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Short communication

Blocking the adverse outcome pathway of skin sensitization through a *N*-acetyl cysteine and lysine-loaded hydrogel

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Skin sensitization is a common adverse effect of a wide range of small reactive chemicals, leading to allergic contact dermatitis (ACD), the most frequent manifestation of immunotoxicity in humans. The prevalence of ACD is increasing, affecting up to 20% of the Western European population. This trend was particularly pronounced in high-risk occupational sectors, including healthcare, food services, metal and construction workers, and hairdressers [1]. The skin sensitization adverse outcome pathway (AOP) comprises 11 elements, with four designated key events (KEs): formation of protein-hapten complexes (KE-1), inflammatory keratinocyte response (KE-2), dendritic cell (DC) activation (KE-3), and T-cell proliferation (KE-4) [2]. As there is no cure for ACD, preventive strategies are of great relevance. In addition to avoiding exposure, preventive measures, such as the use of latex gloves, barrier creams, emollients, and moisturizers, often have limited effectiveness [3]. Here, fitting an AOP-guided rationale, *in chemico* reactivity towards cysteine (CYS) and lysine (LYS), the upregulation of the co-stimulatory molecules CD86 and CD54 (THP-1 cells), and the transcription of nuclear factor E2-related factor 2 (Nrf2)-dependent genes (HaCaT cells) were used to identify molecules capable of counteracting the effects of strong human skin allergens. A hydrogel containing the most promising active molecules was formulated and its preventive potential was evaluated using the murine local lymph node assay (LLNA).

We hypothesized that molecules containing sulfhydryl and/or LYS groups would react with haptens, thereby preventing them from binding to skin proteins. This interruption hinders the cascade of events essential for the sensitization phase of ACD. Additionally, in individuals already sensitized, it helps maintain the concentration of reactive allergens below the threshold necessary to trigger the elicitation phase.

We initiated our investigation by exploring the potential of *N*-acetyl CYS (NAC), CYS, glutathione (GSH), methionine (MET), LYS, and the combination of NAC + LYS to inhibit the interaction of the potent sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB) with peptides that contain CYS and LYS residues. This assessment was conducted using a peptide competitive assay recently developed by our research team [4] and is detailed in the Supplementary data. DNFB was found to strongly bind to CYS (74% depletion) and LYS residues (83% depletion), which was significantly abrogated by *in chemico* pre-incubation of the sensitizer with LYS, NAC, CYS, GSH, and NAC + LYS (Fig. S1). In subsequent experiments, we tested another strong skin sensitizer, methylisothiazolinone (MI), to validate the efficacy of candidate molecules in rescuing other KEs of skin sensitization AOP. As a certain level of cytotoxicity is essential for effective DC maturation (KE-3), the concentrations of DNFB and MI required to reduce cell viability by up to 30% (EC_{30}) were determined. The EC_{30} values obtained were 8.0 μM for DNFB and 100 μM for MI (Fig. S2). Unless otherwise specified, subsequent *in vitro* experiments were performed using these concentrations.

To address KE-2, HaCaT keratinocytes were pre-incubated with

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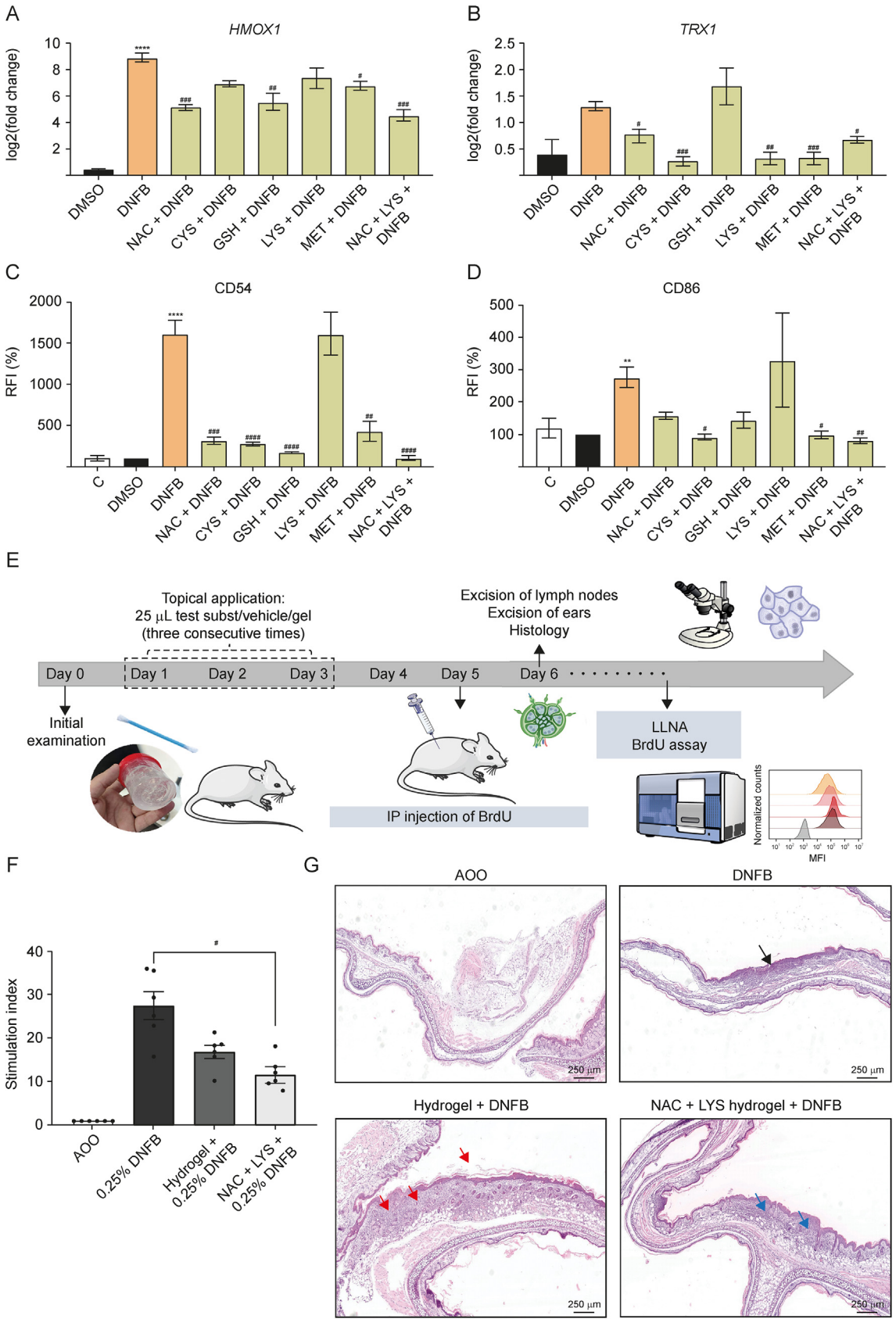

Fig. 1. *N*-acetyl cysteine (NAC) and lysine (LYS)-loaded hydrogel blocks the adverse outcome pathway (AOP) of skin sensitization. (A, B) Effect of NAC, cysteine (CYS), glutathione (GSH), LYS, methionine (MET), and the combination of NAC + LYS on the transcription of nuclear factor E2-related factor 2 (Nrf2)-dependent genes *HMOX1* (A) and *TRX1* (B), after exposure of human keratinocytes to 1-fluoro-2,4-dinitrobenzene (DNFB) during 6 h. The results are presented as relative fold changes compared to dimethyl sulfoxide (DMSO) (vehicle) and represent the mean ± standard error of the mean (SEM) of at least three independent experiments. (C, D) Suppressive effects of NAC, LYS, CYS, GSH, MET, and

the test molecules for 1 h and then stimulated for an additional 6 h with DNFB or MI. The effect of the test molecules on the transcription levels of Nrf2-dependent genes *HMOX1, TRX1, SOD1, NQ01,* and *GSTP1* (primer sequences in Table S1) was analysed using quantitative polymerase chain reaction (qPCR). Pre-incubation with NAC and GSH, as well as the combination of NAC and LYS, significantly decreased *HMOX1* transcription triggered by DNFB (Fig. 1A). The combination of NAC + LYS ($P < 0.05$) also significantly reduced DNFB-induced *SOD1* transcription (Fig. S3A). Regarding *TRX1* gene, NAC ($P < 0.001$), CYS ($P < 0.01$), LYS ($P < 0.001$), MET ($P < 0.05$), and NAC + LYS ($P < 0.05$) significantly decreased sensitizer-induced transcription (Fig. 1B). In contrast, no significant alterations were observed in *NQ01* and *GSTP1* genes (Figs. S3B and C). Concerning the skin allergen MI, NAC ($P < 0.001$), GSH ($P < 0.0001$), and NAC + LYS ($P < 0.001$) significantly decreased sensitizer-triggered *HMOX-1* transcription (Fig. S4A). Similarly, NAC + LYS ($P < 0.05$) decreased MI-induced *TRX1* transcription, and NAC ($P < 0.05$), GSH ($P < 0.05$), and MET ($P < 0.05$) decreased MI-induced *SOD1* transcription (Figs. S4B and C). Despite an unexpected increase caused by pre-incubation with CYS, none of the other tested conditions significantly modulated MI-triggered *NQ01* transcription (Fig. S4D). Regarding *GSTP1,* NAC ($P < 0.01$), MET ($P < 0.01$), and NAC + LYS ($P < 0.05$) attenuated the effects of MI (Fig. S4E).

We then evaluated the ability of the tested molecules to mitigate sensitizer-triggered activation and maturation of DCs, which is KE-3 in the skin sensitization AOP. This was conducted using the Organization for Economic Co-operation and Development (OECD)-approved *in vitro* skin sensitization method, the human cell line activation test (h-CLAT), with minor modifications. In addition to CYS ($P < 0.001$) and GSH ($P < 0.05$), which caused a significant increase in CD54 expression, the remaining conditions tested did not lead per se to a significant modulation of this activation marker or the co-stimulatory molecule CD86, thus confirming their safety profile (Figs. S5A and B). As expected, DNFB induced THP-1 maturation, significantly increasing the levels of CD54 ($P < 0.0001$) and CD86 ($P < 0.01$) (Figs. 1C and D). Except for LYS, all the other molecules tested prevented DNFB (Figs. 1C and D) or MI (Figs. S5C and D) triggered expression of CD54 and CD86.

The combination of NAC + LYS exhibited the highest efficacy in attenuating the initial three KEs triggered by both DNFB and MI. Consequently, these conditions were chosen for further *in vivo* investigations (KE-4). To this end, a hydrogel incorporating both molecules was formulated and subsequently evaluated for its ability to prevent sensitization induced by DNFB using the LLNA bromodeoxyuridine-flow cytometry (BrdU-FCM) assay (Fig. 1E). Our team recently formulated and characterized the hydrogel, demonstrating that it is safe and does not cause skin irritation or sensitization [5]. Pretreatment of mouse ears with the hydrogel containing 100 mM NAC + LYS significantly decreased lymphocyte proliferation (stimulation index (SI)) triggered by DNFB (Fig. 1F). Furthermore, macroscopic (Fig. S6 and Tables S2 and S3) and histological analysis (Fig. 1G) revealed that pre-application of the NAC + LYS hydrogel before the DNFB challenge reduced ear swelling, thickness, eosinophil infiltration, and ulceration indicating its protective potential.

Overall, the present study describes a novel topical formulation carrying LYS and NAC that can chemically sequester skin allergens, thus mitigating the development of ACD. Although additional research is required to ascertain the effectiveness of the developed formulation against other sensitizers, the findings presented herein pave the way for addressing the pressing challenge of managing ACD. Specifically, they highlighted the importance of incorporating amino acids, peptides, and other molecules containing thiol and LYS groups into topical preparations. These molecules effectively counteract the detrimental effects of low-molecular-weight skin allergens, thereby significantly reducing the likelihood of disease recurrence upon subsequent exposure.

Ethical statement

This study was conducted in strict accordance with the ethical guidelines and regulations set forth by the European Directive Regarding the Protection of Laboratory Animals Used for Scientific Purposes (2010/63/EU) (European Parliament, Council of the European Union, 2010) and the Portuguese Law on Animal Welfare (Decreto-Lei 113/2013). The study was authorized by the national entity Direção-Geral de Alimentação e Veterinária, Portugal (Approval No.: 0421/000/000/2020), and efforts were made to reduce the number of animals used and their suffering. All the animal experiments were performed by team members accredited by the Federation of European Laboratory Animal Science Associations, Belgium.

CRediT authorship contribution statement

Gonçalo S. Brites: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Isabel Ferreira:** Writing – review & editing, Investigation. **Ana I. Sebastião:** Investigation. **Cátia Sousa:** Investigation. **Ana Silva:** Investigation. **Mylene Carrascal:** Formal analysis, Data curation. **Rui C. Oliveira:** Formal analysis, Data curation. **Margarida Gonçalo:** Supervision, Conceptualization. **Carla Vitorino:** Methodology. **Bruno M. Neves:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Maria T. Cruz:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest. A component of this work contributed to the development of an international patent WO2024013711.

NAC + LYS on the maturation profile of THP-1 cells evoked by DNFB. Cells were pre-treated for 1 h with test molecules and then stimulated for 24 h with the skin sensitizer. Relative fluorescence intensity (RFI) was then analysed for CD54 (C) and CD86 (D) by flow cytometry (FCM). Data are presented as mean RFI ± SEM for at least five independent experiments normalized to DMSO. Viable cells were gated according to their morphological properties. (E) Workflow of the modified version of the murine local lymph node assay (LLNA) for evaluation of the efficacy of a hydrogel loaded with NAC and LYS to prevent the development of skin sensitization. (F) *In vivo* validation of the efficacy of a hydrogel containing NAC + LYS. Each substance was topically applied to the dorsal skin of the ears of mice for three consecutive days. On day 5, mice were injected with bromodeoxyuridine (BrdU) and on day 6, draining lymph node cell proliferation was determined by quantification of BrdU incorporation, using FCM. Results are expressed as stimulation index (SI). Vehicle (acetone/oil (AOO), 4:1, *V*/*V*), positive control (DNFB), and hydrogel formulations were applied before DNFB challenge. Data are presented as mean SI ± SEM of at least six independent experiments normalized to AOO-treated mice. (G) Representative histological images (hematoxylin/eosin staining) of the different treatment groups. Black arrow shows cutaneous ulceration and acute inflammation, red arrows show moderate subacute inflammation in the dermis between the cutaneous adnexa, and blue arrows show mild subacute inflammation. Two group comparison was performed with a Student's *t*-test for DMSO vs. DNFB. $^{**}P < 0.01$ and $^{****}P < 0.0001$. Multiple group comparison (DNFB vs. DNFB + test molecules in Figs. 1A–C and DNFB vs. DNFB + hydrogel formulations in Fig. 1F) was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$. IP: intraperitoneal injection; MFI: mean fluorescence intensity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101071.

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