Adiponectin inhibits the production of TNF-α, IL-6 and chemokines by human lung macrophages

Short running title: Adiponectin and human lung macrophages

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Word count: 3982/4000

Acknowledgments

The authors thank David Fraser D.Phil (Biotech Communication SARL. Ploudalmezeau, France) for medical writing support.

Funding statement: The work has been funded by grant from Cardif Assistance. The funding source had no involvement in study design, data analysis nor report writing.

Conflict of Interest Statement: The authors declare having no competing interests

Authors contribution

Study conception: H.S., P.D., S.G.D,

Experiments and data analysis: H.S., S.G.D., E.N., M.B., C.A., T.V., P.D

Interpretation of experimental results: H.S, P.D.,

Drafting of the manuscript: H.S., L.J.C., P.D.

Data availability statement: Data are available upon request to the corresponding author

Ethics approval: Approval for experiments on human lung tissue was obtained from the regional investigational review board (Comité de Protection des Personnes Île de France VIII, Boulogne-Billancourt, France).

Bullet point summary

What is already known ?

- Obesity leads to an over-risk of severe respiratory infections and asthma.
- A fall in circulating level of adiponectin is associated with obesity-related inflammatory diseases.

What this study adds ?

- The release of adiponectin by human lung explants is negatively correlated with the donor's body mass index
- Human adiponectin expressed in *E. coli* and a synthetic adiponectin receptor agonist (AdipoRon) inhibit inflammatory cytokine production by human lung macrophages

Clinical significance ?

- The adiponectin receptors may constitute a novel therapeutic target for controlling airways inflammation.

55**ABSTRACT**

56**Background and purpose**: Obesity is associated with an elevated risk of severe respiratory 57infections and inflammatory lung diseases. The objectives were to investigate (i) the 58production of adiponectin by human lung explants, (ii) the expression of the adiponectin 59receptors AdipoR1 and AdipoR2 by human lung macrophages (LMs), and (iii) the impact of 60recombinant human adiponectin and a small-molecule APN receptor agonist (AdipoRon) on 61LMs activation.

62**Experimental approach**: Human parenchyma explants and LMs were isolated from patients 63operated for carcinoma. The LMs were cultured with recombinant adiponectin or AdipoRon 64and stimulated with LPS (10 ng.mL^{-1}), poly(I:C) (10 μg.mL^{-1}) or interleukin (IL)-4 (10 ng.mL^{-} 65^{1}) for 24 h. Cytokines or adiponectin, released by explants or LMs, were measured using 66ELISAs. The mRNA levels of AdipoR1 and AdipoR2 were determined using real-time 67quantitative PCR. AdipoRs expression was also assessed with confocal microscopy.

68**Key results**: Adiponectin was released by lung explants at a level negatively correlated with 69the donor's body mass index. AdipoR1 and AdipoR2 were both expressed in LMs. 70Adiponectin (3-30 μg.mL^{-1}) and AdipoRon (25-50 μM) markedly inhibited the LPS- and 71poly(I:C)-induced release of Tumor Necrosis Factor-α, IL-6 and chemokines (CCL3, CCL4, 72CCL5, CXCL1, CXCL8, CXCL10) and the IL-4-induced release of chemokines (CCL13, 73CCL17, CCL22) in a concentration-dependent manner. Recombinant adiponectin produced in 74mammalian cells (lacking low molecular weight isoforms) had no effects on LMs.

75**Conclusions and implications:** The low-molecular-weight isoforms of adiponectin and 76AdipoRon have an anti-inflammatory activity in the lung environment. Targeting adiponectin 77receptors may constitute a new means of controlling airways inflammation.

78**KEYWORDS**: adiponectin, obesity, human lung macrophages, cytokine, AdipoRon, LPS, 79Poly(I:C)

ABBREVIATIONS

AdipoR: adiponectin receptor

APN: adiponectin

BMI: body mass index

FEV1: forced expiratory volume in one second

FVC: forced vital capacity

IL: Interleukin

LM: lung macrophage

MDM: monocyte-derived macrophage

TLR: toll-like receptor

Introduction

In a worldwide analysis in 2016, it was estimated that about 671 million adults and 124 million children over the age of four were obese, and that a further 1.3 billion adults and 213 million children over the age of four were overweight (NCD Risk Factor Collaboration, 2017).

Overweight and obesity are associated with an increased risk of noncommunicable diseases such as diabetes mellitus and cardiovascular disease (GBD 2015 Obesity Collaborators et al., 2017). Obesity also constitutes a risk factor for respiratory infections such as pneumonia, severe influenza and severe coronavirus disease 2019 (Baik et al., 2000; Morgan et al., 2010; Salvator et al., 2011; Van Kerkhove et al., 2011; Kass et al., 2020; Lighter et al., 2020). Epidemiological studies have demonstrated that (i) asthma is more likely to occur in obese individuals, and (ii) obese people with asthma experience more severe symptoms, worse quality of life, and increased healthcare use (Sutherland, 2014).

Fat tissue acts as an endocrine organ by releasing various bioactive substances referred to collectively as adipokines (Ouchi et al., 2011; Leal and Mafra, 2013). Adiponectin (APN, the most abundant adipokine) is known to deactivate proinflammatory pathways (Ouchi et al., 2011; Ohashi et al., 2014). In lean individuals, APN is present in the circulation at high concentrations (~µg/ml) in various oligomeric states. However, circulating levels of APN are lower in obese individuals, and this fall is thought to contribute to obesity-related inflammatory diseases (Arita et al., 1999; Ouchi et al., 2011).

A growing body of evidence suggests that APN modulates macrophage function. Macrophages can be committed to a continuum of functional phenotypes. The two extremes of the spectrum are the phenotypes often referred as either M1 or M2. Stimulation of toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) or of TLR3 by poly(I:C) to mimic a bacterial or a viral infection respectively, leads to a classical macrophage activation state (M1) with the

production of cytokines (such as tumor necrosis factor-α (TNF-α) and IL-6) and a particular subset of CC and CXC chemokines. The Th2 cytokines (IL-4, IL-13) generate an alternative macrophage activation state (M2) in which a different subset of chemokines is produced (Murray et al., 2014; Abrial et al., 2015; Shapouri-Moghaddam et al., 2018).

Adiponectin's effect on cytokine production by monocytes-macrophages has been examined in nonstimulated or LPS-stimulated preparations. In LPS and interferon-γ (M1)-activated murine bone-marrow-derived macrophages, APN exerted a proinflammatory effect by substantially elevating the expression of TNF-α, IL-6, and IL-12 levels (van Stijn et al., 2015). In human monocyte-derived macrophages (MDMs), the results of transcriptional profiling experiments indicated that APN promotes a pro-inflammatory phenotype which resembles M1 more than it does M2 (Cheng et al., 2012). In contrast to the latter study on unstimulated MDM, experiments in porcine or human MDMs have shown that APN abrogates the LPS-stimulated expression or production of IL-6, TNF-α, CCL2 and CXCL10 (Yokota et al., 2000; Wulster-Radcliffe et al., 2004; Okamoto et al., 2008; Ohashi et al., 2010). At present, however, little is known about APN's actions on lung macrophages. Mouse alveolar macrophages express the two main, specific APN receptors (AdipoR1 and AdipoR2) and inhibits the LPS-mediated expression or release of TNF-α and CCL2 (Summer et al., 2008; Ohashi et al., 2010). Mouse macrophages and human MDMs are both surrogate cell models that do not adequately recapitulate the biology of human primary lung macrophages (LMs). Adiponectin's known role in obesity-related pulmonary inflammation and diametrically opposing effects of APN on different macrophage preparations prompted us to investigate (i) APN production by human lung explants, (ii) the expression of the two AdipoRs on LMs, and (iii) the impact of recombinant APNs and a small-molecule APN receptor agonist (AdipoRon (Okada-Iwabu et al., 2013)) on the regulation of LM activation by LPS, poly(I:C) or IL-4.

Materials and methods

Materials

Human recombinant APNs (expressed either in *Escherichia coli* (*E.coli*) or in human embryonic kidney (HEK) 293 cells) were purchased from Biovendor (Karasek, Czech Republic).

AdipoRon hydrochloride and human recombinant IL-4 (produced in *E.coli*) were acquired from TOCRIS/R&D Systems (Lille, France) and solubilized respectively in DMSO and Roswell Park Memorial Institute 1640 medium (RPMI). Antibiotics, DMSO, L-glutamine, trypan blue dye, heat-inactivated fetal calf serum and LPS (from *E. coli* serotype 0111:B4) were purchased from Sigma (St. Louis, MO, USA). High-molecular-weight poly(I:C) was obtained from InvivoGen (Toulouse, France). Bovine serum albumin and RPMI medium were from Eurobio Biotechnology (Les Ulis, France).

Preparations of human lung explants and macrophages.

Experiments on human tissue were approved by the regional investigational review board (*Comité de Protection des Personnes Île de France VIII*, Boulogne-Billancourt, France). Lung tissue samples were obtained from 51 patients (median age: 63 years [range 49-83]; 32 males and 19 females; current smoker/ex-smoker: 49; never smoker: 2; mean ± standard deviation (SD) pack-years: 40±22; BMI: 25±5; FEV1: 87±16%; FEV_1/FVC ratio: 0.78 ± 0.14) undergoing surgical resection for lung carcinoma and who had not received prior chemotherapy. The lung explants and LMs were isolated from macroscopically normal lung parenchyma obtained from sites distant from the tumor, dissected free of pleura, visible airways and blood vessels, and then finely chopped into 3-5 mm^3 fragments, as previously described (Buenestado et al., 2010, 2012, 2013).

After 2 h, 4 h and 24 h of incubation in RPMI at 37°C (in a 5% CO_2 humidified atmosphere), culture supernatants from the explants were collected and stored at -80°C for subsequent APN assays. Lung macrophages were isolated by adherence, as previously described (Abrial et al. 2015). The adherent cells ($211 \pm 54 \times 10^3$ cells per well, for a 24-well plate) were >95% pure macrophages, as determined by May-Grünwald-Giemsa staining and CD68 immunocytochemistry. Cell viability exceeded 90%, as assessed by trypan blue dye exclusion. Culture plates with adherent macrophages were washed with warm medium. One mL of fresh medium supplemented with 1% fetal calf serum was added per well, and culture plates were incubated overnight at 37°C in a 5% CO_2 humidified atmosphere.

Treatment of LMs and explants

On the day after isolation, macrophages or explants were washed twice, and 1 mL of RPMI was added per well. Classically activated macrophages were obtained by exposure for 24 h to LPS (10 $ng \cdot mL^{-1}$) or poly(I:C) (10 $\mu g \cdot mL^{-1}$), and alternatively activated macrophages were produced by exposure to IL-4 (10 $ng \cdot mL^{-1}$) (see Supplementary Material). Recombinant APN (3-10-30 $\mu g.mL^{-1}$) or AdipoRon (5-10-25-50 µM) was added to the culture medium one hour before exposure to LPS, poly(I:C) or IL-4. The APN concentrations were originally chosen to reflect human plasma concentrations (3-30 $\mu g.mL^{-1}$) (Arita et al., 1999; Lindberg et al., 2013, 2017). In control experiments, the vehicle used for AdipoRon (0.1% DMSO) did not alter cytokine production. Following 24 h of incubation, supernatants were collected and stored at -80°C for subsequent analysis.

Cytokine and APN assays.

The supernatants' cytokine concentrations were measured with an ELISA (R&D Systems), according to the manufacturer's instructions. The limits of detection are indicated in the

Supplementary Material. The supernatants were diluted with RPMI as appropriate, and the optical density was determined at 450 nm using a microplate reader (MRX II, Dynex Technologies, Saint-Cloud, France). Cytokine concentrations are expressed in ng.10^{-6} LMs. The APN levels were measured with an ELISA (R&D Systems; detection range: 62.5 – 4000 pg.mL^{-1}). Cell viability was assessed by measuring LDH release with the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega®, Madison, USA) in the LMs supernatants. (Supplemental material).

Quantitative reverse transcriptase polymerase chain reaction

Lung macrophages were stimulated (or not) for 24 h with LPS, poly(I:C) or IL-4. The RNA was prepared as previously described (supplementary material). Specific TaqMan® arrays based on predesigned reagents (*AdipoR1*: Hs01114951_m1; *AdipoR2*: Hs00226105_m1, Thermo Fisher Scientific, MA, USA) were used to analyze AdipoR1 and AdipoR2 transcripts. Reverse transcriptase-quantitative polymerase chain reaction was performed using Gene Expression Master Mix (Thermo Fisher Scientific) with 20 ng of cDNA in a StepOnePlus thermocycler (Thermo Fisher Scientific). The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The housekeeping gene coding for hypoxanthine phosphoribosyltransferase (*HPRT1*: Hs99999909_m1) was used for signal normalization.

Laser confocal immunofluorescence microscopy

The LMs were fixed in methanol 80% on labteck glass chamber slides. After incubation with 1% bovine serum albumin in phosphate buffered saline solution for 30 min, immunostaining was performed using primary antibodies targeting either AdipoR1 (monoclonal rabbit Ig, dilution 1/100; ENZO, Lausanne, Switzerland) or AdipoR2 (monoclonal rabbit Ig, dilution

141/30; Abcepta, CA, USA). After 1 hour of incubation, a secondary antibody (donkey 215monoclonal anti rabbit Ig) coupled with fluorescent probe Alexa Fluor 488 (green) was added 216for 1 hour before washing. Images were acquired using a SP5 Leica confocal microscope 217(Leica, Nanterre, France) with a 63X objective. A spectral imaging acquisition method was 218used in order to overcome the challenge of spectral overlap with macrophages 219autofluorescence.

220

221**Statistical analysis.**

222The data and statistical analysis comply with the recommendations on experimental design 223and analysis in pharmacology (Curtis et al., 2018). Data are expressed as the mean ± SEM per 22410^6 macrophages or per 100 mg lung explants obtained from *n* patients. Intergroup 225comparisons of APN concentrations were performed with unpaired t-tests, and the 226relationship with BMI was estimated by calculating Spearman's correlation coefficient. The 227effects of various concentrations of APN and AdipoRon on cytokines levels released by LMs 228were compared on log-transformed data using either paired Student's t test or a one-way 229repeated measures ANOVA followed by Dunnett's post-test for multiple comparisons using 230GraphPad Prism® software (version 7, GraphPad Software Inc., San Diego, CA, USA). The 231threshold for statistical significance was set to $p< 0.05$.

232

233**Results**

234**1-Production of APN by human lung explants**

235The APN concentration in the supernatant above nonstimulated explants (n=30 patients) after 23624 h was 185.0±127.3 ng.mL^{-1} per 100 mg of tissue. After adjustment for the volumes of the 237culture medium (3 mL) and the explants (100 mg ≈ 0.1 mL), this corresponded to a

concentration of APN about 6.1 $\mu g.g^{-1}$ of lung tissue (i.e. close to the blood concentration). Neither LPS, poly(I:C) nor IL-4 altered APN production.

Time-course measurements of the APN concentration in explant supernatants highlighted an increase over time, and suggested that APN was released by the tissue (Figure 1a). The APN concentrations were negatively correlated with the explant donor's BMI (Figure 1b). In contrast, APN was not detected in LM supernatants - suggesting that these macrophages were not involved in the release of APN by the explants.

2-Expression of APN receptors on human LMs

An RT-PCR analysis demonstrated that LMs expressed both AdipoR1 and AdipoR2 at the mRNA level. Unstimulated LMs expressed AdipoR1 more strongly than AdipoR2. The expression of both AdipoR1 and AdipoR2 transcripts was enhanced after exposure to LPS for 24 hours, whereas exposure to poly(I:C) only enhanced the expression of AdipoR2. In contrast, IL-4 did not alter the transcription of either APN receptor (Figure 2). The expression of the two types of AdipoRs was also detected by immunostaining and confocal microscopy (Figure 3).

3-Adiponectin and AdipoRon abrogate the LPS- and poly(I:C)-induced production of cytokines by LMs.

3.1 LPS- and poly(I:C)-induced cytokine production by LMs

Incubation with LPS or poly(I:C) during 24h was associated with markedly greater production of various M1 cytokines (Table 1). Exposure to LPS was associated with greater production of TNF-α, IL-6, CXCL1, and CXCL8, whereas exposure to poly(I:C) was associated with greater production of CCL5 and CXCL10. Conversely, LPS and poly(I:C) did not alter (or

only weakly altered) the production of the M2 chemokines CCL13, CCL17, and CCL22 (data not shown) .

3.2- Effect of APN on the LPS- and poly(I:C)-induced production of cytokines by LMs

On unstimulated LMs, APN did not alter the production of cytokines (Supplemental data- Table S1). Adiponectin inhibited both LPS- and poly(I:C)-stimulated cytokine production in a concentration-dependent manner (Figure 4 and Supplemental data- Supplemental data-Table S2). A submaximal concentration (10 $\mu g.mL^{-1}$) of APN had similar effects on the LPS- and poly(I:C)-induced production of cytokines, with the exception of CXCL8. Although APN (10 $\mu g.mL^{-1}$) markedly inhibited the LPS-induced CXCL8 production, it did not change significantly the poly(I:C)-induced CXCL8 production (Figure 4 and Table S2).

At a concentration of 30 $\mu g.mL^{-1}$, APN almost completely inhibited the cytokine production (Table S2) and was associated with a 70% relative reduction in the LPS-induced production of IL-10 (Supplementary Material).

3.3- Effects of various recombinant APNs on the LPS-induced production of cytokines by LMs

In addition to human recombinant APN produced in *E. coli*, we tested recombinant APN produced in HEK293 cells (Biovendor). The main difference between the two was the presence of high-molecular-weight (HMW) isoforms (more than hexameric) in the HEK293 APN. The APN expressed in *E. coli* lacks the characteristic post-translational modifications of eukaryotic APN and particularly fails to form HMW multimers (Tsao et al., 2002). When tested on unstimulated LMs (Table S1), APN from HEK293 cells was devoid of significant effects on the production of TNF-α, CXCL8, CCL3, CCL4, or IL-6 at concentrations up to 30

286μg.mL^{-1} (n=5-6) but significantly inhibited the LPS-induced production of TNF-α and CCL4
287at 30 μg.mL^{-1} (Supplemental data-Table S3).
288A mutant APN (C39A: alanine substituted for cysteine at position 39) expressed in HEK293
289cells (Biovendor) forms trimers but not hexamers or HMW isoforms (Haugen and Drevon,
2902007). At 30 μg.mL^{-1}, the mutant APN did not alter the production of cytokines by
291unstimulated or LPS-stimulated LM (n=5). This latter result agrees with previous reports on
292human blood monocytes and U937 monocytic cells (Haugen and Drevon, 2007; Song et al.,
2932009).
294We also compared the activity of several batches of APN produced in *E. coli* (Biovendor).
295One batch clearly differed in its low-molecular-weight (LMW) isoform content
296(Supplemental data-Figure S1) and was devoid of inhibitory activity on LPS-induced cytokine
297production. Only the batches containing the LMW isoforms (used in the present study)
298inhibited the LPS-induced production of cytokines. As a whole, these findings suggest that
299APN's anti-inflammatory activity can be ascribed to its LMW isoforms - probably the
300hexamers.
301
302***3.4- Effects of AdipoRon on the LPS and poly(I:C)-induced production of cytokines by LMs***
303On unstimulated LMs, AdipoRon inhibited the basal production of TNF-α ($p<0.05$), IL-6
304($p<0.05$), CXCL1 and CXCL8 (Supplemental data-Table S1), AdipoRon also inhibited LPS-
305and poly(I:C)-induced cytokine production in a concentration-dependent manner (Figure 5
306and Supplemental data-Table S4). AdipoRon at concentrations of 5–50 μM activate the APN
307receptors in myotubes to the same extent as APN does (Okada-Iwabu et al., 2013). As
308observed with APN, AdipoRon was less effective in inhibiting poly(I:C)-induced CXCL8
309production. The inhibitory effects of AdipoRon (50 μM) did not differ significantly from
310those of APN (30 μg.mL^{-1}).

4-Effects of Adiponectin and AdipoRon on the IL-4-induced release of cytokines by human LMs.

Incubation with IL-4 was associated with the elevated production of the M2-chemokines CCL13 (basal: 16±10 pg.mL^{-1}; +IL-4: 83±14 pg.mL^{-1}; a 5.2-fold increase), CCL17 (basal: 6.2±2.1 pg.mL^{-1}; +IL-4: 46.6±28.7 pg.mL^{-1}; a 7.5-fold increase) and CCL22 (basal: 0.9±0.7 ng.mL^{-1}; +IL-4: 3.6±1.6 ng.mL^{-1}; a 4-fold increase) on 5-6 paired preparations. Both APN and AdipoRon inhibited IL-4-stimulated chemokine production in a concentration-dependent manner (Figure 6 and Supplemental data-Table S5).

Discussion

The present study is the first to have demonstrated (i) the production of APN by human lung explants and its correlation with the donor's BMI, (ii) that both AdipoR1 and AdipoR2 are expressed by human LMs, and (iii) both APN and the small-molecule agonist AdipoRon reduce the LPS-/poly(I:C)- and the IL-4-induced production of cytokines by LMs.

In patients with asthma, sputum levels of APN were found to be elevated after an allergen challenge (Biagioni et al., 2014). Adiponectin has also been detected in bronchial alveolar lavage (BAL) both in asthma and COPD and was even one of the most strongly expressed cytokines in BAL (Miller et al., 2009; Holguin et al., 2011; Sideleva et al., 2012; Kramer et al., 2017). However, the APN concentrations in BAL were several magnitudes lower than those found in the serum, which probably reflects the dilution of lung fluids by the BAL process. It has been suggested that most of the adipokines in BAL diffuse from the bloodstream, although other researchers have reported disparities between serum and BAL concentrations of APN (Holguin et al., 2011; Kramer et al., 2017). This disparity might be due to the active transport of circulating APN multimers (which might not pass freely through the pulmonary vasculature into the alveolar space) (Sood, 2010) or by local synthesis of APN (independently of the blood concentration). In the present study, we showed that APN is produced by human lung explants but not by latter's constituent LMs. In the lungs, APN may be also synthesized by the epithelial cells, as suggested by the high levels of APN expressed by the human A549 epithelial cell line (Miller et al., 2009), although the involvement of other cell types cannot be ruled out. Adiponectin is not the only adipokine synthetized by the lung, since leptin is reportedly synthetized by bronchial epithelial cells and alveolar type II pneumocytes (Bruno et al., 2009; Vernooy et al., 2009).

It is widely accepted that serum APN levels are positively associated with age and female gender, and negatively associated with BMI (Arita et al., 1999; Lindberg et al., 2013, 2017).

However, BAL levels of APN were seen to be weakly and negatively associated with BMI in a small sample of patients with asthma and healthy controls (Holguin et al., 2011). This weak association may reflect variations in the diluting effect of the BAL. In contrast, we found a clear association between APN concentrations in the supernatants of explants and the donor's BMI – suggesting that lung APN production is related to BMI. After taking account of dilution in the culture medium, the estimated APN concentration in lung tissue is close to the range found in the blood (5-20 $\mu g.ml^{-1}$) and to the concentrations of recombinant APN found to inhibit cytokine production by LMs in the present study.

The expression of the APN receptors AdipoR1 and AdipoR2 has been previously reported on murine macrophage-like cells (RAW264) (Yamaguchi et al., 2005), human monocytes (Kollias et al., 2011), MDMs (Cheng et al., 2012), and the THP-1 cell line (van Stijn et al., 2015). In the present study, we showed for the first time that human LMs express both APN receptors. Unstimulated LMs expressed AdipoR1 more strongly than AdipoR2 – as also reported in human monocytes (Kollias et al., 2011; van Stijn et al., 2015). The activation of mouse bone marrow or peritoneal macrophages by interferon-γ/LPS was associated with downregulation of AdipoR1/R2 transcription (van Stijn et al., 2015); this contrasted with the upregulation observed in human LMs activated by LPS. Hence, APN receptor expression appears to be regulated differently in LPS-activated mouse macrophages vs. human LMs. Activation of murine macrophages by IL-4 or IL-10 was associated with (i) weak downregulation of or no change in AdipoR1 expression, and (ii) upregulation of or no change in AdipoR2 expression (van Stijn et al., 2015). In human LMs activated by IL-4, AdipoR1 mRNA levels did not vary and AdipoR2 mRNA levels rose.

Previous research has shown that incubation of nonstimulated primary human monocytes or MDMs with recombinant APN produced in mammalian cells is associated with elevated mRNA and/or protein levels of M1 and M2 cytokines such as TNF-α and IL-6, and the

chemokines CCL2, -3, -4, and -5, and CCL23 (Neumeier et al., 2011; Cheng et al., 2012). The C-terminal globular fragment of APN has been shown to activate nuclear factor-κB and to increase the production of TNF-α, IL-6 and/or CXCL8 in the U937 monocytic cell line and THP-1 macrophages (Tsatsanis et al., 2005; Haugen and Drevon, 2007). Furthermore, adiponectin's HMW isoforms have been shown to induce the production of IL-6, CXCL-8 and CCL2 in THP-1 cells and human primary monocytes (Neumeier et al., 2006; Haugen and Drevon, 2007; Song et al., 2009). In sharp contrast, incubation of nonstimulated LMs either with APN produced in HEK293 cells (containing HMW isoforms) or with recombinant APN from *E. coli* did not increase the production of TNF-α, CCL2, CCL3, CCL4, or CXCL8 (present study). Hence, the differences in composition (LMW *vs*. HMW isoforms) between recombinant APNs are unlikely to explain the different effects on nonstimulated monocytic cells and LMs. Moreover, the synthetic APN receptor agonist AdipoRon did not induce the production of cytokines by nonstimulated LMs. Therefore, the absence of APN-associated pro-inflammatory effect on LMs is probably due to differences in the cell types rather than in the effects of LMW vs. HMW isoforms. Indeed, monocytes and MDM are surrogate cell models that do not adequately recapitulate the biology of LM - as demonstrated in our laboratory (Victoni et al., 2017).

We also showed for the first time that APN from *E. coli* exerts an anti-inflammatory effect on human LMs by largely decreasing the production of cytokines induced by LPS, poly(I:C) or IL-4 without significant impact on cell viability. Such a reduction level in the cytokine release by human monocytes without any impact on cell viability has already been demonstrated with prostaglandin E_2 (Takayama et al., 2002; Bryn et al., 2006). In addition, we have recently shown a similar magnitude of the APN-induced reduction in cytokine production by human primary bronchial epithelial cells (Salvator et al., 2020). In previous research, eukaryotic recombinant APN was found to have an anti-inflammatory effect (i) in the human THP-1

395monocytic cell line (by repressing several TNF-α-induced proinflammatory genes, including
396cytokines such as CXCL8 (van Stijn et al., 2015)), (ii) in the U-937 monocytic cell line (by
397inhibiting LPS-induced NF-κB activity (Haugen and Drevon, 2007)), (iii) in human
398monocytes or MDMs (by either suppressing the expression or production of LPS-induced
399cytokines such as TNF-α, IL-6, CXCL9-11 (Neumeier et al., 2006; Okamoto et al., 2008;
400Folco et al., 2009)). Although APN expressed in HEK293 cells inhibited TNF-α and IL-6
401production in MDMs (Folco et al., 2009), it is noteworthy that this type of eukaryotic
402recombinant APN weakly suppressed LPS-induced cytokine production by LMs in the
403present study. It is worth noting that HMW-APN did not suppress LPS-induced IL-6
404production in human monocytes and THP-1 cells while LMW-APN reduced LPS-mediated
405IL-6 release (Neumeier et al., 2006). Furthermore, mouse recombinant APN produced in *E.*
406*coli* inhibited the LPS-mediated release of TNF-α by mouse alveolar macrophages (Summer
407et al., 2008). The results of our experiments on batches of APN from *E. coli* that differed with
408respect to the LMW isoform profile suggested that the latter parameter is involved in APN's
409anti-inflammatory effect in LMs. In addition, the observed inhibitory effect of AdipoRon on
410LPS-, poly(I:C) and IL-4-induced cytokine production confirmed that APN receptor
411activation has anti-inflammatory effects on LMs.
412Adiponectin has been shown to stimulate IL-10 expression and production by human blood
413monocytes and MDMs (Neumeier et al., 2006; Folco et al., 2009). However, antagonism of
414IL-10 did not abrogate APN's anti-inflammatory actions - indicating that the inhibition of
415cytokine production in response to various proinflammatory stimuli does not require IL-10
416(Folco et al., 2009). In contrast to APN's stimulatory effect on MDMs, APN decreased the
417LPS-induced production of IL-10 by LMs in the present study. The reasons for this
418discrepancy remain unclear, although the simplest explanation is that we used human LMs

and the previous studies used human monocytes or MDMs (Neumeier et al., 2006; Folco et al., 2009).

Our present findings indicate that LMW APN may act to maintain LMs in a quiescent state and thus protect the lung from dysregulated macrophage activation. These results and our previous results in human bronchial epithelial cells (Salvator et al., 2020) also suggest that the reduction in APN levels associated with an elevated BMI could be a risk factor for the development of inflammatory lung diseases. Asthma associated with obesity is a growing public health problem for which there are few effective treatments (Umetsu, 2017) and recent COVID-19 pandemy highlighted once again susceptibility of obese persons towards serious viral infections. The activation of APN receptors with inhaled small molecule agonists may constitute a new pharmacological means of controlling the airways inflammation both in chronic pulmonary inflammatory diseases and acute pulmonary viral infections.

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Tables

Table 1. Amounts of cytokines in the supernatants of human LMs treated with LPS or poly(I:C).

Cytokine	Baseline	LPS 10 ng.mL-1	poly(I:C) 10 µg.mL-1	LPS vs. poly(I:C)
CCL3	1.9 ± 0.3	191.8 ± 34.5*** [101]	81.9 ± 14.5*** [43]	ns
CCL4	4.1 ± 2.1	197.5 ± 80.4*** [48]	178.1 ± 25.5*** [43]	ns
CCL5	0.07 ± 0.05	1.1 ± 0.4*** [15]	3.5 ± 0.4*** [49]	*
TNF-alpha	0.08 ± 0.1	33.2 ± 5.7 *** [415]	8.4 ± 1.8** [105]	*
IL-6	0.35 ± 0.18	81.3 ± 17.2*** [232]	2.7 ± 0.9* [8]	**
CXCL1	0.85 ± 0.4	155.6 ± 22.9*** [183]	7.1 ± 2.6* [8]	***
CXCL8	23.0 ± 2.9	989.6 ± 152.8*** [43]	72.7 ± 20.5** [3]	***
CXCL10	0.03 ± 0.007	2.33 ± 0.7** [78]	14.6 ±2.3*** [487]	**

Results are expressed as ng/10^6 LMs and reported as the mean ± SEM of 5 to 16 independent experiments. The increase in the production of the cytokines induced by LPS or poly(I:C) is expressed as fold-change relative to baseline and given in brackets. Asterisks indicate significant differences relative to the baseline condition or a significant difference between LPS and poly(I:C) (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Legends of figures

Figure 1: APN release from lung explants.

a) Time-course of APN release from nonstimulated human lung explants cultured for 2, 4 and 24 h. Data are reported as the mean ± SEM of 5 to 11 independent experiments (***: $p<0.001$).

b) Relationship between the APN production from unstimulated human lung explants after 24h incubation assessed as the concentration in the supernantants and the donor's BMI (n=30). Spearman's correlation coefficient r = -0.48; p=0.008.

Figure 2: **Expression of AdipoR1 and AdipoR2 transcripts by human LMs.**

Human LMs were cultured for 24 h in absence or presence of LPS (10 $ng.mL^{-1}$), poly(I:C) (10 $\mu g.mL^{-1}$) or IL-4 (10 $ng.mL^{-1}$). AdipoR1 and AdipoR2 transcript levels were determined by RT-qPCR and normalized against those of a housekeeping gene (*HPRT1*). Data correspond to the mean ± SEM of 6 independent experiments (*: $p<0.05$).

Figure 3: AdipoR1 and AdipoR2 are expressed on human LMs.

AdipoR1 (A) and AdipoR2 (B) staining appears in green (AF488 fluorochrome). Immunostaining was evaluated using a SP5 Leica confocal microscopy (X63) and a spectral imaging acquisition.

Figure 4: Inhibitory effects of APN on the LPS- or poly(I:C)-induced cytokine release by human LMs

Macrophages were incubated with LPS (10 $ng.mL^{-1}$, left column) or poly(I:C) (10 $\mu g.mL^{-1}$, right column) in the absence or presence of APN (3, 10 or 30 $\mu g.mL^{-1}$) for 24 h. Data are expressed as a percentage with respect to LPS or poly(I:C)-induced production. Results are

shown as the mean ± SEM of 5 to 16 different experiments. Asterisks indicate significant effects of APN with respect to LPS or poly(I:C) alone (*:$p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 5: Inhibitory effects of AdipoRon on the LPS- or poly(I:C)-induced cytokine release by human LMs.

Macrophages were incubated with LPS (10 ng.mL^{-1}, left column) or poly(I:C) (10 µg.mL^{-1}, right column) in the absence or presence of AdipoRon (5, 10, 25, and 50 µM). Data are expressed as a percentage with respect to LPS or poly(I:C)-induced production. Results are shown as the mean ± SEM of 5 to 9 different experiments. Asterisks indicate significant effects of AdipoRon with respect to LPS or poly(I:C) alone (*:$p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 6: Inhibitory effects of APN and AdipoRon on the IL-4-induced chemokine release by human LMs.

Macrophages were incubated in the absence or presence of APN (3, 10 or 30 µg.mL^{-1}) or AdipoRon (5, 10, 25 or 50 µM) before being stimulated with IL-4 (10 ng.mL^{-1}) for 24 h. Data are expressed as a percentage with respect to IL-4-induced production. Results are shown as the mean ± SEM of 6 to 12 different experiments. Asterisks indicate significant effects with respect to IL-4 alone (*:$p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site