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ORIGINAL ARTICLE

Advanced Glycated End Products Alter Neutrophil Effect on Regulation of CD_4^+ T Cell Differentiation Through Induction of Myeloperoxidase and Neutrophil Elastase Activities

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Abstract— CD_4^+ T cell subset imbalance plays an important role in the development of diabetic complications. Neutrophils have recently been known as the regulator of CD_4^+ T cell differentiation. However, whether neutrophils affect CD_4^+ T cell population in diabetes is still elusive. In this study, we investigated the effect of neutrophils stimulated with advanced glycated end products (AGEs), the marker of diabetes, on CD_4^+ T cell differentiation and its underlying mechanism. Our data showed that the cultural medium of healthy adult neutrophils treated with AGEs increased expressions of both Th_1 (IFN-γ) and Th_{17} (IL-17) phenotypes and the transcription factors of Th_1 (Tbet) and Th_{17} (RORγt) in naive CD_4^+T cells and $CD_4^+CD_{25}^+FoxP_3^+$ (Treg) T cells *in vitro*. Next, we found that AGEs induced the generations of myeloperoxidase (MPO) and neutrophil elastase (NE) in neutrophils; inhibition of MPO or NE attenuated the effect of AGE-stimulated neutrophils on CD_4^+ T cell bias. Furthermore, receptor for AGEs (RAGE) inhibitor interrupted AGE-induced MPO and NE expressions, but MPO and NE inhibitions did not change AGE-increased RAGE gene expression. These results suggested that AGEs drive the effect of neutrophils on CD_4^+ T cell differentiation into pro-inflammatory program through inducing MPO and NE productions in neutrophils, which is mediated by AGE–RAGE interaction.

KEY WORDS: advanced glycated end products; neutrophils; CD_4^+ T cell subsets; myeloperoxidase; neutrophil elastase.

INTRODUCTION

Diabetes and its complications are still major global health burdens, affecting about 41.5 million peoples in the world in 2015 and expectedly up to 64.2 million in 2040 estimated by International Diabetes Federal [1]. Diabetes is featured by various metabolic disorders, with hyperglycemia as the main derangement [2, 3]. Persistent increased plasma glucose takes non-enzymatic reaction with the amino groups of proteins, lipids, and nucleic acids and progressing into formation of advanced glycated end products (AGEs) [2, 3]. Excessive AGE production induces tissue and organ damages including atherosclerosis, blindness, nephropathy, and Alzheimer's

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Abbreviations AGEs, Advanced glycated end products; MPO, Myeloperoxidase; NE, Neutrophil elastase; RAGE, Receptor for AGEs; Treg, Regulatory T cells; NET, Neutrophil extracellular traps; ROR, Retinoic orphan receptor; IFN-γ, Interferon-gamma; BSA, Bovine serum albumin; PBS, Phosphate-buffered saline; ICS, Intracellular cytokine staining; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

disease [4–6]. In the process of damages, inflammation plays an important role [7].

Inflammation is the response to any change of tissue or cell integrity in order to maintain tissue or cell homeostasis *via* different repair mechanisms under acute or chronic pathological conditions. Proper response is crucial to avoid undue expansion of the initial inflammatory process and consequently corresponding damage and disease development [8, 9]. The maintenance of the internal homeostasis mainly depends on the close cooperation between the two arms of immune system, innate and adaptive immunity [10].

In the adaptive arm, distinctive CD_4^+ T cell plasticity plays a key role [11]. Under environmental stimuli, naive CD_4^+ T cells differentiate into T cell subsets, mainly including Th_1, Th_2, Th_{17}, and Treg cells [12]. Th1 specification is driven by IFN-γ and brings about the activation of T-bet (the lineage-specific transcription factor). Th_1 cells are required for cell-mediated immunity and delayed-type hypersensitivity reactions [13]. Th_2 program is induced by IL-4 and resulting in the activation of the lineage-specific transcription factor GATA3 [14]. Th_2 cells are responsible for B cell help in humoral immunity and resistance of extracellular microbes and intestinal helminthes. Th_{17} cells provide host defense against fungi and certain extracellular bacteria at mucosal surfaces and confer coverage of some microbes that are not targeted in Th_1 or Th_2 responses [15]. Induction of the Th_{17} lineage occurs when IL-6, IL-23, and TGFβ are present in the inflammatory milieu, which induces retinoic orphan receptor (ROR) transcription factors, RORα and RORγT, leading to production of Th_{17} cytokines IL-17, IL-17F, and IL-22 [16].

Regulatory T (Treg) cells are identified by expression of the transcription factor $FoxP_3$ and play a vital role in maintaining self-tolerance, preventing autoimmunity and inhibiting immune responses during viral, bacterial and parasitic infections as well [17].

CD_4^+ T cell subsets balance is essential to prevent tissue damage inducing by excessive inflammation, to sustain tolerance, and to involve in antitumor immune responses [18]. Currently, the Th_{17}–Treg programs are considered as a dominant conceptual framework for comprehending the relationship of immunity/inflammation and tolerance/immune-suppression in a wide spectrum of diseases [19]. Th_{17}–Treg imbalance facilitates the development of either autoimmune disorders or compromised immune diseases, depending on the skew side [20, 21].

Neutrophils are the major immune cells of the innate arm. Neutrophils have ever been viewed as short lifespan and ultimately differentiated phagocytes and lacking interaction with adaptive immunity. However, the traditional notion of neutrophil features is recently challenged by several research reports [22]. Firstly, average circulatory neutrophil lifespan was found 5.4 days in healthy volunteers, at least ten times longer than traditional time (18 h) [23]. Secondly, neutrophils have regulatory roles in adaptive T cell functionalities, such as T cell proliferation or cytokine productions [24]. In addition, neutrophils can extrude their own nuclear or mitochondrial DNA to form neutrophil extracellular traps (NETs), which is possibly involved in killing pathogen, inflammation development, and even tissue damages [25, 26].

Accumulating evidences have been shown that CD_4^+ T cell subset balance is regulated by neutrophil activities [27, 28]. Recent studies have shown that the number and function of neutrophils changed, and neutrophil elastase increased in diabetes patients, thereby indicating that neutrophils are involved in initiation and development of type 1diabetes [29, 30]. These results lead us to hypothesize that neutrophils in the milieu of diabetes influence the balance of CD_4^+ T cell subsets and promoting inflammation development. Here, we investigated naive CD_4^+ T cell differentiation in the culture medium from AGE-treated neutrophils and found that this medium induced naive CD_4^+ T cell differentiation bias Th_1/Th_{17} cell population and facilitated Treg cell transformation into Th_1/Th_{17} cells. Further study demonstrated that the effect of neutrophils was related to increased myeloperoxidase (MPO) and neutrophil elastase (NE) activities through AGE–RAGE interaction. Our results provided the novel insight on the mechanism of inflammatory development related to diabetes and gave us new clue to prevent or treat diabetes inflammation by interrupting neutrophil activity.

MATERIALS AND METHODS

AGE Preparation and Chemicals

AGE preparation followed the protocol described as Lin [31]. Briefly, a mixture containing 50 mg/mL of bovine serum albumin (BSA) (USB Corp., Cleveland, OH, USA) and 0.5 M of glucose was made in 0.2 M of sodium phosphate buffer (pH 7.4). The solution was filtered with sterile syringe filters prior to be incubation in the dark at 37 °C for 60 days. After removing unbound materials by extensive dialysis against phosphate-buffered saline (PBS), the solution was used to detect the concentration of AGEs by measuring AGE-specific fluorescence with excitation at 360 nm and emissions at 440 nm. The fluorescence of

qualified AGEs used in our experiments must be at least 70-fold high than that of the BSA control. Sivelestat (SV, a neutrophil elastase inhibitor, S7198) and 4-aminobenzoic acid hydrazide (ABAH, a specific irreversible MPO inhibitor, A41909) were purchased from Sigma (St. Louis, MO, USA). FPS-ZM1 (Cat. 553030), a RAGE inhibitor, was obtained from Calbiochem (San Diego, CA, USA).

The following fluorochrome-labeled Ab and their respective isotype IgG controls were purchased from Becton-Dickinson, NJ, USA: CD_4-V500 (clone RPA-T4), CD_{25}-BV605 (2A3), IFN-γ-FITC (B27), IL-17A-AF700 (N49-653), $FoxP_3$-APC (clone PCH101), PerCP-Cy™5.5-IL-4 (Clone 8D4-8), Tbet-V450 (O4-46), GATA-3-PECy7 (L50-823), and RORγt-PE (Q21-559).

Isolation of Human Neutrophils and CD4 Cells

Human neutrophils were isolated and purified from adult peripheral blood by density centrifugation using Ficoll–Paque PLUS gradient (GE Healthcare); CD_4^+ T cells or naïve CD_4^+T cells were isolated by negative selection (EasySep™ Human CD_4^+ T cell enrichment kit (Cat. 19052) or EasySep™ Human Naïve CD_4^+ T Cell Isolation Kit (Cat. 19555)) (StemCell Technologies, Vancouver, Canada). Treg cells were isolated from purified CD_4^+ T cells by positive selection (EasySep™ Human CD_{25} Positive Selection Kit) (StemCell Technologies) described as Lin [32]. Viability of cultured cells was determined by trypan blue staining; cell purity was > 95%.

Neutrophils Treatment with AGEs

Isolated neutrophils (2.5×10^6 cells/mL) suspended in T cell medium (TCM, RPMI 1640/10% FBS) were cultured with final concentration of 200 μg/mL of AGEs or in TCM alone at 37 °C, 5% CO_2 for 4 h. After stimulation, cell-free polymorphonuclear cell culture medium (PCM or PCM-AGE) were harvested and stored at − 80 °C until bioassay.

CD_4 Cell Co-cultures

Purified CD_4 cells (2×10^6 cells) were cultured in CTCM (2 mM glutamine, 50 μM β-mercaptoethanol, 10% heat-inactivated human AB type serum, 100 U penicillin/100 μg streptomycin/mL), or in CTCM containing PCM or PCM-AGE (50% of the total culture volume). All CD_4 cells were cultured on 24-well plates coated with anti-CD_3 Ab (2 μg/mL) and in the presence of IL-2 (50 U/mL) at 37 °C, 5% CO_2 for 3 days.

ICS and Flow Cytometry

CD_4 cells were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin for 1 h prior to incubation with 5 μg/mL of Brefeldin A (Golgi-Stop^{plus}, BD) for an additional 4 h at 37 °C. Stimulated cells were stained for surface antigens (CD_4, CD_{25}) and then fixed and permeabilized according to the manufacturer's instructions. Permeabilized cells were subsequently stained using fluorochrome-labeled mAbs or IgG controls. Samples were acquired within 24 h of staining using a 16-color BD LSRII flow cytometer. Acquired samples were analyzed using the FlowJo 7.2.2 software (Tree Star, Ashland, OR, USA). Within the gate of CD_4^+cell population, Th_1, Th_2, and Th_{17} were identified by their expression of IFNg, IL-4, and IL-17, respectively. Tregs were identified as $CD_4^+CD_{25}^+FoxP_3^+$ cells.

Real-Time PCR

Total RNA was extracted from neutrophils and CD_4 T cells using TRI reagent (Sigma). First-strand cDNA was produced using high-capacity RNA–cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Gene expression profiles of nuclear transcription factors or MPO, NE, and RAGE were detected by real-time PCR using the SYBR Green JumpStart Tag Ready Mix (Sigma). Specific primers were designed based on mRNA sequences (Table 1). Each targeted mRNA expression levels were normalized relative to GAPDH expression, and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Table 1. The List of the Primers Used in This Study

TBET	F	5′-ATG TGA CCC AGA TGA TTG TGC-3′
	R	5′-TGG AAA GTA AAG ATA TGC GTG TT-3′
GATA3	F	5′-GAA CCG GCC CCT CAT TAA G-3′
	R	5′-ATT TTT CGG TTT CTG GTC TGG AT-3′
RORC	F	5′-TTT TCC GAG GAT GAG ATT GC-3′
	R	5′-CTT TCC ACA TGC TGG CTA CA-3′
FoxP3	F	5′-CAC CAC CGC CAC TGG GGT CT-3′
	R	5′-TCTGGGGCACAGCCGAAAGG-3′
NE	F	5′-CTG CAG GAG CTC AAC GTG AC-3′
	R	5′-CCC TCA CGA GAG TGC AGA C-3′
MPO	F	5′-GGC ATC ACC ACC GTG TCT AA-3′
	R	5′-AAG CCA GGT TCA ATG CAG GA-3′
RAGE	F	5′-TGG ATG AAG GAT GGT GTG CC-3′
	R	5′-CAC AGC TGT AGG TTC CCT GG-3′
GAPDH	F	5′-CAC TCC TCC ACC TTT GTC GCT-3′
	R	5′-CCT GTT GCT GTA GCC AAA TTC GT-3′

Western Blotting Analysis

Preparation of whole cell lysates, electrophoresis, transblotting, and subsequent immunoreactions were performed as we previously described [33]. β-Actin was used as an invariant control for equal loading. Protein concentrations were determined using the BCA Protein Assay Kit according to the manufacturer's instruction (Pierce, Rockford, IL, USA). Thirty micrograms of protein samples were loaded on each well. Protein bands were visualized by using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotech, Santa Cruz, CA, USA). Mouse anti-human RAGE IgG1κ (sc-365154), mouse anti-human MPO IgG2b (sc-52076), and mouse anti-human NE IgG1κ (sc-365950) were purchased from Santa Cruz Biotech. The densitometry analysis of band was performed with ImageJ software.

Immunofluorescent Staining

Neutrophils were fixed using 3% para-formaldehyde and 0.3% Triton X-100 in PBS for 10 min and blocked with 1% BSA and 0.3% Triton X-100 in PBS. Primary antibodies were added and incubated for 1 h at room temperature and detected using AlexaFluor-488-conjugated secondary antibodies (A32723, Invitrogen, Carlsbad, CA, USA). Nuclei were stained by a mounting solution with DAPI. The slides were observed under a fluorescent microscope (Leica DM 4000 B). Representative views of cell staining were presented.

Statistical Analyses

Differences between means were evaluated using an unpaired two-sided Student's *t* test ($p < 0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with controls were analyzed by ANOVA with the Dunnett's test for *post hoc* analysis.

RESULTS

AGE-Stimulated The Induction of Neutrophils on CD_4^+ T Cell Differentiation Bias Th_1 and Th_{17} T Cells

Neutrophils were derived from human health blood and stimulated with or without 200 μg/mL of AGEs for 4 h (the culture time point refers to the report [34]). The cultural media of neutrophils (PCM) were collected and supplied for incubation of human naive CD_4^+ T cells. To obtain the reasonable effect of PCM on CD_4^+ T cells incubation, we tried to use different ratios of PCM: fresh T cell culture medium to treat CD_4^+T cells and finding that CD_4^+ T cells cultured with 50:50 (*v*/*v*) medium grew better (data not shown here). Therefore, in present study, CD_4^+ T cells were treated with the T cell culture medium containing 50% volume of PCM for 3 days. The cell phenotypes were analyzed by flow cytometry. The data showed in Fig. 1b that the cells treated with PCM-AGE had a pronounced increase in percentage of IFN-γ (+) or IL17 (+) cells, compared to the PCM control ($p = 0.007$ or $p = 0.038$, respectively). Next, the transcription factors representing subtype cell signatures were detected by real-time PCR. The mRNA levels were highly elevated in Tbet (specific for Th_1) and RORγt (specific for Th_{17}) when the cells treated with PCM-AGE (Fig. 1c). These results suggested that neutrophils treated with AGEs affected naive CD_4^+ T cell differentiating propensity to Th_1 and Th_{17} cells, which were considered as the pro-inflammatory Th subtypes.

AGE-Stimulated Neutrophils Promoted $CD_4^+CD_{25}^+FoxP_3^+$ T Cell Transformation into Th_1 and Th_{17} T Cells

Th_1/Th_2 or Th_{17}/Treg cell balance is critical to the immune homeostasis. In some conditions, Th_1, Th_{17}, and Treg cells undergo transformations into each other in response to various stimuli. To observe whether AGE-stimulated neutrophils affect Treg cell transformation, Treg cells with $CD_4^+CD_{25}^+FoxP_3^+$ phenotypes were purified and incubated with PCM or PCM-AGE for 3 days; the cell phenotypes in Fig. 2a showed that PCM-AGE significantly increased the productions of INFγ $^+$ or IL17$^+$ cell population, compared with PCM only or controls, but this increase was not observed in IL-4$^+$ cell population. Similarly, PCM-AGE increased the gene expressions of Tbet and RORγt but not GATA3. Taken together, the results suggested that AGE-stimulated PMNs facilitate the phenotype switch of Treg into Th_1 and Th_{17} cells.

AGE Increased MPO and NE Productions in Neutrophils

Next, we investigated the mechanism underlying AGEs affecting neutrophil's activity which shapes Th cell fates and function. We first hypothesized that AGEs regulated some enzyme productions and functions in neutrophils. To confirm this hypothesis, purified healthy neutrophils were cultured with various concentrations of AGEs (0, 50, 100, 200, 300 μg/mL) for 4 h or 200 μg/mL for different times (1, 2, 4, 8, 16 h); the resultant cells were collected and used to detect gene expressions of MPO and NE by real-time PCR and the protein levels by Western blotting analysis. The data

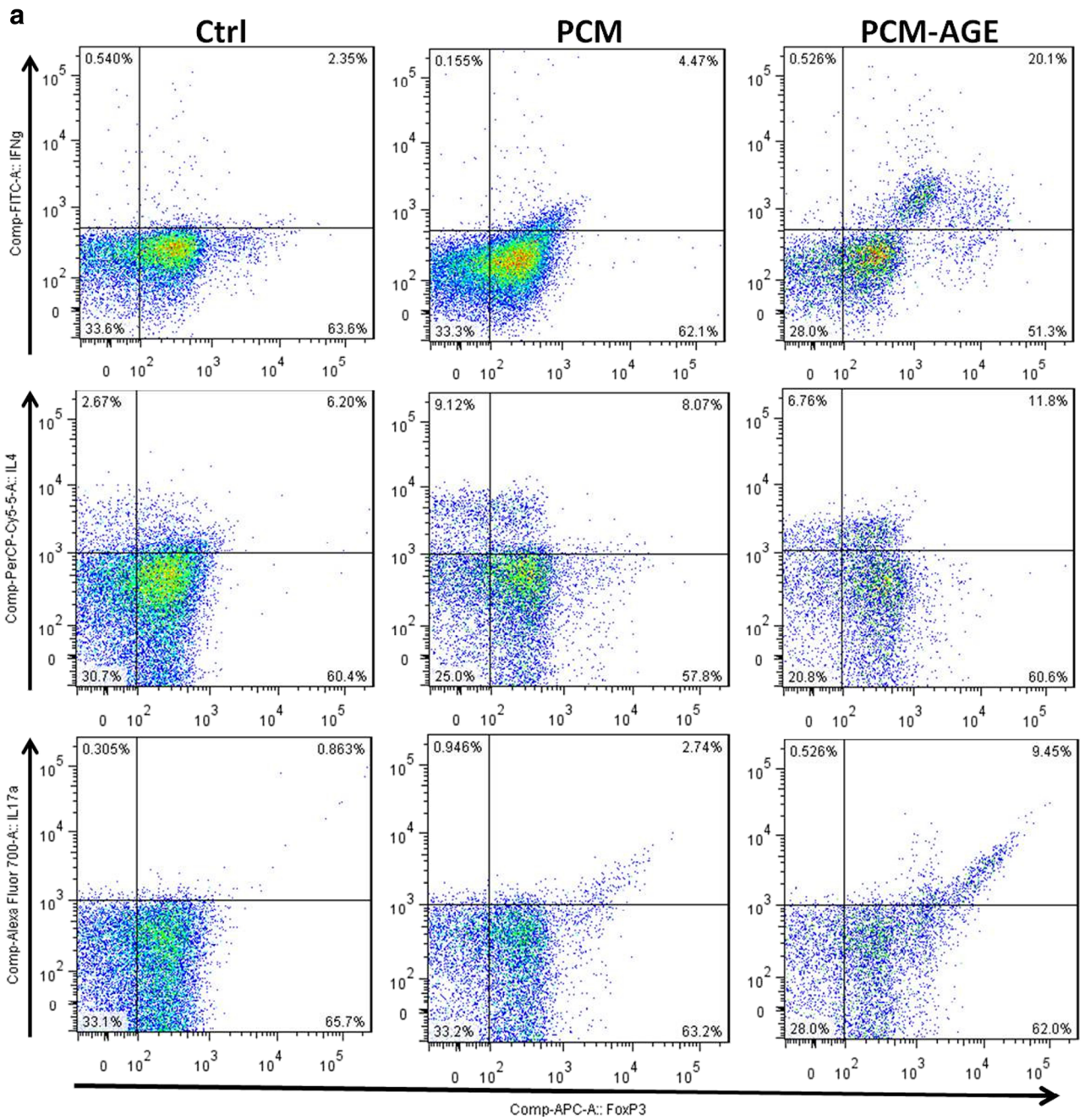

Fig. 1. AGE-stimulated neutrophils induced CD_4^+ T cell differentiation bias Th_1 and Th_{17} T cells. Purified human healthy neutrophils were treated with or without 200 μg/mL of AGEs for 4 h, the culture medium (PCM) were collected and used to treat human CD_4^+ T cells at 50% (vol/vol%) concentration in anti-CD_3 coated plates for 3 days. The resultant cells were used for flow cytometry (**a**, **b**) and real-time PCR analysis (**c**). IL-2 was added to all culture medium with final concentration of 50 U/mL. **a** Representative views of flow cytometry for IFN-γ, IL-4, IL-17, and $FoxP_3$ expressions were presented. **b** The data for flow cytometry were shown, $n = 15$, dot represented individual sample, and dash represented the mean value. **c** Real-time PCR analysis of Tbet, GATA3, RORgt, and $FoxP_3$ were shown as means ± SEM, $n = 5$. $^*p < 0.05$, $^{**}p < 0.01$, *vs* untreated control; $^{\#\#}p < 0.01$, *vs* AGE-treated group.

showed AGE dose-dependently elevated MPO, NE mRNA levels (Fig. 3a), and protein levels (Fig. 3c). In time-dependent experiments, MPO and NE mRNA levels were gradually increased after application of

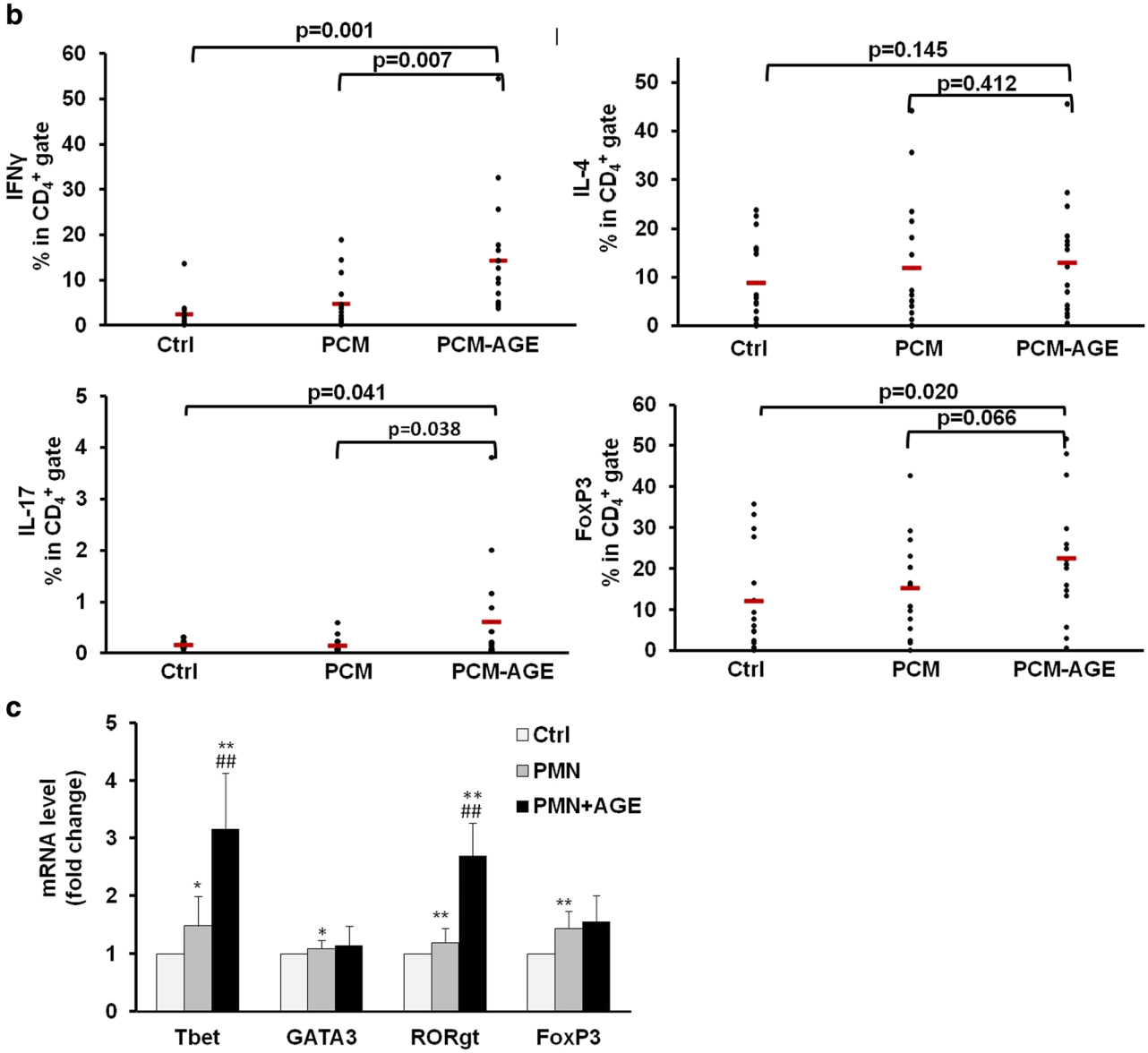

Fig. 1. continued.

200 μg/mL of AGEs, reached the peak value at 4 h point, then dropped at 8 h and 16 h points (Fig. 3b). Furthermore, these time point change trends were also found in the changes of MPO and NE protein levels (Fig. 3d). Additionally, we did immunofluorescence detection for neutrophils treated with 200 μg/mL of AGEs for 4 h. The data indicated that AGEs obviously increased MPO (Fig. 3e) and NE (Fig. 3f) expressions. Taken together, these data supported that AGE modulates enzyme's production which is essential for neutrophil activity.

Inhibition of NE and MPO Blocked AGE-Treated Neutrophils' Effects on CD_4^+ T Cell Differentiation into Th_1 and Th_{17} Cells

To verify the roles of NE and MPO in AGE-treated neutrophils' effect on CD_4^+ T cell differentiation, Sivelestat (SV, a neutrophil elastase inhibitor, CAS#S7198, Sigma, USA) and 4-aminobenzoic acid hydrazide (ABAH, a specific irreversible inhibitor of MPO, CAS# 5351-17-7, Sigma, USA) were used to block NE and MPO activities. Purified neutrophils were co-incubated with 200 μg/mL of AGEs and

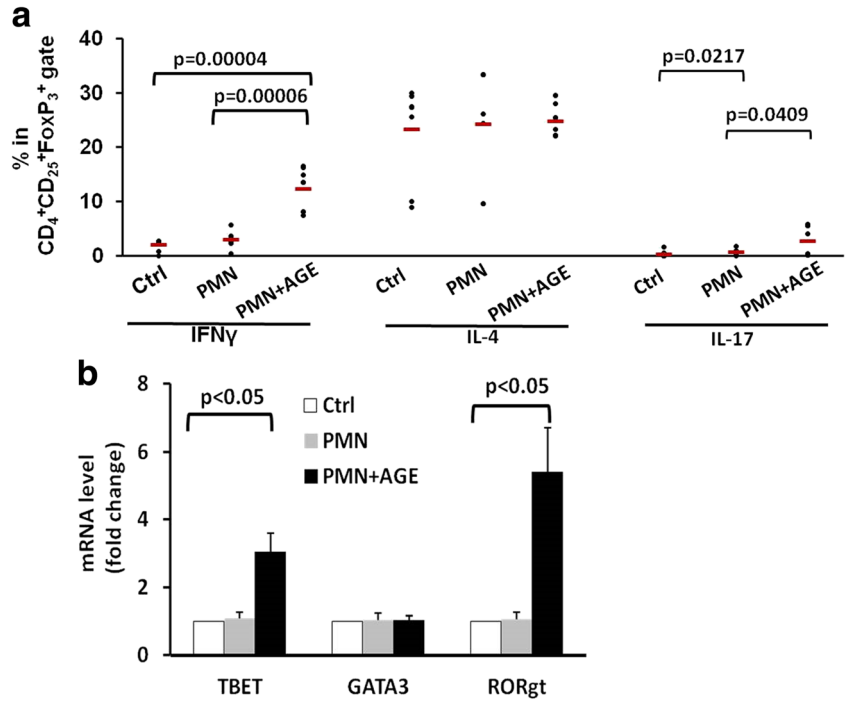

Fig. 2. AGE-stimulated neutrophils promoted $CD_4^+CD_{25}^+FOXP_3^+$ T cell transformation into Th_1 and Th_{17} T cells. Purified human healthy neutrophils were treated with or without 200 μg/mL of AGEs for 4 h; the culture medium (PCM) was collected and used to treat human $CD_4^+CD_{25}^+FoxP_3^+$T cells at 50% (vol/vol%) concentration in anti-CD_3 coated plates for 3 days. The resultant cells were used for flow cytometry (a) and real-time PCR analysis (**b**). IL-2 was added to all culture medium with final concentration of 50 U/mL. **a** Flow cytometry were shown, $n = 8$, dot represented individual sample, and dash represented the mean value. **b** Real-time PCR analysis of Tbet, GATA3, and RORgt was shown as means ± SEM, $n = 8$.

100 nM of SV or 100 μM of ABAH for 4 h. Control cells were incubated in the presence of regular medium. The media were collected and used as cultural medium for naive CD_4^+ T cells for 3 days. The cells were analyzed by flow cytometry. As shown in Fig. 4a, b in CD_4^+ gate (left groups), either SV or ABAH attenuated AGE's enhancing role in IFN^+ or $IL\text{-}17^+$ cell populations. Furthermore, the mRNA level about Tbet or RORγt was significantly reduced in AGE-PCM with SV or ABAH treatment, compared with AGE-PCM treatment only (Fig. 4c, d in CD_4^+ gate, left group). The data suggested that the effect of AGE-treated neutrophils on CD_4^+ T cell differentiation is largely associated with NE and MPO activities.

Inhibition of NE and MPO Suppressed the Role of AGE-Treated Neutrophils in Transformation of Treg Cells into Th_1 and Th_{17} Cells

Similarly, we observed the effect of NE and MPO on the AGE-treated neutrophil's role in Treg cell transformation. Purified PMNs were co-incubated with 100 nM of SV or 100 μM of ABAH and 200 μg/mL of AGE for 4 h. The collected media were used to incubate $CD_4^+CD_{25}^+FoxP_3^+$ cells for 3 days. The cell phenotypes analyzed by flow cytometry are shown in Fig. 4a, b on $CD_4^+CD_{25}^+FoxP_3^+$ gate (on the right group). As expected, PCM-AGE-elevated IFN-γ^+ or $IL17^+$ cell population was reduced by SV or ABAH application. In addition, real-time PCR analysis about Tbet or RORγt mRNA level in Fig. 4c, d in $CD_4^+CD_{25}^+FoxP_3^+$ T cells also supported that SV or ABAH blocked Treg cell transformation into Th_1 or Th_{17} cells.

Interruption of RAGE Inhibited the Effect of AGE on MPO and NE Production in Purified Neutrophils

To further investigate the mechanism about the role of AGE in neutrophils activity, we hypothesized that the role was mediated by the interaction of AGEs with its receptor (RAGE). To test the hypothesis, purified neutrophils were co-incubated with/without 200 μg/mL of AGE and RAGE inhibitors and FPS-ZM1 (Cat. 553030, Calbiochem, USA) at various doses (0, 50, 100, 200 nM) for 1 h. MPO and NE mRNA levels were detected by real-time PCR. The data in

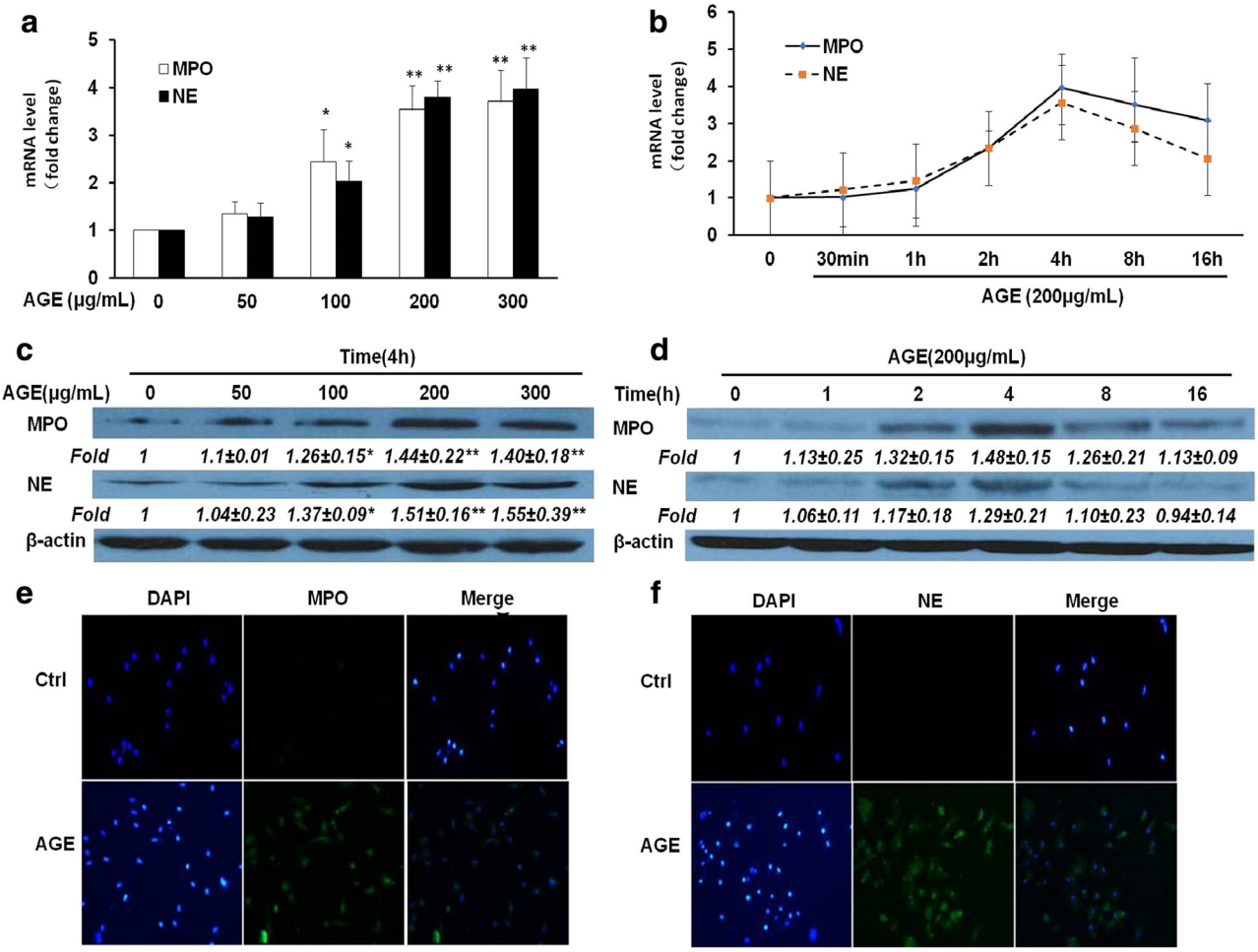

Fig. 3. AGE increased myeloperoxidase (MPO) and neutrophil elastase (NE) production in neutrophils. Purified human healthy neutrophils were treated with various concentrations of AGEs (0, 50, 100, 200, 300 μg/mL) for 4 h (**a**, **c**) or 200 μg/mL of AGEs for different time points (0, 1 h, 2 h, 4 h, 8 h, 16 h) (**b**, **d**). **a**, **b** Real-time PCR analysis for MPO and NE gene expressions as means ± SEM, $n = 8$. *$p < 0.05$, **$p < 0.01$, *vs* untreated control (the first column on the left). **c**, **d** Western blotting analysis for MPO and NE protein levels (30 μg proteins/well were loaded); β-actin was used as an internal control for equal loading. The numbers beneath the blot were the fold change (means ± SEM, $n = 3$) in the densities of the bands compared with control (the first band on the left) in the blot, after normalization with the internal invariable control. **c** *$p < 0.05$, **$p < 0.01$, *vs* untreated control (the first band on the left). **e** Purified human healthy neutrophils were treated with 200 μg/mL of AGEs for 4 h. MPO and NE expressions *in situ* were detected by immunofluorescence assay. Nuclei were stained with DAPI. Typical views were presented (× 40).

Fig. 5a, b showed that FPS inhibited MPO and NE gene expression in a dose-dependent manner. The mRNA levels of MPO and NE were significantly reduced at 100 nM of FPS (the second column from the right), compared with that in AGE treatment only (the second column from left) ($p < 0.01$, $p < 0.05$, respectively). Then, the MPO and NE protein productions were detected by Western blotting analysis; Fig. 5c shows that FPS dose-dependently attenuated the increase in AGE-induced MPO and NE production. These data supported that RAGE played an important role in the effect of AGEs.

Changes of MPO and NE Activities Unaffected RAGE Gene Expression in Purified Neutrophils

Our data indicated that AGE–RAGE interaction regulated neutrophil activities; the further question was whether PMN activities, in turn, affected RAGE expression. To clarify this question, purified neutrophils were co-incubated with 200 μg/mL of AGE and various concentrations of SV (0, 100, 200, 400 nM) or ABAH (0, 100, 200, 400 μM) for 4 h, the resultant cells were used to detect RAGE gene expression. As shown in Fig. 5d, AGE-treated

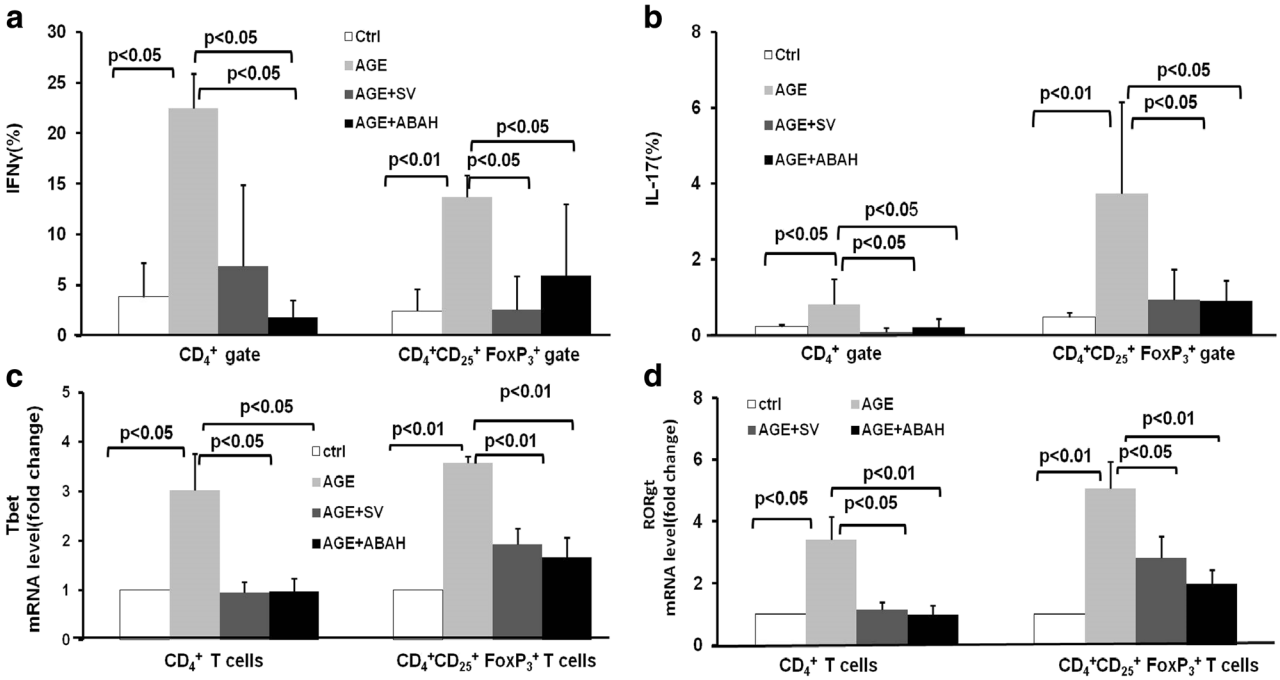

Fig. 4. Inhibition of MPO or NE attenuated PCM-AGE's effect on CD_4^+ differentiation into Th_1 and Th_{17} cells. Purified healthy neutrophils were co-incubated with 200 μg/mL of AGEs and 200 nM of SV or 100 μM of ABAH for 4 h. Control cells were incubated in the presence of regular medium. The culture medium were collected and used to treat healthy naïve CD_4^+ T cells or $CD_4^+CD_{25}^+FoxP_3^+$ T cells at 50% (vol/vol%) for 3 days. **a** Flow cytometry for IFN-γ expression, $n = 6$, means ± SEM. **b** Flow cytometry for IL-17, $n = 6$, means ± SEM. **c** Real-time PCR analysis for Tbet mRNA levels, $n = 6$, means ± SEM. **d** Real-time PCR analysis for RORgt mRNA levels, $n = 6$, means ± SEM.

neutrophils had increased RAGE mRNA level, which was not affected by the pre-incubation with various concentrations of SV or ABAH. And Western blotting analysis also supported the findings; Fig. 5e, f indicated that SV or ABAH did not influence the increased RAGE production induced by AGEs. Taken together, elevation of MPO and NE activities in neutrophils was the consequence of the downstream responses following AGE–RAGE interaction.

DISCUSSION

In this study, we designed to evaluate the effect of AGE-stimulated PMNs on CD_4^+ Th cell differentiation. Our present report demonstrated that PMN treated with AGE drives both naive CD_4^+ T cells and Treg cells developing into Th_1 and Th_{17} cell populations. This PMN activity is carried out through AGE–RAGE interaction and largely associated with MPO and NE production.

AGEs represent one of the main players responsible for the development of [35] diabetes's complications [34, 35]. Usually, this pathological process is initiated with the aberrant interaction between innate and adaptive immune systems induced by AGEs [36–38]. Previous studies indicated Th_1 and Th_{17} cell populations, representatives of pro-inflammatory cells, and their numbers are markedly increased while $CD_4^+CD_{25}^+FoxP_3^+$ cell population, representative of anti-inflammatory cells, are decreased in diabetes patients [39, 40]. The exact mechanism underlying imbalance of CD_4^+ T cell populations in DM patients is still unclear. In this study, we found that AGE-treated neutrophils altered naive CD_4^+ T cell differentiation and Treg cell transformation and provided a possible explanation about the mechanism. To our knowledge, it is the first time to introduce the role of neutrophils in AGE's regulating CD_4^+ T cell dynamic switches and suggest that neutrophils may become a possible target against the development of DM complications.

The effect of AGEs on cell activities is mainly mediated through its receptors on cellular surfaces. The most studied receptor is receptor for AGEs (RAGE). Our present study indicated that interruption of RAGE inhibited AGE's effect on neutrophils. AGE–RAGE interaction activates different signaling pathways, such as ERK/JNK, PI3K, or

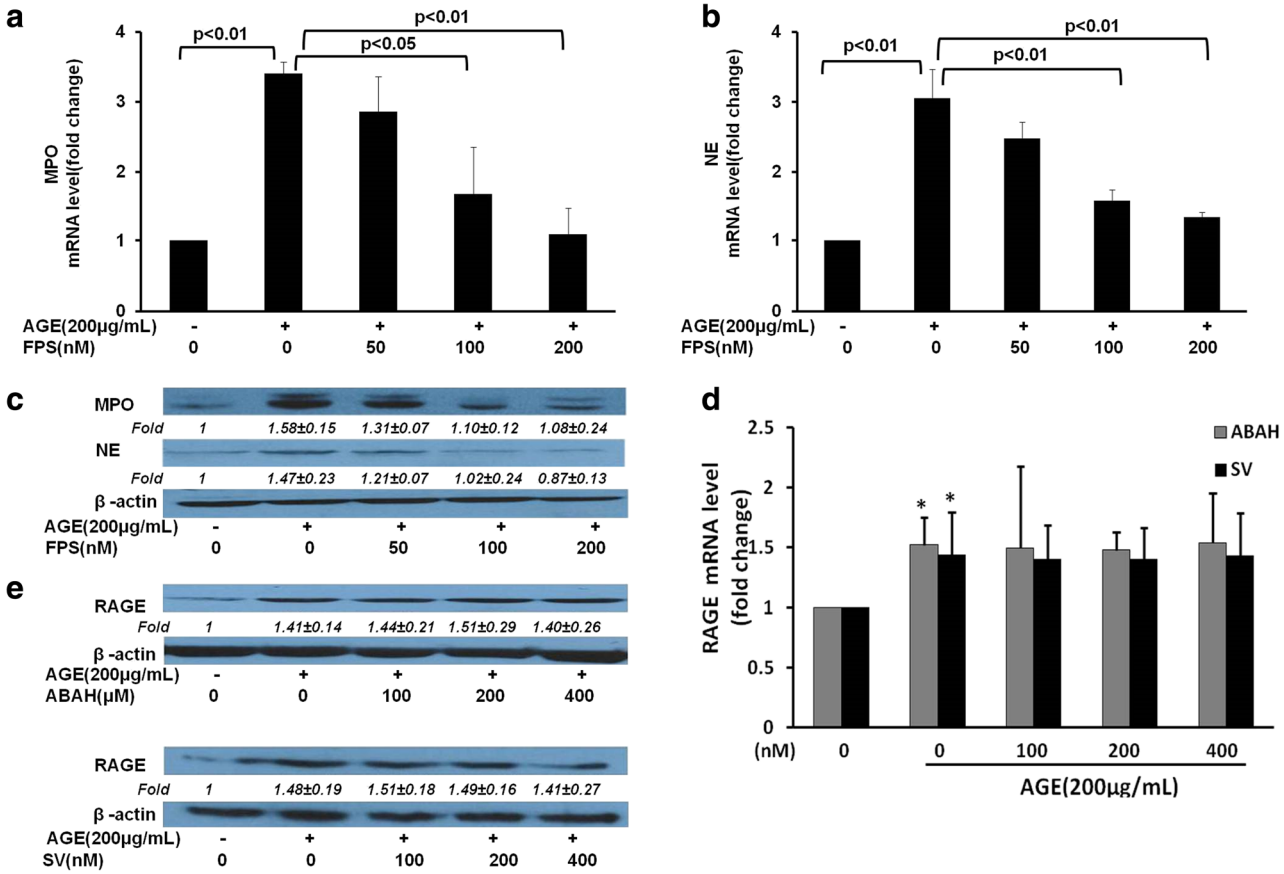

Fig. 5. AGE receptor (RAGE) is required for the effect of AGE on MPO and NE production in neutrophils. **a**–c Purified healthy neutrophils were incubated with AGE and RAGE inhibitor, FPS-ZM1, at various doses (0, 50, 100, 200 nM) for 4 h. **a** Real-time PCR analysis for MPO mRNA level, $n = 6$, means ± SEM. **b** Real-time PCR analysis for NE mRNA level, $n = 6$, means ± SEM. **c** Western blotting analysis for MPO and NE protein levels (30 μg proteins/well were loaded); β-actin was used as an internal invariable control for equal loading. **d**, **e** Purified healthy neutrophils were co-incubated with 200 μg/mL of AGE and different concentrations of ABAH (0, 50, 100, 200 μM) or SV (0, 50, 100, 200 nM) for 4 h. **d** Real-time PCR analysis for RAGE mRNA level, $n = 6$, means ± SEM. $^*p < 0.05$, *vs* untreated control (first column on the left). **e** Western blotting analysis for RAGE protein levels (30 μg proteins/well were loaded); β-actin was used as an internal invariable control for equal loading. **c**, **e** The numbers beneath the blot were the fold change (means ± SEM, $n = 3$) in the densities of the bands compared with control (the first band on the left) in the blot, after normalization with the internal invariable control.

other molecules, which in turn regulating some transcription factor activities [41] and then resulting in the expression of MPO or NE. In addition, AGE–RAGE axis stimulates the production of oxygen radicals. Both the generations of these enzymes and oxygen radicals are important for neutrophils activities in killing pathogens, allergic reaction, inflammatory response, or tissue damages [42, 43]. It is noteworthy that RAGE inhibitor (FPS), even 200 nM, did not completely reverse the AGE's effect (Fig. 5a–c); the possible explanation is that the AGE's action can be taken by the receptors other than RAGE. The other receptors includes, but not limited to, CD36 and AGE receptor (AGER) 1, R2, and R3 [44, 45]. CD36 is a scavenger receptor that can promote rapid activation of caspase-3 leading to neutrophil apoptosis [46]. Our previous study showed that AGER1 counteracted AGE's effect in activation of hepatic stellate cells and suggested that enhanced AGER1 gene expression might be a novel strategy to treat AGE-associated diseases [31]. The change and role of these scavenger receptors in AGE-treated neutrophils need further investigation.

Neutrophils are the major player in the innate immune system and generally considered to play a role in antimicrobial activity and even immunopathology. Recent emerging evidence has confirmed that neutrophils have an additional activity on priming a Th_1- and Th_{17}-acquired immune response [47, 48]. In present study, neutrophils treated with AGEs facilitated naive CD_4^+ T cell differentiation into Th_1

and Th_{17} cell population. The design of this study is limited to use cultural medium of neutrophils to incubate naive CD_4^+ T cells; the effect of neutrophils, therefore, is considered through soluble mediators. MPO and NE are two enzymes abundantly expressed in neutrophil granulocytes and play important roles in destroying pathogens and host tissues, and forming neutrophil extracellular traps (NETs), which can bind pathogens [49]. In this study, we found MPO and NE inhibitions blocked neutrophil–CD_4^+ T cell crosstalk, suggesting MPO and NE may act as the mediators between neutrophil–CD_4^+T cell communication. How MPO and NE activities affect CD_4^+ T cell differentiation is still elusive. It is noted that other factors or cytokines are not ruled out in mediating this process. Besides the soluble mediator, neutrophil–CD_4^+T cell direct contact has been reported to regulate CD_4^+ T cell differentiation [32]. Our ongoing study is to investigate the effect of cell–cell contact culture on CD_4^+ T cell differentiation.

Treg cells are recently recognized as $CD_4^+CD_{25}^+FoxP_3^+$ T cell population and play a dominant role in maintaining immune homeostasis and tolerance. Treg cells have plasticity, in autoimmune and inflammatory diseases, such as diabetes, multiple sclerosis, and rheumatoid arthritis; Treg cell activity has been found reduced [50]. The underlying mechanisms at least include the reduction of differentiated Treg cell number and increase of Treg transformation into other cells. In this study, we demonstrated that the cultural medium from AGE-treated neutrophils enhanced naive CD_4^+ T cell differentiation into pro-inflammatory cells, relatively, reducing Treg cell population. On the other hand, these cultural media induced $CD_4^+CD_{25}^+FoxP_3^+$ T cell transformation into Th_1 and Th_{17} cells. Combined these two pathways, Th_{17}/Treg ratio loses its homeostatic equilibrium, which in turn accelerates the development of inflammation and autoimmune diseases.

In summary, our study confirmed that neutrophils mediate AGE-related CD_4^+ T cell differentiating bias into pro-inflammation, partly through the generation of MPO and NE. The study provides novel insight on the mechanism about the development of diabetes-associated inflammation. Because of huge number of neutrophils existing in the circulation, it is possibly expected to use neutrophils as a therapeutic target in the prevention and treatment of diabetes complication.

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COMPLIANCE WITH ETHICAL STANDARDS

This study was approved by the Ethical Review Committee of Guangdong Second Provincial General Hospital. Informed consent of blood donation was given by healthy adult volunteers who had no record of food allergy or sensitization.

Conflict of Interest. The authors declare that they have no conflict of interest.

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