Different cross-presentation pathways in steady-state and inflammatory dendritic cells

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Presentation of exogenous antigens on MHC class I molecules, termed cross-presentation, is essential for the induction of CD8 T-cell responses and is carried out by specialized dendritic cell (DC) subsets. The mechanisms involved remain unclear. It has been proposed that antigens could be transported by endocytic receptors, such as the mannose receptor (MR) in the case of soluble ovalbumin, into early endosomes in which the cross-presentation machinery would be recruited. In these endosomal compartments, peptides would be trimmed by the aminopeptidase IRAP before loading onto MHC class I molecules. Here, we have investigated the contribution of this pathway to cross-presentation by steady-state CD8$^+$ DC and inflammatory monocyte-derived DC (moDC) generated in vivo. We demonstrate that IRAP and MR are dispensable for cross-presentation by CD8$^+$ DC and for cross-priming. Moreover, we could not find any evidence for diversion of endocytosed antigen into IRAP-containing endosomes in these cells. However, cross-presentation was impaired in moDC deficient in IRAP or MR, confirming the role of these two molecules in inflammatory DC. These results demonstrate that the mechanisms responsible for cross-priming by steady-state and inflammatory DC are different, which has important implications for vaccine design.

antigen presentation | inflammation

Priming of CD8 T-cell responses requires the presentation of exogenous antigen on MHC class I molecules by antigen-presenting cells, a process called cross-presentation. Dendritic cells (DC) are the main cross-presenting cells in vivo (1, 2), but only certain subsets of DC have this ability, primarily the CD8$^+$ resident DC found in the spleen and lymph nodes and the CD103$^+$ migratory DC found in lymph nodes (2–4). Monocyte-derived DC (moDC) that differentiate during inflammation (5) have also been shown to mediate cross-presentation and activation of memory T cells (6, 7), but their contribution to CD8 T-cell priming in the absence of inflammation is unclear.

Several mechanisms for cross-presentation have been proposed. The 'cytosolic pathway' involves the transfer of internalized antigens to the cytosol where they are degraded by the proteasome. The resulting peptides are translocated into the endoplasmic reticulum (ER) by TAP transporters and loaded onto MHC class I molecules (8). It has also been proposed that some antigens are degraded in endosomal compartments for loading onto MHC class I molecules in a TAP-independent manner (9). A variation of this 'endosomal pathway' has been recently proposed based on the study of soluble ovalbumin (OVA). In this model, OVA is transported into specialized early endosomes by the mannose receptor (MR) and can be stored in those compartments for several hours (10, 11). The MHC class I presentation machinery (including TAP) seems to be selectively recruited to these early endosomes, where peptides are trimmed by the insulin-regulated aminopeptidase (IRAP) for direct loading onto MHC class I molecules without trafficking to the ER (12, 13).

In this study, we have investigated the involvement of the endosomal cross-presentation pathway in splenic CD8$^+$ DC and in vivo-generated moDC. We show that cross-presentation of OVA is independent of IRAP and MR in CD8$^+$ DC. We analyzed the subcellular trafficking of OVA in splenic DC and could not find any evidence for its diversion and storage into specialized early endosomal compartments. However, we observed that cross-presentation was impaired in IRAP$^{-/-}$ moDC generated in vivo and that uptake of OVA was diminished in MR$^{-/-}$ moDC leading to decreased antigen presentation. These results show that the mechanisms of cross-presentation differ between steady-state and inflammatory DC.

Results

IRAP Is Dispensable for Cross-Presentation by Splenic CD8$^+$ DC. To analyze the contribution of the endosomal pathway to cross-presentation by CD8$^+$ DC, we first examined the role of the aminopeptidase IRAP. IRAP was similarly expressed in CD8$^+$ and CD8$^-$ DC subsets (Fig. 1*A*). To address its role in cross-presentation, purified wild-type or IRAP$^{-/-}$ CD8$^+$ or CD8$^-$ DC were incubated with soluble OVA (Fig. 1*B*) or with OVA-coated irradiated splenocytes (a model of cell-associated antigen), and cocultured with CFSE-labeled OVA-specific transgenic CD8 OT-I T cells. T-cell proliferation was measured 3 days later as a read-out for cross-presentation (Fig. 1*C*). Wild-type and IRAP$^{-/-}$ CD8$^+$ DC cross-presented soluble or cell-associated OVA with similar efficiency. We also investigated the role of IRAP in the in vivo induction of cytotoxic T lymphocytes (CTL), a process mainly dependent on cross-presentation by CD8$^+$ DC (2, 14). Wild-type and IRAP$^{-/-}$ mice were immunized with OVA-coated irradiated splenocytes and the differentiation of effector CD8 T cells was assessed 5 days later. In vivo killing of target cells by CTL (Fig. 1*D*) and proliferation of endogenous specific CD8 T cells in the spleen (Fig. 1*E*) were similar in both groups. These results show that IRAP is not required for cross-presentation of antigens by splenic CD8$^+$ DC.

MR Is Dispensable for Cross-Presentation of Soluble OVA by Splenic CD8$^+$ DC. We then addressed the role of MR in splenic DC subsets. As reported previously (15), MR was not detected on splenic CD8$^+$ or CD8$^-$ DC, although it was detected on macrophages copurified from the same spleens (Fig. 2*A*). By contrast, MR was highly expressed by bone-marrow derived DC (BM-DC) generated in the presence of GM-CSF (Fig. 2*A*), consistent with a previous report (10). However, we could not discard the possibility that MR was expressed on splenic DC at low levels that were undetectable by flow cytometry. To definitively address the requirement for MR for uptake and presentation of soluble OVA by splenic DC, we analyzed purified CD8$^+$ and CD8$^-$ DC from WT or MR$^{-/-}$ mice. DC were

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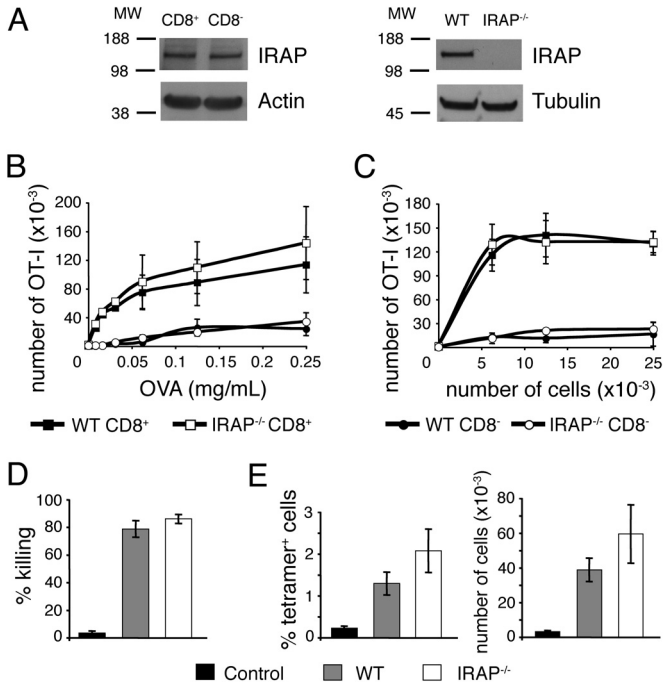

Fig. 1. IRAP is dispensable for cross-presentation of OVA by splenic dendritic cells. (*A*) Total cell lysates of wild-type $CD8^+$ and $CD8^-$ DC or wild-type and $IRAP^{-/-}$ total splenic DC were analyzed by Western blot for the presence of IRAP, actin, or tubulin. Molecular weights are indicated (kDa). (*B–C*) Purified wild-type or $IRAP^{-/-}$ $CD8^+$ and $CD8^-$ DC were incubated with soluble OVA and washed (*B*) or were cultured with OVA-coated splenocytes (*C*) and cocultured with CFSE-labeled OT-I T cells. After 3 days, proliferation of OT-I T cells was analyzed by flow cytometry. The number of OT-I T cells that had undergone at least one division is shown (mean ± SEM of three independent experiments). (*D–E*) wild-type or $IRAP^{-/-}$ mice were immunized with OVA-coated splenocytes. Control wild-type mice were injected with uncoated splenocytes. After 5 days, $CD45.1^+$ CFSE-labeled target cells were injected and spleens were analyzed the next day. (*D*) The percentage of killing was determined by comparing the number of remaining SIINFEKL-pulsed target cells and unpulsed target cells (mean ± SEM, $n = 6$ in two independent experiments). (*E*) The proliferation of OVA-specific endogenous T cells was analyzed by tetramer staining. The percentage among $CD8^+$ T cells and total number of $tetramer^+$ $CD8^+$ T cells in the spleen are shown (mean ± SEM, $n = 6$ in two independent experiments).

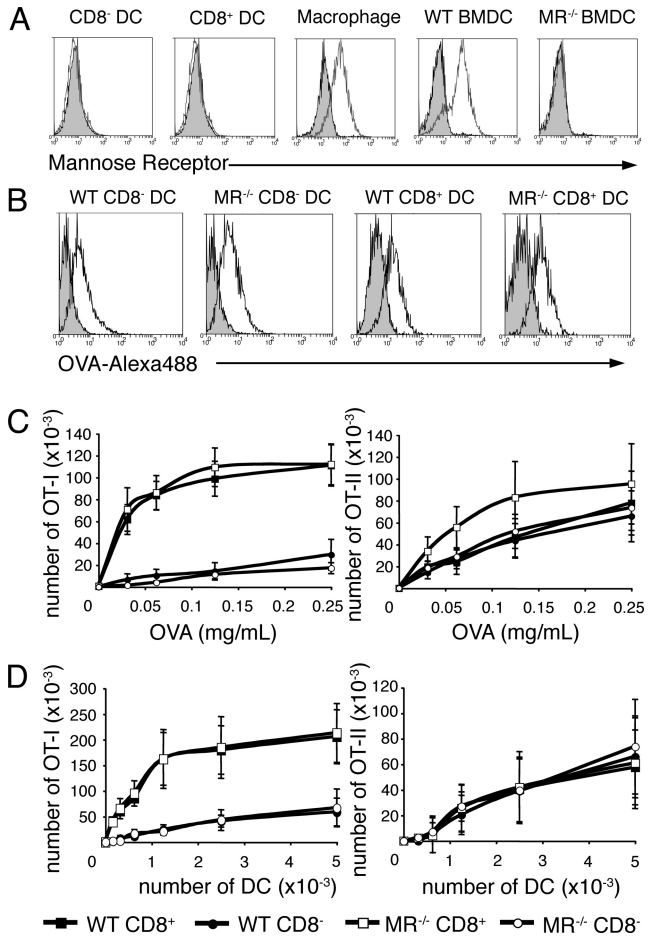

Fig. 2. The mannose receptor is dispensable for presentation of OVA by splenic dendritic cells. (*A*) Splenocytes were stained for CD11c, MR, CD11b, and either F4/80 or CD8. MR expression is shown for $CD8^-$ DC (gated as $CD11c^+CD11b^+CD8^-$), $CD8^+$ DC (gated as $CD11c^+CD11b^-CD8^+$), and splenic macrophages (gated as $CD11c^-CD11b^+F4/80^+$). Bone marrow-derived DC (BMDC) from GM-CSF cultures of wild-type or $MR^{-/-}$ bone marrow were stained for MR. Gray shaded histograms represent control isotype staining. (*B*) $CD8^+$ or $CD8^-$ DC from wild-type or $MR^{-/-}$ mice were incubated with OVA-Alexa488 for 30 min at 4 °C (gray shaded histogram) or 37 °C (black histogram) and analyzed by flow cytometry. (*C*) Purified wild-type or $MR^{-/-}$ $CD8^+$ and $CD8^-$ DC were incubated with soluble OVA, washed and cultured with CFSE-labeled OT-I or OT-II T cells. T-cell proliferation was assessed by flow cytometry after 3 days of culture. The number of OT-I or OT-II cells that have undergone at least one division is shown (mean ± SEM of four independent experiments). (*D*) Wild-type or $MR^{-/-}$ mice were injected i.v with 2 mg OVA and DC were isolated 3 h later. Serial dilutions of sorted $CD8^+$ or $CD8^-$ DC were cultured with CFSE-labeled OT-I or OT-II T cells and T-cell proliferation was assessed by flow cytometry after 3 days of culture. The number of OT-I or OT-II cells that have undergone at least one division is shown (mean ± SEM, $n = 3$ in three independent experiments).

incubated with OVA-Alexa488 and OVA uptake was assessed by flow cytometry (Fig. 2*B*). $MR^{-/-}$ and WT DC subsets internalized similar amounts of OVA. $CD8^+$ and $CD8^-$ DC were also incubated with OVA, washed, and cocultured with CFSE-labeled transgenic OVA-specific OT-I or OT-II T cells (Fig. 2*C*). $MR^{-/-}$ DC were as efficient as WT DC at inducing T-cell proliferation, both for cross-presentation and MHC II-restricted presentation. To rule out the possibility that the binding of MR to its ligand was affected by our splenic DC purification process, we injected OVA intravenously into WT or $MR^{-/-}$ mice and splenic DC were purified 3 h later to assess *ex vivo* presentation. We obtained similar results (Fig. 2*D*), demonstrating that MR is not required for uptake and presentation of soluble OVA by splenic DC.

OVA Is Not Stored in IRAP-Containing Endosomal Compartments in Splenic DC. We then investigated whether exogenous antigen is sequestered and stored in early endosomal compartments in cross-presenting splenic DC. Trafficking of soluble OVA along the endocytic route was examined by subcellular fractionation. $CD8^+$ and $CD8^-$ DC were incubated with OVA, washed, and subcellular fractions were prepared at different time points. As shown by the presence of classical markers, intracellular organelles were separated into early and recycling endosomes [$RAB4^+$ $syntaxin13^+$ fraction (16)], late endosomes ($RAB7^+$ fraction) and lysosomes ($Cathepsin\ S^+$ fraction) (Fig. 3*A*). IRAP was predominantly detected in the early and recycling endosomes fraction (Fig. 3*A*), suggesting that this fraction contained the proposed cross-presenting early endosomal compartment (12). However, we did not observe any accumulation of internalized OVA in early endosomes in $CD8^+$ DC and there was no major difference in the trafficking of OVA between $CD8^+$ and $CD8^-$ DC (Fig. 3*B*). These results argue against the diversion and storage of OVA into specialized cross-presenting endosomal compartments in splenic $CD8^+$ DC.

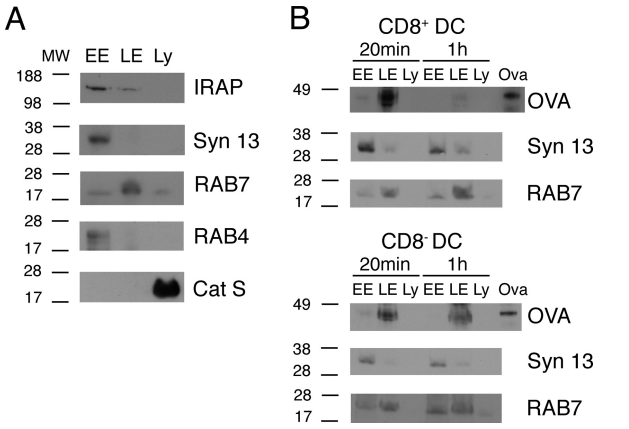

Fig. 3. Internalized OVA does not accumulate in IRAP-containing endosomes in splenic dendritic cells. (*A*) Subcellular fractions containing early endosomes (EE), late endosomes (LE) or lysosomes (Ly) were prepared from splenic CD8$^+$ DC. Fractions were analyzed by Western blot for the presence of RAB4, RAB7, cathepsin S (Cat S), syntaxin 13 (Syn 13), or IRAP. (*B*) Splenic CD8$^+$ or CD8$^-$ DC were incubated with soluble OVA for 45 min, washed, and further incubated for 20 min or 1 h. Subcellular fractions were prepared and the presence of OVA was analyzed by Western blot. Syntaxin 13 and RAB7 stainings are shown as a loading control. Results are representative of three independent experiments.

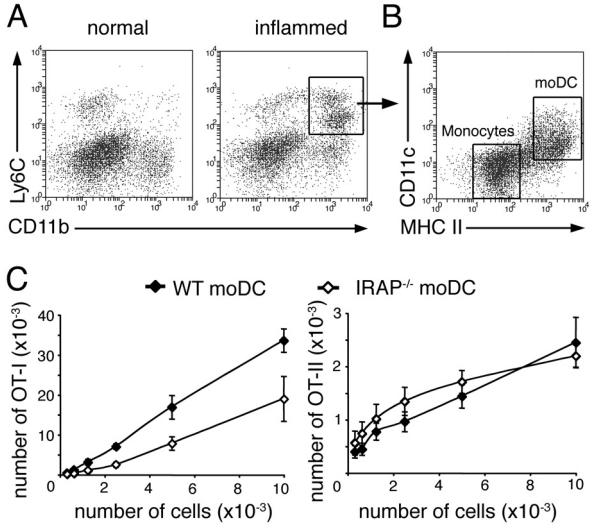

Fig. 4. IRAP is involved in cross-presentation of OVA by monocyte-derived dendritic cells. Inflammatory peritonitis was induced in mice by two successive intradermal injections of mBSA emulsified in CFA followed by i.p. injection of mBSA. (*A*) Splenocytes from normal or inflamed mice were stained for Ly6C and CD11b. (*B*) Ly6C$^+$CD11b$^+$ cells from inflamed mouse spleen were analyzed for their expression of CD11c and MHC class II molecules. (*C*) Inflamed wild-type or IRAP$^{-/-}$ mice were injected i.v with 2 mg OVA and splenocytes were harvested 3 h later. Serial dilutions of purified monocyte-derived DC were cultured with CFSE-labeled OT-I or OT-II T cells and T-cell proliferation was assessed by flow cytometry after 3 days of culture. The number of OT-I or OT-II cells that have undergone at least one division is shown (mean ± SEM, $n = 6$ in two independent experiments).

IRAP and MR Are Involved in Cross-Presentation of Soluble OVA by moDC. Most of the experiments supporting a model for cross-presentation occurring in specialized early endosomes have been conducted on in-vitro-generated BM-DC, and our results show that these findings do not apply to CD8$^+$ DC. Because BM-DC are thought to be the in vitro equivalent of inflammatory moDC (17, 18), we explored the relevance of this cross-presentation model in in-vivo-generated moDC. To obtain these cells, we used a model of GM-CSF dependent inflammatory peritonitis that allows the differentiation of monocytes into moDC in the spleen (19, 20). The spleen of inflamed mice contained a population of Ly6C$^+$CD11b$^+$ cells that was absent from normal spleen (Fig. 4*A*). Within this population, moDC were characterized by high expression of MHC class II molecules and intermediate levels of CD11c (20, 21) (Fig. 4*B*). To assess the role of IRAP in antigen presentation by moDC, inflammatory peritonitis was induced in wild-type or IRAP$^{-/-}$ animals and mice were injected intravenously with soluble OVA. Splenic moDC were isolated 3 h later and cocultured with CFSE-labeled OT-I or OT-II T cells. Cross-presentation of soluble OVA was impaired in IRAP$^{-/-}$ moDC, but MHC II-restricted presentation was not affected (Fig. 4*C*) indicating that IRAP is involved in cross-presentation by moDC. We also sought to address the role of MR in OVA presentation by moDC. Unlike monocytes, in-vivo-generated moDC expressed MR (Fig. 5*A*). MR has been shown to mediate the internalization of OVA by BM-DC (10), so we analyzed the role of MR in OVA uptake by incubating splenocytes from wild-type or MR$^{-/-}$ inflamed mice with OVA-Alexa488. Internalization of OVA was decreased in MR$^{-/-}$ moDC as compared to wild-type moDC (Fig. 5*B*). Consistent with diminished uptake, cross-presentation and MHC II-restricted presentation of OVA were impaired in MR$^{-/-}$ moDC (Fig. 5*C*). These results show that MR is involved in the uptake and presentation of OVA by moDC.

Discussion

Cross-presentation by DC plays a crucial role in anti-viral and anti-tumor immunity (cross-priming) and in the inactivation of autoreactive T cells (cross-tolerance) (22). Moreover, vaccine formulations designed to target cross-presenting DC can result in tolerance or immunity (23, 24), providing opportunities for clinical exploitation of this pathway. Cross-presentation can also contribute to disease, allowing DC to induce autoimmunity, hyperresponsiveness, graft rejection, tumor immunoescape, and immunopathology (22). To fully characterize the role of cross-presentation in vivo, identify potential targets for pharmacological intervention and design effective vaccines, it is important to define which DC type(s) mediate this function and which mechanisms are involved. In this manuscript we demonstrate that the endosomal cross-presentation pathway requiring MR and IRAP is functional in inflammatory moDC but not in steady-state CD8$^+$ DC.

Our results are largely in agreement with previous reports on the role of IRAP and MR in cross-presentation, which examined primarily BM-DC (10–12), but some discrepancies were also apparent. First, Saveanu et al. reported a small, albeit significant, reduction in the number of endogenous CD8 T cells that responded to cross-presented OVA in IRAP$^{-/-}$ mice as compared to control mice (12), whereas we did not detect significant differences (Fig. 1*E*). We also compared the induction of CTL activity by cross-priming in WT and IRAP$^{-/-}$ mice, which supported our conclusion that IRAP played little or no role in cross-priming by steady-state DC (Fig. 1*D*). Second, the report by Burgdorf et al. (11) did not detect reduced MHC II presentation of soluble OVA by MR$^{-/-}$ DC, but we did (Fig. 5*C*). These discrepancies may be due to subtle differences in the genetic background of the mice used or in the protocols used by each laboratory.

Although multiple types of DC and non-DC are capable of cross-presentation under some circumstances (22, 25), the resident CD8$^+$ DC subtype has been shown to play the dominant part in cross-priming against multiple viruses, bacteria and tumours, and in cross-tolerance (3, 26). Furthermore, vaccines targeting this subtype are more effective at inducing CTL

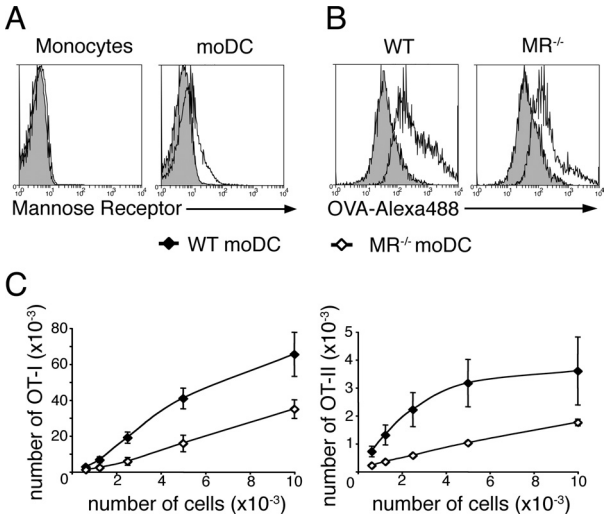

Fig. 5. The mannose receptor is involved in uptake of OVA by monocyte-derived dendritic cells. (*A*) Splenocytes from inflamed mice were stained for Ly6C, CD11b, CD11c, MHC class II molecules, and MR. MR expression is shown for monocytes (gated as Ly6C$^+$CD11b$^+$MHCII$^-$CD11c$^-$) and monocyte-derived DC (moDC) (gated as Ly6C$^+$CD11b$^+$MHCII$^+$CD11c$^+$). Gray shaded histograms represent control isotype staining. (*B*) Splenocytes from wild-type or MR$^{-/-}$inflamed mice were incubated with OVA-Alexa488 for 30 min at 4 °C (gray shaded histogram) or 37 °C (black histogram) and analyzed by flow cytometry. Alexa488 fluorescence is shown for monocyte-derived DC. (*C*) Inflamed wild-type or MR$^{-/-}$ mice were injected i.v with 2 mg OVA and splenocytes were harvested 3 h later. Serial dilutions of purified monocyte-derived DC were cultured with CFSE-labeled OT-I or OT-II T cells and T-cell proliferation was assessed by flow cytometry after 3 days of culture. The number of OT-I or OT-II cells that have undergone at least one division is shown (mean ± SEM, $n = 5$ in three independent experiments).

responses by cross-priming than those targeting other subtypes (24, 27). The importance of CD8$^+$ DC for cross-priming in vivo has been dramatically demonstrated with the analysis of mice deficient in the transcription factor Batf3, which lack CD8$^+$ DC and are severely impaired in their capacity to elicit immune responses by cross-priming (2). Since our results indicate that the MR/IRAP-dependent mechanism of cross-presentation is not operative in CD8$^+$ DC, our results suggest that pharmacological manipulation of this mechanism, or vaccines designed to exploit this pathway, would not work in steady-state conditions. It is during infection or inflammation, situations in which moDC are recruited to the infected or inflamed site and have been shown to contribute to cross-priming (6, 7), that the IRAP/MR-dependent pathway most likely plays a role in vivo. Although the human equivalent of mouse CD8$^+$ DC has yet to be characterized, it is worth noting that human splenic DC seem to lack expression of MR (28) while human moDC have been shown to express MR (29), which can serve for uptake and presentation of antigen (30). These observations suggest that our findings in the mouse system have a correlate in the human dendritic cell network.

MoDC lacking MR or IRAP were not completely impaired in their capacity to cross-present, suggesting that they possess at least another mechanism, in addition to the endosomal pathway, to carry out this function. Presumably this is the same mechanism used by the CD8$^+$ DC, namely the cytosolic pathway. Indeed, some of the mechanisms that are thought to play a role in cross-presentation are found in both moDC and CD8$^+$ DC. For instance, in these two DC types endosomal compartments containing captured antigens are acidified slowly, favoring cross-presentation, and this mechanism is not found in noncross-presenting CD8$^-$ DC (31, 32). Further studies of this pathway in CD8$^+$ DC will be required to fully characterize the mechanisms of cross-presentation under steady-state conditions in vivo, which may yield clues for the development of approaches for pharmacological intervention or vaccination.

Methods

Mice. C57BL 6 (B6), B6.CH-2bm 1 (bm1), OT-I (33), OT-II (34), mannose receptor deficient (35), IRAP deficient mice (36), and wild-type littermates were bred and maintained under specific pathogen-free conditions at The Walter and Eliza Hall Institute or Howard Florey Institute animal breeding facilities according to institute guidelines. Where indicated, B6 mice were injected s.c. with 5×10^6 B16 melanoma cells secreting murine Fms-like tyrosine kinase ligand (Flt3-L) (37) and killed after 9–10 days.

Induction of Inflammatory Peritonitis. Induction of inflammatory peritonitis was performed as described in ref. 19. Briefly, mice were injected intradermally at the base of the tail with 100 μg of mBSA (Sigma–Aldrich) emulsified in an equal volume of CFA containing 5 mg/mL heat-killed *Mycobacterium tuberculosis* (H37 Ra; Difco Laboratories) and were subjected 2 weeks later to the same treatment. Seven days later, mice were injected intraperitonally with 100 μg mBSA in PBS for the induction of inflammation. Mice were used 2 days later.

Cell Isolation. OT-I and OT-II T cells were isolated from the lymph nodes of OT-I or OT-II transgenic mice as described in ref. 38. Total DC from normal mouse spleens were isolated as described (39, 40). Light-density splenocytes from inflamed mouse were isolated after digestion of the spleens with DNase I (0.1%, Roche Molecular Biochemicals) and collagenase (1 mg/mL, type II, Worthington Biochemical) and centrifugation in Nycodenz medium (density 1.082 g/cm^3, 1700 × *g* for 10 min). Cells were further purified by cell sorting with a FACS Aria (Becton Dickinson) or Mo-Flo (Cytomation) instrument. For fractionation experiments, total DC were prepared from the spleens of Flt3-L treated mice and CD8$^+$ DC were further isolated by positive selection using immuno-magnetic beads (MACS, Miltenyi Biotec) after staining with anti-CD8 (YTS 169.4) antibody. Remaining cells were depleted of CD205$^+$CD24^{high} DC precursors (38) after staining with anti-CD205 (NLDC-145) and anti-CD24 (M1/69) antibodies and MACS beads depletion, and finally CD8$^-$ DC were isolated by positive selection using MACS beads after staining with anti-CD11b (M1/70) antibody.

BM-DC Culture. After lysis of red blood cells, bone marrow cells were cultured for 7 days in RPMI medium 1640 supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (DC medium) in the presence of 20 ng/mL GM-CSF (PeproTech).

Subcellular Fractionation. DC were incubated with OVA (1 mg/mL) for 45 min at 37 °C in DC medium. After three washes in PBS, cells were further incubated for 20 min or 1 h at 37 °C in DC medium. Cells were homogenized with a cell-cracker (HGM Laboratory Equipment) in homogenization buffer [PBS, 0.25 M sucrose, 10 mM Tris, 1 mM EDTA supplemented with proteases inhibitors (Roche), pH 6.8)]. Postnuclear supernatant was prepared by centrifugation (1,000 × *g* for 10 min) and loaded on top of a 10% Percoll (GE Healthcare) solution in homogenization buffer. After ultracentrifugation (50,000 × *g* for 45 min), 1 mL fractions were collected. The top three fractions (early endosomes) were pooled and concentrated by ultracentrifugation (130,000 × *g* for 1 h). The bottom two fractions were pooled and loaded on top of a 45% Percoll solution in homogenization buffer. After ultracentrifugation (50,000 × *g* for 45 min), the top three fractions (late endosomes) ant the bottom two fractions (lysosomes) were pooled and concentrated by ultracentrifugation (130,000 × *g* for 1 h).

Western Blot Analysis. Total cell lysates were run on a 4–12% SDS/PAGE gel (Invitrogen) and transferred onto nitrocellulose membrane. Membranes were stained with serum against mouse actin (Sigma), IRAP (41), antibodies against mouse tubulin (Abcam), syntaxin 13 (15G2, Stressgen), or polyclonal antibodies against RAB4 (Stressgen) or Cathepsin S (M-19, Santa Cruz). Horseradish peroxidase (HRP)–conjugated anti-rabbit IgG antibody (Sigma), anti-mouse IgG (Pierce), anti-goat IgG (Abcam) were used for protein detection.

Flow Cytometry Analysis. The following antibodies were used and produced in-house unless otherwise indicated: anti-CD11c (N418), CD8 (YTS 169.4), CD4 (GK1.5), CD11b (M1/70), CD45.1 (A20–1.1), MHC class II (M5/114), F4/80, Ly6C (5075–3.6), H-2K^b gB$_{497–505}$ tetramer, mannose receptor (MR5D3, AbD Serotec), and Vα2 (B20.1, BD). Cells were labeled with propidium iodide (PI) to

assess viability and analyzed on a FACSCalibur or LSR instrument (Becton Dickinson). Flow cytometry data were analyzed with the in-house software WEASEL.

Antigen-Presentation Assay. For in vitro presentation of soluble OVA (Sigma), splenic DC populations (5×10^3 cells per well) were incubated in 96-well plates (Costar-Corning) with different concentrations of OVA at 37 °C in DC medium. After 45 min, cells were washed twice in DC medium. For presentation of cell-associated antigen, bm1 spleen cells were γ-irradiated (1500 rads), washed, incubated with 10 mg/mL OVA in RPMI medium 1640 for 10 min at 37 °C, and washed three times with RPMI medium 1640 supplemented with 3% FCS. Splenic DC populations (25×10^3 cells per well) were plated with different numbers of bm1 OVA-coated splenocytes in DC medium. For *ex vivo* presentation, mice were injected intravenously with 2 mg of OVA. After 3 h, splenic DC populations were sorted and different numbers of DC were plated in 96-well plates in DC medium. Fifty thousand CFSE-labeled OT-I or OT-II cells were added in each well in DC medium supplemented with 10 ng/mL GM-CSF. Proliferation was analyzed by flow cytometry after 60–65 h of culture as described in ref. 40. Each determination was performed in duplicate.

In Vivo CTL Assay. WT or IRAP$^{-/-}$ mice were immunized intravenously with 20×10^6 OVA-coated bm1 splenocytes and LPS (Sigma) (1 μg/mouse). Control WT mice were injected with 20×10^6 uncoated bm1 splenocytes and LPS (1 μg/mouse). Five days later, mice were injected intravenously with 5×10^6 CD45.1$^+$ splenocytes that had been pulsed with 33 ng/mL SIINFEKL peptide (Genscript) for 40 min and labeled with 2.5 μM CFSE, and 5×10^6 unpulsed CD45.1$^+$ splenocytes labeled with 0.25 μM CFSE. The next day, spleens were analyzed for the presence of CD45.1$^+$ target cells.

OVA Uptake Assay. OVA was conjugated to Alexa488 using Alexa488-labeling kit (Invitrogen) according to manufacturer's instructions. Cells were incubated with 30 μg/mL OVA-Alexa488 in DC medium for 30 min either on ice or at 37 °C. Cells were washed three times, then stained for FACS analysis.

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