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Antigen-Conjugated Human IgE Induces Antigen-Specific T Cell Tolerance in a Humanized Mouse Model

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Dendritic cells (DCs) play an important role in immune homeostasis through their ability to present Ags at steady state and mediate T cell tolerance. This characteristic renders DCs an attractive therapeutic target for the induction of tolerance against auto-antigens or allergens. Accordingly, Ag-conjugated DC–specific Abs have been proposed to be an excellent vehicle to deliver Ags to DCs for presentation and tolerance induction. However, this approach requires laborious reagent generation procedures and entails unpredictable side effects resulting from Ab-induced crosslinking of DC surface molecules. In this study, we examined whether IgE, a high-affinity, non–cross-linking natural ligand of FcεRI, could be used to target Ags to DCs and to induce Ag-specific T cell tolerance. We found that Ag-conjugated human IgE Fc domain (Fcε) effectively delivered Ags to DCs and enhanced Ag presentation by 1000- to 2500-fold in human FcεRI-transgenic mice. Importantly, this presentation resulted in a systemic deletion of Ag-specific T cells and prevented these mice from developing delayed-type hypersensitivity, which is critically dependent on Ag-specific T cell immunity. Thus, targeting FcεRI on DCs via Ag-Fcε fusion protein may serve an alternative method to induce Ag-specific T cell tolerance in humans. The Journal of Immunology, 2014, 192: 3280–3288.

Because Abs are bivalent, their binding to cells can cross-link cell surface molecules. Surface molecule cross-linking often triggers stimulatory signaling in cells, the outcome of which varies depending on cell type (16–19). Importantly, clinically development of human Abs is challenging, and it requires laborious manufacturing procedures, including the initial generation of mAbs in vivo, followed by extensive modifications of the Abs in vitro (20). Thus, there is a need for an alternative method to target DCs and for an animal model to better gauge its targeting efficacy in humans.

Targeting the high-affinity IgE receptor FcεRI with Ag-conjugated IgE could be a promising alternative method. Whereas FcεRI is expressed only by mast cells and basophils in steady state mice, it is additionally expressed by DCs and monocyes in humans (21). The FcεRIα-chain, a member of the Ig superfamily, binds IgE Fc domain at a 1:1 ratio of receptor to IgE with unusually high affinity (Kd = \(10^{-10}\) M) (22). When IgE/FcεRI complexes are cross-linked by multivalent Ags, a strong inflammatory signal is transmitted to cells, resulting in degranulation of mast cells and basophils and Ag presentation and cytokine production in DCs and monocytes (21). When bound by IgE alone, however, FcεRI does not transmit signaling in general; however, certain IgE clones, namely cytokinergic IgE, appear to induce signaling through IgE Fv domain (23).

In this study, we examined whether the human IgE Fc domain could be used to induce Ag-specific tolerance in vivo by using the previously described hFcεRIα-transgenic (Tg) mice (24), which express human FcεRIα under the control of its own promoter. We demonstrate that Ag-Fce fusion proteins strongly bind both human DCs and hFcεRIα-Tg mouse DCs, that such Abs are presented to Ag-specific T cells with a 1000–2500 fold greater efficiency than unlinked Ag are, and that this presentation leads to Ag-specific T cell deletion and tolerance.

Materials and Methods

Mice and cell lines

hFcεRIα transgenic mice (24), SMARTA mice (25), Foxp3<sup>GFP</sup> mice (26), and U937 cells transfected with hFcεRI (27) have been described previously. The human IgE secreting myeloma cell line U266 was purchased.
from American Type Culture Collection. B6.SJL-PtprcaPepcb/Brej, OT-II, and OT-F1 mice were purchased from Jackson Laboratory. All mice were housed in the University of California San Francisco (UCSF) animal facility, and all experiments and procedures were performed according to protocols approved by the UCSF Institutional Animal Ethics Committee.

**Human blood**

Healthy human blood was obtained through an ongoing study under the UCSF Institutional Review Board–approved protocol 10-02596 with informed consent.

**Abs**

Abs to human CD3 (HIT3a), CD19 (HIB19), CD56 (MEM188), CD14 (HCD14), HLA-DR (L243), BDCA1 (L161), CD123 (6H6), hFcRls (CRA-1), IgE (MHE-18), murine CD49b (Dx5), CD11b (M1/70), GR1 (RB6-8C5), Thy1.1 (HIS51), TCRV (CRA-1), and isotype control Ab. Cells were gated as described in (A) and flow cytometry as shown in Supplemental Fig. 1. Anti-hFcRI Ab staining is shown black, and isotype control Ab staining is shown gray. Data are representative of five independent experiments.

**Flow cytometry**

Human PBMCs were isolated from whole blood via density gradient centrifugation using Ficoll Plaque Plus (GE Healthcare) (28). Murine blood was collected via cardiac puncture and PBMCs were isolated. Cells were labeled with various cell surface Abs and acquired on a FACs LSRII system (BD Biosciences). Only live (propidium iodide–negative [BioLegend]), singlet cells were used for all data analysis. Murine cells were gated as follows: conventional DC (cDC), CD11c+MHCII+; plasmacytoid DC (PDC), BDCA1+ DCs, PDCs, basophils, and monocytes, but not BDCA3+ DCs, B cells, T cells, or NK cells. PBMCs were isolated and stained using cell-identification Abs (anti-CD11c, MHCII, Dx5, CD131, CD11b, GL1, and mPDCA1) and hFcRI or isotype control Ab. Cells were gated as described in Materials and Methods. Anti-FcRls Ab staining is shown black, and isotype control Ab staining is shown gray. Data are representative of five independent experiments.

**In vitro Ag presentation assay**

cDCs, PDCs, monocytes, basophils, and B cells were isolated from OVAp-Fc–injected hFcRls-Tg mice by FACS. Isolated cells (1 × 10^6 cells) were cultured with CTV (Invitrogen)–labeled CD4+ OT-II T cells at a 1:5 APC/T cell ratio in a 96-well plate for 3 d. CTV dilution was analyzed by flow cytometry. When T cell proliferation was measured using 3H-thymidine, isolated cells were cocultured with CD4+ OT-II T cells at a 1:10 APC/T cell ratio in a 96-well plate for 4 d. H3-thymidine was added for the last 15 h of the culture. As a positive control, CD4+ OT-II T cells were

**FIGURE 1.** FcRls is expressed on BDCA1+ DCs, PDCs, monocytes, and basophils among human PBMCs. (A) Identification of FcRls-expressing cells in human PBMCs by flow cytometry. PBMCs were isolated from healthy blood donors and stained with a panel of cell-identification Abs (anti-CD14, HLA-DR, BDCA1, CD123, CD3, CD19, and CD56) and FcRls or isotype control Ab. (B) FcRls expression in human blood BDCA1+ DCs, PDCs, basophils, and monocytes, but not BDCA3+ DCs, B cells, T cells, or NK cells. PBMCs were stained as described in (A) and gated by flow cytometry as shown in Supplemental Fig. 1. Anti-hFcRls Ab staining is shown black, and isotype control Ab staining is shown gray. Data are representative of three independent experiments with blood collected from multiple donors.

**FIGURE 2.** hFcRls-Tg mice show hFcRls expression pattern similar to those in humans. Expression of hFcRls in hFcRls-Tg mouse blood (A) and spleen (B). Blood leukocytes and splenocytes were isolated from hFcRls-Tg mice and stained using cell-identification Abs (anti-CD11c, MHCII, Dx5, CD131, CD11b, GL1, and mPDCA1) and hFcRls or isotype control Ab. Cells were gated as described in Materials and Methods. Anti-FcRls Ab staining is shown black, and isotype control Ab staining is shown gray. Data are representative of three independent experiments.
incubated with 10 μg/ml anti-CD3 and 20 μg/ml anti-CD28 Abs (BD Biosciences).

**In vivo Ag presentation assay**

Indicated mice were injected i.v. with 0.5–1 × 10⁶ CTV or CFSE-labeled Ag-specific CD4⁺ or CD8⁺ T cells. One day later, mice were injected i.v. with the indicated amounts of fusion proteins, OVA peptide (323–339; China peptides), or OVA protein (Fisher Scientific). Three, seven, or twelve days later, a single-cell suspension was prepared from spleen, lymph nodes, or blood, and stained for flow cytometry using fluorophore-labeled Abs directed against CD4, CD8, TCRβ, and Thy1.1, and analyzed by flow cytometry. GFP⁺CD4⁺Thy1.1⁺TCRβ⁺ cells were gated and cell numbers were determined.

**T cell restimulation assay and delayed-type hypersensitivity assay**

Naive CD4⁺ T cells (0.5–1 × 10⁶) from OT-II mice were injected into hFcreRα-Tg mice or Tg⁺ control mice. After 24 h, 0.5 μg OVA-Fc fusion proteins or PBS were injected. Seven days later, mice were injected s.c. with 30 μg OVA peptide (323–339) mixed with IFA (Sigma). An additional 7 d later, mice were examined for the development of OVA-specific T cell immunity by performing the T cell restimulation assay and delayed-type hypersensitivity (DTH) assay. For the T cell restimulation assay, 2 × 10⁵ splenocytes were isolated and incubated with or without 20 μg/ml OVA peptide (323–339) in a 96-well plate for 4 d. [³H]Thymidine (1 μCi) was added to each well for the last 15 h of incubation. [³H]Thymidine incorporated into cells was determined using Microbeta counter (1450 Microbeta Trilux; PerkinElmer). For DTH assay, mice were injected onto the right footpads with OVA peptide (323–339) mixed with IFA and onto the left footpads with IFA alone. Twenty-four hours later, thickness of each footpad was measured using a caliper rule.

**Results**

**FcreRI is expressed on BDCA1⁺ DCs, PDCs, monocytes, and basophils in human PBMCs**

To determine which cells express FcreRI in human blood, we performed flow cytometry on human PBMCs using an anti-FcreRI Ab and a panel of cell type-specific Abs. We found that FcreRI was expressed on a small fraction of PBMCs and that this fraction was composed of more than one cell population, as indicated by heterogeneous labeling by FcreRI and CD14 Abs (Fig. 1A, upper panel). Further analysis revealed that these FcreRI-expressing cells consisted of basophils, plasmacytoid DCs (PDCs), BDCA1⁺ DCs, and monocytes (Fig. 1A, lower panel). To confirm that these cell types indeed expressed FcreRI, we gated them independently of FcreRI expression using cell type–specific markers (Supplemental Fig. 1) and subsequently examined FcreRI expression. We found that BDCA1⁺ DCs, PDCs, and basophils all expressed FcreRI homogeneously, whereas monocytes appeared to express it heterogeneously (Fig. 1B). We also examined BDCA3⁺ DCs, T cells, B cells, and NK cells by gating them using specific markers (Supplemental Fig. 1). None of these cells expressed FcreRI at appreciable levels (Fig. 1B). Thus, FcreRI is specifically expressed in BDCA1⁺ DCs, PDCs, basophils, and a subpopulation of monocytes in human PBMCs.

**hFcreRα-Tg mice have a similar pattern of hFcreRI expression compared with humans**

To determine whether hFcreRα-Tg mice express hFcreRI in a similar fashion to humans, we performed flow cytometry of blood cells from hFcreRα-Tg mice using the same anti-human FcreRI Ab. We found

**FIGURE 3.** Generation of OvAP-Fce recombinant protein. (A) A schematic diagram of OvAP-Fce cDNA. The Vκ signal sequence cDNA is positioned at the 5’ terminal end of an extended sequence containing OVA peptide (323–339; OvAP) linked to the Cε2-Cε4 domains of hlgE with a 6×His tag cDNA at the 3’ terminal end. (B) A schematic diagram of OvAP-Fce recombinant protein. Disulfide bonds predicted to form intramolecularly and intermolecularly are indicated by † and |, respectively. (C-F) Generation and purification of OvAP-Fce. OvAP-Fce cDNA—shown in (A)—was transfected into 293T cells cultured in serum-free media. OvAP-Fce was purified via Ni-NTA column chromatography via Imidazole (Im) elution. Culture supernatant and the chromatography fractions were both analyzed with nonreducing SDS-PAGE (C) followed by immunoblotting (IB) (D and E). Culture supernatant was also analyzed with a reducing SDS-PAGE (F). Nonreducing and reducing SDS-PAGE was labeled as β-mercaptoethanol (β-ME) – and +, respectively. An asterisk and an arrowhead indicate the nonreduced and reduced OvAP-Fce, respectively. (G) Binding of OvAP-Fce to hFcreRI determined by flow cytometry. hFcreRI-transfected (left panel) and untransfected U937 cells (right panel) were incubated with 1 μg/ml OvAP-Fce at 37°C for 1 h, and stained with anti-hlgE Ab (black) or isotype control Ab (gray). Experiments shown in (C)–(G) were performed at least three times.
that basophils, monocytes, and PDCs expressed hFcRI; cDCs also expressed it, although levels were significantly lower than for other cell types (Fig. 2A). No FcεRI was found on lymphocytes, including B cells (Fig. 2A). A similar expression profile of hFcRI was found in the spleen (Fig. 2B). Thus, hFcεRIα-Tg mice express hFcεRI in cell types similar to humans, albeit with slightly different expression levels.

**Generation of OVAp-Fc recombinant protein**

Having determined that hFcεRIα-Tg mice express hFcεRI similarly to humans, we decided to use these mice to examine whether IgE could be exploited to target Ags to DCs for presentation in vivo. For this study, we generated an OVAp-Fc fusion protein. First, a cDNA was cloned encoding a V_{H} signal sequence linked sequentially to OVA peptide (323–339; OVAp), human IgE Cε2-Cε4 domains (Fce), and 6×His tag (Fig. 3A). In this study, the signal peptide was included to let the recombinant protein be secreted into the cell culture media. OVA peptide (323–339) was included as a model Ag, the presentation of which can be readily determined by using OT-II transgenic mice, which express OVA-specific TCRs. The 6×His tag was added to ease purification of recombinant protein via affinity chromatography. This cDNA construct was transfected into 293T cell lines cultured in protein-free media. Media was subsequently examined for the presence of the recombinant protein by using nonreducing SDS-PAGE followed by Western blot analysis using an anti-hIgE Ab and an anti-His Ab. A single protein band was detected at the m.w. of OVAp-Fc dimers (Fig. 3D, 3E, “Supermarket”), which was expected because IgE heavy chains dimerize via intermolecular disulfide bonds (Fig. 3B) (29). To confirm dimerization, we ran the same sample on a reducing SDS-PAGE. As predicted, the reduced protein band appeared at about half the m.w. of the nonreduced protein band (Fig. 3F). We enriched and purified the recombinant protein by collecting media and running it through a Ni-NTA column. We found that the anti-IgE and anti-His reactive band was eluted from the column at high purity (Figs. 3C–3E, “Eluates”).

Next, we determined whether OVAp-Fc specifically binds to hFcεRI. OVAp-Fc was incubated with U937 cells that were either untransfected or transfected with hFcεRI (27); flow cytometry was performed using an anti-hIgE Ab that specifically reacts with hIgE Fc domain. We found that anti-hIgE Ab stained hFcεRI-transfected U937 cells but not untransfected cells (Fig. 3G), indicating that OVAp-Fc specifically binds to FcεRI. We also determined whether OVAp-Fc cross-links FcεRI. OVAp-Fc was incubated with hFcεRIα-Tg mouse bone marrow–derived mast cells, and degranulation was quantified by hexosaminidase release assay (30). No hexosaminidase was released upon incubation with the construct (Supplemental Fig. 2), indicating that OVAp-Fc does not cross-link FcεRI.

**OVAp-Fc binds to human DCs in vitro and to DCs of hFcεRIα-Tg mice in vivo**

Next, we examined whether OVAp-Fc binds to human DCs. OVAp-Fc was incubated with human PBMCs, and its binding to cells was determined by flow cytometry with an anti-His Ab to avoid unintentional signal from endogenous IgE bound to FcεRI. We found that anti-His Ab bound to all FcεRI-expressing cells, including BDCA1+ DCs, PDCs, basophils, and monocytes, but not to FcεRI-nonexpressing cells such as BDCA3+ DCs (Fig. 4A). This finding indicates that OVAp-Fc specifically binds to human DCs and other FcεRI-expressing cells.

Next, we examined whether OVAp-Fc binds to DCs of hFcεRIα-Tg mice in vivo. OVAp-Fc was injected i.v. into hFcεRIα-Tg mice. One hour after injection, we isolated blood cells and performed flow cytometry using anti-His Ab and cell type specific Abs. We found that anti-His Ab bound to cDCs and other hFcεRI-expressing cells such as PDCs, but not to FcεRI-negative cells such as B cells (Fig. 4B, upper panel). Like in blood, anti-His Ab stained cDCs and other FcεRI-expressing cells, but not B cells in the spleen (Fig. 4C). To confirm that OVAp-Fc binding to DCs is mediated by hFcεRI, we performed the same experiment using Tg-negative control mice and found no anti-His Ab binding in any cell type (Fig. 4B, lower panel). Thus, OVAp-Fc binds to DCs in hFcεRIα-Tg mice in an hFcεRI-dependent manner.

**Ag targeting to hFcεRI enhances Ag presentation in hFcεRIα-Tg mice by 1000–2500 fold in vivo**

To determine whether Ag targeted to FcεRI through our Ag-Fc fusion protein is presented in vivo, we injected OVAp-Fc into hFcεRIα-Tg mice that had been adoptively transferred with a mixture of CTV-labeled OT-II and CFSE-labeled SMARTA mouse CD4+ T cells. We included SMARTA T cells, which are specific
for an irrelevant Ag, to determine whether T cell proliferation is Ag-specific or whether it is the result of nonspecific T cell activation. Proliferation of OT-II and SMARTA CD4+ T cells was examined 3 d after OVAp-Fcε injection by determining the dilution of CFSE and CTV by flow cytometry. As expected, dilution of CTV was readily detected while dilution of CFSE was not observed (Fig. 5A). This finding indicates that Ag targeted to FcεRI through Ag-Fcε fusion protein is presented to Ag-specific T cells in hFcεRα-Tg mice, resulting in the restricted proliferation of only Ag-specific cells.

Next, we examined the degree to which targeting Ags to FcεRI enhances Ag presentation. For this study, CTV-labeled OT-II CD4+ T cells were transferred into hFcεRα-Tg and Tg− control mice. One day later, mice were injected i.v. with 0.05–0.5 μg OVAp-Fcε. Spleens were harvested 3 d later, and the dilution of CTV was examined by flow cytometry. We found that OTII CD4+ T cells proliferated in hFcεRα-Tg mice, but not in control mice (Fig. 5B). To determine whether the lack of OT-II T cell proliferation in control mice is due to a general defect in Ag processing or presentation of these mice, we injected hFcεRα-Tg and non-Tg control mice with OVA Ags at high doses, such as 10 μg OVA peptide (323–339) or 50 μg OVA protein. We found that OT-II CD4+ T cells proliferated in control mice and hFcεRα-Tg mice to a similar degree (Fig. 5C, 5D), indicating that Ag processing and presentation are not significantly different between Tg+ and Tg− mice. Thus, it is the targeting of OVA to FcεRI that dramatically enhances OTII T cell proliferation.

To quantify the increase in Ag presentation efficiency when Ags were targeted to FcεRI via IgE, we compared the degree of OT-II T cell proliferation in hFcεRα-Tg mice injected with OVAp-Fcε...
versus OVA protein or peptide (323–339). We found that 0.1 μg OVAp-Fc (which contains an equivalent molar quantity of 0.004 μg OVA peptide [323–339]) induced OTII T cell proliferation to a degree similar to 10 μg of OVA peptide (323–339; Fig. 5E), and that 0.05 μg of OVAp-Fc elicited an OT-II T cell proliferation equivalent to that of 50 μg OVA protein (Fig. 5F). These findings indicate that Ag presentation increases by 1000–2500 fold when Ag is targeted to FcRI in hFcRIα-Tg mice.

It is noteworthy that targeting Ags to FcRI did not appear to enhance Ag presentation to CD8+ T cells. We generated OVA (257–264)- Fce and injected it into hFcRIα-Tg and Tgε control mice that had received OT-I CD8+ T cells expressing OVA (257–264)-specific TCR. We found that OT-I CD8+ T cells proliferated in Tgε and Tgε mice to similar degrees (Supplemental Fig. 3), indicating that DC targeting of Ags via FcRI only enhances Ag presentation to CD4+ T cells in this mouse model.

_FcRI-targeted Ags are specifically presented by cDCs in hFcRIα-Tg mice_

To investigate which FcRI-expressing cells present OVA targeted to FcRI, we i.v. injected hFcRIα Tg-mice with OVAp-Fc. After 24 h, cDCs, PDCs, basophils, monocytes, and B cells were isolated from spleen by FACS. Each of the isolated cell types was cultured with CTV-labeled OT-II CD4+ T cells, and the dilution of CTV was determined by flow cytometry 4 d later. We found that dilution of CTV was limited to when OT-II CD4+ T cells were cultured with cDCs, PDCs, basophils, and monocytes that express FcεRI, indicating that cDCs, PDCs, basophils, and monocytes all express FcεRI. These findings indicate that although cDCs, PDCs, basophils, and monocytes all express FcεRI and bind OVAp-Fc in hFcRIα-Tg mice (Figs. 2, 4), cDCs are the only cells that present OVA.

_Ag targeting to FcεRI results in Ag-specific CD4+ T cell deletion in hFcRIα-Tg mice_

Next, we examined the fate of OT-II CD4+ T cells following proliferation in hFcRIα-Tg mice injected with OVAp-Fc. For this study, we adoptively transferred CD45.1 expressing CTV-labeled OT-II CD4+ T cells into hFcRIα-Tg mice, injected the mice i.v. with OVAp-Fc the following day, and determined the frequency and CTV dilution of CD45.1+ T cells in the spleen at days 3, 7, and 12 after injection. We observed substantial dilution of CTV on day 3, indicating OT-II CD4+ T cell proliferation. However, proliferation did not continue; in fact, there were fewer CD45.1+ cells observed on day 7, and they completely disappeared by day 12 (Fig. 7A). When we quantitated the frequency of CD45.1+ cells in the spleen, lymph node, and blood, we found that these cells were disappearing over time from all these organs, indicating systemic deletion (Fig. 7B). To determine whether hFcRIα-Tg mice have an intrinsic defect in expanding Ag-specific T cells, we examined OT-II CD4+ T cell expansion in these mice after injection of OVAp-Fc together with the immunostimulatory agent LPS. We found that OT-II CD4+ T cells proliferated substantially and continuously for the entire 12 d of examination in spleen, LN, and blood (Fig. 7C, 7D). This finding indicates that hFcRIα-Tg mice are capable of expanding Ag-specific T cells when Ag is targeted to hFcRI in the presence of inflammatory stimuli, but delete them when Ag is targeted to hFcRI in the absence of such stimuli.

We also examined whether targeting Ags to hFcRI induces Ag-specific regulatory T cells (Tregs) in hFcRIα-Tg mice. Mice were adoptively transferred with GFP CD4+ T cells that were isolated from Foxp3GFP OT-II mice by FACS (Fig. 8A) and labeled with CTV. OVAp-Fc was injected i.v. the next day, and GFP expres-

**FIGURE 7.** Ag targeting to FcεRI results in Ag-specific CD4+ T cell deletion in hFcRIα-Tg mice. (A and B) Rapid deletion of OT-II CD4+ T cells in hFcRIα-Tg mice injected with OVAp-Fc. hFcRIα-Tg mice were adoptively transferred with CTV-labeled CD45.1+ OT-II CD4+ T cells 1 d before they were injected i.v. with 0.2 μg OVAp-Fc. Three, seven, and twelve days after injection, spleens, inguinal lymph nodes, and blood were harvested. Cells from each tissue were stained and analyzed by flow cytometry for the frequency and extent of CTV dilution of CD45.1+ T cells. OVAp-Fc was injected i.v. the next day, and determined the frequency of CD45.1+ T cells in the spleen, lymph node, and blood (Fig. 7C, 7D). This finding indicates that hFcRIα-Tg mice are capable of expanding Ag-specific T cells when Ag is targeted to hFcRI in the presence of inflammatory stimuli, but delete them when Ag is targeted to hFcRI in the absence of such stimuli.

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sion by transferred OT-II CD4+ T cells was monitored by flow cytometry. Very few GFP+ OT-II CD4+ cells were found at any days of examination following injection with OVAp-Fc at high or low doses (Fig. 8B, 8C). Thus, Ag-targeting to hFcRI hardly induces Tregs.

**Ag-Fc fusion protein inhibits development of Ag-specific T cell immunity in FcεRIa-Tg mice**

Lastly, we investigated whether targeting Ags to FcεRI could inhibit development of Ag-specific T cell immunity and induce T cell tolerance in vivo. For this study, we examined whether OVAp-Fc could prevent hFcεRIa-Tg mice from developing delayed-type hypersensitivity against OVA. hFcεRIa-Tg mice adoptively transferred with OT-II CD4+ T cells were injected i.v. with either OVAp-Fc or PBS. Seven days later, mice were immunized with OVA peptide mixed with IFA. An additional 7 d later, OVA-specific T cell immunity was determined using either in vitro restimulation assay or DTH assay. For the in vitro restimulation assay, splenocytes were isolated from mice, incubated with OVA peptide, and examined for proliferation using [3H]thymidine. We found that cells from OVAp-Fc–injected mice incorporated [3H]thymidine at significantly lower levels than cells from PBS-injected mice did. This finding indicates that OVAp-Fc inhibits the development of OVA-specific T cell immunity in hFcεRIa-Tg mice (Fig. 9B). To verify that this inhibitory effect was mediated by hFcRI, the same experiment was performed using control mice that do not express hFcεRI. Splenocytes from OVAp-Fc–injected control mice incorporated [3H]thymidine as much as those from PBS-injected hFcεRIa-Tg mice did (Fig. 9B). Thus, OVAp-Fc inhibits development of OVA-specific T cell immunity only when hFcRI is expressed.

For DTH assay, mice were injected into the left footpads with OVA peptide mixed with IFA, and into the right footpads with IFA alone. Twenty-four hours after injection, the thickness of each footpad was measured. The thickness of the left footpad was subtracted from that of the right footpad to determine swelling induced by OVA rechallenge (Fig. 8A). We found that both PBS and OVAp-Fc–injected mice showed appreciable swelling, but the degree was markedly less in OVAp-Fc–injected mice, suggesting that OVAp-Fc injection significantly inhibited mice from...
developing DTH (Fig. 8C). Importantly, OVAp-Fce failed to inhibit Tg ε control mice from developing DTH (Fig. 8C) indicating that the inhibitory effect of OVAp-Fce in DTH development requires hFceRI expression. Taken together, these findings demonstrate that Ag targeting to FceRI inhibits the development of Ag-specific T cell immunity and induces T cell tolerance.

Discussion

In this study, we have found that Ag-Fce fusion proteins can be exploited to target Ags to DCs and induce Ag-specific T cell tolerance. We found that Ag-Fce bound to human DCs in vitro and to DCs of hFceRI-εTg mice in vivo, and that injection of hFceRI-εTg mice with Ag-Fce resulted in Ag presentation by DCs and subsequent systemic deletion of Ag-specific T cells. Importantly, injection of Ag-Fce into hFceRI-εTg mice significantly inhibited these mice from developing DTH.

Previous studies have indicated that the efficiency of Ag presentation can be increased by 100–1000 fold when Ags are conjugated with DC-specific Abs such as anti-DEC-205, Dectin-1, DNGLR1, DCIR, Langerin, Treml4, or DC-SIGN (9, 14, 31–33). We found in hFceRI-εTg mice that Ags conjugated with human Fce were presented by DCs at 1000–2500 fold higher efficiency than unconjugated Ags were. We predict that the efficiency of targeting of this construct to human DCs will be even higher than in hFceRI-εTg mice, because human DCs express much higher levels of FceRI than hFceRI-εTg mouse DCs. Thus, the efficiency of Ag presentation in humans may increase markedly when Ags are conjugated with Fce than when it is conjugated with DC-specific Ab. In addition, generation of Ag-Fce fusion protein is more straightforward, as described in this study, and perhaps more cost-effective than the generation of Ag-DC-specific Ab fusion protein. A single cDNA cloning and transfection will generate Ag-Fce; there is no need to immunize animals, generate and select hybridomas, and humanize selected Abs.

The mechanism by which Fce-linked Ag is presented to CD4+ T cells remains to be determined. It has been known that IgE/FceRI cross-linking using multivalent Ags results in the entry of Ag/IgE/FceRI complexes to endocytic compartments in DCs and subsequent processing and presentation of the Ags to CD4+ T cells (34, 35). However, whether non–cross-linking Ag could follow the same pathway of DC entry has never been examined. Our finding (34, 35) that the injection of Ag-Fce into FceRI-εTg mouse DCs do. Thus, the efficiency of Ag presentation in humans may increase markedly when Ags are conjugated with Fce than when it is conjugated with DC-specific Ab. In addition, generation of Ag-Fce fusion protein is more straightforward, as described in this study, and perhaps more cost-effective than the generation of Ag-DC-specific Ab fusion protein. A single cDNA cloning and transfection will generate Ag-Fce; there is no need to immunize animals, generate and select hybridomas, and humanize selected Abs.

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histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J. Exp. Med. 196: 1627–1638.


**Suppl Fig. 1. Human cell gating strategy.** Human PBMCs were stained with various antibodies for flow cytometry analysis.

BDCA1+ DCs and BDCA3+ DCs were gated as shown in (A). Basophils and PDCs were gated as shown in (B). B cells and T&NK cell mixtures were gated as shown in (C). Monocytes were gated as shown in (D).
**Suppl. Fig. 2. OVAp-Fce does not induce mast cell degranulation.** hFceR1α-Tg mouse bone marrow-derived mast cells were equilibrated with Tyroid buffer and mixed with increasing amounts of OVAp-Fce or anti-hFceRI Ab:anti-mouse IgG Ab complexes in a 96 well plate. After incubation at 37 °C for 1 hr, plate was centrifuged, supernatant was collected, and cell pellet was lysed with 0.1% Triton X-100. The supernatant and cell lysates were mixed with the hexosaminidase substrate, p-nitrophenyl-N-acetyl-b-D-glucosaminide (1mM). After incubation at 37 °C for 1 hr, 0.1 M sodium acetate buffer was added to stop the reaction. Absorbance was read at 400 nm. The percentage of hexosaminidase released is indicated with mean ± SEM. *denotes the p value < 0.05. ns is ‘not significant’.
Suppl. Fig. 3. Antigen targeting to FcεRI does not enhance antigen presentation to CD8+ T cells in hFceRIα-Tg mice. (A) Schematic of SIINFEKL (OVA (257-264))-Fcε. (B-C) hFceRIα-Tg mice (Tg+, upper panel) and Tg-negative control mice (Tg-, lower panel) were adoptively transferred with CTV-labeled CD45.1+CD8+ OTI T cells one day before iv injection with 0.2 µg or 0.02 µg SIINFEKL-Fcε. Three days later, spleens were harvested and cells were stained and analyzed by flow cytometry. The percentage of proliferating CD45.1+TCRα2+CD8+ OTI T cells was determined by gating CTV-diluted cells. Shown in (B) are data from one representative mouse for each group. Shown in (C) are data from 5 mice for each group injected with 0.2 µg SIINFEKL-Fcε with mean ± SEM. ns denotes ‘not significant’.