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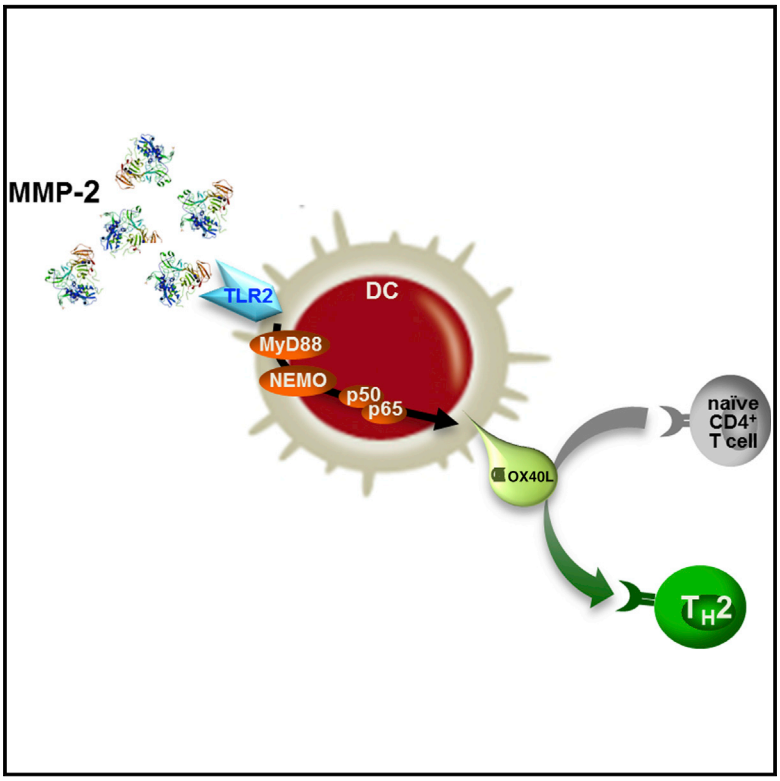
# Activation of Toll-like Receptor-2 by Endogenous Matrix Metalloproteinase-2 Modulates Dendritic-Cell-Mediated Inflammatory Responses

## Graphical Abstract

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## In Brief

Godefroy et al. now demonstrate that matrix metalloproteinase-2 (MMP-2) directly interacts with and activates dendritic cells (DCs) via Toll-like receptor-2. MMP-2-exposed DCs upregulate OX40L, promoting type 2 polarization both in vitro and in vivo. This may represent a key immune regulatory mechanism involved in a variety of inflammatory disorders.

## Highlights

MMP-2 is shown to be a ligand for the Toll-like receptor 2

MMP-2-dependent TLR2 triggering induces type 2 polarization via OX40L upregulation

MMP-2 triggers TLR2 independently of its usual coreceptors, i.e., TLR1, 6, and 4

MMP-2 polarizes $T_H2$ immune responses in vivo in a TLR2-dependent manner

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# Activation of Toll-like Receptor-2 by Endogenous Matrix Metalloproteinase-2 Modulates Dendritic-Cell-Mediated Inflammatory Responses

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## SUMMARY

**Matrix metalloproteinase-2 (MMP-2) is involved in several physiological mechanisms, including wound healing and tumor progression. We show that MMP-2 directly stimulates dendritic cells (DCs) to both upregulate OX40L on the cell surface and secrete inflammatory cytokines. The mechanism underlying DC activation includes physical association with Toll-like receptor-2 (TLR2), leading to NF-κB activation, OX40L upregulation on DCs, and ensuing $T_H2$ differentiation. Significantly, MMP-2 polarizes T cells toward type 2 responses in vivo, in a TLR2-dependent manner. MMP-2-dependent type 2 polarization may represent a key immune regulatory mechanism for protection against a broad array of disorders, such as inflammatory, infectious, and autoimmune diseases, which can be hijacked by tumors to evade immunity.**

## INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. They can degrade extracellular matrix proteins, participate in the cleavage of cell-surface receptors and chemokines or cytokines, and modulate cell proliferation, migration, differentiation, and angiogenesis. MMP-2, a member of the gelatinase subfamily of MMPs, participates in the remodeling and resolution of tissue injury (Bian and Sun, 1997; Brooks et al., 1998), embryonic development and morphogenesis (Seshagiri et al., 2003), infection clearance (Atarashi et al., 2011; D'Angelo et al., 2001; Lima et al., 2012), and tumorigenesis (Coussens and Werb, 2002; Egeblad and Werb, 2002; Hanahan and Weinberg, 2000; Liotta et al., 1980; Westermarck and Kähäri, 1999).

We recently identified an unexpected role for MMP-2 in the modulation of innate immune function and in the differentiation of inflammatory $T_H2$ responses. MMP-2 pre-exposure inhibits interleukin-12 (IL-12) function and upregulates OX40L expression by human dendritic cells (DCs) (Godefroy et al., 2011). Enzymatically active MMP-2 causes degradation of the IFNAR1 chain of the type 1 interferon (IFN) receptor, reducing the ability of IFN-β to enhance transcription of the IL-12p35 subunit through STAT1 phosphorylation (Godefroy et al., 2011). In the absence of IL-12, OX40L now functions as a key costimulatory molecule for the priming of $T_H2$ cells (Ito et al., 2005; Soumelis et al., 2002). However, the mechanism by which MMP-2 upregulates OX40L is not known, and the role of MMP-2-driven $T_H2$ cells in vivo has not been determined.

MMP-2 overexpression is observed in certain infections where eradication and control of immunopathogenesis rely on the development of protective type 2 responses (Oakley et al., 2013; Sauer et al., 2013). For instance, various parasites including plasmodium (Lima et al., 2012) and toxoplasma (Lu and Lai, 2013) species can trigger MMP-2 overexpression. As another example, MMP-2 plays a central role during wound healing and repair (Bian and Sun, 1997; Brooks et al., 1998). Inflammatory $T_H2$ cytokines (tumor necrosis factor alpha [TNF-α], IL-4, and IL-13) have been described as essential components in this process (Chen et al., 2012). Last, MMP-2, which is overexpressed in tumors, promotes cancer progression (Egeblad and Werb, 2002; Hofmann et al., 2000), and our prior studies suggest that this may in part be due to its ability to skew type 2 polarization (Godefroy et al., 2011). These observations suggest that MMP-2, through its ability to drive $T_H2$ cells, plays a unique role in modulating effector T cell responses.

In this study, we specifically investigated mechanisms by which MMP-2 upregulates OX40L on DCs to drive type 2 polarization. We identified a physiological receptor for MMP-2 on DCs that, upon activation, leads to $T_H2$ polarization. Therefore, extracellular MMP-2 has the potential to locally affect DCs leading to

modulation of immune responses during infectious, inflammatory, or malignant diseases.

## RESULTS

### MMP-2 Induces Human DCs to Upregulate OX40L and Secrete Inflammatory Cytokines

To better understand how MMP-2 influences DC function, we first characterized levels of OX40L expression on DCs pre-exposed to MMP-2. Unexpectedly, heat-inactivated (HI) MMP-2 or MMP-2 preincubated with a specific inhibitor induced human monocyte-derived DCs (moDCs) to upregulate OX40L at 48 hr, and to a lesser extent at 24 hr (Figure 1A), similar to what was observed with enzymatically active MMP-2. Enzymatic inactivity of HI-MMP-2 was verified as previously described (Godefroy et al., 2011). MMP-9 and the MMP-2 vehicle control did not affect OX40L expression (Figure 1A), suggesting that MMP-2 protein, but not its closely related family member MMP-9, specifically upregulates OX40L.

MMP-2-exposed moDCs also secreted significant levels of inflammatory cytokines such as TNF-α, IL-6, and IL-8 ($p < 0.02$ compared to vehicle control or inhibitor alone; Figure 1B), but not IL-12 (data not shown). Once again, both active and inactivated MMP-2, but none of the controls, stimulated moDCs (Figure 1B). Purified blood DCs were similarly activated by MMP-2 (Figure S1). These experiments show that MMP-2 induces human DCs to secrete inflammatory cytokines and that activation of DCs is independent of MMP-2's enzymatic activity.

### MMP-2 Activates the Canonical NF-κB Pathway

OX40L expression is modulated by molecules such as TSLP (Ito et al., 2005), Toll-like receptor 2 (TLR2) and TLR4 agonists, e.g., zymosan, lipopolysaccharide (LPS) (Han et al., 2011), and short ragweed pollen (Li et al., 2011). Upregulation of OX40L requires activation of the NF-κB pathway, which is also integral to the production of proinflammatory cytokines. Using a cell-permeable NEMO-binding domain (NBD) peptide, which inhibits the proximal NEMO molecule of the canonical NF-κB signaling pathway (May et al., 2000), we confirmed the significance of this pathway in MMP-2-induced inflammatory cytokine production (Figure 2A) and OX40L upregulation (Figure 2B) by moDCs ($p < 0.05$).

To better characterize which components of the NF-κB pathway were implicated, we monitored the translocation of NF-κB transcription factors into the nucleus of DCs after MMP-2 stimulation. MMP-2 induced significant translocation of p50 and p65/RelA ($p < 0.05$), but not c-Rel, p52, or RelB from the cytosol to the nucleus (Figures 2C and 2D). Thus, MMP-2 activates the canonical pathway in human DCs, which underlies the upregulation of OX40L and production of inflammatory cytokines.

### MMP-2 Triggers the Toll-like Receptor 2

We next evaluated which receptors facilitated MMP-2-mediated upregulation of inflammation and OX40L. TSLP as well as TLR2 and TLR4 agonists can increase OX40L expression through NF-κB activation (Arima et al., 2010; Han et al., 2011; Ito et al., 2005; Li et al., 2011). Neither TSLP (Arima et al., 2010) nor TNF-α (a control for NF-κB activation) induced OX40L upregulation on moDCs (Figure S2). Compared to active and HI-MMP-2, only zymosan and MALP2 (TLR2/6 agonists), but not $Pam_3CSK_4$ (TLR2/1 agonist) or other agonists (for TLR3, TLR4, TLR5, and TLR7/8), upregulated OX40L expression on moDCs (Figure 3A). These results suggest that MMP-2 stimulates DCs via TLR2/6. TLR2 expression on moDCs and mDCs was confirmed by flow cytometry (Figure 3B). A blocking antibody for TLR2, but surprisingly not an anti-TLR6, significantly inhibited MMP-2-induced cytokine production and OX40L upregulation by moDCs (Figures 3C and 3D). Importantly, the vehicle control did not activate moDCs indicating that MMP-2 itself rather than a contaminant was responsible for this effect. A role for heat-resistant contaminants was further excluded using proteinase K-degraded MMP-2, which failed to activate NF-κB or upregulate OX40L (Figures S3A and S3B) in TLR2-expressing HEK-Blue cells. MMP-2 degradation was confirmed by SDS-PAGE and Coomassie staining (Figure S3C). Furthermore, supernatants from MMP-2-secreting melanoma cells directly induced NF-κB activation and OX40L upregulation (Figures S3D–S3G), further reducing the likelihood of potential contaminant involvement. Together, these results show that MMP-2 specifically triggers TLR2, independently of TLR6, on moDCs, which leads to NF-κB activation, inflammatory cytokine secretion, and OX40L upregulation.

We also used HEK-Blue cells stably cotransfected with human *tlr* genes and the secreted embryonic alkaline phosphatase gene placed under control of the NF-κB promoter ("Blue" technology, InvivoGen) to test MMP-2's specificity. MMP-2 and various TLR2/6 (zymosan and MALP2) or TLR2/1 ($Pam_3CSK_4$) agonists significantly activated NF-κB and IL-8 production ($p \leq 0.0001$) in TLR2-transfected HEK-Blue cells, which express endogenous TLR1 and TLR6 (Figure 4A, black bars). As in moDCs (Figure 3), MMP-2-mediated activation of TLR2-transfected HEK-Blue cells was inhibited upon TLR2 blocking ($p < 0.005$ and $p < 0.00003$ for NF-κB and IL-8 inhibition, respectively; Figure 4A, gray bars). The anti-TLR6 blocking antibody had no effect on MMP-2-activated HEK cells but blocked zymosan and MALP2 stimulation ($p < 0.05$; Figure 4A, striped bars). Moreover, neither MMP-2 nor the TLR2 agonists activated NF-κB in the $TLR1^+/TLR6^+$ parental HEK-Blue cell line (Null-HEK-Blue), which was not transfected with TLR2 (Figure 4B), highlighting the specificity of MMP-2 for TLR2. To further control for contaminants (e.g., endotoxins), we used TLR4-transfected HEK-Blue cells. MMP-2 did not activate NF-κB in these cells (Figure 4C). Likewise, MMP-2-induced OX40L upregulation was observed in TLR2-expressing HEK cells and inhibited with a blocking antibody to TLR2 (Figure 4D). As expected, MMP-2 did not upregulate OX40L on either Null-HEK (Figure 4E) or TLR4-transfected HEK cells (Figure 4F).

Altogether, these data indicate that MMP-2 specifically triggers TLR2 independently of TLR6 or TLR1, which leads to NF-κB activation, inflammatory cytokine secretion, and OX40L upregulation.

### MMP-2 Is a Ligand for TLR2

We next assessed whether MMP-2 directly interacts with TLR2. Both MMP-2 and MyD88 immunoprecipitated with TLR2 when TLR2-transfected HEK cells were pretreated with MMP-2 (active or inactive), but not with MMP-9 (Figure 5A; $p < 0.02$ compared

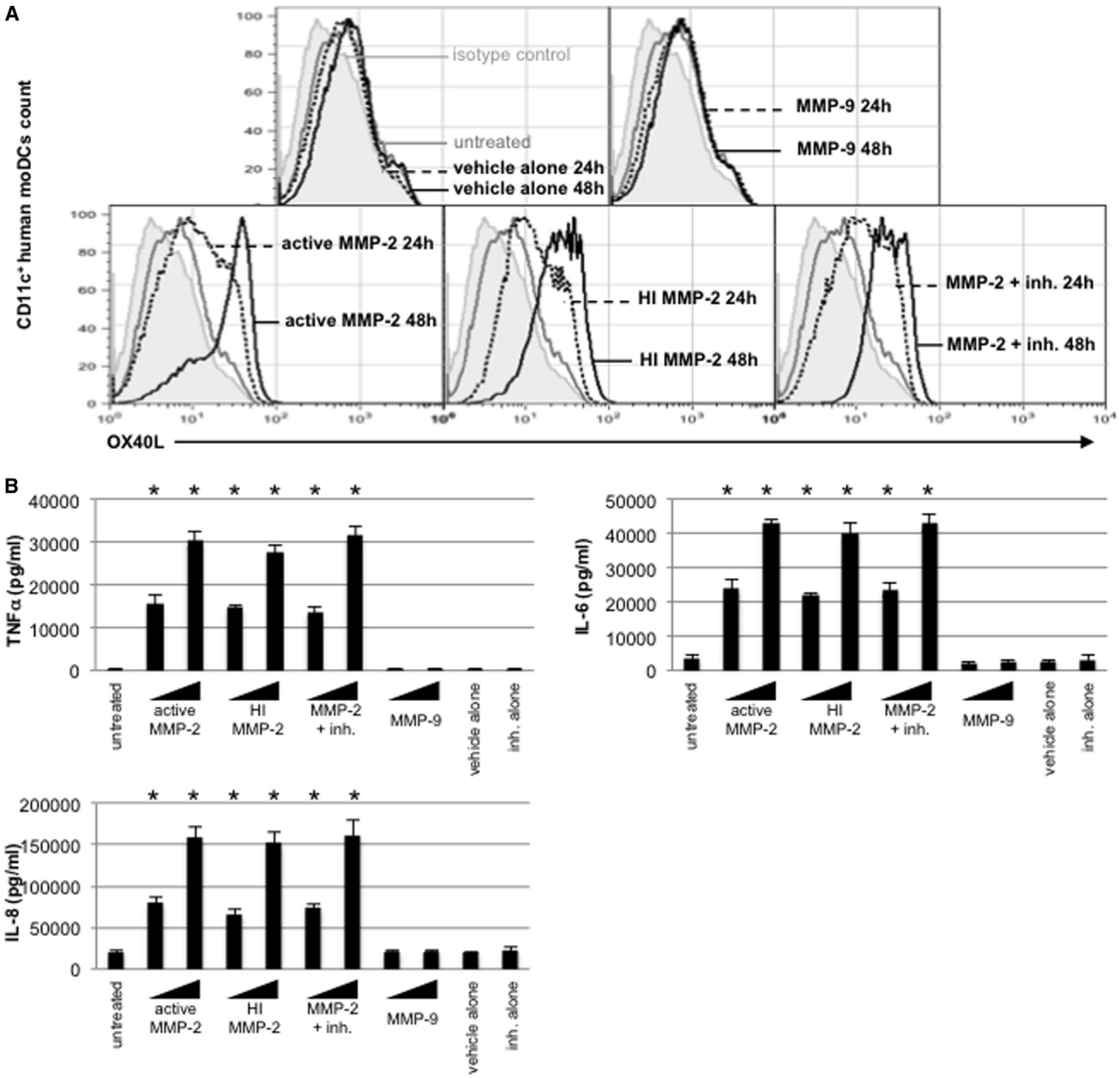


**Figure 1. MMP-2 Upregulates OX40L Expression and Inflammatory Cytokine Secretion by Human moDCs**

Immature moDCs were cultured with enzymatically active or inactive MMP-2 (0.5 and 5 μg/ml). MMP-2 was inactivated either by heat treatment (HI) or by adding a specific inhibitor (MMP-2+inh.) as described in Experimental Procedures. The vehicle control or MMP-9 (0.5 and 5 μg/ml) served as controls.

(A) After 24 and 48 hr, OX40L expression was assessed by surface staining on $CD11c^{high}$ gated cells. An isotype mAb was used as a negative control. MoDCs from a representative donor out of three is shown.

(B) Inflammatory cytokines (TNF-α, IL-6, and IL-8) were measured in the supernatants after ON stimulation (with CBA technology). Experiments were performed in triplicate and are representative of three independent experiments (error bars, SD). Two-tailed Student's t tests were used to compare MMP-2-treated DCs to DCs incubated with the vehicle control (for active and HI MMP-2) or the inhibitor alone (for MMP-2+inh).

See also Figure S1.

to MMP-9-treated cells). Specificity for TLR2 was controlled using TLR2-negative cells (Figure 5A). Similarly, TLR2 and MyD88 immunoprecipitated with MMP-2 when moDCs were pretreated with MMP-2 (Figure 5B; ($p < 0.03$ compared to MMP-9-treated cells). Immunoprecipitation assays using an isotype antibody control confirmed binding specificity (Figure 5C). Total cell lysates were used as positive controls to demonstrate TLR2 specific blotting (Figures 5D and 5E).

Biacore experiments further confirmed a physical association between MMP-2 and TLR2. A high-affinity interaction with an

equilibrium affinity constant ($K_D$) of 3.22 × $10^{-8}$ M, which falls within the range for other TLR/ligand binding affinities (Akashi et al., 2003; Rutz et al., 2004) was measured. Altogether, these results confirm that MMP-2 directly interacts with TLR2, which leads to the recruitment of and association with MyD88.

### TLR2 Expression Is Sufficient for MMP-2-Mediated DC Activation

TLR2 forms heterodimers with TLR1, TLR6, and potentially TLR4 (Lee et al., 2004; Takeda et al., 2002). We addressed whether TLR2 required heterodimerization with one of these additional TLRs in order to be triggered by MMP-2. Untransfected wild-type (WT) HEK cells as well as HEK cells transfected with plasmids encoding human TLR1, TLR4, or TLR6 were not activated by MMP-2 (Figures 6A–6D). On the other hand, HEK293 cells transfected with the TLR2 plasmid alone or cotransfected with plasmids encoding TLR2 and TLR1, TLR4, or TLR6 (Figures 6E–6H) produced comparable amounts of IL-8 in response to MMP-2, suggesting that TLR1, TLR4, and TLR6 are not required for optimal cytokine production. Moreover, blocking TLR2 but not TLR6 significantly inhibited MMP-2 stimulation of TLR2-expressing HEK293 cells (Figures 6E–6H; $p < 0.000045$). Overall, these data strongly suggest that MMP-2 triggers TLR2 independently of TLR1, TLR6, or TLR4.

We next formally demonstrated that MMP-2 requires TLR2 to activate DCs by using TLR-deficient C57/BL6 mice. Bone-marrow-derived DCs (BM-DCs) were cultured with murine MMP-2 or controls. In the presence of either active or HI-MMP-2, WT BM-DCs as well as $TLR6^{-/-}$ and $TLR7^{-/-}$ BM-DCs secreted significant amounts of inflammatory cytokines such as TNF-α (Figure S4A) or IL-6 (Figures S4B and S5A) and upregulated OX40L (Figures S4C and S5B). $TLR4^{-/-}$ BM-DCs treated with MMP-2 secreted little to no inflammatory cytokines (Figure S5A) but upregulated OX40L (Figure S5B), suggesting a possible role for TLR4 in MMP-2-dependent triggering of cytokine production in murine BM-DCs, unlike the human condition. $TLR2^{-/-}$ and $MyD88^{-/-}$ BM-DCs were not activated by MMP-2 or the respective TLR2/6 and TLR2/1 agonists, MALP2 and $Pam_3CSK_4$ (Figures S4A–S4C). As expected, MALP2 stimulated WT BM-DCs, but not $TLR2^{-/-}$, $TLR6^{-/-}$, or $MyD88^{-/-}$ BM-DCs, whereas $Pam_3CSK_4$ stimulated WT and $TLR6^{-/-}$ BM-DCs, but not $TLR2^{-/-}$ or $MyD88^{-/-}$ BM-DCs (Figures S4A–S4C). These results were confirmed using CD11c-purified splenic DCs (Figures S6A and S6B). Of interest, human MMP-2 strongly activated TLR2-expressing murine DCs (Figures S7A and S7B). Therefore, both active and HI-MMP-2 induce DCs to produce inflammatory cytokines and to upregulate OX40L in a TLR2/MyD88-dependent manner.

### MMP-2 Triggers TLR2-Dependent Inflammation In Vivo

We next evaluated whether MMP-2 triggered TLR2 in vivo. First, TLR-deficient C57/BL6 mice were injected intravenously (i.v.) with human MMP-2, which we found induced the strongest response by murine DCs in vitro. In addition, MMP-2 was heat-inactivated before injection to follow its influence on TLR2 triggering, without concern for effects due to its enzymatic activity. Sera obtained from WT mice contained TNF-α (Figure 7A), IL-6 (Figure 7B), and MCP-1 (data not shown). However, sera derived from $TLR2^{-/-}$ or $MyD88^{-/-}$ mice had no inflammatory cytokines ($p \leq 0.000000001$ and $p \leq 0.000000000002$ for TNF-α and IL-6, respectively; Figures 7A and 7B). Therefore, as shown in vitro, HI MMP-2 induces an inflammatory response in vivo in a TLR2/MyD88-dependent manner.

MMP-2 was then evaluated for its ability to physiologically skew type 2 responses in vivo. $CD45.1^+$ C57/BL6 mice were injected i.v. with $OVA_{323–339}$-specific $CD45.2^+$ $CD4^+$ OT-II cells. The next day, mice were injected subcutaneously (s.c.) footpad with MMP-2 or with type 1 or type 2 polarizing adjuvants (Poly[I:C] and alum, respectively) together with OVA protein. At day 10, $CD4^+$ cells from draining lymph nodes were purified and restimulated in vitro with $OVA_{323–339}$ peptide. Priming with OVA and Poly(I:C) elicited high frequencies of OT-II cells producing IFN-γ (Figure 7C), but not IL-13 (Figure 7E). In contrast, OT-II cells primed with alum or either active or HI-MMP-2 produced little IFN-γ, but significant levels of IL-13 (Figures 7C and 7D). Serum collected at day 10 demonstrated higher levels of OVA-specific immunoglobulin G2a (IgG2a) levels than IgG1 in response to Poly(I:C) adjuvant. On the contrary, higher levels of OVA-specific IgG1 than IgG2a where observed when either alum or MMP-2 was used (Figure 7E). Significantly, these MMP-2-induced $T_H2$ responses were abrogated in TLR2-deficient animals (Figure 7F). Together, these results establish that MMP-2 polarizes type 2 immune responses in a TLR2-dependent manner in vivo, and that this property is independent of its enzymatic activity.

In summary, our data demonstrate that MMP-2 is a physiological TLR2 ligand/agonist responsible for cytokine production and OX40L upregulation on DCs through MyD88 and NF-κB activation, which has the capacity to skew $T_H2$ responses in vivo.

## DISCUSSION

MMP-2 modulates many physiological conditions including promoting wound repair, tissue remodeling, and metastasis. In the latter case, tumor-associated MMP-2 expression correlates with later tumor stages, increased dissemination, and poorer survival/prognosis (Egeblad and Werb, 2002; Hofmann et al., 2000). General MMP inhibitors have failed in the clinic probably because they were designed to block enzymatic activity, and it was not appreciated that enzymatically inactive MMPs can directly dysregulate innate and adaptive immunity as shown here.

We demonstrated that MMP-2 cannot only function as an antigenic target of tumor infiltrating T cells (Godefroy et al., 2005) but can drive $T_H2$ differentiation through inhibition of IL-12p70 production and upregulation of OX40L expression by human DCs (Godefroy et al., 2005, 2011). Interestingly, although MMP-2 enzymatic activity is necessary to block IL-12p70 production by DCs (Godefroy et al., 2011), we demonstrated here that it was not required for OX40L upregulation (Figure 1A) or inflammatory cytokine production (Figure 1B). Strikingly, both active and inactive forms of MMP-2 induced the same degree of inflammatory response in DCs (Figure 1). These findings indicate that MMP-2 uses a multitude of mechanisms to modulate the immune system, some of which are clearly independent of its catalytic activity and speak to its ability to incapacitate effective

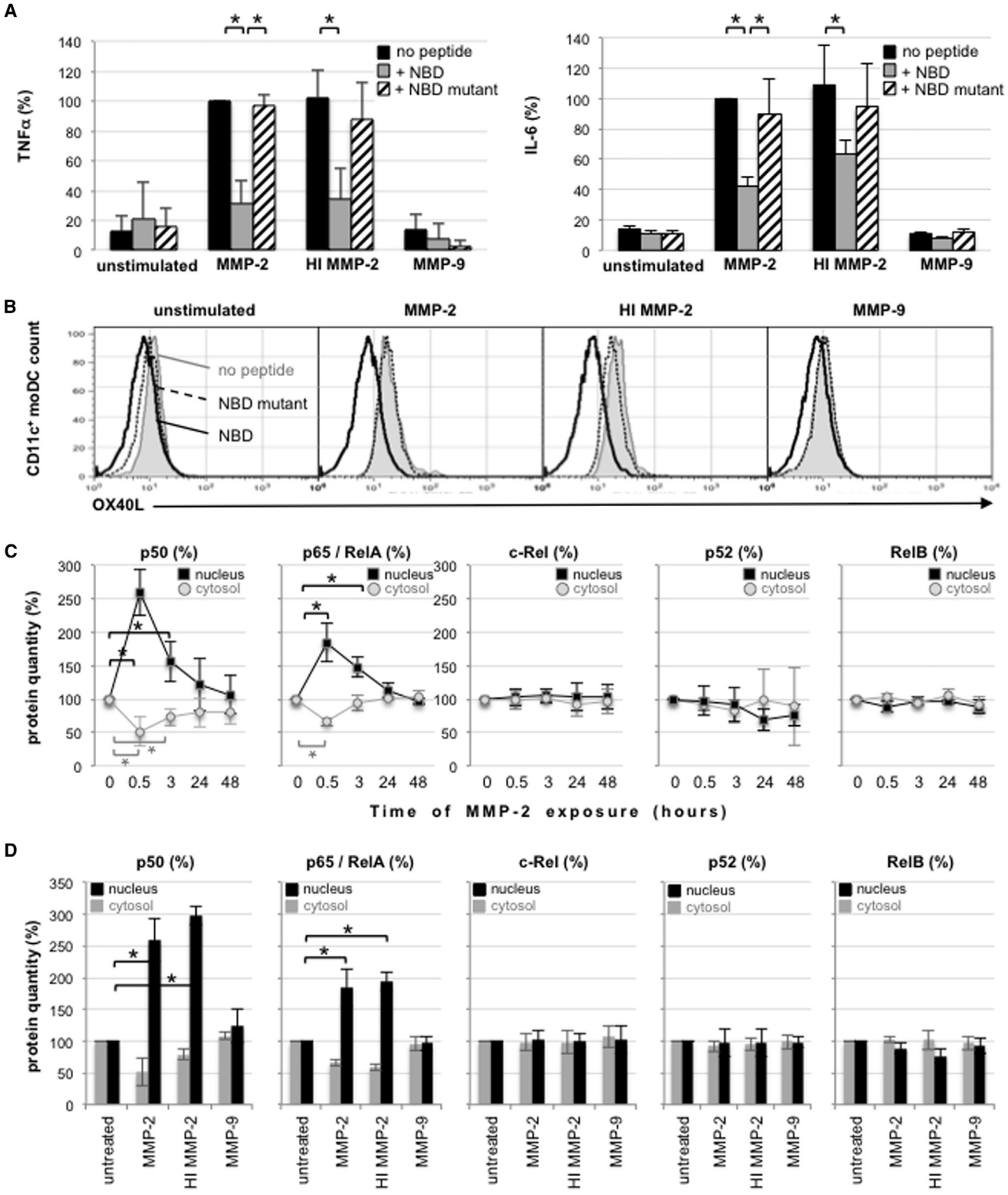


**Figure 2. Involvement of the NF-κB Pathway in MMP-2-Mediated Activation of Human moDCs**

(A and B) MoDCs were incubated with or without either the WT or the mutant NEMO-binding domain (NBD) peptide (4 μM) for 30 min, prior to addition of MMP-2 (5 μg/ml) or controls. (A) Inflammatory cytokine levels were measured in supernatants the following day using CBA technology. (B) OX40L expression was assessed by immunostaining at day 2.

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immune responses through multiple mechanisms. MMP-2 is not alone in this sense as other MMPs possess noncatalytic functions (reviewed in Kessenbrock et al., 2010). MMP-9, through its enzymatically inactive hemopexin domain, promotes both epithelial cell migration probably via MAPK and PI3K activation (Dufour et al., 2008) and B cell survival after triggering $\alpha4\beta1$ and CD44v, which induces intracellular signaling involving Lyn and STAT3 (Redondo-Muñoz et al., 2010). The hemopexin domain of MMP-12 plays a major role in its antimicrobial function (Houghton et al., 2009), and pro-MMP-1 (Conant et al., 2004) and MMP-14 (Rozanov et al., 2004), respectively, inhibit the complement cascade and induce neuronal death in a noncatalytic manner through inhibition of AKT dephosphorylation. Therefore, numerous nonproteolytic functions are mediated by MMPs, and the property of MMP-2 described in this study adds a new dimension.

Our study is distinguishable from those cited above in that MMP-2 specifically polarizes T cell immune responses through manipulation of innate immune cells through pattern recognition receptors: MMP-2 is a physiologic ligand for TLR2 on DCs. This interaction, which does not require catalytic activity, leads to OX40L upregulation and ensuing $T_H2$ polarization (Figure 7; Godefroy et al., 2011). This is consistent with previous work, demonstrating that TLR2 triggering by microbial or synthetic ligands can also skew immune responses toward a $T_H2$ profile (Agrawal et al., 2003; Dillon et al., 2004). Thus, MMP-2 appears to function similar to endogenous signals released in response to infection or tissue remodeling and injury, also referred to as ''alarmins.'' Alarmins are endogenous mediators, produced by several cell types, that can activate and recruit effectors of innate and adaptive immunity (Oppenheim et al., 2007). Interestingly, some of these share similar mechanistic pathways with MMP-2, suggesting that alarmins in general serve an important physiological function in the host, one that may become usurped in certain conditions such as inflammation and tumorigenesis.

In some respects, our observation that MMP-2 modulates DC function to polarize $T_H2$ responses is analogous to what has been described for TSLP, typically produced by epithelial cells in response to helminths (Allen and Maizels, 2011). After triggering its receptor (TSLPR), TSLP induces target cells to produce and release type 2 cytokines such as IL-4 (Siracusa et al., 2011) and upregulate OX40L (Ito et al., 2005), hence promoting protective antiparasite inflammatory $T_H2$ cell differentiation. However, we showed that TSLP-induced signaling differs from MMP-2's as the former was shown to activate NF-κB through translocation of p50 (canonical pathway) and RelB (non-canonical pathway) into the nucleus, where they bind to atypical NF-κB-like sites in the OX40L promoter (Arima et al., 2010). In contrast, MMP-2 only activates components of the canonical NF-κB pathway, i.e., NEMO, p50, and p65 (Figure 2). This divergence might in part be related to the fact that MMP-2 induces OX40L upregulation on both mDCs and moDCs (Figures 1A and S1A), whereas TSLP does so only on primary mDCs (Akamatsu et al., 2008; Arima et al., 2010), but not on moDCs (Figure S2). Notably, TSLPR seems to be expressed by mDCs, but not or minimally by moDCs (Akamatsu et al., 2008), whereas TLR2 is expressed by both subsets (Figure 3B). These different receptors and their expression patterns could therefore account for distinct signaling patterns between MMP-2 and TSLP.

Other $T_H2$-skewing alarmins include cytokines such as IL-25 (Saenz et al., 2010) or IL-33 (Moro et al., 2010; Neill et al., 2010), which drive the production of type 2 cytokines when mounting antiparasite immune responses or during various disorders, such as allergic and autoimmune diseases. IL-33 can be rapidly released during tissue injury (Cayrol and Girard, 2009; Ohno et al., 2009) and is involved in tissue repair after infection by the hookworm parasite *Nippostrongylus brasiliensis* (Wills-Karp et al., 2012). Similarly, HMGB1 has been described not only as an alarmin that can drive the pathogenesis of inflammatory and autoimmune diseases (Harris et al., 2012), but also as a potential mediator in the skin wound healing process in a collagen synthesis-dependent manner (Zhang et al., 2012).

In addition to HMGB1, other alarmin-like molecules stimulate TLRs in vivo. Similar to MMP-2, some of these trigger TLR2. Interestingly, two of them, versican and biglycan, are also components of the extracellular matrix. Versican, a large proteoglycan produced by tumor cells, can activate myeloid cells and induce the production of proinflammatory cytokines through TLR2/TLR6 complexes thereby inducing metastasis (Kim et al., 2009). MMP-2, in contrast, seemingly triggers TLR2 independently of coreceptors including TLR6, both in human (Figures 3, 4, and 6) and murine cells (Figures 7 and S4). TLR4 has also been shown to dimerize with TLR2 (Lee et al., 2004; Popovic et al., 2011; Schaefer et al., 2005). Indeed, the proteoglycan biglycan seems to activate MyD88 and TRIF pathways through TLR2/TLR4 heterodimers, leading to enhancement of T cell priming and autoimmune perimyocarditis (Popovic et al., 2011; Schaefer et al., 2005). However, TLR4 did not seem to be involved in MMP-2-dependent triggering of human TLR2 because HEK293 cells transfected with the plasmid encoding TLR2 alone or in combination with TLR4 responded similarly to MMP-2 (Figure 6). Likewise, WT and $TLR4^{-/-}$ splenic DCs exhibited comparable MMP-2-induced responses in terms of cytokine production (Figure S4A) and OX40L upregulation (Figure S4B). However, $TLR4^{-/-}$ BM-DCs displayed partially impaired cytokine production (Figure S3A), even though they still upregulated OX40L (Figure S3A), suggesting that certain murine DC subsets may utilize TLR4 for optimal MMP-2-mediated TLR2 signaling. MMP-2 did not trigger TLR2/TLR1 heterodimers as HEK293 cells transfected with a plasmid encoding both TLR2 and TLR1 versus TLR2 alone did not further increase MMP-2-mediated responses (Figure 6). Furthermore, $Pam_3CSK_4$ (TLR2/TLR1 agonist) did not induce OX40L in DCs (Figure 3A), suggesting that this heterodimer does not participate in OX40L regulation. Altogether, these results suggest that MMP-2 triggers TLR2 homodimers or heterodimers with an unidentified

(C) MoDCs were stimulated with 5 μg/ml MMP-2 and were harvested at indicated times. Levels of NF-κB components were measured in moDC cytosolic and nuclear fractions. Results are represented as percentages of untreated cells.
(D) MoDCs were stimulated for 30 min with 5 μg/ml MMP-2 or controls, and NF-κB components were measured and represented as in (C). Experiments were performed in triplicate. Two-tailed Student's t tests were used to assess inhibition by the NBD peptide and NF-κB translocation into the nucleus (error bars, SD).

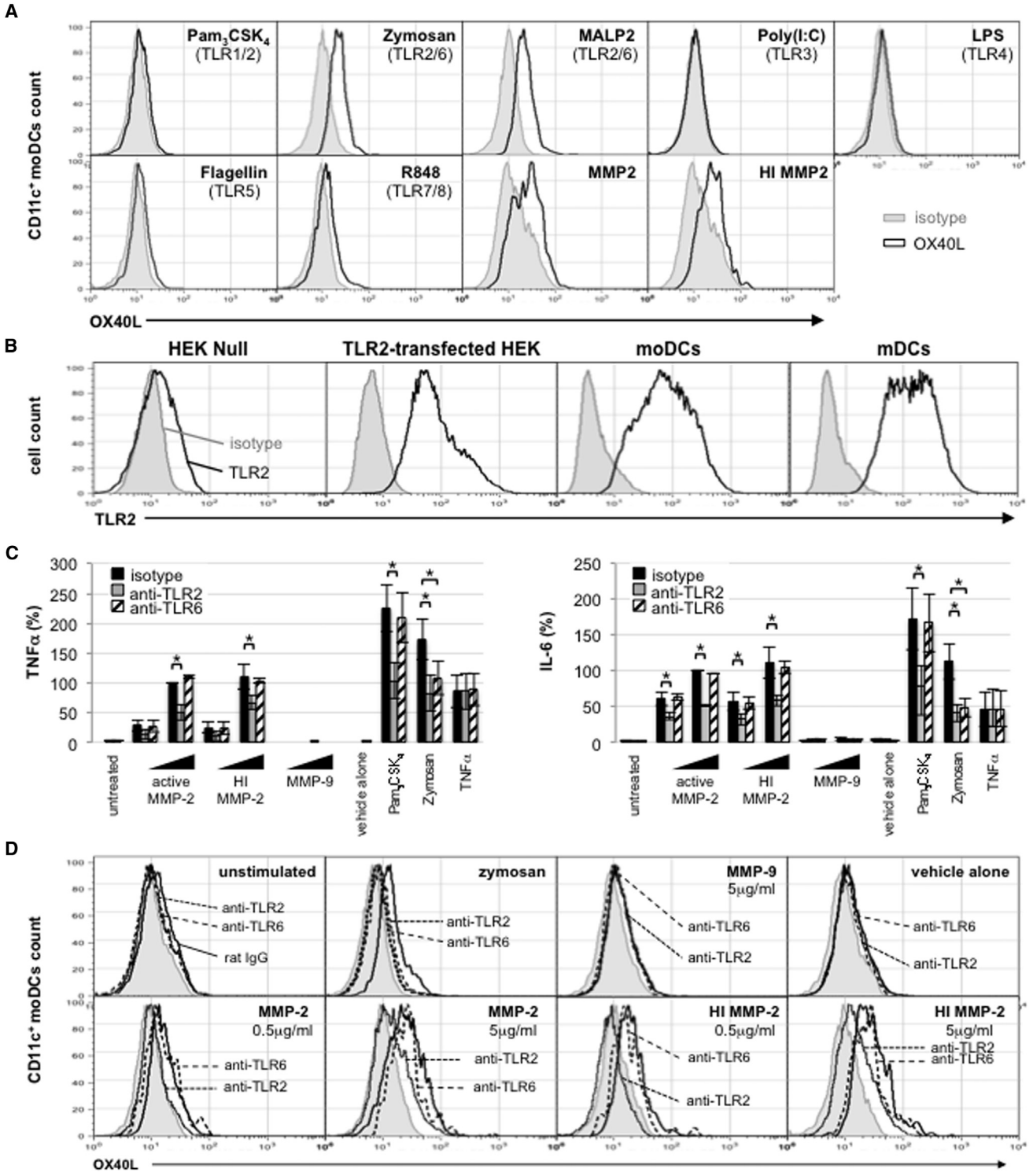


**Figure 3. MMP-2-Induced Activation of moDCs Depends on TLR2**

(A) MoDCs were incubated with 5 μg/ml MMP-2 or various TLR agonists: $Pam_3CSK_4$ (100 ng/ml), Zymosan (1 μg/ml), MALP2 (100 ng/ml), polyI:C (10 μg/ml), LPS (500 ng/ml), Flagellin (100 ng/ml), and R848 (50 μM). OX40L expression was assessed on moDCs 60 hr later.

(B) TLR2 expression by moDCs and mDCs was assessed by immunostaining. TLR2-transfected HEK cells and null HEK cells ($TLR2^-$) were used as positive and negative controls, respectively.

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receptor. Analogously, the eosinophil-derived neurotoxin (EDN), that is generated at sites of inflammation and associated with parasitic infection and allergy, activates TLR2 independently of TLR1 or TLR6 in DCs to enhance $T_H2$ immune responses (Yang et al., 2008). Finally, TLR10 is yet another TLR that can dimerize with TLR2 (Guan et al., 2010; Hasan et al., 2005). Phylogenetic studies indicate that TLR10 is most related to TLR1 and TLR6 (Chuang and Ulevitch, 2001). But, in comparison with these two latter TLRs, TLR10 is expressed in a highly restricted fashion, essentially by B cells and pDCs (Hasan et al., 2005), making it unlikely to be involved in MMP-2/TLR2-dependent $T_H2$ polarization. Furthermore, TLR10, alone or in cooperation with TLR2, failed to activate typical TLR-induced signaling, including NF-κB or IL-8 (Guan et al., 2010).

In addition to the coreceptors described above, TLR2 can utilize accessory receptors that are thought to enhance ligand delivery and/or contribute to cytoplasmic signaling. These include CD14, CD36, and integrin β3. CD14 directly binds $Pam_3CSK_4$ and delivers it to TLR2, substantially increasing the sensitivity of TLR2 responses (Vasselon et al., 2002, 2004). CD14 is not expressed on moDCs (data not shown) and is therefore unlikely to be involved in TLR2 triggering by MMP-2. CD36 can mediate the sensing of some agonists through triggering of the heterodimer TLR2/TLR6 (Hoebe et al., 2005). MMP-2-dependent triggering of TLR2 does not involve TLR6, and thus we postulate that CD36 is not implicated. The integrin chain β3 can participate in sensing bacterial lipopeptides when associated with TLR2 (Gerold et al., 2008). It can also interact with MMP-2 directly via its hemopexin domain (Brooks et al., 1996; Godefroy et al., 2005). A recombinant version of this MMP-2-derived domain, rhPEX, induced neither inflammatory cytokine production nor OX40L upregulation by moDCs (data not shown), hence making β3 unlikely to participate in the MMP-2-mediated model.

Biacore experiments, which demonstrated a direct high-affinity interaction between MMP-2 and TLR2 ($K_D = 3.22 \times 10^{-8}$ M) also support a MMP-2/TLR2 direct interaction. Although additional potential receptors could further increase this affinity, such a $K_D$ value falls within other TLR/ligand binding ranges. For instance, the $K_D$ for the interaction between TLR9 and CpG DNA is around 200 nM (Rutz et al., 2004), which is significantly higher than for the MMP-2/TLR2 interaction. As another example, the $K_D$ for LPS binding to TLR4 (taking into account the interaction with MD2) is approximately 3 nM (Akashi et al., 2003).

Of note, the doses of MMP-2 used both in vitro and in vivo (from 0.5 to 5 μg/ml) are consistent with the physiological concentrations reported in pathological conditions such as in tumors and in the blood of cancer patients (Incorvaia et al., 2007; Schmalfeldt et al., 2001). Therefore, it is not surprising that supernatants derived from MMP-2-secreting melanoma cells induced NF-κB activation and OX40L upregulation in both TLR2-expressing HEK cells and moDCs (Figures S3C–S3G), further reinforcing the physiological relevance of this mechanism.

A fundamental question highlighted by our study is why MMP-2 triggers inflammatory $T_H2$ responses. In addition to participating in controlling parasitic infections, $T_H2$ cells may generally contribute to wound healing following inflammation (Bian and Sun, 1997). MMP-2 is upregulated by several stromal cells of repairing tissues. MMP-2-induced inflammatory $T_H2$ cells produce cytokines (TNF-α, IL-4, and IL-13) that are essential for major steps during wound healing, such as angiogenesis (Kulbe et al., 2012; Larsen et al., 2012), enhancing the production of growth factors like EGF (DeNardo et al., 2009), and antiapoptotic functions (Di Stefano et al., 2010). Therefore, tissue repair functions of MMP-2 might, at least in part, be due to its ability to drive $T_H2$ immune responses. In this respect, it should be pointed out that other alarmins including IL-33/TFF2 and HMGB1 are also involved in wound-healing processes (Wills-Karp et al., 2012; Zhang et al., 2012).

Our findings suggest that MMP-2 could be uniquely targeted in numerous conditions. Blocking antibodies to MMP-2 and/or TLR2 as well as small molecule inhibitors of the MMP-2/TLR2 interaction could be applied to prevent $T_H2$ skewing in metastatic disease. By using it as an adjuvant, and in combination with antigens, MMP-2-triggered OX40L expression and ensuing $T_H2$ polarization could limit the development of destructive $T_H1$ or $T_H17$ inflammatory or autoimmune diseases. Similarly, MMP-2-mediated signaling either through direct injection or through mimics could promote wound healing. The confirmation of these proposed functions will require further investigation in vivo in disease-specific models. In summary, the discovery of these MMP-2 functions adds to the significance of stroma-derived alarmins as potentiators of inflammatory responses in vivo.

## EXPERIMENTAL PROCEDURES

### Monocyte-Derived Dendritic Cell Preparation and Activation

Peripheral blood mononuclear cells (PBMCs) were purified from anonymous healthy donor blood (purchased from the New York Blood Center). PBMCs were plated at $40 \times 10^6$ cells/10 ml/dish in complete RPMI with 5% pooled human serum. Cells were allowed to adhere for 2 hr at 37°C. Nonadherent cells were removed. The adherent monocyte-enriched fraction was supplemented with 100 UI/ml rhGM-CSF and 300 UI/ml rhIL-4 (R&D Systems) on days 0, 2, and 4. Immature DCs were harvested on day 5 and matured/stimulated using various TLR agonists or MMP-2. Secretion of IL-12p70, TNF-α, IL-6, and IL-8 by DCs was assessed using the Human Inflammatory Cytokine Cytometric Bead Array kit (CBA; BD Pharmingen).

### Mice

$TLR2^{-/-}$, $TLR4^{-/-}$, $TLR6^{-/-}$, $TLR7^{-/-}$, $MyD88^{-/-}$, and WT C57/BL6 were provided by Bali Pulendran (Emory Vaccine Center) and Sergio Trombetta (Cancer Institute, NYULMC) and initially originate from Taconic and Jackson Laboratories. Mice were housed under specific-pathogen-free conditions and

(C and D) MoDCs were incubated with blocking Abs or isotype control for 30 min before adding MMP-2 (0.5 and 5 μg/ml) or controls. (C) Inflammatory cytokine secretion was measured in supernatants from three donors the following day. Results are represented as percentages of 5 μg/ml MMP-2-treated cells preincubated with an isotype control. Two-tailed Student's t tests were used to assess TLR-dependent inhibition. (D) OX40L expression was evaluated by immunostaining at day 2.
Error bars, SD. See also Figures S2 and S4.

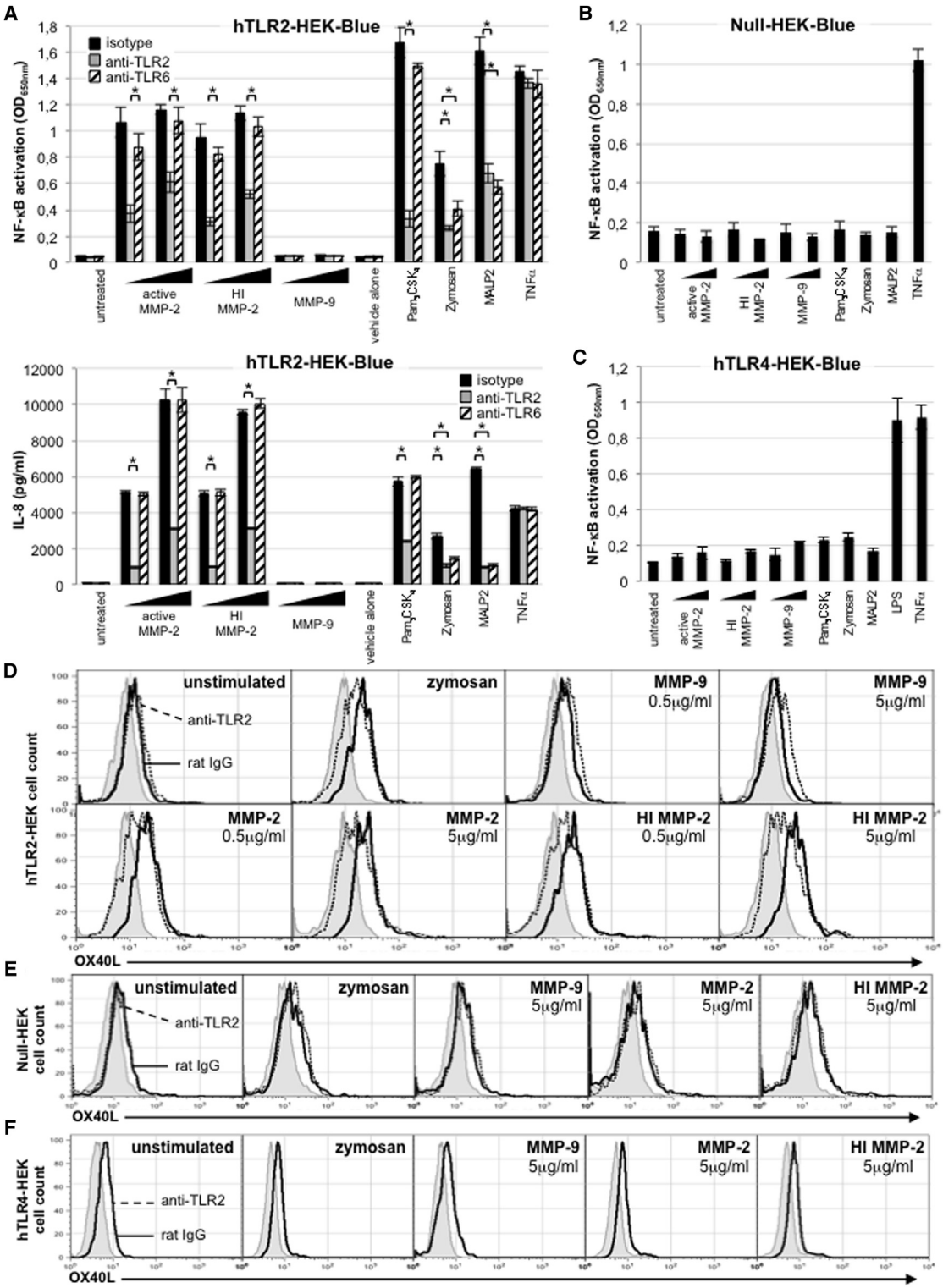



**Figure 4. MMP-2-Induced Activation of HEK Cells Depends on TLR2**

(A–C) (A) TLR2-transfected HEK-Blue cells, (B) parental Null-HEK-Blue cells, or (C) TLR4-transfected HEK-Blue cells were preincubated with TLR-blocking Abs or an isotype control and subsequently stimulated with MMP-2 (0.5 and 5 μg/ml) or controls. After 20 hr, IL-8 production and NF-κB activation were measured as described in Experimental Procedures. Two-tailed paired Student's t tests were used to assess TLR-dependent inhibition.

(D–F) OX40L expression was evaluated by immunostaining at day 2 after indicated treatments.

Error bars, SD. See also Figure S4.

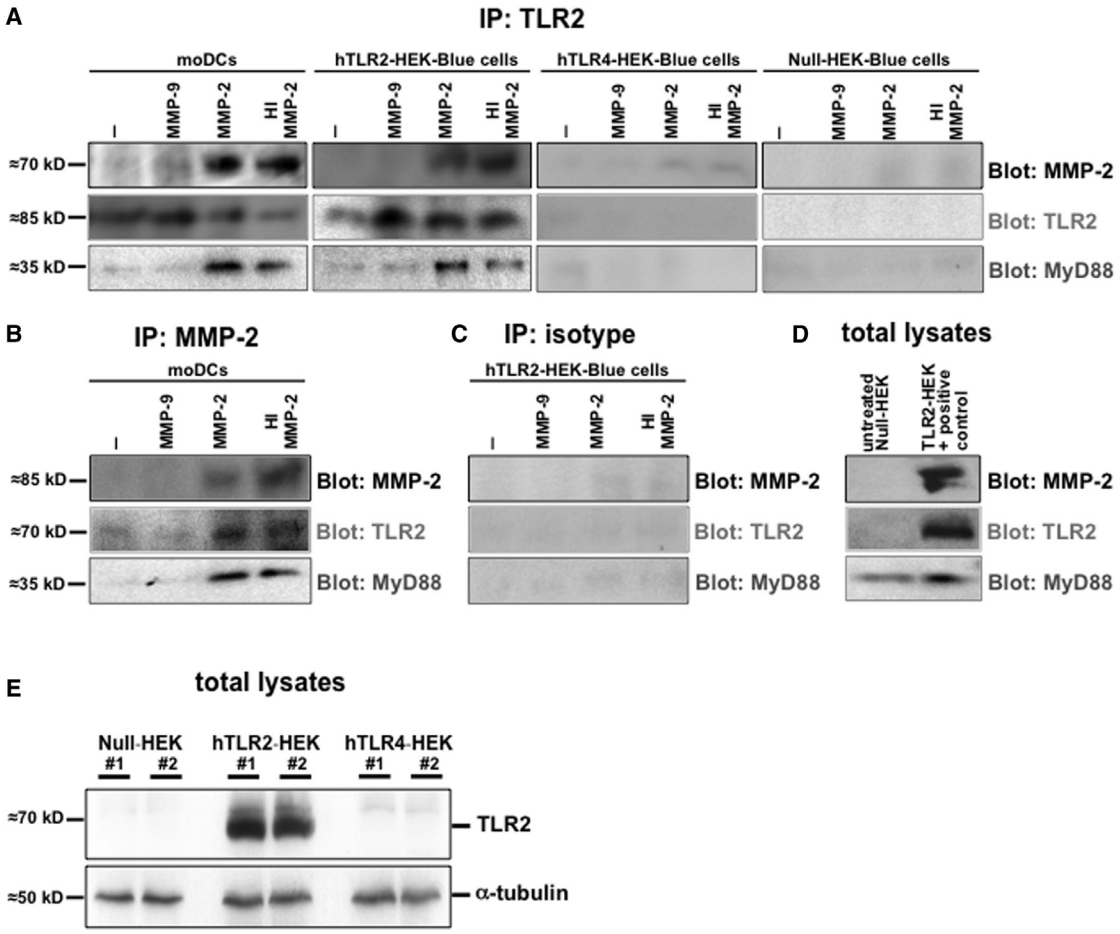



**Figure 5. MMP-2 and TLR2 Coimmunoprecipitate**

(A and B) $10^6$ moDCs or TLR2-transfected HEK cells (equivalents per lane) were incubated for 20 min with or without 5 μg/ml MMP-2 or MMP-9 before being lysed. Untransfected and TLR4-transfected HEK cells were used as negative controls. TLR2 (A) and MMP-2 (B) were immunoprecipitated using specific Abs and protein G-agarose.

(C) An isotype control was used to control the specificity of the immunoprecipitations. Western blots for MMP-2 (≈70 kDa), TLR2 (≈85 kDa), and MyD88 (≈35 kDa) were then performed.

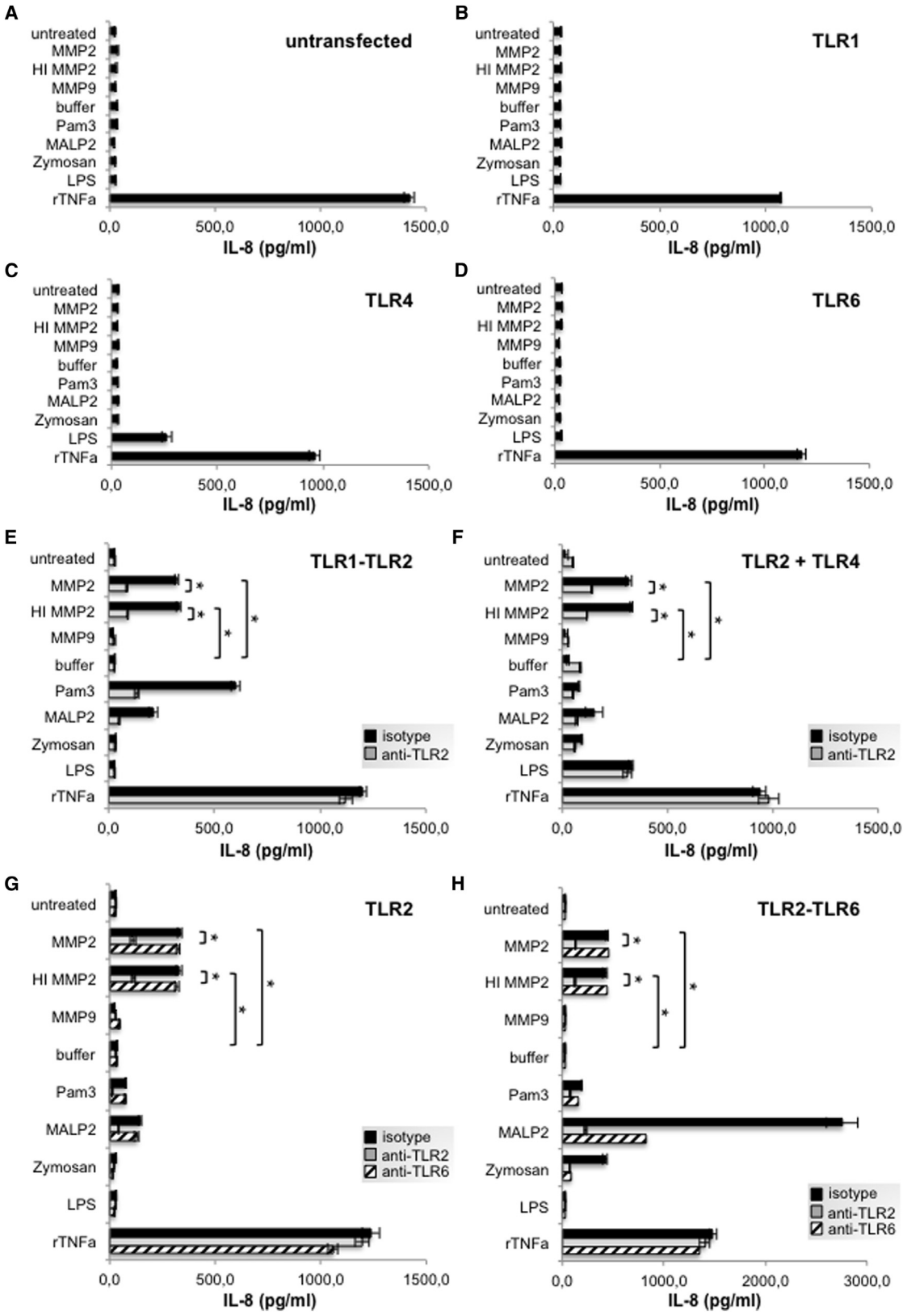
(D and E) Total lysates were used to confirm Ab specificity. Null-HEK-Blue and TLR4-HEK-Blue cells were used as a negative control (D, lane 1; E, lanes 1, 2, 5, and 6). TLR2-HEK-Blue cells preincubated with 5 μg/ml MMP-2 were used to control for MMP-2, TLR2, or MyD88 blotting, respectively (D, lane 2; E, lanes 3 and 4).

maintained in compliance with institutional and federal regulatory guidelines. A IACUC approval was obtained for this study. Mouse BM-DCs were generated as described previously (Lutz et al., 1999). Briefly, $2 \times 10^6$ bone marrow cells were cultured in media supplemented with 20 ng/ml rmGM-CSF (Invitrogen) and differentiated DCs were used for experiments between days 8 and 9. BM-DCs were incubated overnight with 1 or 5 μg/ml of recombinant murine MMP-2 or MMP-9 (active and heat-inactivated forms) as well as TLR agonists for controls. Cytokines released in the supernatant were quantified by CBA analysis. OX40L expression was assessed 2 days later by immunostaining.

For in vivo experiments, 5 μg HI hMMP-2, 100 μg $Pam_3CSK_4$, 2 μg LPS, or controls were administrated i.v. into WT, $TLR2^{-/-}$, or $MyD88^{-/-}$ C57/BL6 mice. Blood was drawn 2 hr later, and sera were tested for IL-6, TNF-α, and MCP-1 contents by CBA analysis. In in vivo skewing experiments, $CD45.1^+$ C57/BL6 mice were transferred i.v. with $3 \times 10^6$ $CD45.2^+$ OVA-specific $CD4^+$ OT-II cells. One day later, mice were inoculated s.c. footpad with 100 μg of LPS-free OVA protein (Seikagaku) together with MMP-2, 50 μg Poly (I:C) (InvivoGen), or 1 mg Alum (InvivoGen). Ten days later, blood and footpad-draining lymph nodes (popliteal, inguinal, and axillary lymph nodes) were harvested. In some experiments, lymph nodes cells suspension was re-stimulated with 2 μg/ml $OVA_{323–339}$ peptide (produced by the proteomics resource center at The Rockefeller University) in the presence of 10 μg/ml BFA (Sigma) for 6 hr, followed by intracellular IFN-γ staining. In other experiments, $2 \times 10^5$ bead-purified CD4+ T cells (Miltenyi) were coculture in vitro with $4 \times 10^4$ splenic $CD11c^+$ bead-purified (Miltenyi) DCs in the presence of 2 μg/ml $OVA_{323–339}$ peptide, and IL-13 ELISA (eBioscience) was performed on the culture supernatants 18 hr later. OVA-specific IgG1 and IgG2a levels were assessed in the blood by ELISA (R&D Systems).

## Reagents

Purified endotoxin-free human and murine MMP-2 as well as its corresponding buffer (also referred as vehicle control herein) were purchased from Enzo/Biomol. MMP-2 was inactivated either by heating at 56°C for 45 min or by pre-incubation with the MMP-2 inhibitor III at 100 nM (Calbiochem) for 20 min. rhMMP-9 and rmMMP-9 were purchased from Calbiochem and R&D Systems, respectively. rhGM-CSF and rhIL-4 were purchased from Immunex and R&D Systems, respectively. $Pam_3CSK_4$, MALP2, and Flagellin were from Imgenex.

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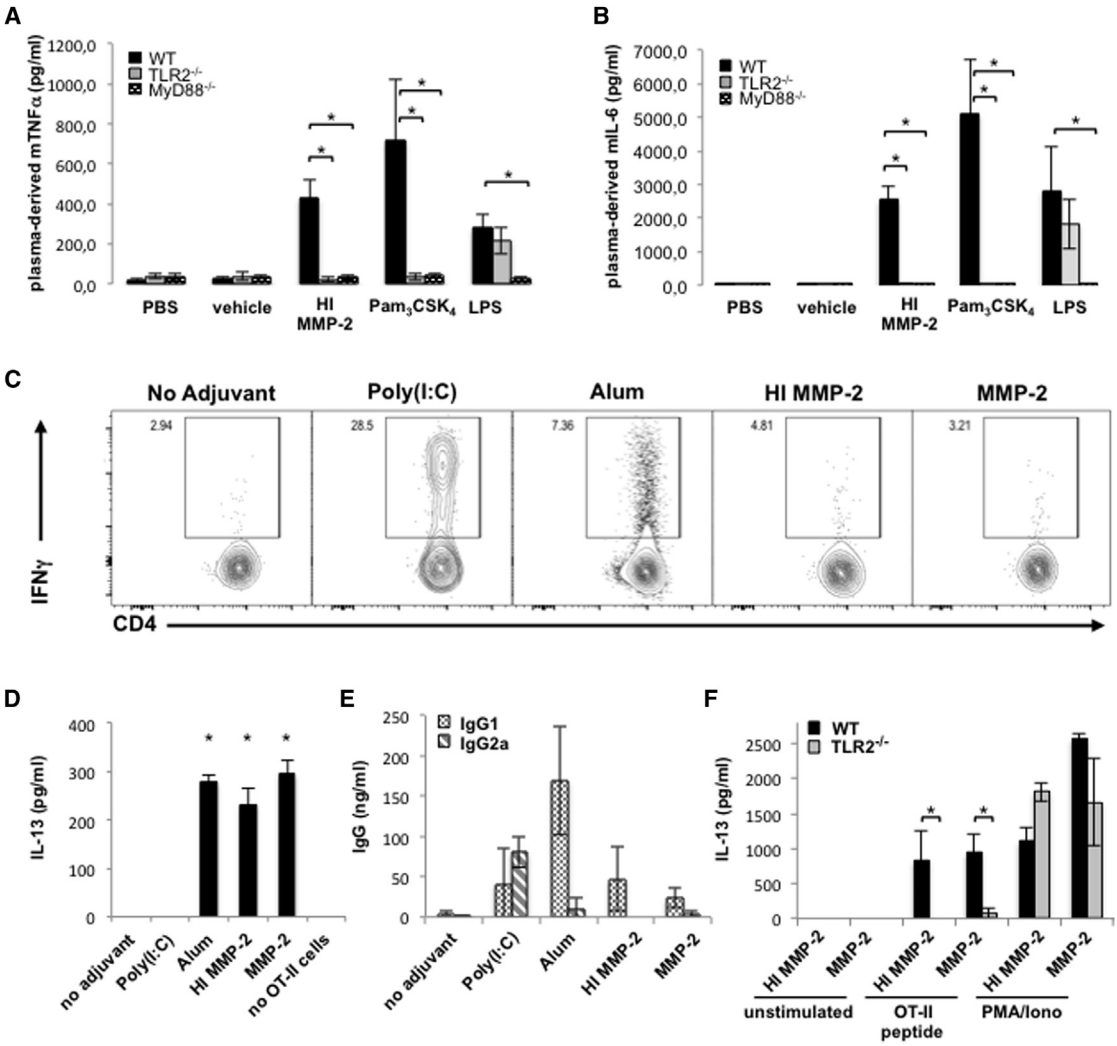


**Figure 7. MMP-2 Triggers In Vivo Inflammation and $T_H2$ Polarization in a TLR2-Dependent Manner**

(A and B) WT, TLR2$^{-/-}$, or MyD88$^{-/-}$ C57/BL6 mice were injected i.v. (tail vein) with 5 μg/ml MMP-2 or controls and plasma was analyzed for TNF-α and IL-6. (C–E) CD45.1$^+$ C57/BL6 mice were transferred i.v. with 3.10$^6$ CD45.2$^+$ OVA-specific CD4$^+$ OT-II cells. The next day, mice received 100 μg OVA protein alone or together with 5 μg/ml MMP-2, 50 μg Poly (I:C), or 1 mg Alum s.c. (footpads). Blood and footpad-draining lymph nodes (popliteal, inguinal, and axillary) were harvested 10 days later. (c) Draining lymph node cell suspension was restimulated for 6 hr with 2 μg/ml $OVA_{323–339}$ in the presence of 10 μg/ml BFA. Shown is IFN-γ production by DAPI$^-$/CD3$^+$/CD8$^-$/CD45.2$^+$/Vα2$^+$ gated cells. Graphs are representative of two similar experiments (with four animals total). (D and F) Bead-purified lymph node-derived CD4$^+$ T cells were restimulated in vitro with bead-purified CD11c$^+$ DC (1:5 ratio) and 2 μg/ml $OVA_{323–339}$ peptide. PMA/ionomycin was used as a positive control for cytokine secretion. Eighteen hours later, IL-13 secretion was measured by ELISA. Shown is the mean ± SD of two experiments with a total of five to six animals. (E) OVA-specific IgG1 and IgG2a levels were assessed in the serum by ELISA. Shown is the ratio of IgG1/IgG2a as the mean ± SD of five to six animals in two different experiments. In all cases, two-tailed paired Student's t tests were used to compare MMP-2 conditions to the vehicle control (buffer).

Zymosan and ultrapure LPS were from InvivoGen. Poly(I:C) was from Amersham. R848 was from 3M Pharmaceuticals. Inflammatory cytokines were quantified using CBA kits (BD Biosciences). The nuclear extraction as well as the TransAM NF-κB ELISA kits (Active Motif) were used according to the manufacturer's instructions. The cell-permeable NEMO-binding domain inhibitory peptide (DRQIKIWFQNRRMKWKKTALDWSWLQTE) and its mutant (DRQIKIWFQNRRMKWKKTALDASALQTE) were from Calbiochem. Protein G-agarose (Pierce) was used according to the manufacturer's instructions.

**Figure 6. MMP-2 Activates TLR2-Transfected HEK Cells**

WT HEK cells were or were not transfected with plasmids encoding human TLR1, TLR2, TLR4, TLR6, TLR1, and 2, alone or in combination using FuGene HD transfection reagent. Twenty-four hours later, cells were preincubated with blocking Abs before addition of 5 μg/ml MMP-2 or indicated controls. IL-8 production in supernatants was evaluated after O/N culture. Results are represented as a mean of triplicates ±SD. Two-tailed paired Student's t tests were used to compare MMP-2 conditions to the vehicle control (buffer).

### Plasmid Preparation and Transfection

Plasmids encoding both GFP and TLR1, TLR2, TLR4, or TLR6 (pUNO-hTLR-GFP), TLR2, and TLR1 (pDUO-hTLR1/TLR2) as well as TLR2 and TLR6 (pDUO-hTLR2/TLR6) were purchased from InvivoGen. To limit endotoxin contamination, plasmids were purified using the EndoFree Plasmid Maxi kit (QIAGEN). FuGene HD transfection reagent (Promega) was used at a reagent/DNA ratio of 3:1.

### Antibodies

PE-conjugated antibody (Ab) to OX40L (mouse mAb 11C3.1) was from BioLegend. Flow cytometry Ab to TLR2 (mouse mAb 1030A5.138) was from Abnova. Abs to TLR2 (mouse mAb TL2.1) and MMP-2 (mouse mAb 101724) used for immunoprecipitation were from Abcam and R&D Systems, respectively. Western blot Abs to TLR2 (rabbit pAb), MMP-2 (rabbit pAb H-76), and MyD88 (rabbit pAb) were from Abcam, Santa Cruz Biotechnology, and Cell Signaling Technology, respectively. HRP-linked anti-rabbit IgG (Cell Signaling) were used as a secondary Ab before chemiluminescent detection (ECL Plus, Amersham Biosciences). Blocking Abs for TLR2 (rat IgG pAb) and TLR6 (rat IgG pAb) were from InvivoGen. Abs against murine CD11c (Armenian hamster mAb N418, allophycoerythrin conjugated) and OX40L (rat mAb RM134L, PE conjugated) were from BioLegend.

### Biacore

Binding experiments were performed on Biacore 3000 at 25°C by Precision Antibodies Company. 400RU of TLR2 (R&D Systems) was captured on the flow cell 2. MMP-2 was flowed over the chip at variable concentrations. Binding of MMP-2 to TLR2 was monitored in real time. Full kinetic analysis was performed using 50 nM MMP-2, followed by serial dilutions. The equilibrium affinity constant ($K_D$) was calculated by steady-state kinetic analysis.

### Statistical Analysis

Separate analyses were performed for each experiment individually and take into account paired observations within donors when appropriate. For three-group comparisons (e.g., isotype, anti-TLR2, or anti-TLR6), analyses of variance were performed for an overall comparison among independent groups, and t tests were then used for specific pairwise comparisons between groups. Two-sided statistical tests were performed at an overall alpha-level of 0.05. Details for each analysis are provided in the figure legends. p values $\leq$ 0.05 (*) were considered statistically significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.067.

## AUTHOR CONTRIBUTIONS

E.G. initiated the study as well as performed, analyzed, and interpreted all human-related experiments, participated in mouse-related experiments, and wrote the manuscript; A.G. did and analyzed most of the mice-related experiments as well as contributed to the study organization and discussions; J.I. performed most in vivo experiments and contributed to study design and discussions; N.T. performed control in vitro assays and participated in in vivo experiments; N.M. provided technical help with mice; B.P. and R.N. provided spleens and legs of WT, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, and TLR7$^{-/-}$ mice; F.J. contributed to the study organization and discussions; M.M. contributed to discussions; S.T. provided most WT, TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice, handled the mice, and participated in study design and discussion; N.B. supervised the project. E.G., A.G., J.I., S.T., and N.B edited the manuscript.

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