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Endothelial integrity may be regulated by a specific antigen via an IgE-mediated mechanism*

Integralność śródbłonka naczyniowego regulowana w sposób IgE-zależny

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Human vascular endothelial function and integrity may be regulated by many non-specific factors. However, the potential influence of specific antigens via an IgE-mediated mechanism remains unknown. The aim of the study was to determine the expression of the IgE receptors FcεRI and FcεRII in the human vascular endothelium and to assess their relevance in the IgE--mediated regulation of endothelial integrity.

Material/Methods: FcεRI and FcεRII expression in human umbilical vein endothelial cells (HUVEC) was genetically assessed by PCR with respective primers and sequencing. HUVEC were cultured with IL-4, and changes in FcεRI and FcεRII mRNA expression were analyzed by real-time PCR. Changes in the integrity of endothelium pre-treated with anti-BSA-DNP IgE following exposure to the specific BSA-DNP antigen was assessed using the Real-time Cell Electric Impedance Sensing system (RTCA-DP).

Results: PCR and sequencing revealed the expression of FcεRI and FcεRII receptors in the human vascular endothelium. IL-4 caused respective 2- and 3-fold increases in FcεRI and FcεRII mRNA expression. Exposure of endothelium pre-treated with anti-BSA-DNP IgE to specific BSA-DNP antigen led to a 20% increase of endothelial integrity ($p<0.05$) after 24 hours, but only in cells pre-incubated with IL-4.

Conclusions: The presence of FcεRI and FcεRII may allow the human vascular endothelium to respond to a specific antigen by increasing its integrity via an IgE-mediated mechanism.

Keywords: **FcεRII • FcεRI • IL-4 • endothelial cells**

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Abbreviations: **EC** – endothelial cells, **FcεRI** – the high-affinity IgE receptor, **FcεRII** – the low- affinity IgE receptor, also known as CD23, **HUVECs** – primary human umbilical vein endothelial cells, **MCs** – mast cells, **MCP-1** – monocyte chemotactic protein 1, **NADPH oxidase** – nicotinamide adenine dinucleotide phosphate oxidase, **NO** – nitric oxide.

Introduction

The vascular endothelium, which tightly covers the inner surface of blood vessels, forms a barrier that controls the flow of solutes and proteins, as well as the entry of leukocytes into tissue [4]. It is a source of cytokines and other mediators that contribute to either aggravation or resolution of tissue inflammation. In addition, it also responds to vasoactive, hemostatic and immunologic factors, as well as adhesion and recognition molecules for leukocytes (P4). However, the presence of receptors for IgG (FcγRs) and IgE immunoglobulins (FcεRI) [11,12,21] indicates that the endothelium may potentially be affected by specific antigens.

IgE is a well-described immunoglobulin produced by B cells and plasma cells in response to parasite antigens and allergens. It mediates the immune reactions to parasitic infections and allergens in allergic diseases, and leads to the release of potent pro-inflammatory mediators, such as histamine, leukotrienes, heparin, matrix proteases and cytokines, mostly by mast cells, basophils and eosinophils [15,18]. Its action requires the cross-linking of two neighboring IgE antibodies, which opsonize to the cell surface covered by FcεR receptors: FcεRI with high affinity to IgE and FcεRII (CD23) with low affinity [17]. Their expression is regulated by IL-4, a pro-inflammatory cytokine mostly engaged in anti-parasitic and allergic responses [2]. Cross-linking of IgE bound to FcεRs initiates intracellular signaling, leading to the immediate release of mediators already pre-stored in cytoplasmic granules. Following this, newly synthesized cytokines and chemokines are secreted [1,3,20].

Although the functional role of IgE-mediated involvement of immune cells in physiological and pathological circumstances is well understood, its role in the functioning of the endothelium requires elucidation. Therefore, the aim of our study was to confirm whether FcεRI and FcεRII expression takes place in human endothelium by genetic methods, and to assess whether this FcεR expression may be induced by a pro-inflammatory cytokine. Finally, the functional relevance of the IgE-mediated regulation of endothelial integrity by a specific antigen was determined using an *in vitro* model especially developed for this purpose.

Materials and Methods

Cells

Endothelial cells (HUVEC) (Lonza C2517A) were expanded in endothelial basal medium-2 (EGM-2) (Lonza, Clonetics, CC-3162), supplemented with EGM-2 BulletKit (Lonza, Clonetics, CC-3156 and 4176). After reaching 80–90 % confluence, the HUVECs were trypsinised with 0.05% trypsin and 0.02% EDTA (SAFC Biosciences, 59417C, Kansas, USA) for four minutes. The mixture was then neutralized by Trypsin Neutralizing Solution (Clonetics, Lonza, CC-5002, Basel, Switzerland) for use in further experiments.

Cell culture in monolayers

The HUVECs were seeded on 12-well plates at a density of 50,000 cells per well in proper media. After reaching 80-90% confluence, HUVEC were induced with IL-4 (rh IL-4, Biolegend, 574004) at a final concentration of 1 ng/ml for 48 hours in order to analyze the expression of particular receptor mRNAs by real-time PCR. The optimal concentration of IL-4 used in this study had been determined in a set of pilot experiments.

mRNA extraction, complementary DNA (cDNA) preparation, real-time polymerase chain reaction (RT-PCR)

mRNA was isolated using the RNeasy Mini Kit (Qiagen) kit, following the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion, and 10 µg of mRNA was reverse transcribed with a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA). PCR was then carried out using an Applied Biosystems 9700HT Fast Real-Time PCR System (Applied Biosystems). The PCR mixture consisted of cDNA solution, SYBR-Green PCR Mastermix (Applied Biosystems) and both sense and antisense primers. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. EF1-α was amplified as a house-keeping gene. FcεRI and FcεRII mRNA expression was normalized to Elongation factor-1α (EF1-α) using ΔΔCt calculation. The following primers were used:

1. FcεRI forward: CTCCATTACAAATGCCACAGT, reverse: CACGCGGAGCTTTTATTACAGTA;

2. FcεRII forward: CTGTGGCACTGGGACACCACA, reverse TTCAGGGCACGTGTTGCACACA; 3. EF-1α forward: CTG AAC CAT CCA GGC CAA AT, reverse: GCC GTG TGG CAA TCC AAT.

Sequencing

The presence of FcεRI and FcεRII mRNA in HAEC cells was demonstrated by mRNA transcript sequencing. mRNA sequence information for the FcεRI and FcεRII genes was obtained from GenBank (accession no. NC_018912 and NC_018930 respectively). The mRNA was reverse transcribed with a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA) as described above. cDNA sequencing was performed using a BigDye Seq kit v3.1 (Applied Biosystems, Foster City, CA, USA). Prior to sequencing, *polymerase chain reaction* (PCR) was performed with the same specifically designed oligonucleotide primers used in the real-time PCR. The reaction was performed in a volume of 32 µl of 10x buffer mixture, 200 µM of each dNTP, oligonucleotide primers, water, the cDNA template and 1 U of HotStar polymerase HiFidelity Polymerase (Qiagen, Germany). The reaction was conducted as follows: 15 min at 95°C, followed by 42 cycles of 30 s at 94°C, 30 s at 61°C and 45 s at 72°C, followed by final elongation for 7 min at 72°C and storage at 4°C.

The PCR products were checked for integrity and purity using 2% agarose gel electrophoresis and purified using commercially available kits (QIAquick PCR Purification Kit, Qiagen, Germany) according to the protocol. The purified PCR products served as template for the *polymerase chain reaction* with fluorescently labeled dideoxynucleotides and one specific oligonucleotide primer. The reaction mixture was composed of 5x buffer, BDX64 buffer, BigDye Sequencing Kit (Version 3.1, Applied Biosystems USA), one specific primer, water and the purified PCR product in a total volume of 20 µl. The reaction was conducted as follows: 3 min at 96°C, followed by 36 cycles of 10 s at 96°C, 5 s at 60°C and 3 min at 60°C and storage at 4°C. The labeled PCR product was purified by ethanol precipitation.

Each product was added to a solution consisting of 96% ethanol, 0.125 mM EDTA and 3M sodium acetate (pH 5.2), before being centrifuged for 50 minutes at 4°C at 4000 revolutions per minute. After removing the supernatant, the cDNA was washed in 75% ethanol and spun again for 20 minutes at 4°C at 4000 revolutions per minute. After drying, the DNA was dissolved in HiDi Formamide (Applied Biosystems, Foster City, CA, USA) and separated by electrophoresis using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting sequence was compared to the reference gene (BLAST).

Cell culture in the Real-time Cell Electric Impedance Sensing system (RTCA-DP, xCELLigence)

The RTCA-DP xCELLigence system (Roche Applied Science), which operates by tracking electrical impedance signals, enables cell growth to be monitored in real time on microelectrode-coated plates. The general procedure of cell culturing in the RTCA DP system is described by Chalubinski et al. [5]. Briefly, the impedance readout is expressed in arbitrary units known as the cell index (CI), which reflects changes in barrier properties, monolayer permeability, cell number, viability and adhesion, as well as morphology. The normalized cell index (nCI) at a certain time point is acquired by dividing the CI value by the value at a reference time point.

At the first stage, endothelial cells cultured in 25 cm^2 T-flasks were pre-incubated with IL-4 in an optimal concentration of 1 ng/ml for 48 hours. The next pool of endothelial cells was not pre-treated with IL-4.

Both pools were then trypsinized and seeded on separate E-16 plates at a density of 20,000 cells per well in culture medium to reach the CI plateau on the second day. The cells were first pre-treated with anti-DNP-BSA IgE immunoglobulin (250 ng/ml) (Sigma-Aldrich, D8406) for 24 hours, and, secondly, specific antigen DNP–BSA (dinitrophenylated bovine serum albumin) (100, 250, 500 ng/ml) (Biosearch Technologies, D-5050) was added to the cultures. The CI changes reflecting changes in endothelial integrity were monitored for 24 hours and measured accurately after 3, 6, 12 and 24 hours of culture. The optimal concentrations of stimulatory agents used in this study had previously been determined in pilot experiments.

Statistical analysis

The results are presented as mean ± SEM for variables with a normal distribution of values. The distribution of particular variables was verified by the Shapiro-Wilk W-test, whereas the Levene test was performed to test homogeneity of variances. The differences between the two groups were analyzed using either the Student's t-test for independent trials in the case of normal distribution and homogeneous variance, or the Mann-Whitney U-test if either of these criteria was not fulfilled. All statistical evaluations were performed using the Statistica package (StatSoft, Inc., Tulsa, Oklahoma).

Results

FcεRI and FcεRII mRNA expression in the human endothelial cells (EC)

A product identified by electrophoresis was obtained in the PCR amplification reaction (Fig. 1A). It was then purified of excess reactants and used in cycle sequencing (Fig. 1B). The transcript sequencing was used to identify the FcεR mRNA in EC. The resulting sequence was compared to the reference gene. Our results reveal the presence of FcεRI and FcεRII(CD23) mRNA in human endothelial cells (Fig. 1).

As FcεR mRNA expression was found to be present in EC,

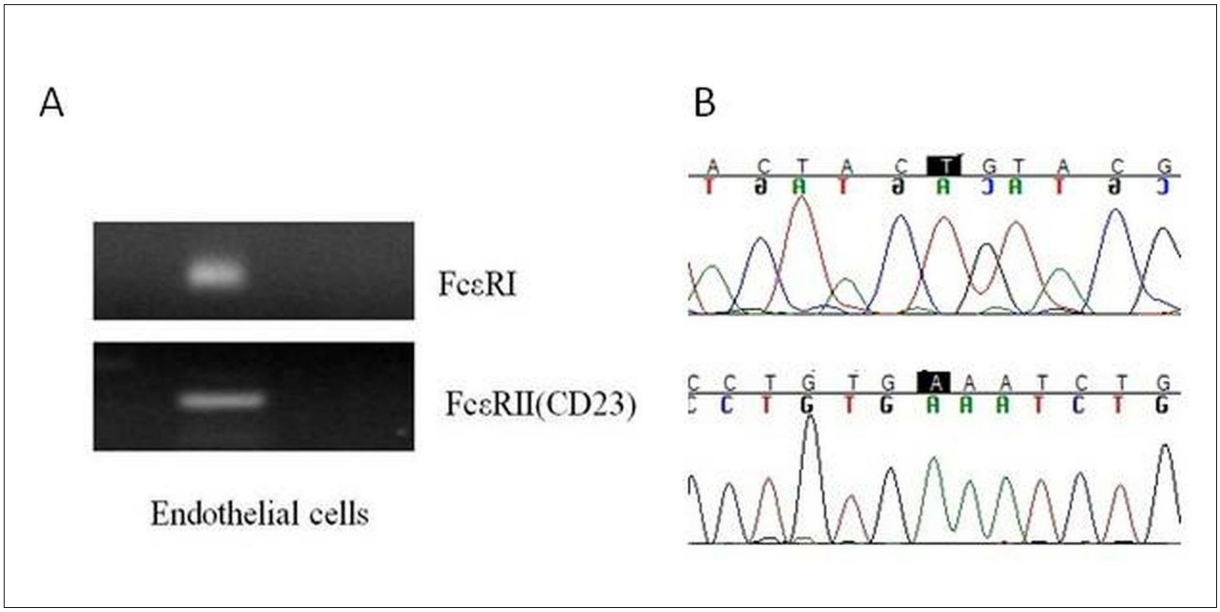

Fig. 1. mRNA expression of FcεRI and FcεRII in human vascular endothelial cells revealed by polymerase chain reaction (PCR) (B) and genetic sequencing (A)

the next step of the study was to examine the effect of IL-4, the cytokine responsible for IgE antibody synthesis, on the *in vitro* expression of mRNA for FcεRI and FcεRII in endothelial cells. The effect of 48-hour IL-4 exposure (1 ng/ml, 10 ng/ml, 100 ng/ml) on FcεR mRNA expression in EC was assessed by real-time PCR (n=6, from three independent experiments).

The effect of IL-4 on FcεRI and FcεRII mRNA expression in human endothelial cells

Incubation of endothelial cells with IL-4 (1 ng/ml) resulted in a 2-fold ($p<0.01$) and a 3-fold ($p<0.05$) increase of mRNA expression for FcεRI and FcεRII, respectively (Fig. 2). IL-4 induced a 2-fold increase of FcεRI, but not FcεRII mRNA expression at a concentration of 10 ng/ml. An IL-4 concentration of 1 ng/ml was chosen for further experiments.

The effect of DNP-BSA on the integrity of human endothelium analyzed in the RTCA-DP system

The RTCA-DP system was used to monitor dynamic changes occurring in the barrier properties of EC monolayers following exposure to the specific antigen DNP-BSA (100, 250, 500 ng/ml), either with or without pre-stimulation by IL-4 (1 ng/ml) (Fig. 3, 4).

Upon the addition of DNP-BSA (100, 250 and 500 ng/ml) to endothelium previously pre-treated with anti-BSA-DNP IgE, the normalized cell index (nCI) did not change and was comparable to untreated cells at 3 hours (1.00±0.24, 1.06±0.14 and 0.94±0.22 vs. 0.95±0.23 respectively), 6 hours (0.97±0.18, 1.01±0.08 and 0.92±0.016 vs. 0.91±0.17 respectively), and 12 hours (0.99±0.014, 1.06±0.08 and 0.96±0.011 vs. 1.00±0.11 respectively) of culture. However, after 24 hours of culture, a significant increase in nCI of 20% was observed compared to untreated cells (1.04±0.1 vs. 0.88±0.15, respectively) (Fig. 3). This suggests that DNP-BSA may significantly enhance endothelial integrity at an optimal concentration of 250 ng/ml.

In contrast to endothelium pre-incubated with IL-4, the addition of the BSA-DNP antigen to endothelium pre-treated with anti-BSA-DNP IgE antibodies, but not IL-4, did not appear to affect endothelial integrity at any of the analyzed time-points. (At 3 hours: 0.92±0.02, 0.88±0.008 and 0.93±0.02 vs. 1.01±0.05, respectively. At 6 hours: 0.98±0.04, 0.98±0.02 and 0.98±0.06 vs. 1.00±0.01, respectively. At 12 hours: 0.96±0.06, 0.99±0.18 and 0.95±0.009 vs. 0.94± 0.03, respectively. At 24 hours: 0.81±0.03, 0.84±0.01 and 0.82±0.01 vs. 0.76±0.01, respectively.) (Fig. 4)

Discussion

The present study shows for the first time that the human vascular endothelium may express both FcεRI and FcεRII: IgE receptors of high and low affinity, respectively. Additionally, our findings indicate that mRNA expression of both receptors could be enhanced by IL-4. It also incorporates an *in vitro* model enabling the assessment of the IgE-mediated effect of a specific antigen on endothelial integrity.

In order to assess the expression of FcεRs in endothelial cells, specific mRNA products were obtained by PCR with respective primers and then they were analyzed by mRNA sequencing. The sequence of both products

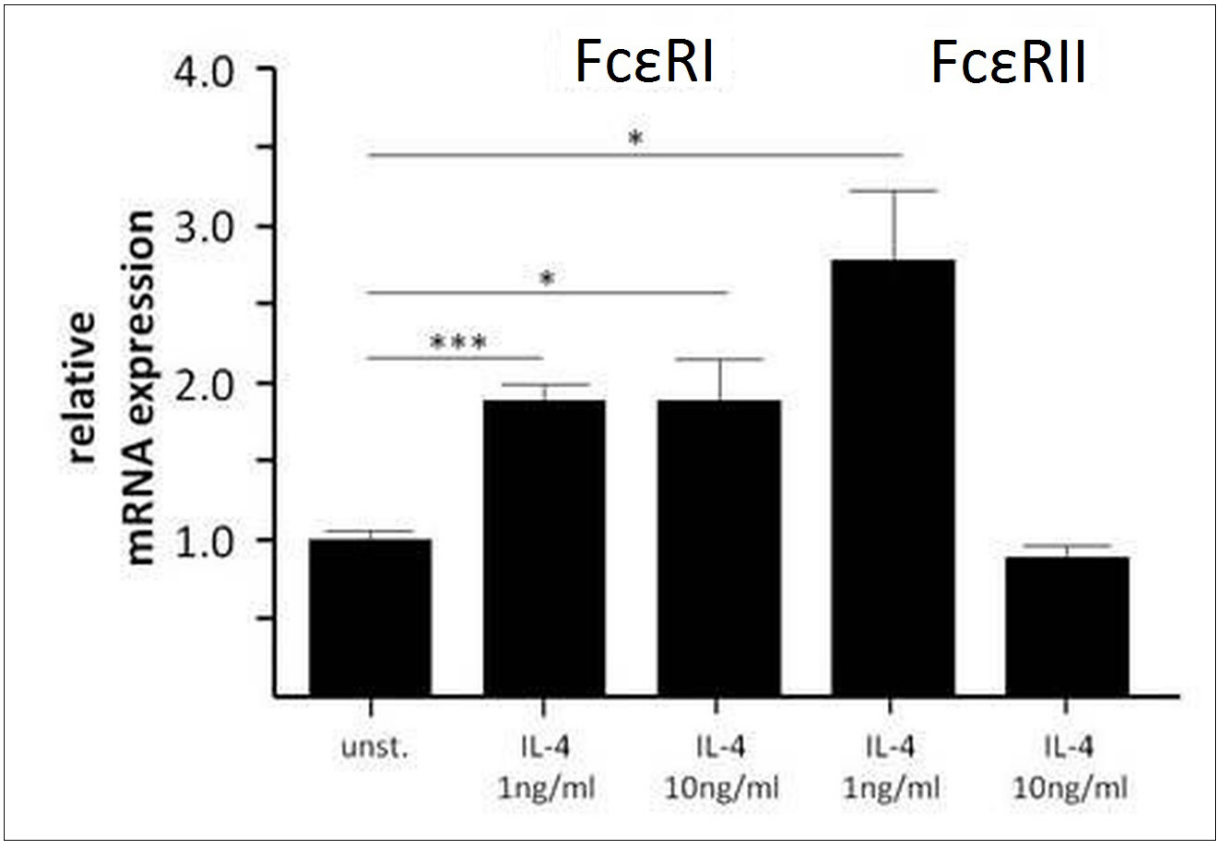

Fig. 2. The effect of IL-4 (1 and 10ng/ml) on the mRNA expression of FcεRI and FcεRII in human vascular endothelial cells (n=6 from 3 independent experiments) (mean±SEM), unst.- unstimulated control, *$p<0.05$, ***$p<0.001$

was found to correspond to known sequences of FcεRI and FcεRII present in various locations, such as mast cells. The expression of high affinity FcεRIα has already been demonstrated in endothelial cells of human atherosclerotic lesions, but not FcεRII [21].

As the constitutive expression of FcεR mRNA was found to be relatively low in the endothelial cells, our next aim was to assess whether FcεR expression may by inducible and enhanced. For this purpose, endothelial cells were treated with IL-4, as this cytokine is involved in the co-ordination of FcεRIα expression on immune cells [2]. This exposure to IL-4 was found to induce a several-fold increase of FcεRI and FcεRII mRNA expression in endothelial cells. Previously, IFN-γ had been evidenced to up-regulate endothelial FcεRIα expression [21], which together with our data indicates that FcεR expression may be inducible in pro-inflammatory conditions.

Our findings suggest that the vascular endothelium has novel functions. Typically, FcεRs are found in mast cells and basophils, where they play a crucial role. In these cells, the cross-linking of IgE bound to FcεRs by specific antigens leads to the release of preformed mediators stored in cytoplasmic granules, such as histamines, leukotrienes, and, later on, newly synthesized cytokines [3,17,18]. Based on these data, it may be assumed that the mechanism of endothelial activation via the antigen-IgE-FcεRs pathway may be similar.

Considering the above, the study used an *in vitro* model based around the Real-time Cell Electric Impedance Sensing system (RTCA-DP) to assess the potential functional relevance of antigen-IgE-FcεRs interaction in endothelial cells. This system enables the assessment of adherent cell status based on a continuous analysis of their integrity and allows observation of both the immediate and late response. As our previous results showed that IL-4 may enhance the expression of FcεRs, endothelial cells were initially pre-incubated with IL-4. Following this, IgE anti-BSA-DNP antibodies were added to the culture in the first step and BSA-DNP antigen with affinity to IgE was added in the second step to enable activation of the endothelium by a specific antigen mediated by IgE.

Our findings indicate that exposure of anti-BSA-DNP IgE-pre-treated endothelium to BSA-DNP antigen caused a 20% increase of endothelial integrity as compared to cells unexposed to the antigen. Interestingly, this effect was observed only in endothelium pre-incubated with IL-4, which suggests that the resting expression of FcεRs is not potent enough to obtain this effect. As endothelial cells do not possess any preformed mediators in the

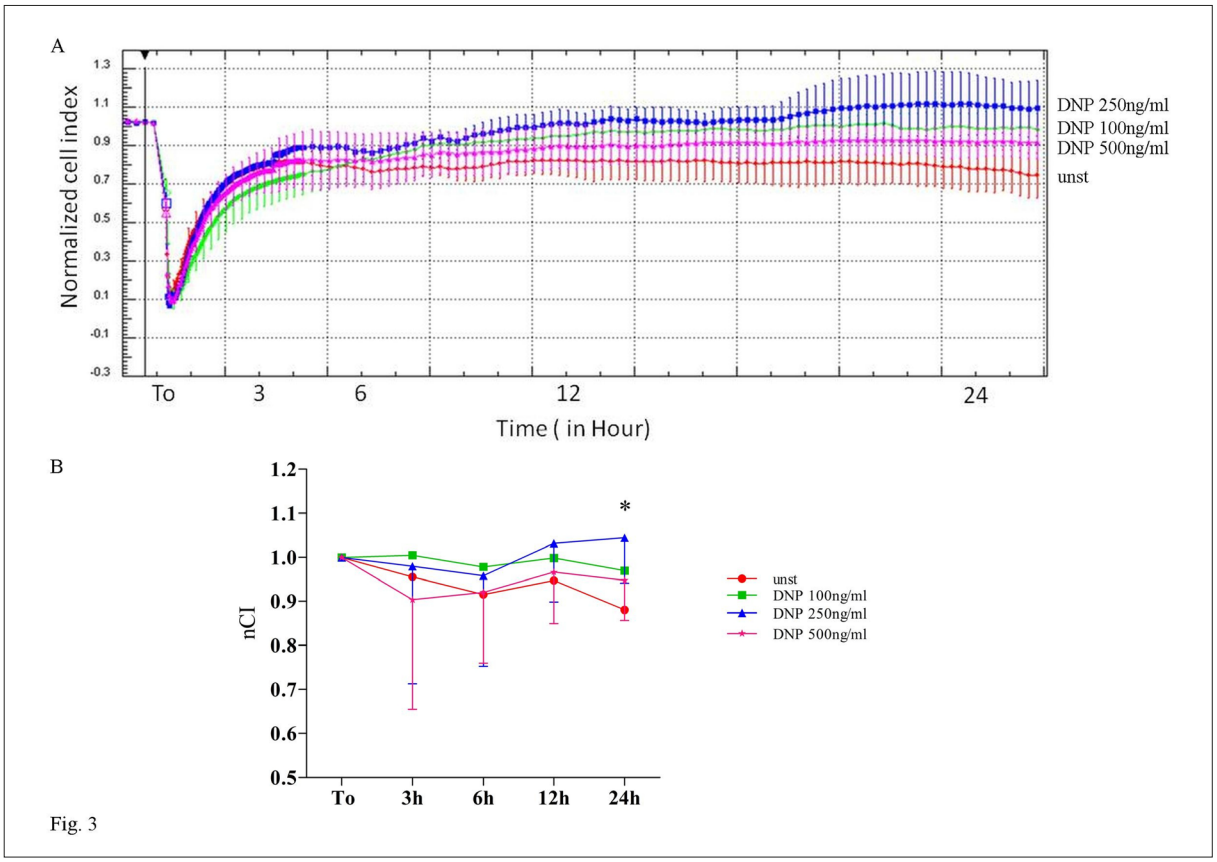

Fig. 3. The effect of the exposure of anti-BSA-DNP IgE-pre-treated human vascular endothelial cells to BSA-DNP antigen on the integrity of endothelium pre-incubated with IL-4 as measured by RTCA-DP. Representative plots from RTCA-DP (A) and analyzed results (B), n=6 from 3 independent experiments; mean ± SEM; unst.- unstimulated controls; nCI - normalized cell index, To - time point of exposure of anti-BSA-DNP IgE-pre-treated endothelium to BSA-DNP antigen; *$p<0.05$ in BSA-DNP (250ng/ml)-treated endothelial cells *vs*. unstimulated controls

cytoplasm similar to mast cells, it would be natural to assume that there would not be any immediate reaction to the activation of the IgE-FcεRs pathway by the antigen. Indeed, changes of endothelial integrity were observed only after 24 hours of incubation with the antigen.

The observation that a specific antigen may regulate endothelial integrity via an IgE-mediated mechanism is a novel one. Although Wang et al. report that IgE stimulation of the endothelium led to the phosphorylation of p38 and JNK, increased levels of caspase-3 and increased apoptosis, no disintegration of endothelium in the RTCA-DP system was observed in the present study after treatment with IgE. However, it should be emphasized that while Wang et al. assess apoptosis after four days of incubation, and only treat the endothelium with IgE [21], our experimental model models the natural activation of FcεRs by cross-linking IgE with antigen more precisely, as it was performed by adding an IgE-specific antigen bound to the FcεRs. The discovery that IgE mediation influences the action of specific antigens on endothelial integrity leads to interesting conclusions. Firstly, it may lead to the enhanced expression of tight junction proteins responsible for the tight binding of cells to each other. Secondly, it may induce cell proliferation by a simple increase of cell number. Finally, it may stimulate the synthesis of cytokines responsible for both of these phenomena.

The results of our study may have fundamental significance. They indicate that apart from responding to a number of varied unspecific factors, such as cytokines, chemokines and tissue factors, the endothelium may have the ability to react specifically to very specific antigens through the IgE-FcεR pathway. This idea, based on our findings and those of Wang et al., places the endothelium in the position of an element of the immune system, one that is even more involved in human immunity than was previously believed.

At present, it is difficult to determine the significance of the role played by the IgE-mediated influence of specific antigens in endothelial function. Physiologically, as IgE is one of the key factors in fighting parasites [9], this mechanism may allow the endothelium to play a more specific role in the immune response to parasite infection. But it also introduces the possibility of the endothelium playing a potential antigen-specific role in pathologies, such as allergic diseases, in which IgE plays a key role.

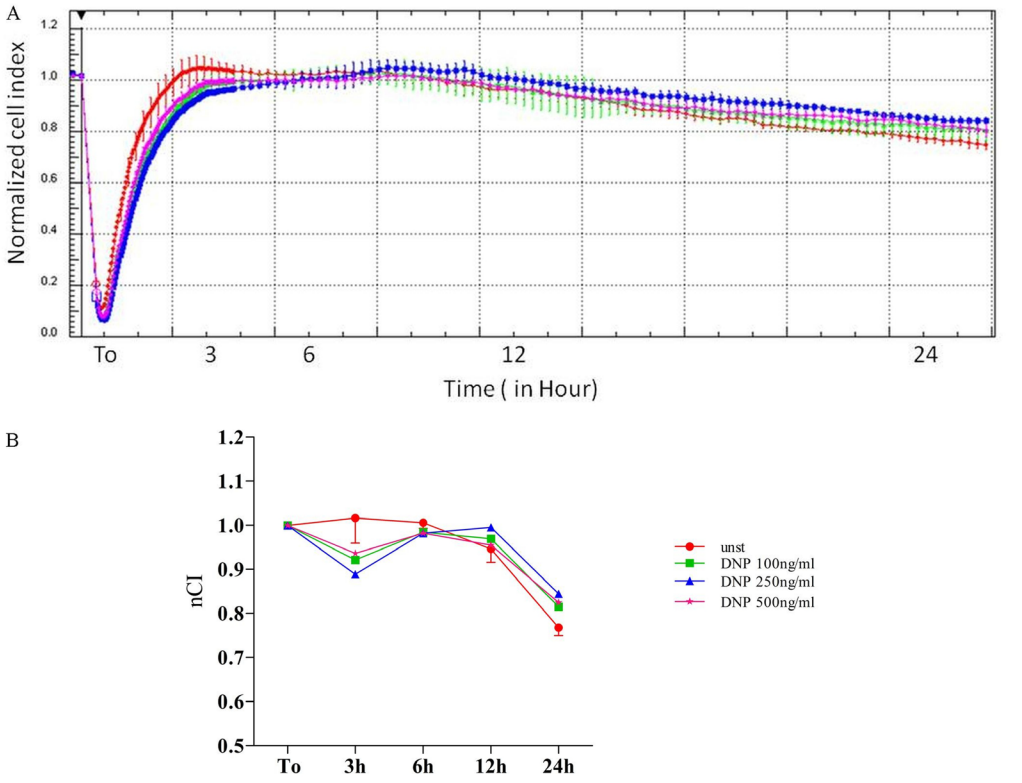

Fig. 4. The effect of the exposure of anti-BSA-DNP IgE-pre-treated human vascular endothelial cells to BSA-DNP antigen on the integrity of endothelium not pre-incubated with IL-4 as measured by RTCA-DP. Representative plots from RTCA-DP (A) and analyzed results (B), n=6 from 3 independent experiments; mean ± SEM; unst.- unstimulated controls; nCI - normalized cell index, To - time point of exposure of anti-BSA-DNP IgE-pre-treated endothelium to BSA-DNP antigen

Many new studies analyze the place of IgE and its main receptor, FcεRI, in different asthma endo/phenotypes and discuss the potential interest of IgE among biomarkers in asthma [10].

The anti-IgE antibody omalizumab, the first approved biological agent recommended for asthma, is currently available for the treatment of patients with severe allergic asthma. Interestingly, this treatment reduces free serum IgE and expression of the high-affinity FceRI receptor present on mast cells and basophils [6,8]. Eosinophilia and elevated IgE in the blood and tissues are characteristically associated with eosinophilic granulomatosis with polyangiitis (EGPA), also known as Churg-Strauss syndrome. The recent results show that anti-IgE antibody may be clinically beneficial also for EGMA patients [7]. In 2015, Lippi and his colleagues found that in patients suffering from anaphylaxis, angioedema, urticaria and urticaria-angioedema, the troponin I levels, which are specific for diagnosing acute myocardial injury, are significantly increased in comparison with healthy controls. This indicates that the heart and especially the coronary arteries constitute primary targets in anaphylaxis [13,16]. Wang et al. show that allergic asthma accelerates atherosclerosis by modulating the balance of Teff/Treg cells in apoE(-/-) mice, which is associated with increased numbers of Th2 and Th17 cells but not Th1 cells [22]. As both IgE and IgE receptors are found in atherosclerotic plaque on the intima [21], coronary artery spasm and plaque rupture may be associated with "allergic myocardial infarction" (Kounis syndrome) [14], and as patients after myocardial infarctions have increased serum total IgE concentrations [19], the IgE-mediated endothelial response could be involved in atherosclerosis.

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The authors have no potential conflicts of interest to declare.