

FcεRI and FcγRIII/CD16 Differentially Regulate Atopic Dermatitis in Mice¹

Georges Abboud,^{2*} Delphine Staumont-Sallé,^{2*†} Akira Kanda,* Thomas Roumier,* Nathalie Deruytter,* Céline Lavogiez,* Sébastien Fleury,* Patrick Rémy,* Jean-Paul Papin,* Monique Capron,* and David Dombrowicz^{3*}

The high-affinity IgE receptor FcεRI and, in some models, the low-affinity IgG receptor FcγRIII/CD16 play an essential role in allergic diseases. In human skin, they are present on APCs and effector cells recruited into the inflamed dermis. FcRγ is a subunit shared, among other FcRs, by FcεRI and CD16 and is essential to their assembly and signal transduction. Using an experimental model reproducing some features of human atopic dermatitis and specific FcR-deficient mice, we have herein delineated the respective contribution of FcεRI and FcγRIII/CD16 to the pathology. We demonstrate that symptoms of atopic dermatitis are completely absent in FcRγ-deficient animals but only partially inhibited in either FcεRI- or FcγRIII/CD16-deficient animals. Absence or attenuation of the pathology is correlated to increased skin expression of regulatory IL-10 and Foxp3. While FcεRI controls both Th1 and Th2 skin response, mast cell recruitment into draining lymph nodes and IgE production, CD16 regulates only Th2 skin response, as well as T cell proliferation and IgG1 production. This isotype-specific regulation by the cognate FcR is associated to a differential regulation of IL-4 and IL-21 expression in the draining lymph nodes. FcεRI and CD16 thus contribute to atopic dermatitis but differentially regulate immune responses associated with the disease. Targeting both IgE/FcεRI and IgG/CD16 interactions might represent an efficient therapeutic strategy for allergic diseases. *The Journal of Immunology*, 2009, 182: 6517–6526.

Atopic dermatitis (AD)⁴ is a common chronic inflammatory skin disease that often begins in infancy and frequently occurs in subjects with a personal or family history of atopic disease (1). Skin lesions in AD display increased epidermal thickness and infiltration with inflammatory cells: activated memory CD4⁺ T cells, macrophages, mast cells, and eosinophils. Acute lesions are rather associated to a Th2 response, while a Th1 profile, with an accumulation of IFN-γ-producing cells, is predominant in chronic phase. Most AD patients show elevated serum IgE levels, with specific IgE directed against environmental allergens or microbial proteins as well as allergen-specific IgG (particularly IgG1 and IgG4) (2, 3).

The Ig Fc portion interacts with α subunits of the various FcRs, exerting pleiotropic effects within the immune system (4, 5). IgE binds to 2 FcεR, including the multimeric high-affinity FcεRI (4) and the low-affinity, lectin type FcεRII/CD23 (6). FcεRI is ex-

pressed on the surface of mast cells and basophils and triggers IgE-mediated degranulation and cytokine release (4). In humans, it is also expressed on professional APCs such as dendritic cells (including epidermal Langerhans cells and inflammatory dendritic epidermal cells), monocytes/macrophages, eosinophils, and platelets (4), where it plays a role in antigenic presentation and/or Ab-dependent cellular cytotoxicity reactions (4). Three FcγR-associated, ITAM-containing activating receptors are found among FcγR family: the high-affinity FcγRI/CD64, the low-affinity FcγRIII/CD16 (in humans, the nonsignaling, glycolipid-anchored CD16b is expressed by neutrophils), and, in mice, the low-affinity FcγRIV. The FcγR family also comprises one monomeric, ITIM-bearing inhibitory receptor: the low-affinity FcγRII/CD32b (in humans, CD32a is also an activating receptor) (5). In mice, CD16, CD32, and FcγRIV also act as low-affinity IgE receptors (4, 7). Most FcγRs have a widespread cell distribution. Activating FcγRs trigger phagocytosis, cytokine release, Ag presentation, and Ab-dependent cellular cytotoxicity reactions (5).

Polymorphisms in FcεRI and CD16 were found to be correlated with atopy. Hasegawa et al. demonstrated the presence of a polymorphism in human *FcεRIα* gene promoter (chromosome 1q21) related to AD in a Japanese population (8). The α subunit of FcεRI plays an important role in IgE-mediated allergic reactions as an amplifier for cell surface expression and signal transduction of FcεRI (4). Polymorphisms in *FcRβ* gene located on human chromosome 11q12–13 have been associated to atopy. Indeed, different groups found single nucleotide polymorphisms in the *FcRβ* promoter in atopic patients (9). Those single nucleotide polymorphisms were causally linked with atopy via regulation of FcεRI expression (9). Likewise, Zeyrek et al. showed that a V158V genotype in CD16a gene polymorphism may represent a genetic risk factor for the development of atopic diseases (10).

IgE has also been shown to increase expression of both FcεRI and CD23 (4, 6), in the former case by preventing proteolytic

*Institut National de la Santé et de la Recherche Médicale Unité 547, Institut Pasteur de Lille, and Université Lille 2, Lille, France; and [†]Department of Dermatology, Centre Hospitalier Régional Universitaire de Lille, Lille, France

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² G.A. and D.S.-S. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. David Dombrowicz, Institut National de la Santé et de la Recherche Médicale Unité 547, Institut Pasteur de Lille, 1, rue Prof. Calmette BP245, 59019 Lille Cedex, France. E-mail address: david.dombrowicz@pasteur-lille.fr

⁴ Abbreviations used in this paper: AD, atopic dermatitis; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; DC, dendritic cell; MHC-II, MHC class II; WT, wild type.

Table I. Primers used for real-time PCR

Gene	Primer	Size	Sequence
GAPDH	Forward	20	5'-CGT CCC GTA GAC AAA ATG GT-3'
	Reverse	20	5'-GAA TTT GCC GTG AGT GGA GT-3'
IL-1β	Forward	20	5'-AGG TGC TCA TGT CCT CAT CC-3'
	Reverse	20	5'-CAG GCA GGC AGT ATC ACT CA-3'
IL-4	Forward	20	5'-GCA TGG AGT TTT CCC ATG TT-3'
	Reverse	20	5'-AGA TGG ATG TGC CAA ACG TC-3'
IL-5	Forward	22	5'-CTC ACC GAG CTC TGT TGA CAA G-3'
	Reverse	22	5'-GAA CTC TTG CAG GTA ATC CAG G-3'
IL-6	Forward	20	5'-CCA CGG CCT TCC CTA CTT CA-3'
	Reverse	22	5'-CCA CGA TTT CCC AGA GAA CAT G-3'
IL-10	Forward	21	5'-TTC ATG GCC TTG TAG ACA CCT-3'
	Reverse	20	5'-TGA ATT CCC TGG GTG AGA AG-3'
IL-13	Forward	20	5'-GGA ATC CAG GGC TAC ACA GA-3'
	Reverse	20	5'-AGG AGC TGA GCA ACA TCA CA-3'
IL-17	Forward	22	5'-AAG TCT TTA ACT CCC TTG GCG C-3'
	Reverse	19	5'-AGG GTC TTC ATT GCG GTG G-3'
IL-18	Forward	21	5'-GGA ATC AGA CAA CTT TGG CCG-3'
	Reverse	22	5'-CCT CGA ACA CAG GCT GTC TTT T-3'
IL-21	Forward	20	5'-AGG AGG GGA GGA AAG AAA CA-3'
	Reverse	20	5'-GGG AAT CTT CTC GGA TCC TC-3'
IL-21R	Forward	20	5'-ACA GCG GGA ACT TCA AGA AA-3'
	Reverse	20	5'-CAG GCT CAG ACA TTC CAT CA-3'
IL-31	Forward	20	5'-CAG CTG TTT CAA CCC ACT GA-3'
	Reverse	20	5'-CAG TTC TGC CAT GCA GTT TG-3'
IL-33	Forward	19	5'-ATC AGG CGA CGG TGT GGA T-3'
	Reverse	21	5'-GAC GTC ACC CCT TTG AAG CTC-3'
IFN-γ	Forward	20	5'-ACC CTG TCG TAT GCT GGG AA-3'
	Reverse	21	5'-GTT GGT GCA GGA ATC AGT CCA-3'
TSLP	Forward	19	5'-TAT CCC TGG CTG CCC TTC A-3'
	Reverse	21	5'-TGT GCC ATT TCC TGA CCG-3'
CCL20	Forward	20	5'-CTT GCT TTG GCA TGG GTA CT-3'
	Reverse	20	5'-CTT CAT CGG CCA TCT GTC TT-3'
CCL22/MDC	Forward	20	5'-TGG TGC CAA TGT GGA AGA CA-3'
	Reverse	21	5'-GGC AGG ATT TTG AGG TCC AGA-3'
CXCL10/IP-10	Forward	19	5'-ACC CAA GTG CTG CCG TCA T-3'
	Reverse	20	5'-CAT TCT CAC TGG CCC GTC AT-3'
CCR3	Forward	20	5'-TGA CCC CAG CTC TTT GAT TC-3'
	Reverse	24	5'-CTG GAC TCA TAA AGG ACT TAG CAA-3'
TGF-β	Forward	21	5'-TAC TCT GGA GAC GGT TTG CCA-3'
	Reverse	20	5'-CAT GAA GAA AGT CTC GCC CG-3'
Foxp3	Forward	21	5'-CCC AGG AAA GAC AGC AAC CTT-3'
	Reverse	18	5'-TTC TCA CAA GGC CAC TTG-3'

degradation and receptor internalization. Indeed, FcεRI surface expression is increased in Langerhans cells, inflammatory dendritic epidermal cells, monocytes, and circulating dendritic cells (DCs) from patients with AD (4). In DCs, this increased expression has been associated to increased FcRγ expression, the limiting factor for receptor surface expression (11). In humans, FcεRI engagement has antiapoptotic effects on monocytes (12). An increased expression of CD64 and CD16 was also demonstrated in acutely and chronically inflamed skin, compared with healthy and nonlesional AD skin (13). However, the respective contributions of IgE and IgG receptors to the physiopathology of AD remain unclear. Thus, using a mouse model reproducing some features of the human disease and FcR-deficient animals, we have examined the role of FcγR-associated FcεRI and CD16 in AD.

Materials and Methods

Animals

BALB/c, C57BL/6, and OT-II (14) mice, FcRγ^{-/-} mice (BALB/c background) (15), and CD16^{-/-} mice (C57BL/6 background) (16) were from Charles River Laboratories, Taconic, and The Jackson Laboratory, respectively. DO11.10 mice (17) were a gift from Dr. C. Verwaerde (Institut Pasteur de Lille). FcεRI^{-/-} mice (BALB/c background) (18) were bred at the Institut Pasteur de Lille. Animals were kept under specific pathogen-free conditions. Eight- to 12-wk-old female mice were used for all the

experiments, performed following approval by the Ethics Committee for Animal Experimentation from the Nord-Pas-de-Calais region. PBS-treated groups were composed of three to seven animals, while OVA-treated groups included six to nine animals. Equal numbers of gene-proficient and gene-deficient animals were used in every experiment.

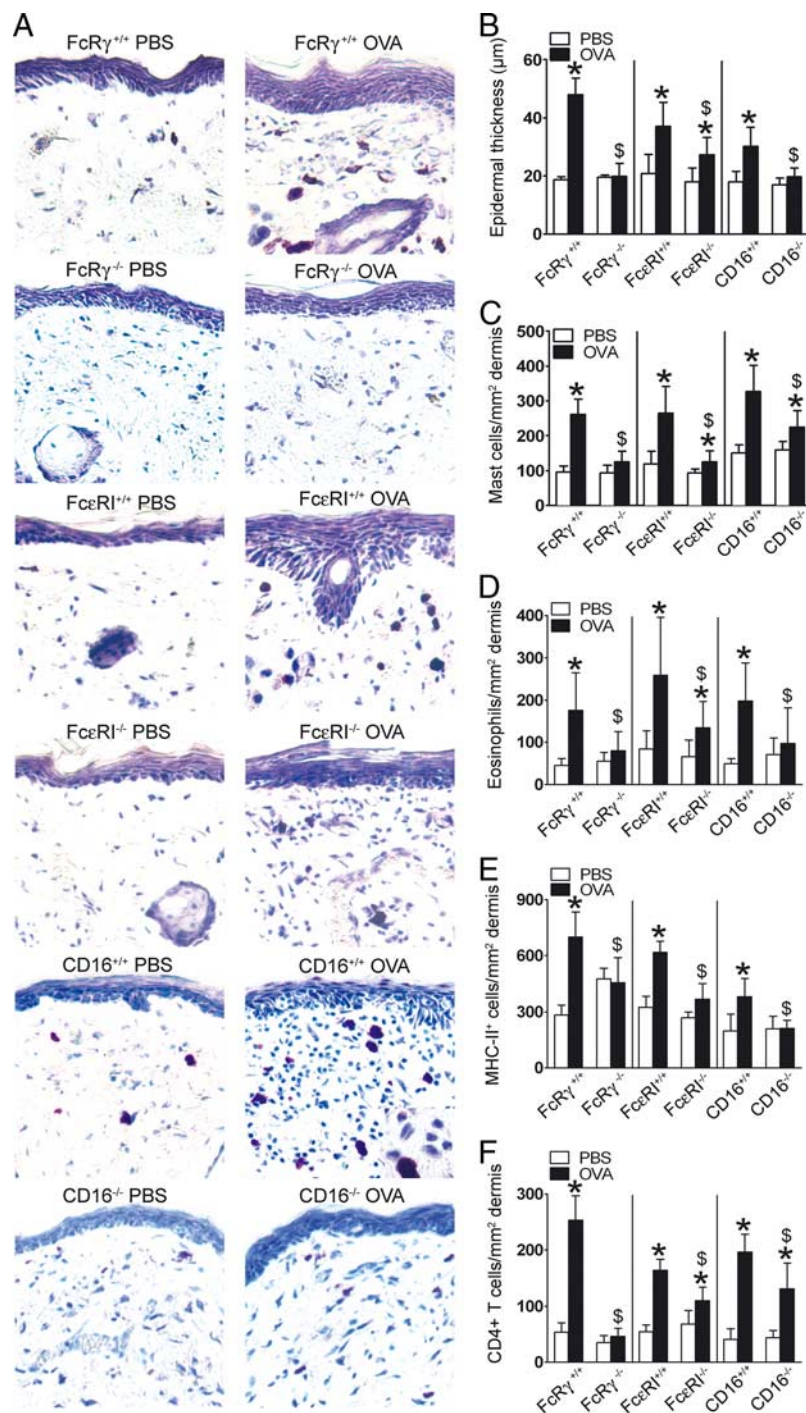
Induction of AD

AD was induced by epicutaneous OVA sensitization (19). Two paper discs (gift from Epitest) soaked with 25 μl of OVA solution (2 mg/ml in PBS) or PBS were applied on abdominal skin 24 h after shaving. Patches were secured with a bioocclusive dressing (Visulin; Hartmann), which was protected with an elastic bandage (Optiplaste; Smith & Nephew). Patches were left on for three 1-wk periods with a 2-wk interval between applications. Animals were sacrificed by cervical dislocation after bronchoalveolar lavage. Skin and inguinal lymph nodes were collected in fixative for histology and/or directly frozen in liquid nitrogen for RNA analyses.

Analysis of lung function and inflammation

At the time of last patch removal (day 49) mice were challenged for 20 min by aerosol nebulization with OVA (1% in PBS) using an ultrasonic nebulizer (Ultramed; Medicalia) and serum was collected (19). On the next day, airway hyperreactivity (AHR) to increasing concentrations of methacholine was measured by whole-body plethysmography (Emka Technologies). Results were expressed as percentage of increase in enhanced pause over baseline value. Following pentobarbital anesthesia, lung samples were used for bronchoalveolar lavage (BAL). BAL fluid was analyzed on cytopsin preparations following RAL 555 staining.

FIGURE 1. Decreased skin inflammation in $Fc\gamma R^{-/-}$, $Fc\epsilon RI^{-/-}$, and $CD16^{-/-}$ mice in a model of AD. **A**, MGG staining of skin sections from OVA-sensitized mice or PBS-treated mice $Fc\gamma R^{-/-}$, $Fc\epsilon RI^{-/-}$, and $CD16^{-/-}$ mice and their corresponding WT counterparts (original magnification $\times 100$). Epidermal thickness (**B**), mast cell (**C**), eosinophil (**D**), MHC-II⁺ (**E**), and $CD4^{+}$ T cell (**F**) density in dermis at the site of sensitization. Data presented are from one out of three independent experiments ($n = 4$ –9 animals/group) and are expressed as means $\pm$ SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from OVA-sensitized WT mice ($p < 0.05$).



Histology and immunohistochemistry

Tissue biopsies were fixed in ImmunoHistoFix (Intertiles) and embedded in ImmunoHistoWax (Intertiles) (20). Serial sections (5 μm) were stained with May-Grünwald-Giemsa for measurement of epidermal thickness as well as eosinophil and mast cell counts. Epidermal thickness was determined at a $\times 250$ magnification with an ocular micrometer; an average of 10 measures were calculated for each sample. Cells were enumerated using an eyepiece equipped with a calibrated grid, by examining 20 random fields at $\times 1000$ magnification; results were expressed as cell number per mm². For immunohistochemical analysis, 5- μm sections were dewaxed in acetone for 5 min and immunostained with anti-MHC class II (MHC-II), anti-CD4, anti-IL-10 mAbs (BD Pharmingen) and anti-Foxp3 (clone FJK-16s) (eBioscience), as previously described (20).

Identification of cell types expressing Foxp3 and IL-10 was performed by immunofluorescence on skin biopsies fixed in 4% paraformaldehyde in PBS and embedded in tissue freezing medium (Leica). Acetone/methanol-

treated 5- μm cryosections were used in multiple staining with anti-Foxp3 revealed by Alexa 488-conjugated anti-rat IgG (Invitrogen) and biotin-conjugated anti-CD4 revealed by Alexa 555-conjugated streptavidin (Invitrogen). IL-10 was identified in T cells using anti-IL-10 revealed by Alexa 555-conjugated anti-rat IgG and biotin-conjugated anti-CD4 revealed by Alexa 488-conjugated streptavidin. Identification in DCs and mast cells was performed using anti-MHC-II revealed by Alexa 488-conjugated anti-rat IgG or FITC-conjugated anti-c-kit (BD Pharmingen), respectively, and biotin-conjugated anti-IL-10 revealed by Alexa 555-conjugated streptavidin. Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich).

Measurement of Ig concentrations

Total IgE concentrations were measured in mouse serum by ELISA, using anti-IgE Abs (BD Pharmingen) (20). Total IgG1 and IgG2a were measured

with the immunoassay kit SBA Clonotyping System/HRP (SouthernBiotech). Anti-OVA IgE was measured using anti-IgE (BD Pharmingen) as capture Ab and biotinylated OVA and HRP-conjugated streptavidin (Amersham Biosciences) for detection. Anti-OVA IgG1 and IgG2a were measured using OVA-coated plates and HRP-conjugated anti-mouse IgG1 and IgG2a (SouthernBiotech). Two-fold serial dilutions were prepared for each serum (starting dilution 1/25 for IgE, 1/5000 for IgG1, and 1/250 for IgG2a titrations). Ab titers were calculated as the dilution corresponding to twice the mean absorbance value obtained for nonsensitized mouse sera.

Quantitative RT-PCR

Tissue RNA extraction was performed as described (21). Reverse transcription was performed with 1 μ g of RNA using SuperScript reverse transcriptase (Invitrogen). cDNAs were amplified by PCR in triplicate assays for 40 cycles in SYBR Green PCR Master Mix (Molecular Probes) on an ABI PRISM 7000 (Applied Biosystems). Oligonucleotide primers specific for mouse cytokines, chemokines, and their receptors and nuclear transcription factors are listed in Table I. Primers were used for amplification in triplicate assays. PCR amplification of GAPDH was performed to control for sample loading and to allow normalization between samples. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method (22). Since expression levels were comparable in PBS-treated wild-type (WT) and corresponding FcR-deficient animals, results for OVA-sensitized animals were expressed as the mean fold induction compared with the mean expression level in PBS-treated mice.

Determination of T cell proliferation in draining lymph nodes

Naive T cells purified from TCR_{OVA} transgenic mice (DO11.10 or OTII) were adoptively transferred into sex- and background-matched recipient mice. CD4⁺ cells were purified from spleens using a Dynal Mouse CD4 Negative Isolation kit (Invitrogen) (purity >95%). CD4⁺ cells were labeled with CFSE (Sigma-Aldrich) (23). Two days before the first sensitization, each recipient mouse received 2×10^6 labeled cells via the lateral tail vein. At day 0 and day 3, mice were epicutaneously sensitized with 100 μ l of OVA (2 mg/ml in PBS) or PBS. Two days after the second sensitization mice were sacrificed and inguinal lymph nodes were harvested and homogenized and processed for flow cytometry.

Flow cytometry analysis

Cells were washed twice in ice-cold PBS, and after incubation with anti-mouse CD16/32 2.4G2 were incubated in 100 μ l of PBS containing the appropriate mAb (2.5 μ g/ml for 1×10^6 cells) for 30 min on ice. At least 2×10^6 cells were analyzed on a FACSCalibur flow cytometer. Unless otherwise mentioned, Abs were from BD Biosciences. For analysis of CFSE-labeled DO11.10 T cells, lymph node cells were stained with anti-CD4-PE and biotinylated KJ1-26 Ab (Caltag Laboratories) followed by incubation with streptavidin-allophycocyanin. For analysis of CFSE-labeled OT-II T cells, lymph node cells were stained with anti-CD4-PE/Cy7, anti-V α 2 TCR-PE, and biotinylated anti-V β 5 TCR followed by incubation with streptavidin-allophycocyanin. Propidium iodide was added for dead cell exclusion, immediately before FACS analysis. CD4⁺CFSE⁺KJ1-26⁺ and CD4⁺CFSE⁺V α 2⁺V β 5⁺ cells were gated and analyzed in mice on a BALB/c and C57BL/6 background, respectively.

Statistical analysis

Statistical significance was determined using Mann-Whitney *U* test. Results were expressed as mean $\pm$ SD; *p* < 0.05 was considered significant.

Results

Both FcεRI and CD16 contribute to skin inflammatory responses in a model of AD

Using a well-established model of AD based on Ag sensitization by repeated epicutaneous OVA applications, we assessed the effect of FcR γ , FcεRI, and CD16 deficiency on skin histology and inflammatory responses. In the absence of OVA sensitization, no inflammation was detected regardless of the mouse genotype (Fig. 1A). Compared with OVA-sensitized FcR $\gamma^{+/+}$, skin inflammatory response was abolished in OVA-sensitized FcR $\gamma^{-/-}$ mice, with an absence of hyperkeratosis, spongiosis, and dermal cellular infiltrates. Upon Ag sensitization, FcR $\gamma^{-/-}$ mice exhibited no significant increase in either epidermal thickness (Fig. 1B) or recruitment of inflammatory cells, mast cells (Fig. 1C), eosinophils (Fig. 1D), APCs (MHC-II⁺) (Fig. 1E), and CD4⁺ T cells (Fig. 1F),

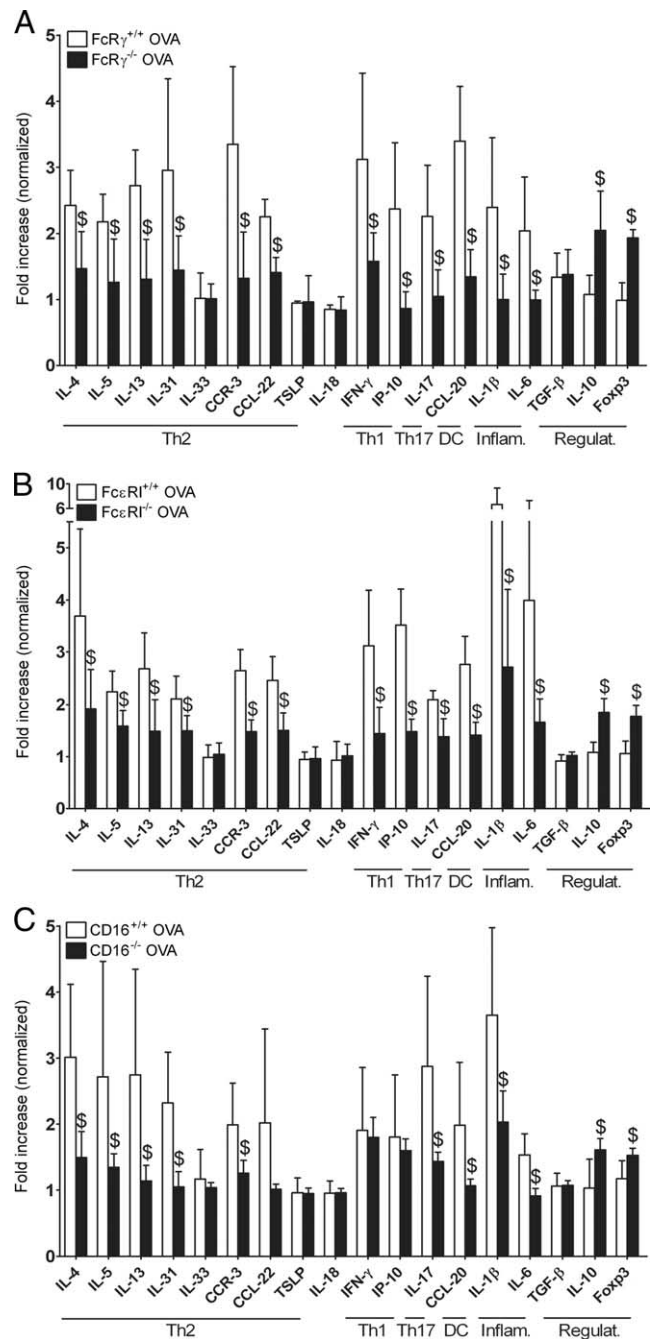


FIGURE 2. Differential decrease in skin cytokines, chemokines, and receptors expression in FcR $\gamma^{-/-}$ (A), FcεRI $^{-/-}$ (B), and CD16 $^{-/-}$ mice (C) compared with their WT counterparts in a model of AD. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. Since expression levels were comparable in PBS-treated WT and corresponding FcR-deficient animals, results for OVA-sensitized animals were expressed as the mean fold induction compared with the mean expression level in corresponding PBS-treated WT mice. Data presented are from one out of three independent experiments (*n* = 4–9 animals/group) and are expressed as means $\pm$ SD. *, Statistically different from PBS-treated mice (*p* < 0.05); \$, statistically different from OVA-sensitized WT mice (*p* < 0.05).

demonstrating that at least one FcR γ -associated receptor is essential for induction of AD. On the other hand, OVA-sensitized FcεRI $^{-/-}$ and CD16 $^{-/-}$ mice showed significant but partial decrease of skin inflammatory response compared with their WT counterparts (Fig. 1A). Compared with FcεRI $^{+/+}$ mice, OVA-sensitized FcεRI $^{-/-}$ mice showed a 45% average decrease in epidermal thickening (Fig. 1B) as well as 78%, 61%, 66%, and 62%

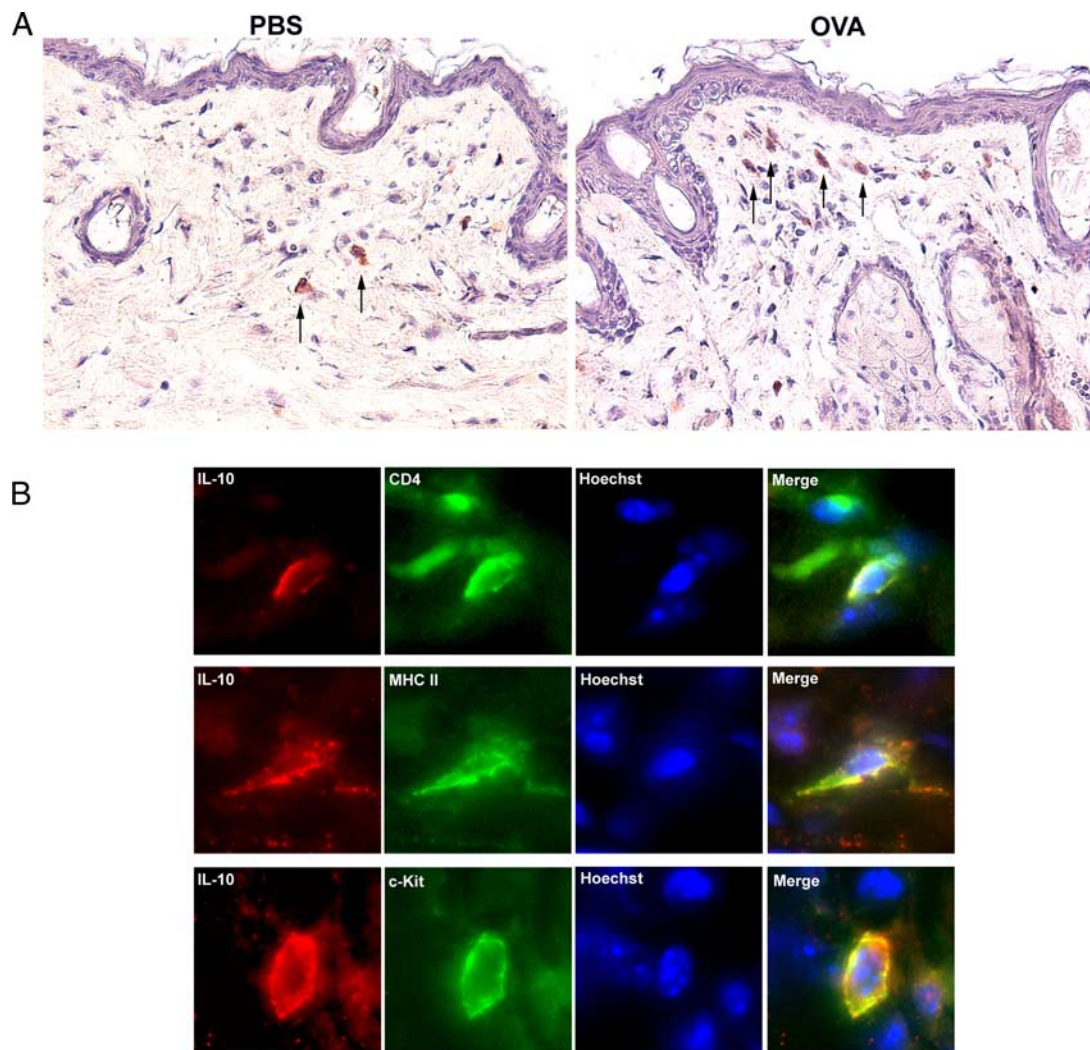


FIGURE 3. Expression of IL-10 by cutaneous immune cells in a model of AD. Immunolocalization of IL-10 in bright field (A) and fluorescence (B) microscopy. A, PBS and OVA treated in $\text{Fc}\gamma\text{RI}^{-/-}$ mice (original magnification $\times 20$). Positive cells are indicated by arrows. B, Identification of IL-10⁺ T cells, DCs, and mast cells by double staining with anti-CD4, anti-MHC-II, and anti-c-kit Abs. Nuclei were counterstained with Hoechst 33258 (original magnification $\times 100$).

average decrease in mast cell, eosinophil, APC (MHC-II⁺), and CD4⁺ T cell recruitment respectively (Fig. 1, C–F). Interestingly, OVA-sensitized CD16^{-/-} mice showed no increase in epidermal thickness or in eosinophil and APC infiltration compared with their PBS-treated counterparts (Fig. 1, B, D, and E). This represents a 63% and 44% average decrease of mast cell and CD4⁺ T cell recruitment compared with CD16^{+/+} mice. Despite the different genetic background used for the experiments, these results nevertheless suggest that both $\text{Fc}\epsilon\text{RI}$ and CD16 contribute to AD skin lesions but differentially control dermal recruitment of inflammatory cells.

$\text{Fc}\epsilon\text{RI}$ and CD16 differentially regulate expression of Th2-, Th1-, and Th17-related molecules in OVA-sensitized mouse skin

We next examined, by real-time PCR, mRNA expression for lesion-, inflammation-, or immune response-associated molecules in the skin from unsensitized or OVA-sensitized $\text{Fc}\gamma\text{RI}^{-/-}$, $\text{Fc}\epsilon\text{RI}^{-/-}$, and CD16^{-/-} mice as well as from their WT counterparts. For each gene examined, skin expression was comparable in PBS-treated gene-deficient animals and in their WT counterparts (not shown). In WT animals, OVA sensitization increased mRNA expression of several genes associated to Th2, Th1, Th17, as well

as to inflammatory response with the exception of IL-18, and thymic stromal lymphopoietin (TSLP), which have been associated to AD in other experimental models, and of IL-33, a Th2 cell chemoattractant and Th2 associated-cytokine inducer, as well as of immunoregulatory TGF- β (24, 25) (Fig. 2). In contrast, OVA sensitization of $\text{Fc}\gamma\text{RI}^{-/-}$ mice did not significantly show increased expression of most of these genes (Fig. 2A). Importantly, this inhibited response coincided with a 2 fold-increase in the expression of immunoregulatory IL-10 and Foxp3 (Fig. 2A), while in $\text{Fc}\gamma\text{RI}^{+/+}$ mice, expression of these latter genes was not affected by OVA sensitization. This thus further indicates that at least one $\text{Fc}\gamma\text{RI}$ -associated receptor is a key regulator of skin immune and inflammatory response in this model of AD.

In skin from OVA-sensitized $\text{Fc}\epsilon\text{RI}^{-/-}$ mice, IL-13, IL-6, and IL-17 expression was comparable to that found in corresponding PBS-treated animals, while a partial decrease of expression of several genes was observed compared with OVA-sensitized WT animals (Fig. 2B): Th2-associated molecules (IL-4, IL-5, IL-31); CCR3, which contributes to eosinophil chemotaxis; CCL22 (macrophage-derived chemokine, MDC), a chemoattractant for Th2 lymphocytes into the inflammatory skin; Th1-associated molecules (IFN- γ and IP-10); CCL20, an APC-attracting chemokine; and

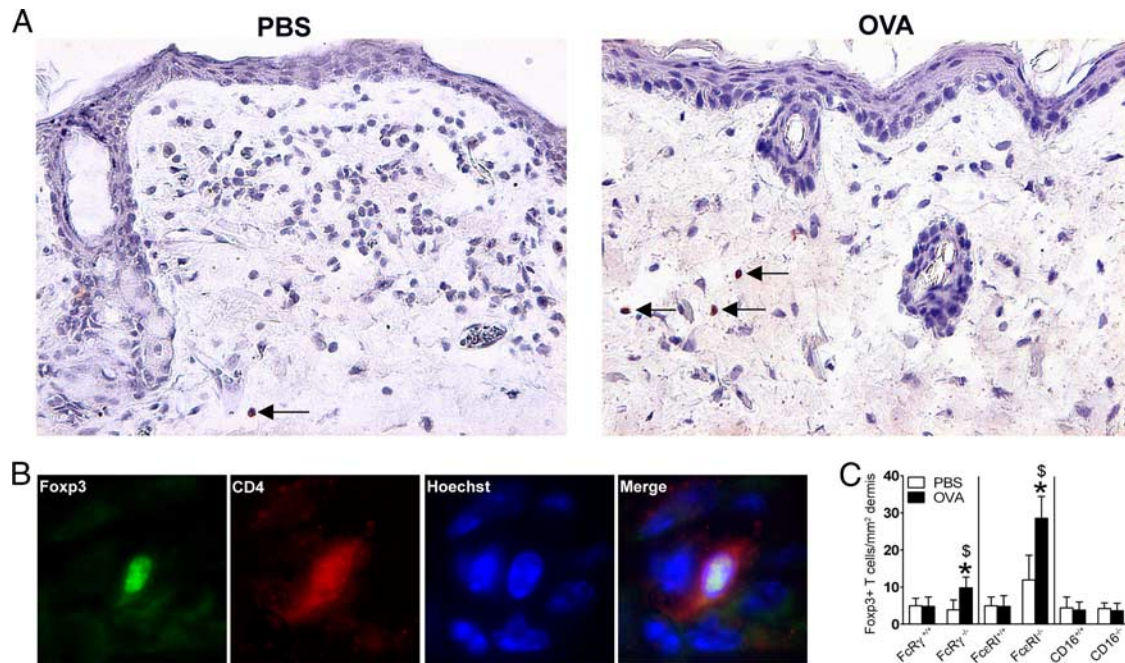


FIGURE 4. Expression of Foxp3 by skin CD4⁺ T cells in a model of AD. *A* and *B*, Immunolocalization of Foxp3 in bright field (*A*) and fluorescence (*B*) microscopy. *A*, PBS and OVA treated in FcγR^{-/-} mice (original magnification ×20). Positive cells are indicated by arrows. *B*, Identification of Foxp3⁺ T cells by double staining with an anti-CD4 Ab. Nuclei were counterstained with Hoechst 33258 (original magnification ×100). *C*, Foxp3⁺ cell density in skin from FcγR^{-/-}, FcεRI^{-/-}, and CD16^{-/-} mice compared with their WT counterparts. Data ($n = 4-9$ animals/group) are expressed as means ± SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from OVA-sensitized WT mice ($p < 0.05$).

proinflammatory IL-1β. As for FcγR^{-/-} mice, FcεRI^{-/-} mice showed a 1.8-fold increase of IL-10 and Foxp3 mRNA expression following OVA sensitization.

Surprisingly, in CD16^{-/-} mice, OVA sensitization did not lead to a significant increase of Th2-related molecules and of CCL20 expression. IL-17, IL-1β, and IL-6 mRNA expression was also decreased compared with OVA-sensitized CD16^{+/+} animals. In contrast, Th1 response was comparable to that of corresponding OVA-sensitized CD16^{+/+} animals. Inhibition of Th2, Th17, and skin inflammatory response in OVA-sensitized CD16^{-/-} mice coincided with a moderate increase of IL-10 and Foxp3 mRNA expression (1.6- and 1.5-fold increase, respectively).

Since IL-10 can be produced by various immune and nonimmune cells types, in skin, we performed double immunohistochemistry to identify cellular source of IL-10. While keratinocytes were devoid of staining, IL-10-containing lymphocytes (CD4⁺IL-10⁺), DCs (MHC-II⁺IL-10⁺), and mast cells (c-kit⁺IL-10⁺) were identified (Fig. 3). A minor increase in IL-10⁺ skin cell density was found between OVA- and PBS-treated FcγR^{+/+}, FcγR^{-/-}, FcεRI^{+/+}, and FcεRI^{-/-} mice. However, no differences were observed between gene-deficient animals and their WT counterparts (data not shown).

As expected, only CD4⁺ cells expressed Foxp3 in skin (Fig. 4, *A* and *B*). No significant increase in Foxp3⁺ cell density was found between OVA- and PBS-treated FcγR^{+/+}, FcεRI^{+/+}, CD16^{+/+}, and CD16^{-/-} mice. However, Foxp3⁺ cell density was increased, respectively, by 2.5- and 2.4-fold in OVA-treated FcγR^{-/-} and FcεRI^{-/-} mice compared with their PBS-treated counterparts, and by 2.1- and 6-fold compared with OVA-treated FcγR^{+/+} and FcεRI^{+/+} mice (Fig. 4C).

Taken together, these data suggest that FcεRI and CD16 differentially regulate skin expression of DA-associated cytokines, with FcεRI controlling both Th1 and Th2 responses while CD16 only regulates Th2 response.

FcεRI and CD16 differentially regulate the humoral response in a mouse model of AD

Serum levels of IgE and IgG1, both associated to a Th2 response, were decreased in OVA-sensitized FcγR^{-/-} mice compared with OVA-sensitized FcγR^{+/+} animals, with a 64% and 55% average decrease for total IgE and OVA-specific IgE, respectively, and a 58.5% decrease for OVA-specific IgG1 (Fig. 5, *A-C*). IgG2a and

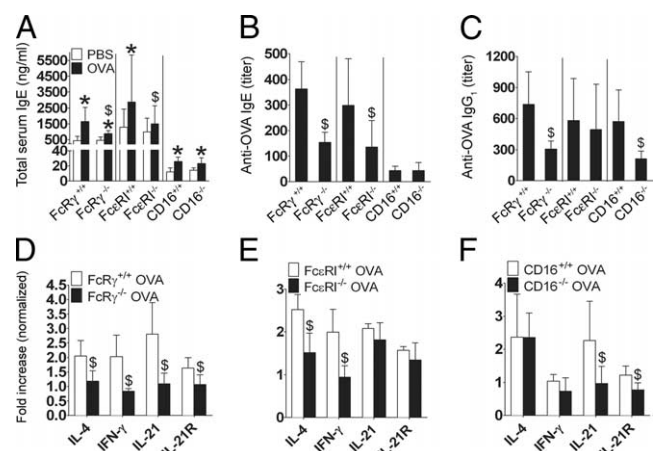
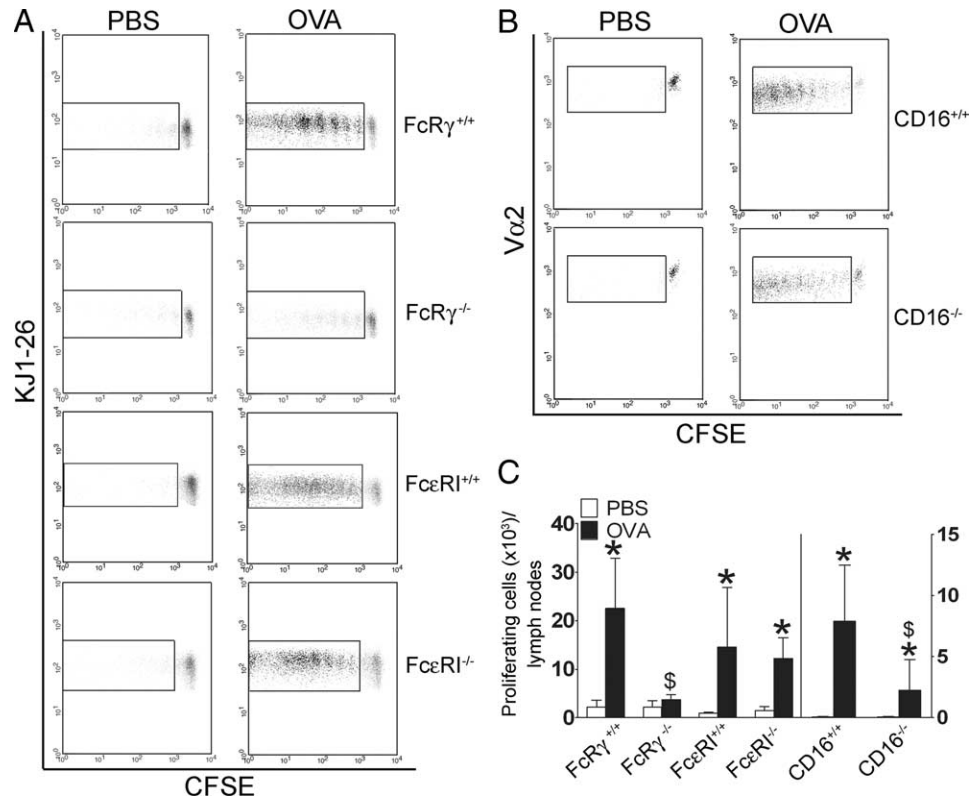


FIGURE 5. Differentially decreased humoral response and lymph node cytokine expression in FcγR^{-/-}, FcεRI^{-/-}, and CD16^{-/-} mice in a model of AD. *A*, Total IgE serum concentrations. *B*, OVA-specific IgE serum concentrations. *C*, OVA-specific IgG1 serum concentrations. *D-F*, Expression of IL-4, IFN-γ, IL-21, and IL-21R in inguinal lymph nodes from FcγR^{-/-} (*D*), FcεRI^{-/-} (*E*), and CD16^{-/-} (*F*) mice compared with their WT counterparts. Data presented are from one out of three independent experiments ($n = 4-9$ animals/group) and are expressed as means ± SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from WT mice ($p < 0.05$).

FIGURE 6. Decreased Ag-induced T cell proliferation in $\text{Fc}\gamma\text{R}^{-/-}$ and $\text{CD16}^{-/-}$ but not $\text{Fc}\epsilon\text{RI}^{-/-}$ mice in a model of AD. **A** and **B**, Flow cytometry analysis of $\text{CFSE}^+\text{KJ1-26}^+\text{CD4}^+$ (**A**) or $\text{CFSE}^+\text{V}\alpha 2^+\text{V}\beta 5^+\text{CD4}^+$ (**B**) T cells from inguinal lymph nodes from $\text{Fc}\gamma\text{R}^{-/-}$ (**A**), $\text{Fc}\epsilon\text{RI}^{-/-}$ (**A**), and $\text{CD16}^{-/-}$ (**B**) mice and their WT counterparts. Dividing cell populations are squared. **C**, Total number of dividing $\text{CFSE}^+\text{CD4}^+$ T cells in inguinal lymph nodes ($n = 3\text{--}6$ animals/group), which are expressed as means $\pm$ SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from WT mice ($p < 0.05$).



IgG2b Abs, associated to a Th1 response, were undetectable in OVA-sensitized WT or $\text{Fc}\epsilon\text{RI}^{-/-}$ mice (data not shown). Interestingly, compared with their $\text{Fc}\epsilon\text{RI}^{+/+}$ controls, only IgE response was affected in OVA-treated $\text{Fc}\epsilon\text{RI}^{-/-}$ mice: 67% and 54% average decrease for total IgE and OVA-specific IgE, respectively (Fig. 5, A–C). In contrast with the latter mice, in OVA-sensitized $\text{CD16}^{-/-}$ mice, only OVA-specific IgG1 response was affected (63% decrease). Despite the different genetic background used for the experiments, these results nevertheless suggest that, in this model, $\text{Fc}\epsilon\text{RI}$ and CD16 specifically regulate the production of their own ligand.

To determine, at the molecular level, the factors that account for this differential regulation, we analyzed, by real-time PCR, cytokine expression in draining inguinal lymph nodes. In particular, we examined the expression of IL-21 and its receptor. Indeed, these latter are essential for IgG1 production (26). In OVA-sensitized $\text{Fc}\gamma\text{R}^{-/-}$ mice, expression of IL-4, IFN- γ , IL-21, and IL-21R were fully inhibited compared with $\text{Fc}\gamma\text{R}^{+/+}$ animals (Fig. 5D). IL-13 expression was comparable to that found in WT animals (data not shown). In OVA-sensitized $\text{Fc}\epsilon\text{RI}^{-/-}$ mice, IL-4 expression was reduced and IFN- γ completely inhibited compared with $\text{Fc}\epsilon\text{RI}^{+/+}$ animals, while the IL-21/IL-21R axis remained intact (Fig. 5E). In contrast, when compared with $\text{CD16}^{+/+}$ animals, IL-4 and IFN- γ expression was comparable in OVA-sensitized $\text{CD16}^{-/-}$ mice, but expression of IL-21 and IL-21R was fully inhibited (Fig. 5F).

Important contribution of CD16 to antigenic presentation in a mouse model of AD

We then investigated whether defective Ag presentation during early stages of epicutaneous sensitization was responsible for the reduced inflammatory skin response observed in OVA-sensitized $\text{Fc}\gamma\text{R}^{-/-}$, $\text{Fc}\epsilon\text{RI}^{-/-}$, and $\text{CD16}^{-/-}$ mice. We thus examined Ag-induced T cell proliferation *in vivo* by transfer of CFSE-labeled CD4^+ T cells from DO11.10 mice to recipient animals that were epicutaneously sensitized for 1 wk. In the absence of OVA sensi-

tization, both percentage and total number of proliferating T cells were very low for each mouse strain (Fig. 6, A, left panel, and C). Compared with $\text{Fc}\gamma\text{R}^{+/+}$ animals, OVA-sensitized $\text{Fc}\gamma\text{R}^{-/-}$ mice showed a 64% decrease in the percentage of proliferating T cells (Fig. 6A, right panel). They exhibited no significant increase in the total number of proliferating cells, compared with corresponding PBS-treated animals (Fig. 6C), indicating that $\text{Fc}\gamma\text{R}$ is essential for Ag presentation and thus T cell proliferation in this model. T cell proliferation following OVA sensitization was comparable in $\text{Fc}\epsilon\text{RI}^{+/+}$ and $\text{Fc}\epsilon\text{RI}^{-/-}$ mice (Fig. 6, A, right panel, and C), in agreement with the absence of $\text{Fc}\epsilon\text{RI}$ expression on WT mouse DCs. In contrast, compared with $\text{CD16}^{+/+}$ animals, OVA-sensitized $\text{CD16}^{-/-}$ mice showed 33% and 72% decrease in proliferating T cells by percentage and total cell number, respectively (Fig. 6, B, right panel, and C). These results suggest that, in this model of AD, CD16 plays a major role by controlling Ag presentation.

CD16 deficiency leads to complete decrease of lung inflammation in a mouse model of AD

Since this mouse model is characterized (as for AD in humans) by Ag-induced lung inflammation, we examined AHR and recruitment of inflammatory cells in the BAL fluid following epicutaneous sensitization and a single nebulization. While OVA-sensitized and -challenged $\text{Fc}\gamma\text{R}^{+/+}$ mice, upon nebulization with increasing doses of methacholine, displayed increased AHR compared with PBS-sensitized animals, OVA- and PBS-sensitized $\text{Fc}\gamma\text{R}^{-/-}$ mice responded similarly (Fig. 7A), demonstrating the essential role of $\text{Fc}\gamma\text{R}$ in airway inflammation following epicutaneous sensitization. Along these lines, BAL inflammatory infiltrate in OVA-sensitized and -challenged $\text{Fc}\gamma\text{R}^{-/-}$ mice was virtually absent compared with $\text{Fc}\gamma\text{R}^{+/+}$ mice (Fig. 7B). Cellular inflammation in BAL was comparable in $\text{Fc}\epsilon\text{RI}^{+/+}$ and $\text{Fc}\epsilon\text{RI}^{-/-}$ mice (Fig. 7C). In contrast, lung inflammatory response was equally abolished in OVA-sensitized and -challenged $\text{CD16}^{-/-}$ mice compared with

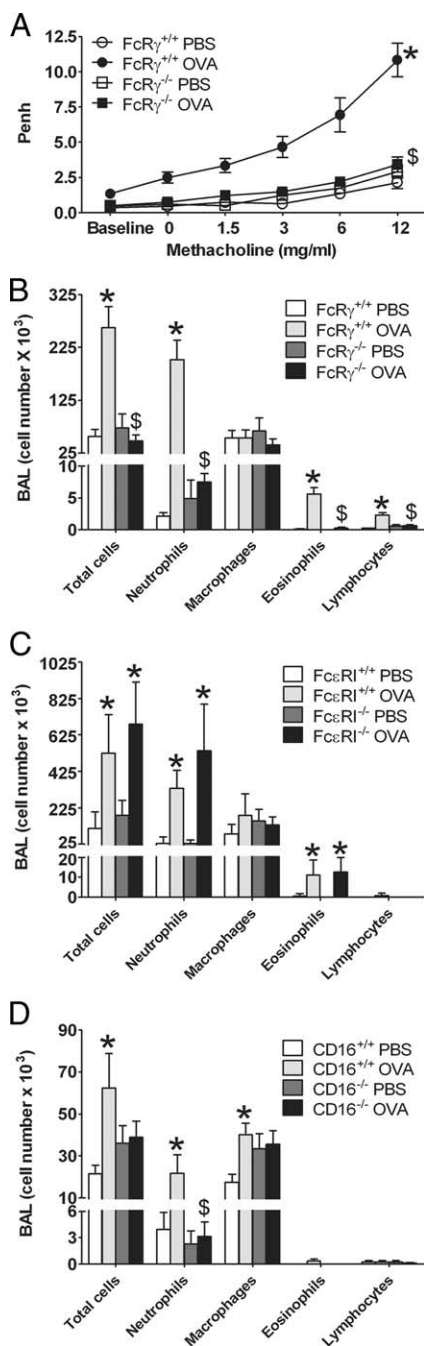


FIGURE 7. Decreased lung inflammation and airway hyperresponsiveness in $Fc\gamma R^{-/-}$ and $CD16^{-/-}$ but not $Fc\epsilon RI^{-/-}$ mice in a model of AD. **A**, Whole-body plethysmography in $Fc\gamma R^{-/-}$ mice and their WT counterparts. **B–D**, Cellularity in BAL from $Fc\gamma R^{-/-}$ (**B**), $Fc\epsilon RI^{-/-}$ (**C**), and $CD16^{-/-}$ (**D**) mice and their WT counterparts. Data ($n = 3–6$ animals/group) are expressed as means $\pm$ SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from OVA-sensitized WT mice ($p < 0.05$).

$CD16^{+/+}$ animals (Fig. 7D), indicating that CD16 is one key $Fc\gamma$ -associated receptor for induction of lung inflammation in this model of AD.

FcεRI regulates mast cell migration to the draining lymph nodes in a mouse model of AD

Since $Fc\epsilon RI$ exerted a significant effect on skin inflammation without regulating APC function, we reasoned that mast cells would be

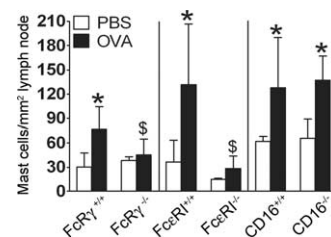


FIGURE 8. Decreased mast cell number in inguinal lymph nodes of $Fc\gamma R^{-/-}$ and $Fc\epsilon RI^{-/-}$ but not in $CD16^{-/-}$ mice in a model of AD. Mast cell density in draining lymph nodes. Data presented are from one out of three independent experiments ($n = 3–7$ animals/group) and are expressed as mean $\pm$ SD. *, Statistically different from PBS-treated mice ($p < 0.05$); \$, statistically different from OVA-sensitized WT mice ($p < 0.05$).

the most likely cell type directly affected by $Fc\epsilon RI$ deficiency. We thus examined mast cell recruitment into draining lymph nodes following sensitization. In contrast to OVA-sensitized $CD16^{+/+}$ and $CD16^{-/-}$ mice, which displayed a similar increase in inguinal lymph nodes compared with corresponding PBS-sensitized animals, $Fc\gamma R^{-/-}$ and $Fc\epsilon RI^{-/-}$ mice showed no significant Ag-induced increase in mast cell recruitment into lymph nodes (Fig. 8). This further suggests that $Fc\epsilon RI$ and CD16 differentially regulate distinct, but partially overlapping, sets of parameters from the AD-associated immune response.

Discussion

In this paper, we provide evidence for the differential contribution of $Fc\gamma$ -associated $Fc\epsilon RI$ and $Fc\gamma RIII/CD16$ in a murine model of AD. Absence of $Fc\gamma$ -associated activating receptors led to a virtual absence of pathology with abolished skin and lung inflammation and drastic decrease of humoral response that were associated to an inhibition of Th2, Th1, Th17, and inflammatory cytokine expression. In contrast, a 2-fold increase of regulatory IL-10 and Foxp3 was observed. An increase in density of Foxp3⁺ cells was also evidenced. These later findings are likely linked to activation of the remaining inhibitory $Fc\gamma RII/CD32b$ expressed by APC, which induces IL-10 production and Foxp3⁺ regulatory T cells (27). $Fc\gamma RII/CD32b$ -independent mast cell activation might also lead to IL-10 production and/or induction of Foxp3 expression (28–30). Indeed, besides CD4⁺ lymphocytes, IL-10 expression was found in both DCs and mast cells but not in keratinocytes, known as a potential source of IL-10 (31). An increase in IL-10 and Foxp3 expression was also found in $Fc\epsilon RI$ - and, to a slightly lesser extent, in $CD16$ -deficient animals. Foxp3⁺ cell density was also increased in skin from $Fc\epsilon RI$ -deficient animals. Defective Ag presentation during early sensitization phase, and thus abrogated Ag-induced T cell proliferation, accounts, at least in part, for the inhibition of pathology as previously observed in models of delayed hypersensitivity (32) and asthma (33). The lack of $Fc\gamma$ expression on effector cells such as mast cells might further inhibit development of inflammation as shown in a mast cell-dependent asthma model (34).

Lack of $Fc\epsilon RI$ led to a partial decrease of epidermal thickening and cellular recruitment into dermis upon induction of AD but, surprisingly, had no effect on lung inflammation. $Fc\epsilon RI$ contribution to the pathology might be slightly underestimated since we have shown that CD16 expression on $Fc\epsilon RI$ -deficient mast cells, and probably basophils, is up-regulated and leads to more severe IgG-mediated anaphylaxis (35). In our AD model, the absence of $Fc\epsilon RI$ also led to impaired skin expression of Th1, Th2, Th17, and inflammatory cytokines but to an increased expression of Foxp3 and IL-10. As observed in some asthma models (36, 37), $Fc\epsilon RI$

involvement in this AD model results from its high expression levels on mast cells, which are very abundant in skin. However, Fc ϵ RI does not contribute the sensitization process at a distal inflammatory site such as lung.

Basophil contribution to the pathology cannot be ruled out, as this cell type contributes to IgE-induced chronic allergic dermatitis (38, 39), and since a basophil influx has been reported during patch tests in atopic patients (40). Histamine release by mast cells promotes Langerhans cell migration to draining lymph nodes through binding to H2 receptors (41). Thus, lack of Fc ϵ RI might indirectly explain the decreased skin APC number compared with sensitized WT animals. Following sensitization, mast cells also migrate to draining lymph nodes, where they induce T lymphocyte recruitment via MIP-1 β production. In our model of AD, mast cell number in draining lymph nodes appears to be controlled by Fc ϵ RI. As mast cells undergo Fc ϵ RI-, IgE-, and Ag-dependent chemotaxis (42), an effect not reported for IgG isotypes, it is likely that the lack of Fc ϵ RI expression accounts for this defective migration and thus for indirect effects on other cell types besides Langerhans cells, particularly T and B cells and eosinophils. Indeed, mast cells also release Th2 and proinflammatory cytokines such as IL-4, IL-5, IL-13, and IL-6 upon Fc ϵ RI activation (43). These cytokines are known to regulate IgE production and eosinophilia, two hallmarks of AD. IL-6 has been shown to regulate IL-17 production (44), recently associated to AD (45). Thus, decreased IL-6 production by mast cells might indirectly account for the decreased IL-17 expression. Nevertheless, IL-17 expression in AD is low compared with psoriasis (46). Surprisingly, in Fc ϵ RI-deficient mice, Ag-specific IgG1 response was unaffected while Ag-specific IgE response was decreased (Fig. 3). We had previously reported a tendency to lower Ag-specific IgE concentrations but normal IgG levels following active anaphylaxis-inducing immunization (35). This regulation of IgE synthesis by Fc ϵ RI thus seems to complete a regulatory loop. Indeed, the ability for IgE to up-regulate expression of both its receptors has been previously documented (4, 6). IL-21 seems to be the only factor known to differentially regulate IgE and IgG1 synthesis (26). Expression of both IL-21 and its receptor were unaffected in draining lymph nodes from Fc ϵ RI-deficient animals, while expression of IL-4, the key cytokine for the regulation of IgE synthesis in this model, was reduced. Thus, the differential effect of Fc ϵ RI and CD16 on IgE and IgG1 production might be attributed to a distinct regulation of IL-4 and IL-21 (see below).

Compared with the pathology developed on the BALB/c background, AD is significantly less severe on a C57BL/6 background. Indeed, most of the histological, cellular, and biochemical parameters are quantitatively lower in the latter case. This renders direct comparisons between Fc γ R- and Fc ϵ RI-deficient mice, on the one hand, and CD16-deficient mice, on the other hand, impossible. Thus, CD16 contribution to AD is only discussed by comparing CD16-deficient and -proficient mice on a C57BL/6 background.

CD16 deficiency led to a complete inhibition of epidermal thickening, dermal and lung recruitment of inflammatory cells following sensitization. Surprisingly, in our model of AD eliciting both a Th1 and a Th2 response (1), skin Th1-associated cytokine response was unaffected in CD16-deficient mice while Th2 response was completely abolished. These results on CD16 selective contribution to the Th2 response, and to AD, are in agreement with those obtained in a (strongly Th2-polarized) asthma model, in which CD64 does not play a role (47). Both IL-17 and IL-6 expression was inhibited while IL-10 and Foxp3 expression was enhanced in CD16-deficient mice, as it was the case for Fc ϵ RI-deficient animals. In contrast, CD16-deficient mice showed a major decrease in Ag-induced T cell proliferation, indicating that CD16 plays an essential role in antigenic presentation and initiation of immune

response in this model of AD. Indeed, CD16 is highly expressed on DC and is known to preferentially bind IgG1 (immune complexes) (48), the major Th2-associated isotype in mouse, whose levels are very high following OVA sensitization in this AD protocol (Fig. 3). Besides its expression on APC, CD16 is also expressed on mast cells (29) and basophils (49). It is thus possible that CD16 deficiency directly contributed to the decreased mast cell number and to a dampened mast cell activation in skin following Ag sensitization. However, mast cell number in draining lymph nodes from CD16-deficient animals was not affected compared with WT animals, suggesting that, in contrast with Fc ϵ RI, CD16 is not involved in chemotactic activity. Finally, as observed for Fc ϵ RI and IgE, CD16 deficiency led to a decreased specific IgG1 response, while IgE production remained similar. Consistent with this result, expression of both IL-21 and its receptor was abrogated in draining lymph nodes from CD16-deficient animals, while IL-4 expression was comparable to that found in WT animals. This contrasts with an asthma model where production of both isotypes was decreased (47). This discrepancy might be due to our less Th2-prone experimental setting, to the different pathology (AD vs asthma), and/or to a different genetic background (C57BL/6 vs BALB/c).

Among Fc γ R-associated receptors, Fc ϵ RI and CD16 were a priori the likeliest to be involved in the regulation of AD, hence our study. Indeed, the only other two Fc γ R-associated receptors are Fc γ RI/CD64 and Fc γ RIV, which are mainly expressed on macrophages and preferentially bind IgG2a and IgG2b (48), two Th1-associated isotypes. In our model, OVA-specific IgG2a and IgG2b were not detected. Fc γ RIV has also been reported to bind IgE^b, found in C57BL/6 but not in BALB/c mice, with an even lower affinity than CD16 (7). Due to this cell distribution and isotype specificity, it is thus unlikely, but cannot be completely ruled out, that either of these receptors plays a major role in AD physiopathology.

In conclusion, the present study delineates for the first time a differential but partially overlapping role of Fc ϵ RI and CD16 in a mouse model reproducing some features of AD physiopathology, where the contribution of IgG immune complexes was documented but poorly understood (50). Even if considerable differences exist between mice and humans regarding identity, structure, cell distribution, and/or isotype specificity of Fc γ R and Fc ϵ RI, we might nevertheless speculate that the partial inhibition obtained by targeting only IgE/Fc ϵ RI interactions, using anti-IgE, in asthma (51) or atopic skin reactions (52) might be due to a significant contribution of CD16 or other Fc γ R to the pathology. Thus, a therapy preventing both IgE/Fc ϵ RI and IgG/Fc γ R interactions might represent a further improvement over the present ones.

Disclosures

The authors have no financial conflicts of interest.

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