Primary immune responses are impacted by persistent herpesvirus infections in older people: results from an observational study on healthy subjects and a vaccination trial on subjects aged more than 70 years old

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Supplemental Material

1. Supplementary information for Cohort 2
2. Descriptive statistics
3. Study flow diagrams
4. STROBE checklist Abstract
5. STROBE checklist Cohort
6. CYTEL (Cohort 2) protocol

1. Supplementary information for cohort 2

Inclusion criteria, baseline characteristics of study participants and adverse events

Of 183 screened individuals 137 healthy elderly (≥ 70 years) were included in the study population, 69 were CMV-positive (CMV+) and 68 CMV-negative (CMV-). Participants had to be ≥70 years old, healthy according to an adapted Cornell medical index and TBEV-naïve. Forty-six participants were excluded because of age (n=1), co-morbidities (n=3), BMI ≥30 kg/m^2 (n=2), high blood pressure (n=1), haemoglobin <12 g/dl (n=4), calculated creatinin clearance <50ml/min (n=1), positive TBEV-serology at baseline (n=16) or CMV-serology results incompatible with balanced recruitment (n=18). To ensure balanced recruitment of CMV+ and CMV- individuals, differences in group size were set at +/- three individuals during the recruitment phase, leading to exclusion of 7 CMV+ and 11 CMV- elderly despite fulfilling all other inclusion criteria.

Baseline characteristics were well balanced between the CMV+ and CMV- group (suppl. Table 1). All participants received three intramuscular immunisations with a licensed TBE-vaccine (FSME Immun®, Baxter, Austria) at week 0, 4 and 24. Plasma and PBMCs were collected for further analysis at week 0, 2, 4, 6, 8, 24, 26 and 28. All 137 included participants finished the study according to the protocol.

The vaccine was generally very well tolerated. During 411 immunisations we recorded 47 (11.4%) vaccine-related adverse events, none of which was serious. Nine (2.2%) moderate but self-limiting and 38 (9.2%) minor adverse events occurred preferentially after the first application (6.3%). The rate of adverse events was similar in CMV-positive and CMV-negative individuals. Transient local reactions like pain, swelling and/or erythema, subsiding within 24-48h, were most frequently reported by 9.8% of all participants. Mild systemic reactions, such as headache, nausea, swelling of lymph nodes and/or fever were reported by 3.85% of all participants. Serious adverse events occurred in 6 individuals. One person was hospitalized with a minor stroke with complete recovery. Three individuals were treated for traumatic injuries and two were diagnosed with malignancy during the 28 week observation time of the study participation. None of these events were considered to be vaccination or study related and all individuals resumed study participation on time.

Table S1: Baseline characteristics of cohort 2 (n=137)

Parameter	**CMV-negative N= 68**	**CMV-positive N= 69**
Age (median; range)	73 (70-86)	74 (70-87)
Gender (female : male)	30:38	38:31
Body Mass Index (BMI) (kg/m^2, mean, range)	24 (18-30)	24 (18-30)
Weight (kg; mean, range)	68.9 (45–97)	68.2 (49–100)
Physical examination		
- Proportion with abnormality	1/67	1/68
Creatinine clearance[1]		
(ml/min; median, range)	76 (50–109)	74 (50–110)
Mean activity score[2]		
Low	17	19
Intermediate	36	33
High	15	17
Co-morbidities		
- Proportion with co-morbidity	26/42	33/36
Medication		
- Proportion with medication	26/42	32/37
Number of drugs		
0	42	37
1	19	26
2	7	6
Recreational drugs		
Alcohol (units/day; median, range)	0 (0-4)	1 (0-4)
Nicotine (Active/Ex-/Non-smoker)	7/15/46	8/20/41
- Active smoker (n)	7	8
- Ex-smoker (n)	15	20
- packyears (mean; range)	28 (5 – 60)	25 (2 - 63)
Yellow Fever vaccination	17/68	26/69
No (incl. unlikely[3])	51	43
Yes (incl. likely[3])	17	26

[1] The Creatinine-Clearance was calculated according to the Cocroft-Gold formula.
[2] Usual physical activity was assessed by self-report questionnaire at baseline containing a) median number of walks > 15min per week and b) median walking-distance without pausing. The two components generated a Physical Activity Score with three categories: low (0-4 walks/week and a walking distance of < 5km), intermediate (5-6 walks/week and a median walking distance of 5-9km) and high (≥ 7 walks/week and a median walking distance ≥ 10km).
[3] If vaccination booklets were not available for review: Yellow fever vaccination was considered "likely" if study participants had previously travelled to endemic countries and/or countries requiring proof of yellow fever vaccination and sought advice at a travel clinic before their trip. Yellow fever vaccination was seen as "unlikely" if study participants had never travelled to the countries mentioned above.

2. Descriptive statistics

Table S2.

Regression equations for data shown in Figures 1, 2a, 2b, 2c and 3b

	Number of infections			
	0	1	2	3+
IL-6 (pg/ml) (Figure 1)	**y=0.0029 + 0.02x**	**y=0.18 + 0.013x**	**y=-0.57 + 0.034x**	**y=-1.1 + 0.043x**
TNF (pg/ml) (Figure 1)	y=1.3 + 0.02x	y=2 + 0.0074x	**y=0.72 + 0.03x**	**y=0.25 + 0.045x**
IL-10 (pg/ml) (Figure 1)	y=0.73 - 0.00091x	y=0.78 - 0.0026x	y=0.39 + 0.0079x	**y=0.27 + 0.0084x**
IFN-α (fg/ml) (Figure 1)	y=0.94 + 0.00038x	y=0.44 - 0.00093x	Y=-0.094 + 0.019x	**y=-1.8 + 0.053x**
EM $CD8^+$ T cells/mm^3 (Figure 2a)	y=32 - 0.16x	y= 69 - 0.52x	**y=-11 + 1.7x**	**y=41 + 2x**
EM $CD4^+$ T cells/mm^3 (Figure 2a)	**y=53 - 0.43x**	y=45 - 0.02x	y=40 + 0.64x	**y=38 + 1.1x**
N $CD8^+$ T cells/mm^3 (Figure 2b)	**y=140-1.7x**	**y=160 - 1.8x**	**y= 170 - 2x**	**Y=160 - 1.8x**
N $CD4^+$ T cells/mm^3 (Figure 2c)	y=220 + 0.076x	y=270 - 1.5x	**y=320 - 2.8x**	**y=280 - 2.1x**
sjTRECS/150,000 PBMCs (Figure 3b)	y=2442 -33.81x	y = 1723 - 21.48x	y=1218 - 14.66x	y=1412 - 17.04x

Strenght of correlation	Very weak	Weak	Moderate	Strong	Very strong
R Value	-0.2<R<0.2	-0.4<R≤-0.2; 0.2≥R>0.4	-0.6<R≤-0.4; 0.4≥R>0.6	-0.8<R≤-0.6; 0.6≥R>0.8	R≤-0.8; R≥0.8

Table S3.

Corresponding to data shown in Figures 3a, 3c and 3d

Naive T cells	Counts of CD4+ T cells (cells/mm3)		Counts of CD8+ T cells (cells/mm3)		TRECs in CD4+ T cells (sjTREC/150,000 naive T cells)		TRECs in CD8+ T cells (sjTREC/150,000 naive T cells)		Ki67 expression in CD4+ T cells (% Ki67+ in naive T cells)		Ki67 expression in CD8+ T cells (% Ki67+ in naive T cells)	
(Figures 3a, c, d)	mid aged donors	older donors	mid aged donors	older donors	mid aged donors	older donors	mid aged donors	older donors	mid aged donors	older donors	mid aged donors	older donors
Minimum	22,64	1,47	0,37	0	268	10	1031	609	0,1	0,23	0,01	0,01
Maximum	505,3	468,9	361,7	49,01	3741	2904	6100	3488	2,07	3,29	0,63	1,28
Range	482,6	467,5	361,4	49,01	3473	2894	5069	2879	1,97	3,06	0,62	1,27
Mean	200,1	115	83,08	8,775	1891	634,9	3330	1588	0,8013	1,288	0,2788	0,5987
Std. Deviation	117	93,7	68,79	10,64	1184	895,9	1469	1299	0,6249	0,884	0,1659	0,4063
Std. Error of Mean	13,7	12,1	8,052	1,373	328,3	270,1	407,4	530,5	0,1562	0,2283	0,04148	0,1049
Lower 95% CI of mean	172,8	90,79	67,03	6,027	1175	33,03	2442	224,1	0,4683	0,7984	0,1903	0,3737
Upper 95% CI of mean	227,5	139,2	99,13	11,52	2606	1237	4217	2951	1,134	1,778	0,3672	0,8237
Coefficient of variation	58,47%	81,48%	82,81%	121,2%	62,61%	141,1%	44,12%	81,84%	77,98%	68,64%	59,53%	67,87%

Table S4.

Corresponding to data shown in Figures 5a, 5c, 5d, 5e

Antigen-specific T cells	TBEv-specific T cells (number of IFNγ+ SFU / 106 PBMC)		TBEv-specific CD4+ T cells (% of IFNγ+ CD4+ T cells)		VZV-specific CD4+ T cells (% of IFNγ+ CD4+ T cells)		SEB-stimulated CD4+ T cells (% of IFNγ+ CD4+ T cells)		TBEv-specific CD8+ T cells (% of IFNγ+ CD8+ T cells)		VZV-specific CD8+ T cells (% of IFNγ+ CD8+ T cells)		SEB-stimulated CD8+ T cells (% of IFNγ+ CD8+ T cells)	
(Figures 5a, c, d, e)	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+
Minimum	5	0	0,028	0,014	0,009	0,008	0,605	3,64	0,03	0,01	0	0,01	3,35	7,18
Maximum	870	670	0,435	0,224	0,369	0,126	15,23	32,04	0,36	0,14	0,46	0,1	20,25	46,26
Range	865	670	0,407	0,21	0,36	0,118	14,62	28,4	0,33	0,13	0,46	0,09	16,9	39,08
Mean	122,4	57,51	0,1267	0,08416	0,08204	0,04323	5,361	11,18	0,07958	0,06115	0,0725	0,03731	11,89	23,09
Std. Deviation	177,2	108,3	0,08808	0,05298	0,09783	0,02895	3,532	8,683	0,07056	0,03734	0,1039	0,02491	5,084	11,5
Std. Error of Mean	21,49	13,04	0,01837	0,0106	0,01997	0,005678	0,721	1,703	0,0144	0,007324	0,02122	0,004885	1,038	2,256
Lower 95% CI of mean	79,47	31,48	0,08861	0,06229	0,04073	0,03154	3,869	7,668	0,04979	0,04607	0,02861	0,02725	9,738	18,44
Upper 95% CI of mean	165,3	83,53	0,1648	0,106	0,1234	0,05492	6,852	14,68	0,1094	0,07624	0,1164	0,04737	14,03	27,74
Coefficient of variation	144,8%	188,4%	69,52%	62,95%	119,2%	66,97%	65,89%	77,70%	88,66%	61,07%	143,4%	66,77%	42,78%	49,83%

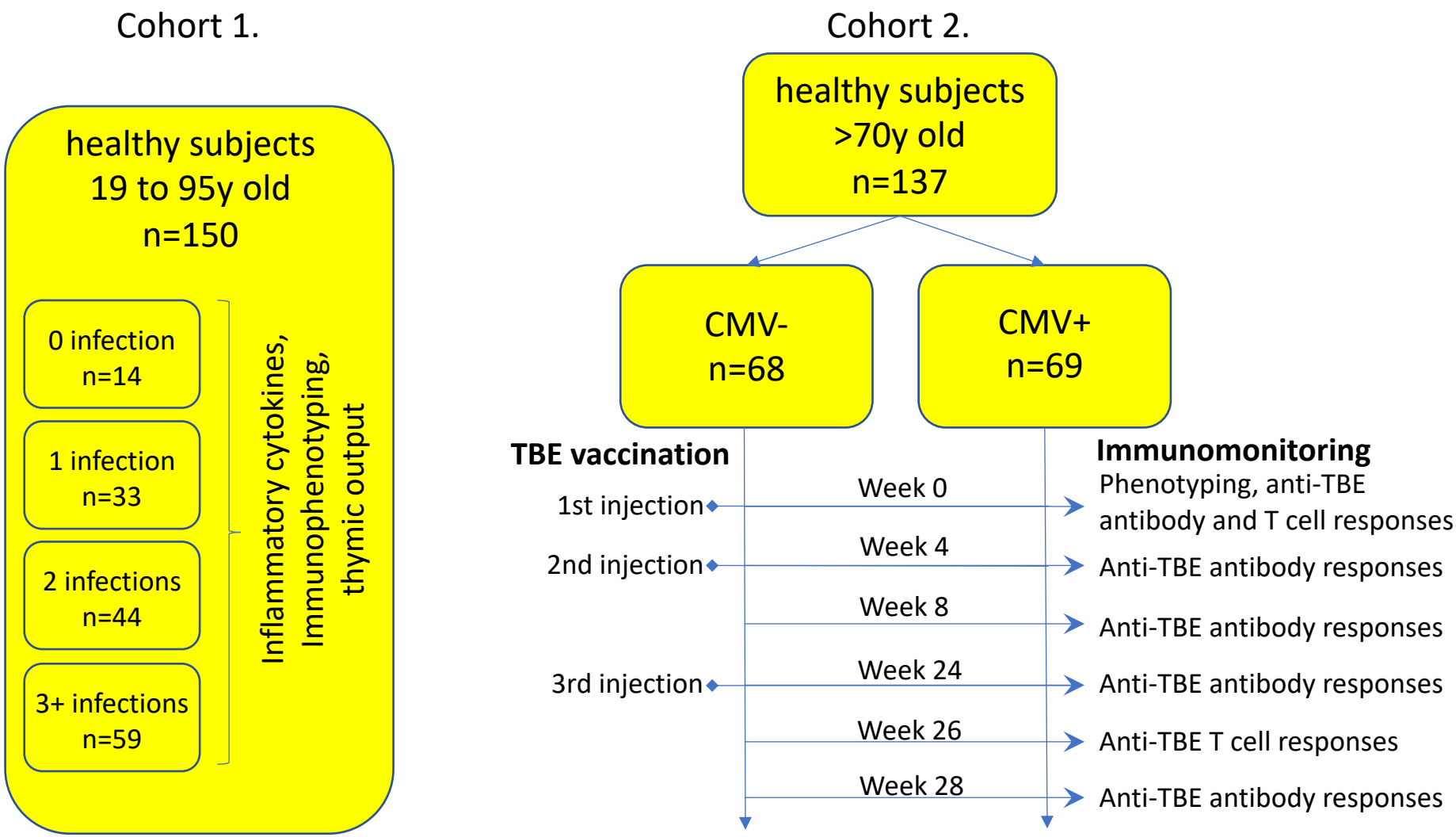
Table S5.

Corresponding to data shown in Figures 6a and 6b

TBEv-binding IgG (VIU/ml)	Wk 0		Wk 4		Wk 8		Wk 24		Wk 28	
(Figure 6a)	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+
Minimum	50	50	50	50	50	50	50	50	262	50
Maximum	189	143	2530	752	22720	5800	3740	1130	95600	21900
Range	139	93	2480	702	22670	5750	3690	1080	95338	21850
Mean	62,43	57,81	293,1	156,5	2276	1213	504,3	280,1	6223	3274
Std. Deviation	33,02	23,59	440,2	148,6	3477	1366	591,8	252,4	12491	4729
Std. Error of Mean	4,005	2,84	53,39	17,88	421,7	164,5	71,77	30,38	1515	569,3
Lower 95% CI of mean	54,43	52,14	186,6	120,8	1435	884,3	361	219,5	3199	2138
Upper 95% CI of mean	70,42	63,48	399,7	192,2	3118	1541	647,5	340,7	9246	4410
Coefficient of variation	52,90%	40,81%	150,2%	94,93%	152,7%	112,7%	117,4%	90,09%	200,7%	144,4%

TBEv-Neutralizing IgG (VIU/ml)	Wk 0		Wk 4		Wk 8		Wk 24		Wk 28	
(Figure 6b)	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-	CMV+
Minimum	5	5	0	5	0	0	0	5	5	5
Maximum	5	5	40	40	640	160	240	40	5120	1280
Range	0	0	40	35	640	160	240	35	5115	1275
Mean	5	5	9,706	8,986	58,82	28,62	15,15	8,913	232,3	96,45
Std. Deviation	0	0	9,379	7,601	90,76	33,77	30,42	8,526	681,2	173,3
Std. Error of Mean	0	0	1,137	0,9151	11,01	4,065	3,689	1,026	82,61	20,87
Lower 95% CI of mean	5	5	7,436	7,159	36,85	20,51	7,784	6,865	67,38	54,81
Upper 95% CI of mean	5	5	11,98	10,81	80,79	36,74	22,51	10,96	397,2	138,1
Coefficient of variation	0,000%	0,000%	96,64%	84,59%	154,3%	118,0%	200,8%	95,66%	293,3%	179,7%

3. Study flow diagrams



STROBE Statement
Strengthening the reporting of observational studies in epidemiology

4. STROBE statement - Abstract

Item	Recommendation
Title	Primary immune responses are impacted by persistent herpesvirus infections in older people: results from a observational study of healthy subjects and a vaccine clinical trial on subjects aged more than 70 years old.
Authors	Francesco Nicoli, Emmanuel Clave, Kerstin Wanke, Amrei von Braun, Vincent Bondet, Cécile Alanio, Corinne Douay, Margaux Baque, Claire Lependu, Peggy Marconi, Karin Stiasny, Franz X. Heinz, Margot Muetsch, Darragh Duffy, Jacques Boddaert, Delphine Sauce, Antoine Toubert, Urs Karrer, Victor Appay Correspondence: urs.karrer@ksw.ch (U.K.) or vappay@immuconcept.org (V.A.)
Study design	Assessment of immunological parameters in two independent cohorts
Objective	Establish the influence of persistent viral infections on the naïve T-cell compartment and primary immune responsiveness in older adults
Methods	Assessment of serological status for common herpesviruses, inflammation related cytokine serum levels, T lymphocyte immunophenotyping, antigen specific T cell responsiveness, and vaccine antibody titers
Setting	The first cohort (observational study) consisted of healthy adults recruited among blood donors or at the geriatric department of the Pitié-Salpêtrière Hospital (Paris, France) between 2015 and 2016. The second cohort (clinical trial) consisted of individuals who received a full TBE vaccination course at the University of Zürich Switzerland between 2007 and 2010.
Participants	Cohort 1. Healthy individuals aged 19y to 95y (n= 150) Cohort 2. Primo-vaccinated individuals above 70y old (n= 137)
Variables	Differences in naïve T-cell counts and primary immune responsiveness according to herpesvirus serological status
Statistical methods	Univariate statistical analyses using the non-parametric Mann-Whitney test to compare groups, and the Spearman's rank test to determine correlations.
Main results	Effect of age and CMV infection on $CD8^+$ and $CD4^+$ naïve T cell count decline respectively Association between CMV seropositivity and blunted $CD4^+$ T-cell and antibody responses to primary vaccination
Conclusions	Age and persistent infections have distinct impacts on the generation of primary immune responses

STROBE Statement
Strengthening the reporting of observational studies in epidemiology

5. STROBE Statement— Observational cohort study

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5
Objectives	3	State specific objectives, including any prespecified hypotheses	6 (last paragraph of intro)
Methods			
Study design	4	Present key elements of study design early in the paper	7. "Study design, setting and participants" paragraph in Methods section
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	7. "Study design, setting and participants" paragraph in Methods section
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	(a) 7. "Study design, setting and participants" paragraph in Methods section
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	(b) not applicable.
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	8. "Variables and biases" paragraph in Methods section
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	8-10. The following paragraphs in Methods section: "Herpesvirus serological assays", "Measurement of inflammation associated cytokines", "Phenotypic analysis", "Analysis of TBEv-specific humoral and cellular immune responses", "DNA extraction and TREC analysis on PBMCs", "DNA and RNA extraction on sorted cell populations", "sjTREC digital droplet PCR", "TCR repertoire analysis".

Bias	9	Describe any efforts to address potential sources of bias	8. “Variables and biases” paragraph in Methods section
Limitations	10	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	8. (Second last paragraph of the Discussion)
Study size	11	Explain how the study size was arrived at	7-8. “Sample size” paragraph in Methods section
Quantitative variables	12	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	10. “Statistical analysis” in Methods section.
Statistical methods	13	(*a*) Describe all statistical methods, including those used to control for confounding (*b*) Describe any methods used to examine subgroups and interactions (*c*) Explain how missing data were addressed (*d*) If applicable, explain how loss to follow-up was addressed (*e*) Describe any sensitivity analyses	(a-b) 10. in Methods section. (c-e) not applicable.
Results			
Participants	14*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	(a) 26-28. Figure legends (b) not applicable. (c) Supplemental material.
Descriptive data	15*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	(a) 20. Table 1 (b) 8. (c) not applicable.
Outcome data	16*	Report numbers of outcome events or summary measures over time	12-16. All paragraphs of the Results section
Main results	17	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (*b*) Report category boundaries when continuous variables were categorized (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	12-16. All paragraphs of the Results section and Figures, when applicable. (b) not applicable. (c) not applicable.
Other analyses	18	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	not applicable.
Discussion			
Key results	19	Summarise key results with reference to study objectives	17-19
Generalisability	20	Discuss the generalisability (external validity) of the study results	18. (fourth paragraph of the Discussion)
Interpretation	21	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	18-20. (last paragraph of the Discussion)

Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	22. "FUNDING SOURCES" section

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

6. CYTEL Study Protocol: Version 1/Amendement 1

Influence of persistent Cytomegalovirus-infection on immune senescence evaluated with a prospective vaccination trial against tick-borne encephalitis virus in healthy elderly individuals (CYTEL-Study)

Principal investigator:
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Co-investigator:
Prof. Robert Steffen[2], MD

Study sites:
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[2]Institute for Social and Preventive Medicine
Vaccination Centre
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Running title: CMV-infection and immune senescence

Aims:
1. Direct evaluation of the influence of persistent Cytomegalovirus (CMV) infection on the immune response to tick-borne encephalitis virus (TBEV) vaccination in the healthy elderly (the response to TBEV-vaccination serves as a surrogate marker for immune senescence)
2. Elucidation of mechanisms of interference between persistent CMV-infection and de novo immune responses
3. Provision of data concerning efficacy and safety of TBEV-vaccination in the elderly

Design:
Prospective, single centre, phase IV evaluation of the efficacy and safety of TBEV-vaccination using one of the currently licensed TBEV-vaccines for adults (Encepur® N or FSME-Immun® CC) in 120 healthy elderly individuals, subdivided into two equal groups of 60 CMV-seropositive and 60 CMV-seronegative subjects.

Study population:
120 TBEV-naïve, healthy, elderly individuals (> 70 years), willing to be vaccinated against TBEV. Screening of 250-300 individuals will be necessary for the recruitment of 120 suitable subjects (estimated CMV-seroprevalence of 70-80%).

Time frame:
Screening period: 1.1.2007 - 31.10.2007
Duration of clinical study: 1.1.2007 - 30.6.2008
Laboratory and statistical analysis: 1.1.2007 - 1.12.2008
Publication of results: 1.4.2009

Place, date printed name signature

_____ _____ _____

Protocol of the CYTEL-study

1. Study rationale

1.1. Scientific background

Infectious diseases such as pneumonia, urinary tract and soft tissue infections are more frequent, more severe and more difficult to treat in aging individuals [1]. Since the population of elderly people will continuously and substantially increase during the next 50 years, efficient prevention and treatment strategies for infectious diseases in this population are of primary importance from an individual and a public health perspective.

The ageing of the immune system, usually termed immune senescence, is one of the most important factors contributing to the increased vulnerability of elderly individuals to infectious diseases [2]. Immune senescence is usually defined as the age-related reduction and dysregulation of immune function. However, the definition remains vague in terms of the precise immunologic parameters which are affected by the ageing of the individual. Therefore, a direct functional *in vivo* readout based on the cooperative action of antigen-presenting cells, T cells and B cells is currently the best predictor to estimate the degree of immune senescence of the adaptive immune system. Analyzing the de novo immune response after vaccination exactly fulfils these demanding criteria.

It is clear that adaptive immunity in particular is influenced by immune senescence at various stages of immune cell development, maintenance and function. As a consequence, elderly individuals are more susceptible to viral respiratory infections (Influenza virus and respiratory syncytial virus) and viral reactivations (Varicella Zoster virus, VZV) [3, 4]. Moreover, vaccines are generally less protective in the elderly leading to the dilemma that those in most need for protective immunity have the poorest vaccine response [5]. Therefore, it is crucial to increase our knowledge about the parameters that are responsible for decreased immune reactivity in the elderly. Such knowledge should pave the way to improve vaccine responsiveness and to enhance resistance to infection in the last decades of life.

1.2. Current status of research

It has recently been suggested that persistent viral infections, especially infection with persistent herpesviruses like Cytomegalovirus (CMV) and Epstein Barr Virus (EBV), may be important driving forces for premature immune senescence [6]. A substantial amount of circumstantial evidence has been accumulated supporting this concept particularly for persistent CMV-infection. Longitudinal studies of very elderly individuals demonstrated an association between CMV-seropositivity and decreased survival [7, 8]. This so-called ‘immune risk phenotype’ correlated with the accumulation of end-stage differentiated CMV-specific $CD8^+$ T cells [9-11].

Two main hypothesis have been proposed how these expanded CMV-specific $CD8^+$ T cell populations contribute to premature immune senescence: 1) The ‘space’ hypothesis: Large expansions of CMV-specific memory T cells may preferentially occupy the limited space, which is available for cells within the lymphoid system and thus exclude other cells with specificities for non-persisting antigens [9, 10, 12]. 2) The ‘deviation’ hypothesis: expanded CMV-specific memory T cell populations may support a proinflammatory environment, possibly affecting the balance between T helper cell type 1 (Th1) and Th2 cytokines [6, 13-15]. Such a proinflammatory state was reported in CMV-seropositive elderly individuals [16] and was associated with a poorer antibody response to influenza vaccination [17].

Overall, these results clearly imply that persistent infections in general and CMV-infection in particular may have a profound and age-related effect on the propagation of immune senescence by shaping the composition and reducing the remaining reactivity of the T cell compartment in elderly individuals. However, direct evidence for such a role from controlled

human studies performed with sufficiently detailed immunological analysis to elucidate potential mechanisms and strategies for interference are currently lacking.

1.3. Proposed study

To firmly establish whether and how persistent CMV-infection influences immune senescence we will conduct a prospective vaccination trial in healthy elderly individuals using the currently licensed vaccine for protection against tick-borne encephalitis virus (TBEV). The strength and the kinetics of the TBEV-specific humoral and cellular immune response after vaccination will be a direct readout for the immunocompetence of these elderly individuals and will be inversely correlated with the degree of immune senescence. In addition, comprehensive evaluation of vaccination efficacy is of primary importance for future strategies to limit the impact of infectious diseases in the elderly population.

We plan to recruit 60 CMV-seropositive and 60 CMV-seronegative elderly individuals and we will measure their TBEV-specific immune response longitudinally during the course of vaccination. As a primary endpoint, we will compare the TBEV-specific vaccine response between CMV-seropositive and CMV-seronegative individuals. If persistent CMV-infection has indeed an important influence on immune senescence we would expect to measure a poorer vaccine response in the CMV-seropositive group. We will also compare the TBEV-specific immune response with the general phenotype and function of the immune system to define surrogate markers of immune senescence. In the group of CMV-seropositive individuals, we will correlate the CMV-specific cellular immune response with the TBEV-specific response. These detailed analyses will allow us to investigate potential mechanisms of interference between immune reactivity against persistent CMV-infection and a de novo immune response.

We have selected the TBEV-vaccine as our model antigen and not the Influenza vaccine, since we want to analyse the capacity of the ageing immune system to mount a de novo immune response (and not a recall response). In addition, the currently licensed TBEV-vaccines are highly immunogenic, they have an excellent safety record and their use is newly recommended for all persons (>12 years) living in the canton of Zürich with possible exposure to infested ticks. Since elderly individuals are at higher risk for severe and persistent neurologic disability after TBEV-disease, vaccination is especially recommended in the exposed elderly.

1.4. Additional research activities

In parallel with the proposed clinical trial we plan to establish a mouse model to study the influence of persistent viral infections on immune senescence. Currently, such a model does not exist. Based on our previous studies with mouse CMV we will be able to generate a mouse model to investigate potential mechanisms of infection enhanced immune senescence [18, 19]. Such a model will also help to develop and test vaccination strategies, which have the potential to overcome the negative impact of accelerated immune senescence. However, the development of such an animal model will require a minimum two years of intense research. Therefore, it seems reasonable to perform the clinical trial and the mouse studies in parallel. All these immunological analyses will be conducted in the laboratory of the principal investigator generating important synergies and direct knowledge transfer between the human and the mouse studies. Overall, this strategy guaranties a most comprehensive approach to tackle the question of persistent infections and immune senescence with the ultimate aim to develop more potent vaccines for the elderly.

2. Time protocol

Screening period: 1.1.2007 - 31.10.2007
Duration of clinical study: 1.1.2007 - 30.6.2008
Laboratory and statistical analysis: 1.1.2007 - 1.12.2008
Publication of results: 1.4.2009

3. Specific aims of the study

1. Direct evaluation of the influence of persistent Cytomegalovirus (CMV) infection on the immune response to tick-borne encephalitis virus (TBEV) vaccination in the healthy elderly (the response to TBEV-vaccination serves as a surrogate marker for immune senescence)
2. Elucidation of mechanisms of interference between persistent CMV-infection and de novo immune responses
3. Provision of data concerning efficacy and safety of TBEV-vaccination in the elderly

4. Design, predefined endpoints, procedures and analyses of the study

4.1. Study design

Prospective, single centre, phase IV evaluation of the efficacy and safety of TBEV-vaccination using one of the currently licensed TBEV-vaccines for adults (FSME-Immun® CC) in 120 healthy elderly individuals, subdivided into two equal groups of 60 CMV-seropositive and 60 CMV-seronegative subjects.

4.2. Endpoints

4.2.1. Primary endpoint:
Geometric mean titer (GMT) of anti-TBEV-antibodies measured by TBEV-neutralisation assay and ELISA one month after each TBEV-vaccine administration in the group of CMV-seropositive versus CMV-seronegative individuals [20].

4.2.2. Secondary endpoints (*in CMV-seropositive versus CMV-seronegative individuals):
Strength of the TBEV-specific $CD4^+$ T cell response over time (ELISpot, proliferation)*
TBEV-seroconversion rate over time*
TBEV-specific IgM-response (GMT) after primary TBEV-immunization*
Correlation of the TBEV-specific humoral and cellular immune response with the CMV-specific cellular immune response in CMV-seropositive individuals
Multivariable analysis of variables possibly affecting TBEV-vaccination efficacy after 1, 2 or 3 immunizations: age, sex, nutrition (body mass index), physical activity, current drug treatment, co-morbidities, CMV-serostatus, EBV-serostatus, Helicobacter pylori (HP) carriage
Frequency and severity of adverse reactions after TBEV-vaccination in healthy elderly

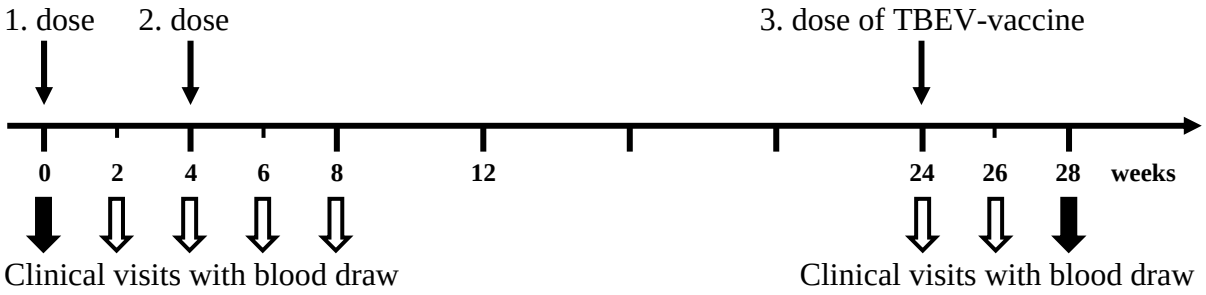
4.3. Intervention, procedures and clinical visits:

Participants will be vaccinated with a licensed vaccine against TBEV for adults (FSME-Immun® CC) at screening and after 1 and 6 months. Blood will be collected at screening and 2 and 4 weeks after each administration of a vaccine dose to monitor humoral and cellular immune responses. Potential adverse vaccine reactions will be assessed at these visits.

Individuals can volunteer to participate in a supplementary study group, if they fulfil all inclusion/exclusion criteria of the study, but cannot be included because of the randomisation process according to CMV-serology and screening date. These individuals receive all

vaccinations according to the study plan but blood for efficacy analyses will only be collected before and after the whole vaccination course (week 0 and 28). Safety analyses will be performed at all visits.

4.3.1. Time schedule of vaccinations and clinical visits:



For immunological analyses 100ml of blood will be taken on week 0 (screening) and on week 28 (study termination). On all other occasions 50 ml of blood will be taken. The total amount of blood of about 500ml taken during the 28-week study period is equivalent to a single blood donation.

4.3.2. Justification for the frequent and substantial blood sampling
The analysis of the cellular immune response against TBEV and CMV is of crucial importance for the results of this study. We need to screen the T cell responses of all patients concerning recognized T cell epitopes derived from TBEV and CMV and we expect to measure only weak cellular immune responses, particularly concerning $CD4^+$ T cell responses against TBEV. To enhance the chance of measuring positive responses in a relevant proportion of participants and to allow such a comprehensive immunological analysis longitudinally we require the suggested amount of blood. The frequent blood sampling two and four weeks after each immunization is necessary, since we expect the peak of the cellular TBEV-specific response two weeks after each immunisation whereas the humoral immune response is usually measured after 4 weeks (allowing comparisons with the published literature).

4.4. Planed analyses of the study
4.4.1. Clinical evaluation
- monitoring of adverse events in relation to vaccine administration
- documentation of adverse reactions in case report forms (CRF)

4.4.2. Laboratory analyses
- separation of PBMCs and cryopreservation of cells and sera for further analysis
- CMV-serology before and after the study (after the study for CMV- only)
- EBV and HP-serology
- measurement of TBEV-specific IgM and IgG by ELISA and of TBEV-neutralizing antibodies by neutralization assays before, during and after the vaccination period.
- analysis of TBEV-specific $CD4^+$ T cell responses by ELISpot, intracellular cytokine staining (ICS) and T cell proliferation
- nalysis of CMV-specific CD8 and $CD4^+$ T cell responses by ELISpot, ICS, degranulation assays and T cell proliferation (if CMV+)
- phenotypic and functional characterization of T cells with FACS using diverse monoclonal antibodies and with polyclonal stimulation for proliferative capacity

4.4.3. Analysis of study results and statistics

Vaccination efficacy will be analyzed longitudinally by TBEV-neutralisation assay and TBEV-specific ELISA [20]. The GMT of these tests are the recognized surrogate markers for protective immunity against TBEV [21].

Analysis of cellular immune responses against TBEV will provide a more direct readout for T cell responsiveness in our study population.

TBEV-specific antibody and T cell responses will be compared between the groups of CMV-seropositive and CMV-seronegative individuals using a T-Test in case of normal distribution or a non-parametric Wilcoxon Test. Univariable and multivariable analyses will be performed to assess the influence of other parameters on vaccination efficacy and immune senescence. These include age, sex, nutrition (body mass index), physical activity, medication, co-morbidities, EBV-and HP-serology. Detailed analysis of the cellular immune response against CMV in CMV-seropositive individuals and correlation of these CMV-related immune parameters with TBEV-specific humoral and cellular immune responses using Pearson's or Spearman's rank correlation will allow to propose immunological mechanisms of interference.

The efficacy and safety of the vaccine within our entire population will be compared with the published literature.

5. Safety measures for study participants

5.1. Risks and side effects

Since we use a licensed vaccine with a long-standing safety record unexpected risks and side effects are unlikely. However, minor side effects may occur more frequently than anticipated from the literature, since we conduct the trial in volunteers of an age group, which may be more vulnerable because of increasing age and where experience with the TBEV-vaccines is more limited. Minor side effects are expected at the following ratio (Documed 2006): *very frequently* (>10%): pain (13%) and tension (30%) at the injection site. *Frequently* (1-10%): headache, nausea, myalgia and arthralgia, fatigue, malaise. *Rarely* (0.1-1%): swelling, induration and erythema at the injection site, vomiting, lymphadenopathy, fever. *Very rarely* (< 0.01%): major side effects.

Nevertheless, study related side effects will be monitored and recorded in the case report form (CRF) at each visit and severe side effects (Grade 3-4) will be immediately reported to Swiss medic and to the manufacturer of the applied vaccine.

Potential adverse effects of the frequent and substantial blood sampling include development of anemia, phlebitis and soft tissue infection. A normal haemoglobin at screening will prevent inclusion of anemic subjects and collection of about 500ml of blood over 28 weeks will not precipitate relevant anemia in a healthy elderly individual. Impeccable hygiene and careful phlebotomy will prevent inflammation and infection.

Overall, the risk of study participation seems minimal and the inconvenience for the participants is limited to the extra visits and to the frequent and substantial blood draws. Long term side effects of study participation are highly unlikely.

5.2. Procedures in case of side effects

In case of minor adverse reactions like local pain, headache or fever we will provide symptomatic treatment and follow-up examinations if required. In case of major adverse reactions appropriate diagnostic and therapeutic interventions will be initiated immediately. These procedures are covered by the insurance of the study.

5.3. Insurance coverage
For study-related risks and side effects all participants and the whole study team are fully covered by an insurance of the sponsor.

5.4. Information and support of the study team
Study physicians and nurses will be trained by oral presentation of the planed conduction of the study. The CRF will provide additional guidance for the correct conduction of the study. All study visits will be recorded in the CRF. We have the advantage of conducting the clinical part of the study at the vaccination centre of the University of Zürich (Hirschengraben 84), where all the staff has extensive experience with vaccination procedures and associated problems. If clinical or logistic problems arise the principal investigator or the co-investigator can be contacted by phone. Their direct intervention will be guaranteed within 15-30 min.

6. Study population

We plan to recruit 120 healthy elderly individuals (>70 years) subdivided into two equal groups according to their CMV-serology (CMV-seropositive versus CMV-seronegative). In case of slow recruitment after 3 months (less than 60 participants recruited) we will reduce the age limit to 65 years. The participation in this study is entirely voluntary and informed consent can be retracted at any time and without justification.

6.1. Counter measures to avoid recruitment bias
Because of a CMV-seroprevalence of 70-80% in this age group we will have to screen 200-300 individuals to recruit 60 CMV-seronegative persons. To ensure unbiased recruitment a CMV-seropositive subject (70-80% of the population) will only remain within the study, if a CMV-seronegative subject (20-30% of the population) was recruited before, leading to a 1:1 recruitment process. Continuation of the study for CMV-seropositive subjects will be decided on the basis of screening date and time.
After 50% of the recruitment is completed a planed interim analysis will compare recruited subjects concerning demographic parameters (age and sex distribution) and drop out rate. If this analysis reveals a substantial inequality between groups the recruitment process may be adjusted and/or prolonged accordingly (after approval of a protocol amendment by the local ethical committee).

6.1.1. Amendment of the randomisation process
During the study, we realized that CMV-seroprevalence in our screening population was in the range of 50% (and not 70-80% as expected). This has caused a transient recruitment bias in favour of CMV-seronegative individuals. Therefore, we adapted the randomisation strategy in October 2007 to allow recruitment into group A or B only, if the other group had already included the same number or more individuals before. To reach a similar group size, recruitment was only closed after definitive inclusion of 68 (group B) and 69 (group A) individuals.

Additional eligible individuals with an unsuitable CMV-serology at the time of randomisation (who fulfil all other inclusion/exclusion criteria) can volunteer to participate in the supplementary study group where we only perform blood analysis for efficacy at visit 1 (screening) and visit 8 (end of study). Vaccine administration and recording of adverse events will also be performed at visit 3 (week 4) and visit 6 (week 24).

6.2. Sample size

Our sample size calculation is based on published data on efficacy and variability of the antibody response after TBEV-vaccination [21]. To detect a two-fold difference in the geometric mean titer (GMT) of TBEV-neutralizing antibodies after the total TBEV vaccination course between the CMV+ and CMV- population we would have to recruit 84 patients in each group to reach a power of 80% with a ONE-SIDED significance level of 5%. Since we will measure TBEV-specific immune responses longitudinally in all patients with separate measurements after one, two and three doses of the vaccine, it will be sufficient to recruit 60 patients for each group to reach a power of > 80% with a ONE-SIDED significance level of < 5%. This calculation includes a safety margin for drop-outs before study completion of 10 subjects per group (15%).

6.3. Inclusion criteria

1. Age >70 years (reduction to 65 years in case of slow recruitment after 3 months)
2. Local criteria for TBEV-vaccination fulfilled (tick exposure possible)
3. Healthy according to a questionnaire based on the modified Cornell medical index (CMI)[22]
4. Capable to understand and sign informed consent form (based on the judgement of the study physician)

6.4. Exclusion criteria

1. Previous exposure to TBEV or TBEV-vaccine
2. Immunodeficiency, history of autoimmune disease or current intake of immune-modulating drugs (corticosteroids a.s.o.)
3. Persistent (> 3 months) pharmacological treatment with more than one drug (exception: combination antihypertensives, i.e. ACE-inhibitor + thiazide)
4. Contraindication for TBEV-vaccination (according to Documed 2006)
5. Past medical history and/or current treatment for one of the following conditions (according to the modified CMI [22]):
   - chronic cardiac disease
   - chronic pulmonary
   - chronic kidney disease
   - diabetes mellitus
   - previous stroke
   - epilepsy
   - dementia (self reported or judged by the interview at screening)
   - Parkinson's disease
6. Laboratory parameters at screening:
   - haemoglobin <12 g/l
   - Blood glucose > 7 mmol/l
   - calculated Creatinin clearance (CCl) <50ml/min

     Formula: CCl = [150-age] x weight (kg) / Creatinin (µmol/l)
     +10% for males; -10% for females

6.5. Recruitment strategy

The study will be advertised in the local media. The advertisement will contain the most important information about the aim of the study, about eligibility, about possible risks and benefits and about availability of further information. General practitioners and organisations of the elderly will be informed separately.

Study participants will be reimbursed for each visit with blood collection according to the following scheme (in Swiss Francs):

- screening visit (visit 1): 50.-
- visit 2-7: 20.-
- study termination (visit 8): 100.-
- Total: 270.-

6.6 Information of participants and informed consent form

Participants will be informed about the study by a study information leaflet which will be available on the internet and in print. This leaflet contains detailed information in simple language about the scientific background and the aims of the study, about its practical course, about personal benefits and risks for participants, about insurance coverage, about confidentiality and about voluntariness of participation.

Before formal screening, potential participants will be asked to complete a confidential questionnaire that will cover the most important inclusion and exclusion criteria to avoid unnecessary screening efforts.

If all inclusion and exclusion criteria are met according to the questionnaire and volunteers are still willing to participate, the formal screening visit starts with additional verbal information about the study also addressing potential questions and concerns of the participant. After a thorough interview about the current health, medication and the past medical history including vaccination history, blood pressure, pulse and temperature are recorded. Informed consent will be signed by the study physician and the participant. About 100ml of blood will be taken for screening analyses and the first dose of TBEV-vaccination will be applied.

In case of clinical screening failure before signature of the informed consent according to the above mentioned inclusion and exclusion criteria, TBEV-vaccination is offered to the candidate on private health insurance (if indications and contraindications of TBEV-vaccination are met). Screening failure caused by laboratory values outside of the specified range (Hb, Glucose, Creatinin, CMV-serology) will also occur after signature of informed consent and administration of the first dose of TBEV-vaccine. These participants (mainly CMV-seropositive individuals) will be informed personally before the next scheduled visit and offered continuation of the TBEV-vaccination course outside of the study at the vaccination centre free of charge.

7. Human and infrastructural resources

The clinical part of the study will be conducted at the vaccination centre of the University of Zürich which is located in the very centre of Zürich with extremely easy access by public transport. The facilities are perfectly equipped to conduct a vaccination trial. During the screening period a (part-time) study nurse will work exclusively for the study and will conduct the visits, the vaccinations and the blood collection. Advice and support by study physicians is available within the vaccination centre and principal or co-investigator are reachable by phone at all (office) times to provide support within 30 min.

The blood will be safely transported to the laboratories of the division of infectious diseases at the University Hospital and processed immediately. The laboratories are located within a 5 min walking distance from the vaccination centre.

Two PhD-students and a technician working in the laboratory of the principle investigator will be responsible for the immunological analyses of the patient samples. The principle investigator is funded by the Swiss National Science Foundation with a ‘Förderungsprofessur’ to conduct this vaccination trial with the aim to establish an independent research group in the field of immune senescence and infectious diseases.

Additional funding and provision of all vaccination doses will be requested from the manufacturers of the TBEV-vaccines and from other private foundations (negotiations and additional grant applications are ongoing).
Statistical analyses of the data will be performed with the help of PD Dr. B. Ledergerber from the Division of Infectious Diseases, University Hospital of Zürich. Data management will be performed by the principle investigator and all files and data will be stored at the University Hospital of Zürich for at least 10 years.

8. Study flow chart

CYTEL-Studie: CMV-infektion und immunologische Alterung

Screening Nr. ..... geb. Datum ..... Initialen .....

Datum	Studienwoche / U-Suchung / Labors / Typ - Röhrchen	Visiten Nr	Klinische Evaluation gemäss CRF*	Ent-schädi-gung	Impfung	Blutentnahmen: Zellen / Infekt / EDTA 10ml	Hb / Hämat / EDTA 3ml	Chemie[1] / Klin Chem / Heparin 3ml	Serolog[2] / diverse / nativ 5ml	Total ml/BE
	Woche 0	**1**	Fragebogen	50.-	1. Dosis	9	1	1	1	100
	(Screening)		Anamnese							
			Informed consent							
			BD, P, Temp							
	Woche 2	**2**	Gesundheit, NW	20.-		5				50
	Woche 4	**3**	Gesundheit, NW	20.-	2. Dosis	5				50
	Woche 6	**4**	Gesundheit, NW	20.-		5				50
	Woche 8	**5**	Gesundheit, NW	20.-		5				50
	Woche 24	**6**	Gesundheit, NW	20.-	3. Dosis	5				50
	Woche 26	**7**	Gesundheit, NW	20.-		5				50
	Woche 28	**8**	Gesundheit, NW	100.-		10			1 [3]	100
	(Abschluss)		BD, P, Temp							
Total	**28**	**8**		270.-	3	49	1	1	2	500

*** Abkürzungen:**
CRF, Case report form; Hb, Hämoglobin; BE, Blutentnahme; BD, Blutdruck; P, Puls; Temp, Temperatur; NW, Nebenwirkung
[1] Glucose, Kreatinin
[2] CMV-, EBV- und Helicobacter pylori-Serologie
[3] CMV-Serologie

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