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) functions as a non-histone chromatin-binding protein in the cell nucleus. However, extracellular HMGN1 acts as an endogenous danger-associated inflammatory mediator (also called alarmin). We demonstrated that HMGN1 not only directly stimulated cytokine production but also had the capacity to induce immune tolerance by a TLR4-dependent pathway, similar to lipopolysaccharide (LPS)-induced tolerance. HMGN1-induced tolerance was accompanied by a metabolic shift associated with the inhibition of the induction of Warburg effect (aerobic glycolysis) and histone deacytlation via Sirtuin-1. In addition, HMGN1 pre-challenge of mice also downregulated TNF production similar to LPS-induced tolerance in vivo. In conclusion, HMGN1 is an endogenous TLR4 ligand that can induce both acute stimulation of cytokine production and long-term tolerance, and thus it might play a modulatory role in sterile inflammatory processes such as those induced by infection, trauma, or ischemia.

Keywords: high-mobility group nucleosome-binding protein 1, endotoxin tolerance, sterile inflammation, sirtuin-1, macrophages

INTRODUCTION

High-mobility group (HMG) proteins are non-histone nuclear proteins. They bind to nucleosomes and regulate chromosome architecture and gene transcription (1). However, upon cell stimulation or under stress situations, such as mechanical change and tissue damage, HMG proteins can be either released or excreted into the extracellular space (2). HMGB1 is the best-characterized HMG-family protein: it is released from injured or activated innate immune cells (1), it stimulates cytokine and chemokine production (3), it can induce dendritic cell activation (4), and it is chemotactic and functions an alarmin.

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 maturation, antigen-specific immune responses, and antitumor immunity (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 inflammatory phase, a resolution phase is actively induced in order to limit the potentially deleterious ongoing inflammation, followed 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 inflammatory response [e.g., induced by the Gram-negative E. coli strain O55:B5, Sigma Chemical Co., St. Louis, MO, USA], 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 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 agonist Bartolina 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), EGCG (30 µM, Epigallocatechin-3-gallate, histone acetyltransferase inhibitor, Sigma), and pargyline (3 µM, histone demethylase inhibitor, Sigma) or EX527 (10 µM, sirtuin-1 inhibitor, 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 mice) 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 production (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 sequence 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
HMGN1-Induced Tolerance

**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-α, and IL-1β 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-α and more IL-1β 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-α 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.
**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 TNF-α 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). 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 IL-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** | 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 (*p = 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 \( n = 5–6, *p < 0.05 \) 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 to respond with lactate production upon stimulation with LPS, Sirtuin-1 inhibitor EX527 restored the capacity of monocytes by HMGN1 or LPS) are not able to mount aerobic glycolysis, the data presented here show that tolerant cells (both induced induction of aerobic glycolysis is crucial. In line with this, the involvement of epigenetic modulators for HMGN1-induced tolerance was examined using inhibitors of epigenetic modifiers including ITF2357 (Histone deacetylase inhibitor), EGCG (Epigallocatechin-3-gallate, histone acetyltransferase inhibitor) and pargyline (histone demethylase inhibitor). Only EGCG had a marginal effect on the short-term effects of HMGN1-induced tolerance. By contrast, a different picture emerged when the effect of the NAD+ dependent histone deacetylase Sirtuin-1 was studied. First, HMGN1 induced, just as LPS, Sirtuin-1 expression. Second, inhibition of Sirtuin-1 by a specific inhibitor partially restored cytokine production during HMGN1-induced tolerance. This provides further support for the sharing of the tolerance pathway by endotoxin and HMGN1. Sirtuin-1 is a pivotal downstream signal of this pathway.

An additional interesting observation concerns the interplay between immune activation of the cells and the cellular metabolism of glucose. A recent study demonstrated that induction of aerobic glycolysis (Warburg effect) is necessary for the effective production of cytokines by macrophages during LPS stimulation. Moreover, we have also recently reported that during trained immunity, a process mirroring tolerance that is also mediated by epigenetic reprogramming, namely induction of aerobic glycolysis is crucial. In line with this, the data presented here show that tolerant cells (both induced by HMGN1 or LPS) are not able to mount aerobic glycolysis, as mirrored by defective lactate production. Interestingly, the Sirtuin-1 inhibitor EX527 restored the capacity of monocytes to respond with lactate production upon stimulation with LPS, demonstrating that histone acetylation controls both immune 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. The sterile inflammation 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 paralysis 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 and Use Committee of National Tsing Hua University (Approval number: 10530, Hsinchu, Taiwan).

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 https://www.frontiersin.org/articles/10.3389/fimmu.2018.00526/full#supplementary-material.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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