice were inoculated in the i.c. with $1x10^6$ C26 cells and were analyzed as in (a). The data represent means ± SD of eight mice.

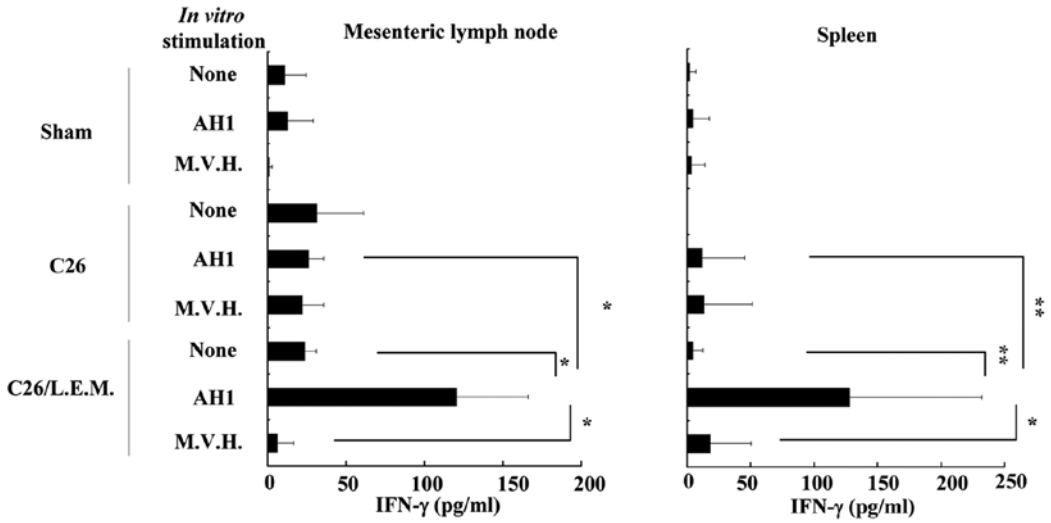

Figure 2. Induction of AH1 peptide-reactive T cells in the i.c. C26-inoculated and L.E.M.-fed mice. BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. The control mice were sham-operated. L.E.M. feeding of the L.E.M. group was initiated 3 days after tumor inoculation. On day 14, the spleen and mesenteric LNs of each group were harvested, pooled and stimulated *in vitro* with each of the indicated peptides in the presence of 20 U/ml IL-2. After 3 days of culture, the level of IFN-γ in the supernatant was determined by ELISA. The data are means ± SD of three values. *Statistical significance ($p<0.05$), **Statistical significance ($p<0.01$).

oral ingestion of L.E.M. extract can suppress the growth of i.c.-inoculated C26 in a T cell-dependent manner.

AH1 peptide-reactive and C26-reactive T cells in the i.c. C26-inoculated mice. We next examined the effect of L.E.M. feeding on T cell reactivity in the draining mesenteric LNs and the spleen of the tumor-bearing mice. An H-$2L^b$-binding AH1 peptide, derived from the envelope protein (gp70) of an endogenous murine leukemia virus (17), was used to test specific T cell responses against a tumor antigen. The M.V.H. peptide (18) was used as an H-$2L^b$-binding control peptide. Mesenteric LN cells and spleen cells from sham-operated, i.c. C26-inoculated or i.c. C26-inoculated and L.E.M.-fed mice were stimulated *in vitro* with or without the AH1 or the M.V.H. peptide, and the level of IFN-γ in the supernatant was determined. Although no AH1-specific IFN-γ production was observed in the mesenteric LN or the spleen cells of sham-operated or C26-bearing but untreated mice, ingestion of the L.E.M. extract resulted in strong induction of an IFN-γ response to the AH1 peptide (Fig. 2). We also determined whether ingestion of L.E.M.

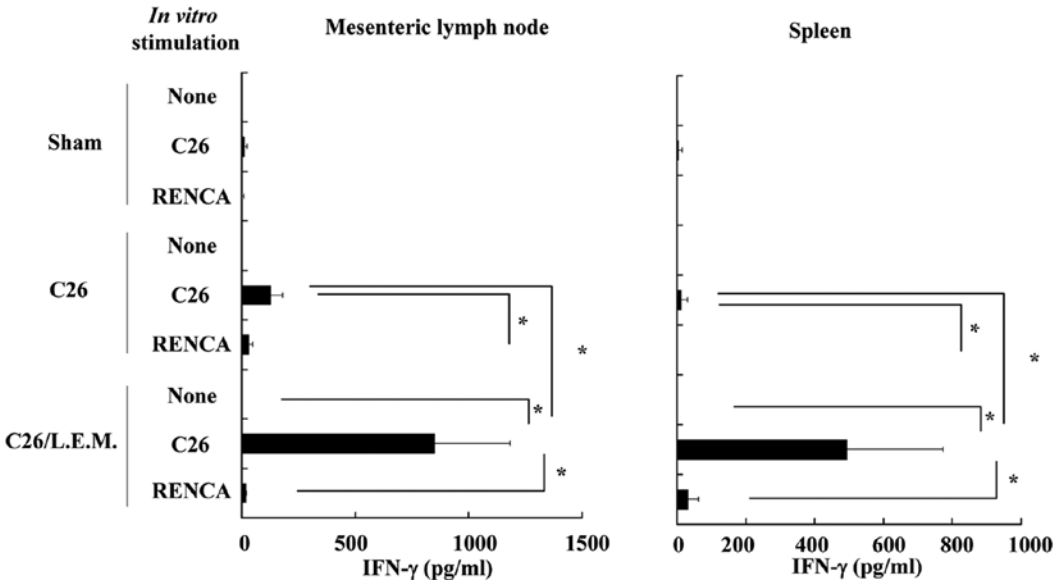

Figure 3. Induction of C26-reactive T cells in the i.c. C26-inoculated and L.E.M.-fed mice. BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. The control mice were sham-operated. L.E.M. feeding of the L.E.M. group was initiated 3 days after tumor inoculation. On day 14, the spleen and mesenteric LNs were harvested, pooled and stimulated *in vitro* with C26 or RENCA cells in the presence of 20 U/ml IL-2. After 3 days of culture, the level of IFN-γ in the supernatant was determined by ELISA. The data are means ± SD of three values. *Statistical significance ($p<0.05$).

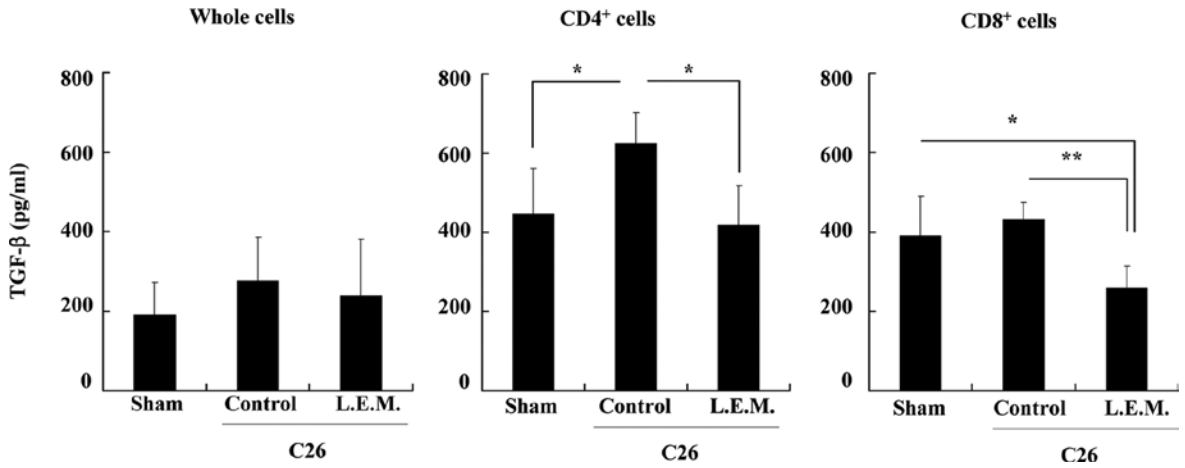

Figure 4. TGF-β production by the mesenteric LN cells of the i.c. C26-inoculated and L.E.M.-fed mice. BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. L.E.M. feeding of the L.E.M. group was initiated 3 days after tumor inoculation. The control mice were sham-operated. Fourteen days after tumor inoculation, the mice were sacrificed and the mesenteric LN cells were collected. Whole population of LN cells, $CD4^+$ T cells or $CD8^+$ T cells ($1x10^5$ cells) were cultured for 3 days without stimulation, and the level of TGF-β in the culture supernatants was determined by ELISA. The data are means ± SD of six mice. *Statistical significance ($p<0.05$), **Statistical significance ($p<0.01$).

extract could increase the number of C26-reactive T cells in the i.c. C26-inoculated mice. As shown in Fig. 3, the mesenteric LN and spleen cells from the i.c. C26-inoculated and L.E.M.-fed mice produced higher levels of IFN-γ in response to C26, but not to control RENCA cells. These results indicate that ingestion of L.E.M. extract can effectively restore MHC class I-restricted and C26-reactive T cells, which are probably $CD8^+$ T cells, in the (local) mesenteric LNs and the (systemic) spleen of the i.c. C26-inoculated mice.

TGF-β production by T cells in GALT of the i.c. C26-inoculated mice. We next determined whether oral ingestion of L.E.M. extract could influence the ability of T cells in GALT to produce TGF-β (Fig. 4). There was no difference in the level of TGF-β produced by the whole population of mesenteric LN cells between the three groups. The i.c. inoculation of C26 cells increased the ability of $CD4^+$ T cells to produce TGF-β, but oral ingestion of the L.E.M. extract prevented this TGF-β production. The i.c. inoculation of C26 cells did not influence the ability of $CD8^+$ T cells to produce TGF-β, but oral ingestion of the L.E.M. extract significantly decreased the production of TGF-β by $CD8^+$ T cell compared to sham and control-inoculated cells. These results indicate that i.c. inoculation of C26 cells increases the ability of $CD4^+$ T cells, but not $CD8^+$

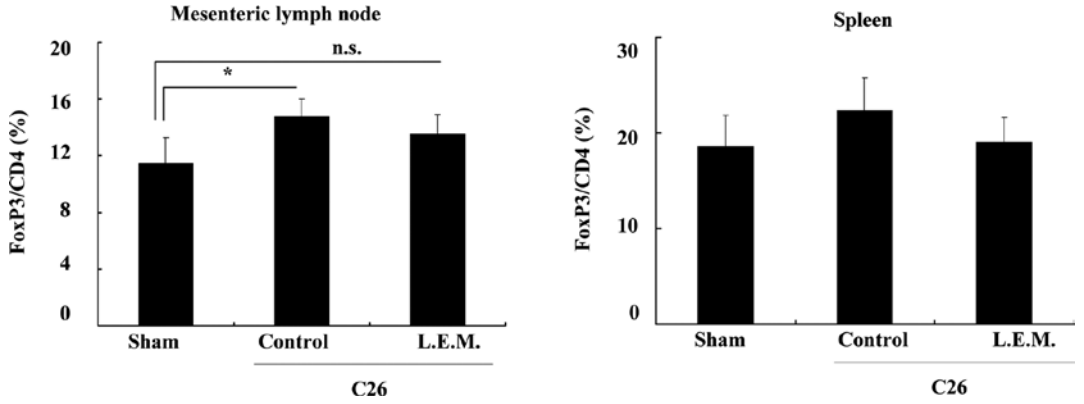

Figure 5. Tregs in the i.c. C26-inoculated and L.E.M.-fed mice. BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. The control mice were sham-operated. L.E.M. feeding of the L.E.M. group was initiated 3 days after tumor inoculation. On day 14, the mesenteric LN and spleen cells were harvested, and the percentage of $CD4^+$ cells that were $Foxp3^+$ cells was determined using flow cytometry. The means ± SD of data from the mesenteric LN and the spleen of eight mice are shown. Similar data were obtained in three independent experiments. *Statistical significance ($p<0.05$). n.s., not significant.

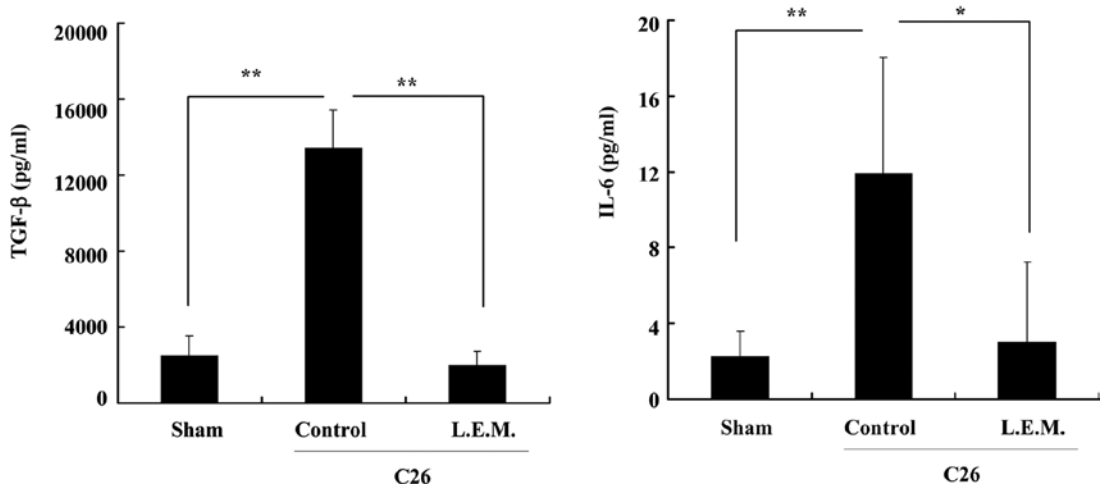

Figure 6. The plasma levels of TGF-β and IL-6 in the i.c. C26-inoculated and L.E.M.-fed mice. BALB/c mice were inoculated in the i.c. with $1x10^6$ C26 cells. L.E.M. feeding of the L.E.M. group was initiated 3 days after tumor inoculation. The control mice were sham-operated. Fourteen days after tumor inoculation, the mice were sacrificed and the plasma was collected. The plasma levels of TGF-β and IL-6 were determined by ELISA. The data are means ± SD of eight mice. *Statistical significance ($p<0.05$), **Statistical significance ($p<0.01$).

T cells, to produce TGF-β, and that oral ingestion of L.E.M. extract can efficiently decrease the ability of not only $CD4^+$ T cells, but also $CD8^+$ T cells, to produce TGF-β.

Tregs in the mesenteric LNs and the spleen of the i.c. C26-inoculated mice. We next examined effects of L.E.M. ingestion on Tregs in the i.c. C26-inoculated mice, because we previously reported that oral ingestion of L.E.M. can mitigate Treg-mediated immunosuppression in mice bearing a subcutaneously established B16 melanoma (12). We therefore evaluated the percentage of $Foxp3^+$ cells in the $CD4^+$ T cell population in the mesenteric LN and spleen cells from either sham-operated, i.c. C26-inoculated, or i.c. C26-inoculated and L.E.M.-fed mice. The percentage of $Foxp3^+$ cells in the $CD4^+$ T cell population in the mesenteric LNs significantly increased in the i.c. C26-inoculated mice (Fig. 5). Oral ingestion of L.E.M. extract slightly decreased this percentage to a level that was not significantly different compared to the level in sham-operated mice. A slight increase in the percentage of $Foxp3^+$ cells was also observed in the $CD4^+$ T cell population in the spleen of i.c. C26-inoculated mice, but this increase was not significantly different compared to the sham-inoculated mice. The combined results suggest that, although i.c. inoculation of C26 cells appears to slightly influence Tregs only in the mesenteric LNs and not in the spleen, oral ingestion of L.E.M. extract does not exert a definite effect on the percentage of Tregs in the spleen of the i.c. C-26-inoculated mice.

Effects of ingestion of L.E.M. extract on the plasma levels of TGF-β and IL-6 in the i.c. C26-inoculated mice. Finally, we examined the effects of ingestion of an L.E.M extract on the plasma levels of TGF-β and IL-6 in the i.c. C26-inoculated mice (Fig. 6). The plasma level of TGF-β significantly increased in

the i.c. C-26-inoculated mice compared to the sham-operated mice, but oral ingestion of L.E.M. extract dramatically reduced the TGF-β level to that of control mice. Similarly, the plasma level of IL-6 was significantly increased in the i.c. C26-inoculated mice, and oral ingestion of L.E.M. extract also reduced the IL-6 level.

Discussion

Since many tumor antigens and their antigenic peptides that can be recognized by CTLs have been identified to date, specific anticancer immunotherapies aimed at the induction of tumor-reactive CTLs in cancer patients have been conducted. However, to date, the efficacy of these therapies has been unsatisfactory (3). To obtain sufficient antitumor effects in cancer patients, a challenging hurdle that has to be overcome is the attenuation of immunosuppression in cancer patients. In particular, immunosuppressive cells, including Tregs and MDSCs, are well known to play a central role in cancer-associated immunosuppression (4,5). In addition, inflammation in the cancer-bearing state promotes the emergence of these cells (6,7). Therefore, new therapeutic modalities to improve the immunosuppression and inflammation that accompany the cancer-bearing state are required.

L.E.M. extract is an immunomodulator that can be orally ingested (8) and has the potential to mitigate inflammation *in vivo* (11). In addition, we recently reported that oral ingestion of L.E.M. extract can inhibit the growth of a subcutaneously established B16 melanoma in a T cell-dependent manner via mitigation of Treg-mediated immunosuppression (12). However, the question remains as to how an orally ingested L.E.M. extract can restore the antitumor immune response in tumor-bearing hosts. The first effect of an orally ingested L.E.M. extract would be exerted at GALT, where immune responses are usually suppressed by Tregs in order to maintain immunological oral tolerance (13,14). We therefore investigated the antitumor effect of an orally ingested L.E.M. extract, as well as the mechanism of this effect, using a mouse model in which murine colon carcinoma cells were inoculated into the i.c. of syngeneic mice and mesenteric LNs were established as the draining LNs.

In this study, we demonstrated that oral ingestion of L.E.M. extract significantly inhibited the growth of i.c.-inoculated C26 cells in a T cell-dependent manner (Fig. 1). In addition, we showed that the number of tumor peptide-specific and C26-reactive T cells significantly increased in both the draining mesenteric LNs and in the spleen of the L.E.M.-ingested mice compared to controls (Figs. 2 and 3). Since the tumor peptide can bind to H-2L^d molecules, these IFN-γ-producing T cells that were induced can be regarded as MHC class I-restricted CD8$^+$ T cells. Unexpectedly, *in vitro* stimulation of mesenteric LN and spleen cells with inactivated C26 cells generated higher levels of IFN-γ compared to stimulation with the AH1 peptide. This result may suggest two possibilities. The first possibility is that oral ingestion of the L.E.M. extract induced CD8$^+$ T cells reactive to C26-derived antigenic peptides other than the AH1 peptide. The second possibility is that CD4$^+$ T cells that recognize C26-derived antigens were induced. The combined evidence indicates that antitumor T cell responses in GALT are usually impaired, as previously reported (15), but that oral ingestion of L.E.M. extract can effectively restore these responses.

TGF-β is a representative of immunosuppressive cytokines in tumor-bearing hosts (19-21). We therefore examined the ability of the draining mesenteric LN cells to produce this cytokine. The ability of CD4$^+$ T cells of the i.c. C26-inoculated mice to produce TGF-β was increased compared to that of the sham-operated control mice, but the level of TGF-β production was significantly lower following oral ingestion of the L.E.M. extract (Fig. 4). On the other hand, the ability of CD8$^+$ T cells of the i.c.-C26-inoculated mice to produce TGF-β was comparable to that of the sham-operated control mice. This observation could be interpreted as evidence to suggest that TGF-β-producing CD8$^+$ T cells pre-existed in GALT, probably in order to maintain immunological oral tolerance (22), and that i.c. inoculation of C26 could not trigger a further increase in their number. However, an important point is that ingestion of L.E.M. extract can significantly decrease the ability of CD8$^+$ T cells to produce TGF-β in i.c. C26-inoculated mice. TGF-β-producing CD8$^+$ T cells can exert an immunosuppressive effect on the antitumor immune response in GALT (15). Our study has shown that oral ingestion of L.E.M. extract is useful for abrogation of this immunosuppressive effect. The combined results indicate that oral ingestion of L.E.M. extract has the potential to reverse the immunosuppressive state induced by TGF-β-producing CD4$^+$ and CD8$^+$ T cells in the GALT of i.c. C26-inoculated mice.

TGF-β is a key cytokine for Treg induction (19), and an increase in Tregs in tumor-draining LNs suppresses the induction of tumor-reactive CTLs (23). We showed that the percentage of Tregs in the draining mesenteric LNs slightly, but significantly, increased following i.c. inoculation of C26 cells, but that there was no difference in the percentage of Tregs between the sham-operated mice and the i.c. C26-inoculated mice that had ingested L.E.M. (Fig. 5). In contrast, the plasma level of TGF-β in i.c. C26-inoculated mice was dramatically decreased by ingestion of the L.E.M. extract (Fig. 6). Based on these results, we conclude that oral ingestion of L.E.M. extract primarily decreased TGF-β production by non-Treg cells, and subsequently inhibited the induction of Tregs. On the other hand, the percentage of Tregs in the spleen showed a tendency to increase in i.c. C26-inoculated mice but this increase was not significant compared to Sham-operated mice. Based on all of these results, it appears that TGF-β-producing cells, other than Tregs, might participate in immunosuppression in the i.c. C26-inoculated mice. However, C26 cells are known to produce IL-6 in addition to TGF-β (24), and IL-6 inhibits the induction of Tregs from naïve CD4$^+$ T cells (25). Therefore, there is a possibility that, although the levels of both TGF-β and IL-6 increased in the i.c. C26-inoculated mice, the increased IL-6 might inhibit the induction of Tregs in these mice.

Th17 cells play a crucial role in inflammation and autoimmune disease, and are induced in the presence of IL-6 and TGF-β (26). Although the roles of Th17 cells in antitumor immune responses have been controversial, some reports show that Th17 cells accumulate in tumor sites and have pro-tumorigenic functions (27,28). It has also been reported that the levels of Th17 cells positively correlate with Tregs in several types of cancers (29). In this study, we showed that the plasma levels

of both TGF-β and IL-6 increased in the i.c. C26-inoculated mice (Fig. 6). This result suggests the possibility that Th17 cells might have been induced in these mice and affected tumor growth. In addition, the level of MDSCs correlates with cancer-associated inflammation and MDSCs promote tumor progression via inhibition of $CD4^+$ and $CD8^+$ T cell activity (30-33). Furthermore, IL-6 has been reported to promote accumulation of MDSCs at the tumor site (31). Consistent with a report that ingestion of L.E.M. extract inhibits the production of proinflammatory cytokines including IL-6 (34), we demonstrated that ingestion of L.E.M. extract decreased the plasma level of IL-6 in i.c. C26-inoculated mice (Fig. 6). These lines of evidence may indicate the possibility that the antitumor effect observed after ingestion of L.E.M. extract was induced via inhibition of Th17 cells and MDSCs. Further studies are needed to elucidate the precise mechanism of this inhibition.

In conclusion, we showed that oral ingestion of L.E.M. extract can restore immunosuppression in i.c. C26-inoculated mice. This antitumor effect is T cell-dependent, and may be mediated by mitigation of TGF-β-mediated immune suppression and inflammation. Our study indicates that oral ingestion of L.E.M. extract can restore the antitumor T cell response of tumor-bearing mice even when the primary antitumor immune response is elicited in GALT.

Acknowledgements

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