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Regulation of bronchial epithelial barrier integrity by type 2 cytokines and histone deacetylases in asthma

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ABSTRACT

Background: Tight junctions (TJs) form a barrier on the apical side of neighboring epithelial cells in bronchial mucosa. Changes in their integrity may play a role in asthma pathogenesis by enabling the paracellular influx of allergens, toxins and microbes to the submucosal tissue.

Objective: The regulation of bronchial epithelial TJs by T_H2 cells and their cytokines as well as their involvement in the epigenetic regulation of barrier function were investigated.

Methods: The expression, regulation and function of TJs were determined in air-liquid interface (ALI) cultures of control and asthmatic primary human bronchial epithelial cells (HBEC), by the analyzes of transepithelial electrical resistance (TER), paracellular flux, mRNA expression, Western Blots and immunofluorescence staining.

Results: Asthmatic HBEC showed a significantly low TJ integrity in ALIs compared to healthy HBEC. T_H2 cells and their cytokines, IL-4 and IL-13 decreased barrier integrity in ALIs of control, but not in asthmatic HBEC. They induced a physical separation of the TJs of adjacent cells in immunoflurescence staining of the TJ molecules, occludin and zonula occludens-1. We observed that the expression of histone deacetylases (HDACs) 1 and 9, and sirtuin (SIRTs) 6 and 7 were significantly high in asthmatic HBEC. IL-4 and IL-13 significantly increased the expression of HDACs and SIRTs. The role of HDAC activation on epithelial barrier leakiness was confirmed by HDAC inhibition, which improved the barrier integrity by increased synthesis of TJ molecules in asthmatic epithelium to the level of control HBEC.

Conclusion: Our data demonstrate that barrier leakiness in asthma is induced by T_H2 cells, IL-4 and IL-13 and HDAC activity. The inhibition of endogenous HDAC activity reconstitutes defective barrier by increasing the expression of TJs.

Clinical implications:

Decreased TJ integrity and altered bronchial epithelial barrier by T_H2 cells and their cytokines in asthma may play a role in increased exposure to environmental factors such as allergens to the inner tissues and contribute asthma pathogenesis.

Capsule summary:

This study describes that T_H2 cells and their cytokines IL-4 and IL-13 decrease barrier integrity of HBEC. The inhibition of HDAC activity reconstituted the epithelial barrier due to increased mRNA and protein expression of tight junctions.

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Key words: asthma, bronchial epithelial cells, epigenetic, tight junctions, T_H2 cells, IL-4, IL-13, histone deacetylase

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Abbreviations:

ALI: air-liquid interface,
AIA: aspirin induced asthma
ATA: aspirin tolerant asthma
BEGM: bronchial epithelial growth medium,
COPD: chronic obstructive pulmonary disease,
EGF: epidermal growth factor,
FITC: fluorescein isothiocyanate,
HDAC: histone deacetylase,
JAM: junctional adhesion molecules,
HBEC: human bronchial epithelial cells,
SIRT: silent information regulator genes (sirtuins),
TER: transepithelial electric resistance,
T_H2: type 2 helper T cell,
TJs: tight junctions,
ZO: zonula ocludens,

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INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by their reversible obstruction.[1, 2] Type 2 inflammation with the major role of T_H2 cells and their cytokines is considered a major pathophysiological mechanisms of asthma.[3, 4] T_H2 cells produce a pattern of cytokines, such as IL-4, IL-5, IL-13, which cooperate in T_H2 cell differentiation, IgE isotype switching and production, tissue and blood eosinophilia, T cell and eosinophil homing to tissues and airway hyperresponsiveness.[4]

Airway epithelial cells constitute the first line barrier to prevent penetration of environmental agents such as allergens and pollutants to the inner tissues, and they mount an innate immune response against infectious agents.[5-7] The epithelial barrier is predominantly formed by tight junctions (TJs) located at the most apical part of the intracellular junctional complexes between the neighboring epithelial cells.[8, 9] They consist of transmembrane proteins: claudins, occludin, junctional adhesion molecules (JAMs), peripheral membrane proteins e.g. zonula occludens (ZO-1, ZO-2, ZO-3) and cingulin, gap and adherens junctions, which allow the transmembrane proteins to organize in the membrane and attach to the cytoskeleton.[8, 9]

First observation of differences in TJ structures in asthma were performed by electron microscopy in 1988 showing that varying degrees of TJ abnormalities were observed in bronchial epithelium of normal, bronchitic and asthmatic subjects.[10] Recent studies have shown that barrier function of the epithelium is impaired in various inflammatory diseases including asthma, atopic dermatitis and chronic rhinosinusitis.[11-16] Disruption of the bronchial epithelial TJ integrity leads to the loss of barrier function, enabling passage of pro-inflammatory and tissue-damaging agents such as allergens and toxins from the lumen into the airway parenchyma.[17]

Epigenetic changes are crucial for the development and differentiation of various cell types and can regulate essential genes, which are known to initiate and maintain asthmatic inflammation.[18-20] The mechanisms of epigenetic regulation comprise the involvement of several families of enzymes, an umbrella of biochemical reactions such as chromatin modifications on histones and other structures and DNA methylation.[21,22] The balance between the actions of these enzymes is a very crucial mechanism for the regulation of gene expression by controlling numerous developmental processes and disease states.[23, 24] Increased expression of proinflammatory genes is regulated by acetylation of core histones, particularly by

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histone acetyltransferases. In contrast, histone deacetylases (HDAC) remove the acetyl groups from hyperacetylated histones, which leads to suppression of gene transcription.[25, 26] They are classified in five groups. Class I includes HDAC1, 2, 3 and 8, class IIa contains HDAC4, 5, 7 and 9, class IIb consists of HDAC6, and 10, class III comprises of sirtuins (SIRT) (1-7) and class IV includes only HDAC11.[27, 28]

The aim of the present study was to investigate the regulation, expression and function of TJs of bronchial epithelial cells in asthma in response to T_H2 cells and their related cytokines, and those present in the epithelial cell microenvironment such as IL-4, IL-5, IL-13, IL-25, IL-33 and TSLP. We hypothesized that there is a relationship between leakiness in epithelium and increased activity of HDAC in asthmatic individuals. Our data demonstrated a mechanism for leakiness in the epithelium in response to T_H2 stimuli and a novel role for IL-4 and IL-13 in increased HDAC expression. The inhibition of HDAC activity reconstitutes barrier integrity in bronchial epithelial cells from asthmatic individuals.

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Methods

Study subjects

Bronchoscopy with epithelial cell brushings was performed on 27 subjects (9 control subjects, 18 patients with asthma). Clinical characteristics of the study participants are presented in Table E1 (Table E1 in the Online Repository). Samples were collected at the University Hospital, Jagiellonian University Medical College, Krakow and the University Hospital Freiburg, Department of Pneumology, Freiburg, Germany. The diagnosis of asthma was performed by a pulmonologist or allergologist according to GINA guidelines. All asthmatics and control subjects were non-smokers. The study was approved by the University Bioethics Committee (KBET/209/B/2011) and the study subjects gave their written consent to participate.

Air-liquid interface cultures

Primary human bronchial epithelial cells (HBEC) from bronchial brushings of asthma patients and control individuals were grown in bronchial epithelial basal medium (BEBM, Lonza) supplemented with the Single Qu ot kit, in T-flask 75 cm^2, in a humidified incubator at 37^0C in 5% CO2. HBEC obtained from controls and asthmatic subjects in passages 2 or 3 were seeded in air liquid interface (ALI) cultures, onto 6.5 mm-diameter polyester membranes with a pore size of 0.4 µm (growth area 0.33 cm^2) (Costar; Corning Incorporated, Corning, NY, USA) at a density of 1.5×10^5 cells per well. BEGM medium supplemented with the Single Quot kit except for retinoic acid and triiodothyronine (Lonza), was mixed 1:1 with Dulbecco's modified Eagle medium (Gibco-BRL, Invitrogen). Fresh all-trans retinoic acid (ATRA) (Sigma-Aldrich, Saint Louis, USA) was supplemented at a concentration of 10 ng/ml and medium was changed every other day. Cells were grown submerged for 5 days to complete confluence, then apical medium was removed and BEGM-DMEM medium at a ratio of 1:1 with ATRA was added basolaterally to differentiate these cells. Experiments were performed between 21-28 days of ALI cultures. Six out of 12 primary cell lines each for asthma and healthy control were used for T_H2 cells and ALI co-culture experiments, other 6 were used for stimulation with T_H2-derived cytokines.

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Cytokine stimulation, co-cultures

ALI cultures were stimulated with different cytokines to the basolateral compartment in the above mentioned BEGM medium. IL-4, (Novartis), IL-5 (PeproTech), IL-13 (eBioscence), IL-25, IL-33, and TSLP (R&D Systems) were all used at concentrations 10 ng/ml and 50 ng/ml. For the blocking experiments, we used anti-IL-4 antibody (2 µg/ml) and anti-IL-13 (3 µg/ml) (both from R&D systems) in the optimum dose according to manufacturer's instructions. These doses were sufficient to neutralize the TER-decreasing effect of exogenously added IL-4 and IL-13 in ALI cultures of control HBECs. T_H2 cells were *in vitro* differentiated from naive T cells into T_H2 cells as described previously.[29, 30] BEGM medium was mixed 1:1 with complete RPMI-1640 medium (Sigma-Aldrich), containing 10 % FCS and antibiotics, for co-cultured HBEC cells and T_H2 cells. HBEC were co-cultured with T_H2 cells, at a concentration of 1×10^5 and 5×10^5 cells per well, added and to the basolateral compartment in the same total volume of 0.6 ml. T_H2 cells were stimulated with anti-CD28 (125 ng/ml), anti-CD3 (125 ng/ml), anti-CD2 (125 ng/ml) and IL-2 (100 U/ml). As a control, epithelial cells alone were cultured using the same conditions.

Inhibition of HDACs

Control and asthmatic HBEC cultured in ALI were treated with the HDAC inhibitor JNJ-26481585 (ApexBio) diluted in BEGM-DMEM medium at different concentrations including 5 nM, 10 nM, 50 nM and 100 nM in basolateral compartment.

Transepithelial electric resistance and paracellular flux measurement

Transepithelial electric resistance (TER) of the HBEC, grown in ALI cultures, was measured daily using the Milicell-ERS volt–ohm meter (Milipore) and results were presented as $\Omega\times cm^2$. Samples from each ALI culture donor were measured in duplicates in multiple well systems. Paracellular flux across the epithelium was measured when fluorescein isothiocyanate (FITC) labeled 4 kDa dextran (2 mg/ml) (Sigma-Aldich) was added apically. 2 hours and 24 hours after addition, 2 hours and 24 hours paracellular flux experiments gave similar results. FITC fluorescence in basolateral medium was assessed with a fluorescence reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm.

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mRNA isolation and RT-PCR

mRNA expression of bronchial epithelial cells of ALIs was analyzed as described earlier.[12] Taqman Low Density Array Micro Fluidic Card PCR (Applied Biosystems, Foster City, Calif.) was performed as described.[31] In the fluidic card PCR EF1α was used as the housekeeping reference gene. The usage of EF1α and GAPDH as a control did not change the results.

Relative quantification was done on a ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and the $2^{-\Delta\Delta CT}$ formula as described previously.[31]

Immunofluorescence staining of TJs

ALI cultures were fixed with 4% paraformaldehyde (Fluka) at 48 hours or 72 hours as described in the results. ALI samples were blocked with goat serum in 1% BSA/PBS and stained for TJs: occludin (mouse anti-occludin antibody, Alexa Fluor 488, 100 µg/ml, Invitrogen) ZO-1 (rabbit, anti-ZO-1 unlabeled antibody, Invitrogen), detected by goat anti-rabbit antibody Alexa Fluor 546 or Alexa Fluor 633 (Invitrogen), claudin-4 (mouse anti-claudin-4 antibody, Invitrogen), detected by goat anti-mouse antibody Alexa Fluor 546, e-cadherin (rabbit anti-e-cadherin antibody, Alexa Fluor 555, Cell Signaling), vimentin (monoclonal mouse anti-vimentin antibody, Dako), detected by goat anti-mouse antibody Alexa Fluor 488 (Invitrogen), HDACs: HDAC1 (rabbit, polyclonal anti-HDAC1 antibody, abcam), HDAC2 (mouse monolclonal anti-HDAC2 antibody, abcam), HDAC7 (rabbit polyclonal anti-HDAC7 antibody, abcam), HDAC9 (rabbit polyclonal anti-HDAC9 antibody, abcam), SIRTs: SIRT1 (mouse monoclonal anti-SIRT1 antibody, abcam), SIRT6 (mouse monoclonal anti-SIRT6 antibody, abcam) detected by goat anti-mouse antibody Alexa Fluor 488 (Invitrogen) and SIRT7 (rabbit polyclonal anti-SIRT7 antibody, abcam), detected by goat anti-rabbit antibody Alexa Fluor 546 (Invitrogen). Samples were mount in the mounting medium ProLong Gold with DAPI (Life Technologies). Samples were analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland). While IMARIS software (Bitplane) was used for three-dimentional visualization of TJs in HBEC and for the calculation of expression intensity.

HDAC activity assay

HBEC from controls and asthmatic donors were cultured in T-flask 75 cm^2 or a 6-well plate, and after 7 days the cells were collected and processed according to the

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manufacturer`s instructions for the EpiQuik™ nuclear extraction kit I (Epigentek) and Epigenase™ HDAC activity direct assay kit (colorimetric) (Epigentek). HDAC activity and the percentage of its inhibition was calculated according to manufacturer`s instructions.

Western Blot

Western Blot was performed as previously described.[32] Briefly, equal amounts of lysate proteins (20 µg) were resolved on a 4-20% Mini-PROTEAN®TGX™ Gel (BIO-RAD) using 10×Tris/Glycine/SDS and deionized H_2O running buffer (BIO-RAD) and then transblotted to the nitrocellulose membranes using Trans-Blot Turbo Blotting System (BIO-RAD). The membranes were blocked with 5% nonfat skimmed milk in PBS containing 0.1% Tween 20 for 1 h and then incubated with the primary antibody overnight at 4^{o}C. Primary antibodies were the same as in immunofluorescence staining. The membranes were subsequently washed and then incubated with a specific secondary antibody conjugated with HRP for 1h (anti-rabbit HRP, anti-mouse HRP, Jackson Immuno Research Laboratories). The blots were developed using the SuperSignal West Femto kit (Thermo Scientific) and visualized on the Luminescent Image Analyzer LAS-1000 (FUJIFilm).

Statistical analysis

The data were analyzed with with GraphPad Prism Version 5 software (GraphPad Software, La Jolla, California, USA). Differences between independent variables were evaluated using the Mann-Whitney *U* test. For differences between paired values the Wilcoxon Rank Sum test and Two-way ANOVA were used. The results were considered as significant at $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***), $P<0.0001$ (****).

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RESULTS

Decreased bronchial epithelial cell TJ integrity in asthma and the role of T_H2 cells

We first investigated whether there was a difference in the TJ barrier integrity of human primary bronchial epithelial cells (HBEC) isolated from patients with asthma and the control subjects. The bronchial epithelial cells from asthmatic patients showed a significantly lower transepithelial electric resistance (TER) compared to controls during the first 7 days development of air-liquid interface (ALI) cultures, as well as after long term cultures (Figure 1A, B). Since asthma is a predominantly T_H2 cytokine dominated disease, we then decided to co-culture T_H2 cells with HBEC to investigate the effect of the T_H2 cells. Control HBEC showed decreased TER after 48 and 72 hours in ALI co-cultured with T_H2 cells. In contrast, ALI cultures of epithelium from asthmatics had an initial low TER, which did not further decrease in response to T_H2 cells (Figure 1C). Measurement of paracellular passage of FITC-labelled 4kD dextran confirmed the link between barrier integrity and TER results, and was significantly increased by T_H2 cells in HBEC from control subjects (Figure 1D). T_H2 cells co-cultured with control HBEC, showed separation and opening of the TJs between neighboring cells, which was illustrated with video of immunofluorescence staining. HBEC from asthmatic individuals already showed a reduced expression of occludin and ZO-1 compared to controls and did not respond to T_H2 cells (Figure 1E, see supplementary video E1, E2 in the Online Repository).

Next, we profiled the mRNA expression of the whole epithelial junction-related complex (TJ molecules, adaptor proteins, gap and adherens junction) in ALI cultures of HBEC from asthma patients and control individuals without any stimulation (see Figure E1 in the Online Repository). We found that claudin-4 and adaptor proteins, gap and adherens junctions, such as plactoglobin, nectin-1, nectin-2 and connexin-26, were significantly low expressed in asthmatic HBEC compared to control epithelium.

IL-4 and IL-13 decrease the TJ integrity of bronchial epithelial cells

During our investigations to identify the responsible factor of Th2 cells that decreases barrier integrity, we demonstrated that, IL-4 and IL-13, the two main T_H2 cytokines induced a significant decrease in TER in control, but not in asthmatic HBEC (Figure 2A). The measurement of paracellular flux was significantly increased by IL-4 and IL-

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13 in parallel cultures of healthy control cells confirming the TER results (Figure 2B). The effects of IL-4 and IL-13 on TJs were also verified by using immunofluorescence staining, presented also in the video, which demonstrated that ALI cultures displayed openings of the TJs between neighboring cells. There was already a weak expression of occludin and ZO-1 in ALI of HBECs with asthmatic origin without any further change by IL-4 and IL-13 (Figure 2C, see supplementary video E3 in the Online Repository). Removal of IL-4 and IL-13 from the culture leads to recovery of TER in bronchial epithelial cells of control individuals (see Figure E2 in the Online Repository). Blocking of IL-4 and IL-13 did not show any significant difference in TER measurement in bronchial epithelial cells from asthma patients (see Figure E3 in the Online Repository). Other T_H2-milieu cytokines such as IL-5, IL-25, IL-33 and TSLP did not induce any change in TER in HBECs of control and asthmatic individuals (see Figure E4 in the Online Repository).

Histone deacetylases and sirtuins are upregulated by IL-4 and IL-13

It was tempting to investigate whether barrier defect of bronchial epithelial cells induced by T_H2 cells and cytokines in asthma is associated with epigenetic processes. Accordingly, HDAC expression in bronchial epithelial cells showed potent regulation patterns in response to IL-4 and IL-13. Most of the HDACs were significantly upregulated both in control and asthma patients upon IL-4 and IL-13, which was confirmed on mRNA (Figure 3A) and protein expression, levels (Figure 3B). After demonstration of their effects on HDACs, we focused more on regulation of HDAC expression and activity in the present study. Accordingly, we investigated the cellular localization of HDACs and SIRTs in ALIs and observed that HDAC1, HDAC2, SIRT1, SIRT6 and SIRT7 were localized in the nucleus, and HDAC7 and HDAC9 in the cytoplasm of HBECs (see Figure E5A in the Online Repository). As an important finding, higher mRNA expression of HDAC1 and 9 was demonstrated in bronchial epithelial cells from asthmatic donors compared to controls (Figure 3A, see Figure E5B in the Online Repository). Western Blot of the ALI cultures showed a significant increase in HDAC1 and 2 protein expressions in control but not in asthmatic ALI cultures, and HDAC7 expression was observed in control and asthmatic epithelium either by IL-4 or IL-13.

Next, we investigated the expression of SIRT family of HDACs, which are considered to be regulatory genes of different biological functions, such as aging, metabolism and stress resistance.[33] Similar to HDAC 1 and 9, higher expressions of

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SIRT 6 and 7 were found in resting conditions of HBECs in asthmatic patients (see Figure E5, E6 in the Online Repository). SIRT 1, 2, 4, 5, 6 and 7 were upregulated by IL-4 and IL-13. Additionally, SIRT 1 and 5 were significantly higher expressed upon IL-13 stimulation in control individuals. These data demonstrate an essential role for IL-4 and IL-13 in the regulation of HDAC and SIRT family of enzymes with epigenetic properties.

Reconstitution of asthmatic bronchial epithelial barrier integrity by the inhibition of HDACs

We further analyzed whether there is functional relevance of increased mRNA and protein expression of HDACs in asthma. As initial evidence suggesting this relationship, we observed a significantly increased HDAC activity in bronchial epithelial cells from asthmatic patients (Figure 4A). This was followed by the investigation of the effects of inhibition of HDAC activity in asthma. Following these experiments, ALI cultures were treated with a global HDAC inhibitor, JNJ-26481585 to demonstrate the role of the regulation of HDACs by the T_H2 response on barrier function. The HDAC inhibitory activity of this compound was confirmed in epithelial cells in comparison to another widely known HDAC inhibitor, Trichostatin A (see Figure E7 in the Online Repository). HDAC inhibition significantly increased TER in bronchial epithelial cells particularly in ALI from asthmatic donors (Figure 4B), but we did not observe any difference between aspirin tolerant (ATA) and aspirin induced (AIA) asthma patients (see Figure E8 in the Online Repository). Reconstitution of TER in asthmatic ALI was associated with an increased expression of occludin and ZO-1 proteins with an intact and honeycomb-like structure in a dose dependent manner by immunofluorescence staining (Figure 4C, D).

During the recovery of epithelial barrier function by HDAC inhibition, the expression of e-cadherin (see Figure E9A in the Online Repository), which plays a role in cell binding, and tissue formation and major intermediate filament-vimentin were also upregulated (see Figure E10A in the Online Repository). In parallel, the expression of TJs occludin, claudin-1, claudin-4, claudin-7 and ZO-1 increased in a dose-dependent manner by HDAC inhibition (Figure 4E, see Figure E9B in the Online Repository). In addition, genes, such as epidermal growth factor receptor (EGFR), amphiregulin and Ki67, which are linked to the growth and regeneration of epithelial cells showed increased expression after HDAC inhibition, suggesting

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increased activation and proliferation of epithelial cells (see Figure E10B in the Online Repository).

Then, the question was posed whether HDAC inhibition induces stable barrier integrity in asthmatic HBECs to protect from IL-4 and IL-13-induced damage. HDAC inhibition from the start of cultures significantly increased TER in ALI cultures of asthmatic HBECs (Figure 5 A, B, C). Normal HBECs did not show any further increase. Afterwards, the HDAC inhibitor was washed away and the ALI cultures were stimulated with IL-4 and IL-13. Stimulation with these T_H2 cytokines significantly decreased TER in control epithelial cells, but asthmatic epithelial cells stayed at the same level and did not decrease to initial low TER levels. (B) Interestingly, the initial effect of TER increase in epithelial cells of asthma patients after treatment with HDAC inhibitor-JNJ-26481585 remained significantly high in the first 24 h. Although statistical significance disappeared after 48 and 72 h, IL-4 and IL-13 combination did not decrease the TER fully to the initial levels. These data suggest that the increased TER by HDAC inhibition is partially sustained and cannot be rapidly and fully decreased by Th2 cytokines. These data demonstrate that a protective effect against T_H2 cytokines is observed on the TJ barrier of asthmatic epithelium by HDAC inhibition. (C) TER was decreased in epithelial cells from control individuals upon IL-4 or IL-13 stimulation, but this effect was reversed when cytokines were added together with the HDAC inhibitor-JNJ-26481585. In contrast, IL-4 and IL-13 alone or in combination with HDAC inhibition did not influence TER measurement in epithelial cells form asthmatic individuals.

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Discussion

Although it is a heterogenous disease with several phenotypes and endotypes, the majority of asthma cases have been linked to type 2 inflammation[34, 35] with a strong evidence that T_H2 cells can initiate and maintain key pathophysiological features through the production of cytokines such as IL-4, IL-5 and IL-13.[4, 36] The barrier integrity of bronchial epithelial cells is a very important component of the defense mechanisms of the airways.[17] Reduced epithelial barrier function might increase the susceptibility to allergen sensitization and lower the threshold of exposure to a certain allergen dose, which is required to drive local antigen-dependent inflammation.[37, 38]

The impairment of this protective function of TJs was suggested in patients with asthma, as a potential causative factor in asthma development. These studies were reviewed by Georas and Rezaee.[7] In some of these studies, the authors did not see any differences in TER between asthmatic patients and nonasthmatic subjects.[39, 40] The reasons for this discrepancy can be listed as a growth factor dependency and the use of cadaveric samples. However, there is substantial evidence consistent with our data showing reduced TER and decreased protein expression of TJs (particularly, ZO-1 was analyzed) in asthmatic individuals.[16, 41-43]

These findings were not only confined in asthma, because irregular, patchy and decreased expression of the TJ molecules, occludin and ZO-1 were described in the epithelial layer of biopsies of chronic rhinosinuitis with nasal polyp and atopic dermatitis patients.[11, 31] Although Th2 cells and cytokines induced a gap between two adjacent epithelial cells, asthmatic epithelium did not typically show this gap. This difference can be due to chronic inflammation and exposure to multiple inflammatory factors in asthma and acute and short time exposure to Th2 cells and type 2 cytokines, and remains to be further investigated. It can be hypothesized that the cellular composition between asthmatic and healthy ALIs may play role in TER differences. However it has been studied in detail that ALI's from healthy and asthmatic HBECs do not show any difference in cellular composition, such as goblet cells, ciliated and non-ciliated epithelial cells.[39-41] In addition, our experiments were performed in a group of asthma patients included aspirin sensitive (AIA), aspirin tolerant (ATA) and controls. Although AIA and ATA is suggested as different endotypes, both groups show sputum eosinophilia[34] and our data demonstrates that there is no difference in barrier leakiness.

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The present study investigates the function and expression of TJs in bronchial epithelial cells obtained from asthmatic patients and control subjects and their link to the T_H2 response and epigenetic regulation. Our data suggests a mechanism for a defective barrier function in asthma, which was observed, when normal epithelial cells were exposed to cytokines produced by effector T_H2 cells. The asthmatic epithelium showed lower barrier integrity already at the start of ALI development as observed with low TER measurements. The protein expression of tight junctions was significantly reduced and disassembled. In contrast, the mRNA expression of TJs did not show any difference between asthmatic and control bronchial epithelial cells in agreement with the findings of Xiao et al.[41] In order to elucidate mechanisms underlying the impaired integrity of asthmatic epithelium, we measured the gene expression of 62 TJs and associated proteins performed in ALI cultures.[31] It appeared that the mRNA expression of TJs, adaptor proteins, gap and adherens junctions in particular claudin-4, plactoglobin, nectin-1, nectin-2 and connexin-26 were expressed at low levels in asthma. Claudin 1, 4 and 7 have been reported to be the most expressed junctional molecules in the lungs.[41]

Interestingly, the leaky barrier function of asthmatic epithelium with decreased TER and increased paracellular flux in human ALI cultures of bronchial epithelium was observed even after 2 to the 3 passages of the primary epithelial cells. These findings have led us to investigate to role of epigenetics in barrier function and the regulation of TJs in asthma. Here, we demonstrate two essential findings of epigenetic regulation in asthma that there is an increased gene expression of HDAC1 and 9 in asthmatic epithelium, and IL-4 and IL-13 are potent upregulators of HDAC mRNA and protein expressions. All HDACs were upregulated by IL-4 and IL-13 except for HDAC1 and HDAC2 in asthmatic individuals, suggesting that increased HDAC activity is a hallmark of T_H2-related asthma. The expression of HDACs has been reported in several gene array studies and in one of them HDAC1 and 9 were upregulated in bronchial epithelial cells of asthma patients similar to our findings. [44-46] Similar to our findings in asthma, HDAC regulation has been reported with decreased HDAC 2, 3, 5, 8 expressions in peripheral lung tissue in comparison to nonsmokers and different stages of chronic obstructive pulmonary disease (COPD).[47] In addition, HDAC 6-mediated selective autophagy was observed in COPD associated with cilia dysfunction.[48]

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In agreement with the data seen for HDACs, the expression of sirtuins (SIRT) showed regulation patterns in response to IL-4 and IL-13 in bronchial epithelial cells. These enzymes play an important role in a cellular response to certain types of stress and toxicity. We showed increased expression of SIRT6 and 7 in asthmatic individuals. The regulation of SIRT 2, 4, 6, and 7 showed a similar response pattern to HDACs by IL-4 and IL-13 stimulation in asthmatic and control ALIs. SIRT1 and SIRT5 were highly expressed after IL-13 stimulation in bronchial epithelial cells from controls patients and also together showed stronger response to IL-13 compared to asthmatics. It was also shown, that filaggrin is regulated by SIRT1, which is critical for skin barrier integrity.[49] SIRT family is involved in gene silencing and several studies have showed that SIRT1, an anti-inflammatory and anti-ageing protein is decreased in the lungs of patients with COPD. They also control the resistance to oxidative stress and DNA repair.[50] SIRT6 was associated with redox state and inhibited cellular senescence and fibrosis, and was reported to have protective effects against COPD.[51]

To complement the mRNA expression, we also observed higher general activity of HDACs in bronchial epithelial cells from asthmatic donors. The treatment of bronchial epithelial cells of asthmatic individuals with a new “second generation” HDAC inhibitor significantly increased barrier integrity, which may be utilized for the prevention and treatment of asthma by correcting the leaky epithelial barrier. This HDAC inhibitor, JNJ-26481585 shows high potency for HDAC1, moderate potency to HDACs 2, 4, 10 and 11, and low potency to HDACs 6 and 7. It has greater than 30-fold selectivity against HDACs 3, 5, 8, and 9.[52, 53] Upon HDAC inhibition increased mRNA and protein expression of junctional molecules: occludin, ZO-1, claudin-4, claudin-7 and e-cadherin, as well as improved TJ staining of epithelial cells, which formed honeycomb like structures was observed both in asthmatic and control bronchial epithelial cells. Together these data suggest that chronic type 2 cytokine exposure and increased HDAC activity maybe one of the key reasons for decreased barrier integrity in asthma.

HDAC inhibition was previously shown to lead to greater expression of TJs like ZO-1, ZO-2 and cingulin in fibroblasts and Hela cell lines.[54] It was also recently shown that CpG-DNA can enhance the barrier function of bronchial epithelial cells in asthma.[55]

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Taken together, the present study showed decreased bronchial epithelial barrier integrity in HBECs from asthmatic individuals, and epigenetic regulation of the epithelial HDACs and SIRTs by IL-4 and IL-13, two of the most potent T_H2 cytokines. The increased expression of particular HDACs and SIRTs in asthmatic epithelium, the correction of the leakiness by the inhibition of HDAC activity and protective effect of HDAC inhibition on barrier integrity opens a new window for the prevention and treatment of asthma.

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628 **FIGURE LEGENDS**

629 **Figure 1**

630 **Barrier defect in ALI cultures of asthmatic HBEC and their response to T_H2**
631 **cells** (A) Transepithelial electrical resistance (TER) during the development of ALI
632 cultures from day 3 to 7 in control and asthmatic HBEC. (B) TER from HBEC after
633 differentation on day 21. (C) TER in response to T_H2 cells in control and asthmatic
634 ALIs. (D) Paracellular flux of 4kD FITC-dextran in response to T_H2 cells in control and
635 asthmatic ALI cultures. (E) Representative immunofluorescence staining of occludin
636 and ZO-1 in ALIs co-cultured with T_H2 cells (5×10^5 cells, 48 hours) in control HBEC
637 and asthmatic ALIs. The arrows point out stratification of TJs. Data are presented as
638 a mean ±SD, (*P < 0.05, **P < 0.01, ***P < 0.001 Wilcoxon Rank Sum test), n=6 per
639 group in duplicates, u.s. - unstimulated.

640 **Figure 2**

641 **Decreased epithelial barrier by IL-4 and IL-13 in ALI cultures** (A) TER from IL-4
642 and IL-13 (50 ng/ml) stimulated control and asthmatic HBEC in ALIs. (B) Paracellular
643 flux of 4kD FITC-dextran after stimulation with IL-4 and IL-13 (50 ng/ml) in controls
644 and asthmatic HBEC. (C) Representative immunofluorescence staining of occludin
645 and ZO-1 in ALIs stimulated with IL-4 and IL-13 (50 ng/ml, 48 hours) in control and
646 asthmatic donors. The arrows point out stratification of TJs. Data are presented as a
647 mean ±SD, (*P < 0.05, **P < 0.01 Wilcoxon Rank Sum test), n=6 per group in
648 duplicates, u.s. - unstimulated.

649 **Figure 3**

650 **Increased expression of histone deacetylases by IL-4 and IL-13**
651 (A) Expression of 11 members of HDAC family of ALI cultures in HBEC from control
652 and asthmatic individuals with IL-4 (50 ng/ml) and IL-13 (50 ng/ml), 48 hours.
653 Measurement was performed with the TaqMan Micro Fluidic Card system. Data
654 represent mean±SD (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001), student`s
655 *t* test, n=5 per group, u.s. - unstimulated. (B) Representative Western Blots of
656 HDAC1, HDAC2 and HDAC7 in control and asthmatic HBEC after stimulation with
657 IL-4 and IL-13, n=3 per group.

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Figure 4

HDAC inhibition increased TJ expression in epithelial cells. (A) HDAC activity in controls and asthmatic donors, n=4 per group. (B) TER in ALIs of HBEC from control and asthmatic individuals treated with 10 nM HDAC inhibitor (JNJ-26481585). (C) The fluorescence intensity for occludin and ZO-1 measured from immunofluorescence staining of asthmatic HBEC treated with indicated doses of JNJ-26481585 for 72 hours. (D) Representative immunofluorescence staining of occludin and ZO-1 in asthmatic HBEC after treatment with HDAC inhibitor (C,D, n=3 per group). (E) mRNA expression of occludin, ZO-1, claudin-7 and claudin-1 in ALIs of HBEC controls and asthmatic donors after HDAC inhibition for 72 hours. Data represent mean±SD, (**P < 0.01, ***P < 0.001), two-way ANOVA test, n=8 per group, u.t. – untreated**.**

Figure 5

HDAC inhibition protects bronchial epithelial cell barrier damage by IL-4 and IL-13. (A) TER in control and asthmatic HBEC pretreated with HDAC inhibitor (JNJ-26481585) 10 nM. After 72 hours of treatment HDAC inhibitor was washed away and all epithelial cells were stimulated with IL-4 (50 ng/ml) and IL-13 (50 ng/ml) for 72 hours. TER measurement was perfomed. Data represent mean±SD (****P* < 0.001), two-way ANOVA test, n=3 per group. (B) TER measurement of bronchial epithelial cells from control and asthmatic donors stimulated with cytokines IL-4 (10 ng/ml) and IL-13 (10 ng/ml) and together with HDAC inhibitor (JNJ) JNJ-26481585 (100 nM) for 72 hours, n=3, Data are presented as a mean ±SD**,** (*P < 0.05, **P < 0.01) unpaired t test, n=3 per group, u.s.:unstimulated. (C) TER measurement of bronchial epithelial cells from control and asthmatic donors stimulated with cytokines IL-4 (10 ng/ml) and IL-13 (10 ng/ml) and together with HDAC inhibitor (JNJ) JNJ-26481585 (100 nM) for 72 hours, n=3, Data are presented as a mean ±SD**,** (*P < 0.05, **P < 0.01) unpaired t test, n=3 per group, u.s.:unstimulated.

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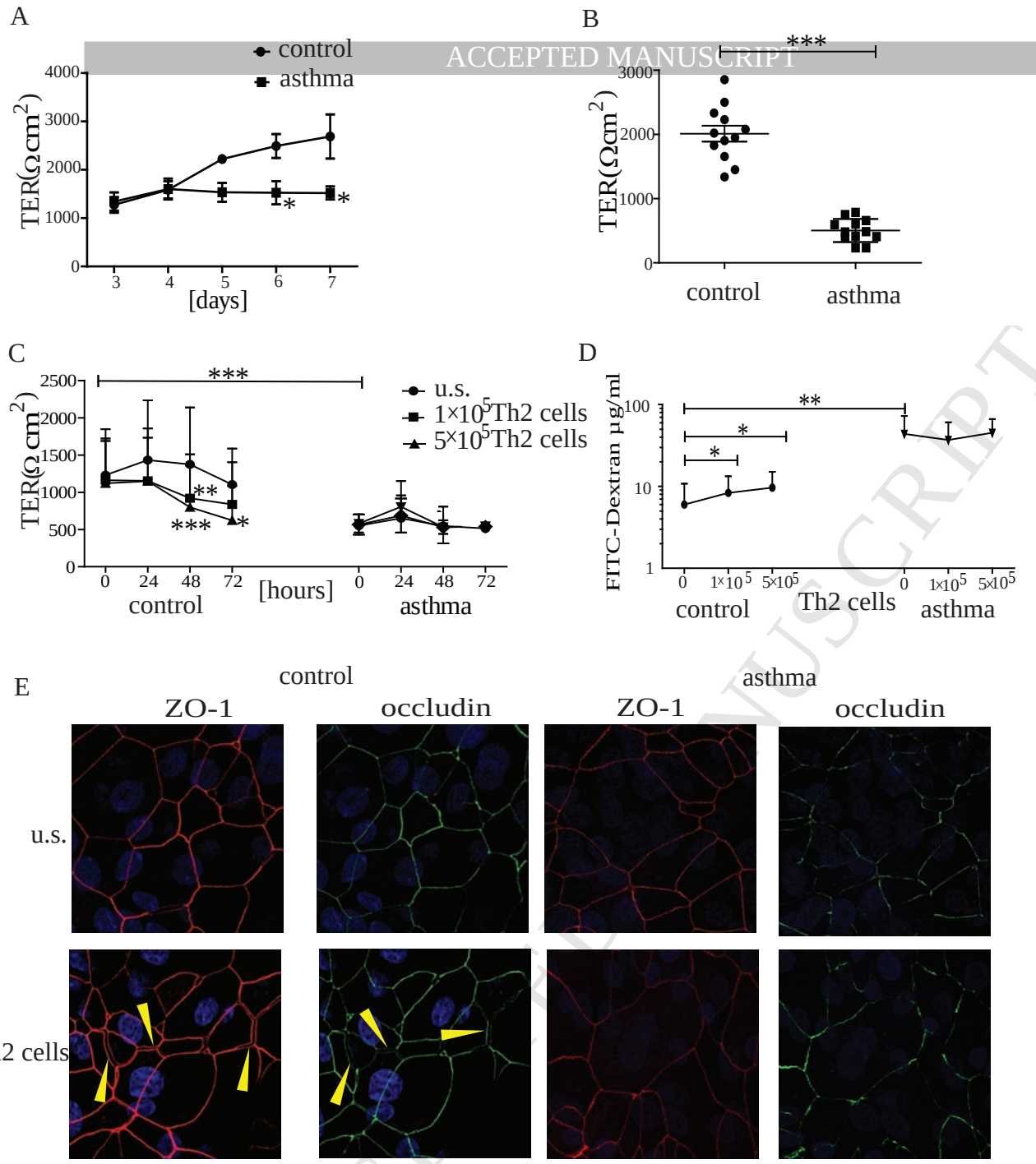
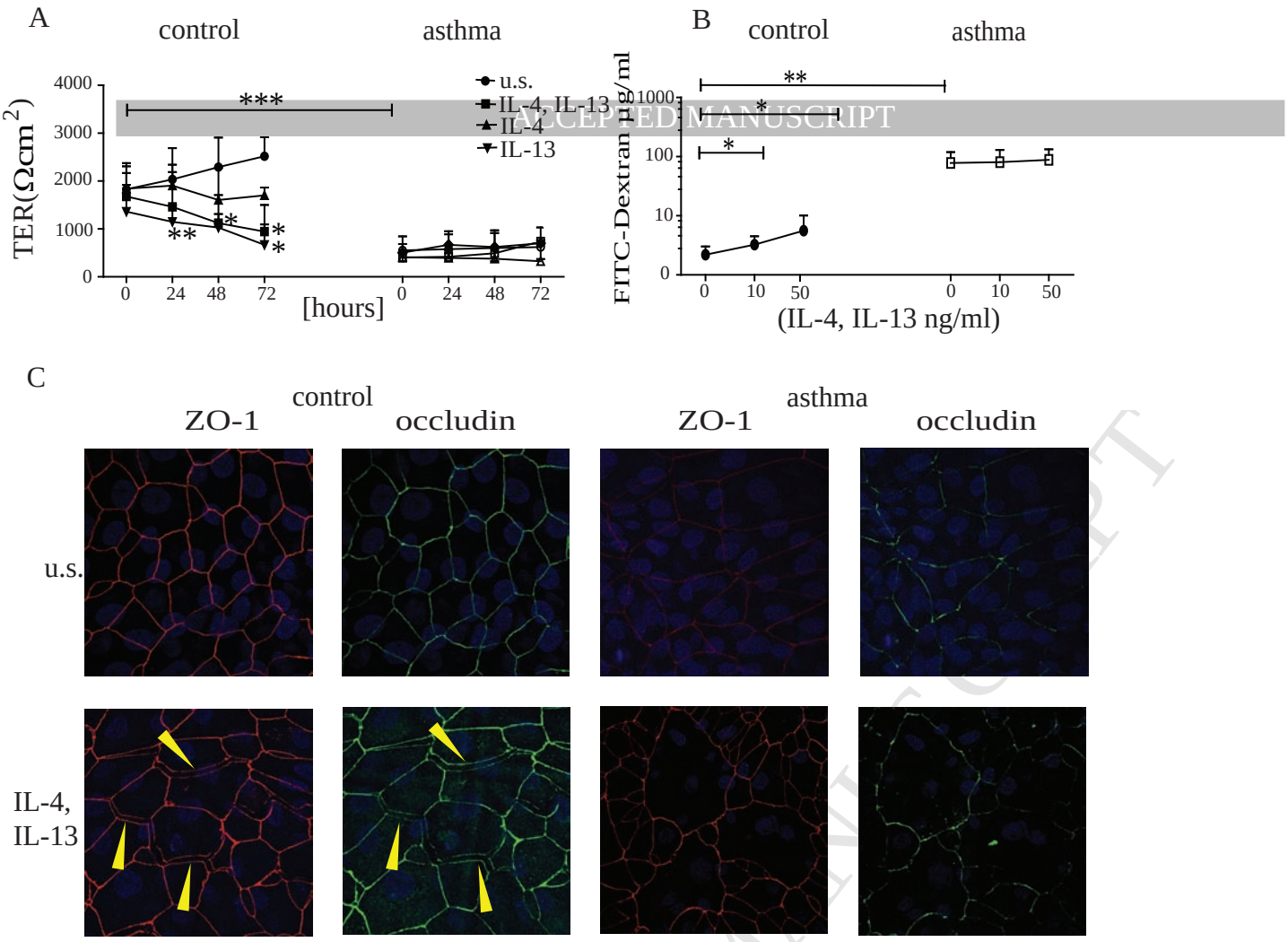


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Figure 2 Wawrzyniak et al.

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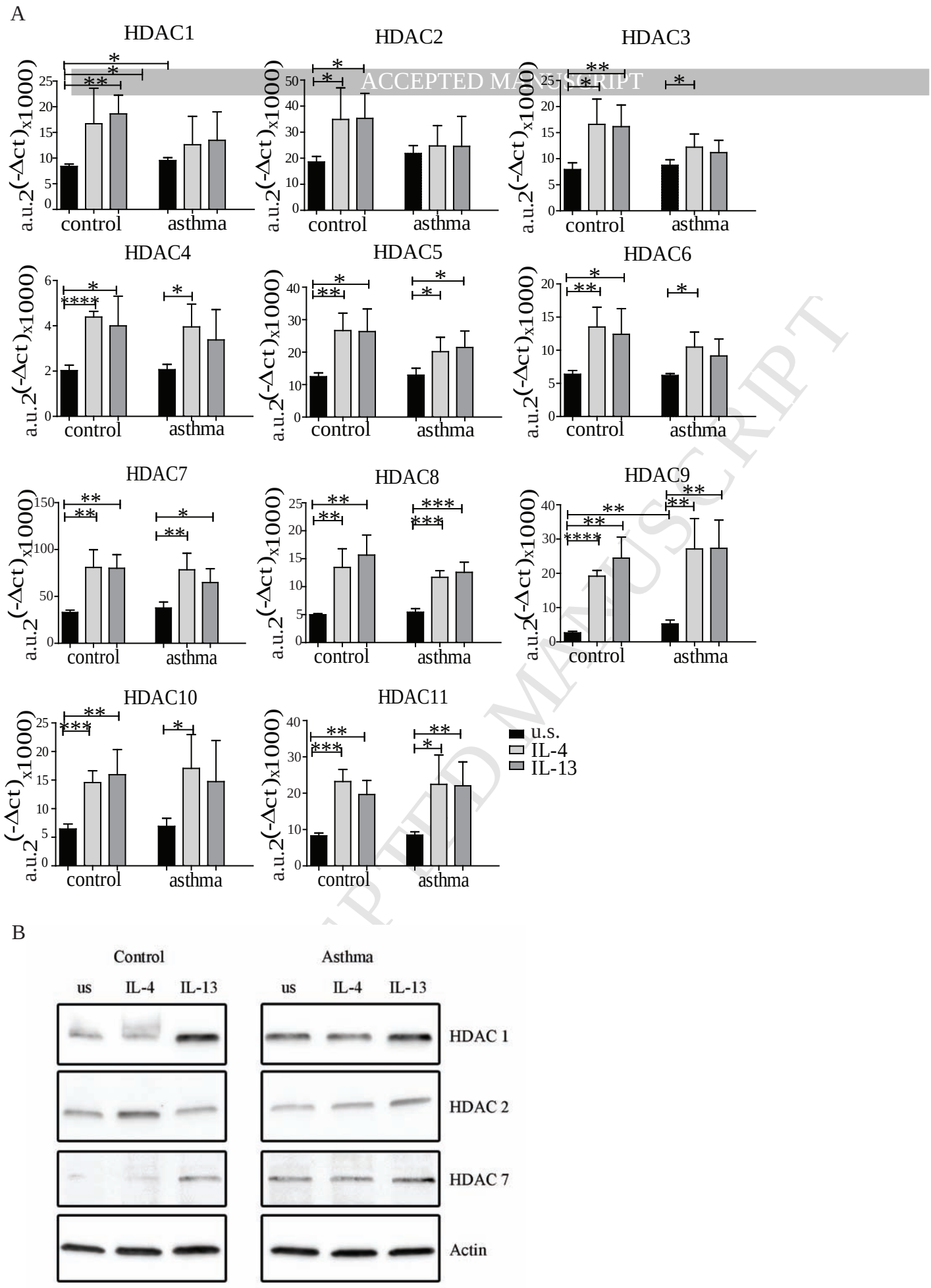


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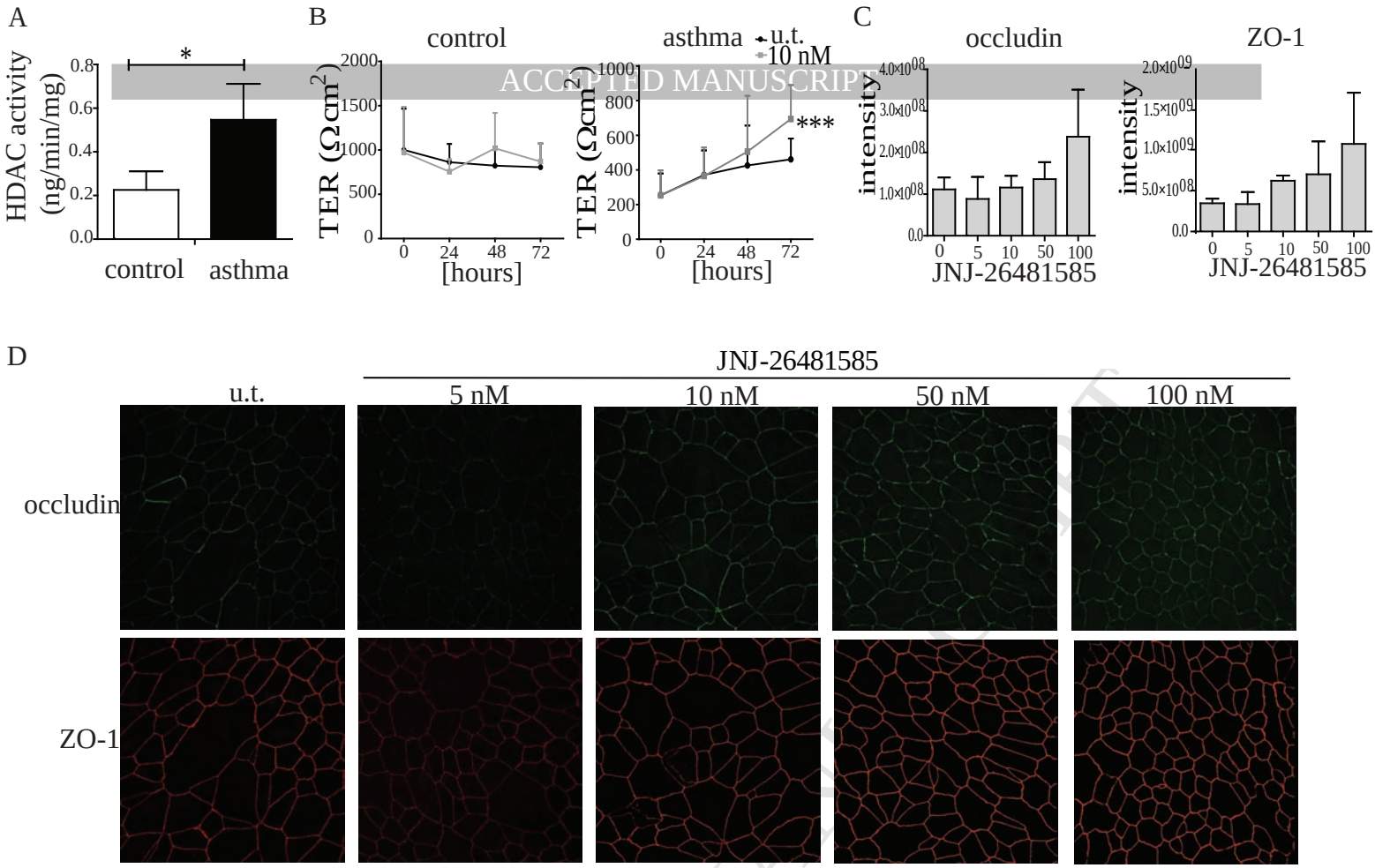


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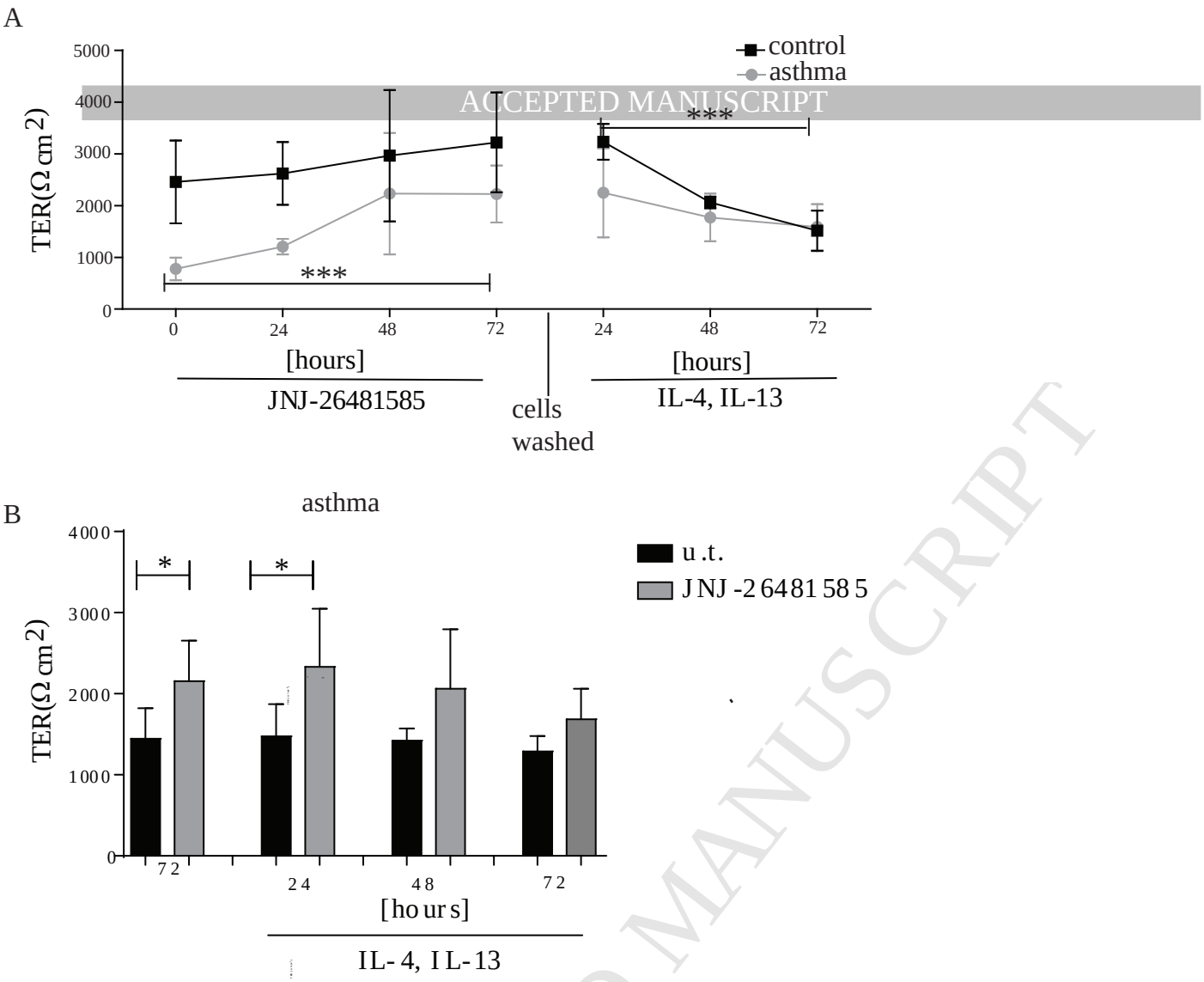


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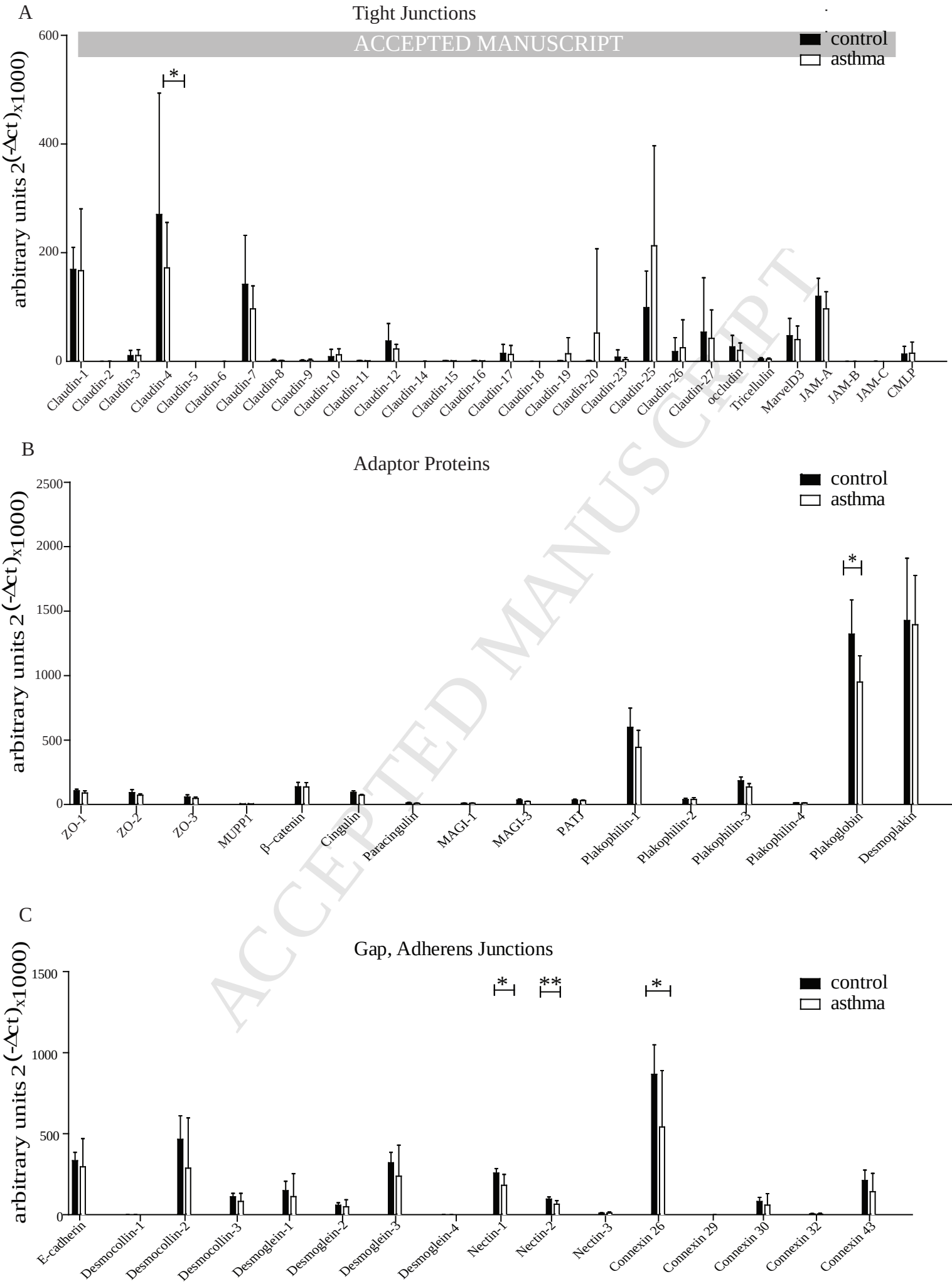
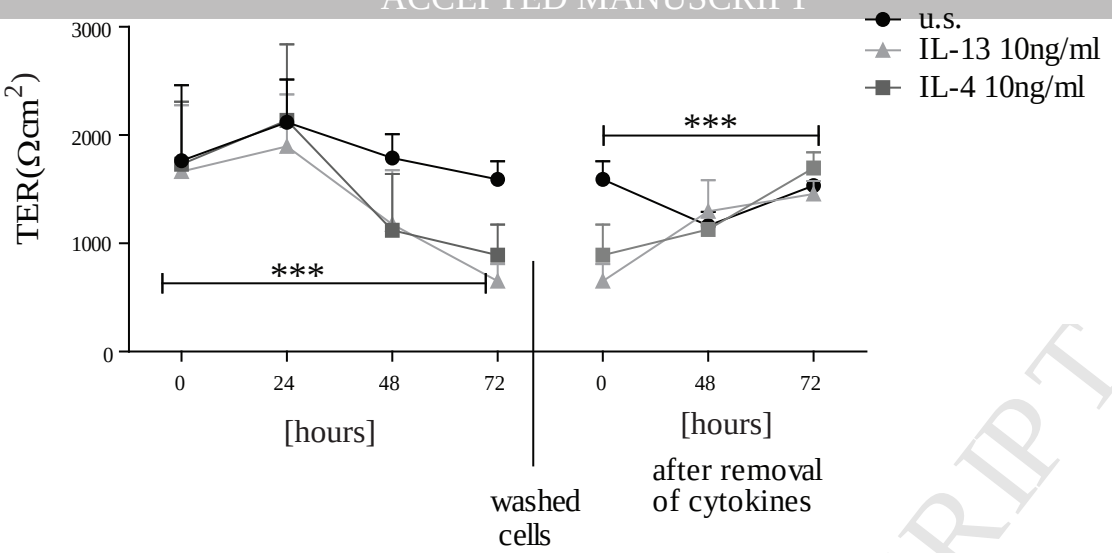


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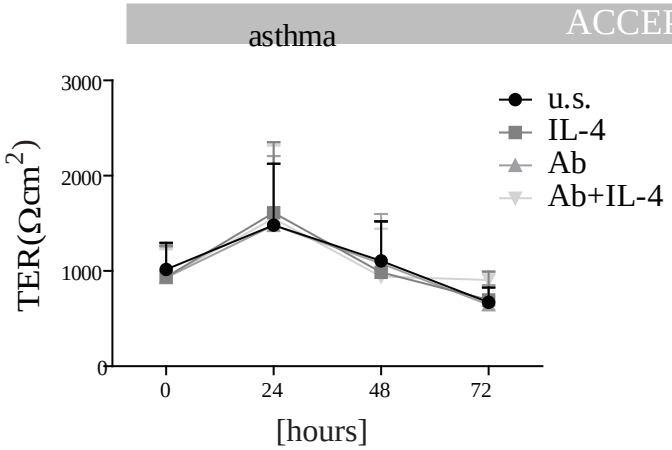
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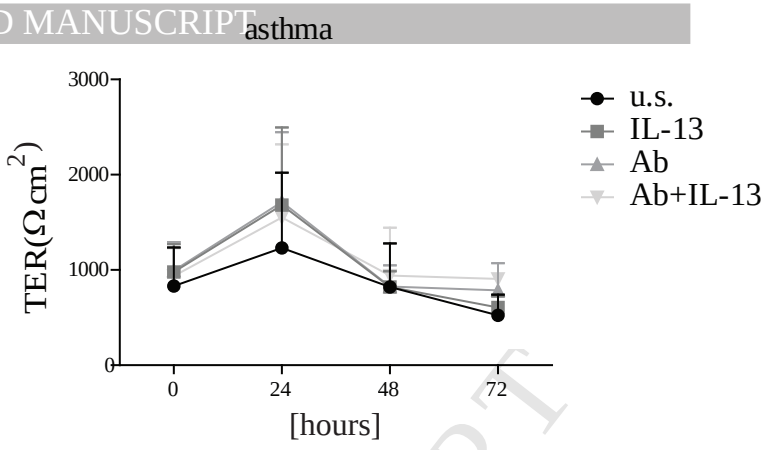


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Figure E2 Wawrzyniak et al.

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Figure E3 Wawrzyniak et al.

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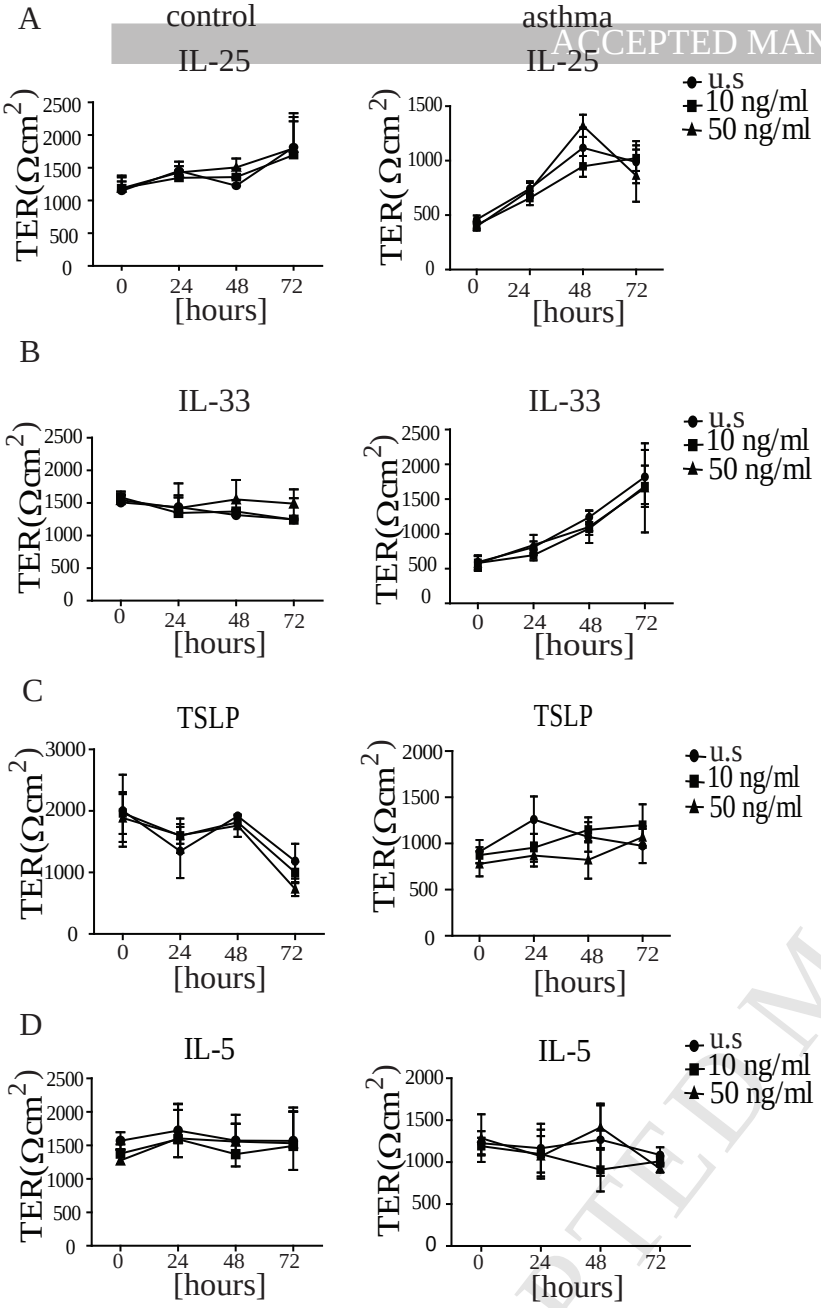
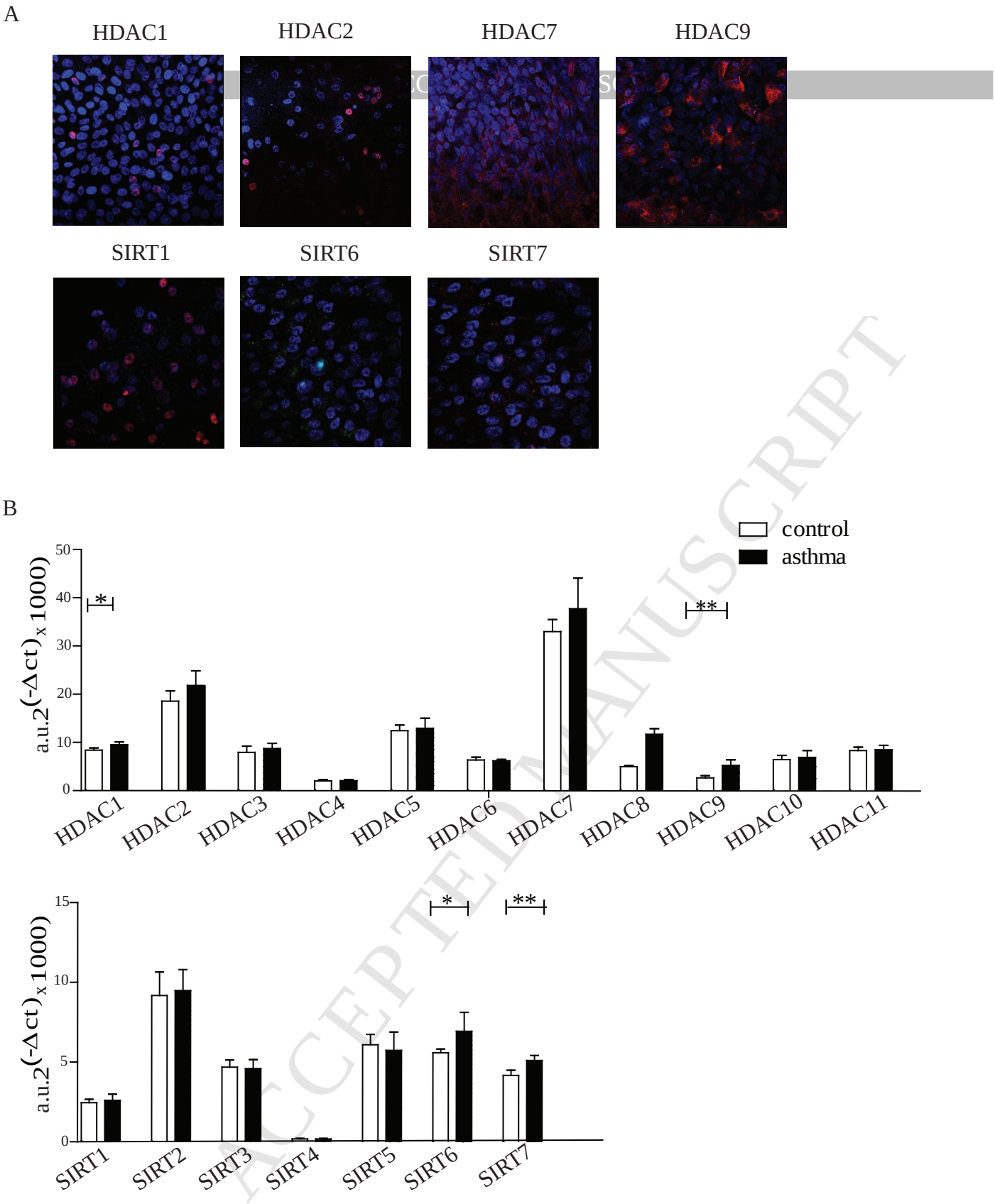


Figure E4 Wawrzyniak et al.



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Figure E5 Wawrzyniak et al.

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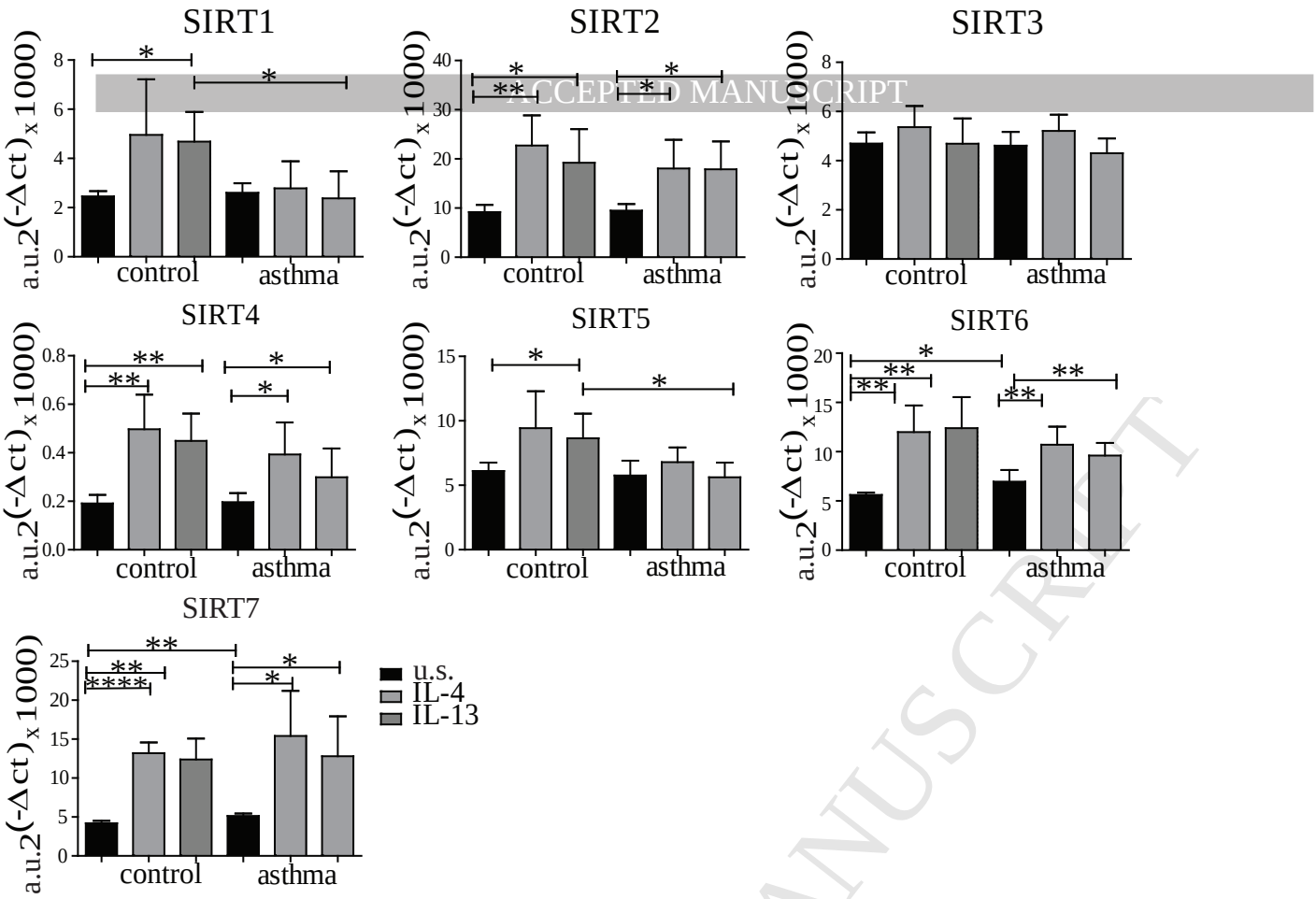
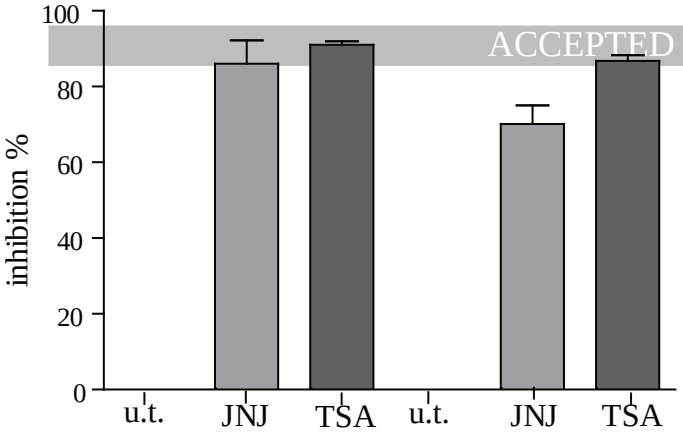


Figure E6 Wawrzyniak et al.

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Figure E7 Wawrzyniak et al.

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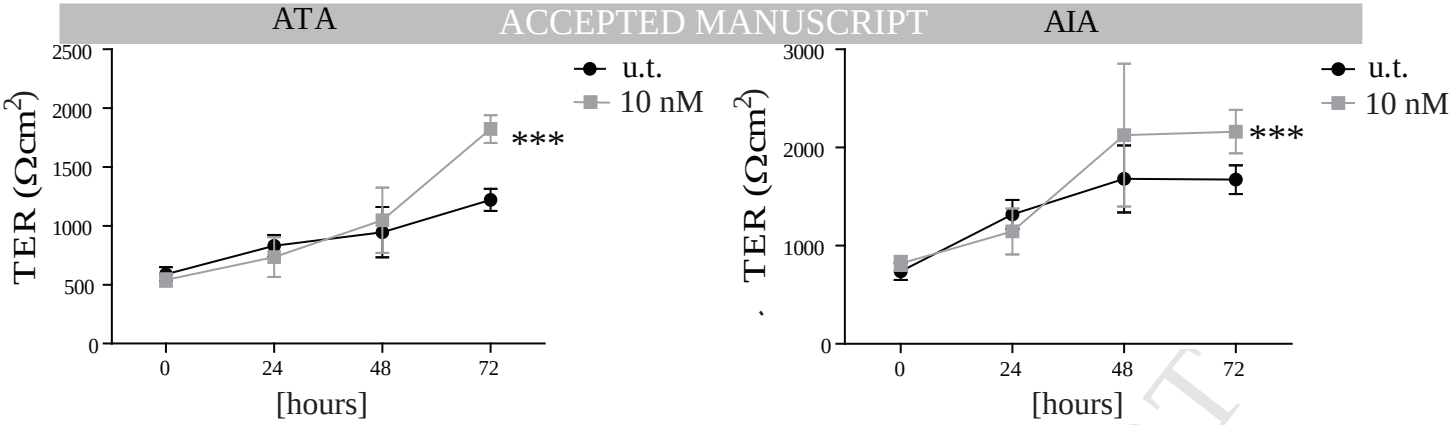
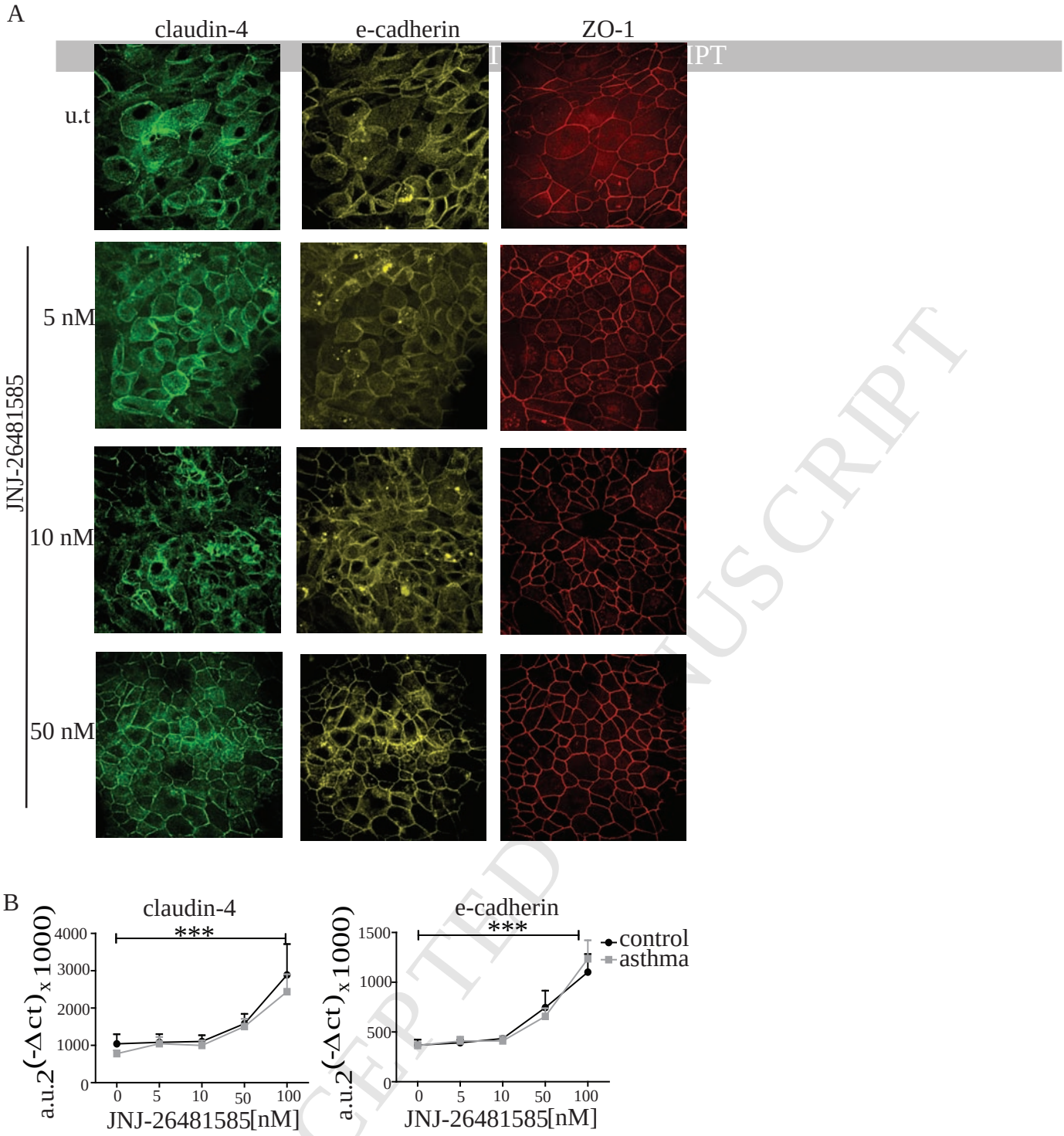
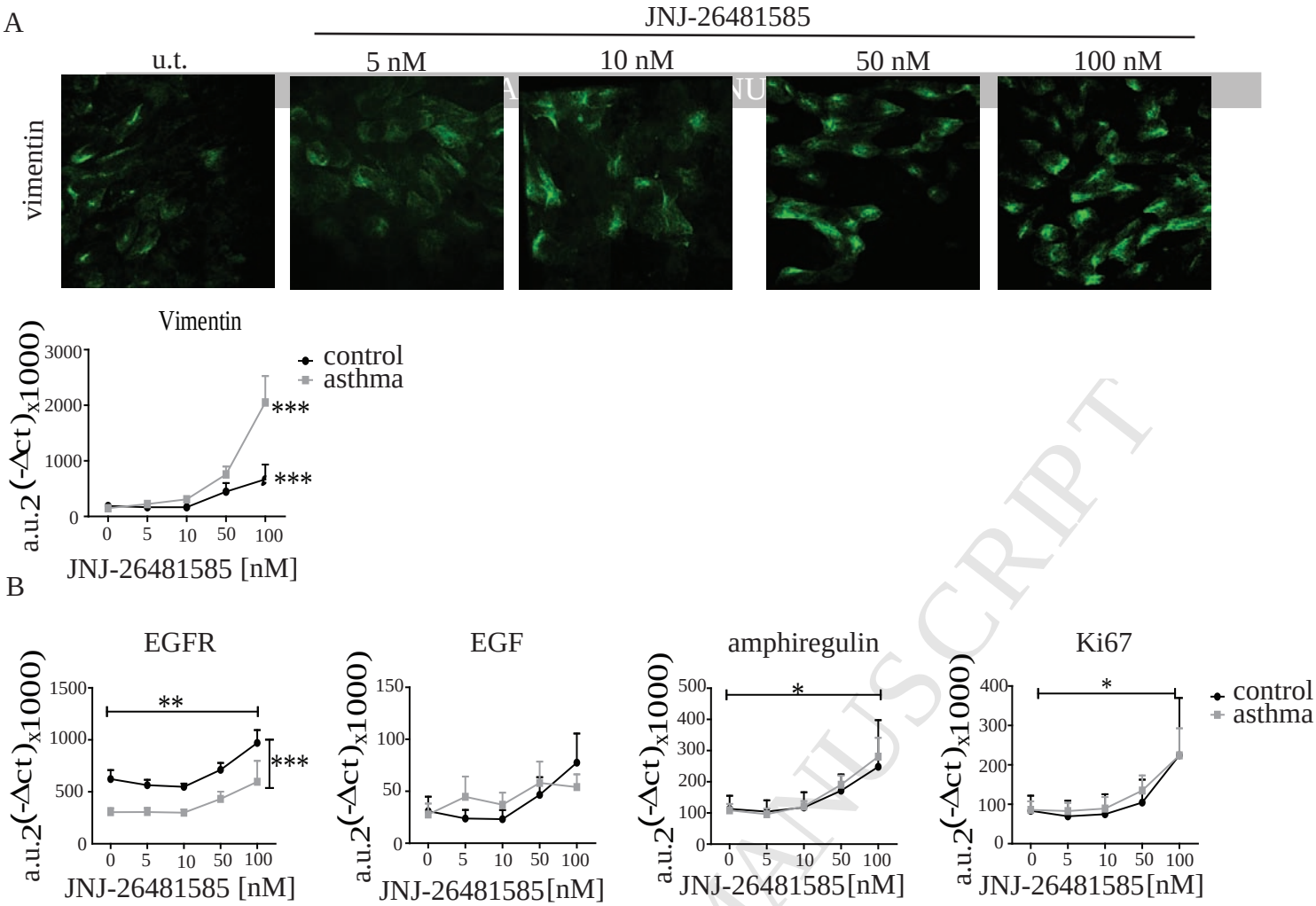


Figure E8 Wawrzyniak et al.



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Figure E9 Wawrzyniak et al.



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