CXCR4 antagonist AMD3100 ameliorates thyroid damage in autoimmune thyroiditis in NOD.H-2^{h4} mice

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Abstract. CXC chemokine ligand 12 (CXCL12) and its receptor, CXC chemokine receptor 4 (CXCR4), are upregulated in mice with autoimmune thyroid diseases. However, whether this interaction is involved in the pathophysiology of autoimmune thyroiditis (AIT) remains to be elucidated. In the present study, the effects of the CXCR4 antagonist, AMD3100, in an iodine-induced autoimmune thyroiditis model were investigated. NOD.H-2^{h4} mice were randomly separated into a control, AIT and AIT+AMD3100 groups. The mice were fed with 0.05% sodium iodide water for 8 weeks to induce AIT. The AMD3100-treated mice were administered with the CXCR4 antagonist at a dose of 10 mg/kg intraperitoneally three times a week during the experimental period. The percentages of $CD19^+$interleukin (IL)10^+ B cells and $CD4^+IL10^+$ T cells, and the mRNA expression levels of IL10 in the splenocytes were reduced in the AIT group, compared with the control group, however, they increased following AMD3100 treatment, compared with the untreated AIT group. The percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD8^+$ interferon (IFN)γ^+ T cells, and the mRNA expression levels of IFNγ increased in the AIT group, compared with the control group, however, these were reduced in the AMD3100 group, compared with the AIT group. The AMD3100-treated mice also had lower serum thyroglobulin antibody titers and reduced lymphocytic infiltration in the thyroid, compared with the untreated AIT mice. These results suggested that inhibition of this chemokine axis may offer potential as a therapeutic target for the treatment of AIT.

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Abbreviations: NaI, sodium iodide; AIT, autoimmune thyroiditis; SAT, subacute thyroiditis; HT, Hashimoto's thyroiditis; TgAb, thyroglobulin antibody

Key words: CXC chemokine ligand 12/CXC receptor 4, AMD3100, autoimmune thyroiditis, interleukin 10, interferon γ

Introduction

Chemokines are a group of peptides of small molecular weight (8-11 kDa). They induce the chemotaxis of various leukocyte subtypes and regulate leukocyte trafficking to sites of inflammation (1). The major function of chemokines is the recruitment of leukocytes to sites of inflammation (1). CXC chemokine ligand 12 (CXCL12), or stromal cell derived factor (SDF-1), is a 68-amino-acid long CXC chemokine, which was originally identified as a growth factor for mouse pre-B cells (2). CXCL12 is constitutively expressed by various types of cell and tissue, and exhibits chemoattractive activity for endothelial cells, epithelial cells, monocytes, bone marrow neutrophils, dendritic cells and, in particularly, T cells and their co-stimulators (3). Following the connecting of the chemokine with CXC chemokine receptor 4 (CXCR4) and its receptor, CXCL12 can exert series of functions, including the transendothelial migration of inflammatory cells and mobilization of leukocytes (4). Previous studies have shown that certain types of immunomodulatory cytokines can affect the expression levels of CXCR4 on T cells. It was reported that interleukin (IL)10 and IL4 significantly upregulate or downregulate the expression of CXCR4 on $CD4^+$ T lymphocytes (5). In addition, the interferon (IFN)γ cytokine induces prompt downregulation in the mRNA expression of CXCR4 and attenuates endothelial cell migration towards CXCL12 (6). Increases in the expression levels of CXCR4 or CXCL12 have been reported in several inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease (IBD) (4,7-9). AMD3100 has been considered as a specific inhibitor of CXCR4 (10). In previous reports, AMD3100 was found to specifically inhibit CXCL12-mediated repercussions and have beneficial effects in several animal models of immune diseases, including diabetes (11), asthma (12) and IBD (8). Thus, this antagonist may provide an effective way of inhibiting thyroid damage in iodine-induced autoimmune thyroiditis in NOD.H-2^{h4} mice,

which express I-A k in their NOD genetic background. These mice spontaneously develop autoimmune thyroiditis (SAT) and produce anti-mouse thyroglobulin (MTg) autoantibodies, and are a prototype murine model of Hashimoto's thyroiditis (HT) in humans (13).

Autoimmune thyroid diseases (AITDs), including HT and Graves' disease are chronic organ-specific autoimmune diseases. They are characterized by the destruction of thyroid follicles by infiltrating inflammatory cells and mononuclear cell infiltration of the thyroid gland (14). It has been shown that T helper (Th)1 cytokines are commonly prevalent in HT, as well as in experimental autoimmune thyroiditis (EAT), by analyzing of the expression of cytokines, including IL1, IL2, IL6, IL10, IFNγ and TNFα, due to the infiltration of T cells and macrophages (15-18). Furthermore, the thyroid follicular cells produce several types of cytokines themselves (19). In previous years, chemokines have been considered to be important in endocrine autoimmune disease, and studies have demonstrated CC and CXC chemokine overexpression in HT and EAT (20-22). It is currently accepted that IFNγ-inducible chemokines (CXCL9, CXCL10, CXCL11, CXCL12, CXCL13 and CCL22) may be the most important mediating chemokines for formation of the germinal center (GC) (23). Thyrocytes are the primary source of CXCL12 in the thyroid, whereas CXCR4 is expressed by T and B cells (13). Thyrocytes can also produce CCL2, CXCL9 and CXCL10. Iodine may induce thyrocyte necrosis, stimulating resident macrophages to produce IL1 and TNFα, which may in turn induce CXCL12 synthesis by the adjacent thyrocytes (24).

To the best of our knowledge, no previous studies have evaluated CXCL12 and CXCR4 in an iodine-induced autoimmune thyroiditis NOD.H-2^{h4} mouse model. The aim of the present study was to measure the levels of CXCL12 in NOD.H-2^{h4} mice, and to investigate the potential effects of the CXCL12 and CXCR4 antagonist, AMD3100, on the inflammation barrier in mouse models of SAT.

Materials and methods

Animals. Male NOD.H-2^{h4} mice (6-week-old; 18-21 g) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Male BALB/c mice (6-week-old; 18-21 g), were purchased from Vital River Laboratories (Beijing, China). The animals were housed under specific pathogen-free conditions in a controlled temperature and humidity environment, with day-night light cycles in the animal facility of China Medical University (Shenyanh, China). The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (25). The experimental protocol was approved by the ethics committee of the Ethics of Animal Experiments of China Medical University (Shenyang, China). All surgery was performed under anesthesia using 10% chloral hydrate (Melone Pharmaceutical Co., Ltd., Dailan, China), and all efforts were made to minimize suffering.

Experimental design. For the induction of autoimmune thyroiditis, the mice were administered with 0.05% (500 mg/l) sodium iodide (NaI) in the drinking water (1,000 times higher than normal iodine intake) during the study period. The CXCR4 antagonist, AMD3100, was obtained from Sigma-Aldrich (St. Louis, MO, USA). For treatment, 10 mg/kg of AMD3100 dissolved in 200 μl phosphate-buffered saline (PBS), or 200 μl PBS alone were administered intraperitoneally three times a week during the study period. Normal control mice received regular drinking water throughout the experiment.

Experimental groups

Experiment one. A total of 12 NOD.H-2^{h4} mice and 12 BALB/c mice were randomly selected and divided into four groups: NOD.H-2^{h4} mice provided with regular drinking water (NOD.H-2^{h4}-CON; n=6); NOD.H-2^{h4} mice provided with 0.05% NaI drinking water (NOD.H-2^{h4}-HI; n=6); BALB/c mice provided with regular drinking water (BALB/c-CON; n=6); and BALB/c mice provided with 0.05% NaI drinking water (BALB/c-HI; n=6). Animals were anesthetized and sacrificed using an overdose of 10% chloral hydrate at week 8 of the experiment.

Experiment two. A total of 36 NOD.H-2^{h4} mice were randomly-selected and divided into two groups: NOD.H-2^{h4} mice with regular drinking water (CON) group and NOD.H-2^{h4} mice with 0.05% NaI drinking water (HI) group, with four mice per group. A total of 4 mice per group were anesthetized and sacrificed at weeks 0, 2, 4, 8, and 16 of the experiment.

Experiment three. A total of 29 NOD.H-2^{h4} mice were randomly selected and divided into three groups: NOD.H-2^{h4} mice with regular drinking water (CON; n=10), NOD.H-2h4 mice with 0.05% NaI drinking water (AIT; n=10) and NOD.H-2^{h4} mice with 0.05% NaI drinking water and AMD3100 treatment (AMD3100; n=9). The animals were anesthetized and sacrificed at week 8 of the experiment.

Assessment of autoimmune thyroiditis. At the experimental end points, the mice were weighed and anesthetized via intraperitoneal injection of 10% chloral hydrate (26). Thyroid tissues were removed and then washed with cold normal saline, dried on a pad of filter paper and weighed on an electronic balance (BS210S; Sartorius, Göttingen Germany). From each mouse, one thyroid lobe was fixed in 10% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for at least 24 h and embedded in paraffin (Huayong, Shanghai, China). Sections of 5-μm thickness were prepared and stained with hematoxylin and eosin (HE; Beyotime Institute of Biotechnology, Hangzhou, China). Histological changes in the thyroid tissue were observed under light microscopy (BX51/BX52; Olympus Corporation, Tokyo, Japan). The extent of lymphocytic infiltration was assessed, as previously described (26,27). Briefly, HE-stained thyroid sections were graded on the following scale, according to the approximate area of lymphocytic infiltration: 0=normal; 1+=1-10%; 2+=10-30%; 3+=30-50%; 4+ >50%. The thyroiditis scores were expressed as the mean of at least three non-contiguous sections from each thyroid gland.

Serum thyroglobulin antibody (TgAb) measurements using ELISA. Blood was collected from the orbital vein, incubated at

Table I. Sequence of primers used for amplification in reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Reference sequence	Forward primer (5'-3')	Reverse primer (5'-3')
CXCL12	NM_021704.3	GCTCTGCATCAGTGACGGTA	ATCTGAAGGGCACAGTTTGG
IFNγ	NM_008337.3	CACGGCACAGTCATTGAAAG	AATCTGGCTCTGCAGGATTT
IL10	NM_010548.2	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
β-actin	NM_007393.3	GGTCATCACTATTGGCAACG	TCCATACCCAAGAAGGAAGG

CXCL12, C-X-C motif chemokine 12; IFN, interferon; IL, interleukin.

room temperature for at least 2 h and the serum was separated by centrifugation at 1,006 x g for 20 min at room temperature and stored at -80˚C until further analysis. MTg was prepared as previously described (26). Thyroid gland tissues were homogenized in PBS and centrifuged at 1,000 x h at 4˚C for 10 min. MTg was obtained from the supernatant using by a salting out as previously described (28), and then purified by repeated gel filtration using Sephadex G-20 (Pharmacia; Pfizer, Inc., New York, NY, USA). Samples were stored at -7˚C until analysis. The TgAb titers were assessed in duplicate by indirect ELISA, using serum from the individual mice, as described previously (26,28). Briefly, sera were diluted to 1:100 with PBS and incubated at 37˚C for 1 h on 96-well EIA/RIA plates (Corning, St. Louis, MO, USA) coated with 10 μg/ml mouse thyroglobulin [prepared from frozen mouse thyroids as previously described (28)]. Peroxidase-labeled goat anti-mouse immunoglobulin G (polyclonal; 1:250 dilution; A0168; Sigma-Aldrich) was used as the secondary antibody. The color change of tetramethyl benzidine was measured at 450 nm using a microtiter plate reader (F200PRO; Tecan Group, Ltd., Zurich, Switzerland), with TgAb levels expressed as optical density (OD) values.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Tissue was stored in RNAlater (Qiagen GmbH, Hilden, Germany) at -80˚C, and homogenized using Tissue Lyser II (Quiagen, Hilden, Germany) at 20˚C. Total RNA was extracted from the splenic cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. For RT, 1 μg total RNA was used to prepare cDNA (26). A reverse transcriptase kit (PrimeScript RT reagent kit; Takara Biotechnology Co., Ltd., Dalian, China) and an ABI 9700 PCR meter (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for cDNA synthesis (37˚C for 15 min, followed by 85˚C for 5 sec). Transcripts were quantified using a Roche LightCycler480 real-time PCR System (version 1.5.0; Roche Diagnostics, Mannheim, Germany) using SYBR® Premix Ex Taq TM II (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. PCR parameters were as follows: 95˚C For 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 20 sec and 65˚C for 15 sec. At the end of the PCR cycles, a melting curve analysis was performed. Experiments were performed in triplicate and data were quantified and analyzed using LightCycler 480 (Roche Diagnostics) analysis software. The reactions were performed using a total volume of 20 μl, in a 0.2-ml flat cap PCR tube (Axygen; Tewksbury, MA, USA). The primers used are indicated in Table I.

Flow cytometry. The spleens were harvested from the mice following sacrifice at week 8 of the experiment, and pressed through a 200-gauge stainless steel mesh (Stainless Steel Nets Factory, Shenyang, China) (26). The tissue was suspended in 10 ml PBS and centrifuged at 111.8 x g for 10 min at room temperature. The erythrocytes were resuspended in 3 ml lysis solution (ammonium chloride; Beyotime Institute of Biotechnology), incubated at room temperature for ~3 min, and centrifuged at 111.8 x g for 10 min at room temperature. The cells were then washed and immunostained with monoclonal fluorescein isothiocyanate-conjugated rat anti-mouse CD4 (RM4-5; 553047), monoclonal PerCP-Cy 5.5-conjugated rat anti-mouse CD19 (1D3; 551001), monoclonal PerCP-Cy 5.5-conjugated rat anti-mouse CD8 (53-6.7; 561109), monoclonal phycoerythrin-conjugated rat anti-mouse IL10 (JES5-16E3; 554467), and monoclonal allophycocyanin (APC)-conjugated rat anti-mouse IFNγ (XMG1.2; 562018). All antibodies were purchased from BD Biosciences (San Jose, CA, USA) and were diluted 1:100. All staining was performed according to manufacturer protocols. For intracellular cytokine detection, the cells were stimulated with a leukocyte activation cocktail in the presence of GolgiPlug (BD Biosciences) for 6 h at 37˚C prior to staining with fluorophore-conjugated anti-IFNγ and anti-IL10 using a Cytofix/Cyto perm plus kit (BD Biosciences) in accordance with the manufacturer's instructions. The stained cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo 7.6.1 software (Tree star Inc., Ashland, OR, USA).

Thyroid immunohistochemistry. The mouse thyroids were embedded in paraffin and sectioned coronally with a microtome into 5-μm sections (29). The sections were dewaxed and rehydrated, and treated for endogenous peroxidase with 3% methanol-hydrogen peroxide (Maixin, Fuzhou, China) for 10 min. All sections were incubated with the mouse anti-CXCL12 primary monoclonal antibody (dilution 1:50; MAB530; R&D Systems, Minneapolis, MN, USA) at 4˚C overnight and were then incubated for 40 min at room temperature. The tissue sections were then incubated at room temperature with biotin-conjugated secondary antibodies (Maixin) for 10 min, and in streptavidin-peroxidase complex (Maixin) for 10 min. Subsequently, the sections were treated with a solution of 3,3'-diaminobenzidine (DAB; Maixin) for 1-3 min, depending upon the staining of the DAB reaction product observed under a light microscope (BX51/BX52; Olympus Corporation). Finally, the sections were counterstained with hematoxylin, dehydrated, rinsed with distilled water, and

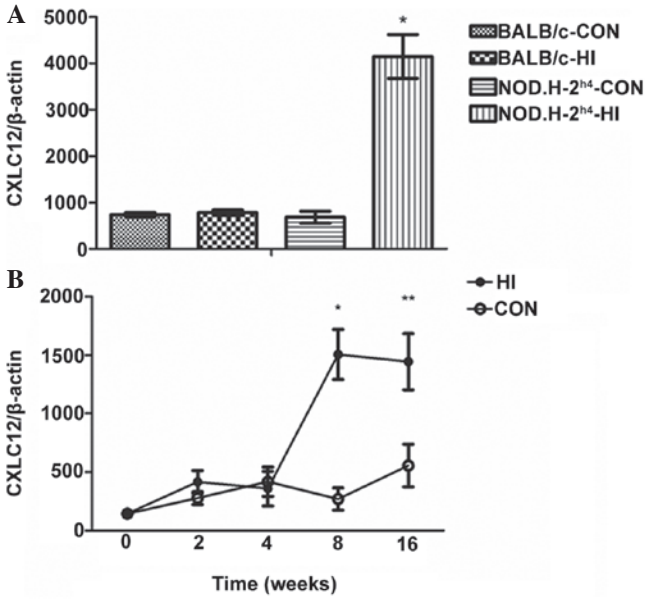

Figure 1. mRNA expression of CXCL12 is increased in thyroids of NOD.H-2 ^{h4}-HI mice and HI mice. (A) mRNA expression levels of CXLC12 were determined in the thyroid using RT-qPCR analysis and expressed relative to β-actin. *P<0.001, compared with the BAL/c-CON group, BAL/c-HI group and NOD.H-2 ^{h4}-CON group. (B) Dynamic changes in the mRNA expression levels of CXLC12 were determined in the thyroid using RT-qPCR and expressed relative to β-actin. *P<0.001 and **P=0.005, compared with the CON group. Data are presented as the mean ± standard error of the mean of four-six mice per group. Statistical analyses were performed consecutively using one-way analysis of variance and Bonferroni's or Dunnett's T3 tests. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CXCL12, CXC chemokine ligand 12; HI, 0.05% NaI drinking water to induce autoimmune thyroiditis; CON, control.

mounted in neutral gum (China National Medicines, Shanghai, China). All sections from each thyroid were viewed at 400x magnification. The integral OD (IOD) values, indicating the expression levels of the proteins were measured using Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Statistical analysis. All results are expressed as the mean ± standard error of the mean. One-way analysis of variance was performed to determine whether there were statistically significant differences among the groups, and post-test comparisons were performed using the Bonferroni's test or Dunnett's T3 test using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). Graphs were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA expression of CXCL12 is increased in the thyroid of NOD.H-2 ^{h4}-HI mice. As shown in Fig. 1A, no difference in the CXCL12 chemokine was observed between the thyroid tissues of the BAL/C-CON group and BAL/C-HI group. However, the mRNA expression of CXCL12 in the thyroid was significantly increased in the NOD.H-2^{h4}-HI group, compared with the NOD.H-2^{h4}-CON group, BAL/C-CON group and BAL/C-HI group.

Subsequently, dynamic changes in the mRNA expression of CXCL12 in the thyroid of NOD.H-2^{h4} mice were examined (Fig. 1B). No differences were observed between the CON group and HI group at 0, 2 or 4 weeks; however, significant differences were apparent at 8 and 16 weeks, in which the mRNA expression levels of CXCL23 were significantly higher, compared with those in the CON group.

Relative thyroid tissue weight is reduced in AMD3100-treated mice. As shown in Fig. 2A, the relative weights of the thyroids in the AIT group, in which NOD.H-2^{h4} mice received NaI in their drinking water for 8 weeks (8.86±1.12 mg/100 g), were increased significantly, compared with those in the CON group (4.88±0.57 mg/100 g) and AMD3100 group (5.20±0.52 mg/100 g). The histological changes of the enlarged thyroids included a lighter color and tougher texture, compared with the normal thyroids. No significant differences were identified between the CON group and AMD3100 group.

Serum TgAb titer is reduced in AMD3100-treated mice. Serum TgAb titers were significantly increased in the AIT group, compared with the CON group (0.625±0.13, vs. 0.18±0.009, respectively; P=0.022). By contrast, the AMD3100 group had significantly reduced serum TgAb titers, compared with the AIT group (0.232±0.03, vs. 0.625±0.13, respectively; P=0.043; Fig. 2B). No significant differences were found between the CON group and AMD3100 group.

Lymphocytic infiltration is less severe in the thyroid of AMD3100-treated mice. In the present study, lymphocytic infiltration was observed in the thyroids in almost 100% of the mice in the AIT group; and the scores of thyroiditis were significantly elevated in the AIT group, compared with the CON group. In the AMD3100 group, the severity of lymphocytic infiltration in the thyroid was lower, compared with that in AIT group. The scores of thyroiditis were not significantly reduced in the AMD3100 group, compared with the CON group (Fig. 2C and D).

mRNA expression of IFNγ is reduced and IL10 is increased in the spleen of AMD3100-treated mice. The mRNA expression of IL10 decreased in the AIT group, compared with the CON group (2.41±0.61, vs. 5.34±0.38, respectively; P=0.018). However, the mRNA expression of IL10 was increased in AMD3100 group, compared with the AIT group (5.79±0.90, vs. 2.41±0.61, respectively; P=0.039; Fig. 3A). The mRNA expression of IFNγ was higher in the AIT group, compared with the CON group (5.67±0.076, vs. 2.44±0.20, respectively; P=0.008). The mRNA expression of IFNγ was reduced in the AMD3100 group, compared with the AIT group (2.43±0.44, vs. 5.67±0.076, respectively; P=0.008; Fig. 3B). However, no significant differences were observed in the expression levels of IFNγ and IL10 between the AMD3100 and NOD groups.

CXCL12 is reduced in the thyroid of AMD3100-treated mice. CXCL12 was distributed predominantly in the thyroid cells. The IOD values of CXCL12 were increased in the AIT group, compared with the CON group; however, the IOD values of CXCL12 were reduced in the AMD3100-treated group,

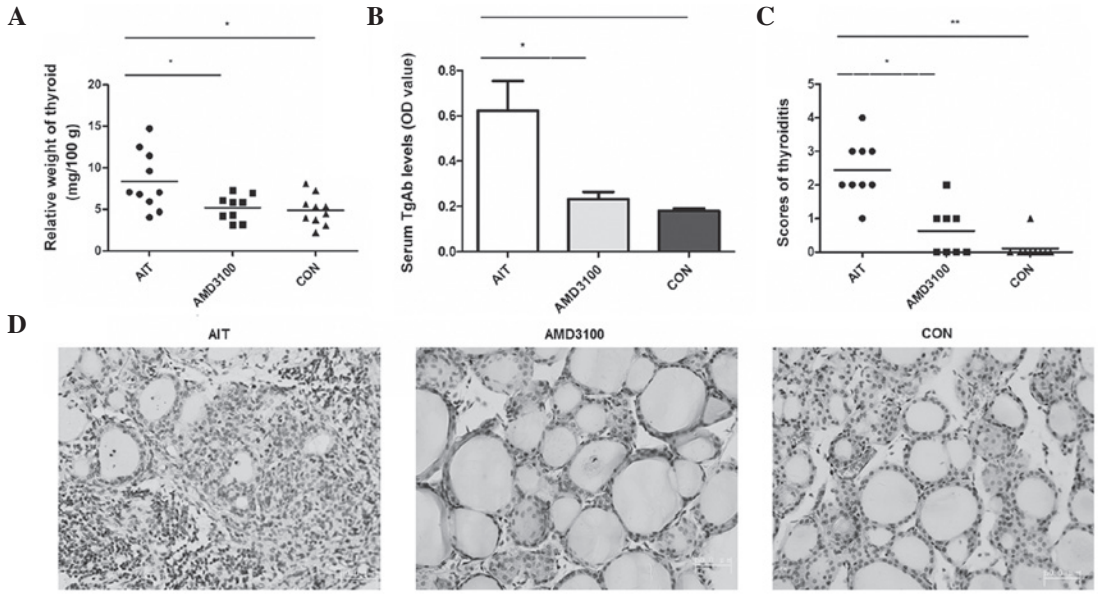

Figure 2. Relative thyroid weight, thyroiditis scores and serum TgAb titers are reduced in AMD3100-treated mice. (A) Relative weights of the thyroid were calculated as thyroid weight (mg)/body weight (g) x 100. (B) TgAb titers in the serum were measured using ELISA and presented by the ODs. (C) Throiditis severity scores: CON=0-1+, AIT=4+, AMD3100=0-2+. (D) Representative images of hematoxylin and eosin-stained thyroid sections from each group (magnification, x400; scale bar=50 μm). Lymphocytic infiltration was accompanied by enlargement of thyroid follicular cells in the AIT group. Lymphocytic infiltration was restored, compared with the AIT mice of the same age. Each data point represents one animal. Data are expressed as the mean ± standard error of the mean of 8-10 mice per group. Statistical analysis was performed using one-way analysis of variance with Bonferroni's or Dunnet"s T3 tests. *P<0.05 and **P<0.001. TgAb thyroglobulin antibody, AIT; autoimmune thyroiditis; CON, control; OD, optical density.

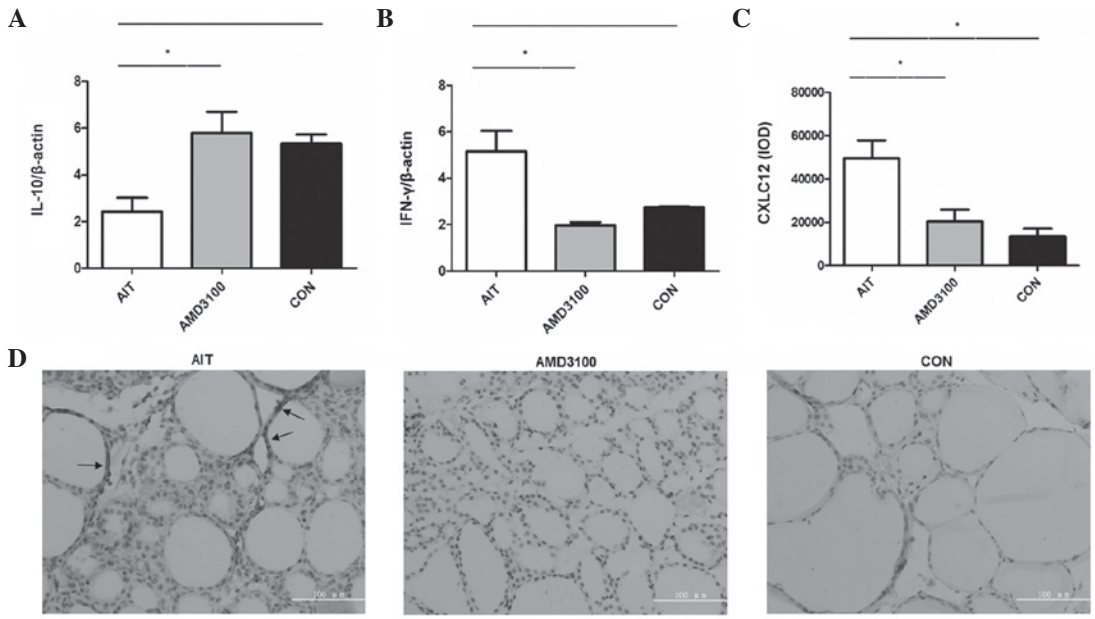

Figure 3. mRNA expression levels of IFNγ, IL10 and CXCL12 in AMD3100-treated mice. (A) CXCL12, expressed as the IOD value; mRNA expression levels of (B) IFNγ and (C) IL-10. IFNγ were determined in the spleen busing reverse transcription-quantitative polymerase chain reaction analysis, and expressed relative to β-actin. Data are presented as the mean ± standard error of the mean of four-five mice per group. Statistical analyses were performed consecutively with one-way analysis of variance and Bonferroni's or Dunnett's T3 tests. *P<0.05 and **P<0.001. (D) Immunohistochemistry-stained thyroid sections from the different groups, arrows indicate CXCL12. (magnification, x400; scale bar=100 μm). CXCL12, CXC chemokine ligand 12; IFN, interferon γ; IL-10, interleukin-10; AIT; autoimmune thyroiditis; CON, control; IOD, integral optical density.

compared with the AIT group. No significant difference was observed between the AMD3100 group and CON group (Fig. 3C and D).

Changes in T cells and B cells in the spleen of AMD3100-treated mice. The present study evaluated the percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD4^+IL10^+$ T cells, $CD19^+IL10^+$ B cells, $CD4^+IFN\gamma^+$ T cells and $CD8^+IFN\gamma^+$ T cells in the splenocyte population (Figs. 4 and 5). As summarized, the percentages of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells were significantly increased in the AIT group, compared with the CON group; however, the percentages were significantly

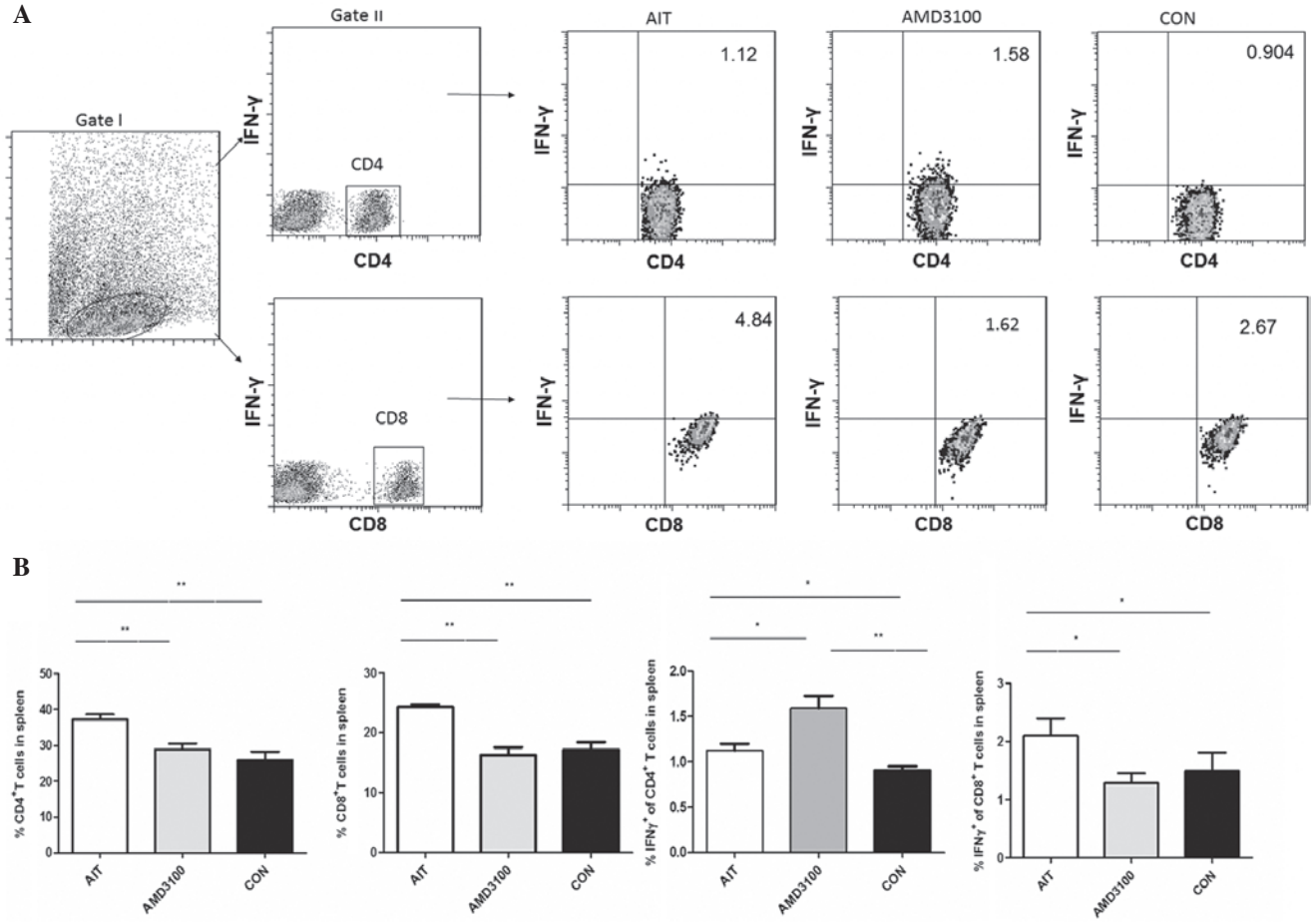

Figure 4. $CD4^+$ T cells, $CD8^+$ T cells and $CD8^+IFN\gamma^+$ T cells are reduced, and $CD4^+IFN\gamma^+$ T cells are increased in AMD3100-treated mice. Lymphocyte cells were identified using flow cytometric analysis of isolated spleen mononuclear cells. (A) $CD4^+$ T and $CD8^+$T cells were gated. (B) IFNγ-producing $CD4^+$ T cells and IFNγ-producing $CD8^+$ T cells were analyzed using flow cytometry in each group and their percentages were determined. Data are presented as the mean ± standard error of the mean of four-five mice per group. Statistical analyses were performed consecutively using one-way analysis of variance and Bonferroni's or Dunnett's T3 tests. $^*P<0.05$ and $^{**}P<0.001$. IFN, interferon γ; IL-10, interleukin-10; AIT; autoimmune thyroiditis; CON, control.

decreased in the AMD3100 group, compared with the AIT group. The percentages of $CD4^+IFN\gamma^+$ T cells were significantly increased in the CON group, AIT group and AMD3100 group, respectively. The percentages of $CD8^+IFN\gamma^+$ T cells were significantly increased in the AIT group, compared with the CON group, but were significantly decreased in the AMD3100 group, compared with the AIT group. For the percentages of $CD4^+IL10^+$ T cells, significantly lower levels were observed in the AIT group, compared with the CON group, but were marginally increased in the AMD3100 group, compared with the AIT group. Similarly, $CD19^+IL10^+$ B cells were reduced in the AIT group, compared with the CON group, and were significantly elevated in the AMD3100 group, compared with the AIT group. No significant differences were observed in the percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD4^+IFN\gamma^+$ T cells, $CD8^+IFN\gamma^+$ T cells, $CD4^+IL10^+$ T cells and $CD19^+IL10^+$ B cells between the AMD3100 group and CON group.

Discussion

Chemokines are involved in autoimmune diseases; however, there are few reports regarding changes in the expression of chemokines in iodine-induced autoimmune thyroiditis NOD.H-2^{h4} mouse models. Following the provision of 0.05% NaI in drinking water to NOD.H-2^{h4} mice, the incidence of subacute thyroiditis (SAT) is almost 100% in female and male mice at 6-8 weeks of age (13), whereas the BALB/c mice strain is not an autoimmune disease-susceptible species. In the present study, the thyroids of NOD.H-2^{h4} mice and BALB/c mice were examined, and almost 100% of the NOD.H-2^{h4} mice developed thyroiditis after 8 weeks pf drinking high iodine water. However, no BAL/C mice developed thyroiditis. The mRNA expression levels of CXCL12 were increased in the NOD.H-2^{h4}-HI group mice.

When the NOD.H-2^{h4} mice did not form thyroid lymphocytic infiltration, there was no difference in the mRNA expression of CXCL12 between the HI and CON groups. However, when the NOD.H-2^{h4} mice developed thyroid lymphocytic infiltration, the mRNA expression of CXCL12 was significantly increased in the HI group, compared with the CON group. This suggested an association between lymphocytic infiltration and chemokine increase in the NOD.H-2^{h4}-HI group. As CXCL12 appeared to be one of the important factors involved in recruiting lymphocytes to the thyroid in NOD.H-2^{h4}-HI group for 8 weeks. Therefore, the present study inhibited the binding of CXCL12 and CXCR4, to investigate the possible mechanisms of CXCL12 in thyroiditis.

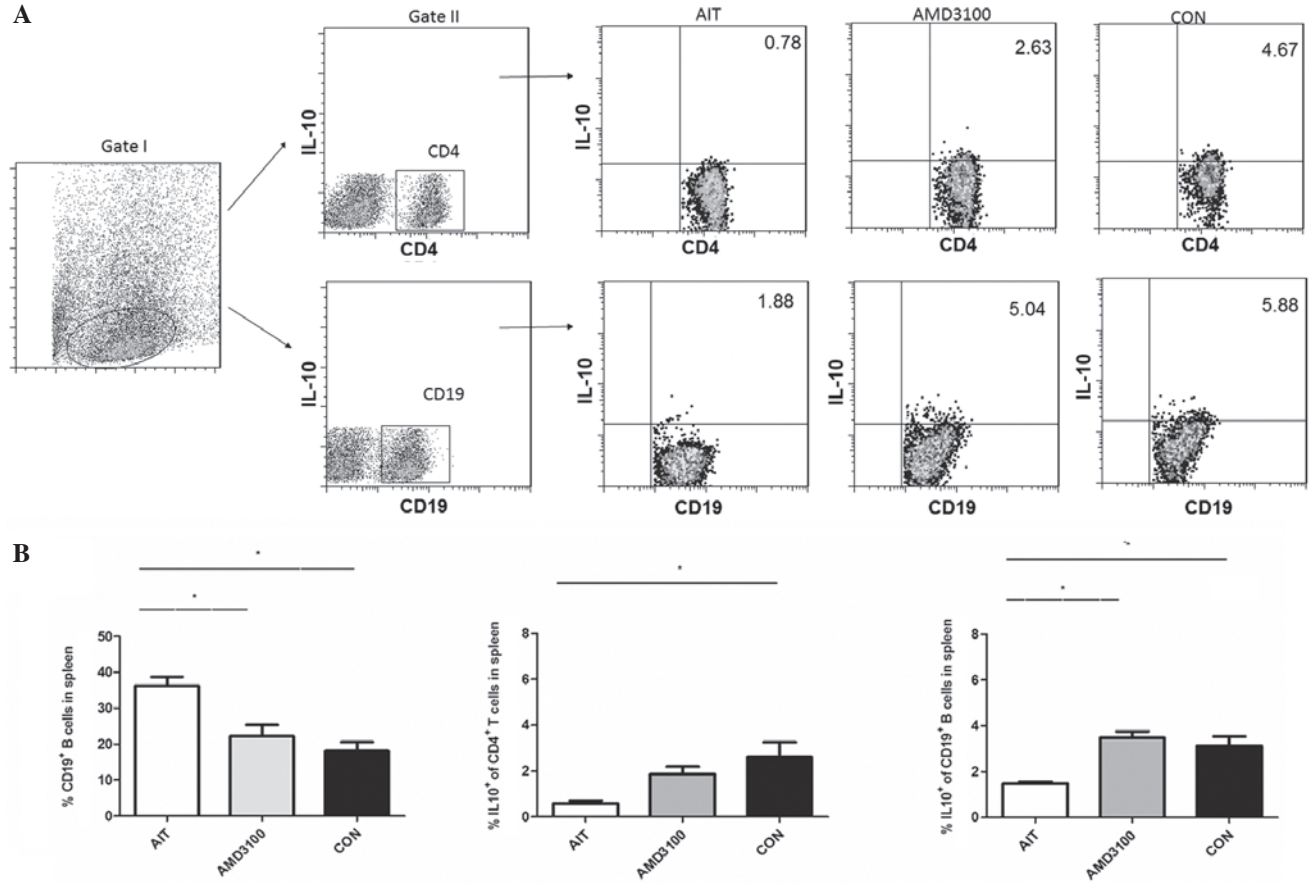

Figure 5. Percentages of $CD4^+IL10^+$ T cells and $CD19^+IL10^+$ B cells increase, and $CD19^+$ B cells decrease in AMD3100-treated mice. IL10-producing $CD4^+$ T cells were analyzed using flow cytometry. (A) $CD4^+$ T and $CD19^+$ B cells were gated. (B) Percentages of each cell type in the treatment groups. Data are presented as the mean ± standard error of the mean of four-five mice per group. Statistical analyses were performed consecutively using one-way analysis of variance and Bonferroni's or Dunnett's T3 tests. $^*P<0.05$ and $^{**}P<0.00$. IFN, interferon γ; IL-10, interleukin-10; AIT; autoimmune thyroiditis; CON, control.

In the present study, elimination of CXCL12-mediated effects by antagonization of CXCR4 through treatment with AMD3100 significantly decreased thyroiditis activity. Using immunohistochemistry, it was found that CXCL12 was upregulated in the AIT group, compared with the CON group, but was almost absent in the AMD3100-treated group. In addition, as the levels of CXCL12 decreased, reduced inflammation of the thyroid gland was observed. This suggested that CXCL12 is one of the important cytokines in AIT, and circulating $CXCR4^+$ leukocytes may be attracted to inflamed tissues. In addition, the level of CXCL12 was increased in the AIT thyroid, and all these mice had lymphocytic infiltration in the thyroids with severity scores of between 1+ and 4+, had increased thyroid relative weights and higher serum TgAb titers in the relative to the CON group, which were decreased in the AMD3100-treated group. This result was in accordance with those observed in experimental colitis (30) and autoimmune collagen-induced arthritis mouse models (31).

Several previous studies have been performed to investigate the role of the CXCL12/CXCR4 chemokine axis in autoimmunity thyroiditis. Specifically, Armengol *et al* (24) previously demonstrated that the mRNA and protein expression levels of CXCL12 in the thyroid glands of patients with HT are higher than, compared with non-autoimmune thyroid glands, and that thyrocytes are the predominant source of CXCL12 in autoimmune thyroiditis. It was also suggested that, as in non-obese diabetic mouse thyroiditis (32), iodine overload may induce thyrocyte necrosis, which stimulates resident macrophages to produce Th1 cell-deriving cytokines, which may induce CXCL12 synthesis by adjacent thyrocytes. Thyrocytes can also produce CCL21 (33), CXCL9 and CXCL10 (20), and can induce lymphoid follicles, which arise in the thyroid gland and even in multinodular goiter, a clinical entity of uncertain etiology. In other animal model autoimmunity diseases, including mouse models of dextran sulfate sodium (DSS)-induced colitis (8), the expression of CXCL12 in the colon of the mice were markedly increased; and application of a CXCR4 antagonist, TF14016, remitted the colonic inflammation in DSS-induced colitis and reduced TNFα and IFNγ production in the mesenteric lymph node cells. This suggested a possible role for the CXCL12/CXCR4 chemokine axis in the pathophysiology of autoimmune diseases.

It has been confirmed that the CXCL12/CXCR4 axis is an efficacious leukocyte chemoattractant, which can attract lymphocytes and mononuclear cells from the bloodstream to sites of inflammation (30). In the present study, it was found that disease progression in NOD.H-2^{h4} mice may be promoted by the elevated expression of CXCL12 through its effect on T cell trafficking, which is partly adjusted by the CXCL12-CXCR4 interaction. It has been documented that $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells are involved in the destruction of thyroid follicles in AIT (32,34). The present

study showed that increased numbers of $CD4^+$, $CD8^+$T cells and $CD19^+$ B cells were present in the spleen of the AIT mice, compared with the CON mice. Following AMD3100 treatment of NOD.H-2^{h4} mice, the percentages of $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells decreased significantly in the spleen. This indicated that elevated expression of CXCL12 may dysregulate lymphocyte trafficking. Thus, CXCR4-expressing T cells may be enlisted to the immune sites of AIT by enhanced expression of CXCL12, and CXCL12 is likely a crucial contributing factor to the development of thyroiditis in NOD.H-2^{h4} mice.

In the present study, the CXCR4 antagonist, AMD3100, significantly reduced the mRNA expression of IFNγ, but increased the mRNA expression of IL10. To further understand the reason for the effect of AMD3100 on IFNγ and IL10 production, the present study investigated its effects on T cells and B cells in the spleen. It was found that NOD.H-2^{h4} AIT mice provided with NaI had lower percentages of $CD4^+IL10^+$ T cells and $CD19^+IL10^+$ B cells. Upon administration of the AMD3100, the percentages of $CD4^+IL10^+$ T cells and $CD19^+IL10^+$ B cells increased. IL10-expressing T cells (T regulatory-1 cells) are involved in the balance of immune responses and the deletion of destructive tissue pathology by limiting and stopping immune responses (35,36). These results are in agreement with a previous report that indicated IL10 can protect against the development of HT (37). IFNγ is a Th1 cytokine produced by $CD4^+$ Th1 cells and is critical for the development of SAT in NOD.H-2^{h4} mice (38), and is also secreted by $CD4^+$ T cells, $CD8^+$ T cells and natural killer (NK) cells. IFNγ alone or in combination with other inflammatory cytokines upregulates the expression of adhesion molecules, and certain chemokines and chemokine receptors to recruit T cells to the site of inflammation. Annunziato *et al* reported that IFNγ downregulates the expression of CXCR4 on the surface of T lymphocytes (39), suggesting that this receptor may be connected with the Th cell. In the results of the flow cytometric analysis in the present study, $CD4^+IFN\gamma^+$ T cells and $CD8^+IFN\gamma^+$ T cells were higher in number in the AIT group, compared with the control group. Following AMD3100 treatment of the NOD.H-2h4 mice, the percentage of $CD4^+IFN\gamma^+$ T cells increased, but the percentage of $CD8^+IFN\gamma^+$ T cells decreased in the spleen. The total mRNA expression of IFNγ was reduced in the AMD3100 treatment group. $CD8^+IFN\gamma^+$ T cells may be important for the decreased expression of IFNγ noted in the AMD3100-treated group. The expression of $IFN\gamma^+$ NK^+ cells was not analyzed and, therefore, requires further verification.

Therefore, recruiting IFNγ- and IL10-producing $CD4^+$ T cells and $CD19^+$ B cells via CXCL12 into an inflamed thyroid site may be an important regulatory mechanism. Taken together, the immune response in AIT is complex, due to the involvement of Th 1 cells, the B cell immune response and pro-inflammatory and regulatory cytokines. To the best of our knowledge, the present study is the first suggesting that the elimination of CXCL12-mediated effects may treat thyroiditis via alterations in Th 1 cells, the B cell immune response, and pro-inflammatory and regulatory cytokines.

AMD3100 was initially developed to treat human immunodeficiency virus infections through the antagonism of the CXCR4 (40). It is a slow, tightly-binding, reversible inhibitor (41). There is a transfer from the pathogenic T helper cytokine profile to the antagonistic T helper cytokine profile in AMD3100-treated mice (42). The present study demonstrated that AMD3100 treatment of AIT NOD.H-2^{h4} mice significantly reduced the infiltration of lymphocytes in the thyroid, and decreased the titer of TgAb. Therefore, CXCL12 may affect IL10 and IFNγ production in AIT mice, and this may be inhibited by AMD3100. Currently, the role of AMD3100 in autoimmune diseases is controversial. For example, in mouse models of type I diabetes melitus (11), collagen-induced arthritis (9), DSS-induced colitis (30) and asthma (12); the treatment of mice with AMD3100 significantly reduced the severity of the disease. By contrast, several studies have reported that AMD3100 treatment promotes disease development (8,43,44). Thus, the results of treatment with AMD3100 have indicated that, in addition to its pro-inflammatory role in autoimmune experimental models and human autoimmune diseases, CXCL12 may also have anti-inflammatory properties.

The precise mechanism underlying the differing conclusions of previous studies remains to be elucidated, however, the findings of the present study lay the foundation for further investigation of AMD3100, for the prevention and/or treatment of autoimmune thyroiditis and possibly other autoimmune diseases with elevated expression levels of CXCL12.

In conclusion, the present study demonstrated that the CXCR4 antagonist, AMD3100, decreased the severity of autoimmune thyroiditis in mice, by inhibiting the production of cytokines and/or the migration of cytokine-producing lymphocytes. This revealed the therapeutic potential for the CXCR4 antagonist in the treatment of thyroiditis.

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