Age-Related Differences in Immune Responses to Inactivated Influenza and Adjuvanted Recombinant Herpes Zoster Vaccines

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Abstract

Immunosenescence, the biological aging of the immune system, leads to dysregulated immune responses, increasing susceptibility to infections and reducing vaccine efficacy in older adults, as seen with flu vaccines. In contrast, the AS01-adjuvanted recombinant herpes zoster vaccine (RZV) maintains high and sustained efficacy, offering 82% protection against herpes zoster at 11-years post-vaccination, in individuals over 50. To identify factors impacting age-dependent vaccine efficacy, we conducted a randomized, partially placebo-controlled clinical study. Young adults (18-35 years, n=84) were randomized 3:3:1:1 to receive either RZV, an inactivated quadrivalent seasonal influenza vaccine (IIV4), placebo for RZV or placebo for IIV4, while older adults (≥60, n=63) were randomized 1:1 to receive RZV or IIV4. RZV elicited robust antibody production, antigen-specific polyfunctional CD4+ T cell responses and IFN-γ from PBMCs in both age groups, while IIV4 increased antibody responses, but induced fewer antigen-specific CD4+ T cells and no elevation of IFN-γ from PBMCs. Interestingly, RZV reduced systemic inflammation in older adults, particularly after the second injection. Baseline inflammation negatively correlated with antibody production and IFN-γ response, especially after RZV. Our findings suggest that RZV—may help overcome immunosenescence, by enhancing cellular responses and potentially decreasing systemic inflammation, deserving further investigation into the underlying molecular mechanisms.

Introduction

Vaccination remains the most powerful and effective strategy for protection against potentially severe infections. Over the years, various vaccine technologies have been developed, including live-attenuated, subunit, inactivated, and mRNA-based vaccines. Despite their distinct development methods, all vaccines share a common goal: to administer a less harmful dose or form of antigens to the host, enabling the immune system to recognize and build a robust, specific response to future exposure with the respective pathogen. Vaccines are estimated to have saved 154 million lives between 1974 and 2024 (1) and continue to protect countless individuals from severe diseases. However, vaccine effectiveness greatly varies across individuals and populations.

Vaccine effectiveness is influenced by various factors, including host factors such as age, sex, and genetic background; perinatal factors such as maternal antibodies; and external factors, e.g., seasonal variations and past infections (2). Among these, aging is particularly notable for its impact on vaccine-induced immune responses. An aging innate immune system displays a bias towards myelopoiesis, yet shows impaired functional innate immune responses including lower capacity for phagocytosis, cytotoxicity, and reactive oxygen species (ROS) production (3). In adaptive immunity, aging results in a decline of T and B cell responses and a reduced pool of naïve lymphocytes, which are essential for recognizing new antigens (4,5). This reduction limits the ability of the immune system to respond to infections and diminishes the effectiveness of vaccines. Additionally, memory T and B cells, which are essential for long-term immunity, experience functional decline, further compromising the immune response. Finally, circulating inflammatory protein concentrations increase with age, contributing to low-grade systemic inflammation and an increased susceptibility to age-related inflammatory disorders (6).

All these changes result in higher susceptibility to infections and lower vaccine efficacy in older individuals. For instance, the efficacy of many vaccines, like those for SARS-CoV-2 and influenza, is generally lower in older adults than in young adults (7,8). According to a recent test-negative design

study, in which vaccination status is compared between patients who test positive vs. negative for influenza, the effectiveness of the annual influenza vaccines against any influenza was 49% for children (<18 years), 37% for young adults (18–64 years), and 31% for older adults (≥65 years) (9). The most commonly used seasonal influenza vaccines consist of three or four inactivated viral strains, are non-adjuvanted, and single-dose. Notably, the effectiveness of the seasonal influenza vaccine varies each year, partly depending on how closely the circulating strains match the strains included in the vaccine (10). Moreover, the segmented, negative-sense RNA genome of the virus allows genetic reassortment, which leads to antigenic drift and shift (11). These processes drive frequent and unpredictable changes in viral surface antigens, contributing to reduced effectiveness of the vaccine within a given season.

In contrast, an adjuvanted recombinant herpes zoster vaccine (RZV), licensed for use in 2017, has shown high efficacy in older adults. RZV, combining the recombinant glycoprotein E (gE) of the varicella-zoster virus (VZV) with the adjuvant $AS01_B$, has demonstrated 97.2% efficacy during a mean follow-up of 3.2 years, 90.9% efficacy at 7 years, and 82% at 11 years in preventing herpes zoster in adults 50 years and older (12–14). In people over 70 years of age, vaccine efficacy was 91.3% at 3.2 years after immunization (15). The vaccine is administered in two doses, separated by 2-6 months, it is well-tolerated and with an acceptable safety profile for both young and older adults (16,17). It is unclear how RZV is able to achieve such high efficacy despite an immunosenescent state, but it is hypothesized that the adjuvant itself and the synergy between the adjuvant and antigen stimulate a persistent response, even in immunocompromised or frail individuals (18). The molecular substrate of this effect is not yet known.

To understand the molecular mechanisms and immunological pathways contributing to age-dependent vaccine effectiveness, we conducted a randomized and partially placebo-controlled vaccination trial in young (18-35 years) and older (≥60 years) adults who received either IIV4, RZV, or a placebo. IIV4 is widely used in older populations but shows variable, age-dependent efficacy, making it suitable for studying reduced protection with age. In contrast, RZV remains highly effective in older adults, and

exploring the underlying mechanisms leading to high protection will give valuable information into how it overcomes age-related decline in immune function.

In this sub-study of the clinical trial, we evaluated the immunological differences between age groups by measuring immune cell subsets, neutralizing antibody titers, peripheral blood mononuclear cells (PBMC)-derived IFN-γ production and CD4+ T cell responses to the specific vaccine antigens, and concentrations of circulating inflammatory proteins before and after vaccination. Finally, we explored how baseline systemic inflammation is associated with vaccine-induced immune responses.

Results

Safety data and participant demographics

84 young and 63 older adults were recruited. Following the initial screening and informed consent process, participants were randomized into different study arms based on age and vaccine type, as illustrated in **Figure 1A**. Due to the extended period between the screening/randomization and vaccination visits (D0), some participants dropped out or withdrew their consent. Ultimately, 22 young and 31 older participants were immunized with RZV, while 30 young and 27 older adults received IIV4. Additionally, 11 young adults in the Placebo-IIV4 and 11 adults in the Placebo-RZV were vaccinated with a placebo. The timeline, sample collection, and performed assays are summarized in **Figure 1B**. The demographics, comorbidities and risk factors of the study cohort are presented in **Table 1**. As expected, older adults had more comorbidities and risk factors, such as hypertension and chronic cardiovascular disease.

Overall, both vaccines were well-tolerated, showing acceptable safety profiles in young and older participants. The most common local adverse reaction (AR) within the first week after vaccination was pain at the injection site, while the most common systemic ARs were myalgia and fatigue (**Supp. Tables 1, 2 and 3).** Most of the ARs were rated mild or moderate by the study participants, whereas some

severe (grade 3) ARs were also reported (**Supp. Tables 4, 5 and 6**). These ARs typically resolved spontaneously within a week. Three serious adverse events (SAEs) were recorded during the study, which were road traffic accident (RZV-Young), atrial fibrillation (RZV-Old) and acute heart failure (RZV-Old), the last SAE being fatal (**Supp. Table 7**). As the participants with atrial fibrillation and acute heart failure both had a prior history of similar conditions and were receiving related medications, and the participant involved in the traffic accident did not develop any condition that contributed to the accident, the SAEs were not considered related to the study vaccines.

Baseline differences in immune parameters between young and older adults

We first evaluated the immune system parameters in young and older adults before they received any study vaccination. The numbers of white blood cells (WBCs), neutrophils, monocytes and lymphocytes were similar between the two age groups (**Supp. Fig. 1A**). Additionally, the baseline concentrations of anti-VZV gE antibodies and the hemagglutination inhibition (HAI) titers against the A/Victoria/H1N1 and B/Phuket virus strains were also comparable in young and older adults (**Supp. Fig. 1B- C**). In contrast, the IFN-γ production of PBMCs from young adults was significantly higher after a 7-day incubation with the IIV4 vaccines and gE antigen compared to that from older counterparts (**Supp. Fig. 1D**). Lastly, many inflammatory proteins were significantly more abundant in the circulation of older individuals than young volunteers (**Supp. Fig. 1E**).

IIV4-induced modulation of immune cell numbers, HAI titers, antigen-specific CD4+ T cells and IFN-γ production in young and older adults

Next, we analyzed changes in circulating immune cells after IIV4 vaccination compared to baseline (D0). In young adults, immune cell counts, except lymphocytes, increased one-day post-vaccination (**Figure 2A**). Although lymphocyte numbers decreased on D1, they remained significantly elevated for the

remainder of the study, compared to baseline. WBC counts remained higher on day 180 compared to baseline, possibly due to the higher numbers of lymphocytes and monocytes. In older adults, changes were minimal (**Figure 2B**). Absolute cell counts after IIV4 and placebo vaccination are presented in **Supp. Fig. 2A-C**.

Subsequently, we measured HAI titers against the viral strains in the 2021/2022 and 2022/2023 IIV4 vaccines before and 60 days post-vaccination. Both age groups showed a significant increase in anti-A/Victoria/H1N1 titers, with a stronger rise in young adults (**Figure 2C-D)**. Similarly, IIV4 also boosted anti-B/Phuket titers in both groups, and the fold induction was not significantly different between the groups (**Figure 2E-F**). Notably, the fold HAI induction to the A/Victoria/H1N1 and B/Phuket strains were significantly higher in older adults vaccinated with the IIV4 2021/2022 season compared to the 2022/2023 season (**Supp. Fig. 2D**).

Seroprotection refers to an antibody titer of ≥1:40, a level associated with a 50% reduction in influenza risk (19). In our study, 23% of young adults had HAI titers ≥40 to A/Victoria/H1N1 before vaccination, which increased to 100% on D60. In older adults, the corresponding rates were 30% before vaccination and 73% after vaccination. All young adults had titers ≥40 against B/Phuket both before and after vaccination. Among older adults, 93% had titers ≥40, rising to 100% after receiving IIV4.

For the A/Tasmania/H3N2 and B/Washington strains (present only in the IIV4 2021/2022), the HAI titers against A/Tasmania/H3N2 increased only in the young group, but seroconversion rates were extremely low in the two age groups (**Supp. Fig. 2E-F**). Both young and older adults showed significant antibody increases to B/Washington with similar fold changes (**Supp. Fig. 2G-H**). No notable changes were observed in antibody levels to the A/Darwin/H3N2 and B/Austria strains 60 days post-vaccination (**Supp. Fig. 2I**).

Previous studies have shown that pre-existing immunity, defined by the presence of specific antibodies before vaccination, negatively correlates with a fold antibody induction following vaccinations like those for influenza, herpes zoster and pneumococcus (20–22). As expected, we observed a negative

correlation between pre-vaccination HAI titers and antibody response for A/Victoria/H1N1 and B/Phuket strains in young adults (**Figure 2G**). In the older group, a moderate-to-high negative correlation was also observed, though not significant for A/Victoria/H1N1 (**Figure 2H**). As such, age may independently impact the relationship between pre- and post-vaccine responses. Additionally, IFN-γ production in PBMCs after 7-day incubation with IIV4 was similar before and after vaccination across all groups (**Figure 2I)**. Subsequently, we explored the potential link between PBMC-derived IFN-γ production and antibody response to IIV4. Fold induction of A/Victoria/H1N1 HAI titers and IFN-γ responses were negatively correlated at D60 in younger adults (**Figure 2J**).

Lastly, we determined the frequency of antigen-specific activated CD4+ T cells that express CD40L, IL-2, TNF, and IFN-γ after IIV4 in young and older adults (**Supp. Fig. 3A-B).** In general, the induction of activated antigen-specific CD4+ T cells by IIV4 was lower in older adults compared to the young individuals. CD40L was the most expressed marker upon incubation with the A/H1N1/Victoria strain. IIV4 led to a small but significant induction of B/Phuket-specific CD4+ T cells that were positive for 2 or 3 markers. There was a significant increase in CD4+ T cells expressing all 4 markers (CD40L, IL-2, TNF, and IFN-γ) in response to A/H1N1/Victoria only in older adults, whereas such an increase to B/Phuket was observed only in young adults (**Figure 2K).**

Overall, young adults showed higher antibody responses to IIV4, but the vaccine did not enhance IFN-γ production from PBMCs in either age group. The vaccine induced polyfunctional antigen-specific CD4+ T cells, although the cell frequencies were low, suggesting the limited ability of IIV4 to boost cellular responses.

RZV-induced modulation of immune cell numbers, antibody concentrations, antigen-specific CD4+ T cells and IFN-γ production in young and older adults

We characterized how RZV vaccination affected immune cell counts after the first and second doses. RZV induced greater fold changes in cell numbers than IIV4. After the first dose, both young and old

groups experienced temporary increases in WBCs, neutrophils and monocytes on day 1, with over 1.7-fold increase in neutrophils (**Figure 3A**). Lymphocyte numbers decreased on D1 only in the young group. Similar changes occurred after the second dose, with significant lymphocyte decreases on day 1 (D61) in both groups, followed by a rise on D67 that stayed elevated until at least D120 (**Figure 3B**). Absolute numbers of cells after RZV and placebo vaccinations are presented in **Supp. Fig. 4A-C**.

RZV vaccination led to strong antibody production after the first dose in both age groups (**Figure 3C**). The second dose further increased anti-VZV gE antibody levels, though the increase in young adults did not reach statistical significance. Young adults showed a greater fold antibody increase only after the first dose (**Figure 3D**). We also found that pre-vaccination antibody concentrations were negatively correlated with a fold increase in antibodies after RZV in both age groups (**Figure 3E**). The concentrations of antibodies before the second vaccination (D60) were negatively associated with fold antibody increase after the second vaccination (**Figure 3F**).

IFN-γ production after gE antigen stimulation increased significantly after two RZV doses (D120) and remained elevated at D240, unlike the placebo, which had no effect (**Figure 3G**). Importantly, prior to RZV, IFN-γ response was detectable in 55% of the young participants, increasing to 92% after the second dose (D120). In older adults, it increased from 20% at baseline to 77% on D120. No significant relationship was found between IFN-γ and antibody production following RZV exposure (**Figure 3H**).

RZV induced a robust and significant increase in antigen-specific polyfunctional CD4+ T cells in both age groups, with a lesser degree in the old (**Supp. Fig 5).** The second dose further increased the frequency of antigen-specific cells. CD40L was the most expressed marker to the antigen among the 4 markers measured. CD40L+ IL-2+ TNF+ IFN-γ+ gE-specific CD4+ T cells had the highest frequency among other marker combinations and were significantly induced both after the first and second RZV dose in young and older adults (**Figure 3I**).

In summary, RZV elicited significant humoral and cellular responses in both young and older individuals.

The impact of IIV4 on circulating immune mediators

We analyzed the impact of IIV4 and placebo on circulating inflammatory proteins using the Olink 96 Target Inflammation Panel. At 60 days post-vaccination, EN-RAGE levels decreased in both IIV4 placebo and young groups (**Figures 4A-B**). In older adults, IIV4 vaccination increased inflammatory mediators such as TNFB, IL-6, CCL25, and TRAIL, while reducing AXIN1, STAMBP, and CD40 (**Figure 4C, Supp. Fig. 6A**). Since older adults received either the 2021/2022 or the 2022/2023 season of the IIV4 vaccine, we analyzed the effect of these two vaccines separately. Interestingly, the 2021/2022 vaccine increased OSM and decreased EN-RAGE (**Supp. Fig. 6B**), while the 2022/2023 vaccine affected other proteins without impacting OSM or EN-RAGE (**Supp. Fig. 6C-D**). These results suggest IIV4 has minimal impact on inflammatory proteins in young adults, but varies in older adults depending on the vaccine year.

The association between baseline inflammation and adaptive immunity responses to IIV4

The immune system's baseline status before vaccination is crucial to predicting vaccine responses (23). Thus, we examined the relationship between baseline inflammation levels as assessed by circulating proteins and the antibody/IFN-γ responses to vaccination in both age groups combined and separately. In the IIV4 cohort, higher baseline inflammation was linked to lower fold increases in A/Victoria/H1N1 and B/Phuket HAI titers, with proteins like CCL11, MCP-4, and HGF negatively associated with antibody responses (**Figure 5A-B**).

Age-specific analysis showed baseline inflammation negatively linked to B/Phuket HAI responses in both age groups in general, while A/Victoria/H1N1 showed mixed associations (**Supp. Fig. 7A-C**). No significant overlapping proteins were found in young and older adults.

Unlike neutralizing antibody titers, fold changes in IFN-γ were positively correlated with baseline systemic inflammation when both groups were combined (**Figure 5C-D**). Higher baseline concentrations of IL-10RA were however linked to lower IFN-γ increases in the young group (**Supp. Fig.**

7D). In older adults, baseline inflammatory proteins like MCP-4, IL-8, CXCL9, and CXCL10 were positively associated with IFN-γ responses (**Supp. Fig. 7E-F**).

Our results indicate that baseline inflammation is generally negatively associated with antibody production and positively linked with specific cellular IFN-γ responses to viral strains in IIV4. However, distinct patterns were observed between age groups.

The impact of RZV vaccination on circulating immune mediators

Next, we analyzed the changes in circulating proteins after placebo and RZV vaccination. Following the first placebo injection, proteins like IL-17C, CXCL5, CXCL1, MCP-4, and MMP-1 increased, but only MMP-1 remained elevated after the second dose (**Figure 6A-B, Supp. Fig. 8A**). RZV had no notable influence on the investigated proteins in young adults (**Figure 6C-D**). Interestingly, in older individuals, the first RZV dose reduced proteins such as MCP-1, MCP-4, CXCL5, and CXCL6, while the second dose broadly downregulated inflammation-related proteins like IL-10, TNF, IL-18R1, and TWEAK, among others (**Figure 6E-F, Supp. Fig. 8B**)

These results suggest RZV reduces systemic inflammation in older adults.

The association between baseline inflammation and adaptive immune responses to RZV

Lastly, we evaluated the relationship between baseline inflammation and immune responses to RZV. Overall, higher levels of circulating immunomodulatory proteins were negatively correlated with antibody responses, even after two doses (**Figure 7A-B**). Proteins like HGF, IL-8, IL-10RA, CSF-1, and OPG were associated with lower antibody responses in both age groups.

Age-specific analysis showed stronger negative correlations in young adults, with proteins like CCL4 and CCL11 linked to lower antibody responses. In older adults, correlations were mixed; higher levels of CXCL9, CXCL10, and IFN-γ were associated with greater antibody induction (**Supp. Fig. 9A-C**).

Baseline concentrations of circulating immune mediators were generally negatively correlated with IFN-γ responses at D60 and D240 post-RZV, although some positive associations emerged after the second dose (D120/D0) (**Figure 7C**). Notably, higher baseline concentrations of CD6 and MMP10 were moderately linked to reduced IFN-γ response to the gE antigen after the first dose. In contrast, elevated CCL19 correlated with an increased IFN-γ fold change at D120 and D240 (**Figure 7D**).

When the age groups were separated, different patterns were observed (**Supp. Fig 9D-E**). In RZV-Young, higher TWEAK concentrations consistently correlated with weaker IFN-γ responses (**Supp. Fig. 9E**). Mirroring the overall trend observed in the age-combined RZV group, baseline CD6 negatively linked to IFN-γ response at D60 in the old group while CCL19 was positively linked to IFN-γ at D120 and D240 (**Supp. Fig. 9F).**

Overall, baseline inflammation was negatively associated with RZV-induced adaptive responses when age groups were combined. However, when analyzed separately, different correlation patterns were seen, with older adults displaying a mix of responses, including more positive associations.

Discussion

Aging substantially impacts vaccine responses, with many vaccines being less effective in older adults. Adjuvants have been developed to address this by enhancing immune activation and memory (24). In this study, we evaluated age-dependent differences in immune system responses to IIV4 and RZV. The results are summarized in **Table 2**. RZV induced strong antibody and IFN-γ responses in both age groups and reduced systemic inflammation in older adults. In contrast, IIV4 primarily increased neutralizing titers, stronger in young adults, but failed to induce effective cellular responses.

These results align with previous reports of strong cellular and humoral responses induced by RZV in both age groups and weak or no cellular responses from seasonal influenza vaccines (17,25,26). Complementing previous findings, we found that both vaccines were well-tolerated with an acceptable safety profile in both age groups (13,27). Although this is a small cohort, it represents valuable information, as safety data on RZV in young adults has been limited compared to older adults (28).

The efficacy of vaccines is primarily mediated through the induction of specific antibodies and T-cell responses. T-cell responses play a key role in fighting infections and providing long-term protection. For example, memory T cells are especially important in controlling varicella-zoster virus infections, which cause shingles (29). In addition, antigen-specific CD4+ T cells and IFN-γ production from these cells have been linked to protection against influenza, but their numbers decrease significantly in older adults (30). IIV4's inability to enhance cellular responses likely contributes to its sub-optimal effectiveness. An important function of adjuvants is to enhance the T-cell memory response (31). For instance, AS01 in RZV contains two components: the TLR4 ligand monophosphoryl lipid A (MPLA) and saponin QS-21 derived from the *Quillaja saponaria* tree (32). MPLA induces the production of pro-inflammatory cytokines through the MyD88 pathway, while QS-21 activates the inflammasome and IL-18/IL-1β release. The synergistic effect of these two components is essential to achieve an optimal T-cell response (33). Overall, AS01 triggers strong innate activation in draining lymph nodes via monocytes/macrophages, dendritic cells, and NK cells and early release of IFN-γ in an IL-12/IL-18 dependent manner (33,34).

Clinical studies with AS01, such as those done with tuberculosis and malaria vaccines, demonstrated strong antigen-specific CD4+ T-cell responses and antibody production (35,36). In this study, we found an RZV-induced robust increase in polyfunctional antigen-specific CD4+ T cells that express different combinations of CD40L, IL-2, TNF and IFN-γ in both age groups. Notably, T cell polyfunctionality is linked to protection by various vaccines, e.g., COVID-19 and yellow fever (37,38). Additionally, CD4+ T-cell-mediated immunity after RZV was associated with better antibody responses for at least three years

post-vaccination (39). Because AS01 induces a robust and durable T cell response, its use could be further explored in vaccines targeting pathogens where strong cellular immunity is essential, as well as in populations with weakened immunity, including immunocompromised or older individuals. Interestingly, the type and magnitude of immune responses induced by AS01-adjuvanted vaccines vary across different antigens, suggesting that both the adjuvant and antigen contribute to forming the overall immunological profile (32).

An important finding of our study is that both vaccines induce CD40L on antigen-specific $CD4^+$ T cells. This is important because recent work shows that CD40-CD40L signaling does more than provide co-stimulation: it can program long-lasting functional changes in human monocytes, similar to trained immunity (40). We found that $CD40L^+$ polyfunctional $CD4^+$ T cells are the main responding population after both IIV4 and RZV, and that these responses persist with aging, suggesting that CD40–CD40L interactions may be a common control point linking innate and adaptive immunity after vaccination. RZV, in particular, induces high levels of $CD40L^+$ IL-2^+ TNF^+ IFN-γ^+ $CD4^+$ T cells in both age groups. In contrast, weaker $CD4^+$ T cell responses to IIV4 may reflect limited CD40-driven support, helping explain its lower cellular immunogenicity and reduced efficacy in older individuals.

Intriguingly, both IIV4 and RZV modulated the circulating inflammatory proteins, particularly in older individuals. We observed different effects of the IIV4 vaccines administered in Fall 2021 and Fall 2022 on the proteins in the circulation of older adults, arguing for variation due to differences in the antigenic composition of the vaccines. However, the absence of a placebo group for the 2022/2023 season and the relatively small sample size limit the conclusions. Notably, RZV reduced several inflammatory proteins in older adults, with stronger effects after the second dose. This age-dependent response may be due to the inherently lower baseline levels of inflammatory mediators in young adults.

Consistent with previous studies, we found higher concentrations of inflammatory markers in older adults than in young adults. This is likely partly due to the higher rates of comorbidities and risk factors, such as hypertension and cardiovascular diseases (e.g. atherosclerosis) (41,42). Elevated

concentrations of inflammatory mediators in older adults indicate a state of chronic, low-grade inflammation, commonly referred to as "inflammaging" (43). This persistent inflammation may impair the immune system's ability to mount a robust antigen-specific response upon vaccination, possibly due to immune exhaustion or overactivation (44) due to continuous stimulation leading to diminished function and reduced responsiveness to new antigens. These mediators might also trigger regulatory feedback that weakens immune responses. Lowering systemic inflammation could prevent immune exhaustion and improve both innate and adaptive responses. As an example, the BCG vaccine reduced systemic inflammation in a Dutch cohort of healthy participants, which was associated with trained immunity induction -the capacity of innate immune cells to develop a memory-like response (45). Consistently, our study found a strong negative association between inflammatory protein concentrations in the blood and antibody fold increases in both vaccine groups.

In our study cohort, baseline inflammation was generally negatively correlated with PBMC-derived IFN-γ response after RZV, while it showed a positive association with IFN-γ response after IIV4. Interestingly, in the older volunteers vaccinated with IIV4, higher circulating concentrations of CXCL9, CXCL10, and IFN-γ were linked to higher IFN-γ production. In the old RZV group, higher CXCL9 and CXC10 were also correlated with greater antibody response. Since CXCL9 and CXCL10 are key chemokines involved in activating NK cells and Th1 immunity, it is tempting to speculate that they contribute to the enhanced type II interferon response observed after vaccination (46) and possibly to improved antibody production. As this study only identifies associations but does not establish causation, future research should aim to understand better how these proteins affect vaccine responses, especially in different age groups. Additionally, future studies should explore strategies to modulate inflammatory proteins prior to or during vaccination, by improving or developing adjuvants, with the aim of enhancing vaccine efficacy, especially in older adults.

This study demonstrated the moderate influence of IIV4 and RZV on immune cell subsets. IIV4 significantly increased WBCs in young adults, driven by lymphocytes and monocytes numbers and

sustained until D180, while older adults showed elevated lymphocytes only at D7. On the other hand, RZV vaccination caused transient but stronger changes in cell counts in both age groups. A temporary increase in neutrophil and monocyte numbers and infiltration to the injected muscle has been reported after other vaccinations, suggesting the immediate innate immune system activation and response (47,48). The similar magnitude of changes in immune cell counts between young and older adults suggests that the AS01-adjuvanted RZV can achieve comparable initial innate immune cell activation in both age groups, supporting its effectiveness (34).

It is important to note that the RZV and RZV-associated placebo injections were administered during spring/summer (first and second dose), while IIV4 and IIV4-associated placebo injections were administered during fall. Seasonal variations are known to influence immune function and therefore vaccine-induced responses. Although the study design partially accounted for this by including two young placebo groups vaccinated in either spring or fall, helping the control for background seasonal effects, we cannot undermine the impact of seasons on vaccine responses.

A limitation of this study is that a placebo group was only available for young participants, while older adults are all vaccinated due to ethical considerations. As a result, direct comparisons between vaccinated and unvaccinated older individuals could not be made, and age-related differences in immune responses must therefore be interpreted with some caution. Particularly for IIV4, where vaccine responses are strongly influenced by prior influenza infections and vaccination history, including an unvaccinated control group of older adults would have helped to better distinguish vaccine effects from those related to pre-existing immunity.

In summary, this study highlighted the age-dependent vaccine responses following IIV4 and RZV exposure, focusing on cellular and humoral immunity, inflammatory mediators, and the influence of systemic inflammation prior to vaccination. While RZV induced potent humoral and cellular immune responses while decreasing systemic inflammation in older adults, IIV4 was able to induce strong antibody production with weaker cellular responses. The incapacity of IIV4 to induce specific cellular

immune responses may contribute to its year-dependent sub-optimal efficacy. Our findings emphasize the role of adjuvants such as AS01 in enhancing vaccine responses in older adults, with modulating low-grade inflammation being a potential strategy. Further research is needed to explore the underlying transcriptional and epigenetic mechanisms driving different vaccine responses by RZV compared to IIV4. Understanding the immunological pathways contributing to increased vaccine immunogenicity could help refine existing adjuvants and develop new ones.

Methods

Sex as a biological variable

Both males and females are included in the clinical study. Sex was considered as a confounder to investigate the differences in vaccine responses in young and older individuals.

Clinical study

The volunteer recruitment in this single-center, randomized, partially placebo-controlled, open-label study was conducted at and sponsored by the Radboud University Medical Center between September 2021 and May 2023. Volunteers aged 18-35 and those 60 years or older were eligible to participate. Exclusion criteria were the use of systemic immunomodulatory drugs, acute or active illness within two weeks before the study, receipt of any vaccination within four weeks before or after the start of the study, receipt of a herpes zoster vaccination in the past year, known allergy to the components of IIV4 or RZV, being immunocompromised, and pregnancy or breastfeeding. Young female participants had to have a negative pregnancy test before participating.

After signing the informed consent form, young participants were randomized to receive either IIV4, RZV, IIV4-associated placebo or RZV-associated placebo (3:3:1:1) (**Figure 1A**). Older participants were

randomized to receive either IIV4 or RZV (1:1); no placebo was administered to older individuals for ethical considerations, as these vaccines are recommended to this population. Blood samples were collected at baseline and volunteers were vaccinated with either a placebo (0.9% NaCl solution), IIV4 (Fluarix Tetra, GSK, UK, 2021/2022 and 2022/2023 seasons), or RZV (Shingrix, GSK). IIV4 vaccinations took place between September and January during the influenza season, whereas RZV vaccinations were done between April-July 2022. Corresponding placebo groups were included in the same period. All vaccinations were administered in the morning between 08:00-12:00 as a 0.5 mL intramuscular injection into the deltoid muscle. The RZV and associated placebo groups received a second dose two months after the first injection. Study participants were followed for 6 months after the first visit in the IIV4 and associated placebo group and 8 months after the first visit in the RZV and associated placebo group (6 months after the second injection). The blood collection times and read-outs for each time point were given in **Figure 1B**. All adverse events and potential immune-mediated disorders were recorded throughout the study. Solicited adverse events recorded in patient diaries within 7 days after vaccinations and SAEs throughout the clinical study were reported in this manuscript.

Of note, all young and 10 of the 27 older participants in the IIV4 group were vaccinated with IIV4 season 2021/2022, while the remaining 17 older participants were injected with IIV4 season 2022/2023. These two vaccines had two common viral strains, which were the A/Victoria/2570/2019 IVR-215 H1N1 (A/Victoria/H1N1 in the manuscript) and the B/Phuket/3073/2013 (B/Yamagata lineage), wild type (B/Phuket in the manuscript). Additionally, the 2021/2022 season contained the A/Tasmania/503/2020 IVR-221 H3N2 (A/Tasmania/H3N2 in the manuscript) and B/Washington/02/2019, (B/Victoria lineage), wild type (B/Washington in the manuscript), while the 2022/2023 season includes A/Darwin/6/2021 IVR-227 H3N2 (A/Darwin/H3N2in the manuscript) and B/Austria/1359417/2021 BVR-26 (B/Victoria lineage), (B/Austria in the manuscript).

Sample collection

Blood samples were collected by venipuncture into EDTA and serum tubes. To obtain plasma and serum from the EDTA and serum tubes, respectively, blood was centrifuged for 10 minutes at 2700g at room temperature (RT). Serum was used for HAI and anti-gE antibody measurements, while plasma was used to measure protein concentrations. All samples were stored at -80°C until analysis.

Measurement of immune cell counts

The number of white blood cells, monocytes, neutrophils, and lymphocytes was determined from whole blood using a hematology analyzer (Sysmex, Japan). This analyzer operates on principles similar to those of a flow cytometer: it uses forward scatter light to determine cell volume, side scatter light to identify cell nuclei and granules, and side fluorescence to detect nucleic acids and organelles.

HAI measurements

Hemagglutination inhibition antibody titers were determined on sera before and 60 days after vaccination using the method derived from the WHO Manual on Animal Influenza Diagnosis and Surveillance (49). Measurements were conducted on thawed frozen serum samples with a standardized and validated methodology. Briefly, serum samples were treated with receptor-destroying enzyme (Sigma-Aldrich, USA, # C8772-1VL) overnight to remove non-specific serum inhibitors, diluted to 1:10, and serial diluted 2-fold in duplicate from 1:10 to 1:10240. After adding an equal volume of standardized virus (4 HAU / 25µL), neutralization was performed for 1 hour at RT, followed by the addition of the red blood cells. Of note, HAU stands for hemagglutinating unit, which is the highest dilution of the virus causing complete hemagglutination. After 60-120 minutes, plates were tilted, and the HAI titer was defined as the reciprocal of the last serum dilution that fully inhibits hemagglutination as compared to an RBC control well. Each sera sample was tested in duplicate within the same assay.

The titer results were reported as the 1ometric mean titer of the duplicates. The assay positivity cut-off value is 10 (1/dilution) and is confirmed for each strain.

Anti-VZV gE antibody measurements

Serum anti-glycoprotein E (gE) antibody concentrations were measured from sera before vaccination and 60 days after each RZV vaccination using a validated GSK in-house ELISA, with a technical cut-off of 97 mIU/mL, as previously described (39). Of note, mIU, milli-international unit, is a standardized quantity of a biological activity or effect. Briefly, GSK-produced, purified recombinant VZV gE was pre-coated on a 96-polystyrene-well microplate at a 2 µg/mL final concentration. The wells were washed and blocked with bovine serum albumin. Diluted serum samples were added and incubated for one hour at RT. In the next step, the plates were washed again and goat antibodies against human IgG conjugated to horseradish peroxidase (a-IgG-HRP conjugate, KPL, ref. 214-1002) were added. After incubation for an hour at RT and subsequent washing, a chromogen-substrate solution (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide) was added. The reaction was stopped with sulfuric acid and the optical density was measured at 450 nm against the reference wavelength, which is 620 nm, using Emax microplate reader (Molecular Devices, USA).

The assay was calibrated against the VZV World Health Organization international reference.

Peripheral blood mononuclear cells (PBMCs) isolation

Venous blood collected with EDTA tubes was diluted with PBS, and PBMCs were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare, IL, USA) in SepMate tubes (Stemcell Technologies, Canada). After centrifugation at 1200g for 10 minutes, the upper layer containing PBMCs was collected and washed twice with cold PBS. The cells were suspended in RPMI 1640 Medium (Dutch modification) (Thermo Fisher Scientific, MA, USA) supplemented with 1 mM sodium pyruvate (Thermo

Fisher Scientific), 2 mM GlutaMAX (Thermo Fisher Scientific), and 50 µg/mL gentamicin (Centrafarm, Netherlands), and then counted using the hematology analyzer. Of note, the Dutch modified RPMI medium has HEPES and a lower concentration of sodium bicarbonate compared to a classical RPMI medium (1 g/L instead of 2 g/L). PBMCs were frozen at $20x10^6$ cells/mL in the Recovery Cell Culture Freezing Medium (Thermo Fisher Scientific) and stored at -150°C until further use.

Ex-vivo stimulation of cytokine production

Frozen PBMCs were thawed in a warm RPMI medium containing 10% calf serum (Capricorn Scientific, Germany) and supplemented as described in the previous section. After washing the cells twice to remove the residual freezing medium, cells were resuspended in RPMI medium with 10% pooled human serum. The pooled human serum is prepared in-house at the Radboud University Medical Center, using the serum provided by the Department of Medical Microbiology. Each serum sample is tested on human PBMCs, and the ones inducing or reducing an immune response are excluded. The remaining samples are pooled to be used for experiments.

Cells were counted and seeded into 96-well U bottom plates (Sarstedt, Germany) at a density of $5x10^5$ cells/well. PBMCs were stimulated with 1 µg/mL of Fluarix Tetra (IIV4, 2021/2022 and 2022/2023 season) and 4 µg/mL of the gE component of the Shingrix (RZV) or left unstimulated. Following a 7-day incubation at 37°C, cell-free supernatants were collected.

IFN-γ concentrations in supernatants were measured using the IFN-γ DuoSet ELISA kits (R&D Systems, MN, USA), following the manufacturer's instructions. The same batch was used to perform ELISA for all samples, and the different time points for each donor were measured on the same plate. Moreover, three control samples with known cytokine concentrations were utilized in measurements to ensure that the reference standards worked correctly. The lower limit of detection (LLOD) for this assay was 93.76 pg/ml.

Antigen-specific CD4+ T cell responses

Antigen-specific T cell responses were measured by intracellular cytokine staining from PBMCs (50,51). In short, thawed PBMCs (10^6 cells/well) were stimulated in 96-well plates either with the A/H1N1/Victoria strain (1 µg/ml), the B/Phuket strain (1 µg/ml), zoster gE– 15mer ov 11 peptides pool (1.25 µg/ml) or left unstimulated (medium only) as a negative control. Following 2 hours of incubation at 37°C, GolgiPlug™ brefeldin A solution at 1/1000 final dilution (BD, NJ, USA) was added for overnight to inhibit cytokine secretion. Then, the cells were harvested, stained for surface markers (a viability dye, CD4 and CD8), fixed and permeabilized with the Cytofix/Cytoperm kit (BD) per manufacturer's instructions. Intracellular staining was performed using the following markers: CD40L, IL-2, TNF, IFN-γ, IL-13, IL-17 and 4-1BB. After washing with the Perm/Wash buffer (BD), cells were analyzed by flow cytometry. Live cells that were positive for CD4+ 4-1BB+ and positive for at least one of the four markers (CD40L, IL-2, TNF, IFN-γ) were quantified. Since the expression of IL-13 and IL-17 was very low, these markers were not used for the analysis. The antigen-specific CD4+ T cell frequency was calculated as the difference between the frequency of CD4+ T cells stimulated with the antigen and those stimulated with medium alone. The frequency was expressed as the number of cells per 10^6 CD4+ T cells. The details of the antibodies used in the flow cytometry staining are given in **Supp. Table 8**.

Proximity extension assay measurements of circulating inflammatory mediators

Circulating proteins in the plasma were measured by proximity extension assay using the Olink Target 96 Inflammation Panel, which includes 92 proteins. All time points from the same participant were measured on the same plate to eliminate potential inter-plate variations in measurements and fold change calculations. Proteins were normalized based on the inter-plate controls, and expression levels were presented on a log2 scale called normalized protein expression (NPX).

18 of 92 proteins were excluded from the analysis due to a more than 20% missing data frequency. Following this, principal component analysis (PCA) was performed, and two apparent outliers were removed. The R package *limma* (v3.60.4) was then utilized to conduct differential expression analysis between the age groups and across different time points within the same group.

Statistical analyses

This clinical trial was designed as an exploratory study, hence no formal sample size calculations were made.

Data comparing young and older participants were adjusted for confounding variables sex and BMI. Comparisons between age groups were made using the Mann Whitney test, while comparisons within the same group across different time points utilized the Wilcoxon signed rank test. When more than two time points were compared, the Friedman test was applied. The baseline concentration of circulating proteins was correlated with fold changes in antibody concentrations and IFN-γ responses using Spearman's correlation. The statistical methods applied to each graph are detailed in the figure legends. A p-value of less than 0.05 was considered statistically significant.

For statistical tests and data visualization, Prism (GraphPad Software Inc., version 10) and RStudio (Posit PBC, v4.4.1) were used. Dot plots were created with Prism (v10), while heatmaps and volcano plots were generated in RStudio using the *pheatmap (v1.0.12)*, *ggplot2 (v3.5.1)* and *ggrepel (v0.9.5)* packages.

Study approval

The local ethics committee Medical Research Ethics Committee (MREC) Oost-Nederland granted ethical approval for this study (NL76061.091.20). Written informed consent was received from all participants

prior to inclusion. All experiments were conducted under the Declaration of Helsinki. The trial is registered at ClinicalTrials.gov with the identifier NCT05082688.

Data availability

All the data that support the findings of this study are available from the corresponding author, upon reasonable request. The numerical data underlying the figures are reported in the Supporting Data Values file.

Author contributions

GK, EJMT, WB, SMB, MMM, and MGN conceptualized the study and designed the experiments. GK, EJMT, and EAD were responsible for the overall conduct of the clinical study, administration and approvals. GK, EJMT, LSH, and EAD were involved in the collection and processing of biological samples. GK, LSH, BG, LvE, HL and SB performed the experiments and curated the data. GK and AB performed data analysis and visualization. GK, MO, YN, WB, SMB, MMM, and MGN were involved in data interpretation. JtO, MMM, and MGN supervised the study. GK prepared the original draft. All authors read, edited and accepted the final version.

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Trademarks

Shingrix and Fluarix Tetra are trademarks owned by or licensed to GSK. AS01 is a GSK proprietary Adjuvant System.

Declaration of conflict of interest

MMM is a scientific founder of Lemba Therapeutics. MGN is a scientific founder of Lemba Therapeutics, Biotrip, TTxD and Salvina. SB, AB, WB and SMB are employed by and hold financial equities in GSK.

References

1. Shattock AJ, et al. Contribution of vaccination to improved survival and health: modelling 50 years of the Expanded Programme on Immunization. *Lancet*. 2024;403(10441):2307-2316.
2. Zimmermann P, Curtis N. Factors That Influence the Immune Response to Vaccination. *Clin Microbiol Rev*. 2019;32(2).
3. Bulut O, et al. Overcoming immune dysfunction in the elderly: trained immunity as a novel approach. *Int Immunol*. 2020;32(12):741-753.
4. Goronzy JJ, Weyand CM. Mechanisms underlying T cell ageing. *Nat Rev Immunol*. 2019;19(9):573-583.
5. de Mol J, et al. The Dynamics of B Cell Aging in Health and Disease. *Front Immunol*. 2021;12.
6. Pereira B, et al. Targeting Inflammation and Immunosenescence to Improve Vaccine Responses in the Elderly. *Front Immunol*. 2020;11.
7. Goodwin K, et al. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*. 2006;24(8):1159-1169.
8. Wang J, et al. The Impact of Age Difference on the Efficacy and Safety of COVID-19 Vaccines: A Systematic Review and Meta-Analysis. *Front Immunol*. 2021;12.
9. Guo J, et al. Real-world effectiveness of seasonal influenza vaccination and age as effect modifier: A systematic review, meta-analysis and meta-regression of test-negative design studies. *Vaccine*. 2024;42(8):1883-1891.
10. Tricco AC, et al. Comparing influenza vaccine efficacy against mismatched and matched strains: A systematic review and meta-analysis. *BMC Med*. 2013;11(1):1-19.
11. Petrova VN, Russell CA. The evolution of seasonal influenza viruses. *Nature Reviews Microbiology 2017 16:1*. 2017;16(1):47-60.
12. Boutry C, et al. The Adjuvanted Recombinant Zoster Vaccine Confers Long-Term Protection Against Herpes Zoster: Interim Results of an Extension Study of the Pivotal Phase 3 Clinical Trials ZOE-50 and ZOE-70. *Clin Infect Dis*. 2022;74(8):1459-1467.
13. Lal H, et al. Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. *N Engl J Med*. 2015;372(22):2087-2096.
14. Strezova A, et al. Final analysis of the ZOE-LTFU trial to 11 years post-vaccination: efficacy of the adjuvanted recombinant zoster vaccine against herpes zoster and related complications. *EClinicalMedicine*. 2025;83.
15. Cunningham AL, et al. Efficacy of the Herpes Zoster Subunit Vaccine in Adults 70 Years of Age or Older. *N Engl J Med*. 2016;375(11):1019-1032.
16. Dagnew AF, et al. Immunogenicity and safety of the adjuvanted recombinant zoster vaccine in adults with haematological malignancies: a phase 3, randomised, clinical trial and post-hoc efficacy analysis. *Lancet Infect Dis*. 2019;19(9):988-1000.

17. Leroux-Roels I, et al. A Phase 1/2 Clinical Trial Evaluating Safety and Immunogenicity of a Varicella Zoster Glycoprotein E Subunit Vaccine Candidate in Young and Older Adults. *J Infect Dis*. 2012;206(8):1280-1290.

18. Heineman TC, et al. Understanding the immunology of Shingrix, a recombinant glycoprotein E adjuvanted herpes zoster vaccine. *Curr Opin Immunol*. 2019;59:42-48.

19. Nauta JJP, et al On the relationship between mean antibody level, seroprotection and clinical protection from influenza. *Biologicals*. 2009;37(4):216-221.

20. Blanchard-Rohner G, et al. Baseline polysaccharide-specific antibodies may not consistently inhibit booster antibody responses in infants to a serogroup C meningococcal protein-polysaccharide conjugate vaccine. *Vaccine*. 2012;30(28):4153-4159.

21. Gilbert PB, et al. Fold rise in antibody titers by measured by glycoprotein-based enzyme-linked immunosorbent assay is an excellent correlate of protection for a herpes zoster vaccine, demonstrated via the vaccine efficacy curve. *J Infect Dis*. 2014;210(10):1573-1581.

22. Gardner EM, et al. Characterization of antibody responses to annual influenza vaccination over four years in a healthy elderly population. *Vaccine*. 2001;19(32):4610-4617.

23. Tsang JS, et al. Improving Vaccine-Induced Immunity: Can Baseline Predict Outcome? *Trends Immunol*. 2020;41(6):457.

24. Zhao T, et al. Vaccine adjuvants: mechanisms and platforms. *Signal Transduction and Targeted Therapy 2023 8:1*. 2023;8(1):1-24.

25. He XS, et al. Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J Virol*. 2006;80(23):11756-11766.

26. Herrero-Fernández I, et al. Effect of homeostatic T-cell proliferation in the vaccine responsiveness against influenza in elderly people. *Immunity and Ageing*. 2019;16(1):1-12.

27. Dos Santos G, et al. Enhanced safety surveillance of GSK's quadrivalent seasonal influenza vaccine in Germany and Spain (2021/2022 season) using an electronic patient-reported outcome system for vaccine safety remote monitoring. *Influenza Other Respir Viruses*. 2023;17(3).

28. GSK. Package Insert - Shingrix. Published online 2025. https://gskpro.com/content/dam/global/hcpportal/en_SG/products/PDF/shingrix/pdf/shingrix-pi-gdsv06si-approved-13dec2021.pdf.

29. Arvin A. Aging, Immunity, and the Varicella–Zoster Virus. *New England Journal of Medicine*. 2005;352(22):2266-2267.

30. McElhaney JE, et al. T-cell immunity to influenza in older adults: A pathophysiological framework for development of more effective vaccines. *Front Immunol*. 2016;7(FEB):180251.

31. Reed SG, et al. Key roles of adjuvants in modern vaccines. *Nature Medicine 2013 19:12*. 2013;19(12):1597-1608.

32. Roman F, et al. Adjuvant system AS01: from mode of action to effective vaccines. *Expert Rev Vaccines*. 2024;23(1):715-729.

33. Coccia M, et al. Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFNγ response promoting vaccine immunogenicity. *NPJ Vaccines*. 2017;2(1).

34. Stylianou VV., et al. Innate immune cell activation by adjuvant AS01 in human lymph node explants is age independent. *J Clin Invest*. 2024;134(22).

35. Leroux-Roels I, et al. Improved $CD4^+$ T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: a randomized trial. *Vaccine*. 2013;31(17):2196-2206.

36. Kester KE, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *J Infect Dis*. 2009;200(3):337-346.

37. Guerrera G, et al. BNT162b2 vaccination induces durable SARS-CoV-2–specific T cells with a stem cell memory phenotype. *Sci Immunol*. 2021;6(66).

38. Gaucher D, et al. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *Journal of Experimental Medicine*. 2008;205(13):3119-3131.

39. Cunningham AL, et al. Immune Responses to a Recombinant Glycoprotein E Herpes Zoster Vaccine in Adults Aged 50 Years or Older. *J Infect Dis*. 2018;217(11):1750-1760.

40. Jacobs MME, et al. Trained immunity is regulated by T cell-induced CD40-TRAF6 signaling. *Cell Rep*. 2024;43(9):114664.

41. Henein MY, et al. The Role of Inflammation in Cardiovascular Disease. *International Journal of Molecular Sciences 2022, Vol 23, Page 12906*. 2022;23(21):12906.

42. Xiao L, Harrison DG. Inflammation in Hypertension. *Canadian Journal of Cardiology*. 2020;36(5):635-647.

43. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci*. 2014;69 Suppl 1:S4-S9.

44. Gao Z, et al. T-cell exhaustion in immune-mediated inflammatory diseases: New implications for immunotherapy. *Front Immunol*. 2022;13.

45. Koeken VACM, et al. BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. *J Clin Invest*. 2020;130(10):5591-5602.

46. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol*. 2011;89(2):207-215.

47. Diks AM, et al. Highly Sensitive Flow Cytometry Allows Monitoring of Changes in Circulating Immune Cells in Blood After Tdap Booster Vaccination. *Front Immunol*. 2021;12:666953.

48. Calabro S, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine*. 2011;29(9):1812-1823.

49. WHO Manual on Animal Influenza Diagnosis and Surveillance. Published online 2002. Accessed August 5, 2025. https://iris.who.int/handle/10665/68026

50. Moris P, et al. H5N1 Influenza Vaccine Formulated with AS03A Induces Strong Cross-Reactive and Polyfunctional CD4 T-Cell Responses. *Journal of Clinical Immunology 2010 31:3*. 2010;31(3):443-454.

51. Essink BJ, et al. Safety and immunogenicity of a modified mRNA-lipid nanoparticle vaccine candidate against COVID-19: Results from a phase 1, dose-escalation study. *Hum Vaccin Immunother*. 2024;20(1).

Figures

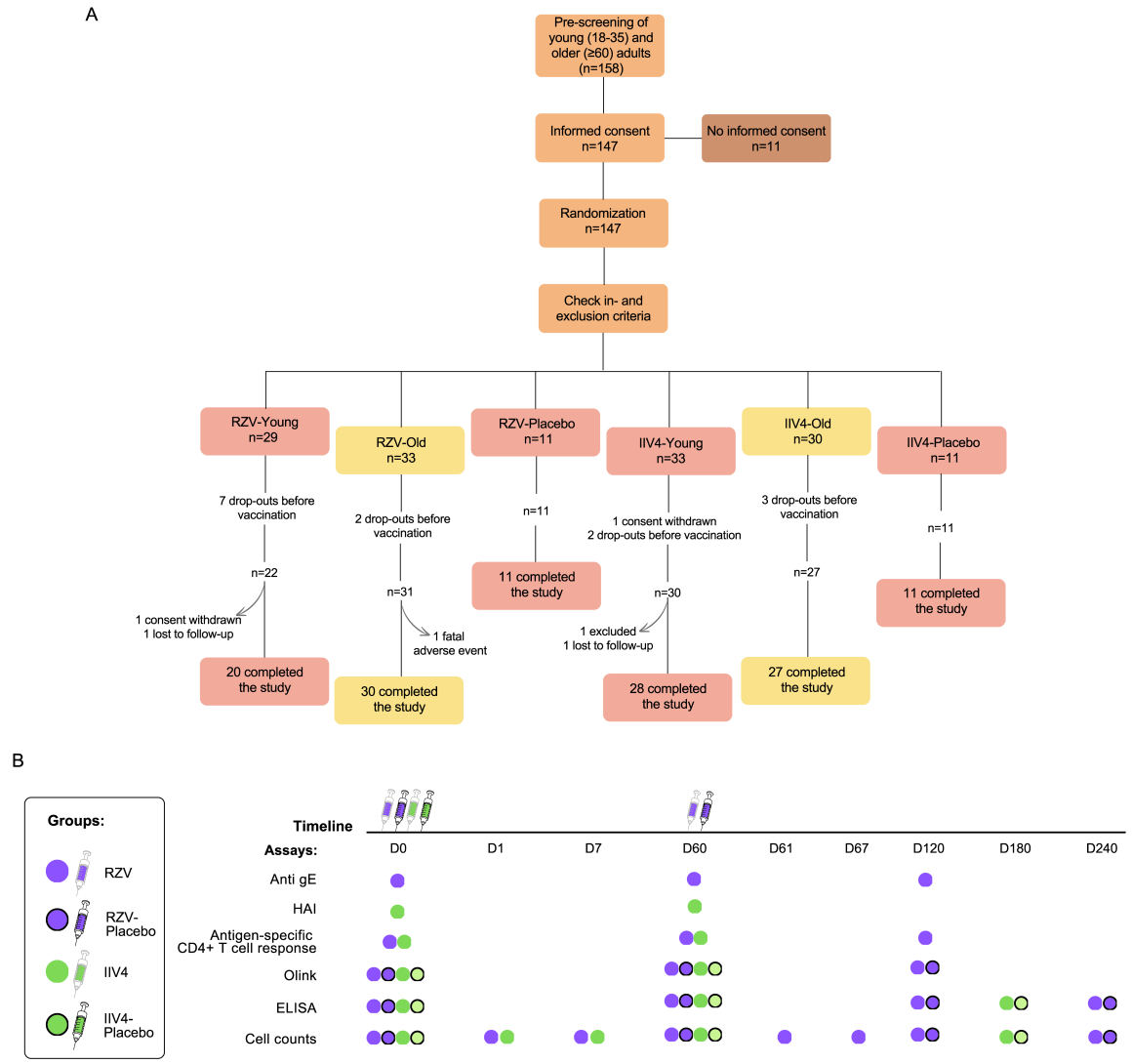


Figure 1: Summary of the clinical study and the time points of each assay.

A) The flow diagram depicts the participant recruitment, randomization, and number of people who started and finished the study. **B)** Vaccination groups in the study and the time points for each injection and experiment.

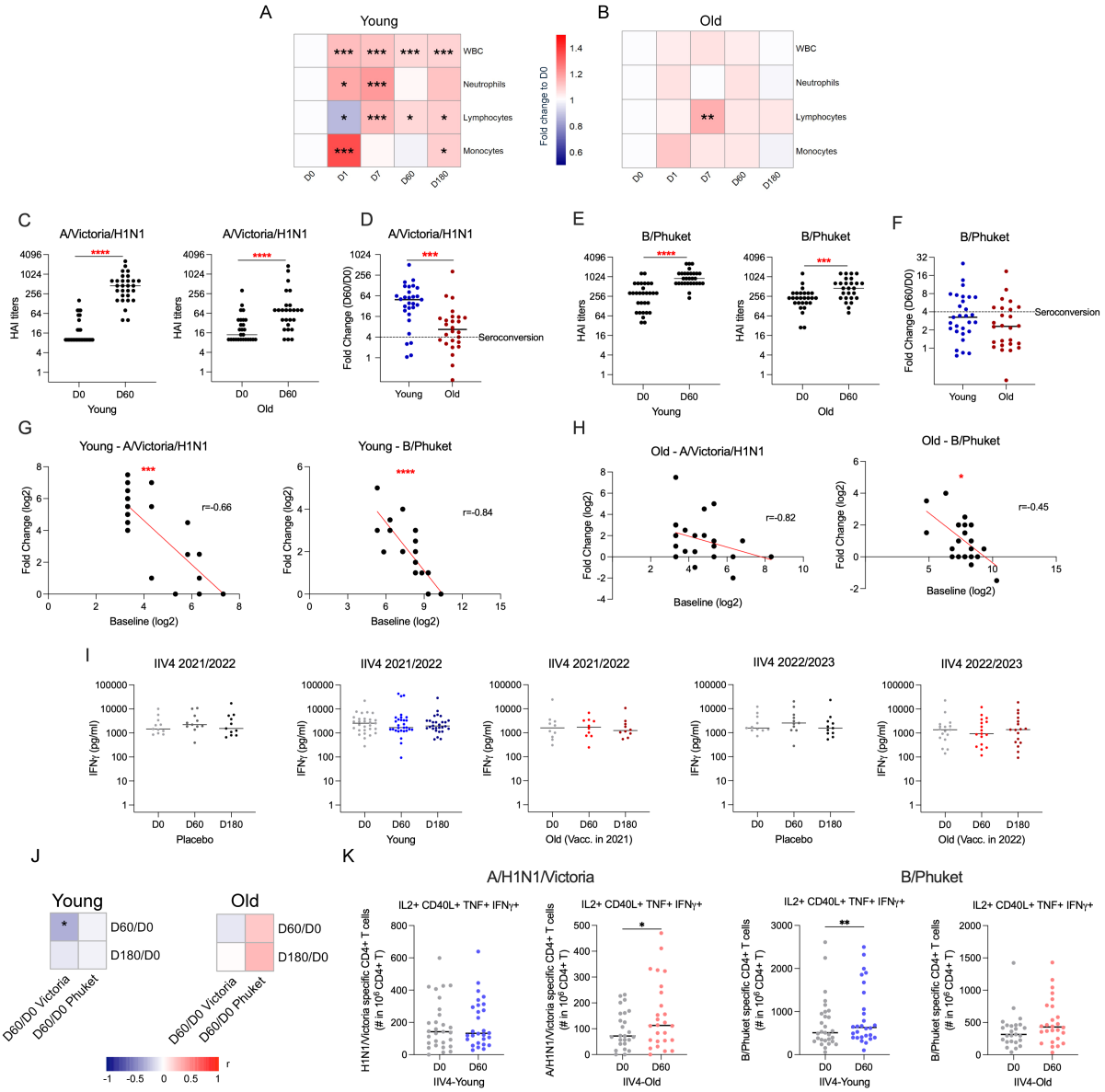


Figure 2: IIV4-induced changes in immune cell counts and adaptive immune responses in young and older adults.

Heatmaps showing the fold changes in immune cell counts after IIV4 vaccination to before vaccination in the **A)** young and **B)** older groups. Fold changes at D1, D7, D60 and D180 were compared to D0, after correction for multiple testing using the Benjamini-Hochberg method. The scale bar displays the fold change values, while the stars on the heatmap represent the FDR values. **C)** HAI titers and **D)** comparison of fold changes in antibody production against the A/Victoria/H1N1 strain. **E)** HAI titers and **F)** comparison of fold changes (D60/D0) in antibody production against the B/Phuket strain.

Spearman correlation between baseline HAI titers against the A/Victoria/H1N1 and B/Phuket strains and fold changes after IIV4 vaccination in **G)** young and **H)** older adults. **I)** IFN-γ production from PBMCs following a 7-day stimulation with 1μg/ml of IIV4 2021/2022 and 2022/2023 seasons. PBMCs from each individual were stimulated with the same season of the vaccine they received. **J)** Spearman correlation of fold IFN-γ response (D60/D0) and fold HAI titers (D60/D0) for the A/Victoria/H1N1 and B/Phuket strains. The scale bar indicates the correlation coefficient (r). **K)** The frequency of activated (4-1BB+) CD4+ T cells per 10^6 CD4+ T cells that were positive for CD40L, IL-2, TNF, and IFN-γ after stimulation with A/H1N1/Victoria and B/Phuket. The y-axis values in panels C-F are displayed on a log2 scale, while those in panel I were shown on a log10 scale. The dashed lines on graphs in panels D and F indicate the threshold of seroconversion (a fold change of 4). Different time points within the same group were compared using the Wilcoxon signed-rank test, while fold changes between young and older adults were compared using the Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

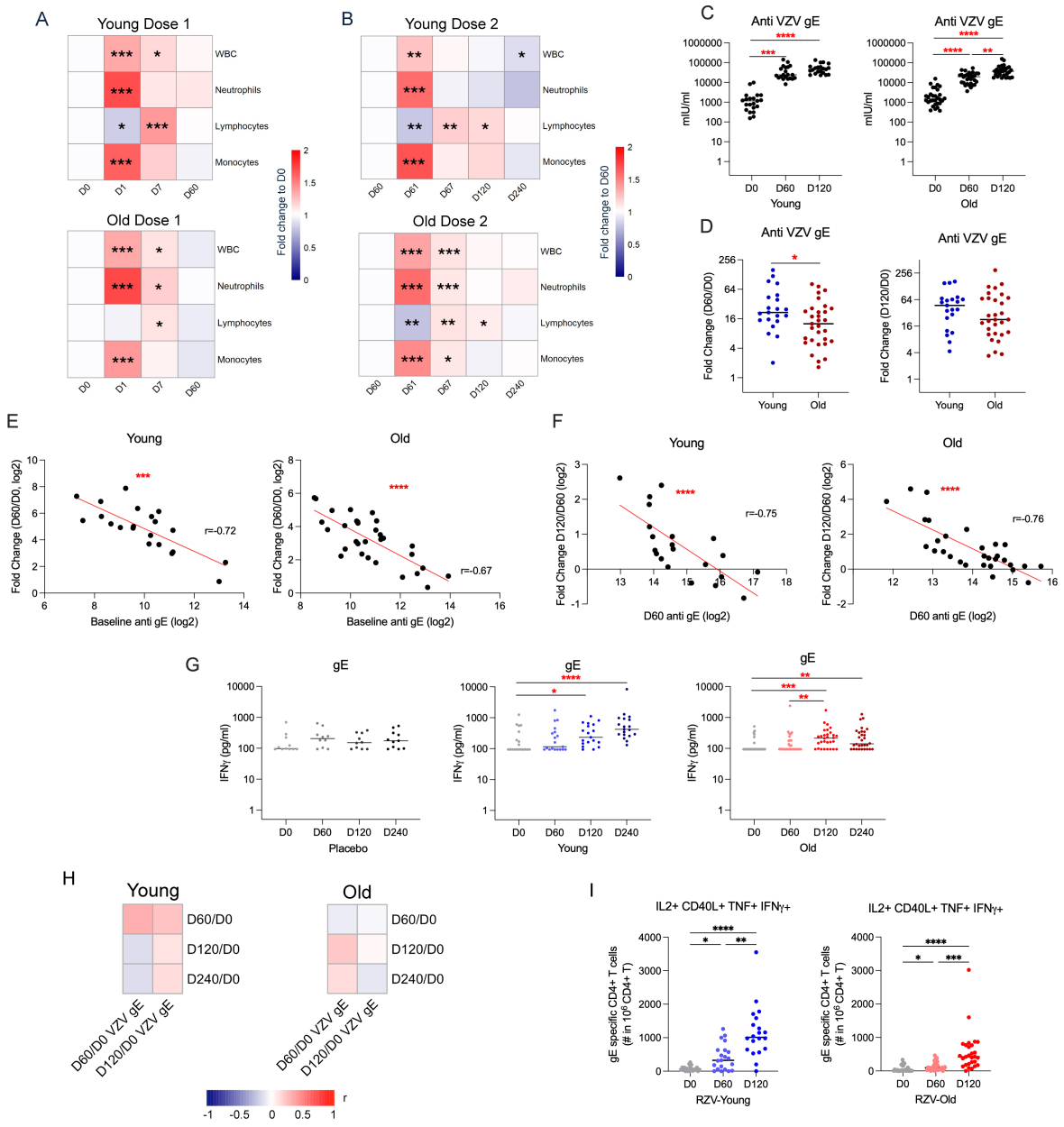
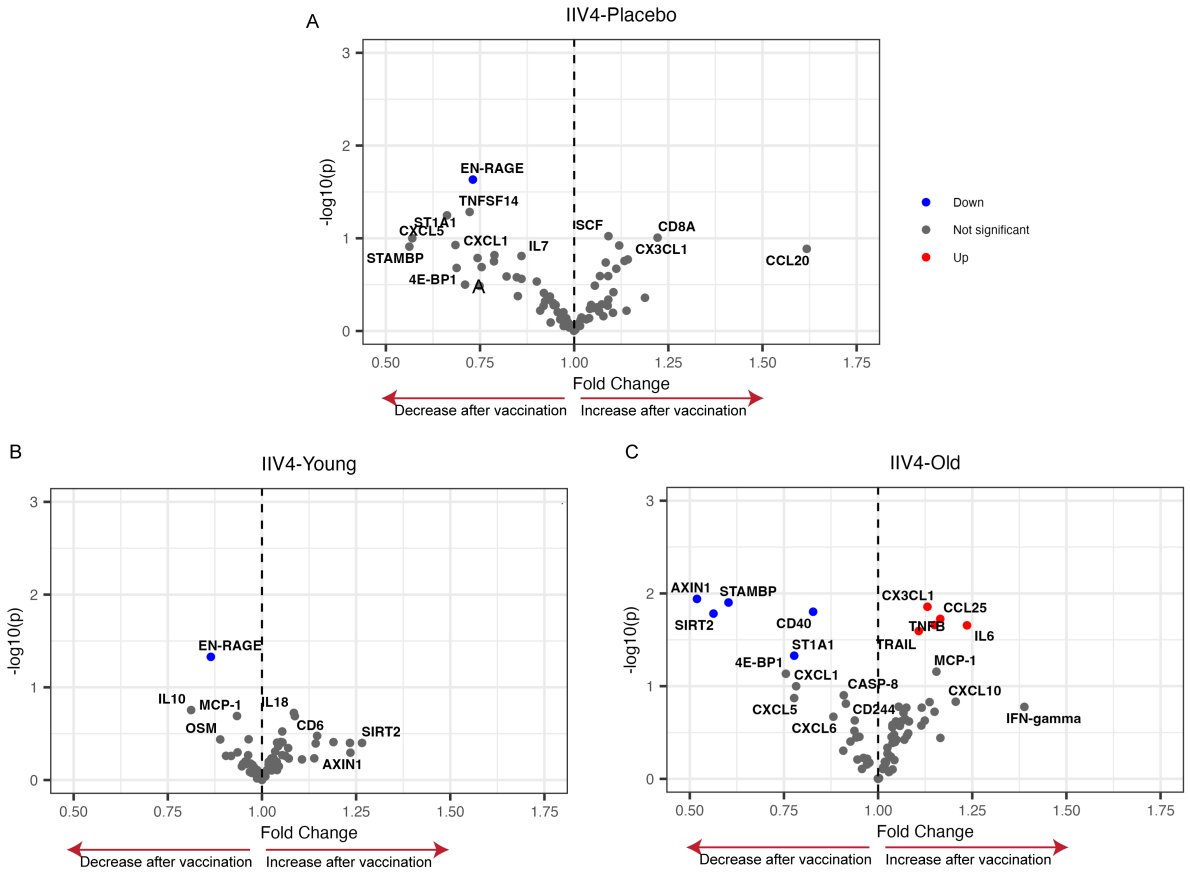


Figure 3: RZV-induced changes in immune cell counts and adaptive immune responses in young and older adults.

Heatmaps showing the fold changes in immune cell counts after the **A)** first and **B)** second dose of RZV vaccination. Fold changes at D1, D7, D60 were compared to D0, while those at D61, D67, D120 and D240 were compared to D60 using the Wilcoxon signed-rank test, with p-values corrected for multiple testing using the Benjamini-Hochberg method. The scale bar displays the fold change values, while the stars on the heatmap represent the FDR values. **C)** Anti HZV gE antibody concentrations and **D)**

comparison of fold changes (D60/D0 and D120/D0) after RZV vaccination in young and older individuals. **E)** Spearman correlation between baseline concentrations of anti VZV gE antibodies and fold antibody response (D60/D0) after RZV vaccination. **F)** Spearman correlation between pre-second vaccination (D60) anti VZV gE antibody concentrations and fold antibody response (D120/D60) after second dose. **G)** IFN-γ production from PBMCs following a 7-day stimulation with the 4 μg/ml of the antigen (gE) in RZV **H)** Spearman correlation of fold IFN-γ responses (D60/D0 and D120/D0 and D240/D0) and fold antibody productions (D60/D0 and D120/D0). The scale bar indicates the correlation coefficient (r). **I)** The frequency of activated (4-1BB+) CD4+ T cells per 10^6 CD4+ T cells that were positive for CD40L, IL-2, TNF, and IFN-γ after stimulation with the gE antigen in young and older adults. The y-axis values in panel C and G are displayed on a log10 scale, while those in panel D are shown on a log2 scale. Different time points within the same group were compared using Dunn's multiple comparisons test, and fold changes between young and older adults were compared using the Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



759

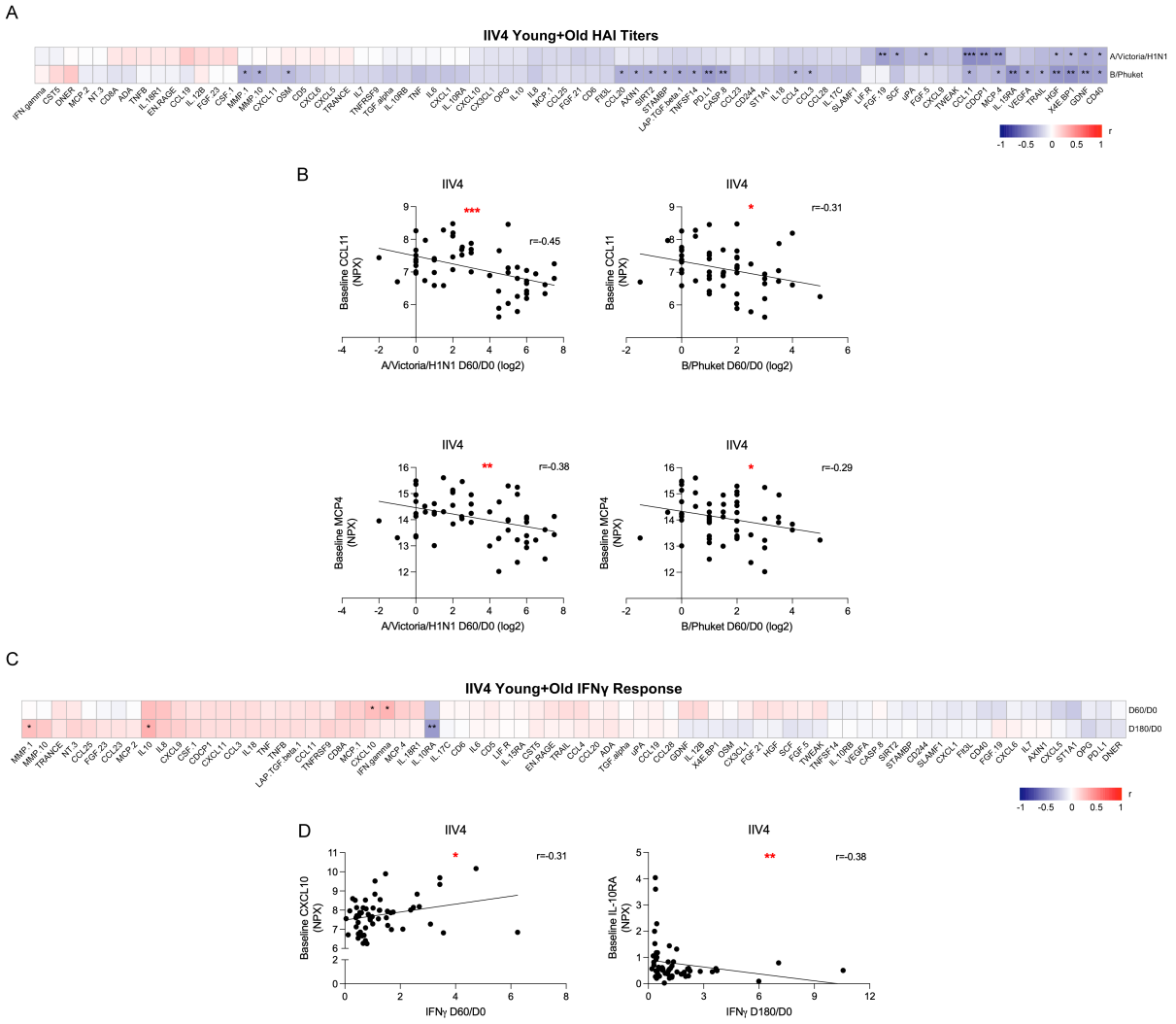
760 **Figure 4: IIV4-induced changes in circulating protein concentrations in young and older adults.**

761 Volcanos displaying the changes in proteins in the **A)** placebo group, **B)** young and **C)** older adults 60

762 days post-vaccination. Blue dots represent the significantly downregulated proteins after vaccination,

763 while red dots show the significantly upregulated ones. A moderated t-test was used in the R package

764 *limma* to compare protein concentrations before and after vaccination. (nominal p-value<0.05).



765

766 **Figure 5: Correlation between circulating protein concentrations at baseline and IIV4-induced**
767 **immune responses across combined age groups.**

768 **A)** Heatmap depicting the association between baseline concentrations of circulating immune-related
769 proteins and the fold antibody response (D60/D0) against the A/Victoria/H1N1 and B/Phuket strains
770 following IIV4 vaccination. **B)** Example correlation plots showing the relationship between CCL11 and
771 MCP4 with the fold HAI titers to A/Victoria/H1N1 and B/Phuket. **C)** Heatmap illustrating the correlation
772 between baseline circulating immune-related proteins and the fold IFN-γ response (D60/D0 and
773 D180/D0) from PBMCs after a 7-day stimulation with 1 µg/ml of IIV4. **D)** Example correlation plots of
774 baseline CXCL10 levels with D60/D0 fold IFN-γ induction, and IL-10RA with D180/D0 fold IFN-γ
775 production. The Spearman correlation was used, the scale bars in panels A and C, along with the ”r”

values on the correlation graphs, represent the Spearman correlation coefficients. Stars on heatmaps indicate the nominal p-values. NPX: Normalized protein expression. *p<0.05, **p<0.01, ***p<0.001

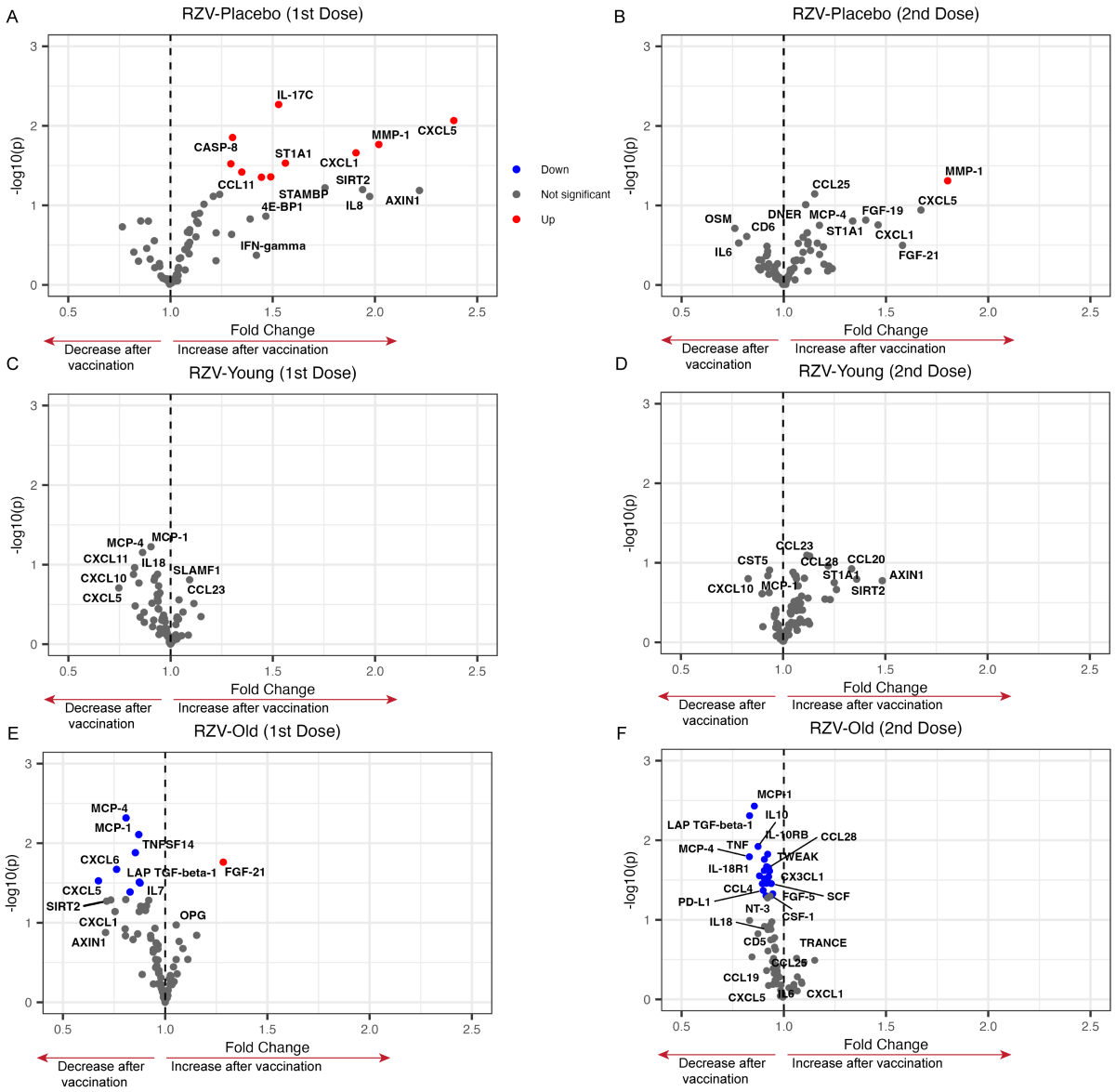


Figure 6: RZV-induced changes in circulating protein concentrations in young and older adults.

Volcano plots displaying the changes in proteins in the **A-B)** placebo group, **C-D)** young and **E-F)** older adults after the first or second dose of vaccination compared to baseline (D0). Panels A, C and E exhibit the protein fold change after the first dose (D60/D0), while panels B, D and F show the protein fold change after the second dose (D120/D0). Blue dots represent the significantly downregulated proteins after vaccination, while red dots show the significantly upregulated ones. A moderated t-test was used

in the R package *limma* to compare protein concentrations before and after vaccination. (nominal p-value<0.05).

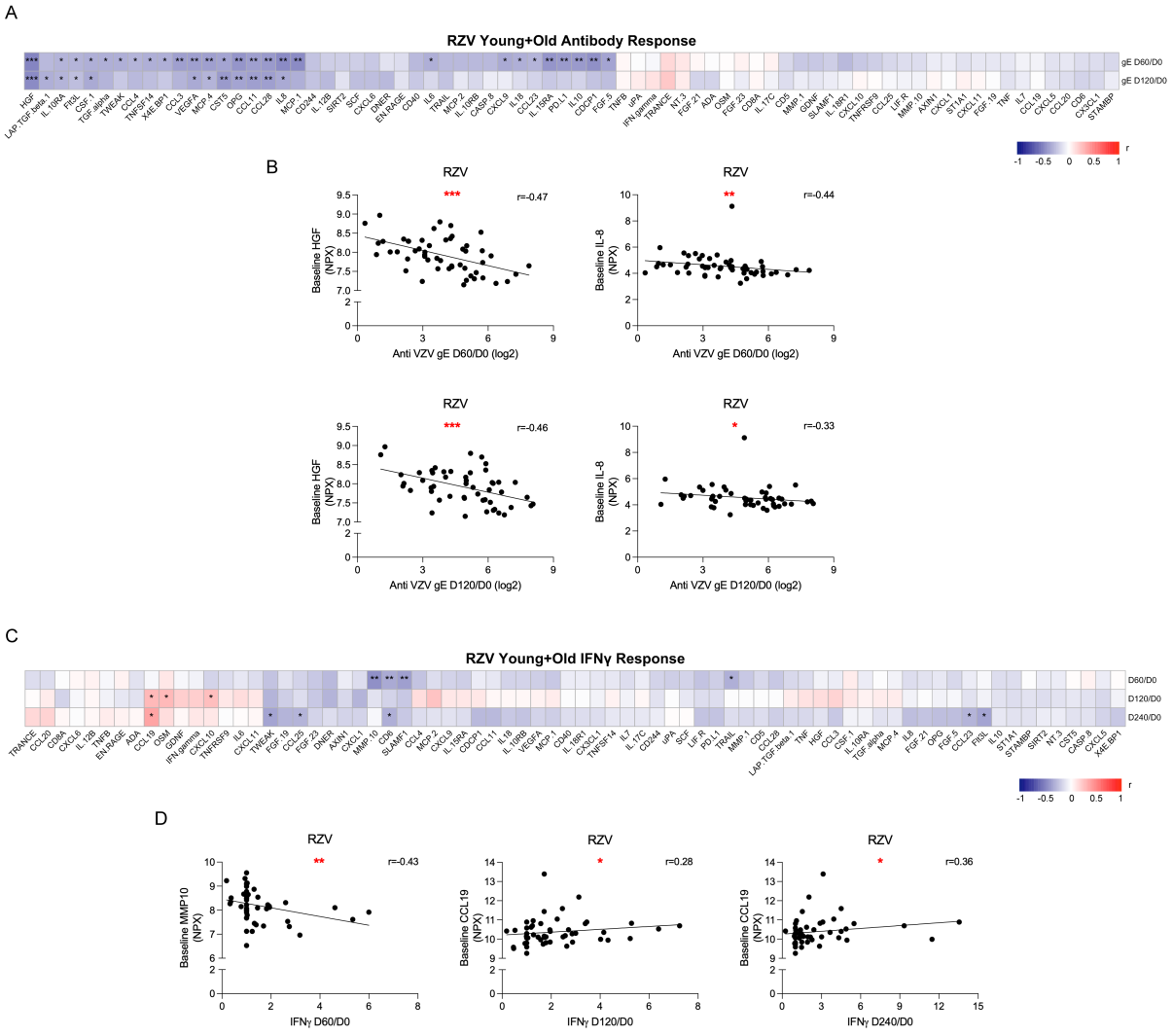


Figure 7: Correlation between circulating protein concentrations at baseline and RZV-induced immune responses across age groups.

A) Heatmap depicting the association between baseline concentrations of circulating immune-related proteins and the fold antibody response (D60/D0 and D120/D0) following RZV vaccination. **B)** Example correlation plots showing the relationship between baseline levels of HGF and IL-8 with the fold antibody response after the first (D60/D0) and second dose (D120/D0). **C)** Heatmap illustrating the correlation between baseline circulating immune-related proteins and the fold IFN-γ response (D60/D0, D120/D0 and D240/D0) from PBMCs after a 7-day stimulation with 4 µg/ml of the gE antigen in RZV. **D)** Example correlation plots of baseline MMP10 levels with D60/D0 fold IFN-γ, and CCL19 with D120/D0 and D240/D0 fold IFN-γ production. The Spearman correlation was used, the scale bars in

panels A and C, along with the ”r” values on the correlation graphs, represent the Spearman correlation coefficients. Stars on heatmaps indicate the nominal p-values. NPX: Normalized protein expression. *p<0.05, **p<0.01, ***p<0.001.

804 ## Tables

805 **Table 1:** Demographics, comorbidities, and reported risk factors of the study cohort.

	RZV-Young (n=22)	RZV-Old (n=31)	RZV-Placebo (n=11)	IIV4-Young (n=30)	IIV4-Old (n=27)	IIV4-Placebo (n=11)
Age	23.7±4.2	69.0±5.3	21.9±2.8	22.9±3.9	68.3±4.9	21.8±3.2
Sex, F (%)	13 59%	15 48%	8 73%	21 70%	14 52%	8 73%
BMI	22.0±2.3	26.5±5.1	22.0±2.8	22.6±2.2	25.1±3.2	24.5±3.3
Comorbidities (n, %)						
Chronic respiratory diseases		2 (6%)	2 (18%)		5 (19%)	
Chronic cardiovascular diseases		7 (23%)			2 (7%)	
Psychiatric diseases					4 (15%)	
Other diseases						
Eczema	1 (5%)					
Rhinitis					1 (4%)	
Polyneuropathy					1 (4%)	
Gout/hyperuricemia					1 (4%)	
IBS					1 (4%)	
Reported Risk Factors (n, %)						
Hypertension		10 (32%)			5 (19%)	
Hypercholesterolemia					2 (7%)	
Type II diabetes		1 (3%)				
PCOS			1 (9%)			1 (9%)

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807 **Table 2:** Summary of the results

	IIV4		RZV	
	Young	**Old**	**Young**	**Old**
Immune cell counts	Strong increase up to 180 days post-IIV4	Temporary increase of lymphocytes	Temporary but strong changes in cell populations	Temporary but strong changes in cell populations
HAI/anti-gE titers	Strong increase to H1N1, moderate or low increase to other strains	Strong increase to H1N1 (lower than young), low increase to other strains	Strong increase	Strong increase
IFN-γ production	No increase	No increase	Strong increase	Strong increase
Cell mediated immunity	Increase in some polyfunctional antigen-specific CD4+ T cell populations	Weak or no induction of polyfunctional antigen-specific CD4+ T cell populations	Strong increase in several polyfunctional antigen-specific CD4+ T cell populations	Strong increase in several polyfunctional antigen-specific CD4+ T cell populations (lower than young)
Circulating proteins	No effect	Vaccine year dependent effects	Less inflammation compared to placebo	Decreased systemic inflammation
Baseline inflammation vs. antibody response	Mainly negative correlations when age groups combined		Mainly negative correlations when age groups combined	
	Both positive and negative associations	Both positive and negative associations	Mainly negative correlations	Both positive and negative associations
Baseline inflammation vs. IFN-γ response	Mainly positive correlations when age groups combined		Mainly negative correlations when age groups combined	
	Both positive and negative associations	Mainly positive correlations	Mainly negative associations	Both positive and negative associations

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