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Organ microenvironment plays significant roles through Fas ligand in vaccine-induced CD4$^+$ T cell dependent suppression of tumor growth at the orthotopic site

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Growth of colon carcinoma cells transfected with mucin 1 (MUC1) was effectively suppressed by vaccination with MUC1 cDNA. The suppression was dependent on the presence of Fas ligand (FasL) in the cecum, whereas it was independent of FasL in the spleen and in the liver, as revealed by the use of *gld/gld* mice as the recipients of vaccination, and transplantation of tumor cells expressing MUC1. CD4$^+$ T cells were transferred from mice immunized with MUC1 cDNA to naive *gld/gld* or C57BL/6 mice, and the suppression of colon carcinoma growth in the cecum was tested. The results clearly showed that FasL in the recipient played a significant role. In the cecum, FasL was associated with intratumoral CD11b$^+$ cells, which are likely to be responsible for vaccine-induced tumor suppression. The T cell response to MUC1 was not influenced by the *gld/gld* status. (*Cancer Sci* 2010; 101: 1965–1969)

Cancer immunotherapy is one of the candidates for the new methods for treatment of cancer and is especially suitable for the eradication of tumor cells left after surgical treatment and micrometastasis. However, there still are many obstacles to overcome before effective agents and optimized protocols become available. There are a few areas where our understanding of the mechanism of immunity against cancer is highly limited. The first is organ selectivity of the effector mechanism suppressing tumor growth in different organ microenvironments. The second is the mechanism of navigation of specific effector cells to the tumor sites. The third is the significance of suppressive mechanisms against tumor specific immunity at the primary and metastatic sites. These questions must primarily be answered by the use of experimental animal models.

In our previous investigations with mice, suppression of colon carcinoma growth was observed both at the site of orthotopic transplantation and liver metastases after mucin 1 (MUC1) DNA vaccination.[1] Using this and other experimental models, depletion assays were performed to estimate the effector mechanisms at different organ sites. To our surprise, CD4$^+$ T cells but not CD8$^+$ cells appeared to be responsible for suppression of tumor growth induced by MUC1 DNA vaccination. As far as this model is concerned, the predominant effector molecules in the orthotopic sites and in the liver metastases were different and Fas ligand (FasL) was suggested to play an important role in the orthotopic sites, although Fas was expressed at similar levels in the tumor cells growing in these different organs.[1] However, conclusive evidence was not previously available.

FasL, a member of the tumor necrosis factor (TNF) super family, induces apoptosis of target cells after ligation with its counter receptor, Fas. This system is known to play an important role in the induction of apoptosis of target cells by natural killer (NK) cells, and CD4$^+$ and CD8$^+$ CTLs.[2] Activated macrophages and non-hematopoietic cells express FasL and are likely to be involved in induction of apoptosis.[3,4] However, the effector cells responsible for this FasL-mediated suppression of tumor growth in the orthotopic site induced by the injection of MUC1 DNA were not previously known.

In the present study, we used FasL mutant *gld/gld* mice to clarify whether FasL in the T cells was essential for the tumor suppressive effects. The results suggest that FasL-expressing cells other than T cells, which are likely to be macrophages residing in the intestinal lamina propria, are the key players. These findings should help to improve the design of novel cancer immunotherapy.

Materials and Methods

Mice. Specific pathogen-free C57BL/6 (B6) mice were obtained from Clea Japan (Tokyo, Japan). C57BL/6J Slc-*gld/gld* mice were purchased from SLC Japan (Hamamatsu, Japan). All experimental animals were housed under specific pathogen-free conditions and handled according to the guidelines of the Bioscience Committee of the University of Tokyo.

Construction of MUC1 cDNA. The full-length human *MUC1* cDNA containing 22 tandem repeats in the pDKOF vector was originally provided by Dr O.J. Finn.[5] The cDNA was recloned into the *Hind*III site of the pCEP4 vector, which includes the CMV promoter (pCEP4-MUC1). The plasmid without the *MUC1* cDNA (pCEP4) was used as a control. The pCEP4 and pCEP4-MUC1 were amplified in the *Escherichia coli* strain JM109 and purified using Qiagen Mega-Plasmid columns (Qiagen, Hilden, Germany).

Immunization. Mice were immunized intradermally three times at weekly intervals in the forelimb with 25 μg DNA dissolved in 50 μL Hanks' Balanced Salt Solution.

Tumor cells. SL4, a highly liver metastatic variant of colon 38 murine colon carcinoma cell line[6] was transfected with human *MUC1* cDNA (pDKOF-MUC1) by lipofection using DOTAP Liposomal Transfection Reagent (Roche, Basel, Switzerland). After selection with G418 (Wako, Osaka, Japan), the limiting dilution method was performed. A clone, which grew at the orthotopic site, was chosen and used as MUC1-transfected cells (SL4-MUC1). MUC1-transfected B16-F10 melanoma cells were prepared as previously described.[7]

Orthotopic transplantation and experimental liver metastasis. One week after the final immunization, SL4-MUC1 cells

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(1×10^6 cells/50 μL) were injected into the space under the cecal serosa of mice in the orthotopic transplantation model. Twenty-one days after the injection, the mice were sacrificed and the increase in the weight of the cecum due to tumor growth was measured. In the liver metastasis model, SL4-MUC1 cells (5×10^5 cells/50 μL) were injected into the spleen. Nineteen days after the challenge, the weights of the spleen and liver were measured.

Interleukin (IL)-2 ELISPOT assays. One week after the final immunization, SL4-MUC1 cells (1×10^6 cells/50 μL) were injected into the space under the cecal serosa of mice. Twelve days after the tumor injection, the spleen was harvested and single cell suspension was obtained. ELISPOT assays were performed on MultiScreen-IP Filter Plates (Millipore, Billerica, MA, USA). The plates were pre-treated with 70 μL of 70% ethanol, washed six times with 200 μL PBS, to which 70 μL of 2.5 μg/mL anti-IL-2 capture antibody (JES6-1A12; Biolegend, San Diego, CA, USA) diluted in PBS was added. This was then incubated at 4°C for 18 h. The IL-2 capture antibody-coated plates were washed six times with sterile PBS and blocked with 150 μL of PBS supplemented with 1% normal mouse serum for 3 h at 37°C. In these plates, splenocytes (5×10^5 cells/well) were incubated with 20 μg/mL 29 mer MUC1 peptide (PDTRPAPGSTAPPAHGVTSAPDTRPAPGK, synthetic peptide) and B16-F10 cells, B16-F10-MUC1 cells, or media alone for 18 h. The B16-F10 and B16-F10-MUC1 cells were treated with 4 ng/mL recombinant mouse interferon (IFN)-γ (PeproTech, Rocky Hill, NJ, USA) for 18 h and 60 μg/mL Mitomycin C (Kyowa Kirin, Tokyo, Japan) for 1 h before use. The plates were washed and incubated with water to lyse remaining cells, and incubated with 0.5 μg/mL biotinylated anti-IL-2 antibody (JES6-5H4; Biolegend) for 2 h at room temperature followed by 1 μg/mL streptavidin-alkaline phosphatase (Mabtech, Nacka Strand, Sweden) for 30 min at room temperature. Spots of deposited IL-2 were visualized with AP-Conjugate Substrate Kit (BioRad, Hercules, CA, USA) and the number was counted using CTL-ImmunoSpot (CTL, Shaker Heights, OH, USA).

Winn assays. B6 and *gld/gld* mice were immunized with pCEP4 or pCEP4-MUC1 as described above. One week after the vaccination, the mice were sacrificed and single cell suspensions were obtained from the spleens. After removal of erythrocytes, the splenocytes were passed through a nylon wool column to enrich T cells. $CD4^+$ T cells were obtained by negative selection using a mixture of biotinylated mAbs CD11b, Ly6G, DX5, B220, and CD8 (eBioscience, San Diego, CA, USA) and streptavidin-microbeads (Miltenyi Biotec, Bergisch Gladhach, Germany) using AutoMACS (Miltenyi Biotec) according to the manufacturer's specifications. $CD4^+$ T cells ($1–1.5 \times 10^6$ cells) were mixed with SL4-MUC1 cells (1×10^5 cells) and injected into the space under the cecal serosa of naive C57BL/6 or *gld/gld* mice. Twenty-five days after the injection, the mice were sacrificed and the tumor growth was evaluated.

Immunohistochemistry. One week after the injection of tumor cells into mice immunized with MUC1 DNA, the ceca were resected and embedded in OCT compound (Sakura, Tokyo, Japan). Frozen sections (6 μm) were fixed with cold ethanol for 30 s, and endogenous biotin was blocked using an avidin/biotin blocking kit (Vector, Burlingame, CA, USA). After the fixation and blocking, the frozen sections were incubated overnight with anti-CD4 antibody (GK1.5, 5 μg/mL; eBioscience) or anti-CD11b antibody (M1/70, 5 μg/mL; eBioscience) and biotinylated anti-FasL antibody (MFL3, 5 μg/mL; BD Bioscience, Franklin Lakes, NJ, USA) at 4°C. Horseradish peroxidase (HRP)-avidin was added and was followed by biotinylated tyramide to amplify the FasL signals as previously described.[8] Finally, Alexa Fluor 488 goat anti-rat IgG antibody (5 μg/mL; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 568 conjugate streptavidin (5 μg/mL, Invitrogen) were used for double staining. The nuclei were labeled with DAPI (Invitrogen). Confocal images were obtained with a Leica TCS SP5.

Results

FasL is essential for tumor rejection in the cecum but not in the spleen or liver. To investigate whether FasL is essential for the antitumor immune response induced by the MUC1 DNA vaccination in the cecum but not in the spleen or in the liver, the effects of the vaccination of B6 or FasL mutant *gld/gld* mice were examined.[9] After intradermal administration of MUC1 DNA, mice were injected with SL4-MUC1 cells. Intrasplenic injections were performed to test for liver metastasis, and intracecal injections were performed to test the orthotopic tumor growth. Nineteen and 21 days, respectively, after the tumor injection, the mice were sacrificed and tumor growth was evaluated by the weight of each organ. In B6 and *gld/gld* mice immunized with MUC1, the growth of tumors in the spleen and the formation of liver metastases were suppressed in a MUC1-specific manner (Fig. 1a,b). Similarly, the weights of the ceca were significantly lower in B6 mice immunized with MUC1 DNA than those in B6 mice with control immunization, indicating that tumor growth was effectively suppressed after injection of tumor cells at the orthotopic sites (Fig. 1c). However, in *gld/gld* mice the weight of the ceca; that is, the tumor growth, did not show any statistically significant difference between MUC1 and the control immunization. (Fig. 1c). The effect of MUC1 DNA immunization was dramatic as the results show that all B6 mice were tumor free, whereas none of the *gld/gld* mice immunized with MUC1 DNA rejected tumor cells. These data strongly reinforce our previous observations wherein antagonizing effects of anti-FasL neutralization mAb on the suppression of tumor growth were only observed with tumors growing in the ceca but not those growing in the spleens or livers. From these results, we concluded that FasL is essential for the antitumor immune responses in the cecum but not in the spleen or in the liver.

MUC1-specific T cell responses were induced by MUC1 DNA vaccination in *gld/gld* mice. To examine whether MUC1-specific T cell responses were induced by the MUC1 DNA vaccination in *gld/gld* mice as in B6 mice, MUC1-specific T cell activation was tested *in vitro*. After MUC1 DNA vaccination, B6 and *gld/gld* mice were injected with SL4-MUC1 cells to augment the MUC1-specific immune responses *in vivo*. After the *in vivo* stimulation, splenocytes from each mouse were collected and incubated *in vitro* with synthetic 29 mer peptide corresponding to the MUC1 tandem repeats (Fig. 2a) or B16-F10 melanoma cells transfected with MUC1 (Fig. 2b). IL-2 production by the splenocytes was evaluated by the ELISPOT assays. As shown in Fig. 2, splenocytes obtained from *gld/gld* mice produced IL-2 in response to naked MUC1 peptide or to melanoma cells expressing MUC1 as observed with spelenocytes from immunized B6 mice.

T cell response in *gld/gld* mice was also supported by the results shown above, indicating that the antitumor effect was observed on the growth of tumor cells in the spleen and on liver metastasis formation. Thus, the impairment of MUC1-specific antitumor immune responses in *gld/gld* mice should be due to the effector mechanisms in these mice.

FasL expression in the cells other than $CD4^+$ T cells was responsible for the antitumor immune responses in the cecum. Based on the notion that $CD4^+$ T cells were responsible for the antitumor immune responses induced by MUC1 DNA vaccine,[1] we further tested the mechanism of FasL-dependent antitumor immune responses in the ceca. To prove or disprove that $CD4^+$ T cells suppress tumor growth through direct

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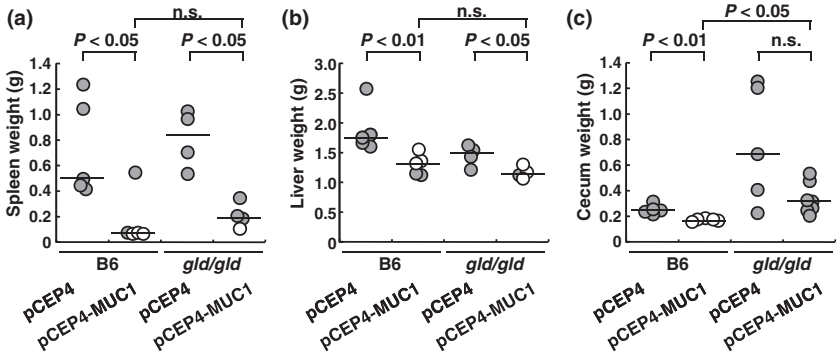

Fig. 1. FasL is essential for the suppression of tumor growth in the cecum but not in the spleen or in the liver. B6 or *gld/gld* mice were immunized with MUC1 DNA three times at weekly intervals. One week after the final immunization with MUC1 DNA, SL4-MUC1 cells (5×10^5 cells/50 μL) were injected into the spleen. Nineteen days after the injection, the weights of the spleen (a) and liver (b) were measured. SL4-MUC1 cells (1×10^6 cells/50 μL) were injected into a space under the cecal serosa of the mice. Twenty-one days after the injection, the mice were sacrificed and the weight of the cecum (c) was examined. Open circles represent tumor-free mice and filled circles represent tumor-bearing mice. Black bars represent median values. Statistical analyses were performed using the Mann–Whitney *U*-test.

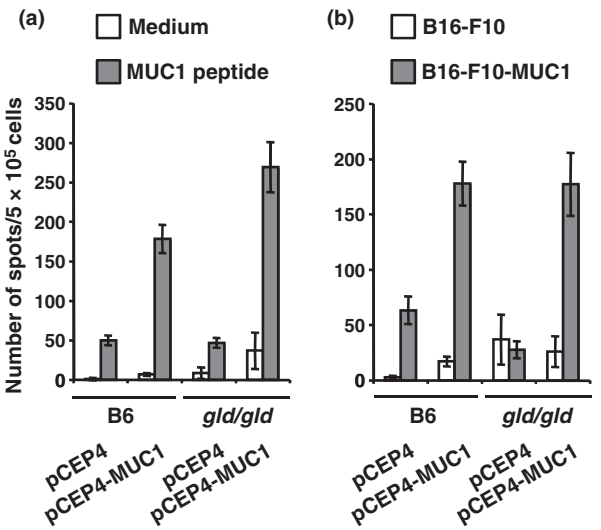

Fig. 2. MUC1-specific T cell responses were induced by the vaccination with MUC1 DNA in *gld/gld* mice. B6 and *gld/gld* mice were immunized with pCEP4 or pCEP4-MUC1. One week after the final immunization, SL4-MUC1 cells were injected into a space under the cecal serosa of the mice. Twelve days after the injection of the tumor cells, the spleen was harvested and single cell suspensions were obtained. (a) Splenocytes (5×10^5 cells/well) were incubated with 29 mer MUC1 peptide (gray bar) or untreated (white bar) and IL-2 production was measured by the ELISPOT assays. (b) Splenocytes (5×10^5 cells/well) were co-cultured with B16-F10 (white bar) or B16-F10-MUC1 cells (gray bar) and IL-2 production was measured by the ELISPOT assays. Data were shown as mean ± SD (n = 3).

involvement of FasL, adoptive transfer experiments were performed. One week after the MUC1 DNA vaccination, B6 and *gld/gld* mice were sacrificed and $CD4^+$ T cells were purified from the spleens. $CD4^+$ T cells were mixed with SL4-MUC1 cells and transplanted into the space under the cecal serosa of naive recipient mice. Twenty-five days after the transfer, the mice were sacrificed and the local tumor growth was evaluated. As shown in Fig. 3, $CD4^+$ T cells obtained from MUC1-immunized B6 mice suppressed tumor growth in the cecum (Fig. 3a) and $CD4^+$ T cells obtained from MUC1 immunized *gld/gld* mice also suppressed tumor growth (Fig. 3b). Thus, we concluded that cells with FasL expression other than $CD4^+$ T cells were important. To verify this hypothesis, MUC1-specific $CD4^+$ T cells were obtained from B6 mice as described above, mixed with SL4-MUC1 cells, and injected into the space under the cecal serosa of naive *gld/gld* mice. Interestingly, when *gld/gld* mice were used as the recipients, the tumor cells were not rejected (Fig. 3c). These results clearly indicated that the growth of tumor cells was suppressed through the Fas–FasL pathway, and that FasL in cells other than $CD4^+$ T cells was responsible.

Intratumoral FasL co-localised with $CD11b^+$ cells. To identify the cell population with FasL within the cecal tumor, immunohistochemical analysis was conducted. Tumor tissues were obtained from mice previously immunized with MUC1 DNA. The mice were injected with tumor cells 1 week prior to the sampling. FasL-expressing cells were visualized by double staining using anti-FasL antibody and various cell surface markers. Confocal images are shown in Fig. 4. $CD4^+$ T cells were shown to have accumulated in the tumor tissue, though anti-FasL antibody did not bind these cells (Fig. 4a). Most of the $FasL^+$ cells were also reactive with anti-CD11b antibody (Fig. 4b). Therefore, $CD11b^+$ cells were likely to be responsible for the suppression of tumor growth through direct interaction with colon carcinoma cells. The Fas–FasL system should play a role in these interactions.

Discussion

Effector mechanisms in vaccine-induced suppression of tumor growth are still unclear even though involvement of antigen-specific $CD8^+$ CTLs have been considered as the gold standard.[10] Contributions of $CD4^+$ cells should also play important role in the orchestration of specific antitumor immunity as revealed by adoptive transfer experiments.[11] During such $CD4^+$ T cell mediated suppression of tumor growth, there seemed to be no direct contact between tumor cells and T cells.[12] Furthermore, MUC1 DNA vaccine-induced suppression of colon carcinoma growth in mice was previously shown to be mediated by $CD4^+$ cells. Mice carrying a point mutation in the FasL, *gld/gld* mice,[9,13] were introduced in the present study to show that FasL was essential in the vaccine-induced suppression of tumor growth in the cecum but not in the liver or in the spleen. The effect of MUC1 immunization demonstrated by the absence of tumors in the ceca of B6 mice was dramatically reduced in *gld/gld* mice, in which all mice had tumors, and this difference was much more prominent than the increase in the size of

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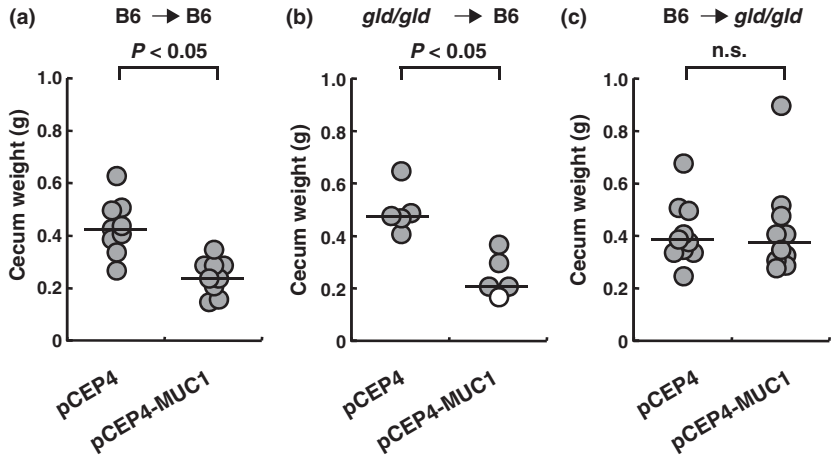

Fig. 3. CD4$^+$ T cells were essential to the antitumor immune responses in the cecum but FasL in these cells was not involved. B6 and *gld/gld* mice were immunized with pCEP4 or pCEP4-MUC1. One week after the MUC1 DNA vaccination, the mice were sacrificed. Single cell suspensions were prepared from the spleens and CD4$^+$ T cells were purified. CD4$^+$ T cells were mixed with SL4-MUC1 cells and injected into the space under the cecal serosa of naive C57BL/6 mice and *gld/gld* mice. Twenty-five days after the injection, the mice were sacrificed and the tumor growth was evaluated. (a) CD4$^+$ T cells purified from immunized B6 mice were transferred to recipient B6 mice. (b) CD4$^+$ T cells purified from immunized *gld/gld* mice were transferred to recipient B6 mice. (c) CD4$^+$ T cells purified from immunized B6 mice were transferred to recipient *gld/gld* mice. Open circles represent tumor-free mice and filled circles represent tumor-bearing mice. Black bars represent median values. Statistical analysis was performed using the Mann–Whitney *U*-test.

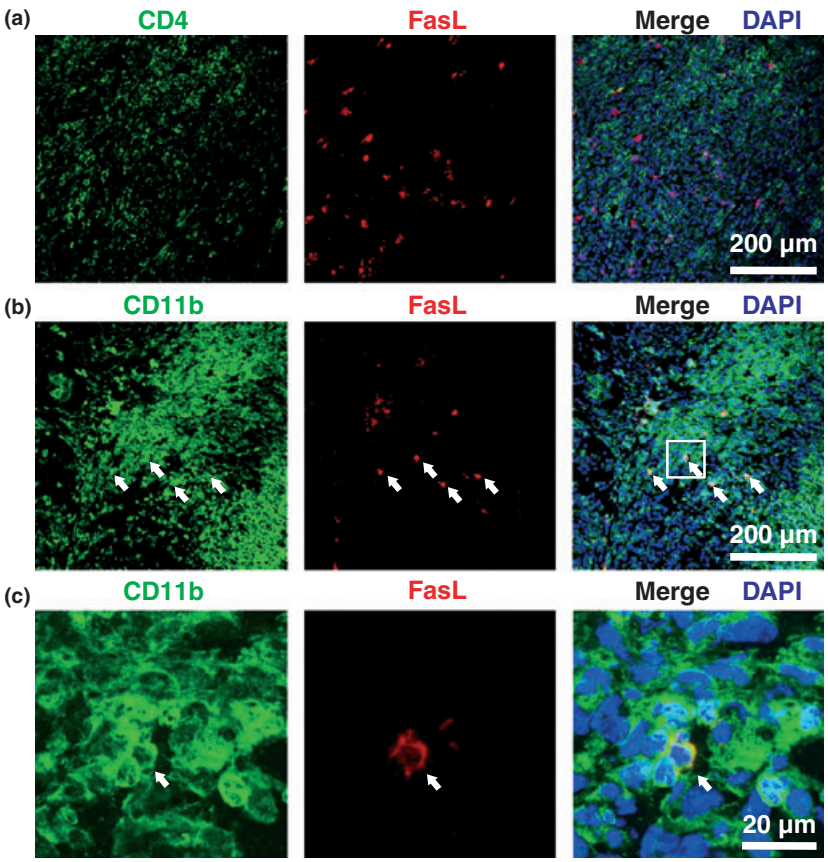

Fig. 4. CD11b$^+$ cells expressed FasL in the cecal tumor. B6 mice were immunized with pCEP4-MUC1. One week after the final immunization, SL4-MUC1 cells were injected into a space under the cecal serosa of these mice. Seven days after the tumor injection, the cecal tumor tissues were resected and frozen sections were prepared. (a) Sections were stained with anti-FasL antibody (red), anti-CD4 antibody (green), and DAPI (blue). (b,c) Sections were stained with anti-FasL antibody (red), anti-CD11b antibody (green), and DAPI (blue). (c) High magnification picture boxed in (b).

tumors in pCEP4-immunized *gld/gld* mice (Fig. 1c). FasL is known to be expressed in activated CD8$^+$ CTLs and NK cells and is potentially involved in the suppression of tumor growth.[14,15] However, transfer experiments clearly demonstrated that FasL on the antigen-specific CD4$^+$ T cells did not play a role (Fig. 3).

As a result of immunohistochemical examinations, FasL seemed to be associated with CD11b$^+$ cells within the tumor growing in the cecum (Fig. 4). The numbers of FasL$^+$CD11b$^+$ cells appeared to be limited and not all CD11b$^+$ cells expressed FasL. They were likely to be macrophages because activated macrophages were shown to express FasL.[3] There are possible

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mechanisms explaining how MUC1-specific $CD4^+$ T cells render $CD11b^+$ cells capable of suppressing tumor growth through the function of macrophages. A plausible explanation is that tumoricidal macrophages developed through IFN-γ secreted by the activated $CD4^+$ cells. However, administration of anti-IFN-γ antibody did not influence the functions of effector cells.[1] Other modes of macrophage activation through direct contact with $CD4^+$ cells should also be considered as previously suggested, such as CD40–CD40L interaction.[16] Tumor-associated macrophages and related populations were proposed to play crucial roles in the regulation of tumor growth. Cells of monocyte-macrophage lineage influence the behavior of malignant cells, determine the microenvironment, and subvert antitumor immunity.[17] These cells have been classified as alternatively activated macrophages associated with tumors.[18,19] Another population, immunosuppressive immature myelomonocytic cells, myeloid-derived suppressor cells, has also been implicated in switching of suppression and promotion of immunity against malignant growth.[20] Development, localization, and maintenance of these different populations of macrophages and related cells, all of which are $CD11b^+$, are regulated by cytokines other than IFN-γ.

Furthermore, it should be considered that the intestinal immune systems consist of unique populations of immune cells, including macrophages and related cells, showing unique characteristics. Origins of intestinal lamina propria dendritic cells were recently reported to differ from those of lymphoid dendritic cells.[21,22] In humans, unique $CD14^+$ intestinal macrophages were reported to contribute to the pathogenesis of Crohn's disease.[23] It is likely that such intestinal-specific $CD11b^+$ cells not only influence T cell functions but also are influenced by T cells, particularly $CD4^+$ T cells through organ-specific mechanisms. In the previous paper we found that effector mechanisms mediated by the MUC1-specific $CD4^+$ T cells were organ-dependent, and FasL was an effector molecule in the cecum but not in the liver. Similar effector profiles were reported by Winter *et al.* where adoptively transferred T cell mediated tumor regression was independent on perforin or FasL in the pulmonary metastasis of B16 melanoma cells.[24] It is important to further examine whether our findings, antitumor immune responses unique to the intestine, may depend on the system employed in the present work; that is, DNA vaccination as the means of immunization, MUC1 as the antigen, SL4 cells as the target, C57BL/6 mice as the recipients, and injection of tumor cell suspension beneath the serosa of ceca as the method of tumor transplantation. The tissue-specific regulation of immunity under a specific organ microenvironment should help us to develop better vaccines for cancer prevention and therapy.

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