

Letter to the Editor

Neuropeptide signaling through neurokinin-1 and neurokinin-2 receptors augments antigen presentation by human dendritic cells*To the Editor:*

Asthma, an inflammatory disease of the airways, is associated with various T_H and innate immune cells.¹ Pulmonary disorders with chronic inflammation induced by infections and severe asthma, including childhood asthma and chronic obstructive pulmonary disease in smokers, are major public health issues. Hypersensitivity pneumonitis (HP) is a complex pulmonary disease caused by repeated exposure to allergens and irritants.² The precise mechanisms mediating severe asthma and HP remain to be elucidated.

Neurotransmitters including substance P (SP) and neurokinin A (NKA) are widely distributed in both the central and peripheral nervous systems, and their receptors, neurokinin-1 receptor (NK1R) and neurokinin-2 receptor (NK2R), are expressed on immune cells. However, the role of the NKA-NK2R axis in immune responses relative to the SP-NK1R signaling cascade has not been elucidated. Previously, we demonstrated that neuropeptide signaling through NK2R was correlated with airway hyper-responsiveness in mouse with severe asthma models.³ Moreover, we found that the NKA-NK2R signaling pathway was involved in the antigen-presenting function of murine dendritic cells (DCs) and that NK2R expression on DCs was enhanced by IFN- γ and LPS stimulation in a signal transducer and activator of transcription 1 (STAT-1)-dependent manner.⁴ In addition, we confirmed that human DCs significantly upregulated gene expression levels of NK2R and tachykinin, precursor 1 (TAC-1), which encoded SP and NKA, after IFN- γ or poly I:C stimulation, suggesting possible roles of neuropeptide signaling in human DCs.⁴ In this study, we investigated the effects of neuropeptide signaling through NK2R as well as NK1R on antigen presentation by human DCs and the subsequent activation of effector T_H cells.

Human DCs (monocyte-derived dendritic cells [MoDCs]), generated from PBMCs in the presence of GM-CSF plus IL-4, demonstrated increased NK2R and NK1R expression after IFN- β , IFN- γ , LPS, or poly I:C stimulation (Fig 1, A and B). The upregulation by poly I:C stimulation was convincing, whereas effects of IFNs were modest. In this study, we found that poly I:C-stimulated MoDCs also induced IFN- α and IFN- β production (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Upregulation of NK1R and NK2R gene expression by poly I:C was STAT-1 dependent (Fig 1, C). We confirmed that surface expression levels of NK1R and NK2R on MoDCs were significantly enhanced by poly I:C stimulation (Fig 1, D). In addition, we found that both NK2R and NK1R were expressed on CD68- or CD163-positive macrophages and HLA-DR-positive cells including DCs in lung tissues from patients with asthma and acute and chronic HP (Fig 1, E and F; see Fig E2 and Tables E1 and E2 in this article's Online Repository at www.jacionline.org). These findings suggest that STAT1 activation by T_H1 cytokines, including IFN- γ and type 1 IFNs induced by Toll-like receptor ligands such as poly I:C, drives the upregulation of NK1R and NK2R on DCs.

To further evaluate whether neuropeptide signaling through NK1R and NK2R was related to poly I:C-induced DC maturation, we analyzed the surface expression levels of HLA and costimulatory molecules after treatment with an NK1R or NK2R antagonist. HLA-DR, CD80, CD86, and CD40 expressions, but not HLA-A/B/C and CCR7 expressions, upregulated by poly I:C stimulation through STAT1 activation (see Fig E1, B), were attenuated in the presence of an NK1R or NK2R antagonist (Fig 2, A). In this experiment, we confirmed that these antagonists did not increase 7AAD⁺ and/or Annexin⁺ cells after the treatments. We further confirmed that SP (25.2 ± 9.2 pg/mL) and NKA (44.6 ± 27.5 pg/mL) were produced in the culture supernatants of MoDCs (2.5×10^5 cells) for 24 hours and HLA class II expression levels on these neuropeptide-treated MoDCs were suppressed in the presence of these antagonists (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus, neuropeptide signaling through NK1R and NK2R may modulate the surface expression levels of HLA class II and costimulatory molecules on MoDCs, suggesting altered antigen presentation to CD4⁺ T cells.

To confirm the effect of NK2R-mediated signaling on DC antigen presentation, we induced Bet-v1-specific IFN- γ - or IL-4-producing T_H cells *in vitro* using white birch-derived antigen (see Fig E4 in this article's Online Repository at www.jacionline.org), as described previously.^{5,6} T-cell proliferation and cytokine production were evaluated after stimulation with antigen-loaded MoDCs. Blocking NK2R-mediated signaling suppressed the induction of antigen-specific CD4⁺ T cells (Fig 2, B). Furthermore, we confirmed the production of cytokines by ELISA for IL-2, IL-4, IL-5, IL-13, and IFN- γ by activated Bet-v1-specific T_H cells after antigen stimulation by MoDCs. The production of T_H1 and T_H2 cytokines was decreased in the presence of NK1R and/or NK2R antagonists or by culturing with NK1R- or NK2R-siRNA-transfected MoDCs (Fig 2, C; see Fig E5, A, and Fig E6 in this article's Online Repository at www.jacionline.org).

Furthermore, MoDC IL-12 production, as detected by ELISA in culture supernatants, was reduced in the presence of NK2R or NK1R antagonists (Fig 2, C). Previous reports demonstrated that DC-T-cell interactions through CD40-CD40L binding induces IL-12 production by DCs.⁷ It has also been shown that SP-NK1R signaling activates IL-12 secretion by DCs.⁸ Thus, neuropeptide signaling through NK2R, as well as NK1R, promotes MoDC activation by antigen-specific T_H cells. Finally, we confirmed that knockdown of NK1R and NK2R or the antagonists attenuated IFN- α , IFN- β , and IL-12p35 gene expression in MoDCs after poly I:C stimulation (see Fig E5, B, and Fig E7 in this article's Online Repository at www.jacionline.org). Notably, the combination of NK1R and NK2R antagonists synergistically inhibited cytokine production (see Fig E7).

Taken together, these findings indicate that NK1R- or NK2R-dependent neuropeptide signaling activates antigen-specific T_H cells by augmentation of DC function in both the induction and effector phases. This suggests that NK1R and NK2R antagonists may have both preventive and therapeutic effects in disorders induced by excessive DC/T_H-mediated airway inflammation. Therefore, these findings may provide potential insights into the

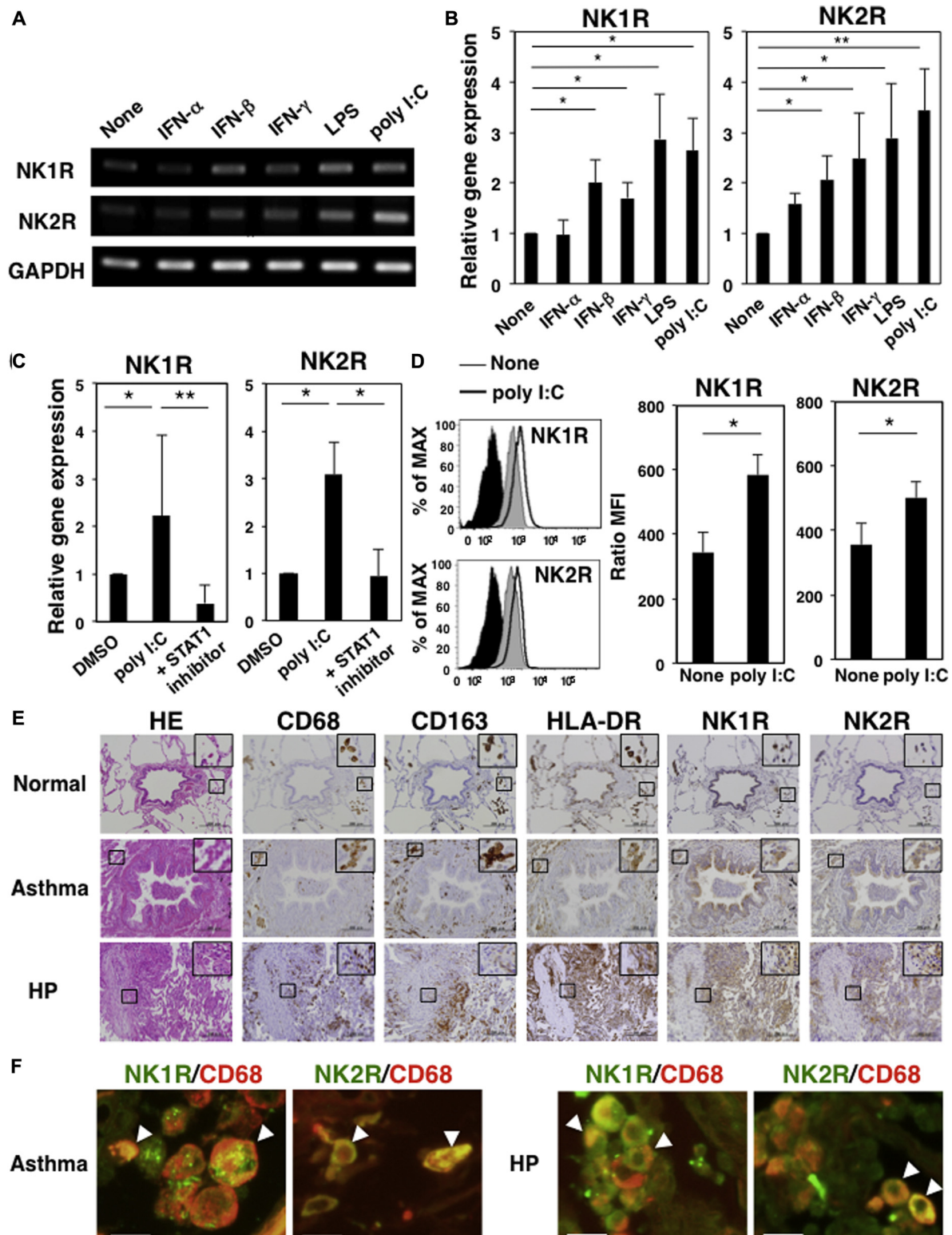


FIG 1. **A** and **B**, NK1R and NK2R gene expression levels in human MoDCs generated from PBMCs ($N = 4$) were evaluated by RT-PCR after stimulation with IFN- α , IFN- β , IFN- γ , LPS, or poly I:C for 24 hours. **C**, MoDC NK1R and NK2R expression levels after stimulation with poly I:C in the presence of a STAT1 inhibitor. **D**, NK1R and NK2R expression on DCs after poly I:C stimulation. Surface expression levels of NK1R and NK2R on CD11c $^{+}$ DCs were evaluated by flow cytometry. Representative profiles and mean and SD ($N = 4$) of the calculated normalized mean fluorescence intensity (ratio MFI = MFI/respective isotype control) are shown. **E**, Hematoxylin-eosin staining and immunostaining of CD68, CD163, HLA-DR, NK1R, and NK2R were performed on sections of normal lung and lesion areas from a representative normal person and a patient with asthma or HP. Scale bar is 200 μ m for all panels. **F**, Dual-immunofluorescence staining of CD68 (red) and NK1R or NK2R (green) was performed for the section from a patient with asthma or HP. Scale bar is 20 μ m for all panels. DMSO, Dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. * P < .05 and ** P < .01.

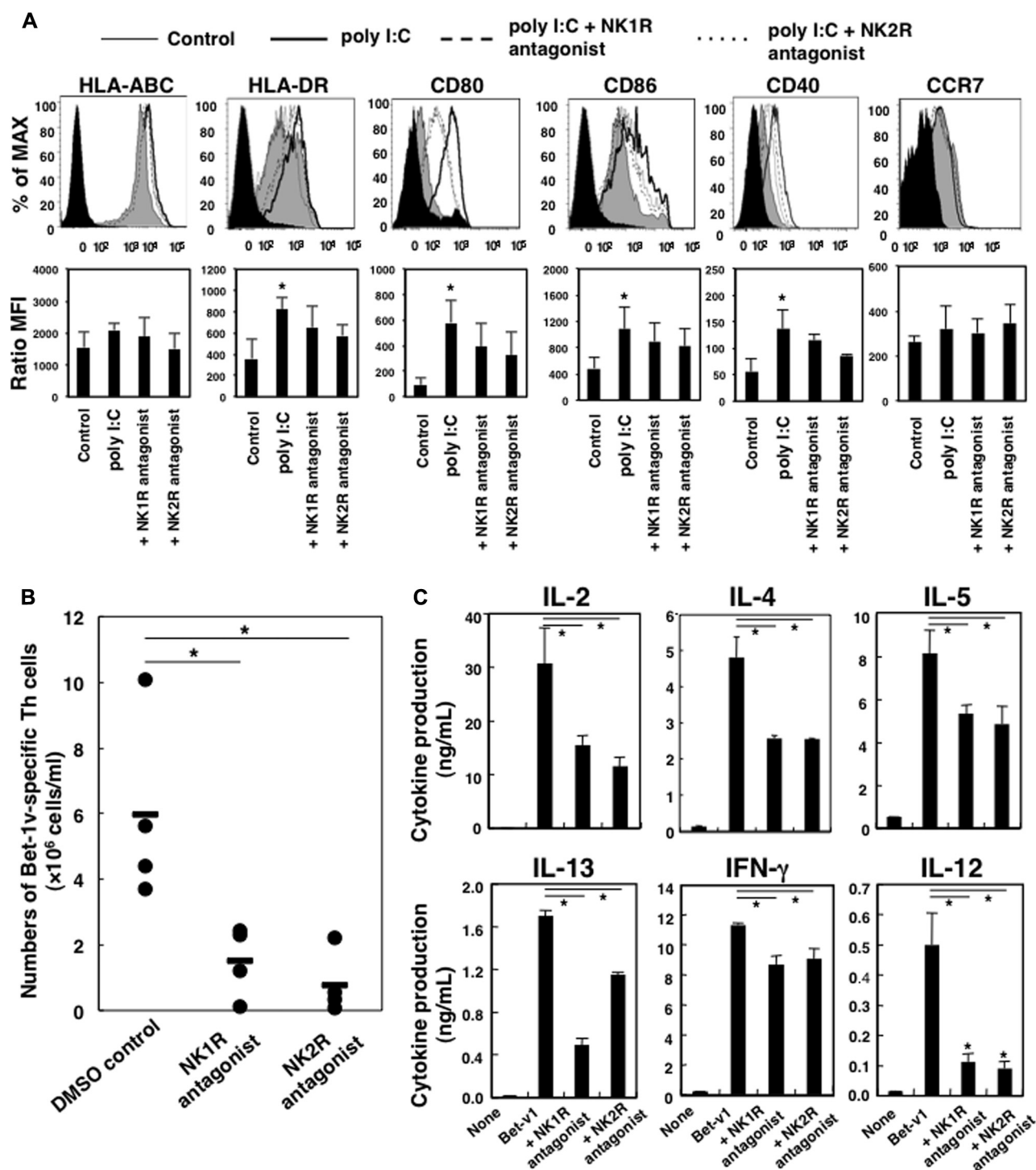


FIG 2. A, DCs (N = 6) were treated with poly I:C in the presence of NK1R or NK2R antagonists for 24 hours. Surface expression levels of HLA-A/B/C, HLA-DR, CD80, CD86, CD40, and CCR7 on CD11c⁺ DCs from PBMCs were evaluated by flow cytometry. Representative profiles and mean and SD (N = 6) of the calculated ratio mean fluorescence intensity (ratio MFI = MFI/respective isotype control) are shown. B, White birch antigen-specific CD4⁺ T cells (N = 4) were induced by Bet-v1 peptide-pulsed MoDCs for 2 weeks. IFN- γ and IL-4 production was evaluated by flow cytometry. Bet-v1-specific T_H cells were activated by peptide-pulsed MoDCs in the presence of an NK1R or NK2R antagonist for 24 hours. Antigen-specific T_H cells were counted, and the mean and SD are shown. C, Bet-v1-specific CD4⁺ T cells were cocultured with antigen-pulsed MoDCs in the presence of NK1R or NK2R antagonist for 24 hours. IL-2, IL-4, IL-5, IL-13, IFN- γ , and IL-12 concentration in culture supernatants was evaluated by ELISA. Three experiments were performed independently. Mean and SD of the representative data are shown. DMSO, Dimethyl sulfoxide. *P < .05.

development of novel treatments for patients with severe inflammatory diseases caused by excessive type 1 immunity.

Neuropeptides, such as SP and NKA, act mainly through their receptors, NK1R and NK2R, respectively, which may also be novel therapeutic targets in asthma. Previous studies demonstrated that antagonism of NK1R and NK2R by receptor antagonists reduced airway hyperresponsiveness and improved lung function.⁹ However, the clinical effects on airway inflammation and asthma symptoms are poorly understood. Recently, we found that human DCs upregulated the expression of TAC-1, which encodes SP and NKA, after IFN- γ or poly I:C stimulation.⁶ Thus, we speculated that type 1 immune-dominant severe asthma, which may be caused by viral infections, will be a promising target for therapy using NK1R and NK2R antagonists.

We conclude that neuropeptide signaling through NK2R and NK1R promotes both innate and acquired immune responses through DC activation, suggesting that neuroimmune cross talk may be associated with various diseases, including infection and chronic inflammation such as severe asthma and HP.

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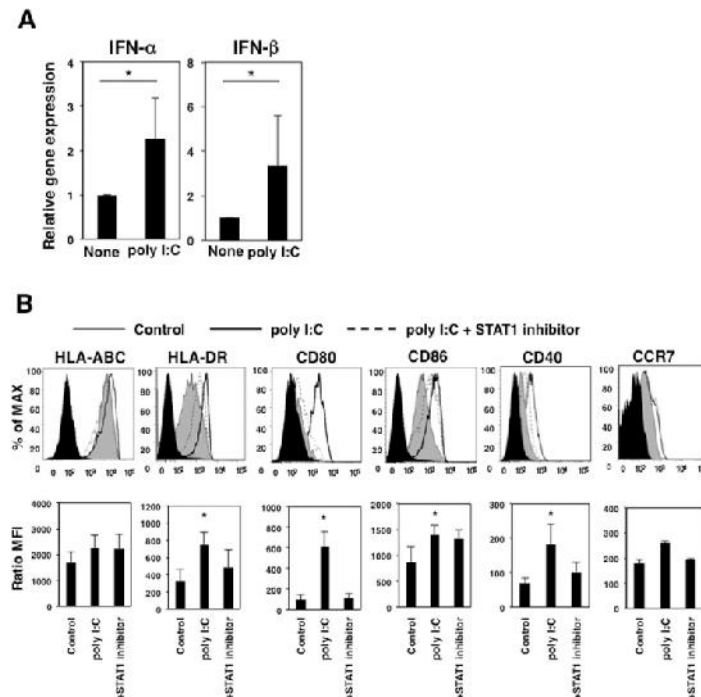


Fig E1.

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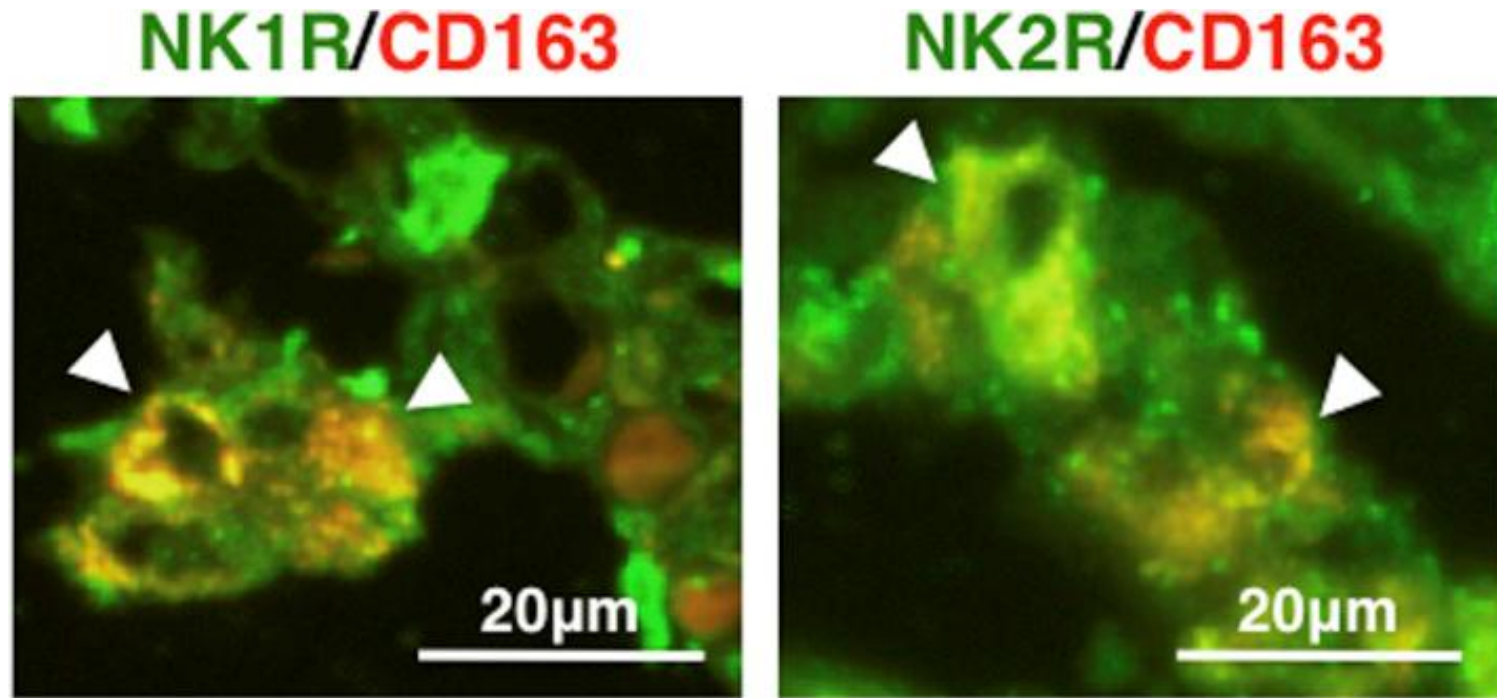


Fig E2.

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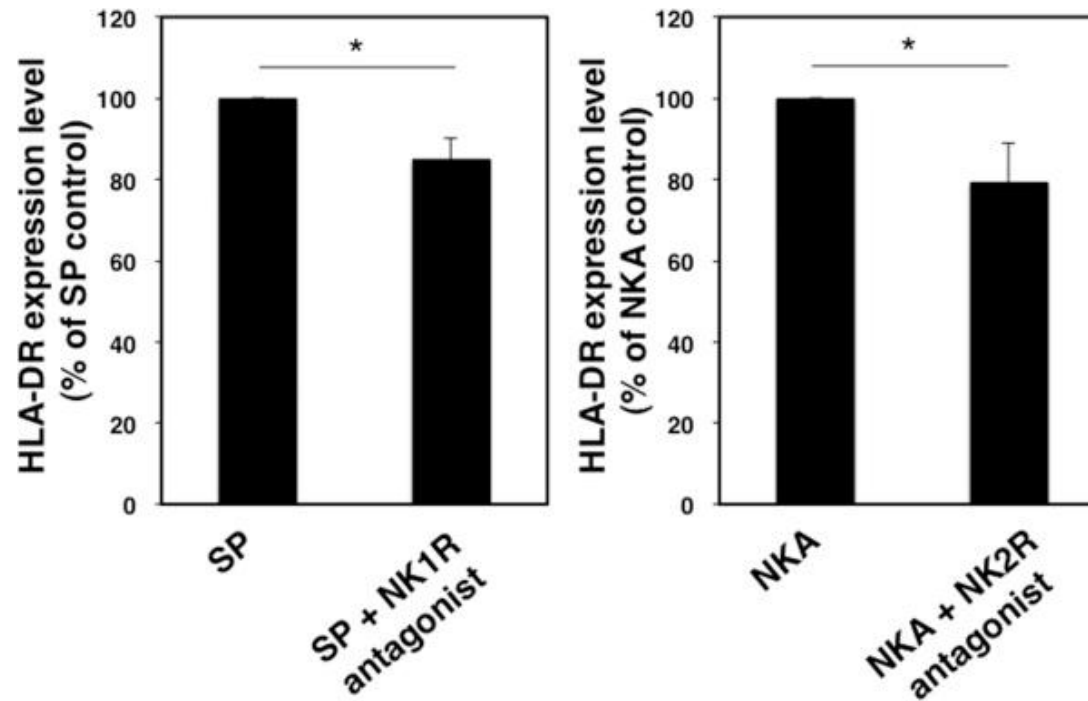


Fig E3.

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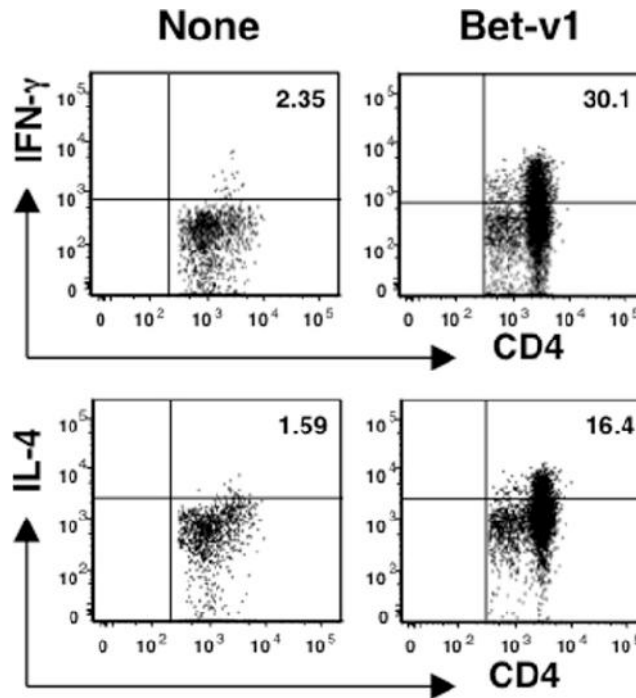


Fig E4.

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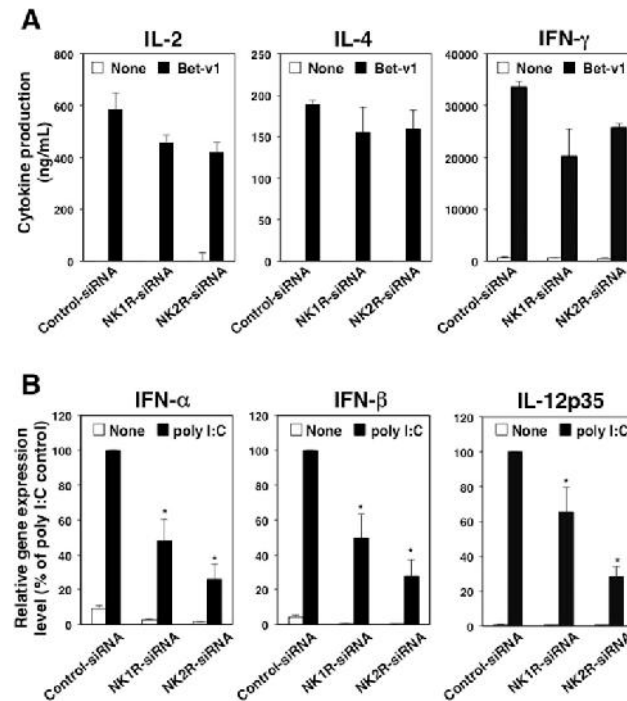


Fig E5.

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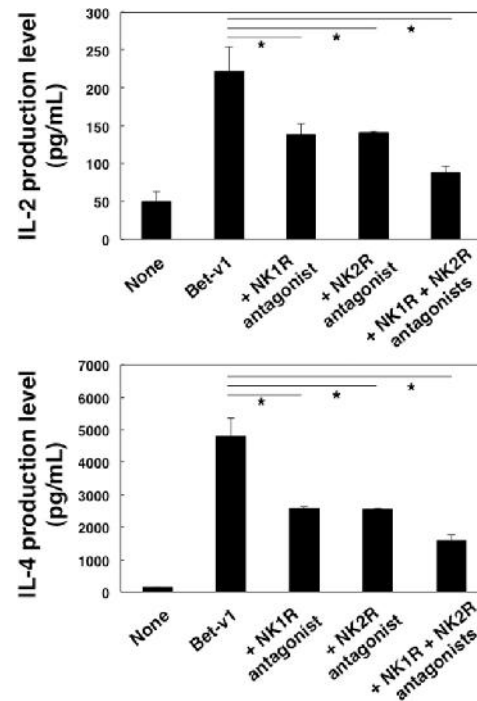


Fig E6.

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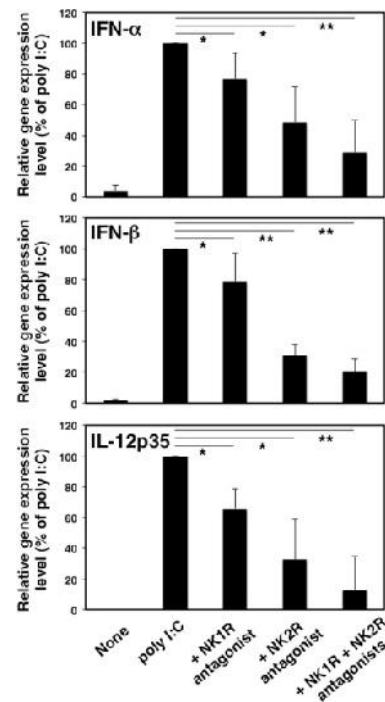


Fig E7.

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5 **METHODS**

6 **Generation of human MoDCs from PBMCs**

7 PBMCs were obtained from healthy individuals by Ficoll-Hypaque (Amersham
8 Bioscience, GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) gradient
9 centrifugation. Adherent PBMCs (2×10^6 cells) were cultured in AIM-V media (Life
10 Technologies, Carlsbad, CA, USA) in the presence of recombinant human IL-4 (50
11 ng/mL, Wako Chemical Industries, Ltd., Osaka, Japan) and GM-CSF (50 ng/mL,
12 Wako Chemical Industries, Ltd., Osaka, Japan) for 7 days as described previously.⁴
13 Purity of the induced CD11c⁺HLA-DR⁺HLA-A/B/C⁺ cells were more than 95 %.
14 MoDCs were treated with DMSO (control), IFN- α (1000 U/mL; Peprotech Inc., Rocky
15 Hill, NJ, USA), IFN- β (1000 U/mL; Peprotech Inc.), IFN- γ (50 ng/mL; Peprotech Inc.),
16 LPS (20 ng/mL; Sigma-Aldrich Co., St Louis, MO, USA), or poly I:C (10 μ g/mL;
17 InvivoGen, San Diego, CA, USA) for 24 h and used for RT-PCR experiments. In some
18 experiments, MoDCs were stimulated with poly I:C (10 μ g/mL) in the presence of a
19 STAT1 inhibitor (Fludarabine, 2 μ g/mL; Selleckchem, Houston, TX, USA), Substance

P agonist (197-12211, Wako Chemical Industries, Osaka Japan), Neurokinin A agonist (140-07171, Wako Chemical Industries, Osaka Japan), NK1R antagonist (RP 67580, 10 μ M; Tocris Bioscience, Bristol, UK), and/or NK2R antagonist (GR 159897, 10 μ M; Tocris Bioscience) for 24 h.

Quantification of gene expression

Total RNA was extracted from human MoDCs using an RNeasy kit (QIAGEN, Hilden, Germany). First-strand cDNAs were synthesized using oligo (dT) primers (Life Technologies) with Superscript III reverse transcriptase (RT; Life Technologies). Genes for human NK1R (*TACR1*), NK2R (*TACR2*), IFN- α (*IFNA*), IFN- β (*IFNB*), IL-12p35 (*IL12A*), and GAPDH (*GAPDH*) were amplified by thermal cycler (LightCycler, Roche, F. Hoffmann-La Roche AG, Basel, Switzerland) with specific primers (see **Table E2** in the Online Repository). Sample signals were normalized to GAPDH according to the $\Delta\Delta C_t$ method: $\Delta C_t = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{reference}}}$. Gene expression relative to the control sample was then calculated for each sample. In some experiments, NK1R, NK2R and GAPDH were amplified by thermal cycling using a GeneAmp PCR System 9700 (Life Technologies).

37

38 **Immunohistochemistry**

39 Lung specimens from six normal, five of asthmatic or seven of acute HP (AHP)
40 and five chronic HP (CHP) patients were formalin fixed, paraffin embedded and
41 sections stained with hematoxylin-eosin. The slides were treated with anti-human
42 CD68 (M0814, Dako ChemMate, Denmark A/S, Glostrup, Denmark), CD163
43 (NCL-CD163, Leica Biosystems, Newcastle, UK), HLA-DR α -chain (TAL.1B5, Dako
44 ChemMate), NK1R (H-83, at 1:100 dilution, Santa Cruz Biotechnology, Inc., Dallas,
45 TX, USA) or NK2R (C-21, at 1:50 dilution, Santa Cruz Biotechnology, Inc.) antibody.
46 Protein expression was visualized using EnVision (Dako ChemMate) for NK1R or
47 N-Histofine Simple Stain MAX PO(G) (Nichirei Biosciences, Inc., Tokyo, Japan),
48 followed by DAB using an Autostainer Plus System (DAKO ChemMate).

49

50 **Immunofluorescence staining**

51 Dual-color immunofluorescence staining was performed for lung tissues of
52 patients with asthma or HP. The sections were incubated with primary antibodies for
53 (M0814, at 1:300 dilution, Dako ChemMate), CD163 (NCL-CD163, 1:300 dilution,

Leica Biosystems), and NK1R (H-83, Santa Cruz, Biotechnology, Inc.) or NK2R (C-21, Santa Cruz, Biotechnology, Inc.) at 4 °C over night. The samples were treated with secondary antibodies, biotinylated anti-rabbit IgG at room temperature for 30 min, and then treated with AlexaFluor 488-conjugated streptavidin and AlexaFluor 594-conjugated anti-mouse IgG at room temperature for 30 min. The fluorescence signals were evaluated by confocal microscopy.

Enzyme immunoassay

Human MoDCs were generated from PBMCs of healthy donors (N=4) and the culture medium was freshly changed. Substance P and Neurokinin A production levels in culture supernatants of MoDCs (2.5×10^5 cells) for 24 h were determined using EIA kits (Substance P: Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA, Neurokinin A: Peninsula Laboratories, San Carlos, CA, USA) according to the manufacture's protocols. Mean and SD of the Substance P and Neurokinin A production levels were calculated, respectively.

Induction of Bet-v1-specific Th cells

Birch pollen-derived antigen, Bet-v1 (GETLLRAVESYLLAHS) helper peptide⁸, was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). PBMCs (2×10^6) were cultured with Bet-v1 peptide ($5 \mu\text{mol/l}$) in AIM-V containing 5% heat-inactivated pooled human AB serum (Hokkaido Red Cross Blood Center) for 7 days and restimulated with mitomycin C -treated autologous MoDCs (1×10^5). Intracellular IFN- γ and IL-4 production in response to the inducing peptide was examined by flow cytometry. T cells (5×10^4) induced by Bet-v1 peptide were cultured with autologous MoDCs (5×10^3) for 20 h. Antigen-specific cytokine production levels in culture supernatants were measured by ELISA (kits manufactured by BD Bioscience, San Jose, CA, USA and eBioscience, San Diego, CA, USA).

Flow cytometry

The fluorescence-conjugated mAbs to IFN- γ , IL-4, CD4, CD8, CD11c, HLA-DR, HLA-A/B/C, CD80, CD86, CD40, CCR7, and the isotype controls for flow cytometry were purchased from Bio Legend (San Diego, CA, USA) or BD Bioscience. MoDCs were stained with 7-amino-actinomycin D (Beckman Coulter Inc., Indianapolis, IN, USA) and fluorescence-conjugated mAbs to examine cell surface molecules.

Antigen-specific Th cells were fixed in BD Cytofix/Cytoperm (BD Bioscience) solution after cell surface staining of CD4, and intracellular staining was performed in BD Perm/Wash buffer using IFN- γ and IL-4 mAbs. Expression levels were evaluated by FACSCanto (BD Bioscience) and results analyzed with Flowjo software (Tree Star Inc., Ashland, OR, USA). Isotype controls are shown as black background.

Statistical analysis

All experiments were repeated independently at least three times. Mean values and standard errors were calculated for flow cytometry and PCR. Mean values and standard deviations were calculated for *in vitro* culture data. Significant differences in the results were evaluated by one-way analysis of variance (ANOVA) and Dunnett's post-test (vs. control group). In some experiments, the two-tailed Student's *t*-test was used for evaluation of the difference between two groups. The *P* values of **P* < 0.05 and ***P* < 0.01 were considered statistically significant.

Study approval

Research protocols in this study of human subjects were approved by the Institutional

105 Review Boards of Hokkaido University Graduate School of Medicine, the Institute for
106 Genetic Medicine, and Japanese Red Cross Medical Center. Written informed
107 consent was obtained from all patients whose samples were collected.

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Figure E1. A, IFN- α and IFN- β expression levels of MoDCs after the stimulation.

MoDCs were generated from PBMCs (N = 4) and stimulated with poly I:C for 24 h.

Gene expression levels of IFN- α and IFN- β were evaluated by RT-PCR.

Representative mean and SD are shown. **B,** Surface expression of HLA and

costimulatory molecules on CD11c⁺ DCs stimulated with poly I:C in the presence of a

STAT1 inhibitor for 24 h. Surface expression levels of HLA-A/B/C, HLA-DR, CD80,

CD86, CD40, and CCR7 on CD11c⁺ DCs from PBMCs were evaluated by flow

cytometry. Representative profiles and mean and SD (N = 4) of the calculated

normalized mean fluorescence intensity (ratio MFI = MFI / respective isotype control)

are shown.

Figure E2. NK1R and NK2R expressions of CD163 positive cells.

Dual-immunofluorescence staining of CD163 (red) and NK1R or NK2R (green) was

performed for the section from a patient with asthma. Representative photos are

shown. Scale bar is 20 μ m for all panels.

Figure E3. Cytokine production of Bet-v1-specific Th cells. White birch antigen-specific CD4⁺ T cells were induced in the presence of Bet-v1 peptide for 2 weeks. IFN- γ and IL-4 productions were evaluated after restimulation with the antigen peptide by flow cytometry. The representative profiles are shown.

Figure E4. Antigen presentation and gene expression of NK1R- or NK2R-knockdown MoDCs. Control siRNA (50 nM, Stealth RNAi siRNA Negative Control Med GC Duplex), NK1R-siRNAs (50 nM, three mixtures of siRNAs designed by Life technologies), or NK2R-siRNAs (50 nM, three mixtures of siRNAs designed by Life technologies) were transfected into MoDCs with Lipofectamine RNAiMAX Reagent (Life technologies) according to the manufactures protocol for 24 h. **A**, White birch antigen-specific CD4⁺ T cells were cocultured with control-, NK1R-, or NK2R-siRNA-transfected MoDCs in the presence of antigen peptides for 24 h. IL-2, IL-4, and IFN- γ production levels in the culture supernatants were evaluated by ELISA. **B**, Control-, NK1R-, or NK2R-siRNA-transfected MoDCs were stimulated with poly I:C for 24 h. Gene expression levels of IFN- α , IFN- β and IL-12p35 in the MoDCs were evaluated by RT-PCR. Representative mean and SD of cytokine and gene levels are

shown.

Figure E5. A, Neuropeptide production by human DCs. MoDCs (N = 4) induced from healthy donors were treated with or without poly I:C for 24 h. Substance P and Neurokinin A production were determined by EIA. Representative mean and SD of the production levels are shown. **B**, Neuropeptide-dependent HLA-class II expression on human DCs. MoDCs were treated with Substance P or Neurokinin antagonist in the presence or absence of the antagonists for 24h. HLA-DR expression levels on MoDCs (N = 4) were evaluated by flow cytometry. Ratio mean fluorescence intensity (Ratio MFI = MFI / respective isotype control) were determined and percentages of HLA-DR expression levels against Substance P (SP) or Neurokinin A (NKA) with SD are shown.

Figure E6. Cytokine production by Bet-v1-specific Th cells in NK1R and NK2R antagonists. White birch antigen-specific CD4⁺ T cells were stimulated by Bet-v1 peptide-pulsed MoDCs in the presence of NK1R and NK2R antagonists for 24 h. IL-2 and IL-4 production levels in the culture supernatants were evaluated by ELISA.

159 Representative mean and SD of cytokine levels are shown.

160

161 **Figure E7.** Gene expression levels of IFN- α , IFN- β and IL-12 in human MoDCs.

162 MoDCs generated from PBMCs (N = 3) were stimulated with poly I:C in the presence

163 of NK1R and NK2R antagonist for 24 h. Gene expression levels of IFN- α , IFN- β and

164 IL-12p35 in MoDCs were evaluated by quantitative RT-PCR. Means and SD of the

165 data are presented.

Table E1. Scores of NK1R and NK2R expressions in lung tissues.

Case number	Underline disease	Age	Gender	NK1R	NK2R
1	Normal	70	F	1+	1+
2	Normal	69	F	1+	1+
3	Normal	51	M	1+	1+
4	Normal	77	M	1+	1+
5	Normal	75	M	1+	1+
6	Normal	52	M	1+	1+
7	Asthma	69	M	2+	1+
8	Asthma	76	F	2+	2+
9	Asthma	65	M	2+	2+
10	Asthma	56	M	2+	2+
11	Asthma	54	F	1+	1+
12	AHP	49	F	2+	2+
13	AHP	69	M	2+	2+
14	AHP	61	M	2+	2+
15	AHP	56	F	2+	2+
16	AHP	53	F	2+	2+
17	AHP	33	F	2+	1+
18	AHP	59	M	2+	2+
19	CHP	53	M	2+	2+
20	CHP	33	M	2+	1+
21	CHP	64	F	2+	2+
22	CHP	58	F	2+	2+
23	CHP	50	F	2+	2+

AHP: Acute hypersensitivity pneumonitis, CHP: Chronic hypersensitive pneumonitis

Table E2. Primers used for PCR.

Gene name	Forward	Reverse	Probe (#)
TACR1	5'-actcctctgaccgctaccac-3'	5'-gtgcacaccacgacaatcat-3'	#53
TACR2	5'-ggaggacatctactgccacaa-3'	5'-ggaatccagagcgaaacct-3'	#42
IFNA	5'-cgggtgcagaatttgtctagga-3'	5'-gcagatcttcaacctctttacca-3'	#84
IFNB	5'-cgacactgttcgtgttgtca-3'	5'-gaagcacaacaggagagcaa-3'	#25
IL12A	5'-tctcttcagaagtgaagggta-3'	5'-cactcccaaacctgctgag-3'	#50
GAPDH	5'-agccacatcgctcagacac-3'	5'-gccaatacgaccaaattcc-3'	#60