

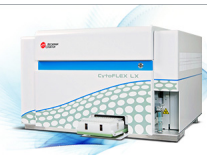


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Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II¹

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Lymphocyte activation gene-3 (LAG-3) is a CD4-related transmembrane protein expressed by regulatory T cells that binds MHC II on APCs. It is shown in this study that during Treg:DC interactions, LAG-3 engagement with MHC class II inhibits DC activation. MHC II cross-linking by agonistic Abs induces an ITAM-mediated inhibitory signaling pathway, involving FcγRγ and ERK-mediated recruitment of SHP-1 that suppresses dendritic cell maturation and immunostimulatory capacity. These data reveal a novel ITAM-mediated inhibitory signaling pathway in DCs triggered by MHC II engagement of LAG-3, providing a molecular mechanism in which regulatory T cells may suppress via modulating DC function. *The Journal of Immunology*, 2008, 180: 5916–5926.

Dendritic cells (DC)³ integrate multiple signals, provided by microbial stimuli and interactions with neighboring cells of the innate and adaptive immune system, to ultimately dictate the qualitative outcome for lymphocyte priming. Much of the work on the functional roles of DCs in immunity has focused on the molecular understanding of the adjuvant effect, namely the inducers of DC activation. Yet, under normal conditions, APCs functionally maintain the tolerant state (1), which might be expected to be similarly enforced upon DCs by neighboring cells in a constitutive or inducible manner. Regulatory T cells (Treg) have been described to negatively regulate DC activation (2–4). Although this process could involve Treg elaboration of immunosuppressive cytokines (e.g., IL-10 and TGF-β), other cell surface molecules expressed on Tregs are likely to contribute (5), because suppression of T cell responses requires both APCs and cell contact (6). Engagement of ITIM-containing receptor pathways with known MHC ligands functionally inhibit DCs, including Ig-like transcript 3 and ILT4 (7, 8) and paired Ig receptor PIR-B (9–11), but their contribution to CD4⁺CD25⁺ T: DC interactions are unclear. In this study, we provide evidence for a cell-contact dependent interaction between Tregs and DCs that involves lymphocyte activation gene-3 (LAG-3/CD223), a membrane protein expressed by Tregs and its ligand on DCs, MHC class II (MHC II).

LAG-3 is a transmembrane CD4-related protein expressed transiently on activated T cells and expressed more highly and persistently after activation of Tregs (12). Initial assessment of LAG-3-deficient mice did not describe major alterations in T lymphocyte development or function in vivo (13). More recently, however, studies of LAG-3-deficient T cells have revealed essential roles in Treg function (12) and in the negative regulation of homeostatic (14) and effector T cell expansion (15). The underlying LAG-3 inhibitory signaling pathways responsible for suppression, either occurring during T:T interactions or in T:DC interactions, have not yet been identified.

A physiological ligand for LAG-3 is MHC II to which it binds with higher affinity than CD4 (16). Cross-linking of LAG-3 on T cells impairs TCR-mediated activation responses in CD4 cells (17), including calcium fluxes, IL-2 production, and Th1 polarization (17, 18). In T cell transductants, ectopic expression of full-length LAG-3 confers regulatory activity, while cytoplasmic truncation LAG-3 mutants do not, arguing for a T cell autonomous inhibitory signaling pathway (19, 20). Thus, it has been proposed that interactions between MHC II-bearing APCs with LAG-3⁺ T cells induces a T cell intrinsic inhibitory signaling pathway, potentially accounting for its effects in limiting T cell activation responses.

We have investigated whether APC:T cell interactions involving MHC II and LAG-3⁺ T cells might also induce reverse inhibitory signaling through MHC II in APCs. Although MHC molecules are known as Ag-presenting structures, additional biological functions have long been reported (21–23). Cross-linking of MHC II on APCs has been shown to induce a variety of disparate cellular responses, including activation/proliferation and/or cell death in B cells (24), monocytes (25), and DCs (26, 27). MHC II cross-linking leads to its recruitment into cholesterol-rich lipid microdomains, activation of src kinases, Ca²⁺-fluxes, protein kinase C activation, and MAPK activation (25). Yet, the short cytosolic domain of MHC II molecules appears inconsistent with this complex signaling transduction pathway, suggesting the presence of membrane-associated signaling components that might provide this functionality. Indeed, recent work by the Cambier group (28) showed that the BCR ITAM-containing subunits, CD79α and β are inducibly associated with MHC II on the membrane upon BCR-mediated B cell activation and provide additional B cell activation programs upon cognate B:T cell interactions.

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³ Abbreviations used in this paper: DC, dendritic cell; LAG-3, lymphocyte activation gene-3; MHC II, MHC class II; BMDC, bone marrow-derived DC; WT, wild type; Treg, regulatory T cell.

We show in this study that MHC II cross-linking on DCs induces suppression of DC maturation and that this inhibitory pathway involves the ITAM adapter Fc γ R γ . In contrast with the activating function of Ig α and β -mediated adapter signaling in B cells, MHC II cross-linking by agonistic Abs or by LAG-3 induces an ITAM-mediated inhibitory signaling pathway, suppressing DC maturation and immunostimulatory capacity. We find that functional inhibition of DCs by MHC II engagement involves downstream recruitment of ERK and SHP-1, thus identifying a novel ITAM-mediated inhibitory pathway in DCs triggered by LAG-3.

Materials and Methods

Mice

C57BL/6 and Shp-1-deficient me^V/me^V mice were purchased from The Jackson Laboratory. SHIP^{-/-} mice were originally generated by G. Krystal (University of British Columbia, Vancouver, CA) and obtained from J. Ravetch (The Rockefeller University, New York, NY). LAG-3^{-/-} mice were obtained from Y.-H. Chen, Stanford University, Palo Alto, CA, with permission from C. Benoist and D. Mathis, Joslin Diabetes Center, Boston, MA. Fc γ R γ ^{-/-} knockout mice, on the C57BL/6 background, were purchased from Taconic Farms. OT-I and OT-II TCR Tg mice specific for OVA/H-2k^b and OVA/I-A^b, respectively, were provided respectively by J. Nikolich-Zugic (Oregon Health Sciences University, Portland, Oregon) and A. Frey (New York University School of Medicine, New York, NY). Syk^{-/-} bone marrow was obtained from radiation chimeras generated by transfer of syk-deficient CD45.2⁺ fetal liver donor cells into CD45.1⁺ hosts (29). The availability of the CD45.2⁺ allotypic marker allowed flow cytometric of DCs as donor-derived. All experimental protocols were performed with prior approval of the Institutional Animal Care and Use Committee at Columbia University.

Abs and reagents

mAbs specific for MHC class II (2G9), LAG-3, CD4, CD8a, CD11b, CD11c, CD45.1, CD40, CD54, CD80, CD86, IFN- γ , and IL-12 were obtained from BD Pharmingen. Foxp3 Abs were obtained from eBioscience. mAbs specific for phospho-tyrosine 4G10 and SHP1 were obtained from Upstate Biosciences. Anti-active ERK and total ERK polyclonal Abs were obtained from Promega. Rabbit anti-Fc γ R γ IgG was provided by J. Ravetch (The Rockefeller University, New York, NY). R406 was a gift from Esteban Masuda (Rigel Pharmaceuticals, South San Francisco, CA).

Bone marrow-derived DCs (BMDCs) and isolated spleen DCs

BMDCs were prepared from bone marrow progenitors by culture (4 \times 10⁵/ml) in GM-CSF-containing media. Supernatant from a cell line J558L (provided by A. Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) transfected with the mouse *gm-csf* gene was used as the source of GM-CSF. BMDCs were harvested at day 7 when typically 80% of CD11c positive cells were immunophenotypically immature (CD86^{low}, MHC II^{low}).

Isolation and in vitro expansion of Tregs

CD4⁺CD25⁺ T cells were FACS sorted from single cell splenocyte/total lymph node suspensions and then stimulated with BMDC pulsed with 50 μ M OTII peptide (chicken OVA peptide 323–339 ISQAVHAAEAEINEAGR) for 24 h at 37°C, before expansion for 7 days in anti-CD3, anti-CD28 coated plates in 10% FBS complete RPMI 1640 medium supplemented with 1000 U/ml IL-2.

To assess inhibition of DC maturation, OT-II expanded Tregs (or Tc cells expanded exactly as above from CD4⁺CD25⁻ FACS-sorted splenocytes) were cocultured with DC for 24 h in the presence or absence of LPS (100 ng/ml) and/or OT-II peptide (10 μ M/ml). DC maturation in these mixed DC:T cell cultures was assessed by flow cytometric analysis of CD86 expression on CD11c⁺ gated populations.

LAG-3-transfected N49 T cell hybridomas

LAG-3 expressing T cell hybridomas were transduced as described (30) using the CD4⁻ variant T hybrid (3A9.N49; H-2A^k-restricted, hen egg lysozyme (48–63-specific) (31). LAG-3 expression was uniformly high in all transductants. T cells and DCs were cocultured at a ratio of 1:1 for 16 h before assessment of DC maturation by flow cytometric analysis of CD11c⁺ gated populations.

Retroviral transduction of conventional CD4⁺ OT-II T cells

Spleens from OTII.LAG-3^{+/+} and OTII.LAG-3^{-/-} mice were removed, and single cell suspensions were made at 2.5 \times 10⁶ cells/ml. The splenocytes were activated with OVA_{326–339} peptide (10 μ M) in culture for 2 days. The activated splenocytes were then incubated on a monolayer of GFP alone, LAG-3, wild type (LAG-3.WT)/GFP, or LAG-3. Δ CY/GFP retroviral producer cells (14) for 2 days in the presence of polybrene (6 μ g/ml). The cells were allowed to rest for 10 days, then sorted for V α 2⁺/CD4⁺/GFP⁺ expression by FACS. Sorted T cells and DCs were cocultured at a ratio of 1:1 in the presence or absence of OT-II peptide (10 μ M/ml) overnight.

In vivo proliferation assays

Naive CD45.1⁺ CD8⁺ OT-I T cells were positively selected by anti-CD8 magnetic microbeads (Miltenyi Biotec) and labeled with 10 μ M CFSE (Molecular Probes). Five \times 10⁶ CFSE-labeled OT-I cells were adoptively transferred i.v. into recipient mice and immunized the next day by i.v. transfer of BMDC pulsed with 10 pM OTI peptide (SIINFEKL). After 3 days, spleens of the recipient animals were analyzed by flow cytometry for intracellular IFN- γ in the gated CD45.1⁺ CD8⁺ OT-I population after 6 h of ex vivo incubation with 100 nM OT-I peptide as described in Ref. 32.

MHC II cross-linking and preparation of cell extracts for Western blot and coimmunoprecipitation

BMDC were stimulated with biotinylated mAb anti-MHC II 2G9 premixed for 15 min at 37°C with streptavidin at 2.5 and 1.5 μ g/ml, respectively. In some cases, BMDCs were stimulated with preformed F(ab')₂ complexes made by incubation of 2.5 μ g/ml biotinylated F(ab')₂ 2G9 with 1.5 μ g/ml avidin. (Inhibition of DCs was also observed with three other anti-MHC II mAbs using rabbit anti-rat IgG F(ab')₂ as a cross-linking agent at 10 μ g/ml, data not shown.) After 24 h of incubation, CD11c⁺ cells were stained for surface expression of CD80, CD86, MHC II, CD54, and CD40, fixed in 4% paraformaldehyde. For IL-12 production, stained cells were permeabilized before intracellular staining for IL-12. For preparation of cell extracts, BMDC were serum starved in 0.5% FBS RPMI 1640 medium for 3 h at 37°C and then stimulated with avidin:biotinylated anti-MHC II complexes. After stimulation cells were quickly chilled on ice and pelleted by centrifugation (2000 rpm for 10 min). Cell extracts were prepared by resuspension of the cell pellet in RIPA buffer for Western blot analysis, or in 0.33% Chaps buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM Na₃VO₄, 10 mM NaF, 0.4 mM EDTA, and 1 \times protease inhibitor mixture for coimmunoprecipitation.

Isolation of lipid raft fraction by sucrose gradient ultracentrifugation

In brief, 10⁸ MHC II cross-linked BMDCs were lysed in 1 ml TNEV buffer containing 0.5% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM Na₃VO₄, 1 \times protease inhibitor mixture and 5 mM EDTA. Cell lysates were then diluted 1/1 with 85% wt/vol sucrose in TNEV solution and then overlaid with 6 ml 35% wt/vol sucrose and 3.5 ml 5% wt/vol sucrose in TNEV in a Beckman polyallomer centrifuge tube (14 \times 95 mm). After centrifugation at 200,000 \times g for 16 h at 4°C, 1 ml fractions were collected from the top of the gradient for Western analysis.

Statistical analysis

In some cases, flow cytometric data is normalized as a "CD86 index %" defined as; MFI CD86 (stimulated – unstimulated)/MFI CD86 unstimulated. This normalization allows for comparison across multiple experiments which vary for CD86 expression on unstimulated DCs. Statistical analysis was performed with a Student's one-sided paired *t* test with *p* values reported by convention, *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

Results

Tregs inhibit DC maturation via LAG-3 interaction with MHC II

Recent work has suggested that Tregs have the capacity to inhibit DC maturation, however this has not been conclusively demonstrated and the mechanism is unknown (2–5). To determine whether Ag specificity contributes to this suppressive activity of Tregs, I-A^b-restricted OVA-specific Tregs were expanded by short term-culture of flow-sorted CD4⁺CD25⁺ OT-II TCR transgenic T cells in the presence of DCs, cognate peptide and high-dose IL-2. To assess their capacity to modulate DC activation, OT-II Tregs

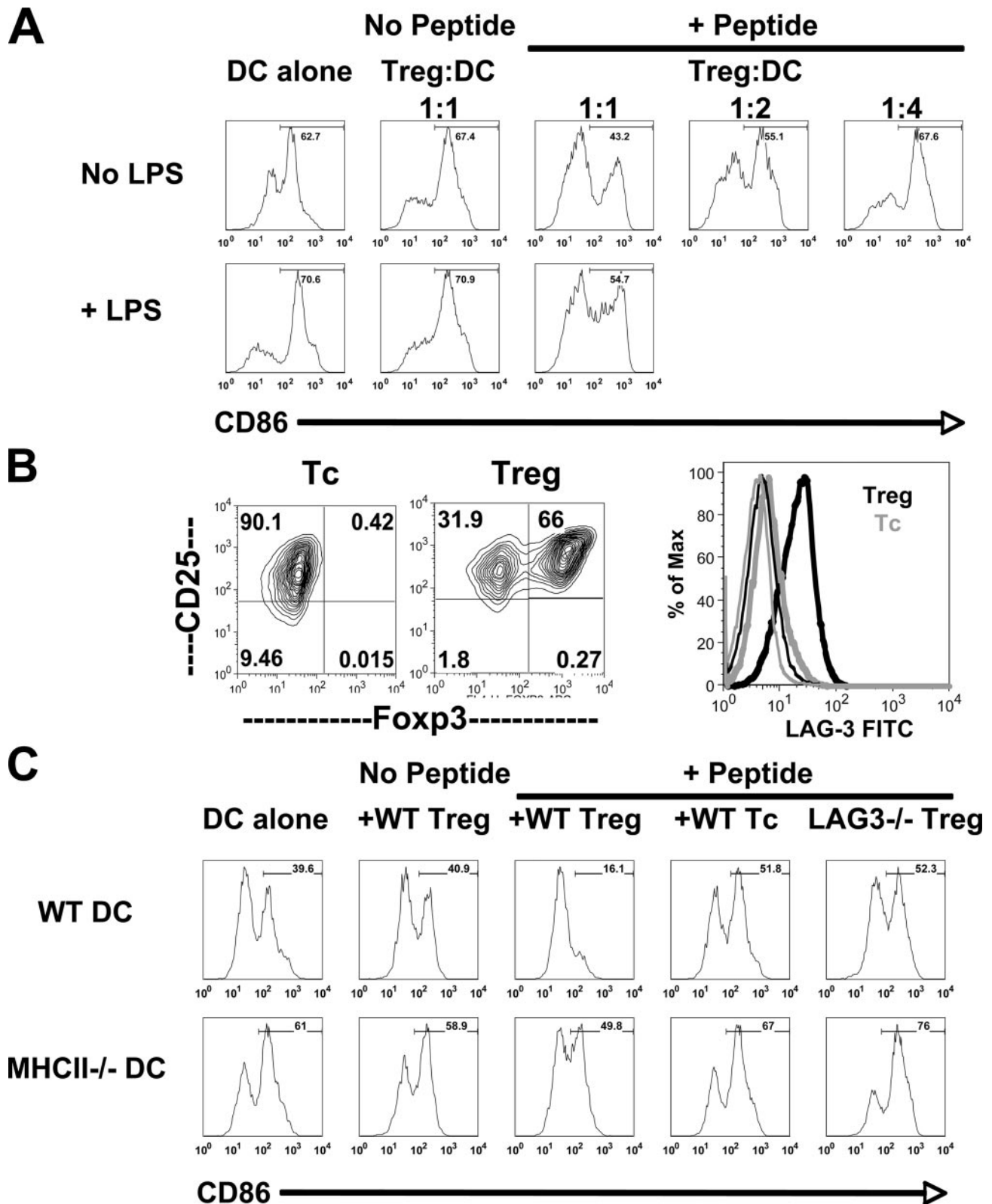


FIGURE 1. Ag-dependent inhibition of DC maturation by Tregs requires MHC II engagement by LAG-3. **A**, Tregs inhibit DC maturation. Regulatory OT-II CD4 cells and DCs were cocultured at the indicated T:DC ratios for 24 h in the presence or absence of 10 μ M OT-II peptide. CD86 expression on the gated CD11c⁺ DC population is shown. Inhibition of both spontaneous and LPS-induced DC maturation in the presence of peptide is evident at Treg:DC ratios of greater than 1:4. **B**, Expanded regulatory OT-II cells but not conventional effector OT-II cells are LAG-3⁺. Sorted splenic and lymph node CD4⁺CD25⁻ (Treg, black) and CD4⁺CD25⁺ (Tc, gray) were expanded in vitro for 7 days and stained for Foxp3, CD25 (left), or LAG-3 (right). Thick lines denote staining with LAG-3 (rat IgG_a) followed by anti-rat FITC, while thin lines denote staining with rat IgG_{2a} isotype control followed by anti-rat FITC. **C**, Inhibition of DC maturation requires both LAG-3 and MHC II expression. LAG-3^{+/+} or LAG-3^{-/-} regulatory OT-II CD4 cells were cocultured together with both WT CD45.1⁺ and CD45.2⁺ MHC II^{-/-} DCs (in the same well) at a ratio of 1:4 for 24 h. CD86 expression on the gated CD11c⁺ DC populations is shown. CD45.1⁺ staining allowed discrimination between CD45.1⁺ MHC II^{+/+} and cocultured CD45.2⁺ MHCII^{-/-} “bystander” DCs. LAG-3^{+/+} but not LAG-3^{-/-} Tregs inhibited DC maturation and bystander DCs MHCII^{-/-} were inhibited to a lesser extent. Representative data of three separate experiments are shown.

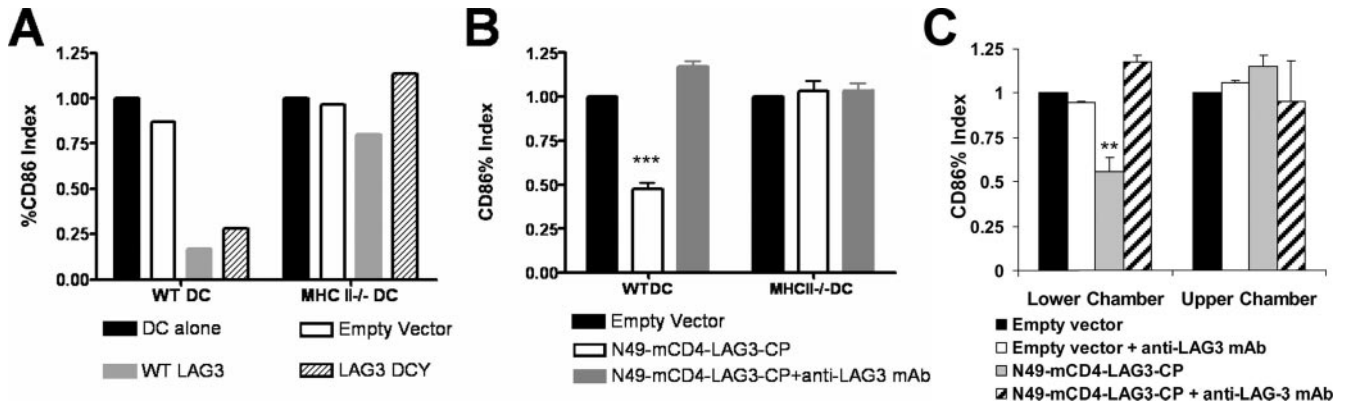


FIGURE 2. Ectopic expression of LAG-3 confers regulatory activity in manner dependent on cell contact and MHC II engagement but independent of LAG-3 autonomous signaling in T cells. *A*, Ectopic expression of LAG-3 confers regulatory activity in primary T cells: CD4⁺ OT-II T cells transfected with either full-length LAG-3 (LAG-3.WT), or a cytoplasmic truncation mutant of LAG-3 (LAG-3.ΔCY) control (empty) vectors, were cocultured with both CD45.1⁺ MHC II^{+/+} and CD45.2⁺ MHC II^{-/-} DCs for 24 h, with or without 10 μM OT-II peptide. CD86 expression on the gated CD45.1⁺ or CD45.2⁺ bystander CD11c⁺ DC populations demonstrates that LAG-3.WT and LAG-3.ΔCY CD4⁺ transfectants inhibited maturation of MHC II^{+/+} DC while vector control transfectants did not. Modest bystander inhibition of MHC II^{-/-} DCs was seen in the presence of LAG-3.WT but not LAG-3.ΔCY transfectants. The data is representative of two different experiments, and DC suppression required the presence of OT-II peptide (data not shown). *B*, Ectopic expression of LAG-3 confers regulatory activity in T cells hybrids: Stable transfectants of the N49.CD4⁻ T cell hybridoma expressing either the noncleavable LAG-3.CP, WT LAG-3, or empty vector were cocultured with both CD45.1⁺ MHC II^{+/+} and CD45.2⁺ MHC II^{-/-} DCs for 24 h in the presence or absence of anti-LAG-3 blocking Abs (10 μg/ml). CD86 expression on the gated CD11c⁺ DC population demonstrates that ectopic expression of LAG-3.CP confers regulatory activity that is inhibitable by blocking anti-LAG-3 Abs. Bystander MHC II^{-/-} DCs were not inhibited. Data is shown as the mean and SD of triplicate wells and is representative of three experiments; *p* values are provided for statistically significant differences between samples vs vector control. *C*, Soluble factors do not mediate inhibitory activity. MHC II^{+/+} BMDCs were cocultured in the upper chambers of transwells in the presence of MHC II^{+/+} DCs and LAG-3.CP transfectants in the lower chambers. Consistent with a requirement for cell contact, MHC II^{+/+} cells in the lower chambers but not in the upper chambers were inhibited. Representative data of three experiments is provided and shown as the mean and SD of triplicate wells; *p* values are provided for statistically significant differences between samples and vector control.

were cocultured for 24 h with DCs in the presence or absence of LPS. Coculture in the absence of OT-II specific peptide did not result in suppression of either spontaneous or LPS-induced DC maturation, as assessed by expression of the costimulatory molecule CD86. However in the presence of OT-II peptide, DC activation was significantly inhibited (Fig. 1A), indicating that cognate Ag recognition was involved. Suppression of DC activation was dependent on the dose of cocultured Tregs, and required ratios of Treg:DC higher than 1:4.

The mechanistic basis of Treg mediated suppression of DC activation is unknown. Because cell-contact mediated interactions are likely to contribute, we considered the possibility that expression of LAG-3 on Tregs plays a role, as LAG-3 is homologous to CD4 and interacts directly with MHC II. After 7 days of expansion with anti-CD3 and anti-CD28, OT-II CD4⁺CD25⁺ Treg populations were largely Foxp3⁺ and LAG-3⁺ positive (Fig. 1B). In contrast effector T cells (Tc) expanded under similar conditions for 7 days from CD4⁺CD25⁻ OT-II cells did not express LAG-3. To assess whether LAG-3 contributes to this suppressive activity, WT or LAG-3-deficient regulatory OT-II T cells were cultured at a DC:T cell ratio of 1:1. OT-II CD4⁺ CD25⁺ Treg obtained from WT mice again showed a peptide-dependent suppressive effect on DC activation with a 2.5-fold reduction in the CD86⁺ population among CD11c⁺ BMDCs while CD4⁺CD25⁻ conventional CD4 effectors (Tc) had no effect. Importantly, this suppressive activity was completely eliminated when DCs were cultured with LAG-3-deficient OT-II Tregs (Fig. 1C).

To address the potential for LAG-3-mediated suppression to promote the inhibition of bystander DCs, MHC II-deficient DCs were cocultured in the same well with both WT DCs and WT regulatory OT-II cells in suppressive conditions. Bystander MHC II^{-/-} DCs were only marginally inhibited, with only 15% fewer CD86⁺ CD11c⁺ cells accumulating after addition of peptide and

WT Tregs (Fig. 1C). Taken together, these data show that Tregs can inhibit DC maturation in an Ag-specific manner and notably requires the expression of both LAG-3 and its ligand, MHC II, on Tregs and DCs, respectively.

LAG-3 signaling is not required for inhibition of DC maturation

Previous studies investigating the suppressive function of LAG-3 have demonstrated impaired functionality of cytoplasmic truncation LAG-3 mutants, arguing for a T cell autonomous inhibitory signaling pathway (19, 20). To examine whether LAG-3 signaling in T cells was required for suppression of DC activation, we compared the suppressive activity of full-length (WT) LAG-3 with a truncated version of LAG-3, that retains the ECD and TM domains but lacks its cytoplasmic domain (LAG-3.ΔCY) (19). OT-II T LAG-3^{-/-} CD4⁺ T cells were transfected with a bicistronic retrovirus encoding GFP alone or GFP and either WT LAG-3 or with LAG-3.ΔCY. Flow sorted GFP-positive transfected T cell populations were incubated with DCs overnight. Interestingly although these CD4 positive populations consisted largely of conventional CD4 cells and few, if any Tregs, transfected LAG-3, but not GFP alone, was sufficient to confer regulatory activity as seen by peptide-dependent suppression of DC maturation (Fig. 2A). Again MHC II expression on DCs was required for the inhibitory activity as cocultured “bystander” MHC II^{-/-} DCs were only modestly suppressed. Furthermore, the direct inhibitory effect did not require the LAG-3 cytoplasmic domain, because CD4 cells transfected with either WT LAG-3 or tailless LAG-3.ΔCY (Fig. 2A) inhibited the maturation of DCs. Thus, LAG-3 signaling within T cells is not required for LAG-3-mediated direct suppression of DC maturation, suggesting instead that it is likely mediated by MHC II signaling in DCs. The limited degree of bystander MHC II^{-/-} DC inhibition observed may be due to elaboration of T cell-derived

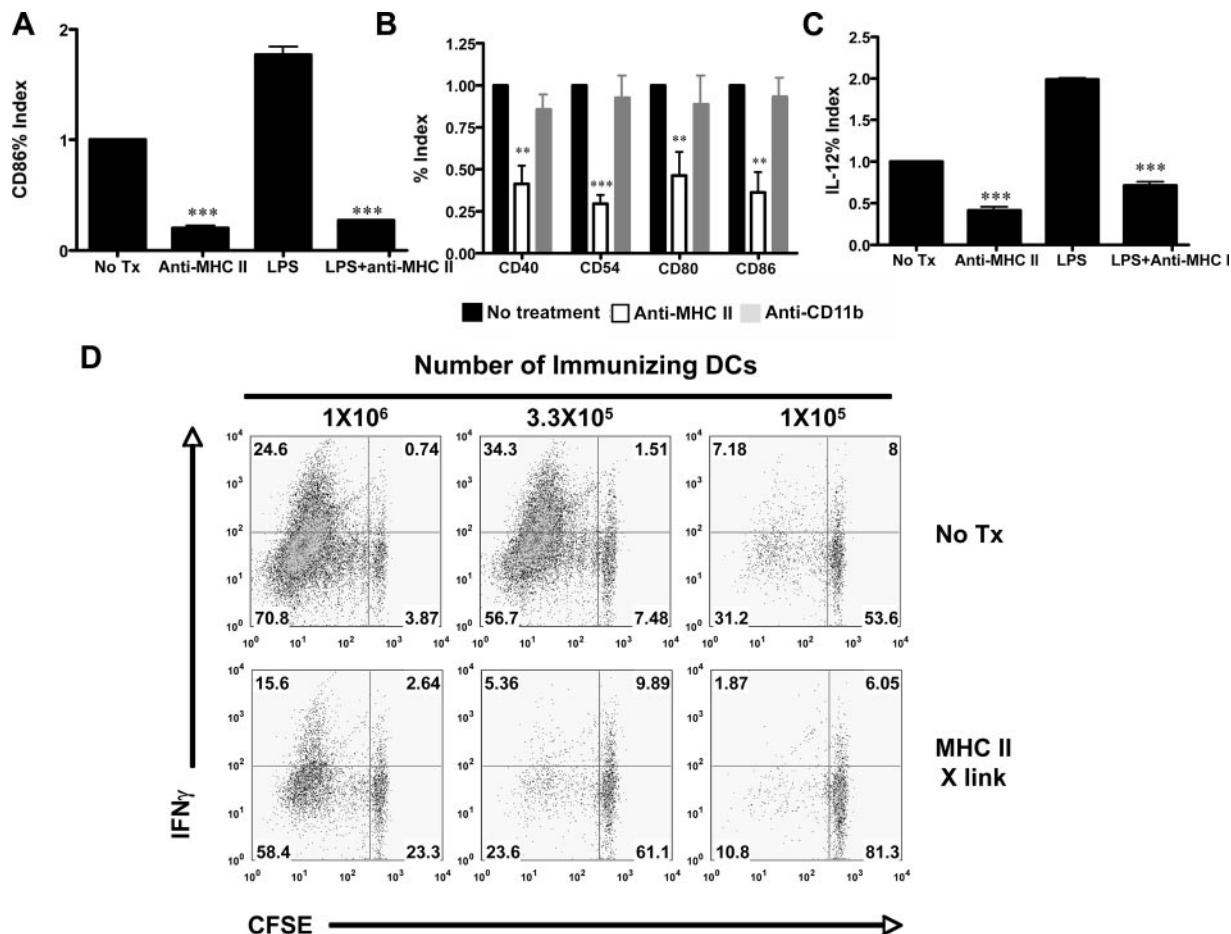


FIGURE 3. MHC II cross-linking inhibits DC function. *A–C*, Cross-linking MHC II on DCs suppresses DC maturation. *A*, Inhibition of CD86 expression. *B*, Inhibition of CD40, CD86, ICAM-1 (CD54) expression. *C*, Inhibition of intracellular IL-12 production. *A*, MHC II on BMDCs were cross-linked with biotinylated 2G9 2.5 $\mu\text{g/ml}$ plus 1.5 $\mu\text{g/ml}$ streptavidin, in the presence or absence of LPS. *B* and *C*, Cross-linking was performed by stimulation instead with avidin and biotinylated 2G9 F(ab')₂. BMDC maturation was suppressed after anti-MHC II cross-linking but not after cross-linking with isotype-matched biotinylated anti-CD11b (M1/70) control Abs. Data is provided as the mean and SD of triplicate wells; *p* values are provided for statistically significant differences between wells with vs without cross-linking Abs. *D*, Cross-linking MHC II suppresses the immunostimulatory capacity of DCs. CD45.2⁺ WT C57BL/6 mice previously transferred with 5×10^6 CFSE-labeled CD45.1⁺ OT-I cells were immunized with graded numbers of BMDCs that were loaded overnight with 10 pM OT-I peptide in the presence or absence of MHC II cross-linking Abs (2G9 biotinylated 2.5 $\mu\text{g/ml}$ plus 1.5 $\mu\text{g/ml}$ streptavidin). After 3 days, activation of proliferating gated CD45.1⁺ CD8⁺ OT-I cells was assessed in splenocytes by intracellular staining for IFN- γ production.

soluble factors triggered by LAG-3 engagement on T cells, because it was induced by full length LAG-3 transfectants but not by cytoplasmic tail-less LAG-3 transductants.

LAG-3 expression confers regulatory activity in a cell-contact dependent manner

Cleavage of LAG-3 from the cell membrane by metalloproteases likely limits autonomous inhibitory T cell signaling (33). Thus, it remained possible that LAG-3 cleavage was obscuring contributions by LAG-3 signaling in T cells. To address this possibility, stable transfectants of hen egg lysozyme-specific CD4⁻ N49 T cell hybrids expressing high levels of surface noncleavable LAG-3 were used (mCD4-LAG-3 CP). This T cell hybrid variant lacks CD4 expression, eliminating potential confounding contributions of CD4 (19) to MHC II engagement. Incubation of BMDCs with N49 T cell hybrids transfected with empty vector was not found to alter either spontaneous or LPS-induced maturation by DCs. However, coculture of DCs with N49 cells expressing a noncleavable LAG-3 molecule inhibited BMDC maturation that was reversible with addition of LAG-3 blocking Abs (Fig. 2*B*). Cocultured “by-stander” MHC II^{-/-} DCs were not inhibited in suppressive con-

ditions, indicating again that MHC II engagement by LAG-3 is required and discounting the importance of soluble mediators elaborated by T cells. Furthermore, cell contact was required for inhibition of DC maturation, as DCs cocultured in the upper chamber of transwells failed to be inhibited, while DCs cocultured with mCD4-LAG-3 CP N49 in the lower chambers were significantly inhibited (Fig. 2*C*). Consistent with this data, transferred supernatants from suppressive conditions did not inhibit DC maturation (data not shown). Taken together these data indicate that ectopic expression of LAG-3 in T cells confers regulatory activity that inhibits DC activation via MHC II engagement.

MHC II cross-linking recapitulates the activity of LAG-3 and inhibits DC maturation and immunostimulatory capacity

We have demonstrated that membrane-expressed LAG-3 engagement of MHC II-bearing DCs is capable of inhibiting DC activation in a contact-dependent manner. Attempts at reproducing inhibitory signaling with soluble LAG-3 Ig were not successful, indicating that oligomerization or MHC II clustering might be required for inhibitory signaling. Indeed, in the absence of cross-linking agents, monomeric anti-MHC II mAbs did not inhibit DC

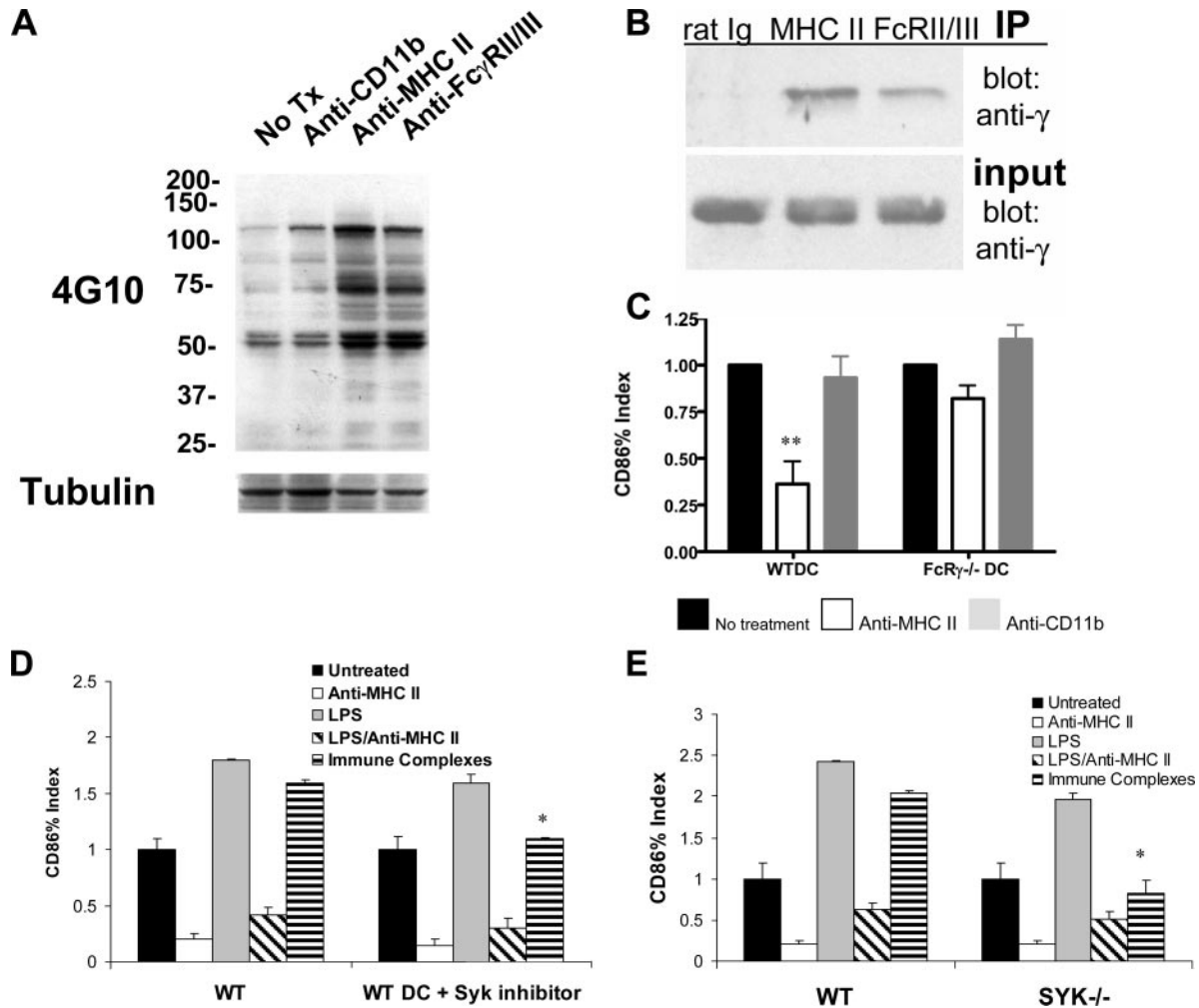


FIGURE 4. MHC II inhibitory signaling involves the ITAM adapters Fc γ R γ -chain but not Syk. *A*, MHC II Cross-linking induces phosphotyrosine activity in DCs: BMDCs were stimulated for 5 min with cross-linking anti-MHC II mAb 2G9, anti-CD11b mAb or anti-Fc γ R/III 2.4G2 mAb. In all three cases, stimulations were performed with biotinylated Abs:avidin at 2.5 μ g/ml:1.5 μ g/ml. Whole cell extracts were immunoblotted for p-Tyr with mAb 4G10. *B*, The Fc γ R γ -chain subunit is physically associated with MHC II. WT DCs extracts were immunoprecipitated with anti-MHC II mAb, anti-FcR/III mAb 2.4G2 and isotype matched anti-CD11b mAb. Immunoprecipitates were immunoblotted with anti-Fc γ R γ . Total cell extracts were immunoblotted with anti-tubulin as a loading control. *C*, Fc γ R γ ^{-/-} BMDCs exhibit impaired inhibitory responses after MHC II engagement. WT and Fc γ R γ ^{-/-} BMDCs were stimulated with MHC II cross-linking Abs and cultured for 24 h before assessing CD86 expression by flow cytometry. Data are provided as the mean and SD of triplicate wells; statistically significant *p* values are provided for WT vs FcR γ ^{-/-} wells. *D*, Inhibitory MHC II signaling is intact in WT DCs treated with the syk-inhibitor R406. WT BMDCs were incubated for 24 h as indicated with LPS (100 ng/ml), R406 (1 μ M), cross-linking anti-MHCII mAb Abs (2.5 μ g/ml biotinylated mAb 2G9:1.5 μ g/ml avidin), and soluble immune complexes (30 μ g/ml rabbit anti-OVA IgG:3 μ g/ml OVA) before assessing CD86 expression by flow cytometry. Data are provided as the mean and SD of triplicate wells. R406-treatment resulted in significantly less CD86 expression after immune complex treatment but not after MHC II cross-linking (*p* values are for comparisons of wells with vs without syk R406). *E*, Inhibitory MHC II signaling is intact in syk^{-/-} BMDC. BMDCs were grown from bone marrow precursors obtained from CD45.2⁺ syk^{-/-}/CD45.1⁺ WT radiation chimeras (syk^{-/-} BMDCs) and from CD45.2⁺ WT/CD45.1⁺ WT radiation chimeras (WT BMDCs). Chimeric bone marrow cells were greater than 90% CD45.2⁺ donor-derived. DCs were treated as described in *D*. CD86 expression on CD45.2⁺ CD11c⁺ gated populations is shown. Data are provided as the mean and SD of triplicate wells; *p* values are provided for statistically significant comparisons of WT vs syk^{-/-}.

maturation (data not shown). However, in the presence of cross-linking agents, anti-MHC II mAbs suppressed both spontaneous and LPS-induced up-regulation of CD86 on BMDCs (Fig. 3A), thus recapitulating the inhibitory effects observed after engagement by membrane-bound LAG-3. Other immunophenotypic markers of maturation behaved similarly, including CD40, CD54, and CD80 (Fig. 3B), all of which were reduced after MHC II cross-linking but not after cross-linking with isotype-matched rat anti-mouse CD11b. The production of IL-12 by maturing DCs was also reduced upon MHC II engagement (Fig. 3C). Inhibition of DC maturation was not due to induction of cellular apoptosis by maturing DCs (data not shown) nor due to Fc-Fc γ R interactions as inhibition was also seen using anti-MHC II F(ab)₂ fragments (Fig.

3, *B* and *C*). Elaboration of inhibitory cytokines by DCs were unlikely to be involved as transferred supernatants were insufficient to transfer suppressive activity and cocultured MHC II^{-/-} DCs were unaffected (data not shown).

To examine the functional consequences of MHC II engagement on the ability of DCs to prime T cells, BMDCs were cross-linked with MHC II mAbs, pulsed with OT-I peptide, and transferred into recipient WT mice that had previously received CFSE-labeled CD45.1⁺ CD8 naive transgenic OT-I T cells (which recognize the ovabumin immunodominant peptide restricted by MHC-K^b). MHC II engagement on immunizing DCs resulted in significant impairment in the induction of both the OT-I proliferative response and in their differentiation into IFN- γ producing effectors (Fig. 3D).

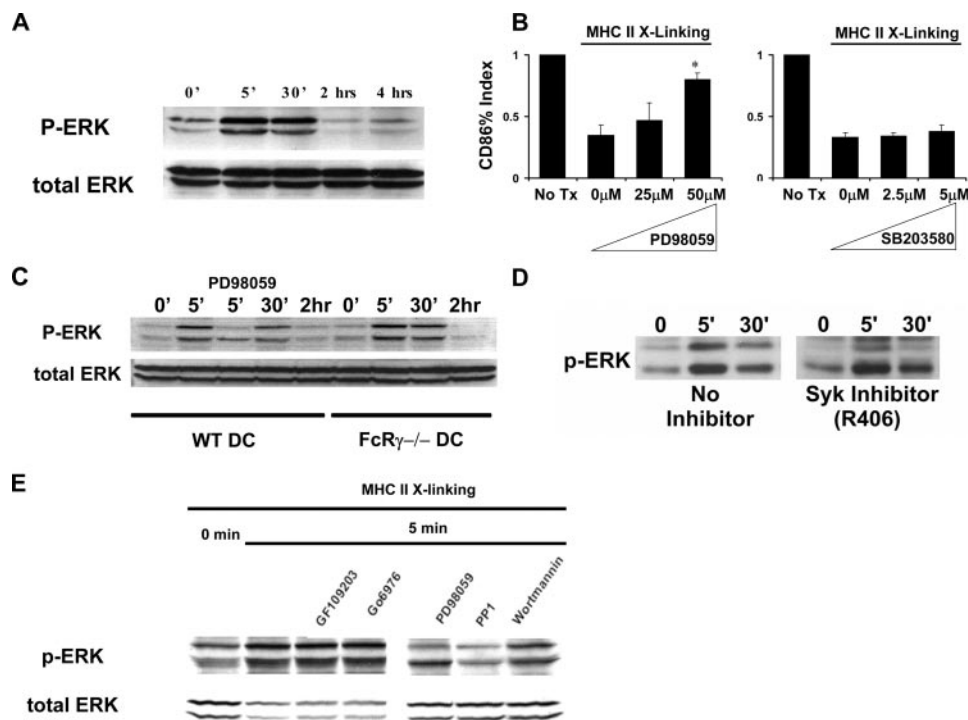


FIGURE 5. ERK activation is required for MHC II inhibitory signaling. *A*, ERK is transiently phosphorylated upon MHC II cross-linking. BMDCs were stimulated as in Fig. 4A for the indicated times and whole cell extracts immunoblotted for p-ERK and total ERK. *B*, Pharmacological inhibition of ERK abrogates MHC II inhibitory signaling in BMDCs. BMDCs were incubated with the ERK inhibitor PD98059 or the p38 inhibitor SB203580 30 min prior and during overnight stimulation by MHC II cross-linking as described in Fig. 3A. Data is provided as the mean and SD of triplicate wells; *p* values are shown for statistically significant differences between samples with vs without PD98059. *C*, ERK activation occurs normally in $Fc\gamma R\gamma^{-/-}$. WT DCs and $Fc\gamma R\gamma^{-/-}$ DCs were stimulated with anti-MHC II cross-linking Abs as in Fig. 4A for the indicated times and the cellular extracts immunoblotted with anti-phosphoERK and anti-total ERK Abs. *D*, ERK activation is not blocked by inhibition of syk. WT DCs were stimulated with anti-MHC II cross-linking Abs as in Fig. 4A in the presence and absence of R406 (1 μ M) for the indicated times and the cellular extracts immunoblotted with anti-phosphoERK and anti-total ERK Abs. *E*, Induction of ERK activation by MHC II engagement is blocked by inhibition of src family kinases. WT BMDCs were incubated for 30 min with the indicated pharmacological inhibitors and then stimulated as in Fig. 4A with anti-MHC II cross-linking Abs for 5 min. Cellular extracts were immunoblotted with anti-phosphoERK and anti-total ERK Abs. Neither PKC inhibitor (GF109203 nor Go6976) nor the PI3K inhibitor (wortmannin) inhibited ERK activation. However the src kinase inhibitor PP1 (as well as the MEKK inhibitor PD98059) blocked ERK activation.

Specifically, >3-fold more immunizing DCs were required after MHC-II cross-linking for comparable degrees of OT-I activation *in vivo*.

MHC II inhibitory signaling involves the ITAM-containing adapter $Fc\gamma R\gamma$ but does not require syk

MHC II engagement has been previously noted to involve the induction of tyrosine kinase activity in cultured B cells, macrophages, and DCs (24–28). As previously demonstrated, MHC II cross-linking of DCs induced rapid induction of tyrosine phosphorylation (Fig. 4A). The induction of protein tyrosine kinase activity suggest the potential involvement of ITAM-mediated signaling, however the short cytoplasmic domains of MHC II chains lack classical ITAM motifs. Alternatively, interacting ITAM-containing adapters could provide this function (28). Indeed, in BMDCs in both steady-state conditions and after MHC II cross-linking, the ITAM adapter $Fc\gamma R\gamma$ was found to interact with MHC II (Fig. 4B). To ascertain the requirement of $Fc\gamma R\gamma$ for functional MHC II inhibitory signaling, WT and $Fc\gamma R\gamma^{-/-}$ BMDCs were stimulated with MHC II cross-linking Abs. Importantly, maturation inhibition was reduced in $Fc\gamma R\gamma^{-/-}$ as compared with WT DCs (Fig. 4C).

The protein tyrosine kinase syk, is critically involved in proximal ITAM-mediated activatory signaling cascades in both B cells and myeloid cells. Syk-deficient B cells and DCs fail to be activated upon BCR (34) and $Fc\gamma R$ cross-linking (35), respectively.

The role of syk in ITAM-mediated inhibitory signaling is less well understood. To investigate the role of syk in MHC II inhibitory signaling, both pharmacologic and genetic approaches were used. Addition of the selective syk-inhibitor R406 (36) did not reduce the functional consequences of MHC II inhibitory signaling (Fig. 4D) on CD86 expression, whereas R406 did inhibit immune complex-triggered up-regulation of CD86 as expected. Furthermore, $Syk^{-/-}$ BMDCs responded normally to MHC II cross-linking and were functionally inhibited by MHC II cross-linking, whereas as expected they failed to be activated by soluble immune complexes (35, 36) (Fig. 4E). Thus, MHC II-triggered inhibition of DC activation is mediated by an $Fc\gamma R\gamma$ -mediated signaling pathway that does not require syk activation.

MHC II inhibitory signaling requires ERK activation

Several reports have linked ERK activation with inhibitory signaling and suppression of IL-12 production in DCs (37–41). ERK was phosphorylated transiently in BMDCs upon MHC II cross-linking, occurring within 5 min after stimulation (Fig. 5A). To examine the requirement for ERK phosphorylation on maturation inhibition, DCs were incubated with increasing doses of the MAPK inhibitors PD98059 and SB203580, which respectively inhibit the specific phosphorylation of ERK and p38. The ERK-inhibitor PD98059 inhibited the suppressive activity of MHC-II

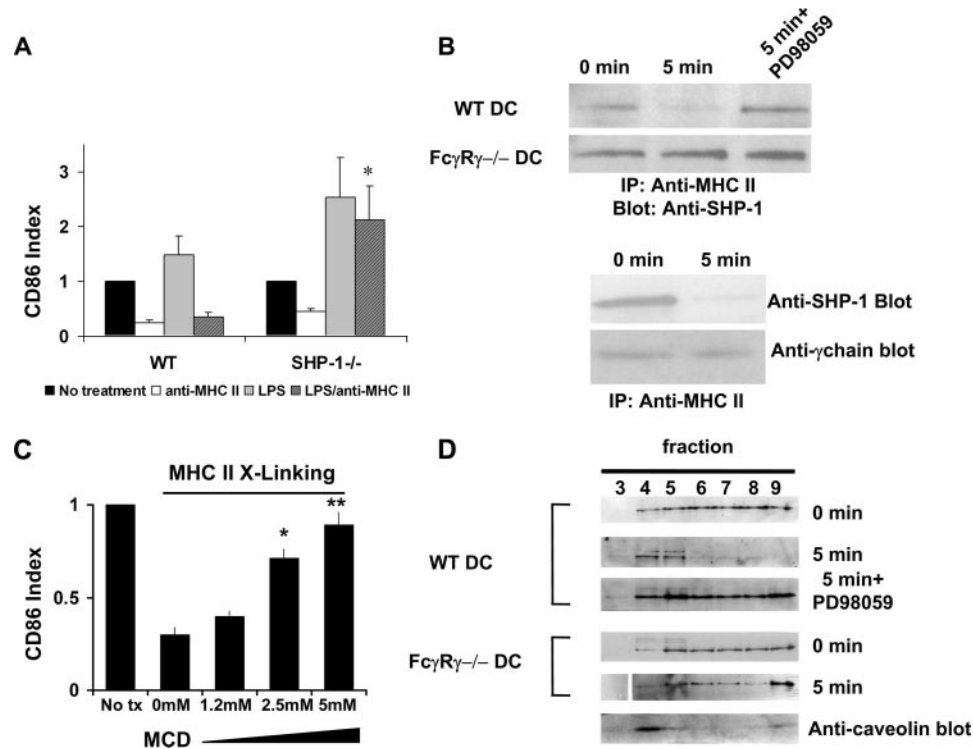


FIGURE 6. SHP-1 participates in MHC II inhibitory signaling via FcR γ and ERK. **A**, SHP-1-deficient (Me^y/Me^y) BMDCs exhibit impaired inhibitory responses to MHC II cross-linking. WT and Me^y/Me^y BMDCs were stimulated with MHC II cross-linking Abs overnight and assessed for CD86 expression by flow cytometry. In the presence of LPS, MHC II cross-linking significantly decreased the number of CD86⁺ mature DCs. In the absence of LPS, SHP-1 deficiency was associated with a modest but statistically insignificant reduction in MHC II-mediated inhibition. Data is provided as the mean and SD of triplicate wells and is representative of three independent experiments; *p* values are provided for statistically significant differences between WT vs SHP^{-/-}. **B**, SHP-1 physically associates with MHC II. MHC II immunoprecipitates obtained from BMDCs were immunoblotted with anti-SHP-1 Abs before and 5 min after MHC II cross-linking with biotinylated anti-MHC II F(ab')₂:avidin (2.5/1.5 μ g/ml) in the presence or absence of the ERK inhibitor PD98059. The membrane was stripped and reprobed with rabbit anti-mouse γ -chain IgG. **C**, MHC II inhibitory responses are abrogated by treatment with methyl- β -cyclodextrin. BMDC were incubated with the indicated amount of methyl- β -cyclodextrin immediately before stimulation with MHC II cross-linking Abs, and then the cells were subsequently incubated for 24 h before flow cytometric analysis of CD86 expression. Data are provided as the mean and SD of triplicate wells; *p* values are provided for conditions with vs without methyl- β -cyclodextrin. **D**, SHP-1 is recruited in an FcR γ -dependent manner to lipid raft upon MHC II cross-linking. Sucrose gradient cellular fractions prepared from unstimulated WT and Fc γ R γ ^{-/-} BMDCs and 5 min after MHC II cross-linking as in Fig. 4A were subjected to Western analysis and probed with anti-SHP-1, and anti-caveolin Abs. SHP-1 was present in all fractions before stimulation but in WT stimulated cells was found enriched in the caveolin-positive fractions 4 and 5. Enrichment of SHP-1 in fractions 4 and 5 did not occur in FcR γ -deficient DCs nor in the presence of the ERK-inhibitor PD98059.

cross-linking, while SB203580 had no effect (Fig. 5B). Thus, phosphorylation of ERK is required for functional competence of the MHC II inhibitory signaling pathway in DCs.

Because ITAM-mediated signaling commences with recruitment of src family PTKs and syk, we assessed the upstream requirement(s) for these PTKs as well as for Fc γ R γ in MHC II-triggered ERK activation. ERK activation occurred normally within 5 min after MHC II cross-linking of Fc γ R γ ^{-/-} DCs (Fig. 5C) or after cross-linking of WT DCs treated with the syk inhibitor R406 (Fig. 5D). However, ERK activation was abrogated by PP1, a pharmacologic inhibitor of src-family PTKs (Fig. 5E). Thus, these data suggest that src-family PTK recruitment/activation is a necessary upstream event preceding ERK activation while neither Fc γ R γ or syk activation are required.

MHC II inhibitory signaling involves SHP-1 recruitment

Because recruitment of phosphatases might be expected to contribute to inhibitory signaling, we investigated the role of SHIP and SHP-1. SHIP^{-/-} BMDCs were found to be competent for inhibitory signaling upon MHC II cross-linking ruling out a major contribution of this inositol phosphatase (data not shown). In contrast, SHP-1-deficient BMDCs were found to be relatively resistant to

the inhibitory effects of MHC II engagement (Fig. 6A), consistent with a functional role for SHP-1 in the MHC II inhibitory signaling pathway. In the presence of LPS, MHC II cross-linking decreased the number of CD86⁺ mature DCs, while SHP-1-deficient DCs were only modestly inhibited. Inhibition of spontaneous maturation in the absence of LPS, was relatively preserved in SHP-1-deficient BMDC. SHP-1 was found physically associated with MHC II in BMDCs (Fig. 6B). Interestingly, SHP-1 interacts with MHC II under steady-state conditions before cross-linking, although this interaction appeared to be diminished transiently 5 min after cross-linking, in an ERK-inhibitable manner. The apparent loss of interaction was potentially due to recruitment of the SHP-1 containing cross-linked MHC II complexes to cholesterol rich lipid microdomains (rafts), predicted to be present in the detergent-resistant insoluble cellular extract fractions. Consistent with a requirement for inhibitory signaling in lipid rafts, and with previous MHC II signaling studies with monocyte cell lines (42), functional inhibition of DCs by MHC II cross-linking was abrogated in the presence of methyl- β -cyclodextrin, which disrupts protein association with lipid rafts (Fig. 6C). Thus, SHP-1 recruitment to lipid rafts was directly assessed, using sucrose gradient subcellular fractionation (Fig. 6D). In unstimulated cells, SHP-1 was present

diffusely across several cellular fractions. In contrast, within 5 min after MHC II cross-linking, SHP-1 was preferentially enriched in caveolin-positive fractions. Interestingly, recruitment of SHP-1 to lipid rafts required the presence of the ITAM adapter Fc γ R γ , because SHP-1 was not found enriched in caveolin-positive fractions obtained from stimulated Fc γ R $\gamma^{-/-}$ BMDCs. Furthermore, SHP-1 recruitment to lipid rafts was found to be dependent on activation of ERK, as it was blocked with incubation with PD98059. Thus, ERK activation is likely a proximal event in inhibitory MHC II signaling and required for subsequent downstream recruitment of SHP-1 to lipid rafts.

Discussion

We show in this study that Tregs functionally inhibit DC activation in an Ag-dependent manner involving the interaction of LAG-3 and its ligand, MHC II. This LAG-3/MHC II molecular interaction provides a novel tolerogenic pathway that may endow Tregs the capacity to enforce tolerance by inhibiting DC function. The ability of Ag-specific Tregs to modulate DC function would potentially allow limited numbers of Ag-specific Tregs to inhibit many potential responding T effectors.

Expression in T cells of LAG-3 lacking its cytoplasmic tail was sufficient to confer regulatory activity, consistent with the notion that “reverse” signaling through MHC II in DCs, rather than LAG-3 signaling in T cells, was responsible for inhibition of DCs. Furthermore, inhibition of DC maturation required cell contact and bystander DCs were only modestly affected, indicating that release of inhibitory cytokines by regulatory cells were not primarily responsible. Prior studies have shown that LAG-3 engagement inhibits T cell activation through the KIELLE motif in the cytoplasmic domain (19) and thus signaling within T cells has been proposed to limit activation and homeostatic responses or induce regulatory activity. Together with our data, the LAG-3:MHC II interaction can now be viewed as a bidirectional inhibitory pathway, with direct inhibitory consequences shared by both cellular partners during the cognate Ag interaction.

A recent study using human DC:Treg cocultures has similarly shown that LAG-3-blocking Abs can block Treg mediated suppression of DCs (43). In contrast to the inhibitory activity of membrane expressed LAG-3, previous reports using a hLAG-3/Ig fusion protein have concluded instead that LAG-3:MHC II interactions induce DC activation (26, 44) and provide immune adjuvant activity (45), findings seemingly inconsistent with a role for LAG-3 in Treg function. Indeed, evaluation in our laboratory confirmed that soluble or plate-bound hLAG-3/Ig (provided by F. Triebel, Châtenay-Malabry, France), induced BMDC activation responses (data not shown). Our data showing DC inhibition by agonistic anti-MHC II mAbs and membrane-expressed LAG-3 might be reconciled by any of several possibilities including the presence of the Fc domain in the LAG-3/Ig fusions that might contribute to biological activity. Indeed, soluble LAG-3, shed after proteolysis of membrane LAG-3 (33), is present at 100–200 ng/ml *in vitro* and *in vivo* (46) but unlike mLAG-3 Ig (30), soluble LAG-3 binds weakly if at all to MHC II *in vivo* and neither agonistic or antagonistic biological activities have been identified (33). Thus, surface LAG-3 is likely the physiologically relevant ligand for MHC II.

Using MHC II cross-linking Abs as a surrogate, we identified an inhibitory reverse signaling pathway involving the ITAM-containing adapter Fc γ R γ , ERK, and SHP-1. ERK was phosphorylated rapidly and transiently upon MHC II cross-linking and its pharmacologic blockade prevented inhibitory responses. ERK activation has been previously associated with inhibition of DCs, IL-10 production, and inhibition of Th1 responses *in vivo* (37–41). A dominant role for IL-10 as a mediator of DC suppression after

MHC II engagement seems unlikely as there was little evidence in our system for soluble factors. Further IL-10 blocking Abs did not block MHC-II mediated inhibition, nor did we see evidence for downstream p-STAT3 activation (data not shown).

Our data indicate a requisite role for src-family PTKs in ERK activation, although Fc γ R γ and syk appear to be dispensable, implicating a potential role for additional MHC II-associated cytosolic adapters (DAP10 or DAP12) and/or other surface immunoreceptors. Indeed, dual involvement of both DAP12 and Fc γ R γ with a single immunoreceptor is not unprecedented, having been recently demonstrated for murine MAIR (47). Alternatively, *cis*-acting interactions between the MHC II signaling complex and SIRPs/Siglecs/integrins (48, 49) may instead contribute to ERK activation and inhibitory signaling.

The inhibitory phosphatase SHP-1 participates in inhibitory signaling as SHP-1 $^{-/-}$ DCs were relatively resistant to inhibition after MHC II cross-linking. Other inhibitory phosphatases, e.g., SHP-2, may also contribute, because inhibition was still partially preserved in SHP-1 $^{-/-}$ DCs. SHP-1 was recruited to caveolin-rich lipid microdomains within minutes after MHC II cross-linking. Recruitment of SHP-1 to lipid rafts was not seen in the absence of Fc γ R γ and was inhibited by pharmacological blockade of ERK phosphorylation, consistent with other reported ITAM-inhibitory signaling pathways (50) defining ERK activation as upstream of SHP-1 recruitment. Taken together, our data suggest the following model: MHC II cross-linking leads to ERK activation and ERK-dependent SHP-1 recruitment to Fc γ R γ in membrane raft microdomains.

Previous studies with MHC II signaling have demonstrated many similarities with ITAM-triggered pathways, yet the short cytosolic domains of the MHC II $\alpha\beta$ -chains lack canonical ITAMs. In DCs, the Fc γ R γ -chain subunit was detected physically associated with MHC II and its loss in Fc γ R $\gamma^{-/-}$ DCs impaired inhibitory responses and SHP-1 recruitment, identifying for the first time a biological role for Fc γ R γ in MHC II signaling. In B cells, the ITAM-containing adapters Ig α, β can associate with MHC II (28). These BCR-related ITAM adapters are not expressed in myeloid cells, which instead express FcR γ , DAP10, and DAP12. Surprisingly, MHC II chains lack a basic charged residue in their transmembrane domains, though required for the salt-bridge interactions between immunoreceptors and ITAM adapters expressing acidic residues in their corresponding transmembrane domains (51). However, this requirement may not be absolute and alternative immunoreceptor:ITAM interacting sites have been identified (52, 53) involving basic residues located in the membrane proximal cytosolic region, rather than in the immunoreceptor transmembrane domains.

In DCs and other myeloid cells, the concept of “paradoxical” ITAM-inhibitory signaling has gathered recent support (54). Engagement of several members of the Ig superfamily, including Fc γ RIII, Siglec-H, TREM-2, NKp44, PIR-A, or ILT-7 leads to impaired TLR-mediated activation through the participation of the ITAM-mediated adapters DAP12 (10, 55–59) or Fc γ R γ (60, 61), respectively. The mechanistic basis for activating vs inhibitory signaling remains unclear. It has been proposed that cellular activation results from high affinity ligands/high signal strength interactions while inhibitory responses result from low affinity ligands/low signal strength ligands, with the level of Fc γ R γ phosphorylation, determining downstream activatory or inhibitory outcome. However, high intensity signal strength is expected by both membrane bound LAG-3 and MHC II cross-linking, discounting the notion that low ligand:receptor signal strength is responsible for inhibitory signaling. Our data suggest a model in

which LAG-3/MHC II ITAM-mediated inhibition involves sequential activation of ERK and recruitment of SHP-1, similar to that proposed for Fc α R1 (50). We speculate that coengagement of MHC II by LAG-3 together with CD4 and TCR at the DC:Treg immunological synapse leads to selective recruitment of inhibitory signaling partners, including SHP-1, that would not otherwise be recruited by TCR and CD4 engagement alone.

Disclosures

The authors have no financial conflict of interest.

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