**Title**
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**Citation**
Journal of Allergy and Clinical Immunology, 136(6), 1690-1694
https://doi.org/10.1016/j.jaci.2015.06.050

**Issue Date**
2015-12

**Doc URL**
http://hdl.handle.net/2115/67660

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**Type**
article (author version)

**File Information**
JAllergyClin13_1690.pdf

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Neuropeptide signaling through neurokinin-1 and 2 receptors augments antigen presentation by human dendritic cells

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Grant Support: This work was partially supported by a Grant-in-Aid for Scientific Research (25460584) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), and by the Joint Research Program of the Institute for Genetic Medicine, Hokkaido University.
Abstract

Background: Neurotransmitters, including substance P (SP) and neurokinin A (NKA), are widely distributed in both the central and peripheral nervous system 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.

Objective: We sought to examine the effect of neuropeptide signaling through NK1R- and NK2R on antigen presentation by dendritic cells (DCs) and the subsequent activation of effector Th cells.

Methods: Expression levels of NK1R, NK2R, HLA-class II and costimulatory molecules of human MoDCs and cytokine production by birch pollen antigen-specific CD4⁺ T cells cocultured with MoDCs in the presence of NK1R and NK2R antagonists were evaluated by quantitative RT-PCR, flow cytometry or ELISA. NK1R and NK2R expression in the lung of patients with asthma and hypersensitivity pneumonitis was evaluated by immunohistochemistry.

Results: Human MoDCs significantly upregulated NK2R and NK1R expression in response to poly I:C stimulation in a STAT1-dependent manner. Both NK2R and NK1R were expressed on alveolar macrophages and lung DCs from patients with
asthma and pneumonitis hypersensitivity. Surface expression levels of HLA-class II and costimulatory molecules on DCs were modulated by NK1R or NK2R antagonists. Activation of birch pollen-derived antigen-specific CD4\(^+\) T cells and their production of cytokines including IL-4 and IFN-\(\gamma\), as well as IL-12 production by MoDCs, were suppressed by blocking NK1R or NK2R after \textit{in vitro} antigen stimulation.

**Conclusions:** NK1R- and NK2R-mediated neuropeptide signaling promotes both innate and acquired immune responses through activation of human DCs.

**Clinical Implications:** Blockade of the SP-NK1R or NKA-NK2R signaling pathway provides a novel strategy for preventing chronic inflammation, such as severe asthma and infectious disease, caused by excessive type-1 immunity.

**Capsule Summary:** An antagonist of neuropeptide signaling through NK1R or NK2R on human DCs may become a promising drug for patients with severe asthma.

**Key words:** antigen presentation; dendritic cells; hypersensitivity pneumonitis; type-1 interferon; neurokinin A; neurokinin-2 receptor; neuropeptide; STAT1; substance P;
neurokinin-1 receptor; severe asthma

**Disclosures:** The authors have declared that no conflict of interest exists.

**Abbreviations:** AHR, airway hyperresponsiveness, DC, dendritic cell, HLA, human leukocyte antigen, HP, hypersensitivity pneumonitis, MoDC, monocyte-derived DC, NKA, neurokinin A, NK1R, neurokinin-1 receptor, NK2R, neurokinin-2 receptor, PBMC, peripheral blood mononuclear cell, STAT, signal transducers and activator of transcription, SP, substance P, TLR, toll-like receptor
To the Editor:

Asthma, an inflammatory disease of the airways, is associated with various Th and innate immune cells. 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. The precise mechanisms mediating severe asthma and HP have remained to be elucidated.

Neurotransmitters including substance P (SP) and neurokinin A (NKA) are widely distributed in both the central and peripheral nervous system 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 hyperresponsiveness in mouse severe asthma models. 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 STAT-1-dependent manner. Additionally, we confirmed that human DCs significantly upregulated gene expression levels of NK2R and TAC-1, which encoded Substance P and Neurokinin A, after IFN-γ or poly I:C stimulation, suggesting possible roles of neuropeptide signaling in human DCs. 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 Th cells.

Human DCs (MoDCs), generated from peripheral blood mononuclear cells (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 were 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. E1A in the Online Repository). 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). Additionally, we found that both NK2R and NK1R were expressed on CD68 or CD163 positive macrophages and
HLA-DR positive cells including DCs in the lung tissues from patients with asthma and acute and chronic HP (Fig. 1, E, F and see Fig. E2 and Table E1 in the Online Repository). These findings suggest that STAT1 activation by Th1 cytokines, including IFN-γ and type 1 IFNs induced by TLR-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 were related to poly I:C-induced DC maturation, we analyzed the surface expression levels of HLA and costimulatory molecules following treatment with an NK1R or NK2R antagonist. HLA-DR, CD80, CD86, and CD40, but not HLA-A/B/C and CCR7 expressions, upregulated by poly I:C stimulation through STAT1 activation (see Fig. E1B in the Online Repository), 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 Substance P (25.2 ± 9.2 pg/mL) and Neurokinin A (44.6 ± 27.5 pg/mL) were produced in the culture supernatants of MoDCs (2.5×10^5 cells) for 24 h and HLA-class II expression levels on these neuropeptide-treated MoDCs were suppressed in the presence of these antagonists (see Fig. E3 in the Online Repository). 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 Th cells in vitro using birch-derived antigen (see Fig. E4 in the Online Repository), as described previously. T-cell proliferation and cytokine production was 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 Th cells after antigen stimulation by MoDCs. The production of Th1 and Th2 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 and see Fig. E5A and E6 in the Online Repository).

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. It has also been shown that SP-NK1R signaling activates IL-12 secretion by DCs. Thus, neuropeptide signaling through NK2R, as well as NK1R, promotes MoDC activation by antigen-specific Th 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. E5B and E7 in the Online Repository). Notably, the combination of NK1R and NK2R antagonists synergistically inhibited cytokine production (see Fig. E7 in the Online Repository).

Taken together, these findings indicate that NK1R- or NK2R-dependent neuropeptide signaling activates antigen-specific Th 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/Th-mediated airway inflammation. Therefore, these findings may provide potential insights into the 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 (AHR) and improved lung function. 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. 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 neuro-immune crosstalk may be associated with various diseases, including infection and chronic inflammation such as severe asthma and HP.

We thank Ms. A. Nishiuchi for her excellent technical and secretarial assistance and Dr. S. Ashino for thoughtful advice on this study.

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References


Figure legends

Figure 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 h. 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 and patient with asthma or HP. Scale bar is 200 µm for all panels. F, Dual-immunofluorescence staining of CD68 (red) and NK1R or NK2R (green) were performed for the section from a patient with asthma or HP. Scale bar is 20 µm for all panels.

Figure 2. A, DCs (N = 6) were treated with poly I:C in the presence of NK1R or NK2R
antagonists 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 = 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 Th cells were activated by peptide-pulsed MoDCs in the presence of an NK1R or NK2R antagonist for 24 h. Antigen-specific Th 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 h. IL-2, IL-4, IL-5, IL-13, IFN-γ and IL-12 concentration in culture supernatants were evaluated by ELISA. Three experiments were performed independently, Mean and SD of the representative data are shown.
METHODS

Generation of human MoDCs from PBMCs

PBMCs were obtained from healthy individuals by Ficoll-Hypaque (Amersham Bioscience, GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) gradient centrifugation. Adherent PBMCs (2 x 10^6 cells) were cultured in AIM-V media (Life Technologies, Carlsbad, CA, USA) in the presence of recombinant human IL-4 (50 ng/mL, Wako Chemical Industries, Ltd., Osaka, Japan) and GM-CSF (50 ng/mL, Wako Chemical Industries, Ltd., Osaka, Japan) for 7 days as described previously.\(^4\) Purity of the induced CD11c\(^+\)HLA-DR\(^+\)HLA-A/B/C\(^+\) cells were more than 95 %. MoDCs were treated with DMSO (control), IFN-\(\alpha\) (1000 U/mL; Peprotech Inc., Rocky Hill, NJ, USA), IFN-\(\beta\) (1000 U/mL; Peprotech Inc.), IFN-\(\gamma\) (50 ng/mL; Peptidech Inc.), LPS (20 ng/mL; Sigma-Aldrich Co., St Louis, MO, USA), or poly I:C (10 \(\mu\)g/mL; InvivoGen, San Diego, CA, USA) for 24 h and used for RT-PCR experiments. In some experiments, MoDCs were stimulated with poly I:C (10 \(\mu\)g/mL) in the presence of a STAT1 inhibitor (Fludarabine, 2 \(\mu\)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 \( \mu \)M; Tocris Bioscience, Bristol, UK), and/or NK2R antagonist (GR 159897, 10 \( \mu \)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-\( \alpha \) (IFNA), IFN-\( \beta \) (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).
Immunohistochemistry

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

Immunofluorescence staining

Dual-color immunofluorescence staining was performed for lung tissues of patients with asthma or HP. The sections were incubated with primary antibodies for (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 peptide, was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). PBMCs (2 × 10^6) were cultured with Bet-v1 peptide (5 µmol/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
Review Boards of Hokkaido University Graduate School of Medicine, the Institute for Genetic Medicine, and Japanese Red Cross Medical Center. Written informed consent was obtained from all patients whose samples were collected.
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 PT-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 µm 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-\(\gamma\) 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-\(\gamma\) 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-\(\alpha\), IFN-\(\beta\) 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.
Representative mean and SD of cytokine levels are shown.

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

MoDCs generated from PBMCs (N = 3) were stimulated with poly I:C in the presence of NK1R and NK2R antagonist for 24 h. Gene expression levels of IFN-α, IFN-β and IL-12p35 in MoDCs were evaluated by quantitative RT-PCR. Means and SD of the data are presented.
Table E1. Scores of NK1R and NK2R expressions in lung tissues.

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AHP: Acute hypersensitivity pneumonitis, CHP: Chronic hypersensitive pneumonitis
Table E2. Primers used for PCR.

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<th>Gene name</th>
<th>Forward</th>
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<th>Probe (#)</th>
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Figure E1.

A

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<tr>
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<td>poly I:C</td>
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Relative gene expression

B

- Control
- poly I:C
- poly I:C + STAT1 inhibitor

**% of MAX**

- HLA-ABC
- HLA-DR
- CD80
- CD86
- CD40
- CCR7

**Ratio MFI**

- Control
- poly I:C
- poly I:C + STAT1 inhibitor
Figure E2.
Figure E3.

The bar graphs show the HLA-DR expression level (% of control) for SP, SP + NK1R antagonist, NKA, and NKA + NK2R antagonist. The graphs indicate a significant decrease in HLA-DR expression level in the presence of NK1R and NK2R antagonists compared to their respective controls.
Figure E4.
Figure E6.

The figure shows the IL-2 and IL-4 production levels in response to different treatments. The x-axis represents the treatments: None, Bet-v1, + NK1R antagonist, + NK2R antagonist, + NK1R + NK2R antagonists. The y-axes represent the production levels in pg/mL for IL-2 and IL-4.

Statistical significance is indicated by asterisks above the bars. The error bars represent the standard deviation.
Figure E7.

The figure shows bar graphs representing the relative gene expression levels of IFN-α, IFN-β, and IL-12p35 under different conditions.

- **IFN-α**: Graphs show bars for 'None', 'poly I:C', '+ NK1R antagonist', '+ NK2R antagonist', and '+ NK1R + NK2R antagonists'. Significant differences are indicated by stars: * and **.

- **IFN-β**: Similar conditions as IFN-α, with significant differences noted by * and **.

- **IL-12p35**: Graphs for the same conditions as above, with significant differences indicated by * and **.

The y-axis represents the relative gene expression level (% of poly I:C). The x-axis lists the conditions: 'None', 'poly I:C', '+ NK1R antagonist', '+ NK2R antagonist', and '+ NK1R + NK2R antagonists'.