Journal of Inflammation Research

Dovepress
Taylor & Francis Group

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ORIGINAL RESEARCH

Understanding the Mode of Action of Several Active Ingredients from the Micro-Immunotherapy Medicine 2LZONA®

Camille Jacques[1], Flora Marchand[2], Mathias Chatelais[2], Adrien Brulefert[3], Ilaria Floris[1]

[1]Preclinical Research Department, Labo'life France, Moncoutant-Sur-Sevre, 79320, France; [2]ProfileHIT, Sainte-Pazanne, 44680, France; [3]QIMA Life Sciences, Gençay, 86160, France

Correspondence: Camille Jacques, Preclinical Research Department, Labo'life France, Pescalis-Les Magnys, Moncoutant-sur-Sevre, 79320, France, Email camille.jacques@labolife.com

Introduction: Varicella-zoster virus (VZV) affects over 90% of the global population. The initial encounter with VZV, often in the early years of childhood, results in varicella. From latency, VZV can reactivate in later stages of life, leading to the development of herpes zoster. Considering the importance of host immune responses in preventing reactivation and clinical manifestations associated with VZV infection, a therapy that sustains the immune system could be of great interest.

Objective: The present work aimed to set the basis of the possible mode of action of 2LZONA®, a micro-immunotherapy medicine composed of five different capsules. Thus, the effects of several active substances employed in this medicine were assessed in human primary immune-related cells.

Results and Discussion: Our results showed that DNA (8 CH) and RNA (8 CH), two active substances used in 2LZONA, displayed phagocytosis-enhancing capabilities in granulocytes and contained sub-micron particles that could explain, at least partially, the observed effect. These two active substances tested singularly and together with other actives of 2LZONA's capsules, modulated the proliferation of immature, transitory, and mature subsets of natural killer (NK) cells in an IL-15-like pattern, suggesting an enhancement of their activation levels. Moreover, the tested items of 2LZONA increased the secretion of IL-2, IL-6, IL-13, and TNF-α in human peripheral blood mononuclear cells (PBMCs). Furthermore, the proliferation of PBMCs-derived NK cells, intermediate monocytes, and neutrophils was slightly increased by this treatment. In CD3 and CD3/CD28 pre-primed conditions, actives present in one capsule of 2LZONA enhanced the secretion of IL-6 and TNF-α. Finally, one capsule of 2LZONA reduced the expression of human leukocyte antigen (HLA) in IFN-inflamed endothelial cells. Overall, these data provide, for the first time, preliminary experimental evidence of the mechanisms of action of some of the active ingredients employed in 2LZONA capsules.

Keywords: varicella-zoster virus, herpes zoster, immune system, cytokines, low doses, ultra-low doses

Introduction

Varicella-zoster virus (VZV), also known as human herpesvirus 3, is a common and highly contagious pathogen responsible for causing both varicella (chickenpox) and herpes zoster (HZ), or shingles. The life cycle of this double-stranded deoxyribonucleic acid (DNA) virus begins with an initial infection, typically occurring during childhood, causing varicella. After a period of latency within the dorsal root ganglia,[1] a reactivation of the virus can ensue years later, leading to HZ, characterized by painful skin rashes and blisters.[2] While acute infections can often be managed effectively, several complications like post-herpetic neuralgia, for instance, and many others, present significant treatment challenges, particularly in immunocompromised individuals and the elderly, and symptoms can persist for months to years after rash resolution.[3–5]

Reactivation from latency occurs when there is a weakening in cell-mediated immunity, partially explaining why increasing age is a predisposing factor for VZV reactivation.[6] Epidemiological analysis indeed reveals that about 70% of HZ cases occur in persons over the age of 50 years old.[7] Apart from the increasing age, there are other risk factors

Received: 11 October 2024
Accepted: 4 March 2025
Published: 21 March 2025

predisposing to VZV reactivation such as concomitant infections with other pathogens, diabetes, female gender, genetic susceptibility, physical trauma, and psychological stress.[8]

Host innate and adaptive immune responses are essential to prevent VZV reactivation and, in cases of reactivation, immune defenses can limit the severity of the clinical manifestations and prevent the onset of complications.[9] Varicella-zoster virus has developed smart mechanisms to evade immune defenses:[10,11] for example, pieces of evidence showed that VZV-infected immature human dendritic cells were unable to upregulate the expression of surface molecules essential for the maturation of these cells.[12]

In addition, the presence of autoantibodies against cytokines such as interleukin (IL)-6, interferon (IFN)-α and IFN-γ has been detected in subjects affected by post-herpetic neuralgia, suggesting their possible causative role in the manifestation of complications derived from the VZV reactivation, and also the importance of these cytokines in controlling VZV infection and reactivation.[13]

The development of antiviral medication has revolutionized the management of VZV infections. Traditional antiviral treatments, such as acyclovir and its derivatives, have been employed with notable success; however, the growing incidence of resistant VZV strains and the side effects associated with the prolonged use of antivirals impose the exploration of new therapeutic avenues.[14]

Therapies that can boost immune responses and sustain the immune system (IS) in the framework of VZV infections can limit the onset of complications and the severity of the clinical manifestations, offering valuable benefits. Thus, the micro-immunotherapy (MI) medicine (MIM) 2LZONA® (also referred to as 2LZONA in the manuscript) was developed to modulate the host immune responses to sustain the IS in fighting against VZV. 2LZONA is composed of five different capsules (2LZONA-1, 2LZONA-2, 2LZONA-3, 2LZONA-4, and 2LZONA-5) and like other MIMs, employs low doses (LD) or ultra-low doses (ULD) of immune mediators, mostly but not solely, human recombinant (hr) cytokines, prepared through a serial kinetic process. These medicines' active substances are expressed in decimal or centesimal Hahnemannian dilutions (DH or CH), or Korsakovian dilutions (K). Depending on the dilution employed, each substance intends to govern the orientation of a particular biological response toward either an activation or an inhibition. Previously published evidence shows that it is possible to apply an immune-boosting effect through the use of LD of cytokines such as IFN-γ (4 CH), tumor necrosis factor-α (TNF-α) (4 CH), or IL-6 (4 CH).[15,16] Conversely, MIM such as 2LARTH® or 2LALERG®, which employ pro-inflammatory cytokines at ULD, have been shown to induce anti-inflammatory effects in vitro and in vivo.[16–21] The starting materials employed at ULD in these medicines include plant-derived total DNA and ribonucleic acid (RNA). These ULD-based actives are intended to target the nucleic acid-sensing machinery to sustain host immune defenses under certain pathological conditions, including diseases related to viral infections. In addition, these medicines can also employ specific nucleic acids (SNA®) at ULD. They intend to act specifically on targets mostly involved in the etiopathogenesis of particular disorders.

In the VZV context, by using hr-IFN-α and hr-IL-2 at ULD (at 7 or 10 CH), together with other immune regulators at ULD, 2LZONA could apply modulatory effects on these mediators of inflammation. In addition, by using DNA (8 CH) and RNA (8 CH), 2LZONA can boost the innate immune response as these actives might activate the immune sensory system similarly to foreign nucleic acids (see also Table 1).

The study has two main objectives: i) perform a fundamental pilot research study on DNA (8 CH) and RNA (8 CH), both tested singularly, to set the basis for their possible mode of action and ii) investigate the effects of four out of the five capsules composing the medicine 2LZONA on different models of human primary immune-related cells.

Thus, the first part of our research focused on the two active substances, DNA (8 CH) and RNA (8 CH), which are not only employed in 2LZONA but also in other MIM like 2LEBV®, a treatment that aims to support the IS in the case of acute or chronic Epstein Barr infection and reactivation.[22]

In the second part of the study, four capsules out of the five composing the sequential medicine 2LZONA (namely 2LZONA-2, 2LZONA-3, 2LZONA-4, and 2LZONA-5) were analyzed in their capacity to modulate the functions and/or the activation status of several human immune cell types, such as natural killer (NK) cells through the same flow cytometry technique.

While the study was limited to some of the capsules of 2LZONA, and further studies are ultimately needed to understand how the medicine modulates immune responses in a more complex system such as an in vivo model, the

Table 1 Composition of the Tested Capsules

Starting Material (CH)	2LZONA-2	2LZONA-3	2LZONA-4	2LZONA-5
hr-IL-2	10	10	7	10
hr-IFN-α	7	10	10	10
RNA	10	8	10	8
DNA	10	8	10	10
SNA-ZONA	10	10	10	10
SNA-HLA-I	10	10	10	10
SNA-HLA-II	10	10	10	18

Abbreviations: CH, centesimal Hahnemannian dilution; DNA, deoxyribonucleic acid; HLA, human leukocyte antigen; hr, human recombinant; IFN, interferon; IL: interleukin; RNA, ribonucleic acid; SNA: specific nucleic acid.

preliminary data obtained in this study indicate that 2LZONA could provide clinical benefits in the context of VZV infections sustaining antiviral responses by enhancing the phagocytosis, the proliferation, the activation, and the cytokine secretion in several types of immune cells.

Materials and Methods

Tested Items and Experimental Controls

The investigational product assessed in this study is 2LZONA®, referred as 2LZONA throughout the manuscript. 2LZONA is a homeopathic medicinal product consisting of a sequence of five different capsules containing sucrose-lactose pillules, also called globules, for an oromucosal administration. These pillules are impregnated with their unique ethanolic preparations of human recombinant (hr) interleukins, plant-extracted DNA and RNA, and specific nucleic acids (SNAs® thereafter named as SNA). The medicine is a sequential treatment intended to be taken according to the order indicated in the blister. In this study, only four out of the five capsules of the medicines were tested (2LZONA-2; 2LZONA-3, 2LZONA-4, and 2LZONA-5). The SNA actives are further referred as SNA-ZONA, SNA-HLA-I, or SNA-HLA-II in the manuscript (as also shown in Table 1). The tested items and the vehicle (Veh.) capsules were manufactured by Labo'life España, avenida des Raiguer, 707330 Consell – Mallorca, Spain, as previously described,[15,23] and have been provided for investigational purposes. Previous publications have already described how the Veh. capsules are produced to provide a suitable control for pre-clinical research.[15,17,18,23,24] In the experiments performed in the context of the current study, the sugar pillules contained in each of the tested capsules of 2LZONA or Veh. were freshly diluted in 100 mL of culture medium to reach the final sucrose-lactose concentration of 11 mm, and this procedure was performed in all the in vitro experiments.

Assessment of the Particle Size, Count, and Concentration, by Tunable Resistive Pulse Sensing (TRPS)

The pillules contained in one capsule of Veh., DNA (8 CH), or RNA (8 CH) were freshly diluted in 1 mL of phosphate-buffered saline (PBS), then the solution was further diluted in PBS 1:2 before being analyzed. The samples, immediately after being prepared, were analyzed by using the qNano Gold tunable resistive pulse sensing (TRPS) instrument (Izon Science, Lyon, France) with the nanopore NP1000, rated for particles having a diameter ranging from 490 to 2900 nm. The pulse signal was calibrated with a 950 nm polystyrene particle standard supplied by Izon Science, with a $5.2_{\times}10^{10}$ particles/mL concentration. Measurements were made with 46.95 mm of stretch being applied to the elastic membrane and a potential 0.10 V being applied across the pore. The volume of 35 µL of diluted sample was loaded into the top fluid cell, and 75 µL of measurement electrolyte PBS was loaded into the bottom fluid cell. 10 Pa of pressure was applied to

the top fluid cell. Izon Control Suite software was employed to collect the data about particle size and concentration. This software also generates histograms and graphs representing the particle distribution.

Assessment of the Phagocytic Capabilities in Human Primary Granulocytes

Granulocytes were isolated from the total peripheral blood of one healthy donor after Ficoll gradient separation. The cells were grown in Roswell Park Memorial Institute Medium (RPMI)-1640 supplemented with L-glutamine 2 mM, penicillin 100 U/mL-streptomycin 100 µg/mL, and bovine serum albumin (BSA) 0.1% at 37 °C and 5% CO_2. The cells were plated in 96-well plates and treated with the Veh., or DNA (8 CH), or RNA (8 CH), for 10 min at 37 °C. Fluorescent beads (Molecular Probes™, FluoSpheres™ Carboxylate-Modified Microspheres, 1.0 µm, yellow-green fluorescent 505/515, Invitrogen Life Technologies, Eugene, OR, USA) were then added and incubated with the cells for the next 45 min. Untreated non-bead-incubated cells were used as a negative control. N-formylmethionyl-leucyl-phenylalanine (fMLP) 10 µM was employed as a positive control for phagocytosis induction. All the conditions were performed in triplicate. After the incubation period, cells were rinsed in PBS/BSA 0.1% and centrifuged. Acquisitions were realized with 10,000 cells/replicate on a BD FACSVerse™ (BD Biosciences, Franklin Lakes, NJ, USA) cytometer. The fluorescence signal in the fluorescein isothiocyanate (FITC) channel (emission beads wavelength = 515 nm) is proportional to the phagocytosed beads. The results were first expressed as FITC-positive percentages, then expressed in percentages of the Veh.-treated cells, the latter being set as 100%.

Collection and Preparation of Blood from Healthy Donors

Healthy volunteers were enrolled by the French Blood Bank Center (Etablissement Français du Sang, [EFS], Pays de Loire, Nantes, France, www.efs.sante.fr, accessed on 9 February 2024), a public institution under the responsibility of the French Ministry of Health, and informed consent was obtained from all individuals. According to French ethics laws, blood donation is based on voluntary participation, non-remuneration, anonymity and non-profitability. A declaration regarding the use of blood samples from blood volunteer donors for non-therapeutic research purposes was submitted to the French Ministry of Research, and the agreement number CPDL-PLER-2023 009 was assigned. Thus, all blood samples were approved by the EFS Blood Bank Center, with written informed consent obtained from all the donors, in accordance with the Declaration of Helsinki.

Natural Killer Cell Activation

Peripheral blood mononuclear cells (PBMCs) were isolated from two blood tubes (ethylenediaminetetraacetic acid (EDTA) tubes, EFS Nantes) using Ficoll density gradient centrifugation (density: 1.077 g/mL). The PBMCs layer was collected, washed with PBS, and the pellet was resuspended in RPMI-1640 medium for automatic cell counting and viability assessment using the LUNA-II™ system (Logos Biosystems, Villeneuve d'Ascq, France). After the counting process, NK cells were isolated employing the "NK Untouched" kit (Dynabeads™ Untouched™ Human NK Cells Kit, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA, ref: #11349D), which facilitates the isolation of NK cells without activation or stimulation. Cells were cultured in RPMI-1640 medium supplemented with 10% human serum (HS, Sigma-Aldrich, Saint-Louis, MO, USA), L-glutamine (2 mM, Sigma-Aldrich), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES buffer (10 mM, Lonza, Bâle, Switzerland). Cells were seeded at a density ranging from 32,000 to 50,000 cells per well. The compounds RNA (8 CH), DNA (8 CH), 2LZONA-2, 2LZONA-3, 2LZONA-4, and Veh. were tested. Activation controls were used to determine the phenotypic characteristics of NK cells in the presence of either IL-15 (20 ng/mL) or an IL-12p70/lipopolysaccharide (LPS) mix (20 ng/mL/200 ng/mL). The compounds were incubated with the NK cells for 24 hours at 37 °C and 5% CO_2. Following a 24-hour incubation period, cells were harvested for multiparametric immunostaining and analyzed on the same day. To enhance the precision of the analysis, the identification of different NK cell populations was performed using CD56/CD16 markers, which define three distinct types of NK cells in culture from PBMCs: (i) $CD56^{bright}$ $CD16^{neg.}$: regulatory NK cells, minimally cytotoxic, primary cytokine producers, minor population (<2 cells/µL); (ii) $CD56^{dim}$ $CD16^{low}$: NK cells known to migrate towards lymphoid organs, transient phenotype, often excluded from the NK analysis;[25] (iii) $CD56^{dim}$ $CD16^{high}$: anti-tumor/anti-viral NK cells, highly cytotoxic, producing few cytokines, major population (50–220 cells/µL). The specific markers used for phenotypic immunostaining thus included:

CD56, CD16, CD69, B- and T-lymphocyte attenuator (BTLA), programmed death receptor 1 (PD-1) and Zombie. Signal acquisition was conducted via flow cytometry using the non-linear system Cytek® Aurora 3-laser V-B-R configuration (Cytek Biosciences B.V., Amsterdam, The Netherlands). The supernatants (SNs) were then collected and frozen at −80 °C for subsequent cytokine assays. The recovery of the SNs enabled a measure of monocyte chemoattractant protein-1 (MCP-1) and IL-8, two cytokines which were analyzed through enzyme-linked immunosorbent assay (ELISA) method (LEGENDplex™, BioLegend, San Diego, CA, USA, ref: #740038).

Experiments in Human Peripheral Blood Mononuclear Cells Under Basal/ Unstimulated Conditions

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of four healthy donors and cultivated in RPMI-1640, added with 2% human serum, non-essential amino acids (1 mM), pyruvate (1 mM), L-glutamine (2 mM) and HEPES buffer (10 mM). Then, the cells were plated at 200,000 cells/well and treated with either the Veh. or 2LZONA-4 for 48 hours. At the end of the experiment, the SNs were collected and the secretion levels of twelve cytokines (IL-2, IL-4, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, and TNF-α) were evaluated by ELISA. The ELISA was run in triplicates for each condition.

Experiments in Human Peripheral Blood Mononuclear Cells Under Pre-Primed Conditions

Freshly isolated human PBMCs were cultivated in RPMI-1640 medium added with 2% inactivated human serum, 1 mM non-essential amino acids, 1 mM pyruvate, and 10 mM HEPES buffer, in the presence of 0.5 µg/mL bottom-coated OKT3 antibody (anti-CD3; ref: #317326, BioLegend), or in the presence of 2 µg/mL of soluble anti-CD28.2 (ref: #302934, BioLegend), together with 0.5 µg/mL bottom-coated OKT3 antibody (anti-CD3 + anti-CD28.2). The treatments and the analysis in these culture conditions were the same as presented in the previous section.

Evaluation of the Proliferation of Peripheral Blood Mononuclear Cells Sub-Populations by Flow Cytometry

Different sub-populations of human PBMCs were discriminated based on flow cytometry analysis according to the expression of the following panel of surface markers: NK: $CD3^-$, $CD11b^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD56^+$, $CD14^-$, $CD16^+$; SSC^{low}; intermediate monocytes (Int. monocytes): $CD3^-$, $CD11b^+$, $CD4^-$, CD8-, $CD19^-$, $CD56^-$, $CD14^+$, $CD16^+$; SSC^{low}; non-conventional monocytes (N.C. monocytes): $CD3^-$, $CD11b^+$, $CD4^-$, $CD8^-$, $CD19^-$, $CD56^-$, $CD14^-$, $CD16^+$; SSC^{low}; neutrophils: $CD3^-$, $CD11b^+$, $CD4^-$, $CD8^-$, $CD19^-$, $CD56^-$, $CD14^-$, $CD16^{\pm}$; SSC^{high}; B-cells: $CD3^-$, $CD11b^-$, $CD4^-$, $CD8^-$, $CD19^+$, $CD56^-$, $CD14^-$, $CD16^-$; SSC^{low}; T-cells: $CD3^+$, $CD11b^-$, $CD4^+$, $CD8^+$, $CD19^-$, $CD56^-$, $CD14^-$, $CD16^-$; SSC^{low}; $CD4^+$ T-cells: $CD3^+$, $CD11b^-$, $CD4^+$, $CD8^-$, $CD19^-$, $CD56^-$, $CD14^-$, $CD16^-$; SSC^{low}; $CD8^+$ T-cells: $CD3^+$, $CD11b^-$, $CD4^-$, $CD8^+$, $CD19^-$, $CD56^-$, $CD14^-$, $CD16^-$; SSC^{low}.

Evaluation of Expression of Human Leukocyte Antigen-DR in Human Umbilical Vascular Endothelial Cells

As a previously published method,[22,26] human umbilical vascular endothelial cells (HUVEC) (passage 5) were purchased from PromoCell (PromoCell, Heidelberg, Germany) as a pool from n = 6 donors and were grown in endothelial cell growth medium (ECBM) media (PromoCell), added with 2% fetal bovine serum (FBS) (PAN-Biotech GmbH, ref: #P170201, Aidenbach, Germany). The cells were plated at 5000 cells/well on day 0 (D0), and treated on D4 with 20 ng/mL IFN-γ (ref: #091927, PeproTech Inc., Thermo Fisher Scientific, Waltham, MA, USA), added with either the Veh., or 2LZONA-5 for 48 hours. Then, the cells were harvested, labeled with an anti-human leukocyte antigen (HLA)-DR antibody, fixed, and analyzed by flow cytometry, on a BD FACS Canto II, configuration 4/2/2. The intensity of the staining was measured as median fluorescence intensity (MFI) value.

Evaluation of Expression of Human Leukocyte Antigens-DP, -DQ, and -DR in CD14$^+$-Derived-Macrophages

Healthy volunteers were enrolled by the Blood Bank Center (EFS, Pays de Loire, France). All blood samples were approved by the Ethics Committee of the EFS Blood Bank Center, with written informed consent obtained from all the donors, following the Declaration of Helsinki. Briefly, PBMCs were isolated from 2 donors, plated in 96-well plate at the density of 500,000 cells/well, and cultivated in complete RPMI medium (ref: #P04-17500, batch number #7131121, PAN-Biotech GmbH), added with 2% decomplemented human AB serum (ref: #H4522, batch number #SLCC1483, Sigma-Aldrich, Saint-Louis, MO, USA). On D1, the cells were concomitantly treated with recombinant human IFN-γ 20 ng/mL (PeproTech Inc.), w/wo either the Veh. or 2LZONA-5. On D4, the media/treatments were renewed, and the cells were challenged with 100 ng/mL LPS (ref: #L6529, batch number #059M4103V, Sigma). On D7, the cells were harvested, immune-stained with anti-HLA-DP (ref: #566825, BD Biosciences, Franklin Lakes, NJ, USA), anti-HLA-DQ (ref: #347453, BD Biosciences), or anti-HLA-DR (ref: #307622, BioLegend) antibodies, fixed, and the viability as well as the three tested HLA expression levels were assessed by flow cytometry.

General Flow Cytometry Analysis

Representative staining and gating strategies for flow cytometry analysis are represented in Supplementary Figure S1.

Statistical Analysis

The graphs in the figures were performed with GraphPad Prism, Version 10.2.3.403 for Windows (GraphPad Software Inc., San Diego California USA, updated on 30/04/2024). Authors have followed the recent recommendations of D.L. Vaux that encourage performing descriptive statistics instead of making statistical inferences when the number of independent values is small.[27] In line with these recommendations, if the results are derived from only one, two, or three (n = 1, or n = 2, or n = 3) experiment(s), it is always better to include a full data set, plotting data points and letting the readers interpret the data for themselves, rather than drawing statistical inferences, showing p values, standard error of the mean (S.E.M.) or results that are not representative. Indeed, no statistical inference has been performed to analyze the results of most of the experiments presented here in this study. However, those data derived from NK cells obtained from n = 6 donors were subjected to inferential statistics, and one-way ANOVA was performed (see corresponding previous sections).

Results

Two Actives of 2LZONA, DNA (8 CH) and RNA (8 CH), Contain Sub-Micron Particles and Increase the Phagocytic Capabilities of Primary Human Granulocytes

To explore the involvement of small particles, possibly containing starting materials in the two ULD-based actives of RNA (8 CH) and DNA (8 CH), we assessed the particle size, the particle count, and their concentration by using the technique of tunable resistive pulse sensing (TRPS). Previous evidence showed that unitary ULD-based cytokine products contain sub-micron particles that might explain their mode of action.[24] The TRPS analysis was performed as described in the Material and Methods section to assess the possible presence of sub-micron particles in DNA (8 CH) and RNA (8 CH). Briefly, sugar pillules were diluted in PBS, and the analysis was conducted with the qNano (Izon Science). As shown in Figure 1A and B, all tested samples, including vehicle (Veh.) contain particles, meaning that the qNano detected the sucrose and lactose molecules used as excipients in MIM in the forms of sugar aggregates of different sizes. Interestingly, the two samples containing the diluted active substances had higher particle concentrations than the Veh. (especially in size ranging from 600 to 1500 nm), possibly due to the presence of DNA and RNA as starting materials. These findings are in line with our previously published data.[24] Nevertheless, more investigations are still needed to ensure that the particles detected by the qNano are the starting materials used to prepare MIM during the first step of the manufacturing process.

We hypothesized that the two active substances, DNA (8 CH) and RNA (8 CH), both employed in 2LZONA, could stimulate the phagocytosis capabilities of immune cells such as granulocytes. To investigate this possibility, an experiment was performed on human granulocytes. Briefly, the cells were pre-incubated for 10 min with either Veh., DNA (8 CH) or RNA (8

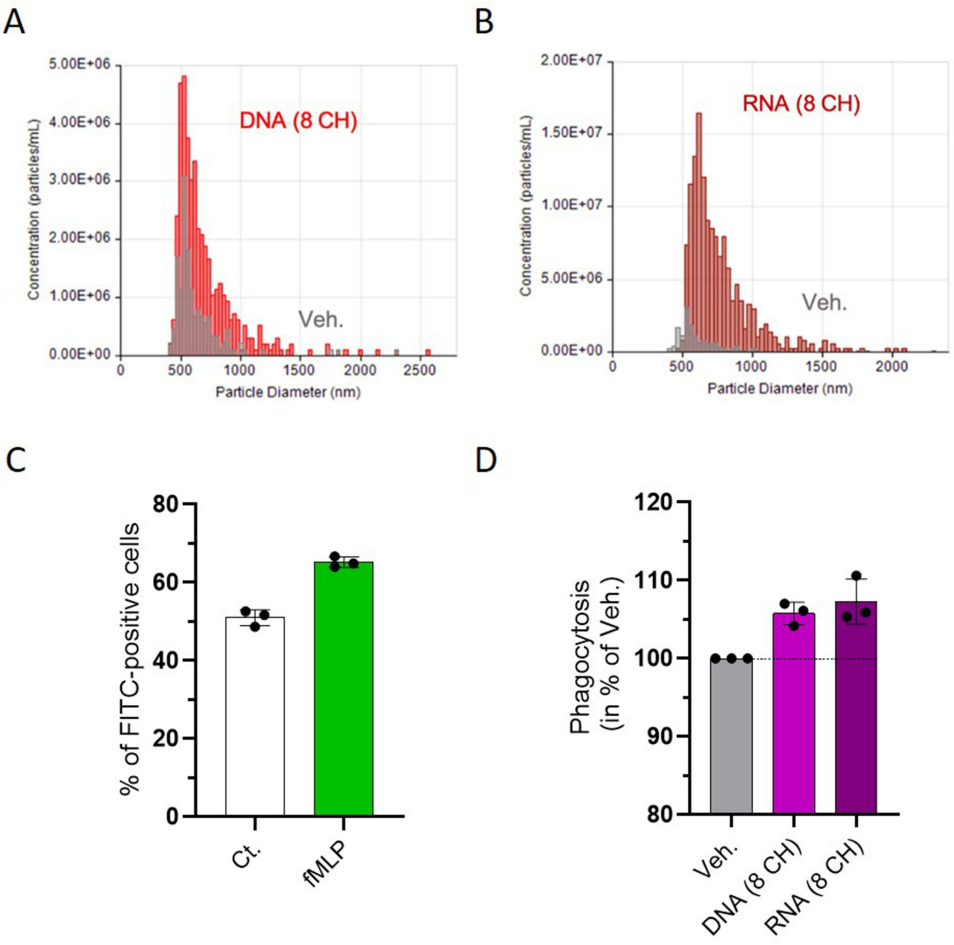

Figure 1 DNA (8 CH) and RNA (8 CH) contain nano-size particles and slightly stimulate the phagocytosis capabilities of granulocytes. (**A-B**) Distribution of particles acquired by using the qNano with the nanopore NP1000. The particle distribution was measured in samples obtained by diluting the pillules contained into one capsule of Veh. (grey histograms), DNA (8 CH) (red histograms), or RNA (8 CH) (dark red histograms) in PBS. A NP1000 nanopore, rated for particles ranging from 490–2900 nm, was employed for the analysis. (**C-D**) The MI unitaries DNA (8 CH) and RNA (8 CH) increase the phagocytosis capabilities of human granulocytes. Human granulocytes were used to assess the effects of the medicine on the phagocytosis. The cells were plated, left untreated (Ct., white histograms), or pre-incubated for 10 minutes with N-formyl-methionyl-leucyl-phenylalanine (fMLP, green histogram) and fluorescent beads were added to the culture medium for an additional 45 min. The percentage of FITC-positive cells was appraised by flow cytometry, and the results are expressed as a percentage of FITC-positive cells (**C**). (**D**) Human granulocytes were treated with either the vehicle (Veh., grey histogram), the MI unitaries DNA (8 CH) (pink histogram), or RNA (8 CH) (purple histogram). Each condition was performed in triplicate and each histogram represents the mean ± standard deviation (S.D.) of the fluorescence as a percentage of the Veh.-treated conditions, set at 100%. The black dot line was drawn to better visualize the effect of DNA (8 CH) and RNA (8 CH) compared to the Veh.

CH). After the pre-incubation time, fluorescent beads were added to the medium, the cells were incubated for another 45 min, and phagocytosis was assessed by flow cytometry. As illustrated in Figure 1C, in the fMLP condition, used as a positive control for the induction of phagocytosis, the basal percentage of FITC-positive cells increased by about 20%, thus validating the cell responsiveness to the experimental setting regarding their phagocytic capabilities. Figure 1D illustrates the effects of Veh.-, DNA (8 CH)- and RNA (8 CH)-treated conditions compared to the untreated control. Our data showed that both actives, DNA (8 CH) and RNA (8 CH), slightly increased the phagocytosis by about 5% compared with the Veh. control group. These results suggest that the tested items might stimulate the granulocytes' function, reinforcing their phagocytic capacities. We are, however, cautious with the conclusions drawn here, as these findings should be confirmed by further experiments.

Capsules 2LZONA-2, -3, and -4, Together with DNA (8 CH) and RNA (8 CH) Slightly Increase the Proliferation of Immature and Mature Natural Killer Cells in vitro

Transitioning from the previously presented section, in which our investigations were performed in human granulocytes, we wanted to subsequently consider using a model of another cell type, natural killer (NK) cells, pivotal defenders in our

https://doi.org/10.2147/JIR.S498930

innate immune arsenal. As these cells are crucial in fighting viral infection, and as VZV is capable of infecting human NK cells from peripheral blood in vitro,[28] DNA (8 CH) and RNA (8 CH), as well as three capsules of 2LZONA (2LZONA-2, 2LZONA-3 and 2LZONA-4) have been analyzed here, on their capabilities to enhance NK cells proliferation. Even if previous findings implied a selective targeting of $CD56^{dim}$ NK cells by VZV,[28] we wanted to have a full picture of the effects of 2LZONA during the maturation process of NK cells; thus, the analysis has been performed within three subsets of NK cells, (i) the $CD56^{bright}$ $CD16^{neg}$ immature support NK cells, (ii) the $CD56^{dim}$ $CD16^{low}$ transitory migratory NK cells, and (iii) the $CD56^{dim}$ $CD16^{high}$ cytotoxic ones. Briefly, PBMCs were retrieved from n = 6 healthy donors, and the NK populations were isolated by immunomagnetic sorting. The cells were treated with either the Veh., DNA (8 CH), RNA (8 CH), 2LZONA-2, 2LZONA-3, or 2LZONA-4 for 24 hours. As IL-15 is known to activate NK cells,[29] a positive control for NK cell activation was obtained by exposing the cells to 20 ng/mL of IL-15. An additional control condition was included in this experiment: thus, the cells were exposed to a mix of IL-12p70/LPS at 20 ng/mL / 200 ng/mL. As illustrated in Figure 2A-C and in Supplementary Figure S2A, the effect of those two controls on the NK cell proliferation differed depending on the analyzed subset. Indeed, while IL-15 significantly increased the proliferation of the immature and the mature NK cells, it reduced the one of the transitory NK. In addition, in the transitory NK cells, IL-12p70/LPS enhanced the cell proliferation but displayed an inhibitory effect in the mature NK. Interestingly, the two tested unitary DNA (8 CH) and RNA (8 CH) (Figure 2D-F and Supplementary Figure S2B) and the three tested capsules of 2LZONA (Figure 2G-I and Supplementary Figure S2C), seemed to have an effect quite similar to the one of IL-15 in terms of tendencies, as they all appeared to increase the proliferation of the immature and the mature NK cells while reducing the one of the transitory NK cells. The statistical significance of the results depended on the subsets and the tested items, but globally, the magnitude of the effect is similar amongst all of them. Overall, this first set of experiments shed light on the potential of DNA (8 CH), RNA (8 CH), 2LZONA-2, 2LZONA-3, and 2LZONA-4 in increasing the proliferation of immature and mature NK cells. An enhanced proliferative capacity could be linked with the activation status of those cells. Further experiments are ultimately needed to confirm these findings.

Capsules 2LZONA-2, -3, and -4, Together with DNA (8 CH) and RNA (8 CH) Activate Natural Killer Cells in vitro, According to Their Expression Levels of CD69, B- and T-Lymphocyte Attenuator, and Programmed Death Receptor 1

In order to provide more information about the effect of the tested items on the activation status of the NK cells, the expression of the activation/inhibition markers CD69, BTLA, and PD-1 was evaluated by flow cytometry within the three previously analyzed subsets of $CD56^{bright}$ $CD16^{neg}$ immature support NK cells, $CD56^{dim}$ $CD16^{low}$ transitory migratory NK cells, and $CD56^{dim}$ $CD16^{high}$ mature cytotoxic NK cells. At first, an initial quantization of the expression levels of those markers was performed at basal level (normal/unstimulated conditions) in the three NK cell subsets to set thresholds of detection (Supplementary Figure S3A-C). Moreover, the two control conditions of IL-15 and IL-12p70/LPS treatments were assessed for their ability to increase/decrease the expression of these markers, and as IL-15 was able to statistically significantly increase the expression of CD69 and BTLA in the three subsets of NK cells, this cytokine was considered as a good control for the activation of the NK cells (Supplementary Figure S4A-F). On the other hand, as IL-12p70/LPS increased the expression of the inhibitory receptor PD-1 in both the transitory and the mature subsets of NK cells, this stimulation condition was considered a good inhibitory stimulus (Supplementary Figure S4G-I). The cells were treated with the Veh., DNA (8 CH), RNA (8 CH), 2LZONA-2, -3, or -4, and the expression of CD69, BTLA, and PD-1 was evaluated by flow cytometry. Concerning CD69, while none of the tested items impacted its expression in the immature NK cells, RNA (8 CH), 2LZONA-2, and 2LZONA-4 significantly increased it in transitory NK cells, and all the tested actives from 2LZONA increased it in the mature NK cells (Figure 3A-C). The same statistically significant upward trend was found regarding BTLA expression in all the NK subsets and for all the tested items, except RNA (8 CH) in the immature NK cells, where the statistical significance was not reached (Figure 3D-F). Interestingly, the up-regulation of those activating markers seemed to be accompanied by a down-regulation of the expression of the inhibitory receptor PD-1, especially within the immature and the mature NK subsets, after treatment with the three tested capsules of 2LZONA (Figure 3G-I). Among all the tested capsules of 2LZONA, the biggest magnitude of the

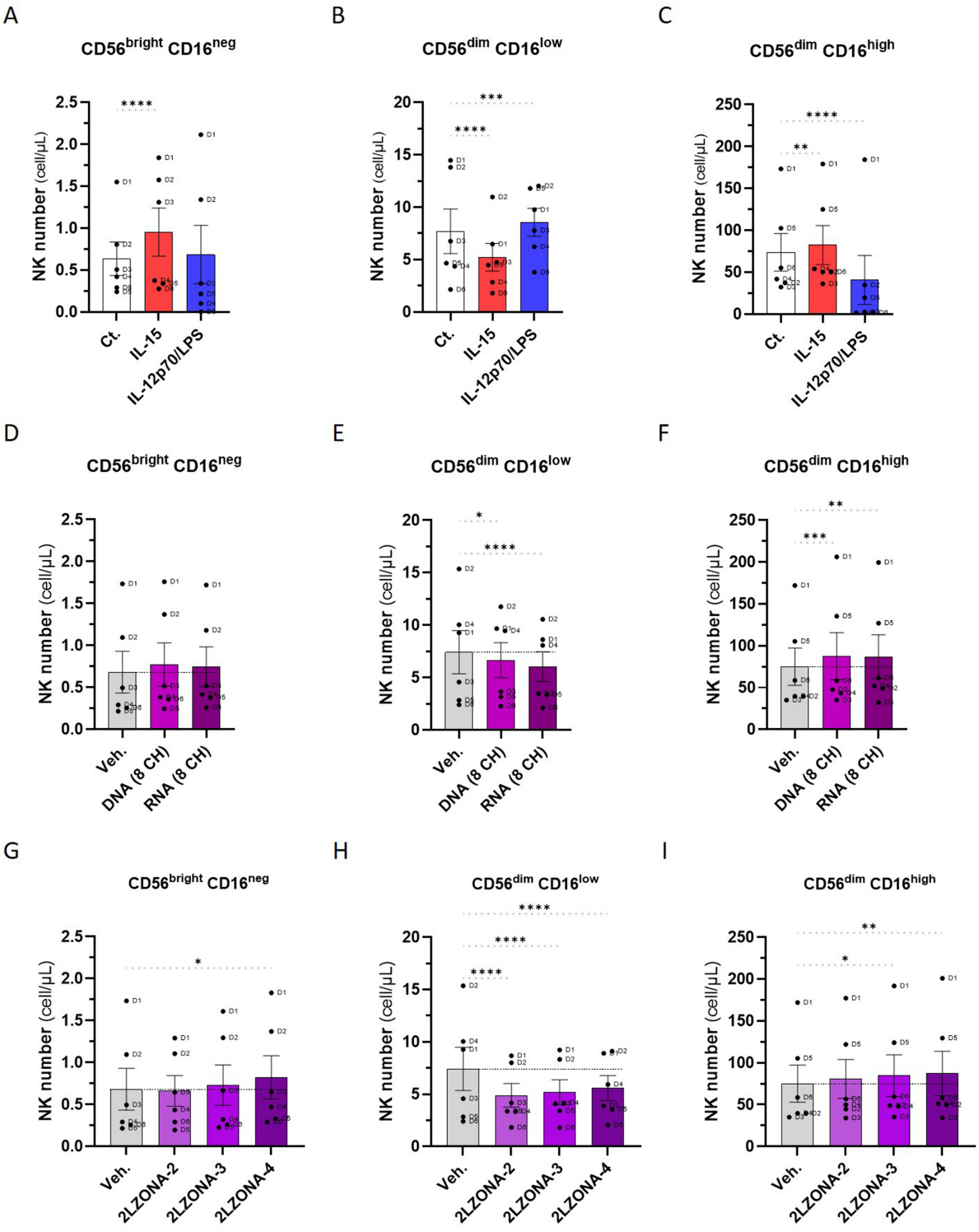

Figure 2 DNA (8 CH), RNA (8 CH), and 2LZONA-2; -3 and -4 slightly increase the proliferation of NK cells in vitro. Briefly, PBMCs were retrieved from n = 6 healthy donors and the NK populations were isolated by immunomagnetic sorting. As depicted in the presented panels, the analysis has been performed within three subsets of NK cells: the CD56^{bright} CD16^{neg} immature support NK cells, the CD56^{dim} CD16^{low} transitory migratory NK cells, and the CD56^{dim} CD16^{high} cytotoxic ones. (**A-C**) The cells were left untreated (Ct., white histograms), or were incubated for 24 hours with 20 ng/mL IL-15 (red histograms) or with a mix of IL-12p70/lipopolysaccharide (IL-12p70/LPS) at 20 ng/mL / 200 ng/mL. The number of NK cells was counted in each subset by flow cytometry. The results are presented as the mean ± S.E.M. cell/µL, obtained from a duplicate measure performed for n = 6 donors (D1-D6), in each condition. One way ANOVA, **** p < 0.0001, *** p < 0.001, ** p < 0.01, compared with the untreated Ct. NK cells. The cells were treated with either the vehicle (Veh.), DNA (8 CH) or RNA (8 CH) (**D-F**), or the Veh., 2LZONA-2, 2LZONA-3, or 2LZONA-4 (**G-I**) in the same experimental setting as presented in (**A-C**). The black dot lines were drawn to better visualize the effect of the tested items compared to the Veh. One way ANOVA, **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 compared with the Veh.-treated NK cells.

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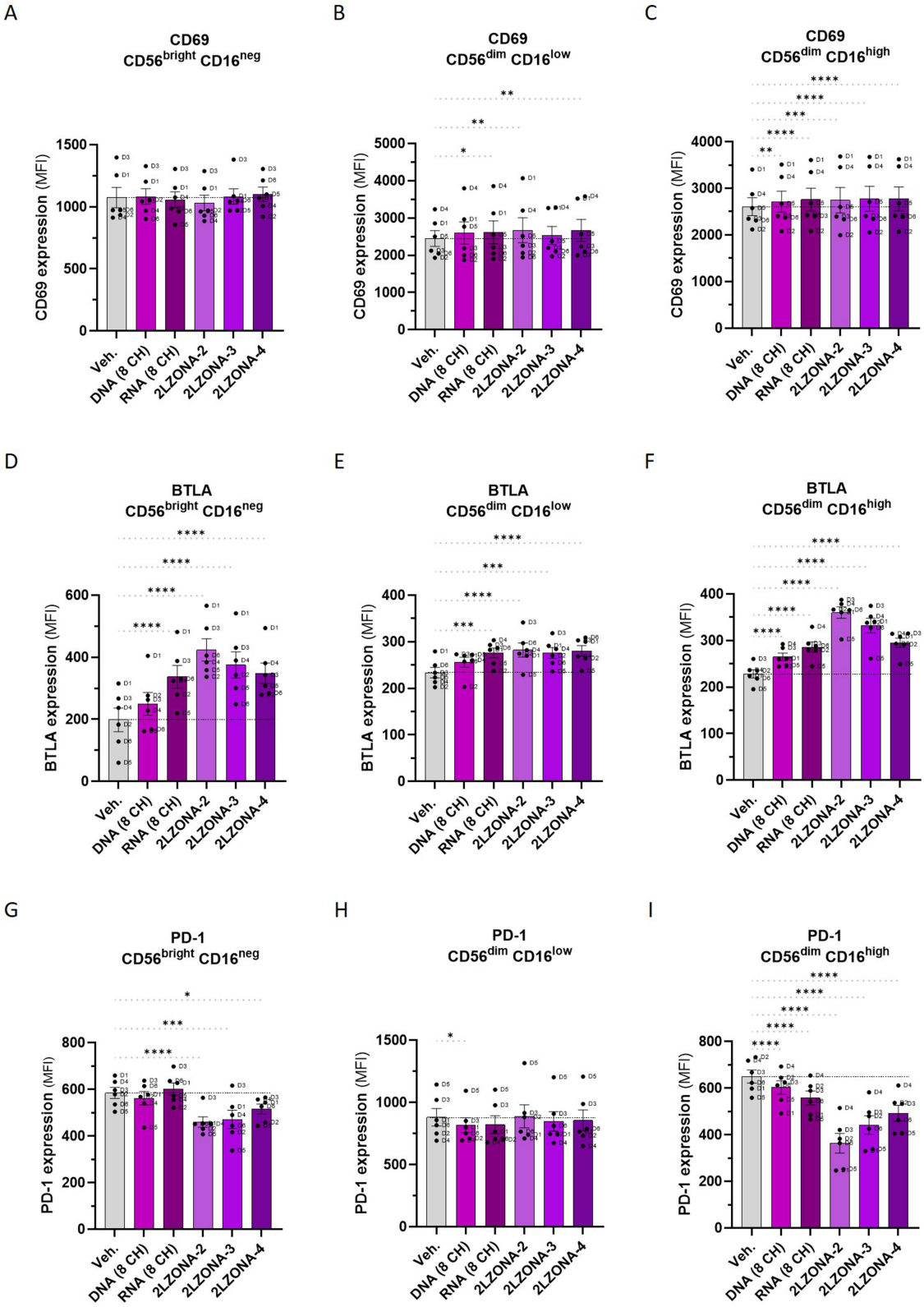

Figure 3 DNA (8 CH), RNA (8 CH), 2LZONA-2; -3, and -4 activate NK cells in vitro. Briefly, PBMCs were retrieved from n = 6 healthy donors and the NK populations were isolated by immunomagnetic sorting. As depicted in the presented panels, the analysis has been performed within three subsets of NK cells: the $CD56^{bright}$ $CD16^{neg}$ immature support NK cells, the $CD56^{dim}$ $CD16^{low}$ transitory migratory NK cells, and the $CD56^{dim}$ $CD16^{high}$ cytotoxic ones. The cells were treated with either the Veh., DNA (8 CH) or RNA (8 CH), 2LZONA-2, 2LZONA-3, or 2LZONA-4 during 24 hours and the expression levels of (**A-C**) CD69, (**D-F**) BTLA, and (**G-I**) PD-1 were assessed by flow cytometry. The results are presented as the mean ± S.E.M. of the median fluorescence intensity (MFI), obtained from a duplicate measure performed for n = 6 donors (D1-D6), in each condition. The black dot lines were drawn to better visualize the effect of the tested items compared to the Veh. One way ANOVA, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with the Veh.-treated NK cells.

effects was observed with 2LZONA-2. Of note, in the CD56^{dim} CD16^{low} transitory NK cells, IL-15, IL-12p70/LPS, DNA (8 CH), and RNA (8 CH) both increased the expression of the activating receptor CD25 in comparison with their respective untreated- or Veh.-controls (Supplementary Figure S5A-B), and IL-12p70/LPS, 2LZONA-2, -3, and -4 all reduced the expression level of the inhibitory receptor T-cell immunoreceptor with Ig and ITIM domains (TIGIT), compared with their appropriate controls (Supplementary Figure S5C-D). Overall, this body of data allows us to conclude that the tested items could increase the activation levels of the NK cell subsets, at least regarding the effects on the expression of the three activator/inhibitor markers CD69, BTLA, and PD-1.

Capsules 2LZONA-2, -3, and -4, Together with DNA (8 CH) and RNA (8 CH) Slightly Modulate the Secretion Levels of Interleukin-8 and Monocyte Chemoattractant Protein-1 in Natural Killer Cells in vitro

The cytokine secretion is another signature of the activation status of NK cells. Thus, to include additional readouts, the SNs were recovered during the experiment realized in PBMCs (described in the previous sections), and the levels of IL-8 and monocyte chemoattractant protein-1 (MCP-1) were analyzed through ELISA. As illustrated in Figure 4A, all the tested actives from 2LZONA slightly reduced the secretion level of IL-8, this inhibitory effect even reaching statistical significance for RNA (8 CH). On the other hand, RNA (8 CH) and 2LZONA-4 slightly increased the secretion of MCP-1 (Figure 4B). Overall, and even if these results are still relatively preliminary, they reveal that the combination of active substances used in 2LZONA-4 may hold the potential to slightly activate NK cells through the modulation of the secretion levels of IL-8 and MCP-1. Even if preliminary, the data presented here provide the basis for the scope of immunostimulatory actions exerted by the tested active substances of 2LZONA. Thus, they not only seemed to confirm the stimulatory effect of the ULD-based unitary DNA (8 CH) and RNA (8 CH) on innate immunity cells, but they could also emphasize the potential role of the capsules of 2LZONA-2, -3, and -4 in activating essential innate cellular responses.

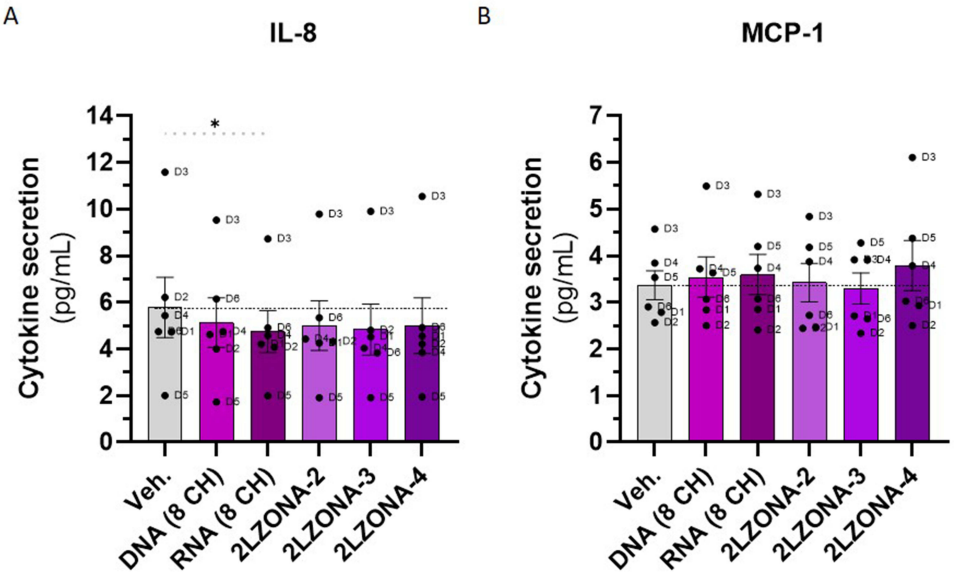

Figure 4 DNA (8 CH), RNA (8 CH), 2LZONA-2; -3, and -4 slightly modulate the secretion of IL-8 and MCP-1, when evaluated in NK cells in vitro. Briefly, PBMCs were retrieved from n = 6 healthy donors and the NK populations were isolated by immunomagnetic sorting. The cells were treated with either the Veh., DNA (8 CH) or RNA (8 CH), 2LZONA-2, 2LZONA-3, or 2LZONA-4 for 24 hours and the secretion of (**A**) IL-8 and (**B**) MCP-1 were assessed by ELISA, after SNs collection and freezing. For each cytokine, the results are presented as the mean ± S.E.M. of the pg/mL, obtained from a duplicate measure performed for n = 6 donors (D1-D6), in each condition. The black dot lines were drawn to better visualize the effect of the tested items compared to the Veh. One way ANOVA, * $p < 0.05$ compared with the Veh.-treated NK cells.

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The Actives Employed in 2LZONA-4 Increase the Secretion of Several Cytokines in Unstimulated Human Peripheral Blood Mononuclear Cells

Considering the composition of the 2LZONA-4 capsule and the employment of IL-2 (7 CH) among other actives at 10 CH, we wanted to assess if this capsule could affect cytokine secretion. Thus, the secretion of IL-2, IL-4, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, and TNF-α was assessed in human PBMCs, in unstimulated (or basal) conditions. Briefly, human PBMCs were retrieved from n = 4 healthy donors and incubated for 48 h in the presence of either the vehicle (Veh.; gray bars) or 2LZONA-4 (purple bars), in normal/basal culture conditions (unstimulated). The results of the ELISA are depicted in Figure 5A-D and Supplementary Figure S6. Figure 5A-D shows that 2LZONA-4 enhanced the secretion of IL-2, IL-6, IL-13, and TNF-α in unstimulated human PBMCs

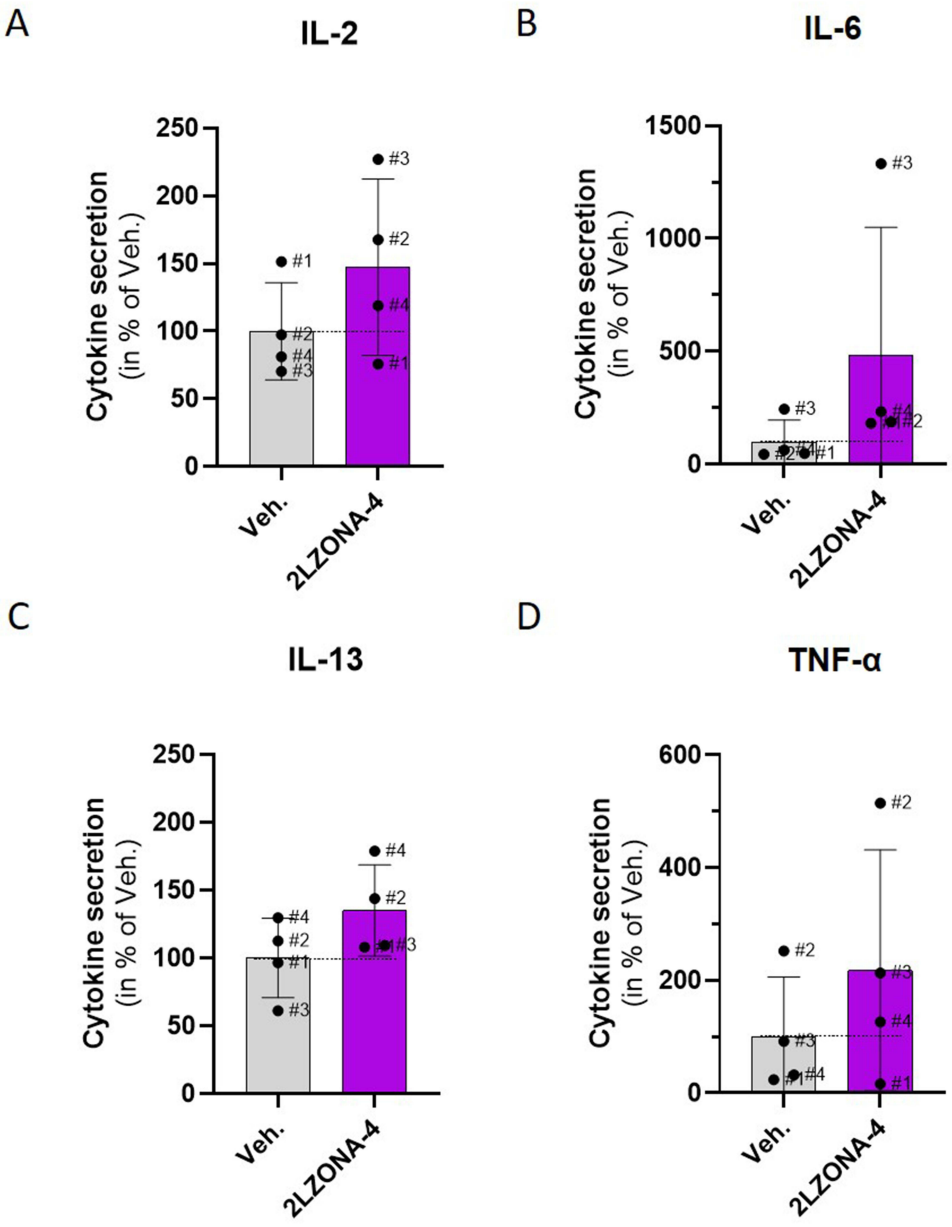

Figure 5 2LZONA-4 upregulates the secretion of several cytokines in unstimulated human PBMCs. Briefly, human PBMCs from four donors (#1, #2, #3, and #4) were retrieved and incubated for 48 h in the presence of either the Veh. (gray bars) or 2LZONA-4 (purple bars), in normal/basal culture conditions (unstimulated). The SNs were collected and an ELISA was performed to assess the secretion of IL-2 (**A**), IL-6 (**B**), IL-13 (**C**), and TNF-α (**D**). The results are presented as the mean ± S.D. of n = 3 measures for each donor, and expressed as a percentage of the values obtained in the Veh.-treated conditions (set as 100%). The dotted lines are drawn to highlight the effect of 2LZONA-4 compared with the Veh.

compared with the Veh. control-treated cells. None of the other analyzed cytokines (IL-4, IL-9, IL-10, IL-17A, IL-17F, and IL-22) seemed to be affected by the treatment under the experimental conditions (Supplementary Figure S6). This first set of preclinical evidence shows that 2LZONA-4 could upregulate the secretion of IL-2, IL-6, IL-13, and TNF-α in unstimulated human PBMCs treated in vitro. Thus, these results could suggest that the specific combination of active substances employed in one capsule of 2LZONA may display immune stimulatory effects in vitro that could be beneficial in the context of VZV infection/reactivation.

The Actives Employed in 2LZONA-4 Increase the Secretion of IL-6 and TNF-α in Human Peripheral Blood Mononuclear Cells Under Pre-Primed Conditions

As VZV T-cells mediated immunity is essential for recovery from varicella, and as the intensity of T-cell responses soon after HZ begins is proportional to the degree of pain related to zoster,[30] a model of pre-activated T-cells was also used to assess the effects of 2LZONA. Thus, human PBMCs were stimulated by the presence of the anti-CD3 antibody alone (referred to as "anti-CD3") or by the presence of the two antibodies together (referred to as "anti-CD3/anti-CD28"). CD3 and CD28, as antigen-independent signaling for the T-cell receptor (TCR) complex, can trigger an immune cell stimulation. The panel of cytokines analyzed was the same as in basal, unstimulated conditions (see previous section). Indeed, the levels of IL-2, IL-4, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, and TNF-α were measured by ELISA.

The results presented in Figure 6 show that, in both tested pre-primed conditions ("anti-CD3" and "anti-CD3 /anti-CD28"), 2LZONA-4 was able to enhance the secretion of IL-6 and TNF-α compared to the Veh.-treated cells. The secretion of IL-6 was the one which was affected the most by 2LZONA, as its average increase was about 300% among all the analyzed donors, while the one of TNF-α was about 45%, in comparison to the Veh., in the anti-CD3-stimulated conditions (Figure 6A and B). With respective increases of about 109% and 16%, the same trend was observed for the above-mentioned cytokines in the anti-CD3/anti-CD28 conditions (Figure 6C and D). The other tested cytokines did not seem to be affected by this MIM treatment, under these in vitro conditions (Supplementary Figure S7).

In addition to the above-reported evidence detailed in the previous section, this body of results on 2LZONA-4 suggests that the combination of active substances included in this capsule could stimulate the secretion of IL-6 and TNF-α, in a model of human PBMCs under both unstimulated and pre-primed conditions.

The Active Substances of 2LZONA-4 Slightly Enhance the Proliferative Capacity of Neutrophils, Intermediate Monocytes, and Natural Killer Cells

Having found that 2LZONA-4 enhanced the secretion of IL-2 in human PBMCs, and knowing the role of this cytokine in promoting immune cell proliferation, we pursued our investigation in unstimulated human PBMCs, assessing the effect of this capsule on the proliferation of different unstimulated sub-populations of PBMCs. As described in Material and Methods, several immune cells' sub-populations were analyzed by flow cytometry according to their specific surface markers expression. Interestingly, our results show that neutrophils, intermediate monocytes (Int. monocytes), and natural killer (NK) cells responded with a slight enhancement of their proliferative capacity after 48 hours of treatment with the tested item compared to the Veh. (Figure 7). The results show that the up-regulatory effect is of about 20%, 15%, and 10%, compared to the Veh., in the sub-populations of neutrophils (Figure 7A), Int. monocytes (Figure 7B), and NK cells (Figure 7C), respectively. The other sub-populations ($CD4^+$ T-cells, $CD8^+$ T-cells, B-cells, non-conventional monocytes (N.C.) do not seem to be affected by the treatment under the tested experimental conditions (Supplementary Figure S8).

The Actives Employed in 2LZONA-5 Reduce the Expression of Human Leukocyte Antigen-DR in Inflamed Endothelial Cells Without Affecting the Expression of Human Leukocyte Antigen Variants and the Viability of Interferon-γ-Stimulated Human Macrophages

Our investigations on 2LZONA finally focused on another combination of active substances, employed in the fifth capsule of the sequence: 2LZONA-5 (see Table 1). SNA-HLA-II at 18 CH is one of the active substances employed in

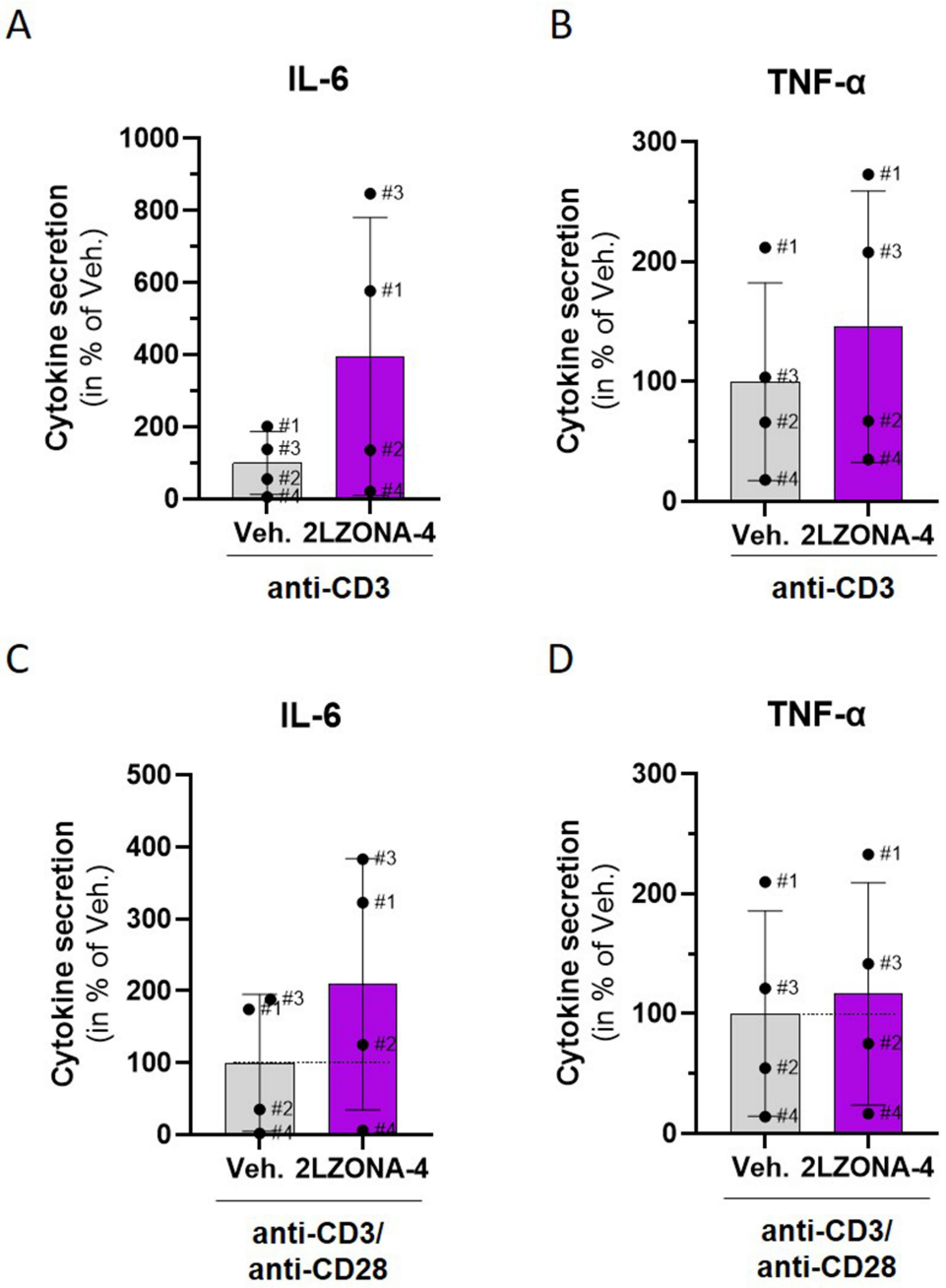

Figure 6 2LZONA-4 upregulated the secretion of IL-6 and TNF-α in human PBMCs under CD3 and CD3/CD28 pre-primed conditions. Briefly, human PBMCs from four donors (#1, #2, #3, and #4) were incubated and stimulated for 48 h by an anti-CD3 antibody alone (referred to as "anti-CD3") or by a combination of the two anti-CD3 and anti-CD28 antibodies together (referred to as "anti-CD3/anti-CD28"). The cells were concomitantly treated with either the Veh. (gray bars) or 2LZONA-4 (purple bars). The SNs were collected and an ELISA was performed. The secretion of IL-6 and TNF-α within the SNs was assessed in the anti-CD3 conditions (**A-B**), and in the anti-CD3 /anti-CD28 conditions (**C-D**), respectively. The results are presented as the mean ± S.D. of n = 3 measures for each donor, and expressed as a percentage of the values obtained in the Veh.-treated conditions (set as 100%). The dotted lines are drawn to highlight the effect of 2LZONA-4 compared with the Veh.

this capsule. Based on previously published preclinical in vitro studies, complex-MIM employing this active substance in association with other actives can down-regulate the expression of HLA-DR in inflamed human umbilical vascular endothelial cells (HUVEC).[22,26] Indeed, to confirm our hypothesis and assess the effect of 2LZONA-5 on the above-mentioned model, we set the same experimental setting previously described. Briefly, 20 ng/mL IFN-γ was used as an inducer of the expression of HLA-DR in this model, and, as it increased the expression of HLA-DR by about 150 times in comparison with the untreated control, the effect of this cytokine as a positive control was validated (Figure 8A).

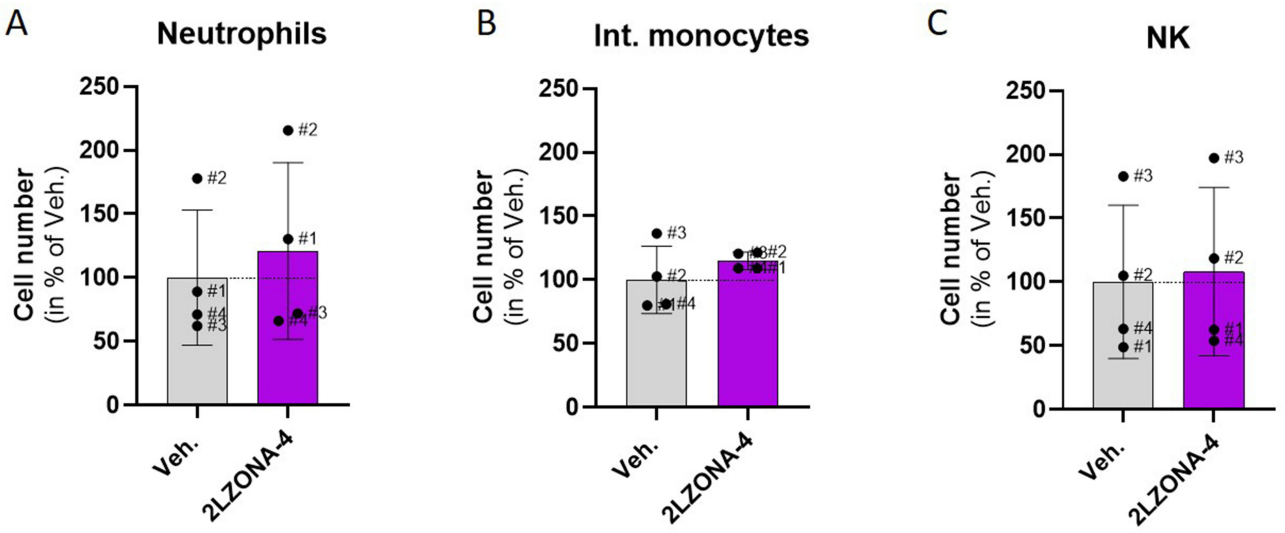

Figure 7 2LZONA-4 increased the proliferation of neutrophils, intermediate monocytes (Int. monocytes), and NK cells derived from human PBMCs, discriminated by flow cytometry. Briefly, human PBMCs from four donors (#1, #2, #3, and #4) were retrieved and incubated for 48 h in the presence of either the Veh. (gray bars) or 2LZONA-4 (purple bars), in normal/basal culture conditions (unstimulated). At the end of the incubation period, the cells were immune-stained and the sub-populations of (**A**) neutrophils, (**B**) Int. monocytes, and (**C**) NK cells were counted by flow cytometry. The results are presented as the mean ± S.D. of n = 3 measures for each donor, and expressed as a percentage of the values obtained in the Veh.-treated conditions (set as 100%). The dotted lines are drawn to highlight the effect of 2LZONA-4 compared with the Veh.

Briefly, the cells were treated with 20 ng/mL of IFN-γ with either the Veh. or 2LZONA-5. The cells were treated for 48 hours, and the expression of HLA-DR was assessed by flow cytometry. As depicted in Figure 8B, the expression of HLA-DR was reduced by about 20% compared with the Veh. These data corroborate previous results and confirm that MIMs employing SNA-HLA-II at 18 CH in their formulation, in association with other actives, can down-regulate the expression of HLA-DR in HUVEC when exposed to IFN-γ.[22,26]

Our investigation on 2LZONA-5 and its effects on HLA-II expression was pursued on another in vitro model, M1 macrophages derived from human PBMCs. Briefly, human PBMCs from four healthy donors were isolated by $CD14^+$ selection and cultivated for six days in the presence of 20 ng/mL IFN-γ, as well as with either the Veh. or 2LZONA-5. The media/treatments were renewed on day 4. On day 6, cells were stimulated with 100 ng/mL LPS to upregulate the expression of HLA-II molecules (HLA-DP, -DQ, and -DR). On D7, the levels of HLA-DP, HLA-DQ, and HLA-DR expression were appraised by flow cytometry. The viability of these IFN-γ- and LPS-stimulated macrophages, which acquired the M1-like phenotype, was also assessed. Supplementary Figure S9A shows the levels of expression of the HLA variants in this model. However, in the assessed experimental conditions, 2LZONA-5 did not impact the expression level of the three measured HLA variants compared to the Veh. (Figure 8C-E, Supplementary Figure S9B-D). Cellular viability was not affected by the treatment, neither (Figure 8F, Supplementary Figure S9E).

Discussion

Varicella-zoster virus (VZV) is a highly contagious herpesvirus responsible for causing chickenpox (varicella) and shingles (HZ). Upon VZV infection, the viral DNA and RNA are crucial in activating the body's innate immune response, the first line of defense against pathogenic infections. These nucleic acids serve as pathogen-associated molecular patterns that are recognized by pattern-recognition receptors in the host cells. Activation of these receptors leads to the initiation of antiviral signaling pathways, resulting in the production of type I IFN and other pro-inflammatory cytokines. These signaling molecules are instrumental in controlling viral replication and spread during the early stages of VZV infection while also orchestrating the adaptive immune response that ultimately contributes to the clearance of the virus and the establishment of long-term immunity. Within the context of our research, which aimed at understanding the potential and mode of action of the MIM 2LZONA and the complex interplay between VZV genetic material and the innate immune system, our approach started with the selective investigation of the two plant-derived

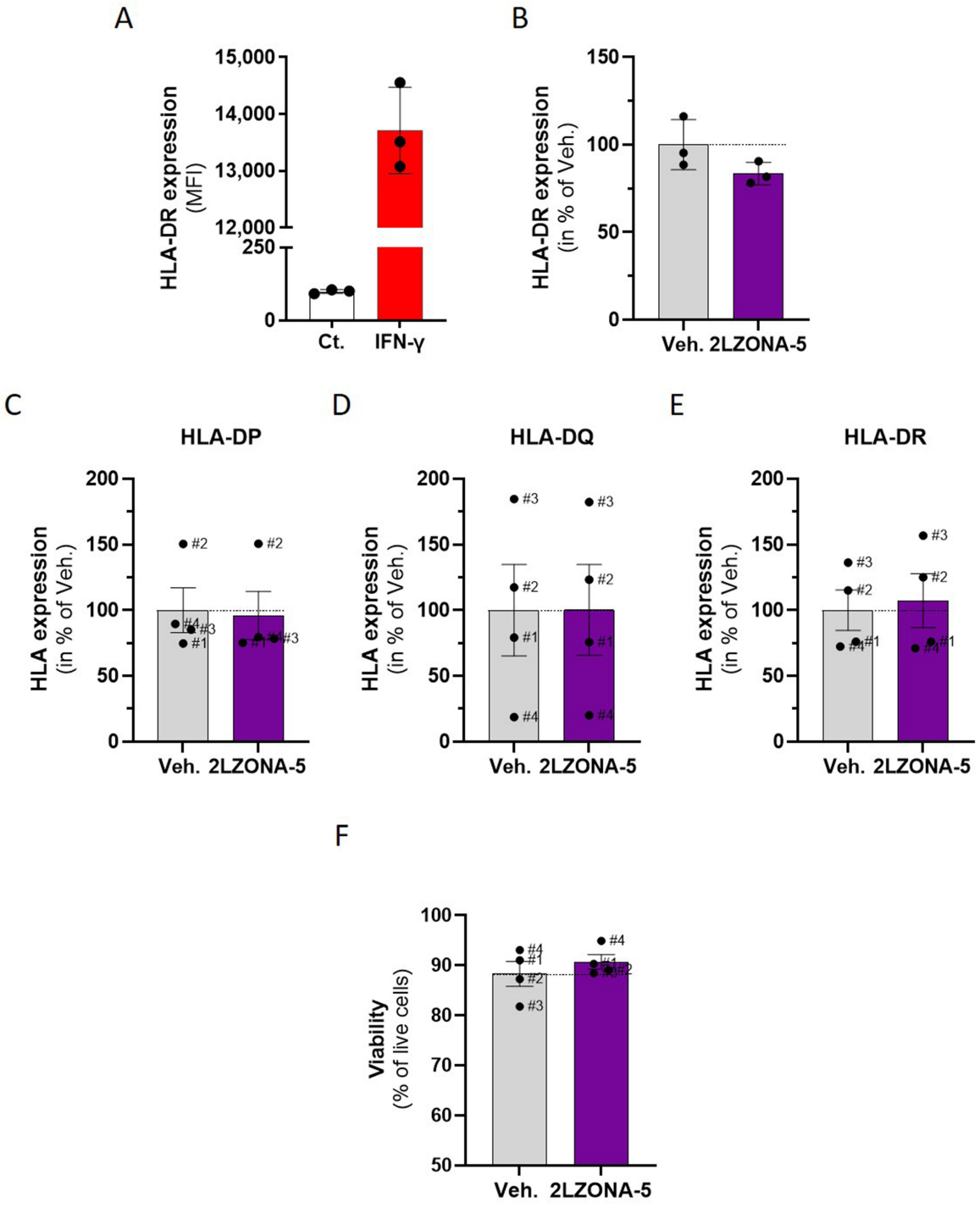

Figure 8 2LZONA-5 reduced the expression of HLA-DR in IFN-γ-stimulated HUVEC, while it did not impact the expression of HLA variants and the viability of IFN-γ-stimulated human macrophages. (**A**) The expression level of HLA-DR was assessed in HUVEC either left untreated as a control (Ct.) (white histogram), or treated with 20 ng/mL IFN-γ (red histogram). (**B**) Interferon-γ-stimulated HUVEC were treated with either the Veh. (gray histogram) or 2LZONA-5 (purple histogram) for 48 h. In (**A**) the results are expressed as the mean MFI ± S.D. for n = 3 replicates per condition and in (**B**), the results are normalized and expressed as the mean percentage ± S.D. of the Veh. conditions, the latter being set at 100%. (**C-E**) The expression level of HLA-DP, HLA-DQ and HLA-DR was assessed in human M1 macrophages treated for six days with 20 ng/mL IFN-γ, in the presence of either the Veh. (gray histogram) or 2LZONA-5 (purple histogram). The measures were done in triplicate, for n = 4 healthy donors (#1, #2, #3, and #4), and the results are normalized and expressed as the mean percentage ± S.E.M. of the Veh. conditions, the latter being set at 100%. (**F**) The cell viability has been appraised by flow cytometry after six days of treatment in the presence of 20 ng/mL IFN-γ added with either the Veh. or 2LZONA-5. The results are presented as the mean percentage of live cells/ total cells ± S.E.M. obtained for the four tested donor. The measures were done in triplicate for each donor. In (**B-F**), the dotted lines are drawn to highlight the effect of 2LZONA-5 compared with the Veh. In (**C**) the results are expressed as the mean MFI ± S.E.M for n = 4 donors (#1, #2, #3 and #4), each measure having been done in triplicate.

nucleic acids active ingredients used in 2LZONA, RNA (8 CH) and DNA (8 CH). In this first part, the goal was to comprehend how these two active substances could affect the IS, in particular the innate immune response, under uninfected in vitro conditions.

Phagocytosis is one of the key mechanisms of innate immunity, and this cellular behavior is particularly important in the context of viral infections such as VZV infections. The phagocytic cells of the innate IS can be activated by toll-like receptor (TLR)-dependent (e.g., TLR3, TLR7, and TLR9) and TLR-independent (e.g., RIG-I/MDA5 and DNA-dependent activator of IFN-regulatory factors) signaling pathways, thus triggering multiple effects, such as increasing their phagocytic abilities or the cytokines secretion.[31] While more investigations are needed to understand the mechanisms behind this effect, our preliminary results from qNano quantization showed that nanoparticles ranging from 600 nm to 1500 nm were detected in the tested samples from the ULD unitary actives DNA (8 CH) and RNA (8 CH) and that these were higher in concentration and size than the ones detected on the Veh. condition (Figure 1A and B). These findings are in line with our previously published data, in which sub-micron particles, possibly containing starting material, were detected in pillules impregnated with ULD of TNF-α and IL-1β, both at 27 CH.[24] In addition, our results showed that the two tested unitary DNA (8 CH) and RNA (8 CH), when employed to treat human granulocytes in vitro, can activate those cells resulting in a slight enhancement of their phagocytic capabilities (Figure 1D). DNA/RNA sensors are well documented and are well-known key innate immunity triggers towards viral DNA/RNA.[32] However, little is known about the effect of plant-derived DNA/RNA on the nucleic acid's sensor machinery. In vertebrates, the ability to discern self from non-self nucleic acids hinges on multiple interconnected aspects. These include the accessibility of nucleic acid triggers, which are subjected to their concentration, decay rate by internal enzymes, and the level of protection through proteins.[33] Moreover, the precise positioning of these nucleic acid triggers—whether outside the cell, within cell compartments, or in the fluid of the cell—plays a role. Lastly, the distinctive structure of these nucleic acids, defined by their unique sequences, shape, and chemical alterations, factors into the process. Collectively, these elements enable the organism to correctly detect foreign nucleic acids that may pose a threat and launch the necessary immune defenses. Mechanistic and biochemical analyses can be useful to unveil how these actives might have acted on granulocytes. In addition, it could be interesting, in future investigations, to assess the capabilities of DNA (8 CH) and RNA (8 CH), as both are employed in several MIM, in triggering phagocytosis in other phagocytosis-prone cell types such as macrophages for instance.

Interestingly, Voigt et al reported that, as the phagocytosis of *C. albicans* contributed to the activation of NK cells,[34] this cellular process, could also lead to the mounting of immune response through NK cells. As it happens, NK cells are important anti-viral effectors of the IS, thus NK cell deficiency is responsible for infection susceptibility, including VZV infection.[35] In particular, they can exert cytolytic activity against virus-infected cells and can massively produce cytokines such as IFNs upon activation, as well as chemokines like chemokine ligand (CCL) 3, CCL4, and CCL5.[36] Thus, some therapeutic strategies aimed at enhancing the anti-viral functions of NK cells were developed and are under investigation. For example, it has been found that the pre-treatment of non-adherent PBMCs with either purified or recombinant IL-2 markedly increased the NK cell response towards both uninfected cells and those infected with the VZV.[37] In addition, studies are showing that NK cell effector responses to viruses can be boosted by using low concentrations of IL-15 (0.75 ng/mL).[38] This body of evidence opens new and interesting avenues for the use of low-dose immune therapies directly or indirectly targeting NK cells to fight against viral infections.

Thus, after discussing how DNA (8 CH) and RNA (8 CH) improved phagocytosis, we then shifted to examine their impact on another key component of the immune response. We delved into the role of NK cells and investigated how these cells, critical in combating viral infections, responded to the products. Since VZV can infect human NK cells from peripheral blood in vitro, this study included an analysis of DNA (8 CH) and RNA (8 CH), as well as three capsules of 2LZONA (2LZONA-2, 2LZONA-3, and 2LZONA-4), to assess their potential to augment the activation of NK cells.

Thus, this investigation, performed on primary human NK cells isolated from PBMCs of n = 6 donors, allowed for the discrimination of three distinct NK cell subsets and their phenotypic characterization, based on the expression of the surface markers CD56 and CD16: (i) the cytokine-producing, low-cytotoxic NK cells ($CD56^{bright}/CD16^{neg}$) immature NK,[39] which were sparsely represented; (ii) the transitional NK cells, which are maturing, characterized by the loss of CD56 expression and acquisition of CD16 expression ($CD56^{dim}/CD16^{low}$),[40] and (iii), the mature NK cells, highly

cytotoxic with low cytokine production ($CD56^{dim}/CD16^{high}$), which were predominantly represented. Overall, the effect of our tested items on NK cell proliferation depended on the NK subset, but we found here that they could increase the proliferation of both immature and mature NK cells while reducing one of the transitory NK cells (Figure 2D-I). The pro-proliferative effect of IL-2 on NK cells has been documented.[41] Among the tested capsules, 2LZONA-4 shows the highest statistically significant effects (Figure 2G–I). Looking at its composition, the presence of IL-2 at 7 CH may have contributed to those slight proliferation-enhancing effects. In addition, the observed subtype-dependent pattern of responses was similar in our IL-15 positive control (Figure 2A-C), and this cytokine was reported to increase NK cell proliferation, too.[41–43] The experiment included an additional control characterized by the incubation of the cells with IL-12-p70/LPS. Under these conditions, the proliferation of the mature NK cells was reduced (Figure 2C). Interestingly enough, our results resonate with the ones from Shemesh et al, who found that, while a 3-day stimulation of primary NK cells with 25 ng/mL IL-12 reduced the number of live cells, the same concentration of this cytokine still led to cell activation, as attested by their increase in IL-18Rα and IL-18Rβ expression.[44] To our knowledge, no literature is available about the combination of IL-12 and LPS as a stimulus to activate NK cells, but while this one was the only one able to reduce mature NK cells' proliferation (Figure 2C), it was also the only one that led to a statistically significant increase in the expression of PD-1 (Supplementary Figure S4I), thus we considered it as another good control condition.

As essential components of the IS, NK cells play a crucial role in controlling viral infections by killing virus-infected cells. To execute their functions effectively and safely, those cells are endowed with a variety of surface receptors that finely tune their activation through a balance of stimulating and dampening signals.[45] Among the activators, NKp30 can bind certain ligands initiating a cytotoxic response.[46] CD69, another key receptor that emerges promptly on the surface of NK cells upon activation, is involved in the regulation of proliferation and other NK cell functions.[47,48] CD25, part of the receptor complex for IL-2, is significant for the growth and further activation of the NK cells.[48] Contrastingly, receptors like Tcell immunoreceptor with TIGIT, BTLA, and PD-1 deliver inhibitory signals that serve to prevent overactivation and attack on normal, healthy cells.[49–51] BTLA and PD-1 dampen the NK cell response, adding to the immune system's ability to discriminate between healthy cells and actual threats, such as cancer cells attempting to evade immune detection, or VZV-infected cells. This delicate equilibrium between activating and inhibitory signals decides NK cells' activation levels. When the activations prevail, an enhanced immune response is seen, leading to the destruction of the abnormal cells. Conversely, when inhibitory influences dominate, it protects normal cells, preventing autoimmune reactions. This interplay is vital for maintaining the body's defense mechanisms. NK cells are also the target of emerging immunotherapies, aiming to amplify the immune system's ability to attack infected cells without harming normal tissues.

To assess the NK cells' potential to transition towards either an activating or inhibitory phenotype, the expression levels of CD69, BTLA, and PD-1 were evaluated, after stimulation with IL-15. For instance, while the literature suggests that IL-15 stimulation typically leads to a global increase in CD69,[52] and PD-1,[53,54] our results showed that the actual response varies depending on the specific NK cell subset and stimulation context (Supplementary Figure S4). Thus, to provide a complete picture of the effects of the tested items on the NK cell populations, DNA (8 CH), RNA (8 CH), and the three 2LZONA capsules (2LZONA-2, -3, and -4) were assessed across the three NK cell subsets. Overall, our findings suggested that, while in immature NK cells, the tested items mainly modulated the expression of BTLA and PD-1 (Figure 3D–G), in the transitory NK cells subsets, CD69 and BTLA were the main upregulated markers in comparison with the Veh. (Figure 3B–E). Finally, in the mature cells, while the expression levels of CD69 and BTLA were statistically significantly increased, the one of PD-1 was statistically significantly decreased (Figure 3C, F, and I).

These two markers interact with herpesvirus entry mediator (HVEM) and programmed death-ligand 1 (PD-L1) respectively, which are expressed on the surfaces of antigen-presenting cells and endothelial cells. The BTLA/HVEM axis is mainly investigated in the realm of immuno-oncology, particularly in the context of leukemias.[50] While mainly characterized as an inhibitory receptor, it is noteworthy that BTLA's effect has also been characterized as pro-inflammatory upon binding to HVEM by activating the NF-kB pathway, among others.[55] Thus, the observed increase in BTLA might be interpreted as an additional activation signal for the three tested populations of NK cells (Figure 3D-F). In addition, the PD-1/PD-L1 interaction is a focal point in immunotherapy research aimed at restoring the immunoreactivity of anergic immune cells within a tumor environment or in the context of chronic viral infection, in which high levels of PD-1 expression can have unfavorable immunological consequences.[56] Furthermore, it has been shown that ex vivo cultures of PD-1 positive

NK cells from Kaposi sarcoma patients were hyporesponsive in the context of a direct activation of NKp30, NKp46, or CD16 receptors, or after brief stimulation with NK cell targets.[57] Thus, the down-regulation of PD-1 receptor expression, as exerted by DNA (8 CH) in transitory NK cells and the three tested capsules of 2LZONA in immature and mature NK cells (Figure 3G-I), could be seen as a positive strategy aimed at restoring the response of NK cells. A previous study of ours focusing on another MIM employing ULD of DNA and RNA, called 2LEID®, shows that the tested actives increased the expression of PD-L1 in a model of human endothelial cells.[23]

Overall, our results could show that in immature, transitory, and mature NK cells, DNA (8 CH), RNA (8CH), 2LZONA-2, -3, and -4 could increase the expression levels of CD69 and BTLA, while reducing the one of the inhibitory receptor PD-1 (Figure 3A-I), possibly attesting of an increase in the global activation of those cells and their functionality. Interestingly, concerning CD69, we had previously reported the effect of ULD-based DNA and RNA in association with other active substances employed in the medicine 2LEID®, in increasing the expression of this receptor.[23] In light of the current results, the effects of the tested actives from 2LEID® could be explained, at least partially, by the employment of RNA and DNA, either at 8 CH or at 10 CH, in its formulation.

Interestingly, the fact that our results suggest that DNA (8 CH), RNA (8 CH), and the three tested capsules of 2LZONA reduced the secreted levels of IL-8 could be interpreted as a mark of activation of NK cells (Figure 4A). Indeed, while resting human peripheral blood NK cells do not spontaneously produce IL-8, such production is a hallmark of decidual NK cells,[58] which, unlike the conventional antiviral and antitumor roles traditionally attributed to NK cells, are regarded as immunoregulatory cells.[59] They play essential roles in tissue remodeling and angiogenesis, functions that could also be put in parallel with the fact that VZV is also implicated in the onset of VZV-related vasculopathies.[60] Moreover, as IL-8 is stimulated by cytomegalovirus (CMV) in the monocytic THP-1 cell line and has also been shown to promote the replication of several viruses, including CMV, encephalomyocarditis virus and poliovirus, the inhibition of this cytokine may help in controlling VZV spreading.[61–64] For what concerns MCP-1, our results showed that DNA (8 CH), RNA (8 CH), and 2LZONA-4 slightly increased the secretion of this factor (Figure 4B). Monocyte chemoattractant protein-1 is a crucial chemokine that governs the movement and infiltration of monocytes and macrophages.[65] As the migration of monocytes from the bloodstream through the vascular endothelium is essential for regular immunological monitoring of tissues and in reaction to inflammation, an increase in the secretion of this cytokine could be beneficial for the organism, as it could contribute to fighting against viral infections, including VZV infection. In light of the publication from Jones et al, human vascular adventitial fibroblasts and human brain vascular smooth muscle cells infected with VZV increased the secretion of IL-8 while reducing the MCP-1 expression in comparison with their mock controls.[66]

Granulocytes and NK cells are not the only immune cells that have been reported to be involved in responses to VZV infection, as additional cell types, such as T-cells or different innate immune cells, might be required to collaborate in coordinating an effective antiviral immune response to neurons infected with VZV.[67] The role of cytokines as crucial key molecules involved in IS functions is continuously under investigation in many disease contexts, such as viral infections including VZV. In particular, it is interesting to know that IL-6 and TNF-α, as well as IFNs, were able to reduce the VZV replication in human neurons.[68] Coherently with that, anti-inflammatory medicines like TNF inhibitors or anti-IL-6 that have been used in several chronic inflammatory diseases are associated with increased risk of infections, and VZV reactivation.[69–71] In line with these findings, a study performed in a small cohort of patients with VZV infection revealed that IL-6 and TNF-α were lower expressed in patients having a more severe clinical picture, suggesting that these two cytokines may be crucial in controlling VZV infection and the associated clinical signs.[72] A therapeutical intervention that can modulate these two cytokines towards an efficient upregulation, without reaching the uncontrolled and high levels associated with chronic inflammatory diseases would be a great therapeutic tool. The results reported in this study are promising because they indicate that 2LZONA-4 could slightly enhance the secretion of IL-6 and TNF-α, under both unstimulated and CD3/CD28 primed conditions (Figures 5B–D and Figures 6A–D). In addition, the secretion of IL-2 and IL-13 was also increased by the tested treatment under basal conditions (Figure 5A–C). While further investigations are needed to confirm this hypothesis, the increase of IL-2 could be explained, at least partially, by the presence of IL-2 at 7 CH in 2LZONA-4. The 2LZONA-4 effects observed on IL-2, IL-6, and IL-13 in our experimental setting are interesting

in regards to a study which showed that the secretion of those cytokines was significantly reduced in VZV-infected quiescent primary human hippocampal astrocytes in comparison to mock-infected cells.[73]

Having determined that 2LZONA-4 boosted IL-2 secretion in human PBMCs and recognizing the role of this cytokine in promoting immune cell proliferation, we continued our investigation using unstimulated human PBMCs. Notably, our findings revealed that neutrophils, intermediate monocytes (Int. monocytes), and NK cells exhibited a slight increase in proliferative capacity after 48 hours of treatment with the tested capsule, compared to the Veh. (Figure 7A-C). Interestingly, IL-2 receptor is expressed on neutrophils, and various functional responses mediated by this cell type are influenced by IL-2. In particular, IL-2 has been shown to extend the lifespan of neutrophils by inhibiting apoptosis, and to foster their functionalities, for example, by increasing the production of TNF-α.[74,75]

The last set of experiments performed in the current study was conducted to investigate the effects of one capsule of 2LZONA, 2LZONA-5, that employs SNA-HLA-II (18 CH) in association with other active substances. Thus, the effects of this capsule were assessed in two in vitro models, both exposed to IFN-γ, on the expression of HLA. Our results showed that 2LZONA-5 could slightly reduce HLA-DR in a model of inflamed endothelial cells in comparison with the Veh. (Figure 8B). These results are in line with those reported in previously published articles, in which other SNA-HLA-II (18 CH)-containing MIM slightly reduced the expression levels of HLA-DR in the same in vitro models.[22,26]

The effect of this capsule of 2LZONA in reducing the up-regulated levels of HLA-DR, if seen in the context of an over-inflammation induced by IFNs during a VZV persistence, might be beneficial. Indeed, in some individuals, in the context of VZV reactivation, VZV can also infect arteries (cerebral and extracranial arteries).[76] This pathologic situation, known as VZV vasculopathy, is associated with transient ischemic attacks, stroke, aneurysm, sinus thrombosis, and giant cell arteritis, as well as granulomatous aortitis. The mechanisms by which the virus induces this pathology are not fully elucidated. However, evidence suggested that viral persistence induces an inflammatory environment with abundant neutrophils and macrophages in the adventitia that could contribute to vascular remodeling and damage observed in patients affected by VZV vasculopathy.[77] While the model used in our study is not VZV-specific, and the inflammatory stimulus used, IFN-γ, can only partially reproduce the local inflammatory environment found in patients,[78] the observed effect of 2LZONA-5 is interesting and merits further investigations.

When 2LZONA-5 effects were assessed in a model of IFN-γ-stimulated human macrophages, this capsule did not impact the HLA variants analyzed, nor the viability of the cells (Figure 8C-F). Regarding the roles of monocytes/macrophages in the IS response against VZV, the current literature is still not exhaustive. According to some evidence, VZV can induce the secretion of IL-6 in human monocytes via TLR2-dependent activation of NF-κB,[79] highlighting the possible role of these cells as a key actor of IS in the fight against VZV infection/reactivation. On the other side, Kennedy et al observed a loss of cell viability in VZV-infected differentiated macrophages, as well as the downregulation of CD14, HLA-DR, CD11b, and M-CSF.[80] Although no cytokine secretion was investigated in this study, the authors suggest the possibility that monocytes/macrophages are affected in their responsiveness and functions due to the VZV evasion mechanisms. Further research is ultimately needed to determine how and to which extent the monocytes/macrophages are affected by VZV. Nevertheless, the increased viability observed in 2LZONA-5-treated cells (Figure 8F) merits further investigations.

While the current study offers interesting insights into the potential mechanisms of action of 2LZONA in modulating immune responses, several limitations warrant consideration. One major limitation is the sample size employed in the experiments, as a larger and more varied pool of human cell models could contribute further to validate the findings and bring robustness to the statistical analysis. Additionally, the study is focused on four out of the five capsules from 2LZONA, leaving 2LZONA-1 and the potential synergistic effects of all the capsules, sequentially tested according to the order of administration, unexplored. This limited scope suggests the necessity for more comprehensive studies involving all the capsules of the medicine and/or more single active substances from the capsules as we did here with DNA (8 CH) and RNA (8 CH). As mentioned in the previous paragraph, another significant limitation is the exclusive use of non-VZV -infected cell models, which may not fully capture the complex interactions between immune cells during VZV infection and/or reactivation. Future in vitro and in vivo studies in VZV-infected systems are still needed to elucidate the therapeutic potential and safety profile of 2LZONA. The integration of longitudinal studies might also offer insights into the sustainability of immune enhancements conferred by this treatment. These steps could significantly advance our

understanding of how to effectively harness host immune responses to prevent VZV reactivation and manage HZ outbreaks, ultimately contributing to the development of robust therapeutic strategies. Nevertheless, the data presented here, as preliminary as they are, represent a crucial first step toward a more comprehensive understanding of the in vitro effects of the MIM 2LZONA.

Conclusion

The human alpha herpesvirus VZV can reactivate and cause HZ, also called shingles. Host immune responses can limit the severity of the clinical manifestations and prevent the onset of complications associated with VZV infections. The MIM 2LZONA, which employs ULD-based active substances, was developed to sustain the IS in fighting against VZV. This study aimed to set the basis for the mechanisms of action of several active substances employed by the MIM 2LZONA, performing several in vitro experiments on various models of human immune cells. Our findings indicate that two ULD-based substances, DNA (8 CH) and RNA (8 CH), tested singularly, potentiate phagocytosis in granulocytes and contain sub-micron particles that may underlie the effects observed. In addition, these two actives, or unitary MI products, as well as three capsules out of the complete formulation of 2LZONA (2LZONA-2; 2LZONA-3 and 2LZONA-4), appeared able to increase the activation levels of NK cells. In human PBMCs, 2LZONA-4 increased the secretion of IL-2, IL-6, IL-13, and TNF-α. Additionally, 2LZONA-4 modestly promoted the growth of PBMC-derived NK cells, intermediate monocytes, and neutrophils. Under pre-activated conditions mimicked by the stimulation with an anti-CD3 and a cocktail of anti-CD3/anti-CD28, the same capsule of 2LZONA amplified IL-6 and TNF-α secretion. Another set of active substances from the complete sequence of 2LZONA, corresponding to 2LZONA-5, was found to downregulate the expression of HLA-DR in endothelial cells exposed to IFN-γ. Collectively, these findings offer novel experimental evidence for the functional and molecular action mechanisms of 2LZONA, potentially mediated through sub-micron particles, possibly containing molecules of plant-extracted DNA and RNA, and suggest promising potential for its use as a therapeutic agent in helping IS in managing VZV reactivation. A more comprehensive study involving in vitro and in vivo experiments is ultimately needed to deeply understand the potential of this medicine in the framework of VZV infections and VZV-related complications.

Abbreviations

BSA, bovine serum albumin; BTLA, B- and T-lymphocyte attenuator; CCL, chemokine ligand; CH, centesimal Hahnemannian dilutions; DNA, deoxyribonucleic acid; ECBM, endothelial cell growth medium, EFS, Etablissement Français du Sang; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; fMLP, N-formylmethionyl-leucyl-phenylalanine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HZ, herpes zoster; HLA-I, human leukocyte antigen-I; HLA-DR, human leukocyte antigen-DR; hr, human recombinant; HUVEC, human umbilical vascular endothelial cells; IS, immune system; IFN, interferon; IL, interleukin; LD, low doses; LPS, lipopolysaccharide; MFI, median fluorescence intensity; MI, micro-immunotherapy; MIM, micro-immunotherapy medicines; N.C., non-conventional monocytes; NK, natural killer; PBMCs: peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD-1, programmed death receptor 1; RNA, ribonucleic acid; RPMI, Roswell Park Memorial Institute medium; S.E.M., standard error of the mean; SN, supernatants; SNA, specific nucleic acids; TCR: T-cell receptor; TGF-β1, tumor growth factor-β1; TIGIT, T-cell immunoreceptor with immunoglobulin and ITIM domains; TLR, toll-like receptor; TNF-α, tumor necrosis factor -α; TRPS, tunable resistive pulse sensing; ULD, ultra-low doses; VZV, varicella-zoster virus.

Acknowledgments

The authors would like to thank Miquel Ensenyat and his team for having prepared, provided, and sent the vehicle and the tested products to ProfileHIT, and QIMA facilities. The authors are thankful to Camille Roesch for her time and expertise in the realization of the tests with the qNano Gold (Izon Science). The authors would like to thank Sofia Frau for her insightful comments and suggestions provided in the last version of the manuscript.

Disclosure

Camille Jacques and Ilaria Floris work for Labo'life France, the company service provider of Labo'life, specialized in preclinical research and regulatory affairs. This professional relationship does not imply any misconduct on the part of the authors. Mathias Chatelais and Flora Marchand work for ProfileHIT, an innovative profiling company involved in the human vascular-immunological crosstalk research field. Adrien Brulefert works for QIMA Life Sciences, a company specialized in biotechnologies. The authors report no other conflicts of interest in this work.

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