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## **The Receptor for Advanced Glycation End-products Enhances Lung Epithelial Wound Repair: an In Vitro Study**

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## **Abstract**

Re-epithelialization of the alveolar surface is a key process of lung alveolar epithelial barrier repair after acute lung injury. The receptor for advanced glycation end-products (RAGE) pathway plays key roles in lung homeostasis, and its involvement in wound repair has been already reported in human bronchial epithelial cells. However, its effects on lung alveolar epithelial repair after injury remain unknown. We investigated whether RAGE stimulation with its ligands high-mobility group box 1 protein (HMGB1) or advanced glycation end-products (AGEs), alone or associated with RAGE inhibition using RAGE antagonist peptide, affects in vitro wound healing in human alveolar epithelial A549 cells. We further asked whether these effects could be associated with changes in cell proliferation and migration. We found that treatment of A549 cells with HMGB1 or AGEs promotes RAGE-dependent wound healing after a scratch assay. In addition, both RAGE ligands increased cell proliferation in a RAGE-dependent manner. Treatment with HMGB1 increased migration of alveolar epithelial cells at 12 h, independently of RAGE, whereas AGEs stimulated migration as measured 48 h after injury in a RAGE-dependent manner. Taken together, these results suggest that RAGE pathway is involved in lung alveolar epithelial wound repair, possibly through enhanced cell migration and proliferation.

**Keywords:** receptor for advanced glycation end-products; lung epithelial injury; wound healing; cell migration; cell proliferation.

## Introduction

Alteration of alveolar epithelial barrier integrity with increased epithelial permeability plays a central role in the pathophysiology of the acute respiratory distress syndrome (ARDS) [1–4], a severe condition associated with significant morbidity and mortality, long-term physical or psychological consequences, and high healthcare-associated costs [5–7]. The lung alveolar epithelial barrier is composed of alveolar type (AT)-I cells, covering 95% of the alveolar epithelial surface, and of AT-II cells. Previous research suggests that re-epithelization of the alveolar surface is a key process that is required to restore the integrity and function of the lung alveolar epithelial barrier [8]. Although this repair process may involve the proliferation and migration of AT-II cells, its precise mechanisms remain underinvestigated [8–10].

The receptor for advanced glycation end-products (RAGE) is a membrane signal transduction receptor that can induce various intracellular pathways by binding to multiple ligands, such as S100 proteins, high-mobility group box 1 protein (HMGB1), and advanced glycation end-products (AGEs), among others [11–16]. RAGE is abundantly expressed in the lung tissue, both as transmembrane full-length and soluble forms, and RAGE pathway may play a key role in lung homeostasis [17,18]. Recent clinical and preclinical research has suggested that soluble RAGE (sRAGE) could be a biomarker of lung epithelial injury associated with severity and outcome in ARDS [19–25]. Moreover, RAGE inhibition or *AGER* gene deficiency decreased lung injury in animal models of experimental ARDS from both septic or non-septic causes [26–29]. Although the RAGE pathway has a major role in initiating and perpetuating nuclear factor-kappa B (NF- $\kappa$ B)-mediated inflammation, it also contributes, among many other potential effects [11], to repair mechanisms such as peripheral nerve regeneration through the recruitment of axonal outgrowth pathways [14,30] or extracellular matrix synthesis and wound repair in human bronchial epithelial cells through stimulation by HMGB1 [31]. However, whether modulation of RAGE pathway might affect lung alveolar epithelial wound repair remains unknown.

In this study, we investigated whether treatment with RAGE agonists (HMGB1 and AGEs), alone or combined with a RAGE antagonist, would affect wound healing of lung alveolar epithelial cells *in vitro*. We further hypothesized that modulation of the RAGE pathway might affect some major processes of wound healing in these cells, such as proliferation or migration.

## **Materials and Methods**

### **Reagents and antibodies**

Cell culture Roswell Park Memorial Institute (RPMI 1640) medium 1640, fetal bovine serum (FBS), antibiotics (streptomycin, penicillin, amphotericin B), and phosphate buffered saline (PBS) were obtained from Fisher Scientific (Illkirch, France). Human recombinant HMGB1 was obtained from Sigma-Aldrich (Saint Quentin-Fallavier, France). AGEs (ab51995) was purchased from Abcam (Paris, France). RAGE antagonist peptide (RAP) was obtained from Calbiochem® Merck Millipore (Molsheim, France).

### **Cell culture**

Human adenocarcinoma-derived alveolar AT-II epithelial cells (A549) were obtained from the American Tissue Culture Collection (CCL-185, LGC Standard®, Molsheim, France). After thawing, cells were cultured in a 95% humidified air atmosphere at 37°C with a CO<sub>2</sub> concentration of 5% in a RPMI-1640 culture medium enriched with 5% FBS and supplemented with 10 mg/mL streptomycin, 10,000 U/mL penicillin, and 25 µg/mL amphotericin B (Fisher Scientific, France).

### **In vitro model of alveolar epithelium wound healing (scratch assay)**

Cells ( $2 \times 10^5$  per well) were cultured for 72 h in six 9.5 cm<sup>2</sup> well-plates (Corning, Samois-sur-Seine, France) until 100% confluence was obtained. The tip of a 200-µL pipette was applied perpendicular to the surface of the cell monolayer to create a thin "wound". After three washes with PBS (1X), wounded cells were either left untreated (control) or treated with RAGE agonists alone or combined with RAP (12.5 µg/mL), before being incubated at 37°C with 95% humidified air and 5% CO<sub>2</sub>. The dose of RAP was chosen based on the findings from previous studies [29,32–34]. The wound area was observed immediately after the scratch (h0) and 12, 24, and 48 h after treatment using a Nikon polarized optical microscope with 40x magnification and a digital camera body. Images were recorded prior to their analysis using the Fiji image processing software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) [35].

Measurements of the wounded area were made from edge to edge by manually delimiting the lesion surface. This experiment was repeated six times (with each condition performed in duplicate). After testing three concentrations of RAGE agonists HMGB1 (0.1, 0.3, and 0.6 µg/mL) and AGEs (10, 30, and 90 µg/mL) for their effect on wound area, subsequent experiments were performed using the minimum effective concentration of each.

### **Cell migration assay**

Epithelial cell migration was assessed with the CytoSelect™ 96-well cell migration assay (CBA-106; 8 µm Fluorometric format; Biolabs, London, United Kingdom). At 12 and 48 h after scratch wounding, cells were suspended in a serum-free medium and placed in the upper chamber. Three hundred microliters of chemoattractant media containing 5% FBS was then added to the lower chamber. After a 24 h incubation, migratory cells that had passed through the polycarbonate membrane (8 µm pore size) were dislodged from the underside of the membrane with a detachment solution. The dislodged cells were then stained with CyQuant® GR Dye (Fisher Scientific, Hampton, USA), diluted in a lysis buffer, and quantified by fluorescence measurement at 480 nm/520 nm (Zeiss LSM 800, Carl Zeiss SAS, Marly Le Roi, France). This experiment, performed in triplicate, was independently repeated three times.

### **Cell proliferation assay**

At 12 and 48 h after scratch wounding, A549 cells were stained with 5-Bromo-2'-deoxy-uridine (BrdU) at 10 µM using a BrdU Labeling and Detection Kit II (11299964001; Roche Diagnostics, Meylan, France). After 45 minutes, they were washed three times with PBS, and fixed with an ethanol fixative solution (50 mM glycine, pH=2, in 70% ethanol) for 20 minutes at -20°C. After three washes in PBS, the cells were incubated with an anti-BrdU antibody (1/25) for 60 minutes at 37°C. The cells were washed three times with PBS, followed by incubation with an anti-mouse-Ig-alkaline phosphatase (1:25 dilution) for 60 minutes at 37°C. The color substrate solution was added to the cells after three washes in PBS, and the bound anti-BrdU antibody was visualized by light microscopy (Zeiss Axio Observer, Carl Zeiss SAS, Marly Le Roi, France). Proliferation was expressed as the ratio of BrdU-positive cells to the total number of cells. This experiment, performed in triplicate, was independently repeated three times.

## Statistical analysis

All analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, USA) and Stata version 15 (StataCorp, College Station, TX, USA). The tests were two-sided, with a type I error set at 5%. Continuous data were expressed as mean  $\pm$  standard deviation (SD) or median [interquartile range] according to statistical distribution (assumption of normality was assessed using the Shapiro-Wilk test). Continuous parameters were compared between experimental groups using analysis of variance or the Kruskal-Wallis test when t-test assumptions were not met (normality studied using Shapiro-Wilk and assumption of homoscedasticity verified by Fisher-Snedecor test). Random effects models were used to analyze the longitudinal evolution of variables: (i) considering between- and within-experiment variability (random subject effects: random intercept and slope) and (ii) evaluating fixed effects: group, time, and time by group interaction. The residual normality was checked for all models. Values were log-transformed for all variables to achieve normality prior to performing random effects models.

## Results

### **Both HMGB1 and AGEs promote dose-dependent and RAGE-dependent wound healing of lung alveolar epithelial cells**

We first tested different treatment concentrations of HMGB1 and AGEs for their effect on wound area after the scratch assay (**Figure 1 and Table 1**). Both RAGE ligands had dose- and time-dependent effects towards better wound healing of epithelial cells. Compared to controls, cells treated with HMGB1 (0.1  $\mu\text{g/mL}$ ) had better wound healing (time by group interaction,  $p = 0.008$ ) at 24 h and 48 h after injury ( $p = 0.04$  and  $p < 10^{-4}$ , respectively, for post-hoc comparisons). A similar effect was found after treatment with AGEs (30  $\mu\text{g/mL}$ ) (time by group interaction,  $p < 10^{-4}$ ), compared to untreated cells, at 24 h and 48 h after injury ( $p = 0.005$  and  $p = 0.0001$ , respectively, for post-hoc comparisons) ( $p = 10^{-4}$ ). The minimum effective doses of HMGB1 (0.1  $\mu\text{g/mL}$ ) and AGEs (30  $\mu\text{g/mL}$ ) were used in subsequent experiments.

Co-treatment with RAP (12.5 µg/mL) significantly reduced the effects of both HMGB1 (time by group interaction,  $p < 10^{-4}$ ) and AGEs (time by group interaction,  $p < 10^{-4}$ ) on wound area, with most detrimental effects on wound healing observed at 48 h for HMGB1 and at 12 h for AGEs (**Figure 2 and Table 2**).

### **HMGB1 and AGEs have distinct effects on the migration of lung alveolar epithelial cells during wound healing**

After injury, cells treated with HMGB1 (0.1 µg/mL) showed higher migration at 12 h, but not at 48 h, compared to controls (**Figure 3A**); RAP did not influence cell migration after treatment with HMGB1 at both timepoints (**Figure 3A**). Cells treated with AGEs (30 µg/mL) had reduced migration at 12 h but increased migration at 48 h after injury, compared to controls. Treatment with RAP significantly counteracted these effects at both timepoints (**Figure 3B and Table 3**).

### **Both HMGB1 and AGEs increase lung alveolar epithelial cell proliferation in a RAGE-dependent manner during wound healing**

Treatment with HMGB1 (0.1 µg/mL) enhanced cell proliferation at both 12 h and 48 h after injury. These effects were significantly inhibited in presence of RAP at both timepoints (**Figure 4A**). Treatment with AGEs (30 µg/mL) increased cell proliferation at both timepoints after injury, which was abrogated by RAP treatment at both timepoints (**Figure 4B and Table 4**).

## **Discussion**

In this study, HMGB1 and AGEs promoted RAGE-dependent wound healing of lung alveolar epithelial cells after a scratch assay. In addition, both RAGE ligands increased cell proliferation in a RAGE-dependent manner. Treatment with HMGB1 increased cell migration at 12 h only, with no effect of RAP, whereas AGEs stimulated their migration in a RAGE-dependent manner 48 h after injury.

There is growing evidence supporting the involvement of RAGE pathway in mechanisms of cell and tissue repair after injury. Indeed, the AGE/RAGE axis promotes wound healing of human corneal epithelial cells [36] and HMGB1/RAGE axis promotes keratinocyte healing in vitro [15], with improved healing of diabetic human and mouse skin [37]. In addition, HMGB1 improves scratch wound healing of human bronchial epithelial cells via toll-like receptor (TLR)-4 and RAGE-mediated increase in extracellular matrix synthesis and modulation of cell-matrix adhesion [31]. However, in our dose-response experiments, higher doses of HMGB1 (>0.1 µg/mL) and AGEs (>30 µg/mL) were associated with no protective effect on wound healing, suggesting a non-linear dose-response to increasing concentrations of alarmins [38], such as RAGE ligands, and subsequent effects that can be either physiological or pathological to multiple cellular processes [11,39,40]. In our study, the activation of RAGE by both HMGB1 (0.1 µg/mL) and AGEs (30 µg/mL) promoted scratch wound healing whereas the use of a RAGE competitive antagonist abrogated these effects, thus supporting for the first time that previous findings of RAGE pathway as a promoter of wound healing may also hold true in the lung alveolar epithelium. Such findings should now be further explored in the clinical setting of ARDS to better understand the complex mechanisms involved in alveolar epithelium repair after injury, including the spreading, migration, and proliferation of alveolar epithelial cells [41].

Here we found that both HMGB1 and AGEs promote A549 cell migration during wound healing. Lung alveolar epithelial cells treated with HMGB1 acquired higher migration, compared to controls, at 12 h but not at 48 h. Although the significance of these discrepant findings remains uncertain, pro-migratory effects of HMGB1 may decrease with time, as previously reported by Huang *et al.* in another study on RAGE activation by HMGB1 [42]. Concurrent treatment with RAP did not decrease the pro-migratory effect of HMGB1, suggesting that the activation of RAGE by HMGB1 may not be involved in epithelial cell migration during wound healing. Indeed, multiple studies support the interaction of HMGB1 with TLR-4, rather than with RAGE, as the main mechanism responsible for smooth muscle cell migration [43] or motility of human non-small lung cancer cells [44]. In contrast, some studies suggest an important role of RAGE activation by HMGB1 in cell migration, such as in mouse endothelial progenitor cells [45], monocytes [46], neural progenitor cells [47] or glial tumor cells [48]. Consistent with this hypothesis that RAGE is implicated in the process of cell migration; Huttunen *et al.* could inhibit transendothelial migration of melanoma cells in vivo and in vitro by blocking the HMGB1-binding site of RAGE [49], while Yang *et al.* reported that HMGB1 could induce migration of human dendritic cells via RAGE pathway [50]. In addition to the RAGE-dependent beneficial effects of HMGB1 on A549 cell wound healing, we also found that treatment by AGEs (30 µg/mL) both decreased cell migration at

12 h and increased migration at 48 h, compared to controls, and that RAGE inhibition with RAP consistently counteracted these effects at both timepoints. Although future studies are needed to understand whether early pro-inflammatory effects of AGEs might explain, at least in part, decreased migration 12 h after AGEs treatment, the pro-migratory effect of AGEs at 48 h after lung alveolar epithelial cell scratch wounding is similar to previous findings in retinal pericytes [51].

Cell proliferation is also an important contributor to wound healing, together with migration. Here, treatment with both HMGB1 and AGEs significantly increased cell proliferation at 12 and 48 h after scratch wounding, compared to controls, whereas simultaneous treatment with RAP counteracted these effects at both timepoints. Although this study is the first to report the importance of RAGE modulation in the proliferation of lung alveolar epithelial cells, these findings are consistent with previous *in vitro* studies in keratinocytes and fibroblasts [15]. Similarly, blocking the HMGB1/RAGE axis through treatment with recombinant sRAGE, acting as a decoy receptor that prevents interaction between RAGE and its ligands, or after transfection of a truncated RAGE, was able to suppress proliferation of glial cells in mice [48]. We also observed that the anti-proliferative effect of RAP could be less pronounced at 12 h after treatment with HMGB1 than with AGEs (**Figure 4**). Indeed, a previous study found synergistic effects of RAGE and TLR activation on human bronchial epithelial wound healing, such that inhibition of both RAGE and TLR-4 was needed to abrogate the proliferative effects of HMGB1 [31]. Our study is, however, unable to support that the effects of AGEs on lung alveolar epithelial cell proliferation might be more dependent on RAGE pathway than those of HMGB1. In particular, whether our use of an S100P-derived competitive antagonist for RAGE (RAP, Calbiochem® Merck Millipore, Molsheim, France) could bring different results than the use of an HMGB1-derived recombinant RAGE antagonist peptide deserves further investigation [52].

This study has some limitations. First, we only used adenocarcinoma AT II-like alveolar epithelial cells as an *in vitro* cell model of the human lung alveolar epithelium. More translational studies of primary human epithelial cell cultures that include proinflammatory conditions and the integration of a more realistic environment (including, but not limited to, the presence of an extracellular matrix and of innate immunity cells) are now needed to further increase our understanding of the roles of RAGE pathway in mechanisms relevant to lung alveolar epithelial repair after injury, including studies of the potential roles of RAGE in cell transdifferentiation [53–55]. In the meanwhile, whether such RAGE-dependent mechanisms favoring lung epithelial wound healing could have clinical relevance in patients with acute lung injury such as ARDS also deserves further investigation. Second, we used RAP as a competitive RAGE antagonist [32]. However, how other strategies of RAGE inhibition, such as RAGE knockout animals, treatment with recombinant

sRAGE, RNA interference or CRISPR-CAS9 methods would affect lung epithelial cell wound healing remains unknown. Finally, in this study, we did not investigate intracellular pathways (such as Mitogen-activated protein kinase ERK 1/2, JAK/STAT, JNK, Rho GTPase, PI3K/Akt, and NF- $\kappa$ B) through which RAGE could affect lung epithelial wound repair, and the mechanisms explaining these effects deserve further investigation [11].

In conclusion, HMGB1 and AGEs promote in vitro wound healing of human alveolar epithelial cells in a RAGE-dependent manner. These effects could be explained, at least in part, by increased cell migration and proliferation. Future studies are now warranted to better understand the precise signaling pathways through which the RAGE pathway affects lung epithelial wound repair.

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## **Data availability**

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Time post-wounding (h)	Experimental conditions				p value for time by group interaction	p value for post-hoc comparisons
	Control (A)	HMGB1 0.1 µg/mL (B)	HMGB1 0.3 µg/mL (C)	HMGB1 0.6 µg/mL (D)		
12	85.8 [84.7 - 87.0]	81.9 [81.0 - 84.5]	83.8 [80.6 - 86.5]	84.9 [81.9 - 88.4]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: >0.05 A versus D: >0.05 B versus C: >0.05 B versus D: >0.05 C versus D: >0.05
24	78.0 [75.7 - 78.0]	73.9 [70.0 - 75.0]	77.0 [74.8 - 79.5]	76.5 [72.3 - 78.2]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: >0.05 A versus D: >0.05 B versus C: 0.04 B versus D: >0.05 C versus D: >0.05
48	64.5 [62.7 - 67.0]	51.8 [49.2 - 54.4]	63.5 [56.9 - 66.0]	64.9 [61.2 - 67.3]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 A versus D: >0.05 B versus C: 0.003 B versus D: 0.004 C versus D: >0.05
Time post-wounding (h)	Control (A)	AGEs 10 µg/mL (B)	AGEs 30 µg/mL (C)	AGEs 90 µg/mL (D)	p value for time by group interaction	p value for post-hoc comparisons
12	84.5 [84.0 - 87.5]	82.0 [81.0 - 85.5]	73.0 [70.0 - 75.2]	81.0 [80.0 - 82.0]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: <10 <sup>-4</sup> A versus D: 0.002 B versus C: <10 <sup>-4</sup> B versus D: >0.05 C versus D: 10 <sup>-4</sup>
24	73.5 [72.0 - 75.0]	66.5 [65.0 - 75.0]	57.0 [53.7 - 60.5]	70.0 [68.8 - 71.3]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: <10 <sup>-4</sup> A versus D: 0.02 B versus C: 0.005 B versus D: >0.05 C versus D: <10 <sup>-4</sup>
48	62.0 [60.8 - 63.0]	59.0 [57.8 - 60.0]	44.5 [41.7 - 47.0]	58.0 [56.8 - 60.0]	<10 <sup>-4</sup>	A versus B: 0.002 A versus C: <10 <sup>-4</sup> A versus D: 0.007 B versus C: <10 <sup>-4</sup> B versus D: >0.05 C versus D: <10 <sup>-4</sup>

**Table 1. Wound area (in %) of A549 cells treated by different concentrations of HMGB1 and AGEs at different time points after a scratch assay.** The wound area is expressed as the fraction of the initial wound (100% at h0). Data are presented as medians and interquartile ranges [IQR]

and were analyzed using random effects models; when time by group interaction was significant, post-hoc comparisons between groups at each timepoint were performed using the same models. *HMGB1*: high-mobility group box 1 protein. *AGEs*: advanced glycation end-products.

Time post-wounding (h)	Experimental conditions			p value for time by group interaction	p value for post-hoc comparisons
	Control (A)	HMGB1 0.1 µg/mL (B)	HMGB1 0.1 µg/mL + RAP (C)		
12	86.9 [84.8 - 88.7]	85.8 [85.4 - 87.7]	81.7 [80.6 - 83.5]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: >0.05 B versus C: >0.05
24	76.4 [73.8 - 78.0]	75.3 [68.3 - 77.7]	72.5 [71.8 - 76.2]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: >0.05 B versus C: >0.05
48	63.2 [62.0 - 65.2]	57.7 [49.2 - 61.5]	59.3 [58.3 - 63.4]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>
Time post-wounding (h)	Control (A)	AGEs 30 µg/mL (B)	AGEs 30 µg/mL + RAP (C)	p value for time by group interaction	p value for post-hoc comparisons
12	83.5 [84.0 - 87.5]	73.0 [70.0 - 75.3]	87.3 [86.5 - 89.5]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>
24	73.5 [72.0 - 75.0]	57.0 [53.8 - 60.5]	79.3 [76.6 - 82.4]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>
48	62.0 [60.8 - 63.0]	44.5 [41.8 - 47.0]	60.0 [55.9 - 63.9]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>

**Table 2. Wound area (in %) of A549 cells treated by different HMGB1 or AGEs, alone or combined with RAP at different time points after a scratch assay.** The wound area is expressed as the fraction of the initial wound (100% at h0). Data are presented as medians and interquartile ranges [IQR] and were analyzed using random effects models; when time by group interaction was significant, post-hoc comparisons between groups at each timepoint were performed using the same models. *HMGB1*: high-mobility group box 1 protein. *AGEs*: advanced glycation end-products. *RAP*: RAGE antagonist peptide.

Time post-wounding (h)	Experimental conditions			p value for time by group interaction	p value for post-hoc comparisons
	Control (A)	HMGB1 0.1 µg/mL (B)	HMGB1 0.1 µg/mL + RAP (C)		
12	6.51 [6.33 - 6.60]	8.40 [7.73 - 8.70]	8.18 [8.09 - 8.27]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: <10 <sup>-4</sup> B versus C: >0.05
48	0.75 [0.65 - 0.84]	0.66 [0.52 - 0.91]	0.64 [0.53 - 0.70]	<10 <sup>-4</sup>	A versus B: >0.05 A versus C: >0.05 B versus C: >0.05
Time post-wounding (h)	Control (A)	AGEs 30 µg/mL (B)	AGEs 30 µg/mL + RAP (C)	p value for time by group interaction	p value for post-hoc comparisons
12	6.51 [6.33 - 6.60]	5.41 [5.27 - 5.67]	5.83 [5.60 - 6.50]	<10 <sup>-4</sup>	A versus B: <10 <sup>-3</sup> A versus C: >0.05 B versus C: 0.03
48	0.75 [0.65 - 0.84]	2.31 [2.11 - 2.65]	0.41 [0.35 - 0.58]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>

**Table 3. Cell migration, as expressed as fluorescence intensity, of lung epithelial A549 cells treated with HMGB1 or AGEs, alone or combined with RAP, at different time points after a scratch assay.** Higher intensity corresponds to higher migration. Data are presented as medians and interquartile ranges [IQR] and were analyzed using random effects models; when time by group interaction was significant, post-hoc comparisons between groups at each timepoint were performed using the same models. *HMGB1*: high-mobility group box 1 protein. *AGEs*: advanced glycation end-products. *RAP*: RAGE antagonist peptide.

Time post-wounding (h)	Experimental conditions			p value for time by group interaction	p value for post-hoc comparisons
	Control (A)	HMGB1 0.1 µg/mL (B)	HMGB1 0.1 µg/mL + RAP (C)		
12	30.0 [28.0 - 33.0]	45.5 [44.3 - 47.3]	42.5 [38.3 - 44.5]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: <10 <sup>-4</sup> B versus C: 0.01
48	22.3 [19.5 - 25.3]	33.2 [30.8 - 38.3]	21.6 [20.3 - 27.9]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: 10 <sup>-4</sup>
Time post-wounding (h)	Control (A)	AGEs 30 µg/mL (B)	AGEs 30 µg/mL + RAP (C)	p value for time by group interaction	p value for post-hoc comparisons
12	30.0 [28.0 - 33.0]	50.0 [42.0 - 54.0]	29.5 [23.5 - 31.5]	<10 <sup>-4</sup>	A versus B: <10 <sup>-3</sup> A versus C: >0.05 B versus C: 0.03
48	22.3 [19.5 - 25.3]	45.9 [42.3 - 48.3]	20.9 [18.6 - 21.9]	<10 <sup>-4</sup>	A versus B: <10 <sup>-4</sup> A versus C: >0.05 B versus C: <10 <sup>-4</sup>

**Table 4. Proliferation capacity, as expressed by the ratio (in %) of proliferating cells to the total number of cells, of lung epithelial A549 cells treated with HMGB1 or AGEs, alone or combined with RAP, at different time points after a scratch assay.** Data are presented as medians and interquartile ranges [IQR] and were analyzed using random effects models; when time by group interaction was significant, post-hoc comparisons between groups at each timepoint were performed using the same models. *HMGB1*: high-mobility group box 1 protein. *AGEs*: advanced glycation end-products. *RAP*: RAGE antagonist peptide.

## FIGURE LEGENDS

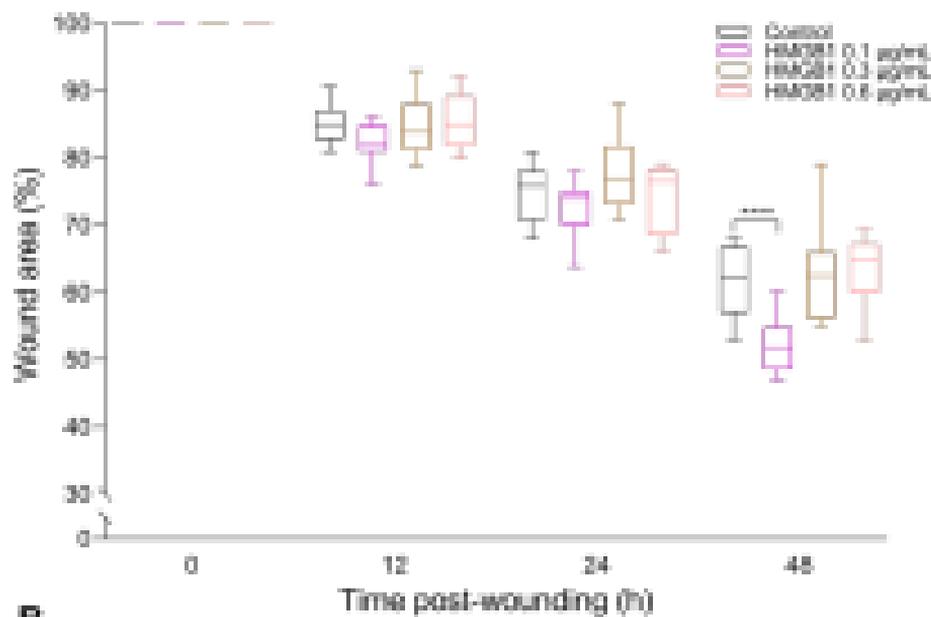
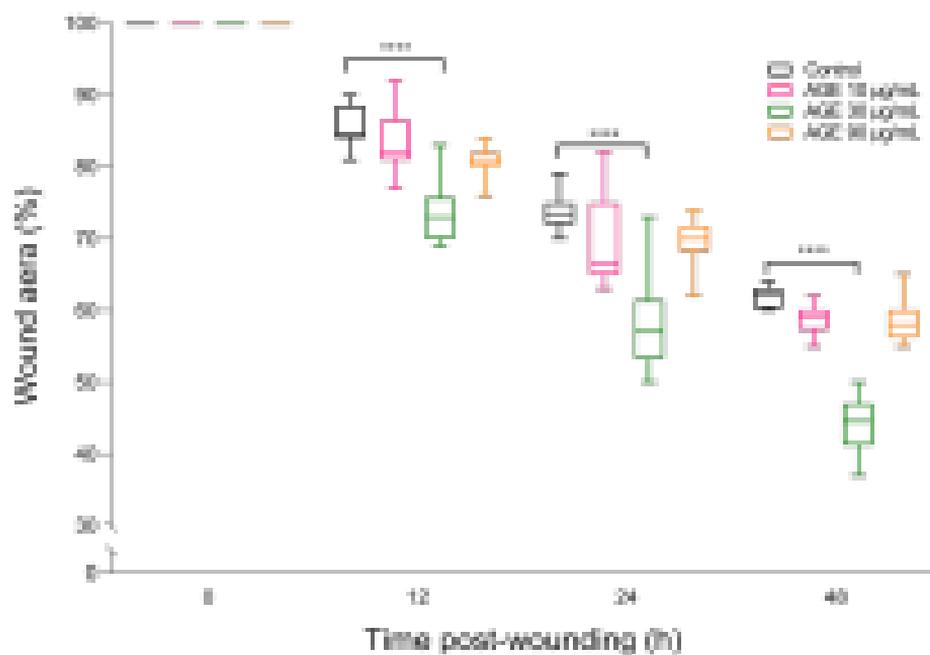
**Figure 1. Dose-response effect of HMGB1 and AGEs on the wound healing of A549 cells. A)** Wound healing was assessed up to 48 h using a scratch assay of A549 cells treated with culture medium (Control) or with different concentrations of recombinant HMGB1 (0.1, 0.3, and 0.6  $\mu\text{g/mL}$ ). HMGB1 promotes wound healing, compared to the control condition within concentrations of 0.1 to 0.6  $\mu\text{g/mL}$ ; its effect on wound area was more pronounced with the minimum effective dose of 0.1  $\mu\text{g/mL}$ , which was therefore used in subsequent experiments. Values are reported as box plots with median values and interquartile ranges, and upper/lower limits (outliers). Measurements were performed in duplicate in six independent experiments. \*\*\*\* $p < 10^{-4}$ . **B)** Wound healing was assessed up to 48 h using a scratch assay of A549 cells treated with culture medium (Control) or with different concentrations of recombinant AGEs (10, 30 and 90  $\mu\text{g/mL}$ ). AGEs promote wound healing, compared to the control condition at the concentration of 30  $\mu\text{g/mL}$ ; this minimum effective dose was therefore used in subsequent experiments. Values are reported as box plots with median values and interquartile ranges, and upper/lower limits (outliers). Measurements were performed in duplicate in six independent experiments. \*\*\*\* $p < 10^{-4}$ . *HMGB1: high-mobility group box 1 protein. AGEs: advanced glycation end-products.*

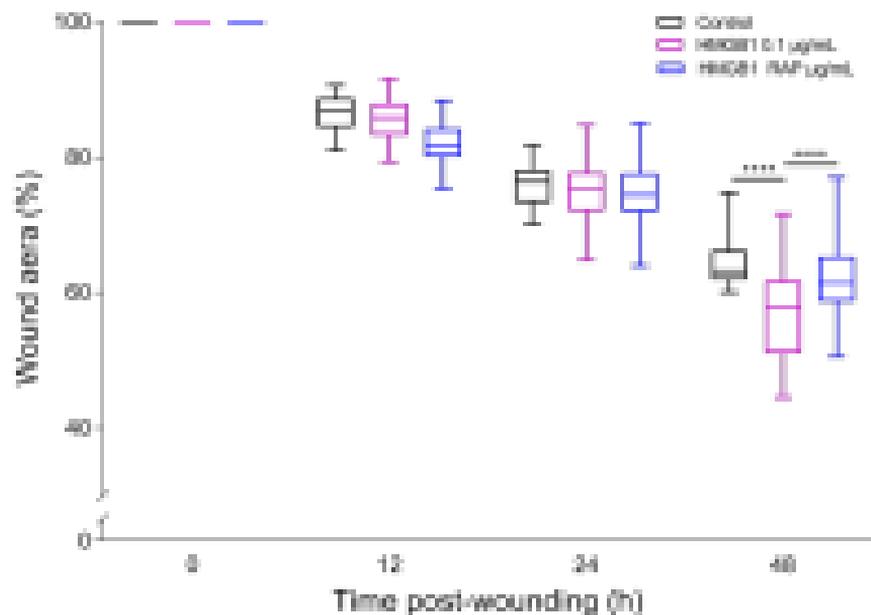
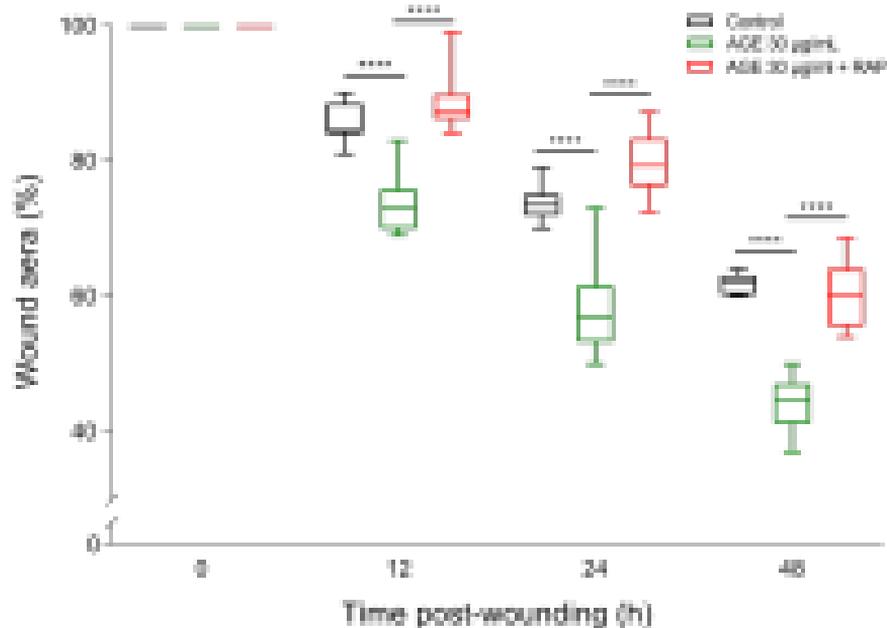
**Figure 2. The effect of RAGE modulation on lung epithelial wound healing in vitro.** Wound healing was assessed in lung epithelial A549 cells treated with **A)** HMGB1 (0.1  $\mu\text{g/mL}$ ) or **B)** AGEs (30  $\mu\text{g/mL}$ ), alone or combined with RAP (12.5  $\mu\text{g/mL}$ ). The wound area, as measured every 12 h, is expressed as the fraction of the initial wound (100% at h0). \*\*\*\* $p < 10^{-4}$ . *RAGE: receptor for advanced glycation end-products. HMGB1: high-mobility group box 1 protein. AGEs: advanced glycation end-products. RAP: RAGE antagonist peptide.*

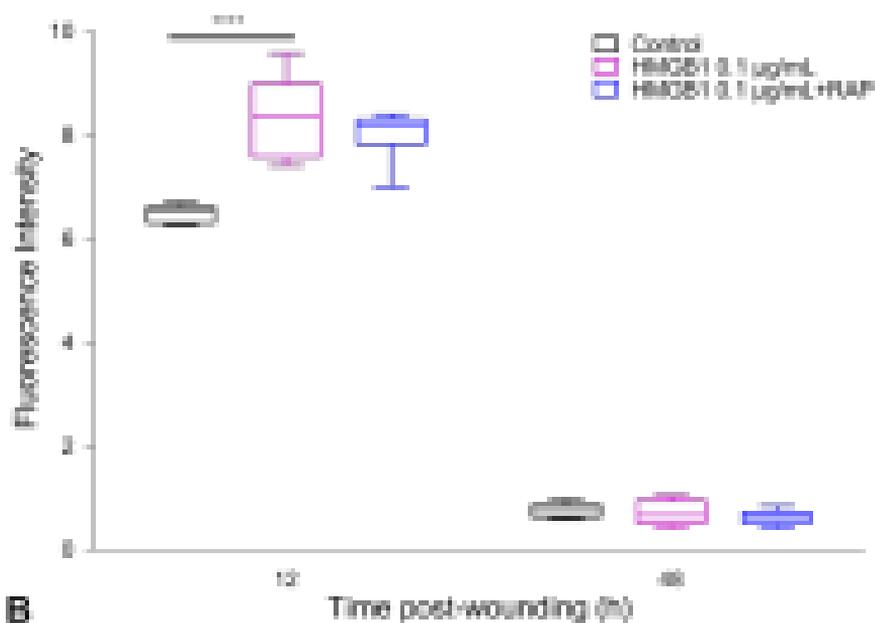
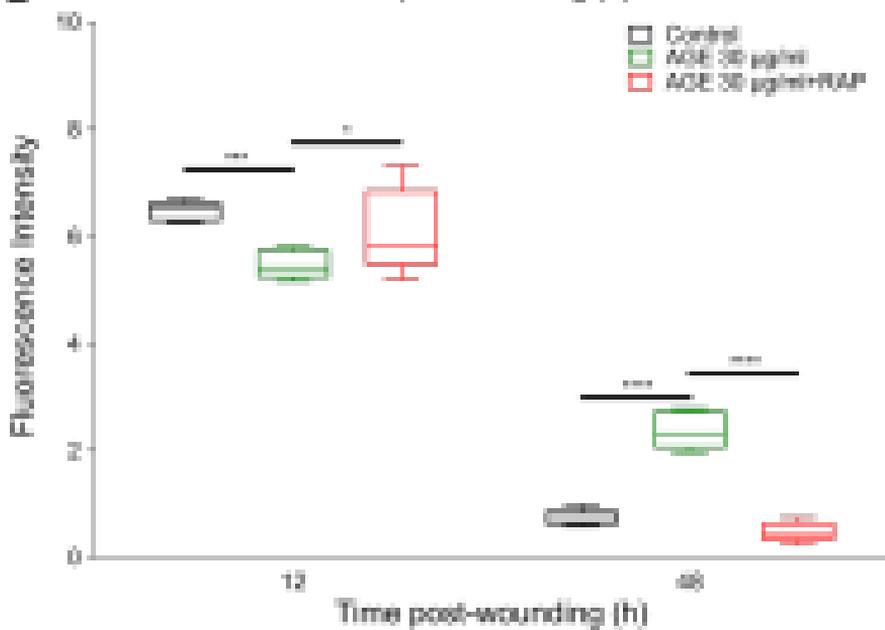
**Figure 3. Effects of treatment with HMGB1 and AGEs, and of RAGE inhibition by RAP, on the migration of A549 cells during wound healing.** The migration of A549 cells, as expressed as fluorescence intensity (with higher intensity corresponding to higher migration) was assessed at 12 and 48 h after a scratch assay using the CytoSelect™ 96-well cell migration assay (CBA-106; 8  $\mu\text{m}$

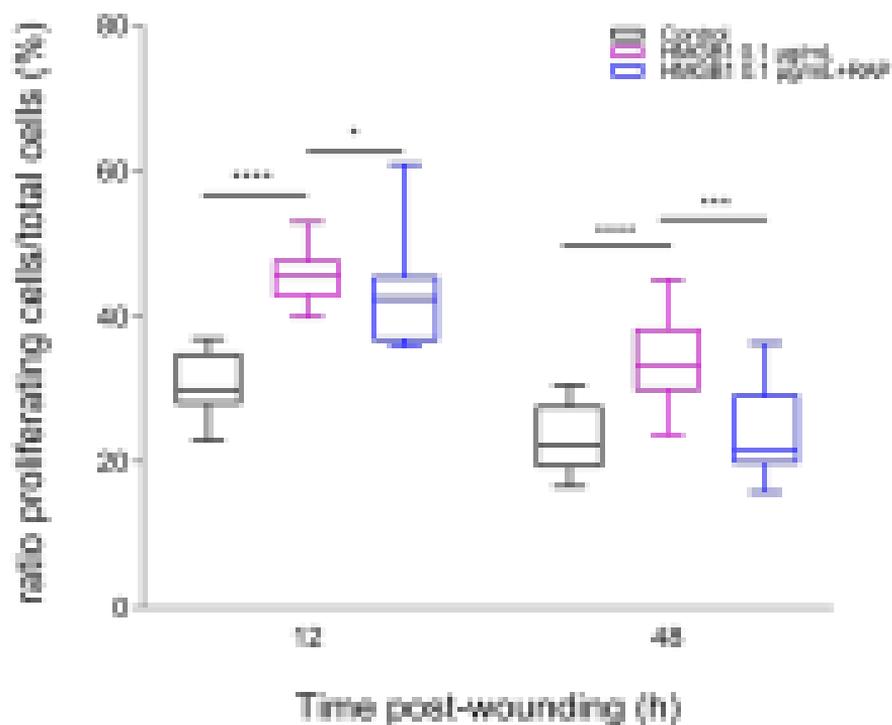
Fluorometric format; Biolabs, London, United Kingdom). \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 10^{-4}$ . **A)** Cells treated with recombinant HMGB1 (0.1  $\mu\text{g/mL}$ ) had increased migration, compared to controls, at 12 h but not at 48 h. Treatment with RAP (12.5  $\mu\text{g/mL}$ ) did not influence this effect. **B)** Cells treated with recombinant AGEs (30  $\mu\text{g/mL}$ ) had decreased migration at 12 h, but increased migration at 48 h, compared to controls. Both effects of AGEs on migration were significantly counteracted after treatment with RAP (12.5  $\mu\text{g/mL}$ ). *RAGE: receptor for advanced glycation end-products. HMGB1: high-mobility group box 1 protein. AGEs: advanced glycation end-products. RAP: RAGE antagonist peptide.*

**Figure 4. Effects of treatment with HMGB1 and AGEs, and of RAGE inhibition by RAP, on the proliferation capacity of A549 cells during wound healing.** Proliferation capacity of A549 cells was assessed using a BrdU Labeling and *Detection Kit II (11299964001; Roche Diagnostics, Meylan, France)*. Results are expressed as the ratio (in %) of proliferating cells to the total number of cells at 12 and 48 h after a scratch assay. **A)** Cells treated with recombinant HMGB1 (0.1  $\mu\text{g/mL}$ ) had increased proliferation capacity, compared to controls, at 12 and 48 h. Treatment with RAP (12.5  $\mu\text{g/mL}$ ) significantly counteracted this effect at both timepoints. **B)** Cells treated with recombinant AGEs (30  $\mu\text{g/mL}$ ) had increased proliferation capacity, compared to controls, at 12 and 48 h. Treatment with RAP (12.5  $\mu\text{g/mL}$ ) significantly counteracted this effect at both timepoints. \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 10^{-4}$ . *RAGE: receptor for advanced glycation end-products. HMGB1: high-mobility group box 1 protein. AGEs: advanced glycation end-products. RAP: RAGE antagonist peptide.*

**A****B**

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