

Interleukin-22 attenuates double-stranded RNA-induced upregulation of PD-L1 in airway epithelial cells via a STAT3-dependent mechanism

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Interleukin-22 attenuates double-stranded RNA-induced upregulation of PD-L1 in airway epithelial cells via a STAT3-dependent mechanism



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ABSTRACT

Double-stranded RNA derived from viruses induces host immune responses. PD-L1, also known as B7-H1, is an immune-checkpoint molecule associated with the escape of viruses from host immune systems, which plays a role in the persistence of viral infection, resulting in exacerbations of underlying diseases such as asthma and chronic obstructive pulmonary disease. Interleukin (IL)-22 is produced from various immune cells and has protective properties on mucosal tissue. The binding of IL-22 to IL-22 receptor induces STAT3 activation. We investigated the effect of IL-22 on the expression in airway epithelial cells *in vitro* and in mouse lungs *in vivo* after the stimulation with an analog of viral double-stranded RNA, polyinosinic-polycytidylic acid (poly I:C).

Stimulation with poly I:C upregulated PD-L1 expression on BEAS-2B cells. This upregulation of PD-L1 was attenuated by IL-22 administration. STAT3 phosphorylation was induced by IL-22 and poly I:C. Treatment of cells with STAT3 siRNA abolished the effect of IL-22 on the poly I:C-induced upregulation of PD-L1. This upregulation of PD-L1 was also attenuated by IL-11, a cytokine inducing STAT3 phosphorylation, in BEAS-2B cells. In mouse lung cells *in vivo*, IL-22 suppressed poly I:C-induced upregulation of PD-L1.

These results suggest that IL-22 attenuates virus-induced upregulation of PD-L1 in airway epithelial cells via a STAT3-dependent mechanism.

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1. Introduction

Viral infections in the airways frequently provoke acute exacerbation of asthma and chronic obstructive pulmonary disease (COPD) [1–3]. This pathophysiological process involves the

activation of innate immune responses triggered by a variety of pathogen-associated molecular patterns (PAMPs) including double-stranded (ds) RNA. Influenza virus has a unique triphosphate-ended dsRNA-like structure at the 5'terminal of genomic single-stranded RNA. Rhinovirus, respiratory syncytial virus (RSV), and human metapneumovirus are known to synthesize dsRNA for their replication in the host cells. These dsRNAs activate innate immune responses through their binding to Toll-like receptor 3 (TLR3) and the family of RNA helicase, namely, the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (Mda5).

The airway epithelial cells are targeted by viruses for their replication. Virus-infected epithelial cells express virus-associated antigens on the major histocompatibility complex (MHC) class I, which is recognized by virus-specific cytotoxic T lymphocytes (CTLs). The activated CTLs lyse the infected cells or induce cell apoptosis [4]. On the other hand, viral species have developed escape mechanisms from host immune responses. One of the

Abbreviations: dsRNA, double-stranded RNA; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; STAT, signal transducer and activator of transcription; poly I:C, polyinosinic-polycytidylic acid; CTLs, cytotoxic T lymphocytes; siRNA, short interfering RNA; BAL, bronchoalveolar lavage.

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escape mechanisms may be the PD-L1/PD-1 axis [5]. PD-L1, also known as B7-H1, belongs to the B7 family and shares its receptor, programmed death-1 (PD-1), with PD-L2, also known as B7-DC. PD-1 is induced in CTLs during their activation, and its ligation to PD-L1 or PD-L2 induces functional exhaustion and then failure of effective virus eradication [6]. We and other investigators reported that polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of viral dsRNA, upregulates the expression of PD-L1 in cultured human airway epithelial cells [7–9].

IL-22 is a member of the IL-10 cytokine family and is produced by various immune cells, including T helper 1 (T_H1) cells, T_H17 cells, T_H2 cells, innate lymphoid cells (ILCs), and natural killer cells [10]. IL-22 contributes to epithelial protection and tissue repair by maintaining lymphoid tissue integrity, decreasing the influx of inflammatory cells, and enhancing the migration and proliferation of epithelial cells [11–13]. Recent studies showed that IL-22 plays various protective roles in mucosal injury caused by airway viral infections [14–18]. The IL-22 receptor is a heterodimeric complex composed of IL-22R1 and IL-10R2 subunits [19]. IL-22 binding to the IL-22 receptor complex leads to activation of the receptor-associated Janus kinases (JAK)1 and tyrosine kinase 2, followed by activation of the signal transducer and activator of transcription (STAT)3 and often STAT1 and/or STAT5 [20]. The STAT molecules are phosphorylated to form homodimers that translocate into the nucleus to induce the expression of specific genes and therefore modulate the cell activities. This raises the possibility that the IL-22/IL-22Receptor/STATs axis may play a regulatory role in virus-induced responses in the airways. In the present study, we investigated the effect of IL-22 on dsRNA-induced upregulation of PD-L1 *in vitro* and *in vivo* and then elucidated the underlying mechanisms.

2. Materials and methods

2.1. Culture of airway epithelial cells

The BEAS-2B cell line, derived from human bronchial epithelium transformed by an adenovirus 12-SV-40 virus, was cultured in six-well plates by using DMEM/F12 containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 ng/ml) at 37 °C with 5% CO₂ in humidified air.

2.2. Treatment of cells with dsRNA, IL-11, and IL-22

When cells reached semi-confluence, they were stimulated by the administration of 0.3–100 µg/ml poly I:C in the culture medium. In several experiments, the cells were treated with 10 or 100 ng/ml recombinant human IL-22, recombinant human IL-11 or vehicle alone, simultaneously with poly I:C stimulation.

2.3. Apoptosis assay

The apoptosis of cultured epithelial cells was assessed via flow cytometric analysis using Annexin V binding in combination with propidium iodide staining, as previously reported [21].

2.4. Mice

Male C57BL/6 mice (5–7 weeks old) were purchased from SLC Japan (Kurume, Japan) and were housed under specific pathogen-free conditions until the experiments commenced.

2.5. *In vivo* experimental protocols

Mice were anesthetized with 80 µg/kg dexmedetomidine intraperitoneal (i.p.) injection and subsequently by inhalation of

3–4% isoflurane. A temporary tracheal cannulation was performed with a 20-gauge catheter. Recombinant murine IL-22 was dissolved in phosphate-buffered saline (PBS). Poly I:C was dissolved in saline before use. Mice received intratracheal (i.t.) instillation of recombinant murine IL-22 (3 µg/50 µl) or PBS alone. Three hours after the administration of IL-22, mice received i.t. instillation of poly I:C (30 µg/50 µl) or saline alone. Sham-treated mice were used as controls. Mice were sacrificed under a mixture of ketamine and sodium pentobarbital i.p. injection 24 h after administration of poly I:C. Their tracheas were cannulated via tracheostomy for collecting bronchoalveolar lavage (BAL) and then the lungs were removed for flow cytometric analysis. All experimental procedures were approved by the animal research ethics committee of Kyushu University (reference numbers: A23-048-1 and A25-007-0).

2.6. Flow cytometric analysis

In the analysis for cultured epithelial cells, 5×10^6 cells were incubated in 100 µl of PBS/0.5% BSA/0.02% NaN₃ containing biotinylated anti-human PD-L1 mAb at room temperature for 30 min. The samples were thoroughly washed and suspended in SA-PE conjugate for 20 min. After washing, the cells were fixed with 4% paraformaldehyde for 20 min. Fixed cells were washed again and then processed for flow cytometric analysis.

In the analysis for murine lung cells, Cells were pre-incubated with anti-mouse CD16/CD32 mAb to prevent nonspecific binding via FcγR and then incubated with FITC-conjugated anti-keratin 5/8 mAb, followed by the addition of biotinylated anti-mouse PD-L1 mAb. Other methodological details are described in the [Supplementary Methods](#).

Flow cytometric analysis was performed using a FACSCalibur flow cytometer with CELLQuest software (BD Biosciences, CA, USA). Ten to thirty thousand events were acquired in a list mode with debris excluded by the forward-scatter threshold. The mean fluorescence intensity (MFI) was compared with control staining using an irrelevant isotype-matched mouse mAb.

2.7. Bronchoalveolar lavage

Both lungs were gently lavaged with 1 ml of 0.9% saline via the tracheal cannula. Methods to count cells in BAL are described in the [Supplementary Methods](#).

2.8. Western blot analysis

Whole-cell protein lysates were prepared from the cell pellets for the analysis of human phosphorylated (p)STAT1, STAT1, pSTAT3, STAT3, pSTAT5, STAT5 and β-actin. Other methodological details are described in the [Supplementary Methods](#).

2.9. RNA interference

Cells were plated at 50–60% confluence in 6-well plates and then incubated for 24 h before transient transfection for 48 h with siRNA mixed with Lipofectamine reagent. The siRNA specific for STAT3 mRNA (5'-UCAUUGACCUUGUGAAAAA-3') as well as a nonspecific siRNA (5'-GUUGAGAGAUUUAGAGUU-3') was obtained from Nippon EGT.

2.10. Reagents

Reagents used in experiments are described in the [Supplementary Methods](#).

2.11. Statistical analyses

Unless otherwise stated, values are expressed as mean \pm SEM. Unpaired *t*-tests were used for comparison of two sets of data. When comparing more than two sets of data analyses were conducted using one-way ANOVA. All data analyses were conducted using JMP (SAS Institute Inc., Tokyo, Japan). Results were considered statistically significant if $p < 0.05$.

3. Results

3.1. IL-22 suppresses poly I:C-induced upregulation of PD-L1 on airway epithelial cells

The effect of poly I:C on the expression of PD-L1 was investigated in BEAS-2B cells. Unstimulated BEAS-2B cells expressed a low level of PD-L1. The expression of PD-L1 was significantly upregulated by treatment with poly I:C in a dose-dependent manner (Fig. 1A) and reached its peak from 24 to 48 h after the stimulation with poly I:C, then gradually attenuated (Fig. 1B). The proportion of apoptotic cells was within 20% until 48 h after the stimulation with poly I:C (Fig. 1C). Taking into account the sufficient upregulation of PD-L1 and minimizing the influence on apoptosis, the time point at 24 h after stimulation with poly I:C at a concentration of 10 μ g/ml was chosen for outcome measurements

in subsequent experiments.

To evaluate the effect of IL-22 on the upregulation of PD-L1 by poly I:C, BEAS-2B cells were treated with recombinant human IL-22 prior to administration of poly I:C and stimulated with poly I:C for 24 h. The expression of PD-L1 was not affected by IL-22 treatment alone. Poly I:C-induced upregulation of PD-L1 was suppressed by IL-22 in a dose-dependent manner (Fig. 1D). Thus, the expression levels of PD-L1 in the cells treated with poly I:C and IL-22 were significantly lower than in those treated with poly I:C alone after 24 h of incubation.

3.2. STAT3 phosphorylation is induced by IL-22 and poly I:C

STAT3 is binding to the IL-22 subunit 1 (IL-22R1), which is a component of the heterodimeric IL-22R complex. It is known that STAT1 and STAT5 can also be involved, but this is cell-type dependent [22]. BEAS-2B cells were treated with recombinant human IL-22 or poly I:C for 30, 60, and 120 min. STAT3 phosphorylation was induced in the cells treated with IL-22 at 30 min, while it was induced at 120 min after stimulation with poly I:C (Fig. 2A). STAT1 phosphorylation was induced by treatment with poly I:C, which was gradually enhanced over time from 30 to 120 min, but was not induced by IL-22 alone. STAT5 phosphorylation was not detectable in the cells treated with IL-22 or poly I:C (Fig. 2A).

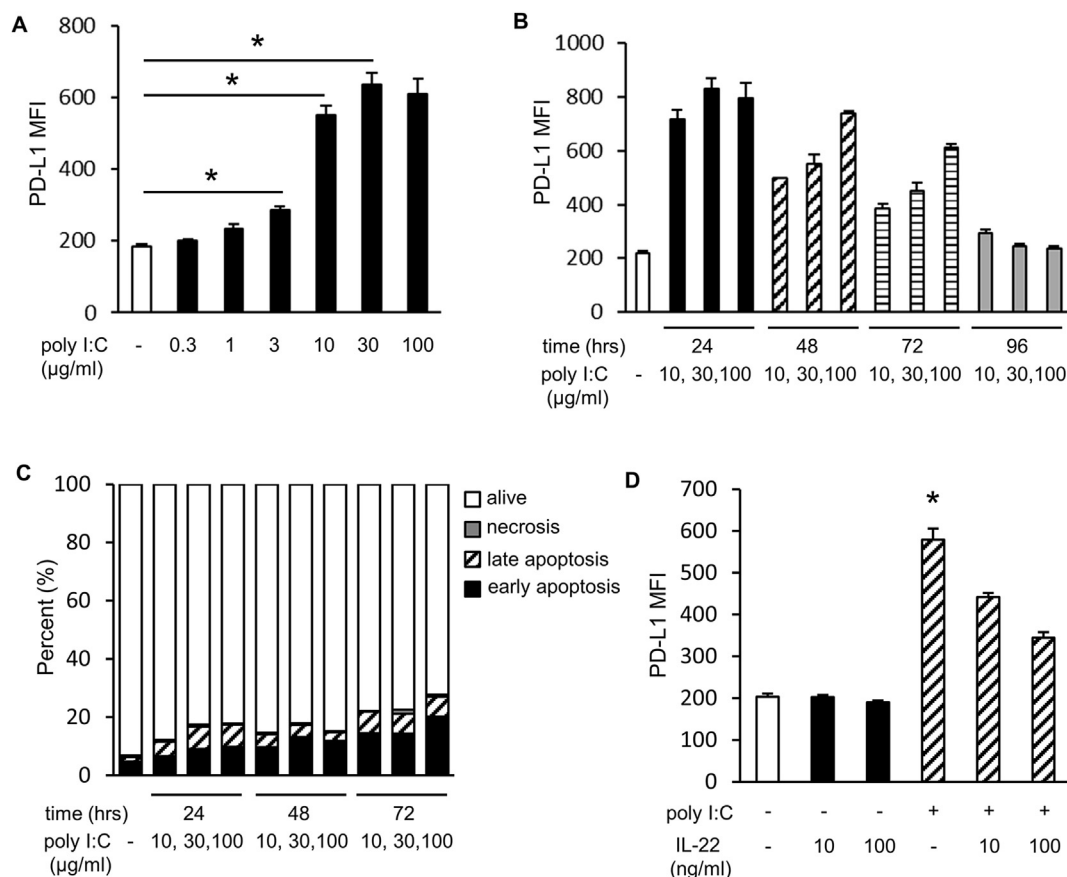


Fig. 1. Effect of IL-22 on the poly I:C-induced upregulation of PD-L1 on BEAS-2B cells. (A) Cells were stimulated with poly I:C at the indicated concentration (0.3–100 μ g/ml) for 24 h. The expression of PD-L1 was analyzed by flow cytometry. (B) Cells were stimulated with poly I:C at the indicated concentration (10–100 μ g/ml) for 24, 48, 72 and 96 h. The expression of PD-L1 was analyzed by flow cytometry. (C) Cells were stimulated with poly I:C at the indicated concentration (10–100 μ g/ml) for 24, 48 and 72 h. Apoptotic or necrotic cells were identified by flow cytometry. (D) Cells were treated with 10 or 100 ng/ml recombinant human IL-22 or vehicle 20 min prior to administration of poly I:C, and then stimulated with 10 μ g/ml poly I:C for 24 h. The expression of PD-L1 was analyzed by flow cytometry. MFI, mean fluorescence intensity. Data are expressed as mean \pm SEM. $n = 6-9$. * $p < 0.05$. Analyses were conducted using one-way ANOVA.

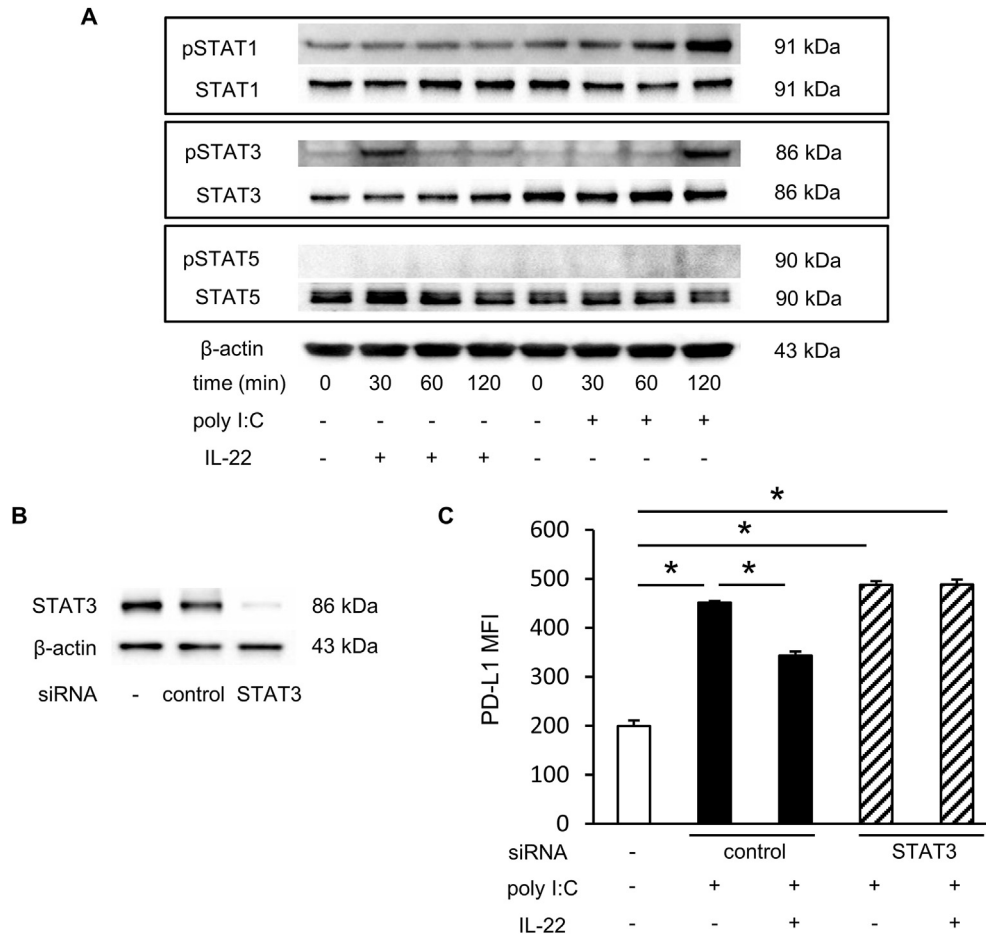


Fig. 2. The suppression of poly I:C-induced PD-L1 upregulation by IL-22 via STAT3 in BEAS-2B cells. (A) After the treatment of 10 μ g/ml poly I:C or 100 ng/ml recombinant human IL-22 for indicated minutes, total cell lysates were assayed for phosphorylated (p)STAT1, STAT1, pSTAT3, STAT3, pSTAT5, STAT5 and β -actin using Western blot analysis. The data is representative of three independent experiments. (B) Cells were transfected with STAT3 siRNA (20 nM/well), non-specific (control) siRNA, or vehicle alone for 48 h. After transfection, total cell lysates were assayed for STAT3 and β -actin using Western blot analysis. (C) After transfection with STAT3 siRNA or control siRNA for 48 h, cells were treated with 100 ng/ml recombinant human IL-22 or vehicle 20 min prior to administration of poly I:C, and then stimulated with 10 μ g/ml poly I:C for 24 h. The expression of PD-L1 was evaluated using flow cytometric analysis. MFI, mean fluorescence intensity. Data are expressed as mean \pm SEM. $n = 9-12$. * $p < 0.05$. Analyses were conducted using one-way ANOVA.

3.3. IL-22 suppresses poly I:C-induced upregulation of PD-L1 via activation of STAT3

To investigate the relevance of STAT3 activation, cells were transfected with STAT3 small interfering RNA (siRNA) or non-targeting control siRNA. Efficient knockdown of STAT3 is shown in Fig. 2B. In the cells transfected with control siRNA, poly I:C-induced upregulation of PD-L1 was significantly attenuated by IL-22 (Fig. 2C). In the cells transfected with STAT3 siRNA, poly I:C-induced upregulation of PD-L1 was not attenuated by IL-22 (data not shown).

3.4. IL-11 also suppresses poly I:C-induced upregulation of PD-L1

Next, we investigated whether IL-11, which also induces STAT3 activation, suppresses poly I:C-induced upregulation of PD-L1. STAT3 phosphorylation was induced by treatment with IL-11 in BEAS-2B cells (Fig. 3A). In the cells treated with poly I:C in the presence of IL-11, the expression level of PD-L1 was significantly lower than in the cells treated with poly I:C alone (Fig. 3B), suggesting attenuation of poly I:C-induced upregulation of PD-L1 via the activation of STAT3.

3.5. IL-22 attenuates poly I:C-induced upregulation of PD-L1 in mouse lungs

The role of IL-22 in PD-L1 expression in the airways *in vivo* was investigated for using an animal model. The expression of PD-L1 was not affected by i.t. administration with IL-22 (Fig. 4A). The expression of PD-L1 on lung cells was significantly upregulated by the i.t. administration of poly I:C compared to the sham-treated control. The expression levels of PD-L1 in poly I:C plus IL-22-administrated mice were significantly lower than those in poly I:C-treated mice (Fig. 4A). Cell profiles in BAL fluid were not affected by IL-22 administration. Poly I:C administration increased markedly the numbers of macrophages and neutrophils in BAL fluid compared to the sham-treated control, which was not affected by treatment with IL-22 (Fig. 4B).

4. Discussion

In the present study using an airway epithelial cell line and an animal model, IL-22 administration attenuated the upregulation of PD-L1 induced by poly I:C, a synthetic analogue of viral dsRNA. Given that the upregulation of PD-L1 in virus-infected epithelial

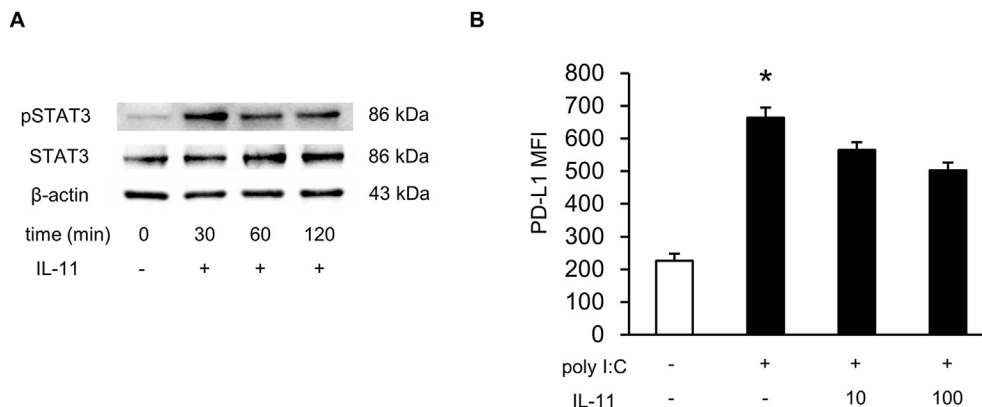


Fig. 3. Effect of IL-11 on STAT3 phosphorylation and poly I:C-induced PD-L1 upregulation in BEAS-2B cells. (A) After the treatment of recombinant human IL-11 (100 ng/ml) for indicated minutes, total cell lysates were assayed for phosphorylated STAT3, STAT3 and β -actin using Western blot analysis. The data is representative of three independent experiments. (B) Cells were treated with 10 or 100 ng/ml recombinant human IL-11 or vehicle 20 min prior to administration of poly I:C, and then stimulated with 10 μ g/ml poly I:C for 24 h. The expression of PD-L1 was analyzed by flow cytometry. MFI, mean fluorescence intensity. Data are expressed as mean \pm SEM. $n = 6$. * $p < 0.05$. Analyses were conducted using one-way ANOVA.

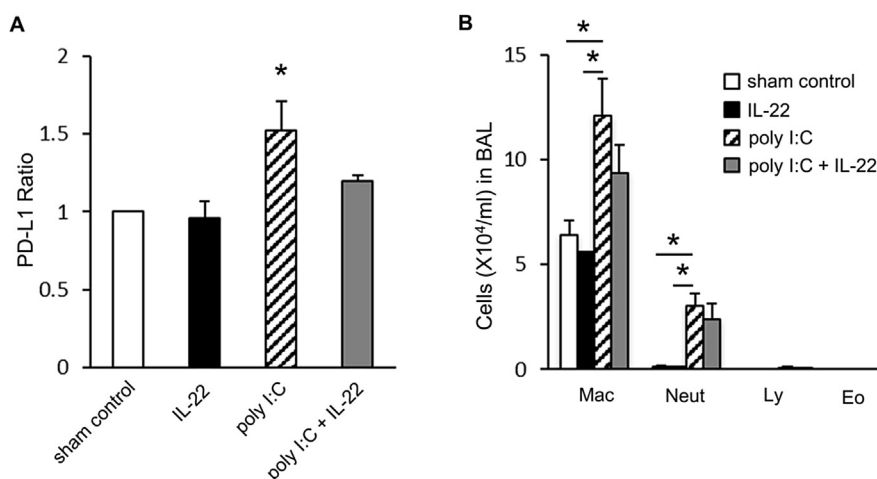


Fig. 4. Effect of IL-22 on the poly I:C-induced upregulation of PD-L1 and airway inflammation *in vivo*. Mice were treated with intratracheal administration of recombinant IL-22 (3 μ g/50 μ l PBS) or PBS alone, which was followed by intratracheal administration of poly I:C (30 μ g/50 μ l saline) or saline alone 3 h after treatment with IL-22. (A) Lung cells were collected 24 h after administration of poly I:C and then processed for flow cytometric analysis to evaluate the expression of PD-L1. (B) Differential cell counts in BAL fluids were analyzed by cytopsin. Mac, macrophage; Neut, neutrophil; Ly, lymphocyte; Eo, eosinophil. Data are expressed as mean \pm SEM and representative of three experiments ($n = 4-5$ mice/group). * $p < 0.05$. Analyses were conducted using an unpaired *t*-test.

cells renders the escape of virus from the attack by CTLs, IL-22 may restore antiviral immunity partly by suppressing the PD-L1/PD-1 axis. Protective properties of IL-22 have been shown against mucosal injury caused by viral infections. IL-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection and protects against lung epithelial damage [14,15]. IL-22-deficient mice showed increased lung injury, decreased epithelial metaplasia, and altered expression of epithelial repair-associated genes following influenza A virus infection [16]. Another study showed that endogenous IL-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection [18]. In addition, the influenza virus promotes IL-22 receptor expression in human airway epithelial cells [17]. It was reported that the serum levels of IL-22 were higher in patients with severe asthma than in patients with mild asthma and healthy control subjects [23]. The levels of IL-22 in serum and sputum were higher in patients with COPD and healthy smokers than in non-smokers [24]. These reports imply that increased IL-22 may have particular relevance to the virus-induced exacerbation of underlying diseases such as asthma and COPD.

The expression of PD-L1 is regulated by multiple mechanisms. Much knowledge has been obtained from tumor cell biology because the PD-L1 on tumor cells is regarded as a promising target for novel immunotherapy [6]. These investigations have shown that the expression of PD-L1 is closely associated with the intracellular signaling pathways directed at cell survival and proliferation, the PI3 kinase/Akt pathway and MAPK pathways, respectively. We recently showed that oncogenic drivers, including the mutant EGFR and the EML4-ALK, induce immune escape in non-small cell lung cancer by upregulating PD-L1 [25,26]. We also showed that dsRNA-induced upregulation of PD-L1 in airway epithelial cells is mediated by the activation of pathways for PI3 kinase/Akt and extracellular signal-regulated kinase (ERK) but not p38MAPK or the c-Jun N-terminal kinase (JNK) [27]. IL-22 is reported to activate ERK, JNK, and p38 MAPK and the JNK pathways in a rat hepatoma cell line [28]. In a preliminary study, treatment of BEAS-2B cells with IL-22 induces the phosphorylation of ERK and p38MAPK but not JNK, whereas treatment with poly I:C induces the phosphorylation of all three types of MAPKs (unpublished observation). With regard to cytokine signaling, T_H1 cytokine, IFN- γ , and T_H2 cytokines, IL-4 and

IL-13, and their putative receptors may be representative axes that mediate PD-L1 expression in various immune cells, tissue structural cells, and tumor cells, via downstream activation of STAT1 and STAT6, respectively [9,29,30].

In the present study, IL-22 attenuated poly I:C-induced upregulation of PD-L1 via the activation of STAT3. IL-11, another cytokine that induces STAT3 activation, also showed a modest but significant attenuation of the poly I:C-induced upregulation of PD-L1. These results are inconsistent with previous reports that STAT3 activation induced PD-L1 expression in immune cells and tumor cells [31–34]. In our recent study, PD-L1 expression in *EML4-ALK* fusion-positive lung cancer cells was not affected by STAT3 inhibition despite the fact that the *EML4-ALK* tyrosine kinase activated STAT3 [25]. Of note, knockdown of STAT3 did not suppress but rather augmented the expression of PD-L1 in BEAS-2B cells treated with poly I:C alone (Fig. 2C). If STAT3 activation is essential for PD-L1 expression, knockdown of STAT3 would show marked suppression on poly I:C-induced upregulation of PD-L1. The expression of PD-L1 might be modulated via different STAT3 signaling pathways in a manner dependent on types of cells and/or stimuli. Elucidation of the underlying mechanisms awaits further investigation.

In summary, our findings suggest that IL-22 attenuates double-stranded RNA-induced upregulation of PD-L1 in airway epithelial cells via STAT3-dependent mechanism. IL-22 may restore antiviral immunity partly by suppressing PD-L1 expression.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2017.10.045>.

Transparency document

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