Advanced glycation end products (AGEs) impair NLRP3 inflammasome–mediated innate immune responses in macrophages

Seunghwan Son1, Inhwa Hwang1, Seung Hyeok Han1, Jeon-Soo Shin1, Ok Sarah Shin1, and Je-Wook Yu†‡§¶

From the †Department of Microbiology and Immunology, Institute for Immunology and Immunological Diseases, Brain Korea 21 PLUS Project for Medical Science, the ‡Department of Internal Medicine, Institute of Kidney Disease Research, Yonsei University College of Medicine, Seoul 03722, Korea and the §Department of Biomedical Sciences, College of Medicine, Korea University Guro Hospital, Seoul 08308, Korea

This work was supported by National Research Foundation of Korea Grants 2014R1A4A1008625, 2015M3A9B6073856, and 2017R1A2B2007467 from the Korean Government and Yonsei University College of Medicine Faculty Research Grant 6-2016-0090. The authors declare that they have no conflicts of interest with the contents of this article.

Advanced glycation end products (AGEs) impair NLRP3 inflammasome–mediated innate immune responses in macrophages. This is an open access article under the CC BY license.

J. Biol. Chem. (2017) 292(50) 20437–20448

Advanced glycation end products (AGEs) are adducts formed on proteins by glycation with reducing sugars, such as glucose, and tend to form and accumulate under hyperglycemic conditions. AGE accumulation alters protein function and has been implicated in the pathogenesis of many degenerative diseases such as diabetic complications. AGEs have also been shown to promote the production of pro-inflammatory cytokines, but the roles of AGEs in inflammasome signaling have not been explored in detail. Here, we present evidence that AGEs attenuate activation of the NLRP3 inflammasome in bone marrow–derived macrophages (BMDMs) as determined by caspase-1 processing and interleukin-1β production. AGEs also dampened the assembly of the NLRP3 inflammasome, but did not affect the NLRC4 or AIM2 inflammasome activation. Moreover, our data indicated that AGE treatment inhibited Toll-like receptor (TLR)-dependent production of pro-inflammatory cytokines in BMDMs. This immunosuppressive effect of AGE was not associated with a receptor for AGEs (RAGE)-mediated signaling. Instead, AGE treatment markedly suppressed lipopolysaccharide-induced M1 polarization of macrophages. Furthermore, AGEs significantly dampened innate immune responses including NLRP3 inflammasome activation and type-1 interferon production in macrophages upon influenza virus infection. These observations collectively suggest that AGEs could impair host NLRP3 inflammasome–mediated innate immune defenses against RNA virus infection leading to an increased susceptibility to infection.

Advanced glycation end products (AGEs) belong to a group of heterogeneous compounds formed by non-enzymatic glycation of proteins (1). AGEs accumulate under hyperglycemic conditions and in the regions associated with diabetic complications (2). Elevated levels of AGEs were frequently found in the samples of patients with type-2 diabetes, aging-related diseases, or obesity (3–5). Accumulated AGEs can cause host tissue damages mainly by direct binding to cell surface receptors such as the receptor for AGEs (RAGE) (2). In this regard, AGEs are considered crucial risk factors for accelerated disease progress or aggravation of many age-related degenerative disorders, including diabetic complications (6, 7). However, the molecular details of AGEs-implicated disease pathogenesis remain poorly understood, although RAGE-mediated signaling is considered pivotal for such pathogenesis. Intriguingly, AGEs were shown to increase the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) in myeloid or non-myeloid cells via binding to RAGE (8–10). The potential impact of AGEs on inflammatory responses could account for AGEs-dependent pathogenesis.

The inflammasome complex plays a key role in the initiation of inflammatory responses by inducing caspase-1-dependent maturation and secretion of IL-1β or IL-18, mainly in myeloid cells (11). Microbial infection or tissue injury could trigger the assembly and activation of inflammasomes, comprising sensor molecules such as nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3), or absent in melanoma 2 (AIM2), ASC, and procaspase-1, leading to caspase-1 activation (11, 12). Inflammasome signaling primarily provides a host innate immune defense against a wide range of microbial infections, including influenza virus (13–15). In addition, NLRP3 inflammasome can also be activated by diverse endogenous risk factors such as palmitate, amyloid β, and cholesterol crystal (16–18). Furthermore, Nlrp3-deficient mice showed a remarkable attenuation of disease symptoms associated with type-2 diabetes, Alzheimer’s disease, and atherosclerosis, indicating that deregulated activation of NLRP3 inflammasome is integral to the pathogenesis of these metabolic or degenerative disorders (18–21). Given that AGEs are closely associated with degenerative disorders, such as type-2 diabetes, it is of interest to investigate the potent role of AGEs in inflammasome-mediated immune responses. Thus far, it has not been explored whether AGEs could modulate the activation of inflammasome signaling in myeloid cells. In this study, we...
examined the potential role of AGEs on inflammasome assembly or activation in macrophages.

Results

**AGEs do not promote the production of pro-inflammatory cytokines**

To examine the effect of AGEs on inflammasome signaling, we first prepared AGEs by incubating bovine serum albumin (BSA) with a high concentration of glucose for 8 weeks, as described previously (22). We subsequently validated the production of AGEs by assessing its unique fluorescence spectrum (excitation 370 nm, emission 440 nm; Fig. 1B). Then, we treated bone marrow-derived macrophages (BMDMs) with AGEs for 6 or 18 h and assessed the secreted levels of pro-inflammatory cytokines from BMDMs (Fig. 1C). Additionally, pretreatment with two commercial AGEs, GA-BSA or glucose-BSA (Glu-BSA) (200 µg/ml) for 18 h, or LPS (0.5 µg/ml) for 3 h (n = 3). D–H, cell extracts were assayed for mRNA levels of IL-1β or IL-6 by quantitative real-time PCR.

**AGEs do not trigger the production of pro-inflammatory cytokines.** A, the fluorescence emission spectra of PBS (green), 8-week incubated AGE-BSA (blue), and 8-week incubated AGE-BSA (red) at 370 nm excitation. B and C, mouse BMDMs were treated with AGEs (10 mg/ml) for the indicated times in the presence of LPS (0.25 µg/ml, final 3 h), or treated with LPS alone (0.25 µg/ml, 3 h), followed by the treatment with ATP (2.5 mM, 30 min). Culture supernatants were assayed for extracellular levels of IL-1β (B) or IL-6 (C) by ELISAs. Asterisk indicates significant difference from LPS alone-treated group (n = 3; B: p < 0.05). D and E, mouse BMDMs were treated with AGEs (0.1–10 mg/ml) for 24 h, or treated with LPS (0.5 µg/ml) for 3 h (n = 4, D; n = 4, E). F and G, THP-1 cells were treated with AGEs (0.1–10 mg/ml) for 24 h, or treated with LPS (0.5 µg/ml) for 6 h (n = 4, F; n = 4, G). H, mouse BMDMs were treated with two commercial AGEs, GA-BSA or glucose-BSA (Glu-BSA) (200 µg/ml) for 18 h, or LPS (0.5 µg/ml) for 3 h (n = 3). D–H, cell extracts were assayed for mRNA levels of IL-1β or IL-6 by quantitative real-time PCR.

**AGEs suppress Toll-like receptor (TLR)-mediated production of proinflammatory cytokines**

Of particular interest, prolonged treatment of AGEs repressed LPS-promoted production of IL-6 from BMDMs (Fig. 1C). Consistent with this data, pretreatment with AGEs (10 mg/ml) for 15 h prior to LPS stimulation significantly attenuated LPS-triggered up-regulation of IL-1β and IL-6 mRNA levels in BMDMs (Fig. 2, A and B). Additionally, pretreatment with two commercial AGEs, GA-BSA and Glu-BSA, also led to a significant suppression of IL-6 mRNA induction in LPS-stimulated BMDMs (Fig. 2C). However, a short incubation with AGEs (up to 3 h) did not cause this inhibitory effect (supplementary Fig. 1, A and B).

To further examine whether AGEs could dampen the production of pro-inflammatory cytokines in macrophages triggered by other TLR agonists than LPS, we stimulated BMDMs with Pam3CSK4, a TLR1/2 agonist, or a poly(I:C), a TLR3 agonist. In agreement with LPS stimulation, AGE pretreatment significantly attenuated Pam3CSK4-stimulated induction of IL-1β and IL-6 mRNA in BMDMs (Fig. 2, D and E). Consistently, poly(I:C)-triggered production of IL-6 mRNA was also diminished by AGE, but not by BSA, pretreatment (Fig. 2F). These findings clearly demonstrated that AGEs could impair TLR-mediated cytokine production in macrophages.

**AGEs inhibit the activation of NLRP3 inflammasome**

We next examined whether AGEs affect caspase-1 and inflammasome signaling. Similar to the findings shown in Fig. 1, AGE treatment failed to induce caspase-1 inflammasome acti-
vation in BMDMs even with costimulation with ATP, as determined by the presence of extracellular cleaved caspase-1 (p20) and IL-1β (Fig. 3A). LPS/ATP treatment was used to stimulate NLRP3 inflammasomes as a positive control. Of more interest, pretreatment with AGES, but not with BSA, showed a robust reduction in the secretion of active caspase-1 and IL-1β from BMDMs in response to LPS/ATP stimulation (Fig. 3B). AGE pretreatment consistently exhibited a dose-dependent attenuation of caspase-1 activation and IL-1β secretion in BMDMs upon stimulation with LPS/ATP (Fig. 3C and supplemental Fig. S2). We also prepared extensively modified AGES by longer incubation of BSA with glucose for 24 weeks. The extensive formation of AGE adducts (24-week incubation) was revealed by a stronger fluorescence intensity of AGES than observed with normal AGES (8-week incubation, Fig. 3D). The 24-week AGE samples, but not 24-week BSA samples, blocked NLRP3 inflammasome activation at a much lower AGE concentration (1 mg/ml, Fig. 3, E and F). To support our observations, commercially available AGES also showed a clear inhibition of LPS/ATP-promoted caspase-1 activation and IL-1β secretion in BMDMs (Fig. 3G).

Additionally, we examined whether glucose in the reaction mixture of the AGE preparation could help suppress NLRP3 inflammasome activation. We performed similar experiments with dialyzed AGE to remove added glucose in the mixture. Dialyzed AGES also showed robust inhibition of NLRP3 inflammasome activation in BMDMs (Fig. 3H). Furthermore, the reaction mixture containing glucose alone (without BSA) did not suppress caspase-1 activation after LPS/ATP stimulation (Fig. 3H). These findings indicate that AGE pretreatment drives lower responsiveness to NLRP3-activated stimulation in macrophages.

Figure 2. Prolonged AGE treatment inhibits TLR-mediated production of pro-inflammatory cytokines. A and B, mouse BMDMs were treated with AGES (0.1–10 mg/ml) for 18 h in the presence of LPS (0.5 μg/ml, final 3 h) (n = 3 or 4, A; n = 5 or 6, B). C, mouse BMDMs were treated with commercial AGES (200 μg/ml) or AGES (5 mg/ml) for 18 h in the presence of LPS (0.5 μg/ml, final 3 h) (n = 4). D and E, mouse BMDMs were treated with AGES (10 mg/ml) for 18 h in the presence of Pam3CSK4 (1 μg/ml, final 3 h) (n = 3). F, mouse BMDMs were treated with AGES (10 mg/ml) for 18 h in the presence of poly(I:C) (10 μg/ml, final 3 h) (n = 3). A–F, cell extracts were assayed for mRNA levels of IL-1β or IL-6 by quantitative real-time PCR. Asterisks indicate significant differences from LPS, Pam3CSK4, or poly(I:C) alone-treated groups (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s, not significant).

AGEs suppress the assembly of NLRP3 inflammasome

We then examined the role of AGES on inflammasome signaling in response to stimulations other than LPS/ATP. NLRP3-mediated inflammasome activation triggered by ATP, nigericin, or alum was clearly attenuated by pretreatment with AGES in LPS-primed macrophages (Fig. 4A). However, poly(dA:dT) transfection-triggered inflammasome activation, mediated by AIM2, was not impaired by AGE pretreatment (Fig. 4B). NLRC4 inflammasome activation in response to Pseudomonas aeruginosa infection was not reduced by pretreatment with AGES (Fig. 4C). Moreover, AGE pretreatment clearly abolished LPS/ATP-triggered oligomerization of ASC, an essential phenomenon of NLRP3 inflammasome signaling (23) (Fig. 4D). In accordance with these data, the formation of speck-like NLRP3 aggregates by nigericin was significantly reduced by AGE pretreatment (Fig. 4, E and F). These results collectively indicate that AGE pretreatment specifically impairs the NLRP3 inflammasome-activating potential of macrophages.

AGEs attenuate TLR4-NF-κB signaling but not mitogen-activated protein kinase signaling

To investigate the mechanism by which AGE pretreatment could modulate NLRP3 inflammasome signaling, we first measured the expression of NLRP3 mRNA. As shown in Fig. 2, AGE pretreatment diminished LPS-triggered IL-1β or IL-6 mRNA production in BMDMs. Similarly, LPS-promoted up-regulation of NLRP3 was significantly repressed by AGE treatment (Fig. 5A). Then, we checked whether AGE pretreatment could interfere with the Toll-like receptor 4 (TLR4)–NF-κB signaling axis. Consistent with the reduced production of cytokines and NLRP3, AGE pretreatment considerably inhibited LPS-stimu-
Suppression of innate immune responses by AGEs

Figure 3. AGE pretreatment attenuates inflammasome activation in macrophages in response to LPS/ATP stimulation. A, mouse BMDMs were treated with 8-week incubated BSA or AGEs at a final concentration of 10 mg/ml for 18 h, or treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min) as indicated. B and C, mouse BMDMs were pretreated with BSA (10 mg/ml) or AGEs (10 mg/ml (A) or 0.1–10 mg/ml (C)) for 18 h. Cells were washed with PBS and fresh medium was added. Cells were then treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min) (n = 3). D, the fluorescence emission spectra of 8-week incubated BSA (blue), 8-week incubated AGE (red), 24-week incubated BSA (cyan), and 24-week incubated AGE (purple) at 370 nm excitation. E and F, mouse BMDMs were pretreated with 24-week incubated AGEs (E) or BSA (F) (0.2, 1, and 2.5 mg/ml) for 18 h, washed and treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min). G, mouse BMDMs were pretreated with commercial AGEs (200 μg/ml) or AGEs (5 mg/ml) for 18 h, washed, and treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min). H, mouse BMDMs were pretreated with AGEs, diazoyed AGEs, BSA (10 mg/ml), or 8-week incubated glucose (40 mg/ml) for 18 h, washed, and incubated with fresh medium containing LPS (0.25 μg/ml) for 3 h, followed by treatment with ATP (2.5 mM, 30 min). A, B, and E–F, culture supernatants (Sup) and cellular lysates (Lys) were immunoblotted with the indicated antibodies. C, culture supernatants were assayed for extracellular IL-1β secretion by ELISAs. Asterisk indicates significant differences from the LPS/ATP-treated group (*, p < 0.05).

lateral phosphorylation and degradation of IκB in BMDMs, indicating that AGEs negatively regulated NF-κB signaling under our conditions (Fig. 5B). We further verified this finding in HEK293 cells stably expressing TLR4, in which LPS stimulation caused a robust activation of NF-κB as determined by performing an NF-κB–luciferase reporter assay. However, pretreatment with AGEs did not inhibit NF-κB activation in TLR4–HEK293 cells in response to LPS stimulation (Fig. 5C). Based on these findings, we inferred that AGE treatment may impair TLR4–NF-κB signaling pathways in macrophages, but not in non-myeloid 293 cells.

Given that extracellular signal-regulated kinase (ERK) was shown to contribute to the activation of NLRP3 inflammasomes in a recent study (24), we examined the potential impact of AGE pretreatment on ERK signaling under NLRP3-activating conditions. LPS/ATP stimulation clearly induced ERK phosphorylation, but AGE pretreatment did not affect ERK phosphorylation (Fig. 5D). Similarly, AGEs did not impair LPS/ATP-triggered phosphorylation of c-Jun N-terminal kinase (JNK) (Fig. 5E). These findings suggest that the anti-inflammatory role of AGEs does not depend on the activation of mitogen-activated protein kinases signaling.

In addition to ERK, many recent findings have suggested that mitochondrial ROS (mtROS) production plays a crucial role in NLRP3 inflammasome activation (25–27). In contrast, data from several studies demonstrated that mtROS production is also a consequence of NLRP3 inflammasome activation (28, 29). In our experiments, pretreatment with AGEs significantly reduced mtROS production in wild-type mouse BMDMs stimulated with LPS/ATP, but not in Nlrp3-deficient BMDMs (Fig. 5F and supplemental Fig. S3). Interestingly, LPS/ATP-triggered production of mtROS was partially attenuated in Nlrp3-deficient BMDMs compared with that in wild-type cells (Fig. 5F). This result suggests that NLRP3 inflammasome activation, at least in part, contributes to mtROS production. AGE pretreatment caused no reduction in mtROS production in Nlrp3-deficient cells. We thus conclude that AGE-mediated reduction in mtROS might be a result of attenuated inflammasome activation. In this context, AGE-mediated impairment of NLRP3 inflammasome activation is not likely to be due to reduced mtROS production.

**AGES suppress M1 polarization of macrophages**

It is well-established that macrophage polarization is a fine-tuning process used by macrophages to adapt to changes in the microenvironment (30). As shown above, our data revealed that AGEs attenuated LPS-triggered production of pro-inflammatory cytokines. In agreement with this finding, we examined
whether AGEs could affect M1 polarization of macrophages. Of interest, AGE treatment exhibited a robust inhibition on LPS- and IFN-\(\gamma\)-induced expression of CXCL11, a distinct marker of M1 polarization (31) (Fig. 6A). Supporting this observation, LPS-driven elevation in the level of M1 markers, including inducible nitric-oxidase synthase and TNF-\(\alpha\), was significantly reduced by AGE pretreatment (Fig. 6, B and C).

Then, we further checked the effect of AGEs on M2 polarization of macrophages. Unlike M1 polarization markers, AGEs did not alter the expression level of M2 genes, such as Arg1 and Ym1, in BMDMs upon IL-4 stimulation (Fig. 6, D and E).

NLRP3 inflammasome activation generally requires two independent stimuli, for example, signal 1 for priming and signal 2 for activation (32). Considering that AGE treatment markedly attenuated LPS-driven priming events and M1 polarization of macrophages, we further examined whether AGEs could inhibit signal 2-induced activation of NLRP3 inflammasome. As reported by a previous study (33), only ATP treatment, a signal 2 stimulus, in the absence of LPS priming was sufficient to induce caspase-1 activation in NLRP3-reconstituted BMDMs (Fig. 6F). Intriguingly, AGEs showed a remarkable suppression of ATP-triggered activation of caspase-1 in NLRP3-reconstituted macrophages (Fig. 6F). These findings suggest that AGEs could interfere with not only the LPS-promoted priming step but also the signal 2-induced activation step for the activation of NLRP3 inflammasome.

AGEs suppress innate immune responses in a RAGE-independent manner

RAGE was first identified as a receptor for AGEs (34). We further examined the potential implication of RAGE in the innate immune-suppressing capability of AGEs. Under our experimental conditions, RAGE-specific antagonist FPS-ZM1 did not affect the AGE-mediated inhibition of LPS/ATP-triggered caspase-1 activation (Fig. 7A). Consistent with this finding, RAGE inhibitor failed to affect the AGE-driven suppress-
Suppression of innate immune responses by AGEs

**Figure 5. AGE pretreatment impairs TLR4–NF-κB signaling, but not mitogen-activated protein kinase pathways.** A, mouse BMDMs were treated with AGEs (0.1–10 mg/ml) for 18 h in the presence of LPS (0.5 μg/ml, final 3 h). Cell extracts were assayed for mRNA levels of NLRP3 by quantitative real-time PCR (n = 3 or 4). B, mouse BMDMs were treated with AGEs (10 mg/ml) for 18 h, washed, and treated with LPS (0.5 μg/ml) for the indicated times. Cell extracts were immunoblotted with the indicated antibodies. C, HEK293–TLR4 cells were transfected with a reporter construct encoding NF-κB-luciferase (0.2 μg). After a 24-h transfection, cells were treated with BSA or AGEs (10 mg/ml) for 12 h, followed by treatment with LPS (0.25 μg/ml, 6 h). Cell extracts were then assayed for luciferase expression (n = 5). D and E, mouse BMDMs were pretreated with BSA or AGEs (10 mg/ml) for 18 h, washed, and treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min). Culture supernatants (Sup) and cellular lysates (Lys) were immunoblotted with the indicated antibodies. F, Nlrp3+/− or Nlrp3−/− mouse BMDMs were pretreated with AGEs (10 mg/ml) for 18 h, washed, and treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2 mM, 30 min). Cells were then stained with MitoSOX and assayed for the MitoSOX-positive cells by flow cytometry. Asterisks indicate significant differences (n = 3, *, p < 0.05, **, p < 0.01, n.s., not significant).

Suppression of IL-6 mRNA induction in BMDMs upon stimulation with LPS (Fig. 7B). However, RAGE inhibitor showed a significant reduction of IL-6 mRNA expression triggered by S100 protein (Fig. 7C). In addition, AGE-mediated inhibition of LPS-promoted IkB phosphorylation was not prevented by RAGE inhibitor (Fig. 7D). As similar, reduction of the M1 marker CxCl11 mRNA level by AGE pretreatment was not reversed by the presence of the RAGE inhibitor (Fig. 7E). These findings collectively indicated that RAGE signaling is not associated with AGE-driven impairment of innate immune responses.

**AGEs impair host innate responses in macrophages in response to influenza virus infection**

As our data demonstrated that AGE pretreatment caused a severe reduction in the assembly and activation of NLRP3 inflammasomes, we further examined whether AGES could affect host inflammasome activation in macrophages upon influenza virus infection. Consistent with the above data, pretreatment with AGES, but not with BSA, clearly abolished both caspase-1 activation and IL-1β secretion in BMDMs upon infection with influenza A virus (Fig. 8A). Next, we measured interferon-β (IFN-β) production by macrophages in response to influenza virus infection. Of note, AGE pretreatment significantly suppressed IFN-β mRNA production and secretion from BMDMs upon influenza virus infection (Fig. 8, B and C). These findings suggest that AGES could impair the host innate immune defense in macrophages against dsRNA-mediated virus infection.

**Discussion**

Accumulating recent evidence has demonstrated that deregulated NLRP3 inflammasome activity is closely associated with the progression of multiple metabolic and degenerative disorders (19–21). Several disease risk factors such as palmitate or cholesterol crystals were previously shown to trigger the activation of NLRP3 inflammasomes (16, 18), indicating that NLRP3 inflammasome activation contributes to the pathogenesis of type-2 diabetes and atherosclerosis. Recent evidence also demonstrated that AGES up-regulated mRNA or protein expression of inflammasome-related molecules such as NLRP3, caspase-1, and IL-1β in non-myeloid cells such as podocytes, nucleus pulposus cells, or placental cells (35–37). However, the detailed molecular impact of AGES on caspase-1 activation or inflammasome assembly has not been tested previously in myeloid cells. In the present study, we found that AGES did not promote nor enhance the activation of NLRP3-dependent inflammasome pathways at least in BMDMs. Instead, AGE treatment unexpectedly suppressed NLRP3-dependent caspase-1 activation.

Contrary to previous findings, our data also demonstrated that AGES failed to induce the production of pro-inflammatory cytokines in BMDMs and THP-1 cells. Although some previous
studies reported that AGEs did not promote the production of cytokines (9, 38), many other findings demonstrated the elevated secretion of cytokines by AGEs (8, 10, 39). At present, we cannot fully explain this discrepancy. One possible explanation could be that different cell types were used, as in the case with inflammasome activity. Most previous studies of AGE-triggered production of pro-inflammatory cytokines were performed using non-myeloid cells and monocytes or peripheral blood mononuclear cells (8, 10, 39), whereas BMDMs were used in our experiments. Further detailed investigations will help clarify the potent roles of AGEs in the inflammatory response of macrophages. In this regard, our data present novel evidence that AGE treatment in macrophages could impair LPS-induced up-regulation of pro-inflammatory cytokine production, as well as NLRP3 inflammasome activation.

The molecular mechanism underlying the anti-inflammatory function of AGEs is not fully understood at present. Given that NLRP3 inflammasome activation requires both a priming signal mediating TLR-associated pathways and an activating signal (12), AGE-induced impairment of cytokine production is possibly in line with attenuated activation of the NLRP3 inflammasome. AGE treatment remarkably suppressed TLR-mediated priming events in macrophages upon LPS, Pam3CSK4, or poly(I:C) stimulations. Of interest, AGEs failed to block NLRC4 or AIM2 inflammasome signaling, which do not require the TLR-mediated priming step for the activation. In this regard, the NLRP3 inflammasome-suppressing effect of AGEs may be due to their inhibition of TLR-associated priming signals. However, AGEs also attenuated ATP-promoted caspase-1 activation in NLRP3-reconstituted macrophages. Therefore, further investigations will be required to elucidate the innate immune-suppressing mechanism of AGEs.

Based on our observations, AGEs inhibited TLR4–NF-kB signaling in macrophages, but not in 293 cells. This finding raises the possibility that the immunosuppressive effect of AGEs may depend on a potential receptor primarily expressed in macrophages. In addition to RAGE, several other AGE receptors such as AGE-receptor 1 (AGE-R1) are expressed in macrophages (2, 40). Of particular interest, AGE-R1 was previously reported to function as an anti-inflammatory molecule via the removal of AGEs (41). It will be thus challenging to determine whether AGE-R1 might be involved in the immunosuppressive effects of AGEs.

Furthermore, we found that AGEs clearly suppressed LPS- or IFN-γ-induced M1 polarization of macrophages. On the other hand, AGE treatment did not affect IL-4-driven M2 polarization. Although AGE alone did not trigger M1 nor M2 polarization of macrophages, AGE treatment definitely alters the polar-
Suppression of innate immune responses by AGEs

Figure 7. RAGE inhibitor fails to block AGE-mediated impairment of innate immune responses. A, mouse BMDMs were pretreated with AGEs (5 mg/ml) in the presence of a RAGE inhibitor (RI, 20 or 40 μM) for 18 h, washed, and treated with LPS (0.25 μg/ml, 3 h), followed by treatment with ATP (2.5 mM, 30 min). Culture supernatants (Sup) and cellular lysates (Lys) were immunoblotted with the indicated antibodies. B, mouse BMDMs were treated with AGEs (10 mg/ml) for 15 h in the presence of RAGE inhibitor (0.2–20 μM), and then further treated with LPS (0.5 μg/ml) for an additional 3 h (n = 4). C, mouse BMDMs were pretreated with RAGE inhibitor (5 or 20 μM) for 30 min, and then further treated with recombinant S100 protein (5 μg/ml) for 3 h (n = 3). D, mouse BMDMs were pretreated with AGEs (10 mg/ml) for 18 h in the presence of RAGE inhibitor (10 μM, 30 min pretreatment before AGEs), washed with PBS, and treated with LPS (0.25 μg/ml, 15 min). Cellular lysates were immunoblotted with the indicated antibodies. E, mouse BMDMs were pretreated with RAGE inhibitor (10 μM) for 30 min, and further treated with AGEs (10 mg/ml) for 18 h in the presence of LPS (0.25 μg/ml, final 6 h) (n = 3). B, C, and E, cell extracts were assayed for mRNA levels of the indicated genes by quantitative real-time PCR. Asterisks indicate significant differences from the S100 alone-treated group (*, p < 0.05; ***, p < 0.001).

Figure 8. AGE pretreatment impairs host innate immune defense of macrophages against RNA virus infection. A, mouse BMDMs were pretreated with BSA or AGEs (10 mg/ml) for 18 h, followed by influenza A virus infection (delNS1/PR8 strain) at an m.o.i. of 3. Culture supernatants (Sup) and cellular lysates (Lys) were immunoblotted with the indicated antibodies. B and C, mouse BMDMs were pretreated with BSA or AGEs (10 mg/ml) for 18 h, followed by influenza A virus infection (m.o.i. = 3). B, cell extracts were assayed for the production of IFN-β mRNA by quantitative real-time PCR (n = 3). C, culture supernatants were assayed for the secreted extracellular levels of IFN-β by ELISAs (n = 3). Asterisks indicate significant differences from the virus-infected group (*, p < 0.05; ***, p < 0.001, n.s., not significant).

Suppression of innate immune responses by AGEs under specific circumstances. Additionally, AGE-treated macrophages showed impaired production of type-1 interferon in response to influenza virus infection. Based on these data, we infer that a chronic exposure of macrophages to AGEs may induce a distinct phenotypic change in macrophages, leading to the decreased responsiveness of macrophages to extracellular pathogen-associated molecular patterns.

Considering that inflammasome signaling occurs mainly in myeloid cells such as macrophages, the accumulation of AGES could have a harmful effect on the activation of host innate defense mechanisms involving NLRP3 inflammasome signaling. In this context, AGES may impair the host immune defense against a wide range of microbial infections. It was previously shown that influenza virus infection causes activation of NLRP3 inflammasomes through viral RNA (15) or its M2 ion channel (42). Inflammasome activation provides a host innate defense against influenza virus infection. Our data demonstrated that AGE pretreatment significantly dampened innate immune responses such as inflammasome activation and...
IFN-β production in response to influenza virus infection, suggesting that AGEs could impair host innate defense mechanisms. Intriguingly, it is interesting to note that patients with obesity or diabetes and obese mice were more susceptible to infectious diseases including influenza virus infection (43–45), thus linking the association of AGE with infection susceptibilities. Our results provide a molecular explanation for this phenomenon. Collectively, our data suggest that AGE, a risk factor for multiple degenerative diseases, could impair NLRP3- and TLR4-mediated innate immune responses, leading to increased susceptibility to infectious diseases.

Experimental procedures

Reagents and antibodies

LPS, ATP, nigericin, poly(dA:dT), and poly(I:C) were purchased from Sigma. BSA was obtained from Affymetrix. Glucosone was obtained from Amresco. Commercially available AGEs were purchased from Merck Millipore (glycoaldehyde-BSA) and BioVision (glucose-BSA). Pam3CSK4 was purchased from InvivoGen. Alum was purchased from InvivoGen. Mouse recombinant IFN-γ and MitoSOX were obtained from Invitrogen. The RAGE antagonist FPS-ZM1 was purchased from Merck Millipore. Recombinant mouse S100A8/S100A9 proteins were obtained from R&D Systems. Mouse IL-1β, IL-6, and IFN-β enzyme-linked immunoassay (ELISA) kits were obtained from R&D Systems. Anti-ASC and anti-phospho-ERK antibodies were purchased from Santa Cruz. An anti-mouse IL-1β antibody was obtained from R&D Systems. Anti-mouse caspase-1 (p20) and anti-NLRP3 antibodies were from AdipoGen. Anti-phospho-JNK1/2 and anti-ERK antibodies were obtained from Cell Signaling. Anti-JNK antibody was purchased from BD Biosciences.

Cell culture

Mouse bone marrow cells were isolated from mouse femurs and differentiated into BMDMs as previously described (14). C57BL/6 mice were obtained from Orient, and bred at the Yonsei University College of Medicine. All mice were maintained under specific pathogen-free conditions, and 8–10-week-old male mice were used for the experiments. The protocols used for the animal experiments were approved by the Institutional Ethical Committee of Yonsei University College of Medicine. All experiments involving BMDM preparation were performed in accordance with approved guidelines from the Institutional Ethical Committee. Immortalized NLRP3–GFP-expressing BMDMs and NLRP3-reconstituted BMDMs (NG5 and N1–8) were provided by E. S. Alnemri (Thomas Jefferson University, Philadelphia, PA). All BMDMs were maintained in L929-conditioned DMEM supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 100 units/ml of penicillin/streptomycin. TLR4-expressing HEK293 cells were provided by I. H. Choi (Yonsei University). TLR4–HEK293 cells were cultured in DMEM supplemented with 10% FBS and 100 units/ml of penicillin/streptomycin.

Suppression of innate immune responses by AGES

Preparation of AGES

AGES were prepared by incubating BSA with glucose, as previously described (22). Briefly, BSA (250 mg/ml) was incubated with glucose (1 mM) in phosphate-buffered saline (PBS) under sterile conditions in the dark at 37 °C for 8 weeks. Alternatively, BSA (50 mg/ml) was incubated with glucose (0.5 mM) for 24 weeks to induce extensive formation of AGEs. As a control for AGES, only BSA was incubated in the absence of glucose under the same conditions. After incubation, samples were filtered to remove aggregated particles, normalized, and stored at −20 °C before use. In some experiments, excessive glucose in the AGE preparations was removed by extensive dialysis against PBS. To measure the formation of AGES, the fluorescence emission spectrum between 385 and 600 nm (370 nm excitation) was scanned using a Varioskan Flash multimode reader (Thermo Fisher).

Assay of inflammasome activation

To stimulate NLRP3 inflammasome activation, mouse BMDMs were primed with LPS (0.25 μg/ml) for 3 h, followed by treatment with ATP (2.5 mM, 30 min), nigericin (5 μM, 45 min), or alum (125 μg/ml, 6 h). To activate AIM2 inflammasomes, BMDMs were transfected with poly(dA:dT). To stimulate NLRC4 inflammasome, BMDMs were infected with P. aeruginosa PAO1 as described previously (46). Culture supernatants were precipitated by the addition of a methanol/chloroform mixture as described previously (47), after which they were immunoblotted with anti-caspase-1 and anti-IL-1β antibodies. Inflammasome activation was determined by the presence of bands corresponding to active caspase-1 p20 and active IL-1β in immunoblots, and was quantified by measuring extracellular IL-1β using a Quantikine IL-1β ELISA Kit (R&D Systems). To measure extracellular levels of IL-6, culture supernatants were assayed using an IL-6-specific ELISA kit according to the manufacturer’s instructions (R&D Systems).

Assay of inflammasome assembly

To determine the oligomerization of ASC, a discuscinimidyl suberate-mediated chemical cross-linking assay was performed as previously described (48). To determine the formation of NLRP3 speck-like aggregates, NLRP3-GFP-expressing BMDMs were observed under a confocal microscope (LSM 700, Zeiss) after the indicated stimulations. The relative percentage of cells containing NLRP3 specks was then counted.

Immunoblotting

Cells were harvested and then lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA and protease inhibitors. After centrifugation to remove cell debris, soluble lysates were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes (Bio-Rad), and then immunoblotted by Western blotting. All blots shown are representative images of at least three-independent experiments. Images have been cropped for presentation.
Suppression of innate immune responses by AGEs

Quantification of mRNA production

To measure mRNA production, quantitative real-time PCR or RT-PCR assays were performed. Briefly, total cellular RNA was isolated using the TRizol reagent (Invitrogen) and reverse transcribed using PrimeScriptTM RT Master Mix (Takara) according to the manufacturer’s instructions. Template DNA was amplified by quantitative real-time PCR using SYBR Premix Ex Taq™ II (Takara). Alternatively, RT-PCR was performed with the AccuPower HotStart PCR premix (Bioneer). Primers were as follows: 5'-GCC CAT CCT CTG TTA TTT TGT CG-3' (mouse Il-1β); 5'-AGT TGC CTT CCT GGG ACT GA-3' and 5'-TCC ACG ATT TTC CAG AGA AC-3' (mouse Il-6); 5'-ATG CTG CTT CGA CAT CTC CT-3' and 5'-AAG CCA TGG GAG ATC CGT AC-3' (mouse Nlrp3); 5'-CGG GTT TCT ATT TTG TTG TT-3' and 5'-AGT CGG CAT CTG TTA TGG TC-3' (mouse Rn18s); 5'-AAC ATG GCC ATT GTG GAA GG-3' and 5'-ACA CAT TGG GGG TAG GAA CA-3' (mouse Gapdh); 5'-TTC CTG CTG TGC TTT AAC TC-3' and 5'-CTT TCC ATT CAG CTG CTC CA-3' (mouse Jnk); 5'-CCG AAC CAA ACA ACA TCA CAT CTA-3' and 5'-GGT CTA AAG GCT CCG GCC T-3' (mouse Nos2); 5'-CGT CAG CCT ATT TGC TAT CT-3' and 5'-CGG ACT CCG CAA AGT CTA AG-3' (mouse Tnfα); 5'-GTG AAG AAC CCA CGG TCT GT-3' and 5'-CTG GTT GTC AGG GGA GTG TT-3' (mouse Arg1); 5'-CAC CAT GGC CAA CCT CAT TCT TGT-3' and 5'-TAT TGG CTC GTC CTT AGC CCA ACT-3' (mouse Ym1); 5'-AGC TGC TCA AGG CCT CTG TCT TA-3' and 5'-CTG CAT TAT GAG GCC AGC TT-3' (mouse Cxcl11); 5'-GGG CCT CAA GGA AAA GAA TC-3' and 5'-TTG TCG TGC TTT AGA GGA GCT GA-3' (human IL-1β); 5'-TAC CCC CAG GAG AAG ATT CC-3' and 5'-TTT TCT GCC AGT GCC TCT TT-3' (human IL-6); 5'-GAG TCA AGG GAT TTG GTC GT-3' and 5'-TTG ATT TTG GGA CCT CG-3' (human GAPDH).

Reporter gene assay

To determine NF-κB promoter activities, 293-TLR4 cells were transfected with an NF-κB promoter luciferase reporter plasmid. After a 24-h transfection, cells were treated with BSA or AGEs for 12 h, followed by treatment with LPS for 6 h. Cell lysates were then assayed for luciferase activity using a Luciferase Assay Kit (Promega) according to the manufacturer’s instructions. To determine IFN-β promoter activity, 293T cells were cotransfected with an IFN-β promoter–luciferase reporter plasmid and an RIG-I plasmid. After a 6-h transfection, the cells were washed and fresh medium containing BSA or AGEs was added to the cells. Cells were incubated for 18 h, followed by transfection of poly(I:C) for 6 h. Cell extracts were then assayed for luciferase expression using a luminometer (Promega).

Measurement of mitochondrial ROS production

To measure the production of mitochondrial ROS, cells were stained with MitoSOX (Invitrogen) according to the manufacturer’s protocol. The fluorescence of cells was then monitored by flow cytometry (FACSVersus, BD Biosciences).

Influenza A virus infection

Human influenza virus A/Puerto-Rico/8/34 (H1N1) PR8 strains lacking the NS1 open reading frame (delNS1) were provided by Dr. Adolfo Garcia-Sastre (Icahn School of Medicine at Mount Sinai, New York) and used. Virus titers were determined by standard plaque assays in Madin-Darby canine kidney cells with a few modifications, as described previously (49). Cells were infected with influenza A virus at a multiplicity of infection (m.o.i.) of 2–4. At 1.5 h post-infection, cells were washed and cultured in regular growth medium for 24 h before being harvested.

Assay of type-I interferon production

To trigger the production of type-I IFN, mouse BMDMs were infected with influenza A virus as described above or transfected with poly(I:C) using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). To measure the production of IFNβ, quantitative real-time PCR was performed to quantify the levels of IFNβ mRNA. Additionally, culture supernatants were assayed for extracellular levels of IFN-β using a mouse IFN-β ELISA Kit (R&D Systems).

Statistical analysis

All values were expressed as the mean ± S.E. of individual samples or independent experiments. Data were analyzed using one-way analysis of variance followed by Dunnett’s post hoc test for comparison of all groups with control group. The level of statistical significance was set at p < 0.05. Analyses were performed with GraphPad Prism.

Author contributions—S. S. and J.-W. Y. designed the study and wrote the manuscript. S. S. and I. H. performed the experiments. S. H. H. and J.-S. S. advised the experiments regarding RAGE inhibitor. O. S. S. coordinated the experiments regarding influenza virus. All authors analyzed the results and approved the final version of the manuscript.

References

1. Singh, R., Barden, A., Mori, T., and Beilin, L. (2001) Advanced glycation end-products: a review. Diabetologia 44, 129–146
2. Ott, C., Jacobs, K., Haucke, E., Navarrete Santos, A., Grune, T., and Simm, A. (2014) Role of advanced glycation end products in cellular signaling. Redox. Biol. 2, 411–429
3. Kilhovd, B. K., Berg, T. J., Birkeland, K. I., Thorsby, P., and Hanssen, K. F. (1999) Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. Diabetes Care 22, 1543–1548
4. Verzijl, N., DeGroot, J., Oldehinkel, E., Bank, R. A., Thorpe, S. R., Baynes, J. W., Bloyll, M. T., Bijlsma, J. W., Lefeber, F. P., and Tekoppele, J. M. (2000) Age-related accumulation of Maillard reaction products in human articular cartilage collagen. Biochem. J. 350, 381–387
5. Uribarri, J., Cai, W., Woodward, M., Tripp, E., Goldberg, L., Pyzik, R., Yee, K., Tansman, L., Chen, X., Mani, V., Fayad, Z. A., and Vlassara, H. (2015) Elevated serum advanced glycation endproducts in obese indicate risk for the metabolic syndrome: a link between healthy and unhealthy obesity? J. Clin. Endocrinol. Metab. 100, 1957–1966
6. Yan, S. F., Ramasamy, R., and Schmidt, A. M. (2008) Mechanisms of disease: advanced glycation end-products and their receptor in inflammation and diabetes complications. Nat. Clin. Pract. Endocrinol. Metab. 4, 285–293
Suppression of innate immune responses by AGEs

7. Stitt, A. W. (2001) Advanced glycation: an important pathological event in diabetic and age related ocular disease. Br. J. Ophthalmol. 85, 746–753

8. Pertyiska-Marzewska, M., Kiriakidis, S., Wait, R., Beech, J., Feldmann, M., and Paleolog, E. M. (2004) Advanced glycation end products upregulate angiogenic and pro-inflammatory cytokine production in human monocyte/macrophages. Cytokine 28, 35–47

9. Liu, J., Zhao, S., Tang, J., Li, Z., Zhong, T., Liu, Y., Chen, D., Zhao, M., Li, Y., Gong, X., Deng, P., Wang, J. H., and Jiang, Y. (2009) Advanced glycation end products and lipopolysaccharide synergistically stimulate pro-inflammatory cytokine/chemokine production in endothelial cells via activation of both mitogen-activated protein kinases and nuclear factor-κB. FEBS J. 276, 4598–4606

10. Rasheed, Z., Akhtar, N., and Haqqi, T. M. (2011) Advanced glycation end products induce the expression of interleukin-6 and interleukin-8 by receptor for advanced glycation end product-mediated activation of mitogen-activated protein kinases and nuclear factor-κB in human osteoarthritis chondrocytes. Rheumatology 50, 838–851

11. Davis, B. K., Wen, H., and Ting, J. P. (2011) The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu. Rev. Immunol. 29, 707–735

12. Yu, J. W., and Lee, M. S. (2016) Mitochondria and the NLRP3 inflammasome: physiological and pathological relevance. Arch. Pharm. Res. 39, 1503–1518

13. Vladimir, G. I., Marty-Roix, R., Ghosh, S., Weng, D., and Lien, E. (2013) Inflammomasomes and hosts defense against bacterial infections. Curr. Opin. Microbiol. 16, 23–31

14. Fernandes-Alnemri, T., Yu, J. W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., Eisenlohr, L., Landel, C. P., and Alnemri, E. S. (2010) The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. Nat. Immunol. 11, 385–393

15. Allen, I. C., Scull, M. A., Moore, C. B., Holl, E. K., McElvania-TeKippe, E., Taxman, D. J., Guthrie, E. H., Pickles, R. J., and Ting, J. P. (2009) The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity 30, 556–565

16. Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T., Brickey, W. J., and Ting, J. P. (2011) Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nat. Immunol. 12, 408–415

17. Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Huang, M. T., Brickey, W. J., Rosenberg, S., Zhang, J., and Alnemri, E. S. (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ. 14, 1590–1604

18. Ghonime, M. G., Shamma, O. R., Das, S., Eldomany, R. A., Fernandes-Alnemri, T., Alnemri, E. S., Gavriliu, M. A., and Wewers, M. D. (2014) Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function. J. Immunol. 192, 3881–3888

19. Zhou, R., Yazdi, A. S., Muen, P., and Tschoopp, J. (2011) A role for mitochnondria in NLRP3 inflammasome activation. Nature 469, 221–225

20. Won, J. H., Park, S.,Hong, S., Son, S., and Yu, J. W. (2015) Rotenone-induced impairment of mitochondrial electron transport chain confers a selective priming signal for NLRP3 inflammasome activation. J. Biol. Chem. 290, 27425–27437

21. Heid, M. E., Keyel, P. A., Kamga, C., Shiva, S., Watkins, S. C., and Saltar, R. D. (2013) Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. J. Immunol. 191, 5230–5238

22. Yu, J., Nagasu, H., Murakami, T., Hoang, H., Broderick, L., Hoffman, H., and Hørgaard, T. (2014) Inflammasome activation leads to caspase-1-dependent mitochondrial damage and block of mitophagy. Proc. Natl. Acad. Sci. U.S.A. 111, 15514–15519

23. Park, S., Won, J. H., Hwang, I., Hongs, S., Lee, H. K., and Yu, J. W. (2015) Defective mitochondrial fission augments NLRP3 inflammasome activation. Sci. Rep. 5, 15458

24. Labonte, A. C., Tosello-Trampont, A. C., and Hahn, Y. S. (2014) The role of macrophage polarization in infectious and inflammatory diseases. Mol. Cells 37, 275–285

25. Porta, C., Rimoldi, M., Raes, G., Bleys, L., Hgezzi, P., Di Liberto, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., Mantovani, A., and Sica, A. (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor-κB. Proc. Natl. Acad. Sci. U.S.A. 106, 14978–14983

26. Bauerfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgeral, K. A., Hornung, V., and Latz, E. (2009) Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J. Immunol. 183, 779–781

27. Juliana, C., Fernandes-Alnemri, T., Wu, J., Datta, P., Solorzano, L., Yu, J. W., Meng, R., Quong, A. A., Latz, E., Scott, C. P., and Alnemri, E. S. (2010) Anti-inflammatory compounds parthenolide and Bay 11–7082 are direct inhibitors of the inflammasome. J. Biol. Chem. 285, 9792–9802

28. Nepeer, M., Schmidt, A. M., Brett, J. Y., Yan, D. S., Wang, F., Pan, Y. C., Elliston, K., Stern, D., and Shaw, A. (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J. Biol. Chem. 267, 14998–15004

29. Shahzad, K., Bock, F., Dong, W., Wang, H., Kopf, S., Kohli, S., Al-Dabet, M. M., Ranjan, S., Wolter, J., Cacci, B., Biemann, R., Stoyanov, S., Reymann, K., Söderkvist, P., Groß, O., et al. (2015) Nlrp3-inflammasome activation in non-myeloid-derived cells aggravates diabetic nephropathy. Kidney Int. 87, 74–84

30. Song, Y., Wang, Y., Zhang, Y., Geng, W., Liu, Y., Gao, Y., Li, S., Wang, K., Wu, X., Kang, L., and Yang, C. (2017) Advanced glycation end products regulate anabolic and catabolic activities via NLRP3-inflammasome activation in human nucleus pulposus cells. J. Cell. Mol. Med. 21, 1373–1387

31. Seno, K., Sase, S., Ozeki, A., Takahashi, H., Okuchiku, A., Suzuki, H., Matsubara, S., Iwata, H., Kyuwama, T., and Shirasuna, K. (2017) Advanced glycation end products regulate interleukin-1β production in human placenta. J. Reprod. Dev. 63, 401–408

32. Valencia, J. V., Mone, M., Koehne, C., Rediske, J., and Hughes, T. E. (2004) Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products. Diabetologia 47, 844–852

33. Miyata, T., Inagi, R., Iida, Y., Sato, M., Yamada, N., Oda, O., Maeda, K., and Seo, H. (1994) Involvement of β2-microglobulin modified with advanced glycation end products in the pathogenesis of hemodialysis-associated amyloidosis. Induction of human monocyte chemotaxis and macrophage secretion of tumor necrosis factor-α and interleukin-1. J. Clin. Investig. 93, 521–528

34. Uribarri, J., Cai, W., Ramdas, M., Goodman, S., Pyzik, R., Chen, X., Zhu, L., Striker, G. E., and Vlassara, H. (2011) Restriction of advanced
glycation end products improves insulin resistance in human type 2 diabetes: potential role of AGER1 and SIRT1. *Diabetes Care* **34**, 1610–1616

41. Lu, C., He, J. C., Cai, W., Liu, H., Zhu, L., and Vlassara, H. (2004) Advanced glycation endproduct (AGE) receptor 1 is a negative regulator of the inflammatory response to AGE in mesangial cells. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 11767–11772

42. Ichinohe, T., Pang, I. K., and Iwasaki, A. (2010) Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat. Immunol.* **11**, 404–410

43. Sheridan, P. A., Paich, H. A., Handy, J., Karlsson, E. A., Hudgens, M. G., Sammon, A. B., Holland, L. A., Weir, S., Noah, T. L., and Beck, M. A. (2012) Obesity is associated with impaired immune response to influenza vaccination in humans. *Int. J. Obes. (Lond)* **36**, 1072–1077

44. Shah, B. R., and Hux, J. E. (2003) Quantifying the risk of infectious diseases for people with diabetes. *Diabetes Care* **26**, 510–513

45. Smith, A. G., Sheridan, P. A., Harp, J. B., and Beck, M. A. (2007) Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J. Nutr.* **137**, 1236–1243

46. Yang, J., Lee, K. M., Park, S., Cho, Y., Lee, E., Park, J. H., Shin, O. S., Son, J., Yoon, S. S., and Yu, J. W. (2017) Bacterial secretant from *Pseudomonas aeruginosa* dampens inflammasome activation in a quorum sensing-dependent manner. *Front. Immunol.* **8**, 333

47. Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., and Alnemri, E. S. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* **458**, 509–513

48. Yu, J. W., Fernandes-Alnemri, T., Datta, P., Wu, J., Juliana, C., Solorzano, L., McCormick, M., Zhang, Z., and Alnemri, E. S. (2007) Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol. Cell* **28**, 214–227

49. Kim, J. A., Seong, R. K., and Shin, O. S. (2016) Enhanced viral replication by cellular replicative senescence. *Immune Network* **16**, 286–295