Dendritic Cell Appearance and Differentiation during Early and Late Stages of Rat Stomach Carcinogenesis

Motoi Takeuchi,1, 2 Masami Yamamoto,3 Masae Tatematsu,1 Kazumasa Miki,2 Yoshiyuki Sakaki1 and Chie Furihata1, 4, 5

1 Human Genome Center, Institute of Medical Science, University of Tokyo, 4-6-1 Sironakanedai, Minato-ku, Tokyo 108-8639, 2 First Department of Internal Medicine, Faculty of Medicine, Toho University, 6-11-1 Okomorisshi, Ohta-ku, Tokyo 143-0015, 3 Laboratory of Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681 and 4 College of Science and Engineering, Aoyama Gakuin University, 1-1 Morinosato-Aoyama, Atsugi, Kanagawa 243-0123

Dendritic cell appearance and differentiation during early and late stages of rat stomach carcinogenesis were studied in the pyloric mucosa. Young male rats were given drinking water with or without N-methyl-N’-nitro-N-nitrosoguanidine (MNNG; 100 mg/liter) for 14 days. Use of competitive RT-PCR and northern blotting showed that MNNG exposure induced 3- to 4-fold greater expression of the genes for integrin β7 and integrin αE2 (identical with antigen OX-62, a dendritic cell marker), as well as three cytokines, IL-4, GM-CSF and TNF-α, in the stomach pyloric mucosa of resistant Buffalo rats compared to sensitive ACI rats. These genes were minimally expressed in control animals. The results confirm the appearance of dendritic cells in the target pyloric mucosa and suggest the possibility that dendritic cell differentiation and maturation are induced by various cytokines, at least in Buffalo rats. Competitive RT-PCR showed expression of integrin αE2 and β7, MHC class II-associated invariant chain (Ii), MHC class II, B7-1, CD28, GM-CSF and TNF-α genes in all 12 examined stomach adenocarcinomas and adenomas induced in male Lewis and WKY rats with 30 weeks’ MNNG exposure, suggesting the presence of dendritic cells in tumors. OX-62 staining and western blotting for OX-62 also confirmed the presence of dendritic cells in tumors. However, the population of dendritic cells in tumors was less than that in the pyloric mucosa after 14 days’ MNNG exposure. The present results suggest that immune defense involving dendritic cells is marshaled from the very early initiation stage during rat stomach cancer development, but is downgraded in developed tumors.

Key words: Dendritic cell — Cytokines — Rat stomach pyloric mucosa — MNNG

There have been few studies on the involvement of the immune response in the initiation stage of chemical carcinogenesis.1–3 Previously we studied rat experimental stomach carcinogenesis induced with N-methyl-N’-nitro-N-nitrosoguanidine (MNNG)4 to clarify gene expression changes during the initiation stage by fluorescent differential display analysis.5 With cloned MHC class II-associated invariant chain (Ii), MHC class II, B7-1, CD28, GM-CSF and TNF-α genes in all 12 examined stomach adenocarcinomas and adenomas induced in male Lewis and WKY rats with 30 weeks’ MNNG exposure, suggesting the presence of dendritic cells in tumors. OX-62 staining and western blotting for OX-62 also confirmed the presence of dendritic cells in tumors. However, the population of dendritic cells in tumors was less than that in the pyloric mucosa after 14 days’ MNNG exposure. The present results suggest that immune defense involving dendritic cells is marshaled from the very early initiation stage during rat stomach cancer development, but is downgraded in developed tumors.

Recently we reported that Ii-mAb-stained cells would also stain with OX-62 mAb (a marker of dendritic cells6,7). Thus, dendritic cells, antigen-presenting cells, were shown to appear in the interstitial tissue of stomach pyloric mucosa 14 days after MNNG exposure. Furthermore, we compared the appearance of dendritic cells in a carcinogenesis-resistant rat strain (Buffalo) and a sensitive strain (ACI).

Different susceptibility to MNNG-induced stomach cancer was reported in Buffalo and ACI rats.8,9 The resistance in Buffalo rats was genetically determined and autosomally dominant.9 A greater number of mature dendritic cells expressing MHC class II, Ii, MHC class I, B7-1 and B7-2 appeared in the interstitial tissue of pyloric mucosa of the resistant Buffalo rats. This suggested the involvement of a dendritic cell response in the resistance to the MNNG induction of stomach carcinogenesis in rats.7 The presence of a susceptibility gene and two resistance genes was suggested,10 but the relationships of these genes to dendritic cells were not elucidated.

The OX-62 gene was recently cloned and the gene product was identified as integrin αE211; the DNA sequence
was released onto DNA databases in 1998. Integrin $\beta$7 is another unit of the hetero-duplex integrin complex containing integrin $\alpha$E2.12) Thus, the expression of integrin $\alpha$E2 and $\beta$7 genes was examined in the present study. E-Cadherin, a cell adhesion molecule, is a ligand of the integrin $\alpha$E2 and $\beta$7 complex,12) so the expression of E-cadherin was also examined in the present study.

Multiple pathways for dendritic cell differentiation and maturation have been proposed, mainly from in vitro studies.13-17) There are at least two possibilities for the source of dendritic cells in the stomach pyloric mucosa. First, dendritic cells may be formed by differentiation and maturation of precursor cells within the tissue by the action of cytokines. Second, mature dendritic cells may migrate from other sources. The first possibility is examined in the present study.

We also examined the presence of dendritic cells in rat stomach tumors to determine whether the appearance of dendritic cells in the target organ is a continuous phenomenon or a temporary event associated only with the early stages of rat stomach carcinogenesis.

MATERIALS AND METHODS

**Animals** Ten male 6-week-old Buffalo rats (BUF/NacIcl; Nihon Clea, Tokyo), ACI rats (ACI/NJcl; Nihon Clea) and Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama) were given MNNG (100 mg/liter, the same concentration as used for long-term stomach carcinogenesis studies; Nacalai Tesque, Inc., Kyoto) in drinking water for 14 days. Ten control rats were given distilled water instead of MNNG. Rats had free access to pellet rodent chow (Oriental MF; Oriental Yeast Co., Ltd., Tokyo). Rats were killed by cervical dislocation under ether anesthesia. Stomachs from five rats in each group were surgically removed. The pyloric mucosa and tumors were isolated by a modified method using acid guanidine thiocyanate/phenol chloroform using TRizol (Life Technologies, Inc., Gaithersburg, MD) and ISOGEN LS (Nippon Gene, Toyama) reagents and stored at $-80^\circ$C until use.18) Total RNAs from stomach tumors were isolated with only TRizol, because the tumors were small and only small amounts of total RNAs were extracted. Proteins were extracted from the residue as described in the ISOGEN LS manual.

**Competitive RT-PCR** The first cDNA strand was prepared with 2.5 $\mu$g of total RNA and 50 pmol of GT$_4$C by using SuperScript II reverse transcriptase (Life Technologies, Inc.). Competitive RT-PCR with specific primers and the first-strand cDNA was performed at high stringency.18) The amplified cDNAs were examined on a 5% polyacrylamide gel stained with SYBR Green I (Molecular Probes, Eugene, OR) and scanned with a fluorescence image analyzer (FluorImager 575; Molecular Dynamics, Sunnyvale, CA). The densitogram was obtained with the same instrument. The respective band area in the MNNG-exposed group lane was compared with the same size area in the control group lane. To compare MNNG-exposed Buffalo rats with ACI rats, the respective band areas of electrophoresed samples from MNNG-exposed animals were compared. cDNA sequence data were obtained from an internet website (http://www.ncbi.nlm.nih.gov/). The oligonucleotide primers were as follows: integrin $\alpha$E2, 5'-GGACATCAACGGCTCTTCTGC-3' (5' primer) and 5'-GGGTGTCTCAACTGGCTCCCT-3' (3' primer) (409 bp); integrin $\beta_7$, 5'-GGTGACGGGTCTCCCTGCAGC-3' (5' primer) and 5'-GGGTGTACGTTAGTGTGACCG-3' (3' primer) (389 bp); E-cadherin, 5'-GGGCTCTTGTAGCCAGGCCGAAG-3' (5' primer) and 5'-CCACTCCCCACATGAAACCC-3' (3' primer) (194 bp); IL-4, 5'-GGGGTTCACCTACG-3' (5' primer) and 5'-GGGTGTACGTTAGTGTGACCG-3' (3' primer) (314 bp); GM-CSF, 5'-GGCTACCCAACACCCTGTCACC-3' (5' primer) and 5'-GGGCTCTTGTAGCCAGGCCGAAG-3' (5' primer) (376 bp); TNF$\alpha$, 5'-GCAGCTGGAGTGCTGGAGC-3' (5' primer) and 5'-GGGCTCTTGTAGCCAGGCCGAAG-3' (5' primer) (393 bp); Ii, 5'-CTCTGTCTCAGGTTGCTGCTTTG-3' (5' primer) and 5'-AGTCTGGTGGTGCTGCTTCTTC-3' (3' primer) (457 bp); MHC class II, 5'-GTCCATTTCCTAGTGTTGCACCC-3' (5' primer) and 5'-GGGCTCTTGTAGCCAGGCCGAAG-3' (5' primer) (400 bp); B7-1, 5'-CCGAGGGTACGGGAAGTTGG-3' (5' primer) and 5'-GGGCTCTTGTAGCCAGGCCGAAG-3' (5' primer) (395 bp); CD28, 5'-GGGTTAGCAGTCCCGCTCC-3' (5' primer) and 5'-CCAGCAACCCGACCGAG-3' (3' primer) (408 bp); and mouse 18S rRNA, 5'-CCAGTAAGTGCGGTTACATAAGC-3' (5' primer) and 5'-CCCTCCGGACGTTACACTAG-3' (3' primer) (218 bp). The competitor for integrin $\alpha$E2 was prepared by RT-PCR with 42 mer-5' primer 5'-GGACATCAACGGCTCTTCTG-3' until use.18) Total RNAs from stomach tumors were isolated with only TRizol, because the tumors were small and only small amounts of total RNAs were extracted. Proteins were extracted from the residue as described in the ISOGEN LS manual.

**RNA and protein isolations** Total RNAs from stomach pyloric mucosa and tumors were isolated by a modified method using acid guanidine thiocyanate/phenol chloroform using TRizol (Life Technologies, Inc., Gaithersburg, MD) and ISOGEN LS (Nippon Gene, Toyama) reagents and stored at $-80^\circ$C until use.18) Total RNAs from stomach tumors were isolated with only TRizol, because the tumors were small and only small amounts of total RNAs were extracted.
CAGCCATGCAGCATGTCC-3' and with 3' primer for integrin αE2 (365 bp). The competitor for integrin β7 was prepared by RT-PCR with 41 mer-5' primer 5'-GGTGTTCCCTGAGCCAGCGGCCCAATTGGATGAGTGG-3' and with 3' primer for integrin β7 (344 bp). The competitor for E-cadherin was prepared by RT-PCR with 42 mer-5' primer 5'-GGGTGCTTAGTGCCAGCATACG-3' and with 3' primer for E-cadherin (147 bp). The competitor for IL-4 was prepared by RT-PCR with 42 mer-5' primer 5'-GGTACGGGTCCCCTGGCAGCGCCCAATTTGGATG-3' and with 3' primer for IL-4 (299 bp). The competitor for GM-CSF was prepared by RT-PCR with 44 mer-5' primer 5'-GCTCACCCAACCCTGTCACCGCCTCCTAAATGACATGCGTGC-3' and with 3' primer for GM-CSF (338 bp). The competitor for TNFα was prepared by RT-PCR with 43 mer-5' primer 5'-GCAGCTGGAGTGGCTGAGCCGGTACCAGCTGTACC-3' and with 3' primer for TNFα (338 bp). The competitor for Ii was prepared by RT-PCR with 48 mer-3' primer 5'-AGTCTGGGTGGGCTGCTTCTCCTCCTTACGCTTCAGATTCTCCGG-3' and 5' primer for Ii (373 bp). The competitor for MHC class II was prepared by RT-PCR with 47 mer-3' primer 5'-TGAGAGCTTGCAAGCCGCTGATGCAATGATGTGCCAACAGAGG-3' and 5' primer for MHC class II (353 bp). The competitor for B7-1 was prepared by RT-PCR with 42 mer-5' primer 5'-CCCGGGGTACCGGAAGTGTGGCTGTACCTTTCAGACAGAG-3' and 3' primer for B7-1 (333 bp). The competitor for CD28 was prepared by RT-PCR with 41 mer-5' primer 5'-GGTGAAGCAGTCCCCGCTGCGCAAAGGAGTTCCGGGCATCC-3' and 3' primer for CD28 (342 bp).

**Northern blot** Total RNAs (50 µg) were resolved on a 1% agarose-2.2 M formaldehyde gel, blotted onto a Hybond-N membrane filter (Amersham, Buckinghamshire, UK), and then hybridized with a 32P-labeled probe. Washed filters were exposed on an imaging plate and analyzed with a BAS 2000 (Fuji Film, Tokyo). The densitogram was determined with the same instrument in the same manner as for competitive RT-PCR.

**Western blot** Protein fractions were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto an Immobilion polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) and stained by alkaline phosphatase-linked immunostaining. OX-62 mAb (dendritic cell marker; Pharmingen, San Diego, CA) was used at 1/1000 dilution. Monoclonal anti-β-actin (clone AC-15, Sigma, Saint Louis, MO) was used at 1/1000 dilution to show the relative amount of protein.

**Histochemistry** Immunohistochemical staining was per-
formed on tissue from 5 rats in each group using monoclonal antibodies as described previously. Five tissue sections from each rat were stained and analyzed. Areas containing severe erosion were avoided for photography. OX-62 mAb (Pharminen) was used on frozen sections fixed with acetone. li-mAb RG11 directed against the carboxyl-terminal segment of the rat invariant γ chain was a gift from K. Reske. Antibody against human E-cadherin (TL-C20820) was obtained from Transduction Laboratories (Lexington, KY). These antibodies were used on ethanol-fixed paraffin sections.

RESULTS

Increase in expression of integrin αE2 and integrin β7 in stomach pyloric mucosa  The results of competitive RT-PCR and northern blotting of integrin αE2 and integrin β7 genes in stomach pyloric mucosa are shown in Fig. 1. The expression of these genes was weak or undetectable in pyloric mucosa of control animals. MNNG exposure for 14 days induced 100- and 20-fold increases in the expression of integrins αE2 and β7, respectively, in the pyloric mucosa of the resistant Buffalo rats, but 4- and

Fig. 3. Immunohistochemical staining of E-cadherin in the stomach pyloric mucosa of resistant Buffalo rats and MNNG-sensitive ACI rats. Samples are (a) MNNG-exposed Buffalo, (b) MNNG-exposed ACI, (c) control Buffalo and (d) control ACI stained with rat E-cadherin-mAb, and (e) unstained control Buffalo. Dark epithelial cells are mAb-stained cells. x200.
10-fold increases in the sensitive ACI rats in northern blots. However, the expression of integrins αE2 and β7 in the MNNG-exposed pyloric mucosa of the resistant Buffalo rats was 3-fold greater than that of the sensitive ACI rats. The present results are consistent with the previous findings that a greater number of OX-62-stained cells appeared in the interstitial tissue of the resistant Buffalo rats than in sensitive ACI rats.

Increase in expression of IL-4, GM-CSF and TNFα genes in stomach pyloric mucosa The results of competitive RT-PCR of IL-4, GM-CSF and TNFα genes in stomach pyloric mucosa are presented in Fig. 2. This shows that expression of these genes was weak or undetectable in pyloric mucosa of control animals. MNNG exposure for 14 days induced a 4-fold increase in IL-4 and GM-CSF expression in Buffalo rats, but no increase was detected in ACI rats. Expression of TNFα was increased 10-fold in both Buffalo and ACI rats. IL-4, GM-CSF and TNFα were reported as inducers of dendritic cell differentiation and maturation in vitro in humans and rats. The present results suggest the possibility that dendritic cells were matured in the stomach pyloric mucosa from peripheral blood precursor cells by IL-4, GM-CSF and TNFα, at least in Buffalo rats.

Expression of E-cadherin gene in stomach pyloric mucosa E-Cadherin is a ligand of the integrin αE2 and β7 complex. The results of competitive RT-PCR and northern blotting of the E-cadherin gene in stomach pyloric mucosa are shown in Fig. 1. The gene was expressed in pyloric mucosa of control animals, with no change after MNNG exposure.

Immunohistochemical demonstration of E-cadherin in stomach pyloric mucosa In order to examine E-cadherin gene expression at the protein level, immunohistochemical staining of pyloric mucosa was conducted with an E-cadherin-specific mAb. Representative results are shown in Fig. 3. Cell membranes of the pyloric mucosa were stained with the antibody in both control and MNNG-exposed ani-
Expression of integrin $\alpha E2$ and $\beta 7$, $Ii$, MHC class II, $B7-1$ (costimulator of dendritic cells), $CD28$ ($B7$ receptor on $T$ cell), and $E$-cadherin in stomach tumors

Having studied a range of markers which may be directly or indirectly associated with dendritic cell presence, we compared the profile of gene expression with that in MNNG-induced rat stomach tumors. Fig. 4 shows competitive RT-PCR of integrin $\alpha E2$ and $\beta 7$, $Ii$, MHC class II, $B7-1$, and $CD28$ genes in six Lewis rat stomach tumors (T2–5, adenocarcinomas and T1 and T6, adenomas) and in six WKY rat stomach tumors (T7–12, adenocarcinomas). Control Lewis and WKY rat (8 weeks old) stomach pyloric mucosa and Lewis rat stomach pyloric mucosa after 14 days’ MNNG exposure served as negative and positive controls, respectively. For integrin $\alpha E2$ and $\beta 7$, $Ii$, MHC class II, $B7-1$, and $CD28$ genes, expression was weak or undetectable in pyloric mucosa of control animals, but it was clearly evident 14 days after MNNG exposure (positive control). In all twelve stomach tumors these genes continued to be expressed, though with different intensities and less strongly than in positive controls, especially for integrin $\alpha E2$, $Ii$, $B7-1$ and $CD28$. The results suggest that differentiated dendritic cells were present in stomach tumors, but to a lesser extent than in pyloric mucosa in the initiation stage of carcinogenesis. In contrast, the $E$-cadherin gene was similarly expressed in control pyloric mucosa, with and without MNNG exposure, and in all stomach tumors.

Competitive RT-PCR of $IL-4$ and $GM-CSF$ and $TNF\alpha$ in stomach tumors

Fig. 5 shows that $IL-4$ gene expression was not detected in stomach tumors induced in Lewis rats (T2–5, adenocarcinomas; T1 and T6, adenomas) or in WKY rats (T7–12, adenocarcinomas) by MNNG, or in control WKY and Lewis stomach pyloric mucosa. $IL-4$ expression was detected in Lewis pyloric mucosa after 14 days’ MNNG exposure (positive control). $GM-CSF$ and $TNF\alpha$ gene expression was low in Lewis and WKY control animals, but was increased in tumors and in MNNG-exposed Lewis pyloric mucosa.

Immunohistochemical demonstration of $OX-62$ and $Ii$ in stomach tumors

To demonstrate the presence of dendritic cells in stomach tumors, the presence of $OX-62$- and $Ii$-mAb-stained cells in stomach tumors was studied immunohistochemically. Only a few stained cells could be seen in control Lewis rat stomach pyloric mucosa (Fig. 6a for $OX-62$ and Fig. 7a for $Ii$). After 14 days’ MNNG exposure, increased numbers of $OX-62$- and $Ii$-mAb-stained cells were observed in the interstitial tissue of pyloric mucosa (Fig. 6b for $OX-62$ and Fig. 7b for $Ii$). $Ii$-mAb-stained cells were seen in a rat stomach tumor (T2, adenocarcinoma) (Fig. 7c), but only a few $OX-62$-stained cells were seen (Fig. 6c). These cells were observed in tissue of interstitial origin.

Western blot of $OX-62$

An $OX-62$-stained protein band was detected in all tumors and in control pyloric mucosa by sensitive western blotting. However, no significant increase in tumors compared to control pyloric mucosa was observed (Fig. 8).
DISCUSSION

The focus of our research program is the very early changes in experimental rat stomach carcinogenesis caused by MNNG.25, 26) Previously we found that dendritic cells, which were stained with OX-62 mAb and which expressed MHC class I and II (MHC class II, Ii) genes, B7-1 and B7-2, appeared in the interstitial tissue of rat stomach pyloric mucosa after 14 days’ MNNG exposure.5, 7) In the present study we confirmed gene expression of integrin αE2, known to be the antigen for OX-62 mAb,11) and demonstrated gene expression of integrin β7, which is another unit of the integrin complex containing integrin αE2.12)

At least three pathways have been proposed for the development of dendritic cells: lymphoid, myeloid and Langerhans cell (in skin) lineages.16, 27) IL-4, GM-CSF and TNFα were proposed as essential cytokines for differentiation and maturation of human dendritic cells in vitro.28, 29) Of these cytokines, IL-4 and GM-CSF have been reported in rats.15) At present, dendritic cell differentiation and maturation and the effects of cytokines are mainly studied with in vitro systems, and there are very few in vivo studies. In normal control animals, only a small number of dendritic cells are found in the stomach pyloric mucosa.7) MNNG exposure for 14 days caused the appearance of dendritic cells in the stomach pyloric mucosa.7) BrdU-labeling showed that cells, including dendritic cells or their precursors, did not proliferate in the interstitial tissue of pyloric mucosa.7) It is likely, then, that the appearance of dendritic cells in the stomach pyloric mucosa can be explained in one of two ways. First, dendritic cells may be formed by differentiation and maturation of precursor cells in the stomach pyloric mucosa under the influence of cytokines. Second, mature dendritic cells may migrate from other sources. This present study examined the first possibility.

Competitive RT-PCR demonstrated that gene expression of IL-4, GM-CSF and TNFα was induced by MNNG.
exposure in pyloric mucosa and that the induction was several times greater in Buffalo rats than in ACI rats. Since MNNG induces a greater number of dendritic cells in interstitial tissue of stomach pyloric mucosa in resistant Buffalo rats, these gene expression results suggest the possibility that dendritic cell differentiation and maturation may be induced in the pyloric mucosa from peripheral blood precursor cells by these cytokines. Dendritic cells appeared after 14 days' MNNG exposure (but not after 3 days' exposure) in rat stomach pyloric mucosa. This period coincided with the time taken for in vitro differentiation and maturation from precursor cells. Recently, rapid recruitment of dendritic cell precursors (OX-62+ MHC II+) within a day from bone marrow cells was reported in rat liver and after rat cardiac transplantation.

We also observed gene expression of integrin αE2 and β7, MHC class II, Ii and B7-1 in MNNG-induced adenocarcinomas and adenomas, suggesting the presence of dendritic cells also in tumors. However, expression of some of these genes was less than that in MNNG-exposed Lewis rat stomach pyloric mucosa. OX-62 immunohistochemistry and western blot also suggested the presence of a smaller number of dendritic cells in tumors. The above results suggest that the population of dendritic cells in tumors is less than that in the pyloric mucosa after 14 days' MNNG exposure.

The presence of dendritic cells in advanced human stomach cancer has been reported, with small numbers of dendritic cells in the primary tumors being associated with reduced lymph node metastasis. Lower dendritic cell presence in tumors was more frequent in lymph node metastasis-negative patients with recurrence.

E-Cadherin is a cell adhesion molecule and a ligand of the integrin αE2 and β7 complex. Recently it was reported that germline mutations in the E-cadherin gene were causes of familial gastric cancer in New Zealand. Mutations in E-cadherin were also reported in human diffuse-type gastric carcinomas. We examined changes in E-cadherin gene expression and protein expression in rat stomach pyloric mucosa after MNNG exposure and in 10 differentiated adenocarcinomas and 2 adenomas. However, no changes in E-cadherin expression were observed in this experimental stomach carcinogenesis system (data not shown).

The dendritic cell is hypothesized to be the strongest antigen-presenting cell to T helper cells, the latter then commanding B cells to make an antibody for a long-term immune response. Although CD4, CD28 and IgM expression was detected by RT-PCR and northern blot, large numbers of T helper cells or B cells were not observed in interstitial tissue of pyloric mucosa examined immunohistochemically (data not shown). There remains the possibility that dendritic cells play another role in interstitial tissue of pyloric mucosa during stomach carcinogenesis caused by MNNG; further studies are required to examine this. For example, endocytotic activity of dendritic cells in rat liver and cytolytic activity of splenic dendritic cells in vitro were reported.

Different susceptibility to MNNG-induced stomach cancer was reported in Buffalo and ACI rats. Some genetic differences and some differences in gene expressions were also revealed. However, further study is required to elucidate the control mechanisms of the different immune responses between the two strains.

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