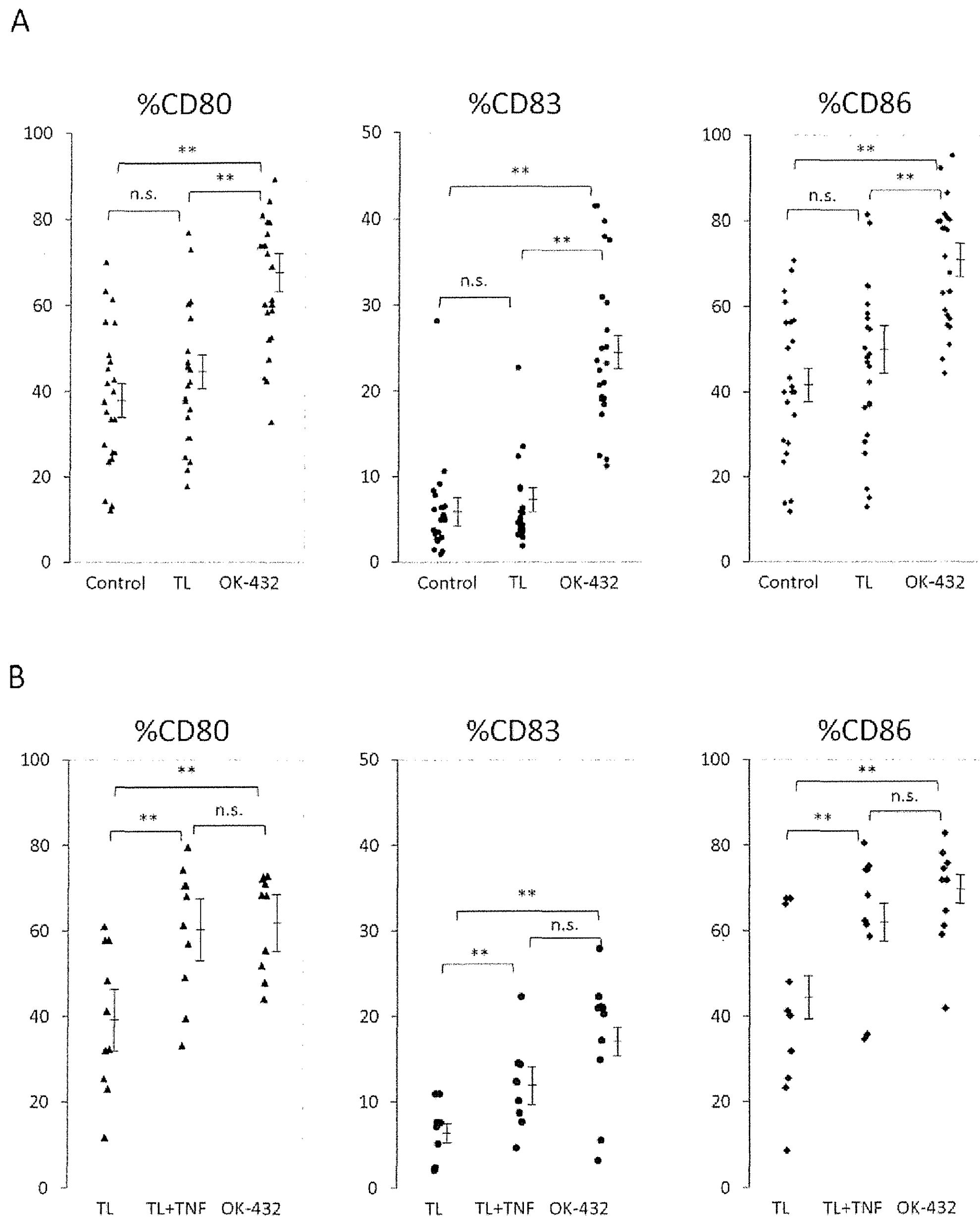

Figure 2. Comparison of %CD80, %CD83, and %CD86 by DC maturation cocktails. TL: tumor lysate, TNF: tumor necrosis factor-α, ** P<0.01, n.s.: not significant **A. Basic DC-preparation Protocol.** Cells treated with tumor lysate (TL) showed slightly higher maturation than control cells (not significant). Cells treated with OK-432 showed greater maturation (P<0.01). **B. Modified DC-preparation Protocol.** Cells treated with a combination of tumor lysate (TL) and TNF-α (TNF) showed markedly higher maturation than cells treated with TL (P<0.01).
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(Beckman Coulter, Fullerton, CA, USA). Cells were analyzed on a FACS Calibur™ flow cytometer. Data analysis was performed with CELLQuestTM software (Becton Dickinson, San Jose, CA, USA). To determine the degree of differentiation and maturation of DCs, $\%CD14^{-}HLA\text{-}DR^{+}$, %CD80, %CD83, and %CD86 were measured.

Immune Response

Blood samples were collected from patients before surgery and one and three months after surgery. IFN-γ and IL-12 were measured in order to determine the magnitude of the immune response following the DC-based immunotherapy.

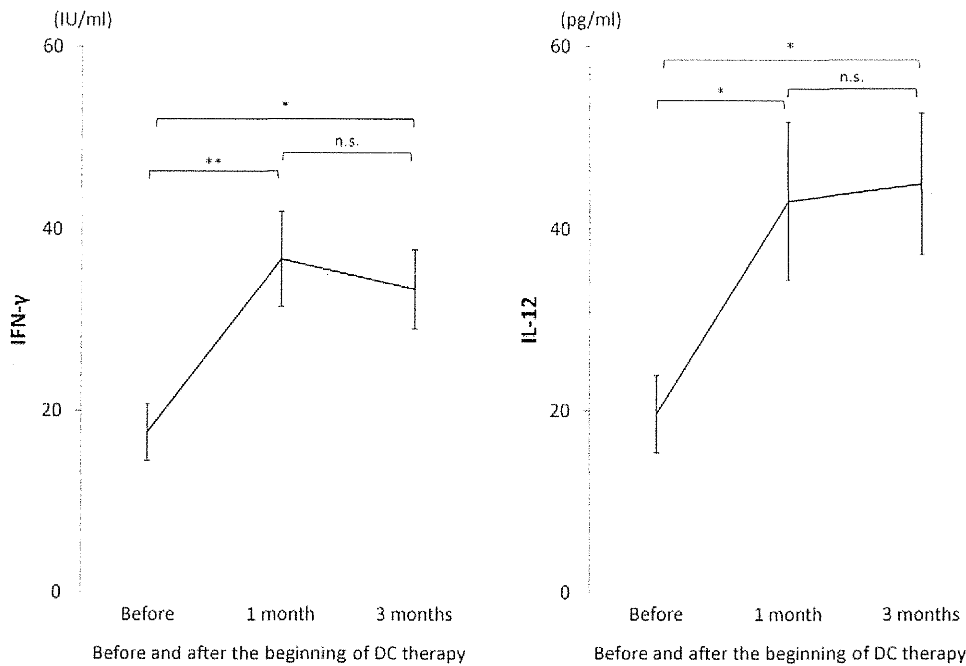

Figure 3. Serum IFN-γ and serum IL-12 before and after DC therapy. * $P<0.05$, ** $P<0.01$, n.s.: not significant.
doi:10.1371/journal.pone.0052926.g003

Overall survival and progression free survival following the DC-based immunotherapy

Overall survival and progression free survival were evaluated by Kaplan-Meier method. Survival was defined as the time from the date of end of DC-based immunotherapy to the date of last follow-up or death due to any cause. Progression free was defined as the time from the date of end of DC-based immunotherapy to the date of last follow-up or tumor progression.

Statistical Analysis

Results are expressed as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) test. Any P-values less than 0.05 were considered statistically significant. Statistical analyses were performed on a personal computer with the statistical package ystat2000 software.

Results

Patients

Thirty-one patients were included in this study. All the patients had metastatic and/or recurrent tumors. There were 14 males and 17 females, with a mean age of 36.3 years (8−64, Table 1). There were 16 patients with bone tumors (13 osteosarcoma and 3 chondrosarcoma,) and 15 patients with soft tissue tumors (3 clear cell sarcoma, 3 synovial sarcoma, 3 malignant fibrous histiocytoma (MFH), 2 leiomyosarcoma, 1 Ewing's sarcoma, 1 liposarcoma, 1

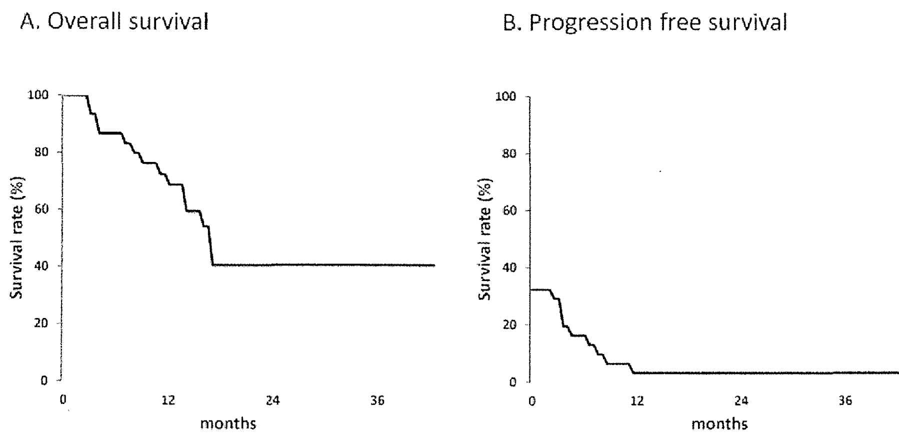

Figure 4. Kaplan-Meier analysis of overall survival and progression free survival distributions for 31 patients with advanced malignant bone and soft tissue tumors.
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alveolar soft part sarcoma (ASPS), and 1 angiosarcoma). None has experienced an adverse event during and after the treatment course.

Differentiation of DCs

To determine the degree of differentiation, %$CD14^-HLA\text{-}DR^+$ was measured by flow cytometry. In total, 52.5±1.2% of cells were classified as $CD14^-HLA\text{-}DR^+$ cells (Table 2). The cells treated with IL-4, GM-CSF, and TL showed more differentiation than the cells treated with IL-4 and GM-CSF only (not significant). The cells treated with IL-4, GM-CSF, and OK-432 showed more differentiation than the cells treated with the other protocols ($P<0.01$). Furthermore, the cells treated with IL-4, GM-CSF, TL, and TNF-α showed more differentiation than the cells treated with IL-4, GM-CSF, and lysate alone ($P<0.05$). These results suggest that TL, TNF-α and OK-432 promoted the differentiation of DCs.

Maturation of DCs

To determine the effects of DC maturation by TL, OK-432, and the combination of TL and TNF-α, %CD80, %CD83, and %CD86 were measured (Figure 2).

The effects of DC maturation by TL and OK-432 (basic DC preparation protocol) were analyzed (Figure 2–A). The %CD80 of the control group, TL group, and OK-432 group were 38.3±3.4, 42.4±3.2, and 64.2±3.1%, respectively. The %CD83 of the control group, TL group, and OK-432 group were 5.9±1.2, 6.2±1.0, and 25.1±1.9%, respectively. The %CD86 of the control group, TL group, and OK-432 group were 41.7±3.6, 45.5±3.9, and 69.9±3.0%, respectively. OK-432 group showed significant increase of %CD80, %CD83, %CD86 comparing with the other groups ($P<0.01$). However, there was no significant difference between TL group and control group. These results indicate that TL slightly induced the maturation of the DCs and that OK-432 markedly induced the maturation of DCs.

The effects of DC maturation by the combination of TL and TNF-α, or OK-432 (modified DC preparation protocol) were analyzed (Figure 2–B). The %CD80 of the TL group, TL + TNF-α (TNF) group, and OK-432 group were 39.1±5.3, 60.3±4.9, and 62.5±3.6%, respectively. The %CD83 of TL group, TL + TNF group, and OK-432 group were 4.5±1.1, 12.2±1.5, and 17.5±2.4%, respectively. The %CD86 of TL group, TL + TNF group, and OK-432 group were 42.0±6.5, 62.5±5.1, and 68.2±3.8%, respectively. Both of TL + TNF group and OK-432 group showed significant increase of %CD80, %CD83, %CD86 comparing with TL groups ($P<0.01$). These results indicate that the combination of TL and TNF-α markedly induced the maturation of DCs.

Immune Responses

Serum IFN-γ and serum IL-12 were compared prior to DC-based immunotherapy, and one and three months after DC injection (Figure 3). Serum IFN-γ before DC injection, and one and three months after the injection was 17.6±3.1, 36.8±5.2, and 33.4±4.4 IU/ml, respectively. Serum IL-12 before DC injection, and one and three months after the injection was 19.7±4.3, 43.1±8.6, and 45.0±7.8 pg/ml, respectively. Both of IFN-γ and IL-12 showed significant increases after DC injection. These results indicate that our DC therapy induced the activation of immune responses one month after the therapy, and that the activated immune response is maintained for at least three months.

Overall survival and Progression free survival

The mean follow-up of all patients was 14.9 months (range 2–43 months). Of the 31 patients, 17 patients were alive and 1 patient was progression free at the time of the final follow-up. Overall survival rates and progression free survival rates of the patients at 3 years were 68.7% and 3.2%, respectively (Figure 4).

Discussion

The introduction of chemotherapy dramatically improved the treatment outcome for patients with primary bone and soft tissue sarcoma [21]. The standard surgery for bone and soft tissue sarcoma before the introduction of chemotherapy was limb amputation [21]. Consequently, the prognosis of patients with bone and soft tissue sarcoma was very poor before the introduction of chemotherapy. The most important prognostic factor before the introduction of chemotherapy was the metastatic lesions that existed before limb amputation. Unlike surgery and radiotherapy, chemotherapy shows systemic antitumor effects, which affect primary tumors and metastatic tumors. However, the continuation of chemotherapy causes many complications, such as renal failure, heart failure, and neuropathy. In cases with local recurrence or metastatic tumors, complications make it difficult to continue the chemotherapy course. Therefore, further systemic treatment of sarcoma is required an improved prognosis of patients with bone and soft tissue tumors. Recently, many experimental and clinical studies concerning immunotherapy for the treatment of malignant tumors have been reported [12]. Additionally, some patients showed a possible tumor suppressive effect of immunotherapy, indicating that this could be a promising approach in the treatment of patients with refractory malignant tumors.

Immunotherapy is classified as either non-specific immunotherapy or tumor specific immunotherapy [22,23]. Non-specific immunotherapy includes cytokine therapy, lymphokine-activated killer cells therapy, natural killer cell therapy, ERM activated killer cell therapy, and CD3-activated T cell therapy. Tumor-specific immunotherapy includes DC-based immunotherapy, cytotoxic T lymphocyte therapy, and peptide-vaccine therapy.

DCs play a critical role in T cell priming, as well as direct and cross-priming [12]. In the steady state, nonactivated (immature) DCs present self-antigens to T cells, which leads to tolerance through different mechanisms. Once activated (mature), antigen-loaded DCs promote T cell proliferation and differentiation into helper and effector cells. DCs are also important in launching humoral immunity. This is partly due to their capacity to directly interact with B cells and to present unprocessed antigens. DCs demonstrate tumor-specific antitumor effects by presenting tumor-specific antigens to immune cells. Furthermore, DCs play a pivotal role in the immunity to tumors. Once a dendritic cell takes up tumor antigens, the dendritic cell will present the tumor antigens to 100– 5,000 lymphocytes. Therefore, DC-based immunotherapy seems to be an efficient treatment for malignant tumors. However, the population of DCs in PBMCs is 0.2% [24], which is too low to make their collection practical for clinical applications [15]. Methods of collection or generation of DCs are required in order to use DC-based immunotherapy clinically. To date, PBMCs-derived DCs have been commonly used for DC-based immunotherapy.

IL-4 and GM-CSF are commonly used for the differentiation of DCs. Nakamoto et al. [25] reported that 45% of PBMCs in patients with hepatocellular carcinoma were differentiated into DCs by the treatment with IL-4 and GM-CSF. This study showed that 52.5% of PBMCs in patients with bone and soft tissue tumors were differentiated into DCs by IL-4 and GM-CSF. Furthermore,

DC maturation requires stimuli such as IL-1β, TNF-α, IL-6, or prostaglandin E_2. Although various DC maturation cocktails have been reported, there is no gold standard method for DC maturation for DC-based immunotherapy. OK-432 (Picibanil), a penicillin-killed Streptococcus pyogenes, is reported to have potent immunomodulation properties in cancer treatment [26]. Hovden reported that OK-432 induces the maturation and migration of DCs and stimulates the secretion of Th-1 type cytokines [27]. Our results show that OK-432 had a strong effect on DCs-differentiation and DC maturation. Although OK-432 has strong effects on DC-differentiation and DC maturation, OK-432-pulsed DCs are not able to activate tumor antigen-specific immunity. Conversely, tumor antigen-pulsed DCs can induce a tumor-specific immune response. Hsu *et al.* [10] reported that DC pulsed with a tumor antigen could elicit specific tumor-reactive T cells, and have clinical efficacy in patients with lymphoma. As with other malignancies, TL-pulsed DCs seem to be effective in the treatment of sarcoma. Our results showed that the TL slightly promoted differentiation and maturation of DCs. The effect of promoting DC-differentiation and DC maturation suggests the existence of tumor-specific antigens in many malignant bone and soft tissue tumors. Additionally, our results showed that TNF-α has a strong effect on promoting DC maturation. The TL/TNF-α pulsed DCs showed 1.6– 1.7 times greater maturation than the TL-pulsed DCs. Although TL-pulsed DCs can present tumor antigens, the TL-pulsed DCs showed only slight maturation. TNF-α promotes the maturation of TL-pulsed DCs. The well-differentiated and well-matured DCs generated by our DCs-generation protocols are considered to have strong tumor antigen-specific effects.

DC-based immunotherapy has been generally ineffective in promoting tumor rejection. The failure may be attributed to the use of insufficiency matured or activated DCs. In patients with advanced and metastatic cancer, immunity is generally suppressed by factors produced by tumors and tumor infiltrating cells such as regulatory T cells [28]. In order to overcome the immunosuppressive circumstances around tumors, inductions of sufficiently activated DCs, which highly express MHC molecules, CD80, CD86, and IL-12, are required. The existence of matured DCs around tumors has a great importance in antitumor immune response and prognostic significance [29,30]. Ménard et al. reported that levels of IFN-γ significantly correlate with progression-free survival [31].

Therefore, evaluations of the DCs-maturation and DCs-related cytokines are important in the DC-based immunotherapy. In this study, levels of IFN-γ and IL-12 were measured before and after the immunotherapy. The immunological responses are commonly evaluated by IL-12 and IFN-γ because these cytokines reflect the activation of the DCs [14,32–35]. Furthermore, it is reported that the existence of tumors does not influence the levels of the serum IL-12 and IFN-γ [32]. IL-12 secreted by DCs acts on IFN-γ production by Th1 cells. IFN-γ activates natural killer (NK) cells and cytotoxic T lymphocytes (CTL) that contribute to optimal antitumor immunity. Although chemotherapy shows rapid effects on tumors, DC-based immunotherapy shows slow tumor regression. The immune response is highest at 8– 10 weeks after DC-injection [36]. This study showed significant elevations of IFN-γ and IL-12 at one and three months after DC-based immunotherapy. However, a limitation of this study is the lack of data about serum IFN-γ and IL-12 in placebo group. Therefore, it seems that tumor progression influenced the increase of serum IFN-γ and IL-12. There are some reports about the correlation between tumor progression and serum cytokine levels [37,38]. Tsuboi et al. [37] reported that mean serum IL-12 level in patients with esophageal carcinoma ($n = 70$) was significantly higher than that in healthy volunteers (15). The levels of serum IL-12 correlated with tumor growth and progression, although the there was no significant correlation between serum IL-12 level and tumor progression. Morreti et al. [38] reported the difference of the serum IL-12 and IFN-γ levels in control population ($n = 45$), patients with localized melanoma ($n = 11$), and patients with metastatic melanomas ($n = 34$). Mean serum IL-12 level of the patients with localized melanomas was lower than that of the patients with metastatic melanomas. Mean serum IFN-γ level of patients with localized melanoma was higher than that of patients with metastatic melanomas, although there is no significant difference. In our study, serum IL-12 and IFN-γ showed significant increases which are much higher than the influence of tumor progression. These results suggest that the increase of the serum IL-12 and IFN-γ levels were influenced by the DC-based immunotherapy.

Conclusions

DCs-based immunotherapy is a promising approach for the treatment of malignant tumors. In this study, about 50% of PBMCs were differentiated into DCs, and markedly matured by the combination of GM-CSF, IL-4, TL and TNF-α. Our results contribute to the development of DC-based immunotherapy for malignant bone and soft tissue tumors.

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Author Contributions

Conceived and designed the experiments: SM HN AT HT. Performed the experiments: SM HN YT MT. Analyzed the data: SM HN. Contributed reagents/materials/analysis tools: NY TS KH HK KI EM YN SK HT. Wrote the paper: SM HN HT.

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GASTROENTEROLOGY

Interleukin-21 and tumor necrosis factor-α are critical for the development of autoimmune gastritis in mice

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autoimmune gastritis, autoantibody production, cytokine.

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Abstract

Background and Aim: Autoimmune gastritis (AIG), an organ-specific autoimmune disease, is accompanied by achlorhydria, pernicious anemia, gastric carcinoid tumors, and gastric cancer. Patients with AIG initially respond to corticosteroids but have a great potential to relapse after treatment is withdrawn. This study examines the roles of cytokines in order to identify potential therapeutic options for AIG patients.

Methods: Using a mouse model of AIG, we monitored disease progression and administered antibodies *in vivo* to block cytokines.

Results: We developed a mouse model of AIG with early onset and rapid progression in which neonatal thymectomy (NTx) was performed on programmed cell death 1-deficient (PD-$1^{-/-}$) mice on the BALB/c background. Using NTx–PD-$1^{-/-}$ mice, we found that in AIG lesions, interferon-γ, and tumor necrosis factor (TNF)-α together with interleukin-21 (IL-21) were highly expressed in the inflamed gastric mucosa. In addition, as with the injection of dexamethasone, *in vivo* administration of either anti-TNF-α or anti-IL-21 suppressed the development of AIG in NTx–PD-$1^{-/-}$ mice.

Conclusions: These data reveal the essential role of IL-21 in the development of AIG and suggest that in addition to corticosteroids, anti-TNF-α as well as anti-IL-21 have the potential to induce the remission of AIG, offering additional therapeutic options for AIG patients.

Introduction

Autoimmune gastritis (AIG), a typical organ-specific autoimmune disease, develops worldwide in diverse ethnic groups. AIG is often accompanied by achlorhydria, which appears to persist asymptomatically for many years, resulting in various severe complications such as pernicious anemia, gastric carcinoid tumors, or gastric cancer.[1–4] The characteristic histological findings of AIG are a chronic mononuclear cell infiltration affecting only or predominantly the corpus mucosa and causing loss of parietal and chief cells from the gastric gland.[1] The serologic hallmark of AIG is the production of characteristic circulating autoantibodies (autoAbs), including an antibody against H^+K^+-ATPase, in the parietal cells of the stomach.[5,6] AIG appears to be a $CD4^+$ T cell-mediated disease, and various inflammatory cytokines, such as interferon (IFN)-γ, and tumor necrosis factor (TNF)-α are present in the inflamed mucosal lesion in AIG.[4,7] However, the roles of these cytokines in the development of human AIG are not fully understood. In addition, although patients with AIG initially respond to corticosteroids, these patients have a great potential to relapse after treatment is withdrawn.[8,9] Additional potential therapeutic options are needed for patients with AIG.

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Mouse models of AIG share many pathological and clinical features with human AIG.[4] One mouse model of AIG comprises BALB/c mice thymectomized 3 days after birth (NTx mice).[10] Adult NTx mice frequently develop AIG, showing lymphocytic infiltration with selective loss of parietal and chief cells from the gastric mucosa, and production of autoAbs to parietal cells.[10] In addition, AIG developed in NTx mice is characterized by a marked infiltration of $CD4^+$ T cells, which produce large amounts of IFN-γ.[11,12] The other mouse model of AIG is programmed cell death 1-deficient ($PD\text{-}1^{-/-}$) mice on the BALB/c background.[13] PD-1 is an immunoreceptor belonging to the CD28/CTLA-4 family and provides negative costimulation to antigen stimulation. Most of the adult $PD\text{-}1^{-/-}$ mice develop AIG and produce autoAbs to parietal cells.[13]

Recently, we found that $PD\text{-}1^{-/-}$ mice thymectomized 3 days after birth (NTx–$PD\text{-}1^{-/-}$ mice) concurrently developed autoimmune hepatitis and AIG.[14–16] In these mice, gastritis and hepatitis simultaneously and sequentially progressed.[14] In 1-week-old NTx–$PD\text{-}1^{-/-}$ mice, immature gastric glands showed no inflammation. However, 2-week-old NTx–$PD\text{-}1^{-/-}$ mice started to show mononuclear cell infiltrations, predominantly in the lamia propria of the gastric gland. These mononuclear cell infiltrations rapidly progressed in 3- or 4-week-old NTx–$PD\text{-}1^{-/-}$ mice.[14] Thus, AIG in NTx–$PD\text{-}1^{-/-}$ mice with early onset and rapid progression seems to be a good model to use to clarify the mechanisms involved in the development of AIG.

In this study using NTx–$PD\text{-}1^{-/-}$ mice, we examined the roles of cytokines and the therapeutic efficacy of neutralizing these cytokines. We found that the inflamed gastric mucosa in AIG highly expressed IFN-γ and TNF-α together with interleukin-21 (IL-21). In addition to the injection of dexamethasone (DEX), *in vivo* administration of either anti-TNF-α or anti-IL-21 suppressed the development of AIG. Thus, these data revealed the essential role of IL-21 in the progression of AIG and suggest that in addition to corticosteroids, anti-TNF-α as well as anti-IL-21 may induce remission of AIG offering the additional therapeutic options for patients with AIG.

Methods

Mice. BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), PD-1-deficient mice and IL-17 deficient mice on a BALB/c background were generated as described previously.[17,18] All of these mice were bred and housed under specific pathogen-free conditions. Thymectomy of the mice 3 days after birth was performed as described previously.[14–16] All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Histological examination. Organs were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin for histopathology. Fluorescence immunohistology was performed on frozen sections as described previously,[14–16] using fluorescein isothiocynate-conjugated anti-CD4, anti-CD11c (eBioscience, San Diego, CA, USA), anti-IL-21 (R&D Systems, Minneapolis, MN, USA) followed by staining using Alexa Flour 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA), anti-TNF-α (abcam, Cambridge, UK) followed by staining using Alexa Flour 488-conjugated anti-rabbit IgG (Invitrogen), or anti-IFN-γ (R&D Systems) followed by staining using Alexa Flour 488-conjugated anti-hamster IgG (Invitrogen). The degree of gastritis was determined according to a modification of a semiquantitative scoring system, as described previously.[19,20] Chronic inflammation, characterized by infiltration of mononuclear cells, was graded from 0 to 3, where 0 = no increase in inflammatory cells, 1 = slight infiltration of the lamina propria by lymphocytes, 2 = moderately dense infiltration, and 3 = very dense infiltration. Atrophic changes were graded from 0 to 3 according to the loss of specialized chief and parietal cells (0 = no loss; 1 = mild loss of specialized cells, limited to half of the corpus glands; 2 = moderate loss of specialized cells, diffusing to more than half the corpus glands; and 3 = severe loss/almost complete loss of specialized cells throughout the gastric body). The degree of foveolar hyperplastic change in mucus neck cells of the corpus glands was scored on a scale of 0 to 3 (0 = no hyperplastic change; 1 = focal hyperplastic change of mucus neck cells of the corpus glands; 2 = moderate hyperplastic change of mucus neck cells, diffusing to the corpus glands with less than twice the height of normal foveolar epithelial layer; and 3 = severe hyperplastic change of mucus neck cells, diffusing to the corpus glands with more than twice the height of a normal foveolar epithelial layer).

ELISA. Serum anti-parietal cell antibody levels were measured by ELISA, as described previously.[19,20] Briefly, duplicate wells of microtiter plates (Nunc, Roskilde, Denmark) were incubated with 10 μg/mL antigens, extracts prepared from the normal gastric mucosa, in phosphate-buffered saline (PBS) for 16 h at 4°C. The wells were blocked with PBS containing 5% nonfat dried milk and then incubated with serial dilutions of sera for 1 h. The wells were then incubated with horseradish-peroxidase-labeled goat anti-mouse IgG (Serotec, Oxford, UK) diluted at a predetermined concentration for 1 h at room temperature. After rigorous washing, each well was reacted with substrate solution (R&D Systems) for 10 min. The reaction was terminated with 50 μL of 2 mol/L H_2SO_4, and absorbency at 490 nm was determined with a microplate reader. Serum cytokine concentrations were measured with a mouse IFN-γ ELISA set (eBioscience) according to the manufacturer's protocols.

Administration of dexamethasone (DEX) in vivo. NTx–$PD\text{-}1^{-/-}$ mice at 1 day after thymectomy were intraperitoneally injected every other day with 1.0 mg/kg of DEX (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS or PBS alone. After 13 injections, mice at 4 weeks of age were sacrificed, and the stomach and sera were harvested.

Administration of neutralizing antibodies (Abs) in vivo. NTx–$PD\text{-}1^{-/-}$ mice at 1 day after thymectomy were intraperitoneally injected every week for neutralizing Abs to mouse TNF-α and IL-21 from R&D Systems. All isotypes were from eBioscience or R&D Systems. After four injections, mice at 4 weeks of age were killed, and their stomach and sera were harvested.

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Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Real-time quantitative RT-PCR was performed as described previously.[20] Gastric tissues were frozen in RNAlater (Qiagen, Hilden, Germany). Total RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Single-stranded complementary DNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using SYBR Green I Master (Roche Applied Science, Basel, Switzerland). The real-time quantitative reactions were performed using a Light Cycler 480 (Roche Applied Science) according to the manufacturer's instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: *GAPDH*: 5′-CAACTTTGTCAA GCTCATTTCC-3′ and 5′-GGTCCAGGGTTTCTTACTCC-3′; *IFN-γ*: 5′-GGATGCATTCATGAGTATTGC-3′ and 5′-CCTTTT CCGCTTCCTGAGG-3′; *TNF-α*: 5′-CCCTCACACTCAGATCA TCTTCT-3′ and 5′-GCTACGACGTGGGCTACAG-3′ *IL-4*: 5′-TCATCGGCATTTTGAACGAG-3′ and 5′-CGTTTGGCACAT CCATCTCC-3′; *IL-13*: 5′-TGGGTGACTGCAGTCCTGGCT-3′ and 5′-GTTGCTTTGTGTAGCTGAGCA-3′; *IL-17A*: 5′-CTCC AGAAGGCCCTCAGACTAC-3′ and 5′-AGCTTTCCCTCCG CATTGACACAG-3′; *IL-21*: 5′-GACATTCATCATCGACCTCG T-3′ and 5′-TCACAGGAAGGGCATTTAGC-3′.

Statistical analysis. Statistical analysis was performed by Student's *t*-test for unpaired comparisons between parametric data. *P*-values below 0.05 were considered significant.

Results

PD-1 deficiency exaggerates histopathology and autoAb production in NTx mice. AIG developed in adult NTx mice and PD-1$^{-/-}$ mice shares many pathological features with human AIG and is characterized by lymphocytic infiltration with selective loss of parietal and chief cells from the gastric mucosa as well as production of autoAbs to parietal cells.[10,13] Recently, we found that NTx–PD-1$^{-/-}$ mice also developed AIG.[14,16] In 1-week-old NTx–PD-1$^{-/-}$ mice, immature gastric glands show no inflammation. However, 2-week-old NTx–PD-1$^{-/-}$ mice started to show mononuclear cell infiltrations, predominantly in the lamia propria of the gastric gland (data not shown). These mononuclear cell infiltrations rapidly progressed in 4-week-old NTx–PD-1$^{-/-}$ mice (Fig. 1a, lower right). Compared with the findings in NTx–PD-1$^{-/-}$ mice, NTx mice and PD-1$^{-/-}$ mice at 4 weeks of age showed milder mononuclear cell infiltrations without any hyperplasia of the foveolar mucus neck cells (Fig. 1a).

These findings were further confirmed by a gastritis scoring system that evaluates (i) chronic inflammation, characterized by the infiltration of mononuclear cells; (ii) atrophic changes based on the loss of parietal and chief cells; and (iii) hyperplastic changes of foveolar mucus neck cells. In contrast to control mice, both the inflammation score and the total gastritis score were significantly elevated in 4-week-old NTx–PD-1$^{-/-}$ mice (Fig. 1b,c).

In addition, using an ELISA to examine the serum levels of anti-parietal cell Abs, we compared the levels of production of anti-parietal cell Abs. In 4-week-old NTx–PD-1$^{-/-}$ mice, serum levels of anti-parietal cell Abs were significantly higher than those in control mice (Fig. 1d). These data suggest that AIG in NTx–PD-1$^{-/-}$ mice, showing early onset and rapid progression of the disease, offer a good model to clarify the mechanisms involved in AIG development.

Elevated gastric expression of IFN-γ, TNF-α as well as IL-21 in NTx–PD-1$^{-/-}$ mice. Because inflammatory Th1 responses are critical for the development of AIG,[11,12] we examined whether 4-week-old NTx–PD-1$^{-/-}$ mice showed enhanced Th1 responses in the inflamed stomach of AIG. We performed real-time quantitative RT-PCR analysis to measure the expression levels of messenger RNA (mRNA)-encoding cytokines such as IFN-γ, IL-4, and IL-17. Inflamed gastric tissues of 4-week-old NTx–PD-1$^{-/-}$ mice expressed a significantly increased level of mRNA expression of IFN-γ together with TNF-α in comparison with those in control mice (Fig. 2, upper panels). In contrast, levels of mRNA expression of Th2 such as IL-4 and IL-13, and Th17-related IL-17, were not significantly affected in NTx–PD-1$^{-/-}$ mice. Interestingly, we found that IL-21 mRNA expression was significantly elevated in NTx–PD-1$^{-/-}$ mice in comparison with that of control mice (Fig. 2, lower right panel). To confirm the enhanced expression of IFN-γ, TNF-α, and IL-21 in the inflamed gastric mucosa in NTx–PD-1$^{-/-}$ mice, the inflamed mucosa was examined by immunohistology. We could detect protein expression of IFN-γ, TNF-α, and IL-21 in inflamed gastric mucosa but not in the normal control mucosa (Fig. 3). Taken together, these data suggest that enhanced Th1 responses as well as IL-21-related inflammation is involved in the development of AIG.

Neutralization of TNF-α or IL-21 as well as DEX injection significantly suppresses AIG development in NTx–PD-1$^{-/-}$ mice. Treatment with corticosteroids can suppress the inflammation of AIG in humans.[8,9] To examine whether corticosteroid treatment suppresses the development of AIG in this mouse model, NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were intraperitoneally injected every other day with DEX diluted in PBS or PBS alone. After 13 injections, mice at 4 weeks of age showed that administration of DEX suppressed the infiltration of mononuclear cells in the gastric mucosa (Fig. 4a, upper panels). These findings were further confirmed by the gastritis scoring system. In contrast to control mice, the inflammation score as well as the total gastritis score were significantly reduced in mice with DEX administration (Fig. 4b, upper panel).

Previously, we reported that in NTx–PD-1$^{-/-}$ mice, neutralization of IFN-γ suppressed the development of AIG, suggesting that IFN-γ is critically involved in the development of AIG.[16] In the present study, we examined whether neutralization of TNF-α or IL-21 can suppress the development of AIG. NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing monoclonal antibody to mouse TNF-α, IL-21, or isotype controls. After four injections, mice at 4 weeks of age were sacrificed. Neutralization of TNF-α or IL-21 suppressed mononuclear cell infiltration in the gastric mucosa as well as gastritis scores at 4 weeks of age (Fig. 4a,b, middle and lower panels). These data indicate that neutralization of TNF-α or IL-21 suppressed the development of AIG in NTx–PD-1$^{-/-}$

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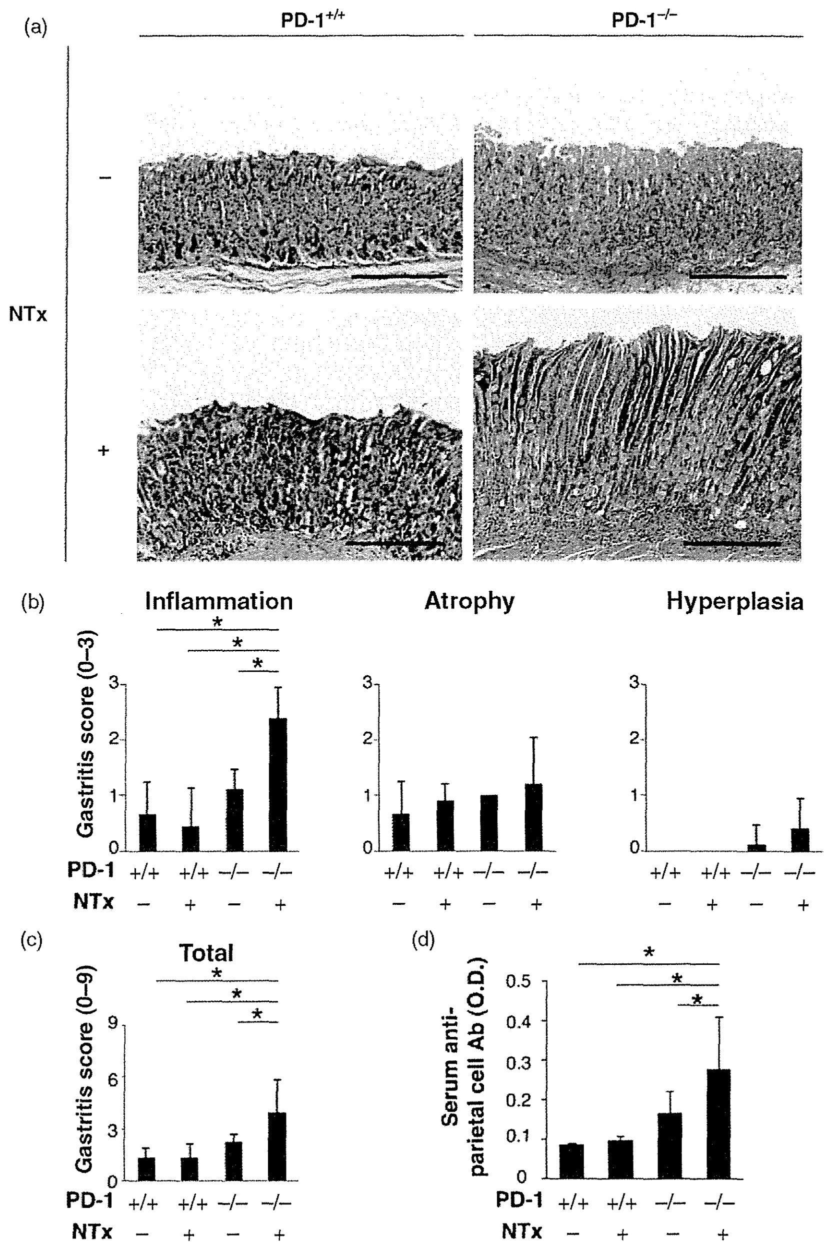

Figure 1 Programmed cell death 1 (PD-1) deficiency exaggerates histopathology and autoantibody (autoAb) production in the mouse model of autoimmune gastritis (AIG). (a) Histologic findings of the gastric mucosa in 4-week-old PD-1$^{+/+}$ and PD-1$^{-/-}$ mice with or without NTx. The sections of tissues were stained with hematoxylin and eosin. All scale bars, 100 μm. (b) Degree of inflammation, atrophic change, and hyperplastic change in the indicated mice were determined by a semiquantitative scoring system, as described in Materials and Methods. (c) Degree of total gastritis score (inflammation, atrophic change, and hyperplastic change) in 4-week-old PD-1$^{+/+}$ and PD-1$^{-/-}$ mice with or without NTx. (d) Serum anti-parietal cell Ab levels were measured by ELISA. Sera were collected from indicated mice. Data are presented as mean and SD of at least five mice from indicated groups. Student's t-test was used for unpaired comparisons. $*P < 0.05$.

mice, suggesting an essential role for TNF-α and IL-21 in the development of AIG.

IL-17 is dispensable for the induction and progression of AIG development. A recent study[21] showed that NTx mice with severe AIG in adult age had the IL-17-producing CD4$^+$ T cells in the inflamed gastric mucosa and that IL-17$^{-/-}$ CD4$^+$ T cells transferred into recipient mice, induced the development of AIG but severity of AIG decreased in comparison with the transfer of wild type CD4$^+$ T cells, suggesting that the production of IL-17 by effector T cells is not indispensable for the initiation of AIG, but may be required for the disease progression.

However, in the AIG model with rapid progression with early onset, we could not find increased IL-17 expression in the inflamed gastric mucosa in the progression (Fig. 2). To further evaluate whether IL-17 is involved in the progression of AIG, we performed thymectomy 3 days after birth in IL-17$^{-/-}$ or IL-17$^{+/+}$ mice in BALB/c background. In 8-week-old mice, anti-parietal cell antibody titers in NTx–IL-17$^{-/-}$ mice were levels similar to those in NTx–IL-17$^{+/+}$ mice (Fig. 5a). Notably, histological examination revealed that the gastric mucosa in 8-week-old NTx–IL-

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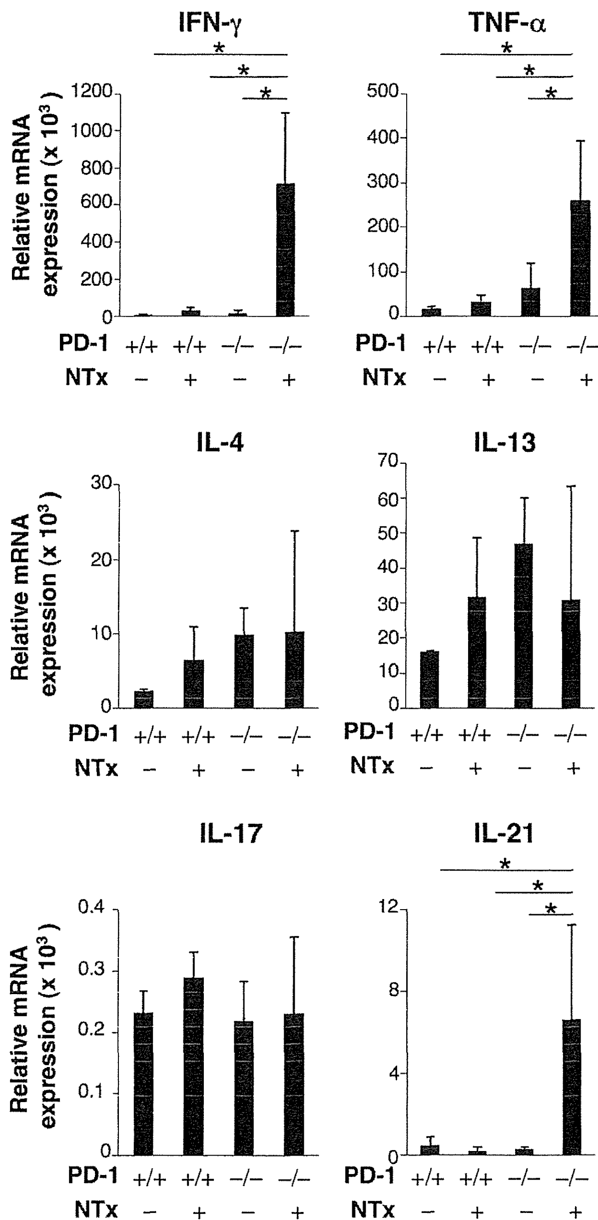

Figure 2 Gastric messenger RNA (mRNA) expressions of interferon (IFN)-γ, tumor necrosis factor (TNF)-α as well as interleukin (IL)-21 are elevated in PD-1$^{-/-}$ mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to measure the expression levels of mRNA-encoding, T-cell–producing cytokines such as IFN-γ, TNF-α, IL-4, IL-13, IL-17, and IL-21 in 4-week-old PD-1$^{+/+}$ and PD-1$^{-/-}$ mice with or without NTx. Data are presented as mean and SD of at least five mice from indicated groups. Student's *t*-test was used for unpaired comparisons. *$P < 0.05$.

17$^{-/-}$ mice had chronic gastritis with mononuclear cell infiltration, and complete loss of parietal and chief cells, accompanied by enhanced hyperplasia of the foveolar mucus neck cells at level similar to that in same aged NTx–IL-17$^{+/+}$ mice (Fig. 5b). These findings were further confirmed by the gastritis scoring system (Fig. 5c). Taken together, these results suggest that IL-17 is dispensable for the induction and progression of AIG development.

DEX injection suppressed the infiltration of CD4$^+$ T cells and CD11c$^+$ cells, whereas neutralization of TNF-α or IL-21 reduced the infiltration of CD4$^+$ T cells but allowed CD11c$^+$ cells to infiltrate into the gastric mucosa. Next, to further investigate infiltrating cells in the gastric mucosa, these cells were examined by immunohistology. The gastric mucosa originally does not have a lymphoid apparatus. In 2-week-old NTx–PD-1$^{-/-}$ mice, CD4$^+$ T cells did not infiltrate the gastric mucosa, whereas CD11c$^+$ cells existed in the lamia propria of the gastric gland.[16] In 4-week-old NTx–PD-1$^{-/-}$ mice, massive infiltrations of CD4$^+$ T cells with CD11c$^+$ cells were observed in the inflamed gastric mucosa (Fig. 6, upper panels). In contrast, administration of DEX suppressed the infiltration of CD4$^+$ T cells as well as CD11c$^+$ cells in the gastric mucosa. However, neutralization of TNF-α or IL-21 reduced the infiltration of CD4$^+$ cells but not CD11c$^+$ cells into the gastric mucosa (Fig. 6, lower panels). Taken together, our results indicate that TNF-α and IL-21 may be involved in CD4$^+$ T-cell accumulation in AIG lesions.

Serum levels of IFN-γ were significantly repressed by neutralization of TNF-α and IL-21. Using NTx–PD-1$^{-/-}$ mice, we previously demonstrated that in the development of AIG, CD4$^+$ T-cell migration into the gastric mucosa is essential for induction and that IFN-γ is critically involved in CD4$^+$ T-cell migration into the gastric mucosa by upregulating local CCL20 expression.[16] Indeed, AIG-bearing NTx–PD-1$^{-/-}$ mice at 4 weeks of age showed markedly increased levels of IFN-γ in the serum. In addition, neutralization of TNF-α or IL-21 suppressed serum levels of IFN-γ in 4-week-old NTx–PD-1$^{-/-}$ mice (Fig. 7). These data suggest that TNF-α and IL-21 may be at least partially involved in the IFN-γ upregulation of local CCL20 expression in the gastric mucosa.

Discussion

In the present study, using a mouse model of AIG in NTx–PD-1$^{-/-}$ mice, we found that IFN-γ and TNF-α together with IL-21 were highly expressed in the inflamed gastric mucosa. In addition, as with injection of DEX, *in vivo* administration of either anti-TNF-α or anti-IL-21 suppressed AIG. These data suggest that IL-21 is essential to the development of AIG and that in addition to corticosteroids, anti-TNF-α as well as anti-IL-21 have therapeutic potential for AIG.

AIG developed in NTx mice and PD-1$^{-/-}$ mice shares many pathological features with human AIG.[10,13] However, AIG manifested in adulthood and the prevalence of AIG development in NTx mice and PD-1$^{-/-}$ mice is about 50% and 70%, respectively.[13,22] In contrast, in this study, we demonstrated that most of NTx–PD-1$^{-/-}$ mice developed AIG with early onset and rapid progression. Thus, NTx–PD-1$^{-/-}$ mice may be an appropriate AIG model for precisely examining and clarifying the mechanisms involved in AIG development.

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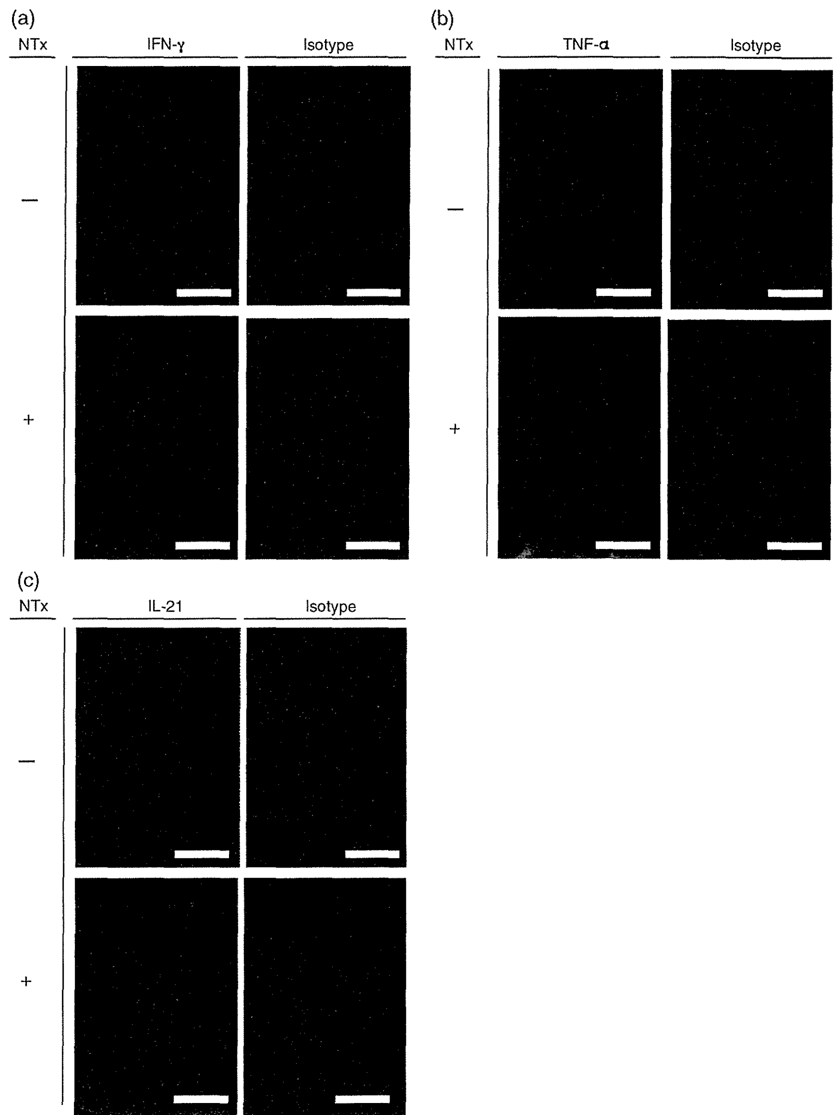

Figure 3 Gastric protein expressions of interferon (IFN)-γ, tumor necrosis factor (TNF)-α as well as interleukin (IL)-21 are elevated in PD-1$^{-/-}$ mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice). Fluorescence immunostainings of the stomach for IFN-γ (a), TNF-α (b), IL-21 (c) and isotype controls in 4-week-old PD-1$^{-/-}$ mice with or without NTx. Data shown are representative of at least five mice in each group. All scale bars, 50 μm.

In this study, using our mouse model of AIG, we observed that intraperitoneal injections of DEX suppressed AIG. Human AIG patients respond well to corticosteroid treatment.[8,9] Thus, our data accord well with the usefulness of corticosteroids in the treatment of human AIG. A previous study to evaluate prednisolone treatment for AIG in mouse models demonstrated that the treatment greatly reduced CD4$^+$ T cells and CD11c$^+$ cells in the gastric mucosa, but some cells remained in the mucosa.[23] However, in this study, intraperitoneal injections of DEX completely suppressed infiltration of CD4$^+$ T cells and CD11c$^+$ cells in the gastric mucosa. It may be that the difference in treatment procedures (intraperitoneal injection of every other day with 1.0 mg/kg of DEX diluted in PBS and 10 mg/kg per day prednisolone in drinking water) affects how efficiently infiltrating cells are reduced in the gastric mucosa.

In this mouse model, we showed that *in vivo* administration of anti-TNF-α suppressed AIG. In addition, we confirmed that the inflamed gastric tissues of AIG highly expressed TNF-α, as described in a previous report.[7] However, the previous study demonstrated that neutralizing anti-TNF-α twice weekly injections for 8 weeks in NTx mice did not suppress the development of AIG.[24] In addition, it showed that neither NTx–TNFR1 deficient mice nor TNFR1 deficient mice with the local transgenic expression of granulocyte-macrophage colony stimulating factor in the stomach developed AIG.[24] Using NTx–PD-1$^{-/-}$ mice, we had previously demonstrated that in AIG development, CD4$^+$ T-cell migration into the gastric mucosa is essential for induction[16] and that IFN-γ is critically involved in CD4$^+$ T-cell accumulation in AIG by upregulating local CCL20 expression.[16] In this study, we found that neutralization of TNF-α suppressed serum levels of IFN-γ in NTx–PD-1$^{-/-}$ mice (Fig. 5). In addition, the previous study reported a critical role for the Fas pathway in mediating gastric mucosal damage associated with AIG.[25] Thus, TNF-α may be mainly involved in the first step of AIG's development to rapid progress, but not in the mucosal cell damage in the target organ. While

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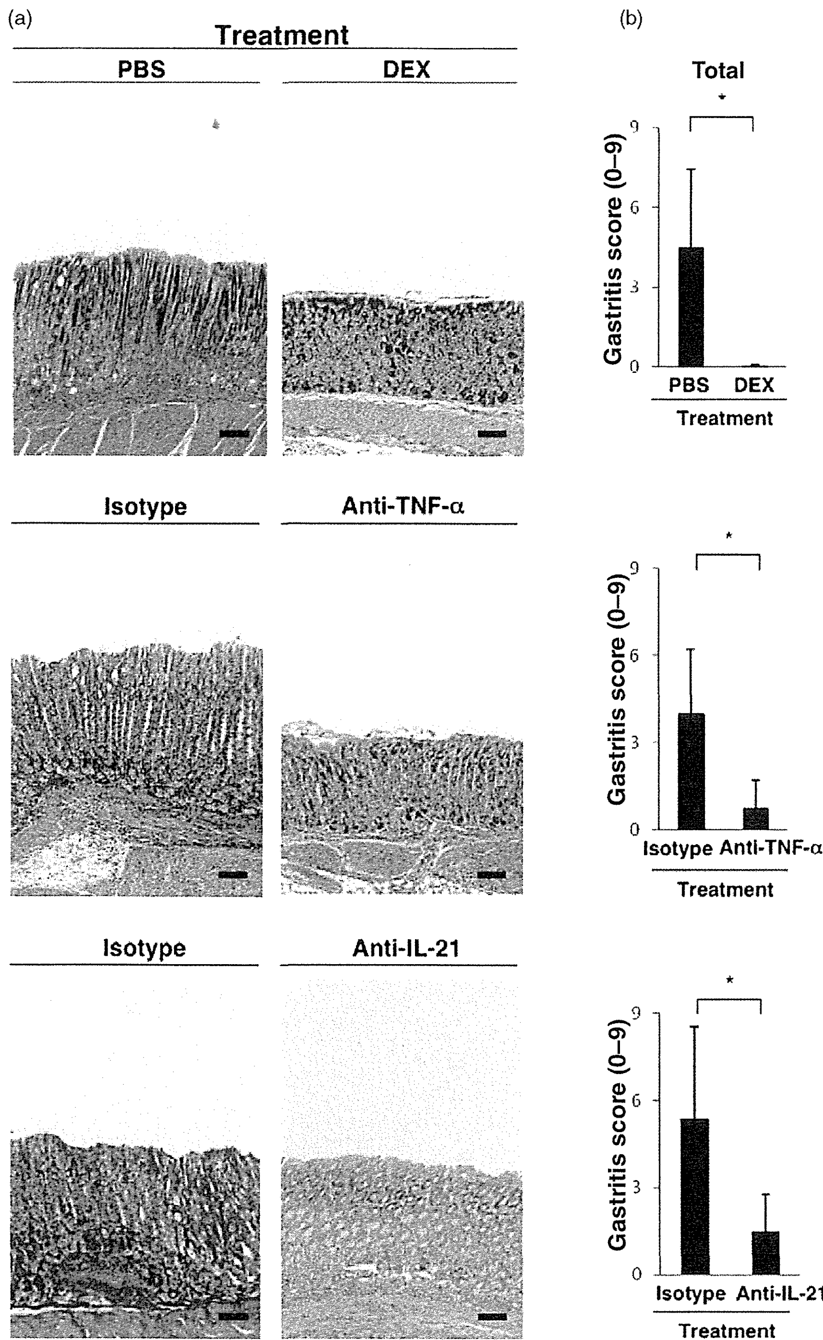

Figure 4 Neutralization of tumor necrosis factor (TNF)-α or interleukin (IL)-21 as well as dexamethasone (DEX) injection significantly suppresses autoimmune gastritis (AIG) development in PD-1$^{-/-}$ mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice). NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were intraperitoneally injected every other day with 1.0 mg/kg of DEX diluted in phosphate-buffered saline (PBS) or PBS alone. After 13 injections, mice at 4 weeks of age were sacrificed, and the stomachs were harvested. For neutralization of TNF-α or IL-21, NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing anti-mouse TNF-α, IL-21, or the isotype control monoclonal antibodies (mAbs). After four injections, mice at 4 weeks of age were sacrificed, and the stomachs were harvested. (a) Histological analysis of the stomach from 4-week-old NTx–PD-1$^{-/-}$ mice treated with indicated treatments. Tissue sections were fixed in formalin and stained HE. All scale bars, 100 μm. Data shown are representative of more than five mice in each group. (b) Degree of gastritis in the indicated mice was determined by a semiquantitative scoring system, as described in Materials and Methods. Data are presented as mean and SD of at least five mice from indicated groups. Student's *t*-test was used for unpaired comparisons. *$P < 0.05$.

blocking TNF-α has therapeutic benefit in patients with certain autoimmune diseases, further studies are required to ascertain whether it may be helpful to AIG patients.

Although AIG is typical organ-specific autoimmune disease mediated by inflammatory Th1 responses, it had not been clear whether IL-21 was involved in the AIG development. In the present study, we found that inflamed gastric tissues of AIG highly expressed IL-21 and neutralization of IL-21 reduced infiltrating CD4 T cells in the gastric mucosa, resulting in the suppression of AIG. IL-21 can activate IL-21R expressing activated T cells and affect their proliferation, differentiation, and survival.[26] *In vivo*, the interaction between IL-21 and IL-21R is critical in viral infection, allergic skin inflammation, and in autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, and type 1 diabetes.[26] However, in humans, it is not clear whether IL-21 can be involved in AIG. Further studies are required

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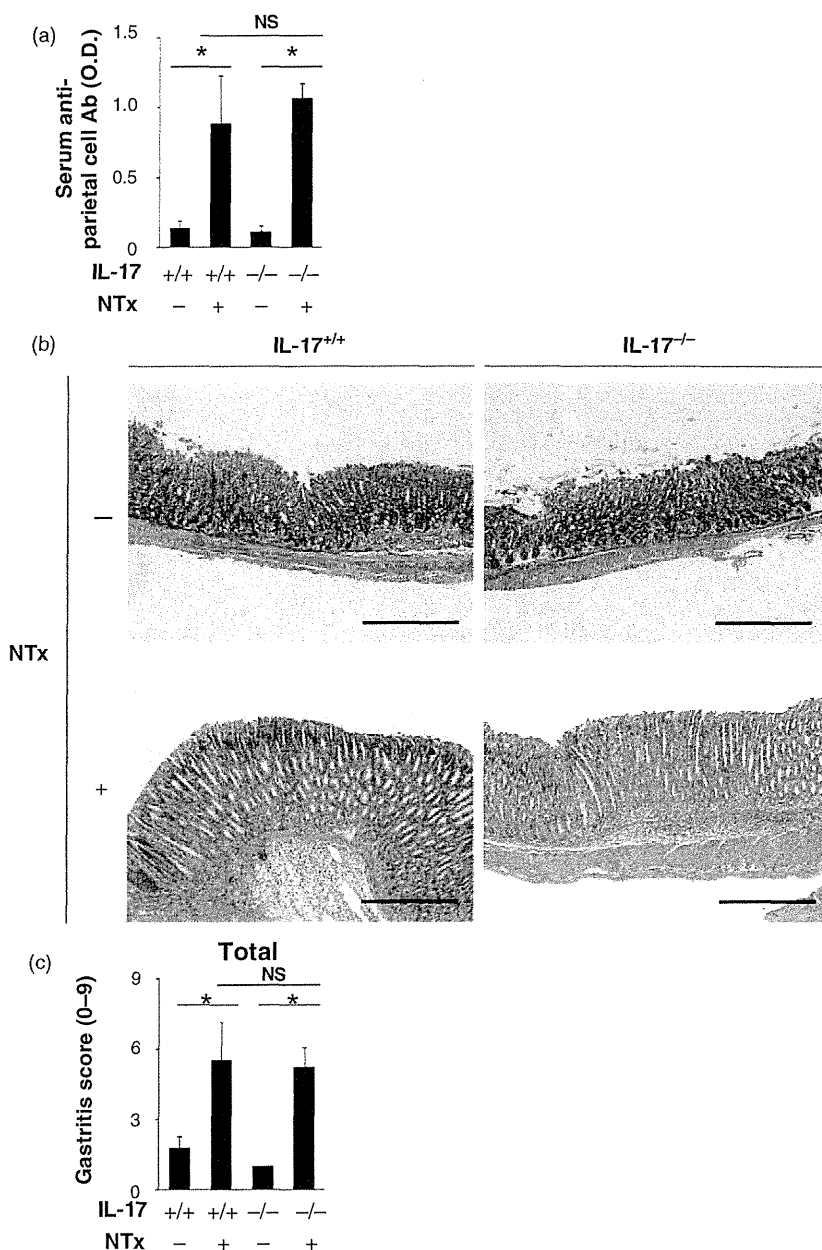

Figure 5 Interleukin (IL)-17 is dispensable for the induction and progression of autoimmune gastritis (AIG) development. (a) Serum anti-parietal cell antibody (Ab) levels were measured by ELISA. Sera were collected from 8-week-old IL-$17^{+/+}$ and IL-$17^{-/-}$ mice with or without neonatal thymectomy (NTx). (b) Histologic findings of the gastric mucosa in indicated mice at 8 weeks old. The sections of tissues were stained with hematoxylin and eosin. All scale bars, 100 μm. (c) Degree of total gastritis score (inflammation, atrophic change, and hyperplastic change) in indicated mice at 8 weeks old. Data are presented as mean and SD of at least five mice from indicated groups. Student's *t*-test was used for unpaired comparisons. $*P < 0.05$. n.s., not significant.

to ascertain whether blocking IL-21R-mediated signaling might be therapeutically useful for patients with AIG.

In recent study, it was suggested that IL-17 plays a role in the progression of AIG.[21] However, in this study, we used AIG model in which mice with BALB/c background were performed thymectomy 3 days after birth and, we found that IL-17 is dispensable for the induction and progression of AIG development. The reason for the apparent discrepancy between our data and the report by Tu *et al.*,[21] who reported IL-17 plays some role in the progression of AIG, is not completely clear at present. However, since they used transfer of $CD4^+$ T cells to induce AIG, it is possible that the difference of the models may have affected the results.

In conclusion, we have demonstrated that IL-21 and TNF-α are essential in the development of AIG. In addition to corticosteroids, anti-TNF-α and anti-IL-21 may have therapeutic uses for AIG in humans.

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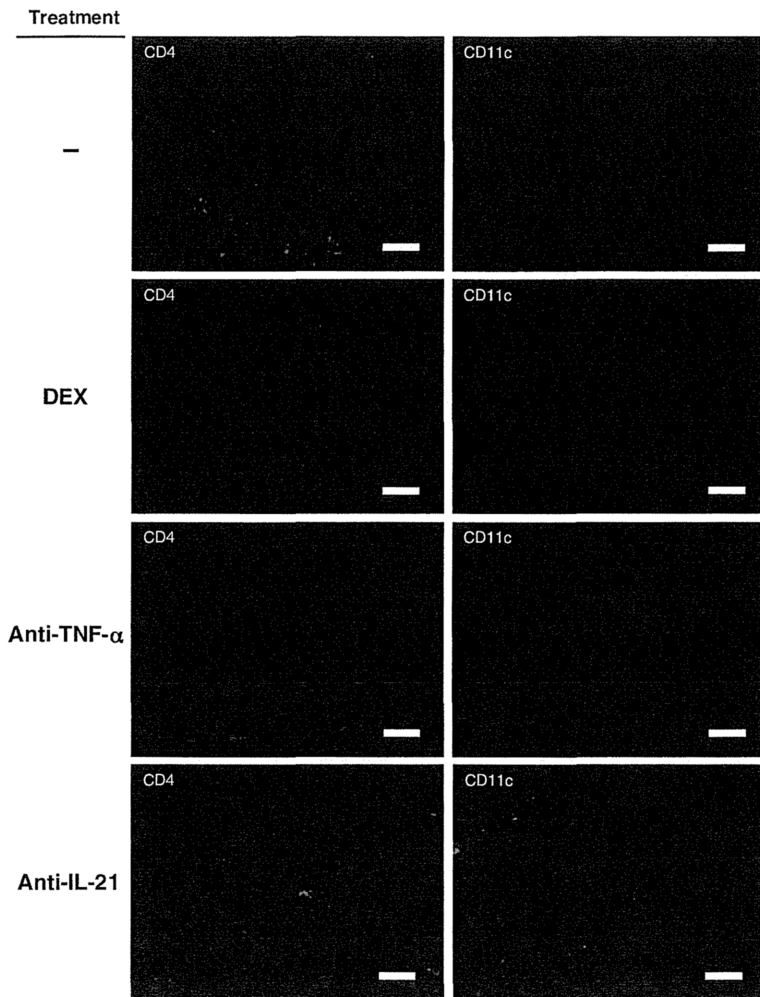

Figure 6 Dexamethasone (DEX) injections suppress the infiltration of CD4^{+} T cells and CD11c^{+} cells, whereas neutralization of tumor necrosis factor (TNF)-α or interleukin (IL)-21 reduces the infiltration of CD4^{+} T cells but allows CD11c^{+} cells to infiltrate into the gastric mucosa. Fluorescence immunostainings of the stomach for CD4 and CD11c in 4-week-old PD-1$^{-/-}$ mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice) treated with indicated treatments. Data shown are representative of at least five mice in each group. All scale bars, 100 μm.

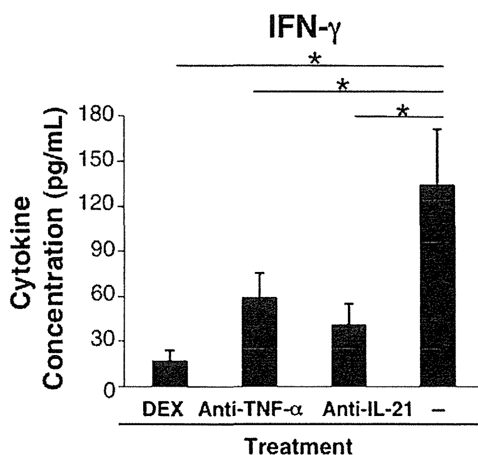

Figure 7 Serum levels of interferon (IFN)-γ are significantly repressed by neutralization of tumor necrosis factor (TNF)-α and interleukin (IL)-21. Serum IFN-γ levels were measured by ELISA. Data are shown as the mean of at least five PD-1$^{-/-}$ mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice) treated with indicated treatments at 4 weeks of age.

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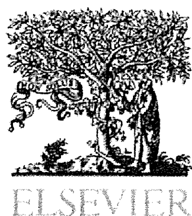

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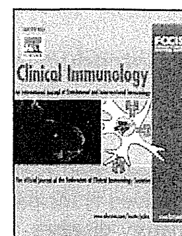

TNF-α is essential in the induction of fatal autoimmune hepatitis in mice through upregulation of hepatic CCL20 expression☆

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Abstract It is unclear what roles TNF-α has in the development of autoimmune hepatitis (AIH) and whether AIH is responsive to anti-TNF-α. We recently developed a mouse model of fatal AIH that develops in PD-1-deficient mice thymectomized three days after birth, finding that CCR6-CCL20 axis-dependent migration of dysregulated splenic T cells is crucial to induce AIH. In this study, we show the indispensable role of TNF-α in the development of AIH. Administering anti-TNF-α prevented the induction, but treatment by anti-TNF-α after the induction did not suppress progression. Administering anti-TNF-α did not prevent splenic T-cell activation, but did suppress hepatic CCL20 expression. In contrast, administering anti-CCL20 suppressed AIH but not elevated serum TNF-α levels. TNF-α stimulation enhanced CCL20 expression in hepatocytes. These findings suggest that TNF-α is essential in the induction of AIH through upregulation of hepatic CCL20 expression, which allows migration of dysregulated splenic T cells.
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Abbreviations: AIH, autoimmune hepatitis; Con A, concanavalin A; GC, germinal center; IFN, interferon; mAb, monoclonal antibody; NTx, neonatal thymectomy; NTx mice, mice thymectomized three days after birth; PD-1, programmed cell death 1; TNF, tumor necrosis factor; T_{FH}, follicular helper T.

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1. Introduction

Dysregulated production of tumor necrosis factor (TNF)-α is involved in the pathology of various immune-mediated inflammatory diseases, including inflammatory bowel diseases and rheumatoid arthritis in humans [1–4]. In the liver, TNF-α directly and indirectly induces cell death of hepatocytes, whereas it can mediate production of inflammatory mediators, hepatocyte proliferation, and liver regeneration [5]. In human diseases, TNF-α is involved in the pathophysiology of viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and ischemia-reperfusion injury [5]. In addition, serum TNF-α levels were elevated in untreated children with type 1 autoimmune hepatitis (AIH) in comparison with those in healthy controls [6]. A polymorphism at position 308 in a promoter region of TNF-α has been reported to be associated with severity of AIH type 1 in Europe and North America [7,8]. However, it is unclear whether TNF-α is critical in the development of AIH or whether TNF antagonists have therapeutic efficacies for AIH in humans.

Several mouse models of AIH have demonstrated the roles of cytokines, costimulatory molecules, T-cell subsets, and autoantigens in the development of AIH. Gorham et al. reported that BALB/c background TGF-β1 deficient mice develop fatal hepatitis characterized by massive $CD4^+$ T cell infiltration [9]. In this model, interferon (IFN)-γ produced by $CD4^+$ T cells drove lethal hepatic damage [10]. Mice deficient in B and T lymphocyte attenuator (BTLA), negative costimulatory molecules expressed on lymphocytes, developed AIH-like disease with hypergammaglobulinemia and production of anti-nuclear antibodies [11]. In addition, mice immunizing human liver autoantigens and mice infected with adenovirus Ad5 expressing human cytochrome P450 2D6 developed persistent immune-mediated hepatitis [12]. Mice vaccinated with dendritic cells loaded with liver antigens and subsequently administered IL-12 also developed persistent immune mediated hepatitis [13]. Furthermore, influenza virus hemagglutinin-specific T-cell receptor transgenic mice further expressing the hemagglutinin specifically in the liver spontaneously developed chronic autoimmune-mediated hepatitis [14]. However, in these models, the roles of TNF-α in the development of hepatitis are not clear.

Concanavalin A (Con A)-induced acute hepatic injury, associated with activation of NKT cells and T cells, is considered to be an experimental model of human AIH [15]. A single intravenous injection of Con A into mice rapidly induces injury of hepatocytes together with increased serum levels of TNF-α [16]. Although one report demonstrated that neutralization of TNF-α by anti-TNF-α significantly suppressed Con A-induced hepatic injury, Tagawa et al. showed that hepatocytes in TNF-α-deficient mice were severely injured by Con A to levels similar to wild-type mice [17]. Thus, an essential role of TNF-α in inducing Con A-induced acute hepatic injury is still controversial.

To clarify mechanisms involved in the development of AIH, we recently developed a new mouse model of fatal AIH [18,19]. Neither programmed cell death 1-deficient mice ($PD\text{-}1^{-/-}$ mice) nor BALB/c mice thymectomized three days after birth (NTx mice), with severely reduced numbers of naturally arising $Foxp3^+$ regulatory T cells in periphery, developed inflammation of the liver. However, $PD\text{-}1^{-/-}$ BALB/c mice with neonatal thymectomy (NTx-$PD\text{-}1^{-/-}$ mice) develop fatal AIH characterized by $CD4^+$ and $CD8^+$ T-cell infiltration, with massive lobular necrosis. Because of the massive destruction of the parenchyma of the liver, these mice start to die as early as two weeks of age, with most dying by four weeks. Notably, the hepatitis in NTx-$PD\text{-}1^{-/-}$ mice was characterized by hyper-gammaglobulinemia and huge production of anti-nuclear antibody, both diagnostic hallmarks of AIH patients [18,19].

In this mouse model, both $CD4^+$ and $CD8^+$ T cells are indispensable for the development of fatal AIH [18,19]. $CD8^+$ T cells are crucially involved in the progression to fatal hepatic damage, whereas $CD4^+$ T cells are responsible for inducing fatal AIH. Initial activation of $CD4^+$ T cells occurs in the spleen. In the induction phase of AIH, splenic $CD4^+$ T cells were localized in B-cell follicles with huge germinal centers (GCs) and showed the $Bcl6^+ICOS^+ IL\text{-}21^+IL\text{-}21R^+$ follicular helper T (T_{FH}) cell phenotype [19]. IL-21 produced by T_{FH} cells has been shown to drive $CD8^+$ T-cell activation [19]. Splenic T_{FH} cells and activated $CD8^+$ T cells expressed CCR6, and CCL20 expression was elevated in the liver. CCR6-CCL20 axis-dependent migration of splenic T cells is crucial to induce AIH in NTx-$PD\text{-}1^{-/-}$ mice [19]. However, it is not known how hepatic CCL20 expression is upregulated in the induction phase of AIH.

AIH-bearing NTx-$PD\text{-}1^{-/-}$ mice at three weeks old showed markedly increased levels of TNF-α in the serum [18]. However, it is not clear whether TNF-α is essential in the development of fatal AIH in this mouse model. This study aimed to identify the roles of TNF-α in the development of fatal AIH. We found that serum levels of TNF-α were markedly elevated from the induction phase and that administration of anti-TNF-α prevented induction of AIH, but treatment by anti-TNF-α after the induction did not significantly suppress the progression of fatal hepatic inflammation. Administering anti-TNF-α did not prevent splenic T cell activation in the induction phase of AIH, but suppressed CCL20 expression in the hepatocytes. In contrast, administering anti-CCL20 suppressed fatal AIH but not elevated serum levels of TNF-α. In addition, we found that stimulation by recombinant TNF-α (rTNF-α) upregulated CCL20 expression in hepatocytes *in vivo* and *ex vivo*. These findings suggest that TNF-α is critically involved in the induction of fatal AIH in mice through the upregulation of hepatic CCL20 expression.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and PD-1 deficient mice on a BALB/c background were generated as described previously [20]. These mice were bred and housed under specific pathogen-free conditions. Thymectomy and splenectomy of the mice three days after birth were performed as described previously [18,19]. All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

2.2. *In vivo* neutralization of cytokines and depletion of T cell subsets, and injection of cytokines

NTx-PD-1$^{-/-}$ mice were injected intraperitoneally every week from day 3 or day 17 after birth with 100 μg of neutralizing monoclonal antibodies (mAbs) to mouse TNF-α (eBioscience, San Diego, CA), or mouse CCL20 (R&D Systems, Minneapolis, MN), and isotype control Abs. After injections, mice at indicated weeks of age were sacrificed, and the livers, spleens, and sera were harvested. For depletion of CD4$^+$ T cells *in vivo*, anti-mouse CD4 from eBioscience was used. After two injections of anti-CD4, flow cytometric analysis was performed. Viable CD4$^+$ T cell numbers were calculated as follows: (the percentage of CD3$^+$CD4$^+$ cells in viable cells) × (the number of viable cells). Numbers of CD4$^+$ T cells in the spleen were reduced less than 5% compared with those in mice without treatment. On the other hand, PD-1$^{-/-}$ mice at four weeks age were injected intraperitoneally with 10 μg/kg of mouse rTNF-α (eBioscience) or PBS. Before and after 1, 2, 3, 6, or 9 h following injections, mice were sacrificed, and their livers and sera were harvested. For immunostaining with anti-CCL20, four-week-old PD-1$^{-/-}$ mice were injected with rTNF-α or PBS four times every 3 h. Three hours after the last injection, mice were sacrificed.

2.3. Histological and immunohistological analysis

Organs were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin for histopathology. Fluorescence immunohistology was performed on frozen sections as described previously [18,19], using FITC-conjugated anti-CD4, and anti-CD8α (eBioscience), peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA), biotin-labeled anti-B220 (BD Biosciences, San Jose, CA) followed by Texas red-conjugated avidin (Vector Laboratories). For CCL20 staining, rabbit polyclonal antibodies to CCL20/MIP3-α (Abcam, Cambridge, UK) were used, followed by FITC-conjugated goat anti-rabbit immunoglobulin (BD Biosciences). The diameter of GC and the number of CD4$^+$ T cells within GC of the spleen were determined in several high-power fields of the spleen in at least 3 sections from each mouse.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of TNF-α in sera and CCL20 in culture supernatants were measured by using mouse TNF-α ELISA set (eBioscience) and CCL20/MIP-3α set (R&D Systems) according to the manufacturer's protocols, respectively.

2.5. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Liver tissues were frozen in RNAlater (Qiagen, Hilden, Germany). Real-time quantitative RT-PCR was performed as described previously [19]. Values are expressed as arbitrary units relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The following primers were used: *GAPDH*: 5′-CAACTTTGTCAAGCTCATTTCC-3′ and 5′-GGTCCAGGGTTTCT TACTCC-3′; *CCL20*: 5′-ATGGCCTGCGGTGGCAAGCGTCTG-3′ and 5′-TAGGCTGAGGAGGTTCACAGCCCT-3′; *CCL25*: 5′-GAGTGCCA CCCTAGGTCATC-3′ and 5′-CCAGCTGGTGCTTACTCTGA-3′.

2.6. Flow cytometry analysis

Single-cell suspensions of mononuclear cells from spleens were prepared as described previously [18,19]. For intracellular cytokine staining, cells were restimulated with 50 ng/ml phorbol myristate acetate (Sigma, St. Louis, MO) + 2 μg/ml ionomycin (Sigma) at a concentration of 1×10^6 cells/ml in RPMI Medium 1640 (Invitrogen, Eugene, OR), supplemented with 10% fetal bovine serum (Sigma), penicillin G, and streptomycin (Invitrogen), and 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan). After 2 h culture with 10 μg/ml brefeldin A (Sigma), cells were collected and stained for cell-surface molecules using PE-anti-CD3ε and APC-Cy7-anti-CD4 (eBioscience) or APC-anti-CD8α. Cells were fixed and permeabilized, using a Fix and Perm Cell Permeabilization™ Kit (Caltag Laboratories, An Der Grub, Austria), and stained with FITC-anti-IFN-γ (eBioscience). For Ki-67 antigen staining, a FITC-conjugated Ab set (BD Bioscience) was used with PE-anti-CD3ε and APC-Cy7-anti-CD4 or APC-anti-CD8α. For CD44 staining, cells were stained with FITC-anti-CD4 or -anti-CD8α and PE-anti-CD44, and PerCP-Cy5.5-conjugated Anti-CD3ε (all from eBioscience). Stained cells were analyzed with a FACSCanto™ II (BD Biosciences). Data were analyzed using Cell Quest Pro™ (BD Biosciences). Dead cells were excluded on the basis of side- and forward-scatter characteristics.

2.7. Primary hepatocyte culture

Primary hepatocytes were obtained from adult PD-1$^{-/-}$ mice using the two-step collagenase perfusion method, followed by centrifugation to collect the mature hepatocytes and cultured as described previously [21,22]. Hepatocytes were resuspended in D-MEM/Ham's F-12 supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells seeded on a 24-well microplate with collagen type 1 coating at a density of 1×10^5 cells/cm^2 and cultured for 72 h with or without 10 or 20 ng/ml of rTNF-α.

2.8. Statistical analysis

The data are presented as the mean values ± standard deviations. Statistical analysis was performed by the Student *t* test for unpairwise comparisons. Survival rates were estimated by the Kaplan–Meier method and compared with the log-rank test. P-values below 0.05 were considered significant.

3. Results

3.1. Production of TNF-α increases in the induction phase of AIH, and neutralizing TNF-α suppresses the development of AIH in NTx-PD-1$^{-/-}$ mice

Previously, we reported that in three-week-old NTx-PD-1$^{-/-}$ mice with severe AIH, serum levels of TNF-α significantly increased [18]. In this study, we performed a time-course study on serum levels of TNF-α from one to three weeks of age. In 1.5-week-old NTx-PD-1$^{-/-}$ mice, the serum level of

TNF-α was significantly higher than in PD-$1^{-/-}$ mice (Fig. 1A). The elevated serum level of TNF-α further increased in two-week-old NTx-PD-$1^{-/-}$ mice, at a level similar to NTx-PD-$1^{-/-}$ mice with severe AIH at three weeks old. These data suggest that TNF-α may play important roles in the process of AIH development.

Next, we examined whether neutralization of TNF-α can suppress the development of fatal AIH. NTx-PD-$1^{-/-}$ mice at one day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing mAb to mouse TNF-α or the isotype control. We found that neutralization of TNF-α showed a significantly increased survival rate (Fig. 1B). After four injections, mice at four weeks of age were sacrificed, and their livers and sera were harvested. Four-week-old NTx-PD-$1^{-/-}$ mice injected with the isotype control developed severe mononuclear cell infiltration in the liver and a massive degeneration of hepatocytes (Fig. 1C left). However, neutralization of TNF-α suppressed mononuclear cell infiltration in the liver and degeneration of hepatocytes (Fig. 1C right). In addition, neutralization of TNF-α showed significantly

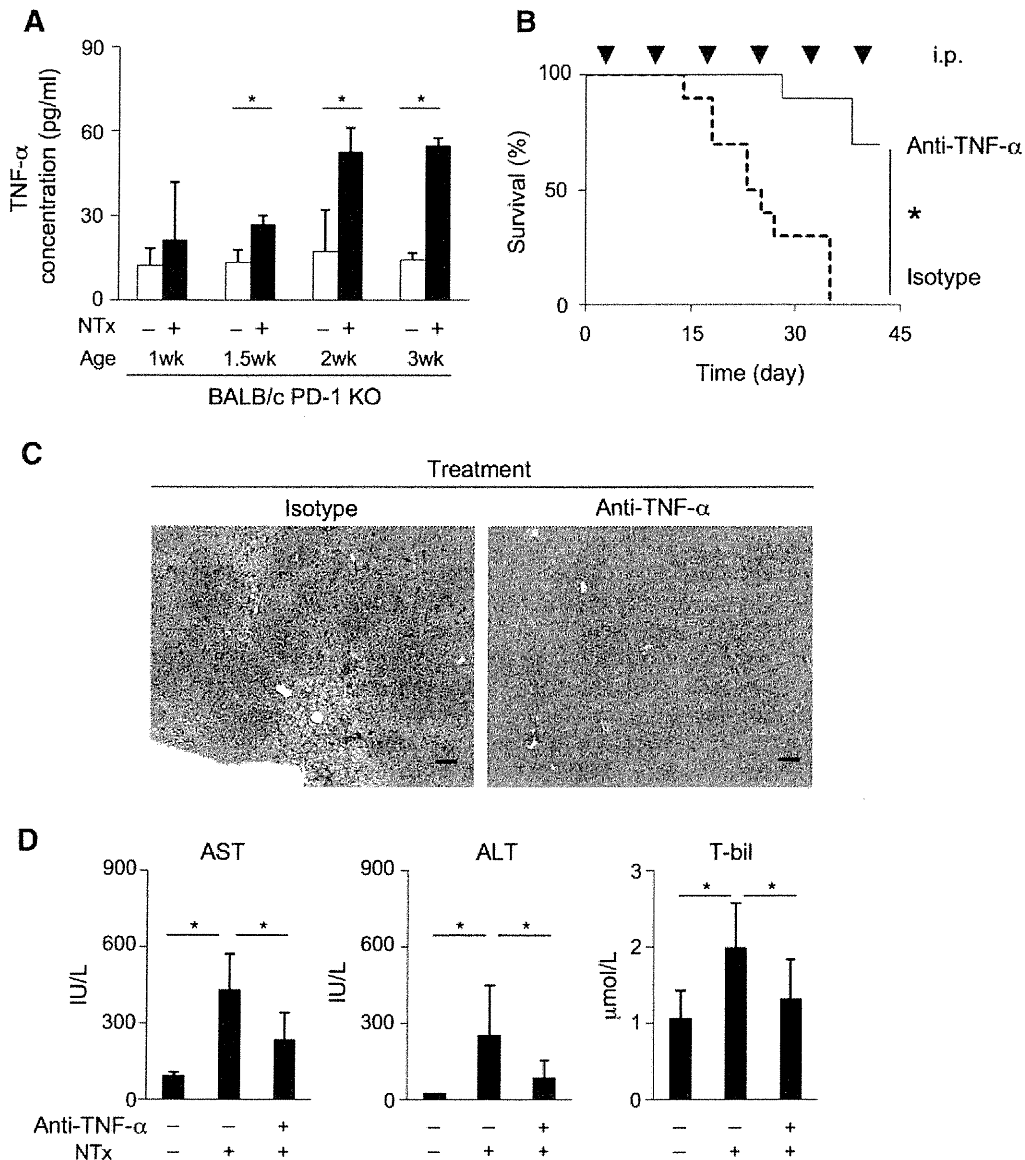

Figure 1 Production of TNF-α increases in the induction phase of fatal AIH, and neutralization of TNF-α suppresses its development in NTx-PD-$1^{-/-}$ mice. (A) Serum TNF-α levels of indicated mice were measured by ELISA. Data are shown as the mean of at least five mice. (B-D) NTx-PD-$1^{-/-}$ mice at one day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing anti-mouse TNF-α or the isotype control mAb. Survival of NTx-PD-$1^{-/-}$ mice treated with anti-TNF-α (solid line, n = 10) or the isotype control (dotted line, n = 10) at six injections *; $P<0.05$ (B). After four injections, mice at four weeks of age were sacrificed, and the liver and serum were harvested. Staining of the liver for hematoxylin and eosin. All *scale bars*, 100 μm (C). Serum levels of the liver transaminases AST and ALT and total bilirubin. Sera from indicated mice were measured. Data are shown as the mean of at least four mice. Error bars represent SD. *; $P<0.05$ (D).

decreased serum concentrations of aspartate aminotransferase and alanine aminotransferase, and total bilirubin at four weeks of age (Fig. 1D). These data indicate that neutralization of TNF-α suppressed the development of fatal AIH in NTx-PD-1$^{-/-}$ mice, suggesting an essential role for TNF-α in the development of AIH.

3.2. Neutralization of TNF-α inhibits the infiltration of T cells into the liver in the induction phase of AIH, whereas it does not suppress the progression of fatal AIH after the development of hepatitis

Next, we examined whether neutralizing TNF-α can suppress the infiltration of CD4$^+$ and CD8$^+$ T cells in two-week-old NTx-PD-1$^{-/-}$ mice at the induction phase of AIH. After two injections of neutralizing anti-TNF-α, NTx-PD-1$^{-/-}$ mice at two weeks of age were sacrificed, and their livers were harvested. Two-week-old NTx-PD-1$^{-/-}$ mice injected with anti-TNF-α did not show any mononuclear cell infiltrations in the portal area or an increased number of CD4$^+$ and CD8$^+$ T cells in the liver, suggesting an essential role for TNF-α in the induction of AIH (Fig. 2A).

In NTx-PD-1$^{-/-}$ mice from two to three weeks of age, mononuclear cell infiltrations in the liver rapidly progressed and were followed by massive destruction of the parenchyma of the liver [18]. To investigate whether neutralization of TNF-α can suppress progression of fatal AIH even after the development of AIH, NTx-PD-1$^{-/-}$ mice at 14 days after thymectomy were injected every week with anti-TNF-α. We found that the neutralizing TNF-α did not significantly increase the survival rate (Fig. 2B). After the second injection, mice at four weeks of age were sacrificed, and we confirmed that the neutralizing TNF-α did not reduce a massive degeneration of hepatocytes or severe CD4$^+$ and CD8$^+$ cell infiltration in the liver (Fig. 2C). Thus, neutralization of TNF-α after the development of hepatitis did not significantly suppress progression of fatal AIH.

3.3. Increased TNF-α production in the induction phase of AIH depends on activation of CD4$^+$ T cells in the spleen

Previously, we reported that in NTx-PD-1$^{-/-}$ mice, CD4$^+$ T cells are responsible for the induction of fatal AIH and initial activation of responsible CD4$^+$ T cells occurs in the spleen [18,19]. Indeed, neonatal splenectomy suppressed elevated serum levels of TNF-α in two-week-old NTx-PD-1$^{-/-}$ mice (Fig. 3A). In addition, *in vivo* depletion of CD4$^+$ T cells suppressed elevated serum levels of TNF-α in two-week-old mice (Fig. 3B). These data indicate that increased TNF-α production in the induction phase of AIH depends on activation of CD4$^+$ T cells in the spleen.

3.4. Neither T_{FH}-cell differentiation nor activation of CD8$^+$ T cells in the spleen is suppressed by neutralization of TNF-α in the induction phase of fatal AIH

In this mouse model, initially activated CD4$^+$ T cells showing T_{FH} cell phenotype were localized in B-cell follicles with huge GCs in the spleen [19]. In this study, we looked at CD4$^+$ and CD8$^+$ T cells in the spleen of two-week-old NTx-PD-1$^{-/-}$ mice treated with anti-TNF-α, finding that splenic CD4$^+$ and CD8$^+$ T cells treated with anti-TNF-α showed Ki-67^{high} activated T-cell phenotype with high proliferation potential at levels similar to those in mice treated with isotype (Fig. 3C left). In addition, expression levels of IFN-γ in these splenic T cells of two-week-old NTx-PD-1$^{-/-}$ mice treated with anti-TNF-α were similar to those in mice treated with isotype (Fig. 3C right). When we looked at expression levels of CD44 in T cells, CD44^{high} cells in the CD4$^+$ T-cell populations in mice treated with anti-TNF-α were similar to those in mice treated with isotype (Fig. 3D). However, CD44^{high} cells in the CD8$^+$ T-cell populations had increased, suggesting further accumulation of activated CD8$^+$ T cells in the spleen. Moreover, when we looked at B-cell follicles in the spleen of four-week-old NTx-PD-1$^{-/-}$ mice injected with or without neutralizing anti-TNF-α, anti-TNF-α did not alter the number of CD4$^+$ T cells within B220$^+$ follicles or the size of PNA$^+$ GC in B220$^+$ follicles (Supplementary Figs. 1A and B). These data suggest that neither T_{FH}-cell differentiation nor activation of CD8$^+$ T cells in the spleen was suppressed by neutralization of TNF-α in NTx-PD-1$^{-/-}$ mice, implying that neutralization of TNF-α predominantly affects the migration step but not the activation of those T cells in the induction phase of fatal AIH.

3.5. Neutralization of TNF-α suppresses upregulated expression of hepatic CCL20

Previously, we reported that CCR6-CCL20 axis-dependent migration of splenic T cells is crucial to induce AIH in NTx-PD-1$^{-/-}$ mice [19]. In this study, we showed that serum levels of TNF-α were elevated from 1.5 to three weeks of age in NTx-PD-1$^{-/-}$ mice (Fig. 1A). In parallel with this observation, expressions of hepatic CCL20 were upregulated at 1.5 to three weeks of age (Fig. 4A). Therefore, we examined whether TNF-α is essential in the upregulated expression of hepatic CCL20 in NTx-PD-1$^{-/-}$ mice. After two injections of anti-TNF-α into NTx-PD-1$^{-/-}$ mice at two weeks of age at the induction phase of AIH, neutralizing TNF-α significantly suppressed mRNA expression of CCL20 in the liver (Fig. 4B left). After four injections of anti-TNF-α, decreased hepatic CCL20 expression was sustained in four-week-old NTx-PD-1$^{-/-}$ mice (Fig. 4B right). These data were further confirmed by immunohistology. Hepatocytes in four-week-old NTx-PD-1$^{-/-}$ mice with injection of the isotype control revealed CCL20 staining, whereas after four injections of anti-TNF-α, continuous neutralization of TNF-α suppressed CCL20 staining in hepatocytes (Fig. 4C). Thus, neutralization of TNF-α suppressed upregulated expression of hepatic CCL20.

3.6. Administering anti-CCL20 suppressed the development of fatal AIH but did not alter elevated serum levels of TNF-α

Conversely, we examine whether administration of anti-CCL20 alters elevated serum levels of TNF-α. Administration of anti-CCL20 suppressed mononuclear cell infiltration and destruction of organ structure in the liver at four weeks of age and showed a significantly increased survival rate in