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Evidence for a differential expression of the FcεRIγ chain in dendritic cells of atopic and nonatopic donors

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While mast cells and basophils constitutively express the high-affinity IgE receptor (FcεRI), it is absent or weakly expressed on APCs from normal donors. FcεRI is strongly upregulated on APCs from atopic donors and involved in the pathophysiology of atopic diseases. Despite its clinical relevance, data about FcεRI regulation on APCs are scarce. We show that in all donors intracellular α chain of the FcεRI (FcεRIα) accumulates during DC differentiation from monocytes. However, expression of γ chains of the FcεRI (FcεRIγ), mandatory for surface expression, is downregulated. It is low or negative in DCs from normal donors lacking surface FcεRI (FcεRI^{neg} DCs). In contrast, DCs from atopics express surface FcεRI (FcεRI^{pos} DCs) and show significant FcεRIγ expression, which can be coprecipitated with FcεRIα. In FcεRI^{neg} DCs lacking FcεRIγ, immature and core glycosylated FcεRIα accumulates in the endoplasmic reticulum. In FcεRI^{pos} DCs expressing FcεRIγ, an additional mature form of FcεRIα exhibiting complex glycosylation colocalizes with FcεRIγ in the Golgi compartment. IgE binding sustains surface-expressed FcεRI on DCs from atopic donors dependent on baseline protein synthesis and transport and enhances their IgE-dependent APC function. We propose that enhanced FcεRI on DCs from atopic donors is driven by enhanced expression of otherwise limiting amounts of FcεRIγ and is preserved by increased IgE levels.

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Introduction

Ligation of the high-affinity IgE receptor (FcεRI) on effector cells of anaphylaxis such as mast cells and basophils induces cell activation and immediate release of allergic mediators. FcεRI on these cells shows a tetrameric structure of a heavily glycosylated α chain of the FcεRI (FcεRIα), two γ chains (FcεRIγ) containing phosphoacceptors for signaling proteins, and a β chain (FcεRIβ), which enhances FcεRI surface expression and signaling (1). In addition, a trimeric form of FcεRI lacking FcεRIβ is found on human dedicated APCs such as DCs, including epidermal Langerhans' cells (LCs), blood DCs, and monocytes (2–7). APCs bearing trimeric FcεRI can efficiently present IgE-bound antigens to T cells in an IgE-mediated delayed-type hypersensitivity reaction (6, 8, 9), putatively playing an important role in the pathophysiology of atopic diseases (10, 11).

The mechanisms regulating FcεRI expression on APCs are of particular interest because, in contrast to constitutive expression on effector cells of anaphylaxis, FcεRI surface expression is associated with the atopic status of the donors. Healthy donors often show low or no surface FcεRI on APCs, depending on the cell type, whereas atopic donors display high levels (5, 7, 12, 13). Only FcεRI expressed in significant amounts, i.e., on APCs of atopic donors, may mediate sufficient signaling and effector functions (11). A role of FcεRI in atopic diseases can be undermined by in vivo observations, such as the emergence of inflammatory dendritic epidermal cells (IDECs), which are present in inflammatory skin, and in atopic dermatitis (AD) show very high FcεRI levels (14). The mechanisms guiding such in vivo phenomena are unknown. Studies about basic mechanisms of FcεRI regulation have been done using in vitro reconstitution systems and effector cells of anaphylaxis. In rodents a tetrameric structure of FcεRI is obligatory, whereas FcεRI expressed in humans requires a minimal trimeric structure without FcεRIβ (15, 16). FcεRIγ is mandatory for in vitro $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ receptor surface expression (16, 17). Regarding FcεRI assembly and maturation (18–21), folding and core glycosylation of immature FcεRIα in the ER are followed by trimming

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Nonstandard abbreviations used: high-affinity IgE receptor (FcεRI); α chain of the FcεRI (FcεRIα); γ chains of the FcεRI (FcεRIγ); β chain of the FcεRI (FcεRIβ); Langerhans' cell (LC); inflammatory dendritic epidermal cell (IDEC); atopic dermatitis (AD); phycoerythrin (PE); protein disulfide isomerase (PDI); peroxidase (POD); monocyte-derived dendritic cell (MoDC); endoglycosidase H (Endo H); anti–4-hydroxy-3-iodo-5-nitrophenyl-acetyl (NIP); NIP-haptenized tetanus toxoid (NIP-TT); relative stimulation index (rSI); negligible FcεRI surface expression on DC (FcεRI^{neg} DC); significant FcεRI surface expression on DC (FcεRI^{pos} DC); brefeldin A (BFA); cycloheximide (CHX); chimeric IgE (cIgE).

of terminal glucose residues. The export of immature FcεRIα from the ER to the Golgi compartment is controlled by correct trimming and association with the FcεRIγ chains. Then terminal glycosylation with complex sugars follows, and mature FcεRI is transported to the cell surface. FcεRIβ enhances this process, leading to increased surface expression of FcεRI.

In APCs, IgE and IL-4 can enhance FcεRI expression on monocytes and THP-1 cells (7, 13, 22). Human LCs are immature DCs forming sentinels of the immune system in the skin and express an intracellular FcεRIα pool irrespective of the atopic status. Increased FcεRI surface levels are associated with upregulation of FcεRIγ (23). However, detailed analyses of FcεRI subunit regulation in LCs are limited because of insufficient availability. In addition, LCs show spontaneous differentiation into mature DCs, which is accompanied by the irreversible loss of FcεRIα expression. To study FcεRI regulation on DCs in detail, alternative systems have become available. DCs can be generated from peripheral monocytes with GM-CSF and IL-4 (24), and the differentiation stages can be controlled more easily. Using this system, we analyzed trimeric FcεRI subunit regulation, localization, and its biochemical status in DCs with regard to the atopic status of donors.

Methods

Reagents. Phycoerythrin-labeled (PE-labeled) T6/RD1 mAb (Beckman Coulter GmbH, Krefeld, Germany) recognizes CD1a. The mAb's 22E7 (a kind gift from J. Kochan, Hoffman-La Roche Diagnostics, Nutley, New Jersey, USA) and 3G6 (Upstate Biotechnology Inc., Lake Placid, New York, USA) detect FcεRIα (25, 26). RAB1 is a kind gift from T. Bjerke (Institute of Anatomy, University of Aarhus, Aarhus, Denmark) and is a polyclonal rabbit Ab against human FcεRIα. Polyclonal rabbit antiserum against FcεRIγ was from Upstate Biotechnology Inc. and mAb 4D8 was kindly provided by J. Kochan (see above). Rabbit polyclonal Ab's for organelle labeling were directed against protein disulfide isomerase (PDI) in the ER (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) and giantin in the Golgi compartment (a kind gift of Y. Misumi and Y. Ikehara, Fukuoka University, Fukuoka, Japan). The mAb against FcγRIII/CD16 (3G8) was from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA). The mAb against FcγRII/CD32 (IV.3) was from Medarex (Lebanon, New Hampshire, USA), and the mAb against FcγRI/CD64 (mAb 10.1) was purchased from PharMingen (San Diego, California, USA). Human myeloma IgE was obtained from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). FITC-, Cy2-, and Cy3-labeled F(ab′)2 fragments of goat anti-mouse Fc Ab as well as Cy2- and Cy3-labeled goat anti-rabbit Ab were purchased from Jackson ImmunoResearch Laboratories Inc. Alexa Fluor 488-nm goat anti-mouse F(ab′)2 fragments were from Molecular Probes Inc. (Eugene, Oregon, USA). PE-labeled anti-CD14 Ab was from Becton Dickinson Immunocytometry Systems (San Jose, California, USA). Peroxidase-conjugated (POD-conjugated) goat anti-mouse Ig Ab was obtained from Bio-Rad Laboratories Inc. (Richmond, California, USA). POD-conjugated goat anti-rabbit Ig Ab, poly-L-lysine, digitonin, and saponin were from Sigma-Aldrich (St. Louis, Missouri, USA).

Monocyte isolation and generation of monocyte-derived dendritic cells. Atopic donors were showing a clinical history of atopic dermatitis, and/or allergic asthma, and/or allergic rhinoconjunctivitis, and elevated serum IgE (>100 kU/l) and were not subject to therapy. Nonatopic donors exhibited none of these parameters. They were selected in accordance with the local ethics committee and gave written informed consent. Monocytes were isolated from peripheral blood with a modified density-gradient protocol using Nycoprep (Nycomed, Oslo, Norway). Briefly, red blood cells were separated from plasma by sedimentation from EDTA blood with one-tenth (wt/vol) 6% dextran 500 in 0.9% NaCl. Plasma was layered over Nycoprep and centrifuged for 20 min at 600 *g*. After separation, the interphase and upper part of the Nycoprep were collected and washed four times with 0.9% NaCl plus 0.13% EDTA plus 1% BSA. Then, CD14 expression was assessed, and isolated monocytes were cultured for up to 8 days with 500 U/ml GM-CSF (Genzyme Pharmaceuticals, Cambridge, Massachusetts, USA) and 500 U/ml IL-4 (Life Technologies GmbH, Eggenstein, Germany) to yield immature monocyte-derived dendritic cells (MoDCs). For analysis of effects on FcεRI regulation, myeloma IgE was initially added at 1 μg/ml at day 0 and 0.5 μg/ml at days 2 and 4. To assess the mechanism of FcεRI upregulation, IgE was added at 1 μg/ml at day 4 ± cycloheximide (Sigma-Aldrich) or ± brefeldin A (GolgiPlug; PharMingen), both at 1 μg/ml. These inhibitors did not affect cell viability determined by 7-aminoactinomycin-D and apoptosis staining. Stimulation of immature MoDCs with TNF-α (100 U/ml) (Genzyme Pharmaceuticals) was performed for 2 days to achieve final maturation. Contamination of monocyte and DC preparations with mast cells or basophils was excluded by staining with anti-CD117 (Becton Dickinson Immunocytometry Systems) and anti-CD203c (Immunotech, Marseille, France) mAb.

Flow-cytometric analysis. Double-staining experiments with saponin or digitonin for the detection of surface or intracellular FcεRI chains were performed as described (3, 14, 27). Saponin permeabilization was used for detection of intracellular FcεRIα, whereas digitonin was used for detection of FcεRIγ expression as reported (23). Cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). For quantitative evaluation, the $CD1a^{pos}$ or $CD14^{pos}$ population was gated manually, and the percentage of FcεRIα- or FcεRIγ-positive was determined using CellQuest software (Becton Dickinson Immunocytometry Systems).

Confocal laser scan microscopy. For staining of FcεRI chains and organelle labeling, 3×10^5 cells were washed with PBS and adhered to coverslips coated with poly-L-lysine solution. Cells were incubated in PBS plus 0.2% BSA, fixed with PBS plus 8% paraformaldehyde, then incubated with PBS plus 0.1 M glycine, separated by washes with PBS. Permeabilization was performed for 30 min with saponin buffer (PBS plus 0.5% BSA plus 0.1% saponin), followed by blocking with 0.5 mg/ml human IgG Fc for 30 min. Incubation with primary Ab was performed overnight at 4°C. After four washes with saponin buffer, the coverslips were incubated with FITC-, Cy2-, or Cy3-labeled secondary Ab for 60 min at room temperature. Then, four washes were performed again. Additional blocking and staining steps as described above were performed with another set of non-crossreactive primary and secondary Ab's to achieve labeling of a second epitope. After staining, the samples were washed with saponin buffer and then PBS. Finally, the coverslips were sealed and analyzed on a Zeiss LSM510 microscope (Carl Zeiss Jena GmbH, Jena, Germany) using Zeiss LSM 510 Image Browser/Examiner software.

Immunoprecipitation of FcεRIα, endoglycosidase H treatment, and Western blotting analysis. For immunoprecipitation of FcεRIα, cells were lysed in 100 mM boric acid with 80 mM NaCl, 0.5% Triton X-100 (1% digitonin for coimmunoprecipitation of FcεRIγ), and protease inhibitors. After preclearing with protein G agarose (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), anti-FcεRIα mAb 3G6 was added for overnight incubation. Then, protein G agarose was added for 2 h. After three washing steps, proteins were eluted with 2x Laemmli buffer. Treatment of FcεRIα with endoglycosidase H (Endo H) (New England Biolabs Inc., Beverly, Massachusetts, USA) was done according to the manufacturer's instructions.

Immunoprecipitates or cell lysates were subject to 10% or 18% SDS-PAGE and blotted to nitrocellulose membranes. After blocking, proteins were identified using the Ab RAB1 for FcεRIα and 4D8 for FcεRIγ (both 1:1,000) or appropriate controls. The bands were visualized with POD-conjugated Ab followed by the ECL system (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Amplification of mRNA and analysis of FcεRI transcripts. MoDCs at day 4 of culture were further incubated with or without the addition of 1 μg/ml human myeloma IgE. After 24 and 48 h, total RNA was extracted from highly purified MoDCs using Trizol (Life Technologies GmbH) following the manufacturer's instructions. RT reactions were performed using 1 μg of RNA. Denaturation at 94°C for 40 s was followed by primer annealing at 55°C for 30 s and extension at 72°C for 30 s. A final extension phase of 5 min was added. Specific primer sequences for each gene were as follows: human β-actin, sense, 5′-GAG CGG GAA ATC GTG CGT GAC ATT-3′; antisense, 5′-GAT GGA GTT GAA GGT AGT TTC GTG-3′, yielding a 240-bp fragment; human FcεRIα, sense, 5′-CTG TTC TTC GCT CCA GAT GGC GT-3′; antisense, 5′-TAC AGT AAT GTT GAG GGG CTC AG-3′ (536-bp fragment), and human FcεRIγ, sense, 5′-CCA GCA GTG GTC TTG CTC TTA C-3′ and antisense, 5′-GCA TGC AGG CAT ATG TGA TGC C-3′(338-bp fragment). Amplification was performed on a Perkin-Elmer Gene Amp PCR System 9600 thermocycler (Applied Biosystems GmbH, Weiterstadt, Germany). The PCR cycle numbers for the amplification of the respective cDNAs were 25 for β-actin and 30 for FcεRIα and FcεRIγ. Specific PCR fragments were separated on a 1% agarose gel and visualized using ethidium bromide staining. The PCR products were evaluated semiquantitatively by comparing the ratio of the specific products versus the β-actin band by digital image analysis using the WinCam system (Cybertech, Berlin, Germany).

FcεRI-mediated antigen uptake and T-cell proliferation assays. For T cell proliferation assays of FcεRI-mediated antigen presentation to autologous T cells, MoDCs were generated from the same donors and assayed under identical conditions. Proliferation assays were performed in a total volume of 200 μl in 96-well round-bottom plastic culture plates using autologous T cells. T cells were isolated from PBMCs using a nylon-wool column (>85% purity was assessed by anti-CD3 staining).

MoDCs from atopic and nonatopic donors (three each) were cultured as described above ± 1μg/ml chimeric human anti–4-hydroxy-3-iodo-5-nitrophenyl-acetyl (NIP) IgE (cIgE) (JW8/1; Serotec, Oxford, United Kingdom) until day 6 of culture. On day 6, FcεRI expression was quantified using flowcytometric staining. MoDCs were washed three times and incubated with 1 μg/ml cIgE for 1 h. After washing, the cells were loaded with NIP-haptenized tetanus toxoid (NIP-TT) (28). As a control, MoDCs were loaded with cIgE, or NIP-TT alone, or left unloaded. Then MoDCs were irradiated (3 Gy) and seeded with autologous T cells at 2×10^4 (1:10 or 1:100 MoDC/T cells) cells/well in 96-well culture plates at 37°C for 96 h. Each condition was done in triplicate. Twenty microliters of ^{3}H-thymidin (Amersham Pharmacia Biotech) was added to each well and incubated for another 16 h. Culture plates were harvested, and the incorporated radioactivity was measured in a liquid scintillation counter. Relative stimulation indices (rSI's) were calculated as follows: rSI = (cpm (SMLR) – cpm (T cell)/cpm (T cell).

Statistical analysis. For statistical evaluation, Wilcoxon or Mann-Whitney *U* tests were performed with SPSS 10.0 for Windows. Results are given in mean percentage of positive cells ± SEM. A *P* value less than 0.05 was considered to be significant.

Results

Differentiation of monocytes toward DCs results in intracellular accumulation of FcεRIα and downregulation of FcεRIγ. FcεRI surface expression on monocytes is upregulated in atopic donors (5, 13), although in another study no direct correlation to the serum IgE level was found (29).

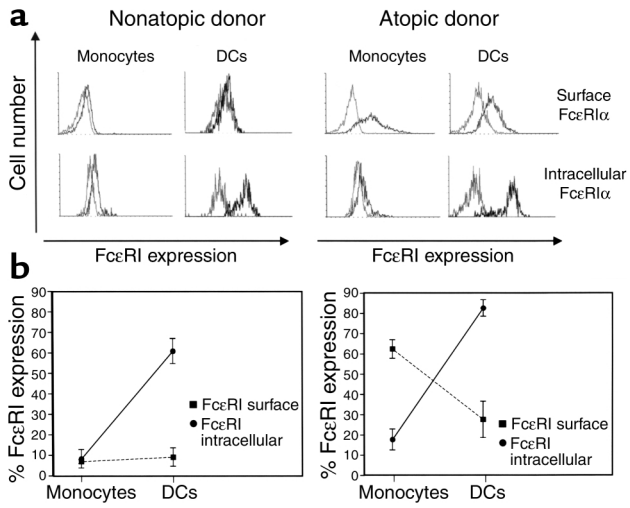

Figure 1

FcεRI surface and intracellular expression in monocytes and DCs from nonatopic and atopic donors. (**a**) For surface staining, cells were subject to double immunolabeling using anti-FcεRIα mAb 22E7, anti-CD14 or anti-CD1a mAb, and 7-aminoactinomycin-D for dead cell discrimination. For intracellular staining, fixation with 4% formaldehyde and permeabilization with 0.5% saponin was done before staining. Acquisition was performed on a FACSCalibur flow cytometer. Shown are histograms from CD14^{pos} or CD1a^{pos} cells (day 8), which represent typical data for at least ten experiments for each donor group. (**b**) Data were obtained as described for (**a**). Shown are at least three experiments for each parameter (mean of percentage of positive cells ± SEM).

In our previous work on FcεRI in epidermal LCs, the paradigmatic immature DCs, high FcεRIα levels were observed intracellularly, irrespective of FcεRI surface expression, which depends on the atopic status of the donors (14, 23). To analyze the expression of FcεRIα during differentiation of monocytes toward DCs, we performed a detailed analysis of surface and intracellular expression of FcεRIα with regard to the atopic status of donors. Figure 1a shows typical data from donors representative for ten experiments for each group. Whereas normal donors showed negligible FcεRI surface expression on monocytes and DCs (FcεRI^{neg} DC) at day 8, monocytes and DCs from atopic donors showed significant FcεRI surface expression (FcεRI^{pos} DC). Interestingly, monocytes from both groups showed very low intracellular expression of FcεRIα, whereas DCs generated from these cells consistently showed high levels of intracellular FcεRIα both in DCs from atopics and nonatopics (Figure 1b). To compare expression of FcεRIα and FcεRIγ subunits during differentiation, we performed immunoblot analyses with FcεRI^{pos} monocytes and MoDCs from atopic donors. Again, we found a strong upregulation of FcεRIα represented by two bands at 50–60 and 60–70 kDa in MoDCs compared with monocytes (Figure 2a). In contrast, FcεRIγ was found to be strongly expressed in monocytes, whereas in MoDCs only low amounts were detected (Figure 2b). Since FcγRI/CD64 and FcγRIII/CD16 can share γ chains with FcεRIα (30, 31), we monitored their expression during differentiation. Figure 2c shows a rapid downregulation of IgG receptors, i.e., FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16, during differentiation into DCs. Since FcγRI/CD64 and FcγRIII/CD16 were absent or showed negligible levels of surface expression in DCs at day 8, an association of FcεRIγ with these structures is unlikely. In LCs we observed a rapid downregulation of FcεRI after spontaneous in vitro differentiation (23). Similarly, Figure 2d shows that maturation of immature MoDCs into CD83^{+} DCs for 2 more days of culture with TNF-α significantly downregulates FcεRI expression on DCs from both atopic and nonatopic individuals ($n = 6$; $P < 0.05$).

Immature FcεRIα represent the majority of preformed intracellular FcεRIα found in MoDCs, but FcεRI^{pos} DCs from atopics also exhibit mature FcεRIα. In models using transfected cells, it has been shown that export of FcεRIα from the ER is dependent upon association with FcεRIγ and a glycosylation-dependent quality control mechanism (18–21). To assess (a) the maturity state of FcεRIα accumulated in DCs and to investigate (b) whether the different FcεRI surface levels observed between FcεRI^{pos} DCs from atopics and FcεRI^{neg} DCs from nonatopics may be associated with different maturity states, we precipitated FcεRIα from DCs. FcεRIα was then treated with Endo H, which deglycosylates immature ER glycoproteins but not mature glycoproteins having undergone complex glycosylation in

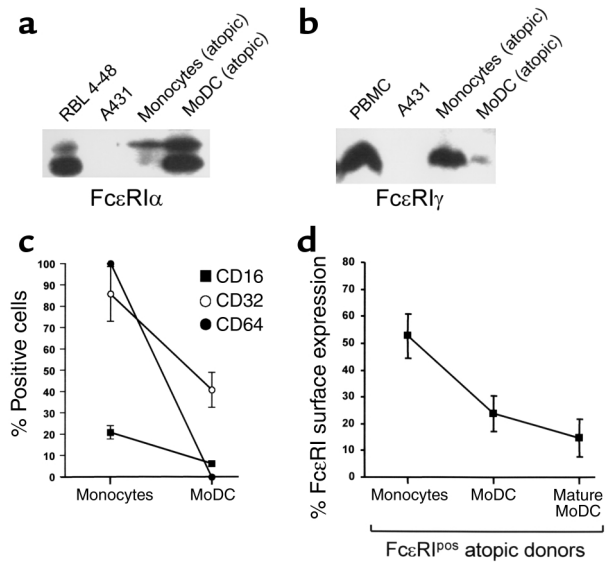

Figure 2

DCs generated from monocytes accumulate FcεRIα, whereas FcεRIγ and Fcγ receptors are downregulated during differentiation. (**a** and **b**) Lysates from monocytes or MoDCs (> 90% purity), as well as RBL 4-48 cells stably expressing human FcεRIα, A431 epithelial cells, and PBMCs used as controls, were fractionated on SDS-PAGE and immunoblotted with Ab for FcεRIα or FcεRIγ. (**c**) Expression of the Fcγ receptors CD16, CD32, and CD64 was determined by flow cytometry as described for **a**. (**d**) Mature DCs were generated by addition of TNF-α to MoDC cultures. Flow cytometric analysis of FcεRI expression of six donors was done as described for Figure 1a.

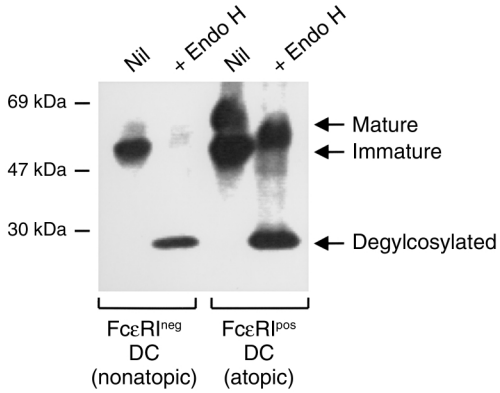

Figure 3
Immature FcεRIα is found both in FcεRI^{pos} from atopics and FcεRI^{neg} DCs from nonatopics, whereas mature FcεRIα is found only in FcεRI^{pos} DCs. Lysates from FcεRI^{neg} and FcεRI^{pos} DCs were subject to immunoprecipitation using 3G6 anti-FcεRIα Ab. Precipitated FcεRIα was treated with Endo H, fractionated by SDS-PAGE, and then immunoblotted using RAB1 anti-FcεRIα Ab to detect immature (Endo H–sensitive) or mature (Endo H–resistant) forms. Nil, without Endo H treatment.

the Golgi compartment (18, 19). As shown in Figure 3, a major proportion of the FcεRIα protein precipitated both from FcεRI^{pos} and FcεRI^{neg} DCs consists of a glycoprotein of 50–60 kDa, which is sensitive to Endo H treatment, yielding a 25–30 kDa core protein band. Thus, this 50–60 kDa protein may represent ER-resident immature FcεRIα. In addition, FcεRI^{pos} DCs from atopics exhibited a band at 60–70 kDa resistant to Endo H, presumably representing a mature form from the Golgi compartment or the cell surface.

The subcellular localization of FcεRI subunits in FcεRI^{pos} and FcεRI^{neg} DCs show a different distribution. The biochemical analyses shown above suggest that most of FcεRIα accumulated during DC differentiation is ER localized. In addition, mature FcεRIα detected in lysates from FcεRI^{pos} DCs should also be detectable in the Golgi compartment. To further confirm these observations, the distribution of the FcεRI subunits was analyzed by confocal laser scan microscopy (see Figure 4). CD14 or CD1a positivity of FcεRI-labeled cells was confirmed using double immunolabeling (data not shown). In contrast to normal donors, freshly isolated monocytes from atopic donors exhibited membrane-localized FcεRIα colocalizing with FcεRIγ subunits. Some few, FcεRIγ-positive but FcεRIα-negative, monocytes were also present in these preparations. After differentiation toward DCs, a strong intracellular FcεRIα expression was observed, while FcεRI surface expression was weaker under these experimental conditions, presumably due to its downregulation and to changes in the plasma membrane architecture. In nonpermeabilized cells, however, it could be detected better (data not shown). While the majority of FcεRI^{neg} DCs from nonatopics showed a strong intracellular expression of FcεRIα localized in the ER (colocalized with anti-PDI), they lacked FcεRIγ expression or showed a discrete staining pattern. In contrast, FcεRI^{pos} DCs from atopics showed colocalization of FcεRIα and FcεRIγ subunits together, mainly in the Golgi compartment (colocalized with anti-giantin) in addition to the ER localization also observed in FcεRI^{neg} DCs. Taken together, these data confirm that DCs acquire an intracellular pool of FcεRIα localized in the ER in FcεRI^{neg} DCs. In FcεRI^{pos} DCs from atopics, which coexpress the γ chain, it is allowed to reach the Golgi compartment and, subsequently, the cell surface.

FcεRIγ controls the surface expression of FcεRIα in DCs. In in vitro reconstitution models and in mast cells, surface expression of FcεRIα is critically dependent on FcεRIγ expression (16, 17, 32). We reported a correlation between FcεRIα surface levels and FcεRIγ expression at mRNA and protein levels in human LCs (23). Since our data show abundant FcεRIα in MoDCs with no or little regard to the atopic status of donors, differential FcεRIγ expression in DCs from normal and atopic individuals could explain the dichotomy observed in FcεRI surface expression. We therefore analyzed both FcεRI

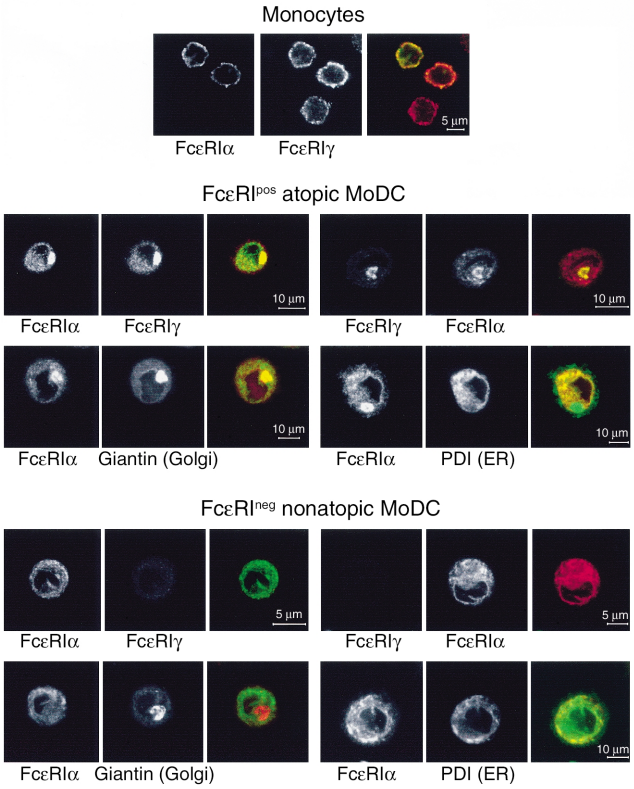

Figure 4
Monocytes, FcεRI^{pos}, and FcεRI^{neg} DCs show a different localization of FcεRI subunits. Cells were adhered to coverslips, fixed, and permeabilized using 0.1% saponin. After blocking using human IgG Fc, sequential indirect immunolabeling with Ab against FcεRI subunits and organelle markers was performed. After mounting, the samples were analyzed by confocal laser scanning microscopy.

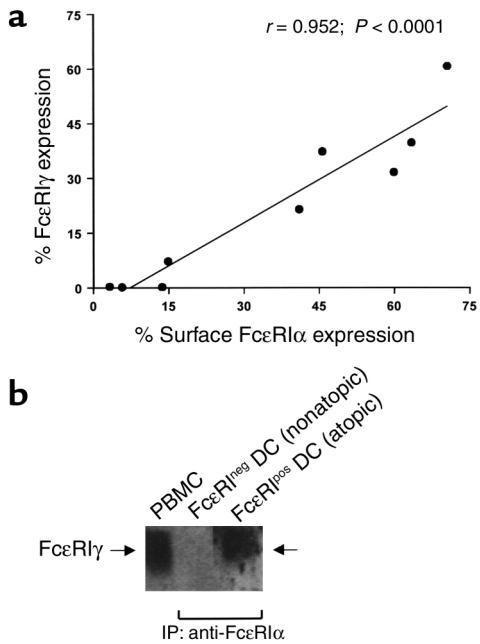

Figure 5

Significant FcεRIγ expression and association of FcεRIα with FcεRIγ can be detected only in FcεRI^{pos} DCs from atopic donors. (**a**) FcεRIγ expression was determined by double immunolabeling of MoDCs using anti-FcεRIγ and anti-CD1a after a mild permeabilization with digitonin to preserve surface-expressed FcεRI complexes. Determination of FcεRI surface expression and flow-cytometric analysis was done as described for Figure 1. (**b**) FcεRI^{neg} (4% positive cells) and FcεRI^{pos} (86% positive cells) DCs were subject to mild lysis with a buffer containing 1% digitonin followed by immunoprecipitation using 3G6 anti-FcεRIα Ab and SDS-PAGE. FcεRIγ coprecipitated with FcεRIα was detected by immunoblotting using 4D8 Ab. PBMC lysates were used as positive controls.

surface expression and FcεRIγ protein levels in 6-day-old MoDCs from donors of atopic (FcεRI^{pos} DCs) and normal backgrounds (FcεRI^{neg} DCs) (n = 9). Figure 5a shows a highly significant correlation between the expression levels of these two structures (r = 0.952; P < 0.0001). To investigate whether FcεRIγ is indeed associated in DCs positive for surface FcεRI, we precipitated FcεRIα from FcεRI^{neg} and FcεRI^{pos} DCs under mild lysis conditions using 1% digitonin (19). Figure 5b shows that FcεRIγ was coprecipitated with FcεRIα in FcεRI^{pos} DCs from atopics, but not in FcεRI^{neg} DCs from nonatopics (despite strong intracellular FcεRIα expression in both groups, data not shown). Thus, we conclude that remaining, moderate FcεRIγ expression in DCs from atopics — as opposed to low or absent FcεRIγ expression in DCs from nonatopics — is sufficient to keep significant FcεRI surface levels on these cells.

An "atopic environment" provided by IgE sustains FcεRI surface expression in DCs. While as yet unknown factors that induce γ-chain expression may provide DCs from atopics with higher baseline FcεRI surface expression, the maximal levels, e.g., seen during active AD, may be regulated by additional factors. In effector cells of anaphylaxis, IgE levels positively regulate FcεRI surface expression (33–35), but it is unclear whether the ligand may influence the level of FcεRI on DCs. We therefore tested the effect of human myeloma IgE on FcεRI expression added every 2 days during DC differentiation. Preliminary experiments showed that an initial dose of 1 μg/ml of IgE was optimal and did not affect the phenotype and apoptosis rate. While addition of IgE failed to induce any FcεRI surface expression at different time points on FcεRI^{neg} DCs from nonatopic donors (data not shown), DCs from atopic individuals exhibited significantly higher FcεRI levels with IgE than DCs generated under control conditions (Figure 6a). We performed a number of additional experiments to investigate the mechanism of IgE-mediated effect: to determine whether IgE induces the transcription of FcεRI chains, we performed semiquantitative RT-PCR with DCs that were incubated with 1 μg/ml IgE for up to 48 h. The experiments showed no significant upregulation of either FcεRIα or FcεRIγ transcripts after addition of IgE in five donors (Figure 6b shows a representative PCR experiment from an atopic donor). Upregulation of surface FcεRI by IgE on MoDCs from three atopic donors was confirmed by flow cytometry (59.5% ± 4.01% FcεRI surface expression with IgE incubation and 14.9% ± 8.7% FcεRI surface expression without IgE incubation), whereas MoDCs from two nonatopic donors showed no upregulation.

In addition, we detected that MoDCs that were incubated with IgE until day 4 display enhanced FcεRIγ protein levels on day 6 of culture (Figure 6c shows one representative experiment of three). To investigate the role of forward intracellular protein transport in the IgE-mediated effect on surface FcεRI, IgE was added at day 4 with or without brefeldin A (BFA) and FcεRI surface expression was analyzed every 24 h. For those experiments, FcεRI^{pos} DCs showing strong downregulation during DC differentiation were selected. As shown in Figure 6d, IgE addition at day 4 of culture restored FcεRI levels almost completely to levels comparable to monocytes at the beginning of culture. BFA abolished this process as early as 24 h after its addition, demonstrating that both basal and IgE-mediated sustained FcεRI surface expression are heavily dependent on forward protein transport. The protein synthesis inhibitor cycloheximide (CHX) also had a strong effect on basal and IgE-mediated sustained FcεRI surface expression (Figure 6e). However, in the first 24 h, IgE was able to counteract the CHX-mediated downregulation of surface FcεRI to a limited extent. The differential effects of BFA and CHX in this early phase suggest that the IgE-mediated upregulation is initially dependent on forward transport (i.e., ER to Golgi compartment) of preformed and/or recycling FcεRI. To differentiate between these two possibilities, we incubated MoDCs for 12 h both with CHX and BFA to inhibit both protein synthesis and forward transport and analyzed total FcεRIα protein by immunoblot. If receptor recycling occurred to a major extent, we would expect total FcεRIα protein not to change much; if forward

transport and processing of intracellular FcεRIα played a major role, we would expect total FcεRIα to go down significantly. These experiments showed FcεRIα protein was massively downregulated, no matter if IgE was added or not (data not shown). In parallel, the FcεRIα surface expression of MoDCs incubated with CHX and BFA was downregulated within 3.5 hours of culture (data not shown). In addition, we excluded that this loss of surface FcεRIα resulted from CHX/BFA–induced apoptosis and necrosis of the cells (data not shown). This argues against a major role of receptor recycling in these processes, although we cannot exclude the possibility that FcεRI is internalized for recycling, but is degraded intracellularly. Taken together, IgE-mediated enhancement of FcεRI surface expression on MoDCs seems to be an accumulation process. It is dependent on continuous basal protein synthesis as well as processing and transport of intracellular FcεRI protein more likely using an intracellular FcεRI pool than recycling FcεRI protein.

IgE-mediated FcεRI upregulation of DCs from atopic donors enhances their capacity to induce proliferation of autologous T cells in IgE-dependent antigen-presentation assays. FcεRI-mediated antigen uptake, processing, and presentation is believed to be a highly efficient mechanism and putatively plays an important role in the pathophysiology of atopic diseases (10, 11). We speculated that IgE-induced preservation of surface FcεRI on DCs from atopics might enhance these IgE-mediated functions. To test this hypothesis, we generated MoDCs from either atopic or nonatopic donors that were incubated with and without 1 μg/ml NIP-specific chimeric IgE (cIgE) during differentiation. To measure IgE-dependent DC antigen-presenting function, these were freshly loaded with NIP-specific IgE and then cocultured with autologous T cells as well as NIP-coupled tetanus toxoid. T cell proliferation was assessed by ^{3}H-thymidine incorporation after 96 h. Figure 7 shows one representative experiment out of three for each donor group. When DCs from nonatopic donors were used, an IgE-induced enhancement of antigen-presenting capacity was hardly detectable, even with IgE preincubation (left panel). In contrast, DCs from atopics targeted by specific IgE and antigen were able to induce higher T cell proliferation (right panel). Stimulatory capacity was even stronger when DCs generated under the influence of IgE and thus expressing higher FcεRI levels, were used. This demonstrates that IgE-mediated sustained surface FcεRI expression can lead to biologically important changes in DC function.

Discussion

The differential FcεRI surface expression on monocytes observed between healthy and atopic donors is in line with previous reports (5, 13), whereas, to our knowledge, no data are available for MoDCs. Another group found no correlation between surface FcεRI and serum-IgE levels on monocytes (29). The donors analyzed in this study contained not only healthy donors and donors with atopic diseases, but also those with hypereosinophilic syndromes, hyper-IgE syndrome, helminth infestation, and IgE myeloma, which may

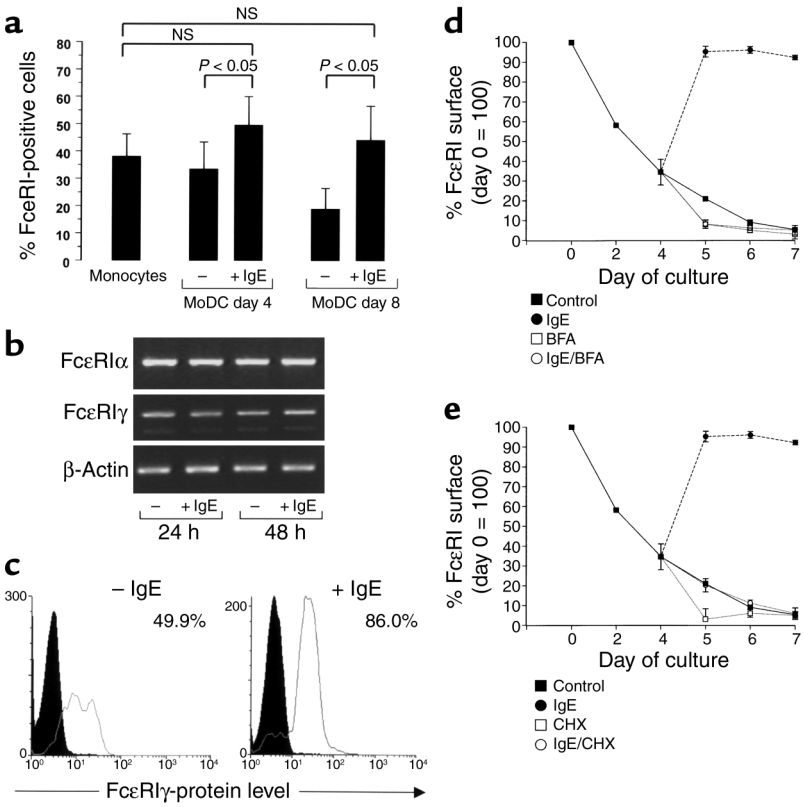

Figure 6

Addition of IgE leads to sustained FcεRI surface expression in DCs from atopic donors in a BFA- and CHX-sensitive process without affecting de novo synthesis of FcεRIα and FcεRIγ chains. (**a**) IgE was added from day 0 of DC culture (atopic donors) with GM-CSF and IL-4. Immunolabeling and flow-cytometric analysis of FcεRI surface expression was performed as described for Figure 1. Percentage of positive cells shown under **a** are the result of six independent experiments. (**b**) MoDCs (day 4) were incubated for 24 and 48 h with or without the addition of 1 μg/ml human myeloma IgE. After RNA isolation from highly purified MoDCs and reverse transcription, FcεRIα and FcεRIγ expression was analyzed by semiquantitative PCR using β-actin as a control. Shown is a representative experiment from an atopic donor of five total experiments. In parallel, FcεRIγ protein levels of one representative experiment of MoDC on day 6 of culture incubated with (+ IgE) and without IgE (– IgE) until day 4 of culture are shown (**c**). (**d** and **e**) MoDCs were generated with GM-CSF and IL-4 until day 4 of culture. Then IgE, CHX, and BFA (all 1 μg/ml) were added as indicated. Flow-cytometric analyses of FcεRI expression were done on days indicated. Mean FcεRI expression ± SEM (n = 7) is shown as percentage of FcεRI expression of monocytes at day 0.

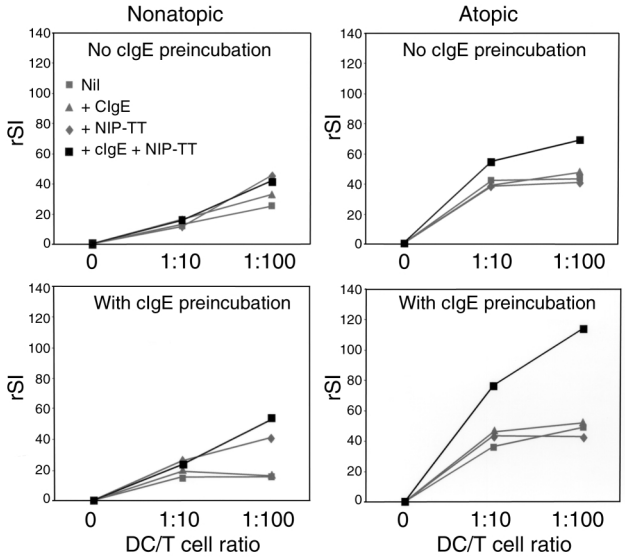

Figure 7

IgE addition during DC differentiation enhances IgE-dependent stimulatory functions of DCs from atopic donors. MoDCs from nonatopic (left panel) and atopic donors (right panel) were cultured for 6 days with GM-CSF and IL-4 with or without NIP-specific cIgE added at days 0, 2, and 4 (1 μg/ml). IgE-mediated surface FcεRI upregulation in atopic donors was controlled by flow cytometry. Then, cells were loaded with 1 μg/ml NIP-specific cIgE. After washing and 96 h of coculture with autologous T cells in a 1:10 or 1:100 ratio of DCs/T cells plus the addition of NIP-TT, T cell proliferation was assessed by ^{3}H-thymidine incorporation and rSI values were calculated from triplicate samples. Negative controls were either IgE or NIP-TT alone or neither, as well as T-cell culture without DCs. Shown are representative data of three experiments for each donor group.

affect FcεRI expression and serum-IgE levels by other mechanisms. They detected, however, significant FcεRI levels on monocytes from some allergic donors, but not from healthy subjects.

The impressive formation of an intracellular FcεRIα pool in immature DCs supports our previous observations, showing constitutive intracellular FcεRIα expression in freshly isolated LCs and a modest induction in DCs generated from CD34$^+$ progenitors, but no surface expression (23, 36). Thus, we propose that preformed intracellular FcεRIα, in contrast to monocytes, is characteristic for immature DCs. However, in the previous studies a detailed analysis of FcεRI subunit regulation, maturity state, and localization was precluded by the limited availability and purity of cells, making it impossible to purify FcεRIα and analyze it by Endo H treatment (23). Intracellular FcεRIα in LC-bound IgE, so we concluded that it may represent mature FcεRIα. While we cannot exclude that LCs may be different from MoDCs at that point, this conclusion may be contrary to the detailed biochemical analyses performed in the present study. However, using an in vitro reconstitution system, immature FcεRIα meanwhile has been shown to bind IgE as well (18).

In FcεRI assembly and maturation (18–21), it is believed that in the ER folding and N-linked core glycosylation, producing the G_3 form of immature FcεRIα that bears three terminal glucose residues on high-mannose chains, are followed by trimming of these residues, resulting in $G_{0/1}$ forms of immature FcεRIα that bear one or no glucose residues. The export of immature FcεRIα from the ER to the Golgi compartment is dependent on correct glucosidase trimming and association with FcεRIγ chains. In the Golgi compartment, terminal glycosylation with complex sugars takes part, and mature FcεRI can then be transported to the cell surface. We detected no Endo H–resistant band at approximately 64 kDa in DCs (Figure 3), which would represent untrimmed G_3 forms of immature FcεRIα (18). Thus, we conclude that the 50- to 60-kDa band represents the correctly trimmed $G_{0/1}$ form of immature FcεRIα, and a glucosidase defect is not responsible for the lacking FcεRI surface expression in FcεRI^{neg} DCs. The Endo H–resistant 60- to 70-kDa band observed in FcεRI^{pos} DCs was considered as mature FcεRIα, in accordance with reports observing two separate maturity forms of FcεRIα with distinct molecular weight (18, 19, 37).

While intracellular FcεRIα protein was present in high amounts, we found a significant correlation of FcεRIγ expression with FcεRI surface levels on DCs. FcεRIγ is strongly downregulated during DC differentiation and only found to be associated with FcεRIα in FcεRI^{pos} DCs from atopic donors. These data were confirmed by immunolabeling and laser scan microscopy, which preferentially showed FcεRIγ/FcεRIα colocalization in the Golgi compartment in FcεRI^{pos} DCs. Thus, we conclude that during DC differentiation, FcεRIγ becomes the limiting factor for surface expression of the whole complex. This limitation may lead to the accumulation of immature, correctly folded, FcεRIα in the ER. Intracellular FcεRIα only in the presence of FcεRIγ is able to leave the ER and acquire full maturity upon terminal glycosylation in the Golgi compartment, thus leading to surface expression of the complex.

Using an in vitro reconstitution system, Albrecht et al. (18) detected immature FcεRIα in the ER in the absence of FcεRIγ, a distribution more similar than that we observed in FcεRI^{neg} DCs. In contrast, monocytes showed very low levels of intracellular FcεRIα but high levels of FcεRIγ, which we think may prevent FcεRIα accumulation while leading to surface expression of the FcεRI complex. However, these cells also express FcγRI/CD64 and FcγRIII/CD16, both known to associate with FcεRIγ. Due to these additional factors, investigations designed to establish a role of FcεRIγ in the regulation of FcεRI surface expression may not be feasible in this system. As shown in Figure 2c, MoDCs lack these molecules, therefore a possible competition between FcγR and FcεRIα for binding to FcεRIγ is unlikely in MoDCs. The mechanisms driving association of FcεRIγ with selected antigen receptors are unclear, however.

To mechanically prove a role of FcεRIγ in the regulation of FcεRI surface levels, it would be desirable to transfect FcεRIγ into FcεRI^{neg} DCs lacking FcεRIγ to

reconstitute FcεRI surface expression. The only efficient transfection methods for DCs so far, however, involve adenoviral vectors, which have been reported to induce DC maturation (38). Since maturation leads to downregulation of antigen receptors and to rapid loss of FcεRIα transcripts and protein (2, 23), this approach is not desirable.

Surface-expressed FcεRI has also been reported on eosinophils from hypereosinophilic patients, where it mediates defense against parasites such as *Schistosoma mansoni* larvae (39). Interestingly, normal human eosinophils contain intracellular FcεRIα with low or negative surface levels (40, 41). Seminario et al. (40) found FcεRIγ protein expression in these cells, so they concluded that deficient FcεRIγ expression was not responsible for that observation. Since eosinophils express all FcεRI subunits, at least at the transcriptional level, regulatory mechanisms different from that in APCs bearing trimeric FcεRI may be in place (40, 41). FcεRIγ detected in eosinophils, however, has not been shown to be associated with FcεRIα. It may also be associated with other structures such as IgαR/CD89 expressed on eosinophils (42, 43). FcεRIα on eosinophils has also been proposed to be secreted, so an association with FcεRIγ would not be required (40). For DCs, FcεRIα secretion is unlikely, since there are no splice variants lacking transmembrane domains both in LCs (23) and MoDCs (S. Kraft, unpublished observation).

In our experiments, IgE sustained FcεRI surface levels during DC differentiation. This effect is known from mast cells and basophils (33–35). To our knowledge, the only study documenting an enhancing effect of IgE on FcεRI surface expression on primary APCs used monocytes (7). In these experiments, CHX and BFA were unable to inhibit the IgE effect, whereas in our experiments inhibition by both of them was observed, suggesting a rapid turnover of FcεRI molecules present at the cell surface. This discrepancy may be due to the different cell systems used. Monocytes quickly undergo apoptosis in the absence of stimuli, so concomitantly de novo synthesis and transport of FcεRI chains may be shut down early. In addition, in our experiments IgE incubation did not change the immunophenotype of DCs, so generation of DCs under an "atopic environment" showing high IgE levels may provide a valuable tool for the analysis of FcεRI function and signaling in DCs. In our experiments, we could not detect increased synthesis of FcεRIα or FcεRIγ upon IgE addition. The IgE effect, however, was observed only in FcεRI^{pos} DCs, suggesting that preexisting surface FcεRI and baseline synthesis of both FcεRIα and FcεRIγ are necessary for that effect. So in our view, increased FcεRIγ-mediated surface expression in DCs from atopic donors needs to be a prerequisite for IgE in order to have an effect. Recently, supporting our data on DCs, Borkowski et al. (44) used FcεRI-transfected U937 cells to show that the IgE-mediated effect does not depend on the presence of FcεRIβ or increased protein synthesis. They demonstrated that IgE exerts its action by stabilizing surface FcεRI, using a preformed FcεRI pool as well as baseline FcεRI protein synthesis.

The FcεRI downregulation observed after terminal maturation of DCs under TNF-α fits to the different functions of immature and mature DCs (2). Immature DCs reside as outposts of the immune system in peripheral tissues and are equipped with receptors facilitating antigen uptake. Upon antigen uptake and additional signals, they migrate to peripheral lymphoid organs, where they present antigens to T cells. This migration is accompanied by a maturation process: it is reflected in a changing immunophenotype with downregulation of antigen receptors and upregulation of surface molecules involved in antigen presentation, such as costimulatory and adhesion molecules.

Atopic diseases such as AD are characterized by strong upregulation of surface FcεRI on DCs, such as epidermal LCs (12). In addition, IDECs are present in inflammatory skin diseases and, in AD, are characterized by very high FcεRI surface expression (14). While it is not clear whether these cells primarily express high FcεRI levels and then migrate into inflamed skin, or whether they acquire it due to signals they receive during or after migration, the question remains, what enables these cells to upregulate surface FcεRI? Upregulation of FcεRIγ synthesis and high IgE levels during AD may be candidate mechanisms. In contrast to FcεRIα, the small γ chain (7–9 kDa) is not subject to extensive posttranslational modification, and so its synthesis presumably is a shorter procedure. The expression of preexisting surface-localized FcεRI could then be further enhanced by IgE binding. The presence of both factors together might lead to enhanced IgE-mediated DC functions further supporting the development of atopic inflammatory reactions, as suggested by our in vitro studies showing enhanced T cell stimulatory capacity of IgE-incubated DCs from atopics (Figure 7).

More information about regulatory factors, however, such as transcription factors determining FcεRIγ expression in APCs, is needed. In addition to established anti-IgE strategies (45), modulation of FcεRIγ expression in DCs may represent a potential target for the management of atopic diseases.

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