Brief Definitive Report

Inducing Tumor Immunity through the Selective Engagement of Activating Fcγ Receptors on Dendritic Cells

Alexis M. Kalergis and Jeffrey V. Ravetch

Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York, NY 10021

Abstract

Induction of tumor-specific immunity requires that dendritic cells (DCs) efficiently capture and present tumor antigens to result in the expansion and activation of tumor-specific cytotoxic T cells. The transition from antigen capture to T cell stimulation requires a maturation signal; in its absence tolerance, rather than immunity may develop. While immune complexes (ICs) are able to enhance antigen capture, they can be poor at inducing DC maturation, naive T cell activation and protective immunity. We now demonstrate that interfering with the inhibitory signal delivered by FcγRIIB on DCs converts ICs to potent maturation agents and results in T cell activation. Applying this approach to immunization with DCs pulsed ex-vivo with ICs, we have generated antigen-specific $CD8^+$ T cells in vivo and achieved efficient protective immunity in a murine melanoma model. These data imply that ICs may normally function to maintain tolerance through the binding to inhibitory FcγRs on DCs, but they can be converted to potent immunogenic stimuli by selective engagement of activating FcγRs. This mechanism suggests a novel approach to the development of tumor vaccines.

Key words: immune complexes • Fcγ receptors • DC maturation • inhibitory/activating receptor pairs • T cell immunity

Introduction

Elucidation of the mechanisms responsible for protective immunity against tumors is a prerequisite to the development of tumor-specific vaccines and effective immunotherapy for cancer. Because of their ability to be specifically captured by dendritic cells (DCs), enhance antigen presentation, and elicit tumor-specific killer T cells, immune complexes (ICs) are emerging as an attractive means of delivering tumor-specific antigens to stimulate protective immunity (1, 2). DCs have been shown to be a key cell in the pathway of antigen capture and presentation to T cells, having the unique ability to directly prime naive $CD4^+$ and $CD8^+$ T cells (3–5), through their ability to efficiently uptake, process, and present antigen on MHC class I and II molecules, together with costimulatory molecules such as B7 and CD40 (3, 6–8). Furthermore, DCs have been shown to efficiently present exogenously derived antigens (e.g., ICs) on MHC class I molecules to naive $CD8^+$ T cells (9–12), accounting for the phenomenon of cross-priming (13). However, for DCs to efficiently stimulate T cells, a process known as maturation is required. Immature DCs, specialized for antigen capture, undergo maturation in response to inflammatory cytokines and bacterial products, leading to the upregulation of costimulatory molecules such as B7 and CD40 (3, 5, 7, 14). This requirement for maturation insures that activation of the immune response be coupled to a mechanism capable of discriminating between endogenous antigens and those derived from exogenous sources, typically microbial pathogens. Thus, in the absence of receiving a maturation signal, the interaction of antigen-presenting immature DCs with T cells can result in peripheral tolerance (14, 15). The challenge of tumor vaccination is to find means of overcoming this protective mechanism and eliciting effective T cell stimulation to endogenous antigens expressed by tumor cells (16–19).

ICs are capable of either enhancing or suppressing the immune response, resulting from their ability to interact with Fcγ receptors on B cells, macrophages, or DCs, thus triggering different cellular responses (20, 21). Engagement of Fcγ receptors by ICs can lead to either activation or inhibitory signaling depending on the specific FcγR being engaged. Activating Fcγ receptors I and III associate with the immunoreceptor tyrosine–based activation motif (ITAM)-containing γ-chain and their engagement by ICs results in src and syk kinase–mediated activation responses. In contrast, the inhibitory Fcγ receptor IIB is a single subunit receptor containing a cytoplasmic immunoreceptor tyrosine–based inhibition motif (ITIM) domain that inhibits

Address correspondence to either J.V. Ravetch or A.M. Kalergis, The Rockefeller University, 1230 York Ave., Box 98, New York, NY 10021. Phone: 212-327-7321; Fax: 212-327-7318; E-mail: ravetch@rockefeller.edu or kalergi@rockefeller.edu

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ITAM-mediated activation signals through the recruitment of the inositol-phosphatase SHIP (22, 23). While expression of the activating/inhibitory receptor pair FcγRIII/FcγRIIB has been described on DCs in mice and humans (10), the role of these signaling pathways in the biology of DCs is unclear. Specifically, the ability of ICs to induce maturation of immature DC through FcγR activation has been variable. Dhodapkar et al. failed to observe IC-mediated maturation in human DCs (2), while Regnault et al. and recently Schuurhuis et al., reported maturation of mouse DCs in response ICs (10, 12). Since the relative expression of activating/inhibitory receptors were not defined in those studies it is possible that IC triggered maturation may depend on the balance between those opposing FcγRs. More importantly, however, the in vivo implications of differential ligation of FcγRs on DC differentiation and T cell priming have not been defined.

Therefore, we set out to test the hypothesis that the balance between activation and inhibitory FcγRs on DCs was critical to induce maturation and effective tumor immunity in vivo in response to ICs. Here we show that DC predominantly express FcγRIIB on the surface, and that interfering with the inhibitory signal delivered by this receptor enhances the ability of ICs to induce DC maturation. By immunizing with FcγRIIB$^{-/-}$ DCs pulsed ex vivo with ICs we have efficiently generated antigen-specific CD8$^+$ T cells in vivo and achieved efficient protective immunity in a murine melanoma model. Our results indicate that expression of inhibitory FcγRs on DCs may be required to maintain tolerance through binding to IgG–ICs. However, selective engagement of activating FcγRs by ICs on DCs works as a strong immunogenic signal promoting DC maturation and T cell priming that could elicit effective antitumor immunity.

Materials and Methods

Analysis of FcγR Expression on DCs and T Cell Priming Assays. Bone marrow–derived DCs were prepared as described previously (24). DCs were grown from bone marrow progenitors in RPMI 1640 containing 5% FCS supplemented at 3% vol/vol with supernatant from J558L cells transduced with murine (m) GM-CSF. Day 6 DC cultures derived from bone marrows of wild-type (WT), γ chain$^{-/-}$, FcγRIIB$^{-/-}$ mice (all in the C57BL/6 background) were double-stained with anti–CD11c-PE (HL3; BD PharMingen) and 2.4G2-FITC (BD PharMingen), 1 μg per 5 × 10^5 DCs, and analyzed by FACS®. For WT DCs, 2.4G2 binds to both FcγRIIB and FcγRIII; for γ chain$^{-/-}$ DCs, 2.4G2 binds to FcγRIIB only; and for FcγRIIB$^{-/-}$ DCs, 2.4G2 binds to FcγRIII only. Background 2.4G2 fluorescence was obtained from DCs in which both FcγRIIB and γ chain have been deleted. For the T cell priming experiments, DCs derived from bone marrows of WT or FcγRIIB$^{-/-}$ mice (C57BL/6 background) were pulsed for 3 h with 50 ug/ml OVA or OVA-IgG ICs. After washing, antigen-pulsed DCs (5 × 10^3–10^5 cells per well) were cocultured with either H-2K^b/OVA– or I-A^b/OVA–specific TCR transgenic T cells (2 × 10^5 cells per well), purified from OT-I and OT-II mice, respectively (25, 26). At 48-h coculture cells were pulsed with ^{3}[H]-thymidine (1 μCi ^{3}[H]-thymidine per well) and harvested 8 h later for determination of ^{3}[H]-thymidine incorporation.

DC Maturation Assays. Day 6 DC cultures derived from bone marrows of WT C57BL/6 and FcγRIIB$^{-/-}$ mice were incubated for 36 h with 20 μg/ml of ICs made of OVA and anti-OVA rabbit IgG (0.5 mg anti-OVA rIgG, ICN, per 1 mg OVA; Calbiochem). For FcγRIIB blocking, WT DCs were incubated simultaneously with OVA-ICs and Ly17.2 mAb (supernatant from K9.361 hybridoma) (27). For LPS-induced maturation, DCs were incubated with 50 ng/ml LPS. After 36-h incubation, DCs were double-stained with anti–CD11c-PE (HL3; BD PharMingen) plus either anti–I-A^b-FITC (AF6–120.1; BD PharMingen) or anti–B7.2-FITC (GL1; BD PharMingen), 1 μg mAb per 5 × 10^5 DCs and analyzed by FACS®.

DC Immunization and Tumor Challenge Experiments. Day 6 DC cultures derived from bone marrows of either WT or FcγRIIB$^{-/-}$ mice (C57BL/6 background) were incubated for 6 h with 50 μg/ml of ICs made of OVA and anti-OVA rabbit IgG (0.5 mg anti-OVA rIgG, ICN, per 1 mg OVA; Calbiochem). DCs were washed in PBS and injected in the footpads of naive syngeneic C57BL/6 mice (2.5 × 10^5 DCs per mouse). As a specificity control mice were immunized with untreated DCs. 2 wk after this single immunization, mice were challenged subcutaneously with a variant of the melanoma B16 tumor line that expresses full-length OVA as a neo-antigen (MO4) (references 28–30) (5 × 10^5 cells per mouse). As an antigen specificity control mice were challenged with the parental B16 tumor line (American Type Culture Collection). Tumor appearance and growth was monitored three times a week, and a mouse was considered positive when palpable tumors were detected. For statistical analysis (Student's *t* test) the Prism™ 2.0 software (GraphPad) was used.

Detection of OVA-specific CD8$^+$ T Cells with H-2K^b/OVA Tetramers. Peripheral blood cells obtained 2 wk after challenging mice with B16-OVA tumor, were double stained with anti–CD8α-FITC (53–6.7; BD PharMingen) and H-2K^b/OVA-PE tetramers. Tetramer staining was done at 4°C, for 1 h with 1 μg of anti-CD8α and tetramers per 10^6 cells. H-2K^b/OVA tetramers carried the immunodominant OVA peptide SIINFEKL and were designed as described previously (31, 32).

Results and Discussion

To evaluate whether the balance between activation and inhibitory FcγRs on DCs is critical to induce maturation and effective tumor immunity in vivo in response to ICs, DCs were generated from bone marrow–derived from C57BL/6 WT and FcγR-deficient mice and characterized for FcγR surface expression. The relative contributions of the low-affinity activating (FcγRIII) and inhibitory (FcγRIIB) receptors on the surface of immature DCs was determined by FACS® analysis of DC isolated from WT and FcγR-deficient animals. FcγRIIB plus RIII expression was determined by 2.4G2 staining, a mAb that binds equivalently to both receptors (33, 34). The FcγRIIB component was determined by 2.4G2 staining on DCs obtained from mice deficient in the common γ chain (RI/RIII$^{-/-}$; reference 35) which only express FcγRIIB. The FcγRIII component was determined by staining DCs obtained from FcγRIIB-deficient mice with 2.4G2, which on these cells would only bind to FcγRIII. As seen in Fig. 1 A, DCs pre-

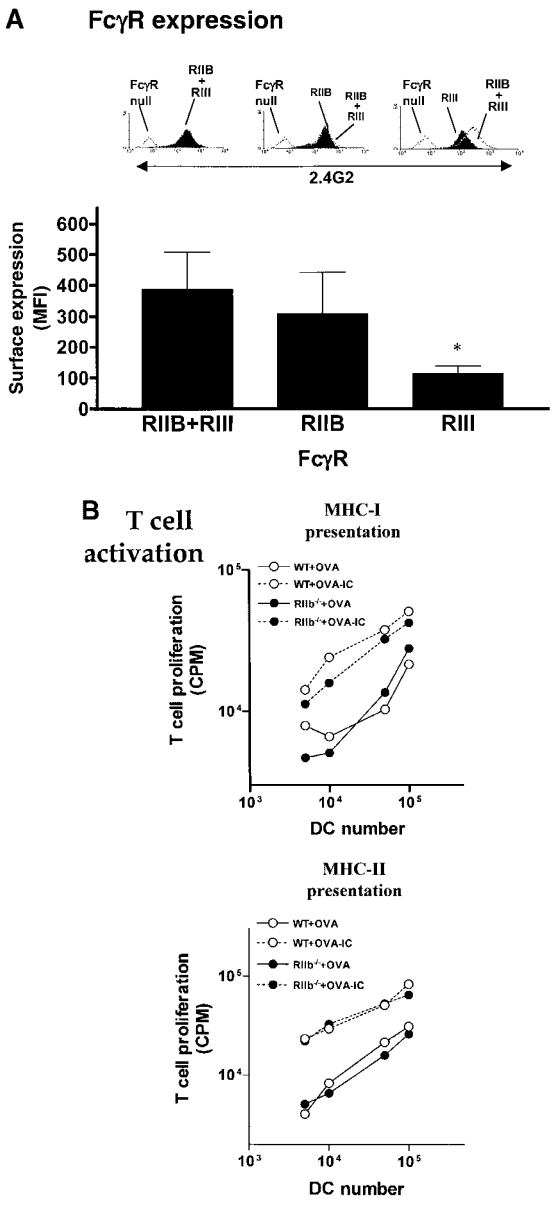

Figure 1. FcγRIIB is the predominant FcγR on the surface of dendritic cells and is not required for IC-mediated enhancement of antigen presentation. (A) Bone marrow–derived DCs were prepared as described previously (reference 24). Day 6 DC cultures derived from bone marrows of WT, γ chain$^{-/-}$, and FcγRIIB$^{-/-}$ mice (all in the C57BL/6 background) were double-stained with anti–CD11c-PE (HL3; BD PharMingen) and 2.4G2-FITC (BD PharMingen), and analyzed by FACS®. For WT DCs, 2.4G2 binds to both FcγRIIB and FcγRIII; for γ chain$^{-/-}$ DCs, 2.4G2 binds to FcγRIIB only; and for FcγRIIB$^{-/-}$ DCs, 2.4G2 binds to FcγRIII only. Background 2.4G2 fluorescence (dotted lines) was obtained from DCs in which both FcγRIIB and γ chain have been deleted (FcγR null). One representative histogram for 2.4G2 staining is shown, gating on the CD11c^{+} DC population. The bar graph shows 2.4G2 mean fluorescence intensity values (CD11c^{+} gate) from four independent experiments ($^{*}P < 0.025$); B). For the T cell priming experiments, DCs derived from bone marrows of WT or FcγRIIB$^{-/-}$ mice (C57BL/6 background) were pulsed for 3 h with 50 ug/ml OVA or OVA-IgG ICs. After washing, antigen-pulsed DCs (5×10^3–10^5 cells per well) were cocultured with either H-2K^{b}/OVA– or I-A^{b}/OVA–specific TCR transgenic T cells, (2×10^5 cells per well), purified from OT-I and OT-II mice, respectively (25, 26). At 48-h coculture cells were pulsed with ^{3}[H]-thymidine and harvested 8 h later for determination of ^{3}[H]-thymidine incorporation.

dominantly express inhibitory FcγRIIB, which accounts for ~75% of total FcγR expression. Consistently, the mean fluorescence intensity on WT DCs observed with the RIIB-specific Ly17.2 mAb (27) also accounted for ~75% of 2.4G2 staining (data not shown). A similar pattern of FcγR expression was observed in DCs purified from spleen (data not shown). These results indicate that resting DCs express mainly inhibitory FcγRIIB on their surface.

Both WT and FcγRIIB$^{-/-}$ immature DCs are equally able to mediate IC presentation to T cells, as shown in Fig. 1 B. DCs isolated from either WT or FcγRIIB-deficient mice were incubated with ICs composed of rabbit IgG anti-OVA and OVA, and then tested for their ability to mediate antigen presentation to MHC class I– and class II–restricted OVA-specific T cells (OT-1 and OT-II, respectively) (25, 26). IC-mediated enhancement of MHC class I and II presentation was equally efficient for both WT and FcγRIIB$^{-/-}$ DCs, indicating that there are no differences between these cells in their ability to capture and present IC-derived antigens to class I– or class II–restricted T cells (Fig. 1 B).

In contrast, DC maturation by ICs was enhanced when activating FcγRs were selectively ligated through the use of FcγRIIB-deficient mice (Fig. 2). In comparison to LPS-induced maturation, WT DCs are inefficiently induced to mature in response to ICs, consistent with previous reports (2, 10, 12). However, a subset of DCs derived from FcγRIIB-deficient mice display a maturation phenotype comparable to that seen for LPS, with upregulation of MHC class II (Fig. 2 A) and B7.2 (Fig. 2 B) surface expression. As seen from the FACS® analysis, this subset of DCs incubated with ICs show a marked increase in MHC class II and B7.2 expression, indicative of maturation of these cells. This effect was not the result of perturbation of DC development by FcγRIIB deletion, as demonstrated by the ability of WT DCs to enhance their maturation in response to ICs when FcγRIIB is blocked by the FcγRIIB-specific mAb Ly17.2 (Fig. 2 A and B). Furthermore, FcγRIIB-deficient DCs show equivalent maturation in response to LPS as compared with WT DCs (Fig. 2 A and B). Conversely, and consistent with previous reports (10), IC engagement of DCs derived from γ chain–deficient mice lacking activation FcγRs does not result in DC maturation (data not shown). These results indicate that the ability of ICs to induce DC maturation is determined by the balance of activation and inhibitory FcγRs. Selective engagement of the ITAM-containing FcγRs by ICs in the absence of coligation of ITIM-containing inhibitory FcγRIIB results in enhanced maturation.

The functional consequence of IC-induced maturation of DCs in vitro was tested in vivo in a murine melanoma model to determine if DCs induced to mature by selectively engaging activating FcγRs resulted in enhanced antitumor immunity. FcγRIIB$^{-/-}$ DCs were pulsed with OVA–IgG ICs as before and then used to immunize C57BL/6 mice. As a control, C57BL/6 mice were immunized with WT DCs treated under the same conditions. 2 wk after a single DC immunization, mice were challenged

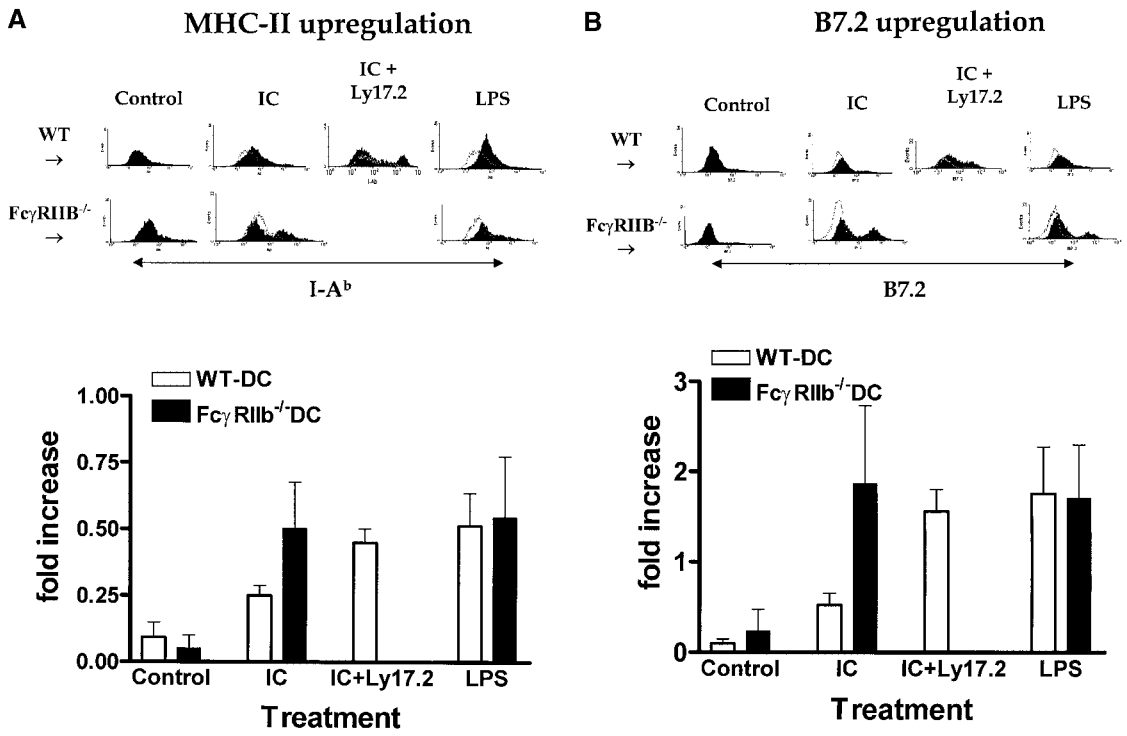

Figure 2. Removal of inhibitory Fcγ receptor signaling from DCs enhances their maturation by ICs. Day 6 DC cultures derived from bone marrows of WT C57BL/6 and FcγRIIB$^{-/-}$ mice were incubated for 36 h with 20 μg/ml of ICs made of OVA and anti-OVA rabbit IgG. For FcγRIIB blocking, WT DCs were incubated simultaneously with OVA-ICs and Ly17.2 mAb (supernatant from K9.361 hybridoma) (reference 27). For LPS-induced maturation, DCs were incubated with 50 ng/ml LPS. After 36-h incubation DCs were double-stained with anti–CD11c-PE (HL3; BD PharMingen) plus either anti–I-A^b-FITC (AF6–120.1; BD PharMingen, data shown in A) or anti–B7.2-FITC (GL1; BD PharMingen, data shown in B), 1 μg mAb per 5×10^5 DCs, and analyzed by FACS®. Histograms show representative I-A^b or B7.2 fluorescence intensities for the CD11c$^+$ DC population gate. Bar graphs show the increase of mean fluorescence intensity for I-A^b and B7.2 (CD11c$^+$ gate) from four independent experiments.

either with an OVA-expressing clone of the melanoma line B16 (28, 30) (Fig. 3 A) or with control B16 cells not expressing OVA. The animals were scored for the kinetics of tumor appearance as well as the absolute size of the tumor mass. As shown in Fig. 3, mice immunized with FcγRIIB-deficient DCs pulsed with OVA–IC developed protective immunity to the tumor challenge, with no detectable tumor appearance, while animals immunized with

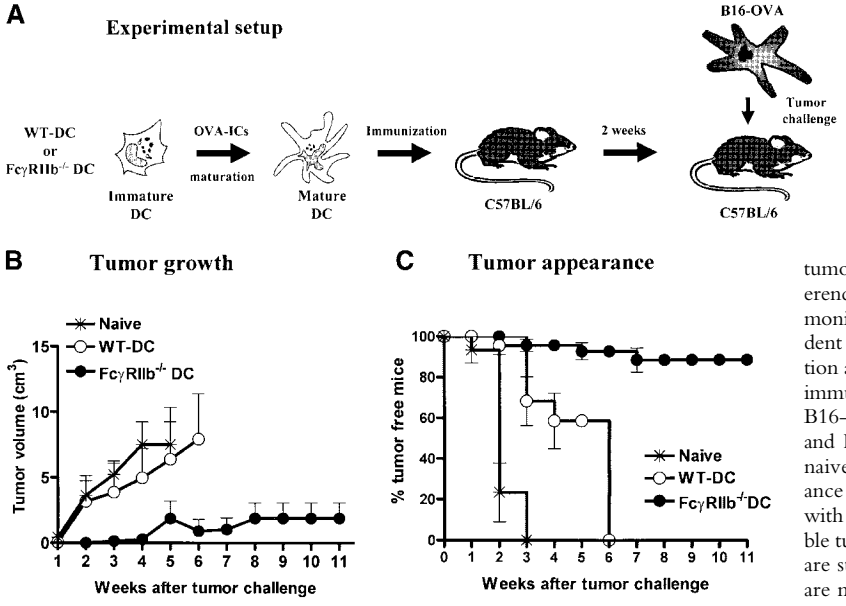

Figure 3. Removal of inhibitory Fcγ receptor signaling on DCs enhances their ability to protect against tumors. Day 6 DC cultures derived from bone marrows of either WT or FcγRIIB$^{-/-}$ mice (C57BL/6 background) were incubated for 6 h with 50 μg/ml of ICs made of OVA and anti-OVA rabbit IgG. DCs were washed in PBS and injected in the footpads of naive syngeneic C57BL/6 mice. 2 wk after this single immunization, mice were challenged subcutaneously with a variant of the melanoma B16 tumor line that expresses OVA as a neo-antigen (MO4) (references 28–30) (5×10^5 cells per mouse). Tumor growth was monitored three times a week and data from three independent experiments are shown. (A) Scheme for DC immunization and tumor challenge. (B) Tumor growth curves for mice immunized with OVA–IC-pulsed DCs and challenged with B16-OVA (mice showing palpable tumors). While WT DCs and FcγRIIb$^{-/-}$ DCs are statistically different ($P < 0.002$), naive and WT DCs are not ($P < 0.80$). (C) Tumor appearance for mice immunized with OVA–ICs and challenged with B16-OVA. Mice were considered positive when palpable tumors were detected. While naive and FcγRIIb$^{-/-}$ DCs are statistically different ($P < 0.0001$), naive and WT-DC are not ($P < 0.079$).

Table I. *Tumor Appearance at 3 wk*

Treatment	Challenging tumor	Tumor$^+$ mice/ challenged mice
Naive	B16-OVA	8/8
WT unpulsed	B16-OVA	4/4
FcγRIIB$^{-/-}$ unpulsed	B16-OVA	4/4
WT plus OVA-IC	B16-OVA	6/8
FcγRIIB$^{-/-}$ plus OVA-IC	B16-OVA	1/8
Naive	B16	4/4
WT unpulsed	B16	4/4
FcγRIIB$^{-/-}$ unpulsed	B16	4/4
WT plus OVA-IC	B16	4/4
FcγRIIB$^{-/-}$ plus OVA-IC	B16	4/4

DC cultures derived from bone marrows of WT or FcγRIIB$^{-/-}$ mice (C57BL/6 background) were pulsed with OVA-IgG ICs and used to immunize naive syngeneic C57BL/6 mice. 2 wk after this single immunization, mice were challenged subcutaneously with a variant of the melanoma B16 tumor line that expresses OVA as a neo-antigen (MO4). Data shown are fraction of mice with palpable tumors at 3 wk after tumor challenge. As antigen specificity controls, some mice were injected with untreated DCs and others were challenged with the parental (OVA negative) B16 tumor line.

WT DCs pulsed with OVA–ICs developed tumors (Fig. 3 B and C), although with a significant delay in appearance (Fig. 3 B and C). No tumor protection was observed when the challenging tumor was the parental B16 line (OVA-negative). Similarly, neither WT nor FcγBIIB$^{-/-}$ unpulsed DCs protected against B16 or B16-OVA. These results indicate that the antitumor response elicited by immunization with OVA–IC-pulsed DCs was antigen (OVA)-specific (Table I).

To determine the mechanism of this protection by DC immunization, we tested peripheral blood of mice immunized with OVA-IC–pulsed FcγRIIB$^{-/-}$ DCs or WT DCs for the presence of OVA-specific CD8$^+$ T cells. Peripheral blood CD8$^+$ T cells from DC-immunized and tumor-challenged mice were stained for H-2K^b/OVA tetramers (H-2K^b/SIINFEKL) (31, 32). OVA-specific CD8$^+$ T cells were only expanded in peripheral blood of mice immunized with FcγRIIB$^{-/-}$ DCs pulsed with OVA–IgG ICs (Fig. 4). These results indicate that FcγRIIB$^{-/-}$ DCs have an enhanced ability to prime antigen-specific CD8$^+$ T lymphocytes in vivo, thus contributing to an effective antigen-specific antitumor immune response.

We conclude by suggesting that effective antitumor immunization by ICs requires that selective Fc receptor engagement be achieved to induce DC maturation and thus efficient CD8$^+$ T cell priming. This selectivity may be accomplished by blocking the inhibitory Fc receptor or by engineering the Fc region of the IgG molecule of the IC to preferentially engage activation and not inhibitory FcγR (36). Conversely, the preferential expression of the

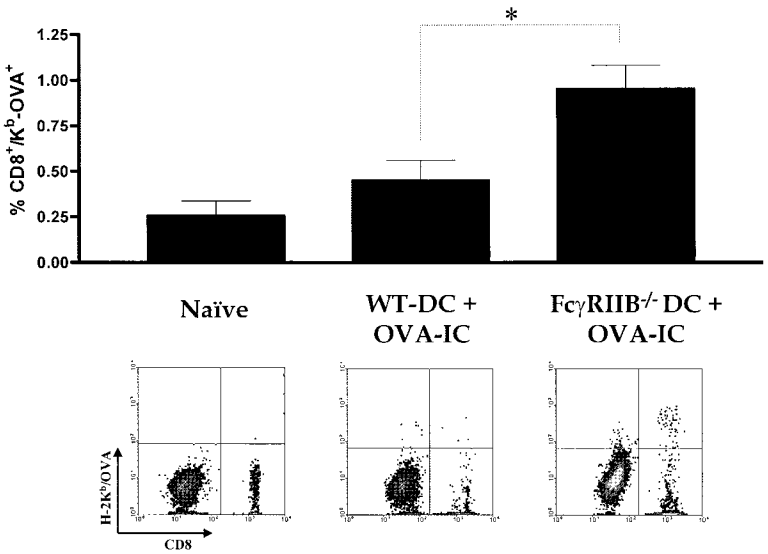

Figure 4. FcγRIIB$^{-/-}$ DCs efficiently induce expansion of antigen-specific CD8$^+$ T cells. Peripheral blood cells obtained 2 wk after challenging mice with B16-OVA tumor, were double stained with anti–CD8α-FITC (53–6.7; BD PharMingen) and H-2K^b/OVA-PE tetramers. Tetramer staining was done at 4°C, for 1 h with 1 μg of anti-CD8α and tetramers per 10^6 cells. H-2K^b/OVA tetramers carried the immunodominant OVA peptide SIINFEKL and were designed as described previously (references 31 and 32). (Left) Naive C57BL/6 mice; (middle) C57BL/6 mice immunized with WT DCs pulsed with OVA-IgG ICs; (right) C57BL/6 mice immunized with FcγRIIB$^{-/-}$ DC pulsed with OVA-IgG ICs. *$P < 0.02$.

inhibitory FcγR on immature DCs suggests that maturation and activation of immune responses may not normally occur in response to IC cross-linking of immature DCs, and may even provide a mechanism to maintain peripheral tolerance to self-antigens. Chronic inflammatory states or reduced inhibitory receptor expression, as has been documented in autoimmune susceptible mouse strains (37, 38), might contribute to the loss of tolerance and expansion of autoreactive lymphocytes by inappropriate activation of DC maturation and subsequent T cell stimulation. Restoring FcγRIIB expression may thus offer an approach to reestablishing tolerance.

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