

Tetraspanins CD9 and CD81 are molecular partners of trimeric FcεRI on human antigen-presenting cells

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Abstract

Background: Most functions of tetraspanins are not related to cell-surface receptor ligand binding, but are mediated by direct interactions with their partner proteins. Functions of trimeric FcεRI, expressed by antigen-presenting cells (APCs), range from amplification of allergic inflammatory reactions to their active suppression. Cell-type-specific protein–protein interactions might play a role in the regulation of these bidirectional tasks. Therefore, we intended to study the interactions of trimeric FcεRI with tetraspanins.

Methods: The expression levels of tetraspanins CD9, CD37, CD53, CD63, CD81, CD82, and CD151 on skin dendritic cells of atopic dermatitis (AD) patients or healthy individuals were detected by flow cytometry. Tetraspanin expression on FcεRI^{pos} and FcεRI^{neg} monocyte subpopulations was evaluated. Flow cytometry, confocal microscopy, immunoprecipitation, and immunoblotting experiments were performed to observe the relationship between tetraspanins CD9 and CD81 and FcεRI. Furthermore, plate stimulation experiments were performed, and cytokines in the supernatants were detected.

Results: We found that human FcεRI^{pos} APCs expressed high amounts of tetraspanins and that the tetraspanins CD9 and CD81 were associated with FcεRI. Concomitant activation of FcεRI and CD9 on human monocytes increased FcεRI-mediated cytokine release.

Conclusion: Taken together, we show for the first time that CD9 and CD81 act as molecular partners of trimeric FcεRI on human APC, which might be of importance in allergic diseases such as AD.

The tetraspanin family consists of 28 members of evolutionally conserved transmembrane proteins, including CD9, CD37, CD53, CD63, CD81, CD82, and CD151 (1). According to their name, all the family members are characterized by four transmembrane domains, which contain polar and nonpolar amino acid residues (2). A small extracellular loop connects transmembrane domain one and two, while a larger extracellular loop links transmembrane domain three and four (2). Protein–protein interactions are organized by the extracellular domains, while the intracellular regions communicate with cytoskeletal as well as signaling molecules (3). Tetraspanins act as linker proteins and organizers of multimolecular membrane complexes and co-regulate a plethora of mechanisms, including cell fusion, adhesion, migration, or membrane signaling (3).

However, most of the functions of tetraspanins are not related to any typical cell-surface receptor ligand binding, but mediated by lateral interactions with their partner proteins creating a 'tetraspanin web' (1). On one hand, members of the tetraspanin web own some common functions, while on the other hand, some unique and specific characteristics are allocated to single network members. Consequently, the pattern of tetraspanins expressed by a cell as well as the associated partner proteins and resulting functions is individually different and cell type specific (2).

The high-affinity receptor for IgE (FcεRI) is a surface receptor, which binds IgE with high affinity and is therefore involved in a multitude of immune responses in allergic diseases (4). FcεRI exists as tetrameric (αβ2γ)-chain complex

on effector cells and as a trimeric ($\alpha 2\gamma$) variant on monocytes, dendritic cells (DCs), and eosinophils (4, 5). The α -chain is a member of the immunoglobulin superfamily and contains an extracellular part that accomplishes IgE binding, while the γ -chain dimers are essential for downstream signaling. The β -chain is part of the tetrameric FcεRI variant expressed by effector cells and responsible for the stabilization of the receptor complex on the cell surface as well as the amplification of FcεRI-mediated signals (6). The γ -chains each contain an ITAM, which is phosphorylated after cross-linking of IgE molecules bound to the receptor (4).

Interaction of tetraspanins with tetrameric FcεRI on effector cells has been demonstrated in two previous studies (7, 8). Negative regulation of FcεRI-mediated degranulation of mast cells by antibodies that recognize CD63 or CD81 was observed, while FcεRI-mediated Ca^{2+} -mobilization and tyrosine phosphorylation remained unaffected (7, 8). Interestingly, CD63-mediated inhibition of degranulation *in vitro* was related to the inhibition of the adhesion of mast cells to fibronectin and vitronectin (8).

Trimeric FcεRI is supposed to play a crucial role in antigen uptake and antigen presentation and is overexpressed by monocytes and human epidermal DCs in atopic dermatitis (AD), the most frequent allergic inflammatory disease of the skin (4). Interestingly, FcεRI expressed by antigen-presenting cells (APCs) seems to fulfill quite opposing tasks, which range from a strong amplification of allergic inflammatory reactions after FcεRI cross-linking (9–13), to the active induction of suppressive, pro-tolerogenic mechanisms (14, 15). So far, it is unclear in which way these bidirectional functions of FcεRI on APCs are co-regulated. However, it is very likely that cell-type-specific protein–protein interactions might play a role. Thus, knowledge about mechanisms aggravating FcεRI-mediated skin immune response would be indispensable to develop directed therapeutic strategies to counteract this process. Therefore, we intended to study the role of highly FcεRI/tetraspanin-expressing cells and putative molecular interactions of trimeric FcεRI with tetraspanins on human APCs.

Methods

Reagents

Mabs against CD9 (M-L13), CD37 (M-B371), CD53 (HI29), CD63 (H5C6), CD81 (JS-81), CD151 (14A2.H1), and FITC-labeled anti-human CD81 (JS-18) antibodies were purchased from Becton Dickinson, Heidelberg, Germany. The mAb mouse-anti-CD82 (B-L2) was purchased from Acris (Hiddenhausen, Germany). Phycoerythrin (PE)-labeled mAb against CD14 (MfP9) was obtained from BD Biosciences (Heidelberg, Germany). PE-labeled anti-FcεRI α (AER37) mAb was from eBiosciences (San Diego, CA, USA). PE-labeled T6RD1 (mIgG1) mAb from Coultertronics (Krefeld, Germany) was directed against CD1a. The high-affinity receptor for IgE, FcεRI, was detected by unlabeled mAb 22E7 (IgG1, generous gift of Dr. J. Kochan, Hoffmann La Roche Co., Nutley, NJ, USA) directed against the α -chain of FcεRI not interfering

with the IgE binding site. FITC-conjugated goat anti-mouse (GaM/FITC) and allophycocyanin (APC)-conjugated goat anti-mouse (GaM/APC) antibodies were from Jackson Laboratories (West Grove, PA, USA). Pacific Blue™-conjugated monoclonal antibody against CD9 (MEM-61) was from BIOZOL (Eching, Germany). Normal mouse serum for blocking purposes was obtained from Dianova (Hamburg, Germany). All other reagents were obtained from Sigma Aldrich (Taufkirchen, Germany) unless otherwise indicated.

Isolation of epidermal DCs and monocytes from the peripheral blood

Shave biopsies were taken from patients with AD according to the criteria of Hanifin and Rajka (16) and healthy control volunteers after obtaining signed informed consent, in accordance with the approval of the local ethics committee and the Declaration of Helsinki Principles. Skin lesion harboring DCs was sampled and prepared by trypsinization as described previously (17). The blood for monocyte isolation was drawn at the Department of Dermatology and Allergy of the University of Bonn. Monocytes were isolated from peripheral blood with a modified density gradient protocol using Nycoprep gradient centrifugation (Axis-Shield PoC AS, Oslo, Norway) as described previously (18). Cells were cultured in very low endotoxin 1640 medium (Biochrom, Berlin, Germany) with 1% antibiotics/antimycotics and 10% inactivated FCS.

Flow cytometry

Flow cytometric staining was performed as described before (11). Finally, the cells were acquired on a FACS-Canto (Becton Dickinson) as described in detail elsewhere and analyzed by FACSDIVA (Becton Dickinson) and FLOWJO (TreeStar Inc., Ashland, OR, U.S.A) software. For quantitative evaluation, dead cells were excluded by 7-AAD staining, and CD1a⁺ or CD14⁺ population was gated out manually and either expressed in percentage of positive cells or by the relative fluorescence index (rFI), determined as follows: $\text{rFI} = (\text{mean fluorescence intensity (MFI) (receptor)} - \text{MFI (isotype control)}) / \text{MFI (isotype control)}$.

Confocal laser scanning microscopy

For staining of FcεRI α chain and tetraspanins CD9 and CD81, 3×10^5 cells were washed with PBS and adhered to coverslips coated with poly-L-lysine solution (Sigma Aldrich). Cells were incubated in PBS + 0.2% BSA, fixed with PBS + 4% paraformaldehyde, then incubated with PBS + 0.1 M glycine, separated by washes with PBS. Permeabilization was performed for 30 min with saponin buffer (PBS + 0.5% BSA + 0.1% saponin) followed by blocking with 0.5 mg/ml human IgG Fc (Calbiochem, San Diego, CA, USA) for 30 min. Incubation with Pacific Blue™-conjugated anti-CD9 (MEM-61), FITC-labeled anti-CD81 (JS-18), and PE-labeled anti-FcεRI α (AER37) antibodies was performed for 1 h at 4°C. After staining, the samples were washed four times with saponin buffer and PBS. Finally, the coverslips were sealed

with Gel Mount™ Aqueous Mounting Medium (Sigma Aldrich) and analyzed with an Olympus FluoView1000 confocal microscope (Olympus, Hamburg, Germany) equipped with an argon–krypton laser. Images with different dyes were scanned sequentially. All scans were acquired with a Plapo 60×, NA 1.4 oil immersion objective (Olympus). The software FV10-ASW 1.4 (Olympus) was used for picture analysis.

Immunoprecipitation and immunoblotting

To examine co-immunoprecipitation of FcεRI with tetraspanins CD9 and CD81, freshly isolated monocytes (approximately 4×10^6 cells/sample) from AD donors were collected. After three rinses with cold PBS, cells were lysed by scraping into ice-cold radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. After 15 min of extraction at 4°C with rocking, insoluble material was removed by centrifugation. Lysates containing equal amounts of protein were incubated with 2 µg mouse IgG isotype control or mouse mAb anti-CD9 (ALMA.1) (Dr. Francois Lanza, Institut National de la Santé et de la Recherche Medicale [INSERM] U.311, Etablissement Francais du Sang–Alsace, Strasbourg, France), anti-CD81 (5A6) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-FcεRI α-chain (22E7) antibody. Immune complexes were collected with protein A/G-Sepharose (Santa Cruz Biotechnology Inc.) overnight incubation at 4°C, separated by SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Blots were blocked with 5% nonfat milk in Tris-buffered saline (20 mM Tris, pH 7.5, and 0.15 M NaCl) containing 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with 1 : 1000 diluted anti-FcεRIα (22E7) antibody overnight at 4°C, followed by 1 : 2000 diluted HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology Inc.). Proteins were detected by enhanced chemiluminescence Western blot detection system (ECL; Amersham Biosciences, Freiburg, Germany). Molecular weights and variations in phosphorylation were determined by Fujifilm LAS 3000 image analysis (Fujifilm Europe GmbH, Düsseldorf, Germany).

FcεRI-ligation

Cross-linking of FcεRI was achieved as previously described (14). Briefly, the cells were harvested and washed with culture medium twice, incubated for 1 h with 1 µg/ml human IgE (Calbiochem), washed twice, and stimulated with 20 µg/ml rabbit anti-human IgE (Dako, Glostrup, Denmark) for the duration of the whole culture.

Plate stimulation and analysis of soluble factors in the cell culture supernatant

For analysis of soluble factors in the cell culture supernatant of CD9- or/and CD81-stimulated monocytes co-activated by FcεRI cross-linking, antibody-coated plates were used as

described previously (19). Briefly, mAb against CD9 (ALMA.1) and mAb against CD81 (5A6) were respectively diluted in 5 µg/ml in coating buffer, plates were covered with the solution and incubated overnight at 4°C, washed with PBS, and blocked with 10% FCS in PBS. Monocytes left unstimulated or stimulated via FcεRI cross-linking were suspended in culture medium and seeded at a concentration of 2×10^5 cells/well to the antibody-coated 96-well plates. After 24 h of stimulation, cells were harvested and cell culture supernatants were kept in –80°C. Amount of IL-10 and TNF-α in the supernatants was quantified with the help of the Flex set kits (Becton Dickinson). Beads were detected with the FACSCanto and analyzed with FACSDIVA software.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Quantitative values were compared between the different groups by using the Mann–Whitney *U*-test. Results are given as mean ± SEM, respectively. Any *P*-values are two-sided and subject to a global significance level of 5%.

Results

Human epidermal DCs from AD patients express high amounts of tetraspanins

Distinct inflammation- and disease-related expression of tetraspanins on APCs has been demonstrated in previous studies (20–22). Increased expression of FcεRI is a characteristic feature of APCs in the peripheral organs such as the skin and blood of patients with allergic inflammatory diseases (4). Co-regulators of FcεRI-mediated signaling on APCs have not been identified so far. Therefore, we analyzed the expression pattern of tetraspanins of human FcεRI^{pos} epidermal DCs in lesional skin of patients with AD, compared to epidermal DCs in the skin of healthy controls. We observed significantly higher expression of FcεRI as well as CD9, CD53, CD63, CD81, and CD82 on epidermal DCs in the lesional skin of patients with AD (Fig. 1A–F), while no difference in the expression of CD37 and CD151 was detected (data not shown).

FcεRI^{pos} blood monocytes from AD patients express high amounts of tetraspanins

To evaluate a putative co-expression of FcεRI with selective tetraspanins, FcεRI^{pos} and FcεRI^{neg} fraction of monocytes isolated from AD patients was subanalyzed for the expression of CD9, CD37, CD53, CD63, CD81, CD82, and CD151. As a result, we found that the subpopulation of FcεRI^{pos} monocytes was highly positive for CD9 and CD81 (Fig. 2A), while only moderate CD9 and CD81 expression was detectable on the respective FcεRI^{neg} monocyte subfraction (Fig. 2B). These data provide evidence for a concomitant upregulation of CD9 and CD81 on FcεRI^{pos} APCs.

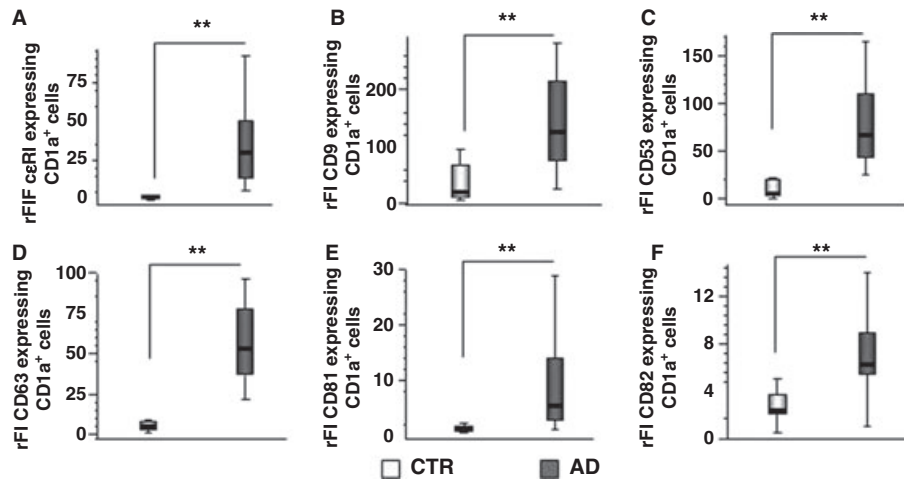


Figure 1 Enhanced expression of tetraspanins on human epidermal DCs from atopic dermatitis (AD) patients. The expression of tetraspanins CD9, CD53, CD63, CD81, CD82, and FcεRI on human

epidermal DCs from AD patients and health controls (A–F) is depicted as relative fluorescence index (rFI). Mean $\pm$ SEM of $n = 8$ is shown; ** $P < 0.01$ vs control.

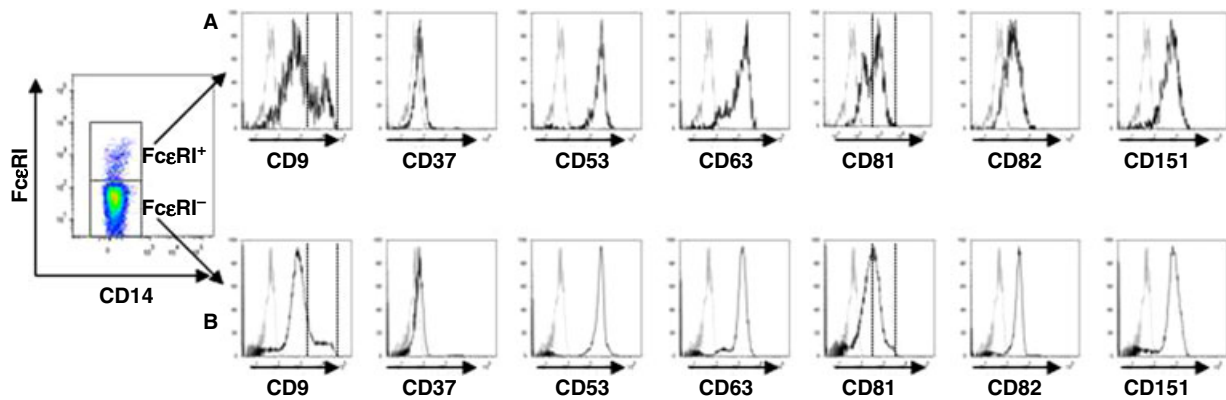


Figure 2 Enhanced expression of tetraspanins CD9 and CD81 on FcεRI^{pos} monocytes. Freshly isolated monocytes were divided into (A) FcεRI^{pos} and (B) FcεRI^{neg} monocytes, and expression of the tetraspanins was analyzed separately for each fraction. One repre-

sentative experiment of $n = 3$ is shown. Gray line: isotype control, Black line: expression of the respective tetraspanin indicated below, expression is shown as log fluorescence intensity. Gating strategy to separate FcεRI^{pos} and FcεRI^{neg} monocytes is shown on the left.

CD9 and CD81 are associated with FcεRI on human monocytes

Our data from freshly isolated human monocytes and DCs suggest that some of the tetraspanins CD9 and CD81 might be co-expressed with trimeric FcεRI on APCs. Therefore, after incubation with CD9, CD81, and FcεRI dye-conjugated antibodies, freshly isolated human monocytes were analyzed by flow cytometry. A subfraction of FcεRI^{pos} blood monocytes co-expressed CD9 and CD81 (Fig. 3A). Furthermore, confocal microscopic analysis of CD9, CD81, and FcεRI triple immunofluorescence-stained monocytes was performed, and colocalization of CD9 and CD81 with FcεRIα was observed (Fig. 3B). Tetraspanins are known to associate with each other and to form multi-levels of interactions with many other surface molecules. To clarify the primary interaction of FcεRI and its tetraspanin partners, we next analyzed whether

tetraspanins co-precipitate with FcεRI on human monocytes. We could show that tetraspanins CD9 and CD81 were tightly associated with FcεRI on freshly isolated human monocytes by co-immunoprecipitation and immunoblotting (Fig. 3C). From these data, we conclude that CD9 and CD81 are molecular partners of trimeric FcεRI on human monocytes.

Increased FcεRI-mediated IL-10 release in response to FcεRI/CD9 co-activation

Previous studies demonstrated that cross-linking of FcεRI on human monocytes promotes both the release of proinflammatory mediators such as IL-1β or TNF-α (13, 14) and the production of pro-tolerogenic, immunosuppressive factors including IL-10 (14). To assess a putative modulatory effect of CD9 and CD81 on signals mediated by FcεRI-activation, human monocytes were either stimulated with stimulatory

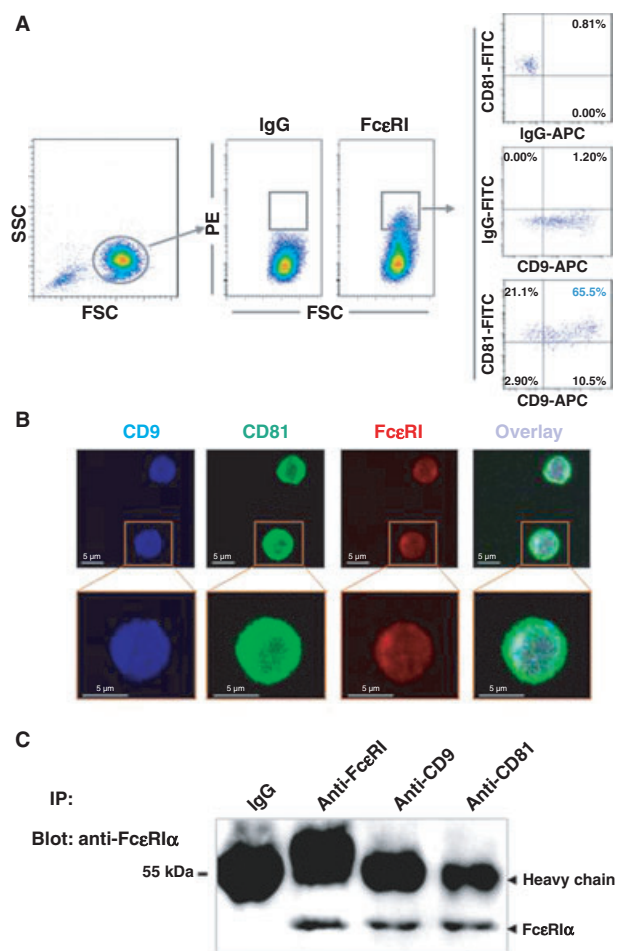


Figure 3 CD9 and CD81 are associated with trimeric FcεRI on human monocytes. (A). Flow cytometric analysis of the co-expression of FcεRI, CD9, and CD81 in human monocytes. Freshly isolated monocytes were incubated with anti-CD9 (ML-13) antibody, followed by incubation with a secondary GaM-APC. After a blocking step, cells were incubated with FITC-conjugated anti-CD81 (JS-18) and PE-labeled anti-FcεRIα (AER37) antibodies. Isotype-matched IgG control antibodies were used. One representative experiment of $n = 4$ is shown. (B). Confocal microscopic analysis of FcεRI, CD9, and CD81 expression of human monocytes. Freshly isolated monocytes were permeabilized and stained with Pacific Blue™-conjugated anti-CD9 (blue), FITC-conjugated anti-CD81 (green), and PE-conjugated anti-FcεRIα (red) antibodies. White color indicates proximity of signals or colocalization. One representative experiment of $n = 4$ is shown. (C) Lysates of human monocytes were immunoprecipitated with mAb against CD9, CD81, and FcεRI or respective isotype control and analyzed with anti-FcεRI immunoblotting. One representative experiment of $n = 3$ is shown.

tetraspanin mAbs against CD9, CD81 or CD9 and CD81 alone or simultaneously co-activated together with FcεRI cross-linking. In line with previous observations, we detected a significantly enhanced IL-10 release by monocytes in response to FcεRI aggregation. This FcεRI-mediated IL-10 release was further increased after FcεRI/CD9 co-activation,

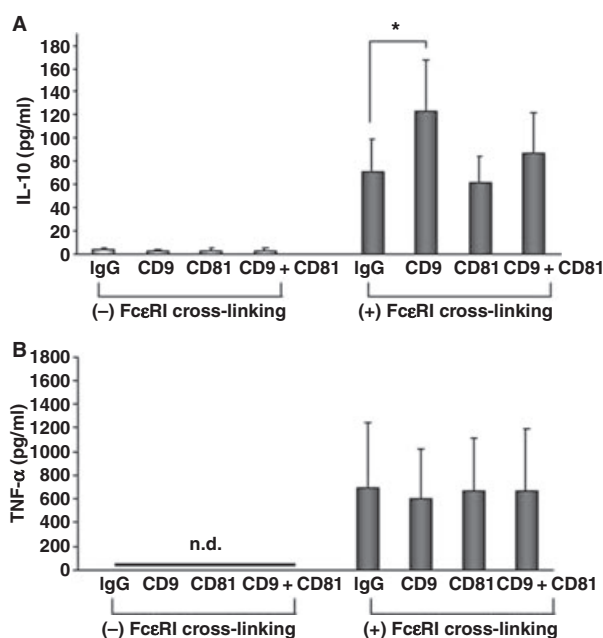


Figure 4 CD9 co-stimulation facilitates FcεRI-mediated IL-10 secretion of human monocytes. Freshly isolated human monocytes were activated by FcεRI-aggregation or left unstimulated, seeded to CD9, CD81, CD9/CD81, or isotype control antibody-coated plates and cultured for 24 h. Supernatants were harvested and amount of IL-10 (A), and (B), TNF-α was measured with cytokine bead assays. Mean value of $n = 5$ experiments + SEM is depicted; * $P < 0.05$ vs control; n.d. = not detectable.

while FcεRI/CD81 co-activation or concomitant CD9/CD81 and FcεRI-activation did not lead to a further upregulated IL-10 secretion (Fig. 4A). In contrast, activation of CD9, CD81 or CD9 and CD81 on monocytes, which were not co-cross-linked via FcεRI, did not induce any detectable IL-10 secretion. No difference in FcεRI-induced TNF-α secretion was observed in monocytes co-activated via CD9, CD81, or CD9/CD81 (Fig. 4B). These data support the concept of tetraspanins as co-modulators of FcεRI-mediated cell activation, without possessing strong proprietary functions.

Discussion

In this study, we demonstrated that the expression of the tetraspanins CD9, CD37, CD53, CD63, CD81, and CD82 on FcεRI^{pos} skin DCs from AD patients was significantly higher than on skin DCs of healthy individuals. In addition, FcεRI^{pos} monocytes expressed more CD9 and CD81 than their FcεRI^{neg} counterparts, suggesting a close link between FcεRI and the tetraspanins CD9 and CD81. Furthermore, flow cytometry, confocal microscopy, and immunoprecipitation/immunoblotting experiments confirmed the association of FcεRI with CD9 and CD81. Thus, stimulation of CD9 together with FcεRI on human monocytes enhanced the release of IL-10, an important immune regulatory cytokine, while the secretion of TNF-α was not affected. These data

provide for the first time evidence for a close interaction of FcεRI with CD9 and CD81 on human APCs.

Pregnancy-specific glycoprotein (16) has been identified as natural ligand to CD9 (23), while the hepatitis C virus envelope protein E2 binds to CD81 (24). The higher expression of tetraspanins on human APCs observed here in AD and concept of CD9 and CD81 as molecular partners is of particular immunological importance. A role of CD81 as amplifier of Th2 immune responses in murine model systems has been described. In these studies, allergen-induced airway hyper-reactivity was reduced in CD81-deficient mice (25) and mice lacking CD81 showed diminished IL-4 secretion and antibody production by B cells following stimulation with antigens, which promoted Th2 immune responses (26). In addition, as a component of the CD19/CD21 complex, CD81 was demonstrated to enhance B cell receptor signaling (27). Furthermore, CD81 expressed by T cells plays a crucial role in cognate T cell/B cell interactions (28). Thus, direct stimulation of CD81 on T cells resulted in stronger Th2 responses and effector functions (25, 29, 30). Taken together, these data indicate that CD81 augments allergic immune responses on various cellular levels, including T cells and B cells. First evidence for CD9 as a facilitator of signaling receptors arose from studies, which described a functional association of CD9 with Fcγ receptors in mouse macrophages, resulting in the activation of macrophages after CD9/FcγR co-ligation (31). Furthermore, studies with human platelets demonstrated Syk phosphorylation after CD9/FcγRII co-aggregation (32). Interaction of Fc receptors, especially FcγRII with another tetraspanin, CD82 also has been described, showing higher Ca²⁺-flux in response to CD82/FcγR coligation (33). Together, these data, in accordance with the data presented here, provide profound evidence for a counter-regulation of Fc receptor functions by tetraspanins, leading to the amplification of FcR-mediated signals. In which way this amplification is accomplished on the molecular level is unclear. This might include higher availability of protein tyrosine kinases or other signal transduction molecules, stabilization of signaling complexes, or facilitated membrane reorganization by S-palmitoylated intracellular domains of CD9 or CD81 and enforced translocation of signaling molecules

to detergent resistant microdomains (34). In addition, the described association of protein kinase C, type II phosphatidylinositol 4-kinase, and phospholipase Cγ with tetraspanins may be of functional relevance in this context (3, 35).

Current knowledge about mechanisms that modulate FcεRI-mediated signaling is mostly based on inhibitory effects and FcεRI-mediated signaling and effector functions of the tetrameric FcεRI complex, which are tightly controlled by inhibitory cell surface molecules. For example, the low-affinity IgG receptor FcγRII (CD32B) binds allergen-specific IgG and down regulates FcεRI-mediated signaling via ITIMs after FcεRI/FcγRII co-aggregation (4). In contrast, not much is known about the regulatory pathways, which might booster FcεRI-mediated signaling of the trimeric FcεRI complex on the cellular level. However, such mechanisms are important in the pathophysiology of allergic diseases including AD, in which FcεRI-overactivation is regarded as an important step.

Altogether, formation of CD9/CD81/FcεRI complex in human APCs, combined with the high expression of these tetraspanins on FcεRI^{pos} APCs in AD, provides an innovative explanation for the versatile character of FcεRI on APCs. Moreover, enhanced IL-10 release after CD9 stimulation might impact on the intensity of the allergic inflammatory reaction, which is often very difficult to control by therapeutic measures in the clinical practice. Hence, knowledge of this molecular interaction provides an important step toward our understanding of the modulation of FcεRI-mediated signaling on APCs.

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Conflict of interest

The authors have no conflicting financial interests.

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