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著者	Iwata Yasunori, Furuichi Kengo, Kitagawa Kiyoki, Hara Akinori, Okumura Toshiya, Kokubo Satoshi, Shimizu Kazuaki, Sakai Norihiko, Sagara Akihiro, Kurokawa Yukie, Ueha Satoshi, Matsushima Satoshi, Kaneko Shuichi, Wada Takashi
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# **Involvement of CD11b<sup>+</sup> GR-1<sup>low</sup> cells in autoimmune disorder in**

## **MRL-*Fas*<sup>lpr</sup> mouse**

Yasunori Iwata, M.D. \*, Kengo Furuichi, M.D. \*, Kiyoki Kitagawa, M.D. \*, Akinori Hara, M.D. \*, Toshiya Okumura, M.D. \*, Satoshi Kokubo, M.D. \*, Kazuaki Shimizu, M.D. \*, Norihiko Sakai, M.D. \*, Akihiro Sagara, M.D. \*, Yukie Kurokawa, M.D. \*, Satoshi Ueha, M.D. <sup>||</sup>, Kouji Matsushima, M.D. <sup>||</sup>, Shuichi Kaneko, M.D. \*, and Takashi Wada, M.D. <sup>¶</sup>

\* Department of Disease Control and Homeostasis, <sup>¶</sup> Department of Laboratory Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, Kanazawa, <sup>||</sup>Department of Molecular Preventive Medicine, University of Tokyo, Tokyo, Japan.

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Short running title; Immunosuppressive monocytes contribute to lupus.

*Corresponding author and reprints request: Dr. Takashi Wada,*  
Department of Laboratory Medicine,  
Institute of Medical, Pharmaceutical and Health Sciences,  
Faculty of Medicine,  
Kanazawa University  
13-1 Takara-machi, Kanazawa 920-8641, Japan  
E-mail: twada@m-kanazawa.jp  
tel +81-76-265-2000 (ext 2498)  
fax +81-76-234-4250

***abstract***

*Objective.* Myeloid derived suppressor cells (MDSCs) have been identified as immunosuppressive cells in tumor related inflammation. However, the pathogenesis of MDSCs for autoimmune disease has not been investigated yet. The aim of this study is to address whether MDSCs contribute to autoimmune organ injury in lupus prone mice.

*Methods.* MDSCs were analyzed by flow cytometric staining of CD11b<sup>+</sup> GR-1<sup>+</sup> in MRL-*Fas*<sup>lpr</sup> mice. CD4<sup>+</sup> T cell proliferation assay was performed by the co-culture with CD11b<sup>+</sup> GR-1<sup>+</sup> splenocytes. The percentage of immunosuppressive cells was examined during disease progression. The expression of chemokine receptor on immunosuppressive cells was analyzed. Moreover, chemotaxis assay was performed.

*Results.* CD11b<sup>+</sup> GR-1<sup>low</sup> cells had a suppressive effect on CD4<sup>+</sup> T cell proliferation, which was restored by an arginase-1 inhibitor. CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased in percentage during disease progression in kidney and blood. The number of migrated CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased in the presence of monocyte chemoattractant protein (MCP)-1/CCL2.

*Conclusion.* We assessed the involvement of CD11b<sup>+</sup> GR-1<sup>low</sup> cells in autoimmune disorder in MRL-*Fas*<sup>lpr</sup> mice. These cells regulate immunological responses via CCL2/CCR2 signaling. The regulation of immunosuppressive monocytes may provide novel therapeutic strategy for organ damage in autoimmune diseases.

## ***Introduction***

Myeloid derived suppressor cells (MDSCs), which show heterogeneous phenotypes including immature granulocytes, monocytes/macrophages (M $\phi$ ), dendritic cells (DCs) and early myeloid progenitors, have been originally identified as immunosuppressive cells in association with tumor expansion. These cells have been reported to express CD11b and GR-1 in mouse models (1-4). In tumor microenvironment, MDSCs inhibit T cell activation via arginase (ARG)-1 and nitric oxidase activation, resulting in tumor growth (4). Moreover, MDSCs play a critical role not only in neoplastic diseases but also in other conditions (5-8). However, the impacts of MDSCs on autoimmune disorder have not been elucidated.

It has been reported that MDSCs exist in peripheral blood and may have immunosuppressive effects in patients with inflammatory bowel disease (8) and melanoma (9). These results indicated that MDSCs may regulate the immune response not only within accumulated organs, but also in systemically. Chemokine system plays critical roles for the migration as well as the activation in various types of cells. Regarding the chemotaxis of MDSCs, recent reports show C-C chemokine receptor 2 (CCR2) regulates the dynamics in tumor environment (10-12).

Autoimmune diseases in MRL/MPJ-*lpr/lpr* (MRL-*Fas<sup>lpr</sup>*) mice resemble to human systemic lupus erythematosus, characterized by the dysregulation of both cellular and humoral immunity (13). In this model, CCR2 has been reported to contribute to the progression of autoimmune kidney injury, followed by the decreased number of macrophage (M $\phi$ ) (14). However, the pathogenesis of MDSCs, especially focused on CCR2 signaling, remains to be investigated. Moreover, it should be elucidated whether MDSCs regulate systemic inflammation as well as peripheral autoimmune organ injury in association with CCR2 signaling.

The aim of the present study is to investigate the phenotype and function of CD11b<sup>+</sup>GR-1<sup>+</sup>

cells in MRL-*Fas*<sup>lpr</sup> mice and to identify the immunosuppressive cells. Moreover, the impact of CCR2 signaling on immunosuppressive cells was examined.

## ***Material and Methods***

### *Mice*

MRL-*Fas*<sup>lpr</sup> mice were obtained from Charles River Japan Inc., Atsugi, Japan. All procedures employed in the animal experiments complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

### *Antibodies*

The following antibodies were used for flow cytometry ; fluorescein isothianate (FITC) conjugated rat anti-mouse CD11b antibody, phycoerythrin (PE) conjugated rat anti-mouse Gr-1 antibody, allophycocyanin (APC) conjugated hamster anti-mouse CD11c antibody, APC conjugated rat anti-mouse CD45R/B220 antibody, biotin conjugated rat anti-mouse I-A<sup>k</sup> antibody (BD Biosciences, San Diego, CA), APC conjugated rat anti-mouse F4/80 antibody (eBioscience, San Diego, CA).

### *Immunohistochemical examination*

Tissues for immunoperoxidase staining were snap-frozen in OCT (Miles Scientific, Naperville, Illinois, USA) and stored at -80°C. CD11b positive cells were examined using biotin conjugated anti-mouse CD11b monoclonal antibodies (BD Biosciences). These positive lesion were identified using the avidin-biotin complex technique (DAKO, Glostrup, Denmark).

### *Cell isolation and separation*

A single cell suspension was obtained from the spleens, kidneys and bone marrow as described previously (15). Following cell isolation, red blood cells were lysed using FACS lysing solution (BD Biosciences). CD4<sup>+</sup> T cells and CD11b<sup>+</sup> cells were purified from spleen using MACS beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. The purity of cells after separation was > 90 %. For separation of CD11b<sup>+</sup> GR-1<sup>+</sup> cells, the purified CD11b<sup>+</sup> cells were resuspended in FACS buffer (1× PBS supplemented with 2mM EDTA and 0.5% bovine serum albumin) and stained with anti CD11b Abs and anti GR-1 Abs. Various myeloid cell populations were sorted by FACSCalibur (BD Biosciences). Cell morphology was examined on May-Giemsa staining (DAKO) in each myeloid cell population.

### *Flow cytometric analysis*

After single cells were collected as described above, the cells were suspended in FACS buffer. Then, cell surface markers were stained with 10% mouse serum blocking. The biotinylated antibodies were visualized with PerCP-Cy5.5 streptavidin (BD Biosciences). FACS data were acquired on FACSCalibur. The percentage of cells expressing cell surface marker were determined by analyzing a minimum of 50000 cells, using FlowJo software (Tree Star, Palo Alto, CA)

### *In vitro cell culture experiment*

Splenic CD4<sup>+</sup> T cells and CD11b<sup>+</sup> GR-1<sup>+</sup> cells were co-cultured as described previously (7). Briefly, CD4<sup>+</sup> T cells and CD11b<sup>+</sup> GR-1<sup>+</sup> cells were purified from MRL-*Fas*<sup>dpr</sup> mice at the age of 12 weeks as described above. These cells were suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) including 100 µg/ml streptomycin and 100 U/ml penicillin. Purified CD11b<sup>+</sup>

GR-1<sup>+</sup> cells were first treated with mitomycin C (Nacalai tesque Inc, Kyoto) at 10 µg/mL for 2 hours at 37°C in 5% CO<sub>2</sub> cell culture incubator. CD4<sup>+</sup> T cells were stimulated by Bio coat anti mouse CD3 coated plate (BD Biosciences) at 1.5 × 10<sup>6</sup> cells/well. CD11b<sup>+</sup> GR-1<sup>+</sup> cells were added to CD4<sup>+</sup> T cells at 1 : 1 or different ratios. To determine the impact of arginase 1 for CD11b<sup>+</sup> GR-1<sup>+</sup> cells, 0.5 mM N(omega)-hydroxy-nor-L-arginine (nor-NOHA) (Carbiochem, Gibbstown, NJ) was added in some experiments. After 48 h, 10 µg/well WST-1 (Premix WST-1 Cell Proliferation Assay System; Takara Bio inc. Tokyo) was added into each well for proliferation assay, and cells were harvested for 4 h. The absorbance was measured at 450 nm with a reference wavelength 650 nm. The proliferative activity of the isolated CD11b<sup>+</sup> GR-1<sup>+</sup> cells was subtracted to determine the activity of CD4<sup>+</sup> T cells.

#### *Chemotaxis assay*

Isolated CD11b<sup>+</sup> GR-1<sup>low</sup> splenocytes were resuspended at 5 × 10<sup>6</sup> cell/mL in RPMI 1640 medium with 0.5 % BSA. The 60 µL cells were added into 8-µm pore size chemotaxis chamber (Chemotaxicell 96; Kurabou, Osaka). Lower chamber of 96-well plate (BD pharmingen) were filled with 100 µL of medium containing 10 ng/mL murine recombinant monocyte chemoattractant protein (MCP)-1 (R&D, Minneapolis, MN). The chamber was incubated for 2 hours at 37°C in 5% CO<sub>2</sub> cell culture incubator. The number of cells which migrated through the filter into lower chamber were counted using cell counter of FACScalibur.

#### *RT-PCR analysis*

Total RNA was purified from isolated CD11b<sup>+</sup> GR-1<sup>low</sup> splenocytes using RNeasy mini kit (Qiagen, Valencia, CA). Reverse transcription was performed with SuperScript II (Invitrogen). Multiple C-C chemokine receptor (CCR) expression was analyzed using MPCR kit for mouse

chemokine receptor CCR set-1 (Maxim Biotec, Inc., San Francisco, CA) according to manufacture's instruction manual.

### *Statistical Analyses*

The data represent the means  $\pm$  SEM. Statistical significance was determined by ANOVA and Kruskal-Wallis analyses. Significance was judged at a  $P$  value of  $<0.05$ .

### **Result**

***MRL-Fas<sup>lpr</sup> mice contained 3 distinct populations of CD11b<sup>+</sup> GR-1<sup>+</sup> cells, which showed the different character in morphology and phenotype.***

To determine whether MDSCs exist in MRL-Fas<sup>lpr</sup> mice, flow cytometric analysis was performed at 10 weeks of age. CD11b<sup>+</sup> GR-1<sup>+</sup> cells contained three distinct populations, showing CD11b<sup>+</sup> GR-1<sup>high</sup>, CD11b<sup>+</sup> GR-1<sup>int</sup> and CD11b<sup>+</sup> GR-1<sup>low</sup> cells (Fig. 1 a). These populations comprised 5.9 $\pm$ 1.0 %, 1.6 $\pm$ 0.1 %, 4.6 $\pm$ 0.3 %, respectively. May-Giemsa stain exhibited that CD11b<sup>+</sup> GR-1<sup>high</sup> cells had lobular shaped nuclei, whereas CD11b<sup>+</sup> GR-1<sup>int</sup> and CD11b<sup>+</sup> GR-1<sup>low</sup> cells were monocytic in morphology (Fig. 1 b). Phenotypic study revealed that CD11b<sup>+</sup> GR-1<sup>high</sup> cells did not express F4/80, CD11c nor B220. CD11b<sup>+</sup> GR-1<sup>int</sup> cells expressed F4/80, but did not express CD11c nor B220. Part of CD11b<sup>+</sup> GR-1<sup>low</sup> cells expressed F4/80, CD11c and B220 (Fig. 1 c). These data suggested that CD11b<sup>+</sup> GR-1<sup>high</sup> cells were granulocyte lineage and that CD11b<sup>+</sup> GR-1<sup>int</sup> cells were F4/80<sup>+</sup> macrophage lineage. CD11b<sup>+</sup> GR-1<sup>low</sup> cells contained heterogeneous populations.

***CD11b<sup>+</sup> GR-1<sup>low</sup> cells suppress T cell proliferation via ARG-1 activity.***



To investigate whether CD11b<sup>+</sup> GR-1<sup>+</sup> cells from MRL-*Fas*<sup>lpr</sup> mice have inhibitory effects on T cell proliferation, each population of CD11b<sup>+</sup> GR-1<sup>+</sup> cells was cultured with CD4<sup>+</sup> T cells at the ratio of 1:1 in anti mouse CD3 coated plate (Fig. 2 a). CD11b<sup>+</sup> GR-1<sup>high</sup> cells had no effect on CD4<sup>+</sup> T cells proliferation, whereas CD11b<sup>+</sup> GR-1<sup>int</sup> cells significantly induced the proliferation of CD4<sup>+</sup> T cells as compared to CD4<sup>+</sup> T cells cultured alone (CD4 only; 0.09±0.01, CD4 + CD11b<sup>+</sup> GR-1<sup>high</sup> cells; 0.08±0.02, CD4 + CD11b<sup>+</sup> GR-1<sup>int</sup> cells; 0.40±0.10). In contrast, CD11b<sup>+</sup> GR-1<sup>low</sup> cells significantly inhibited the proliferation of CD<sup>+</sup>4 T cells (0.03±0.01) (Fig. 2 a). At the increased ratio of CD11b<sup>+</sup> GR-1<sup>low</sup> cells to CD4<sup>+</sup> T cells, 2 : 1, the proliferation of CD4<sup>+</sup> T cells were completely suppressed (CD4 only; 0.38±0.02, CD4 + 2 X CD11b<sup>+</sup> GR-1<sup>low</sup> cells; 0.01±0.00) (Fig. 2 b). However, no inhibitory effect of CD11b<sup>+</sup> GR-1<sup>low</sup> cells was shown at the ratio of 0.2 : 1(0.36±0.01) (Fig. 2 b). To determine the impact of ARG-1 on inhibitory effects of CD11b<sup>+</sup> GR-1<sup>low</sup> cells, the cultured cells were treated with 0.5 mM nor-NOHA, an ARG-1 inhibitor. The supplementation of nor-NOHA restored T cell proliferation (0.25±0.04) (Fig. 2 b). These results implicated that CD11b<sup>+</sup> GR-1<sup>low</sup> cells had the inhibitory effect on CD4<sup>+</sup> T cell proliferation via ARG-1 activation in MRL-*Fas*<sup>lpr</sup> mice.

***The percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells changed according to disease activity in MRL-*Fas*<sup>lpr</sup> mice.***

MRL-*Fas*<sup>lpr</sup> mice show glomerular, tubular and perivascular lesion at 22 weeks age (Fig. 3). To detect the localization of CD11b<sup>+</sup> cells in peripheral organs, immunohistochemistry was performed on the spleens and kidneys at 22 weeks age. In the spleens, CD11b<sup>+</sup> cells were detected at perifollicular lesion (Fig. 3 a). In the kidneys, CD11b<sup>+</sup> cells were observed at perivascular lesion (Fig. 3 b) as well as within glomeruli (Fig. 3 c). The percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells in spleen, bone marrow, kidney and peripheral blood was analyzed during the

disease progression in MRL-*Fas*<sup>lpr</sup> mice. In the spleen and the bone marrow, the percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells significantly increased in early stage of the disease, then decreased according to disease activity (Fig. 3 d, e). In spleen, CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased from 3.5±0.1 % of all splenocytes at 6 weeks of age, to 4.6±0.3 % at 10 weeks of age, then decreased to 2.7±0.5 % at 22 weeks of age. In bone marrow, CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased from 6.7±1.0 % at 6 weeks of age, to 10.8±0.9 % at 14 weeks of age, then decreased to 8.1±0.3 % at 22 weeks of age. In contrast, the percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased during disease progression in the kidney and the blood (Fig. 3 f, g). The percentage increased from 0.1±0.0 % of all kidney cells at 6 weeks of age, to 2.0±0.2 % at 22 weeks of age, and from 2.2±0.5 % at 6 weeks of age, to 15.2±2.8 % at 22 weeks of age in the blood. In MRL<sup>++</sup> mice, the frequency of CD11b<sup>+</sup> GR-1<sup>low</sup> cells did not change in these organs.

***CCR2 signaling contributed to the migration of CD11b<sup>+</sup> GR-1<sup>low</sup> cells.***

To examine whether CCR2 contributes to the chemotaxis of CD11b<sup>+</sup> GR-1<sup>low</sup> cells in MRL-*Fas*<sup>lpr</sup> mice, the expression of CCR2 and MCP-1/CCL2 induced chemotaxis assay were examined. Multiple chemokine receptor expression analysis revealed that CD11b<sup>+</sup> GR-1<sup>low</sup> cells expressed strongly CCR4, CCR5 and weakly CCR2 mRNAs (Fig.4 a). In the chemotaxis assay, MCP-1/CCL2 induced chemotaxis of CD11b<sup>+</sup> GR-1<sup>low</sup> cells (CD11b<sup>+</sup> GR-1<sup>low</sup> cells alone; 1125±41/well, MCP-1/CCL2 added; 1434±25/well) (Fig.4 b).

***Discussion***

In various tumor models, the critical role of MDSCs has been reported. However, the impact of MDSCs on inflammatory disease, especially for the autoimmune organ disease, has not been examined yet. In this study, the proliferation of CD4<sup>+</sup> T cells was markedly inhibited by the

co-cultured with CD11b<sup>+</sup> GR-1<sup>low</sup> cells and restored by ARG-1 inhibitor. These cells were monocytic in morphology and showed heterogeneous populations in phenotype. In spleen and bone marrow, the percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells was increased in early stage of the disease. In kidney and peripheral blood, the percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased according to disease activity. CCL2/CCR2 signaling contributed to the migration of CD11b<sup>+</sup> GR-1<sup>low</sup> cells. Taken all together, these results suggested CD11b<sup>+</sup> GR-1<sup>low</sup> cells as immunosuppressive cells and may contribute to autoimmune response in MRL-*Fas*<sup>lpr</sup> mice via CCL2/CCR2 signaling.

CD11b<sup>+</sup> GR-1<sup>+</sup> cells contained 3 distinct populations in MRL-*Fas*<sup>lpr</sup> mice. Based on morphology and cell surface markers, CD11b<sup>+</sup> GR-1<sup>high</sup> cells are granulocyte lineage. These cells showed no effects on CD4<sup>+</sup> T cells in functional assay. CD11b<sup>+</sup> GR-1<sup>int</sup> cells express F4/80 and activated CD4<sup>+</sup> T cells proliferation, indicating this population as Mφ lineage. CD11b<sup>+</sup> GR-1<sup>low</sup> cells had the suppressive effect on CD4<sup>+</sup> T cells. Therefore, this subset may be responsible for immunosuppressive cells in MRL-*Fas*<sup>lpr</sup> mice. Supporting this notion, some reports exhibited suppressive effects of CD11b<sup>+</sup> GR-1<sup>low</sup> cells in tumor mice models (11, 17). Moreover, the L-arginine metabolism is one of the mechanisms of MDSCs to suppress T cell function (4). In this regard, ARG-1 inhibitor restored CD4<sup>+</sup> T cells function, which was cultured with CD11b<sup>+</sup> GR-1<sup>low</sup> cells in the present study. In addition, CD11b<sup>+</sup> cells were detected in perifollicular lesion of the spleen, indicating that CD11b<sup>+</sup> cells interact with T cells. These results may indicate CD11b<sup>+</sup> GR-1<sup>low</sup> cells as MDSCs in MRL-*Fas*<sup>lpr</sup> mice. However, recent report showed CD11b<sup>+</sup> GR-1<sup>-</sup> F4/80<sup>+</sup> monocytes, which expressed arginase 1, as immunosuppressive M2 Mφ (18-20). Moreover, tumor-infiltrating MDSCs have been reported to bear M1 and M2 Mφ characteristics (11). Therefore, it remains to be resolved to determine distinct and defined markers between MDSCs and M2 Mφ.

The percentage of CD11b<sup>+</sup> GR-1<sup>low</sup> cells increased at 10 weeks of age in spleen, kidney and

blood, suggesting these cells contribute to immune modulation in MRL-*Fas*<sup>lpr</sup> mice. Zhu et al. reported that the percentage of MDSCs in peripheral organs increased after disease induction in experimental autoimmune encephalomyelitis (7). In alopecia areata mice, the injection of MDSCs has been reported to promote hair growth, resulting from inhibiting T cell function (6). These results, together with our present results, indicated that MDSCs play an important role not only in neoplastic diseases but also in inflammatory diseases.

In vitro study, the low expression of CCR2 mRNAs was detected on CD11b<sup>+</sup> GR-1<sup>low</sup> cells. Moreover, MCP-1/CCL2 induced chemotaxis of CD11b<sup>+</sup> GR-1<sup>low</sup> cells. Suggesting this notion, recent reports revealed that some chemokine receptors, including CCR2, CCR5 and CX<sub>3</sub>CR1 were expressed on MDSCs and contribute to chemotaxis (10-12).

In conclusion, we assessed the involvement of CD11b<sup>+</sup> GR-1<sup>low</sup> cells, which were responsible for CD4<sup>+</sup> T cell suppression in MRL-*Fas*<sup>lpr</sup> mice. Immunosuppressive monocytes regulated immunological responses via CCR2 signaling. Further, the regulation of immunosuppressive monocytes may provide novel therapeutic strategy for organ damage in autoimmune diseases.

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### **Figure legend**

#### **Figure 1**

Morphology and phenotype of CD11b<sup>+</sup>GR-1<sup>+</sup> cells in spleen of MRL-*Fas*<sup>lpr</sup> mice. (a) Three distinct populations of CD11b<sup>+</sup> GR-1<sup>+</sup> cells were identified in the spleen of MRL-*Fas*<sup>lpr</sup> mice. They are CD11b<sup>+</sup> GR-1<sup>high</sup>, CD11b<sup>+</sup> GR-1<sup>int</sup> and CD11b<sup>+</sup> GR-1<sup>low</sup> populations. (b) May-Giemsa stain exhibited that CD11b<sup>+</sup> GR-1<sup>high</sup> cells have lobular shaped nuclei, whereas CD11b<sup>+</sup> GR-1<sup>int</sup> and CD11b<sup>+</sup> GR-1<sup>low</sup> cells were monocytic in morphology. (c) Phenotypic study revealed that CD11b<sup>+</sup> GR-1<sup>high</sup> cells do not express F4/80, CD11c nor B220. CD11b<sup>+</sup> GR-1<sup>int</sup> cells express F4/80, but not express CD11c nor B220. Part of CD11b<sup>+</sup> GR-1<sup>low</sup> cells express F4/80, CD11c and B220. Splenocytes from MRL-*Fas*<sup>lpr</sup> mice at 10 weeks of age was analyzed by flow cytometry.

#### **Figure 2**

To investigate whether CD11b<sup>+</sup> GR-1<sup>+</sup> cells from MRL-*Fas*<sup>lpr</sup> mice have inhibitory effect on T cell proliferation, each population of CD11b<sup>+</sup> GR-1<sup>+</sup> cells was co-cultured with CD4<sup>+</sup> T cells at the ratio of 1:1 in anti mouse CD3 coated plate. (a) CD11b<sup>+</sup> GR-1<sup>high</sup> cells had no effect to CD4<sup>+</sup> T cells proliferation, whereas CD11b<sup>+</sup> GR-1<sup>int</sup> cells induced the proliferation of CD4<sup>+</sup> T cells as compared to CD4<sup>+</sup> T cells cultured alone. In contrast, CD11b<sup>+</sup> GR-1<sup>low</sup> cells inhibit the proliferation of CD4<sup>+</sup> T cells. (b) At the increased ratio of CD11b<sup>+</sup> GR-1<sup>low</sup> cells to CD4<sup>+</sup> T cells, 2 : 1, the proliferation of CD4<sup>+</sup> T cells were completely suppressed. However, the inhibitory effect of CD11b<sup>+</sup> GR-1<sup>low</sup> cells were not shown at the ratio of 0.2 : 1. Moreover, the supplementation of nor-NOHA, an ARG-1 inhibitor, restored T cell proliferation. The proliferative activity of the isolated CD11b<sup>+</sup> GR-1<sup>+</sup> cells was subtracted to determine the activity of CD4<sup>+</sup> T cells. # p<0.01; ++ p<0.05



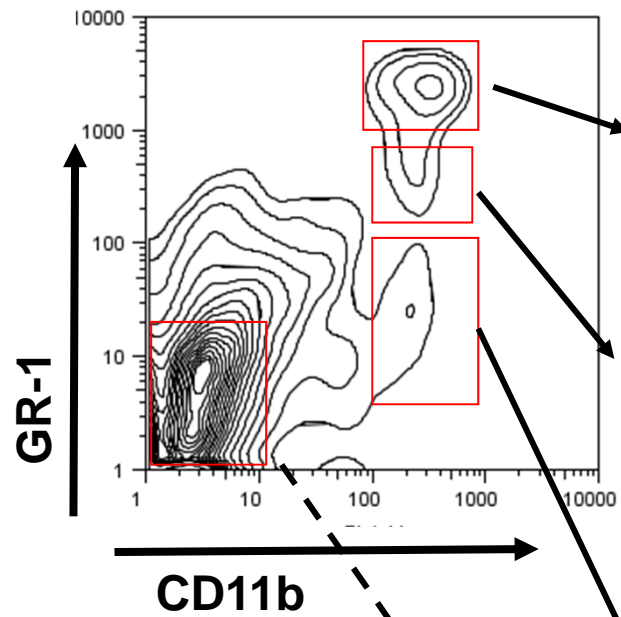
### Figure 3

The localization of CD11b<sup>+</sup> cells and the time course of CD11b<sup>+</sup> GR-1<sup>low</sup> cells were examined in MRL-*Fas*<sup>lpr</sup> mice. MRL-*Fas*<sup>lpr</sup> mice show glomerular hypercellularity, tubular damage (dilatation and casts;\*) and perivascular cell infiltration (arrow heads) at 22 weeks age. (magnification: ×200) (a) In the spleens, CD11b<sup>+</sup> cells were detected at perifollicular lesion. (magnification: ×100) (b) In the kidneys, CD11b<sup>+</sup> cells were observed at perivascular lesion. (magnification: ×100) (c) CD11b<sup>+</sup> cells also localized within glomeruli. (magnification: ×400) Frozen sections were stained with the indirect avidin-biotinylated peroxidase complex method. (d, e) In the spleen and the bone marrow, the frequency of CD11b<sup>+</sup> GR-1<sup>low</sup> cells was significantly increased in early stage of the disease, then decreased according to disease activity. (f, g) In contrast, the frequency of CD11b<sup>+</sup> GR-1<sup>low</sup> cells was increased during disease progression in the kidney and the blood. v; vessel ; # p<0.01; ++ p<0.05 as compared to 6 weeks.

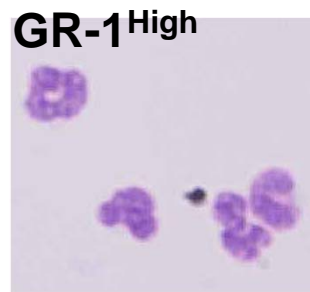
### Figure 4

To test the impact of chemokine receptor for the function of MDSCs, the expression of chemokine receptor and the functional assays were performed. (a) Multiple PCR analysis for chemokine receptor revealed that CD11b<sup>+</sup> GR-1<sup>low</sup> cells expressed strongly CCR4, CCR5 and weakly CCR2 mRNAs. (b) In the chemotaxis assay, MCP-1/CCL2 induced chemotaxis of CD11b<sup>+</sup> GR-1<sup>low</sup> cells. ++ p<0.05

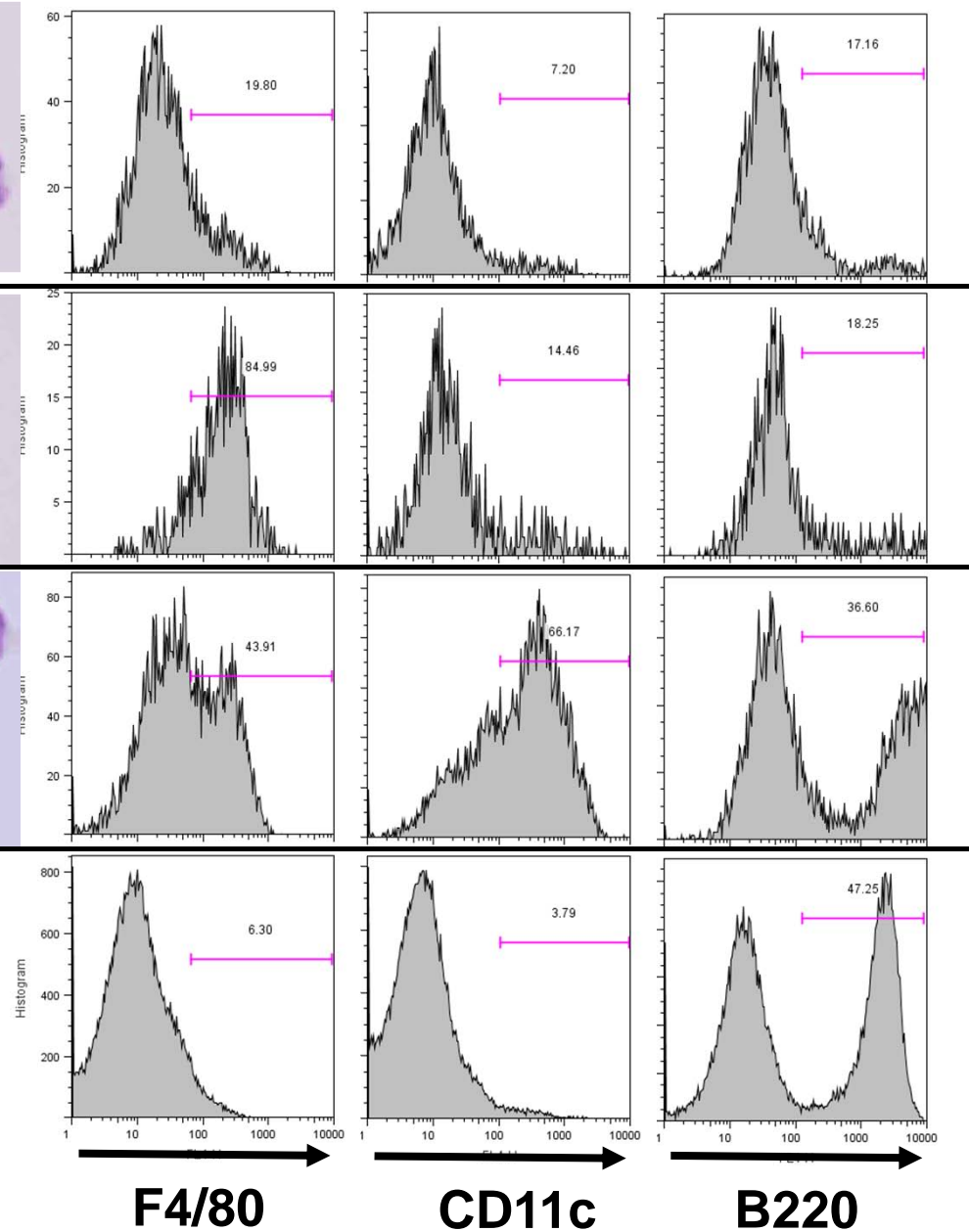
**Fig.1 a**



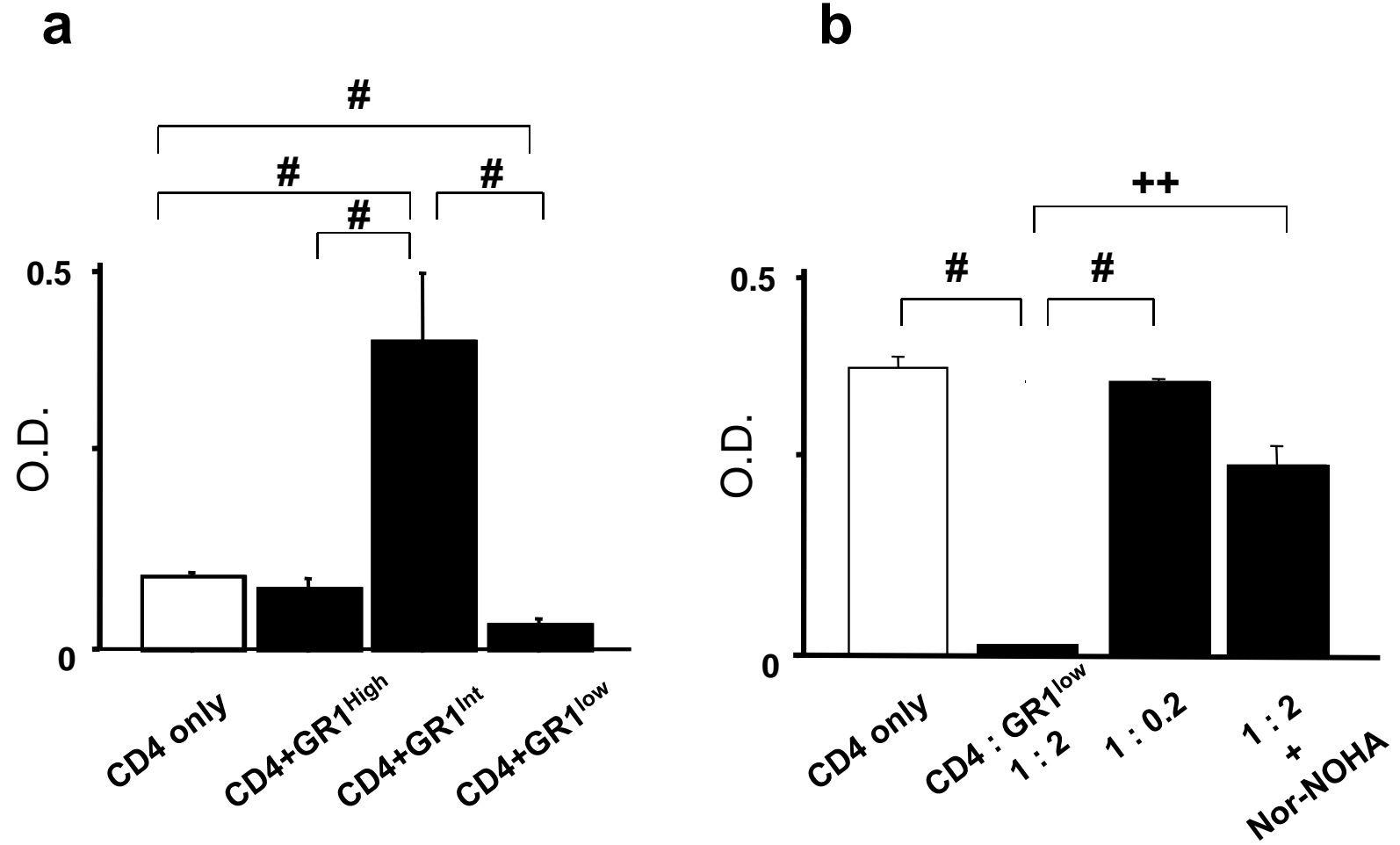
**b**



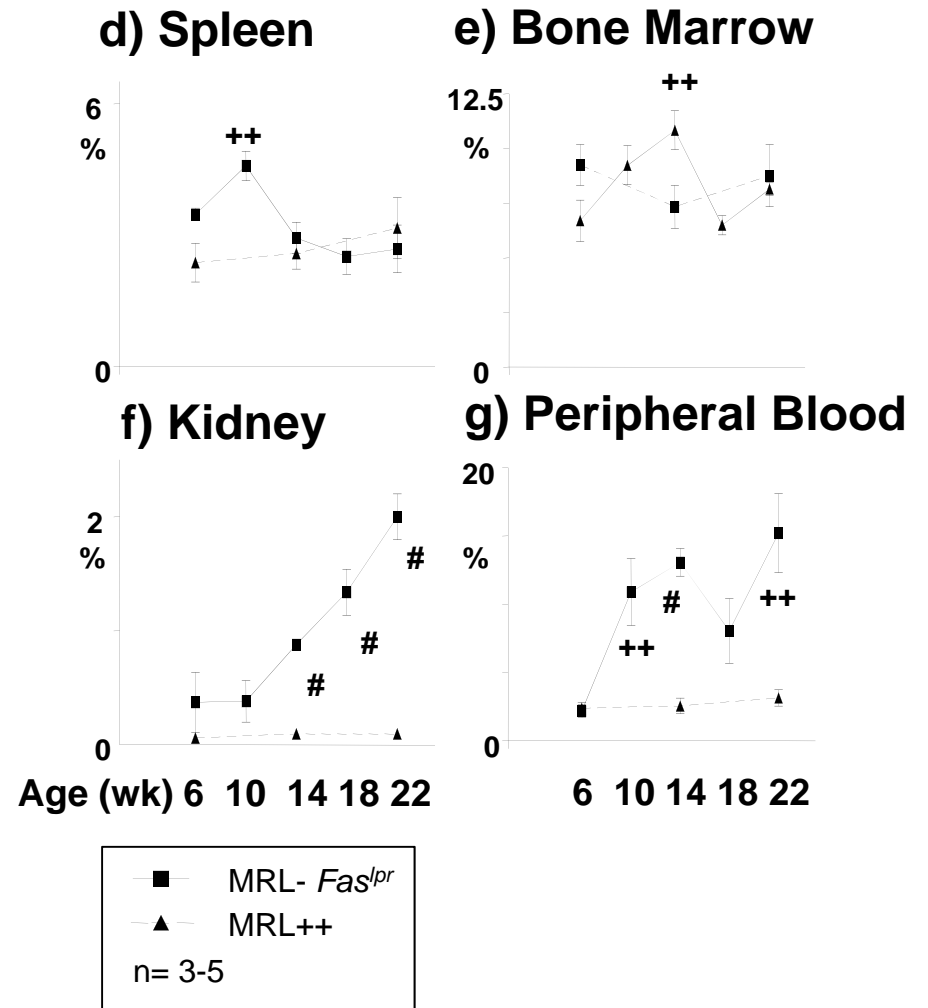
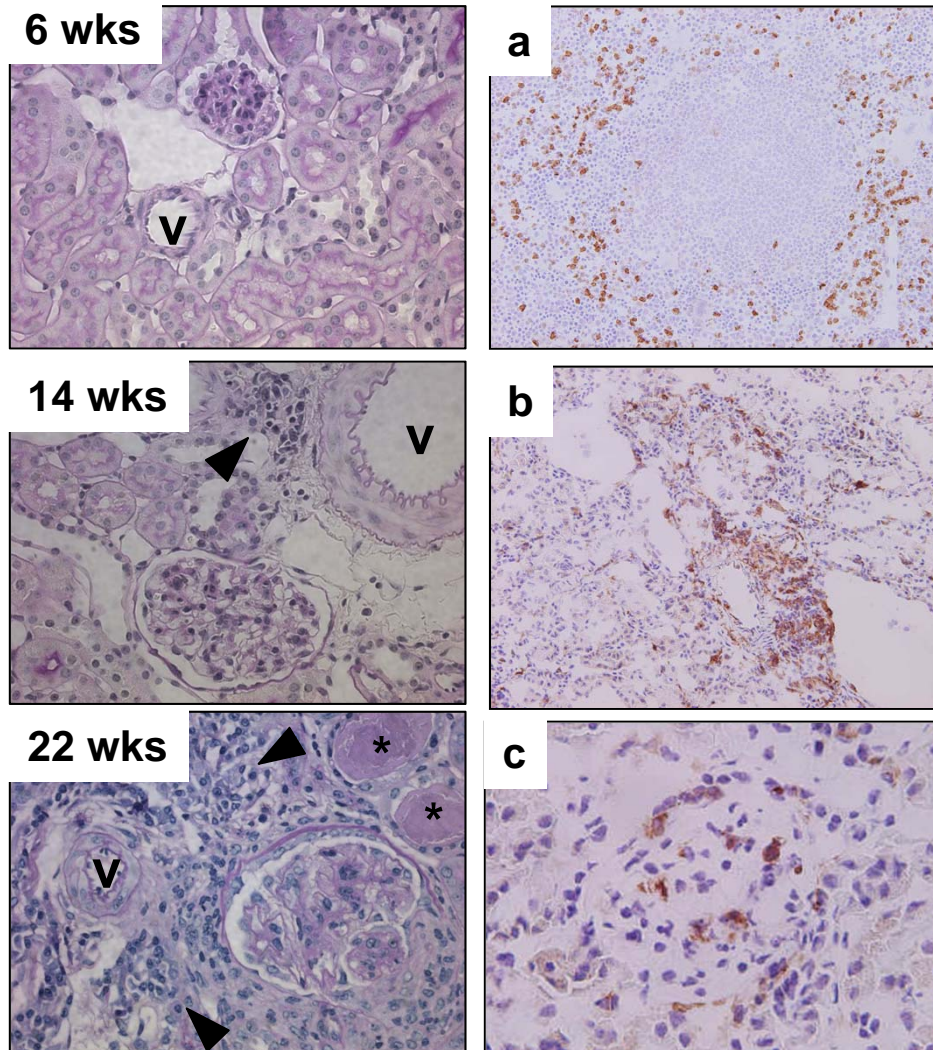
**c**



**Fig.2**

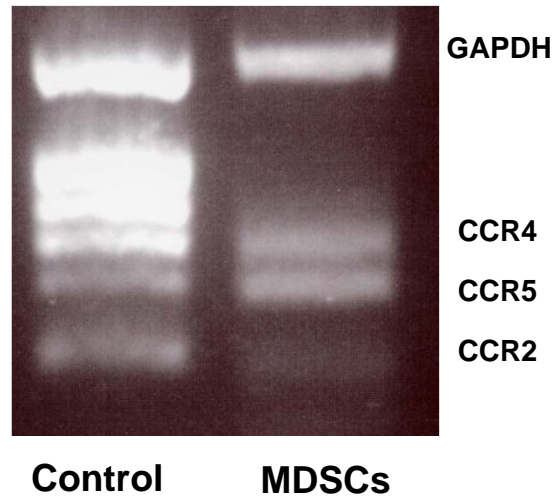


# Fig.3



**Fig.4**

**a**



**b**

