Scientific Report

Project: **Effects of genetic variants associated with eczema herpeticum on herpes simplex virus susceptibility in primary immune cells.**

Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases with over 20% affected children and 2-10% adults. The atopic inflammation leads to vulnerability of the skin leading to an increased susceptibility towards viral infections causing significant morbidity in patients. Most prominently, eczema herpeticum (EH), also known as a form of Kaposi varicelliform eruption caused by a viral infection, usually with the herpes simplex virus 1 (HSV-1), is an extensive cutaneous vesicular eruption that arises from pre-existing skin disease and affects children and adults with underlying AD[1]. EH develops in less than 3% of AD patients, affecting infants and children more frequently than adult patients[2].

Despite the availability of antiviral therapies, EH remains a dermatologic emergency today: EH can be severe, progressing to disseminated infection with organ failure and death if untreated. Systemic viremia, fungal and bacterial superinfection, and bacteremia are usually the complications that cause mortality. Complications of this infection may also include scarring from blisters and infection in the cornea of the eye (herpetic keratitis), which left untreated, can lead to blindness 1. Moreover, EH is associated with a high risk of hospitalization, increased costs of care and significantly affects the quality of life 3. A study by Aronson et al. (2011), which included 1331 children in the age between 2 months and 17 years with EH, showed that 51 patients (3.8%) required intensive care unit admission [4].

The proposed project aims to investigate whether genetic variations contribute to this increased susceptibility to HSV-1 in EH patients. To identify EH susceptibility genes, next-generation whole-exome-sequencing (WES) of representative patients suffering from AD and with a history of EH was performed

in a pilot experiment, resulting in several promising candidates that were re-assessed in a different cohort comprising 117 EH patients, 117 AD patients without a history of EH, and 188 healthy controls (collaboration with Prof. Dr. Stephan Weidinger, Department of Dermatology and Allergy, University Hospital Schleswig-Holstein, Kiel, Germany).

Preliminary analysis showed that a single nucleotide polymorphism (SNP) affecting the gene of a transmembrane collagen XXIII alpha 1 (COL23A1) is significantly associated with higher HSV-susceptibility and higher risk of EH in AD individuals. Transmembrane collagens represent a relatively recently discovered family of proteins, which are highly expressed in skin and in immune cells and play a notable role in tissue development and homeostasis. Nowadays, it is believed that transmembrane collagens closely interact with the extracellular matrix and play an important role in cell adhesion, migration and cancer metastasis spreading. Interestingly, it was discovered that their structure is similar to macrophage scavenger receptor MARCO, suggesting a potential antiviral role. Further on, the expression of transmembrane collagen-associated genes is a marker of M2-polarization, which is connected with allergic inflammation [5–7]. Nevertheless, transmembrane collagens in the skin remain understudied despite their association with epidermal wound healing and dermal fibrotic processes.

Considering this information, we decided to further study this genetic variant, which affects the structure and functions of transmembrane collagen COL23A1. Thus, the current project could help to elicit the possible causes of increased HSV susceptibility in EH patients.

Results

1. Culturing M2-polarized macrophages from human PBMCs

In the first step, we aimed to culture cell lines of M2-polarized macrophages *in vitro* using an established protocol by PromoCell [8]. Human Peripheral Blood Mononuclear Cells (PBMCs) isolated from anonymized donors´ buffy-coat blood

were the main source of cells. A detailed description of the isolation protocol was published earlier by Mommert S. et al. (2018)[9]. After the *in vitro* generation protocol, the expression of the characteristic M2 surface markers (CD68, CD163 and CD206 [8,10]) was confirmed using Flow Cytometry (FACS) (Figure 1). M2-cell lines generated this way were applied in further experiments.

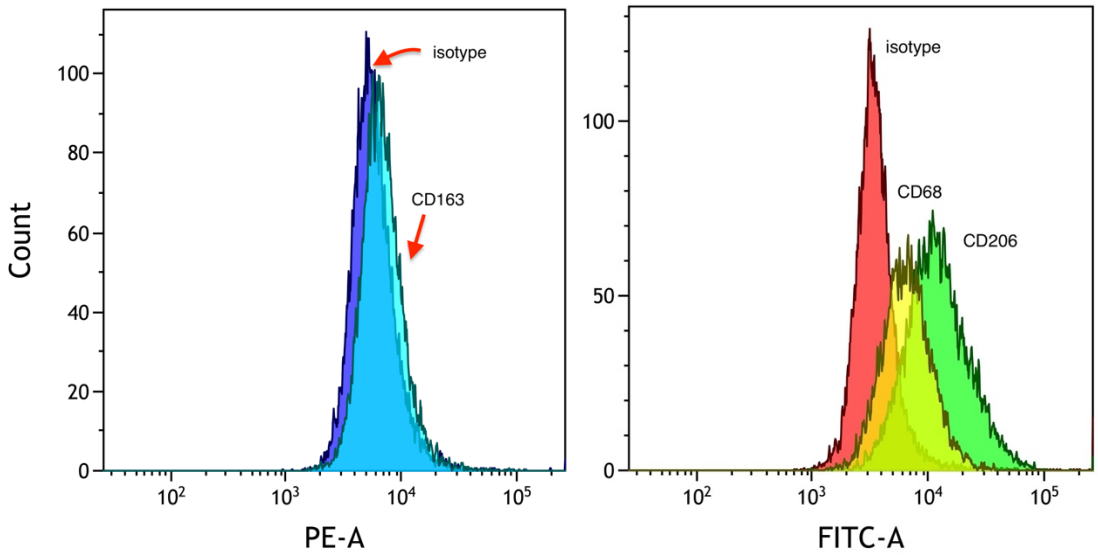

Figure 1. The expression of characteristic surface markers (CD68, CD163, CD 206) on M2-macrophages by Flow cytometry (FACS). FITC - Fluorescein isothiocyanate, PE – Phycoerythrin.

The basal surface expression of COL23A1 on M2 macrophages was assessed using FACS. Cells were stained with commercially available anti-COL23A1 antibody (Proteintech, 14337-1-AP) using the standard protocol (Figure 2).

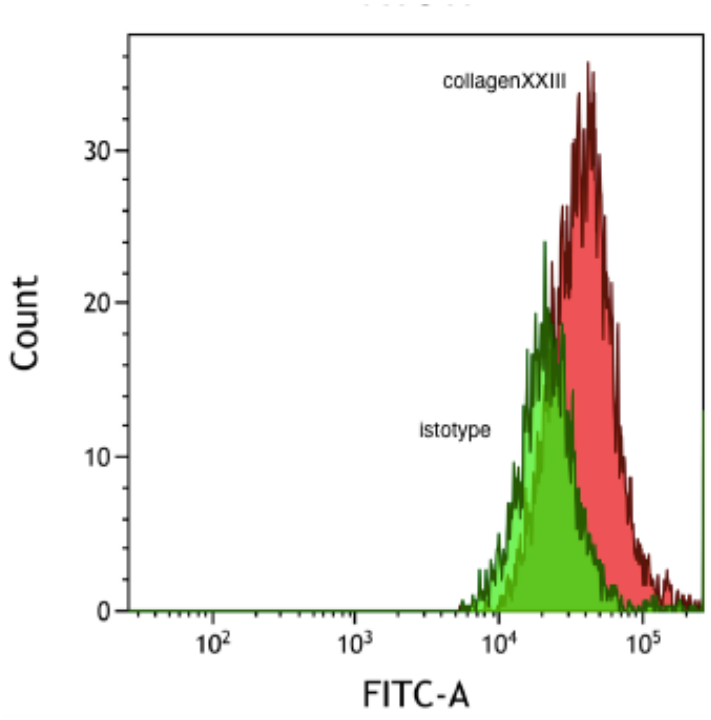

Figure 2. Basal expression of COL23A1 on the surface of M2 macrophages (Flow cytometry, FACS). FITC - Fluorescein isothiocyanate

Pilot experiments with monocytes and inflammatory dendritic epidermal cells (IDECs) showed that these cell lines were not suitable for further investigation because of technical difficulties in culturing, too low expression of the protein of interest, and variable infectibility by the herpes simplex virus 1 (HSV-1) (Data not shown).

2. Functional experiments to induce the higher expression of a protein of interest using physiological stimuli

Serotonin (5-HT), 5-HT2B–specific agonist BW723C86, histamine, and a selective furin inhibitor I (FI) were used to increase the expression of COL23A1 *in vitro* on the surface of M2 macrophages. In the study carried out by de las Casas-Engel M. et al. (2013) it was shown that the above-mentioned stimulants tend to increase the expression of the *COL23A1* gene and to skew the polarization of macrophages towards M2 phenotype [6,7]. According to the study of Veit G. et al. (2007), the shedding of COL23A1 is mediated by the enzyme furin[11]. Inhibition of this protein

by a selective FI could in theory cause the retention of uncut transmembrane collagen on the cell surface and protect it from shedding.

In the current experiment, M2 macrophages were stimulated with 5-HT (10^{-5} M) and histamine (10^{-5} M) for 24 hours or with FI 50 μM/75 μM for 1 and 24 hours. Subsequently, the expression was analyzed on the cell surface by FACS and in the cell culture supernatant by Enzyme-linked Immunosorbent Assay (ELISA) (Figures 3-5).

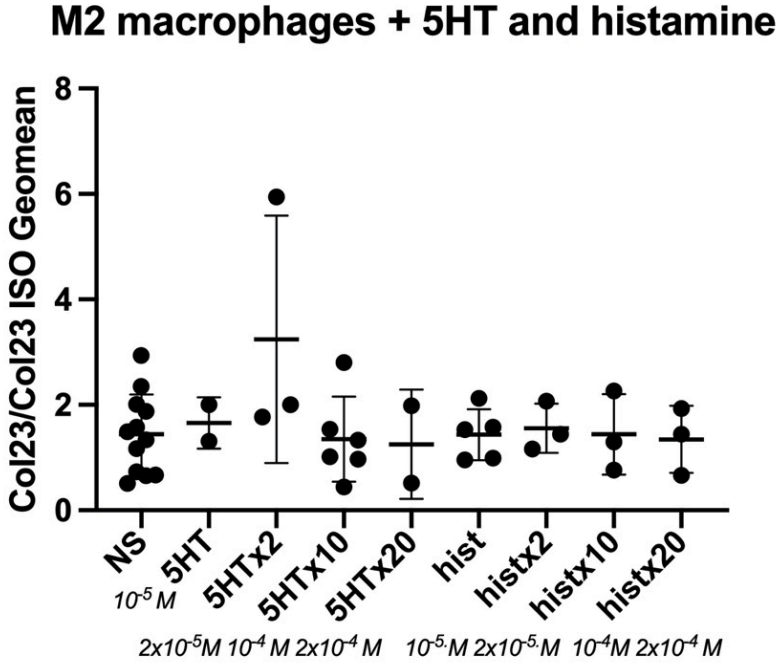

Figure 3. Effects of serotonin (5-HT) and histamine on the expression of the protein of interest according to the FACS measurements (index Geomean of COL23A1-APC/Geomean of APC-isotype). NS – not stimulated, 5HTx1 and histamine x1 – 10^{-5} M, x2 – $2x10^{-5}$ M, x10 – $10x10^{-5}$ M, x20 – $20x10^{-5}$ M. Reagents were added directly to the cell medium, the incubation time was 24h. Median and 95% CI are shown.

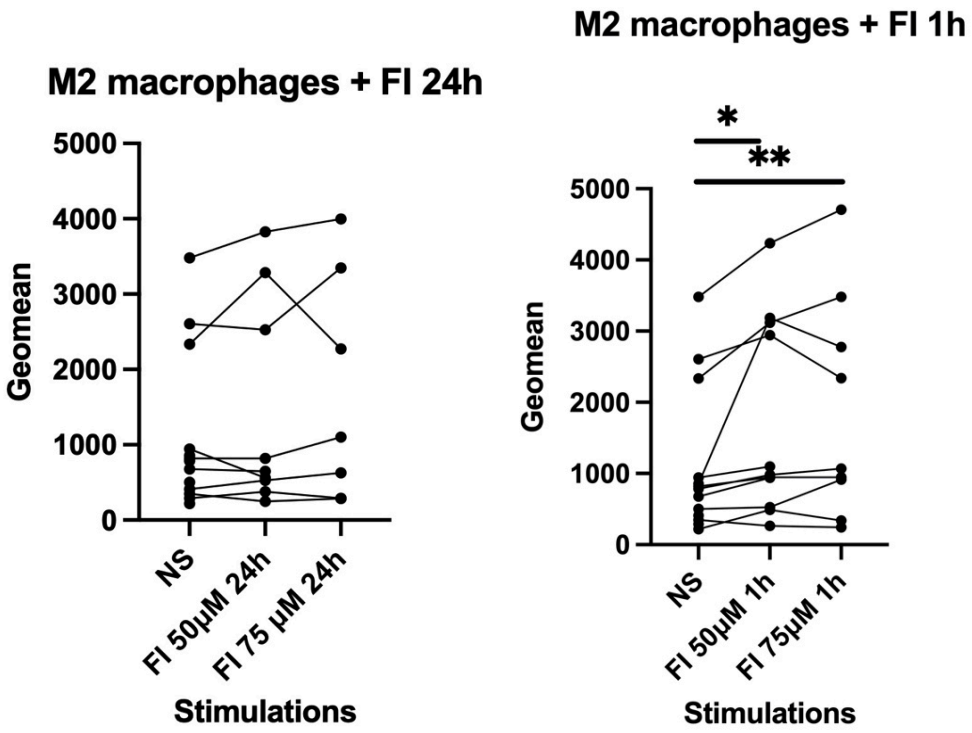

*Figure 4. Effects of furin inhibitor 1 (FI) on the expression of the protein of interest according to the FACS measurements (COL23A1-APC Geomean). * NS vs FI 50 µM 1h (p=0,029), ** NS vs FI 75 µM 1h (p= 0,0391), NS vs FI 50 µM 24h (p=0,6406, close to significance). NS – not stimulated. Wilcoxon matched-pairs signed rank test.*

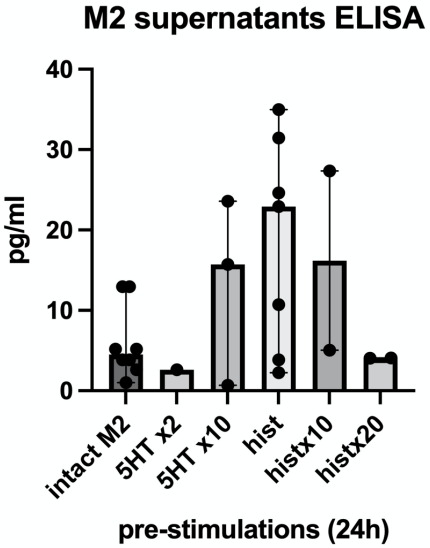

Figure 5. Effects of serotonin (5-HT) and histamine on the amount of the protein of interest according to ELISA. 5HTx1 and histamine x1 – 10^{-5} M, x2 – $2x10^{-5}$ M, x10 – $10x10^{-5}$ M, x20 – $20x10^{-5}$ M. Cell culture supernatants were analyzed. Reagents

were added directly to the culture medium, incubation time - 24h. Median and 95% CI are shown.

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According to the achieved data 5-HT and histamine don't significantly affect the surface expression of COL23A1. Nevertheless, these substances (serotonin in concentration 10^{-4} M (x10) and histamine – 10^{-5}M (x1) and 10^{-4} M (x10) increase the amount of shedded collagen in the supernatant. Interestingly, FI caused an increase in surface expression of the COL23A1 (significant effect for FI 50 and 75 μM for 1h*)*.

Experiments with 5-HT2B–specific agonist showed that this chemical together with the increase of COL23A1 surface expression causes significant cell death (FACS, BV510-A staining) (Data not shown)

3. Functional in vitro experiments to explore the role of protein of interest in HSV-1 infection

In this part of the project infection experiments with genetically modified HSV-1 were carried out. The scheme of experiments is represented in Figure 6.

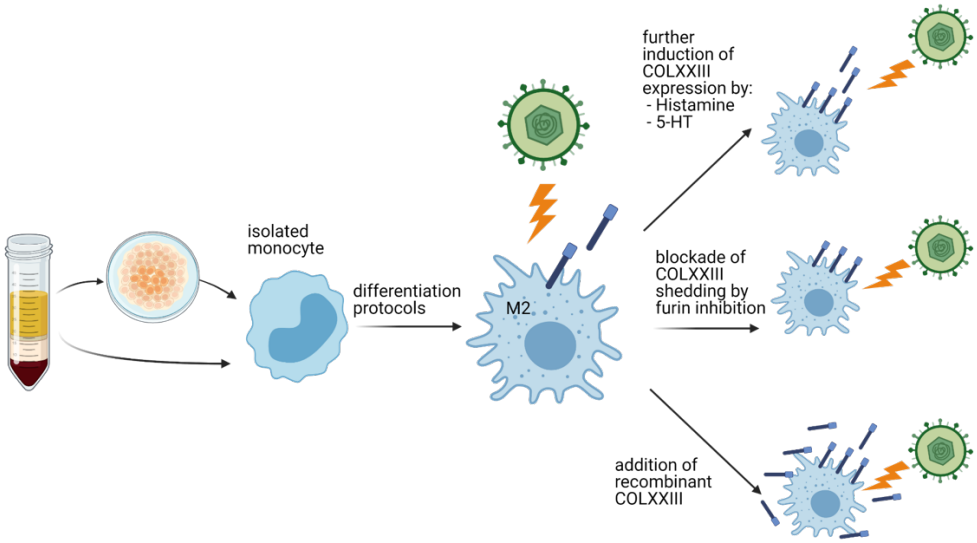


Figure 6. Schematic plan of HSV-1 infection experiments in M2 macrophages.

HSV1(17+)Lox-pMCMVGFP, which expresses soluble green fluorescent protein (GFP) under the control of the murine cytomegalovirus major immediate-early promoter was used in our infection experiments[12,13].

Pilot titration experiments with GFP-tagged genetically modified HSV-1 revealed that multiplicity of infection (MOI) 6 is optimal for macrophages. This result is in agreement with published literature and our preliminary data [14,15]. In all further HSV-1 infection experiments MOI 6 was applied (Figure 7).

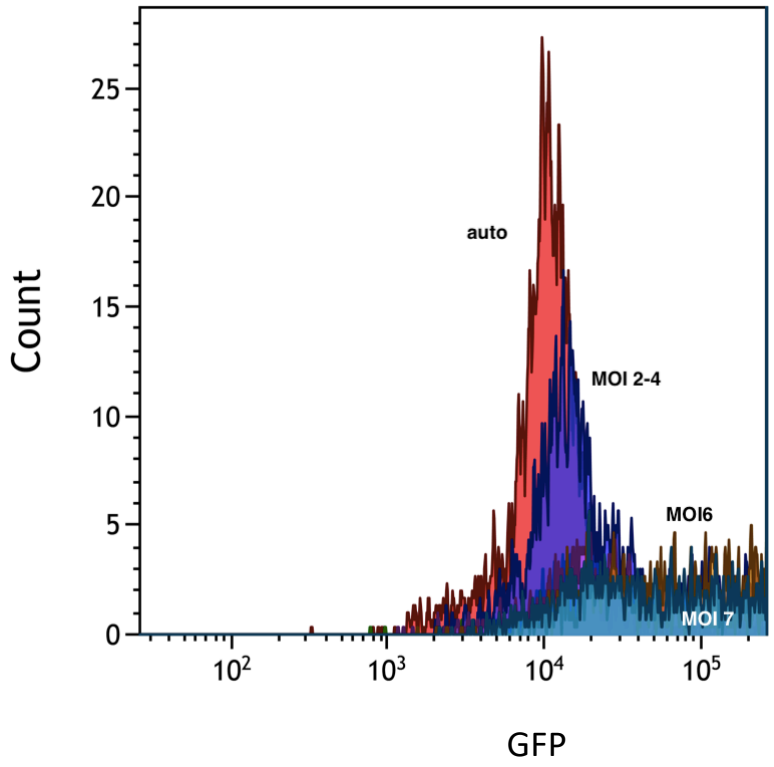


Figure 7. Titration experiment with GFP-tagged HSV-1 in order to reveal optimal MOI for M2 macrophages. MOI 6 and 7 have the same infection rate in M2 macrophages, GFP - Green fluorescent protein.

M2 macrophages were infected with GFP-tagged HSV-1 and then stained with rabbit anti-COL23A1 polyclonal antibody and goat anti-rabbit secondary APC-antibody [12]. The gating strategy for FACS is depicted in Figure 8. This way four cells populations could be distinguished: 1) $COL23A1^-/GFP^+$ (infected), 2)

COL23A1^{-}/GFP^{-} (non-infected) 3) COL23A1^{+}/ GFP^{+}, **4) COL23A1^{+}/ GFP^{-}** (non-infected, with the expression of the protective factor).

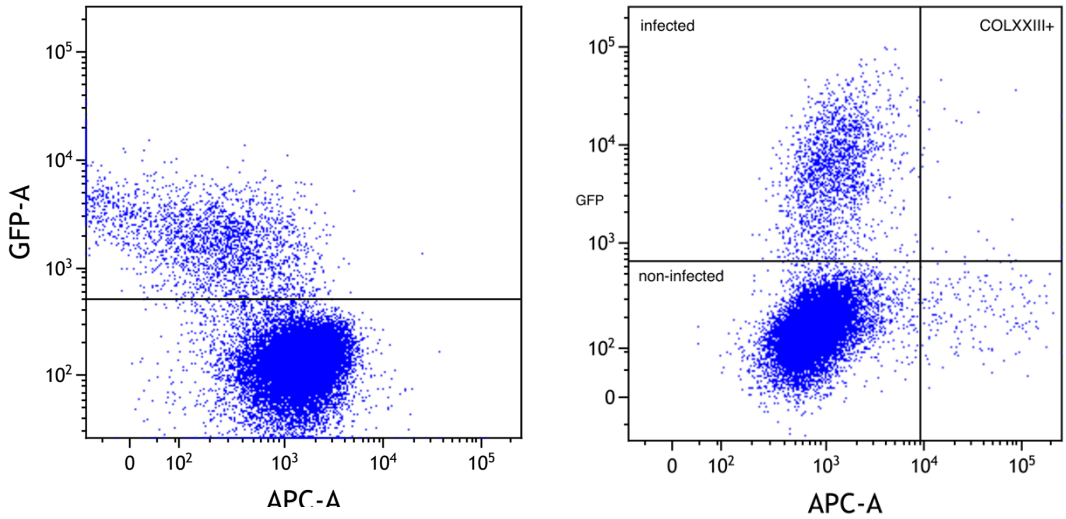


Figure 8. HSV-1 infection in M2 macrophages visualized by FACS. Cells located in the upper quadrant (above the threshold) are considered infected. Macrophages located in the lower right quadrant are non-infected, with a high expression of COL23A1. GFP – green fluorescent protein. APC – allophycocyanin.

3.1. **HSV-1 infection of intact M2-macrophages**

Populations of infected M2 cells with high and low expression of COL23A1 were compared (Figure 9). We found out, that COL23A1^{+} M2 macrophages were less often infected.

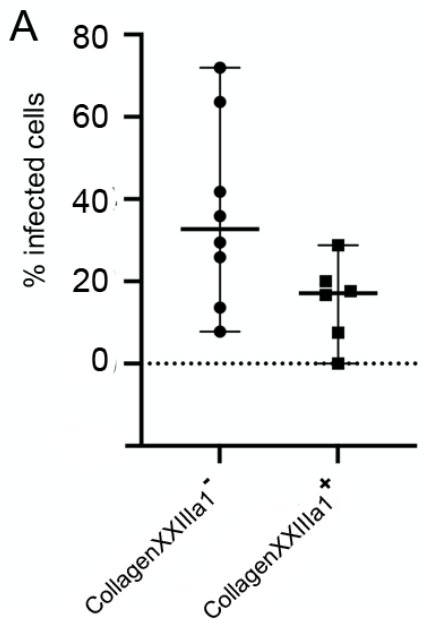


Figure 9. Comparison of the infectibility of M2 macrophages with high and low expression of the protein of interest. Data from several independent infection experiments with M2 macrophages derived from different donors is shown. Median and 95% CI are shown.

In the next step, we compared M2 macrophages from an AD patient carrying the identified *COL23A1* SNP, an AD patient without the SNP (wild type) and a healthy donor. Cells derived from the AD patient with the COL23A1 SNP demonstrated increased susceptibility to HSV-1 infection (Figure 10).

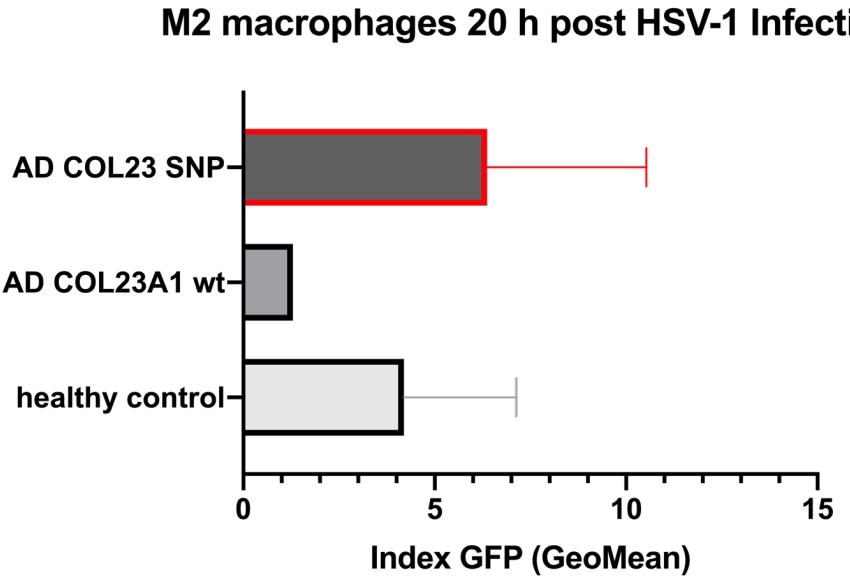


Figure 10. Infection rate (GFP Geomean) in M2 macrophages derived from different patients: SNP-carrier, an AD patient with wild-type (WT) gene and a healthy donor. Geomean of WT-donor is set to 1. Mean + SD are shown.

Additionally, patient with acute EH, patient carrying the identified *COL23A1* SNP and healthy donor with wild-type gene were compared (Figure 11).

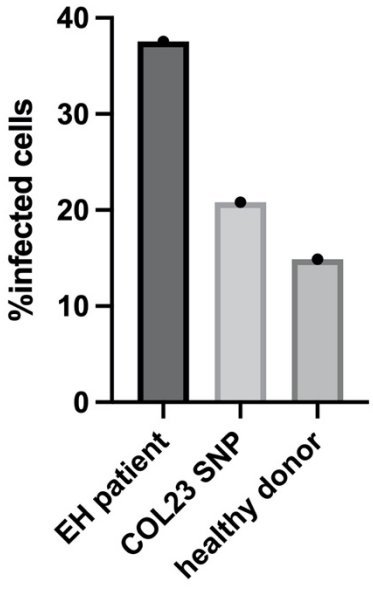


Figure 11. Infection rate (% of HSV-1 infected cells) in M2 macrophages derived from different patients: a patient with acute eczema herpeticum (EH), SNP-carrier and a healthy donor with wild-type gene.

According to the described results we can suggest that COL23A1 may play a role as a protective factor against HSV-1 infection in human M2 macrophages.
There was no significant effect on the infection rate in M2 macrophages after 24h pre-stimulation with 5-HT or histamine.

3.2. Inhibition of furin to prevent shedding of COL23A1 limits HSV-1 infection in M2 macrophages.

According to the literature, furin cleavage leads to the shedding of the extracellular domain of COL23A1 [11]. The addition of a furin inhibitor to the M2

macrophage culture before infection may block the shedding of protein and increase its protective anti-viral potential.

In order to investigate the effects of a furin inhibitor (FI) on the infection rate and population of COL23A1^{+} non-infected (GFP^{-}) cells we incubated M2 macrophages with furin inhibitor 50 (50 μM) and furin inhibitor 75 (75μM) for 1h and 24h before HSV-1 infection (Figures 12-13).

In our set of experiments, we observed that incubation with FI for 24h caused a relative but non-significant decrease in the HSV-infection rate in M2 macrophages. In the case of 1-hour pre-incubation with FI before the addition of GFP-tagged HSV-1 significant decrease in infection susceptibility was observed (Figure 12). Focusing on the COL23A1^{+} M2, FI (in both concentrations) had a significant positive effect on the population of non-infected COL23A1^{+} cells (HSV-1-protected M2 population) (Figure 13).

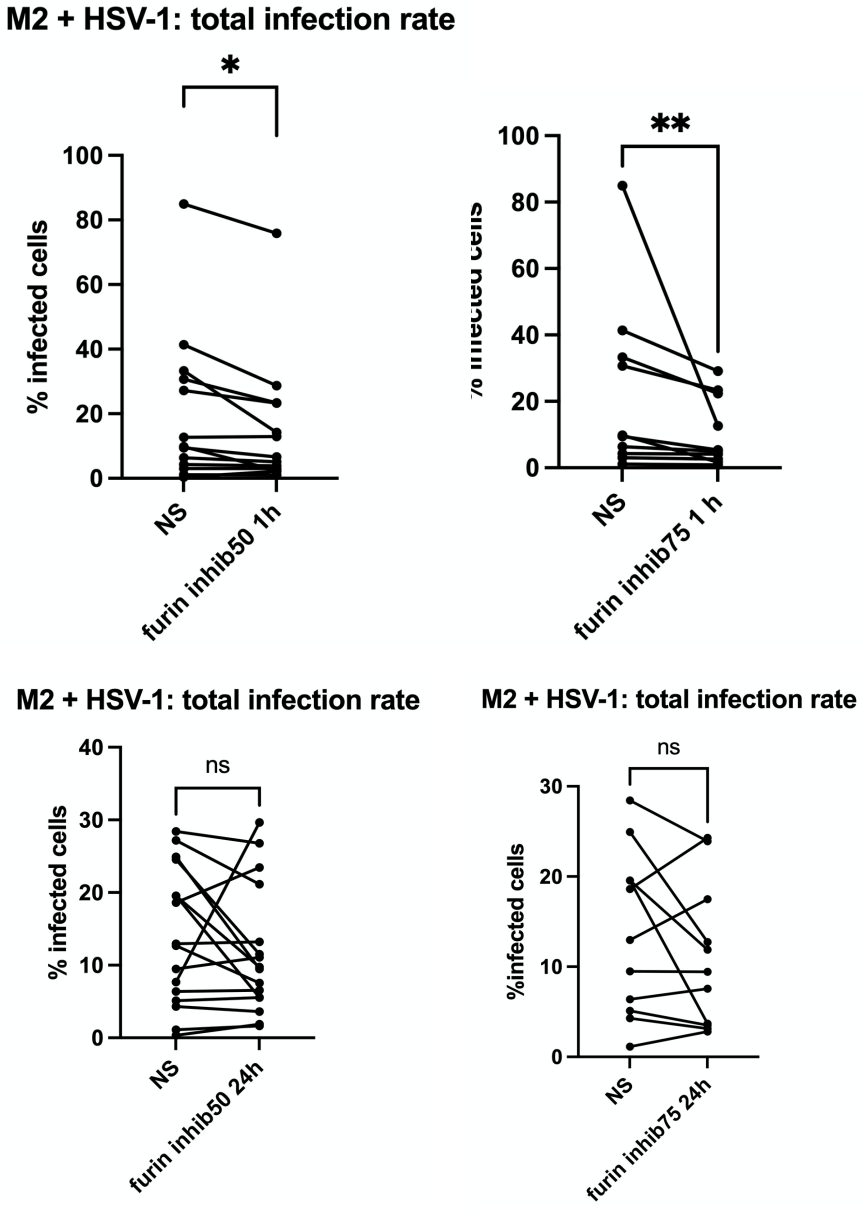
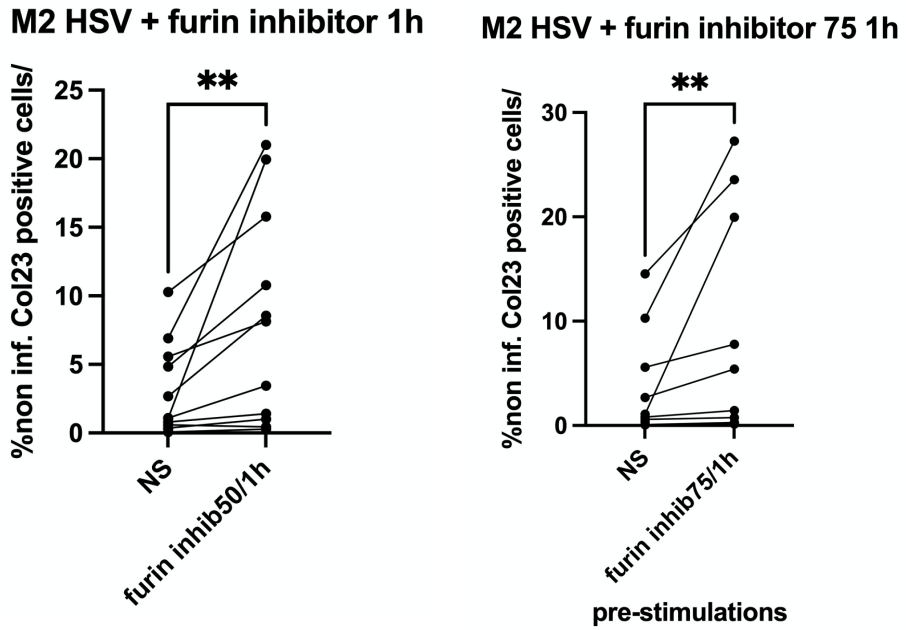


Figure 12. HSV-1 infection rate in M2 macrophages: effects of pre-incubation with furin inhibitor 50 μM and 75 μM for 1 and 24 hours before infection. NS – not stimulated. Wilcoxon matched-pairs signed rank test.



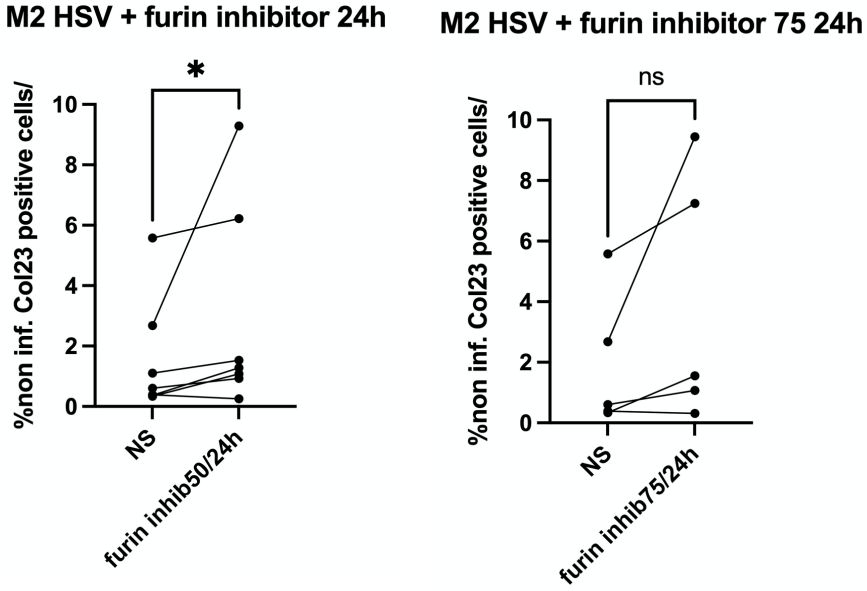


Figure 13. COL23A1^{+} non-infected population of M2 macrophages after HSV-1 infection: effects of pre-incubation with furin inhibitor (FI) 50 μM and 75 μM for 1 and 24 hours before infection. NS – not stimulated. Wilcoxon matched-pairs signed rank test

3.3. Recombinant COL23A1 limits HSV-1 infection in M2 macrophages.

M2 macrophages were incubated with recombinant human COL23A1 (rhCOL) for 1 hour before infection. Two isoforms of the protein were used: a full-length (Cusabio, CSB-MP774831HU) and a truncated one (R&D Systems, 4165-CL-050), with the latter being similar to the extracellular domain of the wild-type COL23A1.

We showed that 1h pre-incubation with both full-length and truncated forms of rhCOL led to a significant decrease of the infection rate in M2 macrophages (p=0,0010 and p=0,0166 respectively, Wilcoxon matched-pairs signed rank test) (Figure 13). Moreover, the long form of recombinant protein induced a statistically significant increase in the COL23A1^{+} non-infected cell population (Figure 14).

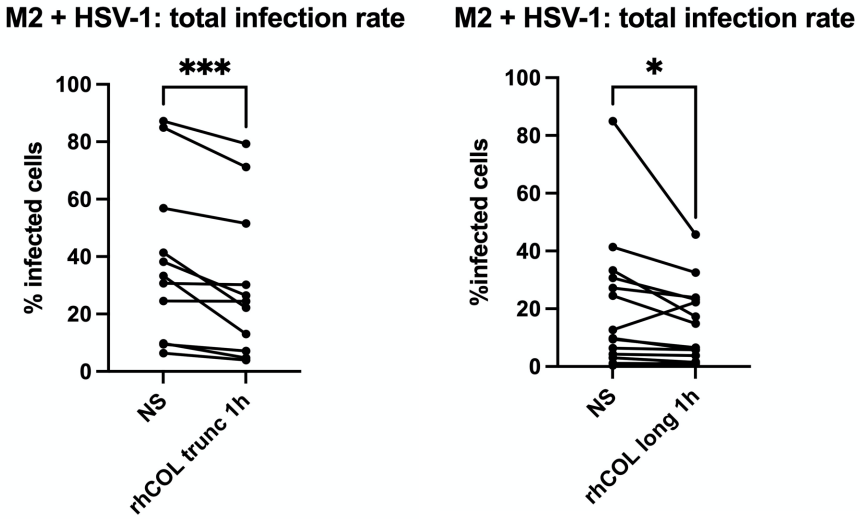


Figure 14. HSV-1 infection rate in M2 macrophages: effects of pre-incubation with long (full-length) and a truncated form of recombinant human COL23A1 (rhCOL) for 1 hour before infection. NS – not stimulated. Wilcoxon matched-pairs signed rank test.

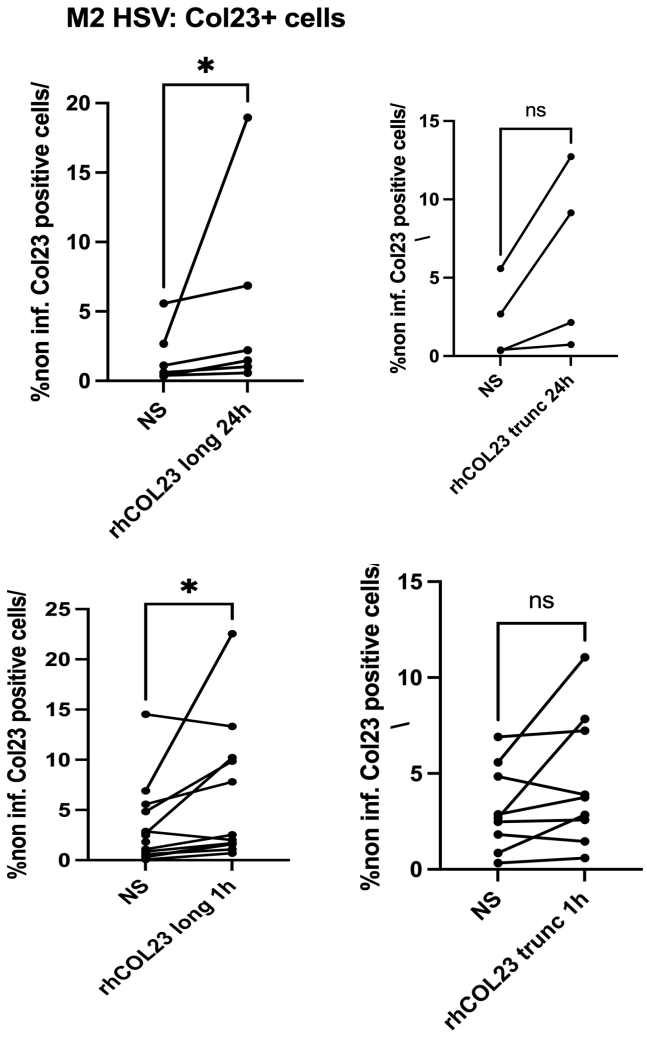


Figure 15. Frequency of COL23A1^{+} non-infected population of M2 macrophages after HSV-1 infection within the M2 population: effects of pre-incubation with long (full-length) and a truncated form of recombinant human COL23A1 (rhCOL) for 1 hour before infection. NS – not stimulated. Wilcoxon matched-pairs signed rank test

We also showed that the addition of a truncated form of rhCOL 1h before HSV-1 lead to a decrease of the infection rate in M2 macrophages, derived from SNP carrier. (Figure 16).

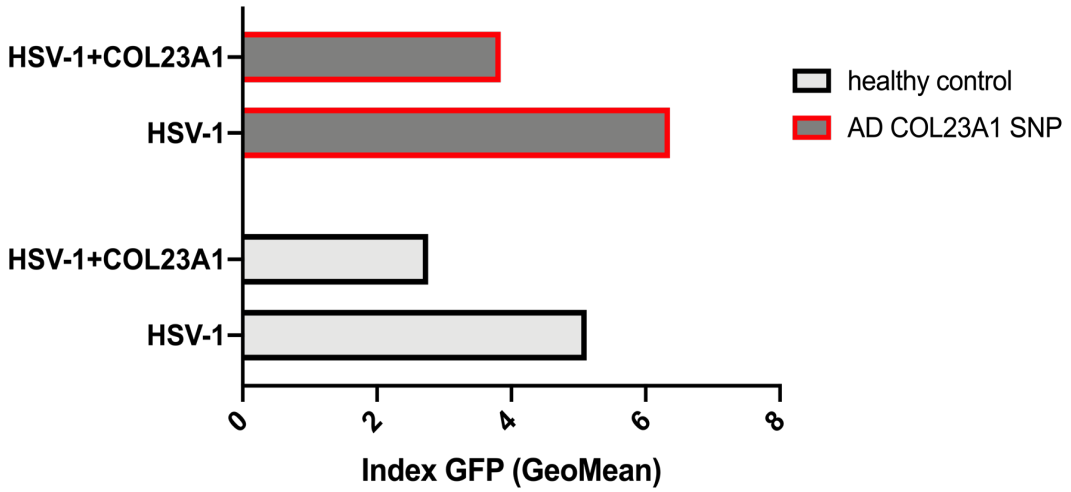


Figure 15. HSV-1 Infection rate detected as GFP Geomean after 1h pre-incubation with (HSV-1 + COL23A1) or without (HSV-1) truncated form of recombinant human COL23A1 in M2 macrophages derived from SNP-carrier and a healthy donor.

Conclusions and future perspectives

During the period of the project following conclusions were formed:

COL23A1 may be a protective factor against HSV-1 in M2 human macrophages, since:

- M2 macrophages significantly express this protein on the cell surface;
- COL23A1^{+} M2 macrophages are less often infected by HSV-1 by a trend *in vitro;*
- macrophages from AD patients with the identified *COL23A1* SNP show an increased susceptibility to HSV-1 infection;
- increased cell surface levels of COL23A1 (as induced by furin-inhibition or by adding recombinant human protein) lead to the decrease of HSV-1 susceptibility in M2 macrophages.

Mikhail Makmatov-Rys

29.12.22

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