FeRγ-Chain ITAM Signaling Is Critically Required for Cross-Presentation of Soluble Antibody–Antigen Complexes by Dendritic Cells


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FcRγ-Chain ITAM Signaling Is Critically Required for Cross-Presentation of Soluble Antibody–Antigen Complexes by Dendritic Cells

Peter Boross,* Nadine van Montfoort,†,‡ Daphne A. C. Stapels,* Cees E. van der Poel,* Christian Bertens,* Jan Meeldijk,*,‡ J. H. Marco Jansen,* J. Sjef Verbeek,§ Ferry Ossendorp,† Richard Wubbolts,‡ and Jeannette H. W. Leusen*

The uptake of Ag–Ab immune complexes (IC) after the ligation of activating FcγR on dendritic cells (DC) leads to 100 times more efficient Ag presentation than the uptake of free Ags. FcγRs were reported to facilitate IC uptake and simultaneously induce cellular activation that drives DC maturation and mediates efficient T cell activation. Activating FcγRs elicit intracellular signaling via the ITAM domain of the associated FcγR-chain. Studies with FcγR-chain knockout (FcγRγ−/−) mice reported Fcγ-chain ITAM signaling to be responsible for enhancing both IC uptake and DC maturation. However, Fcγ-chain is also required for surface expression of activating FcγRs, hampering the dissection of ITAM-dependent and independent FcγR functions in FcγRγ−/− DCs. In this work, we studied the role of Fcγ-chain ITAM signaling using DCs from NOTAM mice that express normal surface levels of activating FcγR, but lack functional ITAM signaling. IC uptake by bone marrow–derived NOTAM DCs was reduced compared with wild-type DCs, but was not completely absent as in FcγRγ−/− DCs. In NOTAM DCs, despite the uptake of ICs, both MHC class I and MHC class II Ag presentation was completely abrogated similar to FcγRγ−/− DCs. Secretion of cytokines, upregulation of costimulatory molecules, and Ag degradation were abrogated in NOTAM DCs in response to FcγR ligation. Cross-presentation using splenic NOTAM DCs and prolonged incubation with OVA-IC was also abrogated. Interestingly, in this setup, proliferation of CD4+ OT-II cells was induced by NOTAM DCs. We conclude that Fcγ-chain ITAM signaling facilitates IC uptake and is essentially required for cross-presentation, but not for MHC class II Ag presentation. The Journal of Immunology, 2014, 193: 5506–5514.

Dendritic cells (DCs) are specialized APCs equipped to effectively induce immune responses. DCs can capture, internalize Ag by several mechanisms, and respond to diverse activation signals, which induce their maturation. The internalized Ag is processed and finally presented in the context of MHC molecules eliciting Ag-specific T cell responses.

DCs can be activated by molecular patterns from pathogens or by endogenous danger signals. It was previously shown that preformed Ag–Ab immune complexes (ICs) efficiently activate DCs and greatly enhance the efficacy of Ag presentation in vitro (1–3).

Importantly, this was recently confirmed by in vivo formed ICs using mice with pre-existent circulating Abs, underscoring the physiological relevance of this mechanism (4).

Interaction of IgG-ICs and FcγRs induces various effector mechanisms depending on the type of FcγR (5). Mouse DCs express three activating FcγRs (FcγRI, FcγRIII, and FcγRIV) and the inhibitory FcγRIIB. The activating FcγRs associate with the Fcγ-chain that initiates signal transduction via the ITAM. ITAM signaling involves the phosphorylation of its tyrosine residues by Src family kinases, which then provides a docking site for the protein tyrosine kinase Syk. Syk activation results in phosphorylation of ERK, influx of extracellular calcium, and ultimately NF-κB activation (6). The inhibitory FcγRIIB is a single-chain receptor that contains an ITIM in its intracellular tail. The balance between activating versus inhibitory FcγR signaling determines the magnitude of the effector response.

Studies with FcγR-chain knockout (FcγRγ−/−) mice showed that IC-enhanced Ag presentation by DCs requires activating FcγRs (1, 7). In contrast, signaling via FcγRIIB inhibits Ag presentation by DCs both in vitro (8) and in vivo (2).

Uptake of ICs by APCs normally results in Ag presentation on MHC class II (MHC II) molecules; however, DCs are equipped to also present these Ags on MHC class I, a process called cross-presentation (9). The mechanism of FcγR-mediated enhancement of cross-presentation is not fully understood. FcγR-mediated IC uptake is very efficient and results in routing of the Ag into specialized intracellular compartments that allow prolonged Ag presentation (10). Furthermore, it was shown that interaction between activating FcγR and IC leads to DC maturation (3) and requires components of Fcγ-chain ITAM signaling cascade, such

*Laboratory for Translational Immunology, University Medical Center Utrecht, 3584 CX Utrecht, the Netherlands; †Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2300 RC Leiden, the Netherlands; ‡Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, the Netherlands; and §Department of Human Genetics, Leiden University Medical Center, 2333 ZA Leiden, the Netherlands.

Current address: Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

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Address correspondence and reprint requests to Dr. Jeannette H.W. Leusen, Immunotherapy Laboratory, F03.821, Laboratory for Translational Immunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands. E-mail address: j.leusen@umcutrecht.nl

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Abbreviations used in this article: BM, bone marrow; BM-DC, BM-derived DC; BMDM, BM-derived macrophage; DC, dendritic cell; IC, immune complex; MHC I, MHC class I; MHC II, MHC class II; WT, wild-type.

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as Syk (11). Syk−/− DCs exhibit abrogated IC internalization and DC activation by ICs (11). This suggests that FcRγ-chain signaling is important in Ag presentation; however, Syk deletion also impairs the signaling of other ITAM-dependent receptors, such as integrins (12). Several studies used FcRγ−/− mice to demonstrate the importance of FcγR signaling, but these mice do, however, lack surface expression of all activating FcγRs. Therefore, the specific role of FcRγ-chain ITAM in Ag presentation could not be studied.

In this study, we investigated the specific requirement for FcRγ-chain ITAM signaling for FcR-facilitated presentation of IC-processed Ag, whereas surface expression of FcγR was normal. To this end, we used DCs derived from previously generated NOTAM mice. These mice express normal levels of activating FcγRs, but, due to a mutation in their FcRγ-chain, ITAM are incapable of canonical FcγR signaling (13).

We show that NOTAM DCs can internalize soluble OVA-IC, albeit less efficient compared with wild-type (WT) DCs. In addition, despite IC internalization and Ag delivery at levels compatible with presentation in the WT cells, Ag presentation on both MHC class I (MHC I) and MHC II in vitro was completely abrogated similar to FcRγ−/− DCs in line with signal-dependent facilitation of the Ag delivery process for both MHC class I and MHC class II.

Materials and Methods

Mice
C57BL/6j/doco mice were obtained from Janvier (Le Genest Saint Isle, France). FcRγ−/−, NOTAM (13), OT-I, and OT-II mice on C57BL/6 background were maintained in the Animal Facility of University Medical Center Utrecht. All experiments were approved by the Animal Ethical Committee of the University Medical Center Utrecht.

Cell culture
A total of 3 × 10^6 bone marrow (BM) cells was seeded in 10-cm plastic petri dishes in 10 ml D1 medium containing IMDM (Biowhitaker) supplemented with 50 μM 2-ME, 200 μM Ultraglutamine I (Biowhitaker), Pen/Strep (Life Technologies), 10% FCS, and 30% conditioned supernatant from GM-CSF–producing NIH 3T3 cells (RII) (14). On day 3, 10 ml fresh medium was added to the dish. On days 6 and 8, the medium was partially refreshed, and, on day 9, the cells were reseeded in fresh medium at 3 × 10^6 DC/ml. On day 13, semiadherent cells were collected and used for experiments.

B3Z T cells, a C57BL/6-derived mouse hybridoma specific for OVA-peptide SIINFEKL in MHC I context (H-2Kb), were cultured in IMDM (Life Technologies, Invitrogen) supplemented with 10% FCS, Pen/Strep, and 500 μg/ml hygromycin B (Invitrogen) (15). BO.97.10.3, a C57BL/6-derived mouse T cell line, recognizing the OVA257-264 epitope in the context of MHC II (I-Ab), was cultured in RPMI 1640 supplemented with 10% FCS and Pen/Strep.

To generate BM-derived macrophages (BMDMs), after the lysis of the erythrocytes, 3 × 10^6 BM cells were seeded in a 6-well plate. Cells were cultured in RPMI 1640 (Life Technologies) with 10% heat-inactivated FCS, Pen/Strep, and 10 ng/ml GM-CSF (Immunex, Seattle, WA). Medium was refreshed on days 2 and 5. At day 8, the adherent cells were harvested with 50 mM EDTA.

Bacterial phagocytosis assay
Phagocytosis of opsonized Streptococcus pneumoniae was measured in a FACS-based assay, as described previously (16). Briefly, opsonization was achieved by a human IgG1 Ab specific for S. pneumoniae, provided by G. Vidarsson (Sanquin, Amsterdam). BM-derived macrophages (2 × 10^6) and 5 μl human IgG1-opsonized S. pneumoniae (serotype 6A) were added together in a total volume of 55 μl using a 96-well plate format. Binding was allowed at 4°C for 1 h, after which the cells were washed once with cold RPMI 1640 medium. Half of the cells were placed at 37°C for 30 min to allow phagocytosis; the other half was kept cold. Binding of bacteria was determined by FACS using a PE-conjugated anti-human IgG Ab. Phagocytosis was defined as the difference between the signals of anti-human IgG1 Ab at 4°C and 37°C.

IC formation
ICs were formed by incubating 100 μg/ml OVA (Worthington) and 400 μg/ml rabbit anti-OVA (Sigma-Aldrich; C6543) in a total volume of 100 μl (RPMI 1640) at 37°C for 30 min. This was used as stock OVA-IC at a concentration of 100 μg/ml and was diluted further. For confocal microscopy and OVA degradation experiments, we used Alexa594- or Alexa488-labeled OVA, respectively (Molecular Probes; O-34781 and O-34783).

IC binding and internalization
A total of 2 × 10^5 DCs was incubated in a U-bottom 96-well plate with different concentrations of OVA-ICs in a total volume of 100 μl for 4 h on ice to measure binding. To measure uptake, BM-derived DCs (BM-DCs) were first incubated with OVA-ICs for 4 h on ice, followed by 1 h at 37°C. To discriminate between binding and uptake, extracellular fluorescence was quenched using trypan blue, and samples were measured on FACS-Calibur and analyzed using CellQuest software.

FIGURE 1. Delayed IC uptake by BM-DCs in the absence of FcRγ-chain ITAM signaling. (A) WT, FcRγ−/−, and NOTAM BM-DCs were analyzed by flow cytometry for FcγR expression. BM-DCs were incubated with OVA-IC for 4 h at 4°C for binding (B) or an additional 1 h at 37°C for uptake (C). Mean ± SD is shown.
**In vitro Ag presentation**

To determine Ag-specific MHC II-mediated activation, BO.97.10.3 T cells that carry a TCR recognizing the I-A\(^\alpha\)/OVA\(_{323-339}\) complex were used (3). A total of 2 \(\times\) 10\(^5\) BM-DCs was incubated with OVA-ICs for 4 h at 37°C in flat-bottom 96-well plates (NUNC). After washing once with RPMI 1640, 4 \(\times\) 10\(^5\) BO.97.10.3 cells were added per well and incubated for 48 h, followed by IL-2 determination in the supernatants.

For MHC I-mediated activation, 5 \(\times\) 10\(^5\) BM-DCs were incubated with various concentrations of ICs for 4 h at 37°C in flat-bottom 96-well plates. After washing once, BM-DCs were incubated with 4 \(\times\) 10\(^5\) B3Z cells overnight. B3Z cells are specific for OVA peptide presented by MHC I and carry a LaZ gene after an IL-2 promoter (15). Ag presentation efficacy was determined after cell lysis and measuring \(\beta\)-galactosidase activity. As a positive control, we used the SIINFEKL peptide or OVA protein.

**Ex vivo Ag presentation**

To determine the Ag-presenting capacity after IC uptake of ex vivo sorted DCs, CD11b\(^+\)/CD11c\(^{\text{bright}}\) splenocytes from WT, FcR\(^{\gamma}\), and NOTAM mice were sorted using FACS AriaII. A total of 10\(^5\) splenic DCs was plated in round-bottom 96-well plates (NUNC) in IMDM supplemented with 10% FCS and Pen/Strep. For MHC II Ag presentation, 1 \(\mu\)g/ml SIINFEKL, 250 \(\mu\)g/ml OVA, or 0.2 \(\mu\)g/ml OVA-IC was added. For cross-presentation, 1 ng/ml SIINFEKL, 250 \(\mu\)g/ml OVA, and 0.02 \(\mu\)g/ml OVA-IC were added. OVA-specific CD4\(^+\) and CD8\(^+\) positive T cells were isolated from the spleens of OT-I and OT-II mice using a CD4 or CD8 T cell isolation kit, according to manufacturer’s instructions (Miltenyi Biotech). OT-I spleen contained \(\approx 97\%\) CD8\(^+\) cells within the CD3\(^+\) fraction, which was enriched to 99.6% after MACS sorting. OT-II spleen contained \(\approx 50\%\) CD4\(^+\) cells within the CD3\(^+\) fraction, and this was enriched to \(\approx 75\%\) after MACS sorting. T cells were labeled with 0.5 \(\mu\)M CellTrace violet (Life Technologies) according to the manufacturer’s instructions. A total of \(\times\) 10\(^5\) T cells was added per well (DC:T ratio of 1:5) and incubated for 4 d. Cells were harvested and labeled with CD3-PE, CD4-PE-Cy7, and Pen/Strep. For MHC II Ag presentation, 1 \(\mu\)g/ml SIINFEKL, 250 \(\mu\)g/ml OVA, and 0.02 \(\mu\)g/ml OVA-IC were added. OVA-specific CD4\(^+\) and CD8\(^+\) positive T cells were isolated from the spleens of OT-I and OT-II mice using a CD4 or CD8 T cell isolation kit, according to manufacturer’s instructions. A total of \(\times\) 10\(^5\) T cells was added per well (DC:T ratio of 1:5) and incubated for 4 d. Cells were harvested and labeled with CD3-, CD4-, and CD8-specific Abs, respectively. The dilution of the dye as measure of proliferation specifically in either the CD4\(^+\) or CD8\(^+\) populations was measured by FACSCantoII and analyzed using FACSDiva software.

**IL-2 ELISA**

Maxisorp plates (NUNC) were coated with 2 \(\mu\)g/ml anti–IL-2 Ab (BD Pharmingen; 554426), in combination with streptavidin-HRP and ABTS substrate. Biotin-conjugated Abs were detected using 2 \(\mu\)g/ml biotin-conjugated anti–IL-2 Ab (BD Pharmingen; 5508 FcR\(^{\gamma}\)). Absorbance at 405 nm was measured for IL-2 determination in the supernatants.

**Statistical analysis**

Parametric data were compared using Student \(t\) test. Significance levels were indicated as follows: *\(p<0.05\), **\(p<0.01\), and ***\(p<0.001\). All error bars represent the SD of the mean.

**Results**

**Normal IC binding, but decreased IC uptake by DCs in the absence of FcRγ-chain ITAM signaling**

To study the role of FcRγ-chain ITAM signaling in Ag presentation, we used BM-DC cultured from NOTAM mice. In these mice, the FcRγ-chain ITAM motif is mutated, resulting in normal surface expression of activating FcγR, but abrogated effector functions, such as Ab-dependent cell-mediated cytotoxicity or secretion of reactive oxygen intermediates (13). WT and FcγR\(^{-}\)-chain knockout (FcγR\(^{-}\)) were used as controls. Flow cytometric

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**Figure 2.** Abrogated MHC II Ag presentation of OVA-ICs in NOTAM BM-DCs. WT, FcγR\(^{-}\), and NOTAM BM-DCs were incubated with OVA\(_{323-339}\) peptide (A), soluble OVA (B), or OVA-IC (C) for 4 h. Thereafter, BM-DCs were washed, and OVA-specific BO.97.10.3 cells were added and incubated for 2 d. IL-2 from the supernatant was measured for T cell activation. Mean \(\pm\) SD is shown. Representative data of five independent experiments.
To investigate the requirement for FcγR-chain ITAM signaling during Ag presentation, we tested the ability of NOTAM BM-DCs to present Ag in the context of MHC II after uptake of OVA-IC. We used T cell clones (BO.97.10.3) that specifically recognize OVA-derived peptides in the context of MHC II, and the efficacy of Ag presentation was measured by the secretion of IL-2 to the supernatant. Ag presentation of the OVA323–339 peptide and soluble OVA was equally efficient in the three types of BM-DCs (Fig. 2A). However, MHC II presentation of OVA-IC was absent in NOTAM and FcγR

\[ \text{WT} \quad \text{FcγR}\text{R}/\text{−} \quad \text{NOTAM} \]

**FIGURE 3.** Abrogated MHC I Ag presentation of OVA-ICs in NOTAM BM-DCs. WT, FcγR

\[ \text{WT} \quad \text{FcγR}\text{R}/\text{−} \quad \text{NOTAM} \]

BM-DCs were incubated with OVA-derived MHC class I immunodominant peptide (SIINFEKL) (A), soluble OVA (B), or OVA-IC (C) for 4 h. Thereafter, BM-DCs were washed; OVA-specific B3Z cells were added and incubated overnight to measure T cell activation (see Materials and Methods). Mean ± SD is shown. Representative data of five independent experiments.

**MHC class II and class I Ag presentation by BM-DCs after OVA-IC uptake is fully abrogated in the absence of FcγR-chain ITAM signaling**

It is known that, next to MHC II presentation, uptake of ICs by FcγR leads to efficient cross-presentation to CD8+ cytotoxic T lymphocytes in the context of MHC I (1). Therefore, we next assessed MHC I-mediated presentation by BM-DCs after IC uptake. All three genotypes of BM-DCs were equally potnet in T cell activation when the immunodominant peptide (SIINFEKL) (Fig. 3A) or high concentrations of free OVA were used (Fig. 3B), the uptake of which is FcγR independent, but rather mannose receptor dependent (19). This indicates that BM-DCs of all genotypes were capable to cross-present. Thousand-fold lower amounts of ICs were induced Ag presentation by WT BM-DCs, but not by FcγR

\[ \text{WT} \quad \text{FcγR}\text{R}/\text{−} \quad \text{NOTAM} \]

BM-DCs (Fig. 3C).

To further establish the essential role of FcγR in Ag presentation, OVA-ICs were disrupted by sonication and tested for their ability to induce Ag presentation. Indeed, Ag presentation was severely diminished upon sonication, further confirming the requirement for intact ICs and excluding the argument that released OVA would be sufficient for Ag delivery (Supplemental Fig. 3).

Together, these data show that FcγR-chain ITAM signaling is essentially required for both MHC II and MHC I presentation of internalized Ags.
Lack of DC activation in the absence of Fcγ-chain ITAM stimulus

FcγRs are required for IC-induced DC maturation (1, 8). Therefore, we assessed DC activation after stimulation with ICs or LPS for 48 h. After IC stimulation, the activation markers CD40 and CD86 were upregulated in WT BM-DCs, but not in NOTAM BM-DCs. However, NOTAM BM-DCs responded similarly to activation by the TLR ligand LPS (Fig. 4A).

WT BM-DCs secrete various cytokines and chemokines after FcγR stimulation, which is greatly diminished in FcγR⁻/⁻ BM-DCs (8). WT BM-DCs secreted MIP-1α, IL-6, and IL-2 after 48-h stimulation with ICs, which was completely abrogated by FcγR⁻/⁻ BM-DCs in line with earlier findings (Fig. 4B). Secretion of these cytokines was also fully abrogated by NOTAM BM-DCs (Fig. 4B).

Taken together, these data suggest that DC activation is abrogated in NOTAM BM-DCs.

Abrogated endosomal routing of ICs in NOTAM BM-DCs after FcγR-mediated uptake

After FcγR targeting on BM-DCs, OVA-ICs are transported to late endosomes, as identified by the presence of LAMP-1 (10, 20–22). The intracellular localization of the Ag is crucial for entering the Ag presentation pathway (9). We performed confocal microscopy studies to examine the role of Fcγ-chain ITAM signaling in intracellular trafficking of OVA. We generated ICs using Alexa594-labeled OVA and determined its localization in all three genotypes of BM-DCs after 4 h of uptake.

In WT BM-DCs, OVA was localized intracellularly and colocalized with LAMP-1-positive late endosomes (Fig. 5) and HLA-DM-positive endosomes (Supplemental Fig. 4). MHC II was transferred to the membrane typical of activated DCs. FcγR⁻/⁻ BM-DCs did not take up OVA at all; sporadically, we observed bound OVA indicated by extracellular signal. In line with our flow cytometric analysis, NOTAM BM-DCs exhibited binding and uptake; however, OVA did not colocalize with LAMP-1–positive compartments. Furthermore, MHC II remained intracellularly after IC challenge, in line with the abrogated activation by FcγRs observed by flow cytometry.

These results confirm that uptake of OVA-IC is greatly reduced in NOTAM BM-DCs. In addition, the results suggest that the internalized OVA-IC is routed toward different compartments in NOTAM DCs compared with WT DCs. These data suggest that the trafficking of OVA-IC to late endosomes after FcγR-mediated uptake is dependent on Fcγ-chain ITAM signaling.

Abrogated Ag degradation in the absence of Fcγ-chain ITAM signaling

In contrast to macrophages, DCs degrade Ag in a regulated manner, which enables efficient presentation of antigenic peptides even days after Ag uptake in the secondary lymphoid organs (23). It was demonstrated that OVA-IC internalized by activating FcγRs is routed to specialized intracellular locations in DCs (Ag depots), and OVA remains incompletely degraded for a few days (10).

In line with this, we observed that OVA-ICs were bound efficiently and partially degraded within hours by WT BM-DCs indicated by the appearance of a dominant cleavage product (indicated by lower arrow) (Fig. 6A). FcγR⁻/⁻ BM-DCs exhibited decreased binding at 4°C, confirming the results obtained by flow cytometry (Fig. 6A, middle panel). Only limited OVA degradation was observed after overnight incubation. NOTAM BM-DCs were able to bind OVA-ICs at 4°C similar to WT BM-DCs; however, OVA degradation was comparable to FcγR⁻/⁻ BM-DCs (Fig. 6A, right panel).

These results together with those showing internalization of OVA-ICs by NOTAM BM-DCs suggest that Fcγ-chain ITAM signaling is required for OVA degradation after FcγR-mediated uptake.

Ag presentation by splenic DCs

To verify the results obtained with BM-DCs, we also performed Ag presentation experiments with ex vivo sorted splenic DCs from WT, FcγR⁻/⁻, and NOTAM mice. Splenic DCs were incubated with OVA-IC (and peptide and OVA as control) and freshly isolated OT-I and OT-II T cells labeled with CellTrace violet. After 4 d, proliferation of T cells was assessed by cyt fluorimetry using CellTrace violet dilution.

MHC II Ag presentation of the OVA323–339 peptide or high concentrations of free OVA was equally efficient in all three strains (Fig. 7A). In contrast to WT DCs, MHC II Ag presentation of OVA-IC was absent in FcγR⁻/⁻ DCs. Surprisingly, NOTAM DCs were able to induce proliferation of CD4⁺ OT-II T cells, suggesting that Fcγ-chain ITAM signaling is not essentially required for MHC II Ag presentation (Fig. 7).

![FIGURE 4. Abrogated DC maturation in the absence of Fcγ-chain ITAM signals.](http://www.jimmunol.org/)

**A** WT, FcγR⁻/⁻, and NOTAM BM-DCs were incubated for 48 h with 1 μg/ml ICs or LPS. Expression of the maturation markers CD40 and CD86 was assessed by flow cytometry and compared with the expression on the same cells before stimulation. **B** WT, FcγR⁻/⁻, and NOTAM BM-DCs were incubated for 48 h with 1 μg/ml ICs. Supernatants were collected after 48 h to assess cytokine secretion. The levels of indicated cytokines were measured by Lumexin bead array.
All three types of DCs induced equal proliferation of CD8+ OT-1 T cells when incubated with SIINFEKL peptide of OVA (Fig. 8). However, presentation of OVA-IC was abrogated by both FcRγ2/2 and NOTAM DCs, demonstrating that cross-presentation of OVA-IC essentially requires FcRγ-chain ITAM signaling.

Discussion
Several studies using FcRγ2/2 DCs have suggested that FcRγ-chain ITAM signaling is important in Ag presentation. However, these DCs also lack surface expression of FcγRs, and therefore are not optimal to specifically study the function of FcRγ-chain ITAM. In this study, we used the unique NOTAM mouse model to specifically address the contribution of FcRγ-chain ITAM motif in the context of intact FcγR expression.

We showed that FcRγ-chain ITAM signaling in BM-DCs is required to facilitate Ag presentation of IC-derived Ags by both MHC II and MHC I. The role of FcRγ-chain ITAM signaling is 2-fold: it enhances uptake of ICs and induces DC activation. Whereas FcRγ2/2 DCs showed abrogated IC uptake, NOTAM DCs exhibited IC uptake, albeit with lower efficacy than WT BM-

FIGURE 5. Intracellular localization of OVA-IC. WT BM-DCs were incubated with 1 μg/ml OVA-Alexa562-IC for 4 h at 37˚C, fixed, permeabilized, and stained for MHC II and LAMP-1, followed by confocal microscopy analysis. Left panels, bright field images; right panels, overlay images. LAMP1 Ab: purple; OVA: red; MHCII Ab: green; original magnification ×63.

FIGURE 6. Degradation of OVA-IC. (A) OVA-Alexa488 (20 μg/ml) or OVA-Alexa488-ICs (1 μg/ml) were incubated with BM-DCs for the indicated time points. BM-DCs were washed, lysed, and imaged with fluorescence imager. Signal indicates bound or intracellular degraded OVA. (B) Quantification of the percentage of degraded OVA using ImageQuant.
DCs. However, Ag presentation by NOTAM BM-DCs was fully abrogated even at high IC concentrations, indicating that FcRγ-chain ITAM signals are specifically required for DC activation and maturation.

In line with data obtained with BM-DCs, cross-presentation of OVA-IC–derived Ags to CD8+ OT-I T cells by NOTAM splenic DCs was fully abrogated. This confirms that the activation of the cross-presentation pathway after OVA-IC uptake can only happen by intact FcRγ-chain ITAM signaling. However, in contrast to NOTAM BM-DCs, NOTAM splenic DCs induced proliferation of CD4+ OT-II T cells after OVA-IC uptake. This discrepancy can be best explained by the experimental setup, as follows: BM-DCs were incubated with OVA-ICS for 4 h, after which excess OVA-IC was washed away. In the experiments with ex vivo DCs, OVA-IC was constantly present for 4 d. OVA-IC uptake by NOTAM BM-DCs after 1 h (Fig. 1C) was decreased compared with WT BM-DCs, and it was even more apparent in other experiments (Fig. 6A). This suggests that in situations when OVA-IC is present for prolonged periods, uptake of OVA-IC via activating FcγRs (most likely via receptor recycling) can lead to MHC II Ag presentation even in the absence of intact FcRγ-chain ITAM signaling. In contrast, cross-presentation of OVA-IC even after prolonged incubation of OVA-IC did not lead to efficient presentation by FcRγ−/− and NOTAM splenic DCs.

Despite the uptake of OVA-ICS by NOTAM BM-DCs, they were not activated, which could account for the defective Ag presentation. TLR triggering can enhance Ag presentation in vitro (24). Therefore, we attempted to restore Ag presentation by NOTAM BM-DCs through providing an extra activation stimulus during Ag presentation. Although TLR triggering induced DC activation, it was not able to restore Ag presentation by NOTAM BM-DCs (P. Boross, D.A.C. Stapels, C. Bertens, and J.H.W. Leusen, unpublished observations). TLR ligand can enhance Ag presentation when directly coupled to the Ag (25). Therefore, it is possible that the Ag and the activating signaling (in this case FcRγ-chain ITAM

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**FIGURE 7.** MHC II Ag presentation by splenic DCs. (A) Ex vivo sorted splenic DCs were incubated with 1 µg/ml OVA323-339 peptide, 100 µg/ml soluble OVA, or 0.2 µg/ml OVA-IC in the presence of CellTrace violet–labeled OT-II T cells (DC:T = 1:5) for 4 d. The proliferation of CD4+ OT-II T cells was analyzed by cytofluorimetry. (B) Quantification of proliferation of OT-II CD4+ T cells after presentation of OVA-IC. Horizontal bar indicates mean. Significance was analyzed by one-way ANOVA; *p < 0.05.

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**FIGURE 8.** Abrogated MHC I Ag presentation by NOTAM splenic DCs. Ex vivo sorted splenic DCs were incubated with 1 ng/ml SIINFEKL peptide, 250 µg/ml soluble OVA, or 0.02 µg/ml OVA-IC in the presence of CellTrace violet–labeled CD8+ OT-I T cells labeled with for 4 d. The proliferation of OT-II T cells was analyzed by cytofluorimetry.
signaling) would have to be in the same intracellular compartment for efficient Ag presentation to induce localized signals. The defective Ag presentation can be explained by the fact that the IC containing Ag does not reach the lysosomal compartments and the Ag remains stable and is not partially degraded (Figs. 5, 6). Noting to note is that we have also previously shown that DC maturation signals including FcγR activation lead to functional upregulation of the PA28 proteasome activator complex involved in enhancing MHC class I processing (26).

It is unclear how IC is internalized by NOTAM BM-DCs. OVA-IC bound on the cell surface of DCs could be internalized in a non-specific manner due to the active pinocytosis or via the turnover of FcγRs. In contrast to uptake via intact FcγRs, this alternative IC internalization is less efficient and results in altered trafficking and abrogated Ag degradation. Therefore, IC uptake and DC activation are principally regulated by FcγR-chain ITAM signaling.

DCs also express the inhibitory IgG Fc receptor FcγRIIB, which can act as an efficient endocytic receptor for soluble OVA-ICs (27). Because no IC uptake was observed in FcγRI γ−/− BM-DCs expressing normal levels of FcγRIIB, it is unlikely that FcγRIIB contributed to IC internalization in our experiments. Dominant expression of FcγRIIB has a detrimental effect on Ag presentation (28, 29), and it is a major regulator of DC activity by controlling the expression of a wide range of genes after IC stimulation (8).

In this study, we did not specifically assess which activating FcγR is important in this effect. Earlier studies using knockout mice demonstrated that deletion of either FcγRI or FcγRII does not diminish IC-enhanced Ag presentation, indicating that the remaining two activating receptors are sufficient. Deletion of both FcγRI and FcγRII resulted in abrogated Ag presentation, suggesting a limited role for FcγRI.

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Disclosures
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