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TLR9- and FcεRI-Mediated Responses Oppose One Another in Plasmacytoid Dendritic Cells by Down-Regulating Receptor Expression[1]

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Plasmacytoid dendritic cells (pDC) express not only TLR9 molecules through which ligation with CpG DNA favors Th1 responses but also possess IgE receptors (FcεRI) implicated in allergen presentation and induction of Th2 responses. This dichotomy prompted an investigation to determine whether TLR9- and IgE receptor-mediated responses oppose one another in pDC by affecting receptor expression and associated functional responses. Results showed that IgE cross-linking reduced TLR9 in pDC and inhibited the capacity of these cells to secrete IFN-α when stimulated with the CpG oligodeoxynucleotide (ODN)-2216. In contrast, an ~15-fold reduction in FcεRIα mRNA and a loss in surface protein were seen in pDC first exposed to TLR9 ligation with ODN-2216. Results indicated that type I IFNs partly mediated this effect, as rIFN-α also caused a significant ~4-fold reduction in FcεRIα mRNA. Finally, this reduction in FcεRIα mediated by ODN-2216 correlated with a selective suppression of allergen-induced $CD4^+$ T cell proliferation, but not of responses resulting from tetanus toxoid. Overall, these results imply mechanisms by which specific innate and IgE-dependent immune responses counterregulate one another at the dendritic cell level and may have significant impact on whether an ensuing response is either of Th1 or Th2 in nature. *The Journal of Immunology,* **2005, 175: 5724–5731.**

Dendritic cells (DC)[3] play an important role in initiating immune responses by activating naive T cells for effector functions. Two major types of DC have been characterized in humans: the $CD11c^+CD123^-$ myeloid (mDC, DC1), for which there are several subtypes, and the $CD11c^-$ $CD123^+$ lymphoid (DC2), also known as the plasmacytoid DC (pDC) (1). Studies have long supported the presence of the high-affinity IgE receptor (FcεRI) on mDC, particularly those from allergic subjects, but have only recently identified this receptor on pDC (2, 3). However, unlike the classical $\alpha\beta\gamma_2$ tetramer that is uniquely expressed on basophils and mast cells and is involved in triggering mediator release from these cells, the role of the $\alpha\gamma_2$ variant on mDC and pDC is less clear, although the data at this time support it having a role in allergen presentation or "focusing" (4).

The TLR have emerged in recent years as vital molecules in innate immunity. Their capacity to recognize specific microbial products can determine the course of adaptive immune responses, including those associated with allergic disease (5). Most TLR are differentially expressed on human mDC and pDC, indicating that ligands for these receptors mediate specific effector functions in these cells. In particular, TLR9, which recognizes unmethylated CpG motifs common in bacterial DNA, is found intracellularly in pDC but not in mDC. As a consequence, human pDC also respond to CpG oligodeoxynucleotides (CpG-ODN) by producing cytokines (e.g., IFN-α, IL-12) that promote Th1 activity rather than the Th2 responses that are a hallmark of allergic disease. These findings have provided the basis for using CpG-ODN as immunostimulatory sequences in allergen immunotherapy (6).

Whether CpG-ODN-TLR9 interactions affect allergen-specific responses by modulating FcεRI expression or whether IgE-FcεRI interactions conversely affect TLR9 expression is currently not known. Therefore, we investigated these hypotheses and found evidence that FcεRI- and TLR9-dependent responses oppose one another in pDC, in part by down-regulating each other's receptor expression. Overall, our results implicate a unique level of counterregulation between IgE-dependent and specific innate immune responses that occur at the DC level, which may have significant impact on the development of effector T cell responses.

Materials and Methods

Special reagents

The following reagents were purchased: crystallized human serum albumin (Calbiochem-Behring; PIPES, FBS, and crystallized BSA (Sigma-Aldrich); gentamicin, IMDM, and nonessential amino acids (Invitrogen Life Technologies; Percoll (Pharmacia Biotec); all recombinant cytokines and anti-mouse PE-conjugated IgG $(Fab')_2$ fragments (BioSource International: tritiated ($[^3H]$) thymidine (New England Nuclear) with specific activity 2 Ci/mmol; CpG-ODN-2216 and -2243, as described previously (7), were synthesized by Invitrogen Life Technologies; PE-labeled Abs to human CD80, CD86 (Caltag Laboratories); and anti-human TLR9, PE-labeled and unlabeled (eBioscience). The 22E7 mAb specific for FcεRIα was provided by J. Kochan (Hoffmann-LaRoche Pharmaceuticals, Nutley, NJ). Human IgE-PS myeloma was provided by T. Ishizaki (8). Use of the polyclonal goat anti-human IgE has been described elsewhere (9).

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[2] Address correspondence and reprint requests to Dr. John T. Schroeder, Unit Office 2, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail address: schray@jhmi.edu

[3] Abbreviations used in this paper: DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC; ODN, oligodeoxynucleotide; BDCA, blood DC Ag; HPRT, hypoxanthine phosphoribosyltransferase; nMFI, net mean fluorescence intensity; TT, tetanus toxoid.

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Special buffers and media

A concentration of 10× PIPES buffer (250 mM PIPES, 1.10 M NaCl, and 50 mM KCl, pH 7.3) was stored at 4°C as a stock solution. PIPES-albumin-glucose was made by diluting one part 10× PIPES with nine parts deionized water and contained 0.003% human serum albumin and 0.1% D-glucose. PIPES-albumin-glucose-EDTA additionally contained 4 mM EDTA. Isotonic Percoll (referred to in this article as 100% Percoll) was prepared by mixing one part 10× PIPES and nine parts Percoll. Working solutions of Percoll at 55% (density, 1.072 g/ml) and 61% (density, 1.081 g/ml) were made by mixing the appropriate amounts of 100% Percoll with 1× PIPES. Conditioned medium (conditioned IMDM) consisted of IMDM supplemented with 5% heat-inactivated (56°C for 30 min) FBS, 1× nonessential amino acids, and 10 μg/ml gentamicin.

Cell purification and culture

Venipuncture was performed on consenting adults (age range, 21–55 years) using a protocol approved by the Western Institutional Review Board (Seattle, WA). Subjects included individuals both with and without a history of allergy. In some instances, cells were prepared from residual cell packs from subjects undergoing platelet pheresis within the Hemapheresis Unit at Johns Hopkins University (Baltimore, MD). Mixed leukocyte suspensions both depleted and enriched for basophils were prepared using a double-Percoll (1.075/1.081 g/ml) density centrifugation protocol as previously described (10). This step served two purposes: to facilitate the purification of both pDC and basophils from a common specimen and, at the same time, help eliminate cross-contamination of FcϵRIα by either cell type. pDC were purified from the basophil-depleted cell suspensions using blood DC Ag (BDCA)-4 positive selection (Miltenyi Biotec). When needed, basophils were purified from the basophil-enriched suspensions using negative selection (StemCell Technologies). Gravity filtration through magnetized LS columns attached to a MidiMACS magnet (Miltenyi Biotec) facilitated the purification of both cell types. For pDC, this involved four washes to remove unbound (i.e., BDCA-4$^-$) cells and then removal of the column from the magnet and dislodging the pDC by gently plunging with buffer. For basophils, this meant collecting the unbound cells, while the contaminating cells remained in the column. Cells were counted using a Spiers-Levy chamber. Assessment of several pDC suspensions prepared in this manner indicated >95% purity as determined by BDCA-2 staining. Basophil counts were determined by Alcian blue staining (11), which indicated purities routinely exceeding >97%.

For all studies investigating FcϵRIα expression, cells were cultured (at 1–2 × 10^6/ml) in uncapped 1.5-ml microcentrifuge tubes placed in a rack and covered with a microtiter plate lid to prevent evaporation. This approach facilitated both the extraction of RNA and the staining necessary for flow cytometry, because it eliminated the potential loss of cells occurring when transferring to fresh tubes after harvesting. All cultures were performed using conditioned IMDM. IL-3 (10 ng/ml) was added during all incubations, since this cytokine not only preserves pDC viability, but also up-regulates and maintains TLR9 expression in these cells (J. T. Schroeder, unpublished data).

RNA isolation

Total RNA was isolated from 0.25 to 1.0 × 10^6 cells using the RNAzol Bee protocol (Tel-Test). Following isopropanol precipitation, RNA was washed with ethanol and nearly dried under vacuum. The RNA was then resuspended in DNase-free water and stored at −80°C.

Quantitative RT-PCR for detection of FcϵRIα and TLR9 mRNA

Single-step real-time RT-PCR was performed in an ABI Prism 7700 Thermocycler using the TaqMan reagent kit (PerkinElmer). The forward primer sequence 5′-GTGAACCTGTGTACCTGGAAGTCTT-3′, reverse primer sequence 5′-CATCCCAGTTCCTCCAACCA-3′, and probe sequence 5′-TGACTGGCTGCTCCTTCAGGCCTC-3′ for detection of mRNA for the α subunit of human FcϵRI have been reported elsewhere (12). Primer/probe combinations for TLR9 have been published elsewhere (13), were checked against accession no. AB045180 and included forward primer 5′-GGACCTCTGGTACTGCTTCCA-3′, reverse primer 5′-AAGCTCGT TGTACACCCAGTCT-3′, and probe 5′-CTGCAGGTGCTAGACCT GTCCCGC-3′. For each probe, the reporter dye and quencher were FAM and TAMRA, respectively. Normalization of FcϵRIα and TLR9 mRNA levels was achieved by comparing to hypoxanthine phosphoribosyltransferase (HPRT) using the following primer/probe sequences: forward primer, 5′-CGGCCGGCTCCGTTA-3′; reverse primer, 5′-TTAGGTAT GCAAAATAAATCAAGGTCAT-3′; and probe, FAM-CCGCAGCCCT GGCGTCGT-TAMRA.

Flow cytometry

Indirect staining of cell surface FcϵRIα protein on pDC was achieved with unlabeled mAb 22E7 followed by PE-conjugated anti-mouse IgG $(Fab')_2$ using methods previously described (14). Likewise, direct staining for surface CD80, CD86, and intracellular TLR9 in fixed (buffered 4% paraformaldehyde) pDC was performed using methodologies previously described (15).

Western blotting

The determination of FcϵRIα protein in freshly isolated or cultured pDC (5×10^5 cells) was done as described for human basophils (12). A lysate of a single passage of rat basophilic leukemia-SX38 cells transfected with the human $\alpha\beta\gamma$-chains (provided by Dr. J. P. Kinet, Harvard University, Boston, MA) was also included with each Western blot experiment.

The determination of TLR9 involved similar protocols. In brief, 500,000 cells were lysed with the addition of 0.020 ml of 1× SDS Tris-glycine sample buffer (Invitrogen Life Technologies) containing 10% 2-ME and preheated to boiling conditions. All lysates were boiled for 5 min before performing electrophoresis on Novex 10% Tris-glycine gels. Gel proteins were then transferred to pure nitrocellulose membranes (Bio-Rad). Membranes were blocked overnight in Tris/Tween 20-buffered saline with 3% Carnation nonfat dry milk at 4°C. Immunoblotting was achieved using rat anti-human TLR9 mAb (eBioscience). Membranes were washed four times for 10 min each in Tris/Tween 20-buffered saline before incubation for 45 min with anti-rat IgG HRP (Amersham Biociences). After four additional washes, membranes were visualized using ECL reagent (Pierce). Intensities of detected bands were measured with a Kodak DC120 digital camera (Eastman Kodak) and NIH Image Software (W. Rasband, National Institutes of Health, Bethesda, MD).

Cytokine measurements

IL-10, TNF-α, IL-6, and IL-12p70 in culture supernatants were determined using commercial ELISA (eBioscience). IFN-α measurements were achieved using an in-house kit, which included the following Abs: mAb (clone MMHA-2) used at 2.5 μg/ml for coating, a rabbit polyclonal (250 ng/ml), both from PBL Biomedical Laboratories in a standard "sandwich" protocol. Detection was achieved using goat anti-rabbit/HRP conjugate at 1/1000 (R&D Systems). rIFN-α (BioSource International) was used for standardization. Plates, coating buffer, blocking reagent, and substrate were all individually purchased and used as suggested (eBioscience).

T cell proliferation assays

Ag-specific proliferation responses of T cells was assayed using PBMC (1×10^5) alone or purified CD4$^+$ T cells (1×10^5) cocultured with either pDC (BDCA4$^+$) or CD14$^+$ accessory cells (1.25×10^4), all purified by positive selection using commercially available immunomagnetic microbead kits with MS columns on a MiniMACS magnet (Miltenyi Biotec). For allergen-specific responses, allergic donors were selected for positive immediate skin tests to dust mites (*Dermatophagoides farinae*; Greer Laboratories). Tetanus toxoid (Cylex) was also used. Accessory cells were irradiated (3000 rad) before culture to prevent their proliferation. Triplicate cultures were performed in U-bottom, 96-well microtiter plates in RPMI 1640 medium/5% human AB serum at 37°C in 5%CO_2 with dust mite (100 allergy U/ml) or with tetanus (1 limits of flocculation units/ml). After a 5-day incubation, [^{3}H]thymidine (1 μCi/well) was added for 16 h before harvesting onto glass fiber filters using a cell harvester. Incorporated radioactivity was assessed using liquid scintillation and reported as cpm.

Statistical analysis

Data are presented as mean ± SEM unless otherwise indicated. Statistical analysis was performed with StatView software (SAS Institute, Cary, NC) and involved the use of the Wilcoxon signed rank nonparametric test unless otherwise stated. Values of $p \leq 0.05$ were considered to be significant.

Results

FcϵRIα expression by human pDC

Fig. 1*A* shows that mRNA for the α subunit of FcϵRI was detected in pDC using quantitative RT-PCR. It is important to note, however, that the relative levels, after normalizing to HPRT, were calculated to be 80- to 300-fold less than those seen in basophils. Fig. 1*B* shows a representative flow histogram indicating that surface staining for FcϵRIα protein was also low (net mean fluorescence

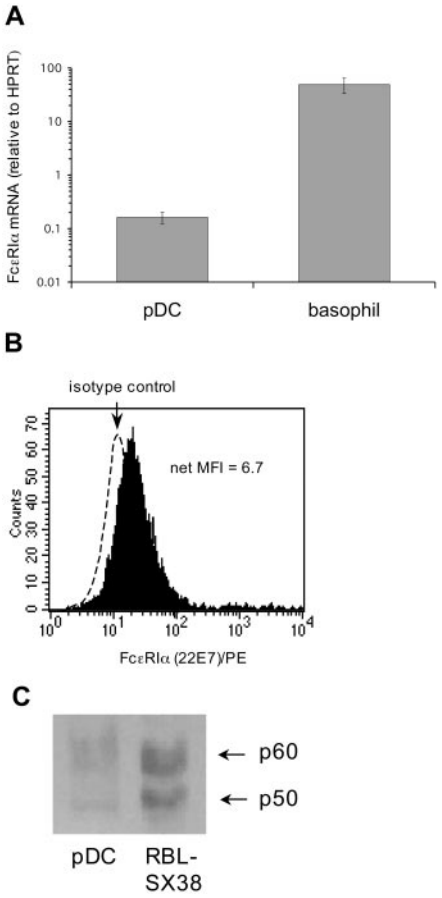

FIGURE 1. FcεRIα expression in pDC. *A,* Quantitative analysis of α subunit mRNA levels in pDC ($n = 11$) vs basophils ($n = 5$) using real-time RT-PCR. Shown are the values (average ± SEM) after normalizing to the housekeeping gene *HPRT* using the equation, $1/1.85^{\Delta ct}$. *B,* Surface expression of the α subunit on pDC using flow cytometry. *C,* Western blot analysis for p50 and p60 forms of FcεRIα protein in pDC lysate and comparison with lysate from rat basophilic leukemia (RBL)-SX38 cells transfected with human FcεRI. Representative experiments shown for *B* and *C*.

intensity (nMFI) = 6.7), yet consistent with values recently reported for these DC (3). Additional analysis of whole cell lysates by immunoblotting identified the presence of both the p60 and p50 variants of FcεRIα (Fig. 1*C*), indicating that pDC, much like basophils and mast cells, express both the mature and immature forms of this receptor subunit (12).

Functional and phenotypic changes in pDC following IgE cross-linking

As shown in Fig. 2*A*, pDC secreted IL-10 and TNF-α when cultured 24 h in IL-3 alone, and the simultaneous addition of anti-IgE significantly induced more of each cytokine. Although this was less evident for TNF-α than for IL-10, neither condition produced detectable levels of IL-12. We further investigated these responses in the absence of IL-3 and using multiple concentrations of anti-IgE. Dose responses were detected after a 24-h incubation, peaking at 3–5 μg/ml for each cytokine, including IL-6, another proinflammatory cytokine secreted by pDC (Fig. 2, *B–D*).

Preliminary array data indicated, however, that IgE cross-linking might have an opposite effect on other cytokines found in these cells, since several constitutively expressed genes appeared downregulated (data not shown). In particular, we investigated whether IFN-α secretion was affected following IgE-dependent activation. As shown in Fig. 3*A*, pDC secreted low-level IFN-α (712 ± 375 pg/10^6 cells) after a 3-day culture in IL-3 (10 ng/ml) alone. Yet, a dose-dependent inhibition of this response occurred with the addition of anti-IgE, which caused nearly 80% inhibition in the production of this type I IFN when used at 5 μg/ml. This inhibition was not observed in cultures receiving an identical concentration of goat isotype control. Moreover, a similar pattern of inhibition was observed when pDC isolated either from allergic or nonallergic subjects were pretreated with anti-IgE for 24 h and subsequently stimulated for 18 h with the type A oligodeoxynucleotide ODN-2216 (Fig. 3*B*). As expected, notable induction of IFN-α occurred with ODN-2216 activation, with levels averaging 107 ± 22 and 97 ± 13 ng/10^6 cells (allergic and nonallergic, respectively). However, these responses were inhibited following pretreatment with anti-IgE (5 μg/ml), and significantly ($p = 0.04$)

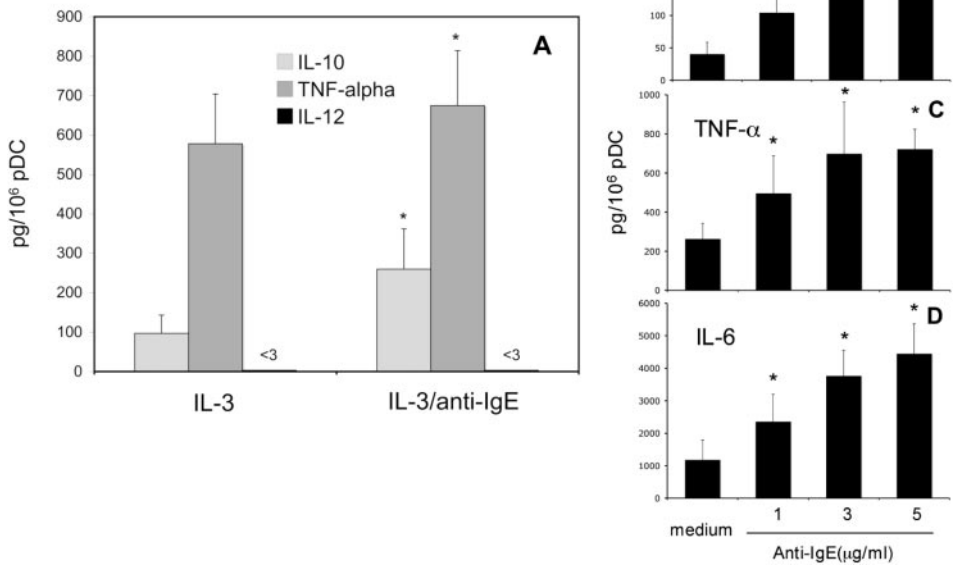

FIGURE 2. Cytokine secretion by pDC following IgE cross-linking. *A,* pDC isolated from blood were cultured in medium containing IL-3 (10 ng/ml) alone and with goat anti-human IgE (5 μg/ml). Supernatants were harvested (after 24 h) and analyzed for IL-10, TNF-α, and IL-12 protein by ELISA. Shown are the average ± SEM values ($n = 10$) using cells from different subjects. *, $p < 0.05$ vs IL-3 control. *B–D*, Anti-IgE dose responses in the absence of IL-3 are shown for pDC secretion of IL-10 ($n = 5$), TNF-α ($n = 8$); and IL-6 ($n = 6$), respectively. Average ± SEM. *, $p < 0.05$ vs medium control.

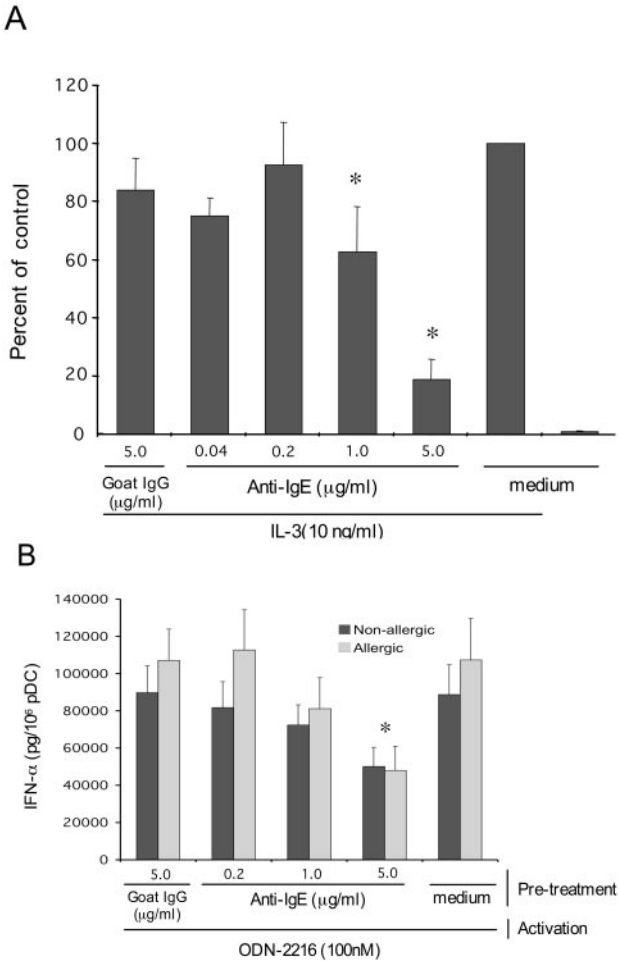

FIGURE 3. IgE cross-linking dose-dependently inhibits IFN-α secreted by pDC. *A,* Isolated pDC were cultured for 3 days with medium alone or with IL-3 and the indicated concentrations of anti-IgE or goat IgG control. Cell-free supernatants were then analyzed for IFN-α protein by ELISA. Values are the average $\pm$ SEM percentage of control with IL-3 alone, which was 712 $\pm$ 375 pg/10^6 cells, $n = 9$. $*$, $p < 0.05$. *B,* pDC from both allergic ($n = 5$) and nonallergic ($n = 8$) subjects were pretreated for 24 h with IL-3 alone or with the indicated concentrations of anti-IgE or goat IgG. ODN-2216 (100 nM) was then added as indicated for another 18-h incubation. Supernatants were analyzed for IFN-α by ELISA. Levels are the average $\pm$ SEM. $*$, $p < 0.05$.

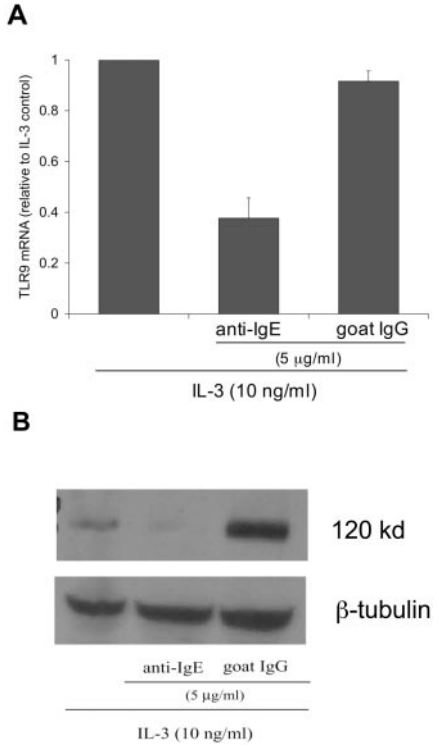

FIGURE 4. IgE cross-linking down-regulates TLR9 expression in pDC. *A,* Isolated pDC were cultured for 18 h as indicated. mRNA levels for TLR9 were then determined by real-time RT-PCR. Shown are values normalized to the housekeeping gene *HPRT* and reported as a fraction (average $\pm$ SEM, $n = 6$) of the IL-3 control. $*$, $p = 0.02$. *B*, Western blot for TLR9 protein in pDC lysates prepared after 72-h incubation in the conditions indicated (representative of three experiments with similar results).

more so for the allergic group (63 $\pm$ 7%, $n = 5$) compared with the nonallergic group (38 $\pm$ 10%, $n = 8$).

We next investigated whether IgE cross-linking induces phenotypic changes in pDC, focusing on TLR9 expression. Fig. 4*A* shows that pDC treated for 18 h with anti-IgE expressed ~60% less TLR9 mRNA than did cells treated with a goat isotype control. Although no consistent changes in TLR9 protein were detected after a 24-h incubation to cross-linking conditions (data not shown), expression of this receptor was markedly reduced after a 72-h incubation (Fig. 4*B*). In contrast to TLR9, no significant changes in the expression of CD80 and CD86 were detected in pDC treated for 24 or 72 h with anti-IgE (data not shown), findings that are consistent with the observations previously reported by Novak et al. (2).

CpG-ODN reduces FcεRIα expression in pDC

Since IgE receptor cross-linking on pDC attenuated TLR9 expression as well as responsiveness to CpG, we then addressed whether cells first exposed to ODN-2216 might conversely show reduced FcεRIα. We began by quantitatively measuring mRNA for this receptor subunit in cells treated for various periods of time with ODN-2216. Fig. 5*A* shows results from a representative experiment where ODN-2216 were added to pDC cultures at the indicated times remaining in an 18-h incubation. Those cultures treated for the full 18 h showed ~15-fold less FcεRIα mRNA than cells receiving the ODN during the last hour of incubation and nearly 10-fold less than those treated for 4 h. We consequently chose 18 h as a preincubation time to conduct additional experiments. Fig. 5*B* shows that ODN-2216 consistently reduced FcεRIα mRNA in pDC, not only causing a ~10-fold reduction in mRNA levels compared with cells cultured for 18 h in IL-3 alone ($p = 0.008$), but also significantly reduced α subunit mRNA expression relative to cells pretreated with its GpC control ODN-2243 ($p = 0.03$). As an aside, it is also important to note that ODN-2216 pretreatment did not affect the levels of FcεRIα mRNA detected in basophils, implying that the ODN-2216 itself was not simply interfering with the primer/probe combinations used in the real-time RT-PCR assay (data not shown).

Down-regulation of FcεRIα mRNA in pDC treated with ODN-2216 prompted us to investigate whether protein expression for this receptor subunit is similarly reduced. As shown in Fig. 5*C*, low-level staining for α protein, as assessed by flow cytometry, was evident on pDC after 18 h in IL-3 alone, with nMFI averaging 4.9 $\pm$ 0.5. However, in cultures also receiving ODN-2216, a significant decrease ($p = 0.01$, $n = 8$) in α expression was observed, with levels (nMFI = 1.2 $\pm$ 0.4) approaching background staining. This decrease in surface FcεRIα following ODN-2216 also proved significant when compared with levels detected on pDC treated with the ODN-2243 control ($p = 0.03$), even though this GpC

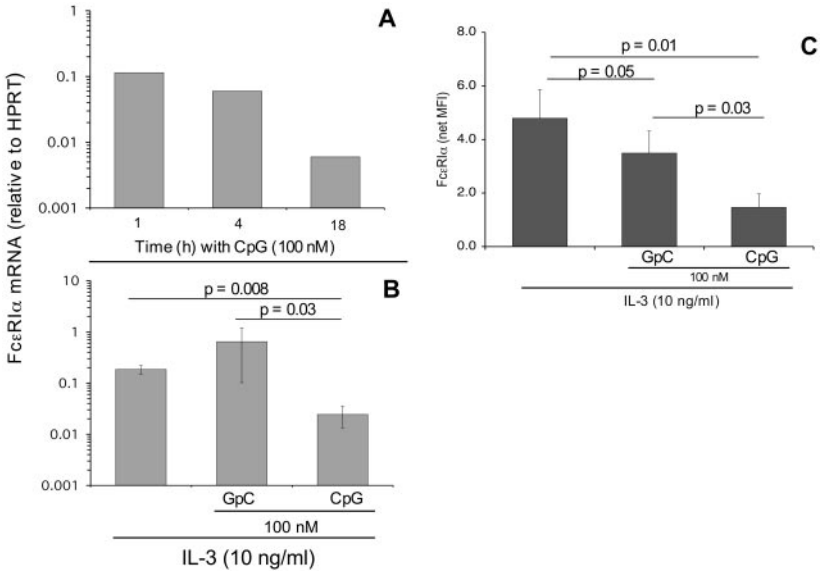

FIGURE 5. Treatment with CpG-ODN reduces FcεRIα expression in pDC. *A,* Representative time course for the effect of CpG-ODN on FcεRIα mRNA. ODN-2216 (100 nM) was added at the indicated times remaining during an 18-h incubation. RNA was isolated and analyzed for α subunit mRNA by real-time RT-PCR and calculated as described in Fig. 1 legend. *B,* pDC were treated as indicated for 18 h before quantifying FcεRIα mRNA by RT-PCR. Values (average ± SEM, $n = 9$) were also calculated as described in Fig. 1 legend. *C,* Flow cytometric analysis of cell surface FcεRIα on pDC treated for 18 h as indicated. Shown are nMFI values (average ± SEM, $n = 9$).

ODN significantly reduced ($p = 0.05$) surface FcεRIα compared with cells cultured in IL-3 alone.

Reduction in FcεRIα mediated by CpG is not reversed by adding IgE

We then questioned whether the addition of IgE would prevent ODN-2216 from down-regulating FcεRIα expression in pDC. The results in Fig. 6*A* indicate that the averaged levels of FcεRIα mRNA (relative to HPRT) in control cultures receiving IgE were ~10-fold greater compared with all previous measurements looking at RNA isolated from cells cultured in IL-3 alone (see Figs. 1 and 5). Despite this apparent up-regulation in the α subunit mRNA occurring with IgE, cells cultured under the same conditions along with ODN-2216 averaged ~15-fold less mRNA for comparison ($p = 0.04$). These findings were further substantiated by experiments investigating whether the CpG-ODN affected FcεRIα expression as assessed by Western blotting. In a representative image shown in Fig. 6*B*, a prominent band for the p60 variant of FcεRIα was detected in pDC cultured for 3 days in medium containing IL-3 (10 ng/ml) and IgE (500 ng/ml). Although little change was observed in the same number of cells also receiving GpC, cells cultured with CpG ODN-2216 showed qualitatively less p60 variant. Densitometry indicated a modest 30–40% reduction, which was consistently seen in three experiments (Fig. 6*C*).

Since type A ODN, such as ODN-2216, are potent activators of type I IFN secretion by pDC, we addressed whether IFN-α and/or IFN-β, when added exogenously, might have a similar inhibitory effect on FcεRIα expression. Because these cells also secreted IL-10 in response to IL-3 alone (see Fig. 2), we also investigated whether this cytokine mediated similar effects. As shown in Fig. 7, levels of FcεRIα mRNA were significantly ($p < 0.05$) reduced ~4-fold in pDC incubated for 18 h with IFN-α (100 U/ml), albeit this effect was considerably less than the ~29-fold reduction observed using ODN-2216. Although cells treated with IFN-β also showed an ~2-fold decrease in α subunit mRNA, the same was not observed for IL-10, which variably affected expression of this receptor subunit.

CpG-ODN inhibits T cell proliferation induced by allergen but not by tetanus toxoid (TT)

Although the exact biological role(s) for FcεRI expression on DC (particularly pDC) remains poorly understood, there is mounting evidence suggesting that it facilitates Ag processing or focusing (4). Therefore, a final series of experiments tested whether ODN-2216 affects allergen-induced proliferation with dust mite (*D. farinae*) compared with an irrelevant Ag, TT. Fig. 8*A* shows a representative experiment in which ODN-2216, when added along with allergen to PBMCs, inhibited the proliferation response induced by

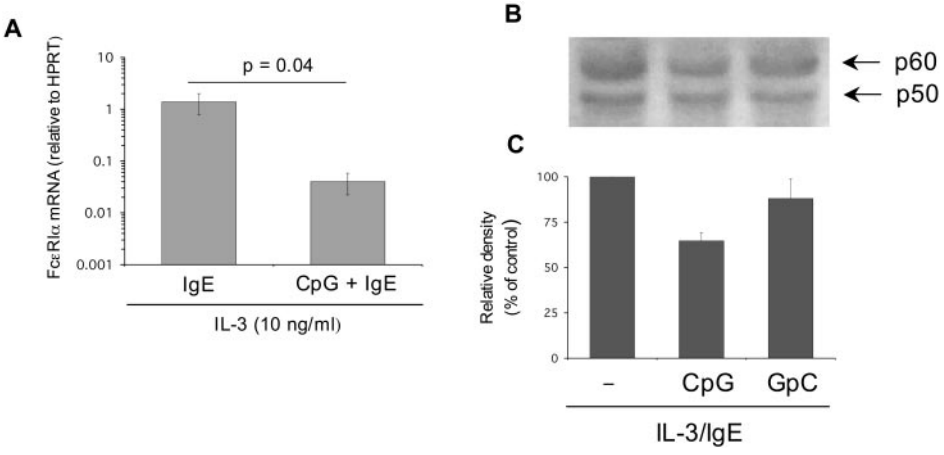

FIGURE 6. IgE added to pDC cultures does not prevent reductions in FcεRIα mediated by CpG. *A,* Real-time RT-PCR analysis of FcεRIα in pDC treated for 18 h as indicated. IgE was 500 ng/ml; ODN-2216 was 100 nM. Shown are the average ± SEM ($n = 5$) values after normalizing to HPRT, as described in Fig. 1 legend. *B*, Western blot analysis showing FcεRIα (p60/p50) in lysates of pDC cultured for 3 days as indicated. *C,* Densitometry (average ± SEM; $n = 3$) of the changes occurring in the p60 α subunit, normalized as a percentage of that seen for cells not treated with ODN.

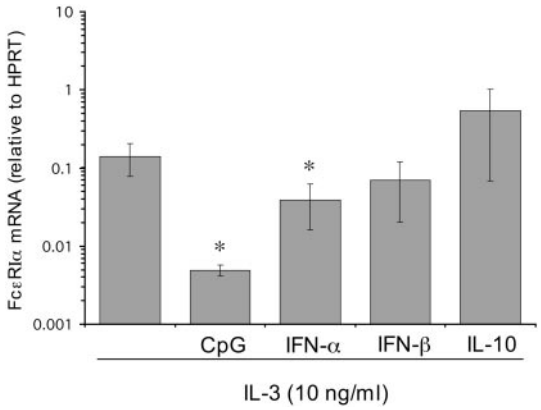

FIGURE 7. Effects of IFN-α, IFN-β, and IL-10 on FcϵRIα expression. pDC were cultured with IFN-α (100 U/ml), IFN-β (100 U/ml), IL-10 (10 ng/ml), or ODN-2216 (100 nM) as indicated in the presence of IL-3 (10 ng/ml). After an 18-h incubation, RNA was analyzed for FcϵRIα expression by real-time RT-PCR. Shown are the average $\pm$ SEM; $n = 5$ values after normalizing to HPRT as described in Fig. 1 legend. $*$, $p < 0.05$.

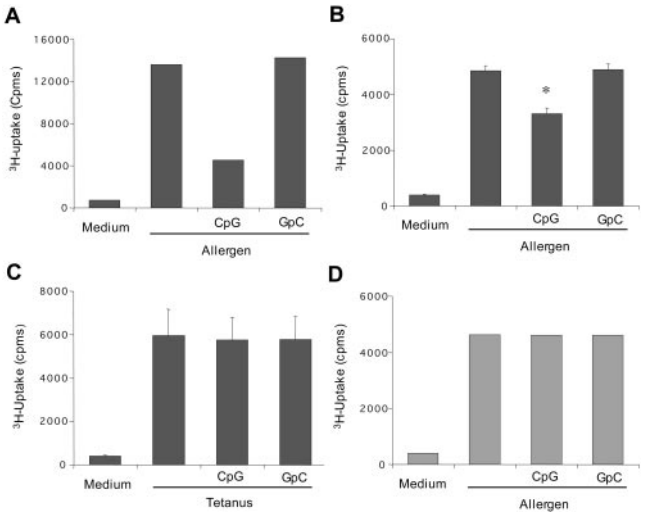

FIGURE 8. CpG inhibits T cell proliferation induced by allergen (*D. farinae*) but not by tetanus. *A,* PBMC were cultured in medium alone or with allergen in the absence and presence of ODN-2216 (CpG) or ODN-2243 (GpC), both at 200 nM. Tritiated thymidine was added for 16 h after a 5-day culture. cpm are shown for a representative experiment. *B* and *C,* $CD4^+$ T cells and pDC (8:1 ratio) were cultured with allergen or with TT with and without ODN as described in *A*. Average $\pm$ SEM cpm are shown ($n = 3$). $*$, $p < 0.05$, *t* test. *D,* $CD4^+$ T cells were cocultured with $CD14^+$ monocytes and allergen as indicated. Results are from a representative experiment.

the allergen alone by nearly 70%. Importantly, this inhibition was specific for this CpG ODN, since its GpC control (ODN-2243) had no effect on allergen-induced proliferation. The specificity for this inhibition was further investigated using cells purified from blood, where pDC and $CD4^+$ T cells added back with one another at a 1:8 ratio (pDC:CD4) showed approximately one-fourth the response to allergen compared with the PBMC cultures. Nonetheless, the addition of CpG consistently inhibited by 30 $\pm$ 5% the proliferation mediated by allergen, while no effect on this response was observed using GpC (Fig. 8*B*). Most importantly, this inhibition mediated by CpG was specific for allergen-induced responses, as proliferation induced by tetanus was unaffected by either CpG or GpC (Fig. 8*C*). Finally, Fig. 8*D* shows that CpG did not affect allergen-induced proliferation when $CD14^+$ monocytes were used as APCs, suggesting that this inhibition is dependent on APCs expressing TLR9, the receptor for CpG.

Discussion

There is a body of evidence suggesting that in humans pDC play a more significant role in inducing Th2 as opposed to Th1 responses. Moreover, these DC have recently been identified in allergic lesions in the nose (16) and are found at higher frequencies in certain allergic conditions (17). In fact, pDC are often referred to as DC2 because of the initial work linking them to the induction of Th2 responses in vitro (18). This concept is further supported by the recent identification that these cells, like many DC in the human system, express IgE receptors that are implicated in capturing allergen for presentation to T cells (2, 3). Despite these findings, there are difficulties in fully adopting this line of thought when it is well known that pDC are an extremely rich source of type I IFN (i.e., IFN-α and IFN-β), which are recognized for their ability to prime for Th1-like responses. Additionally, pDC are currently thought to be the only class of DC in humans that express TLR9, the innate immune receptor responsible for binding CpG-ODN that is so potent at inducing type I IFN production in these cells.

It seemed unusual that pDC coexpress receptors (i.e., TLR9 and FcϵRI) with such opposing functions. This dichotomy therefore prompted the hypothesis that a level of counterregulation occurs between TLR9 and FcϵRI in pDC, with activation of either possibly affecting the expression of the other and its associated functional responses. As a result, we found evidence that IgE cross-linking on pDC markedly reduced TLR9 expression and was correlated with a reduction in IFN-α secreted in response to CpG-ODN. In contrast, TLR9 ligation with CpG-ODN markedly reduced the expression of FcϵRIα in pDC and in theory reduced its ability to bind IgE for subsequent focusing of allergen to T cells. This latter notion is supported by a selective inhibition of allergen-induced T cell proliferation. Overall, these results implicate a unique mechanism by which IgE-dependent adaptive immune responses and innate immunity oppose one another at the DC level by inversely modulating receptor expression and function.

In demonstrating the expression of FcϵRIα on pDC, our results confirm those recently published by other investigators (2, 3). However, we provide additional information worth noting. First, by using a protocol involving double-Percoll density centrifugation combined with positive selection, we essentially eliminate the possibility that the FcϵRIα detected in our pDC suspensions was derived from contaminating basophils. This is particularly relevant since the levels of FcϵRIα mRNA were 80- to 300-fold less in pDC as compared with basophils isolated from the same subjects (Fig. 1*A*). In other words, a seemingly insignificant contamination of just 0.5% basophils could have essentially doubled the levels of FcϵRIα mRNA detected, and most likely would have affected any interpretations of the data obtained. A second important observation pertains to the α subunit itself, in that both the p60 and p50 variants were detected by Western blot analysis of pDC lysates (Figs. 1*C* and 6*B*). Both molecular species have been identified in basophils, with the belief that the p60 variant is a mature form of the α subunit that is expressed on the cell surface and is responsible for binding IgE (12). In contrast, the immature cytoplasmic p50 variant is not expressed on the plasma membrane and cells expressing this form of the receptor (e.g., eosinophils) do not generally bind IgE (19, 20). Therefore, the detection of the p60 variant in pDC only adds to the notion that these DC, like basophils (and mast cells), actually bind IgE that is functionally relevant.

Of equal importance, we also observed through indirect evidence that IgE added exogenously up-regulated FcϵRIα mRNA

expression on pDC (Fig. 1*A* vs 6*A*). Although additional studies are needed to more definitively prove this claim, it could have significant implications in how these cells (and possibly other DC) regulate IgE receptor expression compared with basophils (and mast cells). On basophils, IgE is thought to regulate cell surface expression of FcϵRI only by regulating the rate that the receptor is lost from the cell surface (21). In pDC, this Ig may additionally regulate receptor expression by actually inducing transcription of the α subunit.

Since FcϵRI has only recently been identified on pDC, less is known regarding the functional consequences resulting in these cells following cross-linking of this receptor. Using intracellular detection by flow cytometry, Novak et al. (2) recently reported IL-10 in pDC activated with anti-IgE. In contrast, this same mode of stimulation resulted in fewer cells staining for type I IFNs (2). We confirmed these findings here, measuring secreted IL-10 and IFN-α by ELISA, and additionally showed that IgE cross-linking, while augmenting secretion of proinflammatory cytokines (i.e., TNF-α and IL-6), dose-dependently inhibited the secretion of IFN-α by pDC isolated either from allergic or nonallergic subjects (Fig. 3). This was true both for the low-level IFN-α secreted during a 3-day culture of pDC incubated in IL-3 alone as well as the large quantities produced in response to ODN-2216. Perhaps more importantly, we provide in this study the first evidence that TLR9, which is so prominently expressed by pDC and is the receptor for CpG-DNA, is markedly down-regulated under IgE/FcϵRI cross-linking conditions. As such, this finding may very well account for the reduced responsiveness to CpG seen in pDC pretreated with anti-IgE. Interestingly, neither the expression of CD80 or CD86 on pDC was similarly affected under the same conditions, indicating that these cells retained the capacity to activate T cells.

Naturally, these phenotypic and functional responses resulting from IgE cross-linking on pDC are consistent with allergen favoring Th2 responses. However, our findings challenge the notion of allergen interacting with DC-bound IgE solely as a means for its capture and presentation to T cells. By down-regulating TLR9, the ensuing FcϵRI activation resulting from this interaction conceivably limits the pDC from providing a cytokine environment that normally favors Th1 development. Moreover, any microbial DNA that would normally favor such an environment by inducing type I IFN is rendered less likely to do so as a result of reduced TLR9 expression.

A very different outcome occurred when pDC were first exposed to CpG-ODN. Quantitative RT-PCR data indicated significant reductions of FcϵRIα in pDC treated with ODN-2216 compared with cells treated in IL-3 alone. Whether this attenuation occurred at the level of transcription or that posttranscriptional events were involved remains to be determined. Nonetheless, the inhibitory effect was less evident using a GpC-ODN control, thus satisfying the specificity requirements associated with TLR9-mediated signaling.

Although an expected diminution in surface staining for FcϵRIα protein accompanied the reduction in RNA, the change was less apparent, particularly when assessed by Western blot (Fig. 6*B*). As noted above, this finding could relate to evidence that turnover of the α subunit protein is slow to occur, particularly in vitro (22) and when occupied with IgE, a situation that is thought to provide stabilizing effects on receptor expression (21, 23). Greater time following CpG treatment may be necessary before large reductions in protein are observed.

Cellular expression of FcϵRI is also "driven" by IgE serum levels (24). This is particularly true for basophils and mast cells, but is more recently evident for DC possessing the $\alpha\gamma_2$ variant of the receptor, including pDC (3). As such, with the noted exceptions (Figs. 3 and 8), the pDC used in these experiments were primarily procured from nonatopic subjects. This could explain the relatively low levels of surface FcϵRIα detected on the pDC used in this study. Nonetheless, these levels were consistent with those reported by Novak et al. (2), who found ~3-fold less α subunit on pDC from normal vs cells from atopic dermatitis patients. Although we did not address whether CpG-ODN similarly reduces the higher levels of FcϵRIα expected using pDC of atopic subjects, our findings do indicate that the inhibitory effect of CpG occurs even in the presence of IgE (Fig. 6). These latter observations are of therapeutic relevance, suggesting that the inhibitory effect mediated by CpG on the expression of FcϵRIα is still evident under the high IgE serum conditions that characterize allergic conditions.

Presently, it is difficult to make any substantial conclusions regarding the exact mechanisms responsible for the reduction of FcϵRIα in pDC mediated by CpG-ODN. However, it should be emphasized that type I IFN could play a role in this effect. When added exogenously, IFN-α (100 U/ml) caused a modest but significant 4-fold reduction in the mRNA expressed by pDC. Therefore, it seems possible that the synthesis of type I IFN, which rapidly occurs in pDC treated with CpG-ODN, may function in an autocrine fashion to down-regulate FcϵRI in these cells. In fact, it is generally accepted that type A CpG like ODN-2216 cause pDC to acquire a more mature phenotype. Perhaps this proposed autocrine effect mediated by type I IFN plays an important role in this maturation, making pDC more conducive for Th1 responses by reducing FcϵRI expression, which would normally help facilitate allergen recognition. Although this hypothesis requires more study, it is consistent with the Th1-like promoting capabilities associated with IFN-α. It is also intriguing to think that the clinical efficacy (and switching of Th2 to Th1 immune responses) recently observed in steroid-resistant asthma subjects treated with therapeutic IFN-α (25) may be partially attributed to reduced FcϵRI expression on pDC (and perhaps other DC).

Under the notion that IgE/receptor expression by DC plays an important role in allergen presentation to responsive $CD4^+$ T cells, we asked whether CpG would specifically inhibit allergen-induced T cell proliferation. In fact, this is exactly what was observed and confirmed the findings recently reported by Farkas et al. (26), who showed that ODN-2216 significantly reduced the percentage of $CD4^+$ T cells proliferating in response to timothy grass allergen. However, in keeping with the belief that this inhibition was a result of reduced FcϵRIα expression, we additionally showed that CpG only affected the proliferation induced by specific allergen and that responses induced by an irrelevant Ag (i.e., tetanus) were not affected (Fig. 8). It is also important to note that the percent inhibition in allergen-induced proliferation (~35%), although modest, was identical to the percent reduction in FcϵRIα observed by Western blot analysis (Fig. 6, *B* and *C*). Although these observations are only correlative and do not necessarily prove that the reduction in FcϵRIα directly accounted for reduced allergen-induced proliferation, the fact that the tetanus response was unaffected by CpG is a good indication that other accessory molecules important for Ag presentation remained intact. Indeed, many studies have shown that CpG-ODN actually increases the expression of HLA-DR, CD80, and CD86 on pDC, and all are well known for promoting the stimulatory capacity of many professional APC (27–30). This partially explains the rationale for using CpG as an adjuvant for boosting immunity. Therefore, it seems reasonable to suggest that the inhibitory effect CpG had on allergen-induced proliferation was mediated by the down-regulation in FcϵRIα expression, thus reducing (but not completely inhibiting) the capacity of pDC to present allergen to responsive $CD4^+$ T cells.

Finally, there is currently great interest in the use of CpG-DNA-based vaccines for treating allergic conditions, including seasonal

rhinitis and allergic asthma (31). The rationale for this comes from mounting in vivo and in vitro evidence that CpG-ODN, also known as immunostimulatory sequences, favors Th1-like responses that potentially counteract the Th2-like responses that are hallmark in allergic disease. Based on the work presented here, it seems appropriate to suggest that therapeutic CpG could work by affecting both the efficiency and the cytokine environment by which pDC present allergen to T cells.

In conclusion, these results suggest a level of counterregulation between FcεRI and TLR9 molecules expressed on pDC such that ligation of either attenuates the expression and function of the other. This facet of pDC biology (and perhaps that of other DC) should provide not only a better understanding of how adaptive and innate immune responses interact, but should also provide insight underlying the therapeutic efficacy of CpG-DNA-based vaccines in allergic disease.

Disclosures

The authors have no financial conflict of interest.

References

1. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.-J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18: 767–811.
2. Novak, N., J. Alllam, T. Hagemann, C. Jenneck, S. Laffer, R. Valenta, J. Kochan, and T. Bieber. 2004. Characterization of FcεRI-bearing CD123 blood dendritic cell antigen-2 plasmacytoid dendritic cells in atopic dermatitis. *J. Allergy Clin. Immunol.* 114: 364–370.
3. Foster, B., D. Metcalfe, and C. Prussin. 2003. Human dendritic cell 1 and dendritic cell 2 subsets express FcεRI: correlation with serum IgE and allergic asthma. *J. Allergy Clin. Immunol.* 112: 1132–1138.
4. Novak, N., S. Kraft, and T. Bieber. 2003. Unraveling the mission of FcεRI on antigen-presenting cells. *J. Allergy Clin. Immunol.* 111: 38–44.
5. Janeway, C., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20: 197–216.
6. Horner, A., J. Van Uden, J. Zubeldia, D. Broide, and E. Raz. 2001. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol. Rev.* 179: 102–118.
7. Krieg, A. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20: 709–760.
8. Ishizaka, K., T. Ishizaka, and E. H. Lee. 1970. Biologic function of the Fc fragments of E myeloma protein. *Immunochemistry* 7: 687–702.
9. Adkinson, N. F., Jr. 1980. *Measurement of total Serum Immunoglobulin E and Allergen-Specific Immunoglobulin E Antibody.* American Society of Microbiology, Washington, DC, pp. 800.
10. Schroeder, J. T., D. W. MacGlashan, A. Kagey-Sobotka, J. M. White, and L. M. Lichtenstein. 1994. IgE-dependent IL-4 secretion by human basophils: the relationship between cytokine production and histamine release in mixed leukocyte cultures. *J. Immunol.* 153: 1808–1818.
11. Gilbert, H. S., and L. Ornstein. 1975. Basophil counting with a new staining method using Alcian blue. *Blood* 46: 279–286.
12. Saini, S., J. Richerson, C. Wofsy, S. Lavens-Phillips, B. Bochner, and D. W. MacGlashan. 2001. Expression and modulation of FcεRIα and FcεRIβ in human blood basophils. *J. Allergy Clin. Immunol.* 107: 832–841.
13. Zarember, K., and P. Godowski. 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol.* 168: 554–561.
14. Sin, A., E. Roche, A. Togias, L.M. Lichtenstein, and J. T. Schroeder. 2001. Nerve growth factor or IL-3 induces more IL-13 production from basophils of allergic subjects than from basophils of nonallergic subjects. *J. Allergy Clin. Immunol.* 108: 387–393.
15. Schroeder, J. T., K. Miura, H.-H. Kim, A. Sin, A. Cianferoni, and V. Casolaro. 2002. Selective expression of nuclear factor of activated T cells 2/c1 in human basophils: evidence for involvement in IgE-mediated IL-4 generation. *J. Allergy Clin. Immunol.* 109: 507–513.
16. Jahnsen, F., F. Lund-Johansen, J. Dunne, L. Farkas, R. Haye, and P. Brandtzaeg. 2000. Experimentally induced recruitment of plasmacytoid ($CD123^{high}$) dendritic cells in human nasal allergy. *J. Immunol.* 165: 4062–4068.
17. Hashizume, H., T. Horibe, H. Yagi, N. Seo, and M. Takigawa. 2005. Compartmental imbalance and aberrant immune function of blood $CD123^+$ (plasmacytoid) and $CD11c^+$ (myeloid) dendritic cells in atopic dermatitis. *J. Immunol.* 174: 2396–2403.
18. Rissoan, M., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt, and Y.-J. Liu. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283: 1183–1186.
19. Saini, S., A. Klion, S. Holland, R. Hamilton, B. Bochner, and D. W. MacGlashan. 2000. The relationship between serum IgE and surface levels of FcεR on human leukocytes in various diseases: correlation of expression with FcεRI on basophils but not on monocytes or eosinophils. *J. Allergy Clin. Immunol.* 106: 514–520.
20. Seminario, M., S. Saini, D. W. MacGlashan, and B. Bochner. 1999. Intracellular expression and release of FcεRIα by human eosinophils. *J. Immunol.* 162: 6893–6900.
21. MacGlashan, D. W., H. Xia, L. Schwartz, and J. Gong. 2001. IgE-regulated loss, not IgE-regulated synthesis, controls expression of Fcε in human basophils. *J. Leukocyte Biol.* 70: 207–218.
22. MacGlashan, D. W., J. MacKenzie-White, K. Chichester, B. Bochner, F. Davis, J. T. Schroeder, and L. M. Lichtenstein. 1998. In vitro regulation of FcεRIα expression on human basophils by IgE antibody. *Blood* 91: 1633–1643.
23. Saini, S., and D. W. MacGlashan. 2002. How IgE upregulates the allergic response. *Cur. Opin. Immunol.* 14: 694–697.
24. Malveaux, F. J., M. C. Conroy, N. F. Adkinson, and L. M. Lichtenstein. 1978. IgE receptors on human basophils: relationship to serum IgE concentration. *J. Clin. Invest.* 62: 176–181.
25. Simon, H.-U., H. Seelbach, R. Ehmann, and M. Schmitz. 2003. Clinical and immunological effects of low-dose IFN-α treatment in patients with corticosteroid-resistant asthma. *Allergy* 58: 1250–1255.
26. Farkas, L., E. Kvale, F. Johansen, F. Jahnsen, and F. Lund-Johansen. 2004. Plasmacytoid dendritic cells activate allergen-specific Th2 memory cells: modulation by CpG oligodeoxynucleotides. *J. Allergy Clin. Immunol.* 114: 436–443.
27. Hartmann, G., G. Weiner, and A. Krieg. 1999. CpG-DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* 96: 9305–9310.
28. Kadowaki, N., S. Antonenko, and Y.-J. Liu. 2001. Distinct CpG DNA and polyinosic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c-type 2 dendritic cell precursors and $CD11c^+$ dendritic cells to produce type I IFN. *J. Immunol.* 166: 2291–2295.
29. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z. K. Ballas, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-α in plasmacytoid dendritic cells. *Eur. J. Immunol.* 31: 2154–2163.
30. Kadowaki, N., S. Ho, S. Antonenko, R. Malefyt, R. Kastelein, F. Bazan, and Y.-J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194: 863–869.
31. Creticos, P., Y.-H. Chen, and J. Schroeder. 2004. New approaches in immunotherapy: allergen vaccination with immunostimulatory DNA. *Immunol. Allergy Clin. North Am.* 24: 569–581.