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High-Mobility Group Nucleosome-Binding Protein 1 as Endogenous Ligand Induces Innate Immune Tolerance in a TLR4-Sirtuin-1 Dependent Manner in Human Blood Peripheral Mononuclear Cells

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High-mobility group nucleosome-binding protein 1 (HMGN1) belongs to the HMG N family but it exhibits no homology to HMGB1. The functions of HMGN1 were mainly related to its nuclear...
localization, including modulating histone phosphorylation (5, 6), acetylation (7), methylation preferentially at CpG island-containing promoters (8, 9), and enhancement of DNA damage repair (10). However, two recent studies showed that HMGN1 also has a biological role as an alarmin by inducing DC matura-
tion, antigen-specific immune responses, and antitumor immu-
nity (11, 12).

Upon engaging microbial or endogenous ligands, innate
immune cells either directly clear them by phagocytosis, or they
induce production of cytokines and chemokines for further
activation of the immune system. After the acute inflamma-
tory phase, a resolution phase is actively induced in order to
limit the potentially deleterious ongoing inflammation, fol-
lowed by a return to steady state. Thus, after the initial marked
inflammatory response [e.g., induced by the Gram-negative
cell wall component lipopolysaccharide (LPS)], subsequent
re-stimulation of leukocytes is no longer able to induce the
release of inflammatory mediators, but instead activates anti-
inflammatory and repair proteins, a process termed innate
immune tolerance (13, 14). Interestingly, the first exposure of
monocytes to other microbial stimulants “trains” or “primes”
the cells and they respond in a more robust way to a secondary
stimulation or infection (15).

We have hypothesized that the first exposure of the innate
cells to HMG proteins may also induce their functional repro-
gramming resulting in either tolerance or training. We showed
that HMGN1 functions as an endogenous TLR4 ligand that, on
the one hand, stimulates acute cytokine production and, on the
other hand, induces tolerance in monocytes through a Sirtuin-1-
dependent mechanism.

MATERIALS AND METHODS

Isolation and Stimulation of Peripheral
Blood Mononuclear Cells (PBMCs)

Separation and stimulation of PBMCs was performed from
buffy coats obtained from healthy blood donors after written
informed consent (Sanquin Bloodbank, Nijmegen). PBMCs
were adjusted to a concentration of 5 × 10⁶ cells/ml and incu-
bated at 37°C in flat-bottom 96-well plates (100 µl/well) with
either 100 ng/ml HMGB1, 100 ng/ml HMGN1, 10 ng/ml LPS
(E. coli strain O55:B5, Sigma Chemical Co., St. Louis, MO, USA),
or culture medium. Recombinant HMGN1 was produced using
an insect expression system constructed as previously reported
(5). HMGN1 in the culture supernatant of High Five insect cells
was purified under sterile condition by affinity chromatography.
The endotoxin level in our HMGN1 preparation is <0.02 EU
per µg of protein as determined by Pierce LAL Chromogenic
Endotoxin Quantitation Kit (Cat #88282). To assess direct
stimulation of cytokines, supernatant was removed and stored
for assessment.

To study the potential reprogramming effects of HMGN1 on
the function of monocytes/macrophages, after the initial stimu-
lation for 24 h the cells were washed with warm PBS, allowed
to rest for 24 h in RPMI containing 10% pooled human serum,
and then restimulated with LPS (10 ng/ml), Pam3Cys (10 µg/ml,
EMC microcollections, Tuebingen, Germany), flagellin (2 µg/ml,
Sigma), or co-culture of Pam3Cys and Candida albicans β-1,3-
(β-glucan [10 µg/ml, kindly provided by D. Williams (East
Tennessee State University)] for an additional 24 h. For the
long-term studies, cells were incubated for a period of 6 days after
the initial 24 h exposure to HMG proteins or LPS. On day 7, the
cells were restimulated with the same stimuli for additional 24 h.
Supernatants were collected 24 h after restimulation and stored
at −20°C.

The receptor pathways involved in the biological effects of
HMGN1 were assessed by blocking TLR4 with the natural anta-
agonist Bartollena quintana LPS (16). A potential role for histone
methylation or acetylation in the long-term effects of HMGN1
was assessed using specific pharmacological inhibitors: ITF2357
(100 nM, Histone deacetylase inhibitor, ITALFARMACO S.p.A,
Milano, Italy), EGC2 (30 µM, Epigallocatechin-3-gallate, histone
decetylase inhibitor, Sigma), and pargyline (3 µM, histone
demethylase inhibitor, Sigma) or EX527 (10 µM, sirtuin-1 inhibi-
tor, Sigma) (15, 17).

Animal Experiments

Female C57BL/6J mice (8–10 weeks old, weighing 20 ± 3 g)
were obtained from National Laboratory Animal Center (Taipei,
Taiwan). All mice were housed in a pathogen-free facility.
Animal welfare and experimental procedures were carried out
in accordance with the National Institute of Health Guide for the
Care and Use of Laboratory Animals, with the approval of the
Institutional Animal Care and Use Committee of National Tsing
Hua University (Approval number: 10530, Hsinchu, Taiwan).
Mice were treated with PBS, recombinant HMGN1 (10 µg
per mouse) or E. Coli LPS (20 µg per mouse) by intraperitoneal
injection. A second injection of LPS (20 µg per mouse) was
performed after 6 h post first injection intraperitoneally. Blood
samples were collected 1 h post second LPS injection for serum
cytokine determination.

Cytokine and Lactate Measurements

IL-6, IL-8 (Sanquin, Amsterdam, Netherlands), TNF-α, IL-1β
(R&D, the Netherlands) concentrations in the culture supernatant
were measured by commercial ELISA kits. The lowest detection
limits are 0.78, 0.78, 3.9, and 3.9 pg/ml for IL-6, IL-8, TNF-α,
and IL-1β, respectively. Mouse serum cytokine were measured by
Cytokine Beads Array (Becton Dickinson, NJ, USA) according to
the manufacturer's instructions. Lactate was measured by a Lactate
Fluorometric Assay Kit (Biovision, CA, USA). Delta lactate pro-
duction (lactate concentration in the LPS restimulated sample
minus the RPMI restimulated sample) is depicted in the figures.

mRNA Extraction and RT-PCR

Cells were primed with either HMGN1 or LPS and restimulated
with LPS as described above. mRNA was extracted by Trizol 4 h
post-stimulation. The qPCR primers sequences are listed in the
(Table S1 Supplementary Material) For sirtuin-1 expression,
cells were stimulated for 4 h before RNA was isolated. cDNA
was synthesized from 1 µg of total RNA by use of SuperScript
reverse transcriptase (Invitrogen). Relative mRNA levels were
determined using the Bio-Rad i-Cycler and the SYBR Green method (Invitrogen). Values are expressed as fold increases in mRNA levels, relative to those in unstimulated cells, with HPRT as a housekeeping gene.

**Statistical Analysis**
Results from at least three sets of experiments were pooled and analyzed using GraphPad Prism software. Data are given as means + SEM and the paired Wilcoxon test or one-way ANOVA was used to compare differences between groups. The level of significance was set at \( p < 0.05 \).

**RESULTS**

**HMGN1 Induces Pro-Inflammatory Cytokine Production in PBMCs**
We first examined the capability of HMGN1 to induce pro-inflammatory cytokine production in human PBMCs. HMGN1-induced considerable IL-6, TNF-\( \alpha \), and IL-1\( \beta \) production in PBMCs after 24 h stimulation in a dose-dependent manner (Figure 1). Strikingly, HMGN1 at 100 ng/ml could induce comparable amount of IL-6 and TNF-\( \alpha \) and more IL-1\( \beta \) compared to that induced by LPS at 10 ng/ml.

**HMGN1 Induces Immune Tolerance in PBMCs**
We hypothesized that HMGN1 may induce long-term effects on innate immune cells. To assess this possibility, PBMCs were first stimulated with HMGN1 or LPS (as a positive control). After 24 h stimulation, cells were washed with PBS to remove remaining stimulants and rested for an additional 24 h or 6 days, before secondary LPS stimulation was performed. IL-6 and TNF-\( \alpha \) production upon secondary LPS (TLR4 ligand) stimulation were significantly impaired in HMGN1 pretreated monocytes both in short-term (Figure 2A) and long-term (Figure 2B) tolerance models, suggesting HMGN1-induced considerable tolerance against LPS stimulation. The HMGN1-induced tolerance is similar to LPS-induced tolerance.

![Figure 1](image1.png)
*Figure 1* | High-mobility group nucleosome-binding protein 1 (HMGN1) induces pro-inflammatory cytokine production. Human peripheral blood mononuclear cells were stimulated with recombinant HMGN1 or lipopolysaccharide (LPS) in a dose-dependent manner. HMGN1 concentration used were 10, 100, and 1,000 ng/ml, and LPS concentration was 10 ng/ml. Supernatant was harvested after 24 h stimulation. IL-6, TNF-\( \alpha \), and IL-1\( \beta \) production was determined by ELISA (\( n = 6 \)).

![Figure 2](image2.png)
*Figure 2* | High-mobility group nucleosome-binding protein 1 (HMGN1) induces short- and long-term immunotolerance. Human peripheral blood mononuclear cells were primed with recombinant HMGN1 or lipopolysaccharide (LPS) in a dose-dependent manner. HMGN1 concentration used were 10, 100, and 1,000 ng/ml, and LPS concentration was 10 ng/ml. Supernatant was harvested after 24 h stimulation. IL-6, TNF-\( \alpha \), and IL-1\( \beta \) production was determined by ELISA (\( n = 6 \)).
HMGN1 Induces Tolerance to Different TLR Agonists in PBMCs

To further examine whether the HMGN1-induced tolerance is specific for TLR4 ligands or more general for other microbial ligands as well, we extended the study by also using TLR2 and TLR5 agonists, as well as the dectin-1 ligand β-glucan. We found that both HMGN1 and LPS could induce partial cross-tolerance to other TLRs both in short-term (Figure 3A) and long-term (Figure 3B) experiments. Only a partial tolerance effect was induced on dectin-1-induced TNFα production.

Blocking TLR4 Signaling Attenuates HMGN1-Induced Tolerance

It has been suggested that HMGN1-induced dendritic cell maturation via TLR4 (11, 12). Therefore, we examined whether TLR4 is involved in HMGN1-induced tolerance in PBMCs. To block TLR4 signaling, PBMCs were first incubated with B. quintana LPS, a natural antagonist of TLR4 (16) for 1 h, followed by stimulation with HMGN1 or LPS. Pretreatment of cells with B. quintana LPS resulted in markedly reduced production of IL-6 and TNF-α upon LPS stimulation and a partial reduction upon HMGN1 stimulation (Figure 4A). Thereafter, we assessed both the short- and long-term tolerance effects induced by HMGN1. TLR4 blockade by antagonists blocked or reversed the tolerance effects induced by HMGN1 on IL-6 and TNF-α production (Figures 4B,C).

HMGN1-Induced Tolerance Is Restricted to Pro-Inflammatory Cytokines, But Not to the Antimicrobial Peptides

Lipopolysaccharide priming has been demonstrated to induce transient silencing of pro-inflammatory mediators and priming of genes such as antimicrobial effectors (14). To address whether HMGN1 also induces similar differential gene regulation patterns, the mRNA expression of pro-inflammatory cytokines IL-6 and TNF-α, anti-inflammatory cytokine IL-10, chemokine IL-8 and antimicrobial peptide CAMP (cathelicidin-related antimicrobial peptide, also called LL-37) were determined by quantitative real-time PCR (Figure 5). In line with the cytokine results, pro-inflammatory cytokines TNF and IL-6 expression were significantly downregulated by both HMGN1 and LPS. IL-10 expression was downregulated by HMGN1 in the short-term incubations (albeit the difference was not significant) and recovered to the normal state in the long-term model. However, unlike the experiments earlier reported in mouse macrophages, neither in HMGN1- nor in LPS-induced tolerance could the expression of CAMP be induced in human PBMCs. Surprisingly, the expression of IL-8 was not inhibited, but was even enhanced after long-term incubation. The long-term effects of HMGN1 on IL-8 production were confirmed by ELISA (Figure S1 in Supplementary Material).

HMGN1-Induced Immune Tolerance In Vivo

To assess the pathophysiological role of HMGN1, mice were pretreated with either recombinant HMGN1 or LPS to induce tolerance for 6 h followed by secondary stimulatory LPS injection. TNF and KC production was significantly blunted in the LPS pretreated group compared to PBS control (Figure 6). Similarly, HMGN1 pretreatment also downregulated LPS-induced TNF and KC production, albeit the downregulated level was lower than that of LPS pretreated group.

The Effects of Histone Methylation and Acetylation on HMGN1-Induced Tolerance

Epigenetic modifications have been suggested to play an important role for the LPS-induced tolerance through histone

![Figure 3](http://example.com/figure3.png)

**FIGURE 3** | High-mobility group nucleosome-binding protein 1 (HMGN1) immunotolerance is not specific for TLR4 ligands. Human peripheral blood mononuclear cells were primed with recombinant HMGN1 or lipopolysaccharide (LPS) for 24 h and then washed with PBS. The cells were further rested in RPMI containing 10% serum for (A) 24 h or (B) 7 days then stimulated with Pam3Cys, flagellin, β-glucan, or RPMI, respectively, for additional 24 h and supernatant were harvested. The IL-6 and TNF-α levels were determined by ELISA (n = 4–8 *p < 0.05 vs RPMI control within each group of restimulation).
acetylation and methylation (18). To examine whether epigenetic modifications are also involved in HMGN1-induced tolerance, several enzymatic inhibitors of acetyl- and methyltransferases were added to the PBMCs prior to the priming stage. However, no obvious restoration of cytokine production was observed by the inhibitors we tested, with the exception of the short-term restoration effect induced by blocking histone acetylation by EGCG for IL-6 production (Figure S2 in Supplementary Material).

The Effects on Sirtuin-1 and Immunometabolism by HMGN1 Stimulation

It has been shown before that Sirtuin-1 (a histone deacetylation inhibitor) is a key regulator of LPS tolerance (17, 19). Sirtuin-1 has been shown to be upregulated during the early phase after LPS stimulation and has a driving role in the transition from a glycolytic energy metabolism to a more β-oxidation-dependent
Figure 5

Effect of high-mobility group nucleosome-binding protein 1 (HMGN1) on TNF, IL-6, IL-8, IL-10, and CAMP production. Human peripheral blood mononuclear cells were primed with recombinant HMGN1 or lipopolysaccharide (LPS) for 24 h and then washed with PBS. The cells were further rested in RPMI containing 10% serum for (A) 24 h or (B) 7 days. The cells were stimulated with LPS or RPMI. The total RNA was extracted after 4 h and the different gene expression was measured by RT-PCR. The expression fold of target genes was normalized to the expression of HPRT \( n = 5–6, ^* p < 0.05 \text{ vs RPMI (LPS restimulated) control} \).
metabolism (19). First, Sirtuin-1 mRNA expression was upregulated after HMGN1 stimulation (Figure 7A). Second, the Sirtuin-1 inhibitor EX527 (17) partially restored cytokine production inhibited by HMGN1 (Figures 7B,C). Finally, EX527 also restored the capacity to release lactate after restimulation (as a measure of glycolysis) in both LPS- and HMGN1-tolerant macrophages (Figure 7D).

**DISCUSSION**

Although HMGN1 functions physiologically within the nucleus, the release of extracellular HMGN1 has been demonstrated to possess chemotactic function and to induce DC maturation (11). In the present study, we demonstrate that extracellular exposure of human PBMCs to HMGN1 induces a robust release of pro-inflammatory cytokines, such as IL-6 and TNF-α. This effect is likely to be relevant during sterile inflammation induced by perturbed cellular and/or tissue homeostasis (20), where the release of intracellular HMGN1 may cause acute local inflammation.

HMGB1 is the best-characterized HMG-family protein. It was initially identified as a nuclear protein that is important for the regulation of transcription (21). HMGB1 facilitates the binding of regulatory protein complexes to DNA by causing DNA bending.
demonstrating that histone acetylation controls both immune
Sirtuin-1 inhibitor EX527 restored the capacity of monocytes
as mirrored by defective lactate production. Interestingly, the
by HMGN1 or LPS) are not able to mount aerobic glycolysis,
the data presented here show that tolerant cells (both induced
the effective production of cytokines by macrophages during
lular metabolism of glucose. A recent study demonstrated that
interplay between immune activation of the cells and the cel-
Sirtuin-1 is a pivotal downstream signal of this pathway.
and metabolic function of tolerant monocytes. This suggests
Sirtuin-1 to be an attractive potential therapeutic target in
immune tolerance and paralysis during Gram-negative sepsis
and other severe infections.
In conclusion, HMGN1 induces tolerance in human PBMCs
through a TLR4/Sirtuin-1 dependent mechanism, arguing
that it may contribute to modulation of sterile inflammation
in processes, such as severe trauma and ischemia-reperfusion,
during which high amounts of TNF and IL-6 are released in
the absence of exogenous stimuli (33). The sterile inflamma-
may be caused by the release of endogenous HMGN1
from the damaged cells and the induction of cytokines through
TLR4 signaling. Moreover, the acute inflammation induced
by HMGN1 might later translate into tolerance and even
immunoparalysis, to increase the susceptibility of the patient
to secondary infections. Therefore, blocking these HMGN1
effects may have potential therapeutic benefits in pathological
processes in which hyperinflammation and/or immune para-
sis play a role in pathogenesis.

ETHICS STATEMENT
This study was carried out in accordance with the National
Institute of Health Guide for the Care and Use of Laboratory
Animals, with the approval of the Institutional Animal Care

AUTHOR CONTRIBUTIONS
Conception and drafting of the article: RA, MN, and S-CC. Performed and analysis of experiments: RA and P-KH. Discussions of the data and critical revision of the article: RA, DY, LJ, JM, JO, MN, and S-CC.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at
full#supplementary-material.
REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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