

Inducible MHC Class II Expression by Mast Cells Supports Effector and Regulatory T Cell Activation¹

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In addition to their well-established role as regulators of allergic response, recent evidence supports a role for mast cells in influencing the outcome of physiologic and pathologic T cell responses. One mechanism by which mast cells (MCs) influence T cell function is indirectly through secretion of various cytokines. It remains unclear, however, whether MCs can directly activate T cells through Ag presentation, as the expression of MHC class II by MCs has been controversial. In this report, we demonstrate that *in vitro* stimulation of mouse MCs with LPS and IFN- γ induces the expression of MHC class II and costimulatory molecules. Although freshly isolated peritoneal MCs do not express MHC class II, an *in vivo* inflammatory stimulus increases the number of MHC class II-positive MCs *in situ*. Expression of MHC class II granted MCs the ability to process and present Ags directly to T cells with preferential expansion of Ag-specific regulatory T cells over naive T cells. These data support the notion that, in the appropriate setting, MCs may regulate T cell responses through the direct presentation of Ag. *The Journal of Immunology*, 2009, 182: 4686–4695.

Mast cells (MCs)³ are tissue-resident cells of the immune system that are primarily located at the host–environment interface, making them one of the first cell types to encounter environmental threats. MCs were once believed to participate solely in allergy, owing to their abundant intracellular granules that are rapidly released upon cross-linking of their high affinity IgE receptor. However, the importance of MCs extends far beyond allergic disease, a notion that was initiated by the discovery that MCs are critical effectors in host defense against parasitic infections. Although the mechanisms are not fully understood, MCs contribute to protection against pathogens such as *Leishmania major* (1), *Giardia lamblia* (2), and intestinal helminths (3, 4).

MCs also play a pathologic role in the development of T cell-mediated hypersensitivity disorders such as delayed-type contact hypersensitivity (5, 6) and asthma (7, 8), and in the induction of autoimmune mouse models of inflammatory bowel disease (9) and multiple sclerosis (10, 11). T cells play a vital role in these mouse models, suggesting that MCs may influence T cell activation. In at least some of these models, a direct

correlation between the activation of T cells and the presence of MCs has been established (1, 12), as attenuated activation of T cells was observed in MC-deficient mice. The effect of MCs on T cell responses may also be inhibitory under certain circumstances, as MCs were recently shown to be vital for T cell-mediated skin allograft tolerance (13).

It has been previously suggested that MCs act as APCs and directly stimulate T cells. Both rodent (14, 15) and human (16, 17) MCs have been reported to constitutively express MHC class II (MHC-II), present Ag to T cell hybridomas, and initiate Ag-specific responses *in vivo*. Moreover, induction of costimulatory molecules CD80 and CD86 by treatment of MCs with GM-CSF has been observed (18). However, a follow-up study demonstrated that MHC-II is found only to a limited extent at the cell surface and resides mainly in intracellular lysosomal compartments (19). The initially described activation of Ag-specific T cell responses by MCs was later attributed to the release of immunologically active MC-derived exosomes because the activation of T cells still occurred by MHC haplotype-mismatched MCs (20). This understanding has led to re-examination of whether MHC-II are expressed at all in MCs, as we and others have demonstrated that resting or Fc ϵ RI-activated MCs do not express MHC-II on the cell surface or intracellularly (21, 22). We did find, however, that MCs can indirectly promote T cell activation by internalizing Ags through Fc ϵ RI, undergoing apoptosis, and subsequently providing Ags to other professional APCs (22).

In this report, we extend our analysis of how MCs may regulate T cell responses under specific conditions. We demonstrate that although MHC-II is not detected on resting MCs, stimulation of MCs with LPS and IFN- γ induces robust expression of MHC-II. The expression of MHC-II conferred MCs the ability to process and present Ags directly to previously activated CD4⁺ T cells and to a limited extent to naive CD4⁺ T cells. Furthermore, we show that MCs preferentially expand Ag-specific regulatory T cells (Tregs) over naive T cells, possibly shedding light on one of the mechanisms that governs allograft tolerance induction by MCs. These data suggest that one of the mechanisms by which MCs

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³ Abbreviations used in this paper: MC, mast cell; MHC-II, MHC class II; Treg, regulatory T cell; PD-L1, programmed death ligand 1; BMMC, bone marrow-derived MC; DC, dendritic cell; LN, lymph node; HA, hemagglutinin; TNP, trinitrophenyl; WT, wild type.

regulate T cell responses could be through the direct presentation of Ag.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, MHCII^{-/-}, RAG^{-/-}, and H-2DM^{-/-} mice were obtained from The Jackson Laboratory and bred in the animal care facility at the University of Pennsylvania. OT-II.2a/Rag1 mice (mouse line 4234, Taconic Emerging Model) were obtained through the National Institute of Allergy and Infectious Diseases Exchange Program (23, 24). MyD88-deficient (MyD88^{-/-}) and B6-E α (25) mice were gifts from Dr. S. Akira and Dr. R. A. Flavell, respectively. TS1, TS1 X HA28, and H2E mice were bred and maintained as previously described (26). All animal care and work was in accordance with national and institutional guidelines and the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Chemicals and tissue culture reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. All cytokines and cell culture reagents were purchased from Pepro-Tech and Invitrogen, respectively. The α -chain of I-E^d (aa 46–74) inserted into pTrcHis2-TOPO vector in frame with red fluorescence protein was provided by Dr. M. K. Jenkins (27). E α -red fluorescence fusion protein was purified from bacterial lysate and trinitrophenyl (TNP)-conjugated with picrylsulfonic acid (pH 8.5) overnight at 4°C and removing excess TNP-conjugated with picrylsulfonic acid by size exclusion columns (GE Healthcare).

Flow cytometry

All Abs used for flow cytometry were purchased from BD Biosciences except for YAc and anti-Fc ϵ RI (eBioscience). Anti-MHC-II Ab (clone Y3P) was purified from HB183 hybridoma supernatant and FITC-conjugated. Biotinylated 6.5 TCR clonotype-specific Ab against TS1 T cells was previously described (26). Cells were blocked with anti-CD16/32 Ab, stained with specified Abs (anti-CD117-allophycocyanin, anti-Fc ϵ RI-PE, anti-IA^b-biotin (clone KH74), anti-CD4-allophycocyanin, anti-CD69-PE, anti-CD80-biotin, anti-CD86-biotin, anti-programmed death ligand 1 (PD-L1)-PE, anti-PD-L2-PE, YAc-biotin) followed by streptavidin-PE or streptavidin-allophycocyanin when using biotinylated Abs. The fluorescence intensity was measured on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using Cell Quest (BD Biosciences) or FlowJo software (Tree Star).

Generation of bone marrow-derived MCs (BMMCs), spleen-derived MCs, peritoneal MCs, and bone marrow-derived dendritic cells (DCs)

To generate MCs (28), spleen or bone marrow cells of mice were cultured in MC medium (RPMI 1640, 15% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2.9 mg/ml glutamine, 50 mM 2-ME, 1 mM sodium pyruvate, 1X nonessential amino acids, 10 mM HEPES) containing IL-3 (10 ng/ml) and stem cell factor (12.5 ng/ml) for 6–8 wk, replenishing with fresh medium twice weekly, and used when >95% of cells expressed high homogeneous levels of Fc ϵ RI and CD117. Bone marrow-derived DCs (29) were generated by culturing bone marrow cells in DC medium (DMEM, 15% FBS, penicillin, streptomycin, glutamine) containing IL-4 (10 ng/ml) and GM-CSF (10 ng/ml) for 7 days, and purified by magnetic cell sorting (MACS) using CD11c beads (Miltenyi Biotec). Peritoneal MCs were obtained by peritoneal lavage of mice using 10 ml of PBS containing 2 mM EDTA.

MC stimulation and RT-PCR analysis

MCs were stimulated with LPS (*Escherichia coli* O127:B8) and/or IFN- γ in 96-well U-bottom plates in MC medium containing IL-3 (10 ng/ml), and the expression of surface molecules was measured on CD117⁺Fc ϵ RI⁺ MCs by flow cytometry. For Ag processing experiments, MCs were stimulated with LPS/IFN- γ for 72 h in the presence or absence of TNP-conjugated E α (TNP-E α) protein (50 μ g/ml), E α _{52–66} peptide (ASFEAQGA LANIAVDKA), or anti-TNP IgG1 (10 μ g/ml). In some experiments the MCs were pretreated with anti-TNP IgE (1 μ g/ml) for 24 h before adding the TNP-E α protein.

For RT-PCR analysis, MCs were FACS-sorted by Moflo cell sorter (DakoCytomation) using CD117 and Fc ϵ RI Abs. Cells were stimulated with or without LPS (10 μ g/ml) and IFN- γ (10 ng/ml) for 24 h in MC medium containing IL-3 and washed, and RNA was extracted using

RNEasy kit (Qiagen). RT-PCR was performed using OneStep RT-PCR kit (Qiagen). The following primer sets were used: IA^b- α (sense) 5'-GAA GACGACATTGAGGCCGACCACG-3', (antisense) 5'-TAAAGGCCCG TGGGTGCTCTGGAGGTG-3' (product size: 748 bp) (30); IA^b- β (sense) 5'-GCGACGTGGGCGAGTACC-3', (antisense) 5'-CATTCCGGAACC AGCGCA-3' (product size: 220 bp) (31); H-2DM α (sense) 5'-AAGGTAT GGAGCATGAGCAGAAGT-3', (antisense) 5'-GATCAGTCACCTGAG CACGGT-3' (product size: 768 bp) (32); H-2DM β (sense) 5'-TGAATT TGGGGTGTCTGTATCC-3', (antisense) 5'-TGCTGAACCACGCAGGTG TAG-3' (product size: 395 bp) (30); CIITA (sense) 5'-TGCAGGCGACCA GGAGAGACA-3', (antisense) 5'-GAAGCTGGGCACCTCAAAGAT-3' (product size: 488 bp); IL-3 (sense) 5'-ATAGGGAAGCTCCCAGAACC TGAAGTC-3', (antisense) 5'-AGACCCCTGGCAGCGCAGAGTCA TTC-3' (product size: 206 bp) (33); and β -actin (sense) 5'-TTCTTTGCA GCTCCTTCGTTGCCG-3', (antisense) 5'-TGGATGGCTACGTACATGG CTGGG-3' (product size: 450 bp).

MC, DC, T cell cocultures

A total of 1×10^5 spleen-derived MCs were stimulated with LPS (10 μ g/ml) and IFN- γ (10 ng/ml) in MC medium containing IL-3 (10 ng/ml) in the presence or absence of OVA protein (grade V), live or heat-inactivated influenza PR8 or J1 virus (1/1000 titer), or OVA peptide (ISQAVHAAHAEINEAGR) or influenza S1 peptide (SFERFEIFPK) for 72 h in 96-well U-bottom plates. Heat-inactivation of influenza virus was performed by incubating the virus at 56°C for 30 min. The peptides were added to the MCs 48 h after LPS stimulation. After extensive washing, the MCs were cocultured in 96-well U-bottom plates with T cells in MC medium containing IL-3. A total of 1×10^5 FACS-sorted Thy1.2⁺ T cells from OT-II, TS1, or TS1/HA28 mice were used as a source of T cells. In some experiments, the CD4⁺CD25⁺ and CD4⁺CD25⁻ fraction of TS1/HA28 T cells were FACS-sorted before coculture. For proliferation assays, the TS1 and TS1/HA28 T cells were prelabeled with CFSE. To obtain Ag-experienced cells, OT-2 T cells were expanded for 6 days by culturing OT-2 spleen cells with OVA peptide (1 μ M). In experiments involving DCs, 5×10^4 bone marrow-derived DCs pulsed with OVA peptide were added to the T cells. For CD69 expression, the cocultures were incubated for 48 h. For detection of IFN- γ , the cocultures were incubated for 6 h in the presence of brefeldin A (10 μ M) and intracellularly stained with anti-IFN- γ PE Ab (BD Pharmingen). Proliferation of T cells was analyzed by CFSE dilution 4 days after coculture. When measuring the proliferation of TS1/HA28 Tregs, cells were stained with anti-CD4-PerCP Cy5.5 and 6.5-biotin Ab, followed by streptavidin-PE, and intracellularly stained for Foxp3 using the mouse Treg staining kit (eBioscience), and visualized by flow cytometry.

LPS and L. major inoculation of mice

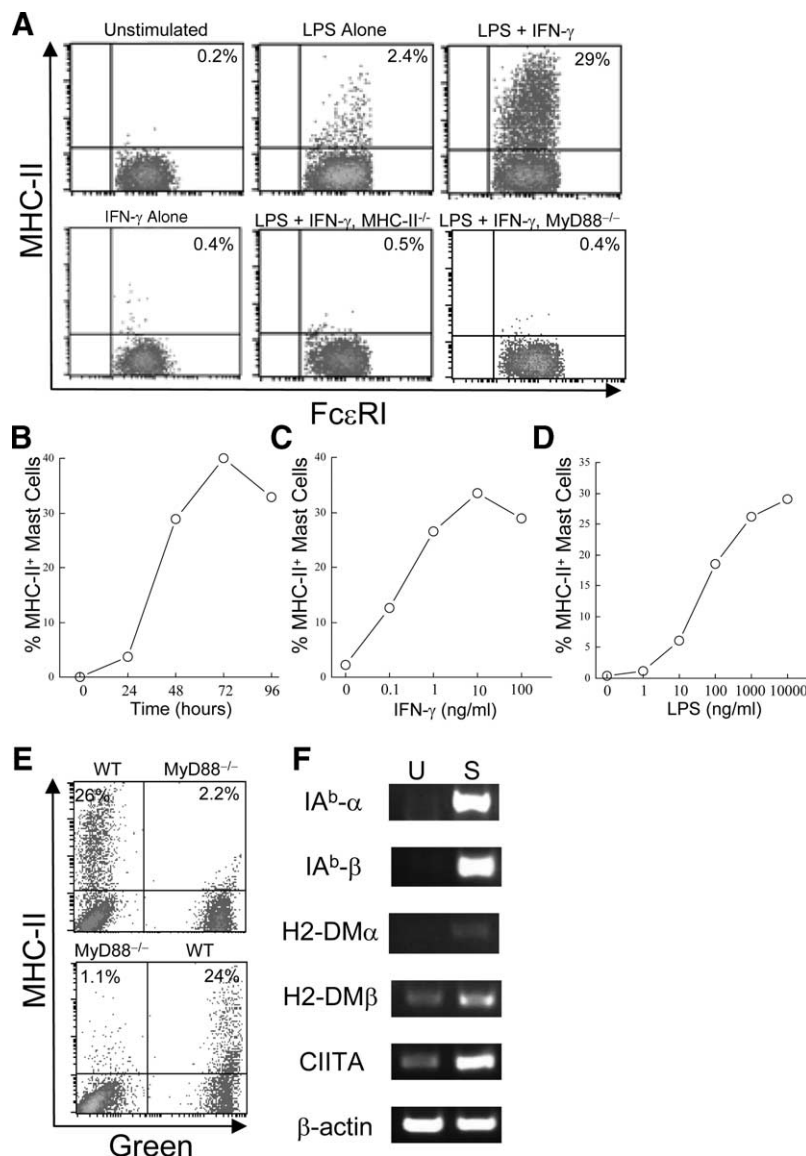
C57BL/6 and RAG^{-/-} mice were injected s.c. in both flanks with 25 μ g/flank of LPS (*S. minnesota* R595 Re platform; AXXORA) in 100 μ l of PBS. Bilateral inguinal lymph nodes (LNs) were harvested at various time points postinjection with LPS, and Fc ϵ RI⁺CD117⁺ MCs were enumerated and analyzed for MHC-II, CD80, CD86, PD-L1, and PD-L2 expression by flow cytometry. *Leishmania* infection was performed by inoculation C57BL/6 mice s.c. in the right hind footpad with 2×10^6 late stationary phase *L. major* promastigotes. At 7 days postinfection, the popliteal LNs ipsilateral and contralateral to the site of infection were harvested. Sections were prepared from LNs fixed in 10% formalin, mounted on glass slides, and stained with toluidine blue to visualize MCs. Representative microscope images were obtained using a Leica DMLB microscope equipped with a SPOT Insight color camera (Diagnostic Instruments) and incorporated using Photoshop computer software (Adobe Systems). Statistical analysis was performed by ANOVA using Microsoft Excel computer software.

Results

Induction of MHC-II on MCs by stimulation with LPS/IFN- γ

BMMCs do not express MHC-II constitutively or after stimulation through Fc ϵ RI (21, 22). Despite lack of MHC-II expression, we previously demonstrated that BMMCs stimulate Ag-specific CD4⁺ T cell responses in an MHC II-independent manner by incorporating Ags through Fc ϵ RI and transferring them to DCs (22). In follow-up studies, we observed experiment-to-experiment variation within our T cell/MC coculture assays. We mapped this

FIGURE 1. LPS and IFN- γ stimulation induces MHC-II expression by MCs. **A**, WT B6 BMMCs were left unstimulated (*top left*) or stimulated with LPS (10 μ g/ml) (*top middle*), LPS plus IFN- γ (10 ng/ml) (*top right*), or IFN- γ alone (*bottom left*). MHC-II $^{-/-}$ (*bottom middle*) or MyD88 $^{-/-}$ (*bottom right*) BMMCs were treated with LPS plus IFN- γ . At 72 h later, MHC-II expression was measured by flow cytometry on Fc ϵ RI $^{+}$ BMMCs. **B**, WT B6 BMMCs were treated with LPS plus IFN- γ . The fraction of MHC-II-expressing BMMCs was assessed and plotted against time or plotted against various concentrations of IFN- γ (with fixed LPS at 10 μ g/ml) (**C**) or LPS (with fixed IFN- γ at 10 ng/ml) (**D**) at 72 h poststimulation. **E**, WT B6 and MyD88 $^{-/-}$ BMMCs were cocultured and treated with LPS + IFN- γ . The BMMCs were distinguished from each other by labeling either the MyD88 $^{-/-}$ (*top*) or WT (*bottom*) with CFSE (green). MHC-II expression was measured 72 h poststimulation on Fc ϵ RI $^{+}$ BMMCs. **F**, RNA was extracted from Fc ϵ RI $^{+}$ CD117 $^{+}$ FACS-sorted B6 BMMCs stimulated with (S) or without (U) LPS plus IFN- γ for 24 h. The presence of IA ^{b} - α , IA ^{b} - β , H2-DM α , H2-DM β , CIITA, and β -actin transcripts were detected by RT-PCR. All results are representative of at least two independent experiments.



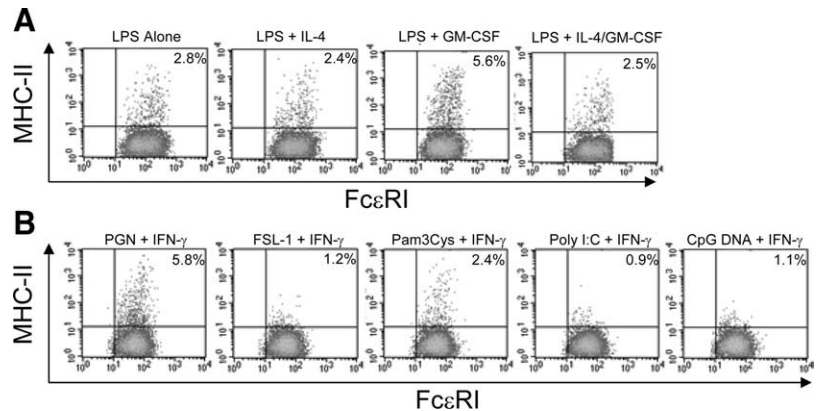
variability to commercial sources of Ag and found that endotoxin-contaminated Ag increased the activation of T cells in an Fc ϵ RI-independent manner, suggesting that endotoxin might influence the way MCs present Ag. To test this notion, we stimulated BMMCs with LPS and found that a fraction of BMMCs expressed MHC-II, a response that was potentiated dramatically by the addition of IFN- γ (Fig. 1A). Bona fide MHC-II expression was confirmed with a second anti-MHC-II Ab (clone Y3P, data not shown) and by the failure to observe this effect in BMMCs lacking the MHC-II gene (Fig. 1A). The induction of MHC-II expression was observed maximally at 72 h poststimulation (Fig. 1B) and occurred at physiological concentrations of both LPS ($\sim$ 10 ng/ml) and IFN- γ ($\sim$ 0.1 ng/ml) (Fig. 1, C and D). Of note, the addition of polymyxin B to LPS/IFN- γ -treated MCs completely abrogated MHC-II up-regulation, suggesting that the effect was not secondary to potential contaminants in our LPS preparation.

Signaling through TLR4 by LPS occurs through two distinct pathways that involve either MyD88 or Toll/IL-1R domain-containing adapter inducing IFN- β (34). In BMMCs, the induction of MHC-II required activation of the MyD88-dependent signaling pathway by LPS because MHC-II expression was not detected on wild type (WT) MCs stimulated with IFN- γ alone or on

MyD88 $^{-/-}$ MCs treated with LPS/IFN- γ (Fig. 1A). One potential explanation for the LPS effect on MHC-II induction is that LPS was indirectly stimulating MHC-II expression via the elaboration of cytokines. To test this possibility, MyD88 $^{-/-}$ and WT BMMCs were labeled with dye (CFSE) to distinguish their genotypes, and cocultured and stimulated with LPS/IFN- γ . MHC-II expression was observed only on WT MCs (Fig. 1E), suggesting that the induction of MHC-II occurred through direct TLR4 stimulation of the MCs. Because IL-1 and IL-18 also signal through MyD88 and LPS-stimulated MCs produce IL-1 β (35), it was still possible that these cytokines contributed to MHC-II expression by MCs. However, neutralization of IL-1 β and IL-18 did not have any effect on LPS/IFN- γ -stimulated MHC-II expression by MCs. Moreover, the addition of IL-1 β and IL-18 failed to induce MHC-II or to enhance LPS/IFN- γ -stimulated MHC-II expression, ruling out the necessity and sufficiency of these cytokines in the induction of MHC-II in BMMCs (data not shown).

To extend our studies on the induction of MHC-II expression and to test whether other MHC-II-associated molecules necessary for Ag presentation were expressed in BMMCs upon LPS/IFN- γ stimulation, RT-PCR analysis was performed. LPS/IFN- γ stimulation of BMMCs induced mRNA expression of MHC-II chains

FIGURE 2. Effects of other cytokines and TLR stimuli on MHC-II expression by MCs. *A*, BMMCs were stimulated with LPS (10 μ g/ml), LPS + IL-4 (10 ng/ml), LPS + GM-CSF (10 ng/ml), or LPS + IL-4 + GM-CSF (plots from left to right). *B*, BMMCs were stimulated with IFN- γ (10 ng/ml) and peptidoglycan (10 μ g/ml), FSL-1 (TLR2/6 agonist, 100 ng/ml), Pam₃Cys (TLR1/2 agonist, 300 ng/ml), polyinosinic-polycytidylic acid (poly(I:C), 10 μ g/ml), and CpG DNA (1 μ M). At 72 h later, MHC-II expression was measured by flow cytometry on Fc ϵ R1⁺ BMMCs. Results are representative of two independent experiments.



IA^b- α and IA^b- β (Fig. 1*F*), suggesting that surface expression of MHC-II was due to de novo synthesis of MHC-II rather than re-localization of internal stores. H2-DM, which is required for efficient peptide exchange on MHC-II (36), and CIITA, the master regulator of MHC-II and MHC-II-associated genes, were also up-regulated by LPS/IFN- γ stimulation (Fig. 1*F*), indicating that LPS/IFN- γ -stimulated BMMCs possess the necessary molecules to present Ags on MHC-II.

We next tested whether other stimuli could up-regulate MHC-II on MCs. Because enhancement of MHC-II expression by IL-4 and GM-CSF was shown in other cell types (37, 38), we tested the ability of these cytokines to enhance the effects of LPS on BMMCs. Unlike IFN- γ , IL-4 was incapable of increasing MHC-II expression (Fig. 2*A*). Moreover, GM-CSF had only a limited effect on MHC-II expression compared with IFN- γ . MCs have been reported to express other TLRs including TLR2, TLR3, TLR5, TLR7, and TLR9 (39). Among the TLR stimuli tested (TLR2, TLR3, TLR9), only TLR1/2 stimulation (peptidoglycan and Pam₃Cys) showed increased MHC-II expression on MCs, albeit to a lesser extent than observed with TLR4 stimulation (Fig. 2*B*). These data suggest that maximal induction of MHC-II expression by MCs occurs by signaling through TLR4 and IFN- γ receptors.

MCs are poor stimulators of naive CD4⁺ T cells

To examine whether MHC-II-bearing MCs stimulate naive CD4⁺ T cells, OVA peptide-pulsed MHC-II-expressing MCs were cocultured with FACS-sorted naive T cells from OVA peptide-specific TCR transgenic (OT-2) mice. Spleen-derived MCs were used in these experiments because a larger proportion of spleen-derived MCs (50–60%) express MHC-II compared with BMMCs (20–30%) when stimulated with LPS/IFN- γ . Upon coculture with OVA peptide-pulsed DCs, OT-2 T cells were strongly activated as measured by CD69 expression. In contrast, coculture of OT-2 T cells with peptide-pulsed MCs showed no effect above background (Fig. 3*A*). To test whether the lack of naive T cell activation by MCs was due to defective peptide binding by MHC-II, we used two approaches: first, binding of biotinylated OVA peptide, and second, staining with an MHC-II-peptide conformation-specific Ab known as YAE, which specifically recognizes MHC-II (I-A^b) bound to a peptide derived from the α -chain of the I-E molecule (E α). With both approaches, peptide binding was detected on LPS/IFN- γ -stimulated WT but not MHCII^{-/-} MCs (Fig. 3, *B* and *C*), suggesting that MHC-II on MCs bind to peptides.

Naive CD4⁺ T cell activation not only requires TCR activation by cognate MHC-II-peptide complexes but also is dependent on costimulatory signals provided by the APC. Therefore,

we examined the expression of several B7 family costimulatory molecules on MCs, using B cells as a positive control. In contrast to B cells, little to no expression of CD80 or CD86 was observed on either resting or LPS/IFN- γ -stimulated MCs (Fig. 3, *D* and *E*). MHC-II expression was also lower on MCs compared with B cells. In addition, compared with B cells, MCs constitutively expressed higher levels of the inhibitory B7 family member PD-L1, which was up-regulated further by LPS/IFN- γ (Fig. 3, *D* and *E*). The expression pattern of costimulatory molecules by MCs could potentially explain the lack of naive T cell activation, despite proper peptide loading of MHC-II on MCs.

MCs restimulate previously activated CD4⁺ T cells

We next examined whether MCs could restimulate Ag-experienced T cells because previously activated cells do not require the same costimulatory signals as naive cells. Peptide-pulsed WT but not MHCII^{-/-} MCs induced IFN- γ production by previously activated OT-2 T cells (Fig. 4*A*), suggesting that MCs could participate in the reactivation of Ag-experienced T cells. However, OVA protein-treated MCs failed to stimulate IFN- γ production by these T cells, suggesting that MCs may lack the ability to process whole Ags and present them on MHC-II.

To test Ag-processing and Ag-presenting ability, specific MHC-II-peptide complexes on TNP-E α protein-treated MCs were examined. YAE staining was detected on TNP-E α -treated WT but not MHCII^{-/-} MCs in an H-2DM-dependent manner (Fig. 4, *B* and *C*), suggesting that MCs were able to process and present protein Ags on MHC-II. To examine whether Ag uptake through Fc receptors would positively impact Ag-processing and -presenting ability, TNP-E α was incorporated into MCs by TNP-specific IgG1 or IgE. Neither YAE staining intensity nor percent-positive fraction increased through Ag incorporation by TNP-specific IgG1 or IgE (Fig. 4*B*), suggesting that internalization by receptor-mediated endocytosis may divert the Ag to compartments that are distinct from macropinocytosis. Endogenously derived proteins were also processed and presented on MCs because YAE staining was detected on LPS/IFN- γ -stimulated MCs derived from mice expressing the E α -transgene (B6-E α) without the addition of exogenous TNP-E α protein (Fig. 5*A*). The MHC-II-E α peptide complexes were derived from an endogenous source in B6-E α MCs because WT MCs mixed with B6-E α MCs did not stain with YAE Ab (Fig. 5*B*).

MCs preferentially activate Tregs

To explore whether MCs could activate other subsets of CD4⁺ T cells, we tested the ability of MCs to activate Ag-specific Tregs because MCs were recently implicated in potentiating allograft tolerance through interaction with Tregs (13). To obtain a large

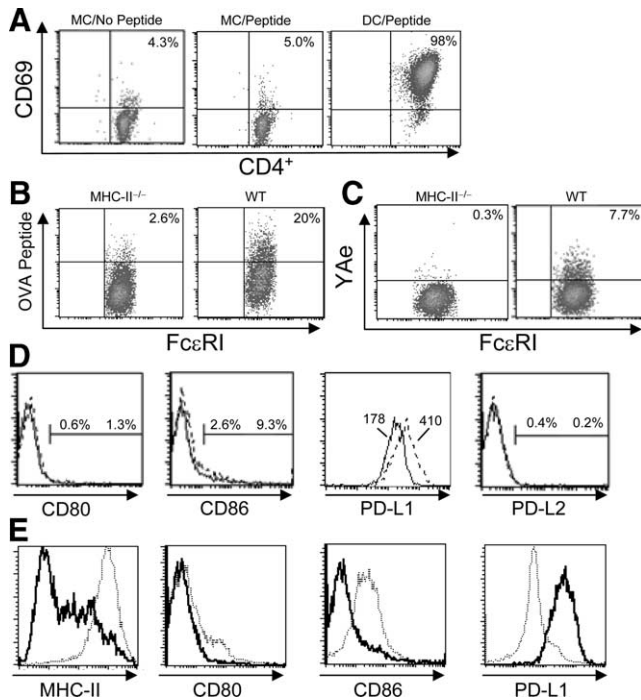


FIGURE 3. LPS/IFN- γ -stimulated MCs cannot support naive T cell proliferation and poorly express costimulatory molecules. *A*, B6 MCs were stimulated with LPS/IFN- γ for 72 h. For the last 24 h, the MCs were pulsed without (*left*) or with (*middle*) OVA peptide (10 μ M). FACS-sorted OT-2 T cells were cocultured with the MCs or with peptide-pulsed BMDCs (*right*) for 48 h. CD69 expression on CD4⁺ T cells was analyzed by flow cytometry. *B*, MHC-II^{-/-} (*left*) or WT B6 (*right*) MCs were stimulated with LPS/IFN- γ for 72 h and pulsed with biotin-OVA peptide (10 μ M) or E α peptide (10 μ M) (*C*) for the last 24 h. Biotin peptide and E α peptide binding were detected by streptavidin-PE and YAc Ab staining, respectively, by flow cytometry. *D*, WT B6 MCs were stimulated with (dotted histogram) or without (solid histogram) LPS/IFN- γ for 72 h and CD80, CD86, PD-L1, and PD-L2 (*left to right*) were analyzed by flow cytometry. The number represents the percentage of costimulatory molecule positive MCs or mean fluorescence intensity (for PD-L1 only) treated with (number on right) or without (number on left) LPS/IFN- γ . *E*, LPS/IFN- γ -treated B6 MCs (solid histogram) and freshly isolated splenic B6 B cells (dotted histogram) were compared for expression of MHC-II, CD80, CD86, and PD-L1 (*left to right*) by flow cytometry. All results are representative of at least two independent experiments.

number of Ag-specific Tregs, we used TS1 X HA28 mice, which express the influenza virus PR8 hemagglutinin (HA) protein as a neo-self peptide and coexpress the TS1 TCR that is specific for the PR8 HA determinant S1. Tregs (CD4⁺CD25⁺Foxp3⁺) comprise ~50% of all HA-specific CD4⁺ T cells from TS1 X HA28 mice (26). When FACS-sorted TS1 X HA28 CD4⁺ T cells were cocultured with influenza peptide-pulsed splenocytes, a similar proportion of proliferating Foxp3⁺ and Foxp3⁻ CD4⁺ T cells was observed (Fig. 6A). In contrast, proliferation of TS1 X HA28 CD4⁺ T cells was heavily skewed toward the Foxp3⁺ fraction after coculture with influenza peptide-pulsed LPS/IFN- γ -stimulated MCs. A similar effect was observed when the LPS/IFN- γ -stimulated MCs were pretreated with live or heat-inactivated PR8 virus but not with influenza virus (J1) lacking the S1 epitope, suggesting that intact influenza-derived proteins could be processed and presented to TS1/HA28 Tregs by MCs (Fig. 6B). Endogenously derived HA protein was also presented by MCs because LPS/IFN- γ -stimulated MCs derived from HACII mice, which express full-length HA protein driven by the I-E α promoter, stimulated the proliferation of TS1 X HA28 Tregs (Fig. 6C).

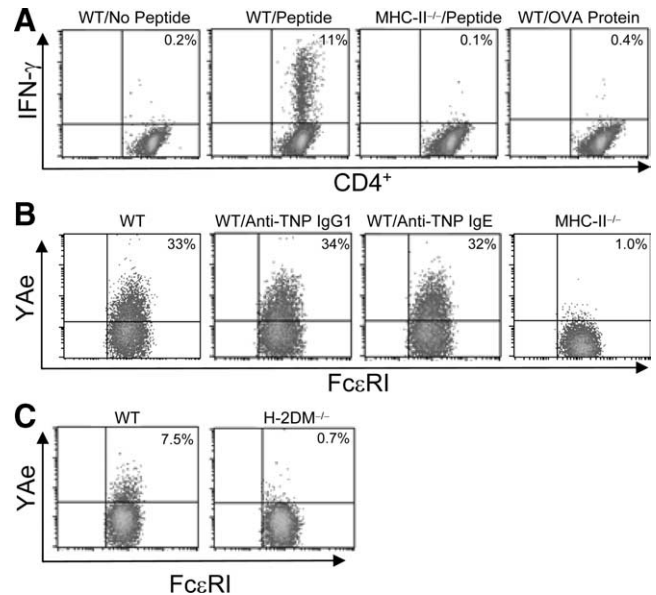


FIGURE 4. Peptide-pulsed but not protein-pulsed MCs support stimulation of previously activated T cells. *A*, WT B6 (first and second plots) or MHC-II^{-/-} (third plot) MCs were stimulated with LPS/IFN- γ for 72 h and pulsed with or without (*left*) OVA peptide (10 μ M) for the last 24 h. Some of the WT MCs were treated with LPS/IFN- γ and OVA protein (50 μ g/ml) for 72 h (*right*). The MCs were washed and cocultured with previously activated OT-2 T cells for 6 h, and IFN- γ production was measured by intracellular staining and flow cytometry. *B*, WT B6 (left three plots) or MHC-II^{-/-} (fourth plot) MCs were stimulated with LPS/IFN- γ and TNP-E α protein (50 μ g/ml) for 72 h. Some of the WT MCs were cotreated with anti-TNP IgG1 (20 μ g/ml, second plot) or pretreated with anti-TNP IgE (1 μ g/ml for 24 h, third plot). *C*, WT B6 (*left*) or H-2DM^{-/-} (*right*) MCs were stimulated with LPS/IFN- γ for 72 h and TNP-E α protein. All results are representative of at least two independent experiments.

The expansion of Tregs by MCs could have resulted from the induction of Foxp3 in previously Foxp3⁻ T cells. To test this possibility, MCs were cocultured with TS1 X HA28 T cells that had been sorted into CD25⁺ and CD25⁻ fractions, which correlated well with Foxp3 expression (data not shown). MCs did not induce Foxp3 expression in Foxp3⁻ T cells because Foxp3⁺ T cell proliferation was only observed with FACS-sorted CD25⁺ TS1 X HA28 T cells but not with FACS-sorted CD25⁻ T cells (Fig. 6D).

MCs were able to stimulate the proliferation of isolated Foxp3⁻ T cells from TS1 X HA28 mice (Fig. 6D), although MCs preferentially stimulated Foxp3⁺ T cells over Foxp3⁻ T cells when both subsets were present in the same culture. In the latter situation,

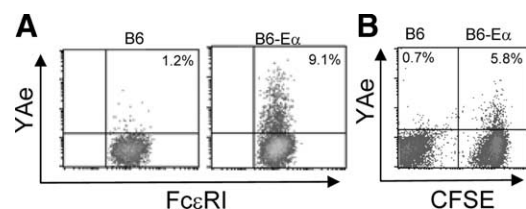
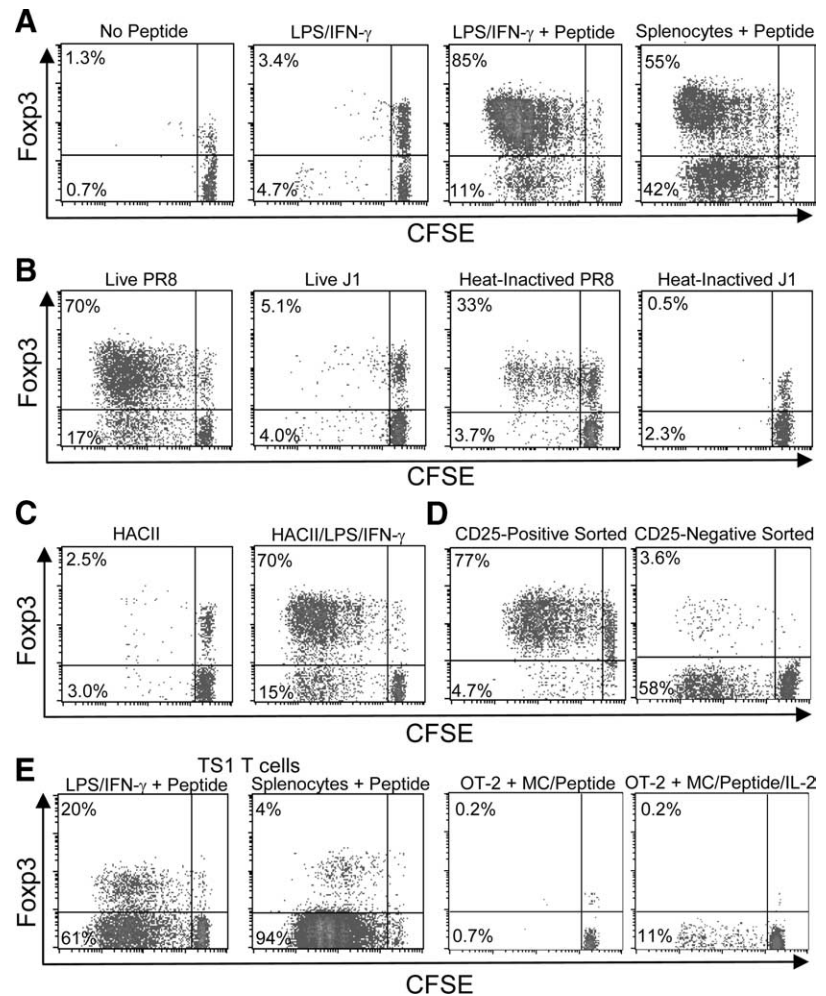


FIGURE 5. Endogenous E α protein is presented on MHC-II by MCs. *A*, WT B6 (*left*) or B6-E α (*right*) transgenic MCs were stimulated with LPS/IFN- γ for 72 h. MHC-II-E α peptide complexes were detected on Fc ϵ RI⁺ BMMCs by YAc staining and flow cytometry. *B*, WT B6 and B6-E α BMMCs were cocultured and treated with LPS plus IFN- γ . The BMMCs were distinguished from each other by labeling the B6-E α BMMCs with CFSE. MHC-II-E α peptide complexes were measured 72 h poststimulation on Fc ϵ RI⁺ BMMCs. Results are representative of two independent experiments.

FIGURE 6. MCs preferentially expand regulatory T cells over naive T cells. *A*, WT BALB/c MCs were stimulated with or without (*left*) LPS/IFN- γ for 72 h and pulsed with (third plot) or without (second plot) S1 peptide (10 μ M) for the last 24 h. CFSE-labeled FACS-sorted TS1 X HA28 T cells were cocultured with the MCs or with irradiated BALB/c splenocytes and S1 peptide (*right*) for 4 days. *B*, WT BALB/c MCs were stimulated with LPS/IFN- γ and live PR8, live J1, heat-inactivated PR8, or heat-inactivated J1 virus (*left to right*) for 72 h and cocultured with CFSE-labeled FACS-sorted TS1 X HA28 T cells for 4 days. *C*, HACII MCs were stimulated with (*right*) or without (*left*) LPS/IFN- γ for 72 h and cocultured with CFSE-labeled FACS-sorted TS1 X HA28 T cells for 4 days. *D*, WT BALB/c MCs were stimulated with LPS/IFN- γ and live PR8 for 72 h and cocultured with CFSE-labeled FACS-sorted CD4⁺CD25⁺ (*right*) or CD4⁺CD25⁻ (*left*) TS1 X HA28 T cells for 4 days. *E*, WT BALB/c MCs were stimulated with LPS/IFN- γ for 72 h and pulsed with S1 peptide (10 μ M). CFSE-labeled FACS-sorted Thy1.2⁺ TS1 T cells were cocultured with the MCs (*left*) or with irradiated BALB/c splenocytes and S1 peptide for 4 days (*right*). *F*, WT B6 MCs were stimulated with LPS/IFN- γ for 72 h and pulsed with OVA peptide (10 μ M). CFSE-labeled FACS-sorted Thy1.2⁺ OT-2 T cells were cocultured with the MCs with (*left*) or without IL-2 (50 U/ml) (*right*) for 4 days. The proliferation of FoxP3⁺ and FoxP3⁻ CD4⁺ T cells was analyzed by CFSE dilution and flow cytometry. *A–E* and *F* are gated on CD4⁺6.5⁺ T cells (TS1 TCR clonotype-positive T cells) and CD4⁺ T cells, respectively. All results are representative of at least two independent experiments.



Treg-mediated active suppression exerted on Foxp3⁺ T cells may be contributing. Indeed, there was an ~2-fold increase in the proportion of dead Foxp3⁺ T cells among TS1 X HA28 T cells cultured with MCs compared with those cultured with irradiated splenocytes. Furthermore, MCs induced the proliferation of Foxp3⁺ naive T cells from TS1 mice, which lack the neo-self HA and are much less enriched for clonotypic Foxp3⁺ T cells (Fig. 6E). These results differ from experiments using OT-2 T cells, which showed no proliferation of naive T cells by MCs (Fig. 6F). However, the proliferation of OT-2 T cells could be slightly induced by MCs in the presence of IL-2 (Fig. 6F). It is possible that the activation of the TS-1 TCR may be less stringent than OT-2 T cells, due to differences in TCR affinity for their cognate Ags. Therefore, depending on the TCR expressed by the T cell, MCs may also be able to prime naive T cells.

LN-localized MCs increase upon inflammation and express MHC-II and costimulatory molecules

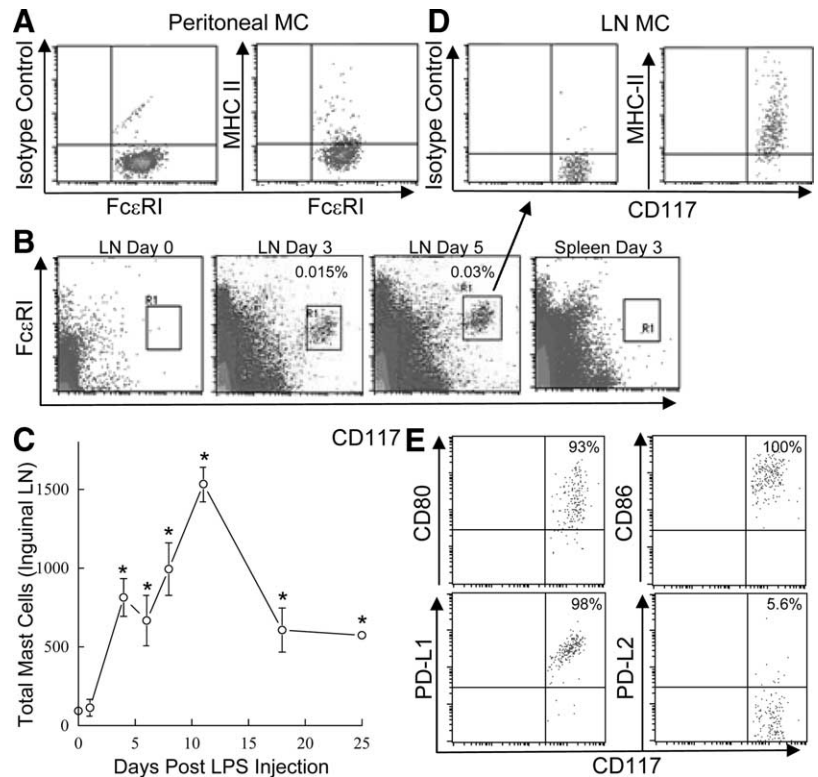
We next asked whether MCs expressed MHC-II in vivo. Similar to cultured MCs, freshly isolated peritoneal MCs were virtually devoid of MHC-II expression (Fig. 7A). We predicted that peritoneal MCs might express MHC-II if stimulated by LPS. However, when mice were injected i.p. with LPS, MCs were no longer recovered from the peritoneal cavity (data not shown), suggesting that MCs might have migrated from the peritoneal cavity to secondary lymphoid organs upon TLR stimulation. To test this possibility, mice were treated with LPS s.c., and the draining LNs were examined for the presence of MCs. Although only few MCs could be seen

residing in the LNs of unchallenged mice, the number of MCs significantly increased after LPS injection peaking at ~11 days postchallenge with LPS (Fig. 7C). The increase in MCs was specific to LNs because no increase in MC numbers was observed in the spleen (Fig. 7B). All LN-localized MCs expressed MHC-II and PD-L1 (Fig. 7, D and E). Moreover, the positive costimulatory B7 family members CD80 and CD86 were also expressed on LN MCs.

The few MCs in the LNs of unchallenged mice localized to the subcapsular and trabecular sinuses (Fig. 8A). Because it was difficult to examine MC localization due to massive B cell hyperplasia and consequent distortion of LN architecture after LPS-challenge, LNs from LPS-challenged RAG^{-/-} mice were analyzed for the presence of MCs. LN architecture was preserved after LPS challenge of RAG^{-/-} mice, and although there was an increase in MC numbers, the localization of MCs was unchanged compared with unchallenged RAG^{-/-} mice (Fig. 8B).

We next tested whether a more physiologic inflammatory stimulus provided by a pathogen would yield similar results to that of LPS. *L. major* was chosen because the cutaneous infection remains localized with defined lymphatic drainage. Therefore, mice were challenged with *L. major* s.c. in one footpad, and the draining popliteal LNs were examined for the presence of MCs. Similar to the findings after treatment with LPS, a significant increase in MC numbers was found in ipsilateral LNs compared with LNs contralateral to the infected footpad (Fig. 8C). Again, localization of MCs was restricted to LN sinuses (Fig. 8D). Collectively, these data suggest that upon inflammation, MCs accumulate in draining

FIGURE 7. LPS increases numbers of LN MCs that express MHC-II and costimulatory molecules. **A**, Freshly isolated peritoneal MCs from B6 mice were stained with isotype control (mouse IgG2a) (*left*) or MHC-II Ab (clone Y3P) (*right*), and analyzed by flow cytometry. Plots are gated on $CD117^+Fc\epsilon RI^+$ MCs. **B**, B6 mice were injected s.c. with LPS (25 μ g/flank), and the inguinal LNs were harvested 0, 3, or 5 days postinoculation (*left to right*). Spleen cells were harvested on day 3 postinoculation (*right*). The cells were analyzed for the presence of $CD117^+Fc\epsilon RI^+$ MCs by flow cytometry. **C**, Total number of $CD117^+Fc\epsilon RI^+$ MCs enumerated in inguinal LNs at various days postinoculation with s.c. LPS of B6 mice ($n = 3$ mice/time point). *, $p < 0.01$ indicates significance compared with MC numbers on day 0 by ANOVA. **D**, LN $CD117^+Fc\epsilon RI^+$ MCs from B6 mice 5 days after s.c. challenge with LPS were stained with isotype control or anti-MHC-II Ab and analyzed by flow cytometry. **E**, LN $CD117^+Fc\epsilon RI^+$ MCs from B6 mice 11 days after challenge with s.c. LPS were analyzed for expression of CD80 (*top left*), CD86 (*top right*), PD-L1 (*bottom left*), or PD-L2 (*bottom right*) by flow cytometry. Plots are all gated on $CD117^+Fc\epsilon RI^+$ MCs. All results are representative of at least two independent experiments.



LNs and express both MHC-II and costimulatory molecules necessary for Ag presentation.

LPS protects against MC death

We have recently reported that IgE cross-linking by cognate Ag induces apoptosis of MCs (22). The Ag-incorporated apoptotic MCs then serve as a source of Ag to be presented to T cells by DCs. However, for MCs to be involved in the direct presentation of Ag on MHC-II, the MCs must survive. Thus, we tested the effects of LPS/IFN- γ on MC survival. In contrast to FcεRI cross-linking, LPS/IFN- γ stimulation protected against MC apoptosis in a MyD88^{-/-} manner (Fig. 9A). The cytoprotective effect did not require IFN- γ and was not a direct effect of LPS on MCs because coculture of WT MCs with MyD88^{-/-} MCs protected MyD88^{-/-}

MCs from apoptosis (Fig. 9B). Upon further investigation, LPS was found to induce IL-3 from MCs (Fig. 9C). Blockade of IL-3 by neutralizing Ab reversed the cytoprotective effect of LPS on MCs (Fig. 9D), suggesting that LPS protects MCs against apoptosis by inducing IL-3. Therefore, we propose that there may be two distinct ways that MCs could be involved in Ag presentation: one involving Ag-incorporated apoptotic MCs through FcεRI cross-linking and another involving LPS-induced survival and MHC-II expression by MCs.

Discussion

We demonstrate in this study that cultured MCs express MHC-II after stimulation with LPS/IFN- γ . Concomitant expression of

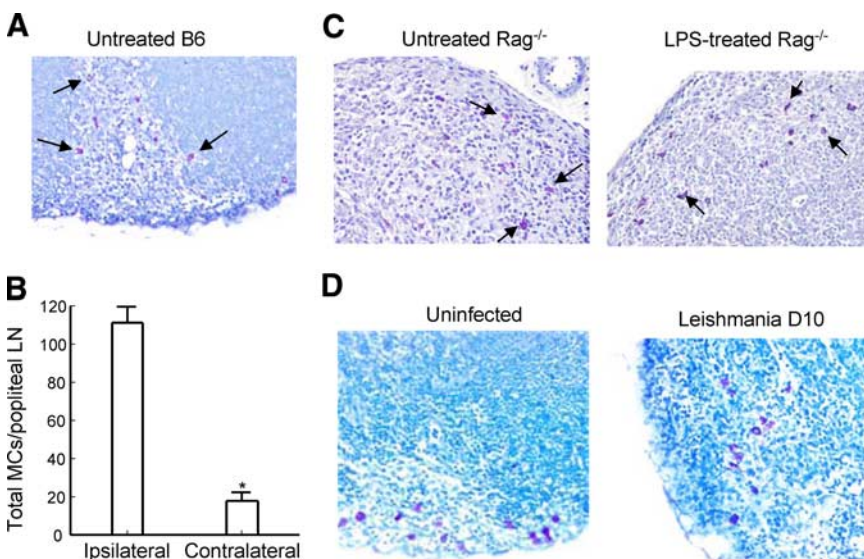


FIGURE 8. MC numbers increase in LNs during *L. major* infection and reside in LN sinuses. Inguinal LNs (**A**) were harvested from LPS-treated B6, unchallenged (**B**), or LPS-challenged (**C**) RAG^{-/-} mice. Sections were taken from fixed LNs, stained with toluidine blue, and visualized with light microscopy at a magnification of $\times 200$. **C**, B6 mice were inoculated with *L. major* s.c. in the right footpad, and the ipsilateral (right LN) and contralateral (left LN) popliteal LNs were harvested separately on 7 days postinoculation. Total MC number in the LN was enumerated by flow cytometry ($n = 4$ mice). *, $p < 0.01$ indicates significance by ANOVA. **D**, Sections from popliteal LNs on days 0 (*right*) and 10 (*left*) postinoculation with *L. major* were stained with toluidine blue and visualized with light microscopy at magnification of $\times 200$. All results are representative of at least two independent experiments.

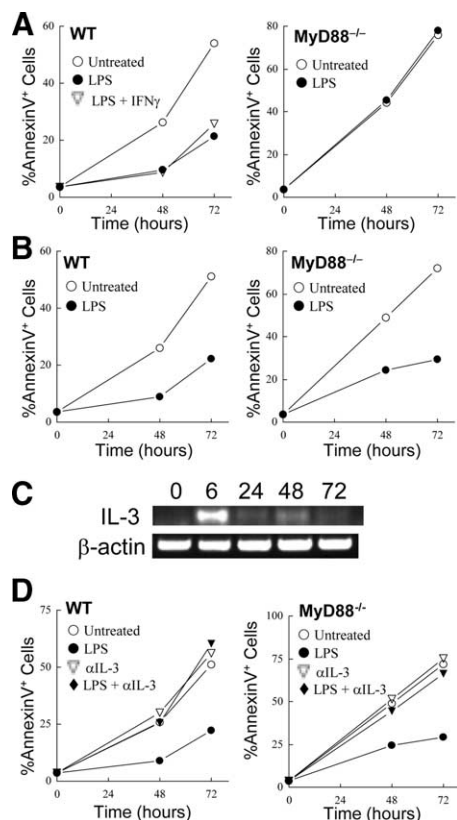


FIGURE 9. LPS promotes survival of MCs by induction of IL-3. **A**, WT (left) or MyD88^{-/-} (right) BMMCs were cultured in IL-3-free MC medium in the presence or absence (○) of LPS (●) or LPS + IFN-γ (open triangles). MC apoptosis was measured by Annexin V staining on days 0, 2, and 3 postculture. **B**, WT (left) or MyD88^{-/-} (right) cells were mixed and cocultured in IL-3-free MC medium in the presence (●) or absence (○) of LPS. The BMMCs were distinguished from each other by labeling the MyD88^{-/-} BMMCs with CFSE. **C**, RNA was extracted from FcεRI⁺CD117⁺ FACS-sorted B6 BMMCs stimulated with LPS for 0, 6, 24, 48, or 72 h. The presence of IL-3 and β-actin transcripts was detected by RT-PCR. **D**, WT (left) or MyD88^{-/-} (right) cells were mixed and cocultured in IL-3-free MC medium in the presence (●) or absence (○) of LPS with (triangles) or without anti-IL-3 Ab. The BMMCs were distinguished from each other by labeling the MyD88^{-/-} BMMCs with CFSE. All results are representative of at least two independent experiments.

MHC-II-associated molecules as well as the inhibitory costimulatory molecule PD-L1 was observed, whereas positive costimulatory B7 family members CD80 and CD86 were not detected. MHC-II-bearing MCs stimulated Ag-specific naive T cells in certain situations, as MCs activated TS-1 but not OT-2 TCR transgenic naive CD4⁺ T cells. However, MCs were fully capable of stimulating previously activated T cells as well as Tregs.

MHC-II expression by MCs has been controversial. Earlier reports claimed that MHC-II is constitutively expressed on cultured MCs (14, 40), whereas more recent studies have failed to observe MHC-II expression on resting cultured MCs (21, 22). Furthermore, OVA peptide-pulsed BMMCs were found to be poor stimulators of OT-2 T cells (41). This study sheds light on this controversy by demonstrating that MHC-II can be induced on MCs when activated with appropriate stimuli such as LPS/IFN-γ. One may speculate that the discrepancies among reports resulted from the potential use of endotoxin-contaminated reagents in some studies. Indeed, an earlier report demonstrated that IFN-γ was contained in WEHI conditioned medium used to grow MCs and that together with LPS further enhanced the constitutive expression of MHC-II

by MCs (14). It is unclear why MCs require both LPS and IFN-γ for expression of MHC-II. IFN-γ receptor was constitutively expressed on MCs and mast cells functionally responded to IFN-γ by increasing MHC class I expression (data not shown), suggesting that LPS is not required for IFN-γ responsiveness.

Previous studies have reported that IL-4 and GM-CSF enhance whereas IFN-γ decreases the Ag-presenting capability of MCs (40). This outcome is in disagreement with our present results, as MHC-II was not observed in MCs cultured with IL-4 and GM-CSF in the absence of LPS. Previous studies have also argued that incorporation of Ag by IgE converts IFN-γ-treated MCs into potent APCs (42). However, we found that Ag incorporation by IgE does not facilitate presentation of Ags on MHC-II. In fact, Ags incorporated by IgE/FcεRI were protected against proteolytic degradation and were preserved as an intact protein much longer than those Ags acquired by macropinocytosis (T. Zou, unpublished observations). Our findings are supported by a recent report demonstrating that *E. coli* incorporated through IgE/FcεRI are protected from proteolysis and remain viable in MCs (43). Perhaps the enhancement of Ag presentation by IgE in IFN-γ-treated MCs occurs in an MHC-II-independent manner, e.g., by transferring IgE-incorporated Ags to DCs by exosomes or as apoptotic bodies (20, 22).

It is puzzling why MCs presented TNP-Eα and influenza but not OVA protein on MHC-II. This discrepancy may be explained by the presence of a specific uptake mechanism for those proteins that were presented on the MHC-II of MCs. Because Eα protein was produced as a recombinant protein in bacteria, TNP-Eα could potentially be coupled to endotoxin or other bacteria-derived products that may facilitate incorporation into MCs through TLRs (44). The influenza virus can be incorporated by receptor-mediated endocytosis using HA/sialic acid interactions. In contrast, the OVA used in our experiments contained low levels of endotoxin (Grade V OVA) and possesses no other means of incorporation into MCs other than macropinocytosis. Thus, it is possible that a specific uptake mechanism of Ags, perhaps those associated with pathogen recognition such as TLRs, is required for efficient processing and presentation of exogenous Ags on MHC-II by MCs.

MHC-II expression by MCs was not merely an *in vitro* phenomenon. Compared with unchallenged mice, LNs of LPS- or *Leishmania*-inoculated mice contained significantly increased numbers of MCs, all expressing high levels of MHC-II and B7 costimulatory family members (CD80, CD86, PD-L1). The ratio of MCs to total LN cells was only slightly increased, suggesting that the increase in MC numbers was proportional to LN hyperplasia. LNs of unchallenged mice contained fewer MCs but all expressed MHC-II and costimulatory molecules (data not shown). These MCs may represent those recruited from skin or mucosal sites after stimulation by normal flora or environmental irritants. Upon infection, more MCs may be recruited to LNs leading to increased numbers of MCs. In support of this argument, MCs were detected in the popliteal LNs of *Leishmania*-infected MC-deficient *Wsh/Wsh* mice that have been reconstituted with MCs in their footpads (T. Kambayashi et al., unpublished observations). Furthermore, previous studies by others have demonstrated migration of MCs to LNs under allergic and bacterial inflammation (45, 46). Alternatively, MCs found in the LN may represent a subtype of MCs that are LN-resident, have Ag-presenting capability, and expand upon inflammation. In support of this hypothesis, all MCs in the LN of LPS-injected mice incorporated BrdU suggesting that these MCs have expanded by proliferation (T. Kambayashi et al., unpublished observations). However, the possibility that the expansion of MCs took place at a remote site and later migrated to the LNs cannot be excluded.

The central question that remains is the function of MHC-II on MCs in vivo. Given the potency of DCs in stimulating naive T cells, it is unlikely that MCs play a major role in initiating primary T cell responses. Indeed, MCs were poor stimulators of naive T cells in vitro, most likely resulting from the absence of costimulatory molecules. It is more likely that MCs participate in the reactivation or propagation of activated T cells, as MHC-II-peptide-bearing MCs stimulated the production of IFN- γ from Ag-experienced T cells, which do not require costimulation for reactivation. However, given that LN-localized MCs express CD80 and CD86, some contribution of MCs to naive T cell priming in vivo cannot be excluded.

The role for MHC-II expression on MCs may be to activate Tregs and dampen the immune response or avoid self-reactivity. MCs stimulated the Ag-specific proliferation of Tregs and favored their activation over naive T cells in mixed cocultures. Activation of Tregs by MCs may contribute to the protective effect of MCs on skin allografts, a process that was proposed to involve IL-9 production by Tregs to recruit MCs to the graft site (13). Bidirectional communication may take place between MCs and Tregs, of which one involves Ag presentation by MCs to Tregs. Endogenous proteins were presented well on MHC-II of MCs, and thus many of the bound peptides may be self-derived, which would favor the notion that MCs activate Tregs. The interaction of T cells and MCs could take place in LNs where MCs are situated to encounter cells that drain through the lymphatic sinuses. In support of this notion, a recent report demonstrated that MCs are in direct contact with Tregs in LNs of mice, an interaction that may contribute to suppression of MC activation (47).

How MCs preferentially stimulate Tregs is uncertain. Like MCs, B cells have also been reported to preferentially expand Tregs through an unknown mechanism (48). A recent study demonstrated that PD-L1 is necessary for the generation of adaptive Tregs by Ag-primed DCs (49). Adaptive Tregs differ from natural Tregs in that they are conventional CD4⁺ T cells that have post-thymically acquired Foxp3. PD-L1 appears not to be involved in our system involving natural Tregs because blockade of PD-L1 by anti-PD-L1 Ab had no effect on the proliferation of Tregs from TS-1 X HA28 mice (data not shown). However, it is possible that PD-L1 on MCs is involved in conversion of CD4⁺ T cells into adaptive Tregs under certain conditions. Further studies involving graft rejection models or infectious disease models will be required to understand how the acquisition of Ag-presenting capability by MCs contributes to the overall function of MCs in physiological and pathologic states.

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Disclosures

The authors have no financial conflict of interest.

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