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## Murine Plasmacytoid Dendritic Cells Initiate the Immunosuppressive Pathway of Tryptophan Catabolism in Response to CD200 Receptor Engagement<sup>1</sup> **FREE**

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# Murine Plasmacytoid Dendritic Cells Initiate the Immunosuppressive Pathway of Tryptophan Catabolism in Response to CD200 Receptor Engagement<sup>1</sup>

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**In this study, using a soluble CD200-Ig fusion protein, we provide evidence that murine dendritic cells (DCs) possess a functional CD200R, whose engagement results in the reinforcement or appearance of immunosuppressive properties in these cells. In particular, the plasmacytoid subset (CD11c<sup>+</sup>B220<sup>+</sup>120G8<sup>+</sup>) of splenic DCs (pDCs) is induced by CD200-Ig to express the enzyme IDO, which initiates the tolerogenic pathway of tryptophan catabolism. As a result, pDCs are capable of suppressing Ag-specific responses in vivo when transferred into recipient hosts after treatment with CD200-Ig. IDO induction in pDCs through CD200R engagement requires type I IFN $\alpha$  signaling. Although the release of IFN- $\alpha$  may contribute to the full expression of CD200-Ig activity, autocrine IFN- $\alpha$  is unlikely to mediate alone the effects of CD200R engagement. These data prospect novel functions for both pDCs and the CD200-CD200R pair in the mouse. At the same time, these data underscore the possible unifying role of the IDO mechanism in immune tolerance. *The Journal of Immunology*, 2004, 173: 3748–3754.**

An emerging theme in immunobiology of tolerance is that the same cells (1), molecules (2), and mechanisms (3) mediate Ag-specific unresponsiveness under a variety of physiopathologic conditions. Although only one of several modes of tolerance induction, the immunosuppressive pathway of tryptophan catabolism has been shown to contribute to successful pregnancy (4) and transplantation (5–7), as well as to effective control of autoimmunity (8–10). We have recently proposed a model of transplantation tolerance that might apply to both transplantation and autoimmunity. According to this model, IFN- $\gamma$  acts on tolerogenic dendritic cells (DCs)<sup>3</sup> to activate the expression of the enzyme, IDO. This leads to the induction of immunosuppressive tryptophan catabolism and to the onset of specific tolerance (11, 12).

Through localized tryptophan deficiency combined with the release of proapoptotic metabolites, DCs can thus exert an IDO-dependent homeostatic control over the proliferation and survival of peripheral T cells, and can promote Ag-specific tolerance (3, 13, 14). However, regulatory T cells will have a directive role in orchestrating the pathway of tryptophan catabolism in DCs, as they constitutively express CTLA-4, which signals DCs through B7 coreceptor molecules. As a result, IFN- $\gamma$  is released, whose autocrine effects lead to activation of IDO (15, 16). Although reports

have indicated that IDO expression can be induced by engagement of multiple receptors on different cell types (2, 17), it is presently unknown whether other T or non-T cell ligands in addition to CTLA-4 may affect IDO expression in DCs or subsets thereof.

CD200 (OX-2) is a broadly distributed cell surface glycoprotein that interacts with a structurally related receptor (CD200R) highly expressed on rodent and human myeloid cells (18, 19). CD200 is involved in the regulation of macrophage (20) and DC (21) function. The observation that CD200-deficient mice show increased susceptibility to different forms of autoimmunity demonstrates that CD200-CD200R interactions play a role in the control of myeloid cellular activity and prevention of autoimmunity (22, 23). A soluble CD200-Ig fusion protein provides protection in models of arthritis (24) and allograft rejection (25). Soluble CD200 also lowers the spontaneous abortion rates in abortion-prone CBA  $\times$  DBA/2 matings, whereas anti-CD200 Ab has the opposite effect in low abortion rate CBA  $\times$  BALB/c matings (2). It has been shown that, in addition to CTLA-4 (26) and B7 (27), placental tissues from successful human pregnancies express significant amounts of CD200 (28). Although these data suggest that CD200 is a tolerance signaling molecule with broad biological activity, the downstream cellular events triggered by CD200-CD200R interactions are largely unknown (19).

In both mice and humans, plasmacytoid DCs (pDCs) represent a subset of phenotypically defined DCs with specific origin and function (29, 30). Murine pDCs, which share the CD11c cell marker with myeloid DCs but also express the B220 marker (31), participate in the regulation of immunity, including induction and maintenance of tolerance, as well as in the defense against viruses (32, 33). In phenotyping IDO<sup>+</sup> splenocytes following treatment with a soluble form of CTLA-4, Mellor et al. (7) found that the CD11c<sup>+</sup>B220<sup>+</sup>CD19<sup>-</sup> plasmacytoid subset of splenic DCs contained cells intensely stained by anti-IDO Ab, as did nonplasmacytoid CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>B220<sup>-</sup> cells, which express IDO under transcriptional control by IFN- $\gamma$  (11). In the present study, by using a soluble form of CD200 (CD200-Ig), we provide evidence

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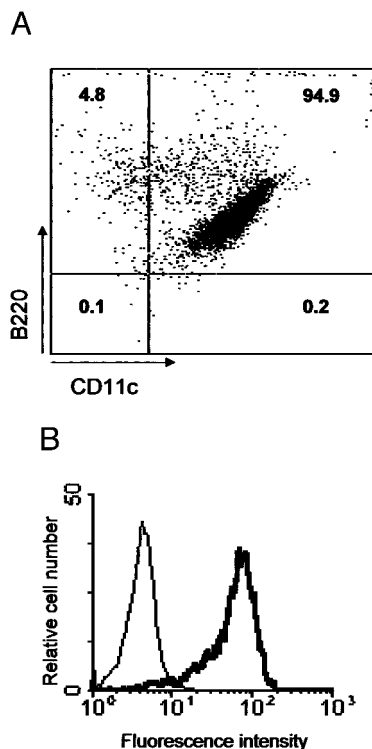
<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; ODN, oligodeoxynucleotide; 1-MT, 1-methyl-D,L-tryptophan.

that otherwise nontolerogenic CD11c<sup>+</sup>B220<sup>+</sup>120G8<sup>+</sup> pDCs are induced by the fusion protein to express IDO-dependent suppressive properties that are contingent upon the autocrine effects of type I IFNs.

## Materials and Methods

### Mice and DC purification

Female DBA/2J mice were obtained from Charles River Laboratories (Calco, Milan, Italy). Mice deficient for the IFN- $\alpha$  receptor (IFN- $\alpha$ BR<sup>-/-</sup>) on an A129 background were obtained as described (30). All *in vivo* studies were done in compliance with National and Perugia University Animal Care and Use Committee guidelines (Perugia, Italy). Splenic DCs were purified by magnetic-activated sorting using CD11c MicroBeads and MidiMacs (Miltenyi Biotec, Bergisch Gladbach, Germany), in the presence of EDTA to disrupt DC-T cell complexes (6, 15). Cells were >99% CD11c<sup>+</sup>, >99% MHC I-A<sup>+</sup>, >98% B7-2<sup>+</sup>, <0.1% CD3<sup>+</sup>, and appeared to consist of 90–95% CD8<sup>-</sup>, 5–10% CD8<sup>+</sup>, and 1–5% B220<sup>+</sup> cells. DC populations were further separated into CD8<sup>-</sup> and CD8<sup>+</sup> fractions by means of CD8 $\alpha$  MicroBeads (Miltenyi Biotec; Refs. 15 and 16). The CD8<sup>-</sup> fraction was ~45% CD4<sup>+</sup> and typically contained <0.5% contaminating CD8<sup>+</sup> cells. Less than 1% CD8<sup>+</sup> and <5% CD8<sup>-</sup> DCs expressed the B220 marker, respectively. For positive selection of B220<sup>+</sup> DCs, CD11c<sup>+</sup> cells were fractionated using B220 MicroBeads (Miltenyi Biotec). More than 95% of the B220<sup>+</sup> cells were stained by 120G8 (see Fig. 1), which is a recently described mAb that selectively recognizes B220<sup>+</sup>Ly6C<sup>+</sup>CD11c<sup>+</sup> pDCs in all lymphoid organs of different mouse strains and has been used to demonstrate strain-specific differences in pDC frequency and IFN- $\alpha$  response *in vitro* and *in vivo* (34). Unless otherwise stated, the pDCs used in this study refer to purified CD11c<sup>+</sup>B220<sup>+</sup> cells. In selected experiments, flow cytometry was used to purify 120G8<sup>+</sup> cells. CD11c<sup>+</sup> cells were stained with rat anti-B220 (CD45R)-PE (BD Pharmingen, San Diego, CA) and 120G8-Alexa 488 for 30 min at 4°C, and 120G8<sup>+</sup> cells were sorted on an EPICS ALTRA (Beckman Coulter, Barcelona, Spain).



**FIGURE 1.** Expression of 120G8 on CD11c<sup>+</sup>B220<sup>+</sup> cells positively selected from the spleens of DBA/2 mice. CD11c<sup>+</sup>B220<sup>+</sup> cells, isolated from total spleen cells by MACS, were assayed by flow cytometry. *A*, Cells were stained with anti-CD11c-FITC and anti-B220-PE. The percentages of cells present in each area are indicated. *B*, Histogram plot showing cells stained with 120G8-Alexa 488 (thick line) or isotype control within the CD11c<sup>+</sup>B220<sup>+</sup> fraction.

### Construction and expression of a fusion protein of CD200 and IgG3Fc (CD200-Ig)

The cDNA encoding the extracellular domain of murine CD200 was generated from splenic T cells stimulated with a combination of plate-bound anti-CD3 and anti-CD28 Abs for 24 h. The cDNA was amplified with primers (sense: 5'-TGCAGCACTAGTATGGGCAGTCTGGTATTC-3'; antisense: 5'-TAAATTGATCAATCCTTTGTCCAGACT-3') containing restriction site *SpeI* (sense) and *BclI* (antisense) sequences. PCR products were digested with the appropriate restriction enzymes and cloned into a modified pEF-BOS plasmid upstream of a region comprising the hinge, CH2, and CH3 domains of murine IgG3 Fc (35). Clones with the correct inserts were stably transfected by electroporation into P815 (clone P511) mastocytoma cells, and supernatants were collected. The fusion protein was affinity-purified by means of protein A-Sepharose and aliquots were stored at -70°C. Fusion protein concentrations in samples were quantified by Coomassie blue staining using a reference scale with known amounts of BSA. In most experiments involving CD200-Ig, native IgG3 was used as a control (35).

### Generation of stable CD200R transfectants

The cDNA encoding murine CD200R was amplified from CD11c<sup>+</sup> cells using sense (5'-CGAAATGTTTTGCTTTTGGAG-3') and antisense (5'-CTTGGTTCTAGATTCCAATG-3') primers and was cloned into the pcDNA3 mammalian expression vector (Invitrogen Life Technologies, San Diego, CA). Clones were introduced into BW5147 thymoma cells by electroporation, and transfectants were selected in G418 (0.5 mg/ml).

### FACS analysis

In all FACS analyses, cells were treated with rat anti-CD16/32 (2.4G2) Ab for 30 min at 4°C to block FcR. Expression of CD11c, B220, and 120G8 was analyzed by the respective use of hamster anti-CD11c-FITC, anti-CD45R/B220-PE, and rat 120G8-Alexa 488. Goat anti-mouse IgG3-PE was from Southern Biotechnology Associates (Birmingham, AL).

### DC treatments

In studies of functional activity, DCs were treated with 5  $\mu$ g/ml CD200-Ig for 24 h at 37°C. Activation of pDCs *in vitro* was achieved by 24-h exposure to 0.2  $\mu$ g/ml oligodeoxynucleotide (ODN) 5'-TCCATGACGTTCTGACGTT-3' (CpG DNA 1826; Invitrogen Life Technologies). The NRP-A7 (KYNKANAFI) peptide was synthesized and purified as described (9). For immunization *in vivo*, cells were loaded with the peptide *in vitro* (5  $\mu$ M, 2 h at 37°C), before irradiation and *i.v.* injection into recipient hosts. A total of  $3 \times 10^5$  CD8<sup>-</sup> DCs were injected either alone or in combination with  $9 \times 10^3$  untreated CD8<sup>+</sup> DCs, or CD200-Ig-treated CD8<sup>+</sup>/CD8<sup>-</sup> DCs or pDCs. The enzyme inhibitor, 1-methyl-D,L-tryptophan (1-MT), was purchased from Sigma-Aldrich (Milan, Italy), and was used *in vitro* at 2  $\mu$ M during cell exposure to CD200-Ig.

### Skin test assay

A skin test assay was used for measuring class I-restricted delayed-type hypersensitivity responses to synthetic peptides, as previously described (9, 16, 36). Peptide-loaded CD8<sup>-</sup> DCs, or combinations of DCs as indicated above, were transferred *i.v.* into recipient hosts that were assayed at 2 wk for the development of peptide-specific reactivity in response to intrafootpad challenge with the peptide. Results were expressed as the increase in footpad weight of peptide-injected footpads over that of vehicle-injected counterparts. Data are the mean  $\pm$  SD for at least six mice per group. The statistical analysis was performed using Student's paired *t* test by comparing the mean weight of experimental footpads with that of control counterparts.

### IDO expression and functional analysis

IDO expression was investigated by immunoblot with rabbit polyclonal anti-murine IDO Ab, as described (16). IDO functional activity was measured *in vitro* in terms of ability of DCs to metabolize tryptophan to kynurenine, whose concentrations were measured by HPLC (6).

### ELISA assessment of IFN- $\alpha$ production

Murine IFN- $\alpha$  was measured by means of specific ELISA (PBL Biomedical Laboratories, Piscataway, NJ). The assay sensitivity was 10–500 pg/ml. Data are the means  $\pm$  SD of triplicate determinations.

## Results

### Binding of CD200-Ig to cell transfectants and splenic DCs

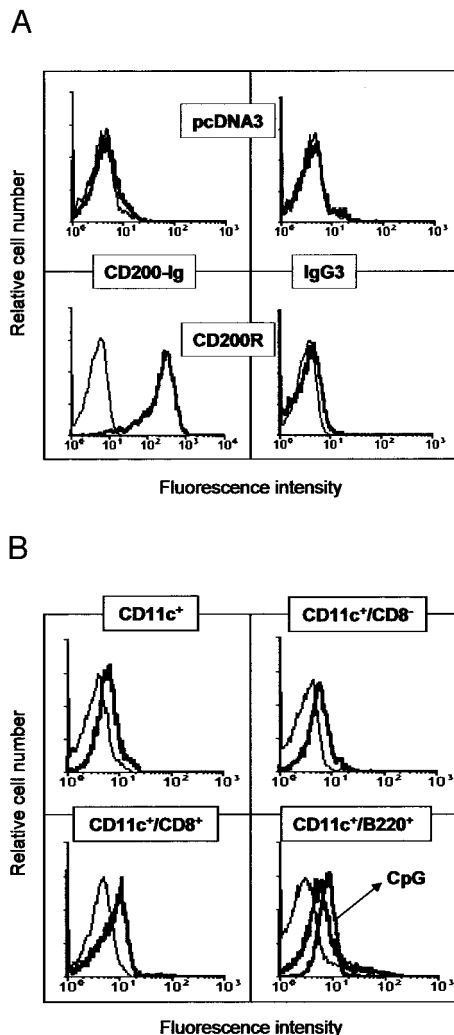
To study the specificity and pattern of CD200-Ig binding to murine cells expressing CD200R, we transfected BW5147 thymoma cells with plasmid containing mouse CD200R, controls involving cells transfected with vector alone. Both types of cell were reacted with 1  $\mu\text{g/ml}$  CD200-Ig or control IgG3, to be assayed by FACS analysis (Fig. 2A). Significant binding was specifically detected with CD200R-expressing cells exposed to CD200-Ig. The examination was extended to splenic DCs and DC subsets (Fig. 2B). Unfractionated DCs, purified CD8<sup>-</sup> and CD8<sup>+</sup> subsets, and pDCs were all incubated with CD200-Ig before staining with anti-mouse IgG3-PE. Because pDCs manifest increased function in response

to viral stimulation (37, 38) or to ODNs containing particular CpG motifs (39), a portion of the pDCs were coexposed to CpG ODN. The results showed that CD200-Ig was capable of binding all types of cells. Binding of CD200-Ig to pDCs was increased by treatment with CpG ODN. In subsequent studies of functional CD200-Ig activity, we made use of pDCs treated with 5  $\mu\text{g/ml}$  CD200-Ig in the presence of 0.2  $\mu\text{g/ml}$  CpG ODN.

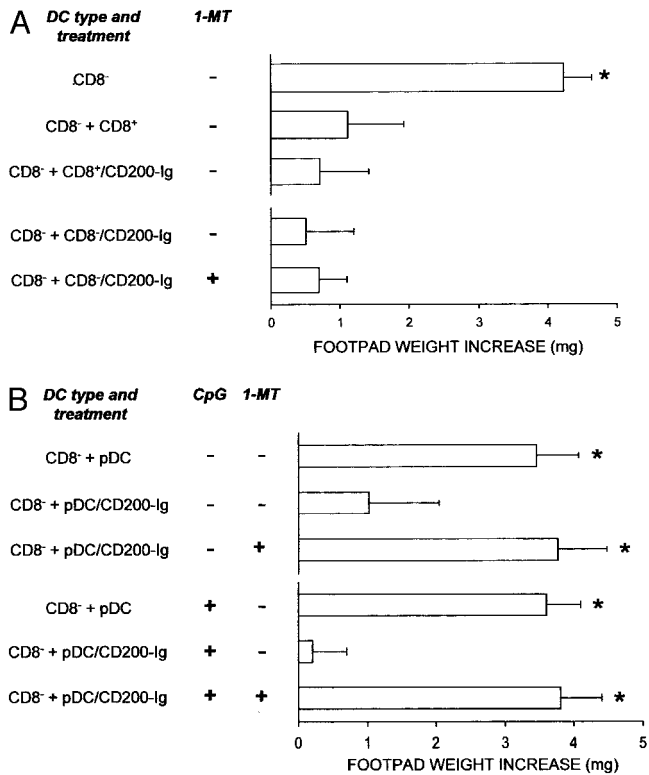
### Functional activity of DC subsets treated with CD200-Ig

Cell populations in the spleens of conventional strains of mice contain variable proportions of mature CD8<sup>-</sup> and CD8<sup>+</sup> DCs that mediate the respective immunogenic and tolerogenic presentation of NRP-A7, a synthetic nonapeptide that acts as a mimotope for autoimmune diabetes in mice (9, 16). Upon transfer into recipient hosts, peptide-loaded CD8<sup>-</sup> DCs initiate immunity, and CD8<sup>+</sup> DCs initiate anergy, when Ag-specific skin test reactivity is measured at 2 wk after cell transfer. The addition of as few as 3% CD8<sup>+</sup> DCs to a population of CD8<sup>-</sup> DCs inhibits priming by the latter cells in this model of class I-restricted reactivity to the synthetic peptide (36, 40). However, otherwise immunogenic CD8<sup>-</sup> DCs become tolerogenic after exposure to the soluble fusion protein CTLA-4-Ig, and such conditioned cells can substitute for CD8<sup>+</sup> DCs in suppressing the induction of immunity by a population of unconditioned CD8<sup>-</sup> DCs (15, 16). The effects of CTLA-4-Ig on CD8<sup>-</sup> DCs are contingent upon the expression of functional IDO (15, 16). Therefore, we became interested in evaluating the effect of CD200-Ig in this experimental setting with NRP-A7.

CD11c<sup>+</sup> DCs were fractionated according to CD8 or B220 expression, and the cells were pulsed with NRP-A7 and transferred into recipient mice to be assayed for Ag-specific skin test reactivity. The otherwise immunogenic CD8<sup>-</sup> DC population was used either alone or in combination with a minority fraction of tolerogenic CD8<sup>+</sup> cells or of DCs (CD8<sup>+</sup>, CD8<sup>-</sup>, or pDCs) conditioned by CD200-Ig treatment. The possible role of IDO in mediating the effect of CD200-Ig was studied by the addition of the enzyme inhibitor, 1-MT, during cell exposure to CD200-Ig. In line with previous results, the default immunogenic properties of CD8<sup>-</sup> DCs were negated by the suppressive function of the CD8<sup>+</sup> subset under conditions of cotransfer, and the activity of CD8<sup>+</sup> cells was not apparently modified by CD200-Ig treatment (Fig. 3A). Although not reported in the figure, we have found that CD200-Ig will indeed reinforce the basal tolerogenic properties of CD8<sup>+</sup> DCs, causing their suppressive effects to overcome the immunogenic potential of IL-12-treated CD8<sup>-</sup> DCs under conditions of cotransfer (36, 40). Fig. 3A also shows that CD200R engagement on CD8<sup>-</sup> DCs imparted suppressive properties to these cells, which were made capable of suppressing the induction of immunity by unconditioned CD8<sup>-</sup> DCs. At variance with the effects of CTLA-4-Ig on CD8<sup>-</sup> cells (15), the effects of CD200-Ig on these cells were not dependent on an intact IDO function. The examination was extended to cotransfer of CD8<sup>-</sup> DCs and pDCs (Fig. 3B). Similar to CD8<sup>-</sup> DCs, CD200R engagement conferred tolerizing ability on pDCs. However, the suppressive properties mediated by CD200R activation on the latter cells were contingent upon functional IDO, as they were negated by the addition of the enzyme inhibitor. One important thing to be noted in these experiments is that treatment of pDCs with CD200-Ig in the absence of CpG ODN still induced suppressive properties in these cells, whereas CpG ODN treatment in the absence of CD200-Ig did not. Thus, CD200 engagement on pDCs endows these cells with suppressive properties that are mediated by the induction of tryptophan catabolism.



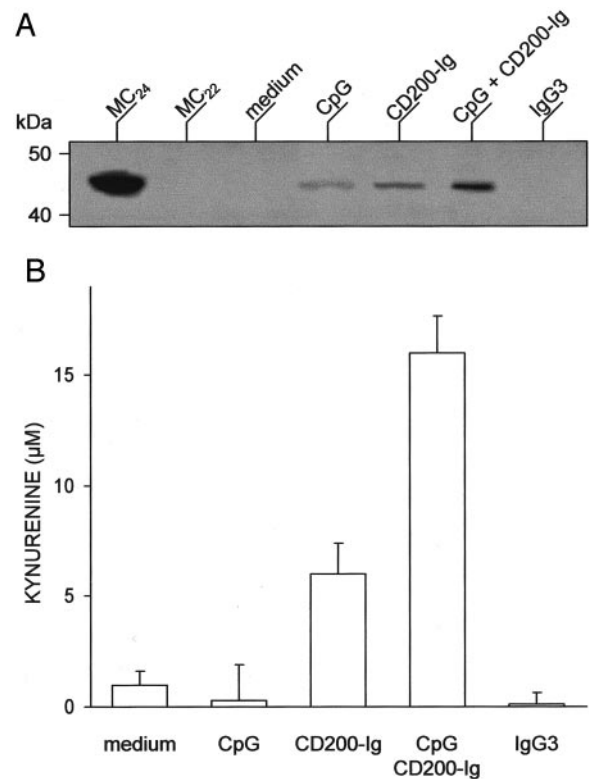
**FIGURE 2.** Cytofluorimetric analysis of CD200-Ig binding to BW5147 transfectants and splenic DCs. *A*, BW5147 cells expressing surface CD200R, as well as control cells transfected with empty plasmid (pcDNA3), were treated with 1  $\mu\text{g/ml}$  CD200-Ig for 30 min on ice, to be reacted with anti-mouse IgG3-PE. Control cells were treated with IgG3 in place of CD200-Ig. Thin lines indicate control cultures treated with anti-mouse IgG3-PE alone. *B*, CD200-Ig binding to unfractionated DCs or subsets thereof. Unfractionated DCs, positively selected CD8<sup>-</sup> or CD8<sup>+</sup> cells, and pDCs were exposed to CD200-Ig as indicated above. For pDCs, CD200-Ig exposure occurred in the presence or absence of CpG ODN. The secondary Ab was anti-mouse IgG3-PE, and controls consisted of cells treated with the secondary reagent alone. No differences were found in the plots of CpG-treated and -untreated cells reacted with the secondary reagent alone.



**FIGURE 3.** CD200R engagement by CD200-Ig confers suppressive properties on pDCs through mechanisms associated with tryptophan catabolism. DCs were fractionated according to CD8 or B220 expression, pulsed with NRP-A7, and transferred into recipient mice to be assayed for skin test reactivity to the eliciting peptide. *A*, The CD8<sup>-</sup> DC fraction was used either alone or in combination with 3% CD8<sup>+</sup> cells or DCs conditioned by CD200-Ig treatment (CD8<sup>+</sup>/CD200-Ig; CD8<sup>-</sup>/CD200-Ig). *B*, The CD8<sup>-</sup> fraction was also used in combination with 3% pDCs either untreated or treated with CD200-Ig (pDC/CD200-Ig). Exposure to CD200-Ig occurred in the presence or absence of CpG. Experimental groups included the use of conditioned DCs treated with 2  $\mu$ M 1-MT during exposure to CD200-Ig. The control treatment for CD200-Ig was IgG3. \*,  $p < 0.001$ , experimental vs control footpads. One experiment representative of three.

#### Ability of CD200-Ig to induce IDO expression and function in pDCs

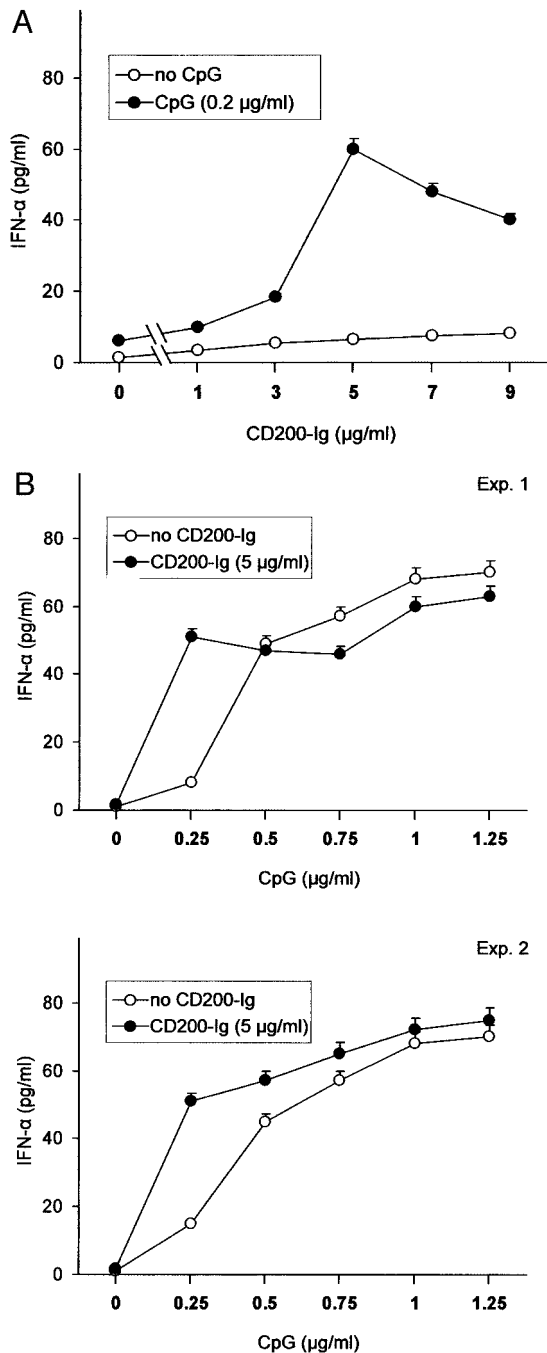
We next examined IDO expression and functional activity in pDCs treated with CD200-Ig. The 120G8<sup>+</sup> cells were purified by FACS as described in *Materials and Methods*. Immunoblot analysis was performed on the sorted 120G8<sup>+</sup> DCs exposed in vitro to CpG ODN, CD200-Ig, or a combination of the two reagents. Expression of IDO protein was studied using a specific Ab. Fig. 4*A* shows that the baseline expression of IDO protein was not altered by control treatment with IgG3 or by CpG ODN alone. Although CD200-Ig treatment would increase IDO expression per se, marked enhancement of enzyme protein was found in pDCs coexposed to CpG and CD200-Ig. On assaying tryptophan conversion to kynurenine (Fig. 4*B*), we found that the differential expressions of IDO protein induced by the different treatments were reflected in distinct abilities to initiate tryptophan catabolism, with CD200-Ig being most effective when combined with CpG treatment. A similar pattern of IDO expression and function, and modulation by CD200-Ig, was observed with CD11c<sup>+</sup>B220<sup>+</sup> DCs purified by magnetic-activated cell sorting (data not shown). It appears therefore that CD200R activation in pDCs is a powerful means of inducing IDO activity, and this property is enhanced by exposure to CpG ODN.



**FIGURE 4.** Ability of CD200-Ig to induce IDO expression and function in 120G8<sup>+</sup> pDCs. *A*, IDO expression was assessed by Western blot analysis. pDCs were treated in vitro with CD200-Ig, either alone or in combination with CpG ODN, IgG3 representing the control treatment. IDO expression was investigated with an IDO-specific Ab. The positive control consisted of IDO-expressing MC<sub>24</sub> transfectants and the negative control consisted of mock-transfected MC<sub>22</sub> cells. Loading controls (not shown) consisted of samples preprobed with  $\beta$ -actin-specific Ab. One experiment of three is shown. *B*, Functional IDO activity in response to CD200-Ig was measured in vitro in terms of the ability to metabolize tryptophan to kynurenine with the use of pDCs treated as indicated above. Kynurenine levels in supernatants were measured by HPLC, and results are the mean  $\pm$  SD of triplicate samples in one of two experiments.

#### Ability of CD200-Ig to modulate IFN- $\alpha$ production in pDCs

The IDO promoter contains a single IFN- $\gamma$ -activated site specific for IFN- $\gamma$  as well as two nonspecific IFN-stimulated response elements, which can respond to IFN- $\alpha$  and IFN- $\beta$  as well as IFN- $\gamma$  (41). Depending on the cell type being cultured, IFN- $\gamma$  has been described as being up to 100 times more potent in inducing IDO expression than IFN- $\alpha$  or IFN- $\beta$  (42). Because of the ability of pDCs to release type I IFNs (37, 38), we measured IFN- $\alpha$  production in pDCs exposed to CD200-Ig. The cytokine was measured by ELISA in supernatants of pDCs cultured with graded concentrations of CD200-Ig in the absence or presence of 0.2  $\mu$ g/ml CpG ODN (Fig. 5*A*) as well as in pDCs exposed to increasing amounts of CpG ODN, with or without 5  $\mu$ g/ml CD200-Ig (Fig. 5*B*). The overall results showed that CD200-Ig induced no significant production of IFN- $\alpha$  when used alone, but its effects were greatly enhanced by 0.2  $\mu$ g/ml CpG ODN, which, in turn, induced little cytokine production per se. Optimal synergic effects were observed on combining this CpG concentration with  $\sim$ 5  $\mu$ g/ml CD200-Ig. Of interest, raising CpG concentrations up to 1.25  $\mu$ g/ml led to IFN- $\alpha$  productions that were not affected, if not reduced, by the copresence of CD200-Ig. In particular, the use of  $\sim$ 1  $\mu$ g/ml CpG-ODN alone yielded IFN- $\alpha$  responses comparable to



**FIGURE 5.** Production of IFN- $\alpha$  in pDCs treated with CD200-Ig and/or CpG ODN. *A*, CD11c<sup>+</sup>B220<sup>+</sup> DCs were exposed for 24 h to different concentrations of CD200-Ig either alone or in the presence of 0.2  $\mu$ g/ml CpG ODN. Culture supernatants were assayed for IFN- $\alpha$  contents by ELISA. Data are representative of three independent experiments. *B*, pDCs were treated with graded concentrations of CpG ODN with or without 5  $\mu$ g/ml CD200-Ig. The results of two independent experiments are shown.

those of 5  $\mu$ g/ml CD200-Ig used in combination with 0.2  $\mu$ g/ml CpG.

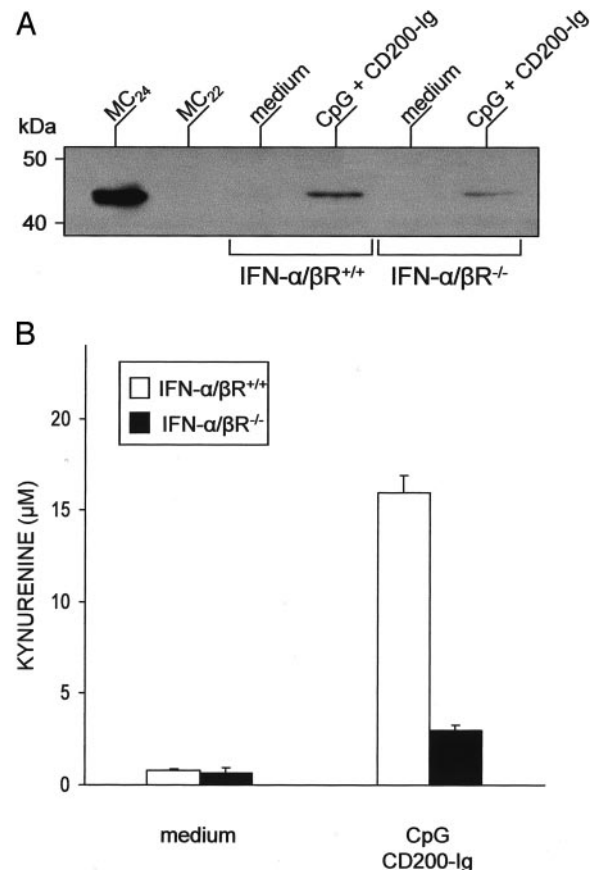
#### Failure of CD200-Ig to induce suppressive properties in pDCs from IFN- $\alpha$ $\beta$ R knockout mice

As shown in Fig. 3*B*, pDCs treated with CD200-Ig in the absence of CpG display suppressive properties. In contrast, the same set of data shows that 0.2  $\mu$ g/ml CpG ODN failed to confer suppressive ability on pDCs when used alone, and CpG was likewise ineffec-

tive when used singly at concentrations of up to 1  $\mu$ g/ml (data not shown). These considerations argue against the possibility of autocrine IFN- $\alpha$  being the single most important factor in determining the effects of CD200-Ig on pDCs. IFN- $\beta$  might contribute to IDO activation and, as a whole, type I IFNs might be necessary, albeit not sufficient, for optimal conditioning of pDCs by CD200-Ig. To investigate the role of autocrine type I IFNs in the effects of CD200-Ig on IDO induction in pDCs, we made use of mice genetically deficient in the expression of type I IFNR. pDCs were purified from the spleens of wild-type and knockout mice, to be used for immunoblot analysis (Fig. 6*A*) as well as assessment of IDO functional activity (Fig. 6*B*). The results showed that the deficiency of IFN- $\alpha$  $\beta$ R negated the induction of IDO protein in pDCs, and also impaired tryptophan conversion to kynurenine. Therefore, type I IFNR signaling appears to play an indispensable role in IDO induction via CD200R engagement in pDCs.

## Discussion

The seminal observation that IDO mediates allogeneic fetal tolerance in mice (4) has recently found mechanistic explanations in the dominant role of regulatory T cells in pregnancy (43) as well as in the ability of these cells to induce IDO expression via B7 signaling



**FIGURE 6.** Failure of CD200-Ig to induce IDO expression and function in pDCs from IFN- $\alpha$  $\beta$ R<sup>-/-</sup> mice. *A*, IDO expression by immunoblot analysis. pDC from wild-type (IFN- $\alpha$  $\beta$ R<sup>+/+</sup>) and knockout (IFN- $\alpha$  $\beta$ R<sup>-/-</sup>) mice were treated in vitro with CD200-Ig plus CpG, MC<sub>24</sub>, and MC<sub>22</sub> transfectants representing the positive and negative controls, respectively. Loading controls (not shown) consisted of samples reprobated with  $\beta$ -actin-specific Ab. One experiment of two. *B*, Functional IDO activity in response to CD200-Ig plus CpG was measured in terms of ability to metabolize tryptophan to kynurenine, using the same mice as above as a source of pDCs. Kynurenine levels in supernatants are means  $\pm$  SD of triplicate samples in one of three experiments.

in DCs (16). Accumulating evidence indicates that IDO-dependent tolerance may be operative not only in pregnancy but also in transplantation (6), autoimmunity (9), and chronic inflammatory diseases (44). There is also evidence to indicate that the same immunoregulatory molecules and mechanisms contribute to maintenance of tolerance in pregnancy and transplantation (2), with possible contributions to successful human pregnancies by CTLA-4 (26), B7 (27), and CD200 (28).

In the mouse, pDCs have been characterized as CD11c<sup>low</sup>B220<sup>high</sup>Gr1<sup>low</sup> cells, capable of producing IFN- $\alpha$  in response to viral stimulation and exhibiting plasmacytoid morphology (30, 32, 45). Autocrine IFN- $\alpha$  signaling in human and murine pDCs has also been reported (46, 47). Recently, a novel rat mAb, 120G8, has been described that recognizes mouse pDCs that produce high amounts of IFN- $\alpha$  in response to viral stimulation and display a phenotype identical with that of the previously described mouse pDCs (34). In the present study, we found that mouse CD11c<sup>+</sup>B220<sup>+</sup>120G8<sup>+</sup> pDCs that bind a soluble form of CD200 respond to CD200R engagement with increased IDO expression and function, which events underlie the emergence of strong immunosuppressive properties in those cells.

However, the induction of IDO-dependent suppressive properties by CD200-Ig treatment was not confined to pDCs. Conventional CD8<sup>+</sup> and CD8<sup>-</sup> DCs were also affected by CD200R engagement. Although not described in the present study, we have found that, similar to IFN- $\gamma$  (48) and CTLA-4-Ig (15), CD200-Ig may indeed act as an enforcer of the default tolerogenic properties of CD8<sup>+</sup> DCs, causing their suppressive effects to prevail over the adjuvant activity of IL-12 acting on CD8<sup>-</sup> DCs (36, 40). With the CD8<sup>-</sup> DCs, we found in this study that the otherwise immunogenic subset could be rendered tolerogenic by CD200-Ig, again resembling the effects of CTLA-4-Ig (15). However, different from the latter, the suppressive properties imparted to CD8<sup>-</sup> DCs by CD200-Ig were not dependent on IDO, suggesting that multiple mechanisms might contribute to the functional plasticity of this DC subset. Although a minority fraction (1–5%) of B220<sup>+</sup> pDCs appeared to contaminate the purified CD8<sup>+</sup> and CD8<sup>-</sup> DC subsets, it is unlikely that pDCs contributed significantly to the tolerizing effects of CD8<sup>+</sup> or CD200-Ig-treated CD8<sup>-</sup> DCs, which were not dependent on IDO activity.

In contrast, the tolerogenic properties conferred by CD200R engagement on pDCs were strictly dependent on an intact IDO function. The mechanisms underlying IDO induction by CD200R engagement in pDCs have not been completely clarified by our current data. Similar to but less potently than IFN- $\gamma$ , type I IFNs are known to induce expression of the IDO gene. Therefore, a likely mechanism of action of CD200-Ig appeared to be autocrine signaling by type I IFNs. However, several considerations suggest that, at least for IFN- $\alpha$ , the cytokine may not be sufficient per se to account for IDO induction by the fusion protein. First, the production of IFN- $\alpha$  induced by CD200-Ig was virtually undetectable in the absence of CpG treatment, and yet pDCs were made tolerogenic by the fusion protein in the absence of ODN. Second, CpG concentrations that resulted in IFN- $\alpha$  production comparable to that of combined treatment with CD200-Ig and CpG did not trigger suppressive properties in DCs. Third, CpG treatment appeared to increase the binding of CD200-Ig to pDCs, suggesting that CpG, at least at the low dosages used in this study, might act primarily to augment CD200R expression. It is known that CD200R associates with SHIP, consistent with a role in the down-regulation of myeloid cell activity (23). Besides synergizing with CpG ODN in inducing IFN- $\alpha$  production, it is possible that CD200R engagement influences the multiple mechanisms that preside over expression of the IDO gene.

Although IFN- $\alpha$  may not be sufficient to activate suppressive properties in pDCs exposed to CD200-Ig, type I IFNs appeared to be necessary for the induction of IDO activity in this setting. Plasmacytoid DCs from mice genetically deficient in the expression of the IFN- $\alpha$ BR manifested limited or no activation of IDO in response to CD200-Ig. IFN- $\alpha$  production was also severely impaired in these mice when treated with CD200 in the presence of CpG ODN (data not shown). This might emphasize the occurrence of an autocrine feedback loop in IFN- $\alpha$  production whereby the cytokine promotes its own release (46). Type I IFNs have antiviral, cytostatic, and prominent immunomodulatory effects, which are all of great importance during viral infections. Our current data of IFN- $\alpha$ BR-dependent ability of pDCs to activate the immunosuppressive pathway of tryptophan catabolism may expand upon the general properties of IFN-producing pDCs. It is tempting to speculate that the tolerogenic properties of pDCs may be particularly important in the context of the local inflammatory reaction associated with viral infection and type I IFN production (49). Under conditions in which IFNs mediate IDO-dependent and -independent antiviral effects (50), pDCs may be instrumental in preserving tolerance to self because of the broad distribution of CD200.

The early studies in the mouse have implicated CD200R in the regulation of normal macrophage/myeloid function (18, 20). Consistent with these studies, CD200R was strongly expressed on peripheral blood monocytes and splenic macrophages. However, in the human, CD200R has been found on the majority of monocyte/myeloid lineage cells in the peripheral blood. In addition to neutrophils and monocytes, CD200R was strongly expressed on basophils and DCs derived from monocytes (19). In murine DCs, recent evidence indicated that the expression of CD200 increases as these cells undergo apoptosis, suggesting that up-regulation of CD200 may represent a novel mechanism whereby immune reactivity to apoptosis-associated self-Ags is suppressed under steady state conditions (21).

In conclusion, the current study demonstrates that mouse DCs express a functional CD200R capable of binding a soluble form of CD200. Receptor engagement in CD8<sup>+</sup>, CD8<sup>-</sup>, and pDCs initiates events that either reinforce (CD8<sup>+</sup>) or induce (CD8<sup>-</sup>, pDCs) tolerogenic functions in these cells. For CD8<sup>-</sup> DCs, the pattern is similar to that induced by CTLA-4 engagement of B7 molecules, one major difference being that CD200-Ig effects are not dependent on an intact IDO function. The induction instead of suppressive properties in pDCs via CD200R engagement requires functional IDO and type I IFNR signaling. Although IFN- $\alpha$  may contribute to the effects of CD200R activation, it is unlikely that autocrine IFN- $\alpha$  signaling is responsible alone for IDO induction in CD200R-activated pDCs. Although pDCs have previously been described as being capable of expressing functional IDO (7), the current study emphasizes that a variety of ligands for DC receptors acting on a variety of DC subtypes may be induced to express IDO-dependent tolerance mechanisms.

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