ITAM signaling in dendritic cells controls T helper cell priming
by regulating MHC class II recycling

Daniel B. Graham1,5, Holly M. Akilesh1,5, Grzegorz B. Gmyrek1, Laura Piccio2, Susan Gilfillan1, Julia Sim3, Roger Belizaire1, Javier A. Carrero1, Yinan Wang1, Gregory S. Blaufuss1, Gabriel Sandoval1, Keiko Fujikawa4, Anne H. Cross2, John H. Russell3, Marina Cella1, and Wojciech Swat1

1Department of Pathology and Immunology
2Department of Neurology and Neurosurgery
3Developmental Biology
Washington University School of Medicine
St. Louis, MO 63110, USA
4Department of Pathology and Immunology
Hokkaido University School of Medicine
060-8638 Sapporo, Japan
5Equal Contribution

Contact: Wojciech Swat
Department of Pathology and Immunology Box 8118
Washington University School of Medicine
660 S. Euclid Ave, St Louis, MO, 63110.
Phone: 314-747-8889, Fax: 314-362-4096
Email: swat@wustl.edu.

Running Head: ITAM signaling in MHCII antigen presentation
Abstract

Immature dendritic cells (DCs) specialize in antigen capture and maintain a highly dynamic pool of intracellular MHC Class II (MHCII) that continuously recycles from peptide loading compartments to the plasma membrane and back again. This process facilitates sampling of environmental antigens for presentation to T helper cells. Here, we show that a signaling pathway mediated by the DC ITAM-containing adaptors (DAP12 and FcRγ) and Vav family GEFs controls the half-life of surface peptide-MHCII (pMHCII) complexes and is critical for CD4 T cell triggering in vitro. Strikingly, mice with disrupted DC ITAMs show defective T helper cell priming in vivo and are protected from experimental autoimmune encephalitis (EAE). Mechanistically, we show that deficiency in ITAM signaling results in increased pMHCII internalization, impaired recycling, and an accumulation of ubiquitinated MHCII species that are prematurely degraded in lysosomes. We propose a novel mechanism for control of T helper cell priming.
Introduction

DCs possess a unique capacity to sample their environment and present exogenous antigens on MHCII for subsequent priming of antigen-specific naïve T lymphocytes\(^1^\)\(^-^\)\(^3\). In the absence of maturation stimuli or danger signals, immature DCs continuously sample their environment by constitutive macropinocytosis and phagocytosis\(^4^\)\(^-^\)\(^6\) while maintaining a dynamic intracellular pool of MHCII for peptide loading\(^7\). Although immature DCs perform a critical role in immune surveillance and are competent to activate naïve T cells\(^8^\),\(^9\), peptide-MHCII (pMHCII) complexes are labile as a result of rapid recycling and degradation\(^7^,^10^,^11\). Upon maturation, DCs downregulate antigen uptake and pMHCII recycling, leading to the accumulation of specific pMHCII complexes on the plasma membrane, thus promoting stable interactions with antigen-specific T cells\(^7^,^10^,^12^,^13\).

Despite intense study, the exact molecular mechanisms regulating MHCII trafficking remain incompletely understood. While the MHCII\(\beta\) chain contains a conserved dileucine motif that promotes clathrin-mediated endocytosis, this motif may not be strictly required for internalization\(^14\). Recently, internalization and sorting of MHCII were shown to be regulated by the MARCH ubiquitin ligases, which polyubiquitinate MHCII\(\beta\) chains\(^15^\)\(^-^\)\(^19\). Following ubiquitination, it is thought that MHCII internalization may require association with ubiquitin-binding clathrin adaptors that promote endocytosis from the plasma membrane and targeting to luminal vesicles within multivesicular bodies, a fate that culminates in lysosomal degradation of MHCII\(^13\). However, it remains unclear at which intracellular locations MHCII is ubiquitinated/deubiquitinated and precisely how this directs MHCII trafficking. In an alternative model, MHCII is thought to be internalized in a clathrin- and dynamin-independent manner in lipid microdomains, perhaps by Arf6-mediated endocytosis\(^20^\)\(^-^\)\(^22\). While these two models are not mutually exclusive, little is known about the signal transduction pathways that regulate pMHCII trafficking and antigen presentation.

Recent work demonstrated that ITAM signaling downstream of several adhesion receptors, including integrins, is essential for effector functions in neutrophils and dendritic cells\(^23^\)\(^-^\)\(^25\). In this context, integrin-mediated adhesion can lead to the activation
of Src family kinases, which phosphorylate ITAMs in the adaptor molecules DAP12 and FcRγ\(^{26,27}\) that serve as docking sites to recruit the tyrosine kinase Syk through its tandem SH2 domains\(^{23,28-30}\). In turn, Syk phosphorylates several substrates orchestrating the assembly of a signaling complex in which SLP-76\(^{31}\) and Vav proteins\(^{32}\) are essential components for initiating cellular responses associated with inflammation\(^{24,25,33}\). However, ITAM signaling in DCs can also inhibit inflammatory responses\(^{34,35}\). For example, DAP12 and FcRγ deficiency was shown to augment \textit{in vitro} maturation of DCs stimulated with TLR ligands and enhance presentation of exogenous antigen on MHCII for activation of CD4 T cells\(^{29}\), even though DAP12 deficiency appears to at least partially protect mice from CNS inflammation in experimental autoimmune encephalomyelitis (EAE)\(^{36}\). Thus, the precise role of ITAM signaling in DCs during T cell priming and MHCII presentation remains unclear.

In this context, recent studies in DCs have demonstrated that active alkalization of the phagosome by the NADPH oxidase NOX2, which itself depends on ITAM signaling, is required for cross-presentation of particulate antigens\(^{23,24,37}\). Thus, the electrogenic activity of NOX2 is thought to delay the induction of lysosomal proteases, so that internalized particulate antigens are rescued from rapid degradation and remain available for loading onto MHC\(I\) following export into the cytoplasm and proteasomal processing. Such alkalization is transient and is followed by phagosome acidification, activation of lysosomal proteases, and generation of peptides loaded onto MHCII molecules. Hence, in this model, MHC\(I\)- and MHCII-restricted presentation of particulate antigens occurs sequentially, with cross-presentation taking place early at more neutral pH, followed by loading of MHCII at more acidic pH. Previously, we have shown that DCs lacking essential components of the ITAM signaling apparatus fail to undergo oxidative burst and cross-present particulate antigens on MHC\(I\) for activation of CD8 T cells\(^{24}\). However, a potential involvement of this signaling pathway in MHCII antigen presentation remains to be determined.

Here, we identify a previously unknown function for ITAM signaling in DCs, which is independent of NOX2, and is critically required for efficient CD4 T cell priming \textit{in vivo} and \textit{in vitro}. We propose a novel model of the regulatory mechanism of MHCII
trafficking in immature DCs and demonstrate that ITAM signaling in DCs is critical for efficient priming of antigen-specific and autoimmune CD4 T cell by promoting salvage of pMHCII through a recycling pathway.
Methods

Mice. Vav\textsuperscript{NULL} mice have been previously described\textsuperscript{38}. DF mice (DAP12\textsuperscript{-/-}FcR\gamma\textsuperscript{-/-}) were provided by M. Colonna (Washington University, St Louis, MO). OT2 mice were a gift from H. Virgin (Washington University, St Louis, MO). WT B6 mice were purchased from Jackson Labs (Bar Harbor, ME). All animal procedures were performed in accordance with institutional guidelines and approved by the Animal Studies Committee at Washington University School of Medicine in St Louis, MO.

Antigen Presentation Assays. Bone marrow derived DCs were cultured in GM-CSF as previously described\textsuperscript{24} and were used on days 5-7 of culture. CD4-positive OT2 T cells were purified from spleens and lymph nodes by negative selection with MACS beads (Miltenyi Biotec) as per manufacturer’s suggestion. Purified T cells were then labeled with CFSE (Vybrant CFDA SE cell tracer kit, Invitrogen) as directed by the manufacturer. T cells (200x10\textsuperscript{3}) were mixed with DCs (50x10\textsuperscript{3}) in round-bottom 96-well plates with the indicated doses of OT2 peptide (a gift from Dr. Paul Allen, Washington University, St Louis, MO) or Ovalbumin-coated latex beads (see\textsuperscript{24} for protocol) for three days of culture. Alternatively, DCs were pulsed with OT2 peptide (100nM) in serum free media for 2 hours at 37°C, washed thoroughly, and cultured at the indicated numbers with OT2 T cells (200x10\textsuperscript{3}) for three days. At the end of culture, T cells were stained with anti-V\alpha2-APC (clone B20.1, eBioscience) anti-CD4-APC (clone GK1.5, Becton Dickinson) and analyzed on a FACSCaliber flow cytometer (Becton Dickinson) with FlowJo software. For analysis of activation marker expression, T cells (without CFSE label) and DCs were cocultured with antigen for 18 hours prior to staining with anti-CD25-FITC (clone PC61.5 from eBioscience) and anti-CD69-PE (clone H1.2F3 from Becton Dickinson).

T Cell Hybridoma Activation. The B11 T cell hybridoma specific for beta-galactosidase (aa429-441) peptide presented by I-Ab was a generous gift from Paul Allen (Washington University, St Louis, MO). Bone marrow derived DCs were pulsed with beta-galactosidase peptide in serum free medium for four hours at 37°C and then washed thoroughly. DCs were then either fixed immediately (0 chase) or cultured for 24 hours prior to fixation (24-hour chase). Fixation consisted of 0.008% glutaraldehyde at room
temperature for three minutes followed by quenching and washing in PBS supplemented with glycine. Fixed DCs were then distributed at the indicated densities into 96-well round-bottom plates with B11 cells (10^5 cells per well). After overnight culture, supernatants were collected for determination of IL-2 production by ELISA. Plates (Nunc Maxisorp) were coated overnight at 4°C with capture antibody (1µg/ml clone JES6-1A12; Biolegend) in PBS, 0.1M NaHCO₃. Plates were washed thoroughly in water and blocked (PBS, 0.5% BSA, 0.1% Tween-20) for one hour. After washing, culture supernatants were added to plates and incubated at room temperature for two hours. Plates were washed and biotin-anti-IL-2 (clone JES6-5H4; Biolegend) was added at 0.5µg/ml in blocking buffer for one hour. Subsequently, excess antibody was removed by washing and streptavidin-HRP (Southern Biotech) diluted 1:10,000 was added to the plates for 30 minutes. After final washing, plates were developed with 1-Step Ultra TMB substrate (Thermo Scientific) as recommended by manufacturer and analyzed with a automated plate reader (TriStar LB 941 Berthold Technologies) equipped with a 450nm filter.

**Ovalbumin Immunizations.** B6 and DF mice (6-8 weeks old) were immunized with 10nmol of the OVA323-339 peptide emulsified with Complete Freund’s Adjuvant (CFA from Sigma Aldrich). Mice were injected subcutaneously with 50µl of prepared emulsion into the hind footpad. At seven days after footpad injection, the popliteal draining lymph nodes were harvested and disrupted into single cell suspensions in DMEM supplemented with 10% FCS (Atlanta Biologicals), 2mM L-glutamine, 1mM sodium pyruvate, 100U/ml penicillin and streptomycin, 1ml/100ml of media of a 100x concentrated nonessential amino acid solution (all from Invitrogen), and 50µM of 2-mercaptoethanol (Sigma Aldrich).

**Proliferation Assays.** Cells were cultured in Microtest™ U-bottom 96-well tissue culture plates from Becton Dickinson Labware at a concentration of 2x10^5 cells per well and stimulated in triplicate with the indicated doses of MOG or OVA323-339 peptides. As a positive control, cells were stimulated with Concanavalin A (2.5µg/ml). During the last 18 hours of the three day culture period, 0.5-1µCi of ^3^H-thymidine was added to each well. At the end of culture, cells were harvested with Micro96 Harvester (from Molecular
Device) on glass fiber filter (Wallac) and next dried and counted with MicroBeta Trilux 1450 LSC & Luminescence Counter Wallac (Perkin Elmer) using dedicated software Wallac 1450 MicroBeta for Windows Workstation (version 4.01.014).

**ELISPOT Assays.** For ELISPOT analysis, MultiScreen™ – IP sterile 96-well filtration plates (with 0.45µm hydrophobic high protein binding Immobilon-P membrane) were purchased from Millipore Corporation. Plates were initially pre-wet with 70% ethanol, washed with PBS, and then coated with primary (capture) anti-mouse IL-2 or IFN-γ antibody (both from Becton Dickinson) at a final concentration of 5µg/ml in PBS for incubation overnight at 4°C. Cells (from spleens or draining popliteal lymph nodes) were cultured in triplicate at a concentration of 2x10^5 per well with MOG or OVA<sub>323-339</sub> peptides at the indicated doses. Cells were incubated for 20hrs at 37°C with 5% CO₂ concentration. After overnight culture, plates were extensively washed with distilled water and blocked with 1% BSA (from Fisher Scientific) in PBS for 1hr at room temperature. Subsequently, the secondary (detection) biotinylated anti-mouse IL-2 or IFN-γ antibody (at a final concentration of 2µg/ml in 1%BSA/PBS) together with streptavidin-AKP from Becton Dickinson (1000x diluted in 1%BSA/PBS) was applied and left for 1hr at room temperature. Plates were extensively washed with distilled water and Sigma Fast™ BCIP/NBT (Sigma Aldrich) was added as per manufacturer’s recommendations. Plates were then left to dry overnight at room temperature, and the number of spots was calculated with an ELISPOT reader provided by Cellular Technology Ltd. (Shaker Heights, OH, USA) using dedicated Immunospot Software for ELISPOT analysis.

**Experimental Autoimmune Encephalitis.** Mice were immunized on day 0 by subcutaneous injection with 50µg of MOG (myelin oligodendrocyte glycoprotein a.a. 35-55) emulsified in IFA containing 50µg *Mycobacterium tuberculosis* (strain H37RA). Intravenous administration of 300ng of pertussis toxin (List Biological Laboratories, Campbell, CA, USA) occurred on days 0 and 3. Mice were subsequently evaluated daily and graded for development of EAE. Clinical scores of 1-5 were assigned as follows: grade 1 =Tail weakness, grade 2 = hind limb weakness sufficient to impair righting, grade 3 = one limb plegic, grade 4 = hind limb paralysis, grade 5 = moribund. Spleens
were harvested from immunized mice on day 21-25 after immunization and single cell suspensions were made after red blood cell lysis. Proliferation assays were set up in 96-well flat-bottom plates with 2.5x10^5 cells per well and 20ug/ml of MOG peptide in DMEM supplemented with 10 percent FBS. Tritiated thymidine at 0.5uCi/well was added to all wells 18 hours prior to harvest and analyzed as described above in proliferation assays.

**Metabolic labeling of MHCII.** Bone marrow derived DCs were starved in Met/Cys-free media at 37°C for 30 minutes before pulsing with 0.5mCi [35S] labeling mix (Perkin Elmer) for 30 minutes. For each sample, 5x10^6 cells were washed and chased for the indicated time points then lysed for 10 minutes on ice in 1ml lysis buffer (0.5% NP-40, 50mM Tris-HCl, 5mM MgCl2, supplemented with complete protease inhibitors (Roche), pH 7.4). Lysates were precleared with protein A/G sepharose (Pierce Biotechnology) and MHCII was immunoprecipitated with 4ug anti-MHCII (clone Y3P, a gift from Emil Unanue, Washington University, St Louis, MO) rotating at 4°C for two hours. Subsequently, samples were washed thoroughly in lysis buffer and eluted in Laemmli sample buffer (2% SDS, 2.5% 2-ME) at room temperature or by boiling for five minutes. Samples were then resolved by PAGE on a 12% gel. The gels were fixed, impregnated with enhancing solution (En3hance, Perkin Elmer), dried, and exposed to film (Kodak biomax MR). After developing films, densitometric measurement of band intensity (in units of mean integrated pixel density) was performed using ImageJ software (NIH). The band intensity of pMHCII at 9 hours chase was normalized to that of 3 hours chase in order to express the extent of pMHCII degradation over time.

**MHCII Surface Retention.** Dendritic cells were plated in 24 well plates at 1 million cells/ml and treated with or without Brefeldin A (GolgiPlug protein transport inhibitor BD Biosciences at 1:1000). At the indicated time points (0, 3, 6, or 9 hours), cells were stained with CD11c-APC (BD Biosciences), I-A^b^-FITC (BD Biosciences), or IgG-FITC isotype control for analysis by FACS. CD11c positive cells were then analyzed for MHCII expression. The mean fluorescence intensity of MHCII at the given time point was then calculated and expressed as a percent of the mean fluorescence intensity at time=0.
**MHCII Ubiquitination.** Dendritic cells (5x10^6/sample) were lysed for 10 minutes on ice in 1ml of lysis buffer (0.5% NP-40, 50mM Tris-HCl, 5mM MgCl₂, 20mM N-ethylmaleimide, supplemented with complete protease inhibitors (Roche), pH 7.4). Lysates were precleared in protein A/G sepharose (Pierce Biotechnology) and immunoprecipitated with 3ug of anti-MHCII (clone M5/114) by rotating at 4°C overnight. Samples were thoroughly washed in lysis buffer, eluted in Laemmli sample buffer by boiling, resolved by PAGE on a 10% gel, and transferred to PVDF membranes (Millipore). Membranes were blotted with antibodies directed towards ubiquitin (clone P4D1, Santa Cruz Biotechnology) or MHCII beta chain (clone KL295, ATCC). Primary antibodies were detected with HRP-coupled anti-mouse antibodies (Zymed) and developed using ECL (GE Healthcare) as recommended by the manufacturer.

**MHCII Recycling and Internalization.** Analysis of MHCII recycling was performed essentially as previously described 39. In summary, DCs were surface biotinylated (50x10^6 cells/ml in PBS pH 8.0) with Sulfo-NHS-SS-Biotin (Pierce Biotechnology) at a concentration of 0.5mg/ml for 30 minutes on ice. Cells were washed with PBS 10mM glycine, and chased for 20 minutes at 37°C in DMEM, at which point cells were stripped by two 15 minute treatments on ice with MESNA buffer (100mM mercaptoethanesulphonic acid (Sigma), 50mM Tris, 100mM NaCl, 1mM EDTA, 0.2% BSA, pH 8.6). Cells were chased again at 37°C for the indicated time points prior to repeating the stripping procedure. Cells were then lysed for 5 minutes on ice in 1ml of lysis buffer (0.5% NP-40, 50mM Tris-HCl, 5mM MgCl₂, supplemented with complete protease inhibitors (Roche), pH 7.4). Lysates were mixed with protein A/G sepharose (Pierce Biotechnology) and immunoprecipitated with 2ug of anti-MHCII (clone M5/114) by rotating at 4°C overnight. Samples were thoroughly washed in lysis buffer, eluted in non-reducing 2x Laemmli sample buffer, resolved by PAGE on a 10% gel, and transferred to PVDF membranes (Millipore). Membranes were then blotted with streptavidin-HRP (Southern Biotechnology) and developed using ECL (GE Healthcare) as recommended by the manufacturer. After developing the blots, densitometric measurement of band intensity (in units of mean integrated pixel density) was performed using ImageJ software (NIH). The relative amount of recycling MHCII that re-emerged on the plasma membrane was determined as follows: 1-(Iₓ/I₀), where Iₓ= band...
intensity at time x minutes of chase, and \( I_{T0} \) = band intensity at time 0 minutes chase. The amount of internalized MHCII relative to total surface MHCII was determined as follows: \( 100 \times \left( \frac{I_{T20} - I_{T0}}{I_{Total}} \right) \), where \( I_{T20} \) = band intensity at time 20 minutes of chase, \( I_{T0} \) = band intensity at time 0 minutes of chase, and \( I_{Total} \) = band intensity of total surface MHCII.

**Statistical Methods.** Data are expressed throughout as mean + standard deviation. Data sets derived from the indicated genotypes were compared using the two-tailed unpaired Student’s t-test. Differences were considered statistically significant when \( p<0.05 \).
Results

Requirement for ITAM signaling in presentation of particulate and soluble antigen by DCs.

Several recent studies indicated that the ITAM-containing “activating” adaptors, DAP12 and FcRγ, can mediate both activating and inhibitory signals in myeloid cells (reviewed in 34,35). In this context, the ITAM-associated signal transduction complex including Syk, SLP-76, and Vav has recently emerged as a central regulatory pathway in myeloid cells23,24. However, a potential requirement for the ITAM signaling module in MHCII presentation by DCs has not been conclusively addressed to date. To approach this issue, we utilized DCs from mice in which ITAM signaling was disrupted by deletion of DAP12 and FcRγ (DF) or deletion of all three Vav proteins (VavNULL). WT and mutant (VavNULL or DF) DCs were incubated with ovalbumin covalently attached to latex beads, and DCs were then co-cultured with freshly isolated MHCII-restricted naïve lymph node OT2 T cells. T cell activation was analyzed by surface marker expression, and T cell proliferation was analyzed by CFSE dye-dilution assays. In these experiments, we found that both WT and VavNULL DCs readily induced expression of activation markers, CD69 and CD25, on OT2 T cells (Fig.1A). Strikingly, however, only WT DCs induced a robust proliferative response of OT2 T cells, whereas VavNULL DCs appeared to lack this ability (Fig.1B). Similar to VavNULL, DF DCs also failed to induce OT2 T cell proliferation (Supplemental Fig. 1). Importantly, defective proliferation of OT2 T cells did not appear to result from diminished antigen uptake or diminished expression of MHCII and/or costimulatory receptors, as our analyses showed that surface MHCII was slightly elevated, while expression of B7.1 and B7.2 and bead internalization by VavNULL and DF DCs were similar to WT (Supplemental Fig. 2 and24,29). Additionally, co-culture experiments showed that antigen-loaded WT DCs were capable of inducing OT2 proliferation in the presence or absence of antigen-loaded, or unloaded, DF or VavNULL DCs (Supplemental Fig. 3, and data not shown) indicating that their defects were intrinsic and did not affect OT2 T cells in “trans”. We also measured cytokine production in culture supernatants from these antigen presentation assays, which showed that production of IL-10 and IL-12 by VavNULL and DF DCs was similar to WT
(Supplemental Fig. 4, and Supplemental Fig. 5). Moreover, we found no differences in viability of Vav$^{\text{NULL}}$ or DF DCs, as compared to WT (Supplemental Fig. 6), and no evidence for the acquisition of a tolerogenic phenotype as evaluated by transcriptional profiling (data not shown). Thus, taken together, these data indicate an unexpected requirement for the intact ITAM signaling apparatus in DCs to induce CD4 T cell proliferative responses to particulate antigens.

To determine if ITAM-deficient DCs could induce OT2 T cell proliferation in response to exogenously supplied antigenic peptides, which do not require processing by a DC and can be passively loaded onto MHCII, WT and DF DCs were incubated with varying doses of continuously supplied OVA$_{323-339}$ peptide and their ability to induce OT2 T cell proliferation was assessed using CFSE dilution assays. In these experiments, both WT and DF DCs readily induced proliferation of OT2 T cells (Fig. 1C). However, when DCs were first pulsed with the OVA peptide for two hours, and then washed to remove excess peptide prior to co-culture with OT2 T cells only WT, but not DF or Vav$^{\text{NULL}}$ DCs, could induce OT2 T cell proliferation (Fig. 1D-F). These results suggest that in immature DCs ITAM signaling is critically required for the maintenance and/or stability of surface pMHCII complexes.

To directly test this scenario, we next utilized the B11 T cell hybridoma specific for β-gal peptide presented by I-A$^b$ which produces IL-2 in a manner proportional to the concentration of specific pMHCII complexes, independently of costimulatory and cytokine signals. In these experiments, WT, DF, and Vav$^{\text{NULL}}$ DCs were first pulsed with a saturating dose of β-gal peptide, the excess peptide was washed away, and DCs were fixed and incubated with B11 cells. Under these conditions, WT, DF, and Vav$^{\text{NULL}}$ DCs induced similar levels of IL-2 production by B11 after 24 hours in culture (Fig. 2A and C). Strikingly, however, when DCs were first pulsed with peptide, but then washed and chased in peptide free media for 24 hours prior to fixation, we observed profound defects in antigen presentation by DF and Vav$^{\text{NULL}}$ DCs, as compared to WT (Fig. 2B and D). Thus, in the absence of ITAM signals, specific pMHCII complexes expressed on the surface of immature DCs appear unstable. These results indicate that ITAM signals are
critical for the ability of immature DCs to maintain surface pMHCII complexes for efficient induction of CD4 T cell proliferation.

**Requirement for ITAM signaling in DCs for priming of conventional and autoimmune CD4 T cells in vivo.**

Given these results, and previously published data indicating that DAP12 deficiency attenuates rather than augments progression of EAE, a murine model of multiple sclerosis that requires CD4 T cell-dependent Th1 and Th17 responses\(^{40-42}\), we hypothesized that the ITAM adaptors may be involved as positive regulators of DC function in antigen presentation in vivo. To test this hypothesis, we first determined the requirement for DAP12 and FcR\(\gamma\) during in vivo priming of CD4 T cell responses with a conventional antigen. Importantly, T-lineage cells developing in DF mice appear phenotypically normal and do not show any obvious developmental or functional abnormalities (data not shown). WT and DF mice were immunized with the MHCII/I-A\(^{b}\)-restricted OVA peptide using a standard protocol, and the antigen-specific T cell response was determined by in vitro restimulation of draining lymph node cells seven days later (Fig. 3). Strikingly, while WT mice generated a robust response to OVA immunization, DF mice showed a reduced response as indicated by diminished frequency of IL-2 producing T cells visualized by ELISPOT assays (Fig. 3A) and decreased T cell proliferation in \[^{3}H\]-thymidine incorporation assays (Fig. 3B). As expected, both DF and WT T cells responded vigorously to stimulation with ConA (Fig. 3A and B) indicating that the attenuation of antigen-specific response in DF mice is not due to defects intrinsic to T cells. Furthermore, expression of MHCII and B7.2 in lymph node DCs was similar in DF and WT mice treated with Complete Freund’s Adjuvant (CFA) (Supplemental Fig. 7). We interpret these results as suggesting that the ITAM-containing adaptors, DAP12 and FcR\(\gamma\), are critically required for efficient priming of CD4 T cells in vivo.

Given these results, we decided to determine if DAP12 and FcR\(\gamma\) are also required for efficient generation of encephalitogenic CD4 T cells in EAE. In this regard, previous studies showed protective effects of the loss of DAP12 expression in EAE\(^{36}\), however susceptibility of mice lacking both DAP12 and FcR\(\gamma\) has not been tested. To address this,
DF and WT mice were immunized with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG\textsubscript{35,55}) and analyzed for development of clinical signs of the disease. As expected, WT mice developed initial signs of disease associated with an ascending paralysis starting at day 14 post immunization, and progressing through day 21 (Fig. 4A). In contrast, DF mice appeared completely resistant to EAE induction and remained free of any clinical signs of disease throughout the duration of the experiment (Fig. 4A). At peak disease, WT mice exhibited notable infiltration of mononuclear cells into the lumbar spinal cord, but such CNS-infiltrates were virtually absent in DF mice (Fig. 4B). Next, we measured antigen-specific T cell responses in MOG\textsubscript{35,55} immunized mice by restimulation of spleen cells \textit{in vitro}. Remarkably, WT mice showed a robust response to MOG as measured by ELISPOT assays of IL-2 and IFN\textgamma-producing T cells and by [\textsuperscript{3}H]-thymidine incorporation, but the MOG-specific T cell response was virtually undetectable in DF mice (Fig. 4C and D). In contrast to DF mice, mice single-deficient in FcR\gamma did not exhibit any detectable defects in CD4 T cell priming, while mice single-deficient in DAP12 showed a mild reduction (Supplemental Fig. 8). In agreement with previous studies, FcR\gamma deficient mice were only marginally protected from EAE whereas DAP12 deficient mice showed partial protection, as compared to WT (Supplemental Fig. 9 and 36,43-48). Taken together, these results show that DAP12 and FcR\gamma play an essential, but functionally redundant, role during \textit{in vivo} priming of both conventional antigen-specific and autoimmune (pathogenic) CD4 T cells. These results highlight the requirement for the ITAM signaling pathway in the regulation of MHCII antigen presentation in DCs.

\textbf{ITAM signaling regulates the stability and half-life of pMHCII in immature DCs.}

While the mechanism of protein antigen processing and presentation through the MHCII pathway is relatively well understood at present, little is known about signaling pathways regulating pMHCII expression, internalization, and re-expression in immature DCs. In these cells, pMHCII complexes are labile and undergo rapid recycling and degradation\textsuperscript{7,10,11} which is thought to be critically important for their capacity to constitutively sample the environment for priming of antigen-specific naïve T cells\textsuperscript{8,9}. Given the requirement for ITAM signals in the activation of naïve CD4 T lymphocytes
both \textit{in vitro} and \textit{in vivo}, we hypothesized that ITAM signals may be involved in the regulation of pMHCII dynamics in DCs. To address this, we first decided to determine the half-life of pMHCII complexes in WT and ITAM-deficient immature DCs. In these experiments, WT, DF, and Vav\textsuperscript{NULL}, DCs were pulsed with $[^{35}\text{S}-\text{Met} \text{ and } -\text{Cys}]$, washed, and chased for 0-9 hours. Subsequently, MHCII complexes were immunoprecipitated with Y3P monoclonal antibody recognizing mature I-A complexes, and immunoprecipitates were then resolved by PAGE and analyzed by autoradiography.

Immediately after pulse (0-hour chase) few newly formed MHCII molecules were immunoprecipitated, presumably because the majority of labeled MHCII molecules were associated with the invariant chain at this time point, and were not recognized by the Y3P antibody (Fig. 5A,B). Following a 3-hour chase, peptide-loaded MHCII complexes emerged as a single SDS-resistant band of 55kDa, which dissociated into I-\(\alpha\) and I-\(\beta\) monomers upon boiling. Remarkably, at 9 hours, SDS-resistant pMHCII complexes decayed by only approximately 20\% in WT DCs, however in DF and Vav\textsuperscript{NULL} DCs these complexes decayed by over 50\% (Fig. 5A,B). Thus, even though the generation of SDS-resistant complexes in DF and Vav\textsuperscript{NULL} DCs at 3 hours was similar if not more robust than in WT DCs, these complexes appeared unstable in ITAM-mutants (Fig. 5A, B, and C). Importantly, the half-life of MHCI was similar in WT and Vav\textsuperscript{NULL} DCs, indicating that the accelerated degradation of MHCII in DF and Vav\textsuperscript{NULL} DCs was not the result of any generalized defect(s) in protein synthesis or degradation (Supplemental Fig. 10).

This finding is consistent with our previous report that ITAM signaling is not required for presentation of soluble protein antigen on MHCI to CD8 T cells\textsuperscript{24}. Taken together, the data indicate that ITAM signals are required to regulate the half-life of SDS-resistant pMHCII complexes in immature DCs. Of note, this process appears to be independent of ITAM-mediated regulation of the NOX2 NADPH oxidase and ROS production, as the half-life of pMHCII complexes in NOX2-deficient DCs was similar to WT (Supplemental Fig. 11).

Given the results of these metabolic labeling experiments, we decided to examine potential contribution of pMHCII internalization and re-expression rates to net surface retention in the absence of \textit{de novo} synthesis. To this end, WT and Vav\textsuperscript{NULL} DCs were treated with Brefeldin A (GolgiPlug) to inhibit transport of newly synthesized MHCII
from the Golgi to the plasma membrane, and surface MHCII levels were analyzed by FACS at 0-9 hours after treatment. Consistent with the results of the metabolic labeling studies, pMHCII complexes in WT DCs treated with Brefeldin A appeared stable and decayed from the plasma membrane with relatively slow kinetics (Fig. 5D). In contrast, pMHCII complexes were lost from the cell surface of Vav\textsuperscript{NULL} DCs significantly more rapidly, as compared to WT (Fig. 5D). These results indicate that ITAM signals are critical for regulation of the half-life of SDS-resistant complexes and contribute to the retention of the pool of mature, recirculating pMHCII complexes in immature DCs.

**ITAM Signaling is Required for Efficient Recycling of MHCII.**

In immature DCs, MHCII is thought to be internalized and sorted by a mechanism activated in response to ubiquitination signals, although this process is incompletely understood at present\textsuperscript{15-19}. To determine if ITAM signaling regulates MHCII ubiquitination and subsequent internalization, MHCII was immunoprecipitated from WT and Vav\textsuperscript{NULL} DCs followed by western blotting for ubiquitin, or total MHCII\(\beta\) chain. Strikingly, we found a significantly higher proportion of MHCII to be ubiquitinated in Vav\textsuperscript{NULL} DCs, as compared to WT (Fig.6A). Together, these results suggest that a disruption of ITAM signaling leads to an accumulation of ubiquitinated MHCII in immature DCs, for example due to dysregulated ubiquitination and/or inefficient routing into the recycling compartment(s). In support of this view, inhibition of lysosomal proteolysis with chloroquine, which has previously been shown to enhance the stability of pMHCII complexes in immature DCs\textsuperscript{17}, led to a significant accumulation of SDS-resistant MHCII complexes in mutant DCs (Fig.6B). Collectively, these results indicate that the impairment in ITAM signaling leads to enhanced ubiquitination and lysosomal degradation of pMHCII in immature DCs. Thus, ITAM signaling appears to be required to extend the half-life of pMHCII complexes via increased trafficking through a recycling compartment(s).

Next, to directly test this notion, we analyzed the kinetics of MHCII recycling (internalization and re-emergence) using an assay in which a cleavable biotin is first used to label surface MHCII, and subsequently chased and cleaved to determine internalization and re-emergence rates. Consistent with published studies\textsuperscript{39}, these experiments showed
that in WT DCs approximately 10% of surface MHCII molecules became internalized at 20 minutes (Fig. 6C). However, the internalization rate was increased approximately two-fold in mutant cells (Fig. 6C). In contrast, the rate of MHCII re-emergence on the plasma membrane over the course of 60 minutes was consistently reduced in DF DCs, as compared to WT (Fig. 6D). Thus, taken together, these data indicate that in the absence of ITAM signaling, pMHCII complexes in immature DCs become excessively ubiquitinated and inefficiently recycled and, as a consequence, may be rerouted to the lysosomal compartment and prematurely degraded resulting in a net loss of MHCII from the recycling pool. We propose a model in which the ITAM apparatus is critically important for the control of internalization and re-emergence of surface MHCII in immature DCs.
Discussion

Considerable effort has been directed towards understanding the cell biology of MHCII presentation\textsuperscript{1,2,13} and significant progress has been made in characterizing the role of MHCII synthesis/degradation during antigen presentation by dendritic cells\textsuperscript{3}. Recently, several microbial PAMPs and cytokines have been shown to enhance antigen presentation by upregulating costimulatory molecules and inducing maturation of DCs, yet the mechanisms governing MHCII trafficking in immature DCs remain obscure\textsuperscript{11,13,21}. Here, we present data demonstrating that ITAM signaling in DCs controls CD4 T cell priming by promoting salvage of MHCII through a recycling pathway. Thus, in the absence of ITAM signaling, MHCII is inefficiently recycled, instead accumulating in ubiquitinated form, and ultimately being degraded in lysosomes. The functional consequences of defective ITAM signaling in DCs are a reduced half-life of mature pMHCII complexes leading to impaired antigen presentation and activation of both conventional antigen-specific and autoimmune CD4 T cells.

Shortly after the first formal demonstration that MHCII can be recycled and reloaded\textsuperscript{39}, a breakthrough study showed that LPS treatment in DCs extends the half-life of pMHCII and induces its redistribution to the plasma membrane\textsuperscript{7}. While much of the research has been focused on the mechanism and the consequences of stabilizing pMHCII in mature DCs\textsuperscript{7,16-19}, our work presented here highlights the importance of the regulation of pMHCII maintenance in immature DCs. Prior to antigen encounter, immature DCs perform the critical task of antigen surveillance and must continuously sample their environment, maintaining a dynamic pool of MHCII for recycling and reloading. By disrupting ITAM signaling, we show here that MHCII recycling is impaired and MHCII is prematurely degraded. Indeed, we can extend the half-life of MHCII in ITAM signaling deficient DCs by treatment with LPS, yet this leads to only partial rescue of antigen presentation defects \textit{in vitro} (Supplemental Fig. 12, and data not shown). Moreover, immunization of DF mice with OVA in the presence of adjuvant fails to induce a robust CD4 T cell response. We interpret these results as an indication that, upon initial antigen encounter, DCs process the antigen quickly, but the upregulation and stabilization of pMHCII may require more time (up to 8 hours\textsuperscript{7}, and our unpublished
observations). Thus, there appears to be a window of time, between antigen encounter and DC maturation, during which robust presentation of specific antigen requires ITAM signaling for MHCII recycling and salvage.

Investigation into the role of ITAM signaling in DCs and other myeloid cells has revealed the complex nature of this pathway. DAP12 deficiency has been shown to increase mortality in mouse models of sepsis as a result of hyper-inflammatory responses49,50. On the other hand, DAP12 is required for survival and expansion of macrophages51. Moreover, deficiency in both DAP12 and FcRγ results in profound functional defects in neutrophils23. Thus, ITAM signaling performs critical positive and negative regulatory functions in myeloid cells35. Most relevant to antigen presentation, a previous report demonstrated that DCs lacking DAP12 and FcRγ could be more efficient antigen presenting cells compared to WT DCs29. Importantly, antigenic peptide was continuously present (not pulsed) in these experiments, and very low doses of LPS or CpG were used to mature DCs. Therefore, since DF DCs respond more robustly than WT to these low dose stimuli, they also induce stronger T cell responses29. While WT and DF DCs respond similarly in terms of upregulation of costimulatory molecules when higher doses of TLR ligands are used, we show here that under the conditions where DCs are pulsed with antigen, rather than antigen being continuously supplied throughout the culture, ITAM signaling is clearly required for antigen presentation. Finally, the most compelling evidence for a critical role of ITAM signaling for antigen presentation by MHCII in vivo is the observation that DF mice fail to prime CD4 T cell responses after immunization with a conventional antigen, or an autoantigen.

Our work presented here reveals a novel regulatory mechanism for MHCII trafficking and raises two important issues: 1. What are the receptors that engage the ITAM-containing adaptors to initiate the signal and, 2. How do these signals converge with the vesicular trafficking machinery in DCs? While we currently do not know the identity of the receptor(s) generating ITAM signals in DCs, our data make some potentially informative predictions. Importantly, DF mice exhibit a remarkably more severe phenotype than either the DAP12 or FcRγ single knockouts with respect to defects in CD4 T cell priming and protection from EAE (Supplemental Figs. 8 and 9 and36,43,48).
This observation suggests that ITAM signaling in DCs is initiated by a class of receptors that utilizes both DAP12 and FcRγ, such as integrins\textsuperscript{23}, or a host of distinct receptors that associate with DAP12 or FcRγ. Several receptors known to pair with DAP12 (TREM2, SIRPβ1) and FcRγ (OSCAR, Mincle) are expressed in myeloid cells, and certainly more will be identified in the future. For example, MHCII was recently reported to interact with FcRγ, and crosslinking of MHCII by specific antibodies or Lag-3 inhibits DC function\textsuperscript{52}. It is also formally possible that ITAM-associated receptors may be dispensable for the generation of ITAM signals in myeloid cells. Rather, expression of ITAM-containing adaptors and “tonic” tyrosine kinase activation may be sufficient to maintain ITAM signaling\textsuperscript{53}. Nevertheless, we favor a model in which interactions between DCs and the extracellular matrix or neighboring cells promotes integrin engagement to initiate ITAM signaling through DAP12 and FcRγ.

The second issue pertains to the mechanism by which ITAM signals regulate MHCII trafficking. ITAM signaling through Vav GEFs has long been recognized as a critical regulator of Rac GTPases and actin dynamics\textsuperscript{54}. Given that endocytosis and vesicle sorting require actin and microtubule remodeling, ITAM signaling may control MHCII trafficking at the level of the cytoskeleton. Another newly identified role for Vav is its regulation of the cytoskeleton by direct interaction with dynamin\textsuperscript{55}. In this context, Vav may exert control over MHCII endocytosis by recruiting dynamin, which facilitates membrane scission/pinching in nascent endosomes\textsuperscript{56}. Alternatively, ITAM signaling may regulate the recruitment of the newly discovered ubiquitin-binding clathrin adaptors that simultaneously bind ubiquitinated transmembrane receptors like MHCII and direct the assembly of clathrin-coated pits during endocytosis. While at present we do not understand exactly how ITAM signaling regulates MHCII trafficking, considerable evidence exists to support a model in which MHCII is ubiquitinated and subsequently endocytosed in a clathrin-dependent manner\textsuperscript{14-17}. However, additional work suggests that MHCII can be endocytosed in a clathrin- and dynamin-independent manner, presumably in lipid microdomains\textsuperscript{20}. In addition, each model may be applicable in different contexts, for example in distinct DC subsets and maturation states. Therefore, it is possible that ITAM signaling regulates a ubiquitin-dependent clathrin-mediated internalization process.
resulting in recycling of MHCII through the H2-DM compartment. While in the absence of ITAM signaling clathrin-mediated internalization and recycling of MHCII may be impaired, lipid microdomain-mediated endocytosis may remain intact. Thus, rerouting of MHCII in the absence of ITAM signaling could result in premature destruction.

Ligation of ITAM-associated receptors could alter expression of the MHCII ubiquitinating ligase MARCH1; however, our analyses of mRNA expression showed similar steady-state levels of MARCH1 in WT and Vav^{NULL} DCs, and similar degree of reduction upon LPS stimulation (Supplemental Fig. 13). Thus, signals emanating from ITAM-associated receptors do not seem to regulate mRNA levels of MARCH1. While it is possible that posttranslational regulation of MARCH1 activity may involve ITAM signaling, it is also possible that regulation of MARCH1 enzymatic activity may be achieved via indirect effects on its E2 partner and/or other components of a complex. Alternatively, MHCII regulation could involve deubiquitinating enzymes, or conceivably, could involve modulation/competition by binding of another ubiquitin homolog such as ISG15^{57}.

Thus, although at present we do not completely understand the molecular mechanism involved, our results presented here provide new insights into the regulatory circuits governing MHCII trafficking by implicating a specific signal transduction pathway in the process. Using mice deficient in ITAM signaling in DCs, we show that this process appears critical for efficient priming of both conventional and pathogenic (autoimmune) CD4 T cells \textit{in vivo}, providing a model in which the molecular machinery driving MHCII trafficking and antigen presentation can be further dissected.
Acknowledgements

We thank Dr. Emil Unanue, Marco Colonna, Ted Hansen, Paul Allen, and Ramnik Xavier for helpful suggestions and providing reagents. We also thank Drs Lisa Denzin and Sergio Grinstein for insightful discussions. This work was supported by National Institutes of Health grants R01AI061077 (to W.S.), R01AI073718 (to W.S.), The Leukemia & Lymphoma Society Scholar Award (to W.S.), a Special Fellowship Award from The Leukemia & Lymphoma Society (to D.B.G.).
Authorship Contributions

Disclosures of Conflicts of Interest

The authors declare no competing financial interests.
References

41. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med.* 2007;13(2):139-145.


Figure Legends

Figure 1. ITAM Signaling is Required for Antigen Presentation by DCs. WT and Vav\textsuperscript{NULL} DCs (50,000) were cultured with the indicated doses of ovalbumin-coated latex beads and purified OT2 T cells. T cells were analyzed for expression of the activation markers CD69 and CD25 by FACS after 18 hours of stimulation (A), or analyzed for proliferation by CFSE dye dilution after 72 hours of stimulation (B). (C) WT and DF DCs (50,000) were cultured with the indicated doses of ovalbumin peptide (continuous peptide) and purified CFSE-labeled OT2 T cells. Proliferation was determined by FACS for CFSE dye dilution on day 3 of culture. (D) Alternatively, WT and DF DCs were pulsed with a saturating dose of ovalbumin peptide (100 nM) for 2 hours and washed thoroughly (pulsed peptide). Subsequently, the indicated numbers of pulsed DCs were cultured with OT2 T cells for analysis of T cell proliferation. Similarly, WT and Vav\textsuperscript{NULL} DCs were cultured with continuous peptide (E) or pulsed peptide (F) and analyzed for their ability to induce OT2 T cell proliferation as described above. Data shown are representative of at least 5 independent experiments.

Figure 2. ITAM Signaling is Required for Maintaining peptide-MHCII Complexes on the Surface of DCs. To assess the presence of pMHCII complexes on the surface of DCs, we utilized the B11 T cell hybridoma specific for beta-gal peptide presented by I-A\textsuperscript{b}. In these experiments, WT and DF DCs were pulsed with a saturating dose of peptide (10 uM), washed, and immediately fixed (A) or chased for 24 hours prior to fixation (B). After fixation, DCs were cultured overnight with B11 cells, after which point, culture supernatants were analyzed for IL-2 by ELISA. Similarly, WT and Vav\textsuperscript{NULL} DCs were pulsed with peptide and immediately fixed (C) or chased for 24 hours prior to fixation (D) and then used to stimulate B11 cells as described above. Data represent mean IL-2 production\textpm{}s.d. for triplicate samples and are representative of 5 independent experiments. * indicates p<0.05 and ** indicates p<0.01.

Figure 3. ITAM Signaling is Required for T Cell Priming \textit{in vivo}. WT and DF mice were immunized in the footpad with ovalbumin peptide in CFA, and 7 days later, the draining popliteal lymph nodes were harvested. Lymph node cells were restimulated \textit{in vitro} with the indicated doses of ovalbumin peptide (continuous peptide) and analyzed
one day later for the frequency of antigen-specific T cells by ELISPOT (A) and 3 days later for proliferation by thymidine incorporation (B). Data shown are the mean±s.d. of triplicate samples. At least 4 mice per group were analyzed. * indicates p<0.05 and ** indicates p<0.01.

**Figure 4. Disruption of ITAM Signaling is Protective in EAE and Inhibits Autoreactive T Cell Priming.** (A) Mice were immunized with MOG\textsubscript{35-55} peptide and pertussis toxin to induce disease. During the course of disease, mice were assigned a clinical score with grade 1 = tail weakness, grade 2 = hind limb weakness sufficient to impair righting, grade 3 = one limb plegic, grade 4 = hind limb paralysis, grade 5 = moribund. Data are expressed as mean clinical score±s.d. for n≥5 mice per group. After the peak of disease (3 weeks), spinal chords were harvested and stained with hematoxylin and eosin (B). In addition, spleens were recovered and restimulated \textit{in vitro} with MOG peptide to determine autoreactive T cell frequencies by ELISPOT (C) and proliferation by thymidine incorporation (D). Data represent the mean±s.d. for n≥5 mice per group. * indicates p<0.05.

**Figure 5. ITAM Signaling Controls the Half-Life and Surface Retention of peptide-MHCII Complexes.** DCs were pulse-labeled with [\textsuperscript{35}S] for 30 minutes, washed, and chased for the indicated time points. Cells were then lysed and mature MHCII complexes were immunoprecipitated with Y3P antibody prior to resolution by PAGE and autoradiography. In WT DCs, stable peptide-MHCII complexes (αβ-peptide), which are SDS-resistant in nonboiled samples, appeared by 3 hours and were slightly reduced after a 9 hour chase. While Vav\textsuperscript{NULL} (A) or DF (B) DCs generated stable peptide-MHCII complexes at 3 hours, they rapidly decayed by the 9 hour time point. (C) Quantification of pMHCII decay from 3-9 hours was achieved by densitometric analysis of pMHCII bands at 9 hours normalized to 3 hours. Data represent the relative mean reduction in pixel intensity±s.d. from 4 independent experiments. (D) WT and Vav\textsuperscript{NULL} DCs were treated with Brefeldin A to block transport of newly synthesized MHCII through the Golgi network, and surface MHCII was detected by FACS at the indicated time points to determine the rate at which MHCII was removed from the cell surface. Data represent the mean percentage of remaining surface MHCII (based on MFI at time 0) ±s.d. from 5
independent experiments. * indicates p<0.05, and ** indicates p<0.01.

**Figure 6. Disruption of ITAM Signaling in DCs Leads to the Accumulation of Ubiquitinated MHCII Species, and Impaired Recycling.** (A) DCs were lysed and MHCII was immunoprecipitated (clone M5/114), resolved by PAGE, and western blots for ubiquitin (clone P4D1) and MHCII beta chain (clone KL295) were performed. (B) The half-life of MHCII was determined in the presence and absence of chloroquine, to inhibit lysosomal proteolysis. DCs were pulse-labeled with [35S] and chased for the indicated time points. Where indicated, chloroquine (50uM) was added 2 hours into the 9 hour chase. After chase, MHCII was immunoprecipitated, resolved by PAGE, and detected by autoradiography. (C) MHCII internalization was determined biochemically by utilizing a surface biotinylating reagent that can be cleaved by MESNA, a cell impermeant reducing agent. DCs were first surface biotinylated, chased for the indicated time points to allow internalization of MHCII, and treated with MESNA to remove biotin from MHCII that remained on the cell surface. Detection of biotinylated MHCII that had been internalized was achieved by immunoprecipitation with anti-MHCII antibody followed by western blotting with streptavidin. Densitometric measurement of band intensity was used to calculate the percentage of internalized MHCII relative to total surface MHCII. Data are representative of 6 independent experiments. (D) Recycling of internalized MHCII back to the plasma membrane was determined biochemically. WT and DF DCs were surface biotinylated and chased for 20 minutes to allow internalization of recycling MHCII. Any biotinylated MHCII remaining on the surface of the cells was then removed by MESNA treatment. DCs were then chased for the indicated time points to allow internalized MHCII to re-emerge on the plasma membrane, at which point biotin was removed with a second MESNA treatment. Detection of biotinylated MHCII that was trapped in intracellular compartments and protected from MESNA was achieved by immunoprecipitation with anti-MHCII antibody followed by western blotting with streptavidin. During the course of the chase, loss of biotinylated MHCII indicates efficient recycling. Densitometric measurement of band intensity was used to calculate the relative amount of recycling MHCII that re-emerged on the plasma membrane at each time point. Data are representative of 6 independent experiments. ** indicates p<0.01.
Figure 1.

A. Ova Beads (18 hours)

B. Ova Beads (72 hours)

C. OT2 Peptide (continuous)

D. OT2 Peptide (pulsed)

E. OT2 Peptide (continuous)

F. OT2 Peptide (pulsed)
Figure 2.
Figure 3.

A. IL-2 ELISPOT

B. Proliferation

OT2 Peptide
Figure 4.

A. Clinical Score vs. Days for EAE.

B. Lumbar Spinal Cord (H&E) images showing Score=4 (WT) and Score=0 (DF).

C. ELISPOT analysis showing spots/250k splenocytes for WT and DF, with significant differences indicated by *.

D. Proliferation analysis showing CPM for untreated (untx) and MOG-treated (MOG) groups.
Figure 6.

A. IP: MHCII, IgG

WT, VavNULL

WB: Ubiquitin

B. Chase (hr): 3, 9, 9

Chloroquine: - - +

WT, VavNULL

WB: l-A beta

C. Surface Biotin:

WT: + + +

Chase (min): 0 0 20

MESNA: - + +

DF: + + +

D. 1st Chase (20min):

WT: + + + + + +

1st MESNA: + + + + + + +

2nd Chase (min): 0 60 5 15 30 60

2nd MESNA: - + + + + +

MHCII Internalization

% Internalized (20min)

WT DF

MHCII Recycling

MHCII Re-emergence

Time (min)

WT DF
ITAM signaling in dendritic cells controls T helper cell priming by regulating MHC class II recycling

Daniel B. Graham, Holly M. Akilesh, Grzegorz B. Gmyrek, Laura Piccio, Susan Gilfillan, Julia Sim, Roger Belizaire, Javier A. Carrero, Yinan Wang, Gregory S. Blaufuss, Gabriel Sandoval, Keiko Fujikawa, Anne H. Cross, John H. Russell, Marina Cella and Wojciech Swat