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Activation of Dendritic Cells Alters the Mechanism of MHC Class II Antigen Presentation to CD4 T Cells

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Abstract

Both immature and mature DCs can process and present foreign antigens to CD4 T cells, however the mechanism by which MHC-II in mature DCs acquires antigenic peptides remains unknown. To address this we have studied antigen processing and presentation of two distinct CD4 T cells epitopes of the influenza virus haemagglutinin coat protein by both immature and mature mouse DCs. We find that immature DCs almost exclusively use newly-synthesized MHC-II targeted to DM^+ late endosomes for presentation to influenza virus-specific CD4 T cells. By contrast, mature DCs exclusively use recycling MHC-II that traffics to both early and late endosomes for antigenic peptide binding. Rab11a knock-down partially inhibits recycling of MHC-II in mature DCs and selectively inhibits presentation of an influenza virus hemagglutinin CD4 T cell epitope generated in early endosomes. These studies highlight a "division of labor" in MHC-II peptide binding in which immature DCs preferentially present antigens acquired in $Rab11a^-$ DM^+ late endosomes whereas mature DCs use recycling MHC-II to present antigenic peptides acquired in both $Rab11a^+$ early endosomes and $Rab11a^-$ endosomes for CD4 T cell activation.

Introduction

Dendritic cells (DCs) are potent antigen (Ag)-presenting cells that initiate adaptive immune responses (1). Resting (immature DCs) constitutively sample their microenvironment and present a diverse repertoire of (mostly self) pMHC-II complexes on their surface (2). After encounter with pathogens or antigens, and in response to inflammatory stimuli, immature DCs become activated and acquire a mature DC phenotype that consists of increased expression of T cell costimulatory molecules and abundant pMHC-II enriched with the peptides that are generated at the time of DC activation (3-5).

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Immature DCs continuously synthesize MHC-II molecules that are directed to late endosomal antigen processing compartments by their binding to the MHC-II chaperone Invariant chain (Ii) (6). These compartments contain the proteolytic enzymes required for proteolysis of both Ii and internalized antigens and the peptide editor DM that assists in the generation of immunodominant T cell epitopes from internalized antigens (7). After their arrival on the immature DC surface, pMHC-II complexes internalize (8, 9), are ubiquitinated by the E3 ubiquitin ligase March-I (10), and are targeted for degradation in lysosomes (9, 11). This process ensures continuous turnover of "irrelevant" pMHC-II in DCs until the cell receives an activation signal. DC activation abrogates expression of March-I (11, 12), thereby preventing pMHC-II ubiquitination (13, 14) and turnover of pMHC-II complexes that were generated at the time of DC activation (15).

Activation of DCs that acquire antigen in their immature state results in a cell that is an extremely potent stimulator of naïve CD4 T cells and, as a consequence of diminished soluble antigen endocytosis and MHC-II biosynthesis it has been proposed that fully-activated DCs are poor antigen sampling, processing, and presenting cells (2, 16, 17). However, receptor-mediated endocytosis and receptor-dependent phagocytosis persist after DC activation *in vivo* (18-21) and there have been reports showing that *in vivo*-matured DCs are indeed capable of foreign antigen presentation (17, 21, 22). To more fully understand these competing models of antigen uptake, processing, and presentation by DCs what is needed is an analysis of the relative efficiency of immature vs. mature DCs to function as APCs for distinct epitopes on a single antigen and to identify molecular pathways that regulate pMHC-II formation in immature vs. mature DCs.

In this study we have investigated the ability of DCs to activate T cells that recognize distinct epitopes on the influenza A virus haemagglutinin (HA) coat protein. We find that one HA epitope is processed and presented in DM-dependent "classical" late endosomal antigen processing compartments in both immature and mature DCs whereas a different HA epitope is presented in both DM-dependent and DM-independent pathways only in mature DCs. The DM-independent antigen processing pathway is regulated by the GTPase Rab11a that controls MHC-II recycling through early endosomes. Ubiquitination blocks the entry of internalized MHC-II into this pathway, thereby suppressing the ability of immature DCs to process and present antigen in recycling endosomes. We conclude that immature and mature DCs are specialized in influenza virus HA processing and presentation from distinct endosomal compartments and that ubiquitination regulates the ability of MHC-II to access recycling endosomes in DCs.

Materials and Methods

Mice and DC culture

March-I knock-out mice and MHC-II $K_{225}R$ ubiquitination mutant knock-in mice on the C57BL/6 background have been described (10). March-I knock-out mice were bred into the H-2^d background by crossing March-I knock-out mice with B10.D2 mice. H2-DM knock-out mice on the Balb/c background (23) were obtained from Liz Bikoff (Univ. of Oxford, England). C57BL/6, B10.D2, and Balb/c mice were from The Jackson Laboratory. Mice were bred and maintained in-house at the NCI-Frederick animal facility. All mice were cared

for in accordance with National Institutes of Health guidelines with the approval of the National Cancer Institute Animal Care and Use Committee. DCs were prepared by differentiating mouse bone marrow cells in medium containing GM-CSF using standard protocols (24) and were activated *in vitro* by incubation with 1μg/ml lipopolysaccharide (LPS) overnight.

Influenza virus and T cells

S1- and S3-specific CD4 T cell hybridomas recognizing distinct epitopes of influenza virus HA have been described previously (25-27). T cell hybridomas were maintained in RPMI supplemented with 10% FBS, penicillin/streptomycin, and 0.05 mM 2-mercaptoethanol. The influenza virus PR8 (H1N1) strain was grown, harvested, and titered from isolates as described previously (28).

Antibodies and reagents

The anti-mouse pMHC-II mAb Y3P (29) was used as a tissue culture supernatant (cells obtained by the American Type Tissue Collection, Rockville, MD) and the anti-I-A^k β-chain rabbit serum has been described previously (30). Mouse mAb anti-Rab11a was from Santa Cruz, rabbit polyclonal anti-Rab11a mAb was from Abcam, and rat-anti-mouse I-A/I-E mAb M5/114 was from BD Biosciences. Biotinylated LAMP-1 mAb 1D4B was obtained from Invitrogen (Carlsbad, CA). mAb Y810C2 was generously provided by Dr. Jon Yewdell (NIAID, NIH).

Venus-Rab11 in the lentivirus vector pLESIP was the generous gift of Dr. Roberto Weigert (NCI, NIH) and has been described previously (31). HRP-conjugated reagents were obtained from Southern Biotech. EZ-Link Sulfo-NHS-SS-Biotin, Lysotracker Red DND-99 and dextran Alexa-fluor 594 were obtained from Thermo Scientific. Reduced glutathione was obtained from Calbiochem.

Lentiviral transduction of DCs

Silencing of Rab11a in mouse DC was achieved using short hairpin RNAs (shRNAs) encoded by lentiviral vectors. Recombinant lentiviruses were produced encoding the following target sequences for Rab11a shRNA; 5’- AGTAGGTGCCTTATTGGTTTA-3’ (32) or a scrambled sequence; 5’- AATTCTCCGAACGTGTCACGT-3’ (33). Lentiviral particles were produced in 100-mm dishes of HEK293T cells which were co-transfected using Lipofectamine 2000 with the lentiviral vector FHUGW (expressing GFP and shRNA), the lentivirus packaging vector Δ8.9, and the vesicular stomatitis virus G envelope glycoprotein vector. Approximately 48 h after transfection, culture media containing lentiviral particles was collected and centrifuged at 100,000 × *g* for 2 h at 4°C. The lentivirus pellet was resuspended in 100 μL PBS, aliquoted, and frozen at −80°C. For DC transduction, concentrated lentivirus Rab11a shRNA and protamine sulfate (10 μg/ml) were added to a culture of bone marrow cells with GM-CSF on day 2 of culture. After culture for an additional 3 days, the medium containing lentivirus was removed and fresh complete DC media containing GM-CSF was added to the cells for an additional 2 days. When indicated, transduced DCs were activated with LPS overnight.

Venus-Rab11 lentiviral supernatant was prepared from HEK293T cells and concentrated Rab11 lentivirus was added to bone marrow cells as described above to generate transduced DCs. Puromycin (5 μg/ml) was added to the medium in the final 24 hr of culture to enrich for transduced cells. Transduced DCs were activated with LPS overnight, stained, and analyzed by immunofluorescence microscopy.

Immunofluorescence microscopy

DCs were stained with anti-pMHC-II mAb Y3P on ice for 30 min, washed, and then incubated either on ice (to allow visualization of surface pMHC-II) or at 37°C for 15 min (to allow visualization of internalized pMHC-II). To reveal only internalized pMHC-II, surface pMHC-II mAb was blocked by incubation of cells with goat anti-mouse IgG on ice for 1 h. Cells were then fixed using 4% paraformaldehyde in PBS, permeabilized using 0.1% saponin in PBS, washed, and incubated with Alexa-fluor-conjugated secondary antibodies to detect pMHC-II mAb. Fixed DCs were plated on poly-L-lysine-coated glass coverslips for 30 min at room temperature for analysis by confocal microscopy. Cells were imaged using a Zeiss LSM 880 confocal microscope using a 63X oil immersion objective lens. Fluorescence intensity of pMHC-II staining was done by masking individual cells and quantitating pixel intensity within the masked area (correcting for variations in cell size) using Zeiss software ZEN 2 (blue edition). At least 20 cells in each condition were analyzed and statistical differences between samples were determined using a Student's t-test. Colocalization of internalized pMHC-II with endosome markers was determined by measuring the Pearson's correlation coefficient using Image J software.

pMHC-II internalization and recycling assays

To monitor pMHC-II endocytosis, DCs were surface-biotinylated with sulfo-NHS-SS-Biotin (1 mg/ml) in PBS for 30 min on ice. After washing in complete medium, the cells were incubated in complete medium at 37°C or kept on ice. At various times cells were harvested, washed in cold HBSS, and surface exposed biotin was reduced by incubating the cells with 50 mM reduced glutathione in a buffer of 75 mM NaCl, 10 mM EDTA, and 75 mM NaOH (pH 7.5) for two treatments of 15 min each. To monitor pMHC-II re-appearance, biotinylated cells were incubated at 37°C for 30 min and surface exposed biotin was removed using reduced glutathione on ice as above to generate a pool of intracellular biotinylated proteins. To quantitate re-appearance, cells were placed in culture again at 37°C for the indicated times before repeating the reduced glutathione procedure. Cells were lysed in 10 mM Tris, 150 mM NaCl, pH 7.4 containing 1% Triton X-100 and proteinase inhibitors (11). pMHC-II was immunoprecipitated using mAb Y3P and analyzed by SDS-PAGE/ immunoblotting using streptavidin-HRP (to detect biotinylated MHC-II) or an MHC-II β-chain antiserum (to detect total MHC-II in the immunoprecipitate) as described previously (15). Following SDS-PAGE and transfer to PVDF membranes, quantitative analysis of protein expression was performed using laser densitometry of immunoblots using Image Lab software.

Measurement of Rab11a expression in DCs

Immature or LPS-matured DCs were lysed in Triton X-100 containing cell lysis buffer and analyzed by SDS-PAGE and immunoblot analysis using Rab11a-specific mAb. The samples

were also analyzed for content of actin as a loading control. Quantitative analysis of protein expression was performed using laser densitometry of immunoblots using Image Lab software. Statistical differences in Rab11a expression between immature and mature DCs were determined using a Student's t-test.

Antigen presentation assays

Influenza virus strain PR8 was inactivated using shortwave UV light (254 nm, 1800 mJ; Stratalinker 1800; Stratagene, La Jolla, CA) for 45 min at 37°C. DCs were pulsed with 0, 2, or 20 haemagglutinating units (HAU) of influenza virus for 45 min at 37°C. Cells were then washed once with complete medium, and DCs (0.1×10^5 cells) were co-cultured with S1- or S3-specific T cell hybridomas (0.5×10^5 T cells) in 96-well well plates in complete medium at 37°C for 16-18 h. T cell activation was measured by monitoring IL-2 release into the culture supernatant using an IL-2 ELISA (R&D Systems).

In some experiments, DCs were treated (or not) with the protein synthesis inhibitor emetine (30 μM) for 4 hr, washed in complete medium, and pulsed with influenza virus for 45 min at 37°C. The cells were then washed, fixed using 1% PFA for 20 min at room temperature, washed once in PBS containing 10 mM glycine, and resuspended in complete medium before the addition of S1- or S3-specific T cell hybridomas. IL-2 release into the medium was measured after culture of the fixed DCs with T cells at 37°C for 48 h.

Statistical analysis

Data in all figures are expressed as the mean ± SD. Differences between pairs of samples were calculated using an unpaired, two-tailed Student't t-test. In all figures, statistical significance is represented as $*p < 0.05$, $**p < 0.01$, ns=not significant.

Results

Processing and presentation of $HA_{107\text{-}119}$ is DM-dependent in DCs.

Mature DCs are capable of antigen capture by receptor-mediated endocytosis and phagocytosis (20), however cessation of MHC-II biosynthesis in mature DCs raises the issue of whether these cells would be capable of efficiently generating stimulatory pMHC-II following antigen uptake and in what intracellular compartment would these complexes be generated. For this study we made use of CD4 T cell hybridomas recognizing two distinct I-E^d-restricted influenza virus HA epitopes termed site 1 (S1, HA amino acids 107-119) and site 3 (S3, HA amino acids 302-313) (25). UV-inactivated influenza virus internalization is mediated by receptor-mediated endocytosis by binding of HA to sialic acid-modified endocytic receptors (34). Under the conditions used in our study both immature and mature DCs capture equivalent amounts of flu virus (Fig. 1A), a finding that is in agreement with previous studies showing that immature and mature DCs have similar capacity for receptor-mediated endocytosis (20).

Activation of S1 T cells by APCs requires HA denaturation and $HA_{107\text{-}119}$ generation and binding to MHC-II is thought to occur in late endosomes (35). Presentation to S1 T cells by immature DCs is DM-dependent (Fig. 1B) and is blocked by the protein synthesis inhibitor

emetine (Fig. 1C), suggesting that newly-synthesized MHC-II binds to $HA_{107-119}$ in late endosomes. Whereas presentation to S1 T cells by LPS-activated mature DCs is also DM-dependent, emetine-treatment does not block HA presentation to S1 T cells by mature DCs. These findings are consistent with the hypothesis that while $HA_{107-119}$ generation and loading onto MHC-II occurs in "classical" DM^+ late endosomes in both immature and mature DCs, newly synthesized MHC-II is responsible for $HA_{107-119}$ presentation by immature DCs and pre-existing (likely recycling) MHC-II is responsible for $HA_{107-119}$ presentation by mature DCs.

$HA_{302-313}$ is generated in early endosomes and binds to recycling MHC-II.

A distinct T cell epitope on HA termed S3 is present in the stalk region of the HA protein and is exposed by partial HA unfolding and $HA_{302-313}$ is thought to be generated by proteolysis in the early endosomal system (36, 37). The partially unfolded stalk region of HA can be recognized by mAb (Y8-10C2) and FACS of mature DC incubated with flu indicate that the mAb Y8-10C2 epitope is generated within 10 min of flu internalization and this epitope is almost completely lost after 120 min of culture at 37°C (Fig. 2A). Immunofluorescence microscopy revealed that unfolded HA is present in $Rab11^+$ early endosomes within 10 min of internalization and remained present in $Rab11^+$ endosomes and late endosomes/lysosomes even after 60 min of internalization (Fig. 2B), demonstrating that HA stalk unfolding occurred in early endosomes and that the open conformation of the HA stalk could persist in late endosomes/lysosomes. Like presentation to S1 T cells, S3 T cell stimulation by immature DCs was largely DM-dependent and was suppressed by blocking protein synthesis (Fig. 2 C,D). In marked contrast, S3 presentation by mature DCs was only partially DM-dependent and did not require new protein synthesis. Curiously, mature DCs were superior to immature DCs as antigen presenting cells for both S1 T cells and S3 T cells. Similar results were obtained using spleen DCs (Fig. S1A), demonstrating that this finding was not restricted to bone marrow-derived DCs as APCs. Superior T cell stimulation by mature DCs was not solely a consequence of enhanced expression of costimulatory molecules in these cells since pre-treatment with LPS for 4 hr prior to addition of T cells enhanced T cell activation by immature DCs and had no effect on mature DCs regardless of whether UV-flu or pre-processed HA peptides were added as antigen (Fig. S1B). These data reveal distinct requirements for MHC-II synthesis for antigen presentation by immature and mature DCs and suggest that in immature DCs both S1 and S3 epitope binding to MHC-II occurs in DM^+ late endosomes whereas in mature DCs S1 epitope binding to MHC-II occurs in DM^+ late endosomes and S3 epitope binding to MHC-II occurs in both DM^+ and DM^- endosomes.

Internalized pMHC-II enters early endosome and late endosomes in mature DCs

Plasma membrane pMHC-II is constitutively internalized via clathrin-independent endocytosis (8) and internalized pMHC-II recycles back to the cell surface of mature (but not immature) DCs (15). Given that new protein synthesis was not required for either S1 or S3 presentation by mature DCs, we set out to determine if surface MHC-II could internalize and enter endosomal antigen processing compartments in mature DCs. Within 15 min of endocytosis a large proportion of surface-tagged pMHC-II was present in $Rab11a^+$ recycling endosomes and had not yet reached $Dextran^+$ late endosomes (Fig. 3). After 45 min was

there significant colocalization of internalized pMHC-II with Lysotracker, demonstrating that in mature DCs internalized pMHC-II can access both early endosomal compartments (for S3 presentation) and late endosomal compartments (for S1 presentation).

Rab11a regulates pMHC-II recycling in mature DCs and MHC-II ubiquitination-deficient immature DCs

To determine if Rab11a directly regulates pMHC-II recycling and antigen presentation in DCs, we reduced Rab11a expression in DCs using lentivirus-encoded shRNA. Expression of Rab11a was routinely reduced by ~90% in the transduced cell population (Fig. 4A) and for this reason we did not attempt to specifically isolate GFP^+ transduced DCs. Lentiviral transduction did not induce phenotypic changes in the DCs, since surface expression of MHC-II and CD86 was not altered in lentivirus transduced DCs and transduced DCs were capable of undergoing TLR-dependent maturation (Fig. S2), thereby allowing us to examine the importance of Rab11a in pMHC-II trafficking and antigen presentation in both immature and TLR-matured DCs.

We monitored the ability of internalized pMHC-II to return to the cell surface of mature DCs using a reversible cell-surface biotinylation assay. In this assay surface MHC-II is reversibly biotinylated on ice and cells are returned to 37°C (or not) to allow a cohort of biotinylated MHC-II to internalize. After 30 min cells are treated with reduced GSH, thereby removing the biotin tag from remaining surface MHC-II. If the internalized MHC-II molecules effectively recycle, re-culture at 37°C for 15 min followed by an additional GSH treatment will reduce MHC-II band intensity, since the biotin-tag on the newly-expressed (i.e. recycled) biotinylated MHC-II will be removed by GSH cleavage. Thus this biochemical assay is an indirect measure of the cell biological process of MHC-II recycling. Rab11a knock-down suppressed recycling of internalized pMHC-II to the plasma membrane in mature DCs by 50% (Fig. 4B) but did not affect endocytosis of pMHC-II from the DC surface (Fig. 4C), highlighting the role of Rab11a in regulating the reappearance of internalized pMHC-II at the plasma membrane but not pMHC-II internalization *per se*.

While internalized pMHC-II recycles very poorly in wild-type immature DCs, pMHC-II effectively internalizes and recycles back to the plasma membrane of immature DCs isolated from March-I deficient mice or MHC-II ubiquitination site-mutant mice possessing a MHC-II $K_{225}R$ substitution, thereby revealing a role for ubiquitination in regulating pMHC-II recycling (15). Lentiviral knock-down of Rab11a in MHC-II $K_{225}R$ immature DCs (Fig. 4D) or March-I deficient immature DCs (Fig. 4E) suppressed pMHC-II recycling in a manner that was indistinguishable from that observed in wild-type mature DCs. Although DC maturation enhanced expression of Rab11a two-fold, even immature DCs possessed significant amounts of Rab11a protein (Fig. S3). These data show that although immature DCs possess the cellular machinery required for pMHC-II recycling, ubiquitination of pMHC-II by March-I diverts internalized pMHC-II away from a Rab11a-dependent recycling pathway.

Rab11a selectively regulates surface pMHC-II expression in mature DCs

Given the role of Rab11a in regulating pMHC-II recycling in mature DCs and the failure of pMHC-II to efficiently recycle in immature DCs, we set out to determine whether Rab11a selectively controls surface expression of pMHC-II in mature DCs. Flow cytometry revealed that Rab11a knock-down had no effect of pMHC-II expression on immature DCs but reduced surface pMHC-II expression on mature DCs by approximately 40% (Fig. S4A). The reduction in surface pMHC-II expression observed by flow cytometry was confirmed by confocal microscopy. Quantitative analysis revealed a 30% reduction in cell surface pMHC-II (Fig. 5A) and a two-fold increase in internalized pMHC-II (Fig. 5B) in the Rab11a knock-down mature DCs as compared to control shRNA transduced DCs. It should be pointed out that the actual amount of internalized pMHC-II “trapped” inside Rab11a-knockdown mature DCs is quite small (as determined by capturing images of surface stained and internalized pMHC-II using identical confocal microscope settings; Fig. S4B). Taken together with our findings that Rab11a knock-down does not affect pMHC-II endocytosis and the pMHC-II does not recycle in immature DCs, these data demonstrate the reduced Rab11a expression leads to the selective accumulation of internalized pMHC-II in mature DCs.

Rab11a selectively regulates presentation of an influenza virus HA epitope in early endosomes by mature DCs

To test the hypothesis that pMHC-II recycling in mature DCs is functionally relevant, DCs were transduced with control or Rab11a shRNA-lentivirus and cultured overnight alone (immature) or with LPS (mature) prior to incubation with flu. Presentation of the HA S1 epitope by either immature or mature DCs was unaffected by Rab11a knock-down (Fig. 6A), a finding that is consistent with the hypothesis that HA S1 epitope generation and binding to pMHC-II occurs in DM^+ late endosomes and that entry into/out of these compartments are independent of Rab11a. By contrast, Rab11a knock-down reduced presentation of the flu HA S3 epitope by mature DCs by 50% but had no effect on the ability of immature DCs to activate S3 T cells (Fig. 6B), revealing a selective role for Rab11a in regulating HA antigen presentation in early endosomes.

As shown above, Rab11a knock-down did not affect S3 presentation by immature DCs, a finding that is consistent with our finding that immature DCs use newly-synthesized MHC-II, and not recycling MHC-II, for S3 epitope capture and presentation to T cells. However, since Rab11a regulates efficient MHC-II recycling in March-I-deficient immature DCs, we asked whether HA S3 epitope presentation was altered in March-I-deficient immature DCs. Unlike results obtained using immature wild-type DCs, Rab11a knock-down dramatically reduced the ability of March-I-deficient immature DCs to stimulate S3-specific T cells (Fig. 6C). Taken together, these data show that Rab11a regulates pMHC-II recycling in DCs, and that pMHC-II recycling allows mature DCs to process and present flu HA peptides to T cells and MHC-II trafficking into/out of these compartments is controlled by the early endosomal recycling regulator Rab11a.

Discussion

There is considerable evidence that DCs activated *in vivo* maintain the ability to internalize antigen by micropinocytosis (17, 20, 22, 38) and receptor mediated endocytosis/phagocytosis (19-21). Despite these findings, it is often assumed that activated (mature) DCs are incapable of foreign antigen uptake, processing, and presentation to CD4 T cells. This idea comes primarily from studies suggesting that MHC-II biosynthesis, a process terminated upon DC activation, is required for efficient antigen presentation (2, 16, 17). However, MHC-II recycling from the cell surface is sufficient for mature DC presentation of select antigens (39) and we have shown that cell surface MHC-II effectively recycles in mature DCs but not in immature DCs (15). For these reasons, in this study we set out to re-examine the issue of mature DC antigen processing and presentation.

We now propose that there exists a “division of labor” in the ability of DCs to process and present antigen to CD4 T cells: immature DCs rely on newly synthesized MHC-II for presentation to T cells and are specialized for antigen processing in DM-containing conventional late endosomes whereas mature DCs rely on recycling pMHC-II molecules for presentation to T cells and are specialized for antigen processing in $Rab11a^+$ recycling early endosomes. These findings were made using T cells recognizing two distinct epitopes on the same flu HA protein and restricted to the same I-E^d MHC-II molecule. Flu endocytosis is mediated by sialic acid-containing receptor-mediated endocytosis (34), and consistent with other studies showing that receptor-mediated endocytosis persists in mature DCs (20, 21) we found that immature and mature DCs internalize identical amounts of flu. The S3 epitope of HA is generated under only mildly acidic conditions and is generated in early endosomes (36, 37). Immature DCs present the S3 epitope of HA to T cells poorly, most likely because Ii-associated MHC-II traverses this compartment quickly *en route* to late endosomal antigen processing compartments (40) and because degradation of Ii is inefficient in early endosomes (41). Unlike immature DCs, mature DCs present the S3 epitope of HA to T cells extremely well. Since mature DCs do not synthesize MHC-II (4, 42) and since blocking protein synthesis did not suppress S3 HA epitope presentation by mature DCs, we attribute the presentation of this CD4 T cell epitope to recycling pMHC-II in mature DCs. Indeed, knock-down of Rab11a, a regulator of protein recycling through early endosomes (43), suppressed both pMHC-II recycling and presentation of S3 by mature (but not immature) DCs, highlighting an early endosomal pathway of pMHC-II recycling that is important for antigen acquisition, processing, and presentation by mature DCs that is not present in immature DCs. Curiously, Rab11a knock-down did not affect presentation of the late endosome S1 HA epitope even by mature DCs, suggesting that MHC-II recycling from late endosomes to the cell surface occurs by a Rab11a-independent recycling pathway in mature DCs. It is important to note that our model highlighting distinct mechanisms of antigen processing and presentation by immature vs. mature DCs is based on analysis of two distinct epitopes of flu HA by CD4 T cells restricted by I-E^d. In expanding on this work it will be important to examine other MHC-II alleles and, more importantly, a wide-variety of model foreign antigens.

Our current study reveals the importance of MHC-II recycling through $Rab11a^+$ endosomes in DC biology. Of note, Rab11a-dependent protein transport is important for different

aspects of APC function, as Rab11a also regulates the ability of MHC-I to recycle from an endosomal recycling compartment that facilitates cross-presentation by DCs (32) and TLR4 to traffic from endocytic recycling compartments to phagosomes in monocytes (44). Although immature DCs possess the molecular machinery required for MHC-II recycling, MHC-II does not recycle in immature DCs because internalized MHC-II is ubiquitinated after internalization by the E3 ubiquitin ligase March-I (9). If MHC-II ubiquitination in immature DCs is blocked (either by genetic deletion of March-I or mutation of the MHC-II ubiquitination site) MHC-II effectively recycles even in immature DCs and these DCs effectively present the S3 HA epitope to T cells in a Rab11a-dependent manner. These data therefore demonstrate that pMHC-II recycling is a “default” pathway in DCs that can be subverted by pMHC-II ubiquitination by March-I.

pMHC-II recycling (15) and receptor-mediated phagocytosis (20) are very efficient in mature DC, and it is these two characteristics that allow recycling MHC-II to efficiently generate MHC-II-HA peptide-complexes from internalized flu virus for presentation to CD4 T cells by mature DCs. As noted above, if this is a general pathway for antigen presentation by mature DCs will require analysis of additional model antigens by different MHC-II alleles. Our data also clearly show that recycling MHC-II can access both early and late endosomes and the partial requirement for DM suggests that both early and late endosomes are important compartments for pMHC-II generation in mature DCs. While Rab11a clearly regulates pMHC-II recycling from early endosomal antigen processing compartments, the molecular machinery that regulates MHC-II recycling from late endosomal antigen processing compartments in DCs, and whether this pathway is identical to that used in immature DCs, remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points:

1. MHC-II recycling through early endosomes in mature DCs is regulated by Rab11a
2. MHC-II synthesis enhances presentation of late endosome antigens by immature DCs
3. MHC-II recycling enhances presentation of early endosome antigens by mature DCs

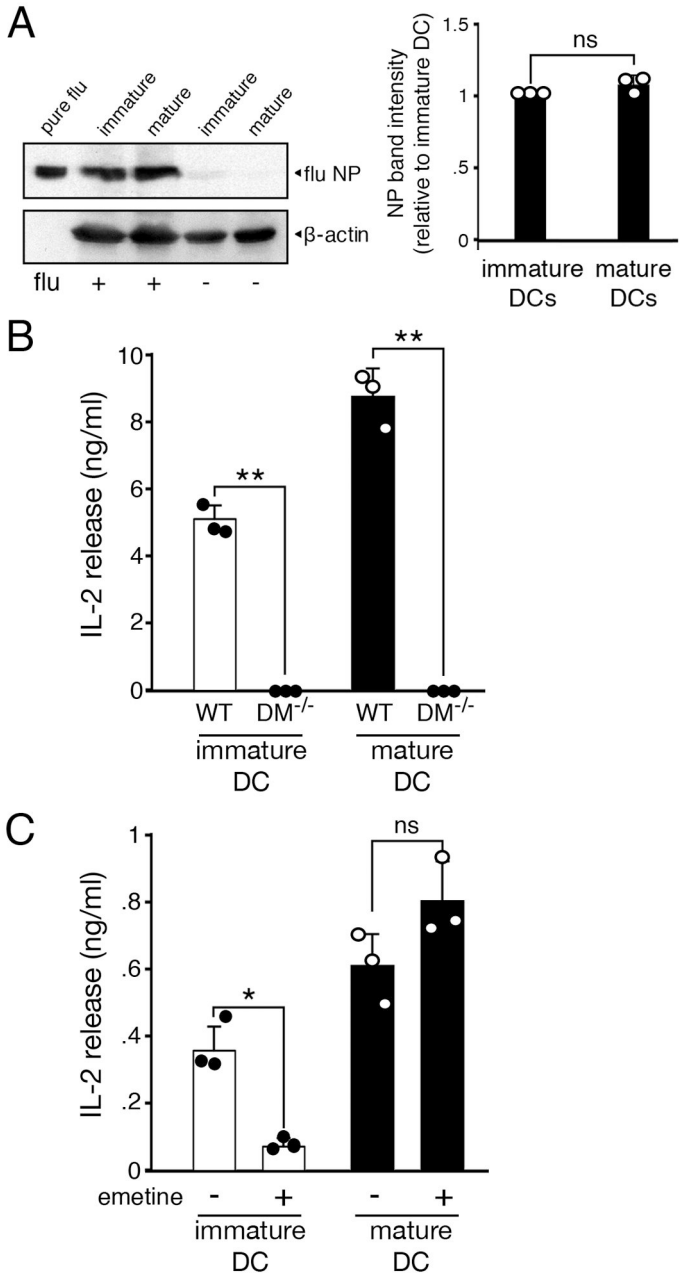

Fig. 1. $HA_{107-119}$ antigen presentation in DCs is DM-dependent.

Immature or LPS-matured DCs were incubated in the absence or presence of 200 HAU of inactivated flu virus for 45 min and washed. (A) Cell-associated flu nucleoprotein (NP) was detected by SDS-PAGE/immunoblot analysis. The amount of flu NP associated with mature DCs was expressed relative to the amount present in immature DCs. (B) Inactivated flu virus-treated immature DCs (open bars) or LPS-matured DCs (filled bars) from wild-type mice or DM-deficient mice were co-cultured with S1 CD4 T cells and T cell activation was monitored by measuring IL-2 release. (C) Immature DCs (open bars) or LPS-matured DCs (filled bars) were pretreated with the protein synthesis inhibitor emetine for 4 h, washed, and pulsed with 20 HAU of inactivated influenza virus for 45 min at 37°C. The cells were then

fixed, washed, and co-cultured with S1 CD4 T cells for 48 hr. T cell activation was monitored by measuring IL-2 release. The data shown are the mean ± SD obtained from three independent experiments.

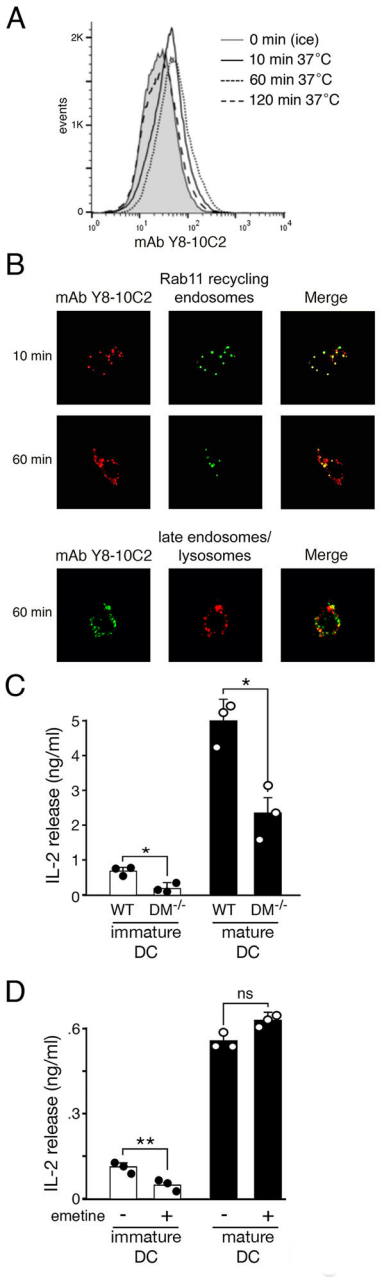

Fig. 2. $HA_{302-313}$ is generated in early endosomes and $HA_{302-313}$ presentation is only partially DM-dependent.

(A) LPS-matured DCs were incubated with UV-inactivated flu virus on ice for 1h, washed, and incubated at 37°C for the indicated time in DC media. The cells were fixed, permeabilized and then stained with a mouse mAb that recognizes the acid-modified form of HA (Y8-10C2) and the appearance of the Y8-10C2 epitope are analyzed by flow cytometry. (B) DCs were transduced with lentivirus encoding venus-Rab11 (*upper and middle panel*) or not transduced (*lower panel*) prior to activation with LPS. LPS-matured DCs were incubated with UV-inactivated flu virus on ice for 1h, washed, and incubated at 37 °C for the indicated time in DC media. The cells were then fixed, permeabilized and stained with a mouse mAb

that recognizes the acid-modified form of HA (Y8-10C2) or the late endosome/lysosome marker LAMP-1 (biotinylated mAb 1D4B; *lower panel*). T cells were then incubated with Alexa-fluor-conjugated anti-mouse antibody (for Y8-10C2) and APC-labeled streptavidin (for 1D4B). The appearance of the Y8-10C2 epitope and localization of Venus-Rab11 and LAMP-1 was determined by fluorescence microscopy. The data shown are representative of that obtained from three independent experiments. (C) Inactivated flu virus-treated immature DCs (open bars) or LPS-matured DCs (filled bars) from wild-type mice or DM-deficient mice were co-cultured with S3 CD4 T cells and T cell activation was monitored by measuring IL-2 release. (D) Immature DCs (open bars) or LPS-matured DCs (filled bars) were pretreated with the protein synthesis inhibitor emetine for 4 h, washed, and pulsed with 20 HAU of inactivated influenza virus for 45 min at 37°C. The cells were then fixed, washed, and co-cultured with S3 CD4 T cells for 48 hr. T cell activation was monitored by measuring IL-2 release. The data shown are the mean ± SD obtained from three independent experiments.

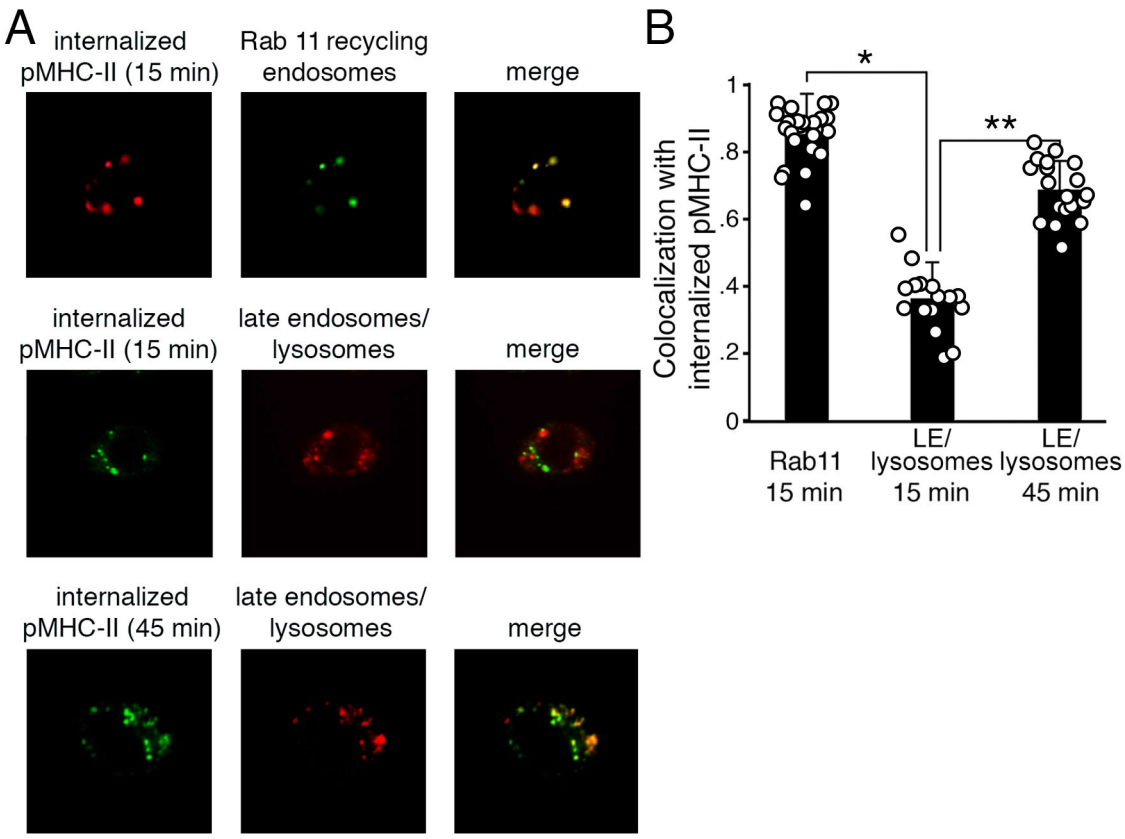

Fig. 3. Internalized pMHC-II is present in both early and late endosomes in mature DCs.

(A, B) DCs were transduced with lentivirus encoding Venus-Rab11 (*upper panel*) or not transduced (*middle and lower panel*) prior to activation with LPS. LPS-matured DCs were stained with pMHC-II mAb on ice, washed, and then incubated at 37 °C for either 15 min or 45 min as indicated. Internalized pMHC-II was specifically visualized by blocking residual surface pMHC-II mAb on ice. Cells were counterstained with markers of recycling endosomes (Venus-Rab11) or late endosomes/lysosomes (Alexa-594 Dextran). (B) The extent of colocalization of internalized pMHC-II with $Rab11a^+$ early endosomes and $Dextran^+$ late endosomes/lysosomes after 15 min or 45 min of internalization was calculated by pixel analysis. At least 20 individual cells in each condition were analyzed for quantitative analysis.

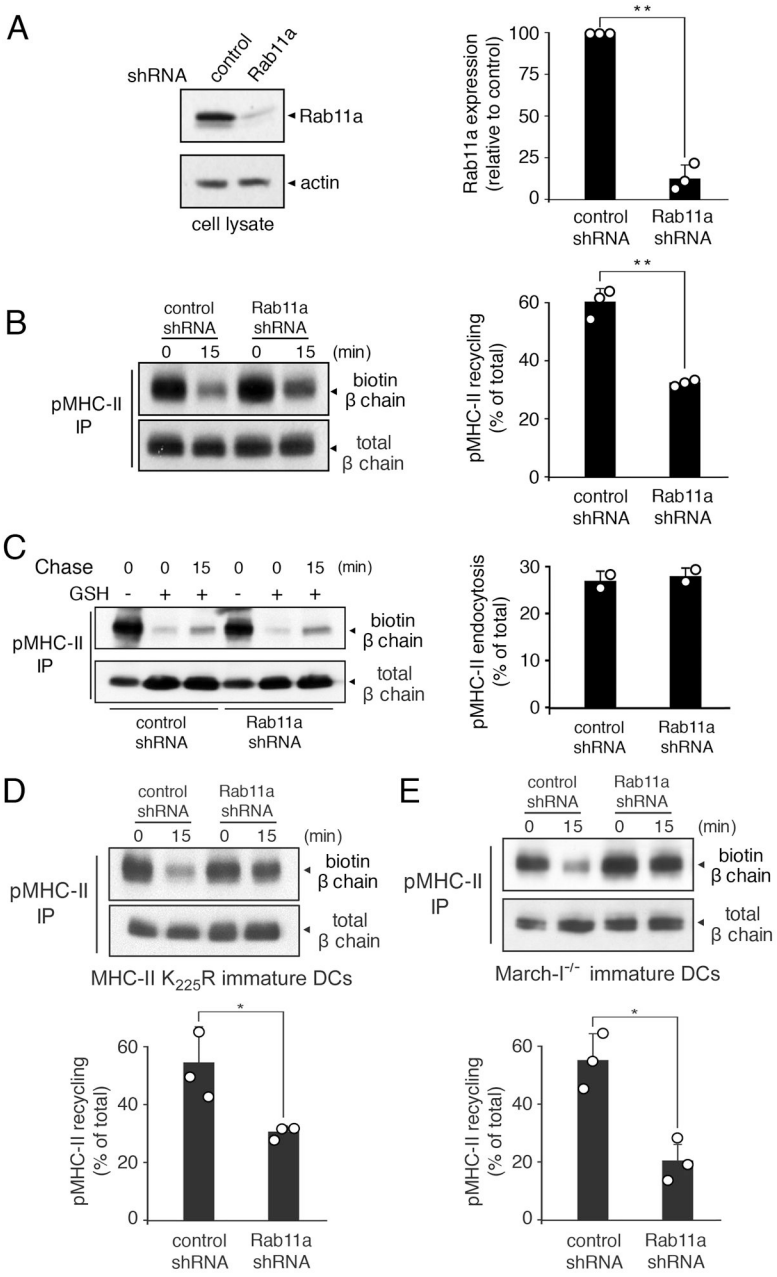

Fig. 4. Depletion of Rab11a inhibits pMHC-II recycling to the plasma membrane.

DCs were transduced with lentivirus encoding control or Rab11a shRNA and activated with LPS. Cells were harvested, biotinylated on ice, and pMHC-II recycling and endocytosis were assayed as described in *Materials and Methods*. (A) The expression of Rab11a in lysates of DCs transduced with control or Rab11a shRNA was assessed by SDS-PAGE/ immunoblotting. Rab11a expression in each condition was quantitated by densitometry and normalized to the amount of actin present in each sample. (B) Recycling of internalized, biotinylated pMHC-II after culture either on ice (t=0) or at 37°C for 15 min was calculated by densitometry of protein band intensity and was expressed relative to the total amount of MHC-II β-chain present in each sample. Note that in this assay effective recycling is

visualized as a *reduction* in the amount of biotinylated MHC-II in the sample. (C) Endocytosis of biotinylated pMHC-II on cells cultured either on ice (t=0) or at 37°C for 15 min was calculated by densitometry of protein band intensity and was expressed relative to the total amount of MHC-II β-chain present in each sample. Note that in this assay endocytosis is visualized as an *increase* in the amount of GSH-resistant biotinylated MHC-II in the sample. (D, E) Immature DCs from MHC-II $K_{225}R$ mice (D) or March-I deficient mice (E) were transduced with lentivirus encoding control or Rab11a shRNA and recycling of internalized pMHC-II was determined. The data shown are the mean ± SD obtained from three independent experiments.

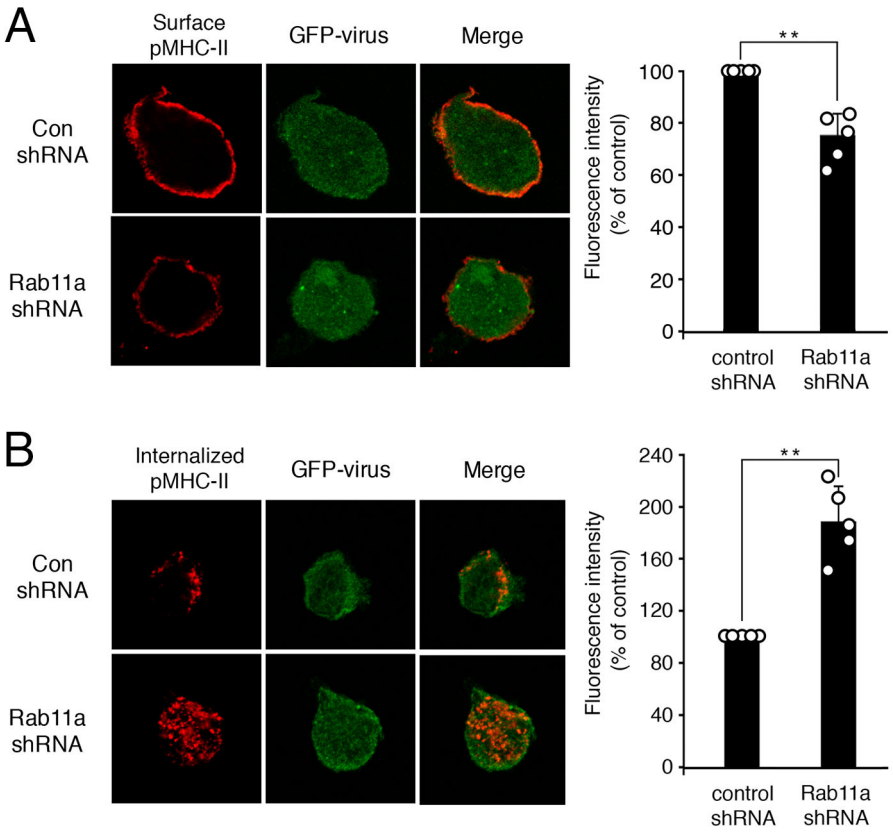

Fig. 5. Rab11a silencing leads to the intracellular accumulation of pMHC-II in mature DCs.

DCs were transduced with lentivirus encoding control shRNA (upper panel) or Rab11a shRNA (lower panel) and activated by culture overnight in LPS. (A) Cells were incubated with mAb recognizing pMHC-II for 30 min on ice and stained with Alexa-conjugated secondary antibodies to reveal surface pMHC-II. (B) After surface pMHC-II staining, the cells were washed and incubated at 37°C for 15 min to allow pMHC-II internalization. Internalized pMHC-II was revealed using Alexa-conjugated secondary antibodies following blocking of residual pMHC-II mAb. The fluorescence intensity of pMHC-II staining on individual cells was calculated, expressed relative to that on control shRNA-transduced cells, and the data shown are the mean ± SD obtained from five independent quantitation experiments.

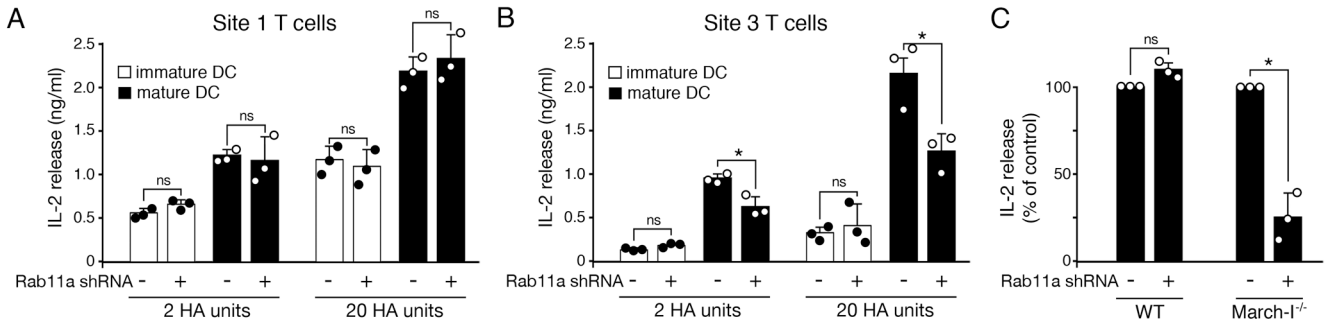

Fig. 6. Rab11a silencing inhibits presentation of an early endosome flu HA epitope.

(A, B) DCs were transduced with lentivirus encoding control shRNA (−) or Rab11a shRNA (+) and then cultured overnight in the absence of LPS (immature DC, open bars) or presence of LPS (mature DC, filled bars). DCs were pulsed with 2 or 20 HAU of inactivated flu virus for 45 min, washed, and then co-cultured with either S1 (A) or S3 CD4 T cells (B). T cell activation was monitored by measuring IL-2 release. (C) Immature DCs from wild-type mice or March-I-deficient mice were pulsed with 20 HAU of inactivated influenza virus for 45 min, washed, and co-cultured with S3 CD4 T cells. T cell activation was monitored by measuring IL-2 release. The data shown are the mean ± SD obtained from three independent experiments.