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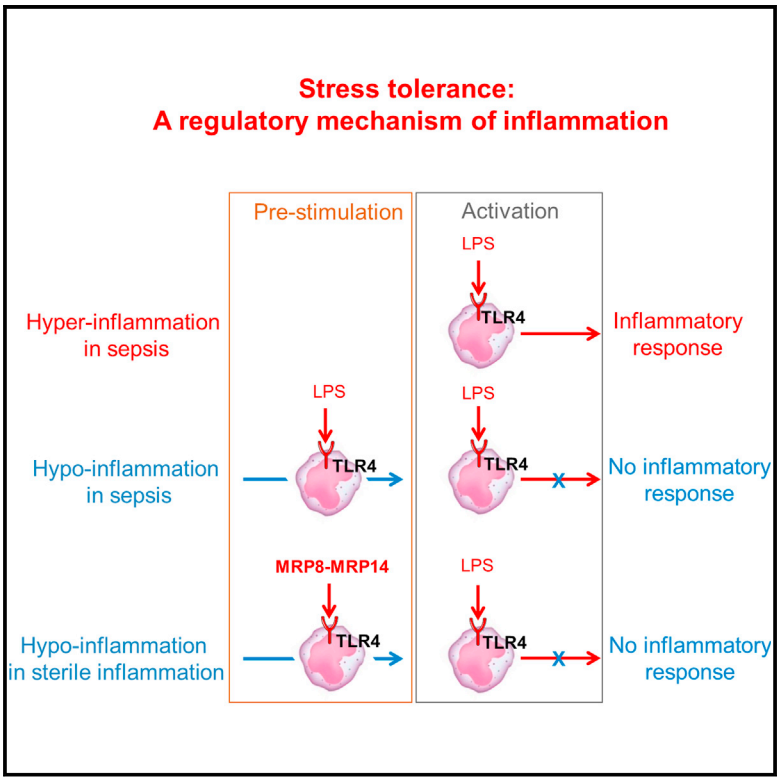
Alarmins MRP8 and MRP14 Induce Stress Tolerance in Phagocytes under Sterile Inflammatory Conditions

Graphical Abstract



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In Brief

Austermann et al. show that the endogenous alarmins MRP8 and MRP14 induce phagocyte hyporesponsiveness, which represents a new regulatory mechanism for the susceptibility of neonates to systemic infections and during sterile inflammation in poly- and burn trauma patients.

Highlights

- Preactivation by alarmins MRP8-MRP14 blocks phagocytes in a TLR4-dependent manner
- MRP-induced stress tolerance is mediated by G9a-dependent chromatin modifications
- Pretreatment with MRP8-MRP14 inhibits inflammation in murine endotoxic shock models
- Sterile stress-induced tolerance is relevant in polytrauma and neonatal sepsis

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Alarmins MRP8 and MRP14 Induce Stress Tolerance in Phagocytes under Sterile Inflammatory Conditions

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SUMMARY

Hyporesponsiveness by phagocytes is a well-known phenomenon in sepsis that is frequently induced by low-dose endotoxin stimulation of Toll-like receptor 4 (TLR4) but can also be found under sterile inflammatory conditions. We now demonstrate that the endogenous alarmins MRP8 and MRP14 induce phagocyte hyporesponsiveness via chromatin modifications in a TLR4-dependent manner that results in enhanced survival to septic shock in mice. During sterile inflammation, polytrauma and burn trauma patients initially present with high serum concentrations of myeloid-related proteins (MRPs). Human neonatal phagocytes are primed for hyporesponsiveness by increased peripartal MRP concentrations, which was confirmed in murine neonatal endotoxinemia in wild-type and MRP14$^{-/-}$ mice. Our data therefore indicate that alarmin-triggered phagocyte tolerance represents a regulatory mechanism for the susceptibility of neonates during systemic infections and sterile inflammation.

INTRODUCTION

Activated phagocytes initiate the first line of defense against infections by sensing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), e.g., lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4). During sepsis, activation of TLRs trigger proinflammatory mechanisms (Rittirsch et al., 2008). However, dysfunction of TLR signaling may lead to hypoinflammation and immune paralysis, especially of the phagocyte system. Interestingly, preactivation of TLR4 by LPS results in transient hyporesponsiveness of phagocytes to subsequent challenge, known as "endotoxin tolerance" (ET). This phenomenon is potentially responsible for the immune paralysis seen in the later course of sepsis, impairing the host's ability to combat subsequent bacterial infection.

Hyporesponsiveness of phagocytes under sterile conditions is a very relevant problem in different clinical conditions where a remarkable sensitivity to systemic infections is shown. For example, polytrauma or burn injury patients develop a state of immune paralysis to secondary infections. In neonates, an impaired response of the innate immune system has been linked to the high susceptibility to infection (Wynn et al., 2008). With all these conditions, an initial microbial trigger is missing, whereas the adjusted state of immune responsiveness appears to be critical (Keel et al., 1996). There is now growing evidence that disseminated inflammation can be modulated by noninfectious TLR ligands (Cavaillon et al., 2003; Yadavalli et al., 2001). Apart from PAMPs, endogenous PRR activators, alias damage-associated molecular patterns (DAMPs) or alarmins, are released in response to tissue damage or danger signals and promote inflammation (Vogl et al., 2012). The phagocyte-specific proteins myeloid-related protein 8 (MRP8) (S100A8) and MRP14 (S100A9) are endogenous ligands of the TLR4-CD14-MD2 complex, amplifying phagocyte activation during sepsis upstream of tumor necrosis factor alpha (TNF-α)-dependent effects (Vogl et al., 2007).

We now demonstrate that endogenous MRP8 and MRP14, similar to exogenous LPS, prime phagocytes for a state of hyporesponsiveness to subsequent microbial challenge in vitro and in vivo. Studies were confirmed by comparative bioinformatic analyses of genome-wide response patterns of human monocytes

Table 1. Epidemiologic and Clinical Characteristics of the Patient Populations

	Trauma	Burns
n	32	31
Age (years)	46.8 ± 18.7	46.8 ± 20.0
Male (%)	75.0	87.1
ISS	32.3 ± 10.8	N/A
TBSA (%)	0	41.4 ± 15.4
With inhalation injury (%)	0	41.9
ICU (8d)	16.4 ± 16.0	34.0 ± 27.7
Sepsis/MOF (%)	20.1	9.4
Mortality (%)	15.6	29.0

Data are means ± SD or percentages. N/A, not applicable.

to tolerizing doses of MRP8-MRP14 or LPS. This MRP-mediated effect depends on aberrations in the nuclear factor (NF)-κB pathway and methyltransferase G9a-dependent chromatin modifications.

This mechanism is of clinical relevance in different conditions of sterile stress and inflammation such as polytrauma and burn trauma or perinatal susceptibility of newborns to infections. Our observations reveal that phagocytes can develop a general "stress tolerance" rather than specific ET, which is of potential relevance for new therapeutic strategies in sterile inflammatory conditions.

RESULTS

MRP8-MRP14 Serum Levels in Sterile Inflammatory Conditions

We analyzed the serum concentrations of MRP8-MRP14 in polytrauma and burn patients (Table 1). We found a biphasic increase of systemic MRP8-MRP14 level in both polytrauma (Figure 1A) and burn trauma patients (Figure 1B) with the early peak at initial presentation representing the innate response to injury. In burn trauma patients, MRP8-MRP14 concentrations at the early peak (day 1) significantly correlated with the percentage of at least second-degree burned total body surface area (TBSA) (Pearson's correlation coefficient 0.47; $p \leq 0.01$). Moreover, survivors showed significantly lower MRP8-MRP14 concentrations than nonsurvivors (Figure 1C). Surprisingly, when we analyzed the value of MRP8-MRP14 as a marker for neonatal sepsis, we found that extremely high serum levels were already detectable in healthy newborns in the first week of life (Figure 1D). Comparably high levels in adults are only found in inflammatory diseases (Frosch et al., 2003, 2009; Viemann et al., 2007).

MRP8 and MRP14 Induce Self- and Microbial Tolerance

We analyzed the production of proinflammatory cytokines in human monocytes prestimulated with different, nonactivating doses of LPS, MRP8, MRP14, or MRP8-MRP14 (24 hr) prior to activation with LPS (4 hr). These prestimulations significantly and dose-dependently attenuated TNF-α release in response to subsequent LPS restimulation (Figures 2A and 2B). In addition, the mRNA level of several cytokines as *TNF*, *IL1B*, and *IL6* were decreased in MRP-pretreated cells after LPS activation (Figure 2C) whereas other transcripts as formyl peptide receptor 1 (*FPR1*) or serine/threonine protein kinase (*SGK*) were increased (Figure S1A). Moreover, genes that have been shown not to be sensitive to endotoxin tolerance (Foster et al., 2007) as inhibitor of DNA binding 3 (*ID3*) or C-type lectin domain family 4 member E (*CLEC4E*) were also insensitive to MRP-induced tolerance (Figure S1B). Quality of MRP protein preparations was validated and endotoxin effects excluded by heat-inactivation experiments (Figure S2). Heat-inactivated MRPs failed to induce tolerance.

Approximately 24 hr after removing the tolerizing stimulus, monocytes lose their tolerance status and respond to LPS with adequate TNF-α production (Figure 2D). Pretreatment with MRP8-MRP14 also induced cross-tolerance to subsequent bacterial lipoteichoic acid (LTA) or MRP restimulation (Figures 2E–2G). Because all MRP derivatives showed a tolerizing effect, we focused on the MRP8-MRP14 complex, which represents the physiologically dominant form (Foell et al., 2007; Roth et al., 1993; Vogl et al., 2004), in subsequent experiments.

To get an unbiased impression about analogies between MRP-induced and LPS-induced tolerance, we performed comparative bioinformatic analyses of genome-wide response patterns of human monocytes to MRP8-MRP14 and LPS. Assessing the variance in gene expression by hierarchical clustering (HC) of the 1,000 most-variable transcripts within the data set clearly revealed a strong difference between the group of prestimulated and activated (MRP8-MRP14-LPS and LPS-LPS) and the prestimulated, but not activated, conditions (MRP8-MRP14-con and LPS-con; Figure 3A). More importantly though, there was shown to be no difference in global gene expression between samples pretreated with either MRP8-MRP14 or LPS, regardless of activation. Visualizing differentially expressed (DE) genes between prestimulated/activated and prestimulated/nonactivated monocytes in a ratio/ratio plot corroborated these findings, placing DE genes on a diagonal line, which is indicative of high correlation between the two different pretreatments (Figure 3B). Figure 3C shows exemplary expression values for candidate genes of both tolerance settings: nontolerized (*IL1RN* and *IL10*) or tolerized (*TLR4*).

High Postnatal MRP8-MRP14 Plasma Levels Attenuate the LPS Response of Monocytes

The LPS response of human cord blood (CB) monocytes was significantly impaired compared to adult blood (AB) monocytes with respect to RNA and protein induction of many proinflammatory genes (Figures 4A and 4B). Preincubation of adult monocytes with CB plasma impaired their LPS response, comparable to the response of CB monocytes, whereas preincubation with medium or AB plasma had no effect (Figure 4C). The same inhibitory effect on proinflammatory gene induction by LPS was seen in medium transfer experiments with supernatants of CB monocytes, but not AB monocytes (Figure 4D). Together, these findings suggest the inhibitory factor in CB plasma as a specific product of CB monocytes. Subsequently, we excluded higher basal secretion of proinflammatory cytokines (interleukin-6 [IL-6], TNF-α, and IL-1β) with potentially exhausting

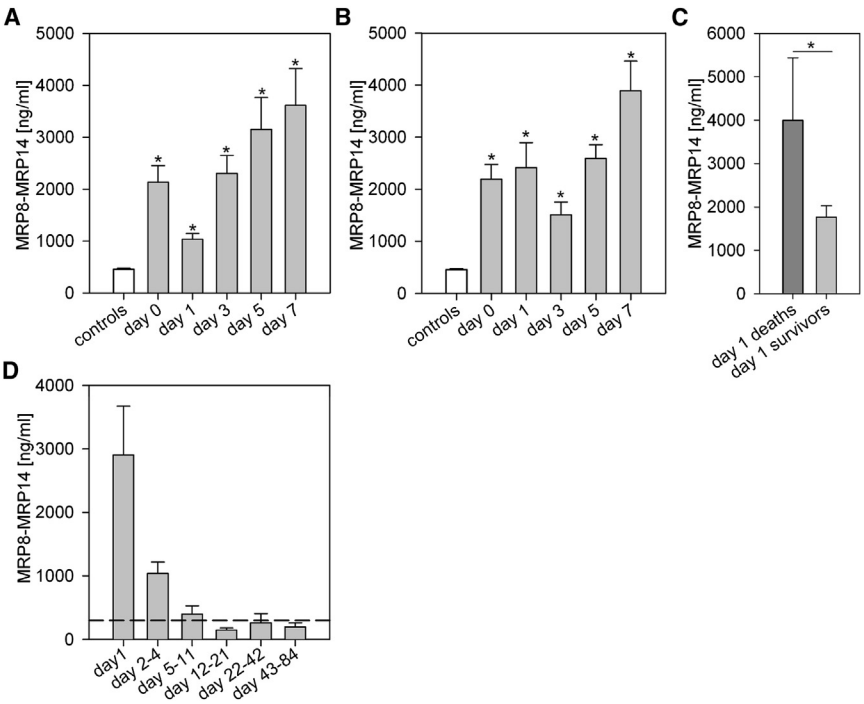

Figure 1. MRP8-MRP14 Concentrations in Sterile Stress and Inflammatory Conditions

(A–C) MRP8-MRP14 levels in (A) polytrauma and (B) burn trauma patients at initial presentation and during the later course of disease and (C) at day 1 in survivors (n = 22) and nonsurvivors (n = 9) after burn injury. Bars represent means ± SD (*p < 0.05). (D) MRP8-MRP14 levels in healthy term newborn during the first 3 months of life (n = 98). The dotted line indicates the mean + two SD of normal adult MRP8-MRP14 serum levels (n = 80).

effects or anti-inflammatory IL-10 by CB monocytes compared to AB monocytes (Figure 4E). However, the basal secretion of MRP8-MRP14 was significantly increased in human CB monocytes compared to AB monocytes (Figure 4E). Similar to AB monocytes (Figure 2D), the LPS response of CB monocytes recovered after 24 hr if the cells were cultured in medium or AB plasma, but not in CB plasma or medium spiked with MRP8-MRP14 (Figure 4F). Inhibition of the proinflammatory LPS response by CB plasma could almost be completely abrogated by either depletion of MRP8-MRP14 from CB plasma (Figure 4G) or pharmacological inhibition of MRP8-MRP14 using paquinimod (Figure 4H; Bengtsson et al., 2012). Our data demonstrate that MRP8-MRP14 in CB plasma is a crucial endogenous tolerizing factor.

Murine Mrp8-Mrp14 Induces Microbial Tolerance In Vivo

To investigate the biological relevance of these phenomena, we analyzed Mrp8-Mrp14-induced ET in a model of endotoxic shock in adult mice (LPS/D-galactosamine [D-Gal] model; Galanos et al., 1979). After intraperitoneal injection of LPS/D-Gal, nonpretreated mice showed acute symptoms, with most dying within 5–12 hr. In contrast, mice pretreated with murine Mrp8-Mrp14 complex showed reduced illness and a significantly higher survival rate (Figure 5A). The tolerizing effect of Mrp8-Mrp14 was stronger when mice were pretreated twice instead of once and comparable to classical LPS-induced ET. In vitro prestimulation of naive murine bone-marrow-derived macrophages (BMDMs) with low doses of LPS and Mrp8-Mrp14 caused no inflammatory response (Figure 5B) but attenuated the release of Tnf-α upon subsequent LPS (Figure 5C) or LTA (Figure 5D) restimulation, comparable to the effects seen in human monocytes. Taken together, these data demonstrate that Mrp8-Mrp14 induces ET, promoting survival during LPS/D-Gal-induced septic shock.

In a second mouse model of neonatal endotoxemia, we analyzed if the high postnatal release of Mrp8-Mrp14 induces LPS tolerance in vivo. First of all, we confirmed excessive high plasma levels of Mrps in healthy neonatal mice that, like in humans, decreased thereafter into the normal range for adult mice (Figure 5E). Already 2 hr after LPS injection, we observed significantly higher increases in Tnf-α and Il-6 in Mrp14$^{-/-}$ mice compared to wild-type (WT) mice (Figures 5F and 5G). Due to this overwhelming inflammation, Mrp14$^{-/-}$ mice showed significantly lower survival after LPS challenge, indicating that Mrp8-Mrp14 promotes a hypoinflammatory LPS response in neonatal WT mice (Figure 5H).

The Tolerance-Inducing Effect of MRP8-MRP14 Depends on Aberrations in the NF-κB Pathway

In order to analyze the molecular mechanisms underlying the induction of MRP-induced tolerance, we investigated the upregulation of negative regulators of TLR signaling like interleukin-1-receptor-associated kinase 1 (*IRAK1*), suppressor of cytokine signaling 1 (*SOCS1*), *SOCS3*, and tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) by quantitative RT-PCR (qRT-PCR). We observed an upregulation of *IRAK1*, *SOCS3*, and *TNFAIP3* as well as a downregulation of *SOCS1* during pretreatment with LPS and MRP (Figure 6A). Because TLR4-dependent signaling requires p38 mitogen-activated protein kinase (p38 MAPK) activation, we analyzed the phosphorylation of p38 MAPK in prestimulated and LPS-restimulated monocytes (Figure 6B). We found no difference in p38 MAPK phosphorylation between naive and pretreated cells during LPS restimulation, suggesting that p38 MAPK activation is unaltered by LPS or MRP prestimulation. Accordingly, the tolerant state of MRP-prestimulated human monocytes was also sustained in the presence of SB202190 inhibitor (Figure 6C).

We went on to analyze the activation of NF-κB, which also plays an important role in LPS-induced tolerance (Biswas and Tergaonkar, 2007). Prestimulated monocytes differed from naive monocytes by an increased nuclear translocation of the NF-κB subunits P65, P50, and RelB upon restimulation with LPS (Figure 6D). We confirmed the relevance of NF-κB signaling in the

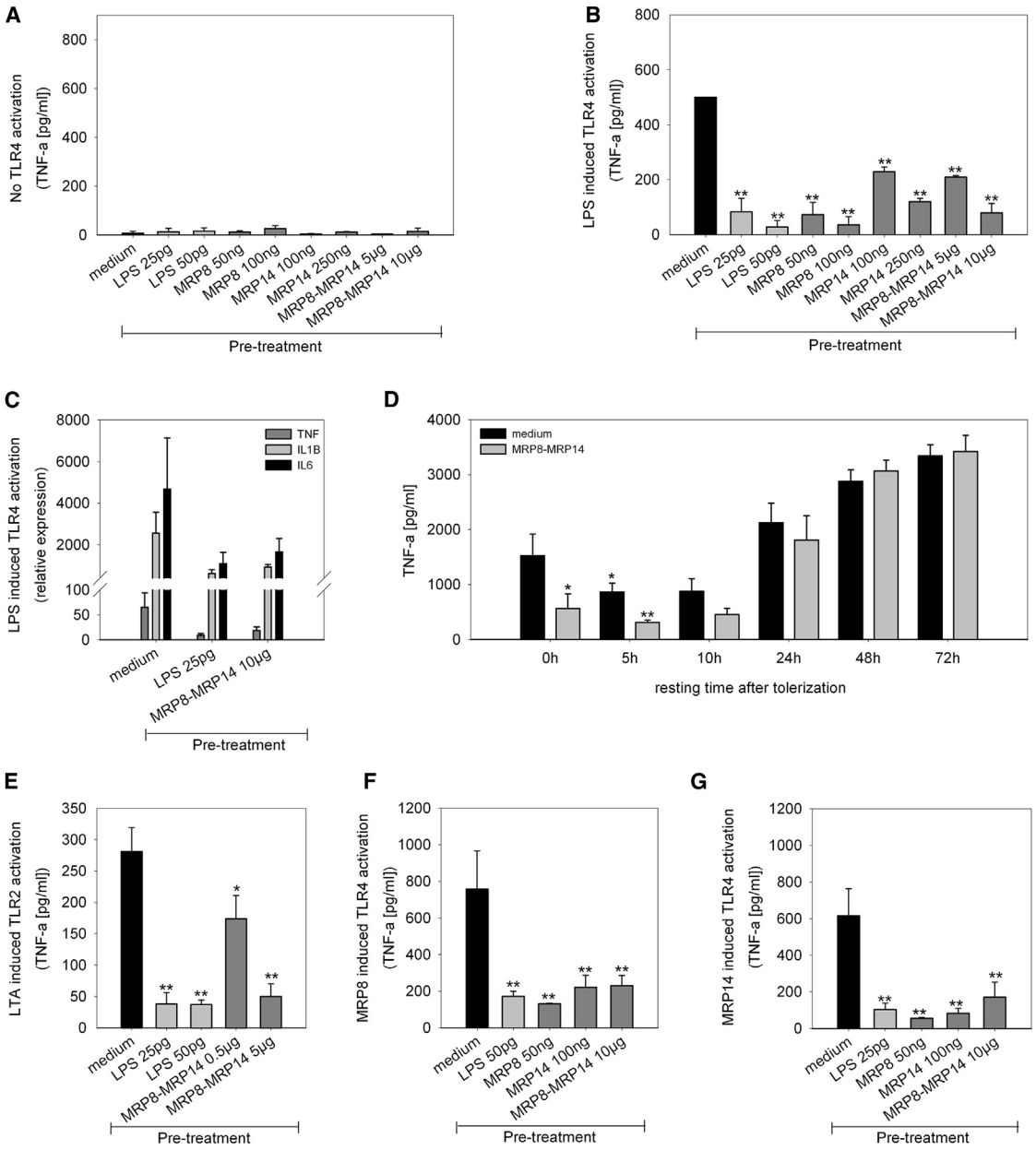

Figure 2. MRP Pretreatment of Monocytes Induces Microbial and Self-Tolerance

(A) TNF-α levels in supernatants of human monocytes after (A) 24 hr pretreatment with medium, LPS, MRP8, MRP14, or MRP8-MRP14 complex (n = 4).

(B) Pretreated cells were activated with LPS (1 ng/ml) for 4 hr, and TNF-α production was analyzed. Data represent mean ± SD and were compared to LPS-activated control monocytes (n = 4; $^{**}p < 0.01$; $^{*}p < 0.05$).

(C) Prestimulated cells were 4 hr activated with LPS (1 ng/ml). mRNA abundance in cells was detected by qRT-PCR. Results are normalized to baseline expression in control cells, set at 1. Data represent mean ± SD (n = 4).

(D) Monocytes were left untreated or 24 hr pretreated with 10 μg/ml MRP8-MRP14. After medium change and indicated resting times, the cells were 4 hr activated with LPS (1 ng/ml) and TNF-α production was analyzed. Data represent means + SEM and were compared to data from untreated control cells (n = 6; $^{**}p < 0.01$; and $^{*}p < 0.05$).

(E) Pretreated cells were activated with LTA (1 μg/ml) for 4 hr, and TNF-α production was analyzed. Naive monocytes activated with LTA were used as a positive control (black bar). Data represent mean ± SD and were compared to LTA-activated naive monocytes (n = 4; $^{**}p < 0.01$; $^{*}p < 0.05$).

(F) Pretreated and naive monocytes (black bar) were activated with MRP8 (1 μg/ml) for 4 hr, and TNF-α production was analyzed. Data represent mean ± SD and were compared to MRP8-activated naive monocytes (n = 4; $^{**}p < 0.01$).

(G) Pretreated cells and naive monocytes (black bar) were activated with MRP14 (1 μg/ml) for 4 hr, and TNF-α production was analyzed. Data represent mean ± SD and were compared to MRP14-activated naive monocytes (n = 4; $^{**}p < 0.01$).

See also Figures S1 and S2.

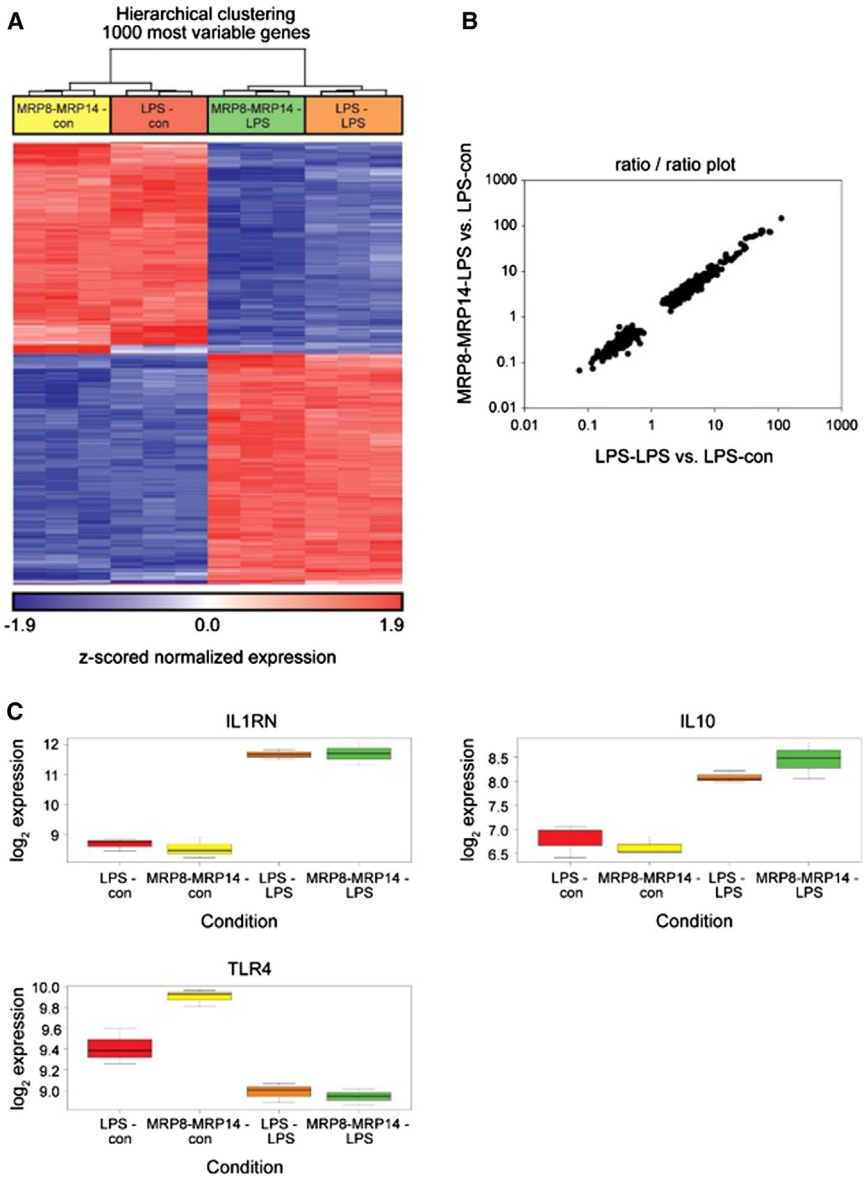

Figure 3. Bioinformatic Analysis of Microarray Data of MRP- and LPS-Tolerance-Induced Monocytes

(A) HC of the 1,000 transcripts with the most-significant variance in samples from MRP8-MRP14-con-, MRP8-MRP14-LPS-, LPS-con-, and LPS-LPS-treated mice.

(B) Ratio/ratio plot for transcripts significantly differentially expressed between LPS-LPS against LPS-con and MRP8-MRP14-LPS against LPS-con (SP; FC > 2; unadjusted p value < 0.05).

(C) Log2 expression values of *ILRN*, *IL10*, and *TLR4* determined by DNA microarray analysis.

process of MRP-induced tolerance by treating monocytes during tolerance induction with the NF-κB inhibitor Bay117085. Inhibiting NF-κB activation during tolerance induction led to a substantial increase in TNF-α secretion upon restimulation with LPS in comparison to nontolerized cells (Figure 6E), indicating that inhibition of NF-κB activation specifically prevents the induction of MRP-induced tolerance.

MRP-Induced Dimethylation of H3K9 Represses TNF-α Transcription

RelB has been described to bind and thereby to recruit methyltransferase G9a to the TNF-α promoter. G9a in turn dimethylates histone H3 on lysine K9 (H3K9), resulting in heterochromatin formation and inaccessibility of the TNF-α promoter to active transcription factors (Chen et al., 2009). Because MRP prestimulation increases nuclear translocation of RelB, we examined dimethylation of the TNF-α promoter nucleosome on H3K9 in MRP8-MRP14-pretreated cells by chromatin-immunoprecipitation (ChIP). We observed increased dimethylation of H3K9 (Figure 6F) and accordingly increased G9a activity in LPS- and MRP-pretreated monocytes compared to controls (Figure 6G). Inhibition of G9a by BIX01294 during induction of tolerance resulted in significantly increased TNF-α secretion in MRP-pretreated cells, but not in LPS-pretreated cells (Figure 6H), which might be due to time-dependent differences in LPS- and MRP-induced tolerance mechanisms.

In summary, our data indicate that prestimulation of human monocytes with MRP8-MRP14 results in an increased, G9a-dependent H3K9 dimethylation of the TNF-α promoter responsible for transcriptional silencing of TNF-α expression.

DISCUSSION

It is still an unsolved question how patients with severe sterile injuries develop a clinical picture similar to microbial-triggered hypoinflammation. In the present study, we demonstrate that polytrauma and burn trauma patients already had significantly elevated levels of the endogenous DAMP proteins MRP8 and MRP14 at initial presentation. We recently classified the phagocyte-specific DAMP proteins MRP8 and MRP14 as two endogenous TLR4 activators (Vogl et al., 2007). MRP8 and MRP14 are known to form noncovalently associated oligomers that represent the physiologically relevant form of these proteins (Roth et al., 1993; Vogl et al., 2004). We now demonstrate that MRP proteins can induce a state of hypoinflammation in phagocytes. Thus, the effect described here resembles classical LPS-induced tolerance and does not reflect an anti-inflammatory effect of these proteins as proposed earlier, because the general effect of these proteins, like for LPS, is a proinflammatory function in many inflammatory conditions. We found wide cross-tolerance between pre-/restimulation of different microbial TLR

activators and endogenous triggers like MRP8 and MRP14. Interestingly, it has been shown earlier that different PAMPs can substitute for each other, inducing cross-tolerance between the TLR2 and TLR4 pathways (Akira and Takeda, 2004). Our present data point to a general mechanism of stress-induced rather than specific microbial tolerance, in which exogenous and endogenous triggers of infection and injury not only amplify inflammatory reactions but also act in concert in a negative feedback loop, preventing overwhelming inflammation and tissue damage. Uncontrolled release of these proteins, on the other hand, may result in severe dysregulation of inflammatory functions like immune paralysis in polytrauma and burn trauma patients. The biological relevance of this phenomenon is confirmed by the fact that pretreatment with Mrp8-Mrp14 complex significantly protects mice against LPS-induced shock similar to classically induced LPS tolerance (Melo et al., 2010).

Whereas undefined endogenous triggers of inflammation have been presumed for the pathogenesis of trauma and burn patients, our data significantly challenge current paradigms regarding the high susceptibility of neonates to infections, which still remains a leading cause of mortality (Kilbride et al., 2003; Stoll et al., 1996, 1998, 2002). The primary LPS response of neonatal monocytes is characterized by strong suppression of the induction of proinflammatory mediators compared to adult monocytes, suggesting innate resistance to infectious triggers. During the postpartum period, human and murine neonates present with significantly increased serum levels of MRP8-MRP14 comparable to those in adults with severe inflammatory processes. In adult monocytes tolerized by MRP8-MRP14 pretreatment, we found a recovery of LPS responsiveness after about 24 hr. Accordingly, after culturing neonatal monocytes in MRP8-MRP14-free medium, we found a recovery of the neonatal LPS response within a similar time frame of 24 hr. Transfer of neonatal plasma to adult monocytes mimicked the ET-inducing effects of purified MRP8-MRP14, which could be abrogated by depletion of MRP8-MRP14 from the plasma or pharmacological inhibition of MRP8-MRP14 by paquinimod (Bengtsson et al., 2012). In addition, LPS-induced systemic inflammation in neonates of WT and Mrp14$^{-/-}$ mice confirmed the biological relevance of our findings in vivo. Taken together, these data point to a molecular mechanism underlying the susceptibility of neonates to infections in which peripartal stress-triggered release of an endogenous TLR4 stimulus induces a state of hypoinflammation in neonatal phagocytes. Whether the hypoinflammatory state of neonatal phagocytes is disadvantageous, promoting the susceptibility of neonates to infections, or whether it is beneficial in preventing infection-induced courses of endotoxic shock needs to be clarified in follow-up studies.

We demonstrate that intracellular expression and cell surface exposition of TLR4 and TLR2 were unaltered by MRP8-MRP14 or LPS-mediated tolerance induction (data not shown), indicating that common TLR4- and TLR2-signaling pathways must be differentially regulated in tolerized and naive monocytes downstream of the receptor level. The "Myd88-dependent" pathway triggers the activation of downstream kinases such as MAPK and ultimately results in translocation of NF-κB to the nucleus, initiating transcription of inflammatory cytokines (Biswas and Tergaonkar, 2007). We present evidence that several regulatory factors in the MyD88-dependent signaling cascade that have been described as playing a role in ET, like *IRAK1*, *SOCS3*, or *TNFAIP3*, are significantly downregulated in MRP prestimulated monocytes comparable to LPS, indicating that similar inhibitory mechanisms dominate the signaling cascades downstream of TLR in "tolerized" phagocytes independent of the initial stimulus. Moreover, bioinformatic analysis of genome-wide transcriptomes of MRP8-MRP14- and LPS-prestimulated human monocytes revealed that MRP and LPS prestimulation induces an almost identical response pattern in human monocytes.

MAPK p38 is one of the intracellular downstream signaling components common to both TLR4 and TLR2, playing an essential role in the induction of proinflammatory cytokines (Zarubin and Han, 2005). However, we observed a similar increase in p38 phosphorylation in both control and MRP8-MRP14-pretreated cells after restimulation with LPS, suggesting that p38 activation is unaltered by MRP8-MRP14 prestimulation. In addition, the tolerant state of MRP8-MRP14-prestimulated cells was sustained in the presence of the p38 inhibitor SB202190, indicating that p38 MAPK activation is not relevant for MRP8-MRP14-induced microbial tolerance.

Several studies have demonstrated impaired NF-κB activity in endotoxin-tolerant murine macrophages and human monocytes. Thus, monocytes from sepsis as well as trauma patients have been described as overexpressing transcriptionally inactive dimers of p50 or p65/RelB (Cavaillon et al., 2003; Ziegler-Heitbrock, 2001). In our experiments, prestimulation of monocytes with LPS or MRP8-MRP14 led to increased nuclear translocation of p50, p65, and RelB proteins. Moreover, we demonstrated that inhibition of NF-κB activation almost completely prevents the induction of ET, which is reflected by increased TNF-α production in "tolerance-induced" monocytes. Therefore, our data suggest that NF-κB seems to have an important function within the TLR4-dependent signaling cascade of MRP8-MRP14- and LPS-tolerant monocytes.

Aside from directly modulating the transactivation of target genes by affecting the binding of NF-κB to its binding sites, epigenetic modifications such as histone and DNA methylation have an important role in selective gene expression in LPS tolerance (El Gazzar et al., 2008; Foster et al., 2007). Interestingly, previous studies analyzing LPS-induced ET reported recruitment of RelB to the TNF-α promoter, where it directly binds to methyltransferase G9a. G9a in turn dimethylates histone H3 on lysine K9, resulting in heterochromatin formation and promoter inaccessibility for transcriptionally active transcription factors (El Gazzar et al., 2008). Consistent with these reports, we observed increased histone methyltransferase G9a activity and dimethylation of the TNF-α promoter nucleosome on H3K9 in MRP8-MRP14-pretreated monocytes. Accordingly, inhibition of G9a activity abolished MRP8-MRP14-induced tolerance. In conclusion, we demonstrate that MRP8-MRP14, an endogenous ligand of TLR4, induces microbial and self-tolerance. Interestingly, concentration levels of these proteins found in the early "vulnerable" phase of sterile inflammation in polytrauma and burn patients point to a clinical relevance of this pathomechanism. Moreover, high peripartal MRP8-MRP14 serum levels seem to be mainly responsible for a hypoinflammatory state in neonatal monocytes,

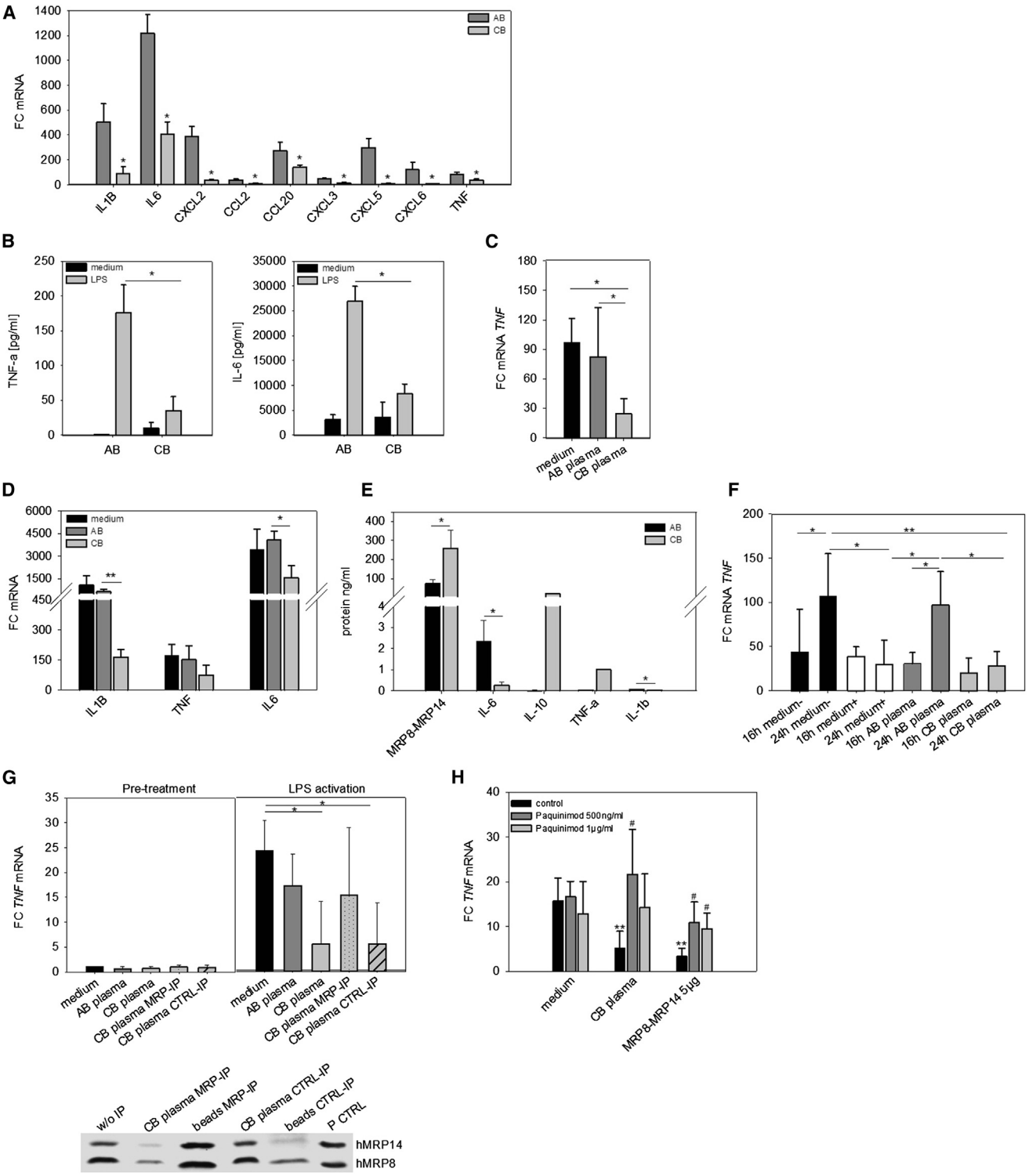

Figure 4. Impaired LPS Responses of Neonatal Monocytes Are Mediated by High Postnatal MRP8-MRP14 Plasma Levels

(A) Human monocytes were isolated from AB (dark gray bars; n = 12) or CB (light gray bars; n = 11) and 4 hr stimulated with LPS. Changes in inflammatory gene expression compared to untreated control cells were analyzed by qRT-PCR and plotted as fold change (FC).

(B) Human monocytes isolated from AB (n = 9) and CB (n = 9) were 16 hr treated with LPS (gray bars) or left untreated (black bars) and IL-6 and TNF-α levels determined in supernatants. Bars represent means ± SD (*p < 0.05).

(legend continued on next page)

supporting the hypothesis that soluble factors released during delivery and not inborn functional deficits of immune cells determine the responsiveness of neonatal innate immune cells upon microbial challenges.

EXPERIMENTAL PROCEDURES

Patients and Study Design

The study comprised 32 polytrauma and 31 burn trauma patients (Department of Surgery of Munich University Hospital). Exclusion criteria were age < 18 years, hospital admission later than 6 hr after injury, severe preexisting infections, and immunologic or cardiovascular diseases requiring long-term medication. Healthy blood donors (n = 61; 51% male) served as controls. All patients received surgical care and intensive care unit (ICU) treatment. Injury severity scores (ISS) were calculated based on the final patient records (Greenspan et al., 1985). Injury severity in burns was assessed by the percentage of TBSA. Severe sepsis and multiple organ failure (MOF) were diagnosed as described earlier (Bone et al., 1992; Majetschak et al., 2008). Sepsis and MOF were combined as sepsis/MOF positive or negative because MOF without positive criteria of severe sepsis or septic shock after polytrauma and burns was expected to be rare. Duration of ICU treatment was documented based on the final patient records.

For the analysis of MRP8-MRP14 serum levels in healthy term neonates, we enrolled 98 neonates (gestational age $\geq$ 37 weeks). Blood was taken on days 1–84 of life for the national screening program for inborn errors of metabolism or for routine blood tests without underlying inflammatory diseases. Normal adult MRP8-MRP14 serum values were determined in blood samples from 80 healthy AB donors.

Reagents and Antibodies

LPS (*Escherichia coli* 055:B5), D-Gal, actinomycin D, and polymyxin B were purchased from Sigma. LTA from *Staphylococcus aureus* was obtained from InvivoGen. Paquinimod (ABR-215757) was a kind gift of Active Biotech AB. BIX01294 and Bay117085 were purchased from Enzo Life Sciences and SB202190 from Merck. The p38 (9212), phospho-p38 (9211), and RelB (4954) antibodies were obtained from Cell Signaling. P50 (ab7971), P65 (ab7970), and anti-HA tag (ab9110) antibodies were purchased from Abcam. A lamin B (C-20) antibody was purchased from Santa Cruz Biotechnology, and MRP8 (8-5C2) and MRP14 (S32-2) antibodies were obtained from BMA. Monoclonal anti-human MRP8-MRP14 (clone 27E10), polyclonal anti-human MRP8, and polyclonal anti-human MRP14 antibodies were purified by T.V.

Purification of MRP8 and MRP14 Derivatives

The human MRP8-MRP14 heterodimer was isolated from granulocytes as described earlier (van den Bos et al., 1998). MRP8, MRP14, Mrp8, Mrp14, and Mrp8-Mrp14 were recombinantly expressed in *E. coli* BL21(DE3) and purified as described earlier (Vogl et al., 2006). Proteins were analyzed by amino acid sequencing and electrospray ionization mass spectrometry. All preparations revealed >98% purity. Endotoxin contaminations were excluded by limulus amebocyte lysate assay (BioWhitaker), heat inactivation (30 min at 80°C), and polymyxin-B-blocking experiments.

Cells and Cell Culture Conditions

Human adult monocytes were isolated from buffy coats and neonatal monocytes from CB by Ficoll-Paque density gradient centrifugation and by using the Monocyte Isolation Kit II (Miltenyi Biotec). The purity of the isolated monocytes was >90% and checked by flow cytometry using phycoerythrin-labeled anti-CD14 monoclonal antibody (BD Biosciences). Cells were cultivated as previously described (Roth et al., 1993). For experiments with AB or CB plasma, monocyte medium was used 1:1 with plasma. For MRP8-MRP14 inhibition experiments, paquinimod was added in indicated concentrations. In medium transfer experiments, supernatant from 24 hr cultured AB or CB monocytes was used 1:1 with medium for preincubation. BMDMs were flushed from femurs and tibias and cultured in Dulbecco medium for 6 days as described earlier (Vogl et al., 2007). For tolerization, 1×10^6 cells/ml were 24 hr incubated with varying concentrations of LPS (0.025–1,000 ng/ml), MRP8 or MRP14 (50–250 ng/ml), or MRP8-MRP14 (0.5–10 μg/ml). Subsequently, cells were stimulated with LPS (1 ng/ml); LTA (1,000 ng/ml), or MRP8 and MRP14 (1,000 ng/ml) for different times.

IP of MRP8-MRP14

For the removal of MRP8-MRP14, CB plasma was precleared two times with protein A/G agarose beads (Pierce/Thermo Scientific). Then, CB plasma was incubated overnight with beads coupled with 27E10 (MRP-immunoprecipitation [IP]) or anti-HA tag antibody (control-IP). Probes from CB plasma before and after MRP-IP and control-IP as well as from the precipitates were retained for cell culture experiments and immunoblotting against MRP8 and MRP14.

Gene Array Expression Studies and Bioinformatic Analyses

Total RNA of human monocytes was isolated (NucleoSpin RNA II kit; Macherey-Nagel) and used for analysis by Illumina human HT-12 Expression BeadChips. The protocol was performed according to the manufacturer's instruction (Illumina). Data were imported into Partek Genomics Suite 6.6 (PGS, version 6.14.0724) using robust multiarray average algorithm prior to batch correction. A one-way ANOVA model was performed to calculate the differentially expressed genes between the different stimulating conditions and the 1,000 most-variable genes. Differentially expressed genes were defined by a fold change (FC) >2 or <−2 and a false discovery rate (FDR)-corrected $p < 0.05$. To visualize the structure within the data, we performed HC on the 1,000 most-variable genes, with default settings in PGS, on FDR-corrected values according to the expression values of the samples across the conditions. In addition, we calculated the ratios between LPS-LPS against LPS-con and MRP8-MRP14-LPS against LPS-con to compare the influence of pretreatment with either MRP8-MRP14 or LPS.

Nuclear Cell Extracts

Nuclear cell extracts were prepared using an extraction kit (Epigentek).

(C) Human AB monocytes were 16 hr cultured in medium (black bars), AB plasma (dark gray bars), or CB plasma (light gray bars) before stimulation with LPS (n = 3, respectively). Induction of *TNF* expression was analyzed by qRT-PCR and plotted as mean ± SD (*$p < 0.05$).

(D) Human AB monocytes 24 hr preincubated with medium (black bars) or with supernatant from 24 hr cultured AB (dark gray bars) or CB monocytes (light gray bars) were treated with LPS (n = 4) and expression of *IL1B*, *TNF*, and *IL6* analyzed by qRT-PCR. Plotted are means ± SD (*$p < 0.05$; **$p < 0.01$).

(E) Supernatants from human AB (dark gray bars; n = 4) and CB monocytes (light gray bars; n = 6) were analyzed after 24 hr for basal secretion levels of MRP8-MRP14 and indicated cytokines. Bars represent means ± SD; *$p < 0.05$.

(F) Human CB monocytes were cultured in medium (black bars), CB plasma (light gray bars), medium spiked with 2 μg/ml MRP8-MRP14 (medium +, white bars), or AB plasma (dark gray bars) for 16 hr and 24 hr before stimulation with LPS (n = 4, respectively). Induction of *TNF* expression was analyzed by qRT-PCR and plotted as mean ± SD (*$p < 0.05$; **$p < 0.01$).

(G) Human CB monocytes were cultured for 24 hr in medium (black bars), AB plasma (dark gray bars), CB plasma (gray bars), MRP8-MRP14-precipitated CB plasma (dotted gray bars), or control-precipitated CB plasma (gray bars). Afterward, they were stimulated for 4 hr with LPS and induction of *TNF* expression analyzed by qRT-PCR (mean ± SD; n = 4; *$p < 0.05$; **$p < 0.01$). Efficiency of MRP8-MRP14 removal was determined by western blot analysis. Results show one representative western blot.

(H) Human CB monocytes were cultured for 24 hr in medium, CB plasma, or medium spiked with 5 μg/ml MRP8-MRP14 without (black bars) or in the presence of 500 ng/ml (dark gray bars) or 1 μg/ml (gray bars) paquinimod. Afterward, they were stimulated for 4 hr with LPS. Induction of *TNF* expression was analyzed by qRT-PCR and plotted as mean ± SD (n = 4; *$p < 0.05$; **$p < 0.01$).

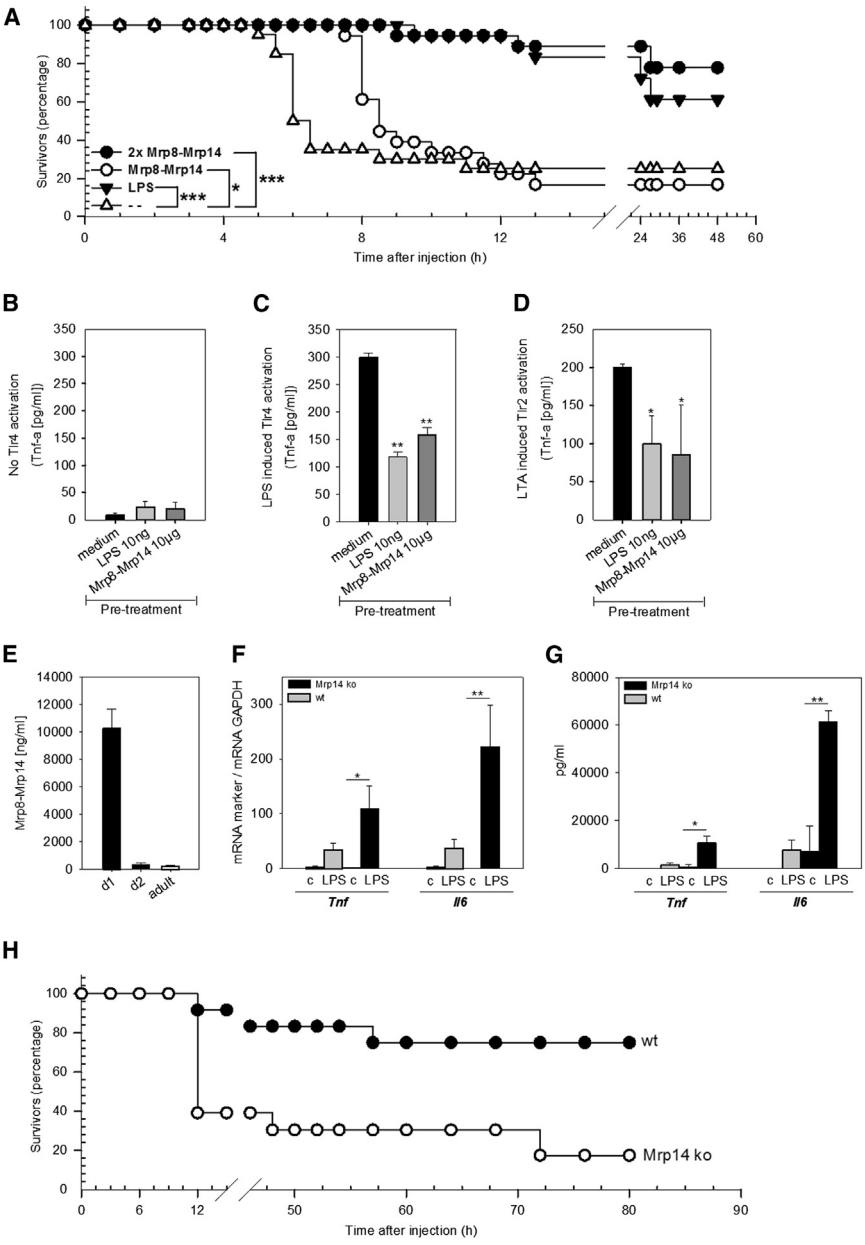

Figure 5. MRP8-MRP14 Induces Microbial Tolerance in Murine Model Systems

(A) WT mice were treated with LPS/D-Gal without pretreatment (n = 18) or after a single- or double-intravenous application (24 hr or 24 hr plus 12 hr before LPS/D-Gal treatment) of Mrp8-Mrp14 complex (300 μg per mouse; n = 18; three independent experiments) or LPS (100 ng per mouse; 24 hr before LPS/D-Gal treatment; n = 18). The figure shows the percentage of survivors over 48 hr (*$p < 0.05$; ***$p < 0.001$).

(B–D) Analysis of Tnf-α in supernatants of WT BMDMs. (B) Cells were 24 hr pretreated with substimulating doses of LPS (10 ng/ml), Mrp8-Mrp14 complex (10 μg/ml), or left untreated and (C) subsequently 4 hr restimulated with LPS (1 μg/ml) or (D) LTA (1 μg/ml). Data represent mean ± SD and were compared to data from LPS/LTA-activated naive BMDMs (black bar; n = 3; **$p < 0.01$).

(E–H) Postnatal release of Mrp8-Mrp14 induces LPS tolerance in vivo. (E) Plasma levels of Mrp8-Mrp14 complex in neonatal WT mice on day 1 and 2 of life (n = 10, respectively) and in adult WT mice (white bar; n = 6) plotted as mean ± SD. (F and G) After treatment of neonatal WT mice (gray bars) and Mrp14$^{-/-}$ mice (black bars) for 2 hr with PBS (control; C) or LPS, RNA was isolated from neonatal liver lysates and plasma samples were obtained (n = 4, respectively). (F) mRNA expression of *Tnf* and *Il6* were analyzed in relation to *Gapdh*, and (G) plasma cytokine levels of Tnf-α and Il-6 were determined. Results are plotted as mean ± SD, respectively (*$p < 0.05$; **$p < 0.01$). (H) Neonatal WT (n = 22) and Mrp14$^{-/-}$ (n = 27) mice were treated with 10 μg LPS. Kaplan-Meier survival curves show the percentage of surviving mice over 80 hr.

Mice

C57BL/6 mice (WT; Harlan Laboratories) and Mrp14 knockout mice (−/−; Manitz et al., 2003) were used and housed pathogen free.

LPS-Induced Septic Shock in the D-Gal Model

Septic shock was induced in 2-month-old WT mice by intraperitoneal injection of 40 μg LPS and 680 μg D-Gal per kg body weight, respectively. In parallel, mice were pretreated by intravenous injection of 100 ng LPS or 300 μg Mrp8-Mrp14 per mouse, followed by a LPS/D-Gal challenge 24 hr later. In additional approaches, Mrp8-Mrp14 complex was injected twice (12 hr and 24 hr) before LPS/D-Gal challenge. The survival of challenged mice was analyzed for 48 hr.

Determination of TNF-α, IL-6, IL10, IL-1β, and MRP8-MRP14 Concentrations

TNF-α/Tnf-α release was determined in supernatants of human monocytes or BMDMs by commercial ELISAs (BD Bioscience). In addition, concentrations of TNF-α/Tnf-α, IL-6/Il-6, IL-10, and IL-1β were determined using the human inflammation cytometric bead array or mouse FlexSet (both BD Bioscience). Serum/plasma concentrations of human/murine MRP8-MRP14 were determined by ELISA as described previously (Frosch et al., 2000). TNF-α and IL-6 analyses were performed after 4 hr of activation and IL-10 and IL1-β analysis after 16 hr of culture time.

qRT-PCR

RNA was isolated using the Nucleospin isolation kit II. qRT-PCR was performed as described earlier (Viemann et al., 2006). Sample data are presented as fold induction compared to control cells.

Mouse Model of Neonatal Endotoxemia

Neonatal WT and Mrp14$^{-/-}$ mice were used at the age of 2 days. Pups were subcutaneously injected with 10 μg LPS or PBS (control). For cytokine expression analysis, blood and organs were harvested 2 hr after LPS treatment. For survival studies, mice were observed for 80 hr.

ChIP Assays

Cells (1 × 10^7 per condition) were fixed with 1% formaldehyde and lysed in 1% SDS for 10 min. The DNA was sheared by sonification and an aliquot kept as

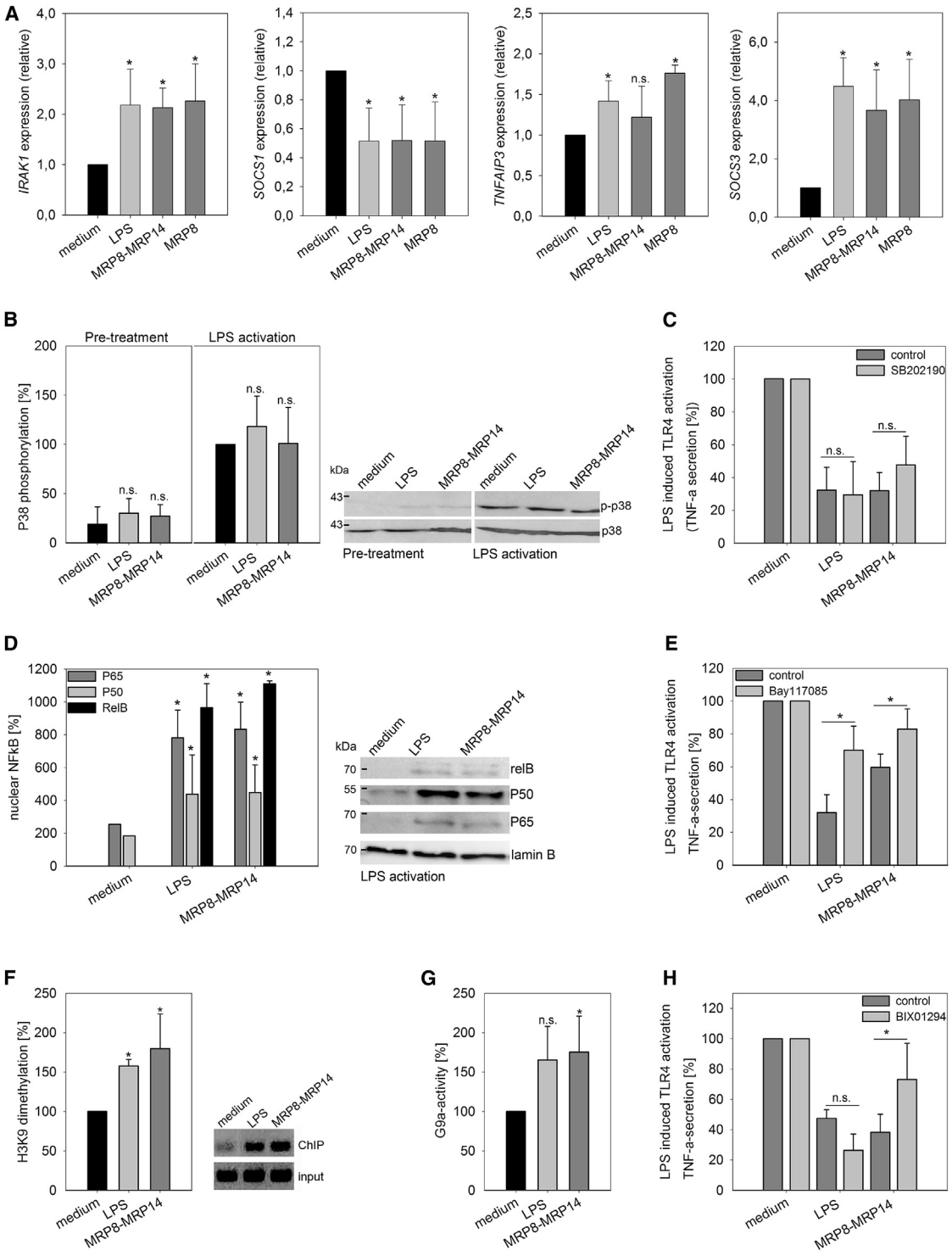

Figure 6. TLR Signaling in Tolerance-Induced Monocytes

(A) qRT-PCR analysis of *SOCS1*, *SOCS3*, *IRAK1*, and *TNFAIP3* expression in human monocytes stimulated for 24 hr with LPS (25 pg/ml), MRP8-MRP14 (10 μg/ml), or MRP8 (100 ng/ml). Results are presented relative to baseline expression in naive cells (black bar), set as one normalized to *GAPDH*. Data represent mean ± SD (n = 4; *p < 0.05).

(B) Monocytes were left untreated or 24 hr tolerized with LPS (25 pg/ml) or MRP8-MRP14 (10 μg/ml). After 30 min LPS activation, p38 phosphorylation was determined by western blot analysis. One representative western blot and densitometric analysis of three independent experiments are shown. p38 phosphorylation in restimulated naive cells (control) was set at 100%.

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input control. The DNA-protein complexes were precipitated with polyclonal anti-dimethyl-histone H3 (Lys9) antibody or immunoglobulin G (both Millipore) and extracted with protein A/G-agarose beads. After intensive washing, the samples were overnight incubated at 65°C and proteins digested with proteinase K. DNA was purified and amplified using TNF promoter primers (*F*: AACCGAGACAGAAGGTGCAGG; *R*: AGAATCATTCAACCAGCGGAAA). PCR products were run on a 1.5% agarose gel, quantified with ImageJ software, and normalized to control cells.

G9a Activity Assay

G9a activity was measured according to the manufacturer's instructions (EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit; Epigentek). Samples were normalized to control cells.

Statistical Analysis

Statistical significance was calculated using the Mann-Whitney U test or paired Student's t test. p values < 0.05 were judged to be significant. Kaplan-Meier survival curves were generated using the Mantel-Cox test.

Ethics Statement

The patient studies were approved by the institutional ethics committees (Institutional Review Board of the University of Munich, the institutional ethics committee of the University of Muenster, and the Hannover Medical School). Written informed consent was obtained from all participating individuals. Mouse experiments were in accordance with German Animal Welfare Legislation and performed as approved by the Lower Saxony State Office for Consumer and Food Safety (LAVES) and the District Government and District Veterinary Office Muenster.

ACCESSION NUMBERS

Microarray data are Minimum Information about a Microarray Experiment compliant and deposited in Gene Expression Omnibus (GSE61477).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.020.

AUTHOR CONTRIBUTIONS

J.A., J.F., T.V., D.V., and J.R. conceived and designed the experiments. J.A., J.F., S.K.F., T.O., J.B., K.B.-K., E.F., S.Z., S.P., C.R., C.M.-T., M.v.K.-B., C.S.v.K., S.B.F., T.V., and D.V. performed experiments. J.A., J.F., C.M.-T., T.V., J.L.S., T.U., J.R., and D.V. analyzed the data. J.A., J.R., D.V., and T.V. wrote the manuscript.

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(C) Human monocytes were preincubated for 1 hr with SB202190 (14 μM). Subsequently, the cells were tolerized with LPS (25 pg/ml) or MRP8-MRP14 (10 μg/ml) for 24 hr. After 4 hr of LPS activation, TNF-α levels in the supernatants were determined. Data represent mean ± SD (n = 4) and were normalized to LPS-dependent activation of control monocytes (100%).

(D) Human monocytes were tolerized with LPS (25 pg/ml) or MRP8-MRP14 (10 μg/ml) for 24 hr. After the second LPS stimulus (30 min), nuclear cell extracts were prepared for western blotting of P65, P50, and RelB. One representative western blot and densitometric analysis of three independent experiments are shown. Data were normalized and compared to unchallenged naive monocytes (100%; *$p < 0.05$).

(E) Human monocytes were preincubated for 1 hr with Bay117085 (2 μM). Subsequently cells were tolerized with 25 pg/ml LPS or 10 μg/ml MRP8-MRP14 for 24 hr. Four hours after LPS restimulation, TNF-α levels were determined in cell supernatants. Data were normalized to naive control monocytes (100%) and plotted as mean percentage ± SD (n = 4; *$p < 0.05$).

(F–H) Human monocytes were tolerized with LPS (25 pg/ml) or MRP8-MRP14 (10 μg/ml) for 24 hr and subsequently activated with LPS (1 ng/ml). (F) H3K9 dimethylation ChIP assays of the TNF-α proximal promoter in tolerant human monocytes. The results show densitometric analysis of n = 8 experiments plotted as mean percentage ± SD. Data were normalized to LPS-dependent activation of naive control monocytes (100%; *$p < 0.05$). (G) After the second LPS stimulus, nuclear extracts were prepared for G9a activity analysis. Samples were normalized to LPS-dependent activation of naive control monocytes (100%; n = 4; *$p < 0.05$). (H) Human monocytes were preincubated for 1 hr with 7 μM BIX01294 inhibitor before tolerance was induced. Four hours after LPS restimulation, TNF-α levels were determined in cell supernatants. Data represent mean percentage ± SD and were normalized to LPS-dependent activation of naive control monocytes (100%; n = 3; *$p < 0.05$).

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