Published in final edited form as:

J Immunol Methods. 2015 August ; 423: 18–28. doi:10.1016/j.jim.2015.04.023.

Quantitating MHC class II trafficking in primary dendritic cells using imaging flow cytometry

Cassandra M. Hennies, Maria A. Lehn, and Edith M. Janssen

Division of Immunobiology, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH, USA

Abstract

Presentation of antigenic peptides in MHC class II (MHCII) on dendritic cells (DCs) is the first step in the activation of antigen-specific CD4⁺T cells. The expression of surface MHCII-peptide complexes is tightly regulated as the frequency of MHCII-peptide complexes can affect the magnitude, as well as the phenotype of the ensuing CD4⁺T cell response. The surface MHCII-peptide levels are determined by the balance between expression of newly generated complexes, complex internalization, and their subsequent re-emergence or degradation. However, the molecular mechanisms that underpin these processes are still poorly understood. Here we describe a multispectral imaging flow cytometry assay to visualize MHCII trafficking that can be used as a tool to dissect the molecular mechanisms that regulate MHCII homeostasis in primary mouse and human DCs.

Keywords

MHC class II; dendritic cells; imaging; internalization

1. Introduction

Activation of adaptive CD4⁺T cells results from direct interaction between the T cell Receptor (TCR) on the T cells and MHCII-peptide complexes on professional antigen presenting cells (APC). While the CD4⁺ T cells require additional membrane and soluble costimulatory signals in order to become adequately activated, the TCR/MHCII-peptide interaction is a prerequisite for the initiation of the CD4⁺T cell response. Moreover, various studies indicate that the quantity of MHC-peptide complexes as well as the duration of the TCR/MHCII-peptide complex interactions can affect the magnitude of the CD4⁺T cells response, as well the CD4⁺T cell phenotype development^{1, 2, 3, 4, 5, 6}.

Corresponding author: Edith Janssen, Cincinnati Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Division of Immunobiology, S5.419, 240 Sabin Way, Cincinnati, OH 45229, USA, Edith.Janssen@cchmc.org, P (513) 803-1055, F (513) 636-5355.

Conflict of interest.

The authors have no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Among the APC, dendritic cells (DC) have the greatest capacity to prime naïve CD4⁺T cells. DCs are uniquely designed to capture antigens via various endocytic pathways and process these antigens for loading in MHC class II^{7, 8, 9}. Upon antigen uptake, the antigen-containing endosomes develop intraluminal vesicles (ILVs) that form multivesicular bodies (MVB) that eventually fuse with lysosomes for lysosomal degradation. Loading of antigen-peptides into MHCII has been reported at nearly every phase of the endocytic process^{10, 11}. However, the majority of the MHCII peptide loading is thought to occur in MHCII containing compartments (MIIC) that resemble late endosomes with multilamellar or multivesicular morphology^{12, 13}.

In the last decades great progress has been made in the elucidation of MHCII peptide loading and trafficking but many questions remain unanswered^{14, 15}.

MHCII molecules are synthesized *de novo* in the endoplasmic reticulum (ER) where they are associated to the invariant chain (Ii)¹⁶. The Ii contains the CLIP region that binds the peptide-binding groove and prevents premature peptide binding¹⁷. The Ii also contains a cytosolic di-leucine-targeting motif that facilitates transportation of the MHCII-Ii complexes to the surface of the DC or directly into MIIC^{18, 19, 20, 21, 22, 23} (fig. 1). The MHCII-Ii complexes that reach the surface are rapidly internalized by clathrin-mediated endocytosis and redirected to MIIC^{24, 25, 26}. Most MHCII loading occurs in MIIC that are characterized by an acidic pH, presence of HLA-DM/H2-M, and a variety of proteases, including Cathepsin S and L^{27, 28}. Peptide loading of MHCII requires the proteolytic processing of Ii into CLIP, followed by displacement of CLIP by other peptides present in the lumen^{29, 30, 31}. The MHCII-peptide complexes are then transported to the cell surface or directly sorted to the lysosomes for degradation (fig. 1). The MHCII-peptide complexes that reach the surface are rapidly internalized and sorted to MIIC followed by degradation in lysosomes or reexpression on the surface.

The processes that govern the formation of the MHCII+ vesicles and facilitate their traveling to and from different membranes/organelles is still unclear. MHCII-peptide complexes are internalized via clathrin- and dynamin-independent pathways, possibly mediated by RhoA/RhoB or Arf6 and Rab35 dependent mechanisms^{32, 33}. Additional studies suggest roles for lipid rafts and tetraspanin-enriched microdomains in these processes^{34, 35, 36}.

The signals that dictate MHCII-peptide surface expression/internalization are also poorly understood. Ubiquitination of the lysine residue at position 225 in the MHCII β chain by ubiquitin-protein ligase E3 membrane-associated RING-CH1 (MARCH1) has been reported to facilitate MHCII-peptide trafficking to ILV and MVB³⁷. Elimination of either MARCH1 or the 225-lysine residue on the MHCII β chain resulted in increased surface MHCII expression and decreased MHCII levels in the ILV/MVB^{38, 39, 40, 41}. However, it is not clear where the ubiquitination of the MHCII occurs³⁷. Several studies suggest that ubiquitination may occur at the surface membrane, thereby providing a mechanism for the internalization of the MHCII^{42, 43}. Other studies found no role for ubiquitination in the internalization process, but observed that ubiquitination prevented the MHCII-peptide complexes to leave the ILV and reach the surface membrane¹⁵.

The dissection of MHCII homeostasis in DCs is complex, as it is affected by the type of DC studied, its maturation state, and the sensing of environmental stimuli^{41, 44}. Dendritic cells are a heterogeneous population that encompasses many blood and tissue-associated subsets. These subsets don't only differ in their capacities to activate CD4⁺ or CD8⁺T cells, they also express different baseline levels of MARCH1, de-ubiquitinases, and molecules associated with vesicle trafficking and fusion (sorting nexins, Rab GTPases)^{45, 46, 47, 48}. To further complicate matters, immature and mature DCs have very distinct MHCII internalization/ expression kinetics. Immature DCs have high endocytic capacity, express high levels of MARCH1, have low levels of surface MHCII, high levels of ILV-associated MHCII, and high MHCII internalization rates. Upon reception of appropriate maturation stimuli, DCs can down-regulate the transcription of MARCH1, reduce their MHCII internalization rate and mobilize MHCII-peptide complexes from the ILV/MVB via tubular structures to the surface membrane^{39, 40, 42, 49, 50}.

This complexity highlights the need for assays that can simultaneously assess multi intracellular parameters while allowing for the identification of subpopulations and maturation/activation levels. Currently most of the MHCII trafficking studies are performed using *in vitro* generated bone marrow-derived DCs using confocal microscopy or scanning disc microscopy. These approaches have the advantage of high resolution data but are limited in parameters, and generally lack the statistical power to compare and identify trends.

Here we describe a multi-color imaging flow cytometry assay to assess MHCII trafficking in primary DCs on a subcellular level. This approach uses an unbiased analysis of MHCII+ vesicles in large populations of DCs and allows for the comparison of trends between various DC subsets. Data obtained from these analyses can further be used to refine confirmatory experiments and reduce observer bias in approaches with higher resolution but poor statistical power.

2. Materials and Methods

2.1. Dendritic cell isolation

2.1.1. Technical considerations—All isolation procedures are performed with buffers, media and equipment at 4°C in order to limit the activation of the DCs. In all buffers BSA was replaced by heat-inactivated FBS to minimize endotoxin contamination. Comparisons between flow-cytometric sorting and magnetic bead sorting in our laboratory indicated that flowcytometric-sorting resulted in higher purity, but a longer processing time, and higher activation status of the DCs that reduced MHCII trafficking. As imaging flow cytometry does not require high purity, magnetic bead sorting (either positive or negative selection) is very suitable for this type of experiment. In our experience no significant differences in experimental outcomes were seen between negative and positive magnetic bead selection approaches.

2.1.2. Primary mouse DCs—All mice were cared for in accordance of the National Institutes of Health guidelines with the approval of the Institutional Animal Care and Use Committee. Mouse spleens were mechanically disrupted and single cells suspensions were

made using complete medium (IMDM, 10% heat-inactivated FBS, 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, and 50uM 2-mercaptoethanol). DCs were further enriched using either directly conjugated CD11c-magnetic beads (Miltenyi), or an in-house generated negative selection kit using biotinylated Ab to CD3, TCR, CD19, IgM, and IgD (all ebioscience/biolegend), followed by anti-biotin magnetic beads (Miltenyi).

2.1.3. Primary human DCs—Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using Ficoll-Paque Plus density gradient centrifugation. DCs were further enriched using a combination of CD11c-biotin (biolegend) followed by anti-biotin beads (Miltenyi) which results in the enrichment of all conventional DCs and monocytes or a BDCA1+DC isolation kit (Miltenyi). Sorted cells were further cultured in RPMI, 10% heat-inactivated FBS, 100U/ml penicillin, 100ug/ml streptomycin, and 2mM L-glutamine.

2.2. DC culturing for MHCII homeostasis assays

The MHCII internalization protocol was adapted from previous publications^{32, 41}. DCs (1×10^6 /ml) were incubated with 5ug/ml biotinylated-Ab to MHCII (M5/114.15.2) on ice for 15 minutes. Human DCs (1×10^6 /ml) were incubated with 5ug/ml biotinylated-Ab to HLA-DR (L243). Subsequently, DCs were washed in cold complete medium, plated into eppendorf tubes (10^5 – 10^6 DC/200–500ul), and cultured at 37°C, 5%CO₂ (fig. 2a). Positive and negative controls for MHCII trafficking included crosslinking of the biotinylated-MHCII by a secondary antibody to induce rapid internalization (anti-Rat IgG for mouse studies, anti-mouse IgG for human studies, 15', on ice) or inclusion of maturation stimuli that reduce MHCII-internalization (LPS 50ng/ml). To prevent *de novo* MHCII synthesis, DCs were cultured in Brefeldin A (BFA, 5ug/ml) for the duration of the experiment. To interfere with lysosomal acidification, Chloroquine (50uM) or Bafilomycin (50nM) were added for the duration of the culture.

2.3. Staining for MHCII homeostasis assays

At different time-points DCs were collected for MHCII/HLA-DR staining (fig. 2a). When working with primary cells it is recommended that a viability staining is included (Molecular Probes/Life Technologies). This can be in the same emission length/channel as the dump channel. The final staining panel will depend on the configuration of the instrumentation and experimental questions addressed. For co-localization studies it is recommended to select colocalizing probes that use different lasers to reduce the amount of compensation needed between your markers or interest. Due to the set-up of our instrumentation, no more than 7 spectral imaging bands were used at any given time.

2.3.1. Surface staining and cell permeable dyes—Cell viability staining with a fixable live/dead staining was performed before the traditional surface staining according to the instructions of the manufacturers (Molecular Probes, Life Technologies). All surface stainings were performed in staining buffer (PBS, 5% heat-inactivated FBS) on ice for 15–20 minutes. Primary mouse DCs were stained with streptavidin-PE to visualize surface biotin-MHC, CD11c, a non-competing Ab to MHCII to visualize total surface MHCII, and a combination of antibodies against CD3, TCR β , NKp46, CD19, IgM, and IgD as lineage staining (table I). Markers for DC activation or subpopulations, CD8 α , CD103, CD11b,

CD4, or SIRP α can be added at this point. For primary human DCs, cells were stained with CD11c, BDCA1, BDCA3, CD14, streptavidin-PE to visualize the surface biotin-HLA-DR, and a combination of antibodies against CD3, CD56, and CD19 as lineage staining (table II).

2.3.2. Quenching and Intracellular staining—Before intracellular staining, samples were incubated with Avidin-D (10ug/ml, Vector laboratories) in staining buffer for 15 minutes on ice to saturate all unbound surface biotin. Subsequently, cells were prepared for intracellular staining using an intracellular staining kit (eBioscience). Cells were stained with streptavidin-Alexa647 to visualize the intracellular biotin-MHCII. Ab to EEA1, Lamp1, and RAB5 or RAB7 followed by the appropriate secondary stain were included for colocalization studies at this point (table III). At the end of the staining, cells were fixed in 1% formaldehyde in PBS for 20 minutes, washed, and resuspended in PBS for acquisition.

2.4. Acquisition, compensation, and data analysis

Multispectral Imaging-based Flow Cytometry was performed using the ImageStream X flow cytometer (Amnis, Seattle, WA) outfitted with the 405, 488 and 658 nm lasers at 60x magnification. The number of events to acquire is dictated by the type of analysis to be performed. Our experience indicates that around 300–600 in-focus events are needed per DC subpopulation to reliably and reproducibly assess co-localization of internalized MHCII with various intracellular organelles. Based on the purity of the DC population (60–80%, when bead sorted), the frequency of the subpopulation of interest within the DCs (10–50%), and the anticipated “in focus” events (70%), the total number of cells for acquisition is determined. In the presented experiments at least 3000 CD11+/lineage^{negative} events (corresponding to approximately 8000 total events) were acquired for each condition. Single stain and secondary Ab staining controls (i.e. samples contain all Ab except the primary unconjugated Ab) were included for each experiment. Post-acquisition spectral compensation and data analysis were performed using ImageStream Data Exploration and Analysis Software (IDEAS) 6.0.

2.4.1. Analysis: Identification of DCs—Live DCs were identified for further analysis after sequential gating for (i) single cells, using bright field area vs. aspect ratio feature gating, (ii) in-focus cells, based on bright field gradient root mean square (GRMS) feature applied to channel 1, (iii) lineage negative cells (intensity feature), (iv) live/dead-stain negative cells (intensity feature), followed by (v) gating on CD11+ and total MHCII+ double positive cells. At this step subpopulations of DCs can be identified based on their surface staining (intensity). For colocalization studies cells were further gated on their expression of fluorochromes of interest (colocalization marker; intensity feature).

2.4.2. Analysis : Generation of Masks—To determine the intensity of the internalized MHCII and surface MHCII on the DCs, the regions of interest were identified through the generation of masks. To generate the standard cytosol mask, the Erode Mask was used on the CD11c staining and eroded 3 pixels. For membrane proximity studies a “narrow” cytosol mask (on CD11c, erode 6) was used. Membrane masks were generated using a Dilate Mask on the CD11c staining (dilate 1) and subtracting the cytosol mask using Boolean Logic (fig. 3).

To analyze the intracellular MHCII+ organelles, a Spot Mask on channel 11 (SA-A647) was generated with a spot to background ratio 2, and spot radius of 4. The spot mask was then combined with the standard cytosol mask to limit the analysis to cytosolic signal and used as input in the Spot Count feature to determine the number of spots. To assess the trafficking of the MHCII+ particles, the “narrow” cytosol mask was used to identify events further from the membrane while a wider membrane mask (CD11c “erode” mask minus the “narrow” cytosol mask) was used to identify events closer to the membrane (fig. 3). For the colocalization analyses, additional spot masks were made for each channel of interest and combined with the MHCII spot masks.

2.4.3. Intensity, spot count, and colocalization analysis—The intensity of the total surface MHCII and the surface biotin-MHCII was assessed using the Intensity feature in combination with the membrane mask while the intensity of the internalized MHCII was determined within the cytosol mask. The spot count and spot size was determined by applying the cytosolic spot mask to the Spot count feature and the Spot Area feature. Co-localization of intracellular MHCII and a single organelle marker (Lamp-1 or EEA1) was measured using the Bright Detail Similarity R3 feature (BDS), that measures the degree of similarity of the brightest details between two fluorescent images using a spot mask for cytosolic MHCII, and the marker of interest. In cases where co-localization of more than two fluorescent probes was required e.g. to assess the localization of MHCII in late endosomes (LAMP1+ RAB7+), spot masks were generated to identify the spots observed for each fluorochrome. These masks were then merged under the Boolean operator “AND”, and used as input to the Bright Detail Similarity R3 Feature (BDS) to identify cells in which MHCII, Lamp1 and Rab7 spots were co-localized. Every BDS was made individually for each combination of channels. All BDS cut-offs were based on the BDS observed in negative control cells that encompassed DCs that were stained for all the fluorochromes (directly conjugated and secondary Ab) but lacked the primary unconjugated Ab to the specific marker.

3. Results

3.1. MHCII internalization in primary human DC subsets

CD11c-positively selected PBMC were incubated with biotinylated-HLA-DR and cultured for 0 and 6 hr. Subsequently cells were stained for BDCA1 (CH12), BDCA3 (CH2), CD14 (CH5), CD11c (CH4), total HLA (CH8), surface biotinylated-HLA-DR via SA-PE (CH3), intracellular biotinylated-HLA-DR via SA-A647 (CH11), in combination with a viability and lineage staining (CH7) (fig. 4a). Within the 3 different DC populations the intensity of the total surface HLA-DR and the biotinylated surface HLA-DR were determined using the “membrane mask” while the intensity of the internalized biotinylated-HLA-DR was determined upon application of the “cytosol mask”. Both BDCA1+ and BDCA3+ DCs showed increases in intensity of total surface HLA-DR upon 6 hr culturing *in vitro*. The intensity of surface biotinylated-HLA-DR decreased over time while the intensity of internalized biotinylated-HLA-DR increased. Compared to BDCA1+DCs, BDCA3+DCs had increased upregulation of total surface HLA-DR expression and reduced HLA-DR internalization. (fig. 4b). CD14+CD11c+ DCs were the most abundant DC population in this

donor (>80%) but showed relatively poor HLA-DR upregulation and limited HLA-DR internalization over time. Due to the large proportion of CD14⁺CD11c⁺ DCs, the total CD11c⁺ population showed little MHCII internalization, thereby underestimating the capacity of DCs to internalize HLA-DR. Similarly, failure to exclude the dead/dying cells results in an underestimation of HLA-DR internalization capacity (fig. 4c). Together these data illustrate the need for the analysis of live subpopulations when using primary DCs.

3.2. Kinetics of MHCII internalization and turn-over in primary mouse DCs

Mouse DCs were incubated with biotinylated-MHCII Ab and cultured for indicated time-points. DCs were stained for CD11c (CH12), CD11b (CH8) CD8 (CH4), total MHCII (CH7), surface biotinylated-MHCII via SA-PE (CH3), intracellular biotinylated-MHCII via SA-A647 (CH11), in combination with a viability and dump staining (CH2) (fig. 5a). Within the 2 different DC populations the intensity of the total surface MHCII and the biotinylated surface MHCII were determined using the “membrane mask” while the intensity of the internalized biotinylated-MHCII was determined upon application of the “cytosol mask”.

In time, the intensity of the total surface MHCII increased on DCs cultured in medium only. Inclusion of BFA, thereby blocking transport of *de novo* generated MHCII from the ER and golgi apparatus, resulted in decreased total surface MHCII expression, implicating a major role of *de novo* MHCII synthesis in the total MHCII surface expression level. Addition of LPS significantly increased MHCII surface levels; likely a result from re-expression of internalized MHCII and *de novo* synthesis (fig. 5b).

The intensity of the biotinylated-MHCII on the surface membrane reduced over time in a linear fashion. Upon stimulation with LPS, DCs maintained their internalization rate for a brief period after which the biotinylated-MHCII levels on the surface stabilized.

Interestingly, in the control DCs the intensity of the internalized MHCII initially increased, but then reached a plateau. Plotting the intensity of internalized biotinylated-MHCII against the intensity of the surface biotinylated MHCII indicated that after 4 hr the internalization and degradation of MHCII started to reach an equilibrium. Inhibition of lysosomal acidification by the addition of either chloroquine or Bafilomycin resulted in a linear increase in internalized MHCII, indicating that under normal conditions internalized MHCII was targeted for lysosomal degradation (fig. 5b). In the presence of LPS the intensity of internalized MHCII sharply declined over time. LPS treatment did not increase the surface biotinylated-MHCII levels, suggesting that the DCs stopped internalizing MHCII and that the already internalized biotinylated-MHCII was sorted to lysosomes for degradation and was not redirected to the cell surface.

Parallel experiments in which CD11bDCs were further subdivided into CD86^{low} and CD86^{high} subsets showed that MHCII-internalization was significantly higher in the less mature DCs than the more mature DCs (fig. 5c/d, CD86^{medium}DCs showed an intermediate phenotype, not shown). These data are in line with the hypothesis that MHCII trafficking is affected by DC maturation and demonstrate the advantage of incorporating maturation assessments into the functional assays.

3.3. Assessment of MHCII containing organelles

While classic flow cytometry can generate most of the information displayed in figure 4 and 5, it cannot provide insight in the internalization and intracellular trafficking patterns of the biotinylated-MHCII. Using a mask function in IDEAS we generated spot masks to identify the number of organelles that contain internalized biotinylated-MHCII (see fig. 3)). Application of these spot masks to the features allowed for the assessment of spot number and spot size. Kinetic studies showed that MHCII internalization started with the appearance of many small MHCII+ particles (fig. 6a/b). In time, the particle number increased only slowly, but the average size of the particles increased significantly, suggesting the fusion of early endosomal particles into larger endosomes or multivesicular bodies (fig. 6b/c). Application of a combination of the spot mask and a “wide membrane” mask or a “cytosol” mask facilitated the analysis of the localization of the spots in relation to their position in the cell (fig. 6d). In mouse CD11bDCs, the smaller MHCII+ particles preferentially resided close to the cell surface membrane at all time points. As the level of surface biotinylated-MHCII is linearly decreasing over time, it is likely that the majority of these small particles represent MHCII that is actively internalized and not MHCII+ particles that are re-cycled from the MVB to the surface via the relatively small tubular structures. In contrast to the small particles, the larger MHCII+ particles were farther removed from the surface membrane (fig. 6d).

Using this approach we next assessed whether LPS treatment affected the trafficking of internalized MHCII. LPS treatment of CD11bDCs resulted in a transient appearance of small MHCII+ particles close to the cell surface. Later time points showed fewer but larger particles with decreasing MHCII intensity that were located towards the center of the DCs (fig. 6b). These observations indicate that LPS treatment inhibited MHCII internalization and that internalized MHCII was not redirected back to the surface via small tubules, but targeted for lysosomal degradation upon vesicle fusion.

3.4. Characterization of MHCII+ organelles by colocalization studies

To further characterize the MHCII+ vesicles, DCs were costained with markers for early, mid, and late endosomes. Specific spot masks were generated for each marker and the colocalization of intracellular MHCII and a single organelle marker was measured using the Bright Detail Similarity (BDS) feature with a combination of the cytosolic spot mask for MHCII and the spot mask for the specific marker. The BDS feature was generated for each combination of markers with the appropriate combinations of masks and the cut-off was determined based on the BDS of the control samples (secondary Ab, no primary Ab). Data was plotted as % of DCs with the particular BDS against the MHCII particle size.

At early time-points, the MHCII+ vesicles showed high BDS similarity ($BDS > 1.5$) with Early Endosomal Antigen 1 (EEA1). After 7 hr, the small MHCII+ vesicles still showed high BDS with EEA1 whereas the larger MHCII+ vesicles had a poor similarity score (fig. 7a). Colocalization studies with Lamp1 showed association of the larger MHCII+ vesicles with Lamp1 (fig. 7b). Additional multi-parameter colocalization studies indicated that at early time points internalized MHCII was found in EEA1+ vesicles but not in Rab5+ vesicles, Rab7+ vesicles, or Lamp1+ vesicles (fig. 7c). At later time points the internalized

MHCII was also found in the larger Rab7+ vesicles, Lamp1+ vesicles, and Rab7/Lamp1 double positive vesicles, but (fig. 7c, and data not shown).

4. Discussion

Imaging of MHCII trafficking has been instrumental in providing insight in the processes that govern MHCII expression, internalization, re-emergence, and degradation in DCs. However, the complexity of DC biology in regard to their subsets and activation/maturation status has highlighted the need for complementary assays that can simultaneously assess multi intracellular parameters in vast number of cells, and correlate the data with additional biological processes. Here we describe a powerful approach to assess MHCII internalization and trafficking in primary DCs using imaging flow cytometry. Our data shows that internalization of MHCII complexes starts with the appearance of multiple small MHCII +EEA1+ vesicles close to the cell surface membrane that acquire Rab5 and migrate away from the cell membrane. The vesicles evolve into larger Rab7+ and Lamp1+ vesicles and eventually fuse with lysosomes that degrade the internalized MHCII. Our data also showed that LPS signaling inhibited MHCII internalization that became noticeable within 3 hr after LPS stimulation. This outcome was expected as LPS stimulation has been shown to reduce mRNA levels of MARCH-I, the E3 ubiquitin-protease that facilitates the ubiquitination of the MCHII β chain. Since MARCHI has a very short half-life (<30 min), MARCHI protein levels should rapidly decrease, thereby preventing MHCII internalization. At the same time, the intensity of the internalized MHCII and the number of MHCII+ vesicles started to decrease, with particle size and position suggesting that these vesicles were targeted for lysosomal degradation. Imaging flow cytometry does not provide three-dimensional data introducing the risk of incorrectly interpreting (co-)localization trends. However, our observations are in line with those of various other groups that used in vitro generated DCs and classic imaging approaches such as confocal microscopy, scanning disc microscopy, or Electron Microscopy, as well as our studies with primary DCs and confocal microscopy. This implies that imaging flow cytometry is an appropriate approach for these types of study ^{14, 15, 40, 41, 42, 43, 51, 52, 53, 54, 55}.

Compared to other state of the art imaging approaches, imaging flow cytometry has significantly lower resolution and is not able to perform live cell imaging. However, it has to the capacity to analyze large numbers of events that are chosen without bias for multi-parameter features and evaluate these data for multi-parameter trends and correlations.

The capacity to analyze large number of cells is important as it provides sufficient significant power to identify trends and processes. The unbiased acquisition, together with the wide dynamic range of the spectral imaging bands eliminates the observer bias that is easily introduced in other methods. While conventional flow cytometry is an unbiased approach, it did not have the discriminative capacity to identify DCs that showed very low levels of MHCII internalization (few small particles), resulting in the incorrect assumption that some specific DC subsets did not have MHCII trafficking/internalization. Similarly, the increased discriminative capacity of the imaging flow cytometry approach allowed for the earlier detection of MHCII internalization as well as the identification of relatively small changes in MHCII trafficking. In our hands, confocal microscopy did allow for the

identification of DCs that had internalized low levels of MHCII and had the important advantage of the generation of three-dimensional images for the accurate assessment of organelle location. However, this approach carries a strong risk of identifying/selecting cells that most closely fit a specific experimental expectation, thereby introducing bias and possibly overlooking other important processes. The use of a motorized stage to image multiple, random cells could overcome this bias, however, the large numbers of events needed to perform reliable and reproducible (colocalization) comparisons between different samples makes this approach very cumbersome. However, once the variation within a sample population has been established using image flow cytometry analysis, biased selection in confocal microscopy can be used as a complementary approach to provide higher quality and more detailed images to further study the biological processes.

The availability of 10 spectral imaging bands for multi-color parameters is another important advantage, especially when analyzing primary DCs. DCs are relatively rare cells and encompass various subpopulations. Manipulations, including sorting, have been shown to induce DC maturation and activation, and affect cell viability, and can therefore negatively impact or limit the experiments. The availability of the many spectral imaging bands allows for the inclusion of DC subset stains as well as a lineage stain, eliminating the need for purified DCs in the assay. Moreover, primary DCs have a relatively short half-life *in vitro* and are highly sensitive to manipulations and chemicals. Loss of viability would significantly confound the interpretation of the data. When chemically interfering with specific pathways of endocytosis we observed that the inhibition of MHCII internalization closely correlated with the loss of viability in the primary DCs and not with the inhibition of the tested pathways. The recent development of fixable life/dead dyes allows for the incorporation of this discriminator in assays that have intracellular stainings. The viability staining can either be assigned to a specific spectral imaging band to assess toxicity of the chemical manipulation, or to the same imaging band as the lineage staining. The multi-color staining also supports direct comparisons between different DC subpopulations from the same experimental conditions or the same donor. We have observed wide variation in DC composition and baseline activation status between different donors. The simultaneous assessment of MHCII trafficking processes in different DC subsets from the same donor provides internal controls in the assay, and thereby allows for the normalization between subsets and the identification of subset specific trends/processes.

The described assay and analysis approaches can be easily adapted to further dissect the basic molecular mechanisms that regulate MHCII expression and internalization. Incorporation of additional Abs to cellular structures, organelles and proteins will further facilitate the identification of MHCII. Abs that recognize specific MHCII-peptide complexes, for example the Y-Aw Ab that recognizes MHCII-E α_{52-68} , can be used to track naturally occurring or experimentally generated (peptide pulsed) antigen-specific MHCII-peptide complexes. In addition, pharmacologic interventions and genetically modified DCs can be used to dissect MHCII trafficking and vesicle formation and fusions under steady-state conditions.

Moreover, this type of approach can be used to dissect MHCII trafficking processes in the context of a wide variety of biologically and immunologically relevant stimuli. The strength

of cytokine or innate receptor signaling can be assessed by the inclusion of stainings for adaptor molecule phosphorylation, translocation of transcription factors to the nucleus, or cycling of the specific receptor. In addition, fluorescently labeled pathogens can be incorporated to determine the relationships between MHCII trafficking, direct pathogenic infection, and the degree of infection.

5. Conclusions

Here we describe a simple and versatile methodology using imaging flow cytometry for the study of MHCII internalization and trafficking that can be used in primary and *in vitro* generated DCs of any species. This approach allows assessment of MHCII trafficking in different cellular structures in the context of biological and immunological relevant stimuli and correlates these processes with DC subtype and their maturation/activation status. This methodology is unbiased and has sufficient power to identify trends and processes and provides complementary data that currently cannot be obtained via the conventional approaches of MHCII trafficking analysis.

Acknowledgments

This work was supported by the National Institutes of Health via National Cancer Institute grant CA138617, the Charlotte Schmidlapp Award, and the LRI (all to E.M.J.).

Abbreviations

APC	antigen presenting cell
BDS	bright detail similarity
CLIP	class II associated invariant peptide
DC	dendritic cell
EEA1	early endosomal antigen 1
GMRS	gradient root mean square
Ii	invariant chain
ILV	intraluminal vesicles
March1	membrane associated RING-CH1 ubiquitin-protein ligase E3
MIIC	MHCII containing compartments
MVB	multivesicular body
TCR	T cell receptor

References

1. Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *The Journal of experimental medicine*. 1995; 182(5):1579–1584. [PubMed: 7595228]

2. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *The Journal of experimental medicine*. 1995; 182(5):1591–1596. [PubMed: 7595230]
3. Farber DL. Differential TCR signaling and the generation of memory T cells. *Journal of immunology*. 1998; 160(2):535–539.
4. Gett AV, Sallusto F, Lanzavecchia A, Geginat J. T cell fitness determined by signal strength. *Nature immunology*. 2003; 4(4):355–360. [PubMed: 12640450]
5. Burton BR, Britton GJ, Fang H, Verhagen J, Smithers B, Sabatos-Peyton CA, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. *Nature communications*. 2014; 5:4741.
6. Rogers PR, Croft M. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *Journal of immunology*. 1999; 163(3):1205–1213.
7. Neefjes JJ, Stollorz V, Peters PJ, Geuze HJ, Ploegh HL. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell*. 1990; 61 (1):171–183. [PubMed: 2156628]
8. Pathak SS, Blum JS. Endocytic recycling is required for the presentation of an exogenous peptide via MHC class II molecules. *Traffic*. 2000; 1(7):561–569. [PubMed: 11208144]
9. Pinet V, Vergelli M, Martin R, Bakke O, Long EO. Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature*. 1995; 375(6532):603–606. [PubMed: 7540726]
10. Griffin JP, Chu R, Harding CV. Early endosomes and a late endocytic compartment generate different peptide-class II MHC complexes via distinct processing mechanisms. *Journal of immunology*. 1997; 158(4):1523–1532.
11. Lindner R, Unanue ER. Distinct antigen MHC class II complexes generated by separate processing pathways. *The EMBO journal*. 1996; 15(24):6910–6920. [PubMed: 9003767]
12. Kleijmeer M, Ramm G, Schuurhuis D, Griffith J, Rescigno M, Ricciardi-Castagnoli P, et al. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *The Journal of cell biology*. 2001; 155(1):53–63. [PubMed: 11581285]
13. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annual review of immunology*. 2005; 23:975–1028.
14. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews Immunology*. 2011; 11 (12):823–836.
15. ten Broeke T, Wubbolts R, Stoorvogel W. MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. *Cold Spring Harbor perspectives in biology*. 2013; 5(12):a016873. [PubMed: 24296169]
16. Jones PP, Murphy DB, Hewgill D, McDevitt HO. Detection of a common polypeptide chain in I--A and I--E sub-region immunoprecipitates. *Molecular immunology*. 1979; 16 (1):51–60. [PubMed: 376435]
17. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990; 345(6276):615–618. [PubMed: 2190094]
18. Bremnes B, Madsen T, Gedde-Dahl M, Bakke O. An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization. *Journal of cell science*. 1994; 107 (Pt 7):2021–2032. [PubMed: 7983165]
19. Castellino F, Germain RN. Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity*. 1995; 2(1):73–88. [PubMed: 7600303]
20. Elliott EA, Drake JR, Amigorena S, Elsemore J, Webster P, Mellman I, et al. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *The Journal of experimental medicine*. 1994; 179(2):681–694. [PubMed: 8294875]
21. Odorizzi CG, Trowbridge IS, Xue L, Hopkins CR, Davis CD, Collawn JF. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *The Journal of cell biology*. 1994; 126(2):317–330. [PubMed: 8034737]

22. Lotteau V, Teyton L, Peleraux A, Nilsson T, Karlsson L, Schmid SL, et al. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature*. 1990; 348(6302):600–605. [PubMed: 2250716]
23. Teyton L, O'Sullivan D, Dickson PW, Lotteau V, Sette A, Fink P, et al. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature*. 1990; 348(6296):39–44. [PubMed: 2234057]
24. McCormick PJ, Martina JA, Bonifacio JS. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(22):7910–7915. [PubMed: 15911768]
25. Dugast M, Toussaint H, Dousset C, Benaroch P. AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. *The Journal of biological chemistry*. 2005; 280(20):19656–19664. [PubMed: 15749704]
26. Benaroch P, Yilla M, Raposo G, Ito K, Miwa K, Geuze HJ, et al. How MHC class II molecules reach the endocytic pathway. *The EMBO journal*. 1995; 14(1):37–49. [PubMed: 7530198]
27. Hsing LC, Rudensky AY. The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunological reviews*. 2005; 207:229–241. [PubMed: 16181340]
28. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, et al. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science*. 1998; 280(5362):450–453. [PubMed: 9545226]
29. Amigorena S, Webster P, Drake J, Newcomb J, Cresswell P, Mellman I. Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *The Journal of experimental medicine*. 1995; 181(5):1729–1741. [PubMed: 7722451]
30. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. *Annual review of immunology*. 2013; 31:443–473.
31. Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell*. 1998; 93(7):1135–1145. [PubMed: 9657147]
32. Walseng E, Bakke O, Roche PA. Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. *The Journal of biological chemistry*. 2008; 283(21):14717–14727. [PubMed: 18378669]
33. Ocana-Morgner C, Wahren C, Jessberger R. SWAP-70 regulates RhoA/RhoB-dependent MHCII surface localization in dendritic cells. *Blood*. 2009; 113(7):1474–1482. [PubMed: 18802007]
34. Knorr R, Karacsonyi C, Lindner R. Endocytosis of MHC molecules by distinct membrane rafts. *Journal of cell science*. 2009; 122(Pt 10):1584–1594. [PubMed: 19383725]
35. Kropshofer H, Spindeldreher S, Rohn TA, Platania N, Grygar C, Daniel N, et al. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nature immunology*. 2002; 3(1):61–68. [PubMed: 11743588]
36. Zilber MT, Setterblad N, Vasselon T, Doliger C, Charron D, Mooney N, et al. MHC class II/CD38/CD9: a lipid-raft-dependent signaling complex in human monocytes. *Blood*. 2005; 106(9):3074–3081. [PubMed: 15941914]
37. Ishido S, Goto E, Matsuki Y, Ohmura-Hoshino M. E3 ubiquitin ligases for MHC molecules. *Current opinion in immunology*. 2009; 21(1):78–83. [PubMed: 19203867]
38. Nathan JA, Lehner PJ. The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. *Experimental cell research*. 2009; 315(9):1593–1600. [PubMed: 19013150]
39. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature*. 2006; 444(7115):115–118. [PubMed: 17051151]
40. van Niel G, Wubbolts R, Ten Broeke T, Buschow SI, Ossendorp FA, Melief CJ, et al. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity*. 2006; 25(6):885–894. [PubMed: 17174123]
41. Walseng E, Furuta K, Bosch B, Weih KA, Matsuki Y, Bakke O, et al. Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(47):20465–20470. [PubMed: 21059907]

42. De Gassart A, Camosseto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, et al. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(9):3491–3496. [PubMed: 18305173]
43. Ma JK, Platt MY, Eastham-Anderson J, Shin JS, Mellman I. MHC class II distribution in dendritic cells and B cells is determined by ubiquitin chain length. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(23):8820–8827. [PubMed: 22566640]
44. Chow A, Toomre D, Garrett W, Mellman I. Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *Nature*. 2002; 418(6901):988–994. [PubMed: 12198549]
45. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome biology*. 2008; 9(1):R17. [PubMed: 18218067]
46. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual review of immunology*. 2013; 31:563–604.
47. Thacker RI, Janssen EM. Cross-presentation of cell-associated antigens by mouse splenic dendritic cell populations. *Frontiers in immunology*. 2012; 3:41. [PubMed: 22566924]
48. Nierkens S, Tel J, Janssen E, Adema GJ. Antigen cross-presentation by dendritic cell subsets: one general or all sergeants? *Trends in immunology*. 2013
49. Walseng E, Furuta K, Goldszmid RS, Weih KA, Sher A, Roche PA. Dendritic cell activation prevents MHC class II ubiquitination and promotes MHC class II survival regardless of the activation stimulus. *The Journal of biological chemistry*. 2010; 285(53):41749–41754. [PubMed: 21047782]
50. Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. *Nature immunology*. 2006; 7(10):1029–1035. [PubMed: 16985500]
51. Cho KJ, Roche PA. Regulation of MHC Class II-Peptide Complex Expression by Ubiquitination. *Frontiers in immunology*. 2013; 4:369. [PubMed: 24312092]
52. Furuta K, Walseng E, Roche PA. Internalizing MHC class II-peptide complexes are ubiquitinated in early endosomes and targeted for lysosomal degradation. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110(50):20188–20193. [PubMed: 24277838]
53. ten Broeke T, van Niel G, Wauben MH, Wubbolts R, Stoorvogel W. Endosomally stored MHC class II does not contribute to antigen presentation by dendritic cells at inflammatory conditions. *Traffic*. 2011; 12(8):1025–1036. [PubMed: 21518167]
54. ten Broeke T, de Graaff A, van't Veld EM, Wauben MH, Stoorvogel W, Wubbolts R. Trafficking of MHC class II in dendritic cells is dependent on but not regulated by degradation of its associated invariant chain. *Traffic*. 2010; 11(3):324–331. [PubMed: 20051049]
55. Young LJ, Wilson NS, Schnorrer P, Proietto A, ten Broeke T, Matsuki Y, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nature immunology*. 2008; 9(11):1244–1252. [PubMed: 18849989]

Highlights

- Assessment of MHCII trafficking in primary human and mouse DCs
- Correlation of activation status with receptor internalization
- Correlating vesicle size, location, and contents with MHCII fate

Figure 1. MHCII trafficking in DCs

The novo synthesized MHCII molecules are transported via the Trans Golgi Network (TGN) to the cell surface or into MHCII containing compartments (MIIC), including the endosomes. Complexes that reach the surface are rapidly internalized and redirected to MIIC. Upon peptide loading the MHCII-peptide complexes are transported to the cell surface or into multivesicular bodies (MVB). Most MHCII in the MVB is degraded upon fusion of the MVB with lysosomes. The MHCII-peptide complexes that reach the surface are rapidly internalized and sorted to MIIC where a small percentage is re-expressed on the surface and the majority is degraded upon lysosomal fusion.

Figure 2. Staining approach for the assessment of MHCII internalization and trafficking

A. The time-line and staining sequence of dendritic cells for the analysis of surface and internalized MHCII. **B.** Representative image of MHCII internalization in primary mouse CD11bDC after 6 hr of culture. Analysis is performed after sequential gating on (i) single cells, (ii) in-focus cells, (iii) CD11c-positive, lineage negative/dead- cells, followed by gating on subtype.

Figure 3.
Single masks and combination masks used in the experiments

Figure 4. MHCII trafficking in human primary DCs

CD11c⁺ cells were sorted from fresh PBMC and incubated with biotinylated-HLA-DR and cultured for the indicated time. Cells were stained with a dye for live/dead cells and antibodies for lineage, CD11c, BDCA1, BDCA3, CD14, total surface HLA-DR, streptavidin-PE for surface biotinylated-HLA-DR and streptavidin-A647 for internalized biotinylated-HLA-DR. **A.** Representative image of BDCA1+DCs, BDCA3+DCs and CD11c+CD14+DCs after 6 hr of culture. Overlays depict the biotinylated surface and intracellular HLA-DR. **B.** Relative expression of total surface HLA-DR, biotinylated surface-HLA-DR (PE), and internalized biotinylated-HLA-DR (A647) in the different DC subsets. PBMC of 3 individual donors were analyzed after 0 hr (open circle) and 6 hr (closed circle) of culture. **C.** Increase in internalized MHCII in cells gated on live CD11c+ BDCA1⁺, live CD11c⁺, and CD11c⁺.

Figure 5. Internalization of MHCII in mouse DCs

Primary splenic mouse DCs were incubated with biotinylated MHCII and cultured for the indicated time. At different time-points cells were stained with a dye for live/dead cells and antibodies for lineage, CD11c, CD11b, CD8a, total surface MHCII, and streptavidin-PE to visualize the surface biotinylated-MHCII. DC were quenched in Avidin D and subsequently intracellularly stained with streptavidin-A647 to visualize the internalized biotinylated-MHCII. **A.** Representative image of CD11bDCs and CD8DCs after 0 and 4 hr of culture. Gating strategy was as in figure 1. Overlays depict the biotinylated surface and intracellular MHCII. **B.** Relative expression of total surface MHCII (blue), biotinylated surface-MHCII (red), and internalized biotinylated-MHCII (green) in time in primary mouse CD11bDCs. Data are expressed as percentage of the intensity at time point $t=0$. **C.** Effect of gating on maturation markers on MHCII internalization data. Cells were stained as in A, except that total MHCII was replaced by CD86. **D.** Intensity of CD86 was used to assess the MHCII internalization signal in the total CD11bDC population as well as the CD86^{medium} and CD86^{high} population. At least 500–1000 events were analyzed for each time point. Data are expressed as mean $\pm$ s.e.m. with 3 independent donors. *, $p<0.05$, student T test.

Figure 6. Assessment of MIIC frequency, size, and location

Primary mouse CD11bDCs were cultured as in figure 3 and the number and size of the MHCII⁺ internalized vesicles were analyzed using the Spot Mask features. **A.**

Representative image of vesicles containing internalized MHCII at different culture times. **B.**

Number of MHCII⁺ vesicles in CD11bDCs cultured with medium (black line) or with LPS (red line). Data are expressed as mean $\pm$ s.e.m of 3 individual donors. *, $p < 0.05$, student T test. **C.**

Size of the MHCII⁺ vesicles in CD11bDCs treated in the presence or absence of LPS. **D.** Membrane and cytosol masks used to analyze MIIC size in relation to the proximity to the surface membrane. **E.**

Particle size of MIIC in the membrane mask (M) or cytosol mask (C), after 3 and 6 hr of culture. Data of one representative experiment are shown as a Tukey boxplot with > 500 events/condition. nd= not detectable

Figure 7. MHCII colocalization studies

Primary mouse CD11bDCs were cultured and stained as in figures 3/4. Ab to EEA1, Rab5, Rab7, or Lamp1 were added to the intracellular staining step. Fixable Lysotracker dye was added before the surface staining. **A.** Colocalization of MHCII+ vesicles with EEA1 after 2 and 7 hr of culture (BDS>1.5). **B.** Colocalization of MHCII and Lamp1 after 2 and 7 hr of culture (BDS>1.5). Data are plotted as vesicle size against the % of DCs with high BDS in the total CD11bDC. **C.** Colocalization of MHCII with indicated markers after 2 and 7 hr of culture. Data are plotted as % of DCs with high BDS (EEA1, Lamp1, BDS>1.5; Rab5, Rab7, BDS>1) in the total CD11bDC population. Data are expressed as mean $\pm$ s.e.m of 3 individual experiments.

Table I

Staining panel for mouse DCs as used in figure 2, 5&6

Marker (clone)	Conjugate	Source	Purpose
CD3 (17A2), TCR β (H57-597), NKp46 (29A1.4), CD19 (6D5), IgM (RMM-1), IgD (11-26C.2A)	FITC (CH2)	1	Lineage stain, dump staining
Viability dye	FITC (CH2)	3	Identification of dead cells
MHCII (NIMR4)	BV421 (CH7)	2	Total surface MHCII
CD11c (N418)	APC-Cy7 (CH12)	1	DC identification
CD8 α (53-6.7)	PE-A610 (CH4)	1	DC subset identification
CD11b (M1.70)	BV510 (CH8)	1	DC subset identification
Streptavidin-PE	PE (CH3)	1	surface staining of labeled MHCII
<i>Streptavidin-A647*</i>	<i>A647 (CH11)</i>	<i>1</i>	<i>intracellular staining of labeled MHCII</i>

Source code: 1= Biolegend; 2= eBioscience; 3= Molecular Probes.

*
intracellular staining

Table II

Staining panel for human DCs as used in figure 4

Marker (clone)	Conjugate	Source	Purpose
CD3 (HIT3A), CD56 (HCD56), CD19 (HIB19)	Pacific Blue or BV421 (CH7)	1	Lineage stain, dump staining
Viability dye	(CH7)	4	Identification of dead cells
HLA-DR (NIMR4)	BV510 (CH8)	2	Total surface MHCII
CD11c (3.9)	PE-A610 (CH4)	2	DC identification
BDCA-1 (L131)	APC-Cy7 (CH12)	1	DC subset identification
BDCA-3 (AD5-14H12)	FITC (CH2)	3	DC subset identification
CD14 (HCD14)	PercP-Cy5.5 (CH5)	1	Monocyte identification
Streptavidin-PE	PE (CH3)	1	surface staining of labeled MHCII
<i>Streptavidin-A647*</i>	<i>A647 (CH11)</i>	<i>1</i>	<i>intracellular staining of labeled MHCII</i>

Source code: 1= Biolegend; 2= eBioscience; 3= Miltenyi; 4= Molecular Probes.

*
intracellular staining

Table III

Staining panel for MHCII colocalization studies in mouse DCs (figure 7)

Marker (clone)	Conjugate	Purpose
Lineage +viability stain (table I)	APC-Cy7 (CH12)	Lineage stain, viability staining
CD11c (N418)	BV510 (CH8)	DC identification
CD8α (53–6.7) or CD11b (M1.70)	PE-A610 (CH4)	DC subset identification
<i>Streptavidin-A647</i> *	<i>A647 (CH11)</i>	<i>intracellular staining of labeled MHCII</i>
<i>EEA1 (C-5, Santa Cruz)</i> *	<i>(DonkeyGoat-A488/CH2)</i>	<i>Early endosomes, secondary Ab Abcam</i>
<i>Rab5 (13253, Abcam)</i> *	<i>(DonkeyRabbit-DL405/CH7)</i>	<i>mid-to-late endosomes, do not combine with Rab7, secondary Ab, Biolegend</i>
<i>Rab7 (Sigma)</i> *	<i>(DonkeyRabbit-DL405/CH7)</i>	<i>Late endosomes, MVB, do not combine with Rab5,, secondary Ab, Biolegend</i>
<i>Lamp-1 (1D4B, Biolegend)</i> *	<i>(PE/CH3)</i>	<i>Late endosomes, lysosomes, MVB</i>

* intracellular staining