Immunomodulation of bone marrow-derived dendritic cells during endotoxin- and bacterial sonicate-induced tolerance

Undergraduate Senior Thesis
Microbiology

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T A B L E   O F   C O N T E N T S

A B S T R A C T

As important members of the innate immune response and conduits for induction of cell-mediated immunity, dendritic cells (DCs) have emerged as integral to understanding the immune response to pathogens. Due to their role in inflammation, ability to stimulate naïve T cells, and widespread presence in the body, the study of their physiological response is key to conceptualize immune function and pathology. While dendritic cells have been shown to influence tolerance in T cell populations, there is a dearth of investigation as to the development of tolerance among DCs themselves. In cells closely related to DCs such as macrophages and monocytes, prior exposure to minute amounts of endotoxin (LPS) can lead to a refractory period where subsequent exposure to higher doses fails to induce an inflammatory response. This study aims to characterize the immunomodulatory response of bone marrow-derived dendritic cells in instances of endotoxin tolerance, as well as tolerance induced by bacterial sonicate containing multiple stimulatory ligands. Murine bone marrow-derived dendritic cells (BM-DCs) were harvested and cultured (tolerized) with 5 ng/ml LPS or 2 μg/ml of either *E. coli* or *H. pylori* strain SS1 sonicate, thoroughly washed, rested, and stimulated with the same component at a higher dose, physiologically mimicking a low-dose primary exposure and a larger secondary exposure. Along with a significant drop in TNF-α and IL-12p70 production, tolerized BM-DCs exhibited increased IL-10 expression. Tolerization and re-exposure to *H. pylori* SS1 sonicate uniquely showed a decreased expression of IL-6 in tolerized BM-DCs. Furthermore, interleukin 1 receptor-associate kinase-M (IRAK-M), a negative regulator of TLR signaling through NF-κB, showed increased intracellular expression during endotoxin tolerance when its transcript was tested with qPCR and protein expression was

evaluated with Western blot. Neutralization of IL-10 by a αIL-10 antibody in culture did not affect the expression of TNF-α in tolerized BM-DCs, indicating that suppressed pro-inflammatory cytokine expression during tolerance is not IL-10-dependent. Surprisingly, when BM-DCs were harvested from IRAK-M knockout mice, tolerance induced by LPS and bacterial sonicate was not prevented, and indeed the expression of TNF-α decreased. IL-10 expression in IRAK-M -/- DCs increased as in wild type tolerized DCs, but this increase in IL-10 was not exhibited by IRAK-M -/- DCs tolerized with bacterial sonicate. IRAK-M therefore seems to play different roles in endotoxin tolerance (single-ligand tolerance) and bacterial sonicate tolerance (multi-ligand tolerance). We conclude that along with endotoxin (LPS), aggregate bacterial components with multiple TLR ligands are able to induce tolerance in BM-DCs, this tolerance is not dependent on autocrine immune suppression by IL-10, and that IRAK-M, as a negative regulator of TLR signaling in related cell lineages, appears to not be essential to tolerance in BM-DCs and may actually enhance tolerance when not expressed. Furthermore, *H. pylori* SS1 sonicate appears to decrease expression of IL-6 in tolerized BM-DCs, whereas LPS and *E. coli* sonicate do not. This indicates that global trends in endotoxin tolerance might shift depending on the components involved in tolerization and subsequent stimulation, and that the function of IRAK-M in tolerance of BM-DCs does not mirror its function in other monocytes-derived cells.

BACKGROUND AND INTRODUCTION

The role of dendritic cells in the innate and adaptive immune response has long been an area of active research. As essential components of the immune system, they are able to interact innately with pathogens as well as serve as mediators to the adaptive immune response in their role as antigen presenting cells (APCs) [1-2]. Since they possess significant migratory capabilities and are able to stimulate the development of naïve T cells, DCs are crucial to developing antigen-specific immune responses [3-4]. Dendritic cells do not proliferate and have a regular course of maturation, serving as critical responders in tumor resistance, preventing metastasis, and combating bacterial challenge [4]. During the innate response, DCs secrete cytokines essential to the inflammatory response and clearance of host infection [3]. Upon encountering an invading pathogen, DCs are competent to ingest antigens via several mechanisms including phagocytosis and receptor-mediated endocytosis [5]. In relation to T cells, DCs are able to skew the T cell subtype proliferation in varying conditions, and are particularly important in regulatory T cell development [2]. Given the key role that dendritic cells possess as mediators of innate and adaptive immune response, special attention has also been given to them as targets of vaccine development by serving as a vaccine vector [4, 6-9]. DCs, as a leukocyte group, are fairly heterogeneous and have complex physiological function, thus underscoring the need for a better understanding of their function.

Despite the knowledge gathered on their role in adaptive tolerance, there is comparatively little known about tolerization of dendritic cells themselves. In monocytes and macrophages, which are closely related in function to dendritic cells, cellular tolerance has been shown to occur in septic patients and with various TLR ligands, particularly the pyrogenic

endotoxin (lipopolysaccharide, or LPS) derived from Gram (-) bacteria [10-14]. Monocytes, macrophages, and dendritic cells all respond to bacterial components such as endotoxin through what have been termed pattern recognition receptors (PRRs) expressed on the surface of their cells [1, 13, 15-17]. Toll-like receptors (TLRs), necessary for the recognition of endotoxin and a wide array of pathogen-associated molecular patterns (PAMPs), are the major PPRs involved in cytokine response and co-stimulatory molecule expression when monocyte-derived cells are exposed to bacterial infection [13]. Extracellular and endosomal signaling by PAMPs through TLRs leads to cytokine activation through NF-κB and MAP kinase pathways, and this signaling is essential to inflammation and eventual activation of the adaptive immune response [1]. Immune response and inflammation is a complex pathophysiological state, and the investigation of endotoxin tolerance (ET) stems from the first descriptions of endotoxin tolerance in patients in the mid-20th century where re-exposure to endotoxin after an initial exposure to minute levels failed to induce fever as it typically does on initial exposure [18-24]. This hyporesponsiveness to otherwise stimulatory bacterial components would suggest the development of intracellular regulation of TLR signaling within the cells that detect bacterial components and release inflammatory signals.

Indeed, regulation of the inflammatory response through adaptive mechanisms is an important means to check autoimmune reactions and cancer, maintain normal metabolic function, and prevent the emergence of over-compensatory immune reaction in the form of a tissue-damaging "cytokine storm" [5, 16, 22, 25-28]. This type of immune regulation would be predicted to serve an important role in a septic infection; development of endotoxin shock during sepsis is avoided if the host develops endotoxin tolerance, or a refractory period after

exposure to minute amounts of endotoxin [18, 20, 22-23, 29-31]. This phenomenon where cells exposed to low concentrations of LPS shift into transient unresponsiveness to further stimulation with LPS would, therefore, seem evolutionarily favorable to survival. However, a delicate balance must be achieved to keep the immune system under control while not suppressing its function so severely as to prevent it from being able to clear the pathogen or combat secondary infections.

This phenomenon has been shown to occur *in vivo* and *in vitro* in human and animal models, with essentially all *in vitro* experiments dealing with monocytes and macrophages [10-11, 14, 23, 32-35]. It was shown that hyporesponsiveness of circulating human monocytes could be induced with as little as one hour of exposure to low-dose LPS, and that this tolerant state was maintained for up to 5 days, after which the cells reverted to a phenotype similar to that exhibited pre-exposure [11]. This tolerant phenotype is correlated with the up-regulation of a number of cell receptors, enhanced phagocytosis, decreased antigen presentation, and a decrease in pro-inflammatory cytokine release [19, 21, 25, 35-36].   ET has been identified as playing a role in diverse pathological settings besides sepsis, including trauma, surgery, pancreatitis, hepatic ischemia, acute coronary syndrome, cystic fibrosis, and even cancer [5, 13, 15, 22, 24, 29, 37]. However, the behavior of dendritic cells during this sort of transient unresponsiveness, as well as how dendritic cells respond to aggregated bacterial components, has been left mostly unexplored.

Further investigation into endotoxin tolerance seems to suggest not just a simple instance of immunosuppression of monocytes and macrophages during transient tolerance, since pro-inflammatory cytokines (such as TNF-α and IL-12) tend to decrease their expression,

whereas as anti-inflammatory cytokines (such as IL-10 and TGF-β) tend to increase their expression. Thus, it has been suggested to describe ET as an instance of "immunomodulation" to better encapsulate the altered, rather than decreased, function of the immune cells [13]. Even though inflammatory cytokines activated by NF-κB and anti-inflammatory cytokines (such as IL-10) are both activated through TLR4 signaling by LPS, they are controlled by different adaptor molecules (MyD88 vs TRIF), and thus their pathways can be differentially regulated [38]. Besides modification of signaling pathways, it has been further demonstrated that ET leads to histone modification within chromatin, which modifies the accessibility of transcription factors and RNA polymerase to genes [39]. Exposure to LPS was shown to lead to histone modification in tolerant and non-tolerant cells, however only in tolerant cells were particular antimicrobial genes activated for transcription, indicating the first LPS exposure as a "warm-up" for the second exposure during which a more robust response of antimicrobial genes developed [16, 26].

A key immunomodulatory molecule that has arisen as important in endotoxin tolerance is interleukin-1 receptor-associated kinase-M (IRAK-M). In human circulating monocytes of human septic patients that display refractory tolerance, IRAK-M is expressed in much higher levels, and it has been shown to be essential for ET *in vitro* in macrophages [12, 14, 34, 40]. IRAK-M has been identified as a potent negative regulator of TLR signaling through MyD88 leading to decreased signaling through NF-κB and consequently decreased expression of the pro-inflammatory cytokines for which it serves as a transcriptional activator (*TNFA, IL1B, IL6, and IL12B,* among others) [40]. It is unclear how exactly IRAK-M functions, but current models describe IRAK-M as inhibiting the separation of IRAK-1 and IRAK-4 from the TLR signaling

complex. This prevents further signaling in the cascade to deactivation of IκB, which allows NF-κB activation, by either stabilizing the TLR/MyD88/IRAK-1/4 complex or inhibiting phosphorylation of IRAK-1 and IRAK-4 [40]. It has been observed in murine macrophages and human monocytes that the ET state correlates with impaired IRAK-1 activity [28, 31], so it is not unusual that IRAK-M might play an important role in maintaining ET in these cells. It has been demonstrated that IRAK-M is up-regulated in macrophages and monocytes, and a few groups have shown it as "essential" for transient tolerance in these cells [10, 38, 40]. In dendritic cells, IRAK-M has not been characterized or shown to be present, nor has it been shown what role, if any, it plays in transient tolerization of dendritic cells to bacterial components.

H Y P O T H E S I S

Based on the inflammatory response of human and murine monocytes and macrophages during endotoxin tolerance, and a few studies that have indicated tolerance in dendritic cells, we hypothesize the following:

- When murine BM-DCs are tolerized with low levels of LPS or bacterial sonicate and then re-exposed to the same component, they will exhibit cytokine modulation compared to an untolerized control

    o Decreased levels of pro-inflammatory cytokine production (TNF-α, IL-6, IL-12)

    o Increased levels of anti-inflammatory cytokine production (IL-10)

- During endotoxin tolerance and bacterial sonicate tolerance, autocrine function of IL-10 will contribute to the decreased expression of pro-inflammatory cytokines

- During endotoxin tolerance, interleukin 1 receptor-associated kinase-M (IRAK-M), a negative regulator of TLR signaling, will be expressed in increased amounts over an untolerized control

- Tolerance with LPS or bacterial sonicate will be ablated in IRAK-M knockout mice due to the role of IRAK-M in negatively regulating inflammatory TLR signaling

MATERIALS AND METHODS

Mice

Specific-pathogen-free C57BL/6 mice aged 8–10 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the animal maintenance facility at the University of Michigan Health System under SPF conditions.. Experiments were conducted on mice between the ages of 10 and 14 weeks. IRAK-M -/- mice with a truncated functional domain were generously provided Ted Standiford (University of Michigan Medical School, Department of Internal Medicine, Pulmonary and Critical Care Medicine), and were the same age as wild type mouse when chosen for experimentation. All animal experiments were approved by the University of Michigan Animal Care and Use Committee.

Media and cytokines

For all experiments, bone marrow-derived DCs were cultured in complete medium consisting of RPMI-1640 (Sigma) with 9% heat-inactivated fetal calf serum (ISC Biosciences), 2 mM added glutamine (4 mM total), 100 U/ml penicillin, and 100 μg/ml streptomycin. The following recombinant cytokines (R&D Systems, Minneapolis, MN) were diluted in complete medium during culturing: mouse granulocyte/macrophage colony-stimulating factor (GM-CSF; 10 ng/ml) and mouse IL-4 (10 ng/ml). After harvest of the cells at day 6 for experimentation, only recombinant mouse IL-4 was not included in the complete medium.

Bacterial strains and culture conditions

Helicobacter pylori was grown on *Campylobacter*-selective agar (BD Diagnostics, Bedford, MA) and supplemented with 5% sterile horse blood, trimethoprim (5 µg/ml), vancomycin (10 µg/ml), and nystatin (10µg/ml) for 2-4 days at 37°C in a humidified microaerophilic chamber (BBL Gas System, with CampyPak Plus packs, BD Microbiology, Sparks, MD). *In vitro* experiments were performed using *H. pylori* SS1 which was grown as described above. *E. coli* was cultured overnight in Luria-Bertani (LB) medium at 37°C. To prepare the bacterial sonicate, bacteria were diluted in PBS (Invitrogen) to a concentration of 1×10^9 /ml and subjected to repeated sonication in an ultrasonic bath to produce a crude bacterial sonicate. Protein levels were assayed using a BSA standard (Bio-Rad), and protein concentration was used as representative of proportional amounts of all bacterial components. LPS was purchased from Sigma and was derived from *E. coli* strain O127:B8. LPS was diluted in PBS.

Generation of bone marrow-derived DCs.

Mice were humanely euthanized with CO_2 and the femur and tibia were promptly excised for extraction of bone marrow. Muscles and tendons were removed from the femur and tibia by gentle scraping with a blade until clean and washed with ethanol. The epiphyses of the bones were removed to expose the marrow. Murine bone marrow cells were suspended in PBS, treated with an ionic lysing buffer in order to deplete red blood cells, and cultured in complete medium with 10 ng/ml GM-CSF and 10 ng/ml IL-4 at 1×10^6 cells/ml. On day 3, 50% of the complete media was aspirated and replaced. On day 6, non-adherent DCs were harvested

by vigorous pipetting and enriched by gradient centrifugation using the OptiPrep density solution (Sigma) according to manufacturer's instruction. The interface between the high and low density solutions containing the DCs was collected by gentle aspiration. The recovered DCs were washed twice with RPMI-1640 and cultured in complete medium with GM-CSF (10 ng/ml).

Tolerization and stimulation of DCs

DCs were tolerized with either 10 ng/ml LPS (unless otherwise indicated) or 2 µg/ml sonicate from *Escherichia coli* or *Helicobacter pylori* produced as described above. Cells were allowed to tolerize for 7-8 hours. After tolerization, culture media was collected and cells were washed 3 times with PBS (Invitrogen). Cells were then re-plated with complete media and allowed to rest overnight (16-18 hrs). This rest period was altered only for the initial dose response and time course experiments, where the rest period was around an hour. A second stimulation was then administered of 100 ng/ml LPS (unless otherwise noted) or 10 µg/ml bacterial sonicate from *Escherichia coli* or *Helicobacter pylori* produced as described above. Cells were collected at 3 hours for mRNA analysis, 8 hours for cytokine analysis, and 8 hours for protein isolation.

mRNA isolation

DCs were collected at three hours by media aspiration and cell scraping. Cells were promptly centrifuged, media aspirated, and treated with TRIzol reagent per instructions of manufacturer (Invitrogen). mRNA pellets were reconstituted in nuclease-free water and purity

analysis was done. All samples used for analysis had A_{260}/A_{280} ratios greater than 1.9 upon spectrophotometric analysis.

cDNA synthesis and qPCR

Total mRNA isolated from TRIzol extraction was adjusted to a concentration of 250 µg/ml and 1 µg of sample was used to synthesize cDNA using iScript cDNA synthesis kit in 20 µl solutions per instructions of manufacturer (Bio-Rad). cDNA was then analyzed by qPCR using iQ SYBR Green Supermix per instructions of manufacturer (Bio-Rad). GAPDH was used as an endogenous housekeeping reference gene. Murine primers (Invitrogen) were:

- GAPDH – Forward: 5' – TCAAGAAGGTGGTGAAGCAGG - 3' reverse: 5' – TATTATGGGGGTCTGGGATGG - 3'

- IL-10 – Forward: 5' - CTTACTGACTGGCATGAGGATCA - 3' reverse: 5'- AGCTGGTCCTTTGTTTGAAAGAAA – 3'

- IRAK-M – Forward: 5' – TGAGCAACGGGACGCTTT – 3' reverse: 5' – GATTCGAACGTGCCAGGAA – 3'

ELISA analysis

Supernatant was collected and centrifuged at 8 hours post second stimulation, and cell-free supernatants were stored in -20^0 for analysis. TNF-α, IL-6, IL-10, and IL-12p70 cytokine measurements were performed through ELISA per instructions of manufacturer (eBioscience, BD Biosciences).

Western blot

DCs were collected through aspiration and plate scraping, centrifuged, washed once with PBS, and lysed with solution containing protease and phosphatase inhibitors (Cell Signaling Technologies) to release intracellular protein. Protein levels were then equilibrated and run through SDS-PAGE. Proteins were then transferred onto PVDF membrane, blotted with primary and secondary antibodies (Abcam), and protein detection was carried out by chemiluminescence. GAPDH was used as an endogenous housekeeping reference protein.

IL-10 neutralization

IL-10 neutralization was performed with purified recombinant goat IgG antibody specific for murine IL-10 (R&D Systems). Antibody was tested to be preservative-, and carrier-free. Neutralizing antibody was diluted in sterile PBS and frozen at -20°, and working concentration in cell culturing conditions was 800 ng/ml, a concentration shown by manufacturer IL-10 neutralization data to exhibit >85% neutralization of IL-10 at concentrations below 2.5 ng/ml of IL-10. ND_{50} is 30-100 ng/ml according to manufacturer data.

R E S U L T S

The results of the current study reveal a number of novel findings related to tolerance within bone marrow-derived dendritic cells.

BM-DCs tolerized with endotoxin and bacterial sonicate exhibit decreased expression of pro-inflammatory cytokines TNF-α and IL-12p70

Bone marrow-derived dendritic cells were cultured for 6 days in complete media supplemented with FBS, GM-CSF, and IL-4 and enriched by density gradient centrifugation. As might be expected in tolerized dendritic cells, levels of the pro-inflammatory cytokines TNF-α and IL-12p70 were significantly decreased when cells were first treated with low dose LPS (10 ng/ml), *H. pylori* strain SS1 sonicate (2 μg/ml), and *E. coli* sonicate (2 μg/ml) for 8 hours, washed thoroughly, allowed to rest overnight, and then subsequently stimulated with the same component they were tolerized with but at higher doses, either LPS (100 ng/ml), SS1 sonicate (10 μg/ml), or *E. coli* sonicate (10 μg/ml) (*Figure 3* and *Figure 4*). Along with demonstrating an adequate model of dendritic cell tolerization with respect to these pro-inflammatory cytokines, this is the first demonstration of dendritic cell tolerization with crude bacterial sonicate. These results indicate that dendritic cells may not only be tolerized with a single TLR ligand (LPS), but also by a crude bacteria extract containing numerous TLR ligands and other PAMPs, and that these additional PAMPs do not prevent the development of tolerance with respect to the inflammatory cytokines TNF-α and IL-12p70. Tolerance occurred to nearly the same degree regardless of whether the bacterial sonicate was derived from *Helicobacter pylori* strain SS1 or *Escherichia coli*.

BM-DCs tolerized with endotoxin and bacterial sonicate exhibit increased expression of the anti-inflammatory cytokine IL-10

As has been shown to occur in macrophages and monocytes, the potent anti-inflammatory cytokine IL-10 was expressed in significantly elevated levels when cells were first treated with low dose LPS (10 ng/ml), *H. pylori* strain SS1 sonicate (2 µg/ml), and *E. coli* sonicate (2 µg/ml) for 8 hours, washed thoroughly, allowed to rest overnight, and then subsequently stimulated with the same component they were tolerized with but at higher doses, either LPS (100 ng/ml), SS1 sonicate (10 µg/ml), or *E. coli* sonicate (10 µg/ml) (*Figure 5* and *Figure 6*). This was shown preliminarily though measurement of IL-10 transcript (*Figure 5*) as well as directly measured protein levels (*Figure 6*). Although IL-10 release has been shown to be increased in LPS-tolerized DCs, increased IL-10 expression in cells tolerized and subsequently stimulated with crude bacterial sonicate represents a novel finding. In keeping with the observed tolerogenic phenotype of sonicate-tolerized dendritic cells with regards to TNF-α and IL-12p70, these tolerized cells express IL-10 in increased amounts with both single ligand stimulation and multiple ligand stimulation from bacterial sonicate. The degree of increased IL-10 expression did not appear to vary significantly between sonicate derived from *H. pylori* or *E. coli.* This trend in IL-10 expression of tolerized DCs further supports the tolerance model of the current experiment.

BM-DCs tolerized with H. pylori *SS1 sonicate, but not endotoxin or* E. coli *sonicate, exhibit decreased expression of the pro-inflammatory cytokine IL-6*

Expression of IL-6 in LPS- and sonicate-tolerized BM-DCs shows a deviation from the relatively uniform trends in expression of TNF-α, IL-12p70, and IL-10. The pro-inflammatory cytokine was not expressed in significantly elevated levels when cells were first treated with low dose LPS (10 ng/ml) or *E. coli* sonicate (2 µg/ml) for 8 hours, washed thoroughly, allowed to rest overnight, and then subsequently stimulated with the same component they were tolerized with but at higher doses, either LPS (100 ng/ml) *E. coli* sonicate (10 µg/ml) (*Figure 7*), as might be predicted from the trend in TNF-α and IL-12p70 in tolerized DCs. However, in DCs tolerized with *H. pylori* SS1 sonicate, IL-6 expression is decreased with relation to non-tolerized DCs. IL-6, IL-12p70, and TNF-α transcription are all influenced by signaling through NF-κB, so this trend in IL-6 expression in LPS- and *E. coli* sonicate-tolerized DCs is interpreted as unusual. This indicates that *Helicobacter pylori* crude sonicate may possess a component that is able to uniquely influence IL-6 expression whereas as LPS and *E. coli* sonicate do not.

Autocrine action of IL-10 does not contribute to suppressed TNF-α expression in BM-DCs tolerance with endotoxin or H. pylori *SS1 sonicate*

To examine the influence of IL-10 in the development of tolerance in bone marrow derived dendritic cells, cells were tolerized and subsequently stimulated in the presence of an IL-10 neutralizing antibody. Tolerization was conducted with LPS and *H. pylori* sonicate and followed the same procedure as previously described. After 8 hours of stimulation, IL-10 was assayed for by ELISA and revealed undetectable levels (data not shown). In dendritic cells tolerized with LPS and *H. pylori* SS1 sonicate, there was no significant alteration in the development of tolerance when IL-10 was neutralized (*Figure 8*).   This indicates that IL-10 does

not act in an autocrine fashion to suppress the expression of the pro-inflammatory cytokine TNF-α. IL-10 has been shown to suppress TNF-α expression, however it appears that TNF-α is suppressed in tolerant dendritic cells through mechanisms independent of IL-10.

BM-DCs tolerized with endotoxin display increased expression of IRAK-M transcript and protein

In order to determine if IRAK-M, a negative regulator of TLR signaling, demonstrated increased expression when BM-DCs were tolerized with low dose LPS (10 ng/ml) and then re-exposed to a higher LPS dose (100 ng/ml), cells were assayed for the presence of IRAK-M transcript and protein. This protein was shown to be up-regulated in macrophages and monocytes when these cells are tolerized, and has even been shown to be "essential" to tolerance, but has not been identified or characterized in dendritic cells. Our results indicate significantly increased expression of IRAK-M transcript in tolerized BM-DCs as determined by qPCR, as well as an increase in IRAK-M protein expression as determined by Western blot (*Figure 9*). Since, in the literature, IRAK-M has not been confirmed to be present in dendritic cells, this represents a novel identification. Given the similar increase in IRAK-M during tolerance as in macrophages and monocytes, these results point to the possibility of IRAK-M having an analogous role in tolerance in dendritic cells

IRAK-M is not essential to tolerance in BM-DCs with respect to TNF-α, but does affect IL-10 expression in BM-DCs tolerized with bacterial sonicate

Utilizing BM-DCs derived from IRAK-M knockout (truncated functional domain) mice, the absence of IRAK-M was shown to not affect the development of tolerance with respect to

TNF-α in BM-DCs tolerized with low dose LPS (10 ng/ml), *H. pylori* strain SS1 sonicate (2 µg/ml), or *E. coli* sonicate (2 µg/ml) for 8 hours, washed thoroughly, allowed to rest overnight, and then subsequently stimulated with the same component they were tolerized with but at higher doses, either LPS (100 ng/ml), SS1 sonicate (10 µg/ml), or *E. coli* sonicate (10 µg/ml) (*Figure 10* and *Figure 11*). In fact, in wild type BM-DCs compared to IRAK-M -/- BM-DCs, tolerized IRAK-M -/- cells showed a significant *decrease* in the expression of TNF-α. This is a peculiar finding in light of the role of IRAK-M as a negative regulator of TLR signaling; if IRAK-M acts as a negative regulator of inflammatory signaling, removing the expression of the protein in the cells should result in the recovery of pro-inflammatory cytokine response in tolerized BM-DCs, yet this was not observed. Indeed, the opposite effect was observed in the absence of expression of IRAK-M. IRAK-M -/- BM-DCs also maintained the tendency to increase IL-10 expression in only endotoxin-tolerized cells; however this ability was not maintained in IRAK-M -/- cells tolerized with *H. pylori* SS1 sonicate or *E. coli* sonicate and subsequently re-stimulated with a higher amount of sonicate (*Figure 11*), as was demonstrated in wild type BM-DCs. These sonicate-tolerized cells showed no significant difference in IL-10 expression regardless of tolerization status. This indicates that IRAK-M may play differing roles as a negative regulator of TLR signaling in cells tolerized with a single stimulatory ligand, such as with LPS alone, or multiple stimulatory ligands, such as with crude bacterial sonicate. Other regulators may also play a more influential role in the development of tolerance in dendritic cells.

TNF-α dose response in DCs tolerized with LPS
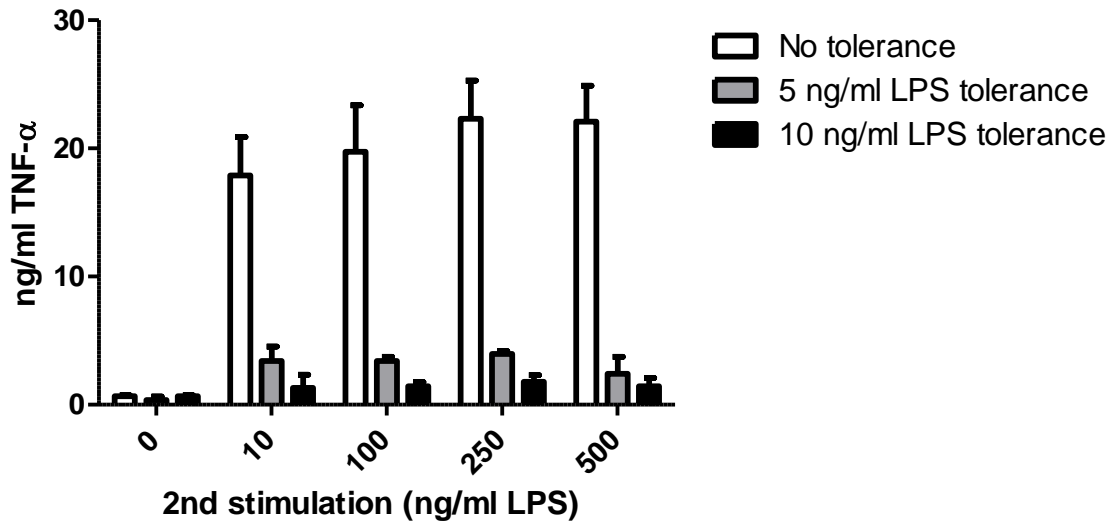
F I G U R E 1 . TNF-α expression with varying low doses of LPS tolerization and subsequent higher dose stimulation. 8 hrs after second stimulation (n=3). Dendritic cells were tolerized with either no LPS, 5 ng/ml LPS, or 10 ng/ml LPS overnight, thoroughly washed, rested for a short period (less than an hour), and re-stimulated with the indicated dose of LPS (x-axis) to physiologically mimic primary and secondary exposures. Tolerance with 10 ng/ml LPS and stimulation with 100 ng/ml LPS was continued throughout the current study. *Note: procedure was modified in later experiments since rest time was deemed to be of inadequate length to allow cells to recover.* Data represent mean ± SD. *, $p < 0.05$

TNF-α time course in DCs tolerized with LPS
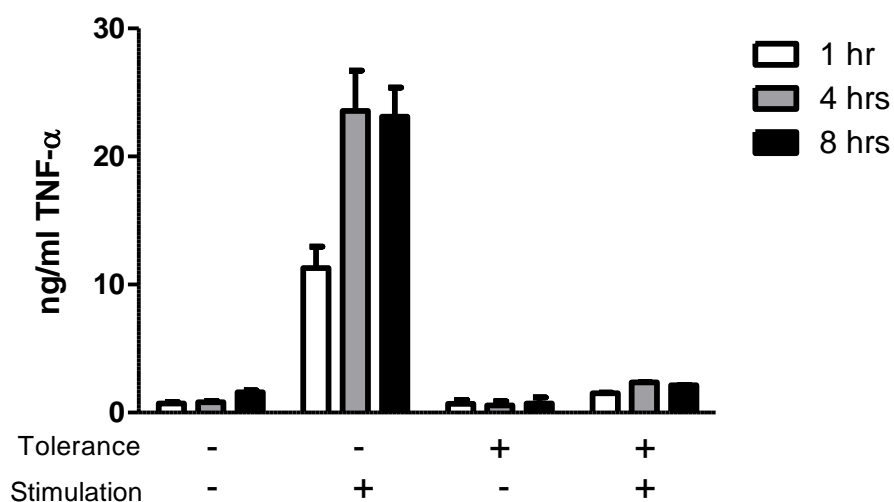
F I G U R E 2 . **TNF-α expression by BM-DCs during tolerance time course.** Cell-free supernatant harvested at 1-8 hrs after second stimulation (n=3). Dendritic cells were tolerized with 10 ng/ml LPS, thoroughly washed, rested for a short period (less than an hour), and re-stimulated with 100 ng/ml LPS to physiologically mimic primary and secondary exposures. Tolerance with 10 ng/ml LPS and stimulation with 100 ng/ml LPS was continued throughout the current study. *Note: procedure was modified in later experiments since rest time was deemed to be of inadequate length to allow cells to recover.* Data represent mean ± SD. *, $p < 0.05$

A TNF-α in DCs tolerized with LPS

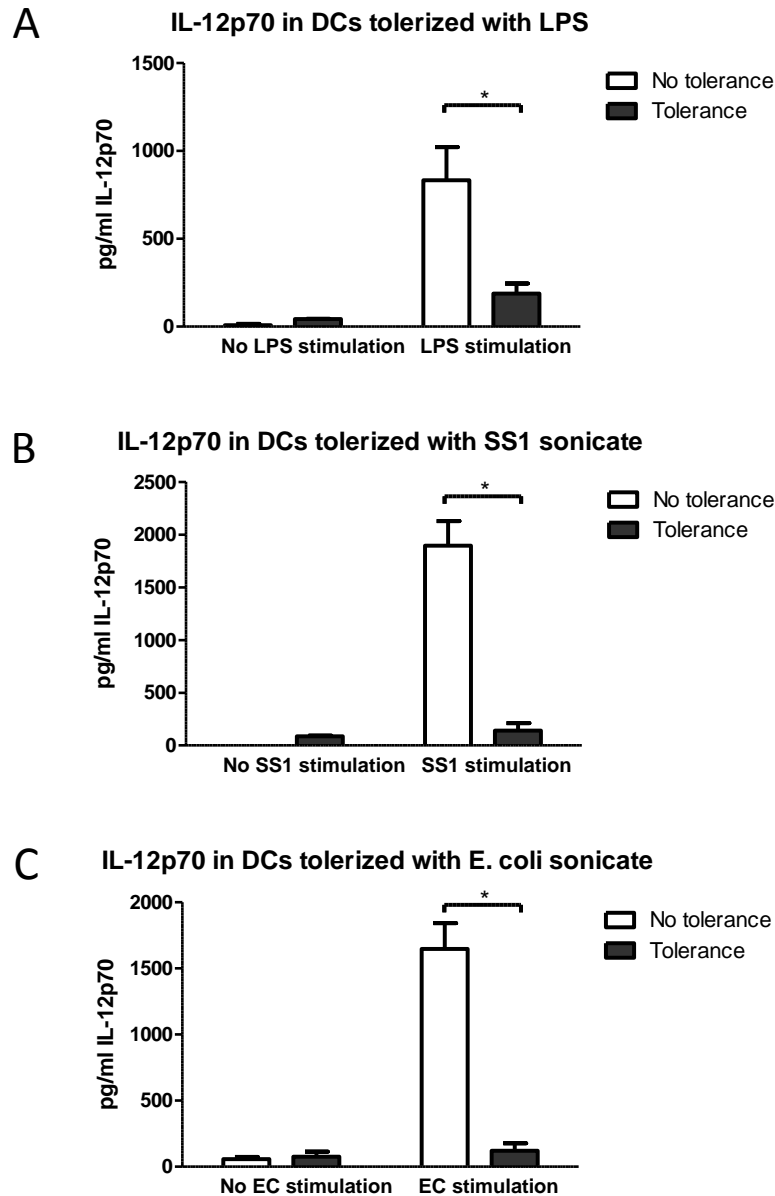**B** TNF-α in DCs tolerized with SS1 sonicate

C TNF-α in DCs tolerized with E. coli sonicate

F I G U R E 3 . **TNF-α expression decreases in supernatant of DCs tolerized with LPS, *H. pylori* SS1 sonicate, or *E. coli* sonicate**. 8 hrs after 2nd stimulation (n=3). *A-C*, Tolerized DCs exhibit a significant ablation in secreted TNF-α (a potent pro-inflammatory cytokine) upon second stimulation by higher dose LPS, SS1 sonicate, or *E. coli* sonicate. Non-tolerized DCs display a robust TNF- α response upon stimulation with higher dose LPS, SS1 sonicate, or *E. coli* sonicate. This confirms that, with respect to TNF-α cytokine expression, BM-DCs behave in a similar manner as monocytes and macrophages under conditions of tolerance due to previous exposure to low-dose stimulation with LPS. A similar effect is observed with tolerance due to low dose bacterial sonicate exposure. $1^{st}/2^{nd}$ dose LPS = 10/100 ng/ml. $1^{st}/2^{nd}$ dose sonicate = 2/10 µg/ml. Data represent mean ± SD. *, $p < 0.05$

A **IL-12p70 in DCs tolerized with LPS**

B **IL-12p70 in DCs tolerized with SS1 sonicate**

C **IL-12p70 in DCs tolerized with E. coli sonicate**

F I G U R E 4 . **IL-12p70 expression decreases in supernatant of DCs tolerized with LPS, *E. coli* sonicate, or *H. pylori* SS1 sonicate**. 8 hrs after 2nd stimulation (n=3). *A-C*, Tolerized DCs exhibit a significant decrease in secreted IL-12p70 (a potent pro-inflammatory cytokine) upon second stimulation by higher dose LPS, SS1 sonicate, or *E. coli* sonicate. Non-tolerized DCs display a robust IL-12p70 response upon stimulation with higher dose LPS, SS1 sonicate, or *E. coli* sonicate. This confirms that, with respect to IL-12p70 cytokine expression, BM-DCs behave in a similar manner as monocytes and macrophages under conditions of tolerance due to previous exposure to low-dose stimulation with LPS. A similar effect is observed with tolerance due to low dose bacterial sonicate exposure. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 μg/ml. Data represent mean ± SD. *, $p < 0.05$

[IL-10/GAPDH] in DCs with LPS tolerance, 3 hrs. after 2nd stimulation

A

[IL-10/GAPDH] in DCs with SS1 sonicate tolerance, 3 hrs. after 2nd stimulation

B
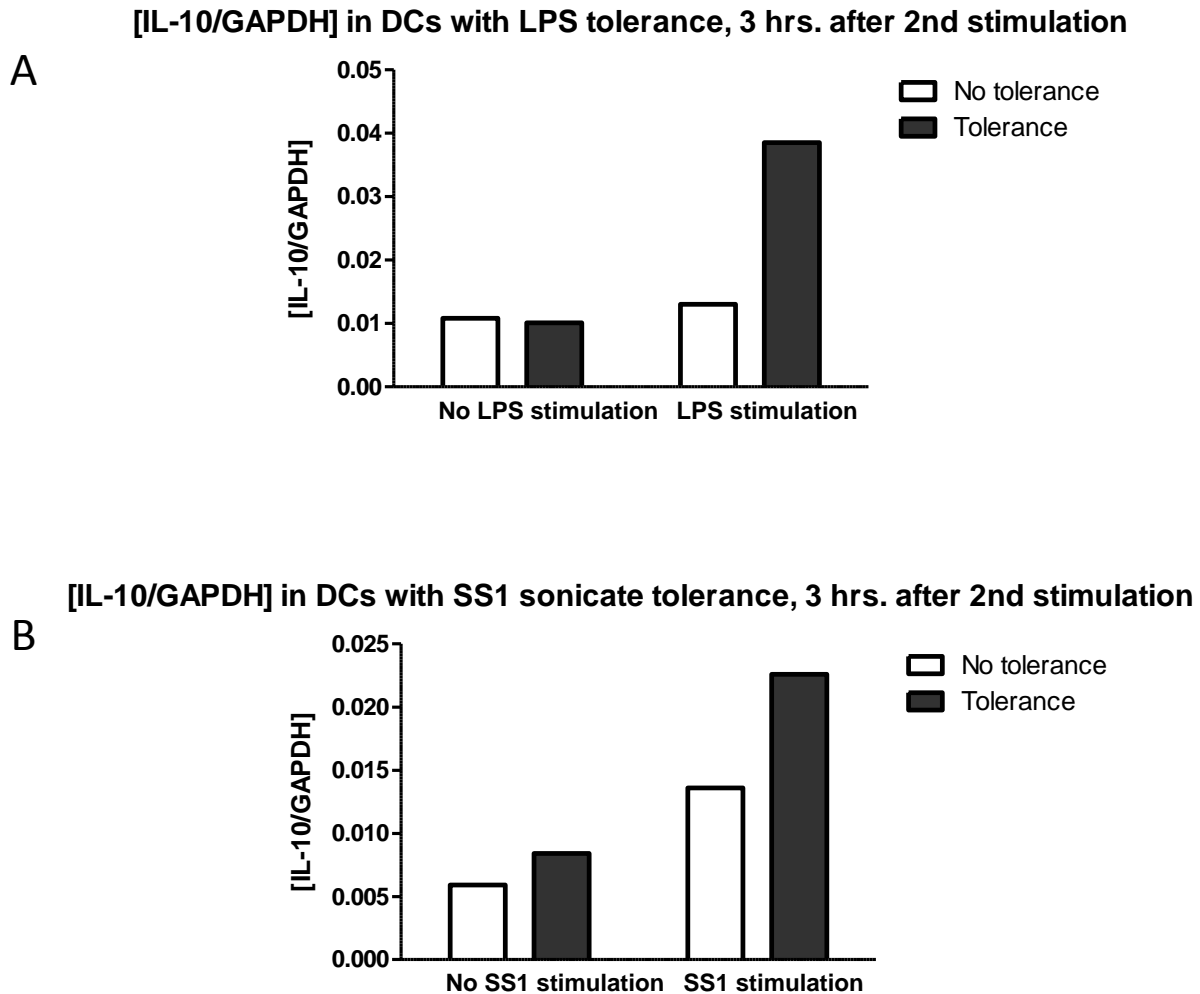
F I G U R E 5 . **IL-10 mRNA and secreted protein expression in DCs tolerized with LPS or *H. pylori* SS1 sonicate**. *A-B*, 3 hrs after 2nd stimulation (n=1). Preliminary experiment to determine IL-10 (a potent anti-inflammatory cytokine) behavior during tolerance. In both the LPS and SS1 tolerized groups, there is increased production of IL-10 transcript upon second stimulation by a higher dose of LPS or SS1 sonicate. Data normalized to GAPDH endogenous housekeeping reference gene. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 μg/ml.

A

IL-10 in DCs tolerized with LPS

B

IL-10 in DCs tolerized with SS1 sonicate

C

IL-10 in DCs tolerized with E. coli sonicate
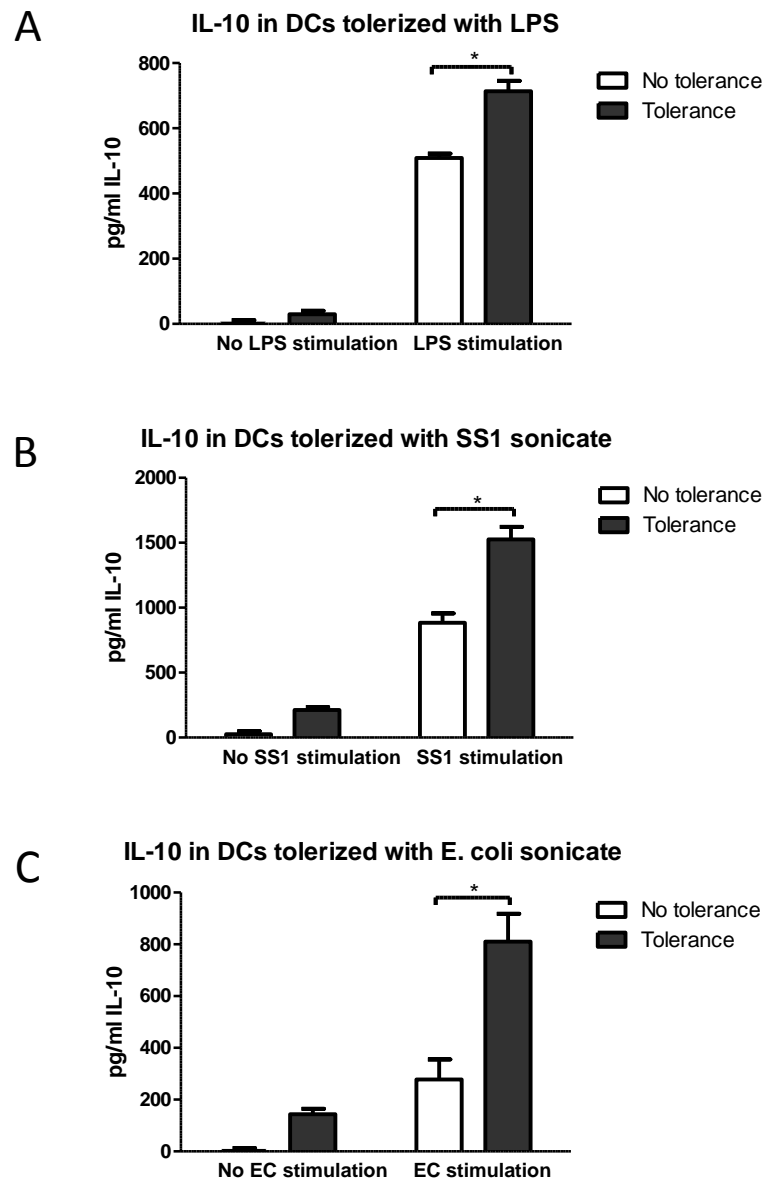
F I G U R E 6 . **IL-10 secreted protein expression in DCs tolerized with LPS, *H. pylori* SS1 sonicate, or *E. coli* sonicate**. *A-C,* BM-DC supernatant 8 hrs after 2nd stimulation (n=3). Closely tracking the mRNA results, tolerized DCs show a significant increase in secreted IL-10 (a potent anti-inflammatory cytokine) upon second stimulation by higher dose LPS, SS1 sonicate, or *E. coli* sonicate. Non-tolerized DCs display less IL-10 expression upon stimulation with higher dose LPS, SS1 sonicate, or *E. coli* sonicate. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 μg/ml. Data represent mean ± SD. *, $p < 0.05$

A **IL-6 in DCs tolerized with LPS**

B **IL-6 in DCs tolerized with SS1 sonicate**

C **IL-6 in DCs tolerized with E. coli sonicate**
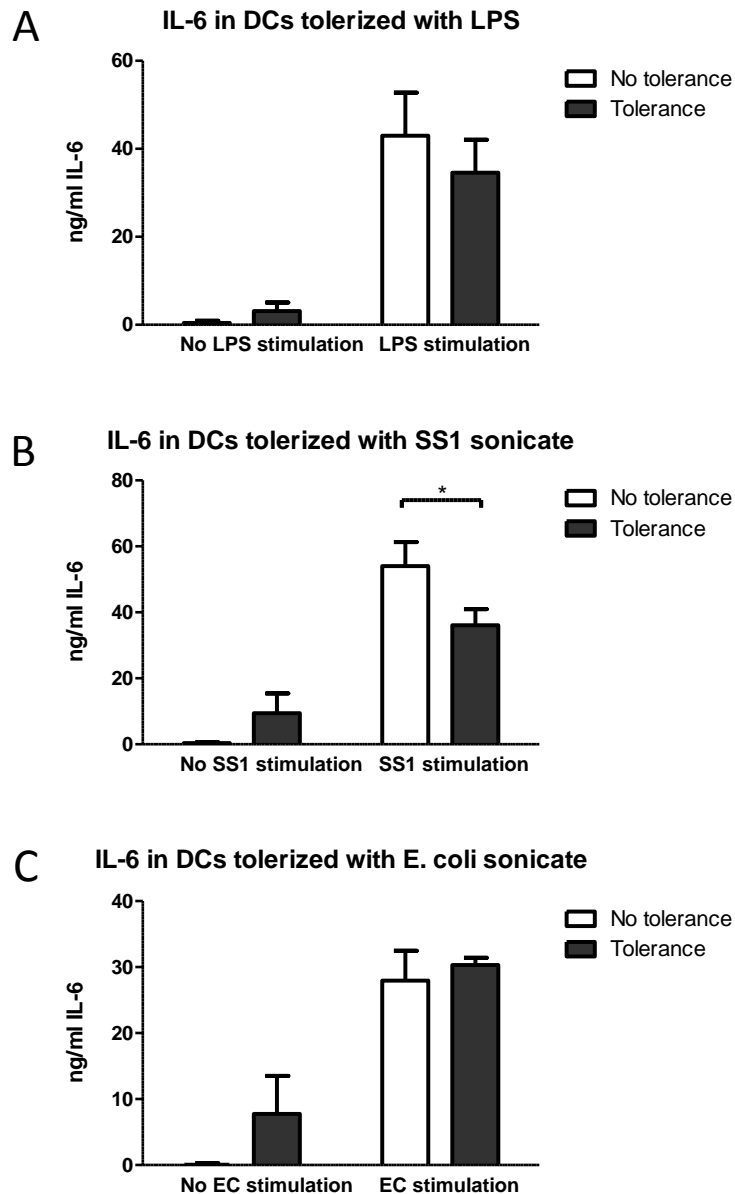
F I G U R E 7 . **IL-6 expression in supernatant of DCs tolerized with LPS, *H. pylori* SS1 sonicate, or *E. coli* sonicate**. 8 hrs after 2nd stimulation (n=3). *A,C,* Tolerized DCs exhibit no significant modulation of secreted IL-6 (a potent pro-inflammatory cytokine) upon second stimulation by higher dose LPS or *E. coli* sonicate as compared with non-tolerized DCs, which display a similar IL-6 response upon stimulation with higher dose LPS or *E. coli* sonicate. *B,* However, IL-6 expression by DCs tolerized with SS1 sonicate showed significantly decreased levels of IL-6 expression as compared to non-tolerized DCs, which show a robust IL-6 response to SS1 sonicate stimulation. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 µg/ml. Data represent mean ± SD. *, $p < 0.05$

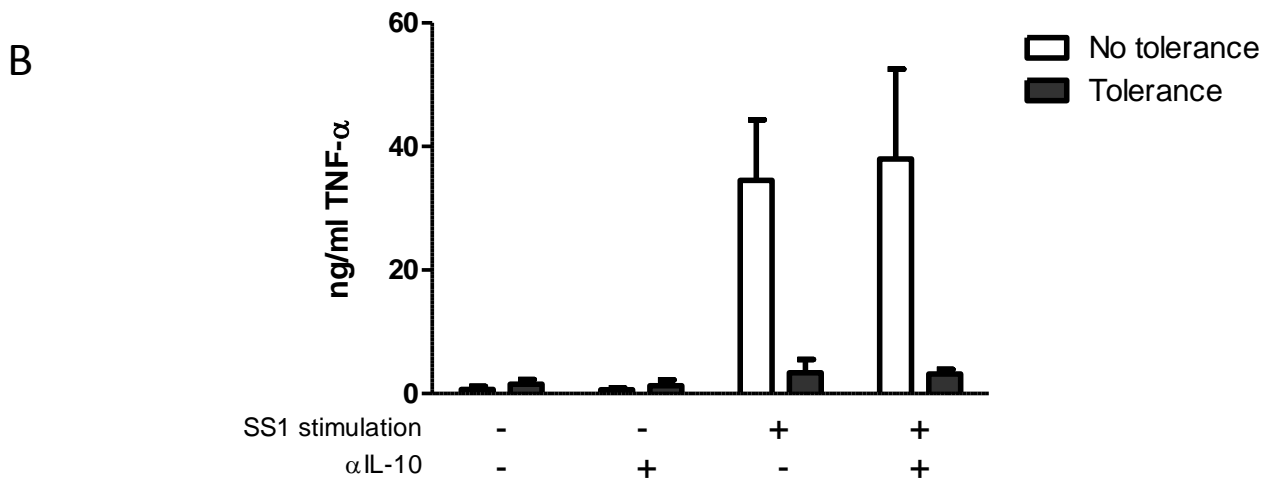## TNF-α in DCs tolerized with LPS + IL-10 neutralizing antibody

A

| LPS stimulation | - | - | + | + |
| αIL-10 | - | + | - | + |
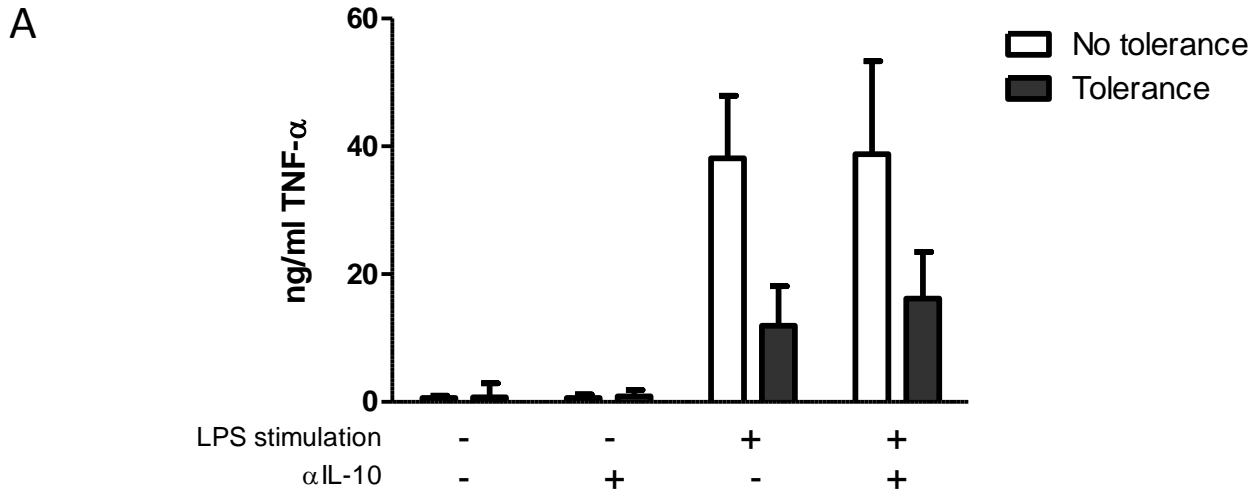
TNF-α in DCs tolerized with SS1 sonicate + IL-10 neutralizing antibody

B

| SS1 stimulation | - | - | + | + |
| αIL-10 | - | + | - | + |

F I G U R E 8 . **TNF-α expression in supernatant of DCs tolerized with LPS and *H. pylori* SS1 sonicate with or without IL-10 neutralizing antibody**. 8 hrs after 2nd stimulation (n=3). *A-B*, Tolerized DCs exhibit a significant ablation in secreted TNF-α (a potent pro-inflammatory cytokine) upon second stimulation by higher dose LPS or SS1 sonicate regardless of whether IL-10 was neutralized by an antibody. IL-10 neutralization was confirmed by ELISA and IL-10 was undetectable (data not shown). This demonstrates that IL-10 does not appear to have an autocrine role in suppressing TNF-α expression in tolerized BM-DCs. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 μg/ml. Data represent mean ± SD.

A **[IRAK-M/GAPDH] in DCs tolerized with LPS, 3 hrs. after 2nd stimulation**
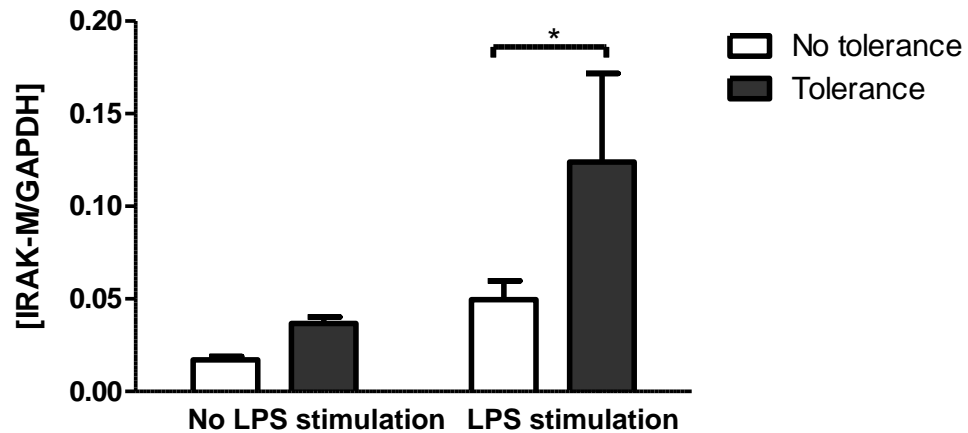
B IRAK-M (68 kd)

GAPDH (35 kd)

| Tolerance | − | − | + | + |
| Stimulation | − | + | − | + |

F I G U R E 9 . **IRAK-M mRNA and protein expression in DCs tolerized with LPS**. *A*, qPCR 3 hrs after 2nd stimulation (n=3). IRAK-M is a negative regulator of TLR signaling and has been shown to be essential to endotoxin tolerance in other monocyte-derived cell lineages. With LPS tolerance there is an observably increased production of IRAK-M transcript upon second stimulation by a higher dose of LPS. Data normalized to GAPDH endogenous housekeeping reference gene. *B*, Western blot 8 hrs after 2nd stimulation. BM-DCs tolerized with low-dose LPS and re-exposed to high-dose LPS appear to show increased intracellular IRAK-M expression compared to untolerized cells. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 µg/ml. Data represent mean ± SD. *, *p* <

A   **TNF-α in DCs tolerized with LPS, wild type vs. IRAK-M -/- mice**
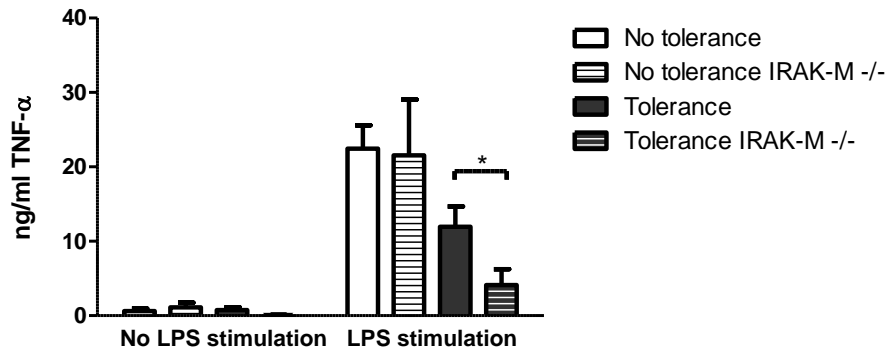
B   **TNF-α in DCs tolerized with SS1 sonicate, wild type vs. IRAK-M -/- mice**
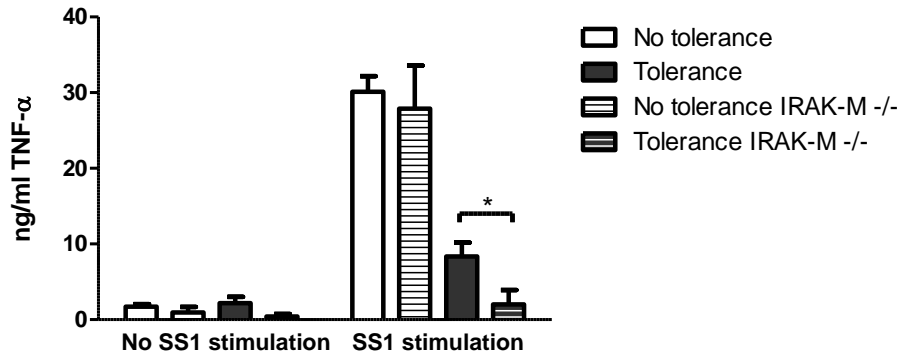
C   **TNF-α in DCs tolerized with E. coli sonicate, wild type vs. IRAK-M -/- mice**

F I G U R E   1 0 . **TNF-α expression in supernatant of wild type vs IRAK-M -/- DCs tolerized with LPS, *H. pylori* SS1 sonicate, or *E. coli* sonicate**. 8 hrs after 2nd stimulation (n=3). *A-C,* Tolerized DCs exhibit a significant ablation in secreted TNF-α (a potent pro-inflammatory cytokine) upon second stimulation by higher dose LPS, SS1 sonicate, or *E. coli* sonicate, regardless of whether cells were derived from IRAK-M -/- or wild type mice. Furthermore, IRAK-M -/- mice showed a greater degree of tolerance than wild type mice in relation to TNF-α release. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 μg/ml. Data represent mean ± SD.
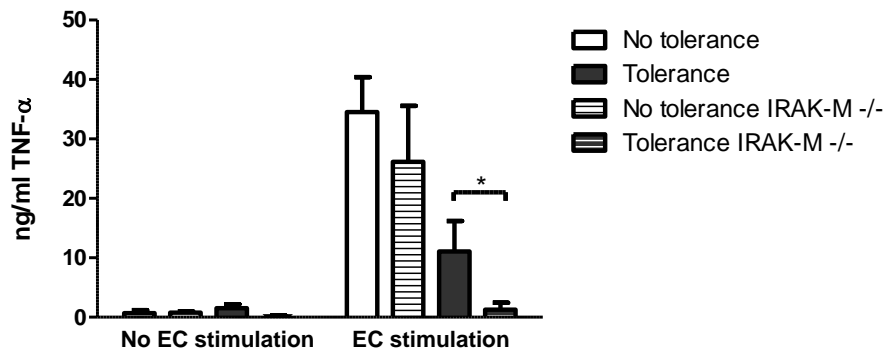
A **IL-10 in DCs tolerized with LPS, wild type vs. IRAK-M -/- mice**
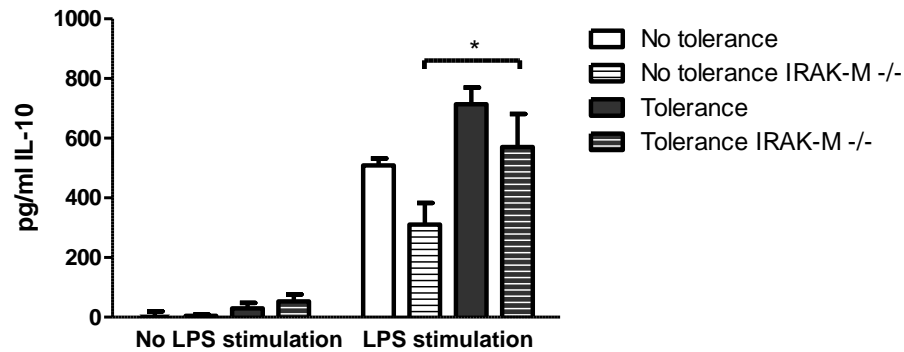
B **IL-10 in DCs tolerized with SS1 sonicate, wild type vs. IRAK-M -/- mice**
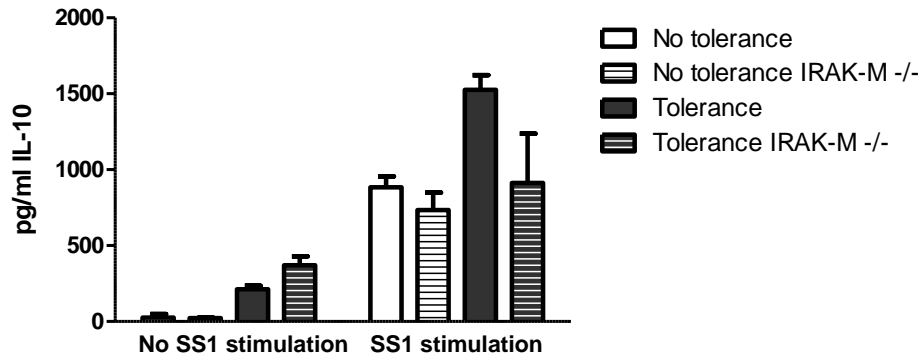
C **IL-10 in DCs tolerized with E. coli sonicate, wild type vs. IRAK-M -/- mice**
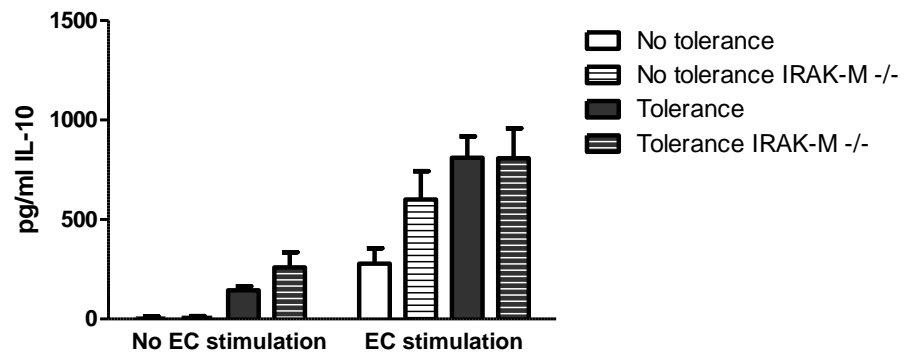
F I G U R E   1 1 . **IL-10 expression in supernatant of wild type vs IRAK-M -/- DCs tolerized with LPS, *H. pylori* SS1 sonicate, or *E. coli* sonicate**. 8 hrs after 2nd stimulation (n=3). *A*, LPS tolerized BM-DCs exhibit increased levels of IL-10 upon second stimulation by higher dose LPS regardless of whether cells were derived from IRAK-M -/- or wild type mice. *B-C*, SS1 sonicate and *E. coli* sonicate tolerized BM-DCs show little modulation of IL-10 in IRAK-M -/- BM-DCs as compared to wild type BM-DCs. This indicates a different influence of IRAK-M in BM-DCs tolerized with a single ligand (LPS) or multiple ligands (sonicate) with relation to IL-10 expression. 1st/2nd dose LPS = 10/100 ng/ml. 1st/2nd dose sonicate = 2/10 µg/ml. Data represent mean ± SD. *, $p < 0.05$

DISCUSSION

Although tolerance within monocytes and macrophages has been extensively explored, tolerance within dendritic cells remains investigated to a far lesser degree. Recent studies in dendritic cell tolerance have demonstrated the behavior of tolerized dendritic cells to individual ligands, as well as the changes in cell surface marker phenotype [17, 41-43]. Yet, more detailed study of regulation during tolerance in dendritic cells, as well as dendritic cells tolerance in the context of multiple ligands, was needed. This study aimed to develop a model of endotoxin tolerance within murine bone marrow-derived dendritic cells (BM-DCs), observe tolerance with crude bacterial components, as well as investigate the role of IL-10 (a potent anti-inflammatory cytokine) and IRAK-M in DC tolerance. IRAK-M was identified as essential to endotoxin tolerance in macrophages and monocytes, yet remained unidentified and uncharacterized in dendritic cells. Overall, the role of dendritic cells in adaptive tolerance has been well-investigated [3, 44], but a scarcity of research remains surrounding how DCs themselves might be tolerized.

After first exploring endotoxin dose response and time-dependent expression of TNF-α in BM-DCs tolerized with low dose endotoxin (*Figure 1* and *Figure 2*), a model was established with an initial low dose tolerization of 10 ng/ml LPS, a thorough wash, and a subsequent stimulation with 100 ng/ml LPS. Although not performed in dose response and time course experiments, an overnight rest was added to allow the cells to adequately recover. Permitting recovery time was deemed prudent after an extensive examination of tolerization in the literature. This model of a low dose followed by a higher dose has been used in several studies to imitate what would, *in vivo,* be primary and secondary exposures [13]. Along with LPS, crude

bacterial sonicate derived from *Helicobacter pylori* strain SS1 and *Escherichia coli* was used to tolerize BM-DCs, of which the tolerization dose (2 μg/ml) and stimulation dose (10 μg/ml) were determined in a similar fashion (data not shown, units of measure are μg/ml protein, taken as representative of proportional amounts of bacterial components). A model of tolerance with bacterial sonicate was developed in order to determine how dendritic cells might behave when exposed to multiple stimulatory ligands, TLR or otherwise, since this multi-ligand tolerance has not been investigated. Understanding how dendritic cells respond to tolerizing stimuli with crude bacterial sonicate, and whether or not they are able to be tolerized with sonicate, might be more representative of *in vivo* conditions than single-ligand tolerance (such as with only LPS) since bacterial components are typically present within the environment in which dendritic cells function. Bacteria and their components are regularly ingested or otherwise inoculated into essentially all organisms, so a model of initial and secondary exposure could mimic repeated exposure in the stomach or bowel, shown to be sites of DC function, or of circulating dendritic cells in a systemic infection. How dendritic cells respond to initial stimuli is critical to how they interact later in modeling the adaptive immune response [45]. *E. coli* was taken as an archetypal model of Gram (-) bacterial exposure, and *H. pylori* was chosen due to its interesting immunomodulatory capabilities and its ability to chronically colonize the gastric epithelium, recently shown to be a site where the bacterium and dendritic cells interact (Kao, awaiting publication). Additionally, *H. pylori* has been shown to demonstrate complex pathologies involving, but not limited to, gastric cancer, esophageal cancer, asthma, gastroesophageal reflux disease, and inflammatory bowel disease [46-51].

Examination of the literature revealed that monocyte-derived cells, such as mature monocytes, macrophages, and to a lesser extent dendritic cells, can be tolerized and display a typical modulated cytokine response, with significantly decreased pro-inflammatory cytokine response (TNF-α, IL-12, IL-6, IL-1β) and increased anti-inflammatory cytokine response (IL-10, TGF-β) [5, 10-13, 17, 23, 28, 30-31, 34, 37, 39, 42-43, 52-59]. Furthermore, these cells display a regular pattern of decreased antigen presentation, enhanced phagocytosis, and expression of negative regulators (particularly IRAK-M) [19, 21, 25, 35-36]. This type of tolerance has been implicated in a wide array of acute and chronic diseases, ranging from sepsis to cystic fibrosis [13]. In addition to LPS derived from the two bacteria used in this study (*Helicobacter pylori* and *Escherichia coli*), the sonicate would also include various cell wall components, flagellin, intracellular proteins, genetic material in the form of RNA and DNA, and lipoproteins, all of which can lead to inflammatory signaling via TLR and other receptors, as well as endocytosis [1]. Although LPS is considered the major source of inflammation through TLR4 signaling, other components that signal through TLR receptors (lipoproteins, TLR1-6; CpG DNA, TLR9; flagellin, TLR 5; RNA, TLR7-8) are highly capable of potentiating inflammatory responses individually [1]. There are also varying signaling cascades/pathways and adaptor molecules through which the TLR stimulation may flow [1], as well as negative regulators of this signaling [38], thus making the question of whether tolerance can occur in DCs with various stimulatory components an interesting one to investigate.

The current study has demonstrated that, in addition to macrophages and monocytes, BM-DCs possess the ability to become tolerized, and that this tolerance can occur not just with endotoxin but also bacterial sonicate with an aggregate of microbial components. With respect

to the inflammatory cytokines TNF-α and IL-12p70, tolerized DCs showed significantly

decreased expression (*Figure 3* and *Figure 4*). Interestingly, though, the inflammatory cytokine

IL-6 only showed a significant decrease during tolerization when the tolerization occurred with

H. pylori sonicate (*Figure 7*). This effect is intriguing since IL-6, IL-12, and TNF-α are all under the

regulation of NF-κB, and the fact that IL-6 is differentially regulated by *H. pylori* sonicate

tolerization may indicate the presence of an *H. pylori*-derived factor that is able to drive the

suppression or IL-6 specifically. It has been shown that *H. pylori*-derived components can

inhibit IL-12 expression by dendritic cells, which may contribute to the means by which this

bacterium is able to suppress local immune response and chronically colonize the stomach of

more than 50% of the world's population [60]. The decreased IL-6 expressed by *H. pylori*

sonicate-tolerized DCs demonstrated in this study may also contribute to an

immunosuppressive effect caused by *H. pylori* that leads to its immune escape and eventually

chronic infection. In a recent study, it was shown that IL-6 was required to activate the

inflammatory T_{H17} response in the recognition of apoptotic cells [61], so the decrease in IL-6

production by *H. pylori* tolerized DCs might aid in preventing an adequate, robust adaptive

immune response and allow the continued presence of the bacterium. It is possible that *H.*

pylori exposure, but not LPS or *E. coli* exposure, leads to cell processes that post-

transcriptionally modify IL-6 in tolerized DCs, however pinpointing the mechanism by which this

occurs would require more in depth investigation (a scan of the literature did not reveal any

research published on this subject).

Concerning the potent anti-inflammatory cytokine IL-10, this study has revealed its

expression as being significantly increased when BM-DCs are tolerized with LPS or sonicate

(*Figure 5* and *Figure 6*). This same phenomenon is exhibited by monocytes and macrophages

tolerized with endotoxin, and our data demonstrates that it can also occur with bacterial

sonicate. IL-10 has wide-ranging effects on tissue, first being recognized for its ability to

suppress the adaptor function of T cells, monocytes, and macrophages by limiting and

eventually terminating the inflammatory response, as well as affecting the development and

differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes,

dendritic cells, keratinocytes, and endothelial cells [62]. Notably, is has a strong influence on

the development of regulatory T cells involved in tolerogenic immune responses [62]. Thus,

increased IL-10 produced by tolerized DCs, as demonstrated in this study, would serve as a

highly potent signal in terminating or limiting immune responses systemically and locally.

Yet another interesting finding from this study is that IL-10, despite its wide-ranging

effects leading to immunosuppression, does not appear to play a role in the development of

tolerance in BM-DCs (*Figure 8*). When we cultured tolerized BM-DCs and stimulated them in

the presence of IL-10 neutralizing antibody, there was no identifiable change in TNF-α

expression. If IL-10 were playing an autocrine role in suppressing inflammatory cytokine

expression in tolerized DCs, a significant increase in TNF-α would be expected to occur in

tolerized cells cultured with the neutralizing antibody and challenged with a stimulus; however,

this study indicates this is not the case. It is possible that another anti-inflammatory cytokine,

such as TGF-β, is at least partially responsible for the immunomodulated cytokine profile of

tolerized dendritic cells in this study. Another possibility is the up-regulation of intracellular

negative regulators of inflammatory signaling, of which a large cohort have been identified [38,

63]. The roles of negative regulators of TLR signaling, specifically, have been recognized for

several years in controlling the inflammatory response; if these proteins are the main source of pro-inflammatory cytokine regulation in BM-DCs, then it would not be particularly surprising that neutralizing the anti-inflammatory cytokine IL-10 resulted in little fluctuation in TNF-α as compared to non-IL-10 neutralized cells that had been tolerized. In order to confirm that IL-10 has little role in immunomodulation through autocrine action, further pro-inflammatory cytokines measurements should be carried out.

Concerning intracellular negative regulators, this study investigated the role of interleukin 1 receptor-associated kinase (IRAK-M), a negative regulator of TLR-signaling, in the tolerance of BM-DCs. IRAK-M was chosen to be investigated since this protein has been demonstrated to be expressed in high amounts in tolerized monocytes and macrophages, and even essential to the development of tolerance within these cells [10-12, 14, 29, 34, 37, 40, 64]. IRAK-M has been shown to interact with IRAK-1 and IRAK-4 to prevent them from dissociating from the TLR4/MyD88 adapter complex [40]. This dissociation is necessary to continue the signal cascade through NF-κB, which serves as a transcriptional activator for an array of inflammatory cytokines [1]. It is not clear how exactly IRAK-M does this, but it is theorized to either participate in modulating phosphorylation or stabilizing the complex to prevent the dissociation [40]. When BM-DCs were tolerized with LPS, our data indicate that IRAK-M does indeed show increased expression (*Figure 9*). This was shown by both increased IRAK-M transcript (qPCR) and protein expression (Western blot). As in monocytes and macrophages, IRAK-M appears to have a correlation to tolerization status in BM-DCs.

However, investigation beyond correlative participation of IRAK-M in tolerance in BM-DCs yielded surprising results. Given the phenotypic and functional relation of dendritic cells to

macrophages and monocytes, it might be hypothesized that IRAK-M would a have a similar role

as being an important member in tolerance development; yet, data from this study seem to

indicate quite the opposite. IRAK-M knockout mice with a truncated and non-functional IRAK-

M protein were obtained through the generous contribution of Ted Standiford (University of

Michigan), and bone marrow cells were taken from these and cultured to produce BM-DCs in

an identical manner as wild type BM-DCs. When these cells were tolerized, they not only failed

to show a lack tolerizability but seemed to *decrease* the expression of TNF-α as compared to

tolerized wild type BM-DCs (*Figure 10*). Since the current understanding of IRAK-M is that its

presence is necessary for decreased TNF-α expression in macrophages and monocytes, these

results are exceedingly unexpected. A few possibilities may underlie this observation; firstly,

IRAK-M function in dendritic cells may be constitutive, that is, the protein may simply always be

expressed at a basal level and any increased expression does not have any sort of noticeable

effect on dendritic cells. This assertion is supported in *Figure 9*, where totally untolerized and

un-stimulated BM-DCs exhibit IRAK-M expression. Thus, DCs would be unresponsive to

additional IRAK-M since its concentration of maximal functionality has already been met or

surpassed in an untolerized state. A second possibility is that IRAK-M functions to suppress the

expression other negative regulators during tolerance in dendritic cells. This up-regulation of

other negative regulators in the absence of IRAK-M might explain why the suppression of TNF-α

is maintained in tolerized DCs, and indeed enhanced. Several negative regulators may behave

this way in dendritic cells [38], such as SOCS1, PI3K, NOD2, TOLLIP, SIGIRR, TRAILR, TRIAD3A,

MyD88s, A20 or sTLR4, but a confirmation of this would require further analysis, such as a chip

array, which was not conducted within the confines of the current study. A third, though

unlikely, explanation would be that IRAK-M merely has an entirely different or unidentified functional role in the tolerance of DCs, doubtful given the close relation between dendritic cells and monocytes/macrophages.

IRAK-M -/- DCs with tolerance also showed peculiar behavior with regards to IL-10. In the DCs tolerized with endotoxin, the typical increase in IL-10 was observed; however, in IRAK-M -/- DCs tolerized with either bacterial sonicate, no increase in IL-10 was seen compared to untolerized IRAK-M -/- cells (*Figure 11*). This would seem to indicate differing roles of IRAK-M depending on whether the cells are interacting with and being tolerized by a single ligand (endotoxin) or multiple ligands (aggregate bacterial sonicate); in cells tolerized with mixed bacterial components, IL-10 expression could not be potentiated above levels expressed by non-tolerized cells without IRAK-M present. Since IL-10 is regulated through a different signaling adapter as compared to TNF-α (MyD88 vs TRIF/JAK-STAT1), it would not be surprising that IL-10 might show a non-uniform trend depending on the tolerization component(s) as compared to the uniform trend displayed by TNF-α, regardless of tolerization component(s). The fact that IL-10 expression can be altered by the absence of a functional IRAK-M protein in dendritic cells is novel, chiefly since IRAK-M has only been researched in the context of MyD88-related signaling. Consequently, these results may indicate additional molecular sites of action under the influence of IRAK-M.

C O N C L U S I O N

This study has demonstrated that BM-DCs are capable of immunomodulation during tolerance involving *E. coli*-derived endotoxin and sonicate containing various stimulatory ligands derived from the Gram (-) bacteria *Escherichia coli* and *Helicobacter pylori*. This immunomodulation is typified by suppressed expression of pro-inflammatory cytokines TNF-α, IL-12, with uniquely suppressed expression of IL-6 in BM-DCs tolerized with *H. pylori* sonicate, and increased expression of the anti-inflammatory cytokine IL-10. IL-10 does not appear to have an autocrine immunosuppressive effect during this tolerance. Although IRAK-M, a negative regulator of immune signaling, showed enhanced expression in BM-DCs tolerized with endotoxin, it does not appear to be necessary for tolerance as in monocytes and macrophages; indeed, the absence of IRAK-M enhanced tolerance with respect to TNF-α expression. IL-10 expression increased in endotoxin-tolerized, but not sonicate-tolerized, IRAK-M -/- BM-DCs, indicating different roles for IRAK-M in BM-DCs tolerized with LPS or multiple bacterial components.

F U T U R E   D I R E C T I O N S

- o Continued cytokine analysis to better reveal cytokine modulation in IL-10 neutralization and IRAK-M -/- tolerization experiments

- o Characterize IRAK-M in sonicate-induced tolerance of BM-DCs

- o Isolation of *H. pylori* LPS, in order to characterize tolerance with *E. coli* LPS vs *H. pylori* LPS

- o Testing for modulation of additional negative regulators of inflammatory signaling and TGF-β to better ascertain the cellular processes leading to DC tolerization

- o Attempt TGF-β neutralization to observe whether it has an autocrine immunosuppressive effect on tolerized DCs

- o Utilize flow cytometry to assess cell maturation and surface marker expression in varying conditions of DC tolerance, as well as intracellular protein expression

- o Conduct experiments with tolerized DCs co-cultured with naïve T cells in order to observe T cell subtype skewing

A B B R E V I A T I O N S

- BM-DC (DC) – bone marrow-derive dendritic cell (dendritic cell)
- LPS – lipopolysaccharide (endotoxin)
- TNF-α – tumor necrosis factor - alpha
- TGF-β – transforming growth factor beta
- IL-#p# - interleukin-# subunit #
- IRAK-( ) – interleukin 1 receptor-associate kinase-( )
- NF-κB – nuclear factor kappa B
- TLR – Toll-like receptor
- qPCR – quantitative polymerase chain reaction
- APC – antigen presenting cell
- PRR – pattern recognition receptor
- PAMP – pathogen associate molecular pattern
- MAP kinase – mitogen-activated protein kinase
- ET – endotoxin tolerance
- MyD88 – myeloid differentiation factor 88
- TRIF - TIR-domain-containing adapter-inducing interferon-β
- IκB – inhibitor of nuclear factor kappa B kinase
- GM-CSF – granulocyte/macrophage – colony stimulating factor
- PBS – phosphate buffered saline
- GAPDH – glyceraldehyde-3 phosphate dehydrogenase
- ELISA – enzyme-linked immunosorbent assay
- SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- IgG – immunoglobulin G
- ND_{50} – 50% neutralizing dose
- NK – natural killer
- SOCS1 – suppressor of cytokine signaling 1
- PI3K – phosphoinositol 3 kinase
- NOD2 - nucleotide-binding oligomerization domain containing 2
- TOLLIP – Toll interaction protein
- SIGIRR - single immunoglobulin IL-1R-related molecule
- MyD88s – short form of MyD88
- sTLR4 – short form of TLR4

REFERENCES

1. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.
2. Palucka, K. and J. Banchereau, *Dendritic cells: a link between innate and adaptive immunity.* J Clin Immunol, 1999. **19**(1): p. 12-25.
3. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
4. Mohamadzadeh, M. and R. Luftig, *Dendritic cells: In the forefront of immunopathogenesis and vaccine development - A review.* J Immune Based Ther Vaccines, 2004. **2**(1): p. 1.
5. Cherayil, B.J., *How not to get bugged by bugs: mechanisms of cellular tolerance to microorganisms.* Curr Opin Gastroenterol, 2003. **19**(6): p. 572-7.
6. Chaussabel, D. and J. Banchereau, *Dendritic cells, therapeutic vectors of immunity and tolerance.* Am J Transplant, 2005. **5**(2): p. 205-6.
7. Kao, J.Y., et al., *Superior efficacy of dendritic cell-tumor fusion vaccine compared with tumor lysate-pulsed dendritic cell vaccine in colon cancer.* Immunol Lett, 2005. **101**(2): p. 154-9.
8. Kao, J.Y., et al., *Tumor-derived TGF-beta reduces the efficacy of dendritic cell/tumor fusion vaccine.* J Immunol, 2003. **170**(7): p. 3806-11.
9. Zhang, M., et al., *Helicobacter pylori-pulsed dendritic cells induce H. pylori-specific immunity in mice.* Helicobacter, 2008. **13**(3): p. 200-8.
10. Liu, Z.J., et al., *Up-regulation of IRAK-M is essential for endotoxin tolerance induced by a low dose of lipopolysaccharide in Kupffer cells.* J Surg Res, 2008. **150**(1): p. 34-9.
11. del Fresno, C., et al., *Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients.* J Immunol, 2009. **182**(10): p. 6494-507.
12. Deng, J.C., et al., *Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M.* J Clin Invest, 2006. **116**(9): p. 2532-42.
13. Biswas, S.K. and E. Lopez-Collazo, *Endotoxin tolerance: new mechanisms, molecules and clinical significance.* Trends Immunol, 2009. **30**(10): p. 475-87.
14. Escoll, P., et al., *Rapid up-regulation of IRAK-M expression following a second endotoxin challenge in human monocytes and in monocytes isolated from septic patients.* Biochem Biophys Res Commun, 2003. **311**(2): p. 465-72.
15. Lemaire, L.C., et al., *Minimally invasive surgery induces endotoxin-tolerance in the absence of detectable endotoxemia.* J Clin Immunol, 1998. **18**(6): p. 414-20.
16. Foster, S.L. and R. Medzhitov, *Gene-specific control of the TLR-induced inflammatory response.* Clin Immunol, 2009. **130**(1): p. 7-15.
17. Chamorro, S., et al., *TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program.* J Immunol, 2009. **183**(5): p. 2984-94.
18. Andreasen, A.S., et al., *Human endotoxemia as a model of systemic inflammation.* Curr Med Chem, 2008. **15**(17): p. 1697-705.
19. Munoz, C., et al., *Dissociation between plasma and monocyte-associated cytokines during sepsis.* Eur J Immunol, 1991. **21**(9): p. 2177-84.
20. Munoz, C., et al., *Dysregulation of in vitro cytokine production by monocytes during sepsis.* J Clin Invest, 1991. **88**(5): p. 1747-54.
21. Wolk, K., et al., *Impaired antigen presentation by human monocytes during endotoxin tolerance.* Blood, 2000. **96**(1): p. 218-23.

22.   Draisma, A., et al., *Development of endotoxin tolerance in humans in vivo.* Crit Care Med, 2009. **37**(4): p. 1261-7.

23.   Cavaillon, J.M., et al., *Endotoxin tolerance: is there a clinical relevance?* J Endotoxin Res, 2003. **9**(2): p. 101-7.

24.   Cavaillon, J.M., *The nonspecific nature of endotoxin tolerance.* Trends Microbiol, 1995. **3**(8): p. 320-4.

25.   Medvedev, A.E., K.M. Kopydlowski, and S.N. Vogel, *Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression.* J Immunol, 2000. **164**(11): p. 5564-74.

26.   Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-8.

27.   Cavaillon, J.M. and M. Adib-Conquy, *Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis.* Crit Care, 2006. **10**(5): p. 233.

28.   Fan, H. and J.A. Cook, *Molecular mechanisms of endotoxin tolerance.* J Endotoxin Res, 2004. **10**(2): p. 71-84.

29.   van 't Veer, C., et al., *Induction of IRAK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model.* J Immunol, 2007. **179**(10): p. 7110-20.

30.   Hotchkiss, R.S., et al., *The sepsis seesaw: tilting toward immunosuppression.* Nat Med, 2009. **15**(5): p. 496-7.

31.   Biswas, S.K. and V. Tergaonkar, *Myeloid differentiation factor 88-independent Toll-like receptor pathway: Sustaining inflammation or promoting tolerance?* Int J Biochem Cell Biol, 2007. **39**(9): p. 1582-92.

32.   Rayhane, N., et al., *Administration of endotoxin associated with lipopolysaccharide tolerance protects mice against fungal infection.* Infect Immun, 2000. **68**(6): p. 3748-53.

33.   Zacharioudaki, V., et al., *Adiponectin promotes endotoxin tolerance in macrophages by inducing IRAK-M expression.* J Immunol, 2009. **182**(10): p. 6444-51.

34.   Hassan, F., et al., *Involvement of interleukin-1 receptor-associated kinase (IRAK)-M in toll-like receptor (TLR) 7-mediated tolerance in RAW 264.7 macrophage-like cells.* Cell Immunol, 2009. **256**(1-2): p. 99-103.

35.   Nolan, A., et al., *Differential role for CD80 and CD86 in the regulation of the innate immune response in murine polymicrobial sepsis.* PLoS One, 2009. **4**(8): p. e6600.

36.   Wolk, K., et al., *Multiple mechanisms of reduced major histocompatibility complex class II expression in endotoxin tolerance.* J Biol Chem, 2003. **278**(20): p. 18030-6.

37.   del Fresno, C., et al., *Inflammatory responses associated with acute coronary syndrome up-regulate IRAK-M and induce endotoxin tolerance in circulating monocytes.* J Endotoxin Res, 2007. **13**(1): p. 39-52.

38.   Liew, F.Y., et al., *Negative regulation of toll-like receptor-mediated immune responses.* Nat Rev Immunol, 2005. **5**(6): p. 446-58.

39.   Chan, C., et al., *Endotoxin tolerance disrupts chromatin remodeling and NF-kappaB transactivation at the IL-1beta promoter.* J Immunol, 2005. **175**(1): p. 461-8.

40.   Kobayashi, K., et al., *IRAK-M is a negative regulator of Toll-like receptor signaling.* Cell, 2002. **110**(2): p. 191-202.

41.   Geisel, J., et al., *IL-6 and maturation govern TLR2 and TLR4 induced TLR agonist tolerance and cross-tolerance in dendritic cells.* J Immunol, 2007. **179**(9): p. 5811-8.

42.   Karp, C.L., et al., *Potent suppression of IL-12 production from monocytes and dendritic cells during endotoxin tolerance.* Eur J Immunol, 1998. **28**(10): p. 3128-36.

43.   Albrecht, V., et al., *Tolerance induced via TLR2 and TLR4 in human dendritic cells: role of IRAK-1.* BMC Immunol, 2008. **9**: p. 69.

44.   Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. **21**: p. 685-711.

45.   Romagnani, S., *Regulation of the T cell response.* Clin Exp Allergy, 2006. **36**(11): p. 1357-66.

46.   Gatta, L., et al., *Sequential Therapy or Triple Therapy for Helicobacter pylori Infection: Systematic Review and Meta-Analysis of Randomized Controlled Trials in Adults and Children.* Am J Gastroenterol, 2009.

47.   Luther, J., et al., *Association between Helicobacter pylori infection and inflammatory bowel disease: A meta-analysis and systematic review of the literature.* Inflamm Bowel Dis, 2009.

48.   Souza, R.C. and J.H. Lima, *Helicobacter pylori and gastroesophageal reflux disease: a review of this intriguing relationship.* Dis Esophagus, 2009. **22**(3): p. 256-63.

49.   Wang, C., Y. Yuan, and R.H. Hunt, *Helicobacter pylori infection and Barrett's esophagus: a systematic review and meta-analysis.* Am J Gastroenterol, 2009. **104**(2): p. 492-500; quiz 491, 501.

50.   Lahner, E., B. Annibale, and G. Delle Fave, *Systematic review: Helicobacter pylori infection and impaired drug absorption.* Aliment Pharmacol Ther, 2009. **29**(4): p. 379-86.

51.   Zhang, Y.Y., et al., *Review article: 'true' re-infection of Helicobacter pylori after successful eradication - worldwide annual rates, risk factors and clinical implications.* Aliment Pharmacol Ther, 2009. **29**(2): p. 145-60.

52.   Fransen, J.H., et al., *Mouse dendritic cells matured by ingestion of apoptotic blebs induce T cells to produce interleukin-17.* Arthritis Rheum, 2009. **60**(8): p. 2304-13.

53.   Fitting, C., S. Dhawan, and J.M. Cavaillon, *Compartmentalization of tolerance to endotoxin.* J Infect Dis, 2004. **189**(7): p. 1295-303.

54.   Wysocka, M., et al., *IL-12 suppression during experimental endotoxin tolerance: dendritic cell loss and macrophage hyporesponsiveness.* J Immunol, 2001. **166**(12): p. 7504-13.

55.   Mages, J., H. Dietrich, and R. Lang, *A genome-wide analysis of LPS tolerance in macrophages.* Immunobiology, 2007. **212**(9-10): p. 723-37.

56.   Kwan, W.H., et al., *LPS induces rapid IL-10 release by M-CSF-conditioned tolerogenic dendritic cell precursors.* J Leukoc Biol, 2007. **82**(1): p. 133-41.

57.   Kobayashi, K.S. and R.A. Flavell, *Shielding the double-edged sword: negative regulation of the innate immune system.* J Leukoc Biol, 2004. **75**(3): p. 428-33.

58.   De Nardo, D., et al., *Down-regulation of IRAK-4 is a component of LPS- and CpG DNA-induced tolerance in macrophages.* Cell Signal, 2009. **21**(2): p. 246-52.

59.   Chang, J., S.L. Kunkel, and C.H. Chang, *Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells.* Proc Natl Acad Sci U S A, 2009. **106**(43): p. 18327-32.

60.   Kao, J.Y., et al., *Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(1): p. G73-81.

61.   Torchinsky, M.B., et al., *Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation.* Nature, 2009. **458**(7234): p. 78-82.

62.   Moore, K.W., et al., Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, 2001. 19: p. 683-765.

63.   Fukao, T. and S. Koyasu, PI3K and negative regulation of TLR signaling. Trends Immunol, 2003. 24(7): p. 358-63.

64.   Mandrekar, P., et al., The opposite effects of acute and chronic alcohol on lipopolysaccharide-induced inflammation are linked to IRAK-M in human monocytes. J Immunol, 2009. 183(2): p. 1320-7.