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Conventional Dendritic Cells Require IRAP-Rab14 Endosomes for Efficient Cross-Presentation

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Dendritic cells (DCs) use cellular pathways collectively referred to as cross-presentation to stimulate CD8+ T cells with peptide Ags derived from internalized, exogenous Ags. We have recently reported that DCs rely on aminoterminal trimming of cross-presented peptides by insulin-responsive aminopeptidase (IRAP), an enzyme localized in a regulated endosomal storage compartment. Considering a report contending that this role is limited to inflammatory DCs (Segura et al. 2009. Proc. Natl. Acad. Sci. USA 106: 20377–20381), in this study, we examined the role of IRAP in steady-state DC subpopulations. Steady-state conventional DCs (cDCs) and plasmacytoid DCs expressed similar amounts of IRAP. IRAP colocalized with the endosomal markers Rab14 and syntaxin 6, both known to be associated with regulated endosomal storage compartments, in CD8+ and CD8− cDCs—however, to a greater extent in the former population. Likewise, IRAP recruitment to phagosomes was significantly stronger in CD8+ DCs. IRAP deficiency compromised cross-presentation of soluble and particulate Ag by both CD8+ and CD8− cDCs, again with a stronger effect in the former population. Thus, the requirement of IRAP in cross-presentation extends to steady-state cDCs. Moreover, these data suggest that increased recruitment of an IRAP+/Rab14+ compartment to Ag-containing vesicles contributes to the superior cross-presentation efficacy of CD8+ cDCs. The Journal of Immunology, 2012, 188: 000–000.
IRAP endosomes for efficient cross-presentation. Our results indicate that cDCs, similar to moDCs, use IRAP and localize with the cross-presentation ability of different DC subtypes. Among various DC subsets with respect to their roles in T cell activation. For example, CD8<sup>+</sup> spleen DCs are more potent than their CD8<sup>−</sup> counterparts in MHC class II Ag presentation and CD4<sup>+</sup> T cell activation (21). Conversely, CD8<sup>+</sup> DCs are the most efficient DC subset for MHC-I Ag cross-presentation and cross priming of naive CD8<sup>+</sup> T cells against West Nile virus (22), HSV (23), and tumor Ags (22).

In this study, we investigated the correlation of IRAP expression and localization with the cross-presentation ability of different DC subtypes. Our results indicate that cDCs, similar to moDCs, use IRAP endosomes for efficient cross-presentation.

Material and Methods

Abs

IRAP-specific Abs were either a polyclonal rabbit serum (a gift from Metabolex) or a mix of three monoclonal murine Abs (generously provided by M. Birnbaum, University of Pennsylvania, Philadelphia, PA) (24). MHC-I immunoblot was performed with a rabbit serum against murine MHC-I (P9; gift of H. Ploegh, Massachusetts Institute of Technology, Cambridge, MA), and MHC-I fluorescence microscopy was performed with anti-H2-K<sup>b</sup> clone AF6-88.5 and anti-H2-D<sup>b</sup> clone B22.249 (both from F. Lemonnier, Institut Pasteur, Paris, France). The other Abs used were: an mAb against mouse β-actin (clone AC-74; Sigma-Aldrich), a rat mAb against MR (AbD Serotec), polyclonal rabbit Abs against Rab14 (Sigma-Aldrich), STX6 (ProteinTech Group, Chicago, IL), OVA (Immunology Consultants Laboratories), EEA1 (Abcam), V-ATPase subunit E (Santa Cruz Biotechnology), and a polyclonal goat Ab against Rab7 (Santa Cruz Biotechnology). Abs for flow cytometry were anti-CD11c-APC (clone N418; eBioscience), anti-CD11b-PE (clone M1/70; BioLegend), anti–B220-Pacific Blue (clone RA3-6B2; BioLegend), anti–CD24-FITC (clone M1/69; BD Pharmingen), and anti–Gr-1–PE (clone RB6-8C5; BioLegend). Secondary Abs used for microscopy studies were: goat anti-mouse F(ab)2 Alexa 594, goat anti-mouse F(ab)2 Alexa 488, goat anti-rabbit F(ab)2-Alexa 594, and goat anti-rabbit F(ab)2-Alexa 488 (all from Molecular Probes). Abs used for cytokine ELISAs were: anti–IL-2 capture, clone JES6-1A12, and anti–IL-2 detection (clone JES6-5H4; BD Pharmingen).

Mice

IRAP-deficient mice (25) were bred in our colony. Sex- and age-matched wild-type (wt) 129SVPasCo mice (Charles River Laboratories) were used for comparison, we checked IRAP expression levels in granulocytes, a cell population present under steady-state conditions in the spleen that efficiently phagocytizes but does not cross-present exogenous Ag. In contrast to DCs, purified splenic granulocytes did not express detectable amounts of IRAP (Fig. 1A, right panel). Considering the similar expression of IRAP in all steady-state DC subtypes analyzed (Fig. 1B), we hypothesized that an IRAP-dependent cross-presentation pathway might exist in all of these cells.

In vitro cross-presentation assays

One hundred thousand DCs were incubated with Ag for 16 h before addition of 2 × 10<sup>5</sup> lymph node cells from OT-I Rag ko mice (26, 27) for 24 h. T cell stimulation was assessed by measuring IL-2 secretion by sandwich ELISA. Ags were 5 × 10<sup>5</sup> Ab- opsonized recombinant yeast-expressing OVA fusion proteins on their cell surface or soluble OVA (Worthington Biochemical, Lakewood, NJ; or EndoGrade OVA from Hyglos, Bernried am Starnberger See, Germany). OVA-yeasts were opsonized by incubation with the polyclonal anti-OVA Abs 30 min at 4˚C. OVA-Alexa Fluor 647 (Molecular Probes) at 0.6 mg/ml was used for quantification of soluble Ag uptake. Recombinant yeast-expressing OVA were prepared as described by Merzougui et al. (28).

Subcellular fractionation

For endosome isolation, 60 million CD11c<sup>+</sup> spleen DCs isolated by magnetic sorting (Milteny Biotech CD11c<sup>+</sup> selection) were incubated for 20 min with 2 mg/ml soluble OVA. After extensive washing, the cells were chased for 20 and 60 min. The endosomes were further prepared by ultracentrifugation in Percoll gradient following the procedure described previously (29).

Fluorescence microscopy

Staining of sorted Flt3L-BMDC was performed in BD BDH slides as described previously (12). For phagosome studies, cells were fed OVA-expressing yeast for 10 min, then noninternalized yeast cells were washed away, and phagocytosis was left to continue for different time periods before paraformaldehyde fixing and staining. Images were acquired on a piezoelectric-driven Leica DMI 6000 microscope (Leica Microsystems) equipped with Optronoptics XF100-2 (FITC), XF102-2 (Texas Red), and XF96 (DAPI) filters and processed for three-dimensional deconvolution. Determination of the percentage of colocalization between two markers was carried out using correlation maps (12) together with the Metamorph 6.3.7 colocalization module.

Statistical analysis

All statistical analyses were performed with the GraphPad Prism software (GraphPad) using the two-tailed Student t test.

Results

Three splenic DC subsets but not granulocytes express IRAP

Our previous studies of IRAP function in vitro were performed with GM-CSF–derived BMDCs, a cell type that resembles inflammation-induced moDCs. To address the question of IRAP presence and function in DC subtypes under noninflammatory conditions, we purified pDC and CD8<sup>+</sup> and CD8<sup>−</sup> eDC equivalents from BMDCs cultured in the presence of Flt3L (17) using the cell-surface markers B220, CD11c, CD11b, and CD24 (Supplemental Fig. 1A) and spleen cDCs (Supplemental Fig. 1B) from mice infected with an Flt3L-secreting tumor.

We first analyzed IRAP protein expression levels in these subsets. Quantification of IRAP via immunoblot showed no significant differences among Flt3L-differentiated DC subtypes (Fig. 1A). Considering the similar expression of IRAP in all steady-state DC subtypes (Fig. 1B), we hypothesized that an IRAP-dependent cross-presentation pathway might exist in all of these cells.
with STX6 and Rab14 was 51 and 76%, respectively) (12). STX6 is a Q-SNARE (soluble N-ethylmaleimide–sensitive factor attachment) localized in trans-Golgi network stacks and endosomes (30). STX6-positive endosomal vesicles, but not trans-Golgi network stacks, contain IRAP in several cell types such as adipocytes, in which IRAP endosomes are called Glut-4 storage vesicles (31), or HeLa cells and mouse fibroblasts (J. Babdor and L. Saveanu, unpublished observations). In contrast to STX6, colocalization of the small GTPase Rab14 with IRAP seems to be limited to specific cell types including BM-DCs because no colocalization is observed in mouse fibroblasts (J. Babdor and L. Saveanu, unpublished observations).

Based on these previous findings, we used the endosomal markers Rab14 and STX6 to test if the intracellular distribution of IRAP in steady-state DC subsets is similar to that in BMDCs. IRAP colocalized with Rab14 and STX6 in all steady-state DC subsets analyzed (Fig. 2). However, among the conventional steady-state DCs, CD8+ DCs displayed an increased percentage of IRAP that colocalized with Rab14 and STX6. The difference between IRAP-Rab14 colocalization in CD8+ DCs (47%) and CD8− DCs (29%) was significant ($p < 0.0002; n = 12$). IRAP-STX6 colocalization was also significantly increased in CD8+ DC (57%) versus CD8− DCs (40%) ($p < 0.0021; n = 12$). In pDCs, the extent of IRAP-STX6 colocalization (40%) was identical to that seen in CD8− DCs, whereas IRAP-Rab14 colocalization (44%) was similar to that seen in CD8+ DCs. Consistent with the results obtained by immunoblot, IRAP was not detected in granulocytes by microscopy.

IRAP is involved in cross-presentation of soluble and particulate OVA by CD8+ and CD8− DCs

We wondered if the differences in IRAP-Rab14 colocalization could be correlated to an increased cross-presentation efficiency for CD8+ DCs when compared with CD8− DCs. We have previously shown that IRAP is involved in the in vitro cross-presentation of OVA-coated latex beads and OVA-expressing necrotic cells by GM-CSF–differentiated BMDCs and CD11c+ splenic DCs (12). We analyzed the ability of IRAP-deficient Flt3L-differentiated DCs to present soluble and particulate OVA to CD8+ DCs and CD8− DCs. We found that CD8+ DCs were more efficient in cross-presentation of OVA than CD8− DCs, and this efficiency was correlated with the increased colocalization of IRAP with Rab14 in CD8+ DCs.
subsets to cross-present soluble and particulate OVA in vitro. DCs were incubated with OVA or OVA-yeast, and IL-2 secreted by activated OT1 cells was measured by ELISA. Conventional IRAP KO DCs displayed reduced cross-presentation of both OVA forms compared with wt DC (Fig. 3A). The presentation of added synthetic peptide and the direct presentation of the peptide encoded by a vaccinia virus was similar in IRAP wt and KO DCs (Supplemental Fig. 1C), demonstrating that the absence of IRAP does not affect the intrinsic ability of DCs to stimulate OT1 cells. These results indicate that both types of Flt3L-differentiated cDCs use an IRAP-dependent pathway to cross-present soluble and particulate Ag. Although CD8+ DCs are thought to cross-present Ags derived from dead or virally infected cells most efficiently (22, 23, 32), we observed similar activation of naive OT1 cells by CD8 and CD8− DCs, consistent with a recent report by others (33). However, cross-priming efficacy will be related to the amount of Ag ingested. Therefore, we analyzed the efficiency of Ag uptake by CD8− and CD8+ DC equivalents. CD8+ DC equivalents phagocytized twice as many OVA-yeast cells as CD8− equivalents (Fig. 3B). The fact that CD8+ DC equivalents, despite reduced uptake of OVA-yeast, activated CD8+ T cells as strongly as CD8− DC equivalents is consistent with the well-documented superior efficiency of the CD8+ DC subtype to cross-present particulate Ag. In contrast, soluble OVA was not cross-presented more efficiently by CD8+ DC than by CD8− DC equivalents, although both cell types ingested similar amounts of protein (Fig. 3C). We hypothesized that this could be due to the absence of GM-CSF in our Flt3L cultures, given that the ability of CD8+ DCs to cross-present has recently been shown to depend on GM-CSF (34, 35). To analyze this possibility, we tested the cross-presentation ability of spleen DC subsets that are physiologically differentiated in the presence of both cytokines, Flt3L and GM-CSF. Both subsets of spleen cDCs, similar to Flt3L-differentiated DCs, required IRAP for efficient cross-presentation of soluble OVA and OVA-yeast Ags (Fig. 3D). Concerning the relative CD8+ and CD8− DCs’ effectiveness, the capacity of CD8+ DCs to cross-present soluble OVA exceeded that of CD8− DCs only in the presence of the TLR4 ligand LPS (Fig. 3D, middle panel).

**IRAP recruitment to Ag-containing vesicles**

We wondered whether the better presentation of Ag derived from OVA-yeasts by CD8+ DCs as compared with CD8− DCs was correlated with the dynamics of IRAP recruitment to phagosomes. To this end, we analyzed by fluorescence microscopy the proportion of OVA-containing phagosomes that acquired IRAP during phagosome maturation in CD8+ and CD8− DCs. After a short pulse with OVA-yeast cells, phagosomal maturation was allowed for 20 (early phagosomes) and 60 min (late phagosomes) before fixation and immunofluorescent labeling. The number of IRAP-enriched phagosomes was consistently higher in CD8+ DCs than in CD8− DCs, and this difference was constant along phagosome maturation (46% IRAP+ late phagosomes in CD8+ DCs against 20% in CD8− DCs) (Fig. 4). Moreover, phagosomal OVA colocalizing with IRAP staining was visible for at least 60 min of phagosome maturation in both subtypes, in agreement with the demonstrated mild proteolytic activity of DC phagosomes (36). In contrast, OVA staining in granulocytes was practically absent after only 20 min of maturation, probably due to the highly aggressive phagosomal environment characterizing these phagocytes (37).

Because cross-presentation of soluble OVA was IRAP dependent in cDCs, we also wondered if soluble OVA is internalized in IRAP vesicles. To this end, we analyzed endocytic trafficking of soluble OVA by subcellular fractionation according to a recently published protocol (29). Immunoblot analysis of endosomal and lysosomal fractions showed an accumulation of OVA in IRAP endosomes

**FIGURE 3.** IRAP is required for cross-presentation of soluble and particulate Ag in both CD8+ and CD8− DC subsets. A, Total of 100,000 IRAP KO or wt CD8+ and CD8− DC equivalents or 2 × 105 pDC equivalents purified from in vitro Flt3L culture were incubated with 0.6 mg/ml soluble OVA (left panel) or 2.5 × 107 OVA-expressing yeast cells (right panel) for 16 h. Subsequently, cells were washed and cocultured with 2 × 105 OT-I cells for 24 h. Activation of Ag-specific OT1 T cells was determined via IL-2 detection in the culture supernatant by ELISA. Graphs show the mean and SD of duplicates from one representative experiment out of three. B and C, Ag uptake under conditions of in vitro cross-presentation assays was compared among different DC subsets. B, Cells were incubated with 0.6 mg/ml Alexa Fluor 647-conjugated OVA for 16 h. After extensive washing, fluorescent OVA uptake was measured by FACS. C, Uptake of yeast Ag during 16 h was quantified by counting phagosomes in at least 140 cells per subtype by microscopy. D, Total of 100,000 ex vivo-sorted DC subsets were incubated with increasing concentrations of endofree soluble OVA (left panel), soluble OVA (Worthington Biochemical) (middle panel), or OVA yeast (right panel) and Ag presentation measured as in A.
early after endocytosis (Fig. 5A). Considering the heterogeneity of early endosome populations (38), we sought to characterize further the OV A-containing endosomes by fluorescence microscopy. Selected cells are representative for the results obtained in three independent experiments. Scale bars, 5 μm. B, IRAP recruitment to phagosomes was quantified as the ratio of the number of IRAP+ phagosomes to the total number of OV A-containing phagosomes. A phagosome was considered IRAP+ if more than half of the phagosomal membrane was stained for IRAP. Bars show mean + SD of 120 phagosomes per cell type from three independent experiments.

Discussion

Our previous identification of the role of IRAP in cross-presentation highlighted a final processing step for cross-presented Ags that can occur in an endosomal environment (12). In this report, we extend these previous findings and present evidence suggesting that IRAP may contribute to the efficacy of CD8+ DCs as cross-presenting APCs. This notion is supported by the strong effect of IRAP deficiency on cross-presentation of soluble and particulate Ag in this DC subtype, the substantial and specific recruitment of IRAP to phagosomes in CD8+ DCs, and the greater colocalization of IRAP with storage endosome markers in CD8+ relative to CD8- DCs.

Because cell-specific external stimuli affect signaling pathways and control the traffic of IRAP vesicles (14), the DC type or the Ag form could influence the contribution of IRAP+ endosomes to cross-presentation. We found that both types of cDCs cross-presented soluble and particulate Ag efficiently. Moreover, we demonstrate that the IRAP-dependent cross-presentation pathway operates in both CD8+ and CD8- steady-state cDCs. The former finding may appear to contradict previous reports underlining the superior cross-presenting efficiency of the CD8+ DC subset (22, 23, 29, 32). Several considerations may reconcile our results with previously published work. First, the efficient cross-presentation of OV A-yeasts by CD8- DCs may, at least in part, be due to increased uptake of yeasts by these cells. Second, the recent finding that CD8+ DCs acquire full cross-presentation capacity only after licensing by GM-CSF, TLR ligands, or CD40 engagement (34, 40) may explain why the CD8+ DC equivalents from our Flt3L-differentiated cultures did not surpass CD8- DCs in their cross-presentation efficiency. Moreover, it has been demonstrated that the cross-presentation capacity of splenic CD8+ DCs is correlated with CD103 expression that varies between mice colonies from 10–100%, according to the hygiene status (35). The low per-

FIGURE 4. Recruitment of IRAP to yeast-containing phagosomes. A, OV A-yeast was fed to purified DCs of different subtypes or granulocytes for the indicated time periods, immunostained for OV A and IRAP, and analyzed by microscopy. Selected cells are representative for the results obtained in three independent experiments. Scale bars, 5 μm. B, IRAP recruitment to phagosomes was quantified as the ratio of the number of IRAP+ phagosomes to the total number of OV A-containing phagosomes. A phagosome was considered IRAP+ if more than half of the phagosomal membrane was stained for IRAP. Bars show mean + SD of 120 phagosomes per cell type from three independent experiments.

FIGURE 5. Recruitment of IRAP to soluble OV A-containing endosomes. CD11c+ spleen DCs (A) or Flt3L-derived DC subsets (B–D) were fed with soluble OV A. Endosome populations isolated with Percoll gradient ultracentrifugation were analyzed by immunoblot for IRAP, OV A, Rab7, and the subunit E of the lysosomal V-ATPase (A). Cells were immunostained for IRAP (B, D), OV A (B, C), or MR (C, D) and analyzed by microscopy.

CD8+ DCs as cross-presenting APCs. This notion is supported by the strong effect of IRAP deficiency on cross-presentation of soluble and particulate Ag in this DC subtype, the substantial and specific recruitment of IRAP to phagosomes in CD8+ DCs, and the greater colocalization of IRAP with storage endosome markers in CD8+ relative to CD8- DCs.
centage of CD8+ DCs expressing CD103 (20%) in mice from our colony may explain the relatively inefficient cross-presentation of soluble OVA by CD8- DCs in our experiments. Consistent with this hypothesis, CD8+ DCs cross-presented far better than CD8- DCs when small amounts of LPS were added to OVA.

Our finding that an IRAP-dependent cross-presentation pathway is operational both in CD8+ and CD8- DCs contradicts a recent report by Segura and associates (29), who suggested that this pathway is limited to moDCs. These authors also failed to observe colocalization of internalized OVA with IRAP, in contradiction to our findings in this and a previous report. We propose that next to a different genetic background, as suggested by these authors, different experimental settings such as the timing of Ag processing, Ag dose, or Ag type (10) may explain these differences.

Timing of Ag processing has a major impact on the efficiency of cross-presentation. When we analyzed during a time course the in vitro cross-presentation capacity of moDC equivalents, we observed that OVA processing times exceeding 6 h erased the IRAP-dependence of cross-presentation (Supplemental Fig. 3). Nevertheless, the differences obtained in vitro for shorter processing times are physiologically relevant, because IRAP ko moDCs that captured Ag in vivo had a compromised cross-presentation ability, as confirmed by Segura et al. (29). The amount of internalized Ag is another factor affecting the magnitude of cross-presentation. When sufficiently high Ag amounts are used, the effect of deficiency not only for IRAP, but also for ERAP and even TAP on cross-presentation of phagocytosed Ag can be overcome (Supplemental Fig. 4). Consistent with published data, we have previously postulated that DCs use multiple pathways for cross-presentation of particulate Ag, which presumably explains this observation (12, 41).

The existence of multiple pathways for cross-presentation and functionally distinct DC populations may be essential in enabling the immune system to respond to a large variety of pathogens. It is well known that different subpopulations of DCs express specific surface receptors that allow them to preferentially take up a given type of Ag, but it is less well known why and how different DC types will use preferentially one of the multiple pathways of cross-presentation. Our analysis of IRAP localization in steady-state DCs before Ag encounter as well as following phagocytosis suggests that the CD8+ and CD8- cDC subtypes may use preferentially different pathways. Among the phagocytic cell types analyzed in this study, CD8+ DCs had the highest recruitment of IRAP to phagosomes and the highest efficacy of cross-presentation if the amount of Ag uptake is taken into account. These results suggest that recruitment of IRAP endosomes to the Ag-containing phagosomes is mandatory for the superior cross-presentation efficiency of CD8+ DCs in comparison with CD8- DCs.

Why do CD8+ DCs acquire higher IRAP levels in their phagosomes, considering that all DC subtypes express similar total amounts of IRAP? Analysis of IRAP endosomes in steady-state DCs by fluorescence microscopy yielded a potential explanation to this phenomenon. CD8+ DCs displayed significantly higher IRAP/Rab14 colocalization than CD8- DCs. Rab14 is a small GTPase that drives IRAP translocation upon activation of the PISK (42). Because PISK is activated early after phagosome formation (43), it is likely that Rab14 is involved in the recruitment of IRAP vesicles to the phagosomal membrane. Therefore, the higher amount of Rab14 in IRAP vesicles of CD8+ DCs, compared with CD8- DCs, could be responsible for the stronger IRAP recruitment to phagosomal membranes in the CD8+ DCs.

In conclusion, in this manuscript, we demonstrated that the role of IRAP in cross-presentation by conventional steady-state DCs is similar to that previously observed for moDCs. These findings are in agreement with the recent demonstration that both classical and in vivo-differentiated moDCs, the physiologic counterparts of GM-CSF–derived BMDCs, efficiently cross-presented Gram-negative bacteria and soluble Ags (18). We demonstrate that all cross-presentation DCs are able to employ IRAP-dependent cross-presentation. Because IRAP endosome trafficking is regulated by cell-specific stimuli (14, 15), it is conceivable that IRAP dependent cross-presentation can become predominant over the endoplasmic reticulum-to-cytosol pathway of cross-presentation in specific circumstances. These circumstances, potentially important for vaccination strategies, might become obvious once specific stimuli and signaling pathways that regulate IRAP trafficking in DCs are identified.

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Disclosures

The authors have no financial conflicts of interest.

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