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Mast Cells Inhibit CD8$^+$ T Cell-Mediated Rejection of a Malignant Fibrous Histiocytoma-Like Tumor: Involvement of Fas-Fas Ligand Axis

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Abstract: Problem statement: Mast cells develop from bone marrow-derived progenitor cells and are distributed in the skin or mucosa where they play proinflammatory roles in the first line of defense. Since some tumors in humans and experimental animals exhibited infiltration of increased mast cells, we investigated the contribution of mast cells to the override of tumor rejection. **Approach:** MRL/N-1 cells are malignant fibrous histiocytoma-like cells established from the spleen of a Fas ligand (FasL)-deficient MRL/Mp-FasL$^{gld/gld}$ (MRL/gld) mouse and are implantable in Fas-deficient MRL/Mp-Fas$^{lpr/lpr}$ (MRL/lpr) mice. MRL/N-1 cells were implanted in MRL/gld, MRL/lpr and MRL/+mice after antibody treatments or with mast cells or macrophages and the tumor growth was observed. **Results:** MRL/N-1 cells were rejected by Fas-intact syngeneic MRL/+ mice in CD8+ T cell-mediated manner. This rejection was inhibited by the co-implanted mast cells. MRL/N-1 cells transfected with FasL were rejected by MRL/+ and MRL/gld mice. **Conclusion:** Mast cells abrogate the rejection of MRL/N-1 tumor cells and that this tumor rejection is mediated by CD8+ T cells and dependent on host Fas-FasL axis.

Key words: Mast cell, syngeneic tumor rejection, Fas, Fasl

INTRODUCTION

Mast cells develop from bone marrow-derived progenitor cells and are distributed in the skin or the mucosa. Mast cells are one of the inflammatory cells that form the first line of defense in the skin or mucosa; they produce chemokines and cytokines to recruit other inflammatory cells to induce inflammation[1]. They are primarily stimulated in response to allergic reactions such as anaphylaxis and asthma, parasitic infections, or wound healing. It was previously reported that mast cells mediate peripheral allograft tolerance[2]. Mast cells can act as antigen-presenting cells and express costimulatory molecules. Mast cells have also been known to play some roles in tumor angiogenesis or tumorigenesis[3-5]. However, it is still not clear whether mast cells contribute to tumor tolerance.

MRL/Mp-Fas$^{lpr/lpr}$ (MRL/lpr) is a lupus-prone strain. MRL/lpr mice show severe lymphadenopathy, splenomegaly and autoantibody production[6]. This abnormality was induced by the mutant Fas gene lpr. The Fas antigen is a member of the tumor necrosis factor receptor superfamily and is known to mediate apoptotic signals[7]. The generalized lymphoproliferative disease gene gld is a mutant developed in C3H/He mice[8]. These mice also show lymphadenopathy and splenomegaly. These abnormalities were revealed to be associated with a Fas Ligand (FasL) defect. The gld-congenic MRL strain of MRL/Mp-FasL$^{gld/gld}$ (MRL/gld) mice has traits similar to that of the MRL/lpr mice[9].

MRL/N-1 cells are malignant fibrous histiocytoma-like cells established from the spleen of an MRL/gld mouse. MRL/N-1 cells are implantable in MRL/lpr and severe combined immunodeficiency mice. MRL/N-1 cells do not metastasize when injected subcutaneously. Therefore, MRL/N-1 transfectants of chemokines or cytokines were used as tools to express these molecules in the circulating blood of MRL/lpr mice via subcutaneous injections[10,11]. We found that MRL/N-1 cells were rejected by MRL/+mice and that the rejection was inhibited by co-implanted mast cells.

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Based on the data obtained, we tried to investigate the mechanisms of tumor development mediated by mast cells.

MATERIALS AND METHODS

Mice: MRL mice were bred under specific pathogen-free conditions in Tohoku University. MRL/+ and MRL/lpr mice were purchased from Charles River Japan, Inc. (Tokyo, Japan). MRL/gld mice were previously described[9]. The N_2 mice were generated by crossing the MRL/+ mice with the MRL/lpr or MRL/gld mice and by subsequent backcrossing of the resulting heterozygous F_1 mice with parental MRL/lpr or MRL/gld mice. The F_2 mice were generated by crossing the MRL/lpr mice with the MRL/gld mice and by subsequent intercrossing of the resulting heterozygous F_1 mice. The N_2 and F_2 mice were genotyped with the following primer sets: 5’-CAATTTTGAGGAATCTAAGGCC and 5’-CAAGACAATATTCCTGGTGCC with StuI digestion for gld and 5’-GTAAATAATTGTGCTTCGTCAG, 5’-TAGAAAGGTGCACGGGTGTG and 5’-CAAATCTAGGCATTAACAGTG for the lpr loci. All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Tohoku University.

Cells: The MRL/N-1 cell is a malignant fibrous histiocytoma-like cell line originally established *in vitro* from the spleen of an MRL/gld mouse without any stimulation or induction[10]. X63Ag8-653.mIL-3 was kindly provided by Dr. Hajime Karasuyama (Tokyo Medical and Dental University, Tokyo, Japan). Hybridomas for anti-CD4 (GK1.5) or anti-CD8 (53-6.72) monoclonal antibodies (mAbs) were provided by Tohoku University, Institute of Development, Aging and Cancer, Cell Resource Center for Biomedical Research. Bone marrow cells were isolated from the femurs of 8-12-week-old mice. Bone marrow-derived mast cells (BMMCs) were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10% FCS, 100 U mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin, 1 mM sodium pyruvate, 1 X non-essential amino acids, 50 µM 2-mercaptoethanol and 2% culture supernatant from X63Ag8-653.mIL-3. Bone marrow-derived macrophages (BMMΦs) were also cultured in DMEM supplemented with 10% FCS, 100 U mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin, 1 mM sodium pyruvate, 1 X non-essential amino acids, 50 µM 2-mercaptoethanol and 10 ng mL^{-1} of murine macrophage colony-stimulating factor.

Tumor implantation and antibody depletion: We used 5 mice per group for all tumor implantation experiments except for N_2 and F_2 experiments. MRL/N-1 cells were suspended in PBS and 100 µL of the suspension was subcutaneously injected into the left flank of the MRL mice. $CD4^+$ or $CD8^+$ T cells or NK cells were depleted using anti-CD4 (GK1.5) or anti-CD8 (53-6.72) mAbs or anti-AGM1 Ab (Wako Pure Chemical Industries, Osaka, Japan), respectively, at day -3, -2, -1, 1, 6, 11, 16 and 21. The depletion efficiency in the peripheral blood was confirmed by using a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ). The major and minor axes of the tumors were measured with a caliper. The sizes of the tumors were determined weekly until 4 weeks after tumor implantation and the surface areas of tumors were estimated using the following formula:

$$\text{The approximate ellipsoidal area } \left(\text{mm}^2\right) = \frac{\pi\times\left(\text{major axis } (\text{mm})\right)\times\left(\text{minor axis } (\text{mm})\right)}{4}$$

Histological analysis: The tumors from MRL/lpr mice were fixed in 10% formalin in 0.01 M phosphate buffer (pH 7.2), embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin or 0.5% toluidine blue in 0.5 M HCl to detect mast cells as previously described[12].

Flow cytometric analysis: MRL/N-1 cells and BMMCs were stained with ant-Fas mAbs (BD Bioscience), according to the manufacturer’s instructions and analyzed using a FACSCalibur flow cytometer as previously described[13].

Apoptosis detection assay: Apoptosis was detected by a flow cytometric assay. Briefly, 5×10^5 BMMCs were incubated in the presence of 5×10^5 MRL/N-1 cells for 15 h, then 3 µg mL^{-1} each of apoptosis-inducing anti-Fas Ab (BD Bioscience) and anti-hamster IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) were added and the cells were cultured another 6 h. Cells in a suspension were washed with PBS and 500 µL of PE-conjugated annexin V and 7-AAD (BD Bioscience) in a calcium containing buffer was added. After incubation for 10 min at room temperature, the samples were immediately analyzed using a FACSCalibur flow cytometer (BD Bioscience). Apoptosis of electrically gated mast cells were analyzed with CellQuest software (BD Bioscience).

RT-PCR, vectors and transduction: Total RNA was isolated from MRL/+splenocytes using Trizol

(Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 µg of total RNA using Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. RT-PCR was performed using KOD plus DNA polymerase (Toyobo CO., Ltd., Osaka, Japan) and the following specific set of primers for FasL with BamHI and EcoRI restriction sites: 5'-AAGGATCCACCTGAGTCTCCTCCACAAGG-3' and 5'-AAGAATTCATTCCTGGTGCCCATGATAAAG-3', respectively. The PCR products were subcloned into the sequence vector pCR4Blunt-TOPO (Invitrogen). The sequences of the inserts were confirmed by sequencing performed using the BigDye Terminator v3.1 cycle sequencing kit and the ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA). The retrovirus vector pMX was kindly provided by Dr. Toshio Kitamura (University of Tokyo, Institute of Medical Science, Tokyo, Japan). FasL and bacterial-β-galactosidase (LacZ) cDNA was subcloned into pMX to generate the pMXFasL and pMXLacZ plasmids, respectively. To generate FasL and LacZ transfectants of MRL/N-1 cells, MRL/N-1.FasL and MRL/N-1.LacZ cells, pMXFasL or pMXLacZ and pCMV-VSV-G (kindly provided by Dr. Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan) plasmids were transfected into Plat-gp packaging cells (kindly provided by Dr. Toshio Kitamura) with FuGENE6 (Roche Molecular Biochemicals, Basel, Switzerland). MRL/N-1 cells were incubated with the viral supernatant and 10 µg mL^{-1} polybrane (Sigma, St. Louis, MO) for 48 h and cell clones were established by limiting dilution method. The cell surface expression of FasL in the cell clones was detected by FACSCalibur flow cytometry. The expression of LacZ in the cell clones was detected by chlorophenol red β-galactoside (CPRG) colorimetric assay as previously described[14]. The pMX empty vector transfectants, i.e., MRL/N-1.(-) cells, were also generated in the same manner. Integration of the vector in the cell clones was confirmed by genomic PCR performed using Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) with the vector specific primer set 5'-CGTCAGTATCGGCGGAATTC-3' and 5'-CTACAGGTGGGGTCTTTCATTCC-3'.

CPRG colorimetric assay: MRL/N-1.LacZ cells were cultured with BMMCs in 96 well plates for 36 h and lyzed with 100 µL of CPRG solution (0.15 mM CPRG, 100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40 in PBS). LacZ activity was determined by colorimetric assay for CPRG substrate conversion by reading absorption of each well at 540 and 620 nm for reference using microplate reader NJ-9000 (Nunc, Roskilde, Denmark).

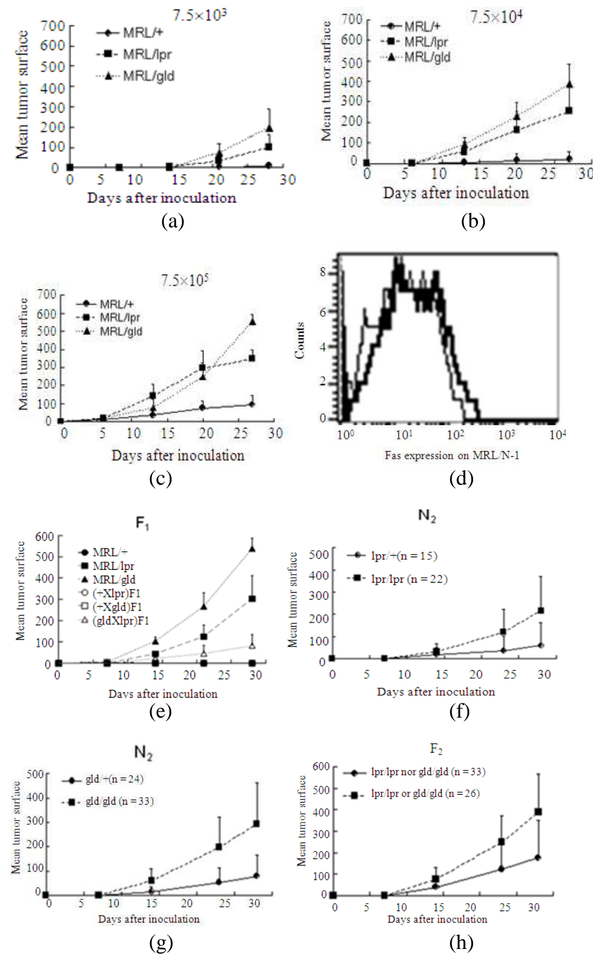

Fig. 1: MRL/N-1 cells were rejected by the MRL/+ mice but not by the MRL/lpr or MRL/gld mice. MRL/+ (filled circles), MRL/lpr (filled squares) and MRL/gld (filled triangles) mice were inoculated with the indicated number of MRL/N-1 cells (a-c). Thereafter, the tumor growth was monitored for 4 weeks. Fas expression in the MRL/N-1 tumor cells was analyzed by flow cytometric analysis (d). Narrow and heavy lines indicate the control and anti-Fas mAb staining, respectively, in the MRL/N-1 cells. MRL/+(filled circles), MRL/lpr (filled squares) and MRL/gld (filled triangles), (MRL/+X MRL/lpr) F_1 (open circles), (MRL/+X MRL/gld) F_1 (open squares) and (MRL/gld X MRL/lpr) F_1 (open triangles) mice were inoculated with 7.5×10^4 MRL/N-1 cells (e): ((MRL/+X MRL/lpr) X MRL/lpr) N_2 mice (f), ((MRL/+X MRL/gld) X MRL/gld) N_2 mice (g) and ((MRL/gld X MRL/lpr) X (MRL/gld X MRL/lpr)) F_2 mice (h) were inoculated with 7.5×10^4 MRL/N-1 cells. Data are presented as mean + SD

RESULTS

Rejection of MLR/N-1 tumor was not allogeneic-recognition but Fas-FasL axis dependent: The MRL/N-1 tumor cells that were inoculated subcutaneously in the MRL/+ mice were rejected. On the other hand, these cells were accepted by the MRL/lpr and MRL/gld mice, i.e., the Fas-deficient and FasL-deficient mice, respectively (Fig. 1a-c). Although the MRL/N-1 cells are syngeneic tumor cells derived from MRL/gld mice, these cells are rejected by the FasL-intact MRL/+ mice. Therefore, it should be verified whether the rejection of MRL/N-1 cells by the MRL/+ mice was mediated via apoptotic signals from the Fas antigen on the cell surface of MRL/N-1 cells. Fas expression in MRL/N-1 cells was analyzed by flow cytometry (Fig. 1d). Fas antigens were not expressed in MRL/N-1 tumor cells. Since MRL/N-1 tumor cells were derived from the MRL/gld mouse spleen, MRL/N-1 cells could not express functional FasL. Thus, the Fas- and FasL-deficient recipients, i.e., the MRL/lpr and MRL/gld mice, respectively, were susceptible to the syngeneic MRL/N-1 tumor, suggesting that this tumor rejection is dependent on the Fas-FasL axis in the recipient, but not in the tumor cells.

The MRL/N-1 tumor cells were rejected in (MRL/+ X MRL/lpr) F_1, (MRL/+ X MRL/gld) F_1 and (MRL/gld X MRL/lpr) F_1 mice (Fig. 1e). If MRL/N-1 cells were recognized as allogeneic graft and rejected by MRL/+ mice, these F_1 mice will accept MRL/N-1 cells. However, these mice rejected the cells, indicating that the possibility of allo-recognition was ruled out. In addition, the tumor rejection pattern of N_2 and F_2 mice derived from MRL/+, MRL/lpr and MRL/gld mice revealed that the rejection was tightly linked to the lpr and gld loci and that the inheritance mode was recessive (Fig. 1f-h, $p = 0.0077$ (f), 0.000012 (g), 0.000098 (H) on the data of the day 28, Mann-Whitney U test). These data indicate that the complete Fas-FasL axis in the recipient was essential for the rejection of syngeneic tumors.

We evaluated the role of $CD4^+$ and $CD8^+$ T and Natural Killer (NK) cells from MRL/+ mice in the rejection of MRL/N-1 cells. In MRL/+ mice, anti-CD8 mAb treatment abrogated the tumor rejection (Fig. 2a). Meanwhile, anti-CD4 mAb treatment had no effect on the rejection. Anti-asialo GM1 (AGM1) Ab treatment partially altered the tumor rejection in MRL/+ mice. A similar Ab treatment study in MRL/lpr and gld mice had no effect as expected (Fig. 2b and c). Thus, the antibody depletion study determined the effector cells in the MRL/+ mice.

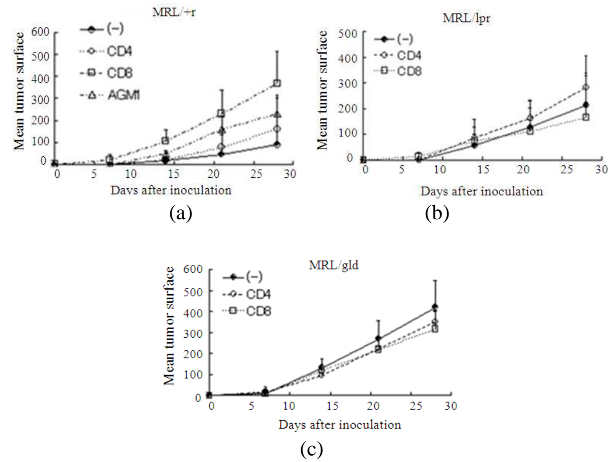

Fig. 2: $CD8^+$ T cells are responsible for the rejection of MRL/N-1 tumor. MRL/+ (a), MRL/lpr (b) and MRL/gld (c) mice were pretreated with anti-CD4 (open circles, GK1.5) or anti-CD8 (open squares, 53-6.72) mAbs or anti-asialo GM1 Ab (open triangles) and inoculated subcutaneously with 7.5×10^4 MRL/N-1 cells. Data are presented as mean + SD

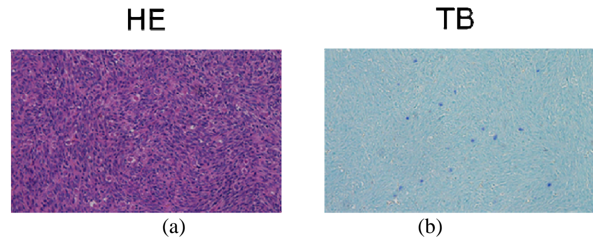

Fig. 3: Histological analysis of mast cell infiltration into MRL/N-1 tumor. Microscopic images of MRL/N-1 tumor stained with hematoxylin and eosin (HE; a) and toluidine blue (TB; b) grown *in vivo* in MRL/lpr mice

Mast cells abrogate the rejection of MRL/N-1 tumor cells by MRL/+ mice: Histological analysis of the MRL/N-1 tumors showed a malignant fibrous histiocytoma-like configuration (Fig. 3a). Toluidine blue staining revealed the focal accumulation of mast cells in MRL/N-1 tumors transplanted into MRL/lpr mice (Fig. 3b). These data suggest that mast cells can be involved in tumor growth in MRL/lpr mice.

To analyze the roles of mast cells in the tumor, MRL/+ mice were inoculated with a mixture of Bone Marrow-Derived Mast cells (BMMCs) and MRL/N-1 tumor cells (Fig. 4a). MRL/+ mice implanted with a mixture of MRL/N-1 cells and BMMCs developed

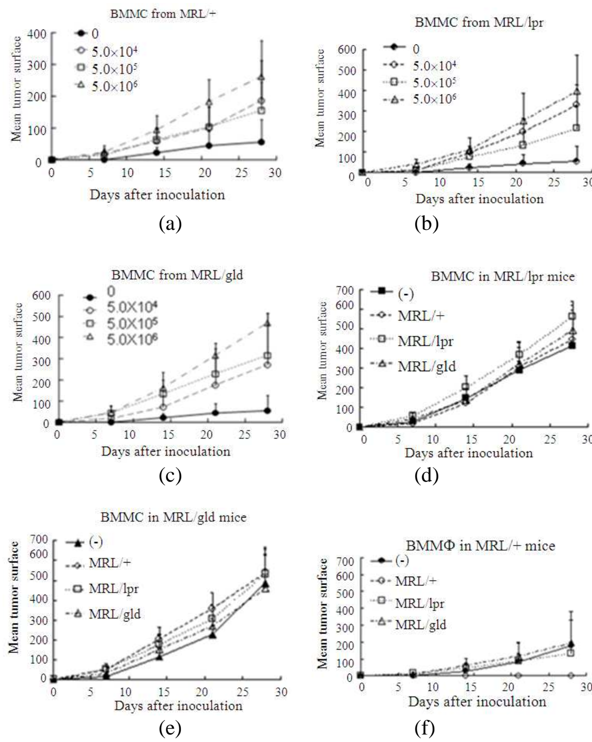

Fig. 4: Mast cells abrogated tumor rejection in MRL/+ mice. MRL/+ mice were inoculated with 7.5 X 104 MRL/N-1 cells and the indicated numbers of BMMCs derived from MRL/+ (a), MRL/lpr (b) and MRL/gld (c) mice. MRL/lpr (d) and MRL/gld (e) mice were inoculated with 7.5×104 MRL/N-1 tumor cells and 5.0×106 BMMCs derived from MRL/+, MRL/lpr and MRL/gld mice. MRL/+mice were inoculated with 7.5×104 MRL/N-1 tumor cells and 5.0×106 of BMMΦ derived from MRL/+, MRL/lpr and MRL/gld mice (f). Data are presented as mean + SD

solid tumors in a dose-dependent manner. The effects of BMMCs derived from MRL/+, lpr and, gld mice on the MRL/+ mice were similar (Fig. 4a-c). MRL/N-1 cells that were implanted with each of the 3 abovementioned types of BMMCs showed similar development in the MRL/lpr and gld mice to that of MRL/N-1 cells without BMMCs (Fig. 4d and e). In contrast, bone marrow-derived macrophages (BMMΦs) had no effect on tumor growth when implanted with MRL/N-1 tumor cells in MRL/+ mice (Fig. 4f). Taken together, these results indicate that BMMCs abrogate the rejection of MRL/N-1 tumor cells by MRL/+ mice.

To elucidate if BMMCs accelerate the growth rate of MRL/N-1cells, cell proliferation studies were performed. The co-cultured MRL/N-1cells with BMMCs proliferate as well as MRL/N-1 cells without co-cultured cells (Fig. 5a-c). BMMCs derived from

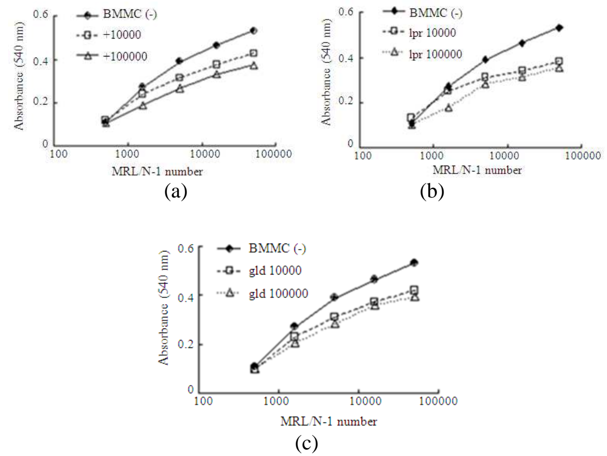

Fig. 5: Mast cells did not accelerate the proliferation of MRL/N-1 cells. Indicated numbers of MRL/N-1.LacZ cells were cultured without or with 1×10^4 or 1×10^5 of BMMCs derived from MRL/+ (a), lpr (b), or gld (c) mice in 96 well plates for 36 h and lyzed with 100 μL of CPRG solution. LacZ activity was determined by colorimetric assay for CPRG substrate

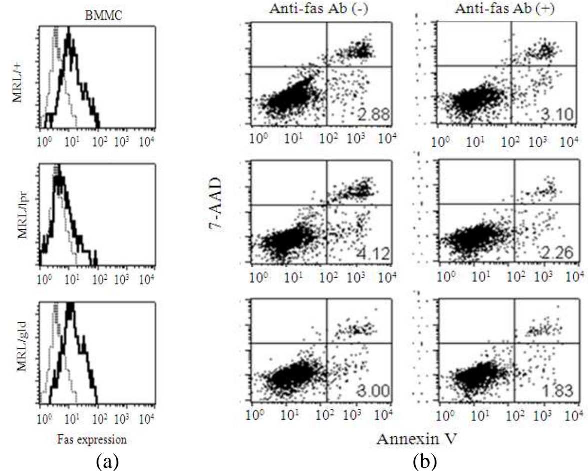

Fig. 6: (a) Mast cells are resistant for Fas-mediated apoptosis. Fas expression in BMMCs from MRL/+, MRL/lpr and MRL/gld mice was analyzed by flow cytometric analysis. (b) Narrow and heavy lines indicate the control and anti-Fas mAb staining, respectively. BMMCs from MRL/+, MRL/lpr and MRL/gld mice were incubated in the presence or absence of anti-Fas Ab after the preincubation with MRL/N-1 cells. The percentage of apoptotic cells was measured by early apoptotic events (annexin V+, 7-AAD)

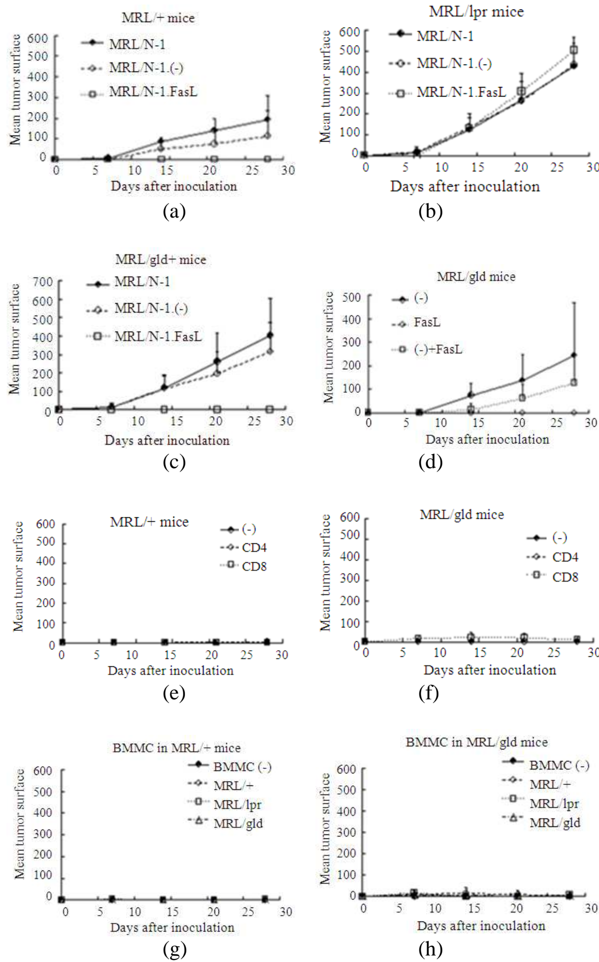

Fig. 7: Effects of FasL-transfection on the rejection of the MLR/N-1 tumor. MRL/+ (a), MRL/lpr (b) and MRL/gld (c) mice were inoculated with 7.5×10^4 FasL-transfected MRL/N-1.FasL cells, empty vector transfectant MRL/N-1.(–) cells and MRL/N-1 cells. MRL/gld mice were inoculated with the mixture of 3.2×10^4 MRL/N-1.FasL and 3.2×10^4 MRL/N-1.(–) cells (d). MRL/+ (e) and MRL/gld (f) mice were pretreated with anti-CD4 (open circles, GK1.5) or anti-CD8 (open squares, 53-6.72) mAbs and inoculated subcutaneously with 7.5 X 10^4 MRL/N-1.FasL cells. MRL/+ (g) and MRL/gld (h) mice were inoculated with 7.5×10^4 MRL/N-1 cells and 5.0×10^6 of BMMCs derived from MRL/+, MRL/lpr and MRL/gld mice. Data are presented as mean + SD

MRL/+, MRL/lpr, or MRL/gld mice had no effects on the proliferation rates of MRL/N-1 cells. These data suggest that mast cells did not accelerate the proliferation of MRL/N-1 cells *in vivo*. The expression of Fas was observed in the BMMC from MRL/+ and MRL/gld mice, but not from MRL/lpr (Fig. 6a). As similar observation was reported from previous studies[15,16], BMMCs from MRL/+, MRL/lpr and MRL/gld mice were resistant for the Fas-mediated apoptosis (Fig. 6b), even after the pre-incubation with MRL/N-1 cells.

Role of Fas-FasL axis in the induction of tumor rejection: FasL was transfected into MRL/N-1 cells to elucidate the role of the Fas-FasL axis in tumor rejection mechanisms. The FasL transfectants of the MRL/N-1 cells, namely MRL/N-1.FasL cells, were implanted into the MRL/+, lpr and gld mice (Fig. 7a-c). The MRL/N-1.FasL cells were rejected by the MRL/+ and gld mice but not by the MRL/lpr mice. MRL/gld mice implanted with a mixture of MRL/N-1.FasL and MRL/N-1.(-) cells developed relatively smaller tumors compared with MRL/N-1. FasL cells (Fig. 7d). In MRL/+ and MRL/gld mice, anti-CD8 or anti-CD4 mAb treatment did not abrogate the tumor rejection (Fig. 7e and f). MRL/+ and MRL/gld mice implanted with a mixture of MRL/N-1.FasL cells and BMMCs did not develop any tumor (Fig. 7g and h). These data indicate that the expression of FasL in tumor cells completely abrogated the tumor growth in MRL/gld mice.

DISCUSSION

In this study, we showed in implantation experiments that mast cells have the ability to abrogate the rejection of MRL/N-1 tumor cells by MRL/+ mice and that this tumor rejection is mediated by $CD8^+$ T cells and dependent on Fas-FasL axis of MRL/+ mice. Some tumors, especially adenocarcinomas, in humans and experimental animals exhibit infiltration of increased mast cells[17-21]. On the contrary, increased or decreased tumor susceptibility has also been reported in mast cell-deficient mice[22-26]. Therefore, it is still controversy if mast cells contribute to the tumor tolerance. Firstly, our data demonstrated that mast cells could override the rejection of a tumor derived from the mesoderm that is dependant on Fas-FasL axis (Fig. 4a-c). Some reports have suggested that mast cells are involved in angiogenesis[3] and, consequently, promote the development of tumors. Recently, it was also revealed that mast cells increase tumorigenesis[4,5]. In this study, the density of capillaries in MRL/N-1 tumors was comparable with that in tumors generated from MRL/N-1 tumor cells implanted along with BMMCs (data not shown). In addition, when mast cells were cultured along with MRL/N-1 in vitro, the growth rate of MRL/N-1 was not augmented (Fig. 5). Tumorigenesis was not able to be observed in our tumor

implantation experiments. Our data suggest that mast cells do not exert an angiogenic or tumorigenic effect on the MRL/N-1 cells; further, they do not accelerate the proliferation rate of these cells. Instead, it was suggested that mast cells tolerize recipient MRL/+ mice to MRL/N-1 tumor cells.

We also demonstrated that the rejection of the syngeneic MRL/N-1 cells by MRL/+ mice was dependent on the Fas-FasL axis in recipient MRL/+ mice. Fas-deficient MRL/lpr and FasL-deficient MRL/gld mice accepted MRL/N-1 cells (Fig. 1a-c). In both these strains, the Fas-FasL axis is nonfunctional because either Fas or FasL is defective. Since the MRL/N-1 cells were derived from MRL/gld mice (gld-congenic MRL strain[9]), it is possible that the MRL/+ mice recognize MRL/N-1 cells as allogeneic grafts and reject them. The gld gene and its neighboring genes on mouse chromosome 1 are derived from C3H/He; thus, gld can act as an alloantigen of the MRL strain. Because (MRL/+ X MRL/lpr) F_1, (MRL/+ X MRL/gld) F_1 and (MRL/gld X MRL/lpr) F_1 mice rejected the MRL/N-1 cells (Fig. 1e), the possibility of the allogeneic recognition of MRL/N-1 by MRL/+ mice was ruled out. Furthermore, the tumor implantation study using N_2 and F_2 mice derived from MRL/+, MRL/lpr and MRL/gld mice revealed that the rejection of syngeneic tumors is dependent on Fas-FasL axis in recipient mice (Fig. 1f-h).

Depletion of $CD8^+$ T cells in MRL/+ mice totally abrogates tumor rejection of the MRL/N-1 cells (Fig. 2a). Stimulated $CD8^+$ T cells express FasL on their cell surface. Therefore, $CD8^+$ T cells are mainly responsible for the FasL-dependent mechanism for the rejection of MRL/N-1 cells, even though the depletion of NK cells partially abrogates tumor rejection. However, the MRL/N-1 cells did not express Fas (Fig. 1d). Thus, $CD8^+$ T cells in MRL/+ mice are main effector cells for the rejection of MRL/N-1 cells, but the MRL/N-1 cells cannot be the direct target of the Fas-FasL dependant killing mediated by $CD8^+$ T effector cells.

As revealed in our study (Fig. 6), it was reported that BMMCs induced in the presence of IL-3 are expressing the Fas antigen on their cell surface but are not sensitive to Fas-mediated apoptotic signals, because of the expression of the Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein[15,16]. IL-4 or IL-10 sensitizes IL-3-induced BMMCs to Fas-mediated apoptosis[27]. Since the MRL/N-1 cells did not express IL-3, IL-4, or IL-10, but did express the kit ligand (data not shown), they could not sensitize the BMMCs to Fas-mediated apoptosis; however, they could promote the proliferation of mast cells in the tumor. In our study, BMMCs were induced in the presence of IL-3 and were not sensitive to Fas-mediated apoptosis *in vitro* (Fig. 6b), even though they were co-cultured with MRL/N-1 cells before the stimulation with apoptosis-inducing anti-Fas Ab. This resistibility of BMMCs can explain why BMMCs derived from MRL/+, MRL/lpr and MRL/gld tolerized MRL/+ mice to MRL/N-1 cells in a similar manner (Fig. 4a-c). Thus, mast cells cannot be the direct target of the Fas-FasL dependant killing mediated by $CD8^+$ T effector cells.

The MRL/N-1.FasL cells were rejected by the MRL/+ and MRL/gld mice but not by the MRL/lpr mice (Fig. 7a-c). When FasL was supplied from MRL/N-1.FasL cells in MRL/gld mice, Fas-FasL axis become intact in the recipient and tumor cells were rejected. Since recipient cells did not express Fas and were resistant to Fas-mediated apoptosis, MRL/lpr mice did not reject the MRL/N-1.FasL cells. MRL/gld mice implanted with a mixture of MRL/N-1.FasL and MRL/N-1.(-) cells developed smaller tumors (Fig. 7d) and the developed tumor did not express FasL on the cell surface (data not shown), suggesting that MRL/N-1.FasL cells supplying FasL on the cell surface were rejected but MRL/N-1.(-) cells without FasL were not. Since MRL/N-1.FasL cells can provide FasL instead of $CD8^+$ T cells, depletion of $CD8^+$ T cells did not abrogate the rejection of MRL/N-1.FasL cells (Fig. 7e, f). $CD8^+$ T cells are not necessary for the rejection of MRL/N-1.FasL cells, but for the MRL/N-1 cells. BMMCs did not abrogate the rejection of MRL/N-1.FasL cells (Fig. 7g and h), because targets of mast cells would be $CD8^+$ T cells; mast cells would directly or indirectly inhibit the function of $CD8^+$ T cells infiltrated in MRL/N-1 tumors, as it was reported that mast cells inhibit the function of $CD8^+$ T cells infiltrated in allograft[2]. Because the MRL/N-1.FasL cells grew *in vitro* as well as MRL/N-1 or MRL/N-1.(–) cells (data not shown), the transfection of MRL/N-1 cells with FasL did not alter the proliferation rate of the MRL/N-1 cells or did not result in the FasL-mediated destruction of these cells. All of these results clearly indicate that the Fas-FasL axis in the recipient regulates tumor rejection and also suggest that the interaction between mast cells and $CD8^+$ T cells may play some roles in the tumor tolerance. The target cells of $CD8^+$ T cells should be sensitive for Fas-mediated apoptosis and could tolerize the recipient MRL/+ mice, but are still unknown. Since MRL/N-1 cells and mast cells are resistant for Fas-mediated apoptosis, they were eliminated from candidates of target cells. Although there are $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and myeloid cells infiltrated in the tumor (data not shown) other than mast cells, Fas-mediated apoptosis-sensitive cells are still unidentified. These Fas-mediated apoptosis-sensitive cells infiltrated in tumor cells were

totally eliminated by $CD8^+$ T cells with FasL or MRL/N-1.FasL cells and could not tolerize the recipient MRL/+ mice. Since these Fas-mediated apoptosis-sensitive cells infiltrated in the tumor in the MRL/lpr mice did not express Fas and was resistant to Fas-mediated apoptosis, MRL/lpr mice did not reject the MRL/N-1cells. The recognition machinery of the tumor antigens from MRL/N-1 cells are unknown, the mechanisms of the induction of the tolerance in MRL/+ mice are also still vague and it is unclear if MRL/N-1 cells were killed by perforin or other tumor necrosis factor superfamily molecules. These should be further investigated. In addition, our findings raise the possibility that drugs targeting tyrosine kinase c-kit, such as Imatinib, did not inhibit only the function of c-kit on Gastrointestinal Stromal Tumours (GISTs), but also that on mast cells infiltrated in GISTs, because some patients with GISTs which did not express c-kit were effectively cured with Imatnib. A novel therapy targeting mast cells would be useful for sarcoma

CONCLUSION

Mast cells abrogate the rejection of MRL/N-1 tumor cells and that this tumor rejection is mediated by CD8+ T cells and dependent on host Fas-FasL axis.

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