

Sublingual vaccination by chitosan-based patch protected aged mice from influenza virus infection

ROVERA Renaud

CNRS, Université Lyon 1, LBTI, UMR 5305

COIFFIER Céline

CNRS, Université Lyon 1, LBTI, UMR 5305

GARAPON Cynthia

CNRS, Université Lyon 1, LBTI, UMR 5305

FERRARO Sandra

CNRS, Université Lyon 1, LBTI, UMR 5305

COLOMB Evelyne

CNRS, Université Lyon 1, LBTI, UMR 5305

Sophia DJEBALI

Centre International de Recherche en Infectiologie, INSERM U1111, CNRS UMR 5308, Université Claude Bernard Lyon 1, Ecole Normale Supérieure de Lyon

Claire MONGE

Claire.monge@cnrs.fr

CNRS, Université Lyon 1, LBTI, UMR 5305

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Abstract

Background

Influenza remains a major global health threat, particularly for the elderly, who exhibit reduced vaccine efficacy. While mucosal vaccination offers a promising strategy to enhance immune protection, the sublingual (SL) route has been underutilized, despite its potential to induce both systemic and mucosal immunity. In this study, we evaluated the protective efficacy of a chitosan-based sublingual mucoadhesive patch delivering a subunit influenza vaccine (PLA-based nanoparticles adjuvanted with a TLR7/8 agonist and functionalized with hemagglutinin) in young (2–3 months) and aged (16–18 months) mice.

Results

Strikingly, the SL patch provided 100% protection in aged mice against an influenza challenge, outperforming injected formulations (75–80% protection), despite lower IgG antibody levels, limited secretory IgA or cytotoxic T cell responses. Conversely, the patch failed to protect young mice, suggesting age-dependent immune mechanisms, possibly linked to IL-6 levels and chronic mucosal inflammation. Mechanistic investigations after vaccination by the chitosan-based patch revealed enhanced B cell activation and neutrophil recruitment in the draining lymph nodes, potentially driven by chitosan, which may contribute to improved antibody affinity.

Conclusions

These findings underscore the potential of chitosan-based SL patches to overcome age-related immune decline and improve vaccine responses in the elderly.

Background

Influenza remains a significant global health burden, particularly among vulnerable populations such as the elderly, who are at high risk of severe complications and mortality due to immunosenescence and age-related immune dysfunction characterized by impaired vaccine-induced immunity [1, 2]. Influenza vaccines have contributed to significant reductions in influenza-associated morbidity and mortality for more than 80 years. However, their effectiveness remains variable and suboptimal [3, 4]. Additionally, vaccine efficacy is age-related, ranging from 70% to 90% in young people but decreasing to 30–50% in people over 65 years [5, 6]. The vaccine effectiveness in this latter population is often suboptimal, with reduced immunogenicity and shorter duration of protection. Recent studies on immune cell dynamics during aging by single-cell RNA sequencing highlighted altered antibody class-switching and impaired antibody functionality [7]. Individuals > 65 years exhibit the highest rates of hospitalization and mortality among all age groups (from 0 to over 65) [8]. This highlights the need for innovative vaccine formulations and delivery strategies capable of eliciting robust and durable immune responses in aged population. Reduction in influenza-related hospitalisations was shown to be possibly attenuated by high-

dose influenza vaccine administration to overcome immunosenescence and enhance protection in this population [9, 10]. Another strategy to improve vaccine responses in elderly individuals is the use of adjuvants [2, 11]. More recently, the development of mucosal vaccine formulations becomes a challenging yet promising strategy to tackle respiratory virus epidemics and pandemics [12].

Mucosal vaccination has emerged as a powerful approach to enhance immune protection by inducing both systemic and mucosal immunity unlike injectable vaccines that mostly induce systemic immunity [13]. The mucosal route not only mimics the natural route of infection but also stimulates local immune responses, including the production of secretory IgA produced by local plasma cells and tissue-resident memory B and T cells (B_{RM} and T_{RM}), which are essential for pathogen clearance and long-term protection directly at the site of infection [12]. Consequently, mucosal vaccines are powerful tools to avoid infection and decrease the risk of pathogen transmission.

Among mucosal delivery methods, buccal administration offers distinct advantages, including ease of use, patient compliance, and the potential to induce broad systemic and local immune responses [14–16]. Interestingly, salivary antibodies have shown considerable neutralizing capacity [17]. Despite its clear advantages, the buccal cavity, and in particular the sublingual mucosa, has received little attention in mucosal vaccine development, mainly because buccal immunity is not yet fully understood [12]. The buccal mucosa is a particularly thin multi-layered tissue and allows easy penetration of antigens and drugs. Once a molecule has permeated through the epithelium, it can be either delivered to the systemic circulation or be directly uptaken by antigen-presenting cells (APCs), and, in particular, dendritic cells (DCs) which play a pivotal role in the linkage between innate and adaptive immunity. Antigens will activate pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that recognize conserved molecular patterns of pathogens. After PRRs activation, DCs will undergo maturation and migration to the draining (submandibular and cervical) lymph nodes, where adaptive immune response will start. Buccal vaccination so far faced two major barriers that impeded its development: poor retention of the vaccine at delivery sites and induction of immune tolerance [15, 16]. Overcoming the tolerance could be achieved by the use of a potent mucosal adjuvant. In order to avoid the dilution of the vaccine in the saliva, mucoadhesive delivery systems have to be employed.. Alike nasal administration, sublingual administration of adjuvanted vaccines induces mucosal immune responses (antibody-mediated and cytotoxic T-cell responses) in the upper and lower respiratory tract, stomach, small intestine, reproductive tract as well as a systemic response [14–16]. Several studies have highlighted the potential of sublingual vaccine administration to induce mucosal immunity against HIV [18, 19] or influenza virus [20, 21]. Yet, as these studies were performed on young animals, there is a possibility that these outcomes may vary during lifetime.

In this study, we investigate the impact of a sublingual patch as a novel delivery system for a subunit influenza vaccine in aged mice. This approach aims to overcome the limitations of traditional parenteral vaccines by leveraging the mucosal immune system to enhance vaccine efficacy in the elderly. By evaluating the immunogenicity and protective efficacy of this sublingual patch, we seek to provide insights into its potential as a viable strategy for improving flu vaccination outcomes in aging

populations. After a viral challenge in young and aged mice, we observed completely different protective effect of the SL patch that was fully protective in aged mice and not in young mice. The investigation of several immunological processes highlighted major discrepancies in vaccine immune response during aging.

Material and methods

Polymeric nanoparticle synthesis and adjuvant encapsulation

All nanoparticle suspensions were prepared using a surfactant-free nanoprecipitation process as described previously [22]. Briefly, PLA polymer was dissolved at 2% (w/v) in acetone and this organic solution was added dropwise to an aqueous phase composed of ethanol and 5 mM sodium bicarbonate (Fisher Scientific, France) under 250 rpm stirring. Organic solvents were then removed under reduced pressure at 30°C with a Rotavapor R-210 (Buchi, France). For the entrapment of either 3M-052 or Red Bodipy TR Methyl Ester (Invitrogen by Fisher Scientific, France) into PLA-NP, the molecule was added to the organic phase containing the polymer prior to conduct the nanoprecipitation. Final PLA-NP suspensions containing 510 µg/ml of 3M-052, were stored at 4°C until use.

Hemagglutinin adsorption

The adsorption of the HA protein was performed either with fluorescent NP-BodipyTR or with NP loaded with adjuvants (NP(3M-052)-HA). The HA protein was diluted at a concentration of 100 µg/mL and incubated with NP for 2 hours at RT, with moderate end-overhead stirring. To determine the adsorption yield of HA protein, the latter was quantified in the supernatant after centrifugation of samples at 10,000 g for 10 min at RT. The quantification was then performed using a micro-BCA™ protein Assay kit from Thermo Scientific and absorbance was measured at 562 nm using a microplate reader.

Particle characterization

Nanoparticle sizes, polydispersity index, and surface charge were determined using a ZetaSizer Nano ZS Plus (Malvern Instruments, Malvern, UK). The characteristics of the PLA-NP are presented in SUPP Table 1.

LbL patch production and PLA-NP adsorption

Chitosan/Hyaluronic acid (CHI/HyA) freestanding patches were produced as described previously [22, 23] using the LbL methodology and a dipping robot (DR-3, Riegler & Kirstein GmbH). Medium molecular weight CHI was purchased from Sigma-Aldrich. CHI was purified by filtering steps and precipitation in water and ethanol, followed by freeze-drying having a final molecular weight of 770 kDa and a degree of

deacetylation (DD) of 78%. Sodium HyA (610 kDa) was purchased from HTL, France, and was used as received. Briefly, polyelectrolyte solutions were freshly prepared at 0.2% (w/v) in a sodium acetate buffer (CH₃COONa 0.2 M, CH₃COOH 0.2 M, pH = 5.5, RT). The substrates were immersed sequentially in CHI and HyA solutions with a washing step in sodium acetate buffer between each deposition in polymers solution. A deposition time of 3 min for polyelectrolytes and 2 min for each washing step was used. These immersions were repeated 100 times and the process was finished by a CHI layer to ensure symmetric mucoadhesion of the produced (CHI/HyA)₁₀₀-CHI patches. Then the membranes were let left to dry at RT. Finally, membranes were easily detached from their respective underlying substrates by being peeled off with a tweezer. A drop of PLA-NP formulations in sodium acetate buffer was added on the surface of the patches and let dry under air-flow during at least 1h.

Mice

In vivo studies were conducted either on 2 to 4-months-old and 16 to 18-months female CB6F1 mice (Charles River Laboratories, France) at the animal facility PBES of Lyon, France. All animals were maintained in pathogen-free conditions. All of the experiments were performed in accordance with animal welfare regulations for their use for scientific purposes governed by European Directive 2010/63/EU. Protocols were validated by the local Animal Ethics Evaluation Committee (CECCAPP: C2EA-15) and authorized by the French Ministry of Education and Research.

Histological analysis

Masson's trichrome staining. After dissection, tongues were embedded and 5µm thick cryostat sections were made. For Masson's trichrome staining, the slides were immersed in 1X PBS to remove the OCT, then the nuclei were stained with Groat's haematoxylin (mixture of v/v of solution A: 50 mL distilled water, 1 g ammonium iron(III) sulfate dodecahydrate 221260-SIGMA, 0.8 mL sulphuric acid and solution B: 50 mL 95° ethanol, 0.5 g haematoxylin H3136-SIGMA and 0.8 mL sulphuric acid) for 2 minutes. After rinsing with running water, the slides were quickly immersed in hydrochloric acid for differentiation. They were washed again under running water and then stained with fuchsin-ponceau (composed of 0.2 g xylidine ponceau 81465-FLUKA, 0.1 g acid fuchsin F8129-SIGMA, 0.6 mL acetic acid and 300 mL distilled water) staining for 3 minutes. They were rinsed with acetic acid 1% before being stained with orange G 2% (O63756-SIGMA) - phosphomolybdic acid 4% (P7390-SIGMA) for 3 minutes. Finally, the slides are stained with light green 0.2% (L1886-SIGMA) for 3 minutes before being dehydrated in two baths of absolute ethanol and mounted with DEPEX mounting medium (06522-SIGMA). For Sirius red staining, the slides are stained after rinsing with PBS1X in Sirius red 1% 365548-SIGMA (diluted in picric acid solution) for one hour at room temperature. The slides are then rinsed with 95° ethanol and dehydrated twice with absolute ethanol before being mounted with DEPEX mounting medium. Photos were then taken on a NIKON TiE inverted epifluorescence microscope with a 4x and 40X objectives lens and analysed using ImageJ/Fiji software.

IgA staining. Tissue sections of turbinates were obtained from the mice after dissection in order to isolate the nasal cavity with the surrounding bone. Under gentle agitation, samples were first fixed in 4% PFA for 3 days at room temperature (RT), then decalcified at 4°C in 10% EDTA (Sigma, St. Louis, MO, USA) at pH 7.4 for 7 days and cryopreserved at 4°C in sucrose 30% (Sigma) for 3 days. After immersion in optimum cutting temperature compound (OCT, TissueTek, Sakura Finetek, The Netherlands), the samples were quickly frozen and stored at -80°C until processing. Tongues were carefully cut, embedded in OCT compound and stored at -80°C. Longitudinal tongue sections, 6 µm thick and nasal cavity sagittal sections, 8 µm thick, were performed using a cryostat microtome (CM1860, Leica). Sections were successively incubated at room temperature with acetone, with 3% hydrogen peroxide (Sigma), with PB containing 3% bovine serum albumin and 5% normal goat serum (Vector Laboratories), and for 1 h with horseradish peroxidase conjugated antibody directed against mouse IgA (1:50, goat, SouthernBiotech, Birmingham, AL, USA). Horseradish peroxidase were revealed using AEC substrate (Vector Laboratories, Burlingame, CA, USA). Sections were finally counterstained with Hematoxylin QS (Vector Laboratories) and mounted with Glycergel (Agilent Technologies, Santa Clara, CA, USA). Images were captured using an inverted microscope (Nikon Ti-E microscope) equipped with a 20x objective for sublingual mucosa and a 4x objective for nasal cavity.

Immunizations

For subcutaneous injections, mice received a single or two injection of formulation in the leg. The liquid formulation consisted either in 100 µl of NP(3M)-HA or 50 µl of Vaxigrip® 2024 (Sanofi, France). For sublingual administration, patches were cut to fit the size of the mouse tongue (2 mm 6 mm) and sterilized by UV light before PLA-NP addition. Patches or liquid formulations were then administered sublingually (ventral part of the tongue) to anesthetized mice (isoflurane 4%). After administration, a gentle pressure was exerted for few seconds on the dorsal part of the tongue to ensure contact of the patch or the liquid solution with the sublingual mucosa. According to the experiments, the animals were either anesthetized (isoflurane 4%) for an additional 15 min. Once the animals recovered from anesthesia, they were left free to swallow or groom. Water and food were removed for the next 30 min after administration.

Viral challenge

Four weeks after the last boost, mice were anesthetized and were infected intra-nasally with a lethal dose of Influenza A virus (strain A/California/07/2009; 10^6 TCID₅₀; 10µL). Mice were monitored daily for survival, body weight, and clinical signs for 10 days post-infection. Mice that lost more than 20% of body weight were euthanized. The protective efficacy of each immunization scheme was determined by survival rate, weight loss of viral load compared to non-vaccinated controls.

Sample collection and processing

Biological fluids were collected before immunization (D0) and at 2, 4, 8 and 12 weeks after the boost to quantify their IgG and IgA content. Serum samples were obtained from whole blood collected by retro-orbital vein puncture. Blood samples were allowed to clot at 37°C for 30 min and sera were isolated after two rounds of centrifugation at 10 000 x g for 10 min. Sera were stored at -20°C until use. Saliva samples, nasal washes (NW), and bronchoalveolar lavages (BAL) were also withdrawn two weeks after the boost. Saliva was collected under ketamine/xylazine anesthesia. After a tracheostomy, 100 µL of pilocarpine (1 mg/mL) was injected intraperitoneally and saliva was collected with a capillary during 30 min. BAL and NW were collected after mice euthanasia by cervical dislocation. Briefly, 600 µL of cold PBS was flushed into the lung or the nasal cavity using a catheter inserted through the trachea. Protease Inhibitor Cocktail (Thermo Scientific) was then added to the mucosal samples to protect Igs from degradation. Mucosal secretions were centrifuged at 10 000 x g for 10 min at 4°C, supernatants were collected and stored at -20°C until further use.

Enzyme-linked immunosorbent assay (ELISA) immunoassay

Ninety-six-well plates (Nunc MaxiSorp, Thermo Fisher Scientific, USA) were coated overnight at room temperature with 100 µL of Influenza A H1N1 (A/California/04/2009) HA protein at concentration of 1 µg.mL⁻¹ in PBS. After removing the solution, the plates were blocked for 1 h at 37 °C with 200 µL of PBS containing 10% non-fat dry milk. Plates were washed 3 times with PBS-0.05% Tween 20 (PBS-T, 300 µL) using an automatic plate washer (also used for the subsequent washings, Thermo Scientific Wellwash Versa). Sera were serially diluted in PBS/BSA 1%. Then, 100 µL of each sample were incubated on blocked plates for 1 h at 37°C. Horse radish peroxidase (HRP)-coupled goat anti-mouse IgG (Southern Biotech, USA) was diluted at 0.1 µg.mL⁻¹ in PBS/BSA 1% and added into the wells after washing. After 1 h at 37°C, wells were washed three times. Finally, antibodies coupled to HRP were revealed using 100 µL of reconstituted TMB substrate reagent (BD Biosciences, USA), and reactions were stopped 30 min later with 1 N sulfuric acid (VWR, USA). The optical density (OD) at 450 nm with a correction at 620 nm was measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific). Antibody titers corresponded to the higher sample dilution, leading to an OD above the predetermined cutoff at day 0.

IgE quantification. Serum concentration of total IgE was quantified using the Mouse IgE enzyme-linked immunosorbent assay kit (ThermoFisher) according to the manufacturer's instructions (ref). Briefly, 100 µL of standards or diluted samples (each serum D0 and D56 diluted 100-fold) were added to pre-coated wells and incubated 2.5 hours at room temperature with gentle shaking. After 4 washes, 100 µL of prepared biotin conjugate were added to the wells and incubated 1 hour at room temperature with gentle shaking. Then, wells were washed again and 100 µL of prepared Streptavidin-HRP solution were added and incubated 45 minutes at room temperature with gentle shaking. Final washes were performed and 100 µL of TMB substrate were added to the wells, incubated 30 minutes in the dark. Reaction was stopped by the addition of 50 µL of Stop Solution. Absorbance was read immediately at 450 nm.

Immunophenotyping

Four weeks after the last immunization, mice were sacrificed, and blood, spleen, lungs, tongue, and draining cervical and submandibular lymph nodes were collected. Single-cell suspensions were prepared by mechanical dissociation and enzymatic digestion (collagenase D, DNase I for tissues). Cells were stained with fluorochrome-conjugated antibodies against surface markers for T cells (CD3, CD4, CD8, CD44, CD62L), B cells (B220, IgM, CD21/CD23), NK cells (NK1.1), neutrophils (Ly6G, CD11b), and monocytes (CD11b, Ly6C). Dead cells were excluded using a viability dye. Samples were acquired on a BD LSRFortessa cytometer, and data were analyzed using FlowJo (version 10.7). Frequencies of effector and memory subsets, as well as innate cell populations, were quantified. Supplementary Table 1 provides the antibodies and fluorochromes used in the study.

Intracellular cytokine staining and polyfunctionality analysis

Splenocytes and lung lymphocytes were isolated from vaccinated and naïve mice four weeks post-boost. Cells were stimulated in vitro with 1 µg/mL HA protein for 5 hours at 37°C in the presence of brefeldin A (10 µg/mL) to block cytokine secretion. Following stimulation, cells were washed, stained with surface markers (CD3, CD4, CD8, CD44), fixed, permeabilized, and stained for intracellular cytokines IFN-γ, TNF-α, and IL-2 using fluorochrome-conjugated antibodies (BD Biosciences). Dead cells were excluded using a viability dye. Samples were acquired on a BD LSRFortessa cytometer and analyzed using FlowJo (version X). Polyfunctional T cell responses were quantified by Boolean gating to identify cells expressing all possible combinations of the three cytokines. Data were further visualized and summarized using SPICE software (version 6.0, NIH), allowing comparison of single- and multi-cytokine-producing T cell populations between immunization schemes and age groups.

Quantification of proinflammatory cytokine secretion

The proinflammatory cytokines IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-10, CXCL1 and TNF-α were analysed from the mice sera by the V-PLEX Proinflammatory Panel 1 (mouse) kit (Mesoscale Discovery, K15048D-2) according to the manufacturer's protocols. Briefly, all mouse sera at D0 and D56 were diluted 2-fold in Assay diluent prior to load 50 µl in MSD plate. Diluted samples were incubated 2 hours at room temperature with orbital shaking at 625 RPM. After that incubation, the plate was washed 3 times and 25 µl of detection antibody solution was added. The plate was incubated 2 hours at room temperature with orbital shaking at 625 RPM. The plate was washed 3 times before the addition of 150 µl of Read Buffer T 2X and read immediately on the MESO QuickPlex SQ 120 instrument. The cytokine concentrations were calculated with the MSD Discovery Workbench software (Version 4.0.13).

Elispot

Splenocytes were isolated from vaccinated and naïve mice four weeks post-boost and plated at 2×10^5 cells/well in 96-well ELISpot plates pre-coated with anti-mouse IFN-γ antibodies (Mabtech). Cells were stimulated with 1 µg/mL HA protein for 24 hours at 37°C. Spots were developed according to the manufacturer's protocol, counted using an automated ELISpot reader (IRIS, Mabtech), and expressed as

spot-forming units (SFU) per 10⁶ cells. This assay quantified antigen-specific CD8 + and CD4 + T cell responses, including single-cytokine-producing populations.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.1.1 (USA). Before statistical analyses, normality was checked using statistical Shapiro-Wilk test. If normality was confirmed, parametric ordinary two-way ANOVA test was used followed by Tukey’s multiple comparison test. Statistical significance is indicated in the figures. A value of $p < 0.05$ was considered statistically significant. In vivo data were obtained from at least 5 animals, unless otherwise indicated in the figure legends.

Results

Design of the vaccine nanoparticle and fonctionnalization of the SL patch

The vaccine designed in this study consisted in poly-lactic acid (PLA) nanoparticles (NP) with encapsulated TLR7/8 agonist (3M-052, telratolimod) as adjuvant, and with hemagglutinin (HA) from H1N1 Influenza A/California/04/2009 adsorbed on the surface (scheme 1). The characteristics of the PLA-NP are presented in Table 1.

Table 1 Physico-chemical characteristics of PLA-NP (+/- HA protein) after encapsulation of 3M-052. PDI: polydispersity index.			
Encapsulated molecule +/- HA adsorption	Diameter (Z-average, nm)	PDI	Zeta potential (mV)
3M-052	139 ± 5	0.14 ± 0.04	-60.2 ± 3.8
3M-052 + HA	180 ± 34	0.31 ± 0.07	- 49.9 ± 2.2

The NPs alone had an average hydrodynamic diameter of around 140 nm, reaching 180 nm after the addition of HA to the surface of the NPs. The measurement of the surface charge of the NPs alone showed a negative zeta potential of around – 60 mV, increasing to around – 50 mV after the adsorption of HA. These changes in physicochemical properties confirmed the efficiency of the HA adsorption step, that was measured to be of around 90%.

The functionalized NP (NP(3M)-HA) were then adsorbed at the surface of a mucoadhesive patch made of polysaccharides (chitosan (CHI) and hyaluronic acid, first described in [23]) (scheme 1). The formulation was then dried under air flow.

The dried patches were administered to mice in the ventral part of the tongue and the floor of the mouth. The immune responses to this formulation was compared to a liquid suspension of NP(3M)-HA

administered by subcutaneous (SC) or SL route to young (2 months) and aged mice (16 months).

Aged sublingual mucosa exhibit chronic inflammation features.

As elderly are at risk population and experience severe flu symptoms, we assessed the potential of a SL boost in aged mice to improve vaccine protection. We first performed histological analysis of the SL tissue of 16-month-old mice, compared to 2-month-old SL mucosa to evaluate morphological differences due to ageing. A Masson's trichrome staining highlighted a smoothing of the basement membrane and an increased keratinised layer (Fig. 1A). Alike in the skin, the collagen density decreased with age (Fig. 1B and D). An increased presence of oedema and MHCII⁺ cells in aged mic compared to young animals witnessed a tissue chronic inflammation (Fig. 1A, C and E). The administration of the SL formulations (SL liquid and SL patch), did not induce an excessive inflammation of the epithelium 10 min and 4 h after administration (Fig. 1F). Yet, a significant increase in the lamina propria thickness was observed 10 min after administration of the liquid formulation but not after patch administration (Fig. 1G). Interestingly, 10 min after patch administration, NP already deeply crossed the sublingual mucosa to reach the submucosa together with chitosan (Fig. 1H).

Sublingual boost with the chitosan-based patch increased quantity and avidity of specific IgG in young and aged mice.

Immunisation studies were carried out with a prime-boost strategy. The immunization of young mice by a SL prime followed by a SC boost did not induced high specific seric IgG titers before the SC boost (Fig S1). Consequently, we decided to use a prime-pull strategy with the SL route as mucosal boost after SC prime injection. Four weeks after immunization, these prime-pull scheme led to higher titers of anti-HA IgG than the single injection, independently of the administration route (Fig. 2A and B, grey line/bars vs other colors). Interestingly, 8 weeks after the last boost, the use of the SL patch led to significantly higher specific IgG titers than compared to SC/SC scheme (Fig. 2B). This result was only observed after patch administration and not after SL liquid formulation (SC/SL liquid), indicating that the patch itself positively impacted the intensity of the induced immune response. Similarly, the boost by the sublingual patch significantly increased the avidity of specific IgG, compared to SC or SL liquid boost (Fig. 2C). However, the Th1/Th2 orientation of the immune response was not affected by the immunisation scheme, as reflected by IgG1/IgG2a ratios (Fig. 2D). In all cases, the IgG1/2a ratios were higher than 2, suggesting a Th2 (antibody mediated)-biased immune response.

Similarly to young mice, an immunization scheme based on a SL prime did not induce significant specific anti-HA IgG production in aged mice (16-month-old at the beginning of the protocol) (Fig S1). Consequently, the same prime-pull strategy was applied to aged mice. The immunization with NP-HA by the combination of SC prime and SL boost did not trigger significant difference in anti-HA seric IgG levels at the final time point (8 weeks post-immunization) compared to the SC/SC scheme (Fig. 2E). Four weeks post-immunization, the SC/SC scheme induced a significant increase (around 2 fold) in specific IgG titers compared to the SC prime alone but also to the SL boost by the patch (Fig. 2F). Yet, at this time point, the avidity of the induced specific IgG was significantly higher after the SC/SL patch scheme than

after all other schemes (Fig. 2G). Additionally, and similarly to young animals, the IgG1/IgG2a ratio indicates a bias towards a Th2 antibody response for all the schemes (Fig. 2H).

The increased avidity after SL patch and not after SL administration of the liquid formulation suggested a role of the patch components, and not the administration route itself. Therefore, we investigated the possible role of chitosan (CHI) in the maturation of the B cells.

Chitosan had an impact on B cell maturation.

B cell maturation was quantified after administration of the SL NP(3M)-HA vaccine in liquid form with or without CHI, and compared with the SL patch formulation. In the draining cervical lymph nodes (cLN), the administration of the SL patch led to an increase in the total B cell content whereas in the spleen this increase is induced by the administration of the liquid formulation with CHI (Fig. 3A). The administration of the SL patch increased the frequency of early activated B cells (CD69⁺ B cells, Fig. 3B) in the cLN and an increase in the frequency of activated CD86⁺ CD69⁺ B cells in the spleen (Fig. 3C); without increased expression of the memory receptor CD27 (Fig S2). At this time point (4 weeks after the end of the immunisation protocol), both formulations with CHI (liquid and patch) induced an increased frequency of non-proliferating activated B cells, also called early memory-like B cells (CD95⁺ CD38⁻) (Fig. 3D). These results suggest an efficient proliferation and activation of B cells after immunisation by the SL patch in young animals.

The protection against viral challenge is different according to the age.

To evaluate the level of protection against a viral challenge, young and aged mice were vaccinated using the NP-HA following a SC/SC or a SC/SL patch scheme, and compared to the benchmark tetravalent VAXIGRIP (2024/2025). The challenge was performed 6 weeks after the last boost by intranasal instillation of the influenza A virus, strain A/California/07/2009 (H1N1), at TDIC₅₀ (Fig. 4A). For the young animals, the SC/SC vaccination schemes with both VAXIGRIP or NP-HA were fully protective (100% survival) (Fig. 4B). However, the SL boost by the chitosan-based patch was deleterious since the survival rate of this group started to decrease earlier than the non-vaccinated group.

On the contrary, for aged animals, the SC/SL scheme showed the highest protection rate with 100% survival after H1N1 infection in mice of 18-month-old (Fig. 4C). Thus, SL boost by the patch triggered a more potent protective immunity than the homologous SC boost (80% survival, Fig. 4C, black line), despite lower IgG levels than SC/SC scheme (Fig. 2B).

To decipher the immunological mechanisms behind the protective effect of the SL patch in aged mice, we performed a comparative study based on IgA titers, cytotoxic T cell activity and immunophenotyping for both the SC and SL route, in the two groups of age.

The sublingual chitosan-based patch did not induce secretory IgA nor IgE.

Based on our hypothesis that mucosal administration induces secretory IgA, we expected that the secretory IgA levels would be high enough to at least partially explain the viral protection. Surprisingly, the levels of secreted IgA in saliva, nasal washes and bronchio-alveolar lavages were lower than expected after sublingual administration [24, 25] independently of the age (Fig. 5A). Even though statistical differences were highlighted in favor of an increased secretion of IgA in saliva with age for both administration routes, the low levels invalidated our first hypothesis of increased mucosal antibody induction after SL administration by the patch. Immunohistological analysis of non-specific IgA production in the nasal cavity showed the presence of IgA within the tissue of naïve mice without secretion (control, Fig. 5B). However, IgA secretion was observed after immunization, independently of the route and scheme used. IgA were also detected within the sublingual tissue, in lower amount in all conditions (naïve and immunized mice). Of note, although total IgE level increased with age in the serum, no further increase was observed after patch vaccination (Fig S3). This result excluded an allergy to chitosan.

SL patch did not induce cytotoxic T cells but modified T cell polyfunctionality compared to SC route.

Enzyme-linked immunospot (ELISpot) was used to quantify specific T cell response in the spleen 4 weeks after immunization on the two immunization schemes (SC/SC and SC/SL) and two ages (3 and 18 months), and compared to the corresponding naïve mice. Interferon gamma (IFN γ)-secreting antigen specific CD8⁺ cells were predominantly observed after SC/SC vaccination, in both ages, indicating that the protection triggered by the SC/SL scheme in aged mice was not based on the cytotoxic activity of CD8⁺ T lymphocytes (Fig. 6A).

The polyfunctionality of specific CD4⁺ and CD8⁺ Th1 cells was assessed by the quantification of cells producing IFN- γ , tumor necrosis factor alpha (TNF- α), and/or interleukin-2 (IL-2). Few multifunctional (IFN- γ , IL-2 and TNF- α) cells were detected in both schemes and ages (Fig. 6B, red color, pie chart). However, after SC/SL immunization, CD8⁺ cell responses were strongly oriented toward the single secretion of IFN- γ (especially in the aged group) or IL-2, according to the total repartition (Fig. 6B), with no statistical difference in the frequency of the cells (Fig. 6C). On the contrary, SC/SC scheme induced a monofunctional response dominated by the expression of TNF- α , in both ages (Fig. 6C). The non-specific stimulation of T cells by PMA/ionomycin also induced a majority of TNF- α -secreting cells (Fig S4, orange external circles), except after the SC/SC immunization in aged mice where the CD4⁺ T cell cytokine secretion was more balanced between IFN- γ , IL-2 and TNF- α (Fig S4).

Aging did not affect the profile of cytokine expression of specific CD4⁺ T cells, whereas the immunization route strongly did (Fig. 6B). Cytokine expression of CD8⁺ T cells was affected by both the age and the immunization route.

Immunisation by the sublingual patch induced an infiltration of neutrophils in the secondary lymphoid organs in aged mice.

Immunophenotyping of the blood, the effector sites (tongue, lungs), the spleen and the draining LN of the SL mucosa (submandibular and cervical LN) revealed significant changes in cellular distribution of immune cells, possibly explaining the difference in vaccine protection against viral challenge according to the immunization route and age. The analysis included two immunization schemes (SC/SC and SC/SL) and two ages (3 and 18 months), with the corresponding naïve mice. The time of analysis (4 weeks after the vaccination) was chosen to give a picture of the immunological landscape 1 week before the viral challenge.

Blood immunophenotyping revealed that both CD4 + and CD8 + memory cells increased with age, with no significant difference between control and vaccinated groups (Fig. 7A and B). Age induced a significant increase in neutrophil frequency, with no incidence of the route of immunization (Fig. 7C).

No changes in the number of resident CD4 + T cells were detected in the lingual tissue after immunization (Fig. 7D), but the SL boost induced a significant increase of CD8 + T cells in both groups of age (Fig. 7E).

Quantification of cell frequencies in the lungs revealed an increase in CD4 + T cells after vaccination for both routes (Fig. 7F), but a decrease in the frequency of CD8 + T cells after SL vaccination at both ages (Fig. 7G). The CD8 + T memory and central memory cell frequencies increased with age but were not affected by the immunization and exhibit similar levels in lung as compared to the non vaccinated groups (Fig. 7H and I). Concerning innate immune cells, there was no statistical difference in neutrophils frequencies after immunization (Fig. 7J), but the frequency of lung NK cells significantly increased after SL boost as compared to SC route, but not compared to the naïve control group (Fig. 7K).

The analysis of immune populations in the secondary lymphoid organs highlighted variations on cell frequencies after immunization by SC and SL routes (Fig. 8A). Notably, SC boost in aged mice induced an increase in the B cell frequency in the submandibular and cervical lymph nodes that was significantly higher than the frequency observed after SL boost (Fig. 8B and C), indicating a possible activation of germinal center in distant lymph node by SC vaccination by the NP(3M)-HA. Additionally, SL boost with the patch increased the frequency of total B cells in the spleen (Fig. 8D) and marginal zone B cells (Fig S4A) in aged mice, compared to the naïve mice. No impact of immunization route was observed on the frequency of spleen follicular B cells (Fig S4B).

Interestingly, an increase in neutrophil frequency was observed in the LN and the spleen after SL boost compared to SC, particularly marked in the aged group (Fig. 8E-G). This general infiltration in the secondary lymphoid organs was also observed with other innate immune cells, NK cells, only in the cLN and not in the submandibular LN or in the spleen (Fig. 8H-J).

SL vaccination by the chitosan-based patch induced increased and probably protective IL-6 levels in the blood of aged mice.

Finally, we quantified the cytokine signatures of young and aged mice before immunisation (D0) and 4 weeks after the end of the vaccination protocol (D56, corresponding to 1 week before the viral challenge) to evaluate if a possible pro-inflammatory status of the blood could have impacted the response and protection to the infection (Fig. 9).

The levels of IL-6 were higher in aged mice after SL vaccination compared to both young SL and aged SC. The levels of IL-2 decreased with age and after SL vaccination. CXCL1 increased with age but returned to basal levels (similar to young animals) after both SC and SL vaccination. IL-1 β levels were lower after vaccination than before in both groups of ages and administration route, contrary to IL-10 in aged groups which showed higher levels after vaccination. No significant difference was observed in all the groups for IFN- γ , TNF- α and IL-5 levels.

The comparison of the general cytokine signature after SC and SL vaccination in young animal groups (grey bars and dotted grey bars, respectively) did not highlight any differences between these two groups. The pro-inflammatory cytokine levels were probably not involved in the discrepancy of protection against the viral infection. The comparison of the administration route in aged animal groups (blue bars and dotted blue bars) showed a significant increase in only one cytokine, IL-6. The increased levels of IL-6 in aged mice after SL patch vaccination could be an important factor that triggered the protective effect against viral infection.

Discussion

In this work, we investigate a vaccine formulation designed to be administered by SL route in two groups of age: young adult mice (2 to 3 month-old) and aged mice (16 to 18 month-old), to evaluate the potency of mucosal vaccines to protect elderly population against influenza virus infection. The SL vaccine consisted on a subunit flu vaccine formulation (polymeric NP of PLA, adjuvanted with a TLR7/8 agonist and functionalized by passive adsorption of hemagglutinin HA from H1N1) deposited on a SL patch. This patch, composed of polysaccharides (chitosan and hyaluronic acid) was fully mucoadhesive, biodegradable and oro-dispersible [23]. The subunit vaccine was either injected (SC) or deposited under the tongue after adsorption onto the mucoadhesive patch. We showed 100% protection of aged mice (18 month-old at the time of infection) against influenza virus challenge, after sublingual vaccination by the mucoadhesive patch. (Fig. 4). A protective effect of 75–80% was quantified after vaccination with injected formulations (VAXIGRIP and NP-HA). Of note, the VAXIGRIP (2024–2025) did not contain the strain A/California/07/2009 (H1N1). The survival rate was thus reflective of the cross protection between strains. When the same SL vaccination protocol was applied to young mice, the survival rate was of 50%, even lower than in the non vaccinated group.

To decipher the immunological mechanisms underlying the protective vs non-protective effect triggered by the SL vaccination patch, several aspects of the immune responses were investigated. Contrary to what was expected, the induction of secreted IgA was found to be too low to explain the viral protection (Fig. 5). Similarly, the antigen-specific cytotoxic effect of T cells was significantly lower after SL

administration than the SC formulation in both age groups (Fig. 6). Given this limited CD8⁺ cytotoxic response, the protective effect of the SL patch appears to operate through mechanisms other than classical cytotoxic T cell activity. Although studies showed that T cell-mediated immunity provides cross-protective immunity to influenza [26], in humans, the protective effect of T cell-mediated immunity against seasonal influenza virus infections is still unclear, and can be counter balanced by the humoral immune response [27]. It has been reported that T cell responses in the blood may serve as stronger correlates of protection against influenza in the elderly compared to serum antibody responses [2]. Currently used inactivated vaccines induce low CD8⁺ T cell responses but efficient CD4⁺ T cells and antibody response [He 2006]. In contrast, live-attenuated influenza vaccines induce both CD4⁺ and CD8⁺ T cells as well as a local immune response by tissue resident memory T cells that exhibit protective capacity against influenza virus infection [28]. Moreover, the ability to protect against a viral challenge is positively correlated to the size of the memory CD8⁺ T cell pool, the phenotype and location of these CD8⁺ T cells [29]. Interestingly, in our study, we found an increased frequency of tissue-associated CD8⁺ T cells in the lingual tissue after SL patch vaccination in both young and aged mice (Fig. 7E), probably due T cell homing at the inductor site. T cell homing after SL immunization was already highlighted in the female genital tract [30, 31] but never reported directly in the buccal tissue.

Both CD4⁺ and CD8⁺ T cells are an important source of pro- and anti-inflammatory cytokines, and their patterns of cytokine secretion have an important role in the shaping of an active immune response against pathogens and vaccines. Single-cytokine-producing cells were shown to have less potential in HIV-1 antiviral immunity as compared to polyfunctional T cells, that have improved functions [32]. In our study, the vaccines (SC or SL) induced predominant single-cytokine-secreting T cells. This could be explained by the antigen dose (1 µg/administration), as it was shown that CD8⁺ and CD4⁺ T cell response are dependant to antigen doses [33], and that low concentrations of antigen induce predominantly single-cytokine-secreting T cells [34]. In our study, if we compare the cytokine secretion of T cells in aged vs young mice after SC/SL scheme, we conclude that 1/ the cytokine profiles of CD4⁺ T cells are the same in aged and young mice, and 2/ the proportion of CD8⁺ T cells secreting IFN-γ is higher in aged mice. IFN-γ, primarily produced by NK cells and adaptive effector T cells, plays a central role in controlling Th1 immunity and promoting cellular responses essential for viral clearance [35]. This difference in cytokine secretion could be involved in the differences in protection against viral infection. Protection mediated by CD4⁺ T cell occurs through antibody response but also by other effector functions, such as cytotoxicity and inflammatory cytokine release [4].

Concerning antibody response, SL boost by the patch in aged mice triggered the mounting of a more efficient protective immunity (100% survival) than the homologous SC boost (80% survival, Fig. 4C, black line), despite lower IgG levels than SC/SC scheme (Fig. 2F). This result suggests a decoreclation between the quantity of antibodies and their protective effect. Besides, eventhough the avidity of IgG was increased by the use of the SL patch in both young and aged animals, only the aged group was protected from the viral challenge, excluding IgG avidity as the main source of protection in aged mice. Since the antibody avidity was only augmented after administration of the SL patch (and not

administration of the liquid formulation by SL route), we hypothesized that a patch component modified the maturation of antibody-secreting cells. Vaccination of young mice by chitosan-based formulations increased the frequency of total B cells and CD69⁺ B cells in cLN, in a with a more pronounced effect when formulated as a SL patch (Fig. 3). These cells represent recently activated B cells that have encountered vaccine antigens and are likely undergoing proliferation and differentiation within the germinal centers of the LN [36]. As they progress, CD69⁺ B cells may further differentiate into plasmablasts (for immediate antibody secretion) or memory B cells (for long-term immunity). Moreover, the presence of chitosan in the vaccine formulation (liquid or patch) induced CD95⁺CD38⁻ cells (early memory-like B cells) in the BM, underscoring the positive impact of the chitosan on B cell activation and maturation.

Concomitantly, a significant neutrophil infiltration was quantified in two lymphoid organs, particularly in the group of aged mice. Neutrophils are the most prevalent leukocytes and substantially contribute to both innate and adaptive immunity [37]. They can migrate to the site of inflammation through the action of several chemoattractive signals. The recruitment of neutrophils can also be induced by vaccine adjuvants, such as MF59. By attracting neutrophils and monocytes at the injection site, MF59 promotes the transport of antigens to the lymph nodes [38]. In our work, we used a synthetic TLR7/8 agonist. The TLR 7/8 is expressed by DCs (one of the most important cell type for TLR7/8 agonists), but also by monocytes, macrophages, neutrophils, mast cells, and eosinophils [39]. However, no chemoattractive properties being described for this adjuvant, we hypothesize that the neutrophil chemoattractant could be chitosan, as it entered the submucosa together with the NP (Fig. 1H). The biological effects of chitosan could be described as different and sometimes antagonists in the literature (pro/anti-inflammatory for example). The complexity of the description of the impact of chitosan as a biomaterial/adjuvant relies on its characterization and specifications. This is why the precise description of the molecular weight and the deacetylation degree (providing information on the positive charges of the polysaccharide) is of most importance to conclude on the beneficial or detrimental effect of chitosan in a formulation. A correlation between chitosan and neutrophil migration was highlighted *in vitro* in some studies [40, 41]. Neutrophils, or precisely polymorphonuclear neutrophils (PMNs), infiltration occurs during inflammation and wound repair. Chitosan was reported to elicit neutrophil infiltration after chitosan-based material implantation in mice, dogs, sheeps and rats [42–44], but no such infiltration was reported after PLA-based materials [45]. It was recently reported that chitosan can also induce activation or differentiation of porcine immune cells [46].

The degree of deacetylation of chitosan was directly correlated to the migration index of neutrophils *in vitro* (differentiated HL-60-PMN) [40, 41]. It was shown that increased DD decreased the migration of neutrophils and IL-8 secretion by an unidentified mechanism. However, Simard et al showed that a 80% DD (similar to the DD of chitosan used in this study), did not induce ROS production, nor myeloperoxidases or lactoferrin in cultures human PMNs, indicating no effect of chitosan on degranulation [40].

Although an excessive neutrophil infiltration can be deleterious for implant integration, in the case of vaccine response several studies have highlighted the important role of neutrophils. When located at the mucosa, neutrophils were shown to bind to proteoglycans and create niches favorable to plasma cell establishment leading to a sustained and local antibody secretion [47]. Also, similarly to our results, a study showed that, after SL vaccination, the rapid recruitment of neutrophils in the SL mucosa and draining lymph nodes inhibited the development of IgA response [48]. The depletion of neutrophils prior to vaccination restored IgA secretion in a model of SL vaccination with *Bacillus anthracis* edema toxin (EdTx). Thus, in our study, neutrophil recruitment at the patch administration site could have induced their migration to the draining lymph nodes and negatively impacted IgA production (Fig. 5). Moreover, neutrophils can directly kill pathogens or, in the case of vaccination, act as antigen-presenting cells (APCs) [49]. Then, neutrophils will migrate to the secondary lymphoid organs [50] where they can act on B-cells by promoting their survival and differentiation by the secretion of APRIL and BAFF [47].

In the spleen, neutrophils were shown to have B cell helper function [51], and activate them from marginal zone more effectively than follicular naive B cells through both contact-dependent and contact-independent mechanisms [52]. Additionally, T-independent B-cell response predominantly involves B cells from marginal zone and is triggered by nonprotein antigens, such as polysaccharides [53, 54]. This could explain the higher affinity of antibodies secreted after patch administration, but not after injection that did not contain chitosan. The observed increase in B cell activation in the cLN, together with local neutrophil recruitment after vaccination by the chitosan-based SL patch, may support enhanced antibody maturation. This increase in frequency could reveal an efficient proliferation/maturation process in the germinal center possibly explaining the high affinity of antibodies. However, the precise contribution of these cells to protection remains to be directly demonstrated.

The protective effect of the SL patch in aged animals and the antagonist and deleterious effect in young animals remains to be elucidated. However, the different IL-6 levels in the blood may constitute an interesting and discriminating parameter. IL-6 contributes to vaccine protection by promoting Th1 responses, coordinating with other cytokines, and supporting both innate and adaptive immunity. This coordinated response is critical for shaping the immune memory recall following influenza virus infection [55]. Additionally, the presence of oedema and MHCII+ cells in aged mice SL mucosa compared to young animals witnessed a tissue chronic inflammation, that could probably have an impact on the quality and intensity of the vaccine response after SL vaccination.

Taken together, our data suggest that the SL patch acts as a potent immune modulator in the elderly. The significant elevation of IL-6, coupled with optimized innate cell recruitment, provides the necessary inflammatory stimulus to overcome age-related immune decline, a phenomenon not observed in young animals.

Conclusion

Our findings contribute to the growing body of research on mucosal and sublingual vaccination, with implications for the development of more effective and accessible vaccine formulations for at-risk groups. This study demonstrates that sublingual vaccination using a mucoadhesive patch conferred 100% protection in aged mice against influenza, outperforming injected formulations, despite lower IgG antibody levels, limited secreted IgA or cytotoxic T cell responses. The protective mechanism could involve enhanced B cell activation and maturation and neutrophil recruitment, potentially driven by chitosan, which may improve antibody affinity and immune memory. The age-dependent efficacy of the patch, with opposing effects in young vs. aged mice, suggests a role for IL-6 and chronic mucosal inflammation in shaping the immune response. These findings highlight the potential of chitosan-based SL patches for elderly vaccination while underscoring the need to decipher the precise mechanisms behind their age-specific protection.

Declarations

Ethics approval

All of the experiments were performed in accordance with animal welfare regulations for their use for scientific purposes governed by European Directive 2010/63/EU. Protocols were validated by the local Animal Ethics Evaluation Committee (CECCAPP: C2EA-15) and authorized by the French Ministry of Education and Research.

Competing interests

The authors declare that they have no competing interests

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Author Contribution

Conception and experimental design: CM, RR, SD. Funding acquisition and supervision: CM. Formal analysis: all authors. Interpretation: all authors. Investigation and methodology: RR, CM, CC, CG, EC, SD. Writing – Original Draft: CM. Writing– Review & Editing: all authors.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

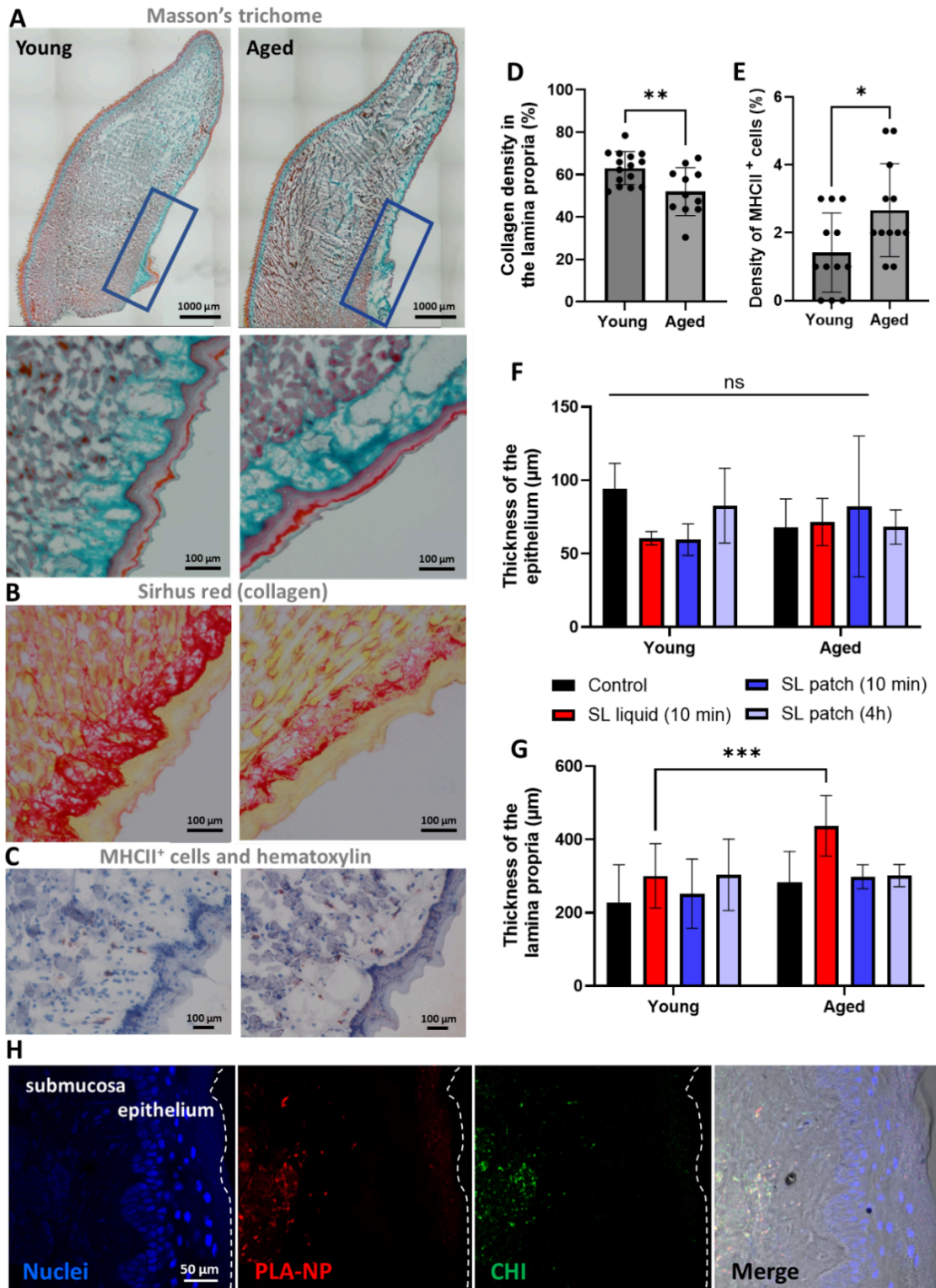


Figure 1

Histological analysis of the suglingual tissue ageing. (A) Masson's trichrome staining of young (3 months) vs aged (16 months) mice. The lower panel is a magnification (40x objective) of the ventral part of the tongue within the blue rectangle in the upper panel (mosaic). (B) Sirhus red staining to visualize the collagen content. (C) Staining of MHCII⁺ cells with an hematoxylin counterstaining. (D) Quantification of the collagen density in the lamina propria (**: $p < 0.001$). (E) Quantification of the MHCII⁺ cells in the lamina propria and

the epithelium (*: $p < 0.01$). (F, G) Thickness of the epithelium (F) and the lamina propria (G) according to the administration route and the time after the vaccine delivery. (H) Longitudinal section of the ventral part of the tongue, with fluorescent staining of the nuclei (DAPI), the PLA-NP (Bodipy-TR) and the chitosan (CHI, FITC). 40x objective (with a 2X digital zoom).

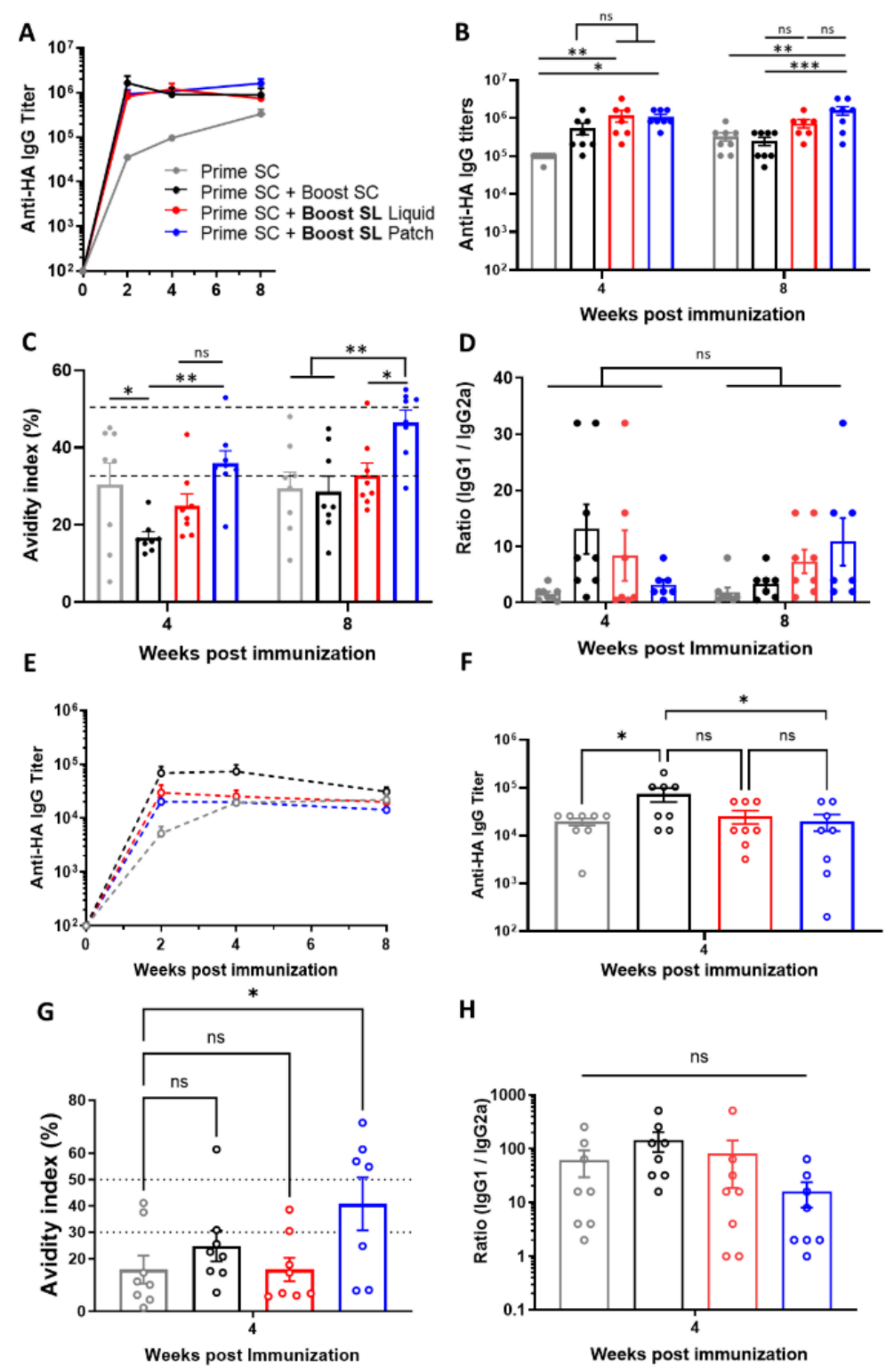


Figure 2

Systemic immune response in young (3 months) (A, B, C, D) and aged (16 months) (E, F, G, H) mice according to the administration route. (A) Kinetics of anti-HA IgG titers determined by ELISA 2, 4, 6 and 8 weeks after SC or SL boost. (B) Comparison of anti-HA IgG titers determined by ELISA at 4 and 8 weeks after SC or SL boost. (C) Quantification of anti-HA antibody avidity towards HA at 4 and 8 weeks after SC or SL boost. Values between 30% and 50% represent an intermediate avidity. (D) Anti-HA IgG1 to IgG2a ratio at 4 and 8 weeks after SC or SL boost. (E) Kinetics of anti-HA IgG titers determined by ELISA 2, 4, 6 and 8 weeks after SC or SL boost. (F) Comparison of anti-HA IgG titers determined by ELISA at 4 weeks after SC or SL boost. (G) Quantification of anti-HA antibody avidity towards HA at 4 weeks after SC or SL boost. Values between 30% and 50% represent an intermediate avidity. (H) Anti-HA IgG1 to IgG2a ratio at 4 weeks after SC or SL boost. N=8, each symbol represents an animal. Data are presented as mean $\pm$ SD and statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (ns: $p > 0.05$, *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$). SC: subcutaneous, SL: sublingual.

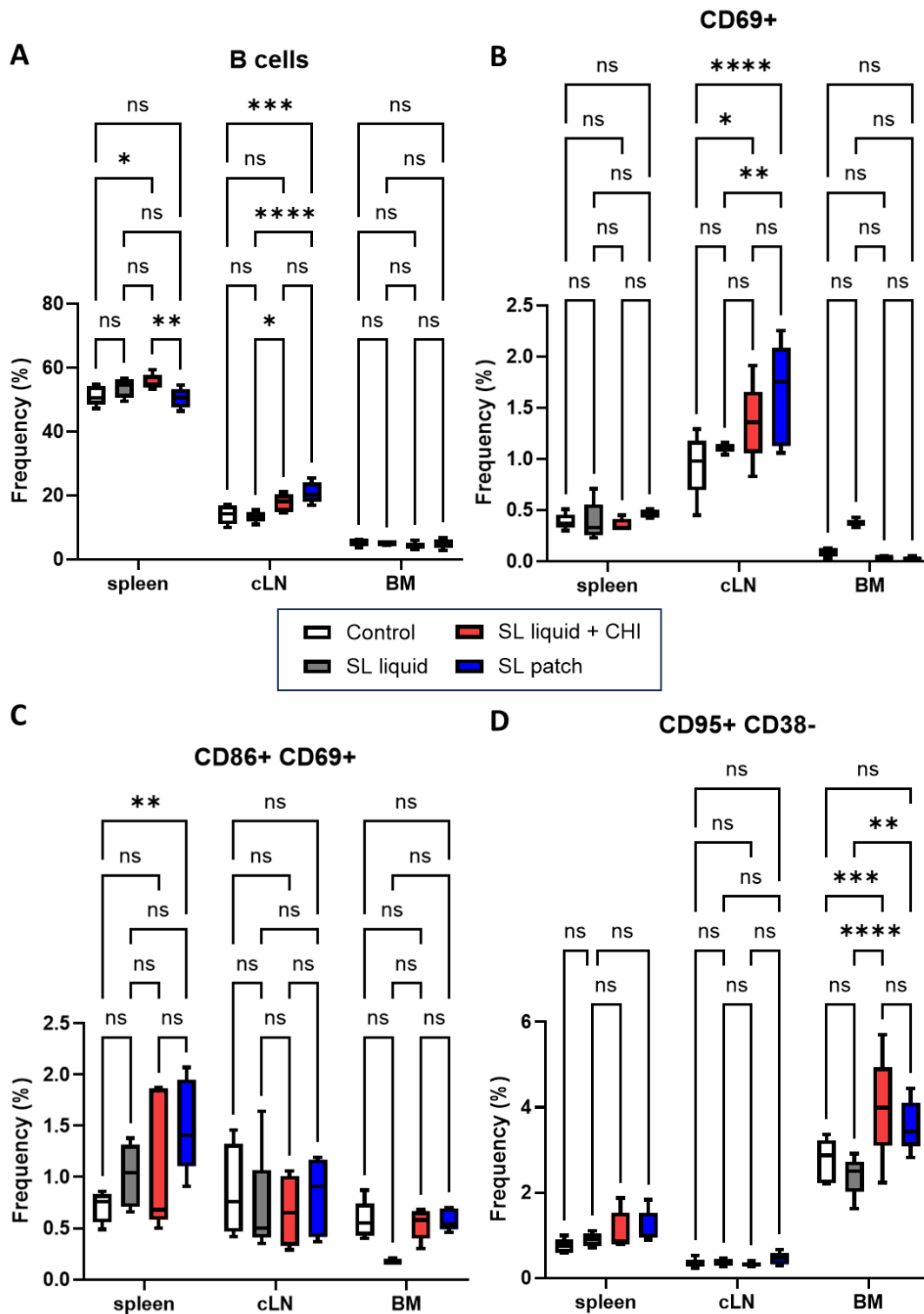


Figure 3

B cell maturation states in the spleen, cervical lymph node (cLN) and the bone marrow (BM), 4 weeks after SL boost in absence or presence of CHI (formulated as liquid or patch) in young mice. (A) Frequency of total B cells (B220⁺), (B) recently activated cells (CD69⁺) and (C) CD86⁺CD69⁺ and (D) early memory-like B cells (CD95⁺ CD38⁻). N=5. Data are presented as mean $\pm$ SD and statistically analysed using one-way

ANOVA followed by Tukey's multiple comparison test (ns: $p > 0.05$, *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, ****: $p < 0.00001$).

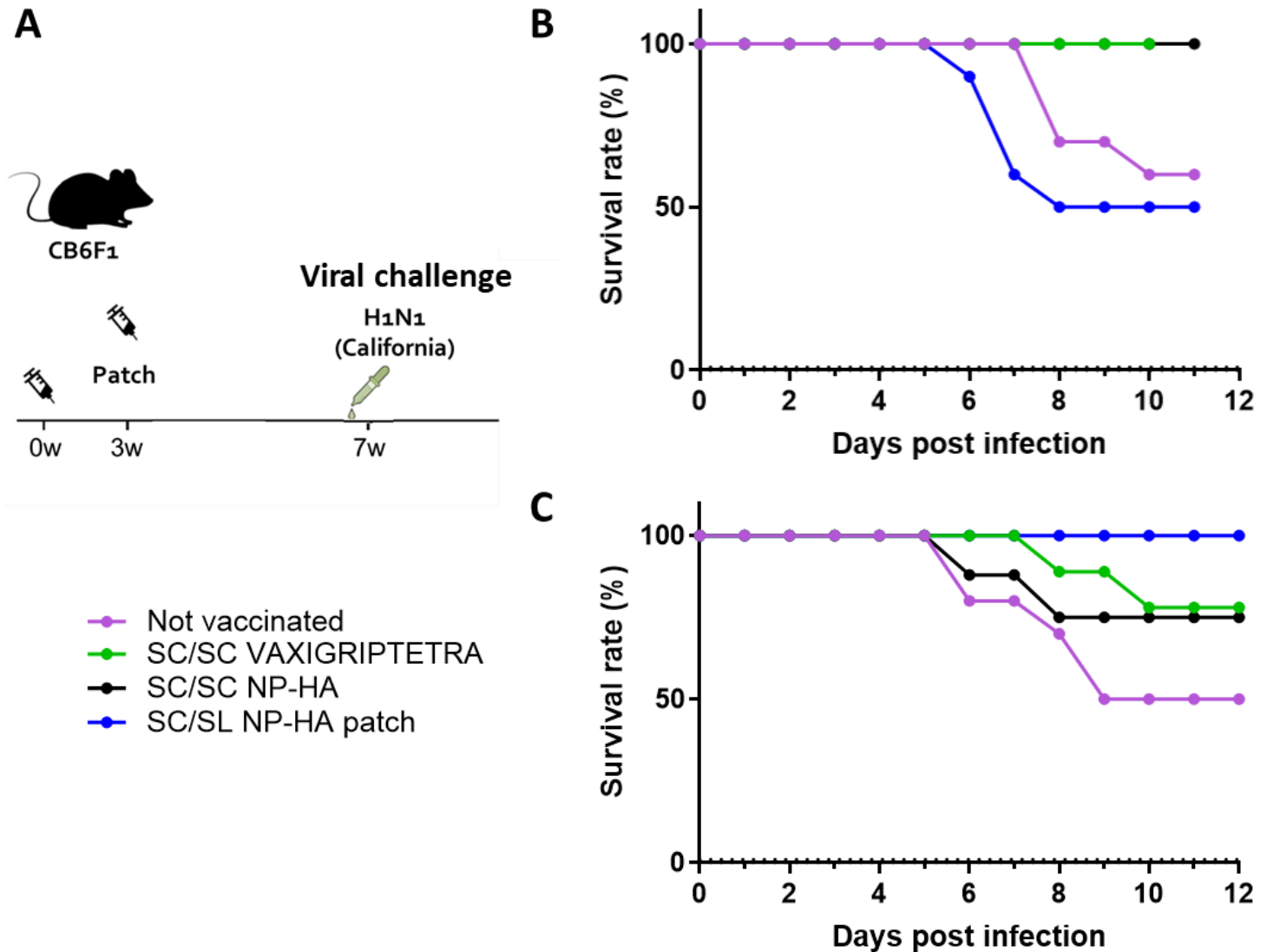


Figure 4

Effect of ageing in vaccine protection against a challenge with H1N1. (A) Immunisation scheme (subcutaneous (SC) prime and SC or sublingual (SL) boost after 3 weeks. The viral challenge was done 4 weeks after the vaccination and consisted in an intranasal administration of a TCID₅₀ dose of H1N1. (B, C) Survival rates of young (B) and aged (C) mice during 12 days after infection. N=8-10.

cavities and sublingual mucosa of mice 14 weeks after the immunisation by subcutaneous (SC) or sublingual (SL) boost. N=3. Objective 4x.

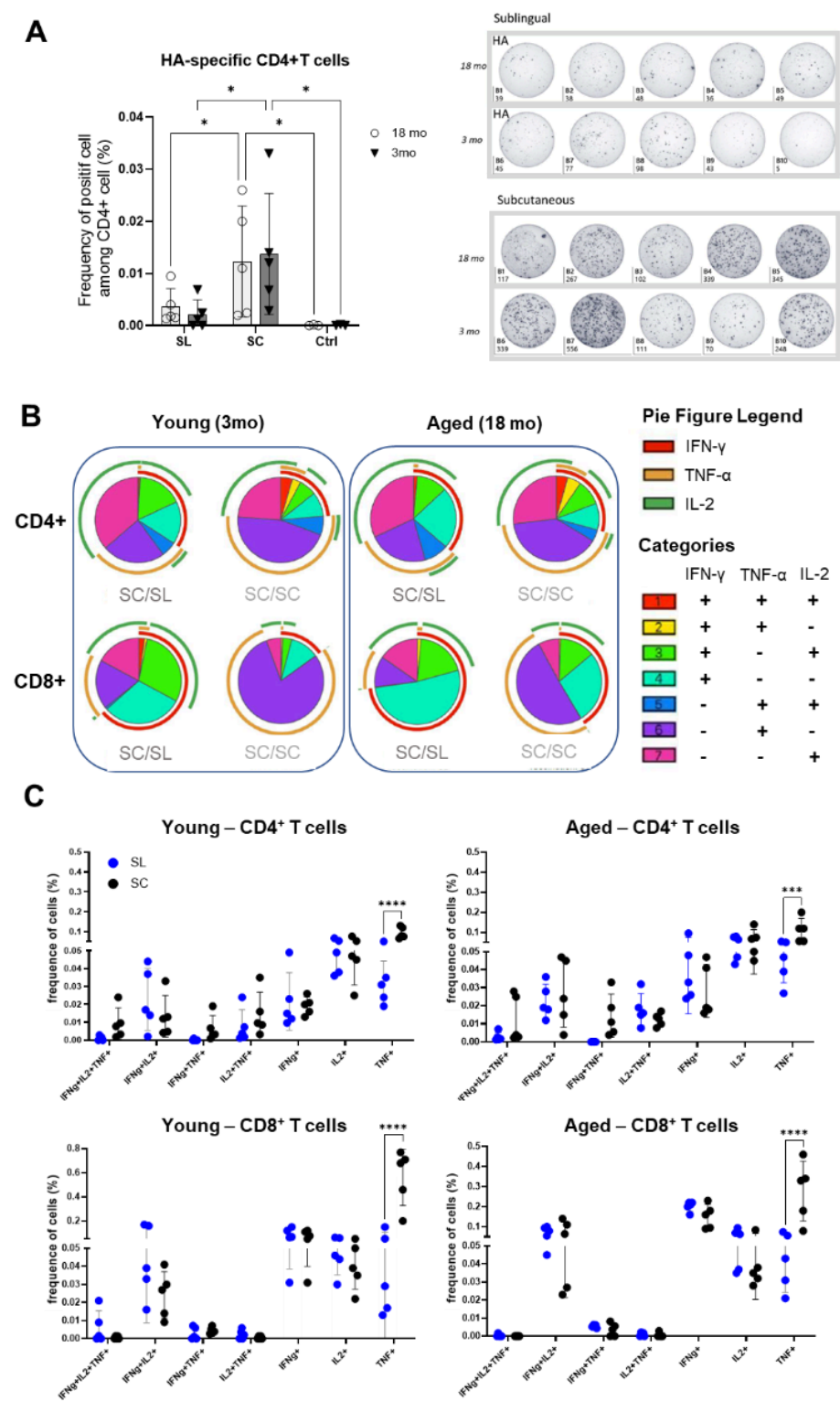


Figure 6

T cell response to the sublingual vaccine in young and aged mice. (A) HA specific CD4⁺ T cell response analysed by EliSpot. (B, C) Multifunctionnall influenza-specific Th1 CD4/CD8 cytokine-secreting response,

represented by pie charts indicating the expression of cytokine combinations from CD4⁺ and CD8⁺ T cells (B) and the frequency of cytokine secreting cells (C). *n*=5, each symbol represents an animal. Data are presented as mean $\pm$ SD and statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (*: *p* < 0.01, ***: *p* < 0.0001, ****: *p* < 0.00001).

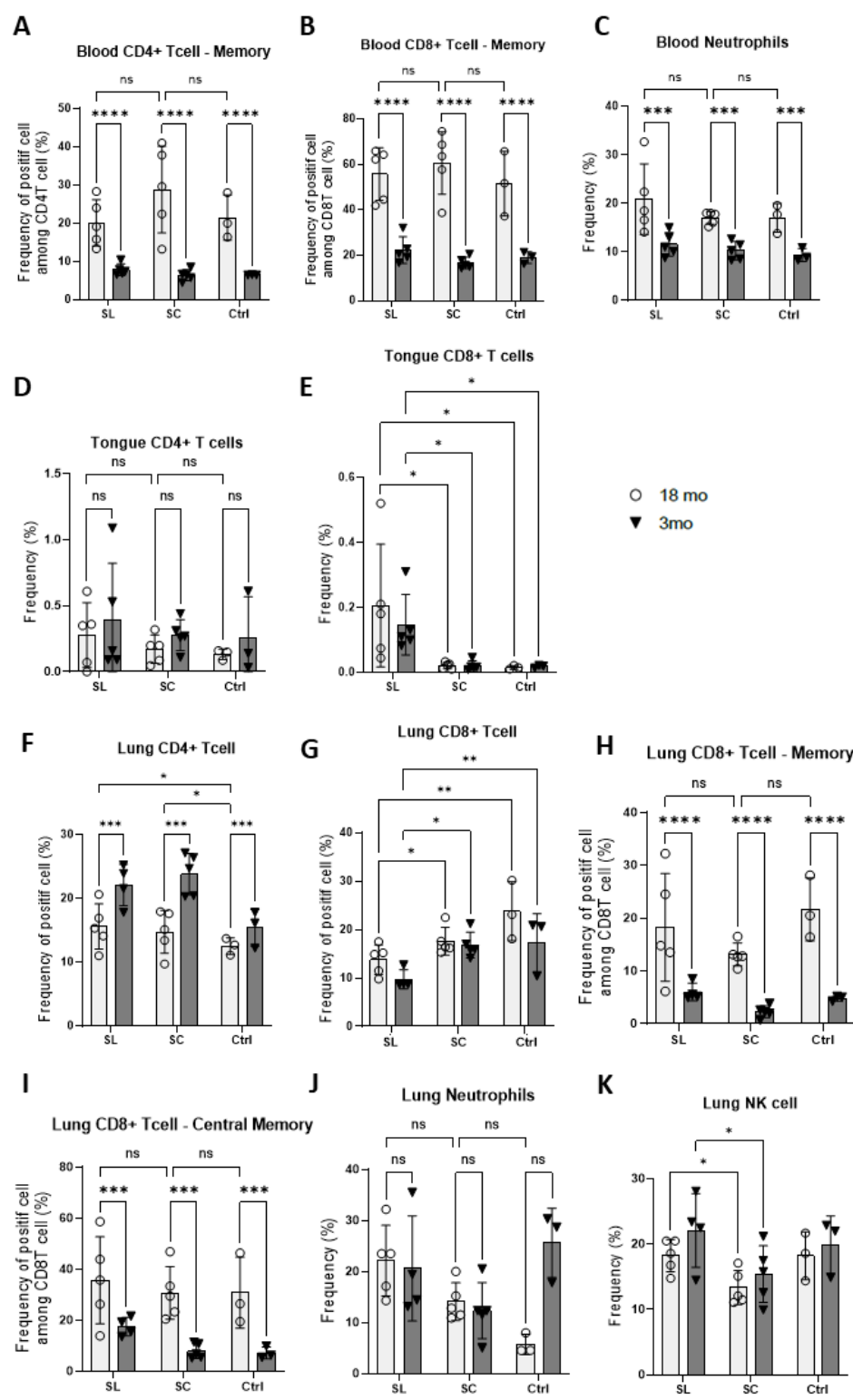


Figure 7

Blood, tongue and lung immunophenotyping after SC or SL boost in young and aged mice. Frequency of memory $CD4^+$ cells (A) among $CD4^+$ T cells and total $CD8^+$ cells (B) among $CD8^+$ T cells in blood. (C) Neutrophil frequency in the total $CD45^+$ cell population in the blood. Frequency of total $CD4^+$ (D, F) and total $CD8^+$ (E, G) T cells in the tongue and the lungs. Frequency of memory (H) and central memory (I) $CD8^+$ cells among $CD8^+$ T cells in lungs. (J) Neutrophil and (K) NK cell frequency in the total $CD45^+$ cell population in the lungs. N=5, each symbol represents an animal. Data are presented as mean $\pm$ SD and statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (ns: $p > 0.05$, *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, ****: $p < 0.00001$).

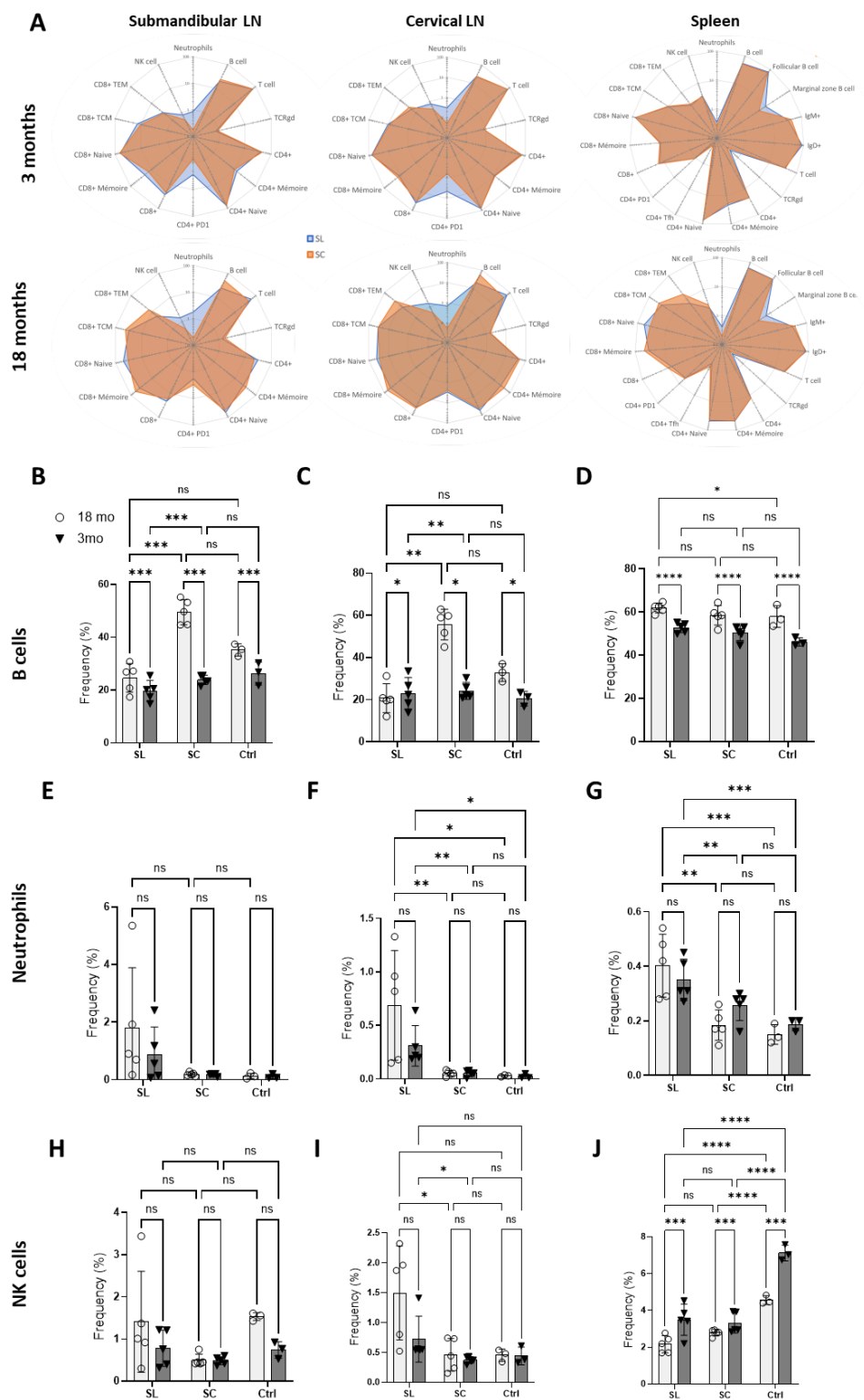


Figure 8

Immunophenotyping of secondary lymphoid organs after SC or SL boost in young and aged mice. (A) Spider's web representation of the representation of immune cells among the draining submandibular and cervical lymph nodes and the spleen. (B, E, H) Frequency of B cells, neutrophils (E, F, G) and NK cells (H, I, J) according to the age and the administration route of the vaccine. N= 5, each symbol represents an animal.

Data are presented as mean $\pm$ SD and statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (ns: $p > 0.05$, *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, ****: $p < 0.00001$).

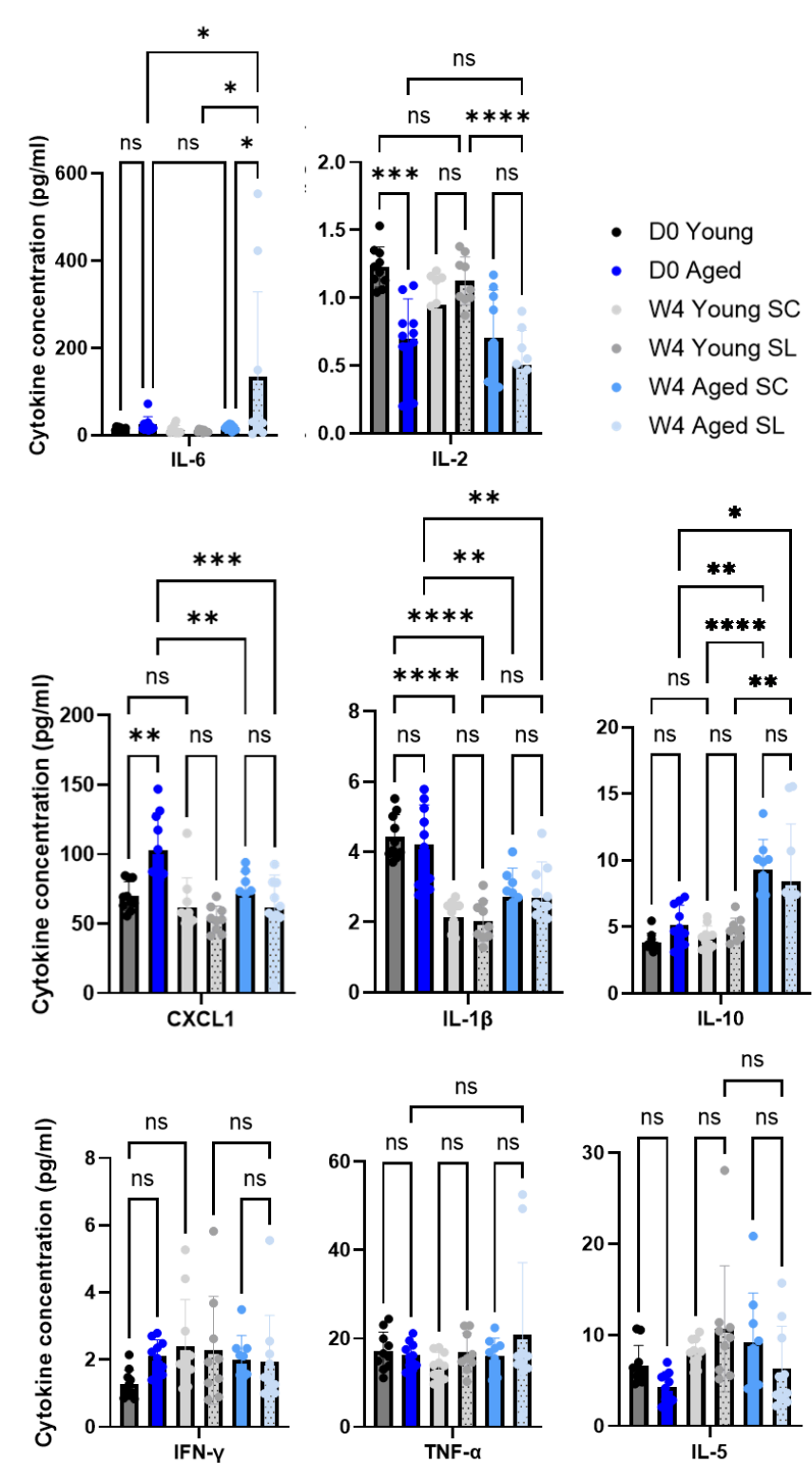


Figure 9

Cytokine signatures of young and aged mice before immunisation (D0) and 4 weeks after the end of vaccination (W4). Cytokine concentrations were quantified from sera. N=8-10, each symbol represents an

*animal. Data are presented as mean $\pm$ SD and statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (ns: $p > 0.05$, *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, ****: $p < 0.00001$).*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarydata.docx](#)
- [Scheme1.docx](#)