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The Myeloid Differentiation Factor 88 (MyD88) Is Required for CD4⁺ T Cell Effector Function in a Murine Model of Inflammatory Bowel Disease

Masayuki Fukata,* Keith Breglio,* Anli Chen,* Arunan S. Vamadevan,* Tyralee Goo,* David Hsu,* Daisy Conduah,* Ruliang Xu, † and Maria T. Abreu² *

Abnormal T cell responses to commensal bacteria are involved in the pathogenesis of inflammatory bowel disease. MyD88 is an essential signal transducer for TLRs in response to the microflora. We hypothesized that TLR signaling via MyD88 was important for effector T cell responses in the intestine. TLR expression on murine T cells was examined by flow cytometry. CD4⁺CD45Rb⁺CD25⁺ regulatory T cells were isolated and adoptively transferred to RAG1⁻/⁻ mice. Colitis was assessed by changes in body weight and histology score. Cytokine production was assessed by ELISA. In vitro proliferation of T cells was assessed by [³H]thymidine assay. In vivo proliferation of T cells was assessed by BrdU and CFSE labeling. CD4⁺CD45Rb⁺ T cells expressed TLR2, TLR4, TLR9, and TLR3, and TLR ligands could act as costimulatory molecules. MyD88⁻/⁻ CD4⁺ T cells showed decreased proliferation compared with WT CD4⁺ T cells both in vivo and in vitro. CD4⁺CD45Rb⁺ T cells from MyD88⁻/⁻ mice did not induce wasting disease when transferred into RAG1⁻/⁻ recipients. Lamina propria CD4⁺ T cell expression of IL-2 and IL-17 and colonic expression of IL-6 and IL-23 were significantly lower in mice receiving MyD88⁻/⁻ cells than mice receiving WT cells. In vitro, MyD88⁻/⁻ T cells were blunted in their ability to secrete IL-17 but not IFN-γ. Absence of MyD88 in CD4⁺CD45Rb⁺ cells results in defective T cell function, especially Th17 differentiation. These results suggest a role for TLR signaling by T cells in the development of inflammatory bowel disease. The Journal of Immunology, 2008, 180: 1886–1894.

Inflammatory bowel disease (IBD)³ is characterized by chronic intestinal inflammation in the absence of a recognized bacterial pathogen. Advances in human genome research have revealed that several candidate genes in IBD are linked to innate immunity (1–6). In contrast, abnormal T cell responses to luminal commensal bacteria are crucial to the pathogenesis of IBD (7–9). The link between genetic defects in innate immunity and sustained activation of T cells remains unclear.

Recognition of commensal bacteria largely depends on TLRs in the intestinal mucosa (10). TLRs are pattern recognition receptors expressed by immune and nonimmune cells in the intestinal mucosa that signal in response to pathogen-associated molecular patterns (PAMPs) expressed by microbes (11). The main purpose of TLR signaling is to provide a rapid response against pathogens to protect the host. Eleven TLRs have been identified and individual or pairs of TLRs recognize distinct PAMPs. Most TLRs, with the exception of TLR3, signal via the adapter molecule MyD88 to the IL-1 receptor associated kinase and TNF receptor-associated factor 6 to TGF-β-activated kinase 1, leading to the nuclear translocation of NF-κB and activation of mitogen-activated protein kinase (12). Previous studies have demonstrated that MyD88-deficient mice develop profound defects in Ag-specific T cell responses, suggesting that TLR signaling plays a role in the development of adaptive immune responses (13, 14), but the effect of MyD88 was thought to be mediated by APCs.

Mucosal immune homeostasis requires a balanced interplay between effector and regulatory T cells (Tregs). Naïve T cells differentiate into effector cells that are divided into three distinctive types, Th1, Th2, and Th17 cells, depending on the type of cytokines secreted. The fate of T cell differentiation into Th1, Th2, or Th17 type T cells or Tregs is largely regulated by the interaction between naïve T cells and dendritic cells (DCs) (15). The recognition of PAMPs by TLRs on DCs promotes stimulation of Ag presentation, up-regulation of costimulatory molecules, and secretion of cytokines, which in turn leads to the induction of T cell differentiation, proliferation, and survival of Ag specific CD4⁺ T cells (11).

Although most TLR functions have been attributed to their role in DC activation, CD4⁺ T cells also express TLRs and may regulate functional responses of CD4⁺ T cells in an APC-independent manner (16–21). For example, TLR2 is a potent costimulatory receptor found on CD4⁺ T cells, which may increase proliferation and IFN-γ secretion following TCR stimulation (22, 23). TLR3 signals have been suggested to prolong CD4⁺ T cell survival (17). TLR5 and TLR7 have been shown to enhance TCR stimulation in memory CD4⁺ T cells (16). TLR4 signaling on T cells mediates adherence to fibronectin and expression of suppressor of cytokine signaling 3 (24). TLR9-mediated CD4⁺ T cell activation has been
shown to be MyD88 dependent (25). Recently, IL-4 receptor-associated kinase, the most proximal downstream signaling molecule after MyD88, has been shown to be essential in eliciting CD4⁺ T cell activation (26). Finally, TLRs modulate Treg proliferation and their ability to suppress T cell activation (27–29). These data suggest a significant role for MyD88 in CD4⁺ T cell activation.

Commensal bacteria are required to initiate chronic intestinal inflammation in most animal models of IBD (30). The CD4⁺ CD45Rbhigh cell adoptive transfer model of colitis is one of the animal models of IBD that most closely reflects human IBD with respect to gene expression profiles (31). In this model of colitis, CD4⁺ CD45Rbhigh T cells are activated in response to commensal bacteria to initiate chronic inflammation in the absence of Tregs (32). This T cell population has also been suggested to be important in the pathogenesis of human IBD (33). Therefore, we hypothesized that TLR signaling via MyD88 is important for the development of colitis in the murine CD4⁺ CD45Rbhigh T cell adoptive transfer model.

In the current study, we first determined the expression of TLRs on CD4⁺ CD45Rbhigh T cells. The finding that CD4⁺ CD45Rbhigh T cells express significant levels of TLRs led us to test their role in the development of colitis. We examined the effects of MyD88 deficiency on CD4⁺ T cell activation in vivo and in vitro using MyD88 knockout (MyD88−/−) mice as donor mice. In the adoptive transfer model of colitis, CD4⁺ CD45Rbhigh T cells from MyD88−/− mice did not induce wasting disease. MyD88−/− T cells also showed defective proliferation in vitro and in vivo. The secretion of IL-2 and IL-17 was significantly reduced in CD4⁺ T cells from mice receiving MyD88−/− T cells compared with mice receiving WT T cells. In vitro, TLR ligands could act as costimulatory molecules in the context of anti-CD3 stimulation. In addition, Tregs from MyD88−/− mice showed a decreased ability to suppress T cell proliferation in vivo and in vitro. These results suggest that MyD88 signaling by CD4⁺ T cells is required for expansion and differentiation in the intestinal compartment. Our results provide a link between an innate immune defect and the sustained activation of T cells in the pathogenesis of IBD.

Materials and Methods

Mouse transfer colitis

MyD88−/− mice were purchased from Oriental BioService and were back-crossed to C57BL/6 mice >10 times. C57BL/6 mice and RAG1−/− mice (C57BL/6 background) were obtained from The Jackson Laboratory. Six- to ten-wk-old gender-matched mice were used in this study. Mice were kept in specific-pathogen free conditions and fed by free access to a standard diet and water. All experiments were performed according to Mount Sinai School of Medicine Animal Experimental Ethics committee guidelines.

Spleen and mesenteric lymph node (MLN) were taken from WT (C57BL/6) mice and MyD88−/− mice. Single cell suspensions from the spleen and MLN were prepared to isolate CD4⁺ T cells using MACS magnetic separation system (Miltenyi Biotec). Enriched CD4⁺ T cells were labeled with FITC-conjugated anti-CD45Rb (BD Biosciences), and allophycocyanin-conjugated CD25 (Biologend). CD4⁺ CD45Rbhigh and CD4⁺ CD45Rblow CD25⁺ cell fractions were sorted with a FACS Vantage flow through, CD4⁺ T cells) were double stained with FITC-conjugated anti-CD45Rb (BD Biosciences) and PE-conjugated anti-CD45Rbhigh (BD Biosciences). The purity of cell separation was over 98% as measured by flow cytometry. Sorted cells were resuspended in stage cell sorter (BD Biosciences). The purity of cell separation was over 99%.

Colon samples were removed, washed in PBS with 5% penicillin/streptomycin, cut into small pieces, and incubated with Ca²⁺ and Mg²⁺ free HBSS containing 0.002 mol/L EDTA for 12 min with gentle agitation to remove epithelial cells. Tissue pieces were then incubated while shaking in complete medium containing 5% FCS, 0.02 mol/L HEPES, 1-g-glutamine, 5% penicillin and streptomycin with 1 mg/ml collagenase, 1.5 mg/ml dispase in RPMI 1640 at 37°C for 60 min. The supernatant was centrifuged and the pellet was washed two times with RPMI 1640 containing 5% penicillin/ streptomycin. LP cells were isolated by lymphocyte separation medium (Mediatech) density gradient centrifugation (800 × g for 20 min) and collected at the interface. DCs were isolated from LP cells by positive selection using the anti-CD11c MACS beads (Miltenyi Biotec). Using the DC isolation flow through, CD4⁺ T cells were further isolated by negative selection with the CD4 MACS system (Miltenyi Biotec).

Real time PCR

Total RNA was isolated from the colonic tissues using RNA Bee (Tel-Test) according to the manufacturer’s instructions. A total of 1 μg RNA was used as the template for single strand cDNA synthesis utilizing the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. Quantitative real-time PCR was performed for IL-23p19, IL-17, and β-actin. The primers and probes used in this study are as follows: (5′→3′ direction) for mouse IL-23p19: sense primer, C TGG GCC TAG GAG TAG TAG CTC; anti-sense primer, A GTC TCT TCA TCC TCT TCT CT; and probe, C TCA GTG CCA GCA GCT TTC TGA GA; mouse IL-6 primers and probe were designed by Applied Biosystems (Mm00446190_m1); for mouse β-actin: sense primer, ATG ACC CAG ATC ATG TTT G; anti-sense primer, TAC GAC CAG AGC CAT AC, and probe, CGT ACG CAC CCA GCC GTC GTG GC. All TaqMan primers and probes were designed using Beacon Designer 3.0 (Premier Biosoft International). The cDNA was amplified using TaqMan universal PCR Master Mix (Roche) on the ABI Prism 7900HT sequence detection system (Applied Biosystems), programmed for 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplification results were analyzed using SDS 2.2.1 software (Applied Biosystems), and the genes of interest were normalized to the corresponding β-actin results.

Western blot analysis

Whole cell lysates were prepared from LP CD4⁺ T cells using a lysis buffer containing 50 mM Tris-HCl, 50 mM NaF, 1% Triton X-100, 2 mM EDTA, and 100 mM NaCl with a proteinase inhibitor mixture (Calbiochem). Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay Dye and SmartSpec 3000 (Bio-Rad). Twenty-five micrograms of the lysates were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membrane was blocked in 5% skim milk and was immobiloblotted with the primary Abs for 1 h, followed by HRP-conjugated goat anti-rabbit IgG, or Streptavidin-HRP (Zymed Laboratories) corresponding to the primary Abs used. The membrane was exposed on x-ray film using an enhanced chemiluminescent substrate SuperSignal West Pico Trial Kit (Pierce), Abs specific for phospho (Ser 727)-STAT3 and phospho (Tyr 705)-STAT3 were purchased from Cell Signaling Technology.

Immunohistochemistry

Paraffin-embedded colon tissue samples (n = 5 each for mice that received WT T cells or mice that received MyD88−/− T cells) were double stained with CD4 and phospho-STAT3. Aq retrieval by microwave was performed after deparaffinization. After blocking the nonspecific binding with 5% skim milk, sections were incubated with rabbit anti-phospho (Ser 727)-STAT3 Ab (1/100, Cell Signaling Technologies) overnight at 4°C. After washing in PBS, sections were incubated with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG1 (1/200, Sigma-Aldrich) for 1 h at room temperature. Sections were then reincubated with 5% skim milk and stained with FITC-conjugated anti-mouse CD4 Ab (1/100, BD Pharmingen, BD Biosciences) overnight at 4°C. Sections were mounted with SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen Life Technologies) after rinsing with PBS. Double stained tissue slides were examined using a Leica TCS-SP (UV) confocal microscope.

In vitro T cell differentiation

The method for in vitro T cell differentiation was adapted from previous studies (36, 37). In brief, CD4⁺ CD45Rbhigh T cells isolated from

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FIGURE 1. TLR expression on CD4^+CD45Rb^{high} CD4^+ T cells. Expression of TLRs on CD4^+ CD45Rb^{high} CD4^+ T cells. Splenic CD4^+ CD45Rb^{high} CD4^+ T cells were examined for TLR2, TLR3, TLR4, TLR5, and TLR9 expression by flow cytometry (black peaks are CD4^+ CD45Rb^{high} CD4^+ T cells of WT mice, gray peaks are CD4^+ CD45Rb^{high} CD4^+ T cells from individual knock-out mice, and white peaks are RAW 264.7 cells). The isotype control Ab is used for TLR5 staining.

MyD88^{−/−} or WT mice spleen were cultured in RPMI 1640 supplemented with 10% FCS, 5% Pen/Strep at 37°C in 5% CO₂. Cells were primed for 5 days with plate-bound anti-CD3 (2 μg/ml; BD Pharmingen), anti-CD28 (2 μg/ml; BD Pharmingen), and anti-IL-4 (4 μg/ml; 11B11 L, Santa Cruz Biotechnology). The following cytokines were added for 5 days as indicated. For Th1 differentiation, IL-12 (4 ng/ml; PeproTech) was added. For Th17 differentiation, IL-6 (100 ng/ml; PeproTech) plus TGF-β (5 ng/ml; PeproTech), or IL-23 (10 ng/ml; R&D Systems) were added. Cells were then washed and stimulated for 24 h with plate-bound anti-CD3 (1 μg/ml; BD Biosciences) at a density of 1 × 10^6 cells/ml. Cell-free supernatants were analyzed simultaneously for IL-17 or IFN-γ production using specific ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Flow cytometry**

CD4^+ CD45Rb^{high} T cells were isolated from spleens. RAW 264.7 cells were used as a positive control for TLR staining. Spleen cells from knock-out mice (TLR4^{−/−}) were used as a positive control for TLR staining. Spleen cells from knock-out mice, gray peaks are RAW 264.7 cells). The isotype control Ab is used for TLR5 staining.

To measure cytokine production, DCs and CD4^+ T cells were isolated from the LP. CD4^+ T cells (1 × 10^6) were cocultured with LP-DCs (5 × 10^5) in RPMI 1640 supplemented with 10% FCS, 5% penicillin/streptomycin in 96-well anti-CD3 precoated (1 μg/ml; BD Biosciences) at a density of 1 × 10^5 cells/ml. Cell-free supernatants were analyzed simultaneously for IL-17 or IFN-γ production using specific ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Cell proliferation assays**

Mice were injected with 120 mg/kg of BrdU (Sigma-Aldrich) i.p. 90 min before sacrificing. Paraffin sections of colon and MLN were incubated with 5% skim milk and stained with FITC-conjugated anti-BrdU (BD Pharmingen), followed by rabbit anti-mouse CD4 and tetramethylrhodamine isothiocyanate-conjugated anti-rabbit IgG. Proliferating CD4^+ T cells were determined by fluorescence microscopy.

In vitro cell proliferation was analyzed in freshly isolated CD4^+ T cells following 3 days of stimulation in anti-CD3 precoated 96-well plates (BD Biosciences) with 1 μg/ml anti-CD28. Single cell suspensions of splenocytes were separated into CD4^+CD25^− and CD4^+CD25^+ subsets using negative selection with the anti-CD4 MACS system followed by positive selection with an anti-CD25 MACS column (Miltenyi Biotec). In certain experiments, we used TLR ligands, Pam3CSK₄ (1 μg/ml; Invivogen), poly I:C (90 μg/ml; Invivogen), LPS (1 μg/ml; Escherichia coli 0111: B4, Invivogen), and CpG-ODN (1 μg/ml; Invivogen) as an alternative to anti-CD28. [3H]Thymidine was added during the last 18 h of culture. To assess Treg suppressor activity, freshly isolated CD4^+CD25^+ Treg cells were cocultured with CD4^+CD25^- T cells at a ratio of 0.125:1 (0.125 × 10⁶ vs 1 × 10⁵ per well). [3H]Thymidine incorporation was analyzed with a beta scintillation counter.

For CFSE labeling, CD4^+ T cells were incubated in 5 μM CFSE (Molecular Probes, Invitrogen Life Technologies) in PBS at 37°C for 15 min. CFSE-labeled cells (5 × 10⁵) were injected i.p. into RAG1^{−/−} mice. After 7 days, CD4^+ T cells were isolated from spleen, MLN, and the colon as described above. Cell division was analyzed by flow cytometry detecting CFSE fluorescence.

**Statistical analysis**

Results were expressed as mean ± SD. Data were analyzed by Student’s t test using the statistics package within Microsoft Excel. The p values were considered significant when <0.05.

**Results**

**CD4^+ CD45Rb^{high} T cells express TLRs**

Several studies have previously demonstrated the expression of TLRs on T cells (16–21). However, most of the previous work only examined expression at the mRNA level. We examined the expression of TLR protein in CD4^+CD45Rb^{high} T cells isolated from spleens of WT mice by flow cytometry (Fig. 1). We focused on TLR2, TLR4, TLR5, and TLR9 because of their relevance to luminal bacterial Ags. We also examined the expression of TLR3 because of viral pathogens. Although TLR expression was lower than that seen in the mouse macrophage cell line RAW 264.7 cells, CD4^+ CD45Rb^{high} T cells expressed significant levels of TLR2, TLR4, TLR9, and TLR3. TLR5 was barely detectable.

**MyD88 signaling modulates T cell proliferation**

We next addressed whether TLR signaling contributes to T cell function using MyD88^{−/−} T cells. To examine T cell activation, CD4^+CD25^- T cells were cultured for 3 days with anti-CD3 and anti-CD28 and proliferation was measured by [3H]thymidine incorporation. MyD88^{−/−} T cells showed 50% less proliferation than WT T cells (Fig. 2A). In addition, we found that exogenous TLR ligands (TLR2, TLR4, and TLR9 ligands) could act as a costimulus to anti-CD3 activation in WT CD4^+CD25^- T cells but not in MyD88^{−/−} CD4^+CD25^- T cells (Fig. 2B). By contrast, The
TLR3 ligand caused similar degrees of proliferation between WT T cells vs MyD88−/− T cells, consistent with being an MyD88-independent TLR.

Given the decrease in proliferation in MyD88−/− T cell with TCR activation, we investigated whether there were differences in total T cell numbers between WT mice and MyD88−/− mice. We isolated CD4+ T cells from spleens and LP of WT or MyD88−/− mice and found similar numbers of CD4+ T cells, CD8+ T cells, B cells (CD19), Gr-1 positive cells, or CD4+CD25+ Tregs. Thus, in the MyD88−/− mouse, other compensatory mechanisms result in normal numbers of cells even in the LP.

To examine the function of MyD88−/− Tregs, WT CD4+CD25+ T cells were cocultured with either WT or MyD88−/− CD4+CD25+ Tregs. MyD88−/− Tregs showed a decreased ability to suppress the proliferation of allogeneic T cells challenged with anti-CD3 and anti-CD28 (Fig. 2C). Thus, in MyD88−/−, mice there are no apparent defects in T cell numbers in the periphery or the intestine under homeostatic conditions, but in vitro functional responses are perturbed.

MyD88−/− CD4+CD45Rbhigh T cells are defective in their ability to induce colitis in RAG1−/− mice

To clarify the role of TLR signaling in CD4+CD45Rbhigh T cells in vivo, we adoptively transferred CD4+CD45Rbhigh T cells isolated from MyD88−/− or WT spleen into RAG1−/− mice. After 9 wk, mice transferred with WT T cells demonstrated significant loss of body weight as previously described (34). However, mice receiving MyD88−/− T cells alone did not induce colitis (Fig. 3A). MyD88−/− T cells transferred with WT CD4+CD45Rbhigh T cells into RAG1−/− mice also failed to induce colitis (Fig. 3B). Histological examination of the colon showed that mice receiving MyD88−/− T cells had significantly decreased severity of colitis compared with that of WT T cell transferred mice (colitis score: 11.1 ± 2.9 vs 3.6 ± 3.7, p < 0.001, max score: 20). Importantly, MyD88−/− lymphocytes did home to the intestine and other compartments, but did not induce colitis. Indeed, MyD88−/− T cells in peripheral blood expressed the mucosal homing marker e4β7 to a similar extent compared with WT T cells (data not shown).

Defective Treg function in TLR2−/− mice has been previously reported (38). We therefore examined MyD88−/− Treg function in...
the colitis transfer model. WT CD4+CD45Rbhigh T cells were co-
transferred with either WT Tregs or MyD88−/− Tregs, and the
severity of the colitis was compared after 9 wk. Tregs from
MyD88−/− mice did not protect against colitis induced by WT
CD4+CD45Rbhigh T cells (Fig. 3, C and D). Histological severity
of the colitis revealed a marginal suppressive effect of the
MyD88−/− Tregs, but the severity score was significantly higher
than in mice cotransferred with WT Tregs (colitis score: MyD88−/−
Tregs 7.7 ± 3.9 vs WT Tregs 3.6 ± 3.1, p < 0.01, max: 20). These results indicate that CD4+CD45Rbhigh T cells
from MyD88−/− mice exhibit an impaired ability to induce
chronic colitis in the adoptive transfer model. In addition, MyD88-
dependent TLR signaling is required for optimal Treg function
in vivo.

CD4+CD45Rbhigh T cells from MyD88−/− mice exhibit
decreased proliferation in MLNs and the LP when transferred
into RAG1−/− mice

Given the in vitro findings that MyD88−/− T cells have a de-
creased ability to proliferate in response to TCR stimulation yet
have normal numbers of T cells in situ, we next addressed whether
this decreased ability to proliferate also occurred when T cells
were transferred to a TLR sufficient host, namely the RAG1−/−
mice. First, we compared the total number of CD4+ T cells iso-
lated from LP between MyD88−/− T cell transferred mice and WT
T cells transferred mice (Fig. 4A). RAG1−/− mice receiving
MyD88−/− T cells had significantly fewer LP CD4+ T cells than
mice receiving WT T cells.

To confirm whether this difference in T cell number was due to
defective proliferation of MyD88−/− T cells in vivo, we examined
local T cell proliferation by BrdU labeling at 9 wk after T cell
transfer (Fig. 4B). Double staining of the colon and MLN with
anti-CD4 and anti-BrdU showed less proliferation of CD4+ T cells
in mice receiving MyD88−/− CD4+CD45Rbhigh T cells than those receiving WT CD4+CD45Rbhigh T cells. We next
examined the dynamic proliferative ability of the CD4+ T cells
using CFSE labeling. CD4+ T cells from WT mice and
MyD88−/− mice were labeled with CFSE and transferred to
RAG1−/− mice. Cell division was analyzed by flow cytometry on
the seventh day after transfer (Fig. 4C). Transferred T cells from
MyD88−/− mice showed fewer cell divisions in MLN than T cells
from WT mice. These results suggest that MyD88−/− CD4+
CD45Rbhigh T cells have defective proliferation in vivo.

Secretion of IL-2 and IL-17 is significantly lower in colonic
CD4+ T cells from RAG1−/− mice receiving MyD88−/− T cells
than in mice receiving WT T cells

Given the results suggesting proliferative defects in MyD88−/−
CD4+CD45Rbhigh T cells, we next addressed whether cytokine
expression and T cell polarization were altered in MyD88−/− T
cells in the colitis model. CD4+ T cells isolated from the LP in
mice receiving MyD88−/− T cells were cocultured with LP DCs,
isolated from the same mouse, and stimulated with anti-CD3 for
48 h. Supernatants were analyzed for cytokine production by
ELISA. There were no differences in the production of IFN-γ or
IL-10 between MyD88−/− CD4+ T cells and WT CD4+ T cells
(Fig. 5). By contrast, the production of IL-2 and IL-17 was sig-
nificantly lower in MyD88−/− CD4+ T cells than in WT CD4+ T
cells (Fig. 5). These results suggest that MyD88−/− T cells
are specifically defective in Th17 polarization. In addition, impaired
expression of IL-2 may be part of the reason for defective T cell
proliferation observed in MyD88−/− CD4+ T cells.

FIGURE 4. Decreased proliferation of MyD88−/− T cells after trans-
fer into RAG1−/− mice. A, The number of CD4+ T cells in the LP of
RAG1−/− mice 9 wks after naive T cell transfer. The data represent the
mean (±SD) of three mice each (*, p < 0.05). B, BrdU labeling of
proliferating cells in the LP and MLN taken from RAG1−/− mice re-
ceiving WT or MyD88−/− naive T cells. Representative pictures show
BrdU positive cells (green) and CD4+ cells (red), appearing yellow.
RAG1−/− mice receiving WT T cells have more proliferating cells in
both the LP and MLN than mice receiving MyD88−/− T cells. C, De-
creased MyD88−/− CD4+ T cell proliferation in MLN of RAG1−/−
mice observed 7 days after transfer. CD4+ T cells isolated from
MyD88−/− and WT mice spleen were labeled with CFSE and injected
i.p. into RAG1−/− mice. Division of CFSE labeled cells was analyzed
by flow cytometry. White peaks show the initial CFSE fluorescence
(before injection). WT CD4+ T cells show more dividing cells than
MyD88−/− CD4+ T cells in the MLN, represented by the broader
shoulder of dividing cells in the WT MLN panel (upper left panel).

Mucosal IL-6 and IL-23 expression are decreased in RAG1−/−
mice receiving MyD88−/− T cells

T cell polarization is regulated by cytokines present during the
initial stage of TCR ligation (39). Th17 polarization is specifically
mediated by DC-derived cytokines IL-23 and IL-6 under the influence of TGF-β (40). To clarify whether defective Th17 differentiation of MyD88−/− T cells is associated with a decrease in these upstream cytokines, we examined the expression of IL-23p19 and IL-6 in the colonic mucosa using real time PCR. Compared with mice receiving WT T cells, mice that received MyD88−/− T cells had significantly decreased mRNA expression of both IL-23 and IL-6 (Fig. 6). Expression of IL-1β and IL-18 mRNA in the colonic mucosa was slightly decreased in mice receiving MyD88−/− T cells but did not reach statistical significance (data not shown). These results suggest that MyD88 function in CD4+ CD45Rbhigh T cells is required to prime LP APCs to produce these cytokines.

**MyD88 is required for the induction of Th17 differentiation in vivo**

Thus far, we found that differentiation of MyD88 CD4+ CD45Rbhigh T cells into Th17 cells was defective in vivo and that expression of IL-6 and IL-23 were also decreased in vivo. We then wished to examine whether the defect in Th17 differentiation could be overcome with exogenous administration of cytokines in vitro. Polarization of T cells was performed as previously described (36, 37). WT and MyD88−/− CD4+ CD45Rbhigh T cells primed with IL-12 and treated with anti-IL-4 expressed equal amounts of the Th1 cytokine IFN-γ (Fig. 7B). In contrast, Th17 differentiation was very different between MyD88−/− CD4+ CD45Rbhigh T cells and WT T cells. Whereas priming with IL-6 plus TGF-β, or IL-23-induced IL-17 expression in WT CD4+ CD45Rbhigh T cells, MyD88−/− CD4+ CD45Rbhigh T cells expressed significantly less IL-17 (Fig. 7A). These results suggest that MyD88−/− CD4+ CD45Rbhigh T cells have an inherent defect in Th17 but not Th1 polarization.

**STAT3 phosphorylation in mucosal CD4+ T cells is decreased in MyD88−/− T cell transferred mice**

STAT3 is active in the colon in IBD (41, 42), and plays a pivotal role in the pathogenesis of colitis following adoptive T cell transfer (43). In addition, activation of STAT3 in response to IL-6 and IL-23 is required for Th17 development by CD4+ T cells (15, 44, 45). IL-17 further induces the activation of STAT3, leading to a positive feedback loop to sustain inflammation (46). We analyzed the phosphorylation state of STAT3 in LP CD4+ T cells of both WT and MyD88−/− T cell transferred RAG1−/− mice (Fig. 8A). Activation of STAT3 is regulated by phosphorylation of Tyr705 and Ser727. Consistent with the altered expression of IL-17, STAT3 phosphorylation was greater in the LP CD4+ T cells from mice receiving WT T cells than the cells from mice receiving MyD88−/− T cells.

We also examined the localization of phosphorylated STAT3 (Ser727) in colonic tissues of those mice by immunohistochemistry (Fig. 8B). Most of the LP CD4+ T cells were positive for phospho-STAT3 in mice receiving WT T cells but very few phospho-STAT3 positive cells were found in the mice receiving MyD88−/− T cells. Cytoplasmic as well as nuclear localization of...
phospho-STAT3 was observed in CD4+ T cells in mice receiving WT T cells but no nuclear localization was seen in mice receiving MyD88−/− T cells. These results suggest that the observed defects of MyD88−/− T cells in the development of the colitis might be due to decreased STAT3 activation.

Discussion

Many aspects of the interface between innate and adaptive immunity remain unexplored. It is particularly interesting to ponder how adaptive immune responses are regulated in the intestine given its coexistence with the microflora. We show that CD4+CD45Rbhigh T cells from MyD88−/− mice exhibit multiple defects in establishing a pathogenic adaptive response to host signals and the luminal microenvironment during chronic colitis. Among the defects we identified, MyD88−/− T cells are unable to be activated and to differentiate into Th17 effector cells, thereby preventing expansion and sustained inflammation. MyD88−/− CD4+ T cells show decreased production of IL-2 and a reduction in proliferation in response to TCR stimulation. Tregs from MyD88−/− mice were also defective in protecting against colitis induced by WT CD4+CD45Rbhigh T cells. Therefore, signaling via MyD88 on CD4+CD45Rbhigh T cells appears to be required for the proliferation and Th17 differentiation of T cells. MyD88 signaling is also required for Treg-mediated suppression of colitogenic T cells. These results suggest a significant role for TLR signaling by T cells in the development of IBD.

The adoptive transfer model of colitis provides an ideal mechanism by which to test the hypothesis that TLR signaling by T cells is important for their function. Extensive previous work in this model has highlighted the dependence of luminal bacteria in the development of colitis (35, 47). In particular, Powrie et al. (34) has shown that intestinal bacteria are necessary in the recipient mouse to develop colitis. Among the defects we identified, the adoptive transfer model of colitis provides an ideal mechanism by which to test the hypothesis that TLR signaling by T cells is important for their function. Extensive previous work in this model has highlighted the dependence of luminal bacteria in the development of colitis (35, 47). In particular, Powrie et al. (34) has shown that intestinal bacteria are necessary in the recipient mouse to develop colitis.

These mice are effectively unable to signal through TLRs. A previous study has found that crossing MyD88−/− mice to IL-10−/− mice protects against the development of colitis (48). But in that study, all the cells of the mouse are null for both IL-10 and MyD88 including APCs and T cells. By contrast, our study allows us to dissect the role of MyD88-dependent signaling within the T cell compartment. Given what was known about T cell development in MyD88−/− mice, we had initially hypothesized that the transfer of CD4+CD45Rbhigh MyD88−/− T cells into a TLR-positive environment, namely the RAG1−/−, would be sufficient to induce colitis. We were surprised by the degree to which MyD88−/− CD4+CD45Rbhigh T cells were unable to cause colitis.

We described significant expression of TLR2, TLR3, TLR4, and TLR9 protein on murine CD4+ T cells. There are several conflicting reports in terms of TLR expression in murine T cells (17, 49, 50). Marsland et al. demonstrated TLR4, TLR7, and TLR9 mRNA expression, whereas Sobek et al. reported TLR1, TLR2, and TLR6 mRNA expression. Another study described expression of TLR2, TLR3, TLR4, TLR5, and TLR9 mRNA (17), but we could not detect TLR5 protein expression. Although the discrepancy may be due to the differences between mRNA and protein expression, others have shown the absence of TLR5 mRNA in CD4+CD45Rbhigh T cells consistent with our finding of absent TLR5 protein (19). Interestingly, it has been reported that TLR4 and TLR2 mRNA expression become undetectable while TLR3 and TLR9 mRNA are up-regulated in naive T cells (CD44+CD25−CD4+) after stimulation with anti-CD3 and anti-CD28 (17). Therefore, TLR expression may vary with T cell activation state and highlights the developmental role of TLRs on T cells.

Our results demonstrate that there is impaired activation of MyD88−/− CD4+CD45Rbhigh T cells after adoptive transfer into RAG1−/− mice. Although this population is meant to represent a population of activation naive cells, clearly this may be a heterogeneous population (51). Nevertheless, the CD45Rb surface Ag permits
us to identify a subpopulation of cells that has been well characterized in this animal model. Several possible mechanisms may underlie the defect in MyD88$^{-/-}$ T cells. APCs express TLRs normally in the RAG$^{1/-}$ mice. The primary defects in T cell function seem most therefore lie within the T cell compartment itself. Previous reports have demonstrated that MyD88$^{-/-}$ T cells have decreased OVA-specific proliferation and IL-2 production compared with WT T cells when Tregs were depleted with anti-CD25 Ab (52). Lack of CD4$^{+}$ T cell memory function in MyD88$^{-/-}$ mice has also been demonstrated (52). These results imply that MyD88$^{-/-}$ effector T cells are defective. In our work, MyD88$^{-/-}$ T cells did not respond properly to TCR stimulation with costimulation by anti-CD28 in vitro. Several previous reports have suggested that TLRs function in costimulation of T cells but conventional costimulation with anti-CD28 can bypass the effect of TLR signaling (17, 25, 53). We could not overcome the TLR defect in MyD88$^{-/-}$ T cells by anti-CD3/CD28 stimulation in vitro. In vivo, APCs expressing CD28 also could not compensate for the absence of a MyD88 dependent signal in CD4$^{+}$CD45Rb$^{high}$ T cell activation.

The second possibility accounting for defective T cell function is impaired cytokine secretion. MyD88$^{-/-}$ mice have been reported to be deficient in the induction of Th1 immune responses (13, 14, 54). Because MyD88$^{-/-}$ T cells express similar amounts of IFN-γ compared with WT T cells, Th1 differentiation can occur in the absence of MyD88-dependent signaling (55, 56). By contrast, expression of IL-17 was decreased in MyD88$^{-/-}$ T cells, suggesting that IL-17 production is regulated by MyD88-dependent signals. Recent reports have suggested the importance of IL-17 producing cells in the pathogenesis of the adaptive transfer model of colitis (57, 58). Previous studies have also found that Th1 cytokines, in particular IFN-γ, are required for colitis (59–61). Our results would suggest that, in the absence of IL-17, the maximal degree of colitis cannot be achieved. These findings do not, however, diminish the importance of IFN-γ since RAG$^{1/-}$ receiving MyD88$^{-/-}$ do develop a mild colitis. Consistent with a reduction in Th17 cells, mucosal expression of IL-6 and IL-23 was decreased in mice receiving MyD88$^{-/-}$ T cells compared with mice receiving WT T cells. These results indicate that there is crosstalk between mucosal DCs and CD4$^{+}$ T cells to establish an appropriate adaptive immune response. Sequential cytokine production is also involved in establishing proper T cell effector function. IL-1β and IL-18 enhance IL-6 production from DCs and potentiate IL-17 production in the presence of IL-23 (62). Given that whether they receive WT T cells or MyD88$^{-/-}$ T cells, STAT3 is involved in IL-17 production, and conversely, IL-17 activates STAT3 (15, 44–46). Therefore, the sequence of events leading to defective STAT3 activation and IL-17 production may underlie the inability of MyD88$^{-/-}$ CD4$^{+}$CD45Rb$^{high}$ T cells to induce chronic colitis in RAG$^{1/-}$ mice.

In this study, we illustrate an important role of MyD88 in the establishment of pathogenic adaptive immunity. In the absence of MyD88 signaling, CD4$^{+}$CD45Rb$^{high}$ T cells can home to the intestine but do not acquire the phenotype of colitogenic T cells characterized by IL-17 production, STAT3 activation, and aberrant, nonhomeostatic levels of T cell proliferation. Therefore, traditional innate immune signaling may have a greater role in adaptive immunity than originally recognized. Our results enhance the understanding of the link between innate immune defects and abnormal T cell activation in response to luminal commensal bacteria in IBD. Inhibition of MyD88 in select T cell compartments may be useful in the treatment of IBD.

Disclosures

The authors have no financial conflict of interest.

References


