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—Original—

A Dominant Trait Linked to Chromosome 1 in DBA/2 Mice for the Resistance to Autoimmune Gastritis Appears in Bone Marrow Cells

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Abstract: Neonatal thymectomy (NTx) induces autoimmune gastritis (AIG) in BALB/c mice, a model for human type A chronic atrophic gastritis, but not in DBA/2 mice and rarely in CDF1 mice (a hybrid of BALB/c and DBA/2 mice). The aim of this study was to clarify the mechanisms of AIG-resistance in mice bearing the dominant trait of DBA/2. Linkage groups associated with, and cells related to AIG resistance were examined with CDF1-BALB/c backcrosses. Intracellular staining and flow-cytometric bead array for several cytokines were performed on NTx BALB/c mice and NTx DBA/2-chimeric BALB/c mice receiving DBA/2-bone marrow cells. In NTx BALB/c mice, IFN-γ-secreting $CD4^+$ T cells were increased, but not in NTx DBA/2 mice. Because $V\beta6^+$ T cell-bearing mice of half of their backcrosses developed AIG, but the other half of $V\beta6^+$ T cell-negative mice developed scarcely, resistance for AIG generation is associated with the presence of the *Mls-1a* locus on chromosome 1 in DBA/2 mice, which deletes $V\beta6^+$ T cells. NTx DBA/2-chimera BALB/c mice showed dominant production of IL-10 and resistance for AIG, although the deletion of $V\beta6^+$ T cells was found not to be a cause of AIG-resistance from *Mls-1a* locus segregation experiments. Although NTx DBA/2-chimeric BALB/c mice did not suffer from AIG, they brought immediate precursors of T cells for AIG. It is concluded that DBA/2 mice generate bone marrow-derived cells that produce anti-inflammatory cytokines to prevent the activation of AIG-T cells.
Key words: autoimmunity, gastritis, mouse, thymus

Introduction

The establishment of a murine model for organ-specific autoimmune diseases, such as gastritis, oophoritis and thyroiditis, induced in mice by neonatal thymectomy (NTx) at 2 to 4 days after birth [22], has substantially contributed to understanding the mechanisms of establishment and breakdown of self-tolerance. Experimental autoimmune gastritis (AIG), known to be a model for human type A chronic atrophic gastritis, can be easily induced in BALB/c mice by NTx [7, 11, 21]. Murine AIG is mediated by $CD4^+$ T cells transferring the disease

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to syngeneic athymic nude mice [25, 30]. IFN-γ, one of the inflammatory cytokines secreted by a Th1 subset of $CD4^+$ helper T cells, is required during the initiation of AIG [4]. The target antigens of these T cells and autoantibodies in the serum of AIG-bearing mice are H^+/K^+-ATPases on the parietal cells of the stomach [1, 18, 33, 34]. In NTx-sham-operated or normal mice, another subset of $CD4^+$ T cells or regulatory T cells (Tregs) is generated in late ontogeny to prevent AIG-T cell development, and Treg generation is disturbed by early removal of the thymus [3, 31].

Kojima and Prehn [21] have reported that various susceptibility genes, including those encoding the H-2 complex, may be involved in the development of organ-specific autoimmune diseases in NTx mice. Regarding AIG, in contrast to BALB/c mice of a susceptible strain, DBA/2 mice are quite resistant [21, 26, 28]. Because both strains have the same *H-2* (*H-2*^{d}), the *H-2* haplotype may not be related to determining susceptibility to disease development, at least in this strain comparison. This finding suggests that the susceptible or resistant gene(s) for AIG-development would be outside the *H-2* locus.

According to a study using recombinant inbred (RI) strains, CXD2, derived from (BALB/c × DBA/2) F_2 mice [12], demonstrated that RI strains carrying the BALB/c-*Mls-1a* locus on chromosome 1 in association with the Hc locus on chromosome 2 were highly susceptible to NTx AIG [26], although the presence or absence of C5 (Hc) is not under the direct correlation to the development of AIG in the mice of BALB/c-genetic background (Fujii M., master's thesis, Niigata University, 2004). BALB/c mice possess T cells bearing the $V\beta6^+$ T cell receptor that could respond to Mls-1a superantigens [15, 24], and these T cells were increased in number along with AIG development [26]. By contrast, DBA/2 mice do not possess these T cells because Mls-1a antigens, as self, deleted $V\beta6^+$ T cells in the thymus by clonal deletion [12, 24]. Concerning the engagement of $V\beta6^+$ T cell-activation in the development of AIG, Clays *et al.* [7] reported that endogenous Mls-1a antigens and $V\beta6^+$ T cells were not involved in the maintenance of and resistance to AIG but may be implicated in the onset of the disease. Indeed, stimulation of NTx BALB/c mice with Mls-1a antigens increased the incidence of AIG, but not of autoimmune oophoritis [26], although an attempt to remove $V\beta6^+$ T cells using the appropriate antibodies from early phase development failed to support this possibility [27].

The different susceptibilities to AIG may be explained by H^+/K^+-ATPase expression in the thymus of 1-day-old DBA/2 mice being higher than that in the thymus of 1-day-old BALB/c mice [6]. Thus, higher H^+/K^+-ATPase expression in the thymus would cause the gastric clones to be tolerant and not aggressive, although deletion of autoreactive T cells in the thymus could not be completed during the first few days after birth [15, 36], Thus, the precise mechanisms involved in determining the different susceptibilities to AIG are very complicated and remained to be clarified.

In the present study, we examined the possible role of Mls-1a antigens in the onset of AIG using BALB/c-DBA/2 hybridization experiments and concluded that Mls-1a antigens are not related to the pathogenesis. However, suppression of AIG development is closely related to a locus other than that of *Mls-1a* on chromosome 1 of DBA/2 mice. Furthermore, bone marrow-derived cells with the genetic traits of DBA/2 produced more IL-10 than those of BALB/c mice, providing an anti-inflammatory state of DBA/2.

Materials and Methods

Animals and thymectomy

Male and female BALB/c, BALB/c-*nu/nu* and DBA/2 mice were originally purchased from Japan SLC (Hamamatsu, Japan) and maintained on a commercial diet (CE-2, Nihon CLEA, Tokyo, Japan) and mated in our animal facility. All mice had free access to food and tap water and housed on sterilized chaw-chips in 0.5μm filter-cleaned air, but not under specific pathogen free conditions, in a temperature-controlled room (25 ± 1°C) with 12 h of light per day. Both sexes of mice were used, because there was no sex difference in the susceptibility for induction of AIG [26]. The day of birth was taken as day 0 of age. Three-day-old mice were thymectomized as previously described [10]. Briefly, the sternum of infants was cut vertically from the salivary glands to the third rib. Thymic lobes were removed by vacuum suction, and the chest cavity was closed. The chest cavities of all animals were analyzed when the mice were sacrificed, and mice having residual thymi were excluded from the experiments. The diagnosis of AIG was made serologically and histologically when sacrificed, generally at twelve weeks of age for NTx mice, when the disease development is almost completed (data not shown). All animal experiments were performed according to the Guidance for Animal Experiments of Niigata University.

Bone marrow cell transfer for myeloid cell chimerism

Single-cell suspensions from the bone marrow of DBA/2 mice were aseptically prepared by flashing femur and tibia bones with minimum essential medium (MEM) using a 24-gauge needle connected to a syringe. The viability of cells was determined using the trypan blue exclusion test. Twenty million cells suspended in 25 μl were intravenously transferred via the anterior facial veins into BALB/c or BALB/c-*nu/nu* mice within 24 h after birth [14]. To evaluate chimerism in the NTx BALB/c mice, we employed the following tests: deletion of $V\beta6^+$ T cells from peripheral blood [16] and inducibility of local Host-versus-Graft reaction in which swelling of popliteal lymph nodes occurred 7 days after an intradermal injection of spleen cells (2×10^7) of the chimera at a hind foot pad of BALB/c mice as host [13]. Because T cell generation from the bone marrow cell inoculum takes more than 2 weeks [14], there should be no DBA/2 T cells of the inoculum origin in the NTx DBA/2-chimera BALB/c mice.

Diagnosis

The diagnosis for AIG was based on histological and immunohistochemical examinations. For the former, stomachs were removed and fixed in Bouin's fluid or 10% buffered formalin. The specimens of the stomach were embedded in paraffin wax. Four-micron thick sections were cut and stained with hematoxylin and eosin (HE), and then were examined by light microscopy. For the immunohistochemical examination, sera from NTx mice were subjected to detect anti-parietal cell autoantibodies, using normal BALB/c mouse stomach sections as previously described [10]. Briefly, dewaxed sections of normal stomach were fixed in Bouin's fluid, rehydrated, incubated overnight with diluted serum (1:100 in PBS) obtained from NTx mice at 4°C, and then washed with PBS three times. All sections were first incubated with goat IgG (Rockland, Gilbertsville, PA) to prevent nonspecific binding. The sections were thereafter incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Calbiochem, San Diego, CA) for 30 min at room temperature. The sections were examined by fluorescence microscopy.

Flow cytometric analysis

Approximately 10^6 spleen cells were first incubated with normal goat IgG to prevent nonspecific binding of antibodies, and then were incubated with phycoerythrin (PE)-labeled rat anti-mouse CD4 antibodies, FITC-labeled hamster anti-mouse TcRβ chain (PharMingen, San Diego, CA, USA), rat anti-mouse Vβ6 antibodies (44-22-1 culture supernatant) or anti-mouse Vβ8.1,2,3 antibodies (F23.1 culture supernatant), followed by PE-labeled goat anti-rat IgG antibodies (Beckman Coulter, Miami, FL, USA). Stained cells were analyzed using an EPICS-XL flow cytometer and EXPO 32 software (Beckman Coulter, Miami, FL).

Culture of spleen cells

Single spleen cells were prepared by teasing the organ and passing the fragments through a 200-mesh stainless steel sieve, followed by lysing red blood cells using ammonium-chloride-lysing buffer. Spleen cells were resuspended in RPMI-1640 medium containing 10% fetal calf serum at a concentration of 4×10^6/ml and cultured in hamster anti-mouse CD3 antibody (145-2C11)-coated 60-mm dishes for 3 days.

Intracellular staining of IFN-γ and cytokine assay using flow-cytometric bead array

To evaluate the production of several cytokines, intracellular staining and flow-cytometric bead array analysis were performed. Spleen cells *in vitro* activated by anti-mouse CD3 antibodies or PMA plus ionomycin were harvested and stained. Monensin (2 μM) was added during the last 4 h before harvesting cells to prevent cytokine secretion. Harvested cells were stained with PE-labeled anti-CD4, and then were fixed using 4% paraformaldehyde-PBS and permeabilized using a permeabilization buffer (50 mM NaCl, 5 mM EDTA, 0.02%NaN_3 and 0.5% Triton X-100 in double-distilled water). Fixed and permeabilized cells were stained with FITC-labeled rat anti-IFN-γ and anti-IL-10 antibodies (PharMingen, San Diego, CA). Stained cells were analyzed using an EPICS-XL flow-cytometer and EXPO 32 software (Beckman Coulter, Miami, FL).

Cultured cell supernatants were collected and frozen at −80°C until ready for analyses. We used 25 μl of the supernatants for the flow-cytometric bead array system (Bender Medsystem, Vienna, Austria). Briefly, 25 μl of each sample or recombinant standards were mixed with 25 μl of the mixed capture beads with distinct fluorescence intensities, and with biotin-conjugated anti-mouse IL-10, IL-17, and GM-CSF antibodies. The samples were incubated at room temperature for 2 h in the dark. After incubation with biotin-conjugated antibodies, samples

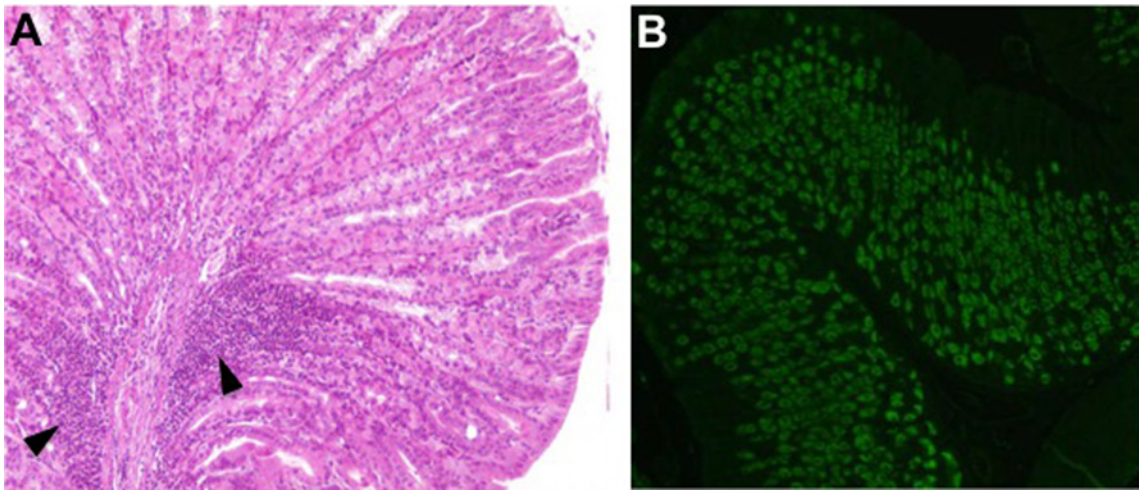

Fig. 1. Histological appearance of gastric mucosa with autoimmune gastritis (AIG). Inflaming, mainly mononuclear cells (*arrowheads*), are infiltrating the submucosa and epithelium (A). Presence of autoantibodies to parietal cells in the sera from AIG mice is demonstrated by indirect immunofluorescence staining of normal gastric mucosa (B). Original magnification, ×100.

were washed, followed by incubation with streptavidin-PE at room temperature for 1 h in the dark. The samples were then washed and resuspended in the assay buffer before acquisition on the EPICS-XL flow cytometer (Beckman Coulter, Miami, FL). Following the acquisition of sample data by flow cytometry, the cytokine concentrations were calculated using cytometric bead array analysis software (Bender Medsystem).

Calculation of the relative amount of intracellular cytokines

To evaluate the capability for production of IFN-γ and IL-10 in $CD4^+$ T cells, we calculated the relative amount of cytokines in the individual $CD4^+$ T cells based on the fluorescent intensity of a given cytokine appearing in the flow cytometric dot plots. At first, we determined the positive level or standard base line cytokine level using negative control cells and then measured the fluorescence level of each cell-dot point in the test cell group.

AIG-adoptive transfer

Spleen cells of NTx BALB/c mice with AIG and those of non-AIG-bearing BALB/c and DBA/2-chimeric BALB/c mice were prepared in MEM. These spleen cells at doses mentioned in the text were injected intraperitoneally into BALB/c-*nu/nu* mice. Mice were sacrificed 2 months later and examined serologically and histologically.

Statistical analysis

The probability of the incidence of AIG was calculated using Fisher's exact probability test. Differences were determined by the paired *t* test or Student's *t* test. A *P* value less than 0.05 was deemed to be a statistically significant difference.

Results

Increase of IFN-γ-secreting $CD4^+$ T cells in AIG-prone NTx BALB/c mice but not in AIG-resistant DBA/2 mice

The strain difference of the incidence of AIG between BALB/c and DBA/2 mice, both being the same *H-2* haplotype, was confirmed. Thus, 33% of NTx BALB/c mice developed AIG with anti-parietal cell autoantibodies as described by indirect immunofluorescence staining of normal stomachs, as always developed AIG-lesions accompanied by infiltration of mononuclear cells into the gastric mucosa and vice versa (Fig. 1 and Table 1). On the other hand, none of the NTx DBA/2 mice developed AIG, and only 8% of their hybrid, CDF1 mice, developed AIG after NTx (Table 1). BALB/c mice are susceptible to AIG, whereas DBA/2 mice are completely resistant, and the latter phenotype would be a dominant trait. Any mouse producing autoantibodies, as detected by immunofluorescence staining, always developed AIG lesions accompanied by mononuclear cell infiltration into gastric mucosa.

Table 1. AIG incidence in NTx BALB/c, DBA/2, and BALB/c-DBA/2 hybrid mice

Strains	AIG	Non-AIG	Incidence	(%)	Probability (*P*)	
BALB/c	90	185	90 / 275	(33)	<0.001	<0.001
DBA/2	0	56	0 / 56	(0)	NS	
CDF1	5	58	5 / 63	(8)		

Mice were thymectomized 3 days after birth, and serological and histological examinations were performed at 3 months of age. NS: non-significant difference.

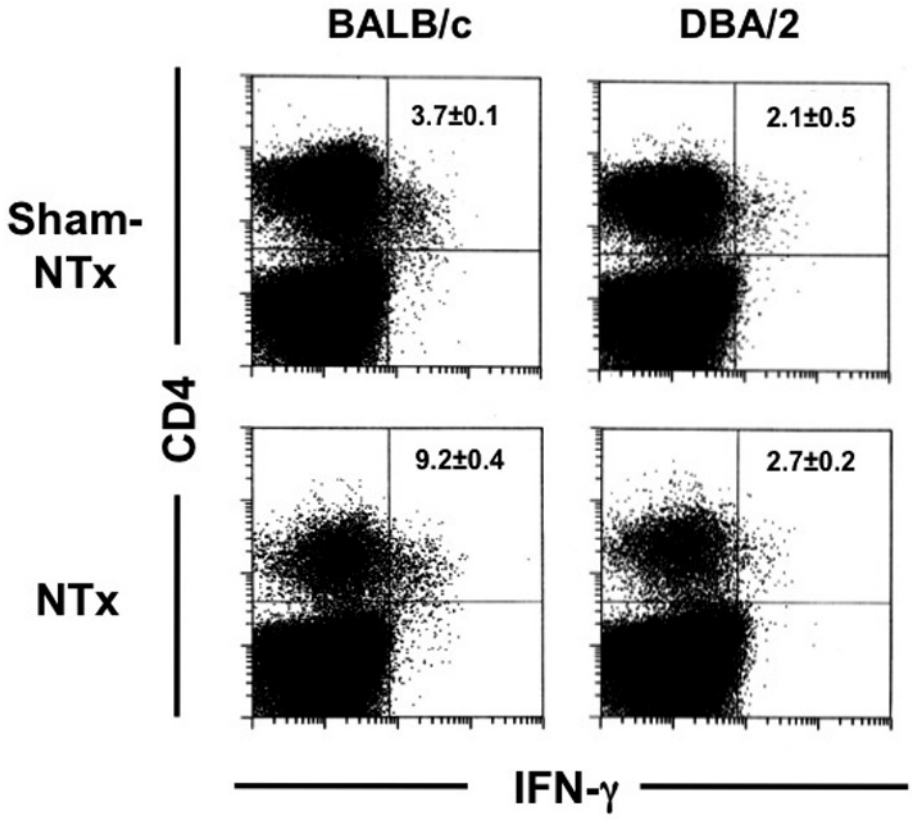

Fig. 2. Increased production of IFN-γ by $CD4^+$ T cells in the spleen of BALB/c mice, but not DBA/2 mice, after neonatal thymectomy. Spleen cells of 4-week-old sham-operated BALB/c (n=5) and DBA/2 (n=3) mice and age-matched NTx BALB/c (n=8) and NTx DBA/2 (n=8) mice that had been pre-activated with PMA and ionomycin, were examined by intracellular staining for IFN-γ. Numbers in figures represent the mean percentages of IFN-γ-producing $CD4^+$ T cells ± SD.

NTx is known to cause lymphopenia and to decrease naturally occurring Treg as observed in many reports [23, 28]. However, relationships between the strain difference in AIG susceptibility and possible changes in IFN-γ production in peripheral $CD4^+$ T cells, which are considered effector cells in AIG, after NTx, have not been well studied. Here, we assessed IFN-γ production by spleen $CD4^+$ T cells *in vitro* at an early age (4 weeks) in normal and NTx mice. As shown in Fig. 2, $CD4^+$ T cells of both normal BALB/c and DBA/2 mice and $CD4^+$ T cells of NTx DBA/2 mice expressed only a negligible level (2–4%) of IFN-γ-positive $CD4^+$ T cells, whereas the $CD4^+$ T cells of NTx BALB/c mice were up-regulated, the percentage of IFN-γ-producing $CD4^+$ T cells was significantly increased (9.2 ± 0.4%).

Table 2. Flow cytometric analyses of cellular components of lymphatic organs in NTx BALB/c mice with or without AIG

Lymphatic organs	Positive cells / T cells (%)			
	V*β*6		V*β*8	
	AIG	Non-AIG	AIG	Non-AIG
Spleen	14.0 ± 2.0 *	8.9 ± 1.1	19.8 ± 0.8	18.4 ± 0.7
Paragastric LN	9.7 ± 0.9	N.D.	20.2 ± 1.2	N.D.
Axillary LN	9.9 ± 1.8	10.1 ± 1.4	22.2 ± 1.2	18.5 ± 1.2

Single cells obtained from the spleen, paragastric lymph node (LN), and axillary LN of NTx BALB/c mice with (n=7) or without (n=3) AIG were stained with anti-V*β*6 or V*β*8 antibodies and analyzed by flow cytometry. Percentage of fluorescence-positive cells in the T-cell population is expressed as the mean ±SD. *: $P<0.05$, N.D.: not determined.

Table 3. AIG incidence in NTx BALB/c-DBA/2 hybrid mice

Mls-1a phenotypes	AIG	Non-AIG	Incidence	(%)	Probability (*P*)
Mls-1a$^-$	14	30	14 / 44	(32)	<0.002
Mls-1a$^+$	2	38	2 / 40	(5)	

Mls-1a$^+$- and Mls-1a$^-$- hybrid mice from BALB/c and CDF1 mating partners were thymectomized 3 days after birth. Serological and histological examinations for AIG development were performed at 3 months of age. These mice were divided into two groups. Mls-1a$^+$ and Mls-1a$^-$: Mls-1a phenotypes were determined by the deletion of peripheral V*β*6$^+$ T cells using flow cytometric analysis as previously described [26].

Increase of Vβ6$^+$ T cells, but not of Vβ8$^+$T cells, in the spleen of AIG NTx BALB/c mice

Because autoimmunity often accompanies an increase in the usage of a given TcR of the TcR repertoire [9, 37], we examined the kinetics of the two largest sets of the TcR repertoire of Vβ6$^+$ and Vβ8$^+$ T cells in the spleen as well as the gastric and axillary lymph nodes. The results are shown in Table 2. A proportion of Vβ6$^+$ T cells, but not Vβ8$^+$ T cells, increased in the spleen of AIG NTx BALB/c mice. Interestingly, Vβ6$^+$ T cells in other lymphoid organs were silent, suggesting some antigenic selection of T cells. Because Vβ6$^+$ T cells are known to be targets of Mls-1a-superantigens of endogenous retroviruses [15, 24], activation or inactivation of Vβ6$^+$ T cells might somehow be involved in the generation of AIG. Because one remarkable difference between these strains of mice concerns the Mls-1a antigens that generate in DBA/2 mice as self, but not in BALB/c mice, and their possible disputable involvement in initiation of AIG [3, 24, 26] we focused first, in the present study, on the significance of Mls-1a antigens in the genetic control of AIG development. Mls-1a antigens in DBA/2 mice might contribute to the resistance of AIG by deletion of Vβ6$^+$ T cells.

The presence or absence of Vβ6$^+$ T cells is well correlated with AIG susceptibility in BALB/c-DBA/2 hybrids

To clarify the involvement of Mls-1a antigens, we made Mls-1a$^+$ or Mls-1a$^-$ back crossed mice that had a genetic background of BALB/c mice of at least one half of each locus by mating BALB/c with CDF1, and examined the incidence of AIG induced by NTx. The results are shown in Table 3. There was a strong difference between the groups. Thus, 14 of 44 (32%) Mls-1a$^-$ mice developed AIG, whereas only 2 of 40 (5%) Mls-1a$^+$ mice developed AIG. These results may indicate that the Mls-1a antigen is related to reduction of AIG generation. Additionally, two Mls-1a$^+$ mice, lacking Vβ6$^+$ T cells in their periphery, suffered from AIG. This strongly suggests that loci other than *Mls-1a* on chromosome 1 are reliable candidates for the retardation of AIG generation.

The insignificance of Mls-1a antigens that delete Vβ6$^+$ T cell generation on AIG resistance in DBA/2-bone marrow chimeric BALB/c mice

To test the significant role of Mls-1a antigens in preventing the development of AIG, we made bone marrow chimeras by transferring bone marrow cells pooled from a large number, greater than 30, of each Mls-1a$^+$ and

Table 4. Comparable AIG incidence between NTx BALB/c mice receiving Mls-1a$^+$ or Mls-1a$^-$ bone marrow cells

Bone marrow cell donors	AIG	Non-AIG	Incidence	(%)
Un-transferred control	19	20	19 / 39	(49)
Vβ6$^+$, or Mls-1a$^-$	11	11	11 / 22	(50)
Vβ6$^-$, or Mls-1a$^+$	9	14	9 / 23	(39)

BALB/c mice were inoculated with 2×10^7 bone marrow cells from either Mls-1a$^+$ or Mls-1a$^-$ backcrossed mice within 24 h after birth, and then were thymectomized at 3 days of age. The bone marrow cells were pooled from more than 30 donors for each group to provide an equal amount of genetic traits except for Mls-1a. The bone marrow transfer was tested by staining the blood cells of each mouse with anti-Vβ6 antibodies at the age of 3 months. There is no significant difference between the groups.

Table 5. Prevention of AIG development in NTx BALB/c mice by DBA/2 bone marrow cell transfer as neonates

NTx mice	AIG	Non-AIG	Incidence	(%)	Probability (*P*)
BALB/c	17	36	17 / 53	(32)	< 0.001
DBA/2-chimeric BALB/c	1	36	1 / 37	(3)	

Mice were inoculated with 2×10^7 DBA/2 bone marrow cells within 24 h after birth, and then were thymectomized at 3 days of age. After the mice had grown, the peripheral blood of each mouse was stained with anti-Vβ6 antibodies and analyzed by flow cytometry to confirm that the bone marrow transfer was successful by the deletion of Vβ6$^+$ T cells. Examination for AIG development was performed at the age of 3 months.

Mls-1a$^-$ BALB/c × CDF1 mouse to provide an equal amount of genetic traits except for Mls-1a, on the day of birth, and treated them with NTx 3 days after birth. To confirm the success of bone marrow transfer, we assessed deletion of Vβ6$^+$ T cells in the peripheral blood of each NTx chimeric BALB/c mouse by flow cytometry. The results are shown in Table 4. There is no difference in AIG inducibility between Mls-1a$^+$- and Mls-1a$^-$- chimeric BALB/c mice. These results clearly show that Mls-1a antigens are not involved in the incidence of AIG.

DBA/2-bone marrow chimerism as neonates causes AIG-prone BALB/c mice to be negative for Vβ6$^+$ T cells and resistant for AIG

Transplantation of bone marrow cells from DBA/2 mice to BALB/c mice as neonates constructed DBA/2 bone marrow chimeric (DBA/2-chimera) BALB/c mice. Successful chimerism of myeloid cells was assessed by the resulting deletion of Vβ6$^+$ T cells of BALB/c origin, and these chimeras could no longer respond to DBA/2 antigens as assessed by local host-vs-graft (HvG) reaction [13] (For details, see Materials and Methods). As shown in Table 5, the result was clear. Although 17 of 53 (32%) NTx BALB/c mice developed AIG, only one of 37 (3%) NTx DBA/2-chimeric BALB/c mice developed AIG. The chimeric mouse showing typical AIG with an enlarged stomach indicates that the presence of Mls-1a antigen may not be critical for suppression of AIG development in NTx DBA/2-chimeric BALB/c mice and that the myeloid cells responsible for AIG development are negative for Mls-1a antigens, a topic this will be later discussed.

DBA/2-bone marrow cells reduced IFN-γ production by CD4$^+$ T cells of NTx-BALB/c mice

DBA/2-chimeric BALB/c mice reduced the AIG incidence, and pathogenic T cells were thought to be suppressed in their functioning because of the anti-inflammatory state of the *in vivo* microenvironment. Thus, we examined this hypothesis by assessing IFN-γ and IL-10 secretion by lymphoid cells *in vitro*. The percentage of IFN-γ-producing CD4$^+$ T cells in the spleen of NTx DBA/2-chimeric BALB/c mice (0.6 ± 0.2%, n=3) was significantly lower than that of NTx BALB/c mice (1.7 ± 0.9%, n=3), and the proportion of CD4$^+$ T cells expressing IL-10 was almost the same level in both groups of mice, 0.4 ± 0.1% and 0.6 ± 0.1%, respectively. This result strongly suggests that DBA/2 myeloid cells normalize the IFN-γ-producing potency once deviated in the NTx BALB/c mice. To clarify the quantitative sig-

Table 6. The relative amount of IFN-γ and IL-10 produced by $CD4^+$ T cells in the spleen

Spleen cells	Relative amount of	
	IFN-γ	IL-10
NTx BALB/c	3.90 ± 2.64 (6)	2.28 ± 0.18 (3)
NTx DBA/2-chmeric BALB/c	1.14 ± 0.47 (7)	1.01 ± 0.36 (3)
Comparison between groups (*P*)	<0.05	NS

Individually prepared spleen cells from 4-week-old NTx BALB/c and NTx DBA/2-chimeric BALB/c mice were activated *in vitro* using PMA plus ionomycin, and then examined using the intracellular staining method for IFN-γ and IL-10. The fluorescence intensity for the cytokines in each cell was evaluated. The relative level is expressed as the mean ± S.D. Figures in the parentheses indicate the number of mice used. NS: non-significant difference.

Table 7. Cytokine production profiles of spleen cells from BALB/c- and DBA/2-chimeric BALB/c-*nu/nu* mice

Bone marow cell donors	Amount of cytokines (pg / mL)		
	IL-10	GM-CSF	IL-17
BALB/c	480 ± 120	520 ± 87	190 ± 88
DBA/2	900 ± 310	320 ± 180	290 ± 180
Comparison between Groups (*P*)	<0.05	NS	NS

Individually prepared spleen cells of 4-week-old BALB/c-*nu/nu* mice receiving either DBS/2- or BALB/c (as a control)-bone marrow cells as neonates were activated *in vitro* using PMA plus ionomycin, and then the culture supernatants were analyzed for IL-10, IL-17, and GM-CSF using the flow-cytometric bead array system. For each spleen, triplicate well incubations were performed. The mean value of each group (n=4) is shown with ± S.D. NS: non-significant difference.

nificance of the reduced amount of intracellular IFN-γ in $CD4^+$ T cells in the DBA/2-chimeric BALB/c mice, we calculated the sum of the relative amount of IFN-γ deposited in the individual $CD4^+$ T cells according to the method of PMA and ionomycin treatments. As shown in Table 6, increased IFN-γ production in NTx BALB/c mice was reduced significantly by DBA/2-chimerism. On the other hand, the difference in the amount of IL-10 between NTx BALB/c and NTx DBA/2-chimeric BALB/c mice was small. These results indicate that NTx BALB/c mice were in a pro-inflammatory state by producing IFN-γ, whereas NTx DBA/2-chimeric BALB/c mice were in a state similar to normal BALB/c mice.

Myeloid cells of DBA/2-chimeric BALB/c mice may produce a higher level of IL-10 than BALB/c mice

DBA/2-chimeric BALB/c mice were thought to be in an anti-inflammatory state, but their own $CD4^+$ T cells produced IL-10 at similar levels to those of BALB/c mice as mentioned above. These phenomena may indicate that cells derived from DBA/2 bone marrow cells would control autoreactive pathogenic T cells to be inactivated. To demonstrate the relationship of myeloid cells but not T cells to the anti-inflammatory state of DBA/2-chimeric BALB/c mice, we made DBA/2-chimeric BALB/c nude mice by transplanting bone marrow cells of DBA/2 or BALB/c, as a control, into BALB/c nude mice as neonates. After these mice had grown, we assessed cytokine production by flow-cytometric bead array and assessed IL-10, GM-CSF and IL-17. Obtained results showed that IL-10 secretion by spleen cells from DBA/2-chimeric nude mice is significantly higher than that from BALB/c nude mice receiving syngeneic bone marrow cells as controls (Table 7). Levels of GM-CSF and IL-17 were not significantly different between the two groups of nude mice. These results suggest that myeloid cells of DBA/2 mice provide an anti-inflammatory state *in vivo* by production of IL-10.

NTx DBA/2-chimeric BALB/c mice have immediate precursors of AIG-T cells

To investigate the mechanisms of inhibition of AIG development in NTx DBA/2-chimeric BALB/c mice, we first tested whether some suppressor cells had been gen-

Table 8. The absence of suppressor cells but the presence of potentially autoreactive T cells in NTx DBA/2-chimeric BALB/c mice

NTx BALB/c spleen cell donors	AIG / Non-AIG	AIG-Incidence (%)
AIG	5 / 0	(100)
AIG + normal	0 / 3	(0)
AIG + non-AIG	3 / 0	(100)
AIG + non-AIG chimera	4 / 0	(100)
Non-AIG chimera	6 / 0	(100)

Groups of BALB/c-*nu/nu* mice received 2×10^7 spleen cells of AIG-BALB/c mice alone (AIG), or either with 2×10^7 spleen cells of normal BALB/c mice (AIG + normal), 4×10^7 spleen cells of non-AIG NTx BALB/c mice (AIG + non-AIG), or 4×10^7 spleen cells of non-AIG NTx DBA/2-chimeric BALB/c mice (AIG + non-AIG chimera). The final group of BALB/c-*nu/nu* mice received 4×10^7 spleen cells of non-AIG NTx DBA/2-chimeric BALB/c mice (non-AIG chimera). All recipients were sacrificed and examined for AIG development 2 months after the cell transfer.

erated in NTx DBA/2-chimeric BALB/c mice in a cell mixture experiment. Suppression of AIG development in nude mice was shown in a cell number-dependent manner when spleen cells of normal BALB/c mice were co-transferred with spleen cells (2×10^7) of AIG NTx BALB/c mice. By contrast, spleen cells (4×10^7) of non-AIG NTx DBA/2-chimera BALB/c mice did not suppress AIG development in this co-transfer experiment (Table 8). Interestingly and surprisingly, all nude mice receiving only spleen cells (4×10^7) of non-AIG NTx DBA/2-chimeric BALB/c mice developed AIG, demonstrating T cell cluster distribution at the lamina propria of the stomach mucosa of these nude mice (Table 8 and Fig. 3-A, B, C). These results indicate that Treg cells do not develop in NTx DBA/2-chimeric BALB/c mice and that AIG-prone T cells are potentially generated but silenced in DBA/2-chimeric BALB/c mice. A similar result was obtained when spleen cells of NTx CDF1-chimeric DBA/2 mice were transferred into BALB/c nude mice (Fig. 3-D, E, F).

These results suggest that an unknown BALB/c genetic trait is involved in the generation of AIG-prone T cells and that AIG-immediate precursors are silenced under the microenvironment constructed by DBA/2-myeloid cells.

Discussion

It is well known that neonatal thymectomy can induce organ-specific autoimmune disease in some susceptible strains of mice via genetic controls [21, 26, 32], although the mechanism remains to be clarified. Generally, autoimmune diseases are primarily thought to be mediated by autoreactive T cells escaping clonal deletion in the thymus, and even if they could escape the purging in the thymus, they would be eliminated by $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in the periphery [23, 27]. In the present study, we demonstrated, using H-2-identical strains of BALB/c and DBA/2 mice, that the numbers of $CD4^+$ T cells producing IFN-γ, one of the predominant inflammatory cytokines, were increased in AIG-prone NTx BALB/c mice but not in AIG-resistant NTx DBA/2 mice (Fig. 2). This result indicates that BALB/c mice would easily be shifted to a pro-inflammatory state by NTx, but DBA/2 mice would not, and AIG-spleen cell mixture experiments using normal BALB/c mice demonstrated that NTx retarding development of regulatory T cells [27] is followed by shifting to an inflammatory state for AIG development. Therefore, clarification of the mechanism involved in AIG resistance of DBA/2 mice is meaningful to understand the control of immune deviation in a fail-safe mechanism.

T cells expressing particular TcR Vβ gene families have been shown to accumulate preferentially in the infiltrated target organ in many experimental models of autoimmune diseases. For example, in experimental autoimmune uveoretinitis [9], experimental allergic encephalomyelitis (EAE) [37], a bias toward TcR Vβ8 usage by effector cells, has been reported. Regarding the relevance of TcR usage and organ-specific autoimmune diseases, $V\beta6^+$ T cells may be thought to be a possible candidate for AIG (Table 2) because Mls-1a antigens

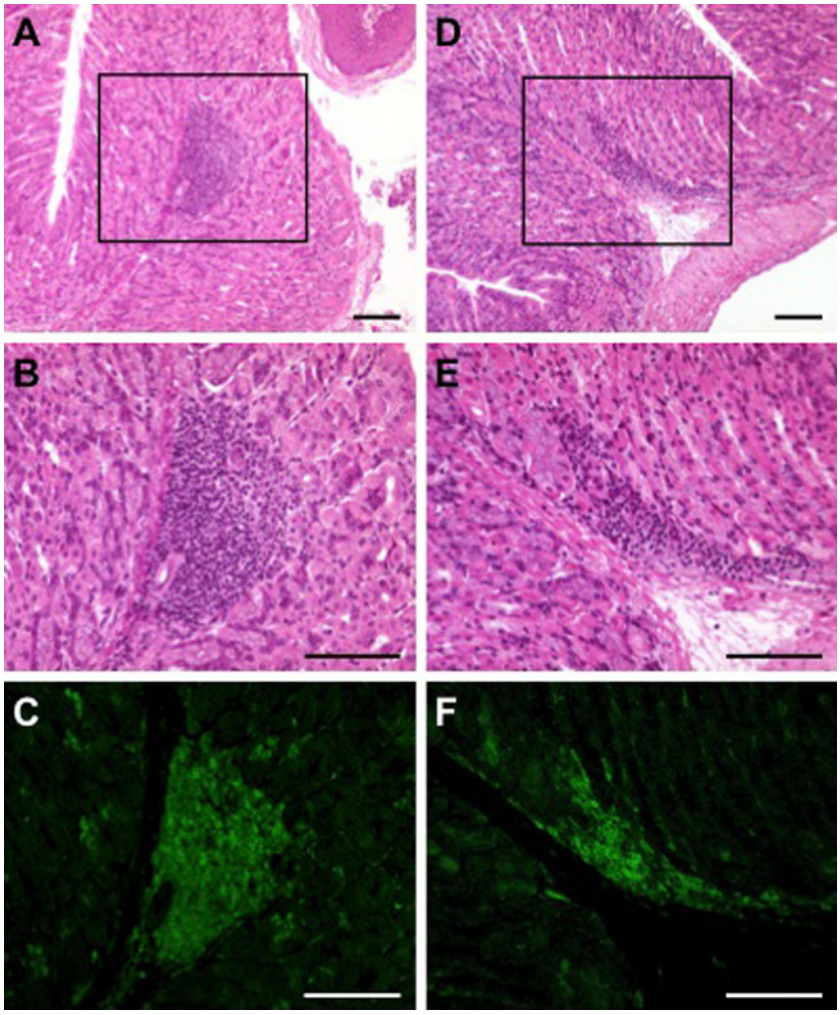

Fig. 3. Histology of the spleen of AIG BALB/c-*nu/nu* mice receiving spleen cells of non-AIG NTx DBA/2-chimeric BALB/c mice (A, B, C) and non-AIG NTx CDF1-chimeric DBA/2 mice (D, E, F) were sacrificed 2 months after the spleen cell-transfer. Their stomach sections were stained with HE (A, B, D, E). B and E are higher magnifications of the squares in A and D, respectively. C and F are immunofluorescent staining of CD3-bearing cells, or T cells on the cut sections adjacent to B and E, respectively. Scale bars: 100 μm.

seemed to be involved in AIG development as shown in our backcross experiments (Table 3). However, Mls-1a antigen was found to be not related to AIG development (Table 4), and some transgenic mice that spontaneously develop AIG used another TcR Vβ chains, such as Vβ4 or Vβ14 [5, 18]. Indeed, without Vβ6$^+$ T cells, AIG was demonstrated in Mls-1a$^+$ congenic BALB.D2 mice [7]. These results exclude the Mls-1a/Vβ6 line as the primary factor for AIG development, although the possible participants of modifiers of AIG generation or expansion remain to be solved because we observed that the ratio of Vβ6$^+$ T cells in the spleen was significantly increased (Table 2) as observed in previous reports [26, 27].

In BALB/c × CDF1 backcrosses, AIG developed in mice bearing the Mls-1a gene (Table 3), indicating that the gene(s) responsible for AIG resistance is closely linked to the *Mls-1a* locus on chromosome 1. The possible site bearing the responsible gene(s) is tentatively speculated from the data of the AIG incidence in NTx CXD2 RI strains presented in our previous report [26] where the data were once discussed on the AIG-prone gene locus. Thus, 4 of 12 CXD2 RI strains were determined to be AIG resistant [26], sharing the *Pep-3* locus of the DBA/2 type among genetic markers for *Idh-1*-,

Pep-3-, *Mls-1a*- and *Akp-1*-loci, distally, on chromosome 1 [12]. From this information, the responsible gene could be identified in the future using recombination experiments on chromosome 1.

Using other AIG-resistant strains of C57BL/6 (*H-2*^{b}) mice as a BALB/c partner for (BALB/c × C57BL/6) F_2 segregation progeny, Silveria *et al.* [32] performed a linkage analysis for genes conferring susceptibility to AIG development and identified two major genes, *Gasa1* and *Gasa2*, on chromosome 4. Because (BALB/c × C57BL/6) F_1 hybrids were AIG susceptible, the genes responsible for the C57BL/6 trait are recessive, in contrast to the dominant genes responsible for the DBA/2 trait, because (BALB/c × DBA/2) F_1 hybrids are AIG resistant (Table 1). These mice produce a congenic BALB/c strain bearing the *Gasa1* gene of C57BL/6, which showed significantly reduced incidence of AIG to one third that of BALB/c mice, and it was reported that activities for Treg development and antigen-presentation for AIG-T cells are similar to those in BALB/c mice [2]. These findings, together with those in the (BALB/c × DBA/2) F_1 hybrid experiments in the present study, indicate that there is a clear difference in the genetic etiology of AIG.

According to our results of adoptive transfer of AIG, NTx DBA/2-chimeric BALB/c mice do possess AIG-prone T cells that were silenced (Table 8 and Fig. 3A–C). This result indicates that DBA/2 myeloid cells could control the development of AIG *in vivo* without promoting clonal deletion of corresponding T cells. In addition, $CD4^+$ T cells from DBA/2-chimeric BALB/c mice were profoundly suppressed to normal or baseline levels for IFN-γ-producing activity (Table 6). It was reported that inhibition of IFN-γ activity prior to detection of a pathological lesion resulted in prevention of AIG [4]. Together with experimental data on the DBA/2- and BALB/c (as the control)-chimeric BALB/c nude mice, we conclude that T cells do not primarily play a role in controlling the different incidences of AIG between the strains. The cells responsible for prevention of AIG development in our case may be neither B cells nor dendritic cells (DCs), both making Mls-1a antigens presentable to T cells [16], because of AIG-generation in NTx DBA/2-chimeric BALB/c mice (Table 5). The cell lineage derived from bone marrow cells transferred as neonates is most likely the myeloid cell lineage producing anti-inflammatory cytokines, known as immunosuppressive cytokines, via inhibition of immune-promoting cytokine production [17].

In experimental autoimmune encephalomyelitis (EAE), Okura *et al.* [29] reported the biological significance of myeloid-derived suppressors, most likely DCs, which produce IL-10 to prevent the pathogenesis of EAE-T cells under suitable physiological conditions. Similarly, treatment of NTx BALB/c mice with IL-10-producing DCs that had been prepared artificially *in vitro* retarded AIG development, most likely with the generation of Treg cells [35]. Other reports showed that the incidence of AIG was increased by innate-immune cells derived from myeloid cells by administration of LPS [20] or poly I:C [19]. These immune modifications may be an extent of regulation during immune responses, whereas myeloid cells involved in the present study shifted from a pro-inflammatory state to an anti-inflammatory state of the physiological environment because the immune trait was determined genetically and developmentally. Thus, myeloid cell suppression would work unsystematically but in an organ-dependent manner in some cases. Indeed, AIG-resistant NTx DBA/2 mice are susceptible to autoimmune oophoritis and prostatitis [26].

Among progressive studies for the molecular mechanisms underlying myeloid cell-mediated regulation [8], those achieving genetic control of disease developments, superior to physiological modifications of the immune responses, provide new insight for understanding the mechanisms of suppression of autoimmunity by bone marrow-derived, non-lymphoid cells.

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