Hallmarks of Improved Immunological Responses in the Vaccination of More Physically Active Elderly Females

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ABSTRACT

Physical inactivity is one of the leading contributors to worldwide morbidity and mortality. The elderly are particularly susceptible since the features of physical inactivity overlap with the outcomes of natural aging – including the propensity to develop cardiovascular diseases, cancer, diabetes mellitus, sarcopenia and cognitive impairment. The age-dependent loss of immune function, or immunosenescence, refers to the progressive depletion of primary immune resources and is linked to the development of many of these conditions. Immunosenescence is primarily driven by chronic immune activation and physical activity interventions have demonstrated the potential to reduce the risk of complications in the elderly by modulating inflammation and augmenting the immune system. Since poor vaccination outcome is a hallmark of immunosenescence, the assessment of vaccine efficacy provides a window to study the immunological effects of regular physical activity. Using an accelerator-based study, we demonstrate in a Singaporean Chinese cohort that elderly women (n=56) who walk more after vaccination display greater post-vaccination expansion of monocytes and plasmablasts in peripheral blood. Active elderly female participants also demonstrated lower baseline levels of IP-10 and Eotaxin, and the upregulation of genes associated with monocyte/macrophage phagocytosis. We further describe postive correlations between the monocyte response and the post-vaccination H1N1 HAI titres of participants. Finally, active elderly women reveal a higher induction of antibodies against Flu B in their 18-month second vaccination follow-up. Altogether, our data are consistent with better immunological outcomes in those who are more physically active and highlight the pertinent contribution of monocyte activity.

Keywords: Aging, Physical Activity, Influenza, Vaccination, Immunosenescence

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1. Introduction

Physical inactivity has been cited by the WHO as the fourth leading risk factor for global mortality (6% of deaths globally) – preceded only by high blood pressure (13%), tobacco use (9%), and high blood glucose (6%) (80). Physical activity, defined as bodily movement directed by the skeletal muscles and resulting in energy expenditure, differs from exercise as the latter refers to planned, structured, and repetitive programmes that are designed to maintain and improve physical fitness (10). Both physical activity and exercise are positively associated with beneficial outcomes in cardiorespiratory (CHD, CVD, stroke, hypertension), muscular (muscle mass, strength and power), metabolic (diabetes, metabolic syndrome), mental (depression) and immunological (immune activation levels, vaccination efficacy and immunosenescence) health (28, 29, 32, 33, 46, 60, 67, 74). Since the elderly are susceptible to a decline in all these faculties, physical activity becomes an attractive prospect of a low-cost intervention, particularly since pharmacological interventions have led to more controversial or limited efficacy in delaying the acquisition of age-related morbidities (6).

The world is aging rapidly and the population of elderly over the age of 65 is expected to approach 1.5 billion by the year 2050 (79). In alleviating the growing demands on healthcare that accompany the aging phenomenon – the elderly impose a disproportionate reliance on these resources – governments must introduce policies that keep the elderly robust. While the benefits of physical activity are relevant throughout lifespan, there is a growing body of literature that describe more pertinent benefits of physical activity in the elderly (14, 75). The age-related loss of immune function is particularly well documented as a driver of age-related causes of morbidity and mortality; the former is widely attributed to the concomitant loss of hematopoietic resources and naïve lymphocytes as well as an overrepresentation of late-stage memory lymphocytes – altogether constituting an immune system that is less poised to respond to both old and new antigens (39). Although this scenario results in poor vaccination outcomes in the elderly, there is compelling evidence that physical activity levels can compensate for these deficiencies (14, 59, 75).

Differences in population demographics or the nature of physical activity have however, contributed to heterogeneous

observations among studies that investigate the modulatory effect of physical activity on vaccination outcomes. Perhaps due to age or uniformed high levels of vaccine-responsiveness, a study on young college students demonstrated that neither physical fitness nor physical activity improved antibody responses after influenza vaccination (70). A separate study found that the antibody response of elite swimmers (aged 17-23) to pneumococcal vaccination encompassed more antibody subtypes than their sedentary counterparts (29). Nevertheless, the impact of physical activity or exercise on vaccination responses in the elderly has generally been described as positive. In the elderly, the augmentation of immune responses by physical activity has been demonstrated in the contexts of pneumococcal, meningococcal and influenza vaccinations (35, 36, 60, 69). The latter may be linked to better immunosenescent profiles in the elderly, since independent studies have revealed reduced frequencies of senescent naïve and memory T-cells as well as a reduced inflammatory signature in those who exercise regularly (46, 52, 73). Unfortunately, studies linking physical activity to advantageous influenza vaccination responses rarely investigate beyond HAI titres. Without the elaborate characterisation of post-vaccination immunity, it is a challenge to determine the nature of physical activity that is required to optimise these benefits.

In summary, while there is a strong case for promoting physical activity as a fundamental component to healthy aging, the results of these studies display a heavy reliance on self-reported physical activity or organised exercise regimes – which may be challenging to interpret and implement on a national or global level (60, 74). Moreover, the specific mechanisms responsible for generating these effects remain elusive, for example, it is unclear whether the enhancement of post-vaccination immunity results from changes in the innate or adaptive immune systems. In this study, we aim to study the value and impact of physical activity on post-vaccination immunity using a more comprehensive and multi-dimensional approach; we also validate the application of an accelerometer in achieving these aims. Using data from an accelerometer-based study of an elderly Singaporean Chinese cohort, we demonstrate that elderly women who walk more display greater post-vaccination expansion of monocytes and plasmablasts in peripheral blood compared with sedentary women. Through the transcriptomic analysis of PBMC-derived mRNA, we further reveal the upregulation of genes associated with monocyte/macrophage phagocytosis in active elderly women. Importantly, we found significant correlations between the post-vaccination expansion of monocytes and H1N1 HAI titres. Finally, active elderly women who walk more mount a stronger antibody response against Flu B in their 18-month second vaccination follow-up. Altogether, our data are consistent with enhanced vaccination outcomes in physically active females, mediated by both the innate and adaptive immune system.

2. Materials and Methods

Study participants

The National Healthcare Group (NHG) Domain Specific Institutional Review Board (DSRB) approved a phase IV clinical trial of Sanofi Pasteur's Vaxigrip© influenza vaccine, which is registered at clinicaltrials.gov under the registration number NCT03266237. We recruited older adults above 65 years of age for participation in an epidemiological study of a second cohort from the Singapore Longitudinal Aging Study (SLAS-2) (51). Participants were community dwellers from eight different housing precincts across Singapore. Volunteers were excluded if they had received an influenza vaccination in the past 6-months. Those with suspected congenital or acquired immunodeficiency as well as those receiving immunosuppressive or long-term corticosteroid therapy were also excluded. All volunteers provided written informed consent for the reception of Vaxigrip©.

Influenza vaccination

Two commercially available inactivated trivalent Vaxigrip© (2013-14) seasonal influenza vaccines were used in this trial. In the first vaccination, vaccine consisted of split virions from three prevalent strains: A/California/07/2009 (H1N1), A/Texas/50/2012 (H3N2) and B/Massachusetts/02/2012. For the second vaccination at the 20-month follow-up, this was updated to A/California/07/2009 (H1N1), A/Switzerland/97/2013 (H3N2) and B/Phuket/3073/2013. Virions were grown in embryonated chicken eggs, inactivated by formaldehyde treatment, and split via anionic detergent. The first vaccine was administered to elderly study participants between January and August 2014. Venous blood specimens were collected from the participants immediately prior to vaccination (Day 0) and on Days 2, 7 and 28 after vaccination. For the second vaccination, blood specimens were collected immediately prior to vaccination and 28-days post-vaccination. Participants with HAI titres >40 (1/dil) were considered seroprotected.

Actigraphy data collection

On the day of vaccination, participants were given a wrist-worn device, Actical© (Phillips, Amsterdam, Netherlands), which they agreed to wear on their wrist for a 14-day period that commenced immediately after vaccination. The device does not interfere with any of the participant's daily activities and participants were not instructed to modify their daily routine and behaviour. The device was used to monitor gross motor activity and collected data were analysed by the software Actical© v3.1 (Philips Electronics, Amsterdam, Netherlands), which in addition to the number of steps completed, provides percentage data on the participant's sedentary period. Although 183 participants consented to actigraphy data collection, we divided participants into quartiles based on their number of steps completed. Participants who belonged to the highest and lowest quartiles were included in the analyses described in this study (Females, n=56; Males, n=34).

Determination of Frailty Status

At the time of recruitment, demographic, medical, psychosocial, behavioural, and neurocognitive variables were collected from study participants by clinical personnel through interviews and on-site clinical assessment. Data specific to the Instrumental Activities of Daily Living (IADL), Short-from Health Survey (SF-12), Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS), Geriatric Anxiety Inventory (GAI) and Montreal Cognitive Assessment (MOCA) were collected as described in validated studies (40,

48, 58, 59, 77, 82). In addition, participants self-reported answers to questions related to their occupational, socio-economic, medical history, exercise and nutritional habits, which were presented in the form of a questionnaire. Physical frailty was defined according to Fried's assessment of five criteria, including unintentional weight loss, slowness, weakness, exhaustion, and low physical activity (25), and scaled to Asian populations as described previously (51).

Characterisation of Sarcopenic Status

Total and regional lean body mass was measured by dual energy X-ray absorptiometry (DXA) scan with a Hologic® densitometer. The participant laid in a supine position on the DXA table with limbs close to the body. DXA scans were performed in the Department of Diagnostic Radiology, National University Hospital (NUH) of Singapore. Appendicular muscle mass was calculated as described by Heymsfield *et. al.* (31). Lower limb strength was assessed using the methods described by Lord *et. al.,* and body mass index (BMI) values were calculated by averaging triplicate measurements (45). Participants were required to complete the 6-meter gait speed test as described by Nelson *et al.,* duplicate measurements were averaged for the determination of gait speed in meters/second (50). In accordance to recommendations by the Asian Working Group for Sarcopenia (AWGS), sarcopenic status was determined using measures of appendicular lean mass, lower leg limb strength and gait speed (11). The appendicular skeletal muscle index (ASMI) was calculated as the ratio between appendicular muscle mass and height^2. Low ASMI refers to values below 7.0 kg/m^2 and 5.4 kg/m^2 for men and women respectively. Cut-off values for low lower limb strength are as indicated: ≤ 18 kg for men and ≤ 16 kg for women. Low gait speed was defined as an average speed of 2 trials ≤ 0.8 m/s. Finally, a participant was categorized as sarcopenic if they had low ASMI and low lower limb strength and/or gait speed.

Vaccine-specific (HAI) antibody titres

Vaccine-specific antibodies were measured using sera collected at Days 0 and 28 after both vaccinations using the Hemagglutination inhibition (HAI) assay. Two independent assays were concluded for the determination of HAI titres. Samples were first heat inactivated and pre-treated with neuraminidase to eliminate non-specific inhibitors and anti-turkey red blood cell (anti-TRBC) hemagglutinins, which may interfere with the test results. Treated sera were then serially titrated, starting at a 1/10 dilution, and incubated with 4 hemagglutinating units/25 µl of the virus. After incubation at 37°C for 1 h, a 0.5% TRBC suspension was added to the plates and was incubated for one hour at ambient temperature. Plates were then read using the tilt method, and the HAI titre was assigned as the reciprocal of the highest serum dilution that exhibited complete inhibition of hemagglutination. The geometric mean titre of both independent runs was used to determine the final titre.

Cytokine Measurements by Luminex

The Milliplex© human cytokine/chemokine panel (Cat: EPX090-12187-901; Merck Millipore, Massachusetts, USA) was used to measure the levels of IFNγ-inducible protein 10 (IP-10) in plasma samples from participants that were collected at baseline and Days 2, 7 and 28 post-vaccination. Eotaxin and high sensitivity C-reactive protein (hs-CRP) were only measured at baseline. Samples and standards were incubated with fluorescent-coded magnetic beads which had been pre-coated with the respective capture antibodies. After overnight incubation at 4°C with shaking, plates were washed twice with wash buffer. Biotinylated detection antibodies were incubated with the complex for 1 hour and Streptavidin-PE was subsequently added for another 30 mins incubation. Plates were washed twice before beads were re-suspended in sheath fluid and read using PCR plates by the Luminex analyser, FLEXMAP© 3D (Merck Millipore, Massachusetts, USA). Data were acquired using the xPONENT© 4.0 (Luminex©, Texas, USA) acquisition software and analysed with a Bio-Plex Manager 6.1.1© (Bio-Rad, California, USA). Standard curves were generated, and a 5-parameter logistic algorithm were used for the estimation of MFI and concentration values.

Immunophenotyping by flow cytometry

Percentages of immune cell subsets were determined using cryopreserved peripheral blood mononuclear cells (PBMCs). Cryopreserved PBMCs were quick-thawed at 37˚C and resuspended in warm RPMI. After a second wash, PBMCs were counted and plated into 96-well plates for subsequent stain-

Table I: T-cell Antibody Panel

Target	Company	Clone	Dye	Catalog
Vd1	Abcam	TS8.2	FITC	ab171097
Vd2	Biolegend	B6	PerCP	331410
CD4	Biolegend	OKT4	PE-Cy5	317412
CD31	Biolegend	WM59	BV605	303122
CTLA4	Biolegend	L3D10	PE-Cy7	349914
CXCR5	Biolegend	J252D4	PE	356904
PD1	eBioscience	eBioJ105	APC-eF780	47-2799-42
CD3	BD	UCHT1	AF700	557943
CD8	BD	RPA-T8	BV711	563677
CD27	BD	L128	BV650	563228
CD28	BD	CD28.2	BUV737	564438
CD45RO	BD	UCHL1	BUV 395	564291
CD95	BD	DX2	PE-CF594	562395
KLRG1	BD	2F1/KLRG1	APC	561620

Table II: B-cell Antibody Panel

Target	Company	Clone	Dye	Catalog
CD10	BD	HI10a	PE-CF594	562396
CD19	BD	HIB19	AF700	557921
CD21	BD	B-ly4	PE-Cy5	551064
CD24	BD	ML5	BV421	562789
CD27	BD	L128	BV650	563228
CD38	BD	HIT2	APC	555462
CD45	BD	HI30	PerCP/Cy5.5	564105
CD138	BD	MI15	BV605	563294
IgD	BD	IA6-2	FITC	555778
IgM	Biolegend	MHM-88	APC-Cy7	314520
CD20	Biolegend	2H7	BV570	302332
CD40	Biolegend	5C3	PE-Cy7	334322
CXCR5	Biolegend	J252D4	PE	356904
HLA-DR	Biolegend	L243	BV785	307642

ing. 0.5, 0.75 and 1.5 million cells were used for T-cell, B-cell and Myeloid phenotyping panels respectively (Tables I, II and III respectively); antibodies were procured from eBioscience (California, USA), Biolegend (California, USA), BD Biosciences (California, USA) and Miltenyi Biotec (Bergisch Gladback, Germany). All panels contain a Thermo Fisher Live/Dead© fixable dye (Cat: L-34957; Thermo Fisher, Massachusetts, USA) to distinguish between live and dead cells. Antibody cocktails were added to aliquoted cells and incubated for 20 minutes in the dark at 4˚C. The cells were then washed twice before resuspension in staining buffer and analysed on the LSR II (BD Biosciences, California, USA) cytometer. Data generated by flow cytometry was analysed with the Flowjo© software (Tree Star, Inc., USA). Events were gated by forward and side scatter followed by subset-specific marker expression.

For deriving absolute counts, the numbers of monocytes, B cells, plasmablasts, CD4 and CD8 T cells, NK cells, and conventional and plasmacytoid dendritic cells were determined using freshly collected whole blood samples. 100 µL of whole blood was stained with antibody cocktail (Table IV) in BD Trucount© Absolute Counting Tubes (Cat: 340334; BD Biosciences, California, USA) for 15 minutes at room temperature. 900 µL of 1X BD FACS Lysing solution (Cat: 349202; BD Biosciences, California, USA) was then added to the tube and incubated for 15 minutes before acquisition on the LSR II Fortessa (BD Biosciences, California, USA) flow cytometer.

Table III: Myeloid Antibody Panel

Target	Company	Clone	Dye	Catalog
CD3 (Lin)	BD	UCHT1	AF700	557943
CD19 (Lin)	BD	H1B19	AF700	557921
CD20 (Lin)	Biolegend	2H7	AF700	302322
CD56 (Lin)	Biolegend	HCD56	AF700	318316
CD2	BD	RPA-2.10	APC H7	562638
CD11c	BD	BLy6	FITC	561355
CD15	BD	HI98	BUV395	563872
CD16	BD	3G8	BUV 737	564434
CD33	BD	WM53	BV421	562854
CD45	BD	HI30	PerCP-Cy5.5	564105
CD80	BD	L307.4	BV650	564158
CD1c	Biolegend	L161	PE-Cy7	331516
CD11b	Biolegend	ICRF44	BV711	301344
CD14	Biolegend	HCD14	PE-Dazzle 594	325634
CD123	Biolegend	6H6	BV510	306022
CD141	Biolegend	M80	APC	344106
HLA DR	Biolegend	L243	BV605	307640
SLAN	Miltenyi	DD-1	PE	130-093-029

Table IV: Trucount Antibody Panel

Target	Company	Clone	Dye	Catalog
CD19	BD	SJ25C1	BV786	563325
CD38	BD	HIT2	APC	555462
CD3	Biolegend	OKT3	PE/cy7	317334
CD4	Biolegend	OKT4	BV510	317444
CD14	Biolegend	M5E2	PerCP	301824
CD16	Biolegend	3G8	A700	302026
CD27	Biolegend	M-T271	PE	356406
CD45	Biolegend	HI30	PB	304029
CD62L	Biolegend	DREG-56	APC/cy7	304814
CD66b	Biolegend	G10F5	FITC	305104
HLA-DR	Biolegend	L243	BV605	307640
CD8	eBioscience	RPA-T8	PE TR	61-0088-42
CD56	eBioscience	CMSSB	PE/cy5.5	35-0567-42
CD123	eBioscience	6H6	BV650	95-1239-42

Determination of Vaccine Specific B-cells by ELISpot

PBMCs were thawed and counted as described in the immunophenotyping section. PBMCs were plated into 4 wells at 0.1 million PBMCs per well, incubated with IL-21, and separately treated with the following conditions: unstimulated, vaccine-stimulated (0.3 µg/mL of trivalent vaccine) in duplicates and positive control as stimulated by both anti-CD40 (Cat: MAB6321; RnD Systems, Minnesota, USA) and CpG oligonucleotides (CpG ODN 2006, Cat: tlrl-2006; Invivogen, California, USA). PBMCs were stimulated for 22 hours in a humidified 37˚C CO2 incubator. Vaccine-specific IgG-secreting B-cells were detected using the Human IgG ELISpot kit© (Cat: 3850-2H; Mabtech, Nacka Strand, Sweden) by adhering to the manufacturer's protocol. ELISpot plates were read using an ImmunoSpot S5 Versa Analyser (Cellular Technology Limited, Ohio, USA). The enumeration of vaccine-specific IgG-secreting B-cells was performed by averaging the number of spots detected from duplicate vaccine-stimulated wells.

Microarray, Differentially Expressed Genes (DEGs) and Ingenuity Pathway Analysis (IPA)

Total ribonucleic acid (RNA) were isolated from PBMCs using the mirVana© miRNA isolation kit (Cat: AM1560; Thermo Fisher Scientific, California, USA). Complementary DNA (cDNA) were generated and purified using the cDNA synthesis kit (Cat: K2561; Thermo Fisher Scientific, California, USA). Gene expression was analysed by microarray using the Illumina human HT-12 V4.0 bead chip platform, TargetAmp©-Nano-g Biotin-aRNA Labeling Kit for Illumina (Cat: TAN07908; Epicenter, Wisconsin, USA). The raw gene expression data output from Illumina Genome Studio© (Illumina, California, USA) were exported in batches of 96 samples (21). Quality control (QC) and pre-processing of data were completed using Bioconductor© packages for the R software. The data were log2 transformed and normalized using the Robust Spline Normalization (RSN) method available in the Lumi package (Bioconductor) (17). Only probes which passed a detection p-value of 0.05 in 90% of the subjects were retained. Differential gene expression analysis between sedentary and active individuals was performed using the Limma package (Bioconductor) with age included as a cofactor within the linear model (68). Differentially expressed genes (DEGs) were selected based on a nominal p-value < 0.05. The list of DEGs along with expression fold changes and p-values were loaded into Ingenuity Pathway Analysis software (IPA®; Qiagen Bioinformatics, California, USA). A standard core analysis was performed in IPA to identify pathways enriched with the DEGs (38). The microarray data is available on Gene Expression Omnibus (NCBI) under the accession number GSE107990 (13).

Statistical Methods

Demographic parameters were compared between sedentary and active individuals by single-factor ANOVA for numerical and Fisher's exact test for categorical data, respectively. Per-

Table 1: Participation Demographics and Serology

Participant Demographics	Female Sedentary (n = 28)	Female Active (n = 28)	p-value	Male Sedentary (n = 17)	Male Active (n = 17)	p-value
Age	75.4 ± 5.1	70.2 ± 3.9	0.00	75.9 ± 3.9	72.1 ± 4.5	0.01
Frail	13 (46%)	10 (36%)	0.59	8 (47%)	5 (29%)	0.48
Frailty Score	2.2 ± 1.5	1.8 ± 1.5	0.37	1.9 ± 1.3	1.6 ± 1.2	0.42
Sarcopenia	10 (36%)	13 (46%)	0.59	7 (42%)	3 (18%)	0.05
BMI	23.1 ± 2.3	22.9 ± 3.5	0.77	23.4 ± 3.8	24.2 ± 2.3	0.43
Waist circumference (cm)	84.1 ± 11.2	81.1 ± 6.5	0.24	90.7 ± 12.00	89.5 ± 6.00	0.73
Smoker (Past or Present)	0 (0%)	1 (4%)	1.00	9 (53%)	7 (41%)	0.73
Currently employed	0 (0%)	6 (21%)	0.02	1 (6%)	2 (12%)	0.60
Number of Comorbidities	2.2 ± 1.5	1.7 ± 1.4	0.77	1.9 ± 1.3	1.4 ± 1.2	0.43
High Blood Pressure	14 (50%)	12 (43%)	0.79	12 (71%)	10 (59%)	0.26
High Cholesterol	18 (64%)	14 (50%)	0.42	10 (59%)	9 (53%)	1.00
Diabetes	8 (29%)	5 (18%)	0.55	5 (29%)	4 (24%)	0.63
Pre-diabetic (abnormal fasting glucose)	11 (39%)	12 (43%)	1.00	4 (24%)	6 (35%)	0.71
Arthritis	7 (25%)	4 (14%)	0.50	4 (24%)	0 (0%)	0.10
Undergone Surgery	19 (68%)	17 (61%)	0.78	6 (35%)	6 (35%)	1.00
Mini Mental State Examination (MMSE)	26.5 ± 2.4	27.8 ± 2.4	0.05	25.4 ± 5.0	28.2 ± 1.4	0.03
Montreal Cognitive Assessment (MoCA)	24.6 ± 4.1	25.9 ± 3.00	0.24	23.4 ± 4.8	25.8 ± 1.9	0.08
Geriatric Anxiety Inventory (GAI)	0.3 ± 1.0	0.4 ± 1.3	0.73	0.5 ± 1.2	0.0 ± 0.0	0.11
Geriatric Depression Score (GDS)	0.8 ± 0.9	1.0 ± 1.5	0.45	0.76 ± 1.7	0.3 ± 0.9	0.32
Immunity and Post-Vaccine Seroprotection						
CD4 T cells (cells/ul)	673.5 ± 232.3	682.8 ± 270.4	0.9	617.9 ± 220.6	763.8 ± 598.2	0.35
CD8 T cells (cells/ul)	307.1 ± 151.8	331.0 ± 221.3	0.64	392.2 ± 272.7	432.8 ± 365.9	0.72
CD4:CD8 Ratio	2.6 ± 1.2	2.8 ± 2.3	0.66	2.0 ± 1.2	2.2 ± 1.4	0.63
Seroprotection H1N1	27 (96%)	27 (96%)	1.00	15 (88%)	14 (82%)	1.00
Seroprotection H3N2	28 (100%)	27 (96%)	1.00	15 (88%)	15 (88%)	1.00
Seroprotection B	28 (100%)	28 (100%)	1.00	17 (100%)	15 (88%)	0.49

Demographic parameters were compared between sedentary and active females (n=56) and males (n=34) by single-factor ANOVA and Fisher's exact test for numerical and categorical data respectively.

Table 2. DEXA indices of active and sedentary participants

Female	Sedentary (n = 28)	Active (n = 28)	p-value
L arm BMD (g/cm²)	0.56 ± 0.07	0.60 ± 0.04	0.01
R arm BMD (g/cm²)	0.58 ± 0.06	0.62 ± 0.05	0.02
L arm BMC (g)	83.00 ± 21.44	93.56 ± 17.87	0.05
R ribs Area (cm²)	129.78 ± 25.86	118.26 ± 18.25	0.06
T spine Area (cm²)	113.88 ± 20.71	123.02 ± 14.37	0.06
Neck BMD (g/cm²)	0.55 ± 0.08	0.59 ± 0.09	0.07
Total % body fat age matched	35.29 ± 25.66	24.74 ± 18.22	0.10
Trunk Fat mass (g)	11525.37 ± 3609.52	10220.56 ± 2114.92	0.11
Neck BMC (g)	2.49 ± 0.37	2.66 ± 0.39	0.11
Total BMC (g)	1481.1 ± 283.18	1609.95 ± 313.26	0.11
Male	**Sedentary (n = 17)**	**Active (n = 17)**	**p-value**
R arm Lean + BMC (g)	2488.85 ± 339.51	2984.23 ± 241.58	0.00
Gynoid % Fat	33.59 ± 4.78	28.62 ± 3.3	0.00
Gynoid Lean + BMC (g)	5966.62 ± 737.74	6766.08 ± 679.93	0.00
Appendicular Lean/height² (kg/m²)	6.24 ± 0.82	7.03 ± 0.51	0.01
L arm Lean + BMC (g)	2150.62 ± 365.14	2477 ± 195.57	0.01
R ribs BMD (g/cm2)	0.56 ± 0.08	0.63 ± 0.06	0.02
Lean/height2 (kg/m2)	14.64 ± 1.71	16.12 ± 1.05	0.02
R leg % Fat	30.83 ± 5.38	26.08 ± 4.73	0.03
R arm BMD (g/cm²)	0.71 ± 0.08	0.78 ± 0.06	0.03
R arm % Fat	31.9 ± 6.78	25.95 ± 6.25	0.03
R arm BMC (g)	156.04 ± 28.16	177.63 ± 17.80	0.03
Average legs %fat	30.58 ± 5.57	25.97 ± 4.74	0.03
Pelvis BMC (g)	155.93 ± 41.87	190.34 ± 39.43	0.04
L leg % Fat	30.34 ± 5.81	25.85 ± 4.77	0.04
Average arms % Fat	32.12 ± 6.55	27.06 ± 5.69	0.05
Subtotal Lean + BMC (g)	38494.85 ± 4374.94	41609.85 ± 3141.58	0.05

Demographic parameters were compared between sedentary and active individuals males (n= 34) and females (n=56) by single-factor ANOVA.

centages of immune cell subsets measured by flow cytometry were compared between sedentary and active individuals using an ANCOVA (analysis of covariance) test with age listed as a covariate. Comparisons across multiple time points within the same set of individuals were performed using the paired Mann-Whitney-Wilcoxon U test. HAI titres and blood counts of immune cells were log2 transformed. Pearson's correlation coefficient between these measurements was calculated across both sedentary and active subjects. P-values of correlation were computed using Pearson's product moment correlation coefficient test in R statistical language using the function cor.test.

3. RESULTS

3.1 Participant demographics and cohort description

A cohort of 183 elderly participants agreed to carry an Actical© (Phillips) device on their non-dominant wrists for a period of 14 days following vaccination with Vaxigrip©. The device records both actigraphy data as well as the number of steps completed by each participant. We divided elderly participants into quartiles based on the number of steps they completed (Females (n=112): LQ <10927 steps/day (n=28), HQ >18509 steps/day (n=28); Males (n=70): LQ <7174 steps/day (n=17), HQ >14770 steps/day (n=17) to group participants into either sedentary or physically active subgroups for further analysis. As the age difference between high and low activity groups was significant across both genders, we adjusted for age in all subsequent analyses. Although the initial plan was to compare individuals from the lowest (sedentary) and highest quartiles (active) in a gender-independent manner, we found that elderly females generally completed more steps than their male counterparts (Figure 1A). Furthermore, the distribution of steps completed among individuals in the highest quartile was more continuous for females than for males. In addition, stratification by sarcopenic status revealed a difference between high activity and low activity males (Table 1). Concordantly, as revealed by Dual-energy X-ray absorptiometry (DEXA) results, active males were more physically robust than their more sedentary counterparts – the most significant differences between active and sedentary males and females are listed in Table 2. Elderly males who walked more had higher bone mineral density (BMD) and content (BMC) in their arms, legs, pelvis and ribs; and lower fat content in their leg and gynoid regions. Although active females displayed trends of higher BMD and BMC, as well as lower fat composition, these differences were statistically

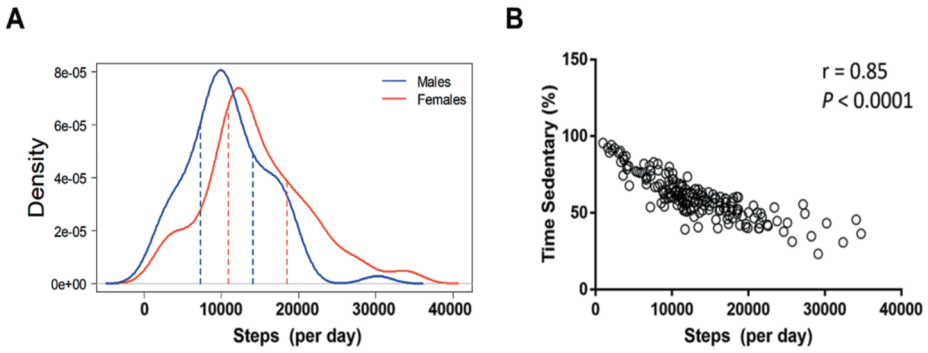

Figure 1. Baseline demographics of active and sedentary elderly participants. (A) Distribution of steps for all male (blue) and female (red) participants in the actigraphy study (n=183). Lines demarcate the boundaries of the interquartile range. (B) Correlation between percentage of sedentary time and the number of steps completed per day for all participants (n=183).

significant only for arm BMD (P < 0.02). The number of steps completed by all participants (n=183) was also well correlated to the sedentary period for each subject (Figure 1B), suggesting that both parameters are closely linked in this study (r = 0.85, P < 0.0001).

Possibly related to differences in ethnicity and infection history, we achieved relatively high post-vaccination seroprotection rates in our cohort of community dwelling elderly subjects (> 96% for females; > 82% in males). We observed no differences in the presence of comorbidities (high blood pressure, high cholesterol, diabetic status, arthritis) between active and sedentary participants. As reflected by their mini mental state examination (MMSE) scores, cognitive functionality was improved for elderly who were more physically active (p = 0.05 for females; p = 0.032 for males), although this difference was not statistically significant when participants were evaluated by their Montreal cognitive assessment (MoCA) scores. Due to fewer male participants (n=34), the lack of a distinct population of highly active male individuals and the large number of confounder variables between sedentary and active groups for males, we focused on female participants (n=56) to identify differences in the post-vaccination immunology of active and sedentary participants in our subsequent analyse.

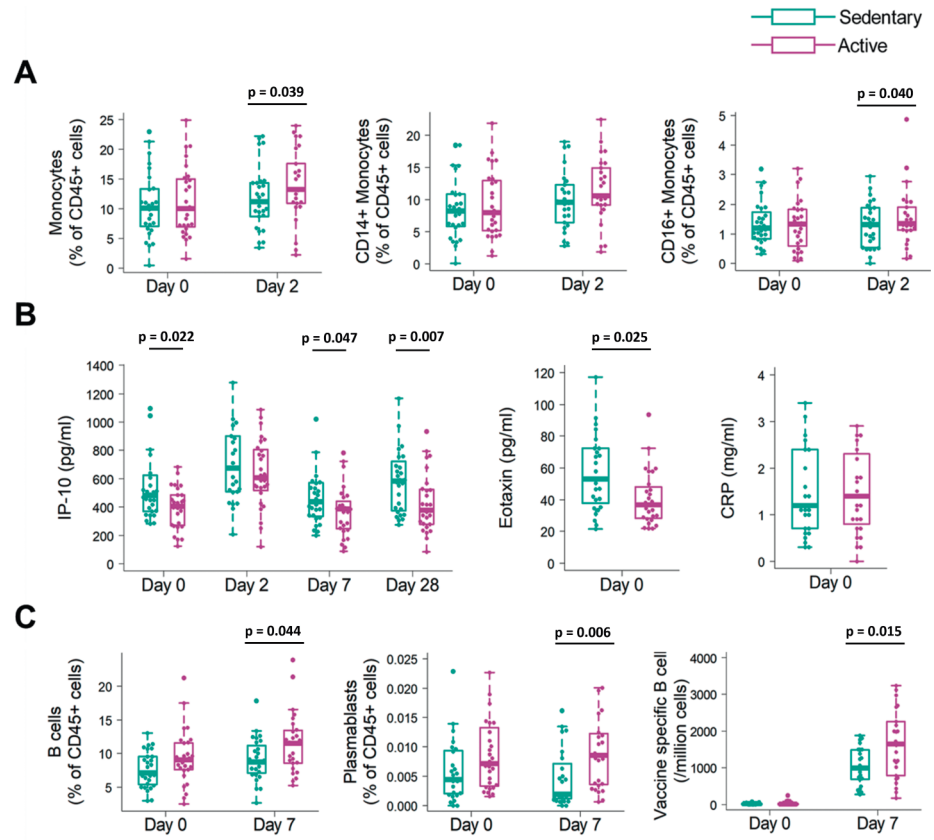

Figure 2. Pre- and Post-Vaccination Immunological Responses in active and sedentary elderly females. (A) Percentages of total monocytes, CD14+ and CD16+ monocytes in active (n=28) and sedentary females (n=28) at D0 and D2. (B) Levels of plasma IP-10 (D0, D2, D7 and D28), Eotaxin (D0) and CRP (D0) in active and sedentary females. (C) Pre- and D7 post-vaccination percentages of B-cells and plasmablasts, as well as the number of vaccine-specific B-cells in active and sedentary females. Percentages of immune cell subsets measured by flow cytometry were compared between sedentary and active individuals using an ANCOVA (analysis of covariance) test with age listed as a covariate. Comparisons across multiple time points within the same set of individuals were performed using the paired Mann-Whitney-Wilcoxon U test. P-values are only indicated where there is a statistically significant difference.

3.2 Kinetics of immune cells and pro-inflammatory cytokines

In active and sedentary females (n=56), we specifically compared the percentages of inflammatory monocytes, classical and non-classical monocytes, eosinophils, neutrophils, dendritic cell subsets and natural killer (NK) cells to identify differences in innate immunity between both populations. Here, the quantity of immune cell subsets as measured by flow cytometry were compared between sedentary and active individuals using an ANCOVA (analysis of covariance) test with age listed as a covariate. The most salient difference was a greater post-vaccination induction of total monocytes (p = 0.039) between active and sedentary elderly females on Day 2 (Figure 2A); this was more statistically significant for non-classical CD16+CD14^{lo} monocytes (ncMono; p = 0.040), although the representation of CD14+CD16- classical monocytes (cMono) was also substantially increased (p = 0.064). We did not observe any changes in the distribution of CD16+CD14+ inflammatory monocytes between active and sedentary elderly women (not shown). In the measurement of pro-inflammatory molecules in

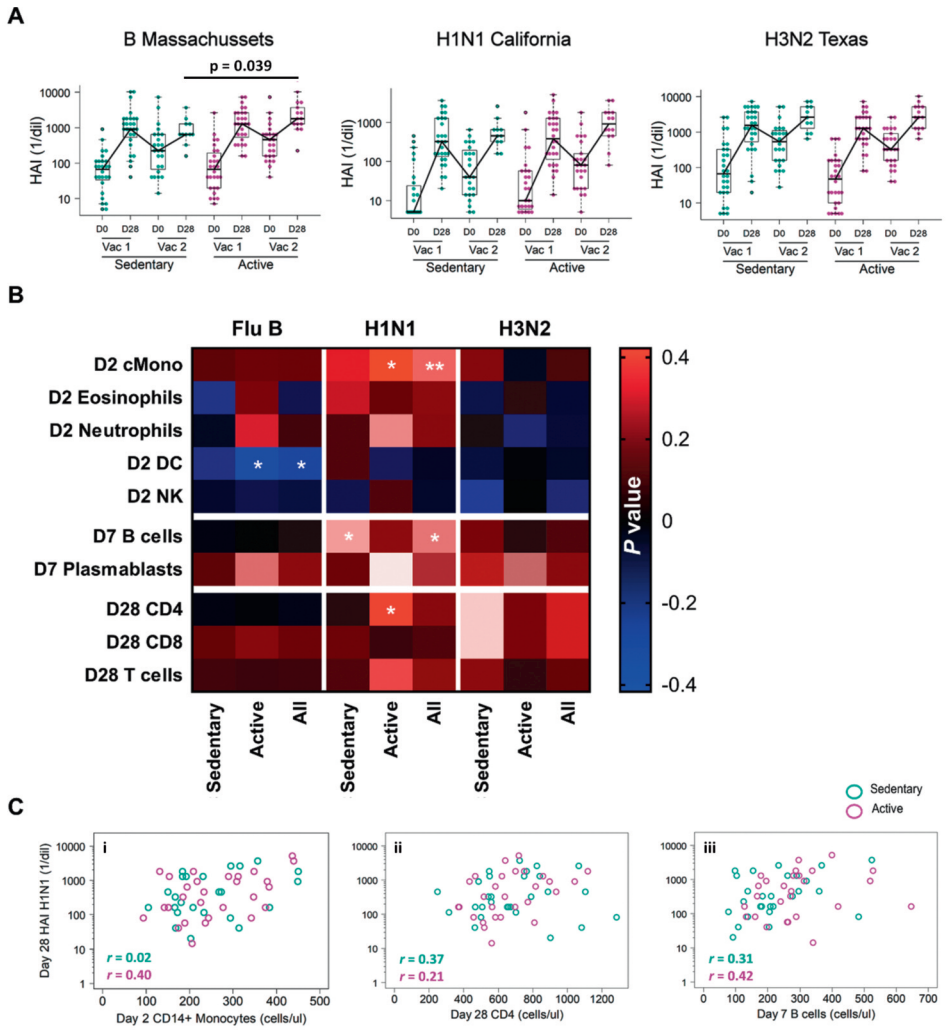

Figure 3. HAI response in active and sedentary elderly females during influenza vaccination. (A) Comparison of strain specific HAI titres between sedentary (green) and active (red) elderly females during the primary (Vac 1) and secondary (Vac 2) vaccination from Baseline (D0) and Day 28 post-vaccination sera (D28). Results for Flu B Massachusetts, H3N1 California and H3N2 Texas are shown. (B) Heatmap corresponding to P-values in the tested correlation between post-vaccination immune cell numbers and D28 HAI titres for all three strains after the first vaccination. P-values of correlation were computed using Pearson's product moment correlation coefficient test in R statistical language using the function cor.test (*P<0.05; **P<0.01). (C) Correlation plots and coefficients for (i) D2 CD14+ monocyte, (ii) D28 CD4 T-cell and (iii) D7 B-cell cell counts versus D28 H1N1 HAI titres. HAI titres and blood counts of immune cells were log2 transformed. Pearson's correlation coefficient between these measurements was calculated across both sedentary and active subject and displayed individually for sedentary (green) and active (red) elderly females.

plasma, there was no statistically significant difference in the levels of high sensitivity C-reactive protein (hsCRP) between sedentary and active elderly females. However, lower levels of the monocyte-derived chemokine, IP-10 (p = 0.022), and eotaxin (p= 0.025) were detected in active elderly females at baseline (Figure 2B).

For adaptive immune cells, we analysed baseline distributions of CD4 and CD8 T-cells and found no statistically significant differences in the numbers of CD4 and CD8 T-cells as well as CD4:CD8 ratios in active and sedentary groups (Table 1). For B-cells, the difference in pre-vaccination percentages of B-cells and plasmablasts (Figure 2C) was not statistically significant between active and sedentary elderly females. However, the Day 7 post-vaccination expansion of B-cells (p = 0.044) and plasmablasts (p = 0.006) was significantly greater in active than in sedentary elderly females. As determined by ELISpot, we also observed an increase in vaccine-specific IgG-secreting B-cells (p = 0.015) within PBMCs that were collected from active females on Day 7 post-vaccination (Figure 2C).

3.3 Post Vaccination HAI Titres

Elderly participants were vaccinated twice in this study, with the second vaccination set at 20 months after receiving the first vaccination. As Vaxigrip® is a trivalent vaccine comprising split virions from three different influenza strains, we investigated HAI titres for each of these strains. Although we did not observe any differences in Day 28 (D28) HAI antibody titres between active and sedentary participants for all three strains in the first vaccination, higher D28 Flu B antibody titres were observed in active females after the second vaccination (Figure 3A; leftmost panel). Subsequently, we assessed whether the post-vaccination numbers of innate and adaptive immune subsets correlated with post-vaccination HAI titres using Pearson's Correlation. Although all three strains were studied (presented as a heat map in Figure 3B), positive correlations between the numbers of immune cell subsets and D28 HAI were only observed for the H1N1 strain after the first vaccination (middle panel; Figure 3B). For active elderly females, the numbers of classical CD14+ monocytes ($p < 0.05$) and CD4 T-cells ($P < 0.05$) at Day 2 (D2) and D28 post-vaccination, respectively, were specifically correlated with D28 HAI titres against the H1N1 strain. The correlation between these immune subsets and H1N1 HAI titres after the first vaccination are resolved in greater detail in Figure 3C, where individual datapoints and r values are individually shown for sedentary (green) and active (red) elderly females (Figures 3C (i) for D2 monocytes and 3C (ii) for D28 CD4 T-cells).

3.4 Transcriptomic Analysis

Finally, we conducted transcriptomic analysis on PBMCs that were collected at baseline to identify differences in gene expression between active and sedentary elderly females. In our microarray analysis, we looked for genes that were differentially regulated between active and sedentary elderly females (Figure 4A). Using a nominal cut off value of $P < 0.05$, we identified 505 DEGs that were further studied using

the Ingenuity Pathway Analysis (34). Here, genetic pathways related to phagocytosis in macrophages/monocytes were highly upregulated in active elderly females relative to their sedentary counterparts (Figure 4B).

4. DISCUSSION

4.1 Summary of Findings and Study Limitations

Opinions on the impact of acute physical activity on the immune system are diverse although many have inferred a state of temporal immunosuppression after acute exercise (76). In addition to lower levels of circulating lymphocytes and antibodies following acute vigorous physical activity, studies have also shown that acute vigorous exercise can increase the risk of infection (8, 53, 54, 60, 76). Although acute physical activity diminishes the frequency of peripheral immune cells, it contributes to the mobilisation of senescent or late differentiated lymphocytes into the periphery (7, 71, 72). Conversely, there is a consensus that frequent physical activity and exercise drives immune competency. Investigations into the relationship between physical activity and improved immunity typically fall into two categories – cross-sectional studies that stratify participants by physical activity level/cardiorespiratory fitness or longitudinal studies that compare immune function after a regular period of physical-activity based interventions. Altogether, these studies describe better responsiveness to mitogen-induced T-cell proliferation, reduced frequencies of senescent cells, higher serum antibody titres following vaccination and cytotoxic activity of NK-cells and neutrophil/monocyte phagocytic function (22, 28, 55, 57, 60, 61).

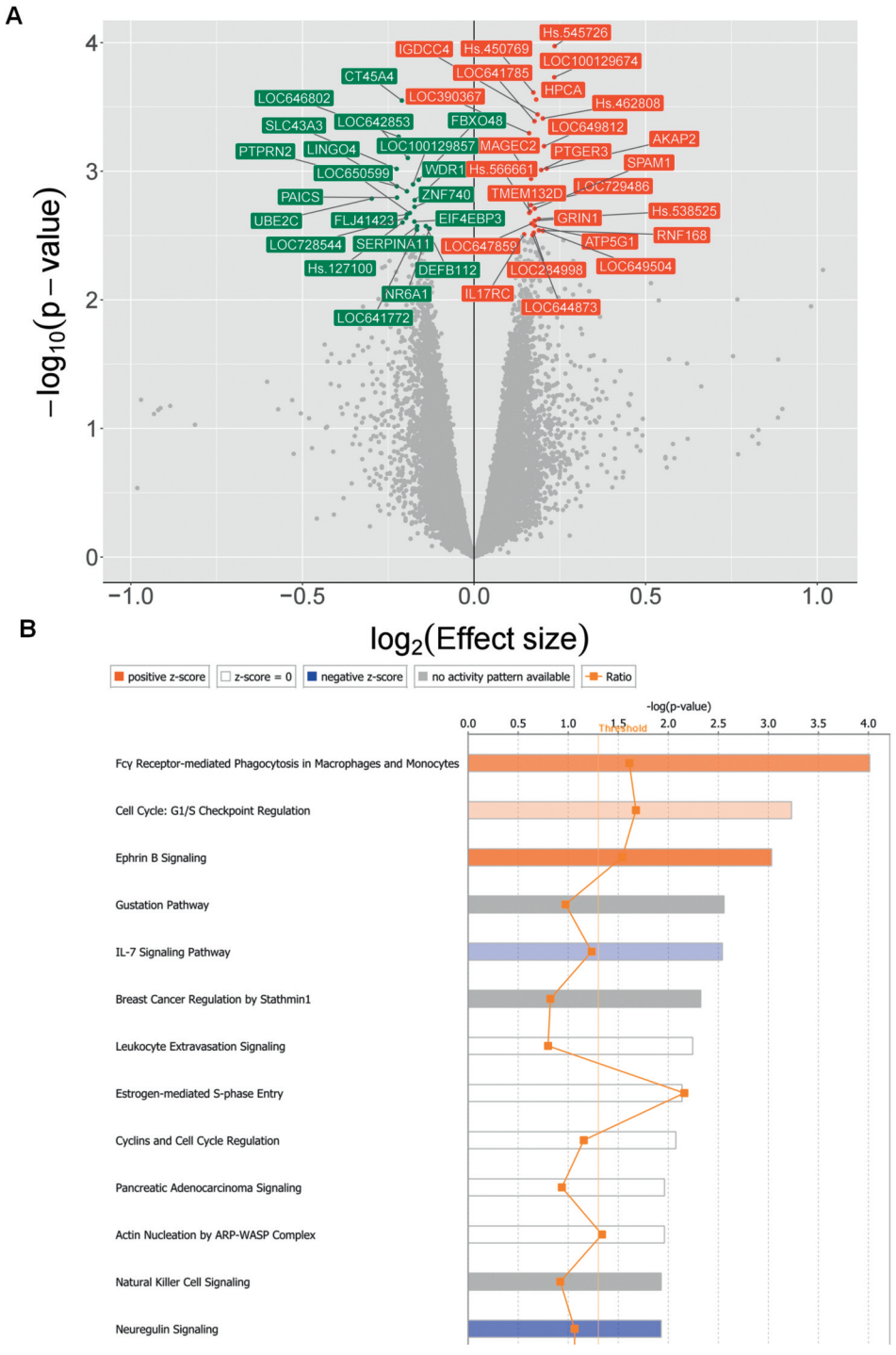

Figure 4. IPA analysis revealing DEGs at baseline in active and sedentary elderly females. (A) 505 DEGs were observed between active and sedentary elderly females using a nominal p value of 0.05 as the cut-off point. (B) Ingenuity Pathway Analysis revealed that genes involved in the regulation of phagocytosis in monocytes and macrophage were most differentially regulated between active and sedentary elderly female participants.

Overall, our findings are consistent with previous literature in describing an augmentation of the post-vaccination immune response in individuals who sustain higher levels of physical activity over extended periods of time. In physically active elderly females, we demonstrate improvements in both arms of the immune response. In addition to a greater post-vaccination expansion of monocytes, we further observed the upregulation of genes related to phagocytic function of monocytes and macrophages, altogether suggesting an enhanced post-vaccination monocyte presence in active elderly females. Furthermore, we describe a stronger post-vaccination plasmablast and vaccine-specific B-cell response in active elderly females as compared to their sedentary counterparts. In active elderly females, the magnitude of the post-vaccination monocyte (D2) and CD4 T-cell (D28) was also found to correlate positively to H1N1 HAI titres, suggesting that the robustness of both innate and adaptive responses is related to the potency of the flu-specific antibody response.

A limitation of this study is that physical activity was only monitored for 14 days after vaccination, however, the design of this study was planned to ensure minimal deviation from their usual routine so that behaviour in these 14 days could be indicative of a prolonged lifestyle in terms of physical activity. Moreover, the generally higher BMC and BMD indices in active females – albeit not statistically significant – suggest long-term differences in physical activity levels between these groups. Thus, it is reasonable to assume that the number of steps completed by each participant are representative of behaviour beyond this 14-day window period. Nevertheless, our study design cannot discriminate whether behavioural history or the amount of physical activity completed within the vaccination window, is responsible for the observed differences in immune activity between active and sedentary elderly women. Since our conclusions are extensively derived from the post-vaccination observation of immune parameters in elderly females, a further limitation is that the improved post-vaccination outcomes influenza vaccination may not be generalisable to males or younger participants.

4.2 Physical Activity and the Immune Response to Influenza Vaccinations

Specific to influenza vaccination, Kohut *et. al.* showed that tri-weekly attendance at a supervised aerobics class contributed to higher antibody titres against H1N1 and H3N2 strains in the elderly (37). While not affecting peak HAI titres, Woods *et. al.* demonstrated that a 10-month cardiovascular exercise-based intervention also benefited the elderly in prolonging the influenza seroprotection period (78). These findings translate poorly to a single bout of cardiovascular exercise, as participation in a brisk walk or a single set of resistance exercise immediately prior to influenza vaccination did not act as an adjuvant to influenza vaccination (20, 44). Although we did not observe any differences in post-vaccination HAI titres between active and sedentary elderly females; the peripheral expansion of monocytes, B-cells, plasmablasts, and vaccine-specific B-cells was more significant in participants who were more active in the immediate post-vaccination period. Curiously, this robust post-vaccination mobilisation of immune subsets was most closely associated with the H1N1 HAI response. A similar strain-specific response has been observed in an earlier trivalent vaccine study, which noted that elderly females mounted stronger antibody responses towards H1N1 when acute exercise was implemented as an adjuvant prior to vaccination (64). In our study, the increased levels of physical activity could contribute to detectable benefits in the immune response against H1N1 as participants had the lowest baseline HAI and seroprotection rates against H1N1.

The lack of convergence with earlier studies, with respect to improvements in post-vaccination HAI titres, could likewise be a result of the high levels of pre-vaccination seroprotection rates and HAI titres against Flu B and H3N2 (>50%) across both active and sedentary groups (36). Since most elderly subjects in our study responded robustly to influenza vaccination and seroconverted, it was less likely for differences in HAI titres between active and sedentary females to be detected. An alternative explanation may relate to the intensity or regularity of physical activity required to boost post-vaccination antibody titres, as participants were not subjected to any organised or specific intervention programmes. Unlike earlier studies that grouped donors based on intervention arms or physical activity levels prior to vaccination, donor stratification was based on post-vaccination physical activity levels in this study (15, 36, 37, 69, 78). Nevertheless, we introduce many important perspectives to the link between physical activity and post-vaccination immunity. We demonstrate firstly that the augmentation of post-vaccination HAI titres by physical activity may be closely related to changes in innate immunity – or more specifically monocyte trafficking and phagocytosis. Consistent with the upregulation of genes associated with macrophage and monocyte phagocytosis in PBMCs from active elderly females, many studies have revealed that physical activity enhances the phagocytic potential of these innate immune cells (22, 57). Since phagocytosis is fundamental to antigen-processing and presentation, improvements in the former are likely to directly benefit the generation of antigen-specific T- and B-cell immune responses (22). Serving as a potential cellular reservoir for subsequent differentiation towards specialised antigen-presenting macrophages and dendritic cells, the greater post-vaccination expansion of the monocyte pool is also likely to have contributed to the increased post-vaccination frequencies of plasmablasts and vaccine-specific B-cells in the active elderly female group.

As participants were administered two rounds of Vaxigrip© vaccination in our study – the second after a 20-month interval – we were able to assess the influence of physical activity on repeated immunisations. That the physically active elderly female group could mount a superior antibody response to Flu B after the second vaccination suggests that they possess better preservation and persistence of memory B-cell plasticity and function. The higher pre-vaccination proportions of B-cells and plasmablasts – and generation of vaccine specific B-cells – during the first vaccination are consistent with this hypothesis and may further reflect improvements in hematopoietic potential in active elderly females. Although not statistically significant, we observed higher HAI titres against Flu B and H1N1 immediately prior to the second vaccination, suggesting that active elderly females may accommodate better antibody persistence in the 20-month post-vaccination period as compared to sedentary participants. The present literature is supportive of the above interpretations. Although the mechanisms are unclear, regular exercise promotes haematopoiesis and contributes to higher numbers of hematopoietic stem cells in the bone marrow and peripheral blood (3, 5). Moreover, cyclists have been reported to possess higher levels of the hematopoietic cytokine, IL-7, and frequencies of recent thymic emigrants in their blood (19). Specific to the B-cell response, de Araújo *et. al.* had also observed more potent and durable antibody responses to influenza vaccination in elderly men with a moderate or intense training lifestyle (15). Finally, reports have highlighted that those who exercise have higher circulating antibody titres (IgM and class-switched isotypes) suggesting that regular physical activity is important for memory B-cell/plasmablast function (49, 56).

4.3 Physical Activity in the Alleviation of 'Inflammaging'

Aging is associated with the persistence of low-grade chronic inflammation – otherwise known as "inflammaging"; this is

characterised by increased levels of pro-inflammatory molecules (IL-6, CRP, IL-1β, IL-15 and TNF-α) and levels of systemic inflammation have shown close association with the development of comorbidities in the elderly – including cardiovascular diseases, type-2 diabetes, Alzheimer's disease, osteoporosis and multiple cancer types (23, 24, 27). Moreover, elevated systemic levels of IL-6 and CRP have been shown to predict mortality in longitudinal studies that include nonagenarians (4, 26, 34). Since immune activation is a key driver of immunosenescence and loss of lymphocyte function, we may well speculate that the lower levels of systemic inflammation (characterised by IP-10 and eotaxin levels) have a critical role to play in driving the higher immune responses observed in active elderly females from our influenza vaccine study. Other studies have highlighted the negative relationships between regular physical activity and systemic IL-6, CRP and TNF-α levels in the elderly (29, 34). Although we did not observe any differences in CRP levels between sedentary and active elderly females, lower levels of pro-inflammatory IFN-γ-induced protein precursors (IP-10 or CXCL-10) and eotaxin were detected in more active females. IP-10 is a potent monocyte- or dendritic cell-derived chemoattractant that recruits activated T-cells to secondary lymphoid tissues and inflammatory sites of action (18). While a decrease in eotaxin levels following a 12-week exercise intervention period has been illustrated with a cohort of Korean women, the relationship between physical activity and IP-10 levels has not been previously described (12).

Since IP-10 is a pro-inflammatory marker that is associated with susceptibility towards infectious diseases, autoimmunity and cancer – lower baseline levels of IP-10 may protect against these pathologies (1, 42, 43). In our study, we further observed that active elderly females achieved better post-vaccination resolution of IP-10 levels – noticeable as early as 7 days post-vaccination in a trend that persists even at 28 days post-vaccination. As we monitored walking activity in the period immediately following vaccination, the swift resolution of IP-10 could be directly related to the levels of physical activity attained by elderly female participants during the period of vaccination. It is important to note that the lower baseline levels of IP-10 in active elderly females do not imply an impaired capacity to secrete IP-10, as active elderly females equally upregulated plasma IP-10 levels on Day 2 post-vaccination. The induction of interferon-induced genes, particularly IP-10 in the first days of vaccination, has been shown to be a critical component of the predictive signature of influenza, yellow fever and Ebola vaccine efficacy (2, 16, 47, 63, 66). In an investigation of the influenza vaccination response, Athale and colleagues also found that vaccine efficacy was highly related to the rapid in vitro secretion of type 1 IFN and IP-10 by innate immune cells (2).

4.4 Improving Health in the Elderly through Physical Activity

While physical activity may have a significant role in lowering the risk of many age-related diseases (10, 75), we did not observe many differences in the frequency of co-morbidities between active and sedentary elderly individuals in our community dwelling cohort. For example, in addition to diabetic and arthritic status, blood pressure and cholesterol levels were not statistically different between active and sedentary elderly individuals in this study. Nevertheless, we observed that sarcopenic status greatly differed between active and sedentary elderly males. While a multitude of cross-sectional and cohort studies have shown that regular physical activity reduces the risk of developing sarcopenia, the cross-sectional nature of our study makes it difficult to determine the directional relationship between sarcopenic status and levels of physical activity among our male participants. Recent evidence, however, indicates that by delaying the decay of skeletal muscle mass and function and improving muscular strength and power, physical activity and exercise are effective intervention strategies that prevent the development of sarcopenia (32, 74). A comparison of DEXA measurements between sedentary and active elderly individuals in our study reveals trends that support this relationship between physical activity and the development of sarcopenia. More specifically, indices related to muscular and skeletal health were strengthened in active elderly participants relative to their sedentary counterparts.

In terms of monitoring the impact of physical activity on vaccination outcomes, our study is the first to describe improved cell-mediated responses using an accelerometer-based study that records physical activity levels in the immediate post-vaccination period. While there is a strong case for promoting physical activity as a fundamental component to healthy aging, the results of previous studies display a heavy reliance on self-reported physical activity or interventions featuring organised exercise regimes. The complexity and type of exercise training (cardiovascular, aerobic training, resistance training, power training) also contribute to contrasting observations, and it is therefore challenging to interpret the value of organised interventions or implement them on a national level (30, 60). The rapidly growing proportion of elderly individuals (aged 65 years or older) worldwide, however, create a pressing need for institutionalised interventions that delay the acquisition of age-related co-morbidities. For the elderly, the WHO has suggested that a weekly recommendation of at least 150 minutes of moderate-intensity aerobic activity or 75 minutes of vigorous-intensity aerobic activity is necessary to achieve the health benefits associated with physical activity (81). Here, the use of pedometers and accelerometers provide practical and reliable methods for tracking and incentivising physical activity among the elderly.

As part of a national initiative to promote physical activity, the Singapore Health Promotion Board has organised a National Steps Challenge, which awards modest financial rewards to motivate participants to become more physically active and has seen 690,000 sign-ups in 2018 alone. Although its impact on national health has not been studied, the high participation rate suggests the feasibility of implementing a pedometer-based programme to boost national health. It could be critical for other countries to implement similar policies to reverse the high prevalence rates of insufficient physical activity (80, 81). Overall, our demonstration of the benefits of physical activity on immune responses to influenza vaccination in elderly women suggest that exercise can be a safe and valuable adjunct to interventions that aim to promote immune robustness in older individuals.

Author Contributions

AL, NBu, BA, TPN and PT conceptualised the design of this vaccine study. GW, Nbo and AL planned the analysis for the exercise component of this study. GW, XC, CT and AL designed and performed the immunological assays. GW analysed the data and wrote the manuscript. VN performed the statistical analyses described in this study. All other authors participated in the collection, analysis and interpretation of data. All authors approved the final version of this manuscript.

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Ethics Approval

The National Healthcare Group (NHG) Domain Specific Institutional Review Board (DSRB) approved a phase IV clinical trial of Sanofi Pasteur's Vaxigrip© influenza vaccine, which is registered at clinicaltrials.gov under the registration number NCT03266237. All volunteers provided written informed consent.

Conflict of Interest Statement

The authors declare a potential conflict of interest: Christope Carre and Laurence Quemeneur are employees of Sanofi-Pasteur and Nabil Bosco is an employee of Nestle Research Centre, Singapore. All other authors declare no competing interests.

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