Macrophage MMP10 Regulates TLR7-Mediated Tolerance

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Using an in vivo model of tolerance to TLR7-induced skin inflammation, we found a critical role for macrophage-derived MMP10 in mediating immune hypo-responsiveness. Cutaneous exposure to Imiquimod (IMQ), a TLR7 agonist, induced acute expression of pro-inflammatory factors (IL1β, IL6, CXCL1) and neutrophil influx equally in both wildtype and Mmp10−/− mice. However, whereas subsequent exposure (11 and 12 days later) to IMQ led to marked abrogation of pro-inflammatory factor expression in wildtype mice, Mmp10−/− mice responded similarly as they did to the first application. In addition, the second exposure led to increased expression of negative regulators of TLR signaling (TNFAIP3, IRAK3) and immunosuppressive cytokines (IL10, TGFβ1) in wildtype mice but not in Mmp10−/− mice. In vitro studies demonstrated that prior exposure of IMQ to bone marrow-derived macrophages (BMDM) made wildtype cells refractory to subsequent stimulation but did not for Mmp10−/− macrophages. These findings expand the critical roles MMP10 plays in controlling macrophage activation to indicate that the development of immune tolerance to TLR7 ligand is dependent on this macrophage-derived proteinase.

Keywords: macrophage, tolerance, toll-like receptor, metalloproteinase, skin immunity

INTRODUCTION

Toll-like receptors (TLRs) are a family of highly conserved Pattern Recognition Receptors that are activated by pathogen-associated and damage-associated molecular patterns. In addition, TLRs contribute to dampening immune responses, which is beneficial in the resolution of inflammation and barring development of autoimmune diseases (1, 2). TLR7, an endosomal receptor that recognizes single-stranded RNAs from viruses or dying cells (3), has a critical role in the induction and modulation of autoimmunity, as in systemic lupus erythematosus (4).

Depending on stimuli within their microenvironment, resident, and infiltrated macrophages can differentiated into pro- or anti-inflammatory cells, referred to as M1 (classically activated) or M2 (alternatively activated) cells, respectively (5–7). Several proteins influence macrophage behavior, including some members of matrix metalloproteinase (MMP) family (8–11). For example, studies by our laboratory demonstrated that MMP28 and TIMP3 mitigate the pro-inflammatory activity of macrophages in response to infection and sterile injury (8, 9), and MMP8 enhances anti-inflammatory function of macrophages by increasing the bioavailability of TGFβ1 (10). We reported that stromelysin-2 (MMP-10), which is expressed by macrophages, promotes the activation of immunosuppressive and matrix-degrading programs in macrophages, including resident skin macrophages (12–14).
TLRs affect macrophage activation typically by promoting their polarization toward pro-inflammatory states (15). Indeed, macrophages deficient in TLR3, 7, 8, 9, or 13 have impaired pro-inflammatory responses to infection (16). Repeated stimulation of TLR7 with synthetic agonists induces tolerance and restrains inflammation in autoimmune and tumor models (17, 18). As macrophages are involved in immune tolerance (19, 20), including tolerance to TLR7 ligands (21, 22), we explored if MMP10 impacts macrophage responses to TLR7 activation. Our findings demonstrate that prior topical treatment with Imiquimod (IMQ), a TLR7 agonist, dampened proinflammatory responses, and promoted tolerance to subsequent application of IMQ at a different site. Using both in vivo and cell-based approaches, we found that these effects were dependent on MMP10 in macrophages. These observations expand the roles for MMP10 in being a critical effector of macrophage activation.

MATERIALS AND METHODS

Animals
Age-matched (8–10 weeks) Mmp10−/− mice (23) and wildtype littermates (C57BL/6J), near equal number of females and males, were used for these studies. All procedures were approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center.

Model of Immune Tolerance
We developed a 2-hit model (QQ; Figure 1A) to induce tolerance to TLR7 signaling. For this, the right ears of mildly anesthetized mice were treated with 25 mg Aldara cream (5% IMQ; Taro Pharmaceutical, Hawthorne, NY). Eleven (11) days later, the back was shaved, and 62.5 mg IMQ was applied on 2 consecutive days. For the 1-hit model (Q), mice received only the two consecutive back treatments (Figure 1A). Mice with mock treatment (shaved, no IMQ) served as controls. One day after the last application (day 3 for Q; day 14 for QQ) mice were sacrificed, and skin at the site of the second application and the inguinal and axillary lymph nodes were collected (Figure 1A).

Assays
Total RNA was isolated from homogenized skin and cultured macrophages, and transcripts were assessed by quantitative real-time PCR (qPCR) as described (24). For flow cytometry, skin samples and lymph nodes were processed to obtain single-cell suspensions (13). Cell surface receptors were stained with conjugated antibodies to CD45 (30-F11), B220 (RA3-6B2), CD4 (GK1.5), CD8α (53-7.3), Ly6G (1A8), CD11b (M1/70), F4/80 (BM8), CD11c (N418), and MHC II (M5/114-15.2; eBioscience, San Diego, CA) and analyzed using a BD LSRFortessa Cytometer and FlowJo software (Tree Star, Ashland, OR). IL10 protein levels were measured using the Mouse IL10 ELISA Ready-SET-Go! Kit (eBioscience).

Macrophage Studies
Isolation, culture, and activation of bone marrow-derived macrophages (BMDM) were done as described (25). Briefly, marrow cells were differentiated to macrophages by culturing in CSF-1-containing medium for 7 or 8 days. For TLR7 activation, BMDM were stimulated with IMQ (InvivoGen, San Diego, CA) in a 1- or 2-hit model. For the 2-hit model, BMDM (4 × 10^5 in 12-well plate) were stimulated with 400 ng/ml IMQ in PBS for 4 h, washed, incubated in fresh medium for 18 h, then restimulated with 1 μg/ml IMQ for 16 h (protein analysis) before harvest of cells and media. For the 1-hit model, BMDM were treated with 1 μg/ml IMQ for 4 or 16 h, and then harvested. For adoptive transfer, recipient mice received 7 × 10^6 wildtype GFP+ BMDM in 200 μl PBS via retro-orbital injection as described (13).

Statistics
Statistical analyses were performed using Prism 5 (GraphPad software, LaJolla, CA). Data are presented as mean ± SEM. Statistical significance was determined using t-test. A p-value of...
≤0.05 was considered statistically significant. In the figures, we
used * to denote a significant difference between the 1-hit
and 2-hit regimens in both wildtype and Mmp10−/− mice and † to
denote significant differences between wildtype and Mmp10−/−
mice (Figure 1B; QQ). Furthermore, whereas expression of Il10
and Tgfb1 increased in wildtype mice treated with the 2-hit
model, expression of neither gene increased in Mmp10−/−
mice (Figure 1D). These findings suggest that development of
immune tolerance to TLR7 ligation was dependent on MMP10.

RESULTS

Model

As described under Methods, we used a 2-hit model (QQ;
Figure 1A) to induce tolerance to TLR7 signaling, IMQ was
applied to the right ears of mice, and 11 days later, IMQ was re-
applied twice to the back. For the 1-hit model (Q), mice received
only the two consecutive treatments (Figure 1A). One day after
the last application, skin at the site of the second application
together and lymph nodes were harvested. To track morbidity,
we monitored weight loss in response to the 1-hit and 2-hit IMQ
treatments. We observed similar weight loss 1 day after the last
back treatment in both 1-hit and 2-hit model in both genotypes
(Figure S1).

MMP10 Moderates Pro-Inflammatory Responses Induced by TLR7 Ligation

To test the immune response to 1-hit IMQ application, we
analyzed expression of Il6, Il1b, and Cxcl1, pro-inflammatory factors
stimulated by TLR activation (26, 27), neutrophil influx,
and expression of Il10 and Tgfb1, two key immunosuppressive
cytokines. As we found in other studies and tissues (12–14), in
intact skin the expression of the cytokines studied here—except
for TGFβ1 (average Ct ∼29)—is low (average Ct >33), and the
levels do not differ between wildtype and Mmp10−/− mice (12–
14). Similarly, the numbers of circulating and tissue leukocytes
does not differ between naïve mice of either genotype (12–14).

In response to the acute 1-hit model, in which IMQ is
applied on two consecutive days to an exposed area of back skin
(Figure 1A), expression of Il6 increased about 50-75-fold, Il1b
about 100-fold, and Cxcl1 about 25-40-fold, with no significant
differences between wildtype and Mmp10−/− mice (Figure 1B).
Consistent with the expression of Cxcl1—a potent neutrophil
chemoattractant—we found significantly more neutrophils in the
back skin of both wildtype and Mmp10−/− mice in response to
the 1-hit model (Q, Figure 1C). Expression of Tgfb1 was not
stimulated by the 1-hit exposure and Il10 increased slightly (∼2-
fold; Figure 1D). These findings indicate that MMP10 does not
influence the initial response to TLR7 activation.

In contrast, we saw significant differences between wildtype
and Mmp10−/− mice in response to the 2-hit (QQ) model.
For this model, mice are sensitized with a single topical
application of IMQ to the ear then challenged 11 days later
with consecutive applications of IMQ to flank skin (Figure 1A).
Whereas expression of Il6, Il1b, and Cxcl1 were close to basal
levels in wildtype skin in response to the 2-hit model, these pro-
flammatory factors were all stimulated in Mmp10−/− mice at
the site of the second exposure, reaching levels comparable to
those seen with the 1-hit (Figure 1B, QQ). In addition, whereas
the neutrophil response was blunted in response to the 2-hot
model in wildtype skin, their influx was stimulated in Mmp10−/−
mice (Figure 1B; QQ). Furthermore, whereas expression of Il10
and Tgfb1 increased in wildtype mice treated with the 2-hit
model, expression of neither gene increased in Mmp10−/−
mice (Figure 1D). These findings suggest that development of
immune tolerance to TLR7 ligation was dependent on MMP10.

MMP10 Mediates Expression of Negative Regulators of TLR

To determine if the hypo-responsiveness we observed in the 2-
hit model was due to tolerance to TLR signaling, we assessed
expression of negative regulators of TLR signaling: Tnfaiip3,
Irak3, and Inpp5d (Ship1) (26, 28). Indicative of immune
tolerance, expression of Tnfaiip3 and Irak3 were elevated in the
2-hit model in wildtype mice (Figure 2). However, expression
of these critical tolerance factors was not altered in Mmp10−/−
mice in response to the 2-hit model. Expression of Inpp5d
did not differ between the 1- and 2-hit models and genotypes.
Similarly, we observed increased levels of TNFAIP3 protein in
the 2-hit model compared to the 1-hit model (Figure S2). These
data indicate that MMP10 promotes tolerance by regulating
expression of Tnfaiip3 and Irak3.

MMP10 Regulates Immune Responses by Modulation of Macrophage Activation

To determine which cell types mediate the tolerance response,
we investigated the possible role of lymphocytes. Although
we found no differences in the number of B (B220+/−) and T
lymphocytes (CD4+, CD8+) in lymph nodes between the 1-
and 2-hit models in wildtype mice (Figure S3A) or in 2-hit
treatment between wildtype and Mmp10−/− mice (Figure S3B),
their activation state could be shaped by other effector cells. To
test if lymphocytes played a role in TLR7 tolerance, we treated
Rag1−/− mice, which lack mature T and B lymphocytes, with
the two models. Following the 1-hit exposure (Q), Rag1−/− lost
about 20% body weight over the next 48 h, due to the systemic
inflammation caused by cream application (29). However,
following the 2nd treatment (QQ), weight loss in Rag1−/− mice
was significant less severe (Figure 3A). In addition, expression
of Il10 (30) were highly expressed in back skin of Rag1−/−
mice in response to the 2-hit model but not in 1-hit treatment
(Figure 3B). We also found a significant increase in expression
of Tgfβ1, TNFAIP3 and IRAK3, although the differences were
not as robust as for Il10 (Figure 3C). Although we observed a
trend for lower expression of pro-inflammatory markers Il6, Il1b,
and Cxcl1 between the 2-hit vs. 1-hit treatments (Figure S4),
the differences were not statistically significant. These findings
indicate that the development of negative regulation of immune
responses to TLR7 ligands does not require B and T cells.

To test the role of myeloid cells, we isolated myeloid cells from
back skin on day 12 after the initial ear treatment (thus, no second
exposure to back skin), and we found—not unexpectedly—
no difference in the numbers of neutrophils, macrophages, or
dendritic cells between genotypes (Figure S5). Compared to
untreated control group we observed significant decrease in
population of macrophages in 1-hit treatment group (both in
wildtype and Mmp10−/− mice; Figure 4). In response to the
second exposure of IMQ to back skin (QQ), the numbers of macrophages in both wildtype and Mmp10−/− mice at the site of administration were significantly elevated compared to mice treated with just the 1-hit regimen (Figure 4A). We isolated skin draining lymph nodes and found a similar increase in the numbers of macrophages in response to the 1st IMQ exposure (Q) in both wildtype and Mmp10−/− mice (Figure 4B), suggesting efflux of these cells from the skin at the site of exposure. However, in response to the 2nd hit, we detected a significant reduction in macrophages recovered from wildtype lymph nodes compared to 1-hit numbers (Figure 4B). In contrast, macrophage numbers remained elevated in Mmp10−/− lymph nodes after the 2nd hit. Dendritic cell numbers did not change among conditions or between genotypes (Figure S6). Similar to what we found in skin wounds (13), these data indicate that MMP10 does not influence macrophage influx to sites of cutaneous inflammation but moderates the efflux of macrophages from sites of inflammation to draining lymph nodes in tolerant condition.

Adoptive Transfer of Macrophages Restores Tolerance in wildtype Mice

To further test the role of macrophages, we applied IMQ to the ears of wildtype mice, then adoptively transferred naïve wildtype GFP+ BMDM 1 day before the back-skin applications of IMQ.
functions and activation status (data in other models indicate that MMP10 regulates macrophage hypo-responsiveness in both the 1- and 2-hit models (Rohani et al. Macrophage MMP10 Regulates TLR7-Mediated Tolerance)). Administration of naïve macrophages had no impact on the immune responses to 1-hit. Evidence of tolerance was seen in control mice (Figures 5B,D; Q vs. QQ, PBS group); however, adoptive transfer of naïve macrophages abrogated the development of tolerance in the 2-hit model as gauged by the elevated levels of Il6, Il1b, and Cxcl1 and suppression of Il10, Tgfβ1, TNFAIP3 and IRAK3 mRNAs (Figures 5C,E; BMDM groups). These findings indicate that macrophages are critical for the development of tolerance to TLR7 activation.

**MMP10 Mediates Tolerance to IMQ in Macrophages**

Pre-sensitized macrophages are tolerant to subsequent stimulation to TLR7/8 ligands (31). Our published *in vivo* data in other models indicate that MMP10 regulates macrophage function and activation status (13, 14), and our data here indicates that this proteinase also controls hypo-responsiveness to TLR7 signaling. As MMP10 is primarily a product of macrophages, we assessed if macrophage MMP10 affects tolerance responses in isolated cells. We treated wildtype and *Mmp10*−/− BMDM with IMQ (1-hit and 2-hit models, exposures modified as described under Methods). As evident by reduced expression of Il6 and Il1b in the 2-hit model, we found that wildtype macrophages were hypo-responsive to subsequent stimulation by IMQ whereas *Mmp10*−/− macrophages were not (Figure 6A), just as we saw *in vivo* (Figure 1B). Furthermore, and as we found *in vivo*, tolerant wildtype macrophages (QQ), expressed higher levels of Il10 (Figures 6B,C). We found no difference in expression of between Tnfaip3 between wildtype and *Mmp10*−/− macrophages, and decreased expression of IRAK3 and TGFβ1 in 2-hit vs. 1-hit treatment in *Mmp10*−/− BMDM, but no change in wildtype BMDM (Figure 5A). In addition, we found no difference in TLR7 expression between wildtype and *Mmp10*−/− macrophages (Figure 5B), suggesting that MMP10 impacts tolerance downstream of TLR7 signaling.

**DISCUSSION**

In this study, we demonstrated that topical application of IMQ, a TLR7 agonist, induced systemic tolerance and dampened the inflammatory responses to subsequent application of this ligand. The hypo-responsiveness in both the expression of pro-inflammatory cytokines and neutrophil influx was associated with increased expression of anti-inflammatory cytokines IL10 and TGFβ1 and the ubiquitin editing enzyme TNFAIP3, which negatively regulates NFκB signaling (32). We found that macrophages were essential effector cells in mediating these responses and that MMP10, a macrophage product, was required for tolerance to develop.

Plasticity and diversity are hallmarks of macrophages, and a range of signaling molecules can modulate their state of activation (33). TLR engagement typically drives macrophage polarization toward pro-inflammatory phenotype (classically-activated macrophages or M1), whereas tolerance seems to be related to alternatively-activated or M2 macrophages (34–37). We reported that Mmp10 drives the conversion of macrophages from pro-inflammatory, M1-biased cells to an anti-inflammatory, M2-biased cells (14). Although we do not yet understand the mechanism of its action, because several *Mmp10*−/− phenotypes seen *in vivo* are duplicated with macrophages in culture (13, 14), we predict that MMP10 cleaves a protein on the surface of M1-polarized macrophages that either by gain- or loss-of-function promote the transition to an M2-biased state. A focus of our lab’s effort is to identify and validate the MMP10 substrate that controls macrophage activation.

In agreement with findings from others (21, 22), our *in vivo* and *in vitro* data support an important role for macrophages in tolerance to TLR7 ligation. Similar to our findings with Rag1−/− mice, Hayashi et al. (17) reported that tolerance to 1V136, another TLR7 agonist, does not require T and B cells. However, Bourquin et al. (18) reported that pre-exposure of plasmacytoid and myeloid dendritic cells to Resiquimod, an analog of IMQ, blocks release...
of IL6, IL12p70, and IFN-α in response to a subsequent exposure, suggesting that these leukocytes also play a role in mediating tolerance in vivo. As we determined that dendritic cells do not express MMP10 (14), the MMP10-dependent function in tolerance would be limited to macrophages. Furthermore, our adoptive transfer studies underscore a critical role for macrophages in mediating tolerance to TLR7 ligation.

Although keratinocytes express MMP10 in response to injury and produce inflammatory markers, such as TNFα and IL8 (38, 39), we do not believe that these cells have a major role in mediating tolerance to TLR7 ligation. Compared to macrophages, these epidermal cells are much less sensitive to TLR7 activation, likely because they do not express TLR7 at meaningful levels. High concentrations of IMQ (100 µm) are needed to induce keratinocyte activation (39). Furthermore, using a IMQ model of psoriasis (40), we found no difference in epithelial thickness between wildtype and Mmp10−/− mice (unpublished data).

Compared to their TLR7-tolerance model, in which systemic administration leads to tolerance within 24 h after stimulation and then dissipating within 5 days (18), our cutaneous-application method required a longer interval for hypo-responsiveness to become apparent. Although the sequence of events that block the function of inflammatory macrophages is not fully understood, we speculate that topical application on the ear induced a systemic response and reprogramming of macrophages to an anti-inflammatory state that made the host tolerant of a second hit. Thus, adoptive transfer of naïve macrophages into pre-treated mice overrode the immunosuppressive activity of tolerant monocytes making the response to a second administration of IMQ appear like that seen in the 1-hit model (Figure 6).

The systemic responses to topical application of Aldara cream have been reported (41). Similarly, in our studies, we observed weight loss as indication of systemic responses. Although the 2-hit regimen induced systemic tolerance in Rag−/− mice, as evidenced by significantly less weight loss compared to 1-hit model, wildtype mice had a similar weight loss in response to both 1-hit and 2-hit treatment (Figure S1). We speculate that in wildtype mice lymphocytes alleviate systemic inflammation in 1-hit model, thereby obscuring differences in weight loss between the two regimens as was observed in Rag−/− mice.

Matrix metalloproteinase modulate a wide range of immune functions and responses (11, 42, 43). For example, MMP7, MMP8, MMP10, and MMP28 serve beneficial functions in response to acute infection or injury by moderating pro-inflammatory responses (10, 12, 14, 25, 44, 45). Here, we found that macrophage MMP10 was needed to induce tolerance to TLR7 signaling. Regulation of macrophage immune tolerance

**FIGURE 6** | Hypo-responsiveness of macrophages in culture to repeated TLR7 stimulation is mediated by MMP10. Wildtype and Mmp10−/− BMDM were stimulated with IMQ for the 1-hit (Q) or 2-hit model. (A,B) mRNA expression levels were quantified by qPCR and normalized to Hprt. Data are normalized to unstimulated wildtype control cells. (C) IL10 was measured in supernatant from cells using ELISA, data are representative of 3 individual experiments (n = 3). (**p ≤ 0.01: WT-Q vs WT-QQ and ****p ≤ 0.0001: Mmp10−/−Q vs. Mmp10−/−QQ; ††p ≤ 0.01; WT-QQ vs Mmp10−/−QQ).
by MMP10 is a novel function for this proteinase (or for any metalloproteinases), but yet we do not know how MMP10 mediates this regulation. As stated, to understand such mechanisms, we need to identify the protein substrate(s) cleaved by macrophage MMP10, which is the focus of our ongoing studies.

ETHICS STATEMENT
This study was carried out in accordance with the recommendations of Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center. The protocol was approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center.

AUTHOR CONTRIBUTIONS
MR, CJ, and WP conceived and designed the research. MR, ED, AB, and YW performed experiments. MR, CJ, and WP analyzed and interpreted data. MR and WP wrote the manuscript and prepared figures. All authors edited and approved final manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02817/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.