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Elarekibep (PRS-060/AZD1402): a new class of inhaled Anticalin medicine targeting IL-4Ra for T2 endotype asthma

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Elarekibep (PRS-060/AZD1402): a new class of inhaled Anticalin medicine targeting IL-4Ra for T2 endotype asthma

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Abstract

Background: T2 endotype asthma is driven by IL-4 and IL-13 signaling via IL-4Ra, which is highly expressed on airway epithelium, airway smooth muscle and immunocytes in the respiratory mucosa, suggesting potential advantages of an inhalable antagonist. Lipocalin 1 (Lcn1), a 16kD protein abundant in human periciliary fluid, has a robust drug-like structure well-suited to protein engineering, but has never been used to make an inhaled “Anticalin” protein therapeutic.

Objectives: to re-engineer Lcn1 into an inhalable IL-4Ra antagonist and assess its pharmacodynamic/kinetic profile.

Methods: Lcn1 was systematically modified by directed protein mutagenesis yielding a high affinity, slowly dissociating, long-acting full antagonist of IL-4Ra designated ‘PRS-060’ with properties analogous to dupilumab, competitively antagonizing IL-4Ra dependent cell proliferation, mucus induction and eotaxin expression *in vitro*. As PRS-060 displayed exquisite specificity for human IL-4Ra, with no cross-reactivity to rodents or higher primates, we created a new triple-humanized mouse model substituting hIL-4Ra, hIL-4, and hIL-13 at their correct syntenic murine loci to model clinical dosing.

Results: Inhaled PRS-060 strongly suppressed acute allergic inflammation indices in triple humanized mice with a duration of action longer than its bulk clearance suggesting it may act locally in the lung.

Conclusion: Lcn1 can be re-engineered into the Anticalin antagonist PRS-060, exemplifying a new class of inhaled topical, long-acting therapeutic with potential to treat T2 endotype asthma.

Clinical implications: Based on its specificity, efficacy, and favorable predictive kinetic profile, Elarekibep (PRS-060/AZD1402) has advanced to human phase 2 clinical trials, in moderate to severe T2 endotype asthma.

Word count: 245/250

Capsule summary:

Human lung lipocalin-1 can be reengineered into a highly effective antagonist targeting IL-4Ra defining a new class of inhaled biological “Anticalin” medicine that has advanced to clinical trials in T2 endotype asthma.

Key words: T2 endotype asthma; Anticalin protein therapeutic; inhaled IL-4Ra antagonist; topical

79 Abbreviations used:

80 BAL: Bronchoalveolar lavage

81 EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

82 ES: Embryonic stem

83 FCS: Fetal calf serum

84 hr: hour

85 KD: Equilibrium dissociation constant is a measure of binding affinity

86 Koff: Rate of dissociation is a measure of retention at the IL-4Ra receptor

87 Kon: Rate of association is a measure of rapidity of binding to the IL-4Ra receptor

88 Lcn: LipocalinNGAL: Neutrophil gelatinase-associated lipocalin

89 NHS: N-Hydroxysuccinimid

90 PAS: Periodic-acid Schiff

91 PFA: Paraformaldehyde

92 Rt: room temperature

93 RU: Resonance Units

94 SPR: surface plasmon resonance

95 SD: Standard Deviation

96 SEM: Standard error of mean

97 TEER: trans-epithelial electrical resistance

98 TLc: tear lipocalin

99 Wt: wild type

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Introduction

Inhalation therapy is preferred for asthma because it delivers high concentrations of drug to the epithelium and mucosa; limits systemic exposure to improve therapeutic index; allows dose flexibility; and enables combination formulations. IL-4Ra is a highly attractive target for inhaled biologicals because it is clinically proven to drive “T2 endotype” asthma; is highly expressed on the airway epithelium¹, airway smooth muscle and inflammatory mucosal immunocytes; and has known ‘on-target’ biomarkers^{2, 3, 4, 5}. However, attempts to develop inhaled biologics to improve clinical utility and overcome the very low lung bioavailability of monoclonal antibodies^{6, 7}, have been frustrated by technical challenges.

Highly stable human lipocalins⁸ Lcn1 (“Tear” lipocalin, Lipocalin 1) and Lcn2 (NGAL, Lipocalin 2) are both naturally found in human airway periciliary fluid and lung tissue^{9, 10}, suggesting potential utility as inhalable drug scaffolds. Unlike monoclonal antibodies, they have a single-chain polypeptide and require no post-translational modifications. Most importantly, their cup-like structure, which is formed by four variable beta-sheeted loops (Figure 1), provides a binding topology that can be systematically mutated to engage diverse targets^{11, 12}. The resulting molecules are known as Anticalin® proteins. Like native Lcn1 and Lcn2, Anticalin proteins retain their low molecular weight (16-20 kDa), rigid structure and high stability, enabling epithelial saturation and mucosal penetration after aerosol delivery⁸. Several intravenously administered Anticalin proteins have proven effective target engagement and favorable safety and tolerability profiles in human clinical trials, but an inhaled Anticalin protein has never been created^{13, 14, 15, 16, 17}.

Here we demonstrate that cycles of directed protein mutagenesis can be used to successfully reengineer Lcn1 into a high-affinity, neutralizing, and inhalable Anticalin protein able to

124 selectively block IL-4Ra with picomolar affinity, very high specificity, rapid onset of target
125 binding and a slow dissociation rate constant conferring sustained target engagement. We
126 further demonstrate the activity of an optimized Anticalin protein, PRS-060, in *in vitro* cellular
127 assays and *in vivo* in a novel “triple humanized” hIL-4/hIL-13 + hIL-4Ra mouse asthma model.
128 These data reveal inhaled Anticalin proteins, exemplified by PRS-060 for T2-endotype asthma,
129 as a novel inhaled drug class. Based on this preclinical profile, PRS-060 has advanced into
130 human Phase 2 clinical trials.

131

Methods

Full details of methods, in particular protein engineering and the creation of a triple humanised mouse model for pharmacokinetic (PK) and ovalbumin-induced asthma-like immunopathology, are presented in the online repository.

Ethics

Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health) and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Use of human epithelial cells was approved by Munich ethics commission (MD034201) in accord with Declaration of Helsinki on biomedical research (Hong Kong amendment, 1989).

Anticalin library generation

The first step in generating an Anticalin protein is to create a massively diverse library of variants that can be screened for target binding using phage display methods. Briefly, a random library of Lcn1 was prepared by concerted mutagenesis of 18 selected amino acid positions located in the 4 loops of human Lcn1 and eliminating the disulfide bridge, as described^{18, 19}. A phagemid display library comprising of 2.0×10^{10} variants was generated by the PCR randomized codons gene method as described^{20, 18}. This process yielded a starting variant hereafter named “**Lcn1 control**”.

Anticalin protein selection and screening

To identify “lead” binders this library was screened against biotinylated human recombinant IL-4Ra. Briefly, four cycles of filamentous phage display selection (2×10^{12} phagemids) against biotinylated IL-4Ra were performed as described^{21, 22}. Enriched clones were

individually expressed in *E. coli* followed by high-throughput ELISA screening leading to an initial candidate (designated '**PRS-A**') that competitively inhibited the interaction between IL-4Ra and its ligand IL-4. The affinity of the PRS-A variant was further improved in two cycles of in vitro evolution. In the first cycle the central part of the PRS-A coding region was amplified via error-prone PCR^{22, 15}. Key positions were refined using a site-directed PCR strategy with degenerated primers^{18, 19} yielding an intermediate candidate (designated '**PRS-B**').

In a second cycle, key residues in loops 1, 2 and 4 of PRS-B contributing most to IL-4Ra binding were identified by saturation site mutagenesis^{23, 24, 19}. By using lower target protein concentration, longer washing and an elevated temperature (37°C) under increased stringency, and affinity conditions, this further improved affinity of the engineered final candidate designated **PRS-060**^{22, 15}.

Structural modeling

Molecular Operating Environment MOE2019.0102 was used to perform homology modeling of intermediates and PRS-060 and based on the published solution NMR structure of human Lcn1 harboring the C101S mutation (PDB ID: 5T43). The unpaired cysteine at position 101 was replaced by a serine to avoid dimerization via inter-molecular disulfide formation.

In vitro assays

Standard and competition ELISA assays; PCR; surface plasma resonance (SPR) affinity and kinetic assays; STAT6-phosphorylation assays revealing IL-4Ra signaling; IL-4- and IL-13-induced proliferation of TF-1 cells; and IL-13-induced epithelial mucus transdifferentiation, were performed by standard, published methods described in detail in the online supplement.

***In vivo* Mouse Models**

Triple humanized mouse mode

A novel IL-4Ra, IL-4 and IL-13 ‘triple humanized’ knock-in mouse model was established because PRS-060 is highly specific for human IL-4Ra and does not cross react with the mouse orthologue. Briefly, the humanized mouse was generated by syntenic replacement of the mouse *il4ra*, *il4* and *il13* genes with the corresponding human gene using homologous recombination. The method ensures that the human DNA segment is present in the mouse gene as a single copy at the correct chromosomal location. These mice were healthy and reproduced normally. The model was primarily used for *in vivo* investigations in acute models of direct IL-13 challenge and acute allergic airway inflammation after precision lung delivery (intratracheal microspray, PennCentury) described in the Supplement. Direct light sheet ‘glassified’ whole lung optical tomography of Alexa-647 tagged Lcn1 confirmed this method exposes the entire respiratory tract epithelium (see Figure E10 in online repository).

Statistical analysis

GraphPad Prism 8.3.1 was used for statistical analysis. AUC was employed for titration curves. IC50 values were determined using nonlinear, variable slope regression. Data are shown as mean $\pm$ SD or SEM ($n = 3-8$ as stated in the figure legends). The appropriate, parametric, non-parametric and multiple comparison corrected statistical tests are stated in each figure legend. $P < 0.05$ was considered statistically significant and P values are indicated by asterisks (* $P < 0.05$, ** $P < 0.001$, and **** $P < 0.0001$).

Results

Generation of a potent IL-4Ra antagonist for inhaled delivery

Anticalin libraries with more than ten billion different combinatorial variants, were constructed via genetic mutation of human Lcn1 leading to randomization of 18 amino acid positions in the structurally variable loop region (Figure 1A). Additional mutations were introduced during the optimization process to further engineer specificity for IL-4Ra yielding the final candidate, PRS-060, illustrated by overlaying residues of Lcn1 and PRS-060 (Figure 1B). The Anticalin libraries were subjected to several cycles of phage display selection against immobilized recombinant human IL-4Ra leading to several human Lcn1 variants. The initial candidate (“PRS-A”) bound IL-4Ra with a dissociation constant (K_D) of 42 nM and competitively inhibited IL-4 binding to IL-4Ra (see Figures E1A and E1B in online repository). To optimize target binding, additional protein engineering was performed enhancing affinity, and extending the dissociation rate constant (k_{off}) of PRS-A by additional cycles of *in vitro* evolution via error-prone gene amplification and selection under conditions of increasing binding stringency combined with phage display selection under limited target concentration and off-rate selection pressure for binding IL-4Ra. Following this campaign, mutational hot spots were identified in loop 1 and loop 2 (Figure 1A) and the most promising Anticalin protein, (“PRS-B”), exhibited an affinity of 140 pM mainly owing to a 50-fold lower dissociation rate constant (k_{off} $1.01 \times 10^{-4} \text{ s}^{-1}$) and a 6-fold faster association rate constant (k_{on} $7.23 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), compared to PRS-A (see Table E1 in online repository). Site-saturation mutagenesis (22) was performed to identify key residues in loop regions of the intermediate candidate with major impact on binding to IL-4Ra by sequentially permutating the codons coding for selected amino acid positions in loop 1 and loop 2 followed by ELISA screening. Once key binding residues were defined a new library with reduced combinatorial size was generated by re-randomizing seven critical positions (26; 32; 34; 55; 56; 58; 63). This library was subjected to a further cycle of

phage display selection under conditions of increasing stringency yielding the final candidate (“PRS-060”) which displayed 10-fold higher picomolar affinity (K_D 14 pM, k_{on} $3.7 \times 10^6 M^{-1} s^{-1}$, k_{off} $5.2 \times 10^{-5} s^{-1}$, see Table E1 in online repository) and retained rapid onset and, slow-dissociation binding.

PRS-060 dissociated 58-fold more slowly from the IL-4Ra complex and binds with 10-fold higher affinity than the clinically tested IL-4 mutein, pitrakinra (hereafter called “IL-4 mutein”, see Table E1 and Figure E1C in online repository). This binding was highly specific for IL-4Ra with no binding observed to the structurally related cytokine receptors IL-6Ra, IL-18Ra, or IL-23Ra (see Figure E1D in online repository). Indeed, PRS-060 was so exquisitely selective for human IL-4Ra that it showed no cross-reactivity to primate or rodent IL-4Ra (see Figures E2 and E3 in online repository), due to differences in primary sequence compared to the human orthologue (see Figure E4 in online repository) at the sites involved in IL-4/IL-4Ra chain interaction²⁵.

To test biological functionality, PRS-060 was assessed in a broad range of *in vitro* assay systems. Phosphorylation of STAT-6 (Signal transducer and activator of transcription 6) is an essential intermediate in IL-4Ra signaling. The PRS-060-mediated reduction in STAT6 phosphorylation was significantly greater than for the IL-4 mutein and was similar to the anti-IL-4Ra antibody AMG317 (Table IA, Figure 2A and 2B).

The TF1 proliferation assay was used to investigate potency of PRS-060 and reference antagonist molecules IL-4 mutein, AMG317²⁶ and dupilumab²⁷. PRS-060 inhibited TF1 proliferation induced by the cytokines IL-4 and IL-13 completely, was 2-3-fold more potent than the IL-4 mutein and had similar potency as the benchmark anti-IL-4Ra antibodies AMG317 (Table IB, Figure 2C and 2D) and dupilumab (Figure 2E and 2F).

IL-4Ra signaling mediates mucus cell metaplasia in the epithelium leading to an increase in goblet cells linked to airway mucus plugging *in vivo*^{28, 29, 30, 31}. PRS-060 was more than twice as potent as the IL-4 mutein to suppress mucus cell transdifferentiation (Figure 3; Table IC).

Generation of a humanized mouse model for characterization of PRS-060 *in vivo*

Figure 4A (and Figures E5-E7 in online repository) describe the creation of the triple transgenic mouse model. The presence of human *il4ra*, *il4*, *il13* genes was evaluated by PCR (described in the online repository) and verified by southern blot analysis (Figure 4B). To confirm functionality of the receptor on immune cells from the humanized mice, human IL-4 induced phosphorylation of STAT6 was similar in wild type bone marrow derived mast cells and humanized cells when stimulated with 50µg/mL of IL-4 (Figure 4C).

PRS-060 inhibits IL-13-induced eotaxin-1 expression in the lung

Eotaxin-1 (CCL11) expression in the epithelium is a critical driver of eosinophilic mucosal inflammation and is therefore a pharmacodynamic (PD) marker of epithelial IL-4Ra engagement *in vivo*. The effect of PRS-060 on IL-13-induced eotaxin-1 expression in the lungs of humanized mice was assessed to determine *in vivo* potency and duration of action when administered via microsprayer i.t. dosing. Efficient biodistribution of an Alexa-fluor labeled Lcn1 in the large and terminal bronchioles of mice at 1 and 24 hr upon intratracheal administration has been demonstrated using the same device as here (Figure E10 in the online repository). Mice were pre-treated with 2/10/98 µg/mouse i.t. doses of PRS-060 at multiple time points (0.5, 1, 2, 4, 8, or 24 hours) prior to the i.t. challenge of hIL-13 (1 µg) which led to an increase of eotaxin-1 mRNA levels. PRS-060 suppressed eotaxin-1 mRNA by 93% after 30 min. The effect was maintained over time with 79% and 38% inhibition at 8 and 24 hours

respectively ($p < 0.01$) (Figure 4D). This sustained effect is remarkable given the relatively short lung half-life of PRS-060 (~4 hours) and may reflect its sustained engagement of IL-4Ra following saturation topical concentrations and the very slow dissociation rate constant combined with some lung retention at these time points. Indeed, 2 $\mu\text{g}/\text{mouse}$ was as effective as a 10 $\mu\text{g}/\text{mouse}$ dose to significantly suppress eotaxin-1 mRNA over all time points (86%, 73%, 58% and 47% for 0.5, 4, 8, 24 hours respectively, ($p < 0.001$ -0.01, Figure 4D). Importantly at these times of marked suppression, PRS-060 was barely detectable in blood (0.1ng/mL = 0.005 nM were measured at 24 hours) which together with the fact that in vitro we could show biological activity of PRS-060 only at IC₅₀ concentrations of 0.1 nM and 0.9 nM in the pSTAT6 assay and TF1 proliferation, respectively, suggests a topical mode of action.

Intratracheal aerosol pharmacokinetics of PRS-060 in broncho-alveolar lavage (BAL), plasma, and lung tissue in “triple-transgenic” humanized mouse.

The humanized mouse was used to assess pharmacokinetic (PK) and lung biodistribution of PRS-060 after precision intratracheal aerosol delivery to the lungs using a microspray device as described in the methods. Increase in systemic exposure was slightly higher than dose proportional. Pharmacokinetic profiles for plasma, BAL and lung tissue across all dose levels appeared comparable, suggesting similar PK following i.t. administration (Figure 5). At the highest dose level (98 μg per animal) achieved maximal levels (C_{max}) in lung tissue were 85-fold higher compared to plasma (20743 ng/g tissue vs 245 ng/mL respectively) 1 hour after i.t. administration (Table II). PRS-060 was shown to have comparable terminal elimination half-lives of approximately 3 to -4 hours in all three compartments at all dose levels and depending on dose approximately 100- to 245-fold lower exposure (AUC_{inf}) of PRS-060 were recovered in plasma compared to lung tissue (Table II). PRS-060 is rapidly excreted by the kidneys and

the observed clearance rate for PRS-060 after single iv dose to mice is close to the glomerular filtration rate, consistent with renal filtration being the major clearance mechanism. Taken together, these results support the hypothesis that in vivo effects can be achieved by topical engagement rather than systemic exposure. At 24 hours, the time point used to assess effects on acute allergic inflammation, no PRS-060 was detected in plasma at the 2 and 10 μ g doses. Levels in BAL and lung tissue fell dose-proportionally with approximately 100-fold less PRS-060 present at 24 hours compared to 1 hour (Figure 5).

Local lung delivery of PRS-060 suppressed ovalbumin-induced acute allergic inflammation in the humanized mouse

Pre-treatment with PRS-060 significantly reduced total counts of all cell types at the 24 and 48 hr post ovalbumin challenge (71 and 78%, respectively, Figure 6A). While a significant 75% inhibition of eosinophils was observed at 24 hr and by 79% at 48 hr, PRS-060 significantly reduced macrophages (55% and 71%), monocytes, lymphocytes, and neutrophils (77% and undetectable) at the 24 and 48 hr time points respectively (Figure 6A). In addition, administration of PRS-060 reduced the severity of all histological indices of inflammation when compared to the wildtype Lcn1 response at 48 hr while the bronchiolar mucosa showed reduced hyperplasia and metaplasia (confirmed by analysis of PAS+ cells, Figure 6B). Twenty-four hours post ovalbumin challenge pre-treatment with PRS-060 reduced BAL hIL-13, mIL-5, and mTarc by 48%, 43%, and 89% respectively (Figure 6C). In lung tissue, hIL-13, mIL-5, mTarc, and mEotaxin were detectable at both 24- and 48-hours post-ovalbumin challenge and mEotaxin and mTarc levels were significantly reduced 48 hours post challenge ($p < 0.001$ and $p < 0.01$ respectively) by PRS-060 (Figure 6D).

Discussion

In this paper we describe the discovery and characterization of PRS-060, a first-in-class IL-4Ra antagonist for inhalation that was generated by recombinantly engineering Lcn1 whose small molecular size enables efficient epithelial absorption and whose structural robustness confers high tolerance to physical stress during formulation and delivery by inhalation devices. We demonstrate that this approach facilitates high local drug exposure in the respiratory tract, in particular the epithelium where IL-4Ra is abundantly expressed. Indeed, the duration of suppression of IL-4Ra dependent mucosal biology exceeded the apparent PK half-life suggesting sustained receptor occupancy consistent with the engineered low dissociation rate constant (k_{off}) of PRS-060. Our data suggest that mucosal exposure alone may be capable of suppressing T2-driven inflammation *in vivo* and supports the potential utility of PRS-060 as an inhaled therapy for T2-driven asthma.

As a drug template for inhaled therapeutics the lipocalin scaffold has several advantages over other types of biologics including antibodies. Its robust structure based on a single polypeptide chain, and small size, facilitate inhaled delivery to the epithelium and airway submucosa. Native Lcn1 is abundant in normal human airway periciliary fluid and physiologically distributes through the epithelium and submucosa³². From a protein engineering perspective, the structurally flexible loop regions of Lcn1 lend themselves to *in vitro* mutagenesis much like the CDRs of an antibody but with larger binding area. This structural plasticity of the epitope binding regions in these loops was particularly important for optimizing PRS-060 from the initial candidate “PRS-A” because it allowed very rapid, high affinity binding and prolonged receptor occupancy, due to a very low dissociation rate constant, to be engineered into the molecule by evolutionary mutagenesis. The affinity and binding kinetics of PRS-060 towards IL-4Ra is directly comparable to therapeutic monoclonals^{33, 34}. There is no evidence PRS-060

interferes with natural Lipocalin *in vivo* in the triple engineered mice. Coupled with these favorable drug-like properties, PRS-060 was highly specific for hIL-4Ra revealing no detectable binding to related or non-related proteins (e.g., IL-23Ra, IL-6R and IL-18Ra) or non-human orthologues. Accordingly, PRS-060 suppressed all *in vitro* models of IL-4 and IL-13 action, including epithelial mucus cell transdifferentiation with a closely comparable pharmacological profile in all models.

This exquisite specificity necessitated the generation of a “triple transgenic” mouse model which was critical to assess the *in vivo* kinetics and therapeutic efficacy of PRS-060. Using a well-established ovalbumin sensitization and challenge model of acute allergic inflammation³⁵, PRS-060 was highly effective in suppressing asthma-like inflammation. The concentrations of PRS-060 in the lung after intratracheal administration were 84-fold higher than those in plasma. This suggests that PRS-060 may act locally in the lung to suppress lung eosinophilia and tissue histopathology. PRS-060 suppressed acute induction of chemokines after lung challenge with IL-13. This is consistent with the previously reported purely topical activity of inhaled SB010/hgd40, an inhaled DNase that blocks T2 responses at the level of the STAT-6 dependent transcription factor, GATA-3, without any systemic bioavailability^{36, 37}. The inhaled IL-4 mutein, pitrakinra, was also clinically active³⁸. Preclinical studies in an allergen challenged primate model have previously indicated potential superior efficacy of local delivery over subcutaneous of pitrakinra³⁹. However, as pharmacologically active PRS-060 delivered there directly from airway inhalation across the respiratory epithelium and endothelium was detectable in plasma a possible contribution from systemic exposure cannot be formally excluded PRS-060, could for example reach splenic immunocytes and modify the response to inhaled antigen. This could, over time, reduce indices of T2 immunity which has been observed with Dupilumab in clinical practice^{27,40}.

Our present results demonstrate that locally administered PRS-060 engages lung IL-4Ra potently for a sustained duration and strongly suppresses IL-13 and OVA induced inflammatory indices. Clinical practice has unequivocally demonstrated that systemically administered dupilumab, which has a near-equivalent *in vitro* pharmacological antagonist profile to PRS-060, is safe and effective in patients with moderate to severe asthma which is not adequately controlled on current standard of care therapies^{27, 41, 35, 42, 43, 44}. While dupilumab is indicated for subcutaneous (s.c.) administration (300 mg, every two weeks) it is unclear what amount of delivered drug is bioavailable to the respiratory epithelium, although typically less than 1% by mass balance of other IgG monoclonals reach the epithelial lining fluid⁶. However, only human clinical studies can resolve whether systemic therapy is non-inferior to inhaled IL-4Ra blockade for T2 asthma that presents without systemic T2 manifestations, such as atopic dermatitis and nasal polyposis. Furthermore, as PRS-060 is a fully human protein we were limited to short-term studies because the mouse immune system recognizes human lipocalins as foreign proteins. Whereas mouse anti-mouse antibodies which do not elicit cross-species immune responses can now be easily made, there is no existing functional mouse ortholog of the human tear lipocalin and thus no technology to make an analogous “mouse anti-mouse anticalin”. Accordingly, we were restricted to short-term *in vivo* experiments. We therefore focused on pharmacokinetic studies to enable human clinical development where the therapeutic benefit of repeated dosing of PRS-060, and its effects on T2 endotype immunity, can be assessed in clinical asthma in the future.

The data presented herein confirm the potency, selectivity, and therapeutic potential of PRS-060, and support its development as a targeted therapy for patients with T2 endotype asthma, where there remains a significant unmet medical need. On the strength of this preclinical profile, and completing relevant preclinical safety studies, PRS-060 entered clinical development and successful phase I clinical trials in healthy volunteers (NCT03384290)⁴⁵ and

394 in patients with mild asthma (NCT03574805) ⁴⁶ have been concluded. PRS-060, now
395 designated “AZD1402/PRS-060”, is currently being investigated in a Phase 2 clinical trial in
396 patients with moderate asthma (NCT04643158). As the first in a new class of inhaled Anticalin
397 proteins, AZD1402/PRS-060 represents a new opportunity to develop effective inhaled
398 biological treatments for respiratory disease and this innovative new technology may prove
399 useful in a range of respiratory diseases. Indeed phase 1 human clinical trials of PRS-220, an
400 anticalin protein which targets the pro-fibrotic matrikine CTGF (Connective Tissue Growth
401 Factor) have commenced (NCT05473533) and PRS-400 which blocks JAG-1 (Jagged-1 a
402 member of the Notch signaling family), a “master epithelial regulator” of goblet cell vs ciliated
403 cell lineage commitment, with potential utility in diverse muco-secretory diseases, is currently
404 in preclinical development presaging a new class of inhaled biological therapeutics^{47–50}.

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Journal Pre-proof

Table I: A) Inhibition of STAT-6 phosphorylation. The table reports IC₅₀ values for each tested IL-4Ra inhibitor on either 0.1 nM of IL-4- or 1 nM IL-13-induced STAT-6 phosphorylation, an index of IL-4Ra receptor activation. Data are shown as mean $\pm$ SD of 3 replicates. IC₅₀ values of PRS-060 and reference curves were compared using Students t-test, **** p<0.0001, ns not significant. **B) Inhibition of hIL-4 or hIL-13 induced proliferation of TF-1 cells.** The table reports IC₅₀ values for inhibition of IL-4Ra inhibitors for either 0.1 nM hIL-4 or 1 nM hIL-13 induced inhibition of TF1 proliferation. Data are show as mean $\pm$ SD of 3 replicates. **C) Inhibition of hIL-13 induced human epithelial cell mucus transdifferentiation.** The table reports IC₅₀ for each tested IL-4Ra inhibitor on blocking the effects of 10ng/mL of IL-13 on goblet cell transdifferentiation of human bronchial epithelial cells in culture. IC₅₀ values (nM) were obtained by non-linear regression of the concentration-response inhibition curves and are reported as IC₅₀ (nM) with 95% confidence intervals).

A

Treatment	hIL-4 inhibition IC ₅₀ [nM] (SD)	hIL-13 inhibition IC ₅₀ [nM] (SD)
PRS-060	0.12 (0.05)	0.10 (0.01)
IL-4Ra benchmark (AMG317)	0.07 (0.02) ns	0.04 (0.00) ns
IL-4 mutein (pitrakinra)	5.74 (2.40) ****	8.01 (0.45) ****

B

Treatment	hIL-4 inhibition IC ₅₀ [nM] (SD)	hIL-13 inhibition IC ₅₀ [nM] (SD)
PRS-060	0.87 (0.03)	0.92 (0.28)
IL-4Ra benchmark (AMG317)	0.71 (0.13)	0.83 (0.23)
IL-4Ra benchmark (Dupilumab)	0.35 (0.06)	0.30 (0.01)
IL-4 mutein	2.10 (0.54)	3.07 (0.00)

C

Treatment	IC ₅₀ [nM] (95% CI)	R ²
PRS-060	5.3 (3.8 - 7.4)	0.98
IL-4 mutein	12.9 (11.2 - 14.9)	0.99

Table II: Multicompartment pharmacokinetics (PK) of precision aerosol delivered PRS-060 in triple transgenic mice *in vivo*. The table reports standard PK parameters of PRS-060 in plasma, BAL, and lung tissue after single aerosol doses delivered by precision microspray directly into the trachea of anaesthetized triple transgenic mice *in vivo*. AUC_{inf} was calculated as area under the mean plasma concentration versus time curve from time zero extrapolated to infinity.

Compartment	Dose level [μg]	C _{max} [ng/mL]	T _{max} [h]	AUC _{inf} [h·ng/mL]	Terminal t _{1/2} [h]
Plasma	2	2	0.5	17.5	3.5
	10	17	0.5	131.9	2.7
	98	245	1.0	1749.0	2.8
BAL	2	N.d.	0.5	N.d	3.4
	10	N.d	0.5	N.d	3.9
	98	N.d	1.0	N.d	4.8
Lung tissue	2	889	0.5	4292.0	3.2
	10	2376	0.5	17741.5	4.2
	98	20734	1.0	171368.5	4.0

Figure Legends

Figure 1. Structural models comparing Lcn1 (wildtype) and intermediates (PRS-A, PRS-B) to PRS-060 showing evolution of residue variations. (A) Structural evolution of PRS-060 from Lcn1 via intermediates (PRS-A, PRS-B) (A: top side view) Lcn1 represents the cup-like structured scaffold protein composed of a beta-sheet barrel (blue) that confers to the molecule's robustness and stability and the four flexible loops (L1-L4) on top that tolerate introducing sequence diversity to engineer new target specificity. Red and orange spheres: peptide randomization sites; blue: retained substitutions; yellow sticks: disulfide bonds. **(B side view)** Superposition of Lcn1 and PRS-060 aligned at C α atoms. Dark red and light blue sticks: residues conferring specificity for IL-4Ra in PRS-060.

Figure 2. Comparative inhibition of TF1 cell proliferation or STAT6 phosphorylation by PRS-060 and references. STAT6: (A) vs 0.1 nM hIL-4; (B) vs 1 nM hIL-13. Proliferation: The hIL-4 (1 nM) (C, E) vs hIL-4 (1nM) (D, F) vs hIL-13 (10 nM). MFI: mean fluorescence intensity. Mean $\pm$ SEM.

Figure 3. Inhibition of IL-13 induced goblet cell metaplasia. (A) Representative images of Alcian blue (AB)-stained ALI cultures after treatment with six different doses of PRS-060 (Anticalin). **(B)** % AB positive cells per total image area were quantified using ImageJ. The inhibitory effect of PRS-060 (blue curve) compared to IL-4 mutein (red curve) is shown. PRS-060 IC₅₀ = 5.33 nM vs IL-4 mutein IC₅₀ = 12.92 nM. Mean values of n=3 $\pm$ SD.

Figure 4. Inhibition of hIL-13 induced eotaxin expression in hIL-4RA/hIL-4/hIL-13 triple transgenic mice. (A) A triple transgenic murine system created by syntenic replacement of segments of the mouse genome for IL4, IL13 and IL4R with the corresponding segments of the human genome in mice with a C129 genetic background were generated. Intercrossing of the two mouse strains led to mice homozygous for both humanized loci. Yellow = Il4r, red = IL4R, blue = IL4/IL13, orange = Il4/Il13; IL4/IL13 or rather Il4/Il13 are indicated by one color due to their localization proximity on the same chromosome. **(B)** Southern blot analysis of transgenic DNA prepared from tail biopsies (n = 5) from mice expected to be homozygous for the human IL4R and human IL13/IL4 loci are shown. Probes have been prepared that correspond to the mouse DNA immediately adjacent to the regions humanized on chromosome 7 (IL4R) and chromosome 11 (IL13/IL4). These probes detect a segment of DNA that spans the transition from mouse to human at each locus. The size of the fragment detected by the probe is different in the humanized mice from the fragment detected in the parental 129 mouse DNA and the parental embryonic stem cell (ES) DNA. **(C)** Bone marrow derived mast cells from triple transgenic mice or wildtype were stimulated with 50 ng/mL of either human or murine IL-4 in vitro for 15 minutes and phosphorylation of STAT6 was analyzed by western immunoblotting. **(D)** Suppression of hIL-13 -induced CCL11 mRNA expression in vivo: PRS-060 (2 or 10 µg/mouse, i.t) (n=5, mean ± SEM; **** p<0.0001 ANOVA).

Figure 5. Pharmacokinetic properties and biodistribution of PRS-060 in human IL-4RA/human IL-4/IL-13 knock-in mice after intratracheal instillation. Levels of PRS-060 were measured in **(A)**, plasma **(A)**, BAL fluid **(B)** and, lung tissue **(C)** at different time points between 0.5h and 24h following i.t. administration of three different doses (red circle 2 µg, blue square 10 µg and green triangle 98 µg per mouse, respectively).

678

679 **Figure 6. PRS-060 PK-PD coupling in acute Ovalbumin (OA)-induced inflammation at**
680 **24 and 48h. A) BAL cells B) H&E histology at 48h. Inset: PAS staining C) BAL cytokines.**
681 **(D) lung tissue cytokine. * $p < 0.05$ and ** $p < 0.001$, Unpaired two tailed t-test. mean $\pm$ SEM,**
682 **n = 9-11.**

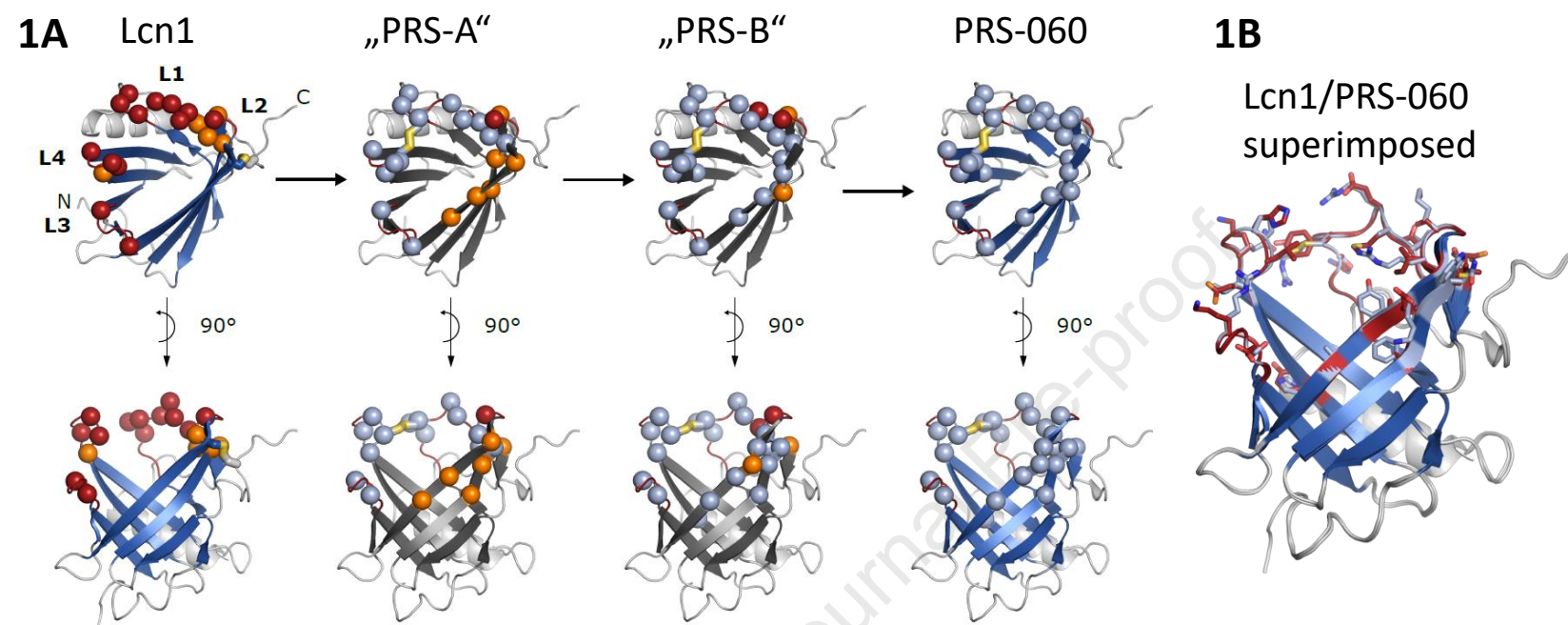


Figure 1

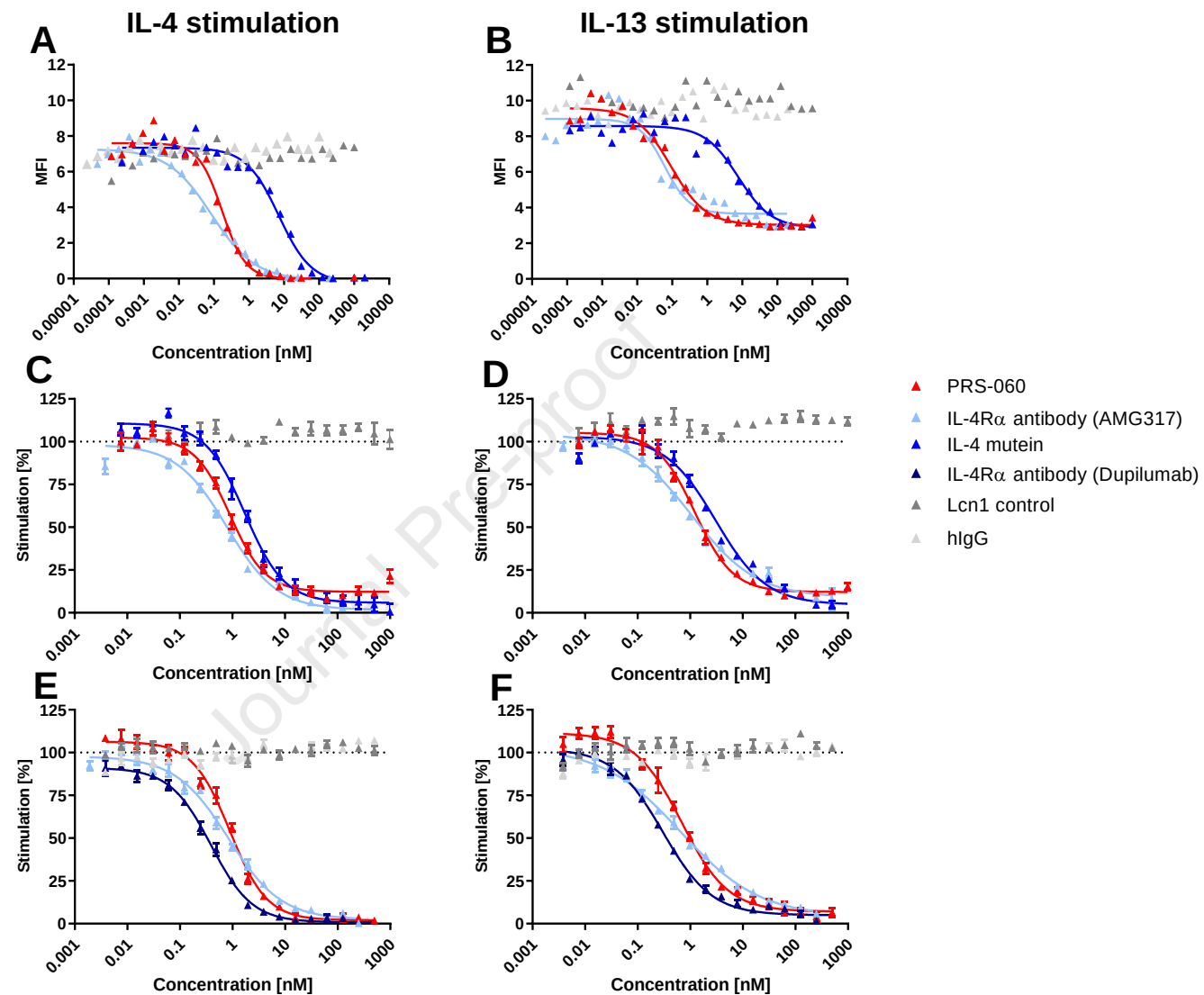


Figure 2

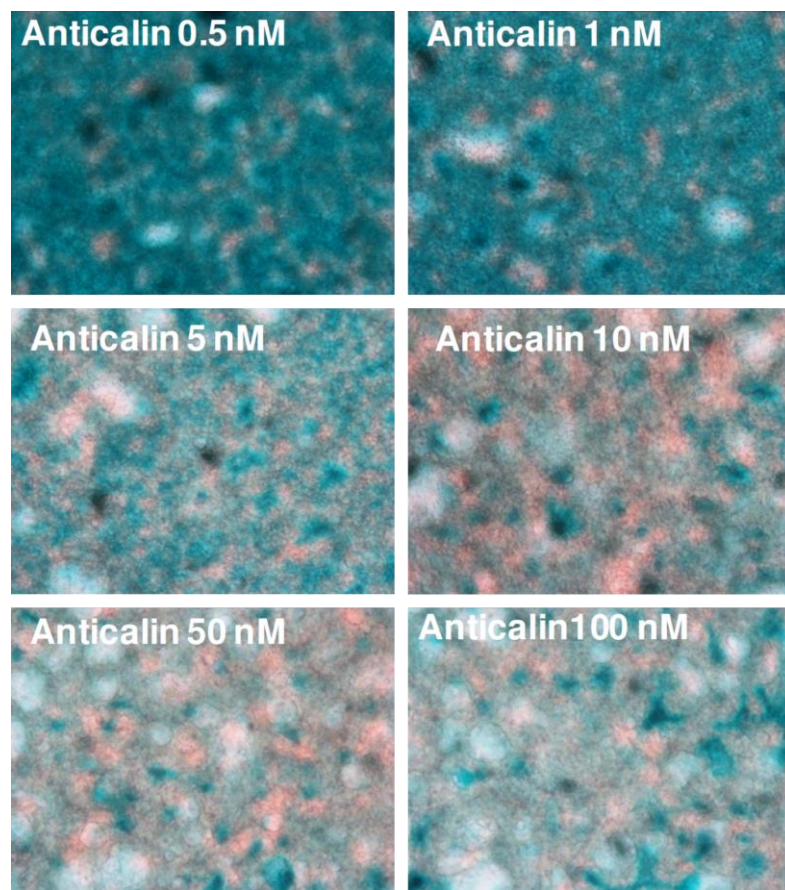
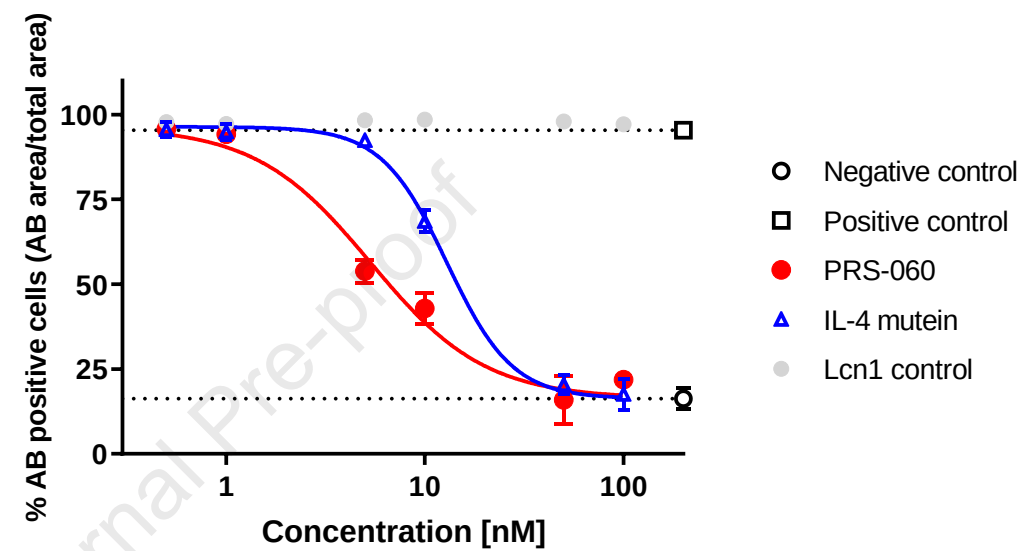
A**B**

Figure 3_revised

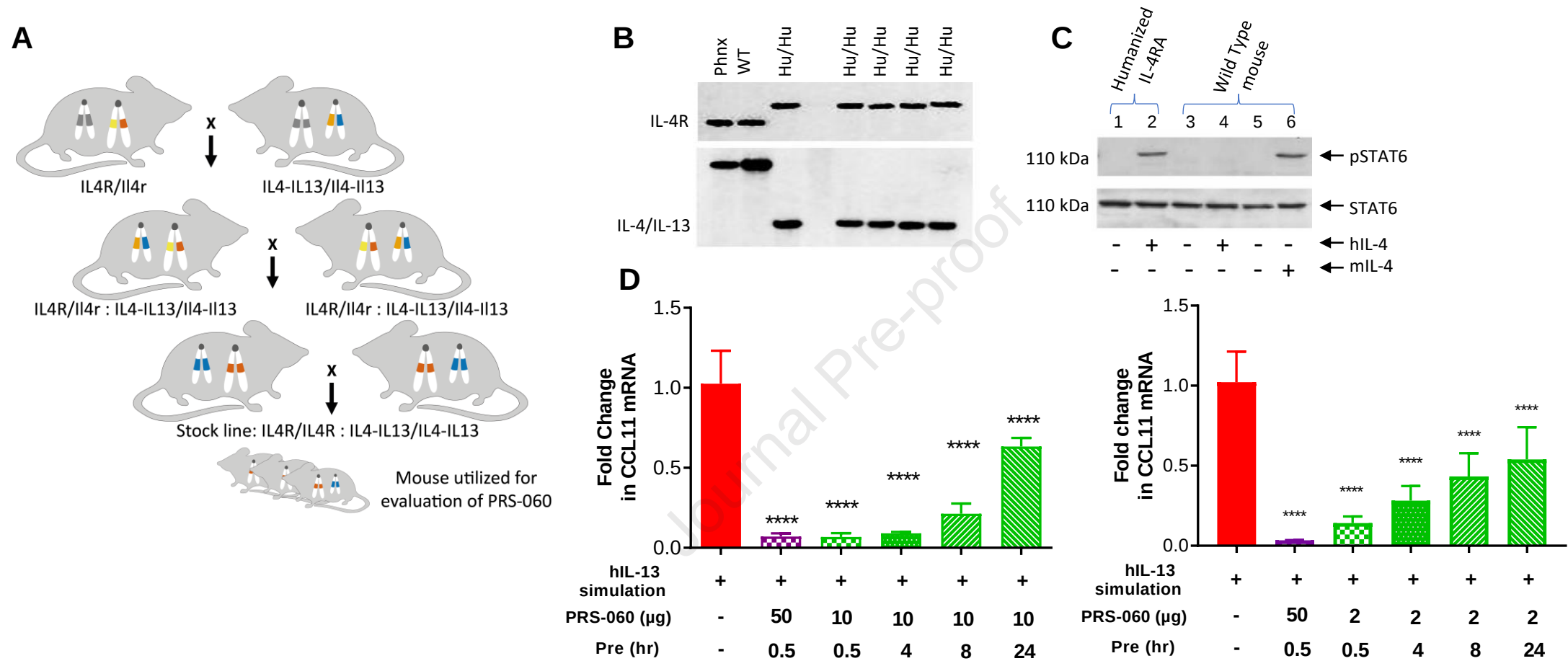


Figure 4

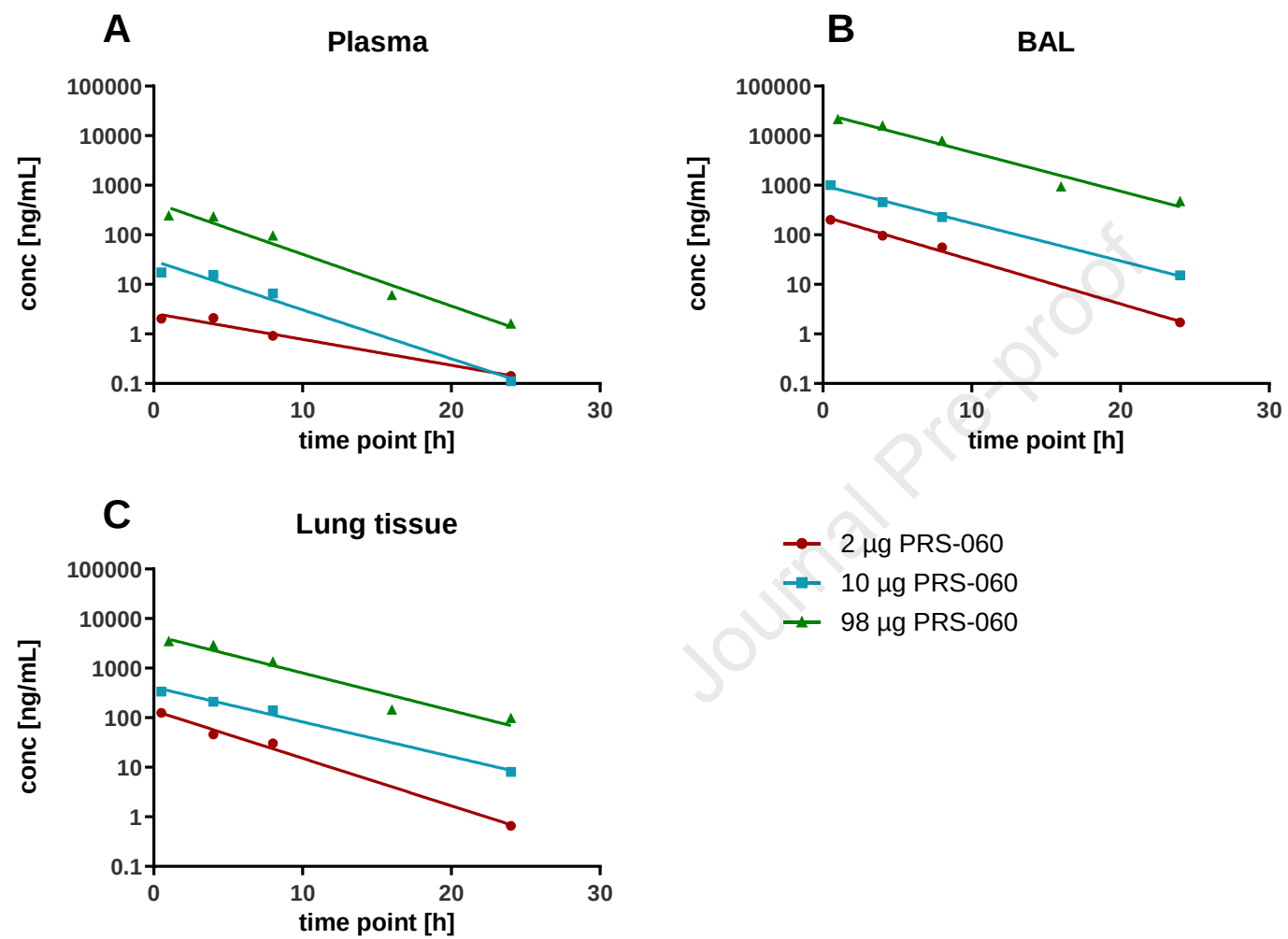


Figure 5

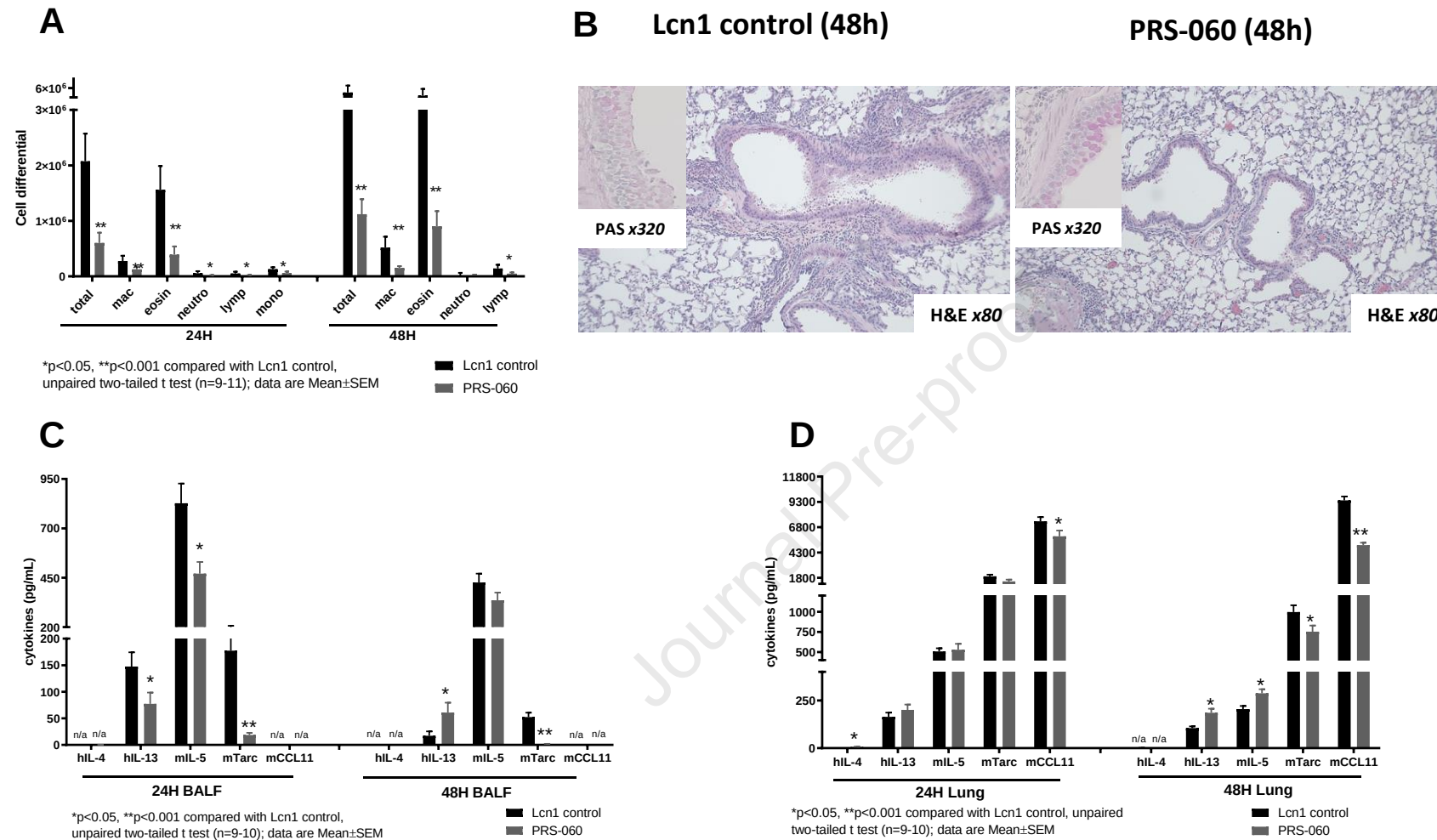


Figure 6