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ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Modulation of allergic inflammation in the lung by a peptide derived from *Mycobacteria tuberculosis* chaperonin 60.1

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Abstract

Background: We have previously demonstrated that *Mycobacteria tuberculosis* chaperonin 60.1 inhibits leucocyte diapedesis and bronchial hyperresponsiveness in a murine model of allergic lung inflammation.

Methods: In the present study, we have investigated the effect of a shorter peptide sequence derived from Cpn 60.1, named IRL201104, on allergic lung inflammation induced by ovalbumin (OVA) in mice and by house dust mite (HDM) in guinea pigs, as well as investigating the action of IRL201104 on human cells in vitro.

Results: Pre-treatment of mice or guinea pigs with IRL201104 inhibits the infiltration of eosinophils to the lung, cytokine release, and in guinea pig skin, inhibits allergen-induced vascular permeability. The protective effect of intranasal IRL201104 against OVA-induced eosinophilia persisted for up to 20 days post-treatment. Moreover, OVA-sensitized mice treated intranasally with 20 ng/kg of IRL201104 show a significant increase in the expression of the anti-inflammatory molecule ubiquitin A20 and significant inhibition of the activation of NF-κB in lung tissue. Our results also show that A20 expression was significantly reduced in blood leucocytes and ASM obtained from patients with asthma compared to cells obtained from healthy subjects which were restored after incubation with IRL201104 in vitro, when added alone, or in combination with LPS or TNF-α in ASM.

Conclusions: Our results suggest that a peptide derived from mycobacterial Cpn60.1 has a long-lasting anti-inflammatory and immunomodulatory activity which may help explain some of the protective effects of TB against allergic diseases.

KEYWORDS

animal models, asthma, eosinophils, regulatory aspects

1 | INTRODUCTION

Heat shock proteins of the HSP60 family are also called chaperonins and form a structurally related subfamily of molecular chaperones. They can be classified into Group I and Group II chaperonins: Group I comprises chaperonins found in bacteria, mitochondria and chloroplasts, whereas Group II chaperonins are found in the cytosol of eukaryotes and in archaea.[1] Group I chaperonins are divided into chaperonin 60 (Cpn60) and chaperonin 10 (Cpn10) proteins, according to the approximate molecular weight (in kDa) of their subunits.

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However, *Mycobacteria tuberculosis* and *Mycobacteria bovis* encode a second chaperonin 60, named chaperonin 60.1.[2]

Hu et al demonstrated that the Cpn60.1 gene is not essential for *M tuberculosis* survival. Furthermore, growth and proliferation of the mycobacteria in culture and in mouse lungs were not affected by the absence of Cpn60.1. However, the mutant strain of *M tuberculosis* did not elicit the expected granulomatous inflammation in the lungs of mice following and that would normally be observed following infection with the wild-type strain. Such observations suggest that Cpn60.1 plays an important role in granuloma formation following infection with tuberculosis.[3]

A number of epidemiological studies have suggested an inverse correlation between allergic asthma and vaccination against tuberculosis with the attenuated form of the bacteria, the bacillus Calmette-Guerin (BCG).[4-7] Data from the literature reveal an inverse correlation between the incidence of tuberculosis and the development of allergic diseases like rhinitis or eczema.[8,9] More importantly, patients exposed to *M tuberculosis* are less likely to develop asthma later in life, strongly suggesting that some component(s) of mycobacteria must have a protective effect against allergy and asthma.[10,11]

The traditional view of molecular chaperones is that of proteins which mediate the folding of other proteins and peptides.[12] However, over the past 20 years, evidence has appeared that some chaperone proteins have distinct, additional roles, notably as intercellular signalling molecules in the immune system. It has also been demonstrated that Cpn60.1 at high concentrations is a potent inducer of inflammatory cytokines, including IL-6, IL-8, IL-12, TNF-α and GM-CSF, and the monocyte-deactivating cytokine IL-10 from human peripheral monocytes in vitro,[13] as well as IL-1 and IL-6 from murine macrophages.[14]

We have previously demonstrated that the chaperonin 60.1 (Cpn60.1) derived from *M tuberculosis* inhibits leucocyte diapedesis, pulmonary eosinophilia, bronchial hyperresponsiveness and associated increases in pro-inflammatory cytokines in a mouse model of allergic lung inflammation.[15,16] In the present study therefore, we have investigated the effect of a shorter sequence of *M tuberculosis* Cpn60.1, named IRL201104, in an attempt to identify the biologically active sequence of this chaperone responsible for manipulating the allergic inflammatory response.[17] We have investigated the effect of IRL201104 on allergic lung inflammation induced by OVA in mice, or induced by HDM in guinea pigs, as well as investigating the action of IRL201104 on human leucocytes and ASM cells in vitro.

2 | MATERIALS AND METHODS

Further detailed methods can be found in the supplemental section.

2.1 | OVA-induced allergic inflammation in mice

Female BALB/c mice (Envigo) were immunized with 30 μg of OVA i.p. (type V; Sigma Chemical Co.) adsorbed to aluminium hydroxide, as described previously.[16] On day 14 of sensitization, all mice were challenged with a 3% aerosol of OVA, once a day for three consecutive days. Experiments were approved by the Home Office under "The Animals (Scientific Procedures) Act (1986)" and by the Ethics Committee of King's College London.

2.2 | Bronchoalveolar lavage in mice

Twenty-four hours after last OVA challenge, mice were anaesthetized with urethane (2 g/kg i.p.; Sigma), a cannula inserted into the exposed trachea, and three 0.5 mL aliquots of sterile saline were injected into the lungs. The total number of cells in the lavage fluid was counted with an improved Neubauer hemocytometer. For differential cell counts, cytospin preparations were stained with Diff Quick (DADE Behring, Switzerland) and cells were counted using standard morphological criteria.

2.3 | Treatment of mice

IRL201104 corresponds to residues 461-491 in the equatorial domain of Cpn60.1[17] (kindly provided by Immune Regulation Ltd) and was prepared by diluting in sterile saline with 0.1% BSA (bovine serum albumin) and administered intranasally (2-200 ng/kg) or intravenously (20 ng/kg) before each OVA challenge in a total of three treatments. Controls received the vehicle only.

2.4 | Cytokine levels

Levels of IL-5 and IL-12 were measured in BAL fluid by standard ELISA (eBioscience) following instructions from the manufacturer.

2.5 | A20 and NF-κB expression measured by immunohistochemistry in mice

Mouse anti-A20 (bs-2803R, Bioss Antibodies), NF-κB p65 (14-6731-81, Invitrogen) or phosphorylated NF-κB (ab86299, Abcam) were used for the immunohistochemistry experiments. Quantification was performed using Image-Pro Plus software, v5.5.0.29 (Media Cybernetics).

2.6 | HDM-induced allergic inflammation in guinea pigs

Immunizations were performed with 50 μg of house dust mite (HDM), *Dermatophagoides pteronyssinus*, (Greer Laboratories Inc) and adsorbed to a saturated solution of aluminium hydroxide s.c. (Sanofi Ltd.). On day 15, animals were challenged with 50 μg HDM intratracheally. Experiments were approved by the UK Home Office under "The Animals (Scientific Procedures) Act (1986)" and by the Ethics Committee of King's College London.

2.7 | Administration of IRL201104 to guinea pigs

IRL201104 was diluted in sterile saline with 0.1% BSA (bovine serum albumin) and administered intravenously (0.3 mL; 200 ng/kg; 80 ng per guinea pig weighing 400 g) immediately prior to HDM challenge on day 15. Controls received vehicle only. Skin testing and BAL were performed 24 hours later.

2.8 | Skin tests and bronchoalveolar lavage in guinea pigs

Evans blue dye was injected intravenously (0.5 mL) followed immediately by intradermal injections of saline, HDM 1:5, 1:50 or 1:500 dilutions from the stock solution of HDM containing 1 mg/mL protein. One hour later, BAL was performed, and treated skin samples removed, weighed and placed in formamide. Optical density of the BAL fluid was measured at 620 nm.

2.9 | Lung function in guinea pigs

Guinea pigs were ventilated (8 mL/kg; 60 breaths/min) through a tracheal cannula connected to a pneumotachograph and a pressure transducer (±2cm H_2O: model MP-45-14-871; Validyne Engineering). Changes in airflow were measured using an automated lung function recording system (Pulmonary Monitoring System, version 5.0; Mumed, London, UK). Airway obstruction was measured [as an increase in R_L and decrease in lung compliance (C_{dyn})] immediately after administration of 100 μg/kg of HDM iv

2.10 | Experiments with human cells

Ethical approval for the biopsies collected from healthy and asthmatic subjects was provided by Guy's and St Thomas' Hospitals Research Ethics Committee (REC: 08/H0804/67). The blood collection from healthy and asthmatic subjects was approved by the National Research Ethics Committee at Guy's and St Thomas' Hospitals (10/H0807/99). Specific demographics regarding these patients can be found in the Supplemental Material and Methods Section. Appropriate consents, permissions and releases were obtained prior collection of all human samples.

2.11 | Isolation and culture of blood leucocytes

Whole blood from healthy or asthmatic volunteers was collected, and some was immediately stained for TNFAIP3(A20) detection by flow cytometry. The remainder was diluted 1:1 with RPMI 1640 supplemented with glutamine (Invitrogen) and cultured with or without IRL201104 (0.1-10 μg/mL). After 24 hours of incubation, cells were harvested for intracellular A20 expression analysis by flow cytometry.

2.12 | A20 detection by flow cytometry

Following treatments, cells were harvested and fixed, permeabilized and incubated with PerCP-conjugated rabbit anti-human A20/TNFAIP3 (1:50, clone EPR2663, ab220511, Abcam). 10 000-100 000 events in a leucocyte gate were collected and analysed using a Beckman FC500 flow cytometer.

2.13 | A20 expression detected by immunohistochemistry in human lung tissues

Frozen human biopsies were incubated with anti-human A20 antibody (1:100, clone 775928MAB75981, R&D). Expression was quantified using Image-Pro Plus software, v5.5.0.29.

2.14 | Preparation of human airway smooth muscle

Airways smooth muscle cells (ASM) were isolated from the biopsies obtained from subjects with asthma and healthy subjects, and seeded at 5000 cells/cm^2 and allowed to adhere to glass for 24 hours. ASM was then growth-arrested in serum-free DMEM (which also served as a control) for 24 hours and then treated with IRL201104 (0.5-5 pg/mL).

2.15 | A20 detection by immunofluorescence in human ASM

Treated ASM was incubated with anti-human A20 (MAB75981, R&D Systems). Fluorescence was quantified using Image J, v 1.45s (NIH, USA).

2.16 | Statistical analysis

Data are expressed as mean ± SEM or as median with 95% CI. Comparisons were made by one-way ANOVA followed by appropriate *post hoc* test or *t* test using GraphPad Prism (version 7.0). The specific *post hoc* test applied in each set of experiments is described in the legend of the figures.

3 | RESULTS

3.1 | IRL201104 inhibits acute allergic lung inflammation induced by OVA in sensitized mice

Our results confirm that immunization and challenge with OVA induce a significant increase in the total number of leucocytes in BAL fluid, reflected by significantly higher numbers of eosinophils in comparison with sham-immunized animals 24 hours after the last OVA challenge (Figure 1A,B). Pre-treatment with IRL201104

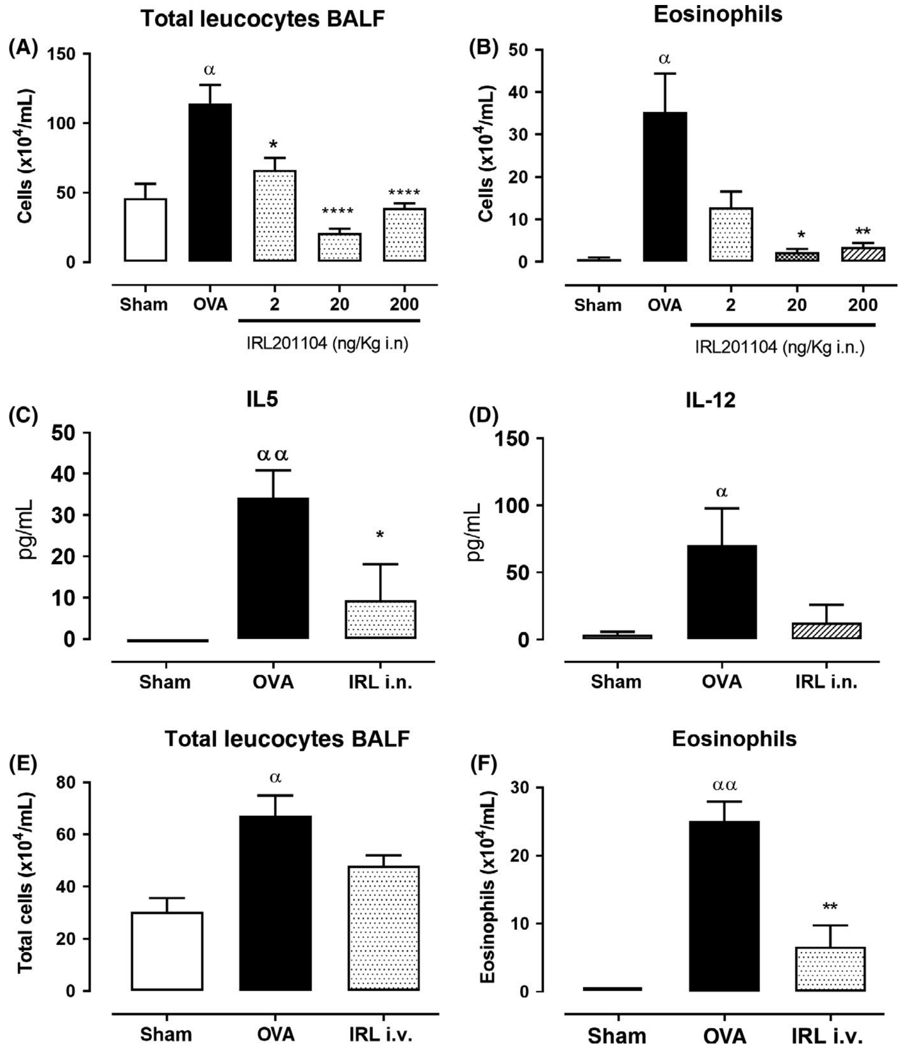


FIGURE 1 Effect of IRL201104 on acute OVA-induced allergic inflammation in the lung. (A) Total cells in BAL fluid; (B) eosinophils in BAL fluid; N left to right: 9, 13, 5, 4, 11. Mice were treated with IRL201104 intranasally 10 min before each OVA challenge (200, 20 and 2 ng/kg i.n). (C) IL-5 levels in BAL of mice treated with 20 ng/kg of IRL201104; N left to right: 5, 8 and 5. (D) Levels of IL-12 in BAL of mice treated with 20 ng/kg of IRL201104, N left to right: 5, 8 and 5. BAL collected 24 h after the 3rd OVA challenge. Data are expressed in mean ± SEM and analysed using one-way ANOVA with *post hoc* Dunnet's test. $^{*}P < .01$, $^{**}P < .001$ $^{****}P < .0001$ vs OVA group, $^{\alpha\alpha}P < .0001$, $^{\alpha}P < .05$ vs sham group. (E) Total cells in BAL fluid, N = 5. (F) eosinophils in BAL fluid collected 24 h after the 3rd OVA challenge N = 4. Mice were treated with 20 ng/kg of IRL201104 intravenously 10 min before each OVA challenge. Data are expressed in mean ± SEM and analysed using one-way ANOVA with *post hoc* Tukey's test (E) and Bonferroni's test (F). $^{**}P < .001$ vs OVA group, $^{\alpha}P < .001$, $^{\alpha\alpha}P < .0001$ vs sham group. Data were obtained in three independent experiments

significantly inhibited the increase of total leucocytes and eosinophil numbers at doses of 20 or 200 ng/kg (Figure 1A,B). Our results also show that intranasal pre-treatment with 20 ng/kg of IRL201104 also significantly inhibited the release of IL-5 and decreased the levels of IL-12 in BAL fluid 24 hours following the last allergen challenge, suggesting that the anti-inflammatory effect of the peptide is not due to a shift from a Th2 to Th1 cytokine profile (Figure 1C,D).

3.2 | IRL201104 inhibits acute allergic lung inflammation induced by OVA independently of the route of administration

In order to assess whether the anti-inflammatory effect of IRL201104 is dependent on the route of administration, we chose a dose (20 ng/kg) of peptide that was significantly effective in inhibiting eosinophil migration when administered via the intranasal route and investigated whether the peptide was still effective when given systemically. Our results show that intravenous pre-treatment with IRL201104 also significantly inhibited the allergen-induced increase in eosinophil numbers in the BAL fluid at a dose of 20 ng/kg (Figure 1E,F).

3.3 | IRL201104 inhibits acute allergic lung inflammation independently of the allergen or the species

Our results show that pre-treatment of guinea pigs with IRL201104 (200 ng/kg intravenously) significantly prevented the increase in the number of eosinophils in BAL fluid 24 hours after a single intratracheal challenge with 50 µg of HDM (Figure 2A). In addition, pre-treatment with IRL201104 also inhibited the extravasation of Evans blue dye into the skin measured 1 hour after the intradermal injection of diluted HDM in saline at ratios of 1:5 and 1:50 in guinea pig skin (Figure 2B). More importantly, we also demonstrated that IRL201104 significantly prevented the increase in airways resistance induced by a single challenge with HDM (100 µg/kg, i.v) (Figure 2C).

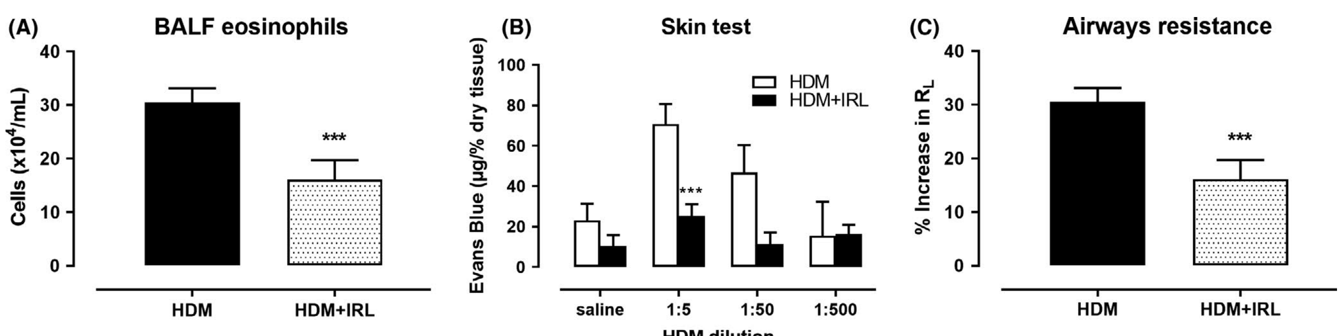


FIGURE 2 Effect of intravenous treatment with IRL201104 on acute HDM-induced allergic inflammation in the lungs of guinea pigs. (A) Eosinophils in BAL collected 24 h after the intratracheal challenge of 50 μg HDM, N = 6. (B) Extravasation of Evan's blue 1 h after subcutaneous injection of a diluted solution of HDM, N = 6. (C) Lung resistance induced by 100 μg/kg of HDM iv, N = 6. Guinea pigs were treated with IRL201104 (200 ng/kg) intravenously 10 min before challenge with HDM. Data are expressed in mean ± SEM and analysed using unpaired *t* test with Welsh's correction (A and C) and two-way ANOVA followed by Sidak's multiple comparisons test (B).****P* < .01 vs HDM group. Data were obtained in two independent experiments

3.4 | The time course of the effect of IRL201104 on allergic lung inflammation

We treated OVA-sensitized mice intravenously or intranasally just before each OVA challenge with the lowest effective concentration of IRL201104, 20 ng/mouse. Ten, 20 or 30 days later, groups of these animals were challenged again with OVA for 3 consecutive days without further treatments with IRL201104. A schematic representation of the protocol of sensitization and treatment can be found in the Supplementary Material accompanying this manuscript (Figure S1).

Our results confirm that IRL201104 inhibits the recruitment of leucocytes to the lung measured 24 hours after the last OVA challenge whether administered intranasally or intravenously (Figure 3A). Ten days later, intranasal treatment still showed a significant effect on eosinophil recruitment when compared to vehicle-treated animals (Figure 3B). Twenty days later, the response to OVA was not as robust as seen at earlier time points, even in vehicle-treated animals, but nevertheless there remained a significantly smaller leucocyte infiltration induced by OVA challenge in the animals that had been pre-treated intranasally with IRL201104 (Figure 3C). At 30 days following treatment with IRL201104, the eosinophilic response following OVA challenge was not significantly different from that observed in the vehicle-treated animals receiving OVA (Figure 3D).

3.5 | IRL201104 inhibits allergen-induced eosinophil infiltration into the lungs of animals that had previously undergone allergen exposure

Mice were sensitized and challenged with OVA following the same protocol as described above. One group of mice then received a single dose of 200 ng/kg of IRL201104 intranasally 24 hours after the last OVA challenge. A second group was intranasally treated with 200 ng/kg of IRL201104, 24, 48 and 72 hours after the last OVA challenge, in a total of three treatments. Ten days after the last OVA challenge, all groups were challenged again with OVA for three consecutive days. No further treatments with IRL201104 were performed at these later time points. Twenty-four hours after the 6th challenge, the number of leucocytes was enumerated in the BAL. A schematic representation of the protocol of sensitization and treatment can be found in the Supplementary Material accompanying this manuscript (Figure S2).

Our results show that regardless of the number of treatments, IRL201104 administered after the third OVA challenge significantly prevented the increase of total number of leucocytes in the lung following further OVA challenges, 10 days later (Figure 4A). This reduction in allergen sensitivity in IRL201104-treated animals is reflected in a significant inhibition of the number of eosinophils in the BAL fluid obtained from mice treated with IRL201104 once or three times after the challenge phase with OVA (Figure 4B).

3.6 | IRL201104 modulates the expression of A20 and inhibits the activation of NF-κB in the lungs of mice sensitized and challenged with OVA

Considering the bacterial origin of IRL201104, we investigated whether this peptide exerts any of its anti-inflammatory or immunomodulatory effects by a A20/TNFAIP3-mediated pathway. We analysed the expression of A20 in the lung tissue of mice sensitized and challenged with OVA, as well as the expression of total and activated NF-κB in these lung samples. Our results show that expression of A20 is reduced in the lungs of untreated OVA-sensitized mice, whilst pretreatment with IRL201104 significantly prevented this decrease in expression of A20 in the lung tissue (Figure 5A). More importantly, the effect of IRL201104 on A20 expression paralleled the time course of the effect of the peptide on eosinophil numbers in the BAL fluid (Figure 3). In addition, we have also shown that whilst IRL201104 does not alter the expression of total NF-κB in the lungs of OVA-sensitized and challenged mice, it does inhibit the activation of this transcription factor (Figure 5B,C).

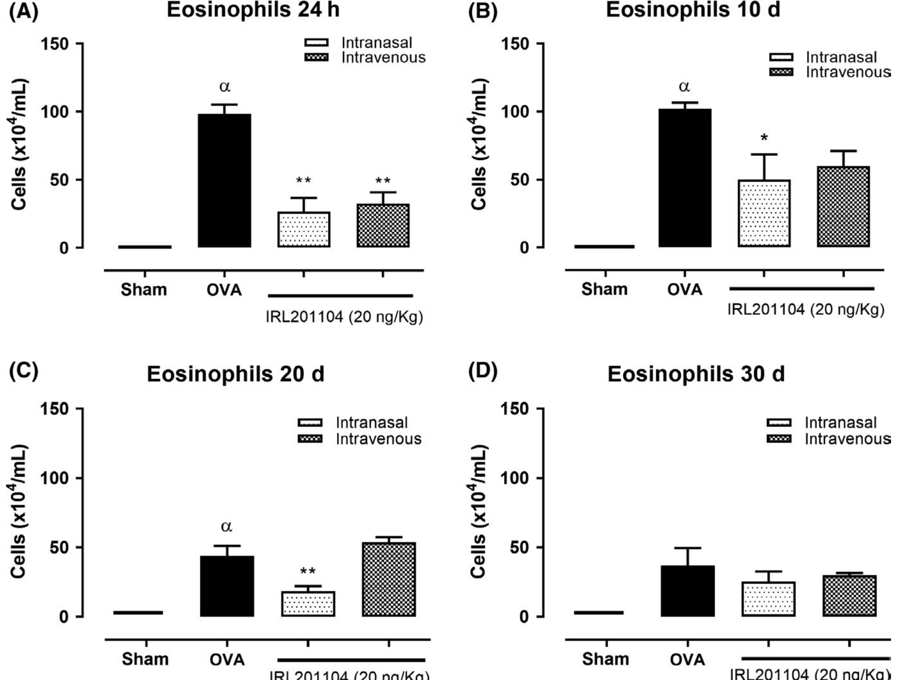


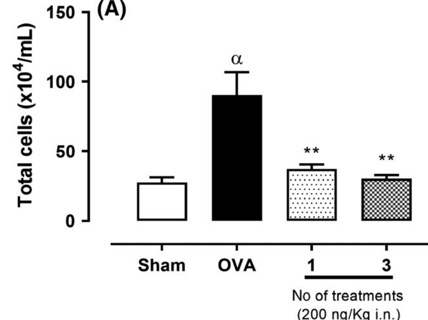
FIGURE 3 Time course effect of IRL201104 on OVA-induced eosinophil infiltration into the lungs of allergic mice. Eosinophils in BAL collected 24 h (A), 10 (B), 20 (C) and 30 days (D) after last IRL201104 treatment. Mice were treated intravenously or intranasally with 20 ng/kg of IRL201104, 10 minutes before the first 3 OVA challenges only. Data are expressed as mean ± SEM and analysed using one-way ANOVA with *post hoc* Dunnett's test. $^*P < .01$, $^{**}P < .001$, vs OVA group, $^{\alpha}P < .0001$ vs sham group. N = 4/group. Data were obtained in one single experiment

3.7 | IRL201104 induces the expression of A20 in human cells

We quantified the expression of A20 in lung biopsies and circulating leucocytes collected from healthy and asthmatic donors. Expression of A20 was measured in lung tissue by immunohistochemistry and in circulating leucocytes by flow cytometry. Results are presented in Figure 6, and we have demonstrated a smaller area of positive expression for A20 in lungs from mild asthmatic patients in comparison with tissue obtained from healthy subjects (Figure 6A,C). Similarly, we observed a significantly lower expression of A20 in leucocytes obtained from severe asthmatic donors in comparison with healthy subjects (Figure 6C,D, Figure S4).

In the next part of our study, we investigated the effect of IRL201104 on the expression of A20 in human circulating leucocytes collected from asthmatic and healthy donors. A20 expression was measured by flow cytometry in full blood cultures incubated for 24 hours with IRL201104. Our results show that IRL201104 at 0.1 µg/mL had a significant effect on the expression of A20 in leucocytes collected from healthy and asthmatic donors (Figure 7A,B, panels C and D), (Additional representative scatter plots can be found in Figure S5A,B).However, pre-treatment with IRL201104 did not significantly alter the mean fluorescence intensity (MFI) in healthy or asthmatic leucocytes (Figure S3).

Given that ASM bundles were found to express A20 in our stained biopsy data (Figure 6A), we also investigated the effect of IRL201104 on the expression of A20 in cultured human ASM which were dissected from the bronchial biopsies collected from asthmatic and healthy donors, and treated in vitro with pro-inflammatory cytokines and stimulant. A20 expression was measured by



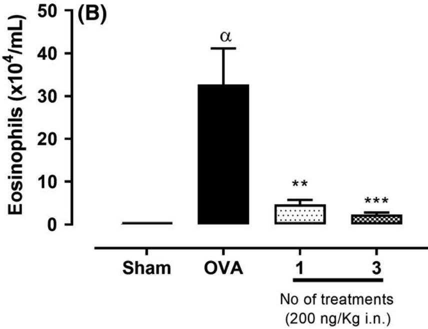


FIGURE 4 Effect of post-treatment with IRL201104 on an established OVA-induced lung inflammation. Total number of cells (A) and eosinophils (B) in BAL collected 24 h after the 6th OVA challenge. Mice were treated intranasally with 200 ng/kg of IRL201104 24 h after OVA challenge (one treatment) or 24, 48 and 72 h after last OVA challenge (three treatments). Challenges 4, 5 and 6 were performed 10 days after challenge 3, and during this subsequent phase of allergen challenge, no further treatment with IRL201104 was performed. Data are expressed as mean ± SEM and analysed using one-way ANOVA with *post hoc* Dunnett's test. $^{\alpha}P < .001$ vs sham group $^{**}P < .001$, $^{***}P < .0001$ vs respective OVA group control. N = 5-6/group. Data were obtained in one single experiment

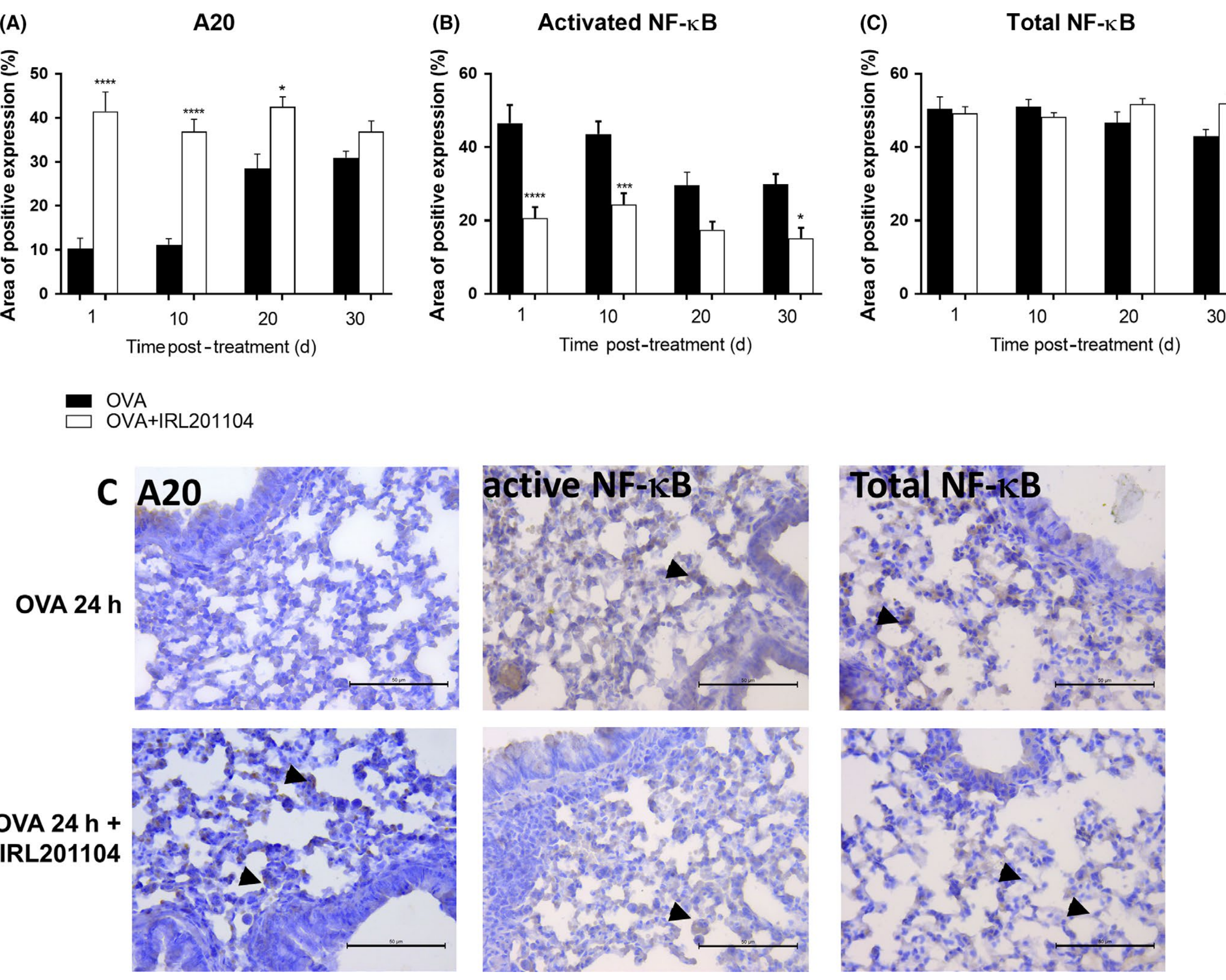


FIGURE 5 Effect of IRL201104 on the expression of A20 and NF-κB in lung tissue. (A) Area of expression of A20, (B) activated NF-kB (phospho S536) and (C). total NF-kB (p65) in paraffin-embedded mouse lung tissue. Lungs were collected 24 h, 10, 20 and 30 d after IRL201104 treatment. Mice were treated intranasally with 20 ng/kg of IRL201104, 10 min before the first 3 OVA challenges only in a total of three treatments. Data are expressed as mean ± SEM and analysed using one-way ANOVA with *post hoc* Tukey's test. $^{*}P < .01$, $^{***}P < .0001$, vs OVA control group; N = 4 mice/group. Data were obtained in one IHC assay

immunofluorescence in cells incubated for 4 hours with IRL201104 alone or in combination with TNF-α or LPS. Our results show that IRL201104 at 0.5 and 5 pg/mL had no significant effect on the expression of A20 in ASM collected from healthy donors (Figure 8A-C and representative photographs of healthy cells a-d). In contrast, pre-treatment with IRL201104 alone significantly increased the expression of A20 in ASM isolated from mild asthmatic patients (Figure 8D) or in combination with 10 ng/mL of TNF-α (Figure 8E) or 100 ng/mL of LPS (Figure 8F). Photographs e-h represent the fluorescent intensity of A20 in ASM collected from asthmatic subjects.

4 | DISCUSSION

We have previously reported that the chaperone Cpn60.1 isolated from *M tuberculosis* is able to exhibit an important anti-allergic effect by inhibiting allergen-induced eosinophil infiltration into the lungs of sensitized challenged mice and the associated bronchial hyperresponsiveness.[15,16] In the present study, we have investigated the effect of IRL201104, a shorter peptide sequence isolated from the equatorial domain region of *M tuberculosis* Cpn60.1, in models of allergic inflammation induced by OVA or HDM.[17] Our results show that pre-treatment of mice or guinea pigs with IRL201104, independently of the route of administration, inhibits the allergic response assessed as a significant inhibitory effect on antigen-induced infiltration of eosinophils into BAL fluid, inhibition of allergen-induced cytokine release in the lung, with doses of the peptide as low as 20 ng/kg. Additionally, in guinea pigs we also observed inhibition of allergen-induced vascular permeability in skin, and more importantly, airways resistance induced by HDM. As such, our results demonstrate that this peptide has similar anti-inflammatory effects as previously observed with the full 60 kDa chaperonin Cpn60.1.[15,16] In addition, we have demonstrated that one single treatment with IRL201104 administered when allergic inflammation in the lung

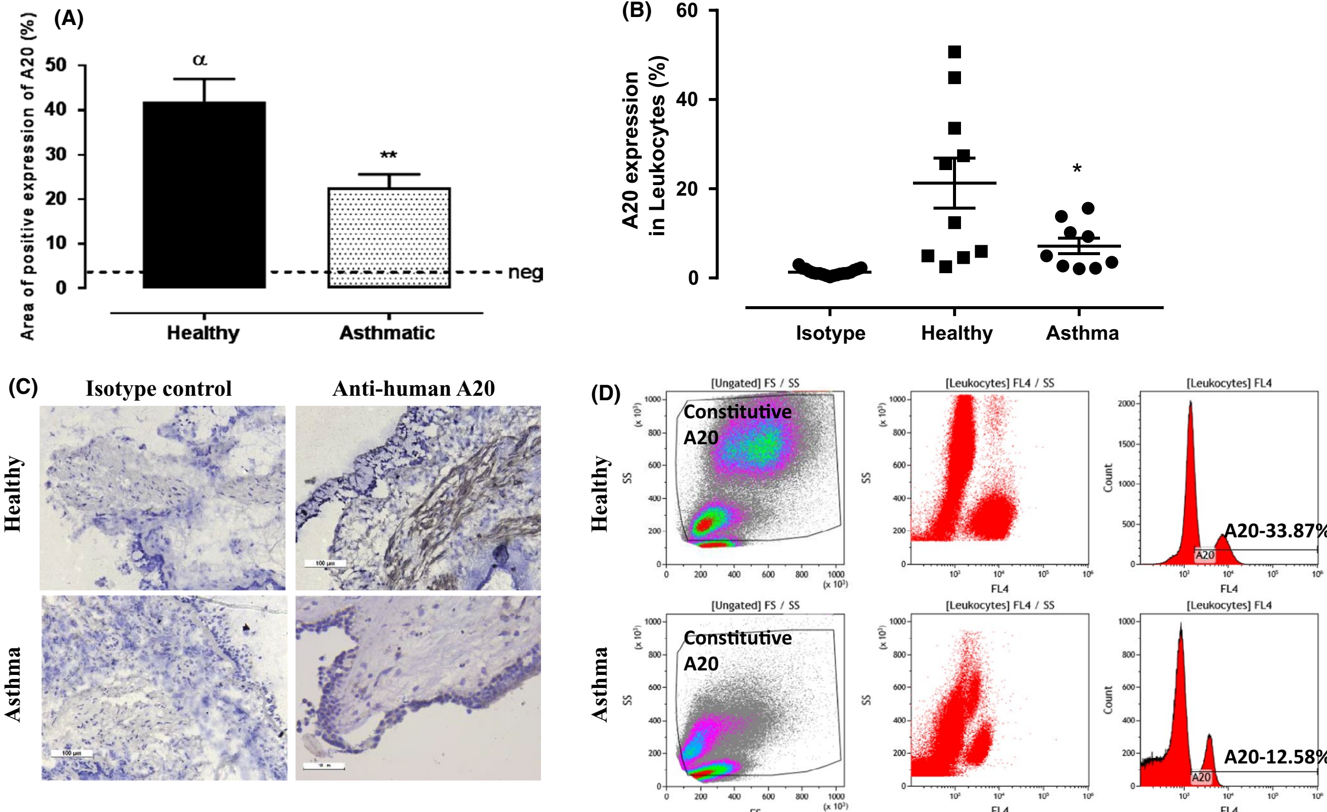


FIGURE 6 Expression of A20 in human lung tissue and circulating leucocytes. (A) Quantification of the regions of positive staining for A20 measured by immunohistochemistry. (B) Percentage of expression of A20 measured by flow cytometry in circulating leucocytes collected from healthy and severe asthmatic patients. (C). Representative photomicrograph of human lung tissue collected from healthy and mild steroid naïve asthmatic donors (20× magnification). (D) Representative scatter plots and histograms of A20 expression from healthy (upper panel) and asthmatic donors (lower panel). Data are expressed as mean ± SEM in graph (B and as median with 95% CI in graph C. Analysed using unpaired *t* test with *post hoc* Mann-Whitney test $^{*}P < .05$ vs healthy, $^{**}P < .001$ vs healthy. N = 3/group in graph B, two independent experiments. N = 10 healthy donors and 9 asthmatics in graph C. Data in graph B were obtained in one single IHC assay. Data in graph C were obtained in 5 independent flow cytometry measurements

has been established is sufficient to significantly prevent a second allergic response 10 days later, demonstrating an important and long-lasting benefit of reducing allergic sensitivity way beyond the short half-life of the molecule (<10 min, unpublished observations). This disparity between the pharmacokinetic and pharmacodynamic half-life of IRL201104 suggests that this molecule has a profound long-lasting anti-inflammatory activity, possibly by influencing the plasticity of cells involved in the initial allergic response (immune memory or immune resetting).

Recent clinical evidence has suggested that people exposed to "barn dust" in early life are also protected against allergic diseases and asthma,[18,19] an observation supported by the demonstration that mice chronically exposed to low doses of endotoxin-rich environments such as farm dust are protected against the development of allergic lung inflammation in response to HDM by a TLR-4-mediated mechanism.[20] TLR-4 signalling in the lung leads to translocation of NF-κB, increasing the expression of attenuators of this response such as the ubiquitin-modifying enzyme A20.[21,22] The A20 ubiquitin, also known as TNFAIP3, is a zinc-finger protein that attenuates NF-κB activation by deubiquitinating key signalling pathways downstream of TLR-4, IL-1 and TNF family receptors.[22-24] It is therefore of interest that in OVA-sensitized mice that have been intranasally treated with 20 ng/kg of IRL201104, there is a significant increase in the expression of A20 in lung tissue in comparison with untreated OVA-sensitized and challenged mice. Moreover, we observed a close correlation between the effect of IRL201104 on A20 expression and the time course of its protective effect against OVA-induced eosinophil migration into the lung. Analysis of the expression of phosphorylated NF-κB in the same lung samples demonstrated that the increase in expression of A20 is accompanied by a significant inhibition of the activation of NF-κB, suggesting that the anti-inflammatory effect of IRL201104 in our model is mediated by A20, similar to results reported with "barn dust".[20]

It has also been shown that A20 expression is reduced in the lungs of subjects with asthma compared with healthy controls.[20] In the present study, we have confirmed this observation demonstrating that A20 expression is significantly reduced in lung tissue biopsies, as well as in circulating leucocytes collected from patients with asthma in comparison with healthy subjects, supporting the concept that A20 is an important endogenous modulator of inflammatory responses.[25,26]

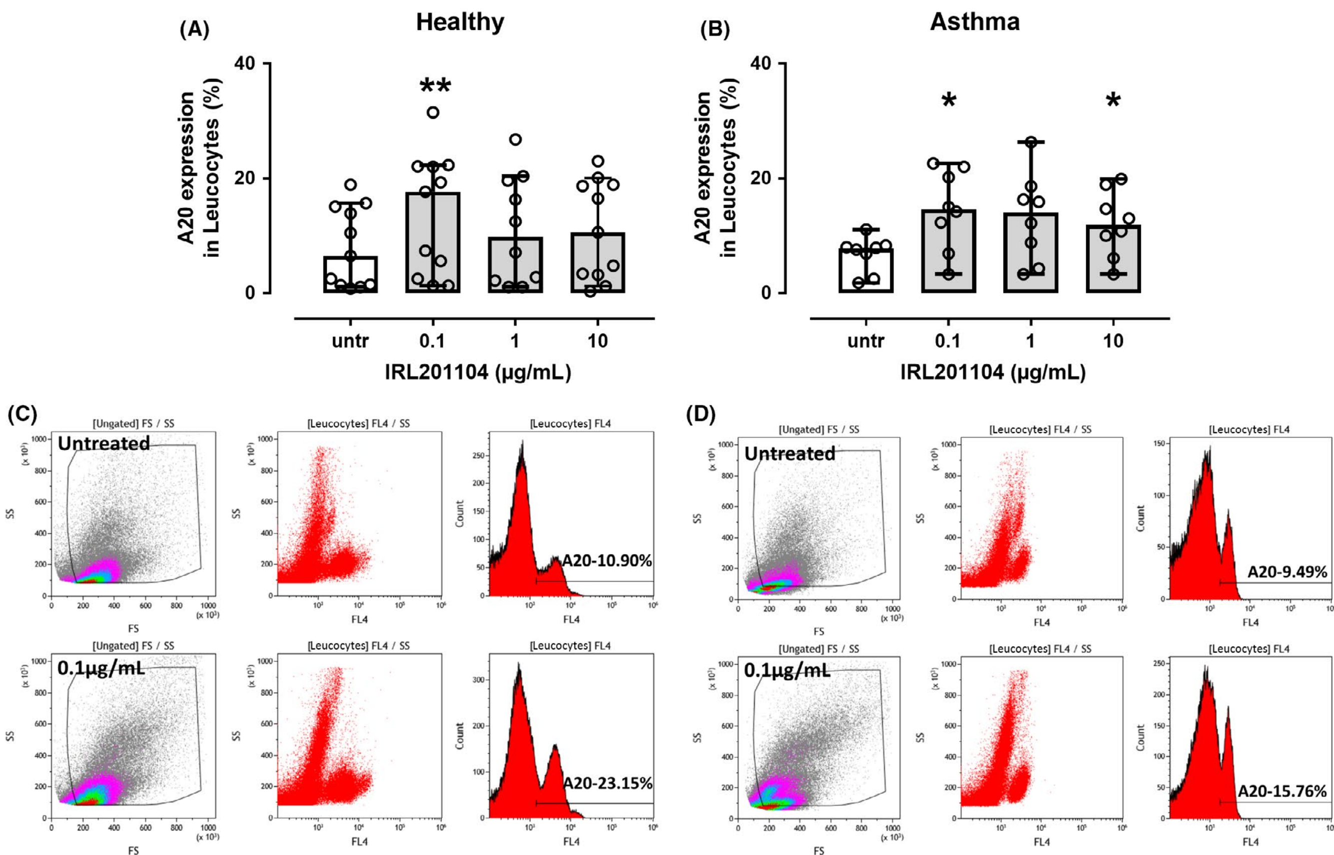


FIGURE 7 Effect of IRL201104 on human circulating leucocytes. Whole blood was obtained from (A). healthy or (B) severe asthmatic donors and incubated for 24 h with IRL201104 (0.1-10 µg/mL) or without (untr). After lysis of red cells and permeabilization, A20 expression in total blood leucocytes was measured by flow cytometry. Representative scatter plots and histograms of A20 expression from healthy (C and D) asthmatic donors untreated or treated with 0.1 µg/mL of IRL201104. FL4 is PerCP A20 label. Values represent median with 95% confidence interval and were analysed using one-way ANOVA with *post hoc* Dunnett's test **P* < .01, ***P* < .001 vs unstimulated cells. N = 8 asthmatic donors and 11 healthy donors. Data were obtained in 10 independent flow cytometry measurements

The role of A20 in inflammatory conditions has been well documented. An important anti-inflammatory role for this ubiquitin has been reported in arthritis, auto-immune diseases, cardiovascular diseases, cystic fibrosis, chronic obstructive pulmonary disease (COPD), allergies and asthma.[20,27-33] In addition, the modulation of the expression of A20 has been demonstrated to be an important mechanism of action of certain drugs including methotrexate, quercetin and the combination of long-acting β_2-adrenoceptor agonistsand glucocorticoids.[34-37] Moreover, it has recently been reported that there is a positive association between a polymorphism in the A20 gene and susceptibility to human asthma in the population investigated in the GABRIELA study, a study that focuses on the effects of farm environments on asthma and allergy in children from central Europe.[20] Furthermore, mRNA levels of A20 measured by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) were shown to be significantly reduced in epithelial cells obtained from subjects with mild or severe asthma when compared with healthy controls, findings confirmed with protein levels.[20]

In the present study, we collected blood samples from healthy and severe asthmatic subjects and measured the effect of IRL201104 on the expression of A20 in circulating leucocytes after 1 and 24 hours of incubation. A 1-hour incubation with the peptide did not significantly alter the expression of A20 in circulating leucocytes in comparison with untreated cells (data not shown). However, after 24-hour incubation of whole blood with IRL201104 we observed a significant increase in the expression of A20 in comparison with untreated cells obtained from both asthmatic and healthy subjects, suggesting that IRL201104 can reverse the loss of expression of A20 in circulating leucocytes. Further experiments are necessary to establish the specific blood cell populations affected by IRL201104.

During our analysis of human lung tissue, we observed the expression of A20 in the epithelial layer, but we also observed a very significant expression of A20 in ASM bundles (Figure 6). This was an unexpected, but interesting observation as little is known about the role of A20 in ASM in asthma. A previous study has demonstrated, by using RNA-based gene knockdown techniques, that A20 is required for the maximal effect of glucocorticosteroids on cytokine release by human ASM in vitro, indicating that ASM can not only express A20, but that this protein may be important in the modulation of ASM function.[36] In the present study, we also investigated whether cultured human ASM, isolated from healthy or mild asthmatic donors, expresses A20 and whether this

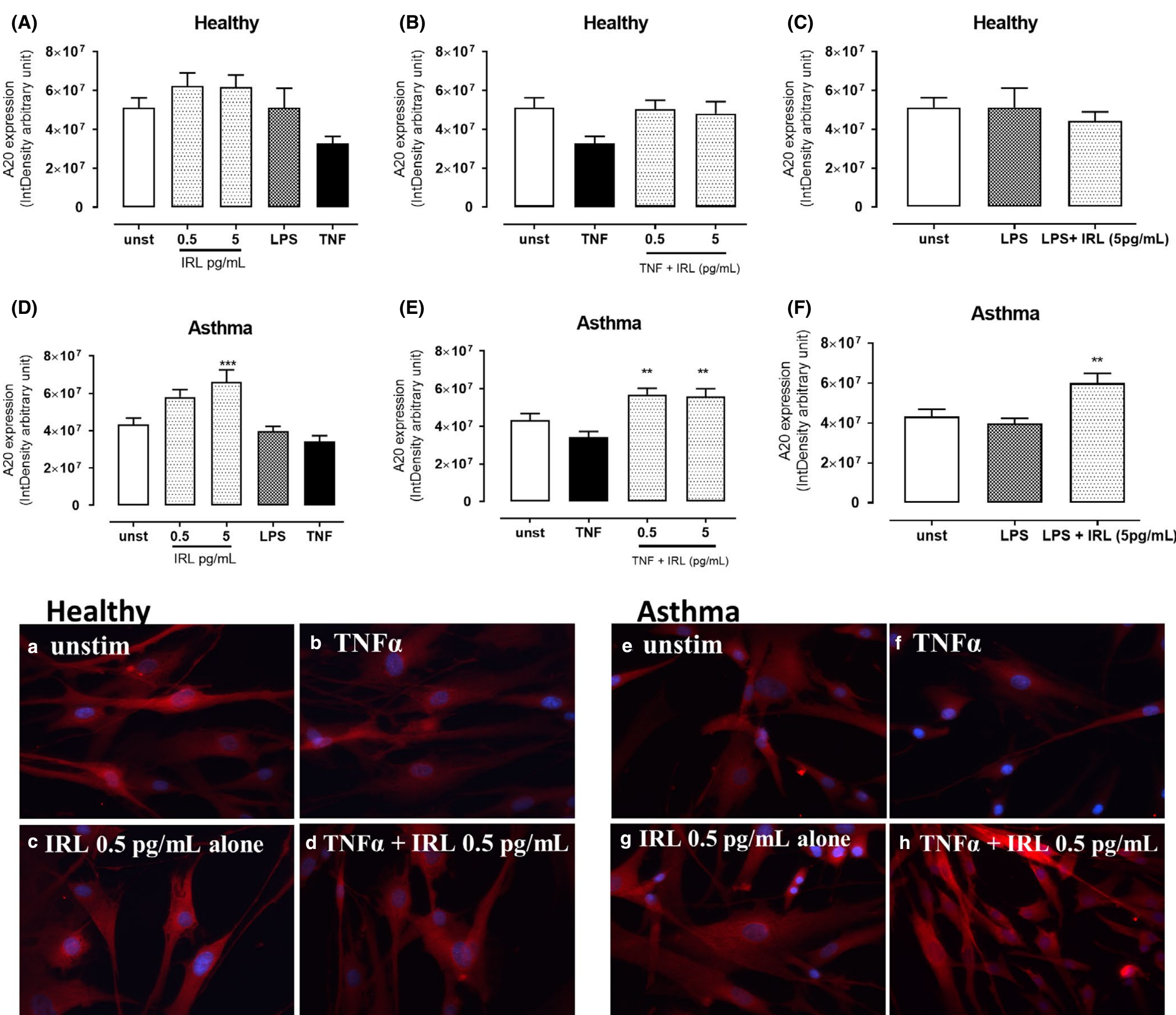


FIGURE 8 Effect of IRL201104 on human ASM. Cells were isolated from healthy or mild asthmatic donors. Cells were cultured until reaching confluence and then incubated for 4 h with IRL201104 alone (A and D), or in combination with 10 ng/mL of TNF-α (B and E) or 100 ng/mL of LPS (C and F). A20 expression was measured by immune fluorescence, and representative images of the intensity of the staining can be seen in the photographs taken with a 40× objective from healthy and asthmatic cells unstimulated and untreated (a and e), treated with 10 ng/mL of TNF-α for 4 h (b and f), 0.5 pg/mL of IRL201104 for 4 h alone (c and g) or the combination of both (d and h). Values represent mean ± SEM of background corrected integrated density and were analysed using one-way ANOVA with *post hoc* Tukey's test ***$P < .0001$ vs unstimulated. **$P < .001$ vs LPS or TNF-α. N = 3 donors/group, two independent experiments. In the lower panel are representative photomicrographs of fluorescent cells. Data were obtained in two independent assays

expression can be modulated by IRL201104. Our results show that constitutive levels of A20 expression in healthy ASM, measured by immunohistochemistry, are not significantly altered by treatment with IRL201104, alone or in combination with LPS or TNF-α. In contrast, ASM from mild asthmatic donors had a significant increase in the expression of A20 when IRL201104 was added to the culture, alone or in combination with LPS or TNF-α. Our results suggest that IRL201104 at very low concentrations can regulate the expression of A20 in ASM from subjects with asthma which may be an important part of the mechanism(s) whereby TB and IRL201104 can inhibit allergic sensitivity.

The results from the present study suggest that IRL201104, a peptide derived from mycobacterial Cpn60.1, can have profound effects on allergen sensitivity that last far beyond the pharmacokinetic half-life of the peptide. These results suggest that this molecule has a long-lasting anti-inflammatory and immunomodulatory activity which may help explain the protective effects of TB against allergic diseases. The observations that this peptide can up-regulate A20, shared with the protective effects of barn dust on allergic diseases, provides a plausible common mechanism as to how exposure to bacterial products in early life protects against the development of allergic diseases. The development of IRL201104 or materials isolated

from barn dust as a novel approach to treating allergic diseases appears warranted.

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CONFLICT OF INTEREST

Professor Clive Page has received grants from Immune Regulation Ltd (formerly Peptinnovate Ltd) to support Dr Yanira Riffo Vasquez and to support the PhD studentship for Francis Man. Professor Page is also a non-Executive Director of Immune Regulation Ltd who are developing the peptide described in this work, and he has equity in the Company. The other authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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