Human endogenous retrovirus HERV-K(HML-2) *env* expression is not associated with markers of immunosenescence

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Abstract

Ageing of the human immune system, or immunosenescence, is characterised by distinct changes in the proportion of the various cell types, e.g., increase of the CD14+ monocytic cells, decrease of CD19+ B lymphocytes, and changes in T cell subpopulations, namely increase of CD4+ and CD8+ cells which have lost the costimulatory CD28 antigen. Currently, it is believed that the lifelong antigenic burden may be one of the inducers of immunosenescence. Thus far, only one exogenous stimulus, cytomegalovirus infection, has shown to be a major factor in this respect. To find other possible candidates, we evaluated the role of the evolutionary youngest group of human endogenous retroviruses, namely HERV-K(HML-2), on immunosenescence. HERVs exist in the genome as proviruses, but their activation has been detected in several immunopathologic conditions. The expression of HERV-K(HML-2) *env* was observed to be lower in the peripheral blood mononuclear cells of nonagenarians (n = 61) than in those of young controls (n= 37). These mRNA levels did not correlate with the age-associated differences in the proportions of CD14+, CD4+CD28- and CD8+CD28- cells, but in the case of CD19+ B cells a strong positive correlation was observed in the nonagenarians. Thus, these data suggest that HERVs do not function as antigenic drivers of immunosenescence. On the contrary, expression of HERV-K(HML-2) *env* is associated with more youthful levels of B cells.

Introduction

The ageing-associated changes in the immune system have been extensively characterised during the last few decades (reviewed in Lang et al. 2013, Goronzy et al. 2013). The hallmarks of immunosenescence are the decreased number of naïve T cells and accumulation of late-stage memory T cells. In the B cell compartment, this decline of naïve cells is also detectable. Ageing is also associated with low-level increase in inflammatory mediators, termed inflamm-aging (reviewed in Franceschi et al. 2014). At the cellular level, one important question has yet to be answered: is immunosenescence an intrinsic ageing process or an adaptive response to an individual pathogen exposure? Thus far, only one pathogen, human cytomegalovirus (CMV), seems to fulfill the criteria of an extrinsic antigen. CMV infection takes place usually in early childhood and the virus remains in a latent form in the body. In elderly individuals, the seropositivity rate is 70-100%. The associations between CMV seropositivity and the ageing-associated changes in the various cell types and functions of the immune system have been extensively demonstrated (reviewed in Söderberg-Naucler et al. 2016, Weltevrede et al. 2016). However, ageing is associated with changes in the function and composition of the immune system also in CMV seronegative individuals (Di Benedetto et al., 2015). In addition, with genome-wide transcriptomic analysis we have identified ageing-associated changes in the immune system in both CMV seronegative and CMV seropositive individuals and have demonstrated that the identified changes differ between CMV seronegative and seropositive individuals (Kuparinen et al., 2013). These results suggest that CMV is only partially responsible for the induction of immunosenescence.

In this study, we analyse the role of an additional virus group, i.e., the human endogenous retroviruses (HERVs) in immunosenescence. HERVs are remnants of ancient infections and have remained in the human genome as proviruses, comprising about 8% of the genome (Magiorkinis et al. 2013). Previous research show that these proviruses, including the evolutionary youngest ones, the HERV-K(HML-2) group members, can be expressed in several pathological conditions, e.g., in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (as reviewed in Magiorkinis et al. 2013). However, no direct causal mechanism has been demonstrated between HERVs and pathological conditions. For instance, no reports show that HERVs have retrotransposonal capacity, and the possible pathogenic effects are thought to be mediated via viral RNA and protein (reviewed in Suntsova et al., 2015). In addition, HERV sequences in the genome may function as regulatory elements and, thus, modify the expression of nearby genes (Magiorkinis et al. 2013, Volkman & Stetson 2014). In this study, we investigate whether the HERV-K(HML-2) RNA could contribute to immunosenescence as shown in exogenous pathogens.

Materials and Methods

The study population consist of 61 nonagenarians born in 1920 (21 males, 40 females) and 37 young controls (healthy laboratory personnel, aged 19-34 years, 12 males, 25 females), who participated in the Vitality 90+ study in 2014. The Vitality 90+ study is an ongoing, prospective, population-based study that includes both home dwelling and institutionalized individuals who are 90 years of age or older, and who live in the city of Tampere, Finland. The recruitment and characterisation of the participants were performed as has been reported previously (Goebeler et al. 2003). The study subjects were all of Western European descent and had not had previous infections or received any vaccinations in the 30 days prior to the blood sample collection. The study participants provided their written and informed consent to participate in the study. This study was conducted according to the principles expressed in the declaration of Helsinki. The study protocol was approved by the ethics committee of the city of Tampere (1592/403/1996).

Blood samples from nonagenarians were collected by a trained laboratory technician during a home visit and the samples from young controls were collected by a trained laboratory technician in the laboratory facilities. All blood samples were drawn between 8 am and 12 am and collected into EDTA containing tubes. Samples were directly subjected to leucocyte separation on a Ficoll-Paque density gradient (Ficoll-Paque™ Premium, cat. no. 17-5442-03, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The plasma layer was collected and stored at -70 °C. The PBMC layer was collected and cells used for RNA extraction were suspended in 150 µl of RNAlater solution (Ambion Inc., Austin, TX, USA). Cells used for FACS analysis were suspended in 1 ml of a freezing solution (5/8 FBS, 2/8 RPMI-160 medium, 1/8 DMSO) (FBS cat. no. F7524, Sigma-Aldrich, MO, USA; RPMI: cat. no. R0883, Sigma-Aldrich, MO, USA; DMSO: cat. no. 1.02931.0500, VWR, Espoo, Finland) and stored in liquid nitrogen.

The anti-CMV IgG titre was measured using a commercial enzyme-linked immunosorbent assay kit (Enzygnost® Anti-CMV/IgG, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Seropositivity for CMV was defined according to the manufacturer's instructions as a plasma anti-CMV IgG titre≥ 230 U/ml. The plasma C-reactive protein (CRP) concentration was measured using Human CRP Immunoassay (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA).

The proportions of different leukocyte subtypes were determined using fluorescence-activated cell sorting analysis (FACS; BD FACSCanto II). The results were analysed with FlowJo software (Tree Star Inc., Ashland, OR, USA). The antibodies used were FITC-CD14 (cat. no. 11-0149), PerCP-Cy5.5-CD3 (45-0037), APC-CD28 (17-0289), PE-CD19 (12-0199) (eBioscience, San Diego, CA, USA), PE-Cy™7-CD4 (cat. no. 557852), and APC-Cy™7-CD8 (557834) (BD Biosciences). Staining was performed in phosphate-buffered saline (PBS) containing 1% foetal bovine serum (FBS) after an incubation step with Fc Receptor Binding Inhibitor (cat. no 16-9161, eBioscience) to minimise non-specific staining of the cells.

RNA was purified using RNeasy mini kit (Qiagen, CA, USA) according to manufacturer's protocol with on-column DNA digestion (Qiagen). The concentration and quality of the RNA were assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 300 ng of RNA was reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The qPCR was performed with PowerUp SYBR Green Master Mix (Applied Biosystems, MA, USA) in 7900HT (Applied Biosystems). Primers (Invitrogen) were used in 500 nM concentration with 6 ng of template per 20 µl reaction. Primers used for HERV-K(HML-2) *env*; forward CTAACCATGTCCCAGTGATG and reverse GGAGACAGACTCATGAGCTTAGAA (Bhardwaj et al., 2014). *GUSB* was used as an endogenous control; primers forward TGCAGGTGATGGAAGAAGTG and reverse TTGCTCACAAAGGTCACAGG (Beer et al, 2015). The PCR reactions were run as 2′ in 50 °C, 2′ in 95 °C and 40 cycles of 15′′ in 95 °C, 15′′ in 57 °C, and 1′ in 72 °C. All samples were run in triplicate. Expression level is expressed as ΔCt value, calculated as Ct(*env*)-Ct(*GUSB*). As a higher Ct value indicates lower expression level, and the expression level of GUSB is higher than that of HERV-K(HML-2) *env*, ΔCt(*env*) is high for low expression levels and *vice versa*.

Results

The age-associated differences in the proportions of the various immune cells in our study population were similar to those reported previously in the literature. The proportion of CD14+ monocytic cells, CD4+CD28-, and CD8+CD28- T cells were increased and the proportion of CD19+ B cells were decreased in the nonagenarians as compared to the young controls (Table 1). HERV-K(HML-2) activity was analysed using the expression of *env*. This expression was detectable in both nonagenarians and young controls, but the expression was slightly higher in the young controls (Table 1). To analyse the biological significance of HERV-K(HML-2) *env* expression, the expression levels were correlated with the cell type proportions. In nonagenarians, the expression of HERV-K(HML-2) *env* was correlated with the proportion of CD19+ B cells, with higher expression level associated with higher proportion of B cells (Table 2). The proportions of other measured cell types were not associated with expression of HERV-K(HML-2) *env* in nonagenarians. In the young controls HERV-K(HML-2) *env* expression was not associated with the proportions of any measured cell type (Table 2).

To analyse the role of HERV-K(HML-2) *env* in inflamm-aging, the expression level was correlated with the plasma level of CRP. However, no association was found in the nonagenarians (Spearman's ρ= -0.100, p= 0.446) or in the young controls (Spearman's ρ= -0.129, p= 0.446). The association of expression level of HERV-K(HML-2) *env* and anti-CMV IgG titer was also analysed, but no association was found among the nonagenarians (Spearman's ρ= 0.012, p= 0.926) or in young controls (Spearman's ρ= -0.017, p= 0.918).

Discussion

Our data reveal that the expression of HERV-K(HML-2) *env* is decreased in nonagenarians as compared to young controls. In addition, the expression of HERV-K(HML-2) *env* is not associated with classical cellular markers of immunosenescence. Thus, HERV-K(HML-2) *env* expression does not contribute to immunosensescence. However, HERV-K(HML-2) *env* expression correlated positively with the percentage of the CD19+ B cells in nonagenarians.

To the best of our knowledge, age-associated differences in HERV-K(HML-2) expression have not been reported in the literature previously. However, Balestrieri et al. (2015) analysed the ageing-associated expression differences in the whole HERV-K group. Contrary to our results, they show that children aged under 12 years have lower expression levels of HERV-K, but in adulthood there are no significant changes in HERV-K expression. As HERV-K(HML-2) is only one of the ten HERV-K HML groups, it appears that different HML groups are expressed independently of each other.

Changes in HERV expression have been reported in various diseases, including various cancers, rheumatoid arthritis, multiple sclerosis and in HIV infected patients. Contrary to ageing, these conditions are associated with increased expression of HERVs (reviewed in Magiorkinis et al., 2013). Also, the difference in HERV-K(HML-2) *env* expression between nonagenarians and young controls was modest compared to some of the reported differences in HERV expression between diseased individuals and healthy controls (Freimanis et al., 2010; Morandi et al., 2017).

The observed positive association between HERV-K(HML-2) *env* and B cells may be connected to the recent findings in Zeng et al. (2015). They observed that, in mice, B cell receptor crosslinking by T-cell independent antigens activates a signaling cascade, involving NF-κB, that leads to transcription of retroviral RNA and further to B cell activation and differentiation. HERV-K(HML-2) transcription was also shown to be upregulated by B cell receptor signaling in human B cells *in vitro* (Zeng et al., 2015). Thus, it may be that this positive or immunostimulatory effect of HERV-K(HML-2) is related to increased survival or proliferation of B cells in elderly individuals. We have shown that both IgG and IgA serum levels are significantly increased in nonagenarians as compared to those of middle-aged controls (Hurme et al. 2005). It is also possible that HERV-K(HML-2) *env* is predominantly expressed in B cells or in some B cell subpopulation.

Previous reports show an association between HERV expression and the inflammatory response, i.e., it is likely that increased production of HERV nucleic acids is able to activate inflammation (reviewed in Volkman & Stetson, 2014). Although increased inflammation, i.e., inflamm-aging (reviewed in Franceschi et al. 2014) is one of the hallmarks of ageing, our results suggest that HERV-K(HML-2) *env* expression does not contribute to this phenomenon, as *env* mRNA levels were not associated with percentage of CD14+ cells (Table 2) or levels of CRP.

Previous report shows that CMV is able to induce expression of HERVs, including HERV-K(HML-2), in cultured cancerous and normal cell lines as well as monocytes derived from healthy individuals (Assinger et al., 2013). However, we did not observe an association between anti-CMV IgG titer and HERV-K(HML-2) expression, suggesting that the reported effects of CMV on immunosenescence (reviewed in Söderberg-Naucler et al. 2016, Weltevrede et al. 2016) are not mediated via HERV-K(HML-2) *env* expression.

Expression of HERV-K(HML-2) *env* was used as an indicator of HERV-K(HML-2) activation. The primers used in this study amplify the *env* sequence of 30 HERV-K(HML-2) group members, yet there are over 90 HERV-K(HML-2) proviruses in the human genome (Subramanian et al., 2011). Although the *env* sequences of some of these proviruses are degraded, we cannot exclude that there might be additional HERV-K(HML-2) *env* expression not detected in our study. It remains to be understood whether the different members of the HERV-K(HML-2) group could have different effects on the immune system, depending on the proviral sequence or the location of the provirus in the genome. In addition, it is still not well understood how the amount of HERV-K(HML-2) *env* mRNA corresponds to the amount of env protein.

Author contributions

All authors contributed to the recruitment of the cohort and sample collection. SM and TN performed the laboratory analyses. SM and MH wrote the manuscript. All authors approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Table 1. Median and interquartile range of HERV-K(HML-2) *env* expression and cell type proportion of PBMCs from nonagenarians (n=61) and young controls (n=37). HERV-K(HML-2) *env* expression is presented as ΔCt value and cell type distributions are presented as percentages.

	Nonagenarians	Young controls	Mann-Whitney U-test p-value
HERV-K(HML-2) *env*	2.88 (2.64 - 3.19)	2.39 (2.18 – 2.69)	<0.001
CD14+[a]	9.28 (2.51 - 14.85)	5.03 (1.82 – 10.50)	0.031
CD19+[a]	2.36 (1.56 - 4.51)	6.18 (4.52 – 8.90)	<0.001
CD3+[a]	60.90 (50.75 - 67.70)	65.40 (58.10 – 69.10)	0.087
CD4+[b]	53.50 (46.20 - 68.25)	57.60 (53.55 – 62.45)	0.695
CD8+[b]	29.70 (21.45 - 43.65)	30.30 (26.75 – 35.45)	0.725
CD4+CD28-[c]	17.20 (7.10– 33.20)	1.50 (0.95 – 4.25)	<0.001
CD8+CD28-[d]	75.00 (62.20 – 82.15)	32.00 (21.65 – 41.60)	<0.001

[a]percentage of live cells
[b]percentage of CD3+ cells
[c]percentage of all CD4+ cells
[d]percentage of all CD8+ cells

Table 2. Correlation between the HERV-K(HML-2) *env* expression and proportions of different immune system cell types. As a smaller ΔCt value represents higher expression level, the negative correlation between the proportion of CD19+ cells and HERV-K(HML-2) *env* expression indicates that higher proportion of CD19+ B cells is associated with higher level of HERV-K(HML-2) *env* expression.

	Nonagenarians		Young controls	
	Spearman's ρ	p-value	Spearman's ρ	p-value
CD14+[a]	0.156	0.229	0.067	0.692
CD19+[a]	-0.334	0.009	-0.188	0.264
CD3+[a]	0.036	0.782	-0.265	0.113
CD4+[b]	-0.022	0.866	-0.079	0.642
CD8+[b]	-0.025	0.848	-0.085	0.618
CD4+CD28-[c]	0.209	0.106	0.088	0.605
CD8+CD28-[d]	0.072	0.581	0.188	0.265

[a]percentage of live cells
[b]percentage of CD3+ cells
[c]percentage of all CD4+ cells
[d]percentage of all CD8+ cells