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終期醣化產物對自體免疫疾病的免疫發炎反應及血管病變引發類似發炎老化反應的分子機制

The Molecular Basis of AGE-ALB on Immunological/ Inflammatory Reactions and Vasculopathy Mimicking Inflamm-aging in Autoimmune Diseases

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本論文係沈玠妤 D03421016 在國立臺灣大學臨床醫學研究所完成之博士學位論文，於民國 113 年 1 月 5 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Graduate Institute of Clinical Medicine on 5th, January, 2024 have examined a Doctoral Dissertation entitled above presented by Shen, Chieh-Yu, D03421016 candidate and hereby certify that it is worthy of acceptance.

口試委員 Oral examination committee:

（指導教授 Advisor）

系（所、學位學程）主管 Director:

誌謝

我從民國 100 年進入碩士班，到 103 年進入博士班就讀，這期間，最要感謝的是余家利教授和謝松洲教授。余教授把我從研究的門外漢，先是手把手帶我建立起對研究的信心，在博士班就讀期間，投稿每每遇到挫折，也是余教授敦促我檢討、思考，一步步修正、重新投稿，才完成博士班的學業。過程中遇到的疑難困惑，老師也都不吝其煩地提出見解，幫助我找到適合自己的解決方式。而謝教授在臨床業務和臨床研究方面，協助我從剛升任主治醫師的跌跌撞撞、心虛茫然，逐步成長到稍能獨當一面。這幾年跟在兩位老師身邊學習，除了學問以外，學習最多的還是師長們思考事情的方法，學習如何獨當一面。詢問問題也從一開始的詢問要做甚麼，要怎麼做，進步成為什麼要這麼做，或是可不可以多做甚麼。回想剛進入博士班的前兩年，剛好遇到科內總醫師斷層，因此身兼總醫師工作，致使臨床業務量驟增，在研究所學業方面便無暇顧及，而在臨床業務略可輕減時，又遇到自己住院手術。那兩三年靠著老師們的幫助勉強度過，才有後面的研究成果。

除了兩位指導老師以外，我也想要感謝中研院的客座研究員，翁啟惠院長及徐翠玲博士。起因是余家利教授和翁院長尚稱認識，剛好翁院長希望將中研院的醣科學研究納入自體免疫疾病的範疇，所以期間也參加了一些中研院主辦或協辦的醣科學方面講座或學會，因此和徐翠玲博士多有接觸。這些大大增廣了我在免疫學以外的見聞及思考線路。而在進行醣科學方面研究，也恰逢中研院

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開始接受委外的實驗委託，一些困難的實驗才在中研院研究人員的協助下完成。

在研究所就讀期間，在實驗進行的部分，我也要感謝 1305 和 1306 實驗室的同仁，還有我前後兩任的研究助理，蔡宗翰先生及陳佳伶小姐及之前余家利教授留下來的助理劉志仁先生。其他實驗室同仁在實驗遇到困難時與我一起討論，需要實驗藥品時也不吝惜借助，因人數眾多就不一一列出。而在臨床業務及病人檢體收集部分，風濕免疫科從 105 年以後歷任的總醫師們，還有 13C 病房的護理人員，也都提供我很多幫助，有他們的協助我才能完成這些研究。

最後也要謝謝我家人的支持，前期因為實驗關係，待在醫院的時間遠遠超過待在家裡的時間，後期投稿不順利時，我想去各處管學習功名的廟宇拜拜，家人也都義不容辭地與我同去。希望這篇論文的研究，能夠對自體免疫疾病的病生理機轉帶來些許貢獻，並且對於未來的治療方向提供些許協助。

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中文摘要

背景：發炎老化反應，是一種長期低度非感染性的發炎，此一現象的持續存在對於老化過程影響甚劇，並且在老化相關疾病的致病機轉中扮演重要角色。終期醣化產物是一群由單醣分子，經過非酵素性的梅納反應修飾而成的大分子。在糖尿病、老化相關疾病和自體免疫疾病的患者，常可見到患者體內終期醣化產物升高的狀況。我們認為終期醣化產物出現在老化相關疾病及慢性發炎疾病患者體內，而發炎老化反應又可能造成老化相關疾病，這兩者之間應有其相關，所以我們假設終期醣化產物可能是誘發發炎老化反應的重要因子之一。我們之前的研究發現終期醣化產物對 Th1/Th2 的抑制以及對單核細胞/巨噬細胞的刺激作用，乃經由 MyD88 和 MAPK-ERK-NF-κB 的訊息傳導路徑。然而，與終期醣化產物引發的發炎老化反應的分子機制仍有待更進一步的闡明。

方法：本研究將人類血清白蛋白和葡萄糖在 37℃、5%CO_2 培養箱中培養 0-180 天，並動態觀察終期醣化產物的生合成。我們用此人工合成的終期醣化產物觀察對免疫細胞，如 T 細胞和巨噬細胞細胞株，及和內皮細胞功能的影響，並研究其分子機制。另外，為了研究發炎現象和醣化之間的交互作用，我們也檢測了和各種不同自體免疫疾病患者血清中的終期醣化產物濃度，並在製作終期醣化產物的過程中加入和免疫老化反應相關的各種不同細胞激素，以評估其影響。

結果：我們發現終期醣化產物在製作過程中其顏色會逐漸由透明最終變為棕

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色，並且其分子量也會逐漸增加。其酸鹼值也從 7.2 逐漸降低到 5.4，但此變化與離子電荷或鈣離子濃度無關。這些變化乃因血清白蛋白分子內的鹼性氨基酸，包括離氨酸和精氨酸的逐漸醣化，從而喪失鹼性特性而趨向酸性溶液相關。我們發現，每毫升 40 微克的終期醣化產物，會經由抑制 p-STAT3、p-STAT4 和 p-STAT6 的訊息傳導路徑，來抑制人類 Jurkat T 細胞株產生第二介白質，同時，也會增加衰老相關β-半乳糖苷酶(SA-βgal) 的表現。但與 Th1/Th2/Treg 亞群的變化無關。另外同樣濃度的終期醣化產物會增加趨化因子 CCL-5、第八介白質、巨噬細胞遷移抑制因子(MIF) 和第一介白質受體拮抗分子(IL-1Ra)的產生，但會抑制巨噬細胞的 SA-βgal 表現量。除此之外，終期醣化產物也會抑制白蛋白對人類冠狀動脈內皮細胞的影響，包括釋放可溶性細胞間質粘附分子 1 (sICAM-1)、可溶性內皮細胞選擇素和內皮素的分泌，並增強了老化相關分子 SA-βgal 的表現量。而在體外研究中，我們發現個別的發炎細胞激素，例如第二介白質、第六介白質、第十七介白質，乙型轉化生長因子，甲型腫瘤壞死因子等，會加速及增加終期醣化產物的生成。本發現佐證了在自體免疫疾病患者體內終期醣化產物增加的現象。

結論：本研究證實終期醣化產物具有免疫抑制、促發炎反應、及引發血管病變等發炎性老化現象。這些病態生理作用乃經由 MAPK-ERK-及 MyD88-STATs-NF-κB 等訊息傳導路徑，並可能與產生衰老相關的 β-半乳糖苷酶相關。另外， AGE-ALB 分子會喪失正常白蛋白對血管內皮細胞的正常生理機能，而引

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發血管病變。而各種不同的發炎性細胞激素因子本身會加速終期醣化產物的形成，而導致惡性循環。

關鍵詞：終期醣化產物、Nε-羧甲基離氨酸、免疫抑制、血管病變、衰老相關β-半乳糖苷酶、發炎性衰老、自體免疫疾病

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English Abstract

Introduction: Inflamm-aging is a chronic, sterile, low-grade inflammation occurred during aging process. Inflamm-aging play a critical role in aging process and contribute to pathogenesis of age-related diseases. Advanced glycation end products (AGEs) are macro-molecules modified by different monocarbohydrates via non-enzymatic Maillard reaction. Increased serum levels of AGEs are commonly found in the patients with Diabetes mellitus (DM), aging-related diseases, and immune-mediated diseases. We thought that both AGEs and inflammaging existed in age-related disease may not be a coincidence, and suggest that AGEs may contribute to inflammaging.

We have already demonstrated that the AGE-BSA would exert inhibitory effects on Th1/Th2 cytokine expression and stimulatory effects on monocyte/macrophage lineage via MyD88- and MAPK-ERK- NF-κB signaling pathways. However, the detailed molecular bases of inflamm-aging and vasculopathy related to AGEs remains elucidation. In addition, the real mechanism(s) of inflammation-related cytokines in enhancing AGE-HSA production in autoimmune diseases need further investigation.

Methods: We incubated human serum albumin (HSA) and glucose at 37℃ in 5% CO_2-95% air incubator for 0-180 days to generate AGE-HSA. The immune-related cell, such as T cell and macrophage cell lines and endothelial cell were incubated with

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AGE-HSA to evaluate their effects on inflamm-aging and the respective signaling pathways. Furthermore, the effects and the possible molecular mechanism(s) of different inflammation-related cytokines including IL-2. IL-6, IL-17, TNF-α, and TGF-β on AGE-HSA formation were also evaluated.

Results: We found the mixture of HSA and glucose gradually changing the color from transparency to brown and increased the molecular weight during incubation. The *p*H value also gradually decreased from 7.2 to 5.4 irrelevant to ionic charge or [Ca^{2+}] concentration, but dependent on progressive glycation of the alkaline amino acids, lysine and arginine, in the HSA protein molecule. Functionally, 40 μg/mL of AGE-HSA decreased IL-2 production from human Jurkat T cell line via suppressing p-STAT3, p-STAT4, and p-STAT6 whereas increasing senescence-associated β-galactosidase (SA-βgal) expression irrelevant to shifting of Th1/Th2/Treg subpopulations. In contrast, AGE-HSA enhanced CC motif chemokine ligand 5 (CCL-5), IL-8, macrophage migration inhibitory factor (MIF), and interleukin 1 receptor antagonist (IL-1Ra) production, but suppressed SA-βgal expression by human macrophage-like THP-1 cells. Interestingly, AGE-HSA abrogated the HSA-induced soluble intercellular adhesion molecules 1 (sICAM-1), sE-selectin and endothelin release from human coronary artery endothelial cells (HCAEC) as well as enhanced SA-βgal expression. The accelerated and increased HSA glycations by individual

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inflammation-related cytokine such as IL-2, IL-6, IL-17, TGF-β, or TNF-α in the *in vitro* study reflect increased serum AGE levels in patients with different immune-mediated diseases.

Conclusions: AGE-HSA can exert immunosuppressive, inflammatory and vasculopathic effects in patients with immune-mediated disease mimicking inflamm-aging via both MyD88-, and MAPK-ERK-STAT-NF-κB signaling pathways and increasing senescence-associated β-galactosidase expression. The inflammatory cytokines *per se* may accelerate AGE-HSA formation as reflected by increased serum AGE-HSA levels in these patients.

Keywords: AGE-modified human serum albumin, Nε-carboxymethyl-lysine, Nε-carboxyethyl-lysine, vasculopathy, senescence-associated β-galactosidase, inflamm-aging, autoimmune diseases.

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Introduction

1.1. Introduction of inflamm-aging

Inflamm-aging, as introduced in year 2000 by Franceschi, C. et al. [1], suggests the chronic, sterile (usually in the absence of infection), low-grade inflammation that occurs during ageing process and contribute to the pathogenesis of age-related diseases. The evolution of the concept was from the “seven pillars” of geroscience suggested by National Institute of Health of United States of America[2]. The seven pillars are about critical area for investigating geroscience, including macromolecular damage, epigenetic dysregulation , adaptation to stress, proteostasis, stem cells regeneration, metabolic disorders, and inflammation. The critical point of inflammation in geroscience included the differentiated maladaptive inflammatory responses, define age-related inflammatory sources and, determine how obesity or metabolic dysfunctions alter inflammation with age. This inflammation is responsive to endogenous signals, such as oxygen free radicals, heat shock proteins, damaged DNAs or macromolecule change [3]. However, besides inflammation *per se*, the other six pillars must attain their biochemical effects via inflammation, which suggest that inflammation is actually one of the central components throughout the aging process and age-related diseases. The term “inflamm-aging” evolved from this observation of that inflammation existed throughout aging process and pathogenesis of age-related

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diseases.

1.2.Inflamm-aging in the cellular levels

From the cellular levels, one of the reasonable ways to investigate inflamm-aging is to focus on senescence of immune cells. Senescence of immune cells may appears in the production of senescence- associated secretory profile (SASP) and senescence-associated β-galactosidase [4], decreased of telomere length and activity of telomerase [4] in immune cells. We have known that SASP may be regulated by NF κ B or p53, and compose tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interleukin (IL)-6, IL-1α, IL-1 β , IL-8, C-C motif ligand (CCL)-2, CCL-5, metalloproteinase (MMP)-3, MMP-9, etc. [5]. Senescence-associated secretory profile composed of many cytokines or chemokines and may have slightly difference among different cells. For example, Minato et al. [6, 7] had found that that SASP of T cells may composed increased osteopontin, TNF-α, TGF-β and IL-6, with memory type surface marker and elevated programed death (PD)-1 expression with irresponsive to T cell receptor activation. SASP of macrophage included IL-1α, IL-6, IL-8, CCL-2, CCL-5, MMP-3, MMP-9, and TNF-α [8-10]. SASP of endothelial cells included not only inflammatory profile, such as IL-6, IL-8, CCL-2 those similar with immune cells and extracellular matrix such as fibronectin. Senescent endothelial cells were found also augmented vascular endothelial growth factor (VEGF), tumor growth factor-β

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(TGF-β) or soluble intercellular adhesion molecules (sICAM-1) [11].

One of the most common applied markers for senescence is the senescence-associated β galactosidase, which is found specifically expressed in senescent cells, rather than quiescent or dead cells [12]. β galactosidase is often used as a quantitative analysis of gene expression. Because of its ability of α complementation, which means two inactive fragments, one lacking the amino-terminal region (the α-acceptor) and the other the carboxy-terminal region (the α-donor), are able to associate to form a tetrameric active enzyme [13]. Senescence associated β galactosidase is an isoform of β galactosidase with the highest activity at *p*H6.0 [14-16]. The reason why senescence associated β galactosidase expressed on the senescent cells remained unclear. But undoubtedly, its ubiquitous existence in the senescent cells renders its biological role in the field of geriatric research.

Telomere length and telomerase activity are also useful biomarkers for investigating cellular aging [17]. Telomere length shortening is correlate with aging process [17] in that telomerase act as repairing enzyme for shortened telomere [18, 19]. It is questioned whether the cell lines are suitable tools for investigating aging process. Recent investigators suggest that with proper experimental designs, such as proper control groups, reverse telomerase activity, detection of short-lived proteins or biomarkers, it remained reliable for investigating aging process with cell lines [20,

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21].

The study on the mechanisms of inflamm-aging can help unveil the myth of aging process and pathogenesis of age-related diseases due to their overlapping [3]. Therefore, we believe that inflamm-aging not only contributes to aging processes but also plays a critical role in age-related disease. Common age related disease include obesity, metabolic syndrome, type 2 diabetes mellitus, cardiovascular diseases, arthritis, osteoporosis, sarcopenia, neurodegenerative disease including Alzheimer disease and dementia, cancer, chronic obstructive pulmonary disease, macular disease and chronic kidney disease[3, 22, 23]. Among them, diabetic mellitus, neurodegenerative disease, cardiovascular disease and metabolic syndrome are known to presemt elevated advanced glycation endproducts (AGEs) and may investigators had suggested that AGEs may contribute to pathogenesis of these diseases[24-37]. We will further introduce advanced glycation endproducts and evaluate the pathognomic effects of AGEs.

1.3.Evidence of AGEs contributing to diabetic complications

Impaired wound healing, involving in both endothelial cell and fibroblast functions, is one of the hallmarks of Diabetic mellitus and hyperglycemia. Dai et al. [38] isolated fibroblasts from human diabetic wounds, and found that these AGE-exposed fibroblasts increased cell apoptosis after NLRP3 inflammasome activation by

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an ROS-induced signal pathway. In short, AGE can enhance both the inflammatory responses and cell apoptosis of fibroblasts via its ROS-inducing capacity to impair fibroblast functions. Negrean et al. [39] focused on the effects of high- and low-AGE meals toward endothelial function of diabetic mellites patients. The data revealed that a high-AGE meal induced a more acute deleterious effect on vascular functions. Yamagishi et al. [40] demonstrated that the cross-talk between the AGE-RAGE axis and the dipeptidyl peptidase-4 (DPP4)–incretin system is intimately involved in the development and progression of diabetes-associated complications, including diabetic microangiopathy and arteriosclerotic CVD.

Aging-related macular disease is one of the major vision-threatening diseases of the elderly and DM patients. Anand Babu et al. [41] proved that AGEs act as a pro-oxidant metabolite to induce production of proinflammatory cytokine IL-6 and IL-8, and vascular endothelial cell growth factor (VEGF) release from human retinal pigment epithelial cells. These cytokines can facilitate macrophage infiltration through oxidative stress, inflammation, chemotaxis, and neovascularization to cause macular degeneration.

1.3.1. Correlation of AGEs with immune-mediated diseases

We reviewed the literatures and found that endogenous factors interfere with AGE including aging, hyperglycemia, obesity, autoimmunity, chronic inflammation,

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chronic kidney disease, oxidative stress and glyoxalase deficiency[42, 43]. elevated AGE levels are frequently found in the immune-mediated diseases including rheumatoid arthritis [44-47], adult-onset Still's disease [48], systemic lupus erythematosus [45, 49-55], Hashimoto thyroiditis [56-58] and psoriasis [59]. Therefore, we measured the AGE levels of these patients and found increase of AGEs levels in these autoimmune diseases as shown in Figure 1. The contribution of AGE to the pathogenesis of immune-mediate diseases remained to be elucidate, but the importance is undoubted.

1.4. Introduction of advanced glycation endproducts (AGEs)

It is conceivable that AGE-modified molecules including proteins, nucleic acids or lipids are known to be generated through non-enzymatic Maillard reaction (MR) between reducing sugars and substrates. Louis Camille Maillard, a French scientist farsightedly observed the brown color change when reducing sugar was heated with amino acids in 1912 [60], and the phenomena is named as Maillard reaction. To date, more than 20 different AGEs have been identified in human tissue and in dietary foods. Arbitrarily, these AGEs can be classified into fluorescent and non-fluorescent [61, 62]. The most pathognomonic non-fluorescent components are N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL), and pyrraline [31, 43] while the most important fluorescent AGEs include pentosidine and methylglyoxal-

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lysine dimer (MOLD) [63, 64]. The key characteristic feature of these AGEs relies on the presence of the glycated lysine, arginine or cystein residue in the molecules. Physiologically, the AGEs are catabolized in renal proximal tubular cells by glyoxalase I or glyoxalase II and are excreted from kidneys [65].

AGE formation is generally a relatively slow process in the physiological conditions. Therefore, AGE deposition usually occurs in the molecules with slow turnover rate, such as albumin, collagen fibers or lens crystalline. To make long story short, the glycation of proteins is induced by MR in three steps: (1) the slow formation of Schiff base. (2) the unstable early formed AGE precursors that underlie Amadori rearrangement, and (3) the late products of irreversible AGEs [66, 67]. Schiff bases are derived from condensation between the carbonyl group of a reducing monosaccharide with the free amino acid, usually lysine or arginine residues [68]. The next step is to rearrange the unstable Schiff bases to the relative more stable Amadori products. Both compounds, Schiff bases and Amadori products, remain unstable and may react with other proteins/glycoproteins in forming protein cross-linkers. These cross-linked proteins would later undergo oxidation, dehydration, or polymerization to finally become a variety of stable advanced glycation end products [68, 69]. AGE levels in blood and tissues are commonly found elevated in patients with Diabetes mellitus (DM) [24-29], aging-associated diseases [27, 30-35], chronic kidney diseases

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[24] and senescence-associated neurodegenerative Alzheimer disease [34, 36, 37, 70]. Not only being a biomarker, AGEs had been proved contribution to diabetic complications, including retinopathy [71, 72], coronary artery diseases [30, 73, 74], cerebrovascular diseases [75] and nephropathy [71, 76]. These complications share a common feature as endothelial cells dysfunction and this suggests endothelial cells may become a target or a victim of AGEs.

1.4.1. AGEs and their receptor signaling

AGE accumulation and the binding of AGEs with their multiligand immunoglobulin superfamily receptors, RAGEs, can induce oxidative stress, inflammatory response, and endothelial dysfunction [66, 77]. However, the other families of cell-surface receptors for AGEs may mediate the opposite functions to RAGE. These may include AGE receptor complex (AGE-R1/OST-48, AGE-R2/80k-H, AGE-R3/galectin-3) and macrophage scavenger receptor family (LOX-1, FEEL-1, FEEL-2 and CD36, SR-A, SR-B, SR-1, SR-E,) those can involve in AGE homeostasis [31, 43, 66]. These homeostatic scavenger receptors act as regulators of clearance by binding with these noxious AGE molecules [78, 79]. Vlassara et al. [65] demonstrated that inverse correlation exists between RAGE to AGE-R1 ratio and the levels of oxidative stress in both humans and mice. Figure 2 depicts the formation and fates of AGEs after binding with different AGE receptors in the body. AGE–RAGE

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interaction transduces signals via the following four pathways: (1) phosphatidyl-inositol 3 kinase (PI3K)–protein kinase B (PKB) pathway, (2) Janus kinase 2 (JAK-2)—signal transducers and activators of transcription 1(STAT 1 pathway), (3) nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase–reactive oxygen species (ROS) pathway, and (4) microtubule associated protein kinases (MAPK)–extracellular signal-regulated kinases (ERK) pathway. The phosphorylated NF-κB enters into the nucleus to finally transcribe the gene of pro-inflammatory cytokines, growth factors, pro-fibrotic cytokines, and oxidative stress [66, 80-82]. Gupta et al. [83] demonstrated that AGE–PMN interactions may upregulate NADPH oxidase expression, and enhanced ROS and RNI generation, resulting in microvascular endothelial cell damage

1.4.2. Effects of AGEs on immune-related cells

Bansal et al. [84, 85] also showed that AGE–HSA enhanced the ROS and RNI production of PMN by the upregulation of NADPH oxidase and inducible nitric oxide synthase (iNOS). Lu et al. [86] found that AGEs activated the release of myeloperoxidase (MPO) and elastase (NE) from neutrophils, and deranged $CD4^+$T cell differentiation via these two granule proteins. Alterations in $CD4^+$T cell differentiation by AGEs resulted in the suppression of both Th1 (denoted by IFN-γ) and Th17 (denoted by IL-17) phenotypes, and diversification in the expression of transcription factor T-bet in Th1, RORγt in Th17, and $FoxP_3$ in Treg cells. van der

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Lugt et al. [87] and Byum et al. [41] demonstrated that dietary AGEs could induce TNF-α secretion from human macrophage-like cells and activated macrophages. This may suggest that AGEs could act as an accelerator in inducing inflammatory response via macrophages. We have also demonstrated in our previous report [88] that AGE–BSA significantly enhanced IL-6 production from normal human monocytes/macrophages, but suppressed Th1 (IL-2 and IFN-γ) and Th2 (IL-10) gene expression. Enhanced IL-6 production from mononuclear cell was proven via MAPK-ERK and MyD88- transduced NF-κB p50 signaling pathways. Accordingly, AGE–RAGE interaction transduces inflammatory and fibrotic signals in immune-related cells to induce somatic cell damage, and finally tissue fibrosis in various tissues and organs.

The pathological effects of AGE–RAGE interactions in different immune-related cells, fibroblasts, and retinal pigmented cells for mediating AGE-related pathology are summarized in Figure 3.

Serum albumin has been reported to possess many physiological effects other than volume expansion. These effects including protection glycocalyx on endothelial cells [89], improvement of endothelial integrity [90], inhibition of endothelial apoptosis [91], modulation of nitric oxide pathway [92], anti-inflammatory effects [93, 94], activation of intracellular process [95], regulation of electrolyte shifts and

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acid-base regulation [96], binding of drugs and other plasma substance [97], and modulation of intracellular volume [98-100]. Albumins from different species origins compose slight difference but binding capacity and immunological characterization remained similar[101]. Human serum albumin is also proved to be glycated and the most frequent glycated lysine sites are believed to be at ^{233}K, ^{276}K, ^{378}K, ^{525}K and ^{545}K on AGE-HSA with 30-day incubation[102].

1.5. Summary of my master's thesis

In our previous report [72], we demonstrated that the AGE-modified bovine serum albumin (AGE-BSA) exerted inhibitory effects on both Th1 and Th2 cells whereas a stimulatory effect on monocyte/macrophage cell lineages. The latter effect was via both MAPK-ERK and myeloid differentiated factor 88 (MyD88) signaling pathways as shown in my master's thesis. However, the exact molecular basis of the non-specific immunosuppressive effects by AGEs was not dissected in that paper. Furthermore, the AGE-BSA does not exist in the human plasma in the real world.

1.6. Aim of my doctoral dissertation

Based on the results of my master thesis, we aim to explore whether AGE-HSA may exert the effects of inducing a scenario composed T cell suppression while macrophage activation effects. These effects, which may present in immunosenescent scenario, composed in inflammaging status. We will use AGE-HSA to stimulate T

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cell, monocyte/macrophage cell lines and coronary artery endothelial cells, with control groups as medium only, HSA and PBS with same *p*H level with AGE. After co-culture, we will collect the supernatant and detect IL-2 in T cell line. For the supernatant from macrophage cell lines, we would detect IL-8, macrophage migration inhibitor factor (MIF), CC motif chemokine ligand 5 (CCL-5), and interleukin 1 receptor antagonist (IL-1Ra), which are known to be markers of macrophage secretion[103-105]. The supernatant from endothelial cells would be used to detect soluble ICAM-1 as one of the markers of endothelial cell function[106], soluble E-selectin[107] and endothelin[108]. It is conceivable that the cell-surface expressed E-selectin and ICAM-1, which binding with CD11a/CD18 (macrophage 1 antigen MAC-1) and CD11b/CD18 (Lymphocyte function-associated antigen 1, LFA-1) expressed on leukocytes and will facilitate the cell immigration from blood circulation into tissues [109]. On the contrary, the soluble ICAM-1 and E-selectin secreted from endothelial cells to the external milieu can help leukocytes free movement in circulation instead of adhesion on the surface of endothelial cells. It is believed that this secretary function by vascular endothelial cells is beneficial to maintain intravascular homeostasis[106].

For molecular basis study, we would focus on the receptor of AGE (RAGE) function and JAK-STAT signaling pathway.. Increased formation of the enzyme can

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raise the cell metabolic activity. Besides, it is well-known that the immune milieu in the various connective tissue diseases may increase their serum AGE-HSA levels [46-48]. We would like to investigate if inflammaging also interfere with AGE formation by incubate AGE with common pro-inflammatory and senescence associated secretory pattern cytokines, such as IL-2, IL-6, IL-17, TNF-α and TGF-β and evaluate if accelerating Maillard reaction to generate AGEs in the immune-related diseases.

Material and Method

2.1. Preparation of AGE-ALB molecules

We followed the method described in our previous report to prepare both AGE-HSA and AGE-BSA [88]. Briefly, 0.5 mol. (9g/dL) of D-glucose (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) was mixed with 0.0746 mmol. (5g/dL) of human serum albumin or bovine serum albumin (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) in phosphate buffered saline (PBS), *p*H7.2. The aqueous solution was incubated in 5% CO_2-95% air at 37℃ for 0-180 days. The same concentration of HSA or BSA (5g/dL) in PBS, *p*H7.2 alone was also incubated as control. After incubation, we put the end products into dialysis tube with a pore size of 10,000 Dalton for intensive dialysis against PBS, *p*H7.2 to remove the residue glucose. In general, the day-180 AGE-ALB product at a final concentration of 40

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μg/mL was used for the ongoing functional assays except particular mention. The following experiments are the kinetic observations of AGE-ALB after incubation for 0-180 days.

2.1.1. Kinetic observation of the color change, detection of molecular weight and *p*H changes

The color of the glucose-albumin mixture was observed regularly every 30 days, both in BSA and HSA. We noted that the color of the mixture gradually changed from transparency at day 0, to light yellow at day 30, orange at day 120, and finally became a brown color at day 180.

AGE-ALB mixture at different incubation periods from day-0 to day-180 were electrophoresed in 10% SDS-PAGE for estimating their relative molecular weights of the individual products.

AGE-HSA products at different incubation periods were determined for their individual *p*H value by pH meter. Incubation in the calcium free (addition of EGTA), in positive (addition of poly-L-lysine) or in negative (addition of dextran) charge environment were also conducted to investigate their effects on the *p*H changes and also the progression of the Maillard reaction.

2.1.2. Lectin array analysis

Mapping of glycans harbored on AGE-ALB was performed with the lectin array technology (GA-Lectin-70, RayBiotech, Inc), which is a semi-quantitative analysis. A

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glass slide with 8 wells of identical lectin arrays consisting of 70 different lectins per well was used. The semi-quantitative analysis was carried out according to the manufacture's instruction [110]. The specific recognition pattern of each lectin is summarized in appendix Table. All steps were carried out under gentle shaking at room temperature. The lectin array slide was saturated by incubation of 100 μL per well of sample diluent for 30 minutes after equilibration and drying. AGE-HSA and HSA (5 μg /100 μL) in a sample diluent were then deposited on the array for 2 hours. After washing 5 times with wash buffer-I, and twice wash with wash buffer-II, the recognition was revealed by adding 80 μL of Cy3 equivalent Dye-Streptavidin conjugate to each well for 1 hour. The wells were then thoroughly washed and dried, and the lectin interactions were finally quantified by scanning the fluorescence signal of Cy3 equivalent Dye at 532 nm. The fluorescence data were extracted using GenePix microarray analysis software.

2.1.3. Quantitation of the % glycated lysine, arginine and cysteine residues by LC/MS proteomics analysis

The different AGE-ALB samples were sent to Mithra Biotechnology Inc. (New Taipei city, Taiwan) for LC/MS identification of glycation number and sites in the amino acid residues. The samples were buffered with 50mM ammonium bicarbonate with a 10kDa molecular weight cutoff filter. Then, we added 1% RapiGest SF

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surfactant to a final concentration of 0.1%, reduced with 5mM DTT at 60℃ for 30 min, and then is alkylated with 15mM IAM in the dark, at room temperature for 30 min. The protein molecules were later digested with two enzymes in the following digestion conditions: (1) chymotrypsin digestion at room temperature overnight (protein: enzyme = 50:1). (2) trypsin digestion at 37℃ overnight (protein: enzyme = 50:1). After digestion, each sample was acidified with 0.5% (v/v) TFA, which was performed at 37℃ and then centrifuged at 14,000 rpm for 30 min. The digested samples were analyzed with a Q-Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA) coupled with an Ultimate 3000 RSLC system (Dionex, Sunnyvale, CA, USA). The LC separation was via a C18 column (Acclaim PepMap RSLC, 75μm x 150 mm, 2μm, 100 Å). The mobile pHSAe A was 0.1% FA in H_2O, and mobile pHSAe B was 0.1% FA in 95% ACN. The flow rate was set 0.25μL/min. The gradient setting was 0 min-1%, 5.5 min-1%, 45 min-25%, 48 min-60%, 50 min-80%, 65 min-1%, 70 min-1%. Full MS scan was performed with the m/z range of 150–2000, and the 10 most intense ions were subjected to fragmentation for MS/MS spectra. Raw data were processed into peak lists by Proteome Discoverer 1.4 for Mascot database search. Database search was performed with Mascot 2.4.1. of the instrument type as ESI-TRAP. We used the ALB Database: ALB. The fixed modification was carbamidomethyl (C) and the variable modifications were glycation

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(K), glycation (R), and glycation (C). The peptide mass tolerance was 10 ppm and the fragment mass tolerance were 0.05 Da. The ion cut-off score was 13.

2.1.4. Quantitation of the % glycated lysine residues in different AGE-HSA preparations by glycation mass spectrometry proteomics analysis

We sent the preparations of AGE-HSA with different incubation periods to Genomic Center Laboratory, Academia sinica, Nangang, Taipei, Taiwan for quantification of lysine glycation by glycation mass spectrometry. FASP (filter-aided sample preparation) method was employed for in-solution chymo-trypsin digestion [111]. In briefly, samples (5 μg protein) were loaded to filter units (Microcon YM-10) and we added with 100 μl of 8 M urea in 0.1 M Tris-HCl pH 8.5 (UB) each one for protein denaturation. The filter units were centrifugated with 14,000 x g for 30 min, followed by adding 100 μl of 25 mM DTT in UB for protein reduction and later incubating at room temperature for 10 min. After centrifugation with 14,000 x g for 30 min, 100 μl of 50 mM iodoacetamide in UB was added to each sample for alkylation, and then the filter units were incubated in the dark for 10 min, followed by centrifugation. 100 μl of 50 mM ammonium bicarbonate in water (ABC) was added to change the buffer system to ABC for in-solution chymotrypsin digestion. After centrifugation to remove the solution, 40 μl of ABC with 0.2 μg chymotrypsin (Promega, sequencing grade) was added and incubated in a wet chamber at 37℃

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overnight. The digested peptides in each sample were spun to the collection tubes, and an additional rinse with 40 µl ABC was performed. The filter units were centrifuged at 14,000 x g for 10 min to collect the flow-through product, which contains digested peptides. Samples were acidified with TFA to a final concentration of 0.1% and heated at 95℃ for 10 min to inactivate chymotrypsin. The peptide samples were cleaned up with C18 ziptips, and dried for analysis with LC-MS/MS.

NanoLC−nanoESi-MS/MS analysis was performed on a Thermo UltiMate 3000 RSLCnano system connected to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanospray interface (New Objective, Woburn, MA). Peptide mixtures were loaded onto a 75 µm ID, 25 cm length PepMap C18 column (Thermo Fisher Scientific) packed with 2 µm particles with a pore of 100 Å and were separated using a segmented gradient in 30 min from 5% to 35% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min. Solvent A was 0.1% formic acid in water. The mass spectrometer was operated in the data-dependent mode. To make long story short, survey scans of peptide precursors from 350 to 1800 m/z were performed at 120K resolution with a 4 $\times 10^5$ ion count target. The highest and lowest charge state m/z ions were isolated for MS2 analysis by isolation window at 3 Da with the quadrupole. EThcD fragmentation was performed by 30% HCD activation with the calibrated charge-dependent reaction

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time supplemented, and ion species were analyzed in the Orbitrap at 30K resolution. The max injection time was 54 ms and the MS2 ion count target was 1×10^5. Only those precursors with charge state 2–8 were sampled for MS2. The instrument was run with 3 s cycles in top speed mode; the dynamic exclusion duration was set to 30 seconds with 10 ppm tolerance around the selected precursor and its iso-topes. Monoisotopic precursor selection was turned on.

Raw files were processed by the MASCOT distiller (Version 2.8.01). MS/MS spectra were searched against the UniProt database (568744 entries) with the criteria of chymotrypsin digest up to 5 miss cleavage. The fragment tolerance and instrument type were 0.6 Da and CID+ETD, and the parameter of MS tolerance was 10 ppm, respectively. Variable modifications of oxidation (M), hexose on Cys, Lys or Arg (Hex-C/K/R), and carbamidomethyl (C) were also included as search parameters. Searched data (DAT) with were imported to Skyline (Version 22.2.0.351, [112]) ($p<0.05$), and the proportions of Hex-C/K/R at sites of glycation were calculated and presented.

2.2.Subculture of human macrophage-like, T cell cell lines and coronary artery endothelial cell

THP-1 (a human macrophage-like cell line), Jurkat E6 cells (a human T cell line), and HCAEC (human coronary artery endothelial cell) were obtained from the

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American Type Culture Collection (ATCC, Manassas, VA) for subculture. The THP-1 and Jurkat E6 cells were subculture in 10% FBS-RPMI 1640 culture medium whereas the HCAEC cells were subcultured in endothelium cell growth medium. Each kinds of cells were then separately incubated with 4ml of medium control, HSA (40μg/mL), AGE-HSA (40μg/mL), and PBS *p*H5.4 for 24 hours for comparison. The concentration of stimulant is we used in master's thesis were 1 to 16 μ g/mL. We adjust the concentration because the normal range of albumin in health adults are 35 to 50μg/mL, and the concentration of 40 μ g/mL is more close to in vivo scenario.

2.2.1. Culture of human Jurkat E6 cell line (human T cell line)

Jurkat T cells ere subcultured following the published literature [113]. Briefly, the cells were suspended in RPMI-1640 culture medium supplemented with penicillin 100 U/mL and streptomycin 100 μg/mL (Thermo Scientific, Waltham, MA, USA) and 10% FBS, and continuously cultured in 5% CO_2-95% air at 37°C until confluence. The cells were put into the wells ($1x10^6$cells/4mL/well) and then pre-activated with anti-CD28+anti-CD3 monoclonal antibodies for 2 hours. These activated cells were then separately incubated with 4ml of medium control, HSA (40μg/mL), AGE-HSA (40μg/mL), and PBS *p*H5.4 for 24 hours for comparison.

2.2.2. Culture of human macrophage-like THP-1 cell line

The culture and the experimental design for THP-1 macrophage-like cells were the same as in Jurkat T cells except THP-1 cell lines were pre-activation with phorbol

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12-myristate 13-acetate (PMA) according to the literature [114]. Briefly, THP-1 cells were pre-activated with PMA at a concentration of 25ng/mL for 48 hours. After confirmation of the cell maturation by morphology change to macrophage, the matured THP-1 cells were washed with 2% FBS-PBS mixture for three times, then 10% FBS in RPMI-1640 culture medium was added and incubated for 24 hours. The cell amount was 80% of culture plate. These activated cells were then separately incubated with medium control, HSA (40μg/mL), AGE-HSA (40μg/mL), and PBS *p*H5.4 for 24 hours for comparison

2.2.3. Culture of human coronary artery endothelial cell (HCAEC)

HCAECs were sub-cultured in endothelium cell growth medium MV2 (PromoCell GmbH, Heidelberg, Germany) at 37°C in 95% air-5% CO_2. This particular culture medium contains epidermal growth factor 5ng/mL, fetal bovine serum 0.05ml/mL, insulin-like growth factor 20ng/mL, basic fibroblast growth factor 10ng/mL, hydrocortisone 0.2μg/mL, vascular endothelial growth factor 0.5ng/mL and ascorbic acid 1μg/mL. The 6–9 generation cells of HCAEC were used in the experiments. The HCAEC were then separately incubated with medium control, HSA (40μg/mL), AGE-HSA (40μg/mL), and PBS pH5.4 for 24 hours for comparison.

2.3.Detection of receptors for AGE (RAGE) expression on the cell surface

The cell surface-expressed RAGE on Jurkat, THP-1 cells and HCAEC was

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detected by indirect fluorescence stain with rabbit anti-human RAGE antibody (Abcam, Cambridge, UK) as 1st antibody followed by FITC-labeled goat anti-rabbit IgG antibody (Proteintech, Chicago, IL, USA) as 2nd antibody. Human erythrocytes were used as the negative control cells.

2.4.Cell cytotoxicity assay detected by CCK-8 assay kit

A CCK-8 assay kit (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) was used to measure the cell cytotoxicity followed by the manufacture's protocol [115]. Briefly, 2.5×10^4 cells/100μL/well of Jurkat T or THP-1 cell, or 6×10^3 cells/100μL/well HCAEC cell were seeded in 96-microwell and cultured for 24 h, followed by treatment with different doses of tannic acid in 100μL DMEM/high glucose medium containing 5% FBS for 24 h. The CCK-8 solution (10μL) was added and was incubated for another 3h. OD450 nm was employed for measuring the cell proliferation ability.

2.5. Effects of RAGE inhibition on Jurkat, THP-1 cells and HCAEC

Preincubation of different cells with RAGE inhibitor (RAGEi) FPS-ZM1 for evaluating the involvement of RAGE on the individual activity by AGE-HSA

The three different cells were preincubated with FPS-ZM1 as RAGEi (R&D Systems, Minneapolis, MN, USA) at a concentration of 5μg/mL for 1 hour, as suggested from literature [116]. Then, AGE-HSA at a final concentration of 40μg/mL

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was added for evaluating the effects on the individual functional assay as previously described.

2.6.Detection of senescence-associated β-galactosidase (SA-βgal)

Senescent cells were detected by using a cellular senescence assay kit (SA-βgal staining; cat. no. CBA-231, Cell Biolabs) [117]. Briefly, cells were washed once with PBS *p*H7.2, suspended in cell lysis buffer for 30 min at 4℃ and then were transferred to a 96-well plate. Equal volume of freshly prepared 2x assay buffer was added to each well and further incubated at room temperature for 3 hours in the dark. After staining, the fluorescence was measured by a fluorescence plate reader at 360 nm (excitation)/465 nm (emission).

2.7.Detection of relative telomerase activity

Telomerase activity assay kit (ScienCell, Carlsbad, CA 92008, USA) was used according to manufacturer's instructions [118]. For each experiment, both negative and positive controls for telomerase in the cell lysates (ScienCell Cat #8928e) were used. Cells were suspended in lysis buffer and incubated 30 minutes at 4°C. After centrifugation at 12,000 g for 20 minutes at 4°C, the supernatant was incubated with telomerase reaction buffer at 37°C for 3 hours followed by heated at 85°C for 10 minutes to stop the reaction. All reactions were run in three replicates. Data analysis was conducted according to the manufacturer's instructions.

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2.8.Detection of relative telomere length

The average telomere length (TL) was measured in the extracted genomic DNA (gDNA) samples from Jurkat cells and HCAEC after culture with AGE-HSA, HSA, medium, or PBS *p*H5.4 for 9 days. After 9-day culture, the gDNA were extracted and diluted with nuclease-free water in a concentration of 20ng/μL. Telomere length measurements were performed by real-time quantitative polymerase chain reaction (qPCR) in a QuantStudio Real-Time PCR system (Thermo Scientific, Waltham, MA, USA) using qPCR assay kits containing telomere primer sets (ScienCell's Absolute Human Telomere Length Quantification qPCR Assay Kit [AHTLQ], Carlsbad, CA, USA), following the instruction of the manufacturer. The qPCR was performed at 95 ℃ for 10 min. followed by 36 cycles at 95℃ for 20s, 52℃ for 20s and 72℃ for 45s. Data analysis was conducted according to manufacturer's instructions.

2.9.Detecting the functional changes of Jurkat, THP and HCAEC cells after AGE-HSA stimulation

2.9.1. Detection of IL-2 (a pluri-potential cytokine for T cell development and homeostasis) production after incubation of anti-CD28+anti-CD3- pre-activated Jurkat cells with AGE-HSA

The anti-CD3 and anti-CD28 antibodies-preactivated Jurkat T cells at a cell concentration of $1x10^6$/4mL/well were cultured with 40μg/mL of AGE-HSA at 37℃

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for 24h. The culture supernatants were obtained for measuring IL-2 by ELISA (R &D Systems, Minneapolis, MN, USA). 40 μg/mL of HSA ,PBS with *p*H5.5 and culture medium were employed as controls. The minimum detection concentration is 7pg/mL.

2.9.2. Detection of transcription factors T-bet (Th1), GATA-3(Th2) and FoxP3 (Treg) by western blot after incubation of Jurkat T cells with AGE-HSA (40 μ g/mL) for 24 hours

Jurkat T cell ($1x10^6$/4mL/well) were incubated with 40μg/mL of HSA or AGE-HSA for 24 hours. The total cell lysates were prepared by sonication. The cell lysates were then electrophoresed in 10% SDS-PAGE followed by immune-blotting with mouse monoclonal antibody against T-bet (BioLegend, San Diego, CA. USA), GATA-3 (Cell Signaling Technology, Danvers, MA, USA), or FoxP3 (BioLegend, San Diego, CA. USA) as primary antibody and HRP-conjugated goat anti-mouse IgGs antibodies (Proteintech, Chicago, IL, USA) as secondary antibody.

2.9.3. Detection of intracellular positive (STATs) and negative (cytokine-induced SH-2 protein, CIS and suppressor of cytokine signaling proteins, SOCSs) regulators for IL-2 by western blot

The Jurkat T cell lysates were dispersed in 10% SDS-PAGE and were then immuno-blotted by the monoclonal antibodies for detecting the total and phosphorylated STAT4 (at site 693), the total and phosphorylated STAT6 (at site 641)

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(R&D Systems, Minneapolis, MN, USA) and the total and phosphorylated STAT3 (at site 727) (Abcam, Cambridge, UK). In addition, monoclonal antibodies for detecting the negative regulators of IL-2 transcription including SOCS1 and SOCS7 (Cell Signaling Technology, Danvers, MA, USA), and CIS (R&D Systems, Minneapolis, MN, USA) were also used in the western immunoblot.

2.9.4. Detection of IL-8, macrophage migration inhibitor factor (MIF), CC motif chemokine ligand 5 (CCL-5), and interleukin 1 receptor antagonist (IL-1Ra) production by PMA-activated human THP-1 cells after reaction with AGE-HSA

The PMA-preactivated THP-1 cells ($1x10^6$cells/4mL/well) were incubated with medium, HSA, AGE-HSA, and PBS, *p*H5.4 in 5% CO_2-95% air for 24h. The supernatants were obtained for measurement of CCL-5, IL-8, MIF, and IL-1Ra concentration by the respective ELISA kit (R&D Systems, Minneapolis, MN, USA). The minimal detection concentration is 15.6pg/mL for CCL-5, 31.2pg/mL for IL-8, 32.2pg/mL for MIF, and 39.1pg/mL for IL-1Ra, respectively.

2.9.5. Detection of soluble intercellular adhesion molecules 1 (sICAM-1), sE-selectin, and endothelin concentration in the culture supernatants of HCAEC by ELISA

The 24h culture supernatants of HCAEC ($6x10^4$/mL/well) in the

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presence/absence of AGE-HSA (40μg/mL) were collected for determination of endothelin, sICAM-1, and sE-selectin by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). The minimum detection concentration is 0.254pg/mL for sICAM-1, 0.027pg/mL for sE-selectin, and 0.207pg/mL for endothelin, respectively.

2.10. Quantitation of N^{ε}-(carboxymethyl)-lysine (CML) and N^{ε}-(carboxyethyl) -lysine (CEL) as the surrogates of AGE-HSA molecule in the culture supernatants and the sera of the patients with immune-related diseases

Commercially available CML and CEL ELISA kits (Cell Biolab, Inc. San Diego, CA, USA) were applied. We followed the manufacturer's instructions to quantify the amounts of CML and CEL as surrogates of lysine glycation in the incubated AGE-HSA mixture. We also checked the CML titer in the sera obtained from various connective tissue diseases. The sera included 9 active systemic lupus erythematosus, 14 active rheumatoid arthritis, 8 connective tissue diseases (2 progressive systemic sclerosis, 1 antiphospholipid syndrome, 2 Adult-onset Still disease, 1 polymyositis, 1 seronegative spondylarthropathy and 1 vasculitis). 19 sera obtained from health patients were the controls. Informed consent was obtained from each patient permitted by the Internal Review Board, National Taiwan University Hospital (201801113RINC and 201904051RINB). The detection limit is 2.25μg/mL for CML and 0.1μg/mL for

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CEL. The range of age in SLE group is from 22 to 52 with median 27, the age of CTD group is from 36 to 69 with median 56, the age of RA group is from 32 to 76 with median 54 and the age of Control group is from 36 to 80 with median 62. There is no significant change among patient age.

2.11. Co-culture of HSA+glucose mixture with or without an inflammation-related cytokine (IL-2, IL-6, IL-17, TGF-β, or TNF-α)

To mimic *in vivo* environment of inflamm-aging or immune-related disease, we add each of the 5 cytokines as SASP in inflammation milieu. These 5 cytokines were obtained from PeproTech Ltd. (Westlake Village, CA, USA). We incubated HSA+ glucose ± individual cytokine for 90 days and 180 days for measuring the amount of CML and CEL as surrogates of AGE-HSA. The concentration of cytokines are the highest level of normal range in health adult, which were 30 pg/mL for IL-2, 15pg/mL for IL-6, 7.5pg/mL for IL-17, 15pg/mL for TNF-α and 70ng/mL for TGF-β. During incubation, the *p*H and relative molecular weight change, LC/MS for glycation and glycation mass spectrometry of the conjugates were concomitantly compared.

2.12. Detection of the residual cytokines in Glucose+HSA+individual cytokine mixture after incubation for180 days.

We measured the amount of IL-2, IL-6, IL-17, TGF-β or TNF-α level in the supernatants by ELISA kit (R &D Systems, Minneapolis, MN, USA) after 180 days

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incubation in the mixture.

2.13. Statistical Analysis

All of the data were presented as mean ± standard deviation. Continuous variables were assessed with a commercially available software package (MedCalc for Windows, MedCalc Software Ltd, Ostend, Belgium) calculated by non-parametric Wilcoxon rank-sum test. A p value < 0.05 was considered statistical significance.

Results

3.1. Progressive changes of color, relative molecular weight, *p*H value, and glycation of amino acid residues during AGE-ALB formation

3.1.1. Progressive color changes during incubation

During the incubation of albumin+glucose mixture at 37℃, we noted that the color of the mixture progressively changed from transparent at day-7, to a light -yellow at day-60, yellow at day-90, orange-yellow at day-150, and finally brown -color with slight viscosity at day-180 as shown in Figure 4. However, the color of albumin alone remained transparency even after incubation for 180 days (data not shown). This finding is noted both in BSA and HSA mixtures.

3.1.2. Progressive increase in relative molecular weight during incubation

The relative molecular weight of AGE-ALB at different incubation periods was

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estimated by 10% SDS-PAGE. We found that the relative molecular weight of AGE-ALB gradually increased along with the incubation period, which may suggest the progressive increase in AGE-ALB formation (Figure 5).

3.1.3. Progressive change of *p*H value toward acidic during AGE-HSA formation

The *p*H value was 7.2 in both HSA alone and HSA+glucose mixture at day-0, which is equivalent to the *p*H value of PBS. Interestingly, the *p*H value of AGE-HSA mixture gradually became acidic with the prolongation of incubation period. Finally, the *p*H value of HSA+glucose solution with the progressive formation of AGE reached 5.35 vs. 6.85 in HSA only in day-180 (Figure 6). It is speculated that the mild acidic change in *p*H value from 7.2 to 6.85 in has only solution was due to the absorption of CO_2 from 5% CO_2 incubator in the long-term culture. In contrast, the accelerated acidic *p*H value in AGE-HSA was due to the progressive loss of alkaline amino acid, lysine and arginine residues, by glycation +CO_2 absorption from CO_2 incubator as shown in Figure 6. The same experiment has been repeated twice with a similar tendency. Accordingly, the *p*H changes toward acidic can be used as a biomarker for HSA glycation formation.

For elucidating the factors contributing to HSA glycation formation by Maillard reaction, we have tested the affection of incubation environment by altering the solution charge or [Ca^{2+}] concentration. The positively charged poly-L-lysine

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(PLL), negatively charged dextran, or calcium ion chelating agent EGTA was individually added to HSA-glucose mixture and incubation for 1-180 days. We use the pH change, as a useful and simple surrogate biomarker for AGE-HSA formation, for measuring AGE formation periodically. We found that the kinetic change or the final pH value was not different after changes the ionic charge or [Ca^{2+}] as shown in Figure 7A, 7B and 7C. Accordingly, these results may suggest that the Maillard reaction between HSA and glucose is independent of charge-charge interaction or [Ca2+] concentration in the solution. The real mechanism is now under investigation

3.1.4. Kinetic glycation of amino acid residues in AGE-HSA molecule

After incubation with glucose, the % glycated lysine in BSA is gradually increased along with the incubation period from 77% at day-30, 100% at day-90, to 100% at day-180 (Figure8, left panel). In HSA molecule, the % glycated lysine is 68% at day-30, 85% at day-120 and 92% at day-180 (Figure8. right panel). In contrast, the % glycated arginine in BSA also progressively increased from 5% at day-30, 8% at day-90, and 16% at day-180 (Figure8, left panel). In HSA molecule, the % glycated arginine is 9% at day-30, 31% at day-120 and 35% at day-180 (Figure8, right panel). However, there is no glycated cysteine in AGE-ALB can be found after 180 days incubation (data not shown). The full table result of LC/MS is

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shown in Table 1.

3.1.5. To detail the degree of glycation in each glycated lysine in HSA molecule, we performed mass glycan study and revealed that not only amount of glycated lysine increased, but also the degree of glycation deepened along with incubation period (Figure 9). We determined > 30% of glycation in a specific lysine site to be deeply glycated. We can see the number of deeply glycated lysine increasing gradually along with incubation period. The detail data of glycan mass study is shown in Table 2.

3.1.6. Difference of lectin- binding capacities between HSA and AGE-HSA

Same with previous result shown in master's thesis, the lectin binding of AGE modified protein differs from the origin protein. In HSA, the lectins with highest binding affinity are DBA, MALECTIN, PSL1A, RS-FUC and VVA. DBA is the abbreviation of dolichos biflorus agglutinin with a carbohydrate specificity toward α-linked N-acetylgalactosamine and is used for determine group A blood typing and some literature suggests it correlate with nature killer cells home with uterus in animal model[119]. Malectin is a membrane-anchored endoplasmic reticulum (ER)-resident lectin and plays an important role in the quality control of newly synthesized glycoprotein through recognizing the Glc2Man9GlcNAc2 (G2M9) oligosaccharide chain[120]. PSL1A is an

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abbreviation of Polyporus squamosus Lectin 1a and possess cytotoxic effect in cancer cell lines with recognition of α2-6 sialic acid[121]. RS-Fuc is a fucose-binding lectin isolated from Ralstonia solanacearum and these fucose-binding lectins are believed to contribute in fucose clearance in liver[122, 123]. VVA is the abbreviation of vicia villosa agglutinin with recognition for α- or β-linked terminal N-acetylgalactosamine and in vitro study suggest it might contribute to breast cancer lymphatic metastasis and Hashimoto thyroiditis[124, 125]. These data suggest that HSA possess effects to clear fructose, maintain glycoprotein synthesis and some anti-cancer activity.

In AGE-HSA, the lectins with the highest binding affinity are BANLEC, CGL2, ConA, DBA, MOA, PSL1A, RS-FUC, SAMB, VVA. BANLEC is abbreviation of Banana Lectin and is a jacalin-related lectin isolated from Musa acuminate with recognition of mannose, Glucose or branched high-mannose containing α1,3-glycoside bond. It is known to be related to inhibit HIV and immune modulation effects[126-129]. CGL2 is a mushroom galectin, Coprinopsis cinereal CGL2, with the recognition of β-Galactosidase, GalNAc-α1-3-Gal (Blood Group A) or Gal-α1-3-Gal (Blood Group B). CGL2 is suspected contribute to defense of fungal infection[130]. ConA is abbreviation of Concanavalin A extracted from the jack-bean (Canavalia ensiformis) with the

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recognition of α-Mannose and α-Glucose. It is known to be a T cell subset stimulator in animal model and may contribute anti-cancer activity[131, 132]. MOA is abbreviation of Marasmius oreades lectin with recognition of non-reducing terminal Gal-α1-3-Gal-carbohydrates and the determinant of blood type B, Gal-α1-3[Fuc-α1-2)-Gal. It is a calcium-dependent cysteine protease and may contribute to defense infection in mushroom. The effect to animal is not yet clear[133, 134]. SAMB is abbreviation of sambucus sieboldiana agglutinin with recognition of NeuAcα2-6Gal/GalNAc. It may be correlate with early lung cancer detection and colon cancer metastasis[135, 136].

Comparing the difference of lectins between HSA and AGE-HSA, we can find that Malectin is loss when modified to AGE but BANLEC, CGL2, ConA, MOA, SAMB binding increased. This may suggest that homeostasis effect of HSA is loss in AGE-HSA but immune cell stimulation effect may be more potent.

The references of total lectin examined and the binding character between the two is shown in appendix Table.

3.2.Expression of RAGE on the cell surface

We have used the indirect immunofluorescence antibody staining method and found RAGE was expressed on the three cell lines with different receptor density. Jurkat T cell exhibited modest whereas THP-1 and HCAEC cells exhibited robust

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stain as shown in Figure 10.

3.3.Suppression of Jurkat T cell IL-2 production by AGE-HSA

3.3.1. No cytotoxic effect of AGE-HSA on human Jurkat T cells

Firstly, we confirmed that AGE-HSA at the final concentration of 40μg/mL was not cytotoxic to human Jurkat T cells detected by CCK-8 assay (Figure 11A).

3.3.2. The IL-2 (a pluripotential cytokine for T lymphocyte homeostasis, development, and proliferation) production was significantly decreased by AGE-HSA at the concentration of 40μg/mL by anti-CD3+ani-CD28 activated Jurkat T cells. The median IL-2 concentration is 565.82pg/mL in HSA vs. 452.1pg/mL in AGE-HSA ($p = 0.05$) as shown in Figure11B.

3.3.3. Molecular basis of suppressive effect of AGE-HSA on Jurkat T IL-2 production

We extracted the total cell lysates in western blot experiment for detection of the expression of phosphorylated STATs and the negative transcription regulators, CIS and SOCSs. We found that the p-STAT3 (at site 727), p-STAT4 (at site 693), and p-STAT6 (at site 641) are significantly down-regulated, but no distinct change of the cytokine negative regulators CIS, SOCS1 and SOCS7 as shown in Figure 11C. These results may suggest that the suppression of Jurkat T on IL-2 production by AGE-HSA is related to the inhibition on the JAK-STAT signaling pathways, but irrelevant to cell

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cytotoxicity. These results are compatible with the already known fact that T cell-mediated immune responses and impaired JAK-STAT signaling are usually found in the aging leukocytes resembling immunosenescence [137, 138].

3.3.4. The effects of RAGE inhibitor on IL-2 production by Jurkat T

The suppressive effect of AGE-HSA on IL-2 production by Jurkat T cell is ameliorated after RAGE block. This may suggest that receptor of AGE contributes a crucial role in the suppressant effect of AGE on T cells. The result is shown in Figure 12.

3.3.5. Increase tendency of senescence associated β-galactosidase (SA-βgal) expression in Jurkat T cells by AGE-HSA

For testing the cellular senescence-inducing capacity of AGE-HSA on Jurkat T cells, we directly measured the SA-βgal expression after incubation with AGE-HSA. We found an increased tendency of the cellular senescence biomarker, SA-βgal, expression in Jurkat T cells, although not reach the statistical significance (Figure13A). However, AGE-HSA exerted neither significant suppression on telomerase activity (Figure13B) nor the relative telomere length (Figure13C) compared to HSA. These results may indicate that AGE-HSA may probably exhibit an immunosenescence activity on Jurkat T cells.

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Nevertheless, the lack of telomere length or telomerase activity in our experiments may be related to insufficient 9 days culture period.

3.3.6. No alteration in the T cell populations

In addition to the potential senescence-inducing capacity as shown above, whether AGE-HSA inhibition of IL-2 production contribute by altering the T cell population differentiation is also a possibility. We detected the T cell subset by the expression of transcription factors, as T-bet for Th1, GATA-3 for Th2, and FoxP3 for Treg by western blot. As shown in Figure14, no change of the expression of these transcription factors could be found. The T cell subset remained to be Th1 after AGE-HSA stimulation. These results may indicate the non-specific immuno-suppressive effects by AGE-HSA *per se* rather than the induction of Treg cell predilection.

3.4. Enhancement of the pro-inflammatory (CCL-5, IL-8, and MIF) and anti-inflammatory (IL-1Ra) cytokines production from human macrophage-like THP-1 cells by AGE-HSA. In addition to the non-specific suppressive effect of AGE-HSA on T cells, macrophage, a major effector cell involving in the inflamm-aging, is another target for investigation.

3.4.1.No cytotoxic effect toward human THP-1 macrophages by AGE-HSA AGE-HSA at a final concentration of 40μg/mL was not cytotoxic to THP-1 cells

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(Figure15A) as confirmed by CCK-8 assay.

3.4.2. Significant enhancement of pro-inflammatory and anti-inflammatory cytokines production by THP-1 macrophages after interaction with AGE-HSA

We found AGE-HSA significantly enhanced inflamm-aging related pro-inflammatory cytokines and chemokines, such as CCL-5 (Figure15B), IL-8 (Figure15C), and MIF (Figure15D) expression as well as the anti-inflammatory cytokine IL-1Ra (Figure 15E) produced by THP-1 macrophages. CCL-5 and IL-8 are also members of senescence associated secretory patterns.

3.4.3. Downregulation of SA- β-gal expression in THP-1 macrophage by AGE-HSA

In consistence with these macrophage-enhancing activities, the senescence marker, SA-βgal expression was also found a tendency of down-regulation by AGE-HSA (Figure 15F). The telomerase activity was not changed compared to HSA (Figure15G). These results may indicate that AGE-HSA can potentially elicit inflammation and increase SASP production in macrophages through activation as found in patients with diabetes and immune-mediated diseases.

3.4.4. Effect of RAGE inhibition on the production of CCL5, IL-8, MIF and IL-1Ra formation by THP-1 macrophages

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The result of RAGE inhibition to the effects of AGE on macrophage is shown in Figure 16. The production of CCL-5 and MIF was interfered by RAGE inhibition, but IL-8 and IL-1Ra are not affected. These results suggested that among SASP, IL-8 and CCL-5 production, may induce immunosenescence via different receptor binding and subsequent signaling pathways.

3.5. Abrogation of HSA-mediated homeostatic effects on HCAEC cells by AGE-HSA

HSA has been demonstrated not only able to maintenance of plasma colloid osmotic pressure, but also possesses anti-inflammatory effects for maintaining vascular homeostasis [139, 140]. These homeostatic effects include reduction-oxidation regulation, transporter of endogenous and exogenous compounds, and acting as a chemical sponge for protection from harmful chemical agents, those can be found defective in the inflamm-aging reaction [11].

3.5.1.No cell cytotoxicity on HCAEC by AGE incubation

We performed CCK-8 assay as previously described and we also found that there is no significance difference was found. The result is shown in Figure 17A.

3.5.2. Abrogation of anti-inflammatory homeostasis effects of HSA on HCAEC by AGE-HSA

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We originally found that some new homeostatic anti-inflammatory effects of HSA on the vascular endothelial cells. These effects include increased release of soluble form E-selectin (Figure17C) and soluble form of ICAM-1 (Figure17B), those are capable of binding with immune cells to prevent immune cells adhesion to the endothelial cells. The increased endothelin release (Figure 17D) from HSA after incubation with vascular endothelial cells can maintain the appropriate vascular tone. These physiological functions of HSA are reversed by its denatured molecules, AGE-HSA.

3.5.3. Enhancement of senescence in HCAEC as reflected by increased SA-βgal expression by AGE-HSA

The effects of AGEs on the cellular senescence-inducing capacity of HCAEC could be reflected by enhancing SA-βgal expression (Figure17E). Similar to Jurkat T cells and THP-1 macrophages, neither decreased telomerase activity (Figure17F) nor shortening of telomere length (Figure17G) could be found by AGE-HSA.

3.5.4. Effects on HCAEC functions by AGE-HSA after RAGE inhibition

The effects of RAGE inhibition on HCAEC functions are shown in figure 18. There is no difference of AGE effects on HCAEC after RAGE inhibition.

3.6. Comparison of AGE-HSA formation between HSA+glucose vs. HSA+glucose+

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individual inflammation-related cytokine mimicking the environment of inflamm- aging in patients with age-related disease and aging process. Our results indicate that various inflammation-related cytokines not only accelerated but enhanced AGE-HSA formation as suggested by the following results.

3.6.1. Faster *p*H toward acidic in the HSA + glucose+ individual cytokine group

The *p*H value progression toward acidic is significantly faster in Day 14 incubation and deeper till D180 incubation, in the HSA+ glucose+ cytokine group rather than HSA+glucose. There is no distinct *p*H changes in the HSA+cytokine group (Figure19A) in the course of 180 days.

3.6.2. No distinct molecular weight change in the presence of various cytokines

There is no distinct molecular weight change in the presence of various cytokines compared to absence of cytokine (Figure19B). The negligible molecular weight increases in the presence of different cytokine as shown in Figure19B may favor the individual glycosylation of HSA and cytokine molecules rather than the conjugation of the two protein molecules following glycosylation via Maillard reaction. This phenomenon may also contributed by the very small amount of cytokines added comparing with HSA concentration.

3.6.3. Accelerated glycation of HSA in the presence of inflammation-related cytokines

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We compared the amount of AGE-HSA after 90 days incubation (left panels of Figure19C and Figure19D) with or without inflammatory cytokine. We found a tendency of increased CML and CEL formation in AGE+cytokine vs. AGE after 90 days incubation. Increased CML and CEL formation of AGE-HSA with and without cytokines at 180-day incubation (right panels of Figure 19C and Figure 19D) were also noted. AGE group without cytokine catching of those incubated with cytokines detected by CML (Figure19C) formation when 180-day incubation. However, while detecting CEL (Figure19D), AGEs without cytokines remained lower than those with cytokines. These results are compatible with the pH level change, which suggest AGE-incubate with cytokines with faster and deeper glycation.

3.6.4. Increase in the intensity of amino acid glycation in the presence of cytokine rather than without cytokine after 30 days incubation

The glycation mass spectrometry detection revealed increased the number of lysine glycation after 30 days incubation in the presence of individual inflammation-related cytokine as shown in Figure 20. The complete glycan mass table is in table 4.

3.6.5. Decrease of residual cytokine in the supernatants after incubation of HSA+glucose+ individual cytokine vs. HSA+ individual cytokine

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To elucidate the mechanism between inflammation related cytokines and AGE formation, we have conducted experiments by comparing the residual cytokines in the supernatants after incubation of HSA+glucose+individual cytokine vs. HSA+ individual cytokine for 14 days and 180 days. We noted that the residual cytokine in the supernatants of the former combination seemed significantly less than the latter combination (Figure 21).. This finding not only suggests the nature degradation of cytokines after long-term incubation but the possible binding or consumption between AGE and the individual cytokine via unknown biochemical effect(s)other than Maillard reaction is also possible.

3.7. The pathological effects and the potential molecular basis of AGE-HSA on the formation of inflamm-aging composing of T cell immunosuppression, macrophage-mediated inflammation, and endothelial cell dysfunction as vasculopathy in patients with age-related disease, aging process and immune-mediated disease are summarized in Figure 22.

Discussion

4.1. Scientific soundness of our findings

In the present study, we clearly demonstrated that AGE-HSA can exert at least 5 pathological effects on inflammation-related, immune-related, and vascular

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endothelial cells *in vitro*. These effects include: (1) Inhibition of IL-2 production by Jurkat T cells via suppressing phosphorylated STAT3, 4, and 6 cytokine signal molecules expression. In addition, the AGE-HSA potentially causes T cell immunosenescence as shown by increasing SA-βgal. (2) Enhanced CCL-5, IL-8 and MIF production by human macrophage-like THP-1 cells for enhancing inflammatory reaction via suppression on SA-β gal expression. (3) Abrogation of normal HSA physiological functions with releasing soluble adhesion molecules, sICAM-1 and sE-selectin to prevent immune-related cells adhesion to vascular endothelial cells. Furthermore, decreased vasoactive endothelin secretion disturb vascular homeostasis. These pathological effects may contribute to the endothelial cell senescence as present by increasing SA-βgal. (4) The 5 inflamm- aging and inflammation related cytokines including IL-2, IL-6, IL-17, TNF-α, and TGF-β not only accelerated but increased Maillard reaction in the *in vitro* study. These deleterious effects of AGE-HSA can lead to a pathological state mimicking inflamm-aging in patients with DM and immune-mediated diseases. (5) The gradually decreases in *p*H value toward acidic can become a useful biomarker for the alkaline amino acid glycation in the AGE-ALB formation.

4.2. Receptors binding and the intraceullar signaling of AGE-HSA in the 3 cell lines.

It has been demonstrated that three kinds of surface receptors expression on the

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cells including AGE-receptors (AGE-R), RAGE, and scavenger receptors (SR) capable of binding with AGEs. The effects of AGE-RAGE axis in activating inflammatory reaction have been extensively reviewed in the literatures [70, 72, 139-141]. In general, the AGE–RAGE axis activation in somatic cells and immune-related cells may transduce signals for inflammatory reactions and oxidative stress that facilitate oxidative stresses and inflammation reactions involving in AGE-related diseases. These inflammatory reactions can damage retinal pericytes, vascular endothelial cells, renal podocyte/mesangial/endothelial/ smooth muscle cells, and cause diabetic triopathy. In our study, we only detected RAGE on the cell surface with modest expression on Jurkat T, but robust on THP-1 and HCAEC cells (Figure 10). However, the AGE-RAGE axis is not entirely corresponded to the effects of AGE-HSA on the 3 cell lines, since some responses are not altered after addition of RAGE inhibitor as shown in Figure 12, 16 and 18. Accordingly, the exact cognate receptor(s) responsible for AGE-HSA on Jurkat T, THP-1 and HCAEC cells needs further investigation. The other receptors, AGE-R1, AGE-R2, AGE-R3 and scavenger receptors currently encounter the difficulty about lack of commercially stable inhibitor and further investigation remained continuing.

We also investigated the cytokine signaling molecules involving in suppressing

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IL-2 gene expression by AGE-HSA. Our results revealed that the molecular basis for IL-2 suppression by AGE-HSA were mainly mediated via inhibition on p-STAT3, p-STAT4 and p-STAT6 expression, but irrelevant to the enhanced negative cytokine regulators. Some investigations have reported that AGEs can promote naïve T cell differentiation [142] and monocyte proliferation [143]. Our results seemed partly compatible with them. Besides, we further demonstrated that AGE-HSA can compromise T cell immunity and elicit macrophage inflammatory reaction, which are commonly found in the patients with DM and inflamm-aging. Interestingly, the impaired JAK/STAT signals have been also reported in the elderly individuals [144, 145]. We suggest that the similar findings between AGE-HSA stimulation and the inflamm-aging in the elderly peoples may suggest a potential effect of AGE-HSA in causing immunosenescence. In addition to induce immunosuppression and inflammation, we firstly demonstrated that AGE-HSA potentially possesses the capacity to induce cellular senescence in both HCAEC endothelial cells (Figure17E) and human Jurkat T (Figure13A) and) by enhancing SA-βgal expression. However, no significant difference of the telomerase activity and relative telomere length can be noted after incubation of AGE-HSA with these cell lines (Figure13B and 13C, Fig 15F and 15G, and Figure 17F and Figure 17G). These results can be explained that the overt cellular senescence is a long-term

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process and can be not induced in a short-term as in the *in vitro* culture for around 9 days as in our experiments. Many authors have demonstrated that the molecules in the different signaling pathways involve in the aging process including mitochondrial DNA mutation signaling [93], AMP- activated protein kinase (AMPK) and its target signaling [89, 90, 146], NF- κ B signaling pathway [147], cAMP response element-binding protein (CREB) pathway[147], autophagy dysfunction signaling [92], and sirtuin signaling [91]. Whether AGE-HSA can elicit these different signaling pathways to accomplish cellular senescence is now under investigation.

4.3. Effects of inflammation-related cytokines on accelerated AGE-HSA Maillard reaction and possible mechanism of inflamm-aging

We also confirmed that the patients with different connective tissue diseases on normal glycemic status such as RA, SLE, PSS, vasculitis, AOSD and SpA contained high AGE-HSA levels in the serum (Figure19A). This scenario is quite similar to age-related diseases with inflamm-aging. For elucidating the molecular basis of it, we added the inflammation-related cytokines including IL-2, IL-17, or those SASP-related cytokines such as IL-6, TNF-α or TGF-β to the HSA+glucose mixture for incubation compare to those without cytokine. We found that AGE-HSA formation can be accelerated and enhanced by these inflamm-aging or

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inflammation-related cytokines as reflected by the accelerated acidic *p*H changes (Figure19B). Furthermore, the increased formation of N^{ε}-carboxyethyl-lysine (Figure19E) and N^{ε}-carboxymethyl- lysine (Figure19D) in 90 days and 180 days incubation *in vitro* were found compared to HSA+cytokine or HSA+glucose alone. Glycation mass spectrometry study also revealed the extent of glycation increased in HSA+glucose+individual cytokine (Figure 20) in D30 incubation. These results may suggest that the inflammatory milieu-related cytokines can potentially increase the total amount of AGE-HSA formation *in vivo*. However, we noted that the molecular weight of HSA+glucose+cytokine combination was not difference from those without cytokines (Figure19C). It is explained that the amounts of cytokines we added were around 70ng/mL, which is far below the lowest detection limit in SDS-PAGE. However, we have compared the cytokines remaining in the supernatants after incubation of HSA+glucose+individual cytokine vs. HSA+individual cytokine alone for 14 and 180 days. We noted that the residual cytokines in the supernatants of the HSA+glucose+individual cytokine are less than the HSA+individual cytokine group (Figure 21). These results may suggest that at least a part of inflammation-related cytokines bound to the AGE-HSA molecule to accelerate the Maillard reaction. However, other modalities such as modifications of Maillard reaction by cytokines *per se* or

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changes of HSA steric structure by cytokine, remain possible. Besides, whether the glycated inflammatory-related cytokines possessing pathophysiological effects are worthy for further investigation.

4.4. AGE-HSA is able to abrogate specific physiological functions of HSA on the vascular endothelial cells and induces senescence of Jurkat T cell and HCAEC

From lectin binding array, we have proved that HSA possess the ability to control glycoprotein synthesis in ER by the lectin MALECTIN. Human serum albumin has been reported not only a nutrient or colloid solution, but also possesses many specific physiological functions on the vascular endothelial cells. These functions include prevention of endothelial apoptosis [91], protection of endothelial cell glycocalyx [89], improvement of endothelial integrity [95], activation of intracellular process [95], modulation of nitric oxide pathway [92], anti-inflammatory effects [93, 94], modulation of intracellular volume [98-100], modulation of electrolyte shifts and acid-base balance [96], and binding of drugs and other plasma substance [97]. In the present study, we originally demonstrated that HSA can enhance the release of sICAM-1, sE-selectin, and endothelin, by HCAECs for preventing the immune cells adhesion to the vascular endothelial cells in causing vasculopathy. It is well known that surface-expressed ICAM-1 molecule on leukocytes is able to facilitate the leukocytes emigration from

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intravascular circulation but not continuously adhere to the vascular endothelial cells [109]. On the contrary, the released sICAM-1 molecule from endothelial cells is able to prevent leukocytes adhesion but keep the cells free movement in the intravascular circulation in maintaining intravascular homeostasis. Another intercellular adhesion molecule, the surface-expressed E-selectin, mediates leukocyte rolling on the endothelial surface for facilitating the recruitment of T cells, neutrophils, and monocytes to the inflammatory foci [107]. On the other hand, the released sE-selectin in the blood stream also facilitate leukocytes free movement within the blood circulation instead of adherence to the blood endothelial cells for maintaining intravascular homeostasis. Endothelin, a potent vasoconstrictor unique in the vertebrae, is responsible for maintaining the intravascular pressure [108] in normal vascular physiology. Obviously, AGE-HSA as a transformed HSA molecule, would abrogate this HSA-mediated physiological homeostasis.

Besides, we have originally identified the cellular senescence-inducing effect of AGE-HSA on vascular endothelial cells (Figure 17E) and T cells (Figure 13A) evidenced by the increased SA-βgal expression. Coleman et al. [148] demonstrated the low expressed VCAM and E-selectin on the cell surface of a human senescent umbilical vein-derived endothelial cells. This is compatible with

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our findings. Furthermore, Prattichizzo et al. [149, 150] and Pantsulaia et al. [151] discovered that the senescent endothelial cells are potential modulators for inflamm-aging. In short summary, serum HSA is crucial for maintaining the endothelial cell functions and vascular homeostasis. Conversely, AGE-HSA possesses the potential capacity to induce endothelial cell senescence. Although the involvement of AGEs in the diabetic vasculopathy have been widely reported [28, 152, 153], the effects of AGE-HSA on endothelial cell senescence remained to be investigated. Obviously, our results can provide direct evidence of AGE-HSA-mediated endothelial dysfunction and aging in patients with diabetes and immune-mediated diseases.

4.5. Alteration of the AGE formation by diets and other potential factors

In addition to hyperglycemia, some environmental factors, such as Mediterranean diet, high dietary AGE content, and cigarette smoking may affect AGE formation. Besides, patients with renal proximal tubular damage may also impair the catabolism and clearance of pre-formed AGE due to glyoxalase deficiency. Recently, a number of phytochemicals and biomolecules were confirmed effective in the cell-based or animal studies including AGE formation inhibitors, AGE breakers, AGE–RAGE axis blockers, or glyoxalase stimulators. In addition to traditional anti-hyperglycemic and anti-hypertensive drugs, these compounds are expected to become novel therapeutic agents in future.

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In conclusion, we have provided substantial data to support the AGE-HSA can induce vasculopathy, non-specific immune suppression, and inflammation in the immune system by accelerating cellular senescence. These adverse effects are quite compatible with the definition of inflamm-aging in different immune-related diseases as well as diabetic patients.

4.6.Drawbacks

Nevertheless, at least 5 drawbacks exist in the present study. (1) The accurate molecular mechanism(s) for inflammation-related cytokines in accelerating and augmenting AGE-HSA Maillard reaction has not been explored. (2) The exact cell surface-expressed AGE-HSA binding receptor(s) responsible for the inflamm-aging has not been identified. The AGE-R1, AGE-R2, AGE-R3 and scavenger receptors are currently lacking commercially stable inhibitors, and we are currently considering to use RNAseq techniques to solve this problem. (3) The real biochemical modifying mechanism of albumin by glucose via Maillard reaction are still under investigation. (4) The signaling pathway(s) of AGE-mediated cellular senescence in HCAEC and Jurkat T cells were not elucidated, and (5) The detailed signaling pathway(s) involving in the macrophage-like THP-1 cells activation by AGE-HSA have not been explored.

Chapter 5. Future prospective

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The following prospective investigations will be conducted for solving the enigmas in the pathological roles of AGE in the daily clinical practice:

(1) The therapeutic strategy for preventing AGE-ALB on inflamm-aging should be established in our future study. We will use different glycolytic enzymes especially the glyoxalase I and II to modify AGE-HSA molecule and evaluate the functions of the modified AGE-HSA. Current candidate enzymes included α-glucosidase, α-galactosidase, β-glucosidase, β-galactosidase, glyoxalase I, glyoxalase II, O-sialoproteinase, neuramidase, and carboxypeptidase etc. We believe that the enzyme-modified AGE-HSA would alter their pathological effects on the inflamm-aging. We expect the effective enzyme(s) will be used as an enzyme therapy for age-related disease in the near future.

(2) As stated in the “Introduction” part, glucose is not the only mono-saccharide underlying glycation via Maillard reaction. The other mono-sacchrides including fructose, sucrose or galactose, are found plenty in the cells and may potentially cause the similar pathologic effects by Maillard reaction. We will prepare the AGE-HSAs by incubating HSA with the sugar moieties of fructose, sucrose, lactose, or even galactose. We will compare the effects of these different preparations on the inflamm-aging with AGE-HSA made up of glucose and evaluate their molecular pathogenesis.

(3) The molecular mechanism of inflammatory/inflammaging-related cytokines in

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accelerating AGE formation remained unknown. We have already demonstrated that the amount of inflammatory cytokine we used in the aforementioned studies was adequate to interfere AGE formation in physiological levels. Furthermore, the residual cytokines remained less in AGE mixture than in HSA solutions. However, it is worthy to know whether the cytokine can bind with HSA or is independently glycated remains elucidation. We will also work up if there is a does dependent effects of cytokines. We will keep working on unveiling the real mechanism of the inflammatory cytokine affecting AGE formation in different autoimmune diseases.

(4) The altered lectin binding in AGE-HSA is noted in our study and most of the lectin binding in AGE-HSA contribute to immune cell activation, anti-cancer activity and fungal infection defense mechanism. We would also exam if AGE-HSA being an effective agent for defensing fungal infection by stimulate fungal cells with AGE-HSA. After stimulation, we will detect the fungal cell survival and cell function.

(5) We will observe the long-term effects of hyperglycemic environment on the immune, inflammation, and aging processes of T lymphocytes. For achieving these purposes, we will long-term culture the immune-related cells with a high glucose medium. According to manufacturer's list, the glucose concentration in RPMI 1640 culture medium is 100mg/dL. We will elevate the glucose concentration to 200mg/dL for culturing Jurkat T and THP-1 macrophage-like cells for at least 3 to 4 generations.

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Then, the cell activation reaction, cell survival, and cell senescence will be evaluated.

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The publications related to the present dissertation by author:

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1. Shen CY, Wu CH, Lu CH, et al. Advanced glucation end products of bovine serum albumin suppressed Th1/Th2 cytokine but enhanced monocyte IL-6 gene expression via MAPK-ERK and MyD88 tranduced NF-κB p50 signaling pathways. Molecules 2019, 24, 2461.

2. Shen CY, Lu CH, Wu CH, et al. The development of Maillard reaction, and advanced glycation end product (AGE)-receptor for AGE (RAGE) signaling inhibitors as novel therapeutic strategies for patients with AGE-related diseases. Molecules 2020, 25, 5591.

3. Shen CY, Li KJ, Wu CH, et al. Unveiling the molecular basis of inflamm-aging induced by advanced glycation end products (AGEs)-modified human serum albumin (AGE-HSA) in patients with different immune-mediated diseases. Clin Immunol 2023, 252, 109655.

4. Shen CY, Lu CH, Wu CH, Li KJ, Kuo YM, Hsieh SC, Yu CL. Molecular Basis of Accelerated Aging with Immune Dysfunction-Mediated Inflammation (Inflamm-Aging) in Patients with Systemic Sclerosis. Cells. 2021, 10, 3402.

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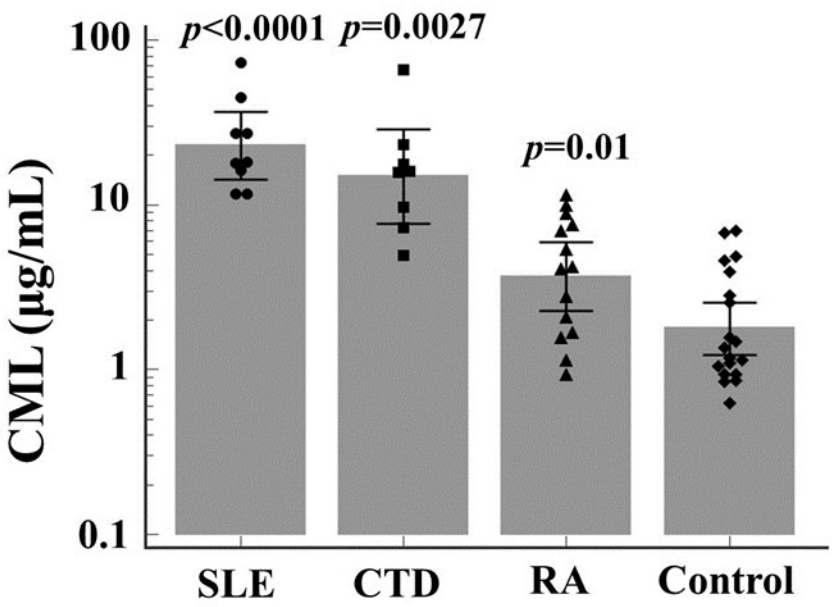

Figure 1. Increased serum levels of CML in patients with SLE, connective tissue diseases, and RA compare to normal health controls. The patients with connective tissue diseases (CTD) include 2 systemic sclerosis, 1 anti-phospholipid antibody syndrome, 2 adult-onset Still's disease (AOSD), 1 polymyositis, 1 seronegative spondylarthritis.

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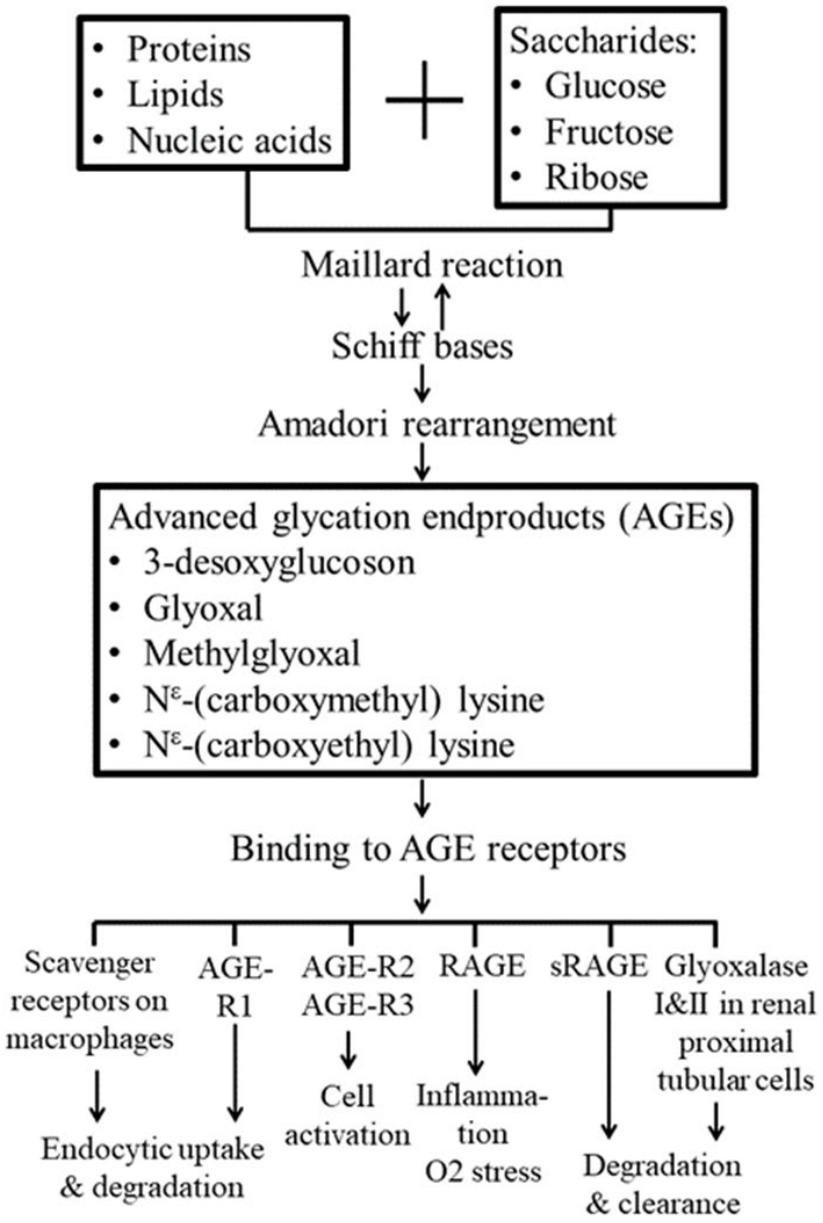

Figure 2. A scheme demonstrating the advanced glycation end-product (AGE) formation via Maillard reaction to form different AGE products and the functions of different AGE-binding receptors after binding with AGEs. The binding receptors for AGE include scavenger receptors on macrophage containing AGE-R1, R2, and R3, and receptor of AGE (RAGE).

Abbrevations: AGE-R1, R2, and R3: AGE receptors R1, R2, and R3; RAGE: receptor

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for AGE; sRAGE: soluble-form RAGE.

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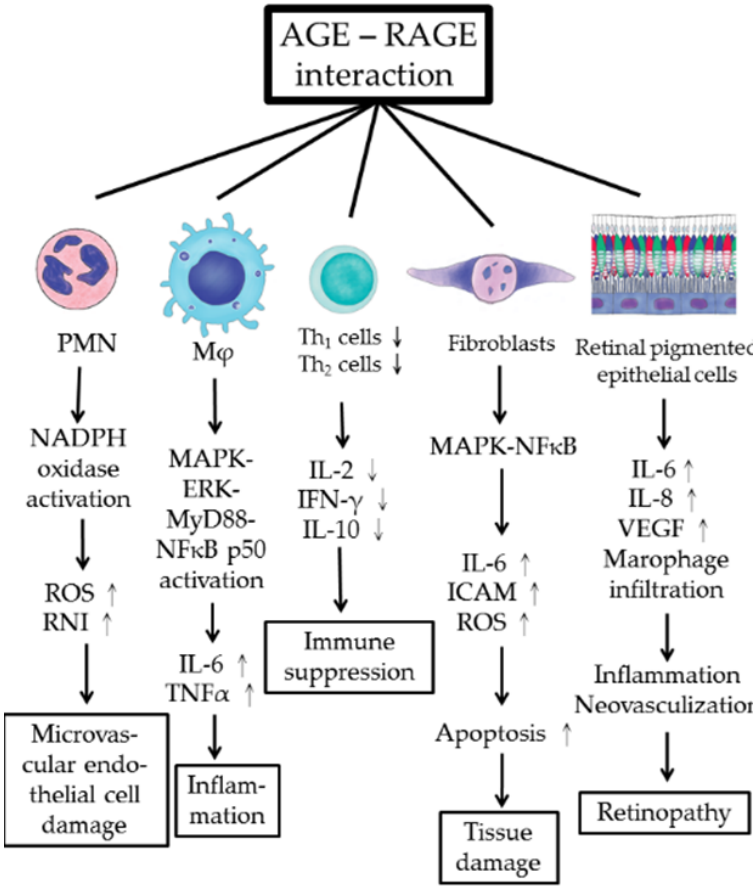

Figure 3. The cellular and molecular pathogenesis of AGE–RAGE axis activation in different cell types to induce different pathological effects.

Abbreviations: ROS: reactive oxygen species; RNI: reactive nitrogen intermediates; ICAM: intercellular adhesion molecule; VEGF: vascular endothelial cell growth factor.

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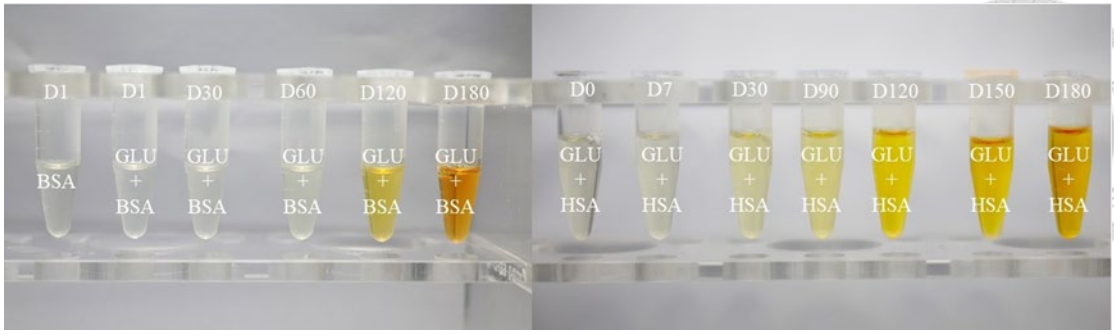

Figure 4: Progressive color change from transparency (before day-7), light yellow (between day-7 to day-60), yellow (between day-60 to day-90), orange-yellow (between day-90 to day-150), and finally brown color with slight viscosity (at day-180). Left panel: dynamic color change of BSA+glucose; Right panel: dynamic color change of HSA+glucose.

Abbreviations: GLU, glucose; BSA, bovine serum albumin; has, human serum albumin

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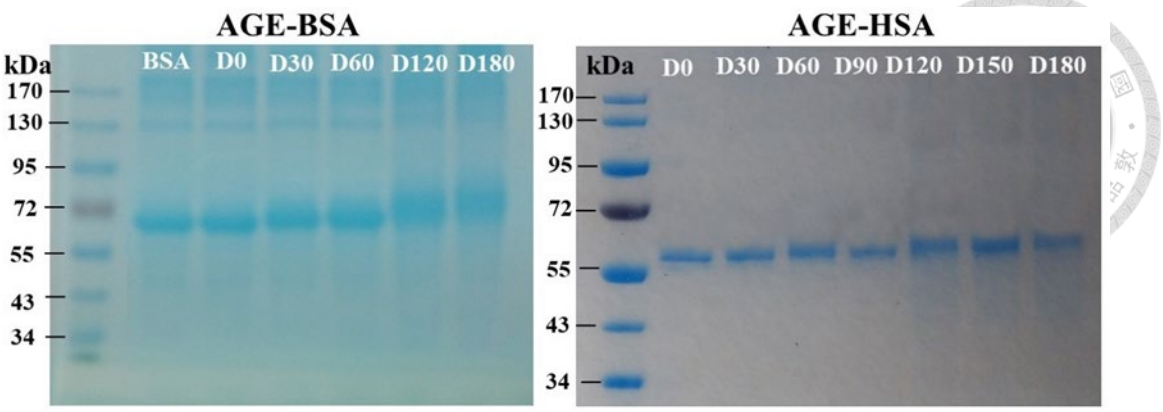

Figure 5. Progressive increase in the molecular weight of AGE-ALB after incubation from day0 to day180 detected by 10% SDS-PAGE. The molecular weight is gradually increased along with incubation periods. The similar pattern can be found in both AGE-BSA (left panel) and AGE-has (right panel).

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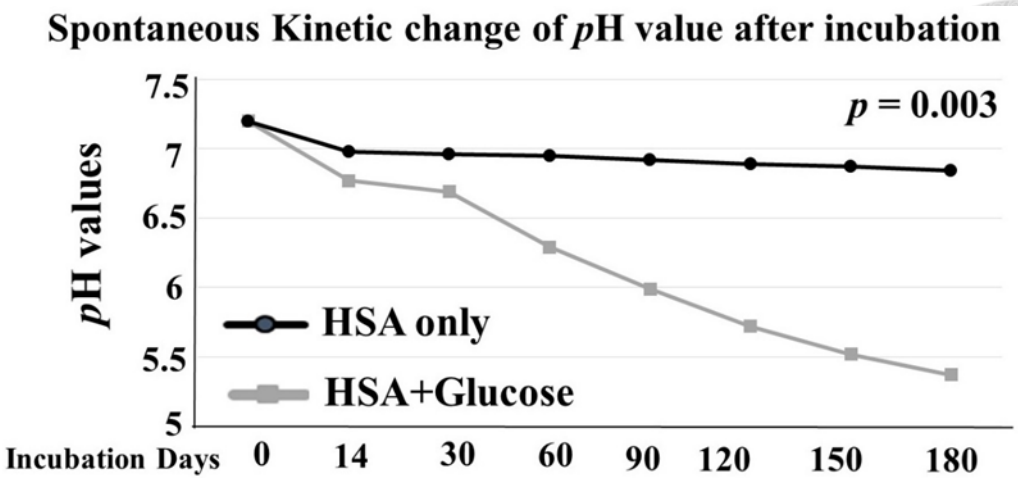

Figure 6: Comparison of *p*H changes between HSA+glucose vs. HSA alone during incubation in 5% CO2-95% air for 0-180 days. The *p*H value of AGE+glucose mixture gradually became acidic from 7.2 to 5.35 with the prolongation of incubation period whereas only modest changes in HSA alone group from 7.2 to 6.85.

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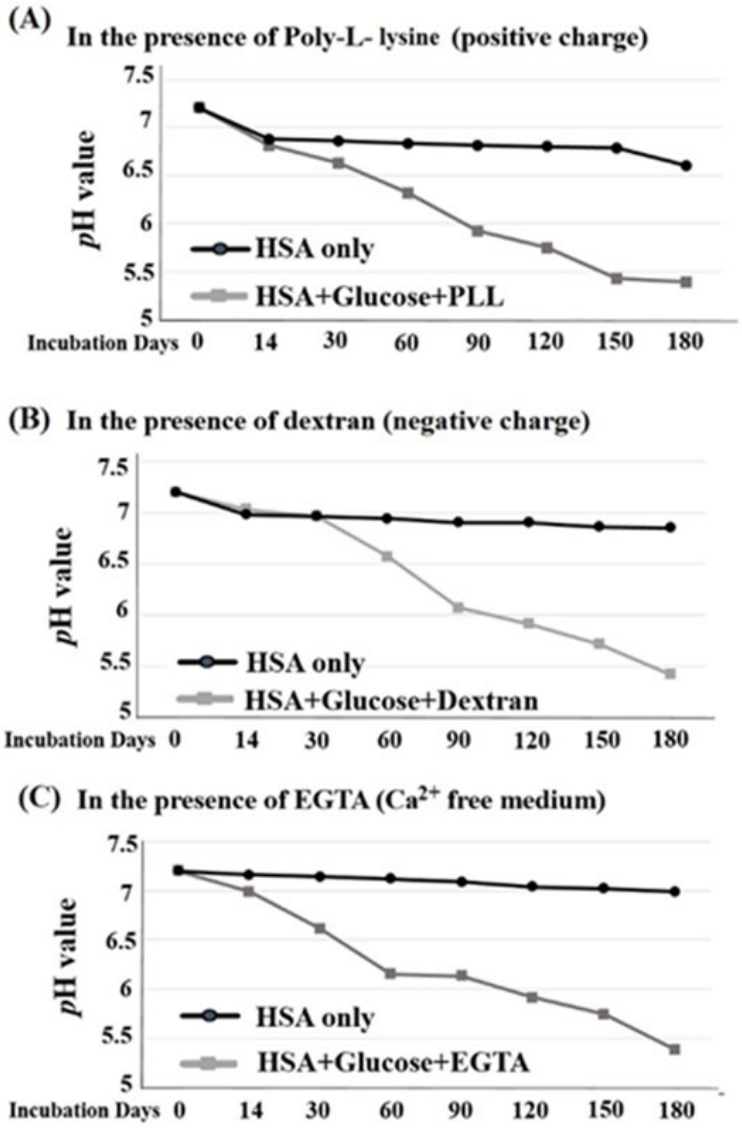

Figure 7: The effect of ionic charge and [Ca^{2+}] concentration on the pH change of HSA+glucose mixture during Maillard reaction for 0-180 days. (A) The addition of poly-L-lysine to form a positively charged milieu. (B) The addition of dextran to form a negatively charged milieu. (C) The addition of EGTA to form a [Ca^{2+}]-free milieu. The pH values in both HSA+glucose and HSA seem no change during 180 days incubation.

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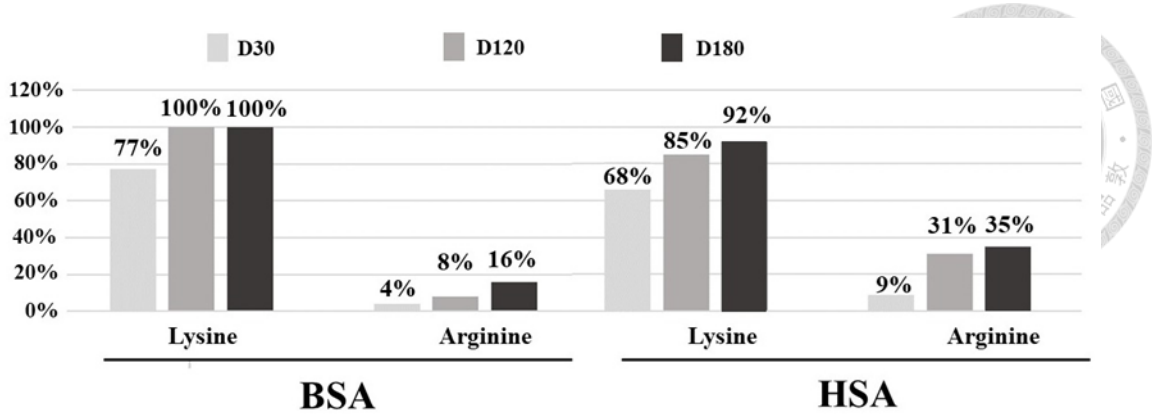

Figure 8. Progressive increase in lysine residue glycation and arginine residue glycation in both BSA+glucose (left panel) and HSA+glucose (right panel) after incubation for 30days, 120 days and 180 days detected by LC/MS. The lysine and arginine residue glycation increased along with the incubation periods compatible with the color and molecular weight changes.

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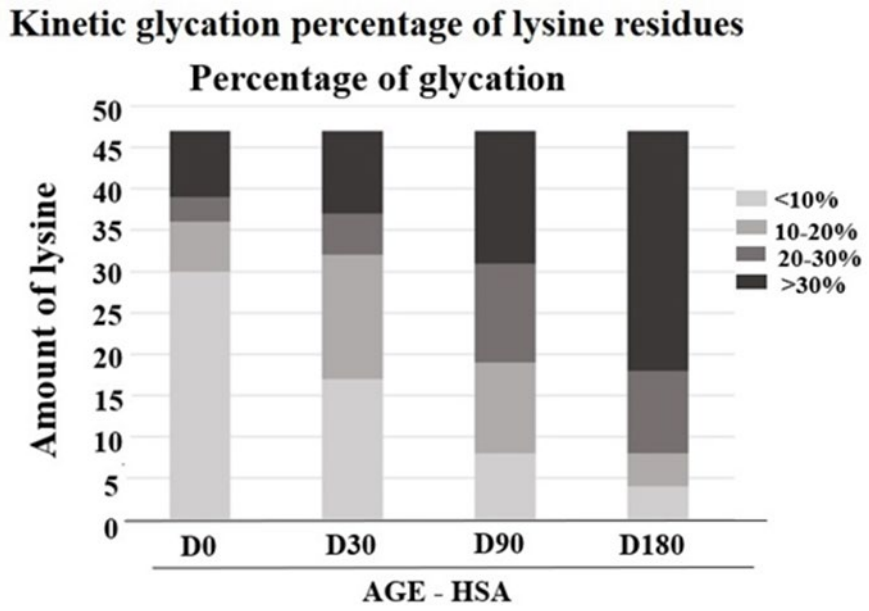

Figure 9. The progressive increased glycation of lysine residues in AGE-HSA molecules after incubation from D0 to D180 as detected by glycation mass spectrometry. In the AGE mixture, the amount of deeply glycated lysine, which suggest the site with >30% lysine glycation, is significant increase after D30. These findings are also compatible with the color changes in Figure 4.

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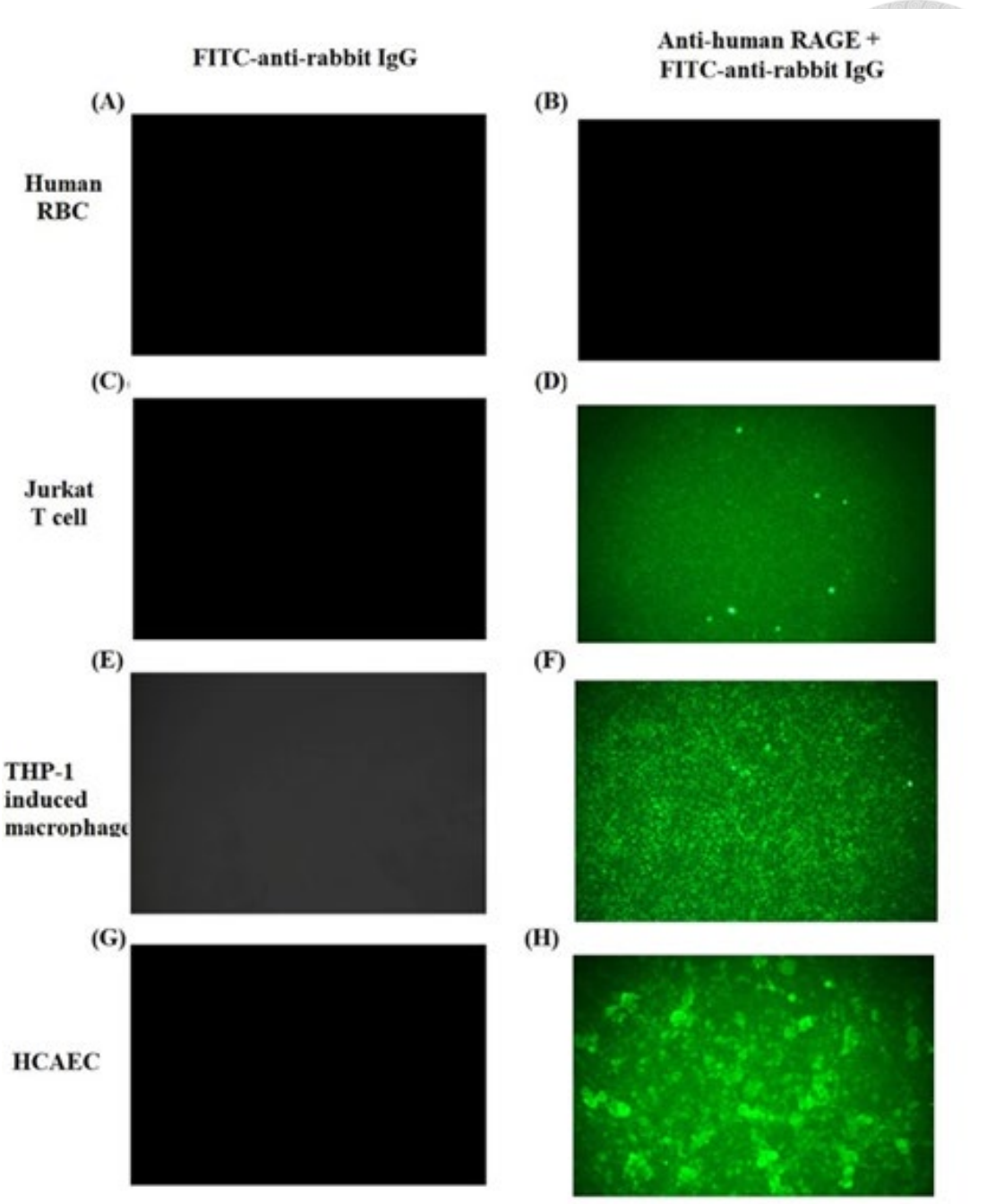

Figure10: Detection of receptor for AGE (RAGE) expression on the cell surface of human coronary artery endothelial cells (HCAEC), human Jurkat T and THP-1 macrophage-like cells by indirect fluorescence antibody staining. Human erythrocytes serve as RAGE (-) cell control. The left panel was stained with FITC-labeled rabbit IgG as negative control. The right panel was stained with rabbit anti-human RAGE

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antibody as primary antibody and then FITC-labeled goat anti-rabbit IgG antibody as secondary antibody. The expression of RAGE on Jurkat T was modest whereas the expression on THP-1 and HCAEC cells are robust.

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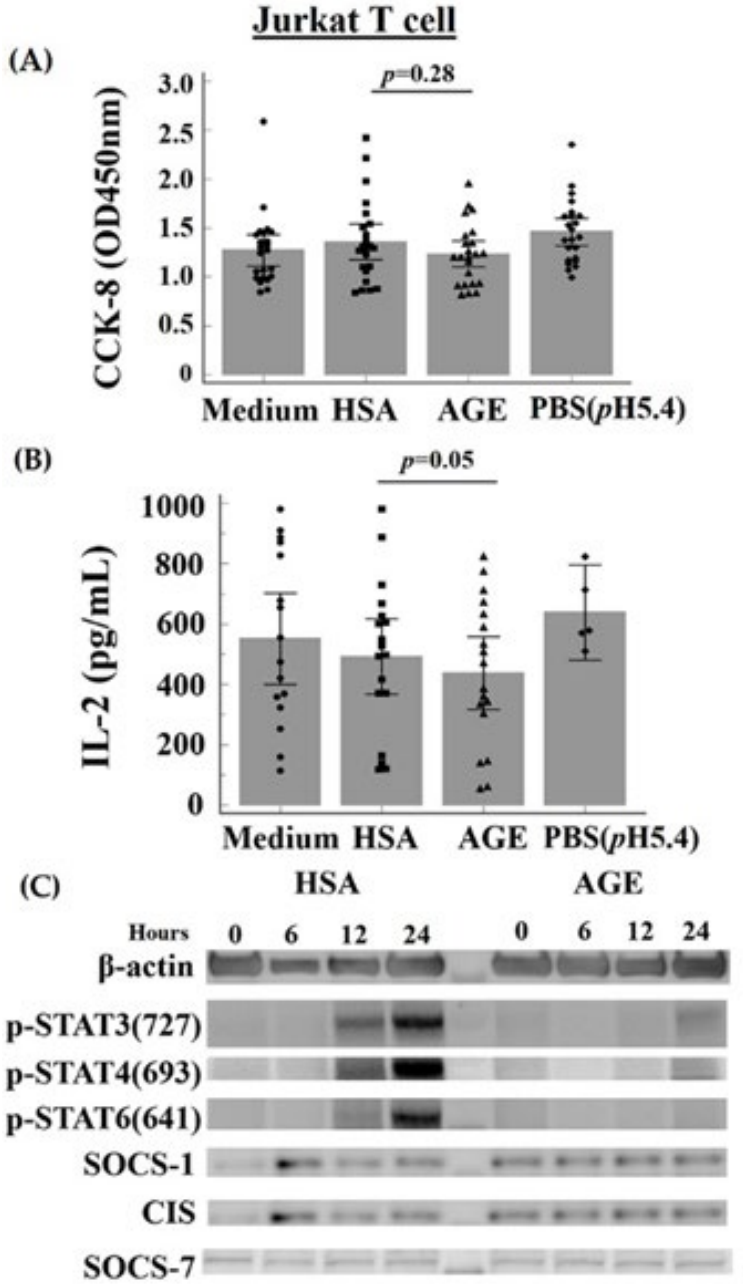

Figure 11: The effect and the molecular basis of AGE-HSA (40μg/mL) on IL-2 (a pluripotential cytokine for T cell development and homeostasis) production from Jurkat T cells. (A) The viability of Jurkat T cells is not different after incubation with medium, HSA, AGE-HSA, or PBS *p*H5.4 as detected by CCK-8 assay kit. (B) The IL-2 production by Jurkat T cells was significantly suppressed by AGE-HSA rather

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than medium, HSA, and PBS pH5.4. (C) Decreased expression of p-STAT 3, 4 and 6, but no significant change on the negative cytokine signaling regulators, CIS, SOCS-1 or SOCS-7 expression in Jurkat T cells by AGE-HSA compared to HSA.

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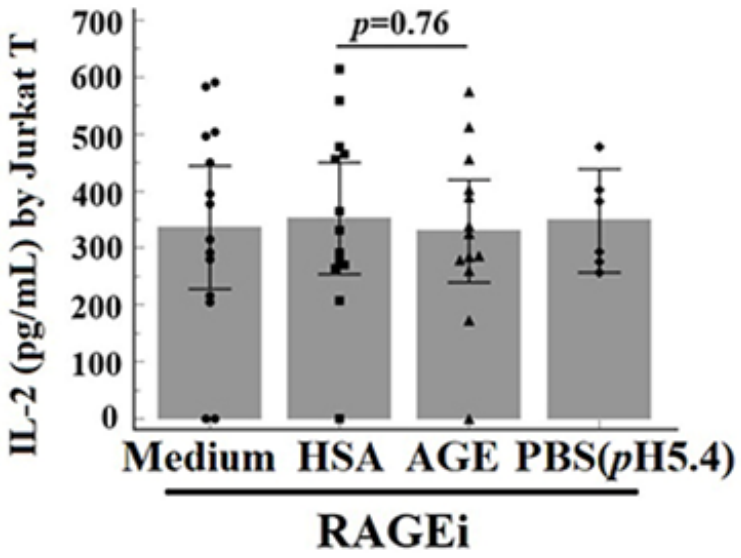

Figure 12: The effect of RAGE inhibitor (FPS-ZM1) on the IL-2 production by Jurkat T cells after incubation with different molecules. The suppressive effect of AGE to Jurkat cell IL-2 production is abrogated after RAGE inhibition.

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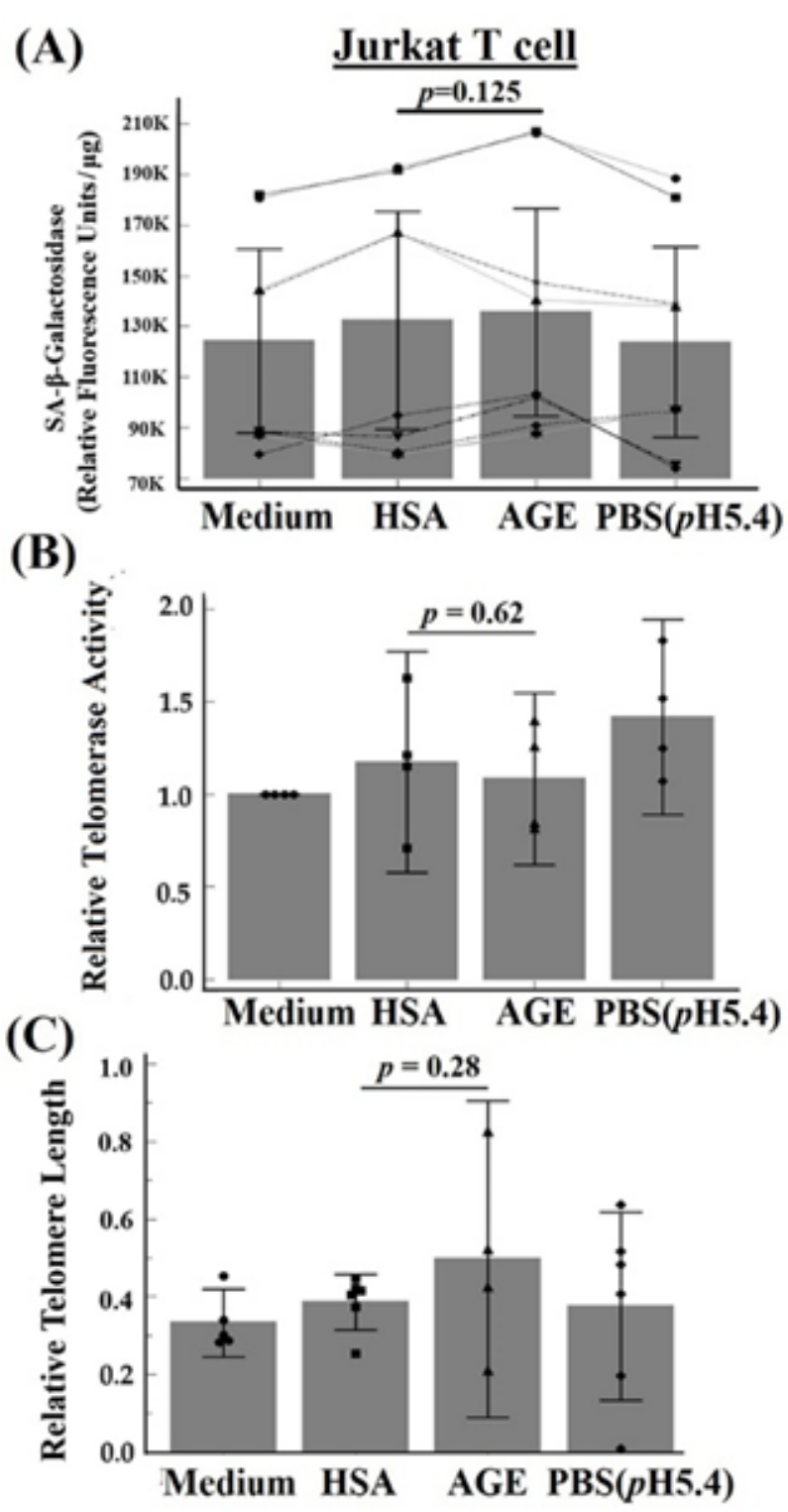

Figure 13: The immunosenescence-inducing activity of AGE-HSA on Jurkat T cells is reflected by an increase tendency of senescence-associated β-galactosidase (SA-βgal) expression, although no significant increase (A). However, no distinct change in

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telomerase activity (B), or relative telomere length (C) can be found after incubation with AGE-HSA or HSA.

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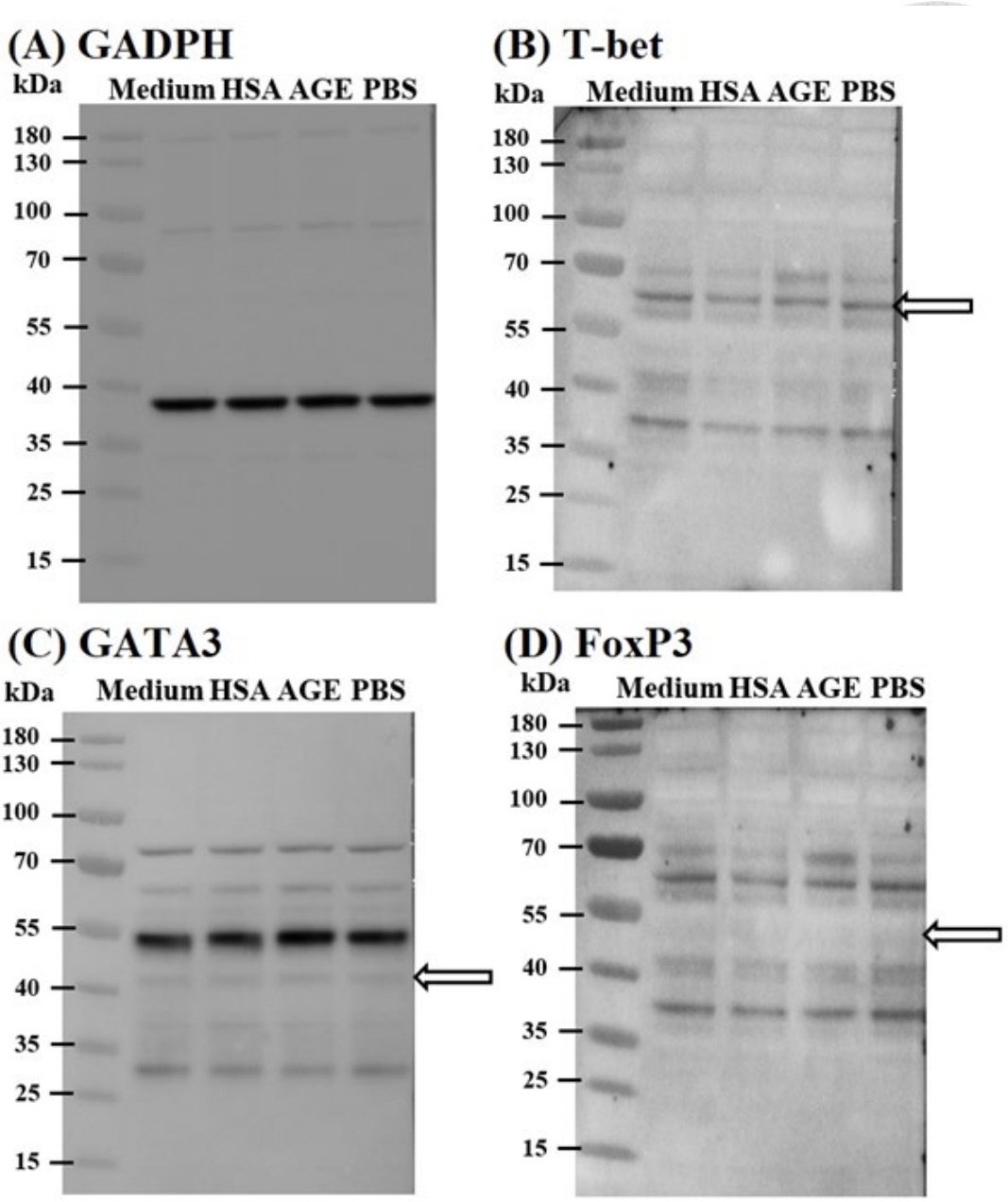

Figure 14: Detection of the transcription factors T-bet (Th1), GATA3 (Th2) and FoxP3 (Treg) in Jurkat T cells after incubation with medium, HSA, AGE-HSA or PBS *p*H5.4 for 48 hours. The results reveal no distinct change of transcription factor expression and no shift of T cell populations after AGEs stimulation. These cells remained to be Th1 cells.

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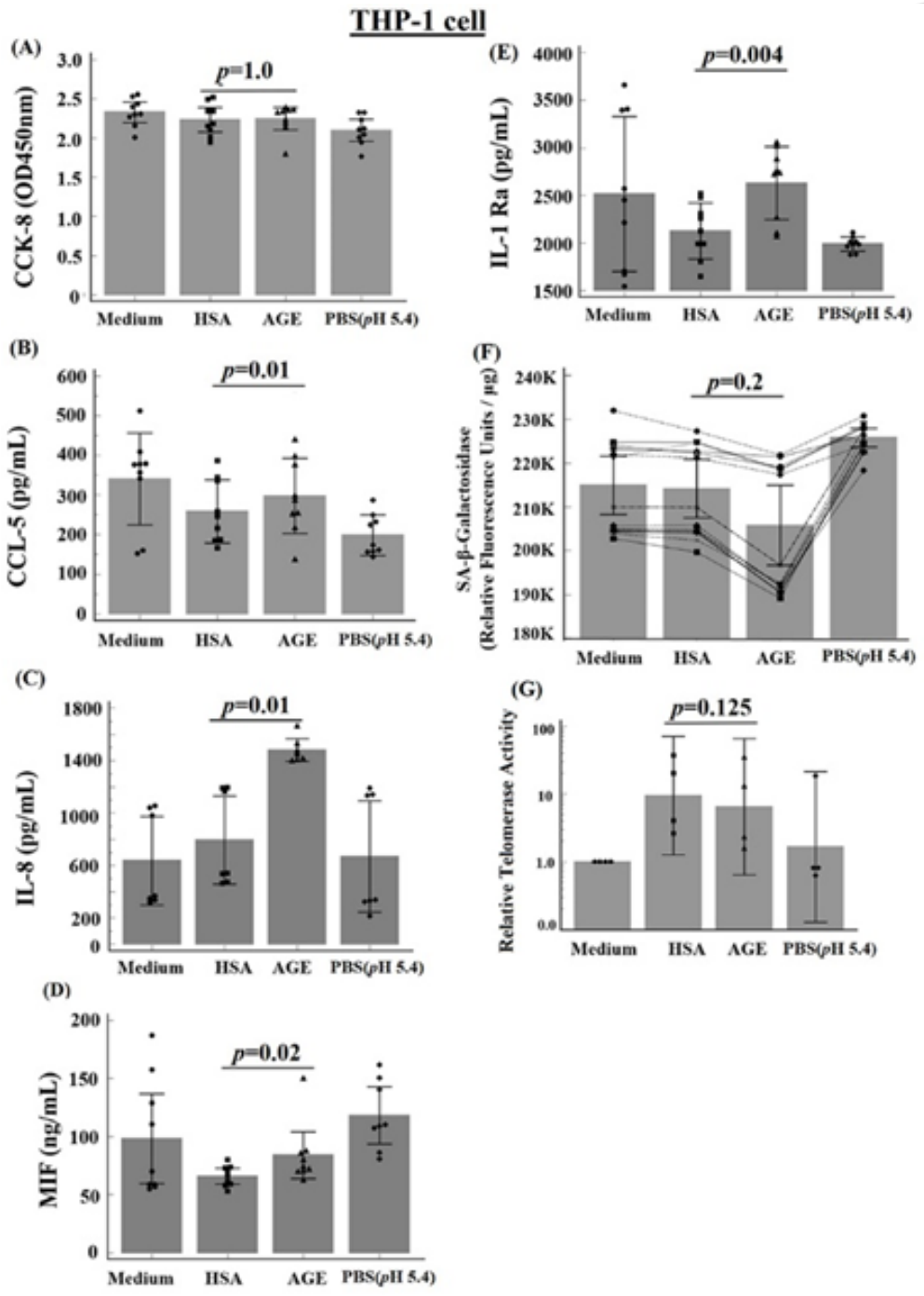

Figure 15: Detection of AGE-HSA on the different cytokine production and immunosenescence biomarker expression by macrophage-like THP-1 cell line. The AGE-HSA did not damage the cell viability as detected by CCK-8 (A). The production of CCL-5 (B), IL-8 (C), MIF (D), and IL-1Ra (E) were all enhanced whereas the SA-β gal (F) was conversely decreased by AGE-HSA. The relative

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telomerase activity (G) seemed no change due to insufficient incubation period as in F

Abbreviations: CCL5: Chemokine CC-motif ligand 5; MIF: Macrophage migratory inhibitory factor

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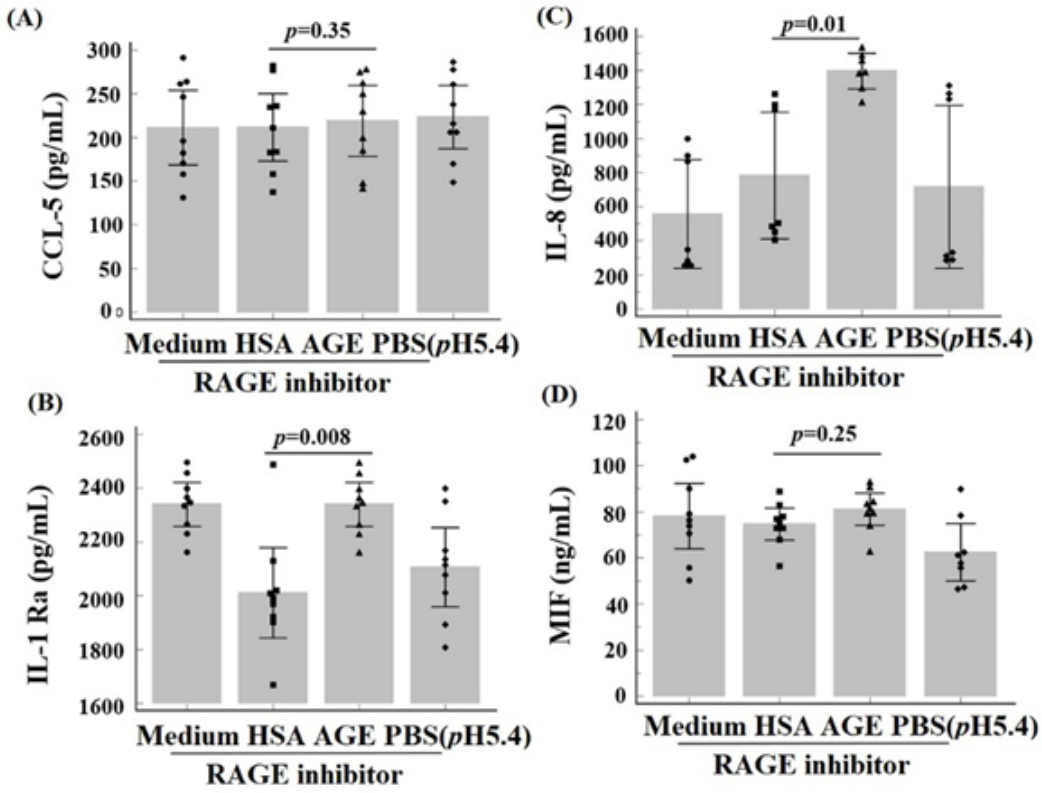

Figure 16: The effects of RAGE inhibitor (FPS-ZM1) on the AGE activation effects toward THP macrophage-like cells. The enhancing effects of CCL5 (A) and MIF (D) are ameliorated after RAGE inhibition. However, the activating effects on IL-1Ra (B) and IL-8 (C) production are not affected by RAGE inhibition.

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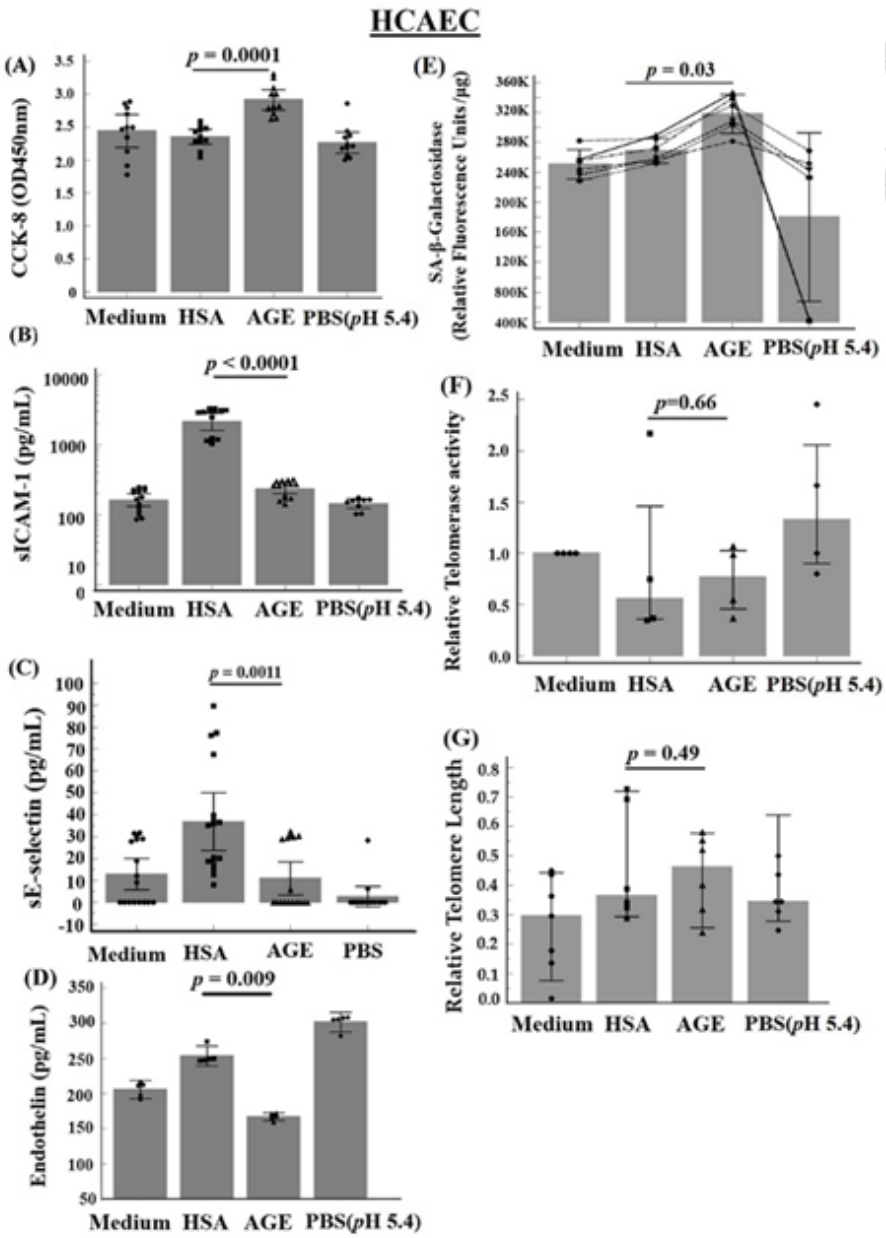

Figure 17: Suppression of AGE-HSA on HSA-enhanced sICAM-1, sE-selectin and endothelin release from human cardiac coronary endothelial cells (HCAEC) via its senescence-inducing activity. The cell viability of HCAEC was not affected by AGE-HSA as detected by CCK-8 (A). HSA *per se* apparently increase the release of sICAM-1 (B), sE-selectin (C), and endothelin (D). However, after AGE-HSA stimulation, the release of sICAM-1 (B), sE-selectin (C), and endothelin (D) are

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significantly suppressed. The senescence biomarker SA-βgal activity is significantly increased after AGE-HA stimulation, as shown in (E). However, the telomerase activity (F) and the relative telomere length (G) are not affected by AGE-HSA at all.

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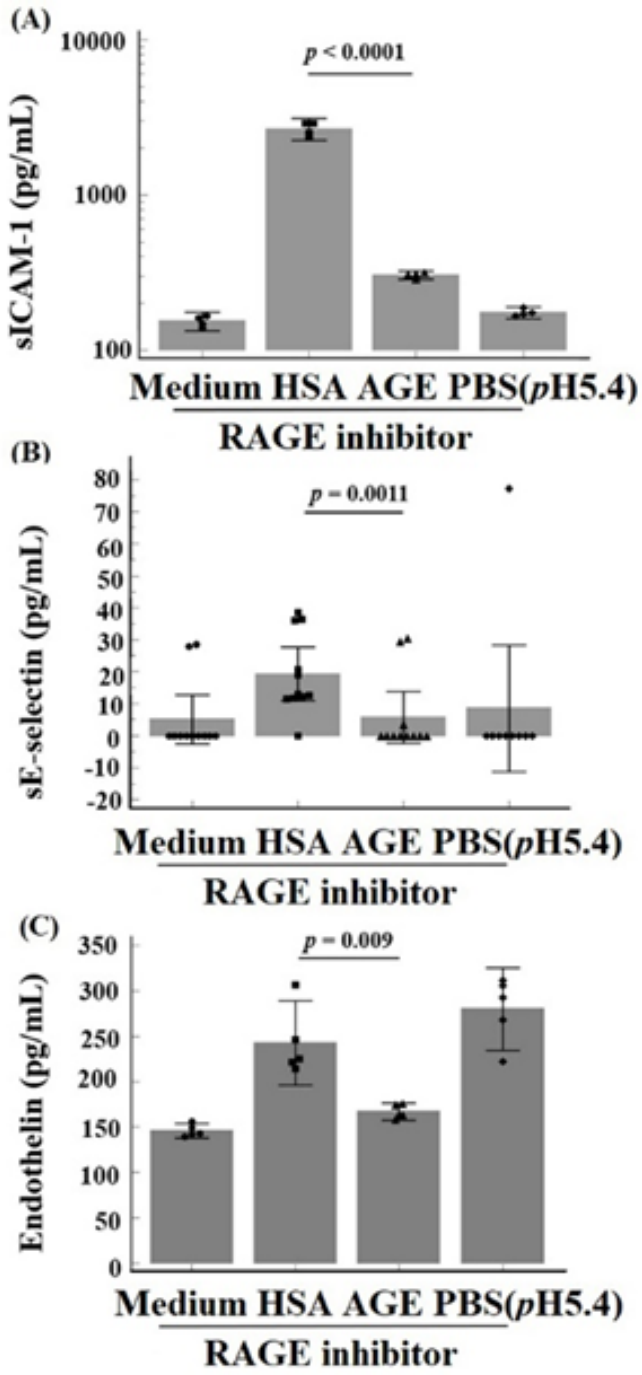

Figure 18: The effects of RAGE inhibitor (FPS-ZM1) on AGE of the HCAEC cells release of soluble adhesion molecules sICAM-1 (A), sE-selectin (B), and endothelin (C) compared to HSA. The effects are not changed after RAGE inhibition.

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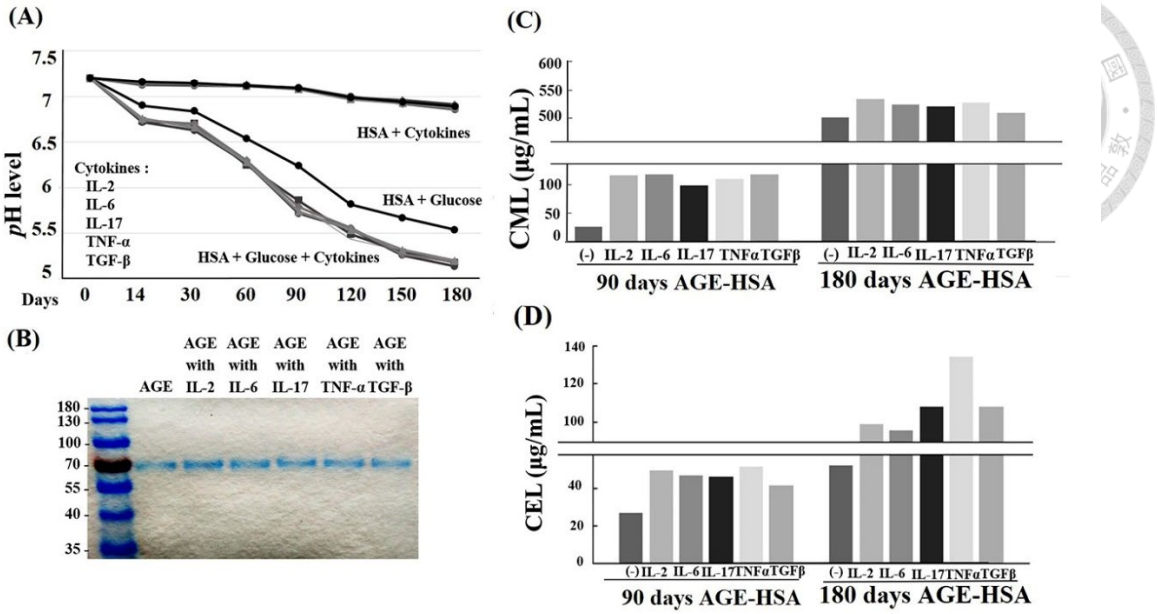

Figure 19: Acceleration and increased formation of AGE-HSA by the inflamm-aging and inflammation-related cytokines. (A) Comparison of kinetic *p*H change among HSA+ individual cytokine, HSA+ glucose, and HSA+ glucose + individual cytokine . The *p*H changes are apparently faster toward acidic in HSA +glucose+ individual cytokine groups. (B) The molecular weight does not change in the presence/absence of different inflammation-related cytokines after 180 days incubation. (C) Comparison of AGE-HSA formation in the presence/absence of different cytokines after 90 days (left panel) and 180 days (right panel) incubation measured by CML amount. The amount of CML is apparently higher after incubation for 90 days in the presence of cytokines. However, the final CML concentrations after incubation for 180 days are not difference. (D) Comparison of AGE-HSA formation in the presence/ absence of individual cytokine after 90 days (left panel) and 180 days (right panel) by

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the CEL amount. The increase of CEL is apparently higher after incubation for 90 days in the presence of individual cytokine. Interestingly, the final CEL concentration after incubation for 180 days are also higher in the presence of different cytokines.

CML: N^{ε}-carboxymethyl- lysine, CEL: N^{ε}-carboxyethyl-lysine.

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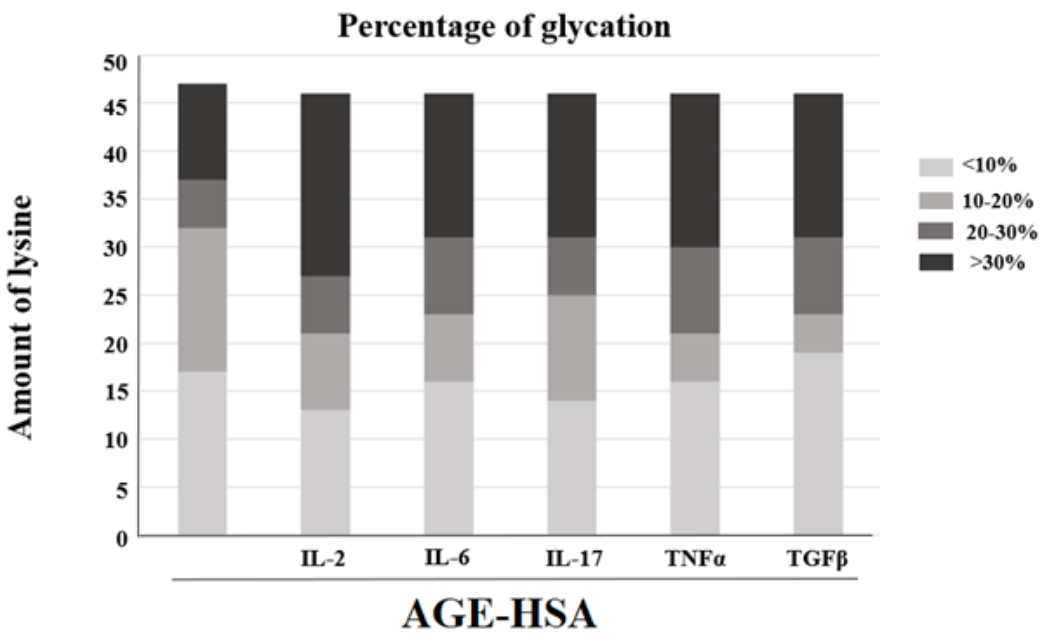

Figure 20. Comparison of lysine glycation in the AGE-HSA molecules after 30 days incubation with or without different inflammation-related cytokine detected by glycation mass spectrometry. It is clear that the amounts of deeply glycated lysine, which indicates a site with >30% lysine glycation, are higher in the presence of cytokine rather than AGE without cytokine.

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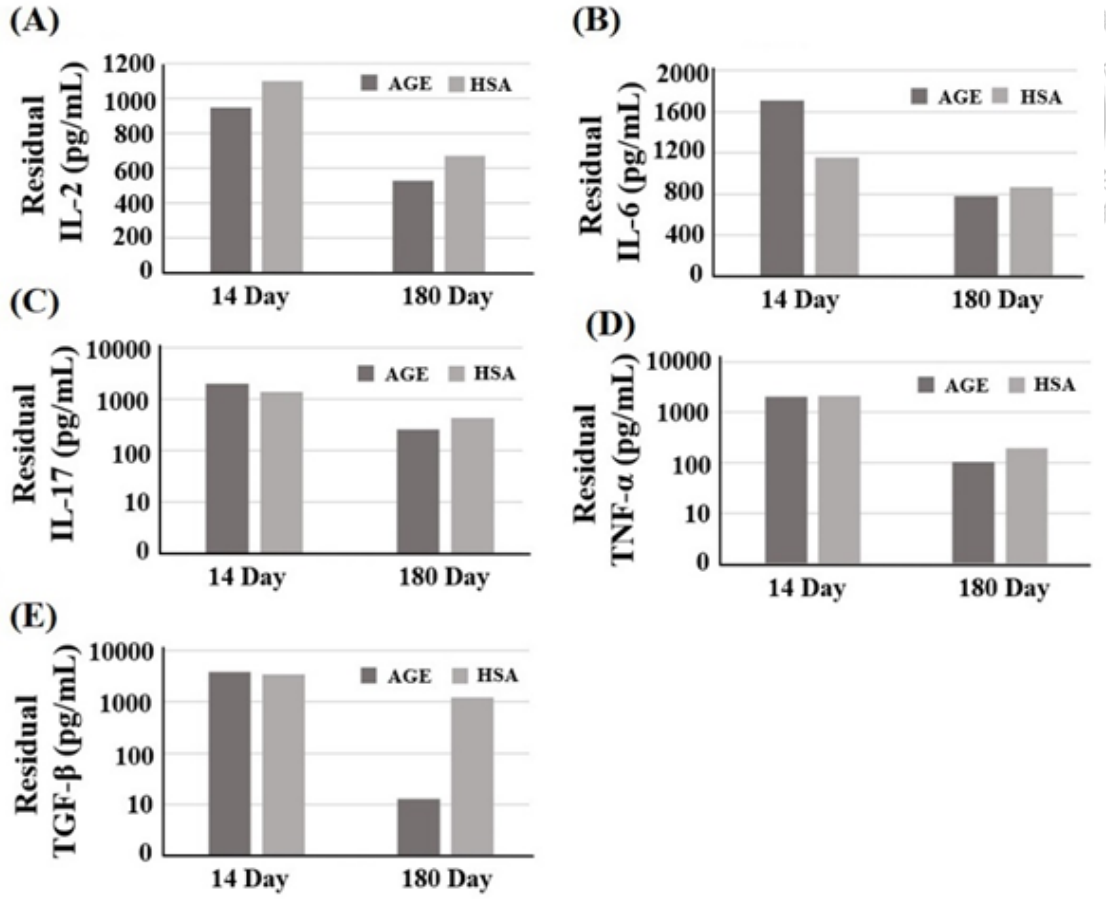

Figure 21. Less amount of the residual cytokines in the supernatants of HSA-glucose-cytokine mixture than HSA-cytokine mixture after 180 days incubation. The decreased residual cytokines are seen in IL-2 (A), IL-6 (B), IL-17 (C), TNF-α (D), and TGF-β (E).

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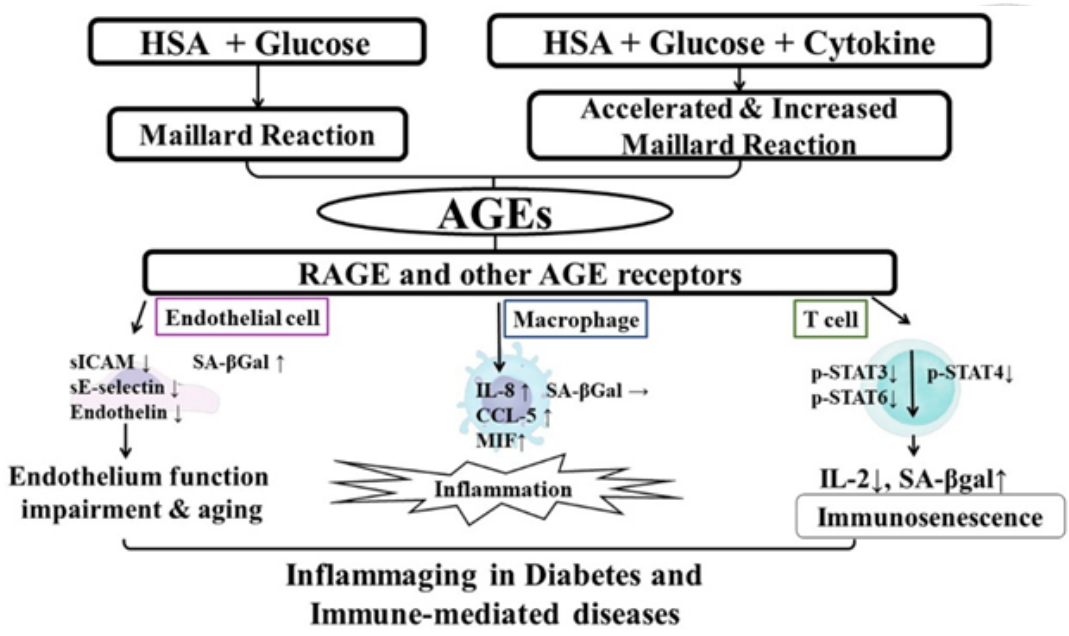

Figure 22: A summary scheme illustrating the regular and the accelerated formation of AGE-HSA by inflammation-related cytokines, and the molecular pathogenesis of AGEs on inflamm-aging commonly found in patients with age-related disease, diabetes, and immune-mediated diseases. AGE-HSA may bind with different receptors leading to the abrogation of the HSA vascular homeostatic functions, activation of macrophage functions and T cell suppression. These adverse effects stimulate the generation of inflamm-aging.

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Table 1.The kinetic glycation in the different lysine sites on the AGE molecule after incubation from D30 to D180. The frequency of glycation sites increased along with incubation periods

K site	AGE D30	AGE D120	AGE D180	K site	AGE D30	AGE D120	AGE D180
N-term	O	O	O	K280	O	O	O
K4	O	O	O	K285	–	O	O
K12	O	O	O	K294	O	O	O
K20	–	O	O	K312	O	O	O
K41	O	O	O	K316	O	O	O
K51	O	O	O	K322	O	O	O
K64	O	O	O	K350	O	O	O
K76	O	O	O	K362	O	O	O
K93	–	O	O	K375	O	O	O
K106	–	O	O	K377	O	O	O
K114	O	O	O	K388	O	O	O
K116	O	O	O	K396	–	O	O
K127	O	O	O	K413	–	O	O
K131	O	O	O	K431	–	–	–
K132	O	O	O	K439	–	–	–
K136	O	O	O	K465	O	O	O
K159	–	O	O	K471	–	O	O
K173	O	O	O	K474	O	O	O
K180	O	O	O	K499	O	O	O
K187	–	–	–	K504	O	O	O
K204	O	O	O	K520	–	O	O
K211	O	O	O	K523	O	O	O
K221	O	O	O	K524	O	O	O
K224	–	O	O	K533	–	–	–
K232	O	O	O	K535	–	O	O
K239	O	O	O	K537	–	O	O
K242	O	O	O	K544	–	O	O
K261	O	O	O	K556	O	O	O
K273	O	O	O	K563	O	O	O
K275	O	O	O	K573	O	O	O

* The mark “O” represents presence of glycation and “-“represents absence of lysine glycation.

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Table 2. Glycation mass spectrometric detection of lysine sites in the AGE-HSA in different incubation period

K position	HSA	AGE D15	AGE D30	AGE D60	AGE D90	AGE D120	AGE D180
36	0.16%	9.47%	15.21%	23.70%	26.85%	32.00%	40.83%
44	0.40%	13.86%	19.73%	23.16%	26.51%	27.08%	25.20%
65	0.00%	4.19%	9.21%	17.81%	24.50%	33.98%	45.15%
75	0.92%	43.72%	27.91%	28.68%	30.53%	27.75%	28.46%
86	0.03%	7.87%	16.41%	27.27%	34.90%	45.77%	51.81%
97	0.30%	2.12%	4.69%	8.26%	11.72%	21.80%	36.43%
130	0.29%	45.67%	31.56%	34.48%	36.74%	35.16%	34.89%
159	0.01%	0.93%	2.13%	2.87%	3.59%	5.11%	5.48%
160	43.65%	23.52%	31.31%	31.42%	30.89%	30.36%	35.13%
161	0.02%	20.72%	29.14%	30.95%	30.57%	33.07%	38.66%
174	0.00%	1.33%	2.39%	4.92%	7.44%	11.84%	20.50%
179	0.00%	0.41%	3.09%	4.41%	11.68%	15.07%	22.44%
186	0.00%	18.73%	32.90%	41.87%	49.12%	52.01%	52.04%
223	0.07%	0.08%	3.02%	9.29%	13.70%	19.54%	34.21%
229	0.42%	18.07%	32.61%	45.22%	53.61%	61.71%	69.66%
246	0.00%	0.26%	0.56%	0.67%	1.21%	2.18%	3.40%
249	0.00%	0.26%	0.56%	0.92%	1.21%	2.18%	3.40%
257	31.13%	43.18%	58.30%	61.82%	61.92%	63.18%	63.60%
307	0.09%	12.87%	23.59%	35.03%	44.81%	50.99%	58.96%
319	0.01%	1.24%	3.12%	4.63%	7.75%	12.28%	18.72%
321	1.74%	10.29%	19.97%	41.52%	23.73%	16.08%	56.22%
337	34.78%	7.58%	14.34%	22.84%	23.55%	25.95%	33.04%
347	1.62%	3.92%	4.61%	8.55%	14.53%	20.98%	35.27%
362	0.05%	9.59%	16.91%	26.37%	29.59%	31.64%	33.27%
375	0.13%	38.46%	50.36%	57.28%	63.09%	64.28%	68.30%
383	3.25%	65.42%	2.59%	91.82%	90.37%	95.56%	92.51%
396	13.49%	3.30%	6.84%	9.78%	11.94%	15.76%	18.78%
402	0.65%	6.23%	13.17%	20.53%	26.26%	31.77%	38.70%
413	8.72%	46.01%	39.69%	39.20%	41.89%	51.69%	60.52%
426	0.55%	7.42%	15.21%	19.34%	25.86%	36.64%	46.58%
434	100.00%	26.86%	74.94%	80.36%	72.99%	69.73%	53.54%
438	0.62%	1.73%	2.77%	3.26%	6.03%	5.58%	8.56%
496	0.40%	7.61%	13.42%	23.32%	24.36%	30.38%	34.81%

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499	0.40%	7.61%	13.43%	23.31%	24.36%	30.39%	34.82%
520	0.00%	7.69%	11.90%	30.80%	51.80%	51.16%	69.99%
524	0.91%	3.99%	29.56%	13.74%	19.88%	21.44%	31.46%
543	0.07%	2.02%	4.40%	5.55%	6.13%	7.84%	10.45%
545	0.07%	1.98%	4.02%	5.55%	6.11%	7.84%	10.45%
548	1.61%	36.24%	32.00%	28.74%	27.55%	25.35%	22.86%
549	1.64%	40.15%	37.22%	35.01%	33.56%	33.08%	36.08%
560	0.23%	3.14%	10.69%	28.37%	16.64%	18.93%	21.66%
562	0.01%	3.02%	10.56%	28.39%	16.66%	18.95%	21.62%
565	0.00%	1.22%	11.48%	31.00%	17.65%	18.83%	21.59%
569	0.07%	8.00%	16.29%	20.49%	21.81%	24.29%	37.41%
581	0.28%	3.37%	6.49%	11.44%	16.45%	19.17%	24.94%
626	0.28%	3.37%	6.49%	11.45%	16.45%	19.14%	24.94%
661	0.07%	13.80%	20.33%	32.06%	41.95%	49.12%	67.42%

*The extent of glycation on different lysine sites in general is increased along with the incubation periods. For example, the extent of glycation on lysine at position 36 (K36) is 9.47% after15 days incubation, 26.8% after 90 days incubation, and finally up to 40.83% after 180 days incubation.

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Table 3. Comparison of lectin binding capacity between HSA and AGE-HSA

Protein	Name of lectin
HSA	DBA, MALECTIN, PSL1A, RS-FUC, VVA
AGE-HSA	BANLEC, CGL2, ConA, DBA, MOA, PSL1A, RS-FUC, SAMB, VVA

*Abbrevioations: DBA: galactose N-Aetylglucosamine(GalNAC) ; MALECTIN: Glucose2-N-biose ; PSL1A:α2-6 Sialic Acid ; RS-FUC: fucose ; VVA: N-Acetylgalactosamine; BANLEC: Mannose, Glucose, branched high-mannose containing α1,3-glycoside bond ; CGL2: β-Galactosidase, GalNAc-α1-3-Gal (Blood Group A) or Gal-α1-3-Gal (Blood Group B) ; ConA: α-Mannose, α-Glucose ; MOA: Gal-α1-3[Fuc-α1-2]-Gal-β1-4GlcNAc (Blood Group B), Gal-α1-3-Galβ1-4GlcNAc, Galα1-3Gal ; SAMB: NeuAcα2-6Gal/GalNAc.

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Table 4 Glycation mass spectrometric detection of lysine sites in the AGE-HSA with different cytokines in 30-day incubation

K site	IL-2	IL-6	IL-17	TNF α	TGF β
36	15.37%	55.08%	19.28%	17.26%	17.87%
44	19.52%	21.21%	21.15%	20.17%	23.84%
65	10.74%	9.33%	9.31%	9.99%	9.08%
75	28.18%	27.38%	29.66%	27.48%	28.56%
86	16.22%	61.37%	15.95%	16.25%	16.15%
97	4.77%	3.42%	5.23%	4.27%	4.19%
130	32.22%	31.51%	34.53%	31.48%	31.11%
159	1.77%	1.51%	1.37%	1.60%	1.45%
160	31.18%	28.23%	31.45%	30.54%	32.70%
161	27.78%	27.94%	28.79%	28.34%	31.47%
174	1.52%	2.47%	3.36%	2.32%	3.00%
179	3.27%	1.90%	5.00%	3.26%	4.21%
186	29.83%	32.55%	32.24%	25.25%	29.19%
223	2.43%	4.60%	2.65%	2.29%	0.62%
229	31.20%	30.86%	32.47%	29.38%	32.93%
240	0%	0%	0%	0%	0%
246	0.56%	0.58%	0.21%	0.44%	0.38%
249	0.56%	0.58%	0.21%	0.44%	0.38%
250	0%	0%	0%	0%	0%
257	55.89%	57.38%	59.85%	55.28%	57.15%
307	27.73%	23.69%	23.15%	19.00%	24.27%
319	2.83%	3.09%	3.18%	1.73%	2.51%
321	16.25%	33.33%	21.07%	22.59%	13.36%
337	13.64%	14.69%	16.41%	51.48%	16.38%
347	2.70%	9.01%	5.31%	3.82%	6.21%
362	16.34%	15.25%	17.65%	17.58%	19.59%
375	52.95%	52.80%	57.83%	48.90%	58.77%
383	91.80%	2.98%	3.03%	2.74%	86.37%
396	7.06%	7.79%	9.10%	8.29%	7.59%
402	12.71%	12.23%	13.40%	12.23%	11.86%
413	27.07%	42.57%	19.47%	19.36%	21.27%

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426	13.12%	13.43%	12.56%	11.47%	13.74%
434	80.48%	100.00%	100.00%	99.96%	100.00%
438	3.12%	2.11%	3.38%	2.62%	2.41%
496	14.41%	16.24%	14.76%	14.21%	16.31%
499	14.42%	16.25%	14.76%	14.39%	16.29%
520	29.16%	19.07%	23.52%	15.81%	14.01%
524	7.19%	7.18%	7.58%	8.99%	7.74%
543	3.19%	4.31%	3.78%	3.22%	2.96%
545	3.19%	4.27%	3.78%	3.22%	2.96%
548	31.80%	31.22%	32.42%	30.31%	31.68%
549	35.60%	36.83%	36.86%	34.49%	37.28%
560	7.73%	11.20%	7.17%	8.96%	6.77%
562	7.73%	11.20%	7.13%	8.95%	6.75%
565	8.73%	11.21%	7.24%	10.22%	7.35%
569	18.03%	14.73%	20.37%	15.38%	15.31%
581	6.78%	5.90%	7.71%	6.82%	6.50%
626	6.77%	5.91%	7.77%	6.83%	6.54%
661	19.93%	19.36%	15.95%	18.85%	25.43%

*The extent of glycation on different lysine sites in general is increased in those AGEs incubated with cytokines.

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Appendix:

Table 1. The sugar moiety abbreviations and the list of 70 key lectins used in the present lectin array assay

Sugar Abbreviations			
Fuc: L-Fucose	Gal: D-Galactose	GalNAc: *N*-Acetylglactosamine	Glc: D-Glucose
GlcNAc: *N*-Acetylglucosamine	Lac: Lactose	Man: Mannose	

VI. Lectin Array 70 Key

	Lectins	Abbreviation	Source	Carbonhydrate specificity
1	*Anguilla anguilla*	AAA	*Anguilla anguilla* (Fresh Water Eel)	αFuc
2	*Aleuria aurantia*	AAL	*Aleuria aurantia* mushrooms	Fucα6GlcNAc
3	*Agrocybe cylindracea lectin*	ACG	E. coli expressed *Agrocybe cylindracea* galectin	α2-3 Sialic Acid
4	*Amaranthus caudatus*	ACL, ACA	*Amaranthus caudatus* seeds	Galβ3GalNAc
5	*Allium sativum*	ASA	*Allium sativum* agglutinin (Garlic)	αMan
6	*Musa acuminata lectin*	BanLec	E. coli expressed *Musa acuminata*	Mannose, Glucose, branched high-mannose containing α1,3-glycoside bond
7	*Burkholderia cenocepacia lectin*	BC2L-A	E. coli expressed *Burkholderia cenocepacia*	High-mannose
8	*Burkholderia cenocepacia lectin*	BC2LCN（AiLecS1)	E. coli expressed *Burkholderia cenocepacia*	Fucα1-2Galβ1-3GalNAc (H type 3), Fucα1-2Galβ1-3GlcNAc (H type 1)
9	*Bauhinia purpurea*	BPA, BLP	*Bauhinia purpurea alba* (Camel's Foot Tree)	Galβ3GalNAc
10	*Calystegia sepium lectin*	Calsepa	E. coli expressed Calystegia sepium	High-mannose
11	*Coprinopsis cinerea lectin*	CGL2	E. coli expressed *Coprinopsis cinerea*	βGal, GalNAcα1-3Gal (Blood Group A), Galα1-3Gal (Blood Group B)
12	*Clitocybe nebularis lectin*	CNL	E. coli expressed *Clitocybe nebularis*	α/βGalNAc, GalNAcβ1-4GlcNAc, GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc (Blood Group A)
13	Coanavalin A	Con A	*Coanavalia ensformis* (Jack Beans) seeds	αMan, αGlc
14	*Dolichos biflorus*	DBA	*Dolichos biflorus* (Horse Gram) seeds	αGalNAc
15	*Dictyostelium discoideum lectin*	Discoidin I	E. coli expressed *Dictyostelium discoideum*	αGalNAc (Tn antigen), LacNAc
16	*Dictyostelium discoideum lectin*	Discoidin II	E. coli expressed *Dictyostelium discoideum*	Gal, LacNAc, Asialoglycans, Gal/GalNAcβ1-4GlcNAcβ1-6Gal/GalNAc
17	*Datura stramonium*	DSA, DSL	seeds	$(GlcNAc)_{2-4}$
18	*Erythrina cristagalli*	ECA, ECL	*Erythrina cristagalli* (Coral Tree) seeds	Galβ4GlcNAc
19	*Eunonymus europaeus*	EEL	*Eunonymus europaeus* (Spindle Tree) seeds	Galα3Gal
20	*E. coli lectin*	F17AG	E. coli expressed *E. coli*	GlcNAc
21	Human galectin1 lectin (stable form)	Gal1	E. coli expressed human galectin1 (stable form)	branched LacNAc, Gal
22	Human galectin1-S lectin	Gal1-S	E. coli expressed human galectin1-S	branched LacNAc
23	Human galectin2 lectin	Gal2	E. coli expressed human galectin2	GalNAcα1-3Gal (Blood Group A), branched LacNAc
24	Human galectin3 lectin (full-length)	Gal3	E. coli expressed Human galectin3(full-length)	poly LacNAc
25	Human galectin 3C-S lectin	Gal3C-S	E. coli expressed Human galectin 3C-S	poly LacNAc
26	Human galectin7-S lectin	Gal7-S	E. coli expressed Human galectin7-S	Galβ1-3GlcNAc
27	Human galectin9 lectin (Stable Form)	Gal9	E. coli expressed human galectin9	poly LacNAc, GalNAcα1-3Gal (Blood Group A)
28	*Galanthus nivalis*	GNA, GNL	*Galanthus nivalis* (Snowdrop) bulbs	αMan
29	*Griffithia sp. Lectin*	GRFT	E. coli expressed *Griffithia sp.*	High-mannose
30	*Griffonia (Banderaea) simplicifolia I*	GS-I, GSL-II, BSL-I	*Griffonia (Banderaea) simplicifolia* seeds	αGal, α3GalNAc
31	*Griffonia (Brandeiraea)*	GS-II, GSL-II, BSL-II	*Griffonia (Banderaea) simplicifolia* seeds	α or βGlcNAc
32	*Hippeastrum hybrid*	HHA, HHL, AL	*Hippeastrum* hybrid (Amaryllis) bulbs	αMan
33	Jacalin	Jacalin, AIL	*Artocarpus integrifolia* (Jackfruit) seeds	Galβ3GalNAc
34	*Phaseolus lunatus*	LBA	*Phaseolus lunatus* (Lima Bean) seeds	GalNAcα(1,3)[αFuc(1,2]Gal
35	*Lens Culinaris*	LcH, LCA	*Lens culinaris* (lenti I) seeds	αMan, αGlc
36	*Lycopersicon esculentum*	LEA, LEL, TL	*Lycopersicon esculentum* (tomato) fruit	$(GlcNAc)_{2-4}$
37	Lentil lectin	Lentil	*Lens culinaris* seeds	D-Mannose, D-glucose
38	*Lotus tetragonolobus*	Lotus, LTL	*Lotus tetragonolobus, Tetragonolobus purprea(* Winged Pea, Asparagus Pea) seeds	αFuc
39	*Laetiporus sulphureus lectin*	LSL-N	E. coli expressed *Laetiporus sulphureus*	LacNAc, poly LacNAc
40	*Maackia amurensis I*	MAA, MAL, MAL-I	*Maackia amurensis* seeds	Galβ4GlcNAc
41	Human malectin lectin	Malectin	E. coli expressed human malectin	Glc_2-*N*-biose
42	*Marasmius oreades lectin*	MOA	E. coli expressed *Marasmius oreades*	Galα1-3[Fucα1-2]Galβ1-4GlcNAc (Blood Group B) Galα1-3Galβ1-4GlcNAc, Galα1-3Gal
43	*Maclura pomifera*	MPL, MPA	*Maclura pomifera* (Osage Orange) seeds	Galβ3GalNAc
44	*Narcissus pseudonarcissus*	NPA, NPL, DL	*Narcissus pseudonarcissus* (Daffodil) bulbs	αMan
45	*Oryza sative lectin*	Orysata	E. coli expressed *Oryza sative*	High-mannose
46	*Pseudomonas aeruginosa lectin*	PA-IIL	E. coli expressed *Pseudomonas aeruginosa*	Fucose, Fucose containing oligosaccharides, Mannose
47	*Pseudomonas aeruginosa lectin*	PA-IL	E. coli expressed *Pseudomonas aeruginosa*	Galα1-3(4)Gal
48	*Phlebodium aureum lectin*	PALa	E. coli expressed *Phlebodium aureum*	High-mannose
			Phaseolus vulgaris Erythroagglutinin (Red Kidney	Galβ4GlcNAcβ2Manα6(GlcNAcb4)

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49	*Phaseolus vulgaris Erythroagglutinin*	PHA-E	*Phaseolus vulgaris Erythroagglutinin* (Red Kidney Bean) seeds)	Galβ4GlcNAcβ2Manα6(GlcNAcb4) (GlcNAcβ4Manα3)Manβ4
50	*Leucoagglutinin*	PHA-L	*Phaseolus vulgaris Erythroagglutinin* (Red Kidney Bean) seeds)	Galβ4GlcNAcβ6(Gl cNAcβ2Manα3)Manα3
51	Phaseolus vulgaris agglutinin	PHA-P	*Phaseolus vulgaris Erythroagglutinin* (Red Kidney Bean) seeds)	Galβ4GlcNAcβ2Manα6(GlcNAcb4) (GlcNAcβ4Manα3)Manβ4, Galβ4GlcNAcβ6(Gl cNAcβ2Manα3)Manα3
52	Peanut	PNA	Arachis hypogaea *Peanut*	Galβ3GalNAc
53	*Pleurocybella porrigens lectin*	PPL	E. coli expressed Pleurocybella porrigens	α/βGalNAc
54	*Pisum sativum*	PSA, PEA	*Pisum sativum* (Pea) seeds	αMan, αGlc
55	*Polyporus squamosus lectin*	PSL1a	E. coli expressed *Polyporus squamosus*	α2-6 Sialic Acid
56	*Psophocarpus*	PTL, PTL-I, WBA-I	*Psophocarpus tretragonoiobus* (Winged Bean)	GalNAc, Gal
57	*Ralstonia solanacearum lectin*	RS-Fuc	E. coli expressed *Ralstonia solanacearum*	Fucose
58	Sambucus Sieboldiana Lectin	SAMB	Japanese elderberry	NeuAcα2-6Gal/GalNAc
59	Soybean	SBA	*Glycine max* (Soybean) seeds	α > βGalNAc
60	*Sophora japonica*	SJA	*Sophora japonica* (Japanese Pagoda Tree) seeds	βGalNAc
61	*Sambucus nigra I*	SNA-I	*Sambucus nigra* (Elderberry) bark	NANAα(2,6)GalNAc > GalNAc = Lac > GalNANAα(2,6)Gal
62	*Sambucus nigra II*	SNA-II	*Sambucus nigra* (Elderberry) bark	GalNAc > Gal
63	*Solanum tuberosum*	STL, PL	*Solanum tuberosum,* (potato) tubers	$(GlcNAc)_{2-4}$
64	*Urtica dioica*	UDA	*Urtica dioica* (Stinging Nettle) seeds	GlcNAc
65	*Ulex europaeus I*	UEA-I	*Ulex europaeus* (Furze Gorse) seeds	αFuc
66	*Ulex europaeus II*	UEA-II	*Ulex europaeus* (Furze Gorse) seeds	Poly β(1,4)Gl cNAc
67	*Vicia faba*	VFA	*Vicia faba* (Fava Bean) seeds	αMan
68	*Vicia villosa*	VVA, VVL	*Vicia villosa* (Hairy Vetch) seeds	GalNAc
69	*Wisteria floribunda*	WFA	*Wisteria floribunda* (Japanese Wisteria) seeds	GalNAc
70	Wheat Germ	WGA	*Triticum volganis* (Wheat Germ)	GlCNAc

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