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The western-type diet induces anti-HMGB1 autoimmunity in *Apoe*^{-/-} mice

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Abstract

Background and aims—Anti-HMGB1 autoimmunity plays a role in systemic *lupus erythematosus* (SLE). Because SLE increases atherosclerosis, we asked whether the same autoimmunity might play a role in atherogenesis.

Methods—We looked for the induction of HMGB1-specific B and T cell responses by a western-type diet (WTD) in the *Apoe*^{-/-} mouse model of atherosclerosis. We also determined whether modifying the responses modulates atherosclerosis.

Results—In the plasma of male *Apoe*^{-/-} mice fed WTD, the level of anti-HMGB1 antibodies (Abs) was detected at ~50 µg/ml, which was ~6 times higher than that in either *Apoe*^{-/-} mice fed a normal chow or *Apoe*^{+/+} mice fed WTD ($p = 0.0005$). The Abs were directed largely toward a novel, dominant epitope of HMGB1 named HMW4; accordingly, compared with chow-fed mice, WTD-fed *Apoe*^{-/-} mice had more activated HMW4-reactive B and T cells ($p = 0.005$ and $p = 0.01$, respectively). Compared with mock-immunized mice, *Apoe*^{-/-} mice immunized with HMW4 along with an immunogenic adjuvant showed proportional increases in anti-HMW4 IgG and IgM Abs, HMW4-reactive B-1 and B-2 cells, and HMW4-reactive Treg and Teff cells, which was associated with ~30% increase in aortic arch lesions ($p = 0.01$) by two methods. In contrast, *Apoe*^{-/-} mice immunized with HMW4 using a tolerogenic adjuvant showed preferential increases in anti-HMW4 IgM (over IgG) Abs, in HMW4-reactive B-1 (over B-2) cells, and in HMW4-specific Treg (over Teff) cells, which was associated with ~40% decrease in aortic arch lesions ($p = 0.03$).

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Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Conclusions—Anti-HMGB1 autoimmunity may potentially play a role in atherogenesis.

Keywords

Atherosclerosis; *Apoe*^{-/-} mice; Western-type diet; Anti-HMGB1 autoimmunity

1. Introduction

Autoimmunity is thought to play an important role in atherogenesis¹⁻³. The spectrum of auto-antigens (Ags) involved in atherosclerosis (“athero-Ags”) is partly known, with only a limited number of auto-Ags such as OxLDL, APOH (β2gp1), and HSP60 being identified thus far^{2, 4-7}. The antigenic epitopes in oxLDL include malonyl dialdehyde and oxidized phosphatidylcholine⁸. Certain antibodies (Abs) to the Ags, for example, the IgM Ab to MDA-LDL, have been shown to modify the course of experimental atherosclerosis, and their titers have also been valuable in monitoring atherosclerosis in patients⁸. Identification of additional athero-Ags may reveal a wider spectrum of the Ag-specific responses and permit a more comprehensive targeting of these responses to improve diagnosis and treatment of atherosclerotic cardiovascular disease.

High mobility group box-1 protein (HMGB1) is a non-histone nuclear protein that, when released from cells, interacts with toll-like receptors (TLR) 2/4 and RAGE (receptor for advanced glycation end products)⁹ as a danger-associated molecular pattern (DAMP). HMGB1 has been shown to stimulate the synthesis of other proinflammatory cytokines such as TNF and IL-1¹⁰. It is a multi-domain DNA binding protein⁹, widely expressed in various cells including those of the evolving atherosclerotic plaque. It is released in the plaque by the macrophages infiltrating the lesions in response to inflammatory cytokines such as TNF-α¹¹ and TWEAK¹². Plasma HMGB1 has been noted as a biomarker predicting unfavorable outcomes in those who have suffered a myocardial infarction¹³. In the *Apoe*^{-/-} mouse model of atherosclerosis, studies have shown that the mice can be protected from severe atherosclerosis by the injection of a rat IgG2a anti-HMGB1 neutralizing Ab, suggesting the role of HMGB1 as an atherogenic mediator¹⁴.

Besides its role as a DAMP, HMGB1 has been shown to act as an auto-Ag. Anti-HMGB1 Abs are detected in a high proportion of patients with autoimmune diseases^{15, 16}. Particularly, in systemic *lupus erythematosus* (SLE), the induction of anti-HMGB1 Abs^{17, 18} and HMGB1-reactive T cells¹⁹ correlate with disease activities in SLE patients. Interestingly, atherosclerosis is accelerated in SLE²⁰, which hints that the same anti-HMGB1 autoimmunity might also promote atherogenesis. Led by this clue, initial ELISA studies in our lab detected anti-HMGB1 Ab in the plasma of atherosclerotic *Apoe*^{-/-} mice.

Our goal is to explore the potential role of HMGB1 as an Ag in atherosclerosis. In the present study, we show that the “western-type diet” (WTD) that accelerates atherogenesis also induces both humoral and cellular immune responses against HMGB1 in *Apoe*^{-/-} mice. Moreover, immunizations with a dominant epitope of HMGB1 can modify the disease outcome. These suggest that besides acting as a proinflammatory mediator, HMGB1 may potentially function as a putative athero-Ag as well.

2. Materials and methods

2.1 Mice, diets, and reagents

The wild-type or *ApoE*^{-/-} C57BL/6 male mice were purchased from The Jackson Laboratory and were used in accordance with The University of Illinois institutional guidelines for animal care. The western-type diet (WTD) was from Research Diets (D12492); the normal chow diet (Chow), from Harlan (Harlan Teklad diet 7001). The human HSP60 was from Enzo Life Sciences. Human HMGB1, human IgG and IgM (both from pooled human sera), DNase I, BrdU, dexamethasone (Dex), and Oil Red O (ORO) were from Sigma-Aldrich. Ficoll-Paque Plus was from GE Healthcare. The horseradish peroxidase (HRP)- and alkaline phosphatase (AP)-based Ig clonotyping kits and the isotype standards for C57BL/6 mice were from SouthernBiotech. The protease-free/Ig-free BSA, streptavidin (SA), and goat anti-human Ig Ab were from Jackson ImmunoResearch Laboratories. The peptides HMW4 (HMGB1₉₈₋₁₁₂, PSAFFLFCSEYRPKI), biotinylated HMW4, randomized HMW4 (rHMW4, IRYFPFCLKS FEAPS), HSS1 (HSP60₄₋₁₉, PTVLRQMRPVSRALA), and HP1 (HSP60₂₉₂₋₃₀₈, KVGLQVVAVKAPGFGDN) were synthesized by Biomatik. MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) of the myelin oligodendrocyte glycoprotein was from AnaSpec. The CD4⁺ T cell purification kit was from Miltenyi Biotec. The fluorephore-conjugated Abs and SA were from Biolegend. Biotin, the complete (CFA) and incomplete (IFA) Freund's adjuvants, and mannan-binding protein agarose were from ThermoFisher Scientific.

2.2 Detection of anti-HMGB1 Abs in blood plasma by ELISA

Ninety six-well EIA/RIA plates were coated in 0.05 M NaHCO₃, pH 9.6 containing human HMGB1 at 50 ng/50 µl/well or HMW4 at 1 µg/50 µl/well, and blocked in the same buffer with 1% Ficoll-Paque Plus. Plasma samples, diluted 1:100 in PBS/0.05% Tween-20 (wash buffer), were plated (50 µl/well) overnight or for 1 h into HMGB1- or HMW4-coated plates, respectively. The plasma Ab bound to the coated Ag was detected using the clonotyping kits and the isotype standards. Total Ig was detected using the anti-Ig detection Ab supplemented with the anti-IgM detection Ab (1:1). The standard for total Ig consisted of IgG1, IgG2b, IgG2c, IgG3, and IgM (1:1:1:1:4). To reduce nonspecific signals, the HRP- and AP-based kits were used for HMGB1- and HMW4-coated wells, respectively; the enzyme-conjugated detection Abs in both kits were applied in 0.05 M Tris/0.15 M NaCl/0.05% Tween-20, pH 8.9. The Ag-dependent signal (ng/well) was calculated by subtracting the signal of non-Ag-coated wells from that of the Ag-coated wells. The result was converted to the Ab concentration in plasma (µg/ml) based on the original volume of the plasma samples in these wells (0.5 µl/well).

2.3 Detection of HMGB1 and HMGB1-IgM complex in blood plasma by ELISA

Ninety six-well EIA/RIA plates were coated in 0.05 M NaHCO₃, pH 9.6, containing the mouse IgG2b anti-human/mouse HMGB1 mAb 3E8 (Biolegend) or a control mIgG2b (100 ng/50 µl/well) and blocked in the same buffer containing 0.5% BSA. Plasma samples, diluted 1:5 in the wash buffer above, were plated (50 µl/well). Bound plasma HMGB1 was detected, in the presence of 200 ng/ml mIgG2b as a blocker, with a rabbit anti-HMGB1 Ab and an HRP-conjugated goat anti-rabbit Ab (Cell Signaling Technology, Inc.). The HMGB1

level (ng/well) was obtained by subtracting the signal of control Ab-coated wells from that of anti-HMGB1 mAb-coated wells. The result was converted to the HMGB1 concentration in plasma (ng/ml) based on the original volume of the plasma samples in these wells (10 μ l/well). For detection of the HMGB1-IgM complex, the plates were coated with 3E8, blocked with 0.5% BSA, loaded with plasma samples, and detected with an HRP-conjugated goat anti-mouse IgM Ab (SouthernBiotech), as described above. Mannan-binding protein agarose-based affinity chromatography was performed as per the manufacturer's protocol.

2.4 Detection of Ag-specific T cells by BrdU incorporation

Mice were injected with HMW4 (25 μ g/injection, i.p.) once on day 1 and once on day 3, and with BrdU twice (1 mg and 0.5 mg in a 4-h interval, i.p.) on day 4. On day 5, CD4⁺ T cells were isolated from the spleen by MACS and stained with anti-CD3 and anti-CD44 monoclonal Abs (mAbs). The cells were fixed (4% paraformaldehyde), permeabilized (0.5% Tween-20), digested with DNase I (0.02 kU/10⁶ cells for 45 min at 37°C), intracellularly stained with anti-BrdU and anti-Foxp3 mAbs, and analyzed by flow cytometry, as we described previously ²¹.

2.5 Detection of Ag-specific B cells by tetramer staining

Blood was taken from the tail (50 μ l/mouse) and pooled (3 mice per pool). Leukocytes were isolated by centrifugation following the lysis of red blood cells with 7 volumes of ACK for 2.5 min. The cells were stained in PBS/2% BSA/5 mM EDTA/0.02% NaN₃ (staining buffer) with 0.2 μ g Fc Block (BD Biosciences), 0.05 μ g anti-CD19-PE/Cy7, 0.025 μ g anti-CD23-APC, 0.125 μ g anti-CD80-FITC, 0.04 μ g SA-PE/Cy5, and 0.8 μ g biotin-blocked SA. After 10 min, the cells were diluted with 10 volumes of staining buffer and centrifuged. The pellet was resuspended in 30 μ l staining buffer containing 0.04 μ g HMW4-tetramers. After 10 min, the cells were washed once with staining buffer and analyzed on a FACScalibur. Cells blocked with goat anti-mouse Ig Ab before the tetramer staining and cells stained with SA-PE (not complexed with HMW4) were used as negative controls. The tetramers used were freshly formed by mixing biotinylated HMW4 and SA-PE at a 2:1 molar ratio for 10 min in a separate tube.

2.6 Immunizations

For conventional immunization (CI), mice were primed with HMW4 in CFA (100 μ g HMW4/200 μ l/injection, s.c.) and boosted 14 days later with HMW4 in IFA (25 μ g/200 μ l / injection, s.c.). For suppressed immunization (SI), mice were injected (i.m.) with Dex at 8, 6, and 4 mg/kg (body weight) on days 1, 2, and 3, respectively; followed by an injection of HMW4 (100 μ g/injection, s.c.) on days 4 and 6. The regimen was repeated once 14 days later. For the Dex treatment, a stock solution of Dex (200 mg/ml in DMSO) was diluted to 5 mg/ml in PBS by vigorous vortexing; the resulting slurry was injected into the mice using a 27G tuberculin syringe.

2.7 Lipid measurement

Plasma triglycerides and cholesterol were measured using the EnzyChrom™ Triglyceride Assay Kit (BioAssay Systems) and the HDL and LDL/VLDL Quantitation Kit (Sigma Aldrich), respectively, as per the manufacturers' protocols.

2.8 Lesion analyses

Lesions in the aortic arch were analyzed by the *En face*²² and the Oil Red O (ORO) solubilization²³ methods, as described. Lesions in the aortic root were analyzed by a morphometric method, as described²⁴.

2.9 Statistic analysis

The unpaired two-sided Student *t* test was used for data involving 2 independent groups. For data involving more than 2 groups, one-way ANOVA and post hoc Turkey's honest significant difference test were used.

3. Results

3.1 Detection of anti-HMGB1 Abs in *Apoe*^{-/-} mice fed WTD

We first assessed the presence of humoral immunity to HMGB1 in the *Apoe*^{-/-} mice. To that end, the mice at age of 8 weeks were fed the atherogenic WTD for 8 weeks to accelerate atherosclerosis. Plasma obtained from the mice was analyzed for anti-HMGB1 Ab by ELISA. To help assess the significance of the result, we also quantified the Ab to HSP60, a known athero-Ag⁶, in the same experiment. The results showed that anti-HMGB1 Ig was present at ~50 µg/ml, which was similar to that of anti-HSP60 Ig (Fig. 1A). But unlike the anti-HSP60 Ig, the anti-HMGB1 Ig was mostly composed of IgM because the levels of the Ig (which includes IgM) and IgM were similar, as assessed by class-specific ELISAs (Fig. 1A), and the Ig-specific signal was reduced by > 80% when the detection Ab for the ELISA was blocked with mouse IgM, but not with a non-mouse Ab (Fig. 1B). The detection of the anti-HMGB1 Ig was HMGB1-specific, as it was blocked by HMGB1, but not by an irrelevant protein (Fig. 1C). Moreover, considering that HMGB1 is a DNA-binding protein, we incubated the plasma samples with either DNase I to eliminate any DNA or an excess amount of dsDNA fragments to block all DNA-binding proteins. Neither treatment affected the ELISA result (Fig. 1C), which excludes the possibility that the positive readings from the ELISA were caused by anti-dsDNA Ab that was tethered to plate-bound HMGB1 via a trace of DNA.

To determine whether the level of anti-HMGB1 Ab is related to the *Apoe* deficiency and WTD, we repeated the ELISA on the *Apoe*^{-/-} mice fed WTD along with two control groups. One consisted of *Apoe*^{-/-} mice of same age fed a normal chow diet (Chow), which have much less lesion at the age of sacrifice; the other consisted of wild-type (*Apoe*^{+/+}) C57BL/6 mice fed WTD, which have very little lesion at this age²⁵ (data not shown). Besides the decreased atherogenicity, the levels of anti-HMGB1 Ig in these two control groups were decreased drastically (by ~6-fold) (Fig. 1D). Collectively, these data indicate that the humoral response to HMGB1 is induced by both *Apoe* deficiency and WTD and may be related to atherogenesis.

To determine whether WTD also promotes the release of extracellular HMGB1, which can serve as the Ag for the anti-HMGB1 response, we compared plasma HMGB1 levels in Chow- and WTD-fed *Apoe*^{-/-} mice by ELISA. The level of HMGB1 was increased by ~8 fold in the WTD-fed group (Fig. 1E). However, most (~78%) of the ELISA-detected plasma HMGB1 molecules were not free ligands, but were rather bound by IgM Ab. As such, they could be enriched with mannan-binding protein (MBP) agarose, which binds specifically IgM (Fig. 1F), and detected as an HMGB1-IgM complex by ELISA (Fig. 1G). In conclusion, WTD increases both the release of extracellular HMGB1 and the Ab response to this DAMP molecule, resulting in an increase in the HMGB1-IgM immune complex.

3.2 Identification of HMW4 as a dominant epitope of HMGB1

Next, we wanted to determine whether there is cellular immunity against HMGB1 as well. To that end, we first searched for MHC II-restricted T cell epitopes in HGMB1, using a combination of bioinformatics^{26–28} and T cell proliferation assays, as we described²⁹. We identified one epitope named HMW4 (HMGB1_{98–112}, PSAFFLFCSEYRPKI), which overlaps partially with two dendritic cell activating peptides Hp91 (HMGB1_{89–107}) and Hp106 (HMGB1_{105–122}) and a TLR4-binding peptide C106 (HMGB1_{89–108}) reported previously^{30, 31}. HMW4 is highly conserved between humans and mice (100% identical). As a T cell epitope, it was predicted to be highly promiscuous, covering the majority of the human class II alleles and the I-A^b haplotype of C57BL/6 mice. Indeed, C57BL/6 mice immunized with the HMGB1 whole protein developed a T cell recall response to HMW4 (Fig. 2A), which suggests that HMW4 matches one of the T cell epitopes released from HMGB1 via Ag processing.

Subsequently, in non-immunized *Apoe*^{-/-} mice, we assessed the relationship between HMW4-specific splenic T cell activities and WTD. Significantly more HMW4-reactive effector T (Teff) cells were present in WTD-fed mice than in Chow-fed mice (Fig. 2B), which suggests the induction of the HMW4-specific cellular immunity that, like the Ab response, coincides with atherogenesis. The number of the CD4⁺Foxp3⁺ regulatory T cells (Treg) reactive to HMW4 was relatively small and was not changed significantly (Fig. 2B).

While T and B cells are classically considered to recognize different parts of an Ag, we showed recently²⁹ that the HSP60-derived epitope HP1 can be recognized by both T and B cells (we termed such epitopes as “dual epitopes”). To determine whether HMW4 is also a “dual epitope,” we tested its ability to specifically block the binding of the anti-HMGB1 Ig and IgM to plate-bound HMGB1 in ELISA. HMW4, but not an irrelevant peptide, blocked the binding effectively (by ~80%) (Fig. 2C). Thus, HMW4 appears to contain one or more B cell epitopes that collectively form a dominant epitope of HMGB1. The finding that both the Ig and IgM were blocked to a similar extent also indicated that the majority of the HMW4-binding Ig was IgM, which is consistent with the observations depicted in Fig. 1A & 1B.

To assess whether IgG, the most abundant component of the plasma Ig, also contributes to the binding of HMW4, we immunotyped the anti-HMW4 IgG in plasma. Regardless of diets, all of the IgG subclasses were detected at sub µg/ml levels; whereas the level of IgM was 30- and 80-fold higher than that of the IgG in Chow- and WTD-fed mice, respectively (Fig. 2D). Among these isotypes, only IgG2c and IgM were increased in response to WTD

(Fig. 2D). Given that the production of Ag-specific IgG2c (in C57BL/6 mice) is induced by Th1 cells^{32,33}, the increased IgG2c response here is consistent with the increased Teff response shown in Fig. 2B.

Intriguingly, the overall Ab response is strongly IgM-biased, with the anti-HMW4 IgM constituting 1.5% and 2.8% of the total IgM in the Chow- and WTD-fed mice, respectively (Fig. 2E). In comparison, the anti-HMW4 IgG2c fraction in the total IgG2c is much smaller, at 0.025% and 0.054% in the Chow- and WTD-fed mice, respectively (Fig. 2E). Thus, HMW4 appears to preferentially induce IgM response.

Because the results above had pointed to WTD as an inducer of the anti-HMW4 Ab in mice, we wanted to determine whether such Ab is also present in humans, given that a significant portion of the population may have consumed high fat and high cholesterol diets. We purchased commercial human IgG and IgM that were isolated from pooled human sera and analyzed their anti-HMW4 activity by ELISA. Because the pooled human Abs were much more heterogeneous than the mouse Abs, we randomized the sequence of HMW4 and used the resulting peptide as a more stringent specificity control in the ELISA. As shown in Fig. 2F, approximately 1.2% of the total IgG and 4.3% of the total IgM were found to be anti-HMW4. In aggregate, these data suggest that HMW4 is a dominant epitope of HMGB1 and that the anti-HMW4 Ab response exists and shows a strongly IgM-bias in both mice and humans.

3.3 Detection of HMW4-reactive B cells

The anti-HMW4 Abs were produced by HMW4-reactive B cells. To assess the presence of the latter directly, we stained total blood B cells with a tetramer formed of biotinylated HMW4 and R-phycoerythrin (PE)-labeled streptavidin (SA) (Fig. 3A). We focused on blood B (B-1 and B-2) cells because they and splenic B cells show a similar degree of sensitivity to Ag stimulation, whereas peritoneal cavity B-1 cells are insensitive³⁴. Thus the change in blood tetramer⁺ B cells reflects the systemic B cell response to the Ag. The results showed that in WTD-fed *ApoE*^{-/-} mice, tetramer⁺ B cells were present at a remarkably high level (~0.5% of circulating B cells) (Fig. 3B). The specificity of the staining was shown by the effective blocking of the staining with either non-labeled HMW4-tetramer as a specific competitor or goat anti-mouse Ig (mIg) Ab as a B cell receptor (BCR) blocker (Fig. 3B). However, compared with the Chow-fed mice, the WTD-fed mice had only a slightly higher level of the tetramer⁺ B cells (Fig. 3C).

B cells upregulate the T cell costimulator CD80 upon their activation³⁵⁻³⁷. We therefore examined the CD80 expression in the tetramer⁺ B cells to identify the activated cells. In the Chow- and WTD-fed mice, about 5% and 10% of the tetramer⁺ B cells, respectively, were CD80⁺ (Fig. 3C). Thus, it was the increased activation (not quantity per se) of the tetramer-binding B cells that was driven by WTD. In both the Chow- and WTD-fed groups, the B-2:B-1 cell ratios were about 2:1 (Fig. 3C). Hence, upon WTD feeding, HMW4-reactive B-1 and B-2 cells both were activated proportionally. This finding is consistent with the WTD-induced, simultaneous increase in both IgG2c and IgM, observed in Fig. 2D. To summarize, these data show that HMW4-reactive B cells are present in *ApoE*^{-/-} mice and are regulated in response to WTD.

3.4 Role of the anti-HMW4 immunity in atherogenesis

Because the data had shown that WTD promotes both atherosclerosis and the anti-HMW4 immunity in *Apoe*^{-/-} mice, we asked whether the immunity actually plays a role in atherogenesis. We reasoned that if it does, manipulating the immune responses to HMW4 would change the disease outcome. Thus far, immunization has proven to be an effective way to manipulate the Ag-specific immunity. Therefore, we devised two immunization strategies that would steer the responses to HMW4 in opposite directions.

One strategy was immunizing *Apoe*^{-/-} mice with HMW4 in the presence of the immunogenic adjuvant CFA (complete Freund's adjuvant), i.e., "conventional immunization" ("CI"), which would intensify the anti-HMW4 responses. As shown in Fig. 4A (y-axis in the log scale), compared with mock-immunized control mice (NT), CI-treated mice indeed produced more anti-HMW4 IgG (IgG2b and IgG2c) and, to a lesser extent, IgM. Of note, the induction of the IgG2b was not seen previously in WTD-fed mice (Fig. 2D). This pattern of the Ab response is consistent with the increased counts of HMW4-reactive B cells derived proportionally from both the B-1 and the B-2 subsets (Fig. 4C). Moreover, CI-treated mice had increased counts of HMW4-reactive T cells derived proportionally from both the Treg and the Teff cell subsets (Fig. 4D). Of note, in this set of experiments, CFA alone did not induce anti-HMW4 immunity; HMW4 alone induced significantly less anti-HMW4 IgG2c and B-2 cells than the CI group.

The other strategy was immunizing the mice with HMW4 in the presence of the immunosuppressant dexamethasone (Dex) serving as a tolerogenic adjuvant, a strategy we developed and termed as "suppressed immunization" ("SI")^{29, 34}, which would diminish the anti-HMW4 responses in an Ag-specific way^{29, 34}. In contrast to the CI-treated mice, SI-treated mice showed an increase in anti-HMW4 IgM, but not in anti-HMW4 IgG (Fig. 4A). The IgM response was specific to the immunizing Ag as the same mice showed no increase in IgM against HP1 (Fig. 4B), an HSP60-derived dual epitope²⁹, which was not used in the immunization. Moreover, these mice showed preferential increases in HMW4-reactive B-1 (over B-2) cells (Fig. 4C) and HMW4-reactive Treg (over Teff) cells (Fig. 4D). As a result, while the levels of the HMW4-reactive B-1 and Treg cells are similar in the CI- and SI-treated mice, the ratios of the B-1:B-2 cells and Treg:Teff cells are increased in the SI treated mice because of the lower levels of the B-2 and Teff cells. Interestingly, both the B-1 and Treg cells are considered to play a protective role in the models of atherosclerosis³⁸⁻⁴¹.

Having succeeded in manipulating HMW4-specific responses via the immunization strategies, we next determined whether we also changed the disease outcome. To that end, we treated additional groups of mice with CI or SI as described above and then challenged them with the atherogenic WTD for 10 weeks. Compared to the control mice (injected with PBS), neither CI- nor SI-treated mice showed statistically significant changes in plasma lipids (Fig. 5A). However, CI-treated mice showed increase (~30%) in lesions at the aortic arch, as quantified by both *en face* analysis²² and Oil Red O (ORO) solubilization²³; whereas SI-treated mice showed decrease (~40%) in lesions (Fig. 5B). Similar, but slightly less pronounced, differences were also seen in lesions at the aortic root, as assessed by a morphometric method (Fig. 5C)²⁴.

In summary, the CI-induced proportional increases in the anti-HMW4 IgG and IgM, HMW4-reactive B-1 and B-2 cells, and HMW4-reactive Treg and Teff cells are associated with increased atherogenesis. On the other hand, the SI-induced preferential increases in the anti-HMW4 IgM, HMW4-reactive B-1 cells, and HMW-reactive Treg cells are associated with decreased atherogenesis. Hence, manipulating the immune responses to HMW4 can, indeed, change the disease outcome.

4. Discussion

HMGB1 is largely known as a DAMP and ligand for RAGE and TLRs 2/4⁹. In the present study, we show that autoimmunity to HMW4, a dominant epitope from HMGB1, is induced by WTD in *Apoe*^{-/-} mice and can also be detected in pooled human sera (Fig. 2F). Moreover, we show that immunizations with HMW4, which change the ratios of the HMW4-reactive T cell (Teff vs. Treg) subsets and B cell (B-1 vs. B-2) subsets, can modify the disease outcome in *Apoe*^{-/-} mice. These point to the possibility that HMGB1 is a disease-relevant Ag in atherosclerosis.

The induction of the anti-HMGB1 immunity depends on both the *Apoe*^{-/-} background and WTD, a combination known to promote atherogenesis. As the resulting lesions can be a source of extracellular HMGB1¹¹ that serves as the Ag, one may speculate that WTD drives the anti-HMGB1 immunity more effectively in *Apoe*^{-/-} mice by promoting lesions. In this scenario, the high fat (21%) and high cholesterol (0.3%) in the WTD are the likely dietary components driving the immunity, which also explains why the control Chow diet (4.4% fat and 0.04% cholesterol) is less immunogenic. In support of this scenario, there is increased release of extracellular HMGB1 in the *Apoe*^{-/-} mice in response to WTD. On the other hand, our immunization experiments show that increased Teff and B-2 cell responses to HMGB1 are associated with increased atherosclerosis, whereas increased Treg and B-1 cell responses to HMGB1 are associated with decreased atherosclerosis. Therefore, the autoimmune responses from these lymphocyte subsets, once initiated, may possibly form feedback loops with the lesions and, thus, regulate the disease progression from that point on. However, while the relevant literature has implicated several specific auto-Ags as atherogenic¹⁻³, it remains possible that autoimmunity may play a causative role in atherogenesis irrespective of what the Ag is.

HMGB1 has been shown to act as an atherogenic inflammatory mediator in the same *Apoe*^{-/-} model¹⁴. Our present study is suggestive of its role as a disease-relevant Ag. Such potential dual function of HMGB1 seems to mirror that of HSP60, which is the prototypical Ag in atherosclerosis (i.e., athero-Ag)⁴². In fact, HMGB1 and HSP60 appear to share a few similarities in the context of atherosclerosis: 1) Both of the proteins are intracellular DAMPs capable of inducing innate inflammation⁴³; 2) Both are upregulated in vascular endothelial cells and activated macrophages under hyperlipidemia and hypercholesterolemia conditions; and 3) Both can stimulate atherogenic immune responses when used as the Ag targets in immunogenic immunizations. Hence, it is conceivable that HMGB1 may impact atherosclerosis both as an athero-Ag and as a proinflammatory mediator, and that the two mechanisms are not mutually exclusive and can be at work simultaneously during atherogenesis.

One may speculate that the development of anti-HMGB1 autoimmunity, the Ab response in particular, represents a protective mechanism that prevents HMGB1 from acting as a mediator. However, the results of our immunization experiments show that the conventional immunization (CI) targeting the HMW4 epitope increases atherosclerosis, whereas tolerogenic immunization (SI) reduces atherosclerosis. These results are rather in line with an overall atherogenic role of the autoimmunity. Similar observations were made previously with HSP60 as well ⁴². On the other hand, the majority of the anti-HMGB1 and anti-HMW4 Abs are IgM, the isotype typically involved in atheroprotection according to the relevant literature ⁴⁴. Thus, the anti-HMW4 IgM Ab might have a protective effect, but such effect may be offset by those atherogenic components of the autoimmunity such as Teff cells and IgG Ab. This might explain partly why SI, which raises the IgM preferentially over IgG, leads to decrease in atherosclerosis. Thus, to explore HMGB1 (or other athero-Ags) for therapeutic purposes, it is necessary to target the protective components of the autoimmunity selectively.

To move toward this direction, we have shown here that while increases in overall anti-HMW4 Abs and HMW4-reactive lymphocytes are associated with atherogenesis, SI-induced preferential increases in anti-HMW4 IgM and HMW4-reactive B-1 and Treg cell subsets are associated with atheroprotection. Additional studies are required to delineate the role that each of these components plays and to understand how the balances among them govern the final outcome of the autoimmunity, i.e., atherogenesis or atheroprotection.

As an epitope, HMW4 is intriguing because it is recognized by a relatively large population of B cells. In WTD-fed *Apoe*^{-/-} mice, up to 0.5% of the circulating B cells bind to the HMW4-tetramer. Moreover, the Ab response to this epitope displays an unusual IgM bias, with the HMW4-reactive IgM constituting ~3% and ~4% of the total IgM in the WTD-fed *Apoe*^{-/-} mice and the human donors, respectively. Even in the presence of highly immunogenic adjuvant CFA, the IgM bias could not be completely overcome; thus, it is likely that the bias represents an intrinsic propensity of the HMW4 epitope, not failure in class switch per se. In atherosclerosis, only oxidized lipids, such as the oxidized phosphatidylcholine in oxLDL, have been shown to display a similar IgM-bias ⁴⁵. In that case, the IgMs are produced by B-1 cells as “natural Abs,” considered to be atheroprotective in general. It remains to be determined whether the anti-HMW4 IgM is produced by B-1 cells and whether the IgM has the same protective efficacy.

To summarize, our study reveals the induction of the anti-HMGB1 autoimmunity in atherosclerosis and suggests that the HMW4 epitope and the HMW4-reactive lymphocytes, the B-1 and Treg cell subsets particularly, may be targeted for anti-atherosclerosis therapy.

In conclusion, this study shows that the WTD that accelerates atherogenesis also induces anti-HMGB1 autoimmunity in *Apoe*^{-/-} mice. Moreover, immunizations with HMW4, a dominant epitope of HMGB1, can modify the disease outcome. This study thus suggests, for the first time, that HMGB1 may potentially affect atherosclerosis by serving as a disease-relevant Ag.

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Fig. 1. Detection of anti-HMGB1 Abs

(A) C57BL/6 *Apoe*^{-/-} mice at age of 8 weeks were fed a western-type diet (WTD) for 8 weeks. Plasma from the mice was then analyzed for anti-HMGB1 and anti-HSP60 Abs by ELISA. Data are shown as mean $\pm$ SD of 8 mice. (B) The plasma samples in A were pooled and analyzed for anti-HMGB1 Ig by ELISA, using the anti-mouse Ig detection Ab that was non-blocked (not shown) or pre-blocked with either mouse IgM (mIgM) or mock-blocked with an irrelevant Ig (i.e. hIgG). Data are expressed as reduction (%) of the ELISA signal (100% - blocked/non-blocked $\times$ 100%). Shown is the mean $\pm$ SD from 4 experiments. **p* = 0.002. (C) The pooled plasma in B was pre-blocked with an indicated protein (1.8 μ g/ μ l plasma) or DNA (average size = 89 bp; 6 μ g/ μ l), or treated with DNase I (0.003 kU/ μ l); and then analyzed for anti-HMGB1 Ig by ELISA. Data are expressed as reduction of the ELISA signal, as defined in B. Shown is the mean $\pm$ SD from 4 experiments. **p* = 0.003 vs. all other groups. (D) *Apoe*^{-/-} and *Apoe*^{+/-} C57BL/6 mice at age of 8 weeks were fed WTD or a normal chow diet (Chow) for 8 weeks. Plasma was then analyzed for anti-HMGB1 Ig by

ELISA. Data are shown as mean $\pm$ SD of 9 – 10 mice per group. $*p = 0.001$ vs. any of the other groups. (E) Plasma samples from *Apoe*^{-/-} mice fed WTD or Chow for 8 weeks were pooled within each group (8 mice/group) and analyzed for the concentration of HMGB1 by ELISA. Data are shown as mean $\pm$ SD of 2 experiments. $*p = 0.04$. (F) The pooled plasma from the WTD-fed *Apoe*^{-/-} mice in E was passed through an MBP agarose column. The amount of HMGB1 in the flow through (Ft) and the bound fractions was determined respectively by ELISA and expressed as a percentage of the total HMGB1 in the starting material. Data are shown as mean $\pm$ SD of 2 experiments. $*p = 0.004$. (G) The pooled plasma from WTD- or Chow-fed *Apoe*^{-/-} mice in E was analyzed for the presence of an HMGB1-IgM complex (IC) by ELISA. Data are shown as mean $\pm$ SD of 2 experiments. $*p = 0.009$.

Fig. 2. HMW4 is a dominant epitope of HMGB1

(A) C57BL/6 mice were immunized with the HMGB1 whole protein and then restimulated with the HMW4 or the MOG₃₅₋₅₅ (as a specificity control) peptide. HMW4-reactive T cells were detected in the spleen by BrdU incorporation and quantified as % of the CD3⁺CD4⁺CD44⁺ memory T cells examined, as described in Materials and methods. Data are shown as mean $\pm$ SD of 4 mice per group from 2 experiments. * p 0.03 vs. all other groups. (B) Non-immunized *Apoe*^{-/-} mice at age of 8 weeks were fed WTD or Chow for 8 weeks and then stimulated with HMW4 or MOG₃₅₋₅₅. HMW4-reactive T cells were detected in pooled (2 mice per pool) splenic CD4⁺ T cells, as described in A. Data are shown as mean $\pm$ SD from 3 – 4 experiments. * p 0.05 vs. the Teff in the other groups. (C) Plasma pooled from 10 WTD-fed *Apoe*^{-/-} mice was analyzed for anti-HMGB1 Ig and IgM by ELISA, performed in the presence of HMW4 or HSS1 (an HSP60-derived peptide) as specific or non-specific blocker, respectively. Data are expressed as reduction (%) of the ELISA signal, as defined in Fig. 1B. Shown is the mean $\pm$ SD from 2 – 4 experiments. * p 0.05 and ** p 0.004, HMW4 vs. HSS1. (D) *Apoe*^{-/-} mice at age of 8 weeks were fed WTD or Chow for 8 weeks, and each of the anti-HMW4 IgG isotypes and IgM in the plasma were then quantified by ELISA. Data are shown in the log scale as mean $\pm$ SD of 10 mice per group. * p = 0.0004; ** p = 0.009. (E) Total and HMW4-binding IgG2c and IgM in the plasma samples in D were quantified by ELISA. The anti-HMW4 fraction was calculated as HMW4-binding/total $\times$ 100%. Data are shown (in the log scale) as mean $\pm$ SD of n = 10 per group. * p 0.008. (F) Commercial hIgG and hIgM, prepared from pooled human sera, were analyzed for the anti-HMW4 fraction by ELISA. Shown is the mean $\pm$ SD from 4 – 8 experiments with 2 lots of hIgG and hIgM. * p = 0.0001; ** p = 0.005 and *** p = 0.0002 in IgG and IgM, respectively, between HMW4 and its randomized control (rHMW4).

Fig. 3. Detection of HMW4-reactive B cells

(A) Gating strategy for detecting tetramer⁺ (R1*R2*R3) and tetramer⁺CD80⁺ (R1*R2*R3*R4) B cells using a FACScalibur. Blood leukocytes were stained with anti-CD19-PE/Cy7 (FL3), anti-CD23-APC (FL4), anti-CD80-FITC (FL1), SA-PE/Cy5 (FL3), and HMW4-SA-PE (FL2). Of note, any B cells that bind PE directly are gated out as FL3-high cells by R2. (B) Blood leukocytes pooled from 5 WTD-fed *Apoe*^{-/-} mice were stained with anti-CD19-PE/Cy7, SA-PE/Cy5, and the indicated other agents. The resulting PE-labeled B cells (R1*R2*R3) were counted by flow cytometry as % of the total CD19⁺ B cells examined (R1*R2). Data are shown as mean ± SD from 2 – 6 experiments. **p* < 0.004. SA, streptavidin; PE, R-phycoerythrin; αmIg, goat anti-mouse Ig Ab; αhIg, goat anti-human Ig Ab. (C) *Apoe*^{-/-} mice at age of 8 weeks were fed WTD or Chow for 8 weeks, and the

HMW4-reactive (tetramer⁺) B cells in the blood were then quantified by flow cytometry as % of the total CD19⁺ B cells (left); then, the CD80⁺ fraction was further quantified as % of the tetramer⁺ B cells (middle). Within the tetramer⁺CD80⁺ fraction, the B-2:B-1 cell ratio was determined (right). Data are shown as mean $\pm$ SD of 11 – 21 mice per group. * $p = 0.02$; ** $p = 0.005$.

Fig. 4. HWM4-targeted immunizations change the immune responses to HWM4

Apoe^{-/-} mice at age of 8 weeks were treated with the HWM4-targeted conventional immunization (CI) or suppressed immunization (SI), mock-immunized with PBS (NT), or control-immunized with CFA or HMW4. (A) The treated mice were restimulated 6 weeks later with HMW4 (25 μg/injection, i.p.) on days 1 and 3. On day 5, blood was collected (from the tail) and separated into plasma and cells. The plasma was pooled (from 9 mice per group) and analyzed for each of the anti-HMW4 IgG isotypes and IgM by isotype-specific ELISA. Only IgG2b, IgG2c, and IgM showed intergroup difference. Data are shown in the log scale as mean ± SD from 2–4 experiments. **p* < 0.01 and ***p* < 0.04 vs. the indicated isotypes in NT and HMW4, respectively. (B) The same plasma samples in A were analyzed for HP1-specific IgM. Data are shown as mean ± SD from 2 experiments. (C) Blood leukocytes were obtained as described in A, pooled (3 mice per pool), and analyzed for tetramer⁺CD80⁺ B cells by flow cytometry. The double-positive cells were quantified as % of the CD19⁺ B cells examined. Data are shown as mean ± SD of *n* = 3–6 from 2 experiments. **p* < 0.05 and ***p* = 0.004 vs. the B-1 and B-2 of NT, respectively. (D) The treated mice were restimulated as in A, and HWM4-reactive T cells were detected in pooled (3 mice per pool) splenic CD4⁺ T cells by BrdU incorporation. Data are shown as mean ± SD from 2–4 experiments. **p* < 0.007 and ***p* = 0.05 vs. the Treg and Teff, respectively, of NT.

Fig. 5. HWM4-targeted immunizations change the severity of atherosclerosis. *Apoe*^{-/-} mice at age of 8 weeks were treated with CI, SI, or PBS injection (NT)

At age of 12 weeks, they were challenged with the atherogenic WTD for 10 weeks. Data are shown as mean $\pm$ SD of 8 – 12 mice per group. (A) Plasma lipoproteins were measured by enzymatic colorimetric assays. (B) Atherosclerotic lesions were quantified by *en face* analysis of the aortic arch stained with ORO; lesion coverage is defined as the percentage of the total surface area stained red with ORO. In addition, the incorporated stain in the aortic arch above was extracted and, then, quantified by spectrophotometry; the result is expressed as the quantity (μ g) of solubilized ORO per mg of the aortic arch tissue. * p 0.03 and ** p 0.01 vs. NT in the lesion coverage (*en face* analysis) and ORO quantity (ORO solubilization analysis), respectively. (C) Sections of the aortic root were stained with ORO. Lesion coverage is expressed as a percentage of the total lumen area. * p 0.04 vs. NT.