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Myeloid-Related Proteins Rapidly Modulate Macrophage Nitric Oxide Production during Innate Immune Response[1]

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S100A8 and S100A9 are intracellular calcium-binding proteins produced by myeloid cells that promote neutrophil/monocyte recruitment at inflamed tissues by enhancing attachment to endothelial cells. Although the intracellular functions of these proteins, i.e., myeloid-related proteins (MRP)-8 and MRP-14, are not completely understood, these proteins exhibit prominent extracellular cytokine-like functions and are considered reliable markers of inflammation in diverse diseases. As S100A8 and S100A9 have been reported to be rapidly released in response to components derived from infectious agents, we hypothesized that they play an important role in the modulation of key microbicidal phagocyte functions. In this study, we report for the first time that MRPs are powerful inducers of NO production by murine macrophages (Mϕ). This increase in NO production was linked to an increased inducible NO synthase expression both at gene and protein level. This induction was concomitant with an important phosphorylation of SAPK/JNK, but also of MEK and ERK kinases. Upon stimulation with MRPs, NF-κB was rapidly translocated to the nucleus (30 min). When Mϕ were treated concomitantly with IFN-γ, another activator of Mϕ functions, we observed a strong synergy in NO production, synergy that resulted from the engagement of exclusive signaling pathways: SAPK/JNK, ERK and NF-κB were involved in signaling of MRPs, whereas IFN-γ uses the JAK/STAT pathway. This suggests that the synergy results from interactions of transcription factors in the promoter region. Finally, we observed this effect to be dependent on TLR4. Collectively, our study unravels the importance of MRPs as potent new inducers of Mϕ NO production. ***The Journal of Immunology,*** **2008, 181: 3595–3601.**

The S100 proteins form a family of small intracellular proteins (10–12 kDa) of nearly 20 members. These proteins are formed of two Ca^{2+} binding domains that are separated by a hinge region (1). Although their action has been mainly reported to occur intracellularly, more attention is now given to their important extracellular roles (2). For instance, S100A8 and S100A9, which are myeloid-related proteins (MRPs)[3] MRP-8 and MRP-14, are found as homodimers or heterodimers (3) and exhibit antimicrobial properties (4). The heterodimeric form is usually translocated to the membrane in response to intracellular Ca^{2+} mobilization before their release through a Golgi-independent secretion system involving cytoskeleton-membrane interactions (5).

S100A8 and S100A9 also exhibit important functions in inflammation (6–10), where S100A8 is known to induce polymorphonuclear cell chemotactism (11). In particular, we have previously reported that S100A8 and S100A9 are important neutrophil recruitment mediators in response to LPS. Treatment with anti-S100A8 and anti-S100A9 considerably reduced neutrophil recruitment, and as MRPs induced the release of neutrophils from bone marrow to the bloodstream (12). Although MRPs are mostly known to be produced by neutrophils where they can represent as much as 30–40% of cytosolic proteins (13), they are also produced by cells of the monocytic lineage (14). It is still unclear which cell receptor is relevant for S100A8/A9 extracellular roles. CD36 was found to bind the heterodimer and facilitate uptake of lipids (15), whereas it was also noticed that S100A8/A9 could bind heparan sulfate glycosaminoglycans (16) or carboxylated glycans (17) on endothelial cells. As RAGE (receptor for advanced glycation end product) is the receptor of S100A12 (18), it is often speculated that RAGE might act as the receptor for S100A8/A9. This topic is still controversial and recently, S100A8 and S100A8/A9 have been shown to be endogenous ligands of TLR4 (19), suggesting an interesting mechanism for their extracellular role.

Knowing MRPs potential to activate polymorphonuclear cells during inflammatory processes and knowing that MRPs secretion can rapidly be induced by pathogen by-products (e.g., LPS and *Plasmodium* hemozoin) (20), we were interested to determine whether MRPs can also be important activator of macrophage (Mϕ) functions, which is a key player of innate immune response. Due to its importance as an antimicrobial agent, NO was of critical interest.

In this study, we report for the first time that MRP homodimers (S100A8 and S100A9) or their heterodimer can induce NO production from Mϕ in an inducible NO synthase (iNOS) expression-dependent manner. Of further interest, IFN-γ and S100A8/A9 showed a synergistic effect on NO generation. In an attempt to delineate the signaling pathways underlying MRP-induced NO by

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[3] Abbreviations used in this paper: MRP, myeloid-related protein; iNOS, inducible NO synthase; Mϕ, macrophage; BMDM, bone marrow-derived macrophage; KO, knockout.

Mϕ, we observed an important phosphorylation of SAPK/JNK kinases as well as a more modest, but conserved phosphorylation of MEK1/2 and ERK1/2, concurrent with NF-κB nuclear translocation. Of importance, IFN-γ and MRPs engage distinct pathways, suggesting that the synergy results from interactions between transcription factors in the promoter region of the iNOS gene.

With this finding, it is clear that MRPs play a crucial role in cytokine-mediated phagocytes activation during innate immune response, and that further investigation concerning its potential as modulator of antimicrobial functions should be considered.

Materials and Methods

Reagents

Murine recombinant S100A8 and S100A9 were produced as previously described (21) and found to contain less than 1 pg of endotoxin/μg of protein as we reported previously (22). Recombinant murine IFN-γ was purchased from Fitzgerald Industries. The anti-iNOS and anti-phospho-JAK2 (Tyr^{1007}/Tyr^{1008}) Abs were purchased from Santa Cruz Biotechnology, whereas anti-phospho-STAT1α (Tyr^{701}), anti-phospho-STAT1α (Ser^{727}), anti-phospho-SAPK/JNK (Thr^{83}/Tyr^{185}), anti-phospho-MEK1/2 (Ser^{217}/Ser^{221}), anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), and their anti-protein Abs were purchased from Cell Signaling Technology. Oligonucleotides containing STAT1, NF-κB, CREB, and AP-1 consensus binding sequences came from Santa Cruz Biotechnology. Inhibitors of MAPKs, SP600125 (25 μM), PD98059 (100 μM), apigenin (50 μM), and SB203580 (1 μM) were bought from Biomol. All material for cell culture was purchased from Life Technologies.

Cell culture and NO assay

B10R murine bone marrow-derived macrophage (BMDM) cell line was grown as previously described (23) and kept in DMEM culture supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine. B10Rs were seeded in 24-well plates at 1×10^5 cells/well overnight. The next day, MRPs were added to the wells at a specified concentration for 24 h. B10R TRL4 knockout (KO) Mϕ obtained as we reported previously (24) were also used in similar conditions. BMDMs were obtained from BALB/c mice using standard protocol (25). In situations in which IFN-γ was used, it was added concomitantly to MRPs at 100 U/ml. At 24 h of incubation, plates were centrifuged and supernatant collected for nitrite measurement by Griess reaction as we reported previously (26). When MAPKs inhibitors were used, they were added 1h before the MRP treatment for 24 h at optimal subcytotoxic concentrations. The maximal subcytotoxic concentration was assessed by the XTT reduction assay. An inhibitor concentration was considered cytotoxic if viability was <90% of untreated control after 24 h.

Western blotting

Western blotting was performed as previously described (27). Briefly, cells were plated overnight at 5×10^5/well in a 6-well plate and stimulated the next day with MRPs or IFN-γ, as described for each experiment. After the defined time, cells were lysed in cold buffer containing 20 mM Tris-HCl (pH 8.0), 0.14 M NaCl, 10% glycerol (v/v), 1% Nonidet P-40 (v/v), 1 mM PMSF, 1 mM sodium orthovanadate (Na_3VO_4), 1 μM NaF, and protease inhibitors (40 μg/ml aprotinin and 20 μg/ml leupeptin). The lysates (30 μg/lane) were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in Tris-buffered saline/0.1% Tween containing 5% milk (or 1.5% fetal bovine albumin for anti-SAPK/JNK Ab), for 1 h at room temperature. Then membranes were washed and incubated with mAb for 1 h (phospho-JAK2, anti-phospho-ERK1/2, anti-phospho-MEK1/2, anti-phospho-STAT-1α Ser, and anti-phospho-STAT-1α Tyr) or overnight at 4°C for anti-phospho-SAPK/JNK. After washing, membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated Ab (GE Healthcare) for 1 h, and proteins were visualized with the use of the ECL Plus Western Blotting reagent (GE Healthcare). Membranes were then stripped using the Restore Western Blot Stripping Buffer (Pierce), and blotting was done again using anti-protein Abs (per the manufacturer of the anti-phospho-protein).

EMSA analysis

EMSA was performed as we previously described (28). Briefly, 1×10^6 cells were plated in a 25-cm^2 flask and let to adhere overnight before treatments. Cells were then treated with IFN-γ or MRPs. Cells were washed with ice-cold PBS then scraped in 1 ml of PBS. After centrifugation, cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF), and incubated on ice for 15 min. The 25 μl of 10% IGEPAL (Sigma-Aldrich) were then added. Tubes were vortexed for 10 s and centrifuged at maximum speed for 30 s. Nuclear fractions were resuspended in 50 μl of ice-cold buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and incubated at 4°C on a shaking platform for 15 min. After centrifugation at $12{,}000 \times g$ for 5 min at 4°C, the supernatants were stored at −70°C until further use. The 6 μg of these nuclear protein extracts were mixed with a γ-^{32}P-labeled oligonucleotide containing a consensus binding sequences for STAT1α, NF-κB, CREB, or AP-1. Complexes were then resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. The gels were dried and visualized by autoradiography. The consensus sequences for NF-κB, STAT1, CREB, and AP-1 were 5′-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3′, 5′-AAG-TAC-TTT-CAG-TTT-CAT-ATT-ACT-CTA-3′, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′, and 5′-CGC-TTG-ATG-ACT-CAG-CCG-GAA-3′, respectively.

Northern blotting

Northern blotting was performed as previously described (29). Cells were plated at 1×10^6 macrophages/25-cm^2 flask and stimulated for 8 h (unless specified otherwise) before being washed in ice-cold PBS. RNA was then extracted using TRIzol (Invitrogen) following the manufacturer's protocol. The 20 μg of RNA were loaded onto 1% agarose gels, and equal loading and RNA integrity were confirmed by ethidium bromide staining. RNA was then transferred onto Hybond-N filter paper and hybridized with random primer-labeled cDNA probes. Equal loading of RNA was also confirmed by hybridization with GAPDH cDNA probe. All washes were performed under stringent conditions. The mRNA hybridizing with the cDNA probe was visualized by autoradiography. Probes have been provided by Dr. D. Radzioch (Montreal General Hospital Research Center, McGill University, Montreal, Québec, Canada).

Statistical analysis

Statistically significant differences were identified using the ANOVA module of StatView from the SAS institute (version 5; SAS Institute). A value for $p < 0.05$ was considered statistically significant. All data are presented as mean ± SEM.

Results

Induction of NO production by MRPs

Initially, we have been interested to test whether MRPs, (S100A8, S100A9, and the heterodimer S100A8/A9) had intrinsic capacity to induce NO generation. In Fig. 1*A*, we show all MRPs used were proven to be a strong inducer of iNOS gene expression to a level even slightly more elevated than our positive control, IFN-γ (100 U/ml). This increase in iNOS gene expression was followed by an increase in protein expression (Fig. 1*B*). Then NO production was investigated (Fig. 1*C*) and MRPs used alone were observed to induce NO production in Mϕ in amounts comparable with IFN-γ-treated Mϕ. This observation paralleled iNOS expression that shows the implication of iNOS in the observed increase of NO in MRP-stimulated Mϕ. Taken together, these initial data showed for the first time that MRPs are important inducer of iNOS and consequently NO.

The concentration of MRPs used initially (10 μg/ml) was in the physiological range as measured previously in our air-pouch model upon stimulation with proinflammatory compound (20), but we decided to perform a dose-response experiment to fully determine MRPs NO induction potency on Mϕ. As can be seen in Fig. 2*A*, iNOS gene expression was increased by all MRP compounds at the level of IFN-γ with doses as low as 1 μg/ml MRPs and increased substantially until our maximal assessed dose of 50 μg/ml. NO production (Fig. 2*B*) matched closely the increase in iNOS gene expression and was undetectable at low doses (0.01 to 0.1 μg/ml), but started to be detected at 1 μg/ml to reach a plateau at the highest physiological doses used (10–50 μg/ml). This trend is

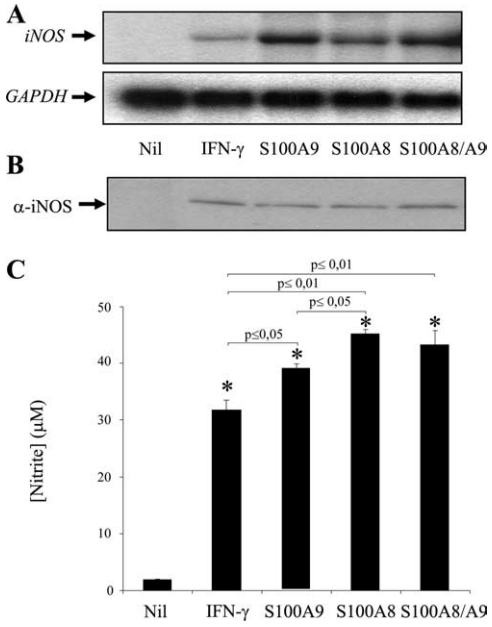

FIGURE 1. Production of NO by B10R Mϕ following stimulation with MRPs. *A*, iNOS gene expression by Mϕ upon stimulation with IFN-γ (100 U/ml) or MRPs (10 μg/ml). Cells were stimulated for 8 h with indicated stimuli, and iNOS mRNA expression was assessed by Northern blotting, using GAPDH as a housekeeping gene. *B*, iNOS protein expression in Mϕ stimulated with IFN-γ or MRPs was assessed after 8 h of treatment. *C*, Mϕ were stimulated with IFN-γ, S100A8, S100A9, or S100A8/A9 for 24 h, and nitrites were measured in the supernatants as reflective of NO. Results shown are representative of at least three independent experiments performed in triplicate. $*$, $p \leq 0.05$ for statistical significance from nil.

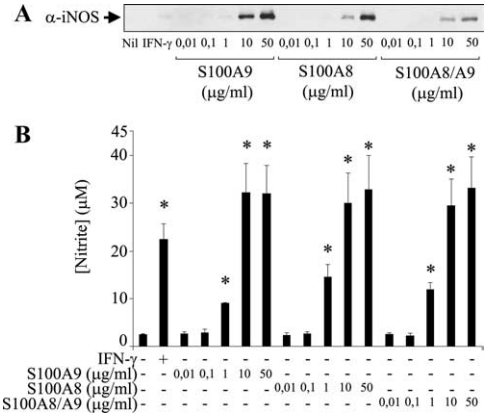

FIGURE 2. Production of NO after MRP stimulation is dose-dependent. *A*, iNOS protein levels were evaluated by immunoblotting after stimulation with doses of MRPs (0.01 to 50 μg/ml) for 24 h. *B*, NO production was measured after the same the same treatment. Results shown are the average of two independent experiments performed in triplicate. $*$, $p \leq 0.05$ for statistical significance from nil.

conserved for all MRPs used; S100A8, S100A9, and their heterodimer. Data reported in this experiment clearly reveal the potency of MRPs as iNOS/NO inducer in comparison to IFN-γ (100 U/ml), a known iNOS inducer in Mϕ (26). Noticeably, even at low doses, MRPs were inducing comparable iNOS expression as IFN-γ-stimulated cells. Because the heterodimer S100A8/A9 was representative of the effects induced by all MRP preparations, this preparation was used throughout the next experiments.

Thereafter we were interested to determine the time-response effect of MRPs on Mϕ to appreciate the rapidity of their modulation over a 24-h period. Additionally, we wanted to compare MRP NO production potency to that of IFN-γ, a known NO inducer, to better appreciate their potency. As revealed in Fig. 3*A*, 100 U/ml IFN-γ stimulation increased iNOS mRNA by 8 h poststimulation, whereas stimulation with 10 μg/ml S100A8/A9 alone induced iNOS expression as soon as 4 h. Most interestingly, stimulation with both compounds resulted in a very rapid (as soon as by 2 h) and dramatic increase in iNOS mRNA production that was sustained for 24 h. The mRNA induction was not restricted to iNOS as we also observed an induction of TNF-α mRNA upon treatment with IFN-γ or MRP. Interestingly, stimulation with both compounds resulted in a stronger and more sustained presence of TNF-α mRNA, correlating observations with iNOS. Protein production was then confirmed by immunoblotting (Fig. 3*B*). Stimulation with IFN-γ induced iNOS production beginning around 8 h, whereas MRPs induced iNOS protein as soon as by 4 h, but this production decreased after an optimum around 8 h, correlating well with mRNA expression. In conrast, when both IFN-γ and MRPs were used, iNOS protein was observed early at 4 h at a level higher than any compound alone, reached an all-condition maximum at 8 h before reducing for 24 h, as opposed to the sustained mRNA presence, suggesting that some posttranscriptional regulation mechanisms limit the iNOS production from mRNA. Final NO production was measured (Fig. 3*C*) and observed at 24 h for IFN-γ stimulation and earlier (8 h) for MRPs stimulation, reflecting observations at gene and protein level. When both compounds were used, NO production began at 8 h at a level higher than any stimulation alone and was also increased at 24 h, suggesting some synergy between the compounds.

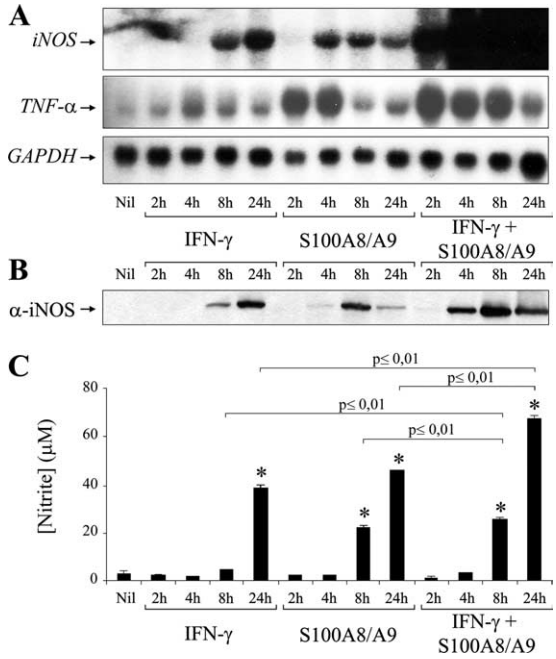

FIGURE 3. NO production after various time points of stimulation with S100A8/A9. *A*, Mϕ were stimulated with IFN-γ (100 U/ml), S100A8/A9, or both for 2, 4, 8, or 24 h. iNOS gene expression was monitored by Northern blot analysis. TNF-α was also investigated for the same time points. GAPDH served as a housekeeping gene. *B*, iNOS protein production was evaluated by immunoblotting after the same time points. *C*, NO production was monitored after the same time of stimulation by Griess reaction. Results shown are representative of at least three independent experiments performed in triplicate. $*$, $p \leq 0.05$ for statistical significance from nil.

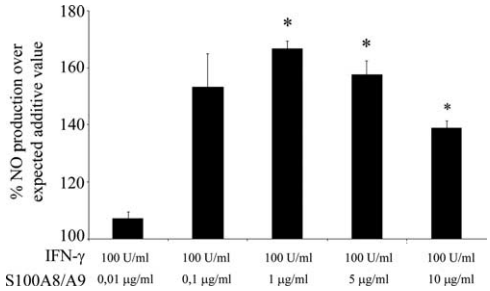

FIGURE 4. Synergistic NO production by Mϕ upon stimulation with IFN-γ and various concentrations of MRPs. Cells were stimulated for 24 h with or without 100 U/ml IFN-γ and various concentrations of S100A8/A9. Results are shown as the percentage of NO production over the additive value of observed NO production of B10R Mϕ stimulated with IFN-γ 100 μg/ml alone and B10R stimulated with MRPs alone at precise concentrations. The percentage is calculated using (NO of IFN-γ (100 U/ml) + MRP (x μg/ml) stimulation)/(NO of IFN-γ (100 U/ml) stimulation + NO of MRP (x μg/ml) stimulation) $\times$ 100, where x is variable. Mean of three independent experiments performed in triplicate is shown. *, $p \leq 0.05$ for statistical significance from nil.

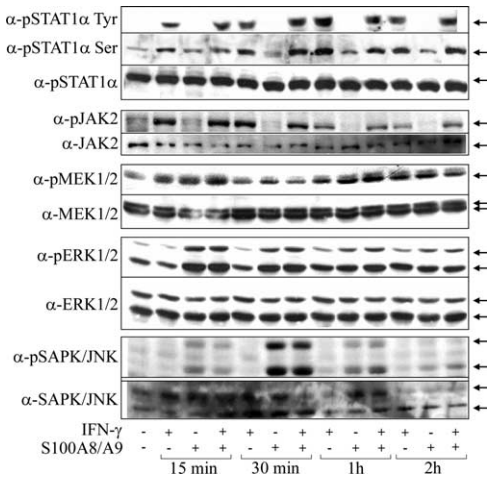

FIGURE 5. MRPs-induced signaling. Mϕ were treated with IFN-γ (100 U/ml), MRP (10 μg/ml), or both during 15 and 30 min, and 1 or 2 h. Protein lysate was then investigated for phosphorylation of STAT1α, JAK2, MEK1/2, ERK1/2, and SAPK/JNK by immunoblotting. Blots shown are representative of at least three separate experiments.

IFN-γ and MRPs synergistic effect on Mϕ-derived NO production

At the view of a possible synergy between IFN-γ and MRPs, we have been interested to further investigate this important mechanism. Using a fixed dose of IFN-γ (100 U/ml), which provides an adequate NO production, and increasing concentrations of S100A8/A9 (0.01–10 μg/ml), we have evaluated whether MRPs and IFN-γ can synergistically induce NO. To illustrate the synergy, cells were stimulated with IFN-γ and MRP alone or in combination. NO production values after stimulations with compounds alone were summed to provide the expected additive value, and the observed NO production upon combined stimulation was expressed as a percentage of this observed additive value. Fig. 4 shows a clear synergistic NO production by cells stimulated with both compounds simultaneously. All doses used result in a clear increase in NO production (more than 100% of observed additive value), but low doses of MRP (0.01 and 0.1 μg/ml) did not reach statistical significance. This observation illustrates that IFN-γ and MRPs can have a synergistic effect on Mϕ NO production that is more than additive.

Deciphering the signaling pathways involved in MRP-induced NO generation

As this report is the first to show MRPs are a powerful inducer of NO, we aimed at determining the pathways engaged in Mϕ after MRP stimulation in Mϕ, a task even more necessary as pathways involved in MRP signaling are not clearly identified. Given the synergy observed with IFN-γ, it was also of paramount importance to determine whether the signaling pathways used by these two S100 proteins were similar to the known signaling events triggered upon stimulation of Mϕ by IFN-γ. As both IFN-γ and MRPs increased NO production, we first monitored whether the IFN-γ-induced JAK2/STAT1 pathway was also induced by MRPs. As shown in Fig. 5, IFN-γ was concurring to its known phosphorylation of Mϕ STAT1α on both Tyr^{701} and Ser^{727} residues, which is a sign of STAT1α activation. This activity was also accompanied by the phosphorylation of JAK2 (Tyr^{1007}/Tyr^{1008}). Interestingly, MRP alone did not induce any phosphorylation on these proteins. A combination of IFN-γ and MRPs did not further increase the phosphorylation observed with IFN-γ alone. Therefore this set of data strongly suggests that JAK2/STAT1α pathway is not involved in MRP-induced signaling concurring to NO generation, even in the context of a synergistic effect.

In parallel, we also investigated the various MAPKs pathways. First we were interested in deciphering the MEK/ERK pathway. We observed that MEK1/2 phosphorylation (Ser^{217}/Ser^{221}) was more induced by MRPs in comparison to IFN-γ. This MRP-induced phosphorylation was observed already at 15 min poststimulation and was sustained up to 1 h to a comparable level in samples stimulated with MRPs alone or in combination with IFN-γ (Fig. 5). In contrast, activation by IFN-γ per se was only noticeable by 1 h poststimulation. Thereafter, downstream phosphorylation of ERK1/2 (Thr^{202}/Tyr^{204}) was also monitored. As observed for MEK1/2, a similar MRP-induced ERK1/2 phosphorylation was seen from 15 min to 1 h after stimulation (Fig. 5). Again, IFN-γ was observed to induce ERK1/2 phosphorylation later in time and to a lesser extent than MRP alone or in combination with IFN-γ. We also investigated the involvement of SAPK/JNK pathway in this experimental context. Of great interest, we discovered that MRPs can induce SAPK/JNK (Thr^{183}/Tyr^{185}) phosphorylation in a similar manner as observed for MEK/ERK kinases from 15 min to 1 h, with an optimal peak at ~30 min poststimulation (Fig. 5). Of utmost importance, IFN-γ was shown not to cause SAPK/JNK phosphorylation. Therefore, at the difference of IFN-γ, the MRPs seem to selectively exploit the SAPK/JNK pathway to induce NO generation. The differential use of JAK2/STAT1 pathway by IFN-γ and of SAPK/JNK pathway by MRPs may concur to amplify the signal leading to NO generation in a synergistic manner. As both molecules use different signaling pathways, these data suggest that the synergistic effect observed does not involve a greater induction of a pathway engaged by both molecules, but might rather depend on engagement of transcription factors.

Pharmalogical inhibition of proteins involved in MRP signaling

As our results point to a role for SAPK/JNK, MEK1/2 and ERK1/2 in the transduction of signal (Fig. 5), we wanted to confirm the implication of MAPKs in NO production upon MRP stimulation. To confirm, we chose to inhibit these kinases using specific inhibitors used at optimal subcytotoxic concentrations (data not shown). Fig. 6 shows that inhibition of SAPK/JNK (SP600125) and ERK1/2 (apigenin) resulted in a complete abrogation of NO production by MRP-stimulated cells, in accordance with the observed

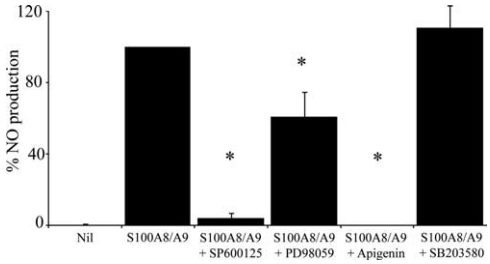

FIGURE 6. Pharmacologic inhibition of MAPKs. B10R Mϕ were treated with various inhibitors 1 h before addition of S100A8/A9 at 10 μg/ml for 24 h after which NO production was measured. SP600125 (25 μM), PD98059 (100 μM), apigenin (50 μM), and SB203580 (1 μM) inhibit SAPK/JNK, MEK1/2, ERK1/2, and p38 inhibitors used. Inhibitors were all used at maximal subcytotoxic doses for a total of 25 h. Average NO production of four individual experiments done in triplicate. *, $p \leq 0.05$ for statistical significance from nil.

increase in their phosphorylation. Interestingly, MEK1/2 inhibition (PD98059) did reduce NO production, but not to the extent of ERK1/2 inhibition, possibly suggesting a mechanism of ERK1/2 activation independent of MEK1/2, a possibility also supported by protein phosphorylation patterns in which ERK1/2 phosphorylation is more prominent than MEK1/2 phosphorylation. As suggested by preliminary immunoblotting (data not shown), p38 MAPK was not involved in MRP-mediated NO generation, as reflected by the conserved NO production upon p38 inhibition (SB203580).

Transcription factors involved in MRP-induced NO generation

To complete the signaling picture of MRPs in Mϕ, we investigated the downstream activation of transcription factors usually involved in IFN-γ-induced iNOS expression (STAT1, NF-κB, CREB, and AP-1). As a prominent inflammatory transcription factor, NF-κB involvement was monitored. As expected, IFN-γ-induced NF-κB translocation was detected at late time points (2–4 h) (Fig. 7), but strikingly, in response to MRPs stimulation, we observed a stronger and quicker induction of Mϕ NF-κB activity that was sustained for 30 min to 1 h, and decreased to a lesser level by 4 h poststimulation. A very similar level of induction was observed for IFN-γ/MRP-stimulated cells, identifying MRPs as a potent inducer of NF-κB. Thereafter, we have been interested to confirm both the induction of STAT1α translocation by IFN-γ stimulation and the

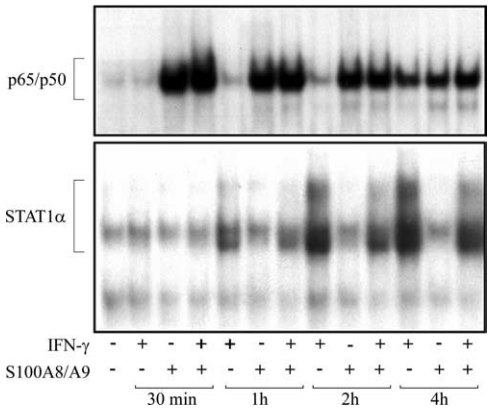

FIGURE 7. NF translocation and binding activity. Mϕ were treated with IFN-γ (100 U/ml), MRPs (10 μg/ml), or both during 30 min and 1, 2, or 4 h. Nuclear proteins were then submitted to EMSA with STAT1α or with NF-κB (p65/p50) consensus oligonucleotide. Results shown are representative of at least three independently performed experiments.

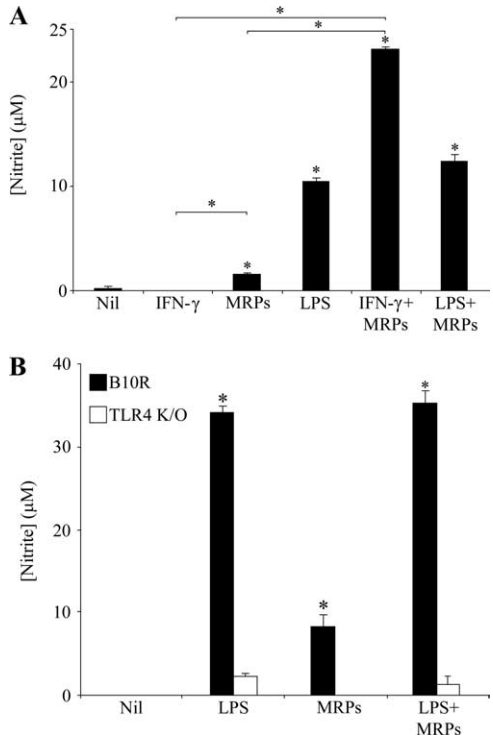

FIGURE 8. NO production in BMDMs and the implication of TLR4 in MRP-mediated NO production in Mϕ. BMDMs (*A*) or B10R and TLR4 KO (*B*) Mϕ were stimulated with IFN-γ (100 U/ml), MRP (25 μg/ml), and LPS (100 ng/ml) or combinations of these compounds. NO was measured 24 h later. Results shown are representative of at least three independently performed experiments. *, $p \leq 0.05$ for statistical significance from nil.

incapacity of MRPs to do so, as suggested by the incapacity of MRPs to induce JAK2/STAT1 phosphorylation. As shown in Fig. 7, STAT1α translocation is, as expected, observable as soon as 1 h upon IFN-γ stimulation and sustained up to 4 h. STAT1α activation was confined to IFN-γ and IFN-γ/MRP-treated cells as MRPs per se were not inducing its activation, therefore confirming that JAK2/STAT1 pathway is exclusively used by IFN-γ in this context. Other transcription factors involved in the regulation of iNOS were investigated (AP-1 and CREB), but MRPs were not significantly modulating these factors (data not shown).

NO production in BMDMs and implication of TLR4 in MRP-induced NO production

Knowing the pathways triggered by MRPs, we were interested to investigate the effect of MRPs on NO production in BMDMs. As shown in Fig. 8*A*, stimulation of BMDMs with MRP-induced NO production. IFN-γ in contrast, did not per se induce NO production, reflective of an IFN-γ priming, as BMDMs could still be activated by LPS. Very interestingly, stimulation with both IFN-γ and MRPs induced a striking synergistic NO production, confirming our observations in B10R Mϕ. It is also noteworthy that MRPs and LPS costimulation does not further increase the NO production induced by LPS alone. As TLR4 was reported to be the receptor for MRPs (19), this observation might suggest that NO production cannot be increased anymore, due to the fact that both compounds signal through TLR4 pathway. To verify this possibility, we used a Mϕ cell line with a TLR4 gene deletion (in the B10R background) and observed NO production after MRP stimulation. As can be seen in Fig. 8*B*, LPS stimulation induced NO production only in wild-type B10R Mϕ. Interestingly, MRPS-induced NO production was observed in wild-type B10R Mϕ, but

was not observed in TLR4 KO Mϕ, revealing a necessary role for TLR4 in sensing extracellular presence of MRPs. Costimulation of both cell lines with MRPs and LPS was not observed to further stimulate NO production, as observed in Fig. 8*A*.

Discussion

The S100 protein family is a wide group of intracellular proteins involved in many cellular events such as contraction, motility, secretion, and cell differentiation (reviewed in Refs. 1, 30). Among this family, S100A8, S100A9, and S100A12 are reported to be important in the immunological context (31). For instance, these proteins were reported to be chemotactic for neutrophils and to be produced by these cells (9, 22, 32, 33), creating a positive feedback loop. More recently, we reported that S100A8, S100A9, and their heterodimer were strongly induced by hemozoin injection (a malaria-related metabolic waste) in the context of an air-pouch model (20). Among the cells recruited in this system were neutrophils, as well as monocytic cells (20). Therefore, in the present study, we have been interested to test whether S100A8 and S100A9 may have immunostimulatory effects on cells of monocytic lineage as Mϕ.

As NO plays a key role in Mϕ microbicidal functions during innate immune response, we investigated this mediator to assess whether MRPs can be an important player in Mϕ activation. Of utmost interest, the present study reports the first observation that MRPs are effectively important inducers of NO, an observation even more interesting given the fact that they were used at physiologically relevant doses (10 μg/ml) as we previously measured in vivo (20). For instance, we observed that all MRPs used in our study were similarly inducing NO production in correlation with equivalent increase in iNOS gene expression. This finding is of paramount importance as epithelial cells are known to secrete MRPs and therefore it suggests that this could be sufficient to rapidly induce microbicidal functions of local Mϕ in response to infectious agents.

The activation induced by MRPs alone showed to be even faster than IFN-γ-driven Mϕ activation (Fig. 3), suggesting an important role in vivo for MRPs. MRPs might have a preponderant role as they can activate freshly recruited cells very quickly in addition to their chemotactic capacity for inflammatory cells. Moreover, we observed that if both IFN-γ and MRPs (S100A8/A9) are present, Mϕ produce even more NO, and as with MRPs alone, begin more quickly. Upon recruitment to the site of ongoing inflammation, a mediator mixture containing IFN-γ and MRPs might be very potent at activating recruited Mϕ. Most interestingly, the interaction of the two products induced a remarkably rapid and strong induction of iNOS gene, beginning at 2 h and quickly resulting in more mRNA expression than with each compound alone. Importantly, the fact that maximal iNOS mRNA expression in response to IFN-γ/MRPs mix does not result in proportional translation of iNOS protein. This result indicates that posttranscriptional events regulate the production of iNOS protein and probably represents a negative feedback loop that prevents excessive production of NO, an event that could potentially drive NO-induced apoptosis in producing cells. This negative feedback mechanism would deserve further investigation in a near future.

The enhanced production of NO proved to be synergistic at doses of MRPs between 1 and 10 μg/ml in which the produced NO is well over the expected additive value. This clearly suggests an important in vivo role as these two mediators could easily be inducing strong proinflammatory capacities of freshly recruited Mϕ and it clearly states that MRPs have more functional extracellular capacities than the mere chemotactic and adhesion inducer effects generally recognized. Although the synergy was consistently observed through our experiments, a variation in its intensity was noted. We attribute this variation in MRP stimulatory activity to variations in biological activity between MRP preparations. We are actually working on a method to standardize our biological activity.

As we established that NO is induced upon stimulation with MRPs, we attempted to identify the signaling pathways involved in response to S100A8/A9. The receptor for S100A8/A9 is still elusive, although it is known that the heterodimer can bind heparan sulfate by the S100A9 subunit (16) and carboxylated N-glycans on endothelial cells (17). Some speculated that RAGE might be the receptor for S100A8/A9 as it is the receptor for S100A12 (18), but other studies were unable to demonstrate its implication in the response induced by S100A8/A9 (34). A recent report also indicates that S100A8/A9 is an endogenous ligand of TLR4 (19). In this study, we focused on identifying the pathways engaged by the recognition of the MRP complex and to determine whether the pathways were entangled with the ones engaged by IFN-γ stimulation, which could help us to understand their synergy. IFN-γ and MRPs induced the activation of different and exclusive pathways. As generally recognized, IFN-γ stimulation resulted in JAK2/STAT1α pathway activation. In contrast, S100A8/A9 engaged MEK1/2 and ERK1/2 pathway at an early time point (15 min) and phosphorylation of this pathway is almost back to normal by 2 h. This finding is in agreement with the recently suggested induction of the ERK/MEK pathway by MRPs in bone marrow cells (19). It is interesting to note that MEK phosphorylation is not as prominent as ERK phosphorylation and that pharmacologic inhibition of MEK does not results in complete abrogation of NO production, whereas inhibition of ERK does. Although MEK/ERK is often viewed as linear, recent results show that some proteins, such as $p56^{lck}$ and protein kinase Cε can bypass MEK to activate ERK (35). Such a mechanism could explain why ERK inhibition is more potent than MEK inhibition in our model (19). Very interestingly, SAPK/JNK pathway was activated rapidly with an optimal phosphorylation around 30 min, but was more prominently phosphorylated than the MEK/ERK pathway. The fact that IFN-γ and MRPs engage exclusive pathways could suggest that the costimulation will result in an additive effect. But as we observed a synergy, it strongly suggests that both pathways collaborate to activate iNOS gene and that this collaboration results in an enhanced capacity to produce iNOS mRNA as observed in Fig. 3*A*, which shows a tremendous increase in iNOS mRNA. This raised the question of which transcription factors are engaged by these signaling pathways.

As iNOS contains promoter binding sequences for STAT1α, NF-κB, CREB, and AP-1, we drew our attention on these transcription factors (36). CREB and AP-1 were not significantly modulated by MRPs (data not shown) and therefore did not retain our attention any further. STAT1α showed to be modulated (Fig. 6) only by IFN-γ and to follow induction of JAK2 and STAT1α phosphorylation (Fig 5). NF-κB (p65/p50 form, consensus sequence) was observed to be modulated only by the heterodimer S100A8/A9. Its induction was very rapid as it showed strong DNA binding capacity after 30 min. Again we show that the ways to activate iNOS expression are distinct. Although other factors might intervene, we can speculate that synergy between the two pathways is the result of the interaction of these two transcription factors that might increase the availability of the binding site for one another. As a matter of fact, their binding sites can be found within 800 bp in the iNOS promoter (36). Recently, our group and other researchers have reported that NF-κB was activated upon stimulation with S100A8/A9 and have shown that this activation was not mediated through RAGE, a proposed receptor for

S100A8/A9 (34, 36, 37). Therefore it appears that NF-κB is consistently involved in cell responses to MRPs. Whereas STAT1α, CREB, and AP-1 are not modulated upon MRP stimulation, suggesting a very specific role of NF-κB in this signaling pathway. Taken together, our work seems to support the recent finding that MRPs are endogenous ligands of TLR4 (19) as NF-κB, SAPK/JNK, and MEK/ERK pathways are activated, which correlates with the effect of TLR4 ligation (38).

After establishing a coherent signaling pathway, we confirmed in BMDMs that MRPs induce NO production. Interestingly, we observed that the synergistic effect between IFN-γ and MRPs is even more striking in this context as IFN-γ only primed these BMDMs, without inducing NO production per se. The resulting NO production upon costimulation was therefore even more potent. As NO production upon LPS and MRP costimulation was not increased when compared with LPS alone, it raised the possibility that MRPs and LPS are signaling through the same receptor. Therefore, we used TLR4 KO B10R Mϕ to verify NO production upon MRP stimulation. As reported by Vogl and colleagues (19), our results also support the role for TLR4 in the recognition of MRPs as TLR4 KO Mϕ were unresponsive to MRPs.

Collectively, our study represents the first demonstration that MRPs can induce Mϕ microbicidal functions through TLR4 as potently as IFN-γ, and that in combination, these cytokines further synergize NO production by Mϕ, suggesting that this activation process may play a crucial role in innate immune response concurring to control infectious agents.

Disclosures

The authors have no financial conflict of interest.

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