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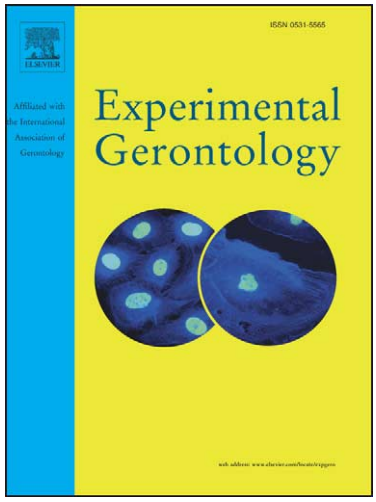
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Impact of CMV and EBV seropositivity on CD8 T lymphocytes in an old population from West-Sicily

Giuseppina Colonna-Romano[1] BioSci, Arne N. Akbar[2] BioSci, Alessandra Aquino[1] PhD, Matteo Bulati[1] PhD, Giuseppina Candore [1] BioSci, Domenico Lio [1] MD, Pietro Ammatuna [3] MD, Jean M. Fletcher [2] PhD, Calogero Caruso [1] MD, Graham Pawelec [4] MA, PhD

[1]Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, and [3]Dipartimento di Igiene e Microbiologia, Università di Palermo, Palermo, Italy; [2]Department of Immunology and Molecular Pathology, The Windeyer Institute For Medical Sciences, Royal Free and University College Medical School, London, UK; [4]University of Tübingen Medical School, Center for Medical Research, ZMF, Tübingen , Germany

Running title: herpes viruses and immunosenescence

Correspondence to: Prof. Calogero Caruso MD, Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metedologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy; e-mail: marcoc@unipa.it; Fax: +390916555933

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ABSTRACT

Herpes viruses (particularly CMV and to some extent EBV) might play a role in accelerating the deterioration of immune functions with age. Indeed, it has been demonstrated that chronic infection with CMV causes an expansion of specific CD8 T lymphocytes and that this is related to a shrinkage of the T cell repertoire in very elderly people, predicting mortality. We have analysed CD8 T cells in young and old healthy Sicilians who were both CMV- and EBV-seropositive. Our data confirm expansions of T cells specific for the HLA-A2-restricted pp65 (495-503) CMV epitope up to nearly 14% of total peripheral CD8 cells in certain elderly individuals (range 0 - 14%). However, the mean percentage of CMV-specific cells in the elderly was not greater than the young (range 0.2 – 3%)., The CMV-specific CD8 cells in the elderly were predominantly CD45RA+, but in the young they were mostly CD45RO+. Our findings are somewhat different from published reports from Northern European populations, both in terms of mean numbers and surface phenotypes.These findings may reflect disparate hygienic and nutritional conditions 70 - 90 years ago, which were very different in Northern and Southern Europe at that time, as well as a different genetic background.

Key Words: CD8, CMV, EBV, Elderly, Immune senescence

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INTRODUCTION

Many alterations of both innate and clonotypic immunity have been described in the elderly, and are generally considered to represent a deterioration of the immune system, designated immunosenescence (Effros, 2005; Vasto and Caruso 2004). A well-functioning immune system in the elderly is thought to be tightly correlated to health status, and some immunological parameters are often notably reduced especially in the frail elderly (Pawelec et al., 2002a). The OCTO longitudinal study of very elderly Swedes selected for good health at >85 yr of age has shown that a cluster of immunological parameters can be used to predict mortality. Increased numbers of CD8 cells, decreased CD4 cells, (CD4/CD8 ratio <1), poor T cell proliferation to mitogens, low B cell numbers and low IL-2 production are related to two year mortality in this very old population (Wikby et al., 1998). This led to the concept of an "immunological risk profile" (IRP) (Olsson et al., 2000; Pawelec et al., 2002b), which was later found to include seropositivity for CMV (Wikby et al., 2002; Pawelec et al., 2004). The fraction of 85 year-olds assigned to the IRP at baseline has been remarkably constant at ca. 20% of subjects at each wave. Retrospectively, several years ago oligoclonal expansions of T cells, especially of CD8 cells, had been reported as a characteristic of human and murine ageing (Pawelec et al., 2002a). Later it was established that CMV seropositivity was associated with many of the same phenotypic and functional alterations to T cell immunity as were being reported as biomarkers associated with ageing (Wang et al., 1995; Wedderburn et al., 2001).

With the advent of tetramer and other technologies, it was discovered that CMV was the driving force behind many or most of the oligoclonal expansions and the altered phenotypes and functions of CD8 cells observed in the elderly (Khan et al., 2002). Importantly, later longitudinal studies in Sweden, examining a representative sample of the very elderly not selected for good health, the majority of whom were actually frail, revealed that the IRP, including CMV seropositivity, still predicted mortality (Pawelec et al., 2005). More recent studies using this longitudinal "NONA" population, now over 90 yr of age, suggested that the number of different clones expanding in the elderly, and particularly the later clonal attrition observed to occur in IRP individuals, was highly correlated with 2, 4 and 6 year survival in this population (Hadrup et al., 2006). However, it is currently not known whether these data are applicable to all populations or whether they are unique to the different environment/genetic 4 background of the Swedish population. Because old Sicilian populations include very few people with an inverted CD4/CD8 ratio (Colonna-Romano et al., 2002), a surrogate for the IRP also observed in other elderly N. European populations (eg. in Britain, ref. Huppert et al., 2005) we asked whether CMV had an over-riding effect on specific immunity in this population as well. We also examined a second

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persistent herpes virus, EBV, which was known to have a similar, but lesser effect, in the Swedish population (Pawelec et al., 2005).

Thus, in the present paper, we have evaluated some parameters of immunity to herpes viruses (CMV and EBV) in a sample of elderly people from the Sicilian population, whose exposure to infective agents over most of their lifetime has presumably been higher than in Sweden. This is due to the general hygienic conditions (eg. bacterial and viral contaminants in the water) in Sicily at that time, which was rather under-developed compared to northern European countries until more recently. The results reveal some subtle differences in the two populations, but in general are in agreement. Differences may be due to the cross-sectional rather than longitudinal study design employed here, to the inclusion of less elderly individuals and to different historical, environmental and genetic factors that influence ageing and longevity (Candore et al., 2006).

2. MATERIALS AND METHODS

2.1 *Subjects*

The donors studied were healthy subjects. None of them was affected by neoplastic, infectious or autoimmune disease, neither were they receiving any drug influencing immune functions at the time of the study. The health status of donors was checked on the basis of both clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins, cholesterol, triglycerides). All the subjects gave informed consent according to Italian law. A total of 86 young and 54 old samples were selected for this study. Mean age of old people was 82.8±5.8 years (range 75-92); control donors were volunteers engaged from student and laboratory employees aged 20-61 years (mean 39.6±22.5). Both young and old samples were screened for CMV and EBV seropositivity. A CMV IgG EIA (Roche, Monza, Italy) test and an Elisa test were performed to determine respectively the CMV and EBV status using serum samples. As expected, the percentage of people seropositive for CMV was higher in the old than in the young group, but nearly a third of the old population remained CMV-seronegative. There was a similar pattern for EBV, but with lower rates of seropositivity in both groups. However, several subjects fall in the “grey zone”, i.e. a borderline levels of antibodies as evaluated by EIA tests. These results, according to the manufacturer’s instructions, must be interpreted in the context of the clinical course. So for the

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purpose of this study we have considered these subjects negative. Accordingly, in some of these subjects we have randomly performed tetramer staining with no positive cells found.

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2.2 ***Typing.***

All the CMV and/or EBV seropositive subjects were HLA class I typed by a microlymphocytotoxicity test (Biotest, Milan, Italy). The HLA-A2+ samples were selected for further analysis.

2.3 ***Peptide – HLA class I tetramers***

The peptide–HLA class I tetramers were provided by Proimmune (Oxford, UK). To evaluate the fraction of CD8 cells specific for CMV, the epitope from pp65 corresponding to residues 495- 503 (NLVPMVATV) was conjugated to HLA-0201; for EBV, peptide GLCTLVAML (from the EBV lytic protein BMLF1 corresponding to residues 280-288) was used.

2.4 ***Flow Cytometric Analysis***

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada). The samples (1 x 10_6 PBMC) were stained with tetrameric complexes (2 ⌠l) for 15 min. at 37°C. After incubation, PBMC were washed in PBS x1, then for three colour immunofluorescence were incubated with different monoclonal antibodies to evaluate different markers. The panel included antibodies for CD8, CD27, CD28, CD45RA, CD45RO, CD57 (Caltag, Burlingame, CA, USA), and Perforin (Pharmingen, BD Bioscience, Mountain View, CA, USA). To evaluate perforin production, cells were fixed and permeabilized according the manufacturer's instructions, using Fix and Perm solution (Caltag, Burlingame, CA). Samples were analysed on a Facscan flow cytometer (Becton Dickinson, Mountain View, Ca, USA). To evaluate the reproducibility of the method we have performed preliminary tests using the same sample twice. Then, we have performed some of these initial tests using both fresh and frozen cells in two different labs (Palermo and London laboratories)

2.6 ***Statistical Analysis***

Values, given as mean ± SD or median (SE), were compared between young and old subjects using one-way Analysis of variance (ANOVA). The phenotype marker distribution was assessed by regression analysis. Differences were considered significant at $p < 0.05$.

3. RESULTS

3.1 ***T cell subsets***

Table 1 shows the frequency of CMV and EBV seropositivity in the subjects evaluated in this study. None of these individuals, old or young, had an inverted CD4/CD8 ratio, regardless of CMV-seropositvity (data not shown). This implies that even though some individuals were up to 92 yr of age, none fell into the IRP category defined in the OCTO/NONA studies (Pawelec et al. 2005). Table 2

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shows the surface phenotypes of the CD8 cells in the young and old donors, clearly delineating significantly lower percentages of CD27+, CD28+ and CD45RA+ cells in the elderly, as expected from previous reports (Pawelec et al., 2002a; Pawelec et al., 2004; Pawelec et al., 2005). Reciprocally, the percentage of CD45RO+ cells was significantly increased in the elderly. On the other hand, although there was a tendency towards increased percentages of CD57+ cells in the elderly, this difference did not achieve significance. There was, however, a significant decrease in perforin+ cells, as previously reported (Rukavina et al., 1998). These data are therefore in general agreement with results previously reported in different populations (Pawelec et al., 2002a; Pawelec et al., 2004; Pawelec et al., 2005). In order to examine the general effect of viral infection on T cell subset distribution, we separately analysed these data for young and old individuals, comparing those seropositive for both EBV and CMV with those seronegative. As shown in Table 3, in seronegative donors, the percentages of CD27+ CD8 cells are significantly lower in the elderly than the young; these percentages are further decreased in seropositive donors in both young and old. Thus, old seropositive subjects have very low levels of CD27+ CD8 lymphocytes. Therefore, EBV/CMV infection influences CD27 expression, that is also decreased in elderly seronegative people, due to ageing *per se* or to other thus far unidentified pathogens. In contrast, analyzing CD28 expression we observed that young and old seronegative donors retained the same percentages of CD28+ CD8 cells. Old donors had significantly lower percentages of CD28+ cells than young donors only when they were seropositive. This was also true for the lower levels of CD45RA+ and higher levels of CD45RO+ CD8 cells in the elderly, but although statistically significant, these differences were not so marked as for CD28. Therefore, only changes in CD27 expression are independent of CMV/EBV infection. Whether changes in CD27 expression can be caused by infections other than with CMV and EBV remains to be determined.

3.2 ***EBV- and CMV-specific CD8 T lymphocytes***

In order to quantify virus-specific cells and examine in greater detail alterations in surface marker phenotypes as influenced by viral infection, we focused on HLA A2+ CMV- and EBVseropositive subjects in order to identify CD8 cells carrying receptors for viral epitopes, by using MHC/peptide multimers. The common immunodominant GLC (EBV) and NLV (CMV) epitopes were used in HLA-A2 tetramers for staining CD8 cells. Figure 1 shows the percentages and absolute numbers of CD8 lymphocytes specific for these epitopes of EBV and CMV in each individual HLA-A2+ subject (the bars show the median values). The percentage of EBV-specific CD8 cells in aged people is significantly lower than in young ($p=0.02$), whereas this was not the case for the CMV-specific cells.

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However, the range of CMV values, both for percentage of CD8 CMV-specific cells and absolute numbers per µl of blood, is much greater in the old than young, with some elderly having large accumulations of CMV-specific cells, as seen in another Italian study (Vescovini et al., 2004) .

3.3 ***EBV- and CMV-specific CD8 T cell phenotypes***

Finally, we have performed surface phenotyping of CD8 CMV- or EBV-specific T lymphocytes. As in the case of total CD8 T cells, here again we have evaluated the expression of CD27, CD28, CD45RA, CD45RO, CD57 and Perforin, because these markers are suggestive of the state of differentiation of the cells.

In Fig. 2, we show the results of the study on CMV-specific CD8 T cells in both young and elderly donors: the percentage of CD27+ cells within the CMV-specific CD8 cells was not significantly different in young and old subjects;. there was also no significant difference in CD28 or CD57 expression, but there was a significant increase in the percentage of CD45RA+ CMVspecific cells, and a reciprocal decrease in CD45RO (but the latter was not statistically significant). This can be partially explained by the expression of CD45RA by terminally-differentiated CMVspecific cells, as reported by others (Appay et al., 2002), but we have no evidence that this is the case also in our subjects. It is, however, also consistent with a trend towards an increase in perforin expression by the CMV-specific CD8 T lymphocytes of the elderly, again contrary to what is seen in the entire CD8 population (Table 2).

The situation with EBV-specific cells is slightly different, but none of the comparisons between young and old reached statistical significance (Fig. 3). Nonetheless, there is a trend towards decreased levels of CD27 and especially CD28, as expected from data of others. There is no increase in CD45RA, also as expected from the previously published phenotypes of EBVspecific cells (Appay et al., 2002).

4. DISCUSSION

CD8 T lymphocytes have been extensively studied since it was observed that in the elderly they are oligoclonally expanded (Posnett et al., 1994). Moreover, longitudinal studies on a very elderly Swedish population have suggested that an inverted CD4/CD8 ratio can be considered a mortality risk factor (Wikby et al., 1998; Wikby et al., 2002). This has been confirmed in another North European elderly population (Huppert et al., 2003). However, this was not seen in a very large sample of old subjects from western Sicily (age 86-102) (Colonna-Romano et al., 2002). There are many differences between these Northern and Southern European populations, and these would have been even more noticeable during the subjects´ fetal and childhood development three quarters of a century ago. For whatever reason, whether genetic, nutritional, different pathogen exposure, or other lifestyle factors, none of the

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subjects studied here fell into the IRP category established from the Swedish longitudinal studies, as defined by a CD4:8 ratio < 1. It is in these individuals that the expansion of CD8 clones, predominantly driven by CMV, is most strikingly observed; monitoring these individuals indicates that following an increase in the number of different clones expanded and the absolute number of CMV-specific cells present, there is a period of clonal attrition representing a contraction of the available T cell repertoire. This is a highly significant predictor of incipient mortality (Hadrup et al., 2006). A similar collapse of the TCR repertoire has been observed by others, without taking CMV specificity into account (Nayor et al., 2005). However, the present findings are somewhat different from published reports from Northern European populations both in terms of mean numbers and surface phenotypes of the cells. How to reconcile the data from the Sicilian population presented here with those from the Swedish longitudinal studies? First and foremost, the present study is not longitudinal. Some individuals did present with large CMV-driven clonal expansions; they may be about to enter the IRP and experience a shorter remaining survival period although none of them at the time of the blood collection shows an inverted CD4/CD8 ratio (the subject with 5% of CMV-specific cells had a CD4/CD8 ratio 1.8, the subject who has expanded the CMVspecific CD8 lymphocytes up to 13.3% had a CD4/CD8 ratio of 2.8) . Other subjects showing essentially no CMV-specific cells (Fig. 2) may already be at the stage of repertoire collapse; the loss of their CMV-specific CD8 cells predicts mortality but could also normalize the CD4:8 ratio at the terminal phase. An alternative explanation may be that CMV is the main driving force for ageassociated clonal expansions of CD8 cells in Northern European populations (or was 80 yr ago) but that this preponderance of CMV was not the case on Sicily. Rather, a different hygiene environment involving different pathogens may be critical in this population. In our subjects, as shown, the incidence of CMV infection is about 70% whereas in the North of Europe it is about 80% in the elderly. This does not seem to be a difference sufficiently large to explain these findings. We can also mention the possibility that the viral epitopes chosen for the study are not really the immunodominat epitopes in Sicily. Moreover we have studied HLA-A2 subjects as this allele is highly represented in our population, in fact it is expressed in 42% of individuals HLA-typed by us. It is to mention that The higher phenotype frequency of the HLA-A2 allele found in Scandinavian countries decreases significantly as one moves further south in Europe, but does not influence our results (except to make it more difficult to recruit subjects for the study). However, it still remains the most frequent HLA-allele in Sicily (Piazza et al., 1989).On the other hand other HLA antigens usually considered for these studies (HLA B35 and B7) were expressed respectively in 23 and 2% of the subjects that we have studied. Regarding EBV, the primary infection, in Sicily, occurs prevalently early in life as reported

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(Ammatuna et al., 1989), whereas few data are available on the occurrence of primary CMV infection in Sicilian population (de Montalebert et al., 1992). An increased number of subjects studied longitudinally over the coming years should provide a definitive answer to these questions.

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Legend to figures

Figure 1. Percentage and absolute number (10^3/µl) of EBV- and CMV-specific CD8 T lymphocytes in young (Y) (n= 18) and old (O) (n= 13) subjects. Horizontal bars represent median values.(Y % GLC Median Value 0.97 P25 0.5, P75 1.98; O % GLC Median Value 0.61, P25 0.21, P75 1.42; Y % NLV Median Value 1.01 P25 0.45, P75 1.92; O % NLV Median Value 0.85, P25 0.26, P75 1.72; Y Absolute number GLC Median Value 5.9 P25 1.18, P75 8.05; O Absolute number GLC Median Value 2.9, P25 1.44, P75 4.21; Y Absolute number NLV Median Value 4.63 P25 2.37, P75 6.8; O Absolute number NLV Median Value 3.04, P25 1.05, P75 7.48). Both for EBV and CMV specific CD8 lymphocytes differences were not significant by ANOVA .

Figure 2 Evaluation of phenotypic markers on CMV-specific CD8 lymphocytes obtained from N= 16 young (Y) and N= 12 old (O) subjects. The longer horizontal bars represent median values; the shorter horizontal bars rapresent P25 and P75. Significant result, Significance was obtained by ANOVA test was obtained for CD45RA ($p = 0.01$). p value obtained for CD45R0 was 0.08 (marginally significant)

Figure 3 Evaluation of phenotypic markers on EBV-specific CD8 lymphocytes obtained from 16 young (Y) and 12 old (O) subjects. The longer horizontal bars represent median values; the shorter horizontal bars rapresent P25 and P75. No significance was obtained by ANOVA test.

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Table 1 Samples evaluated in the study. At the beginning of the study a total of 140 samples from young or old donors were screened for CMV and EBV seropositivity. Only seropositive subjects were HLA typed. Thereafter HLA-A2 positive subjects were selected for tetramer staining.

Total Samples evaluated n=140							
Results of CMV screening				**Results of EBV screening**			
OLD (n=54)		**YOUNG** (n=86)		**OLD** (n=54)		**YOUNG** (n=67)	
CMV Positive	**CMV Negative**	**CMV Positive**	**CMV Negative**	**EBV Positive**	**EBV Negative**	**EBV Positive**	**EBV Negative**
39 (72.3%)	15 (27.7%)	39 (45.3%)	47 (54.7%)	29 (53.7%)	25 (43.3%)	23 (34.3%)	44 (65.7%)
13 A2+		**18 A2+**		**13 A2+**		**18 A2+**	

Table 2. CD8 phenotype in blood samples of young and old subjects not selected on the basis of seropositivity to CMV and EBV (data are present as Mean + SD; P values were calculated by ANOVA *p<0.05 significant).

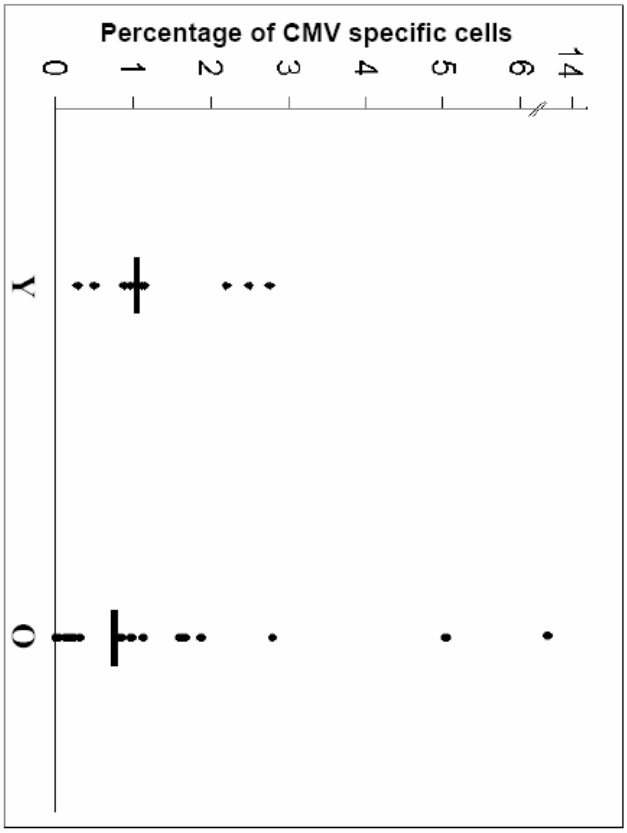
	%CD27	**%CD28**	**%CD45RA**	**%CD45R0**	**%CD57**	**%PERF**
Young (26)	61.8±17.6	64±16.1	65.4±12.9	40.2±14.8	44.2±18.6	15.2±8.1
Old (22)	42±17.6	54.3±18.4	55.1±18.2	49.7±16.4	53.6±21.4	10.4±7.9
P	**0.0005***	**0.04***	**0.02***	**0.04***	0.11	**0.05***

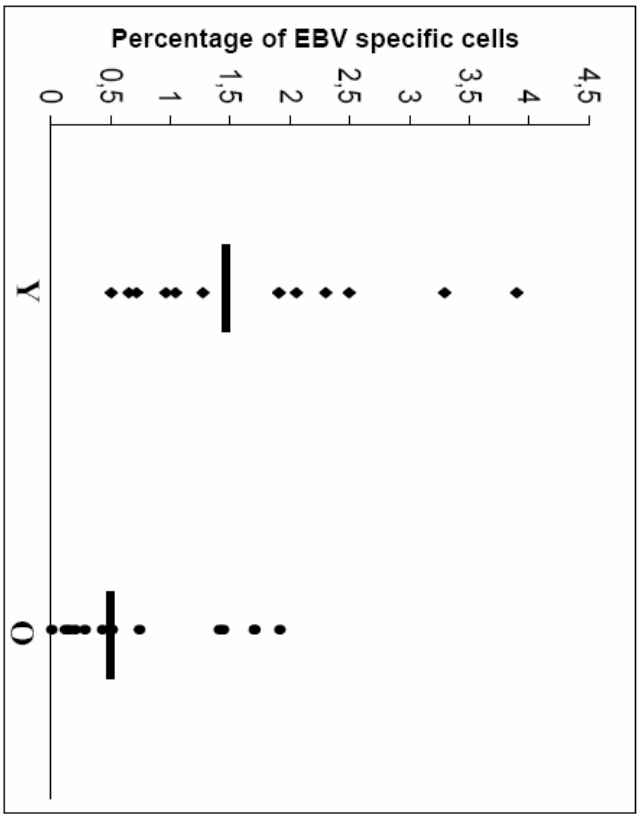
ACCEPTED MANUSCRIPT

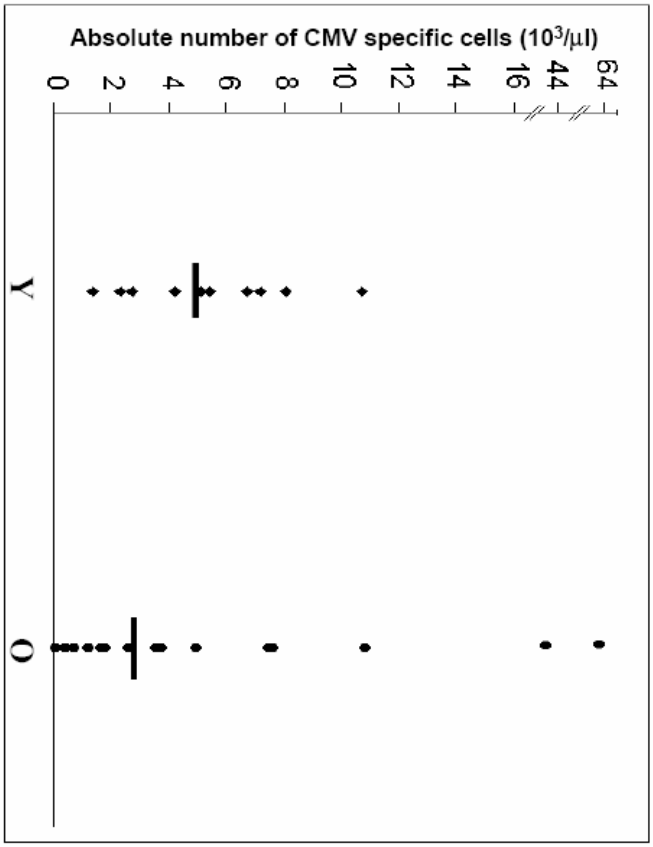
Table 3. CD8 phenotype in blood samples of young and old subjects assembled according to the seropositivity or seronegativity to CMV and EBV (data are present as Mean + SD; P values were calculated by ANOVA *p<0.05 significant).

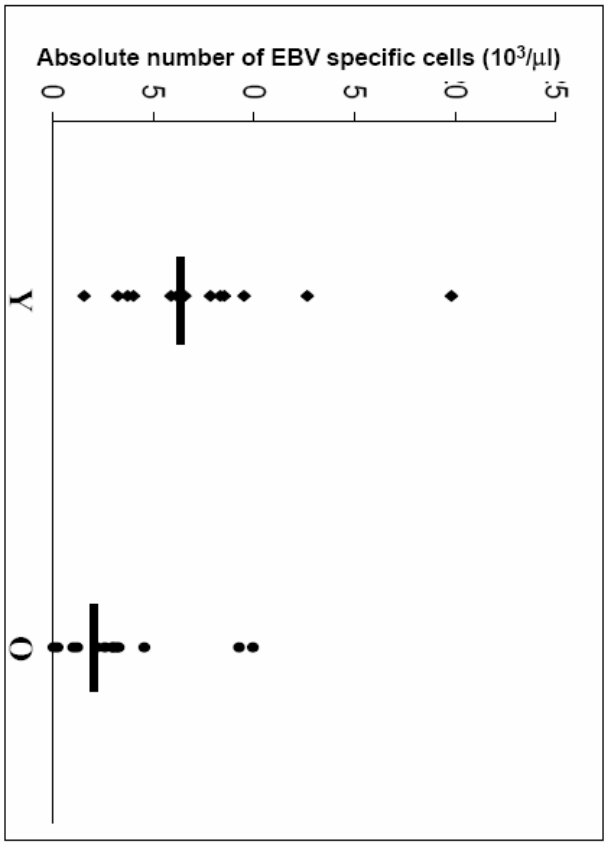
SERONEGATIVE SAMPLES						
	%CD27	**%CD28**	**%CD45RA**	**%CD45R0**	**%CD57**	**%PERF**
YOUNG(n=10)	75.8±11.4	77.3±15.3	72.8±15.5	39.3±22	33.3±23.3	12.1±6.2
OLD (n=10)	48.1±18.5	72.9±7.01	63±14	49.6±18.4	55.8±23.2	6.4±8
P	**0.001***	0.4	0.1	0.3	0.08	0.1
SEROPOSITIVE SAMPLES						
	%CD27	**%CD28**	**%CD45RA**	**%CD45R0**	**%CD57**	**%PERF**
YOUNG(n=16)	53.05±14.9	57.04±11.2	60.8±8.7	40.79±7.2	49.83±13.8	17.25±8.6
OLD (n=12)	37.9±16.45	42.33±11.9	49.91±19.4	50.25±15.8	52.16±21.1	13.16±7
P	**0.013***	**0.0016***	**0.05***	**0.04***	0.71	0.17

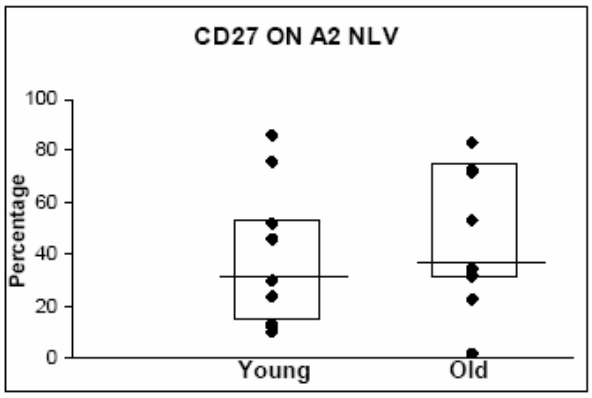
ACCEPTED MANUSCRIPT

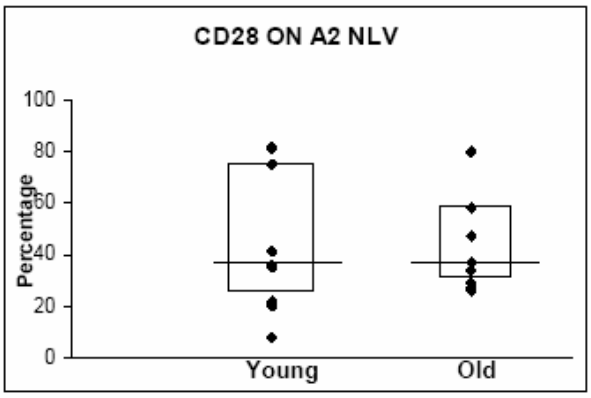


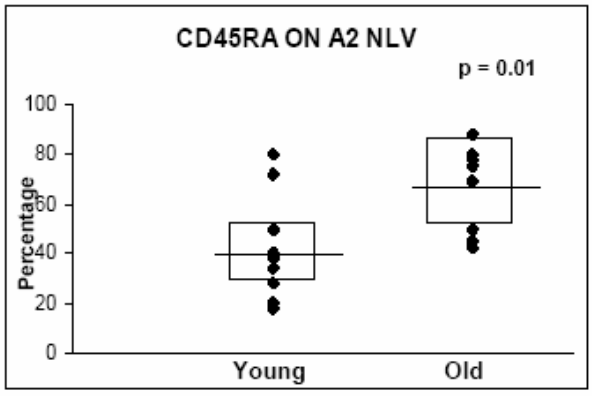


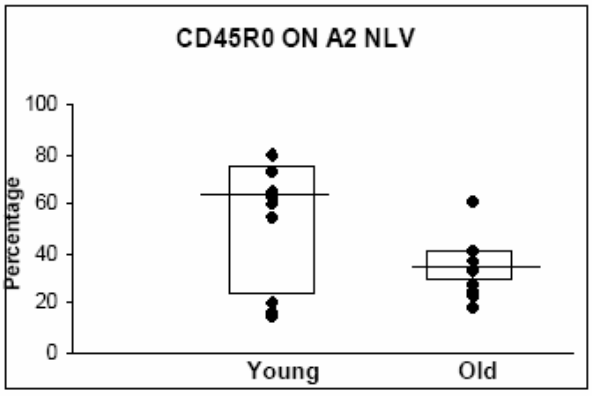


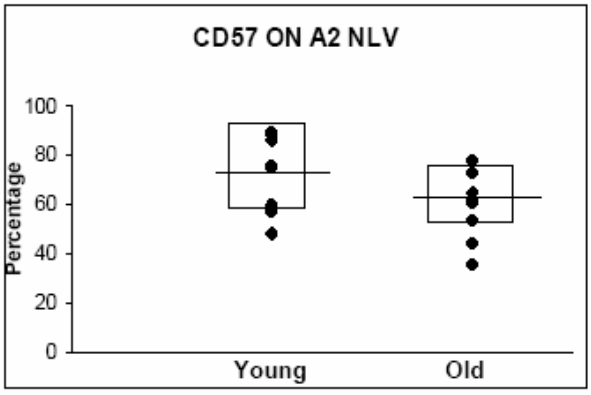


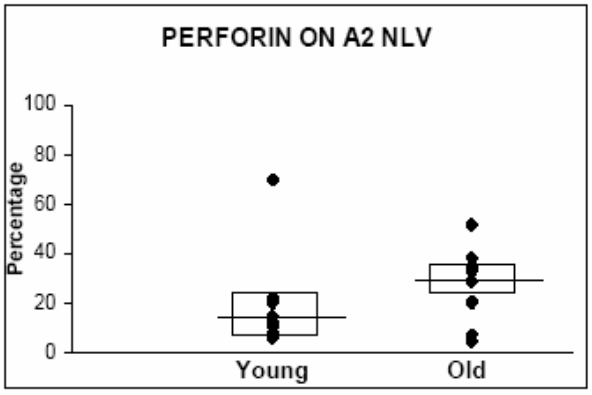

CD27 ON A2 NLV
Percentage
100
80
60
40
20
0
Young
Old

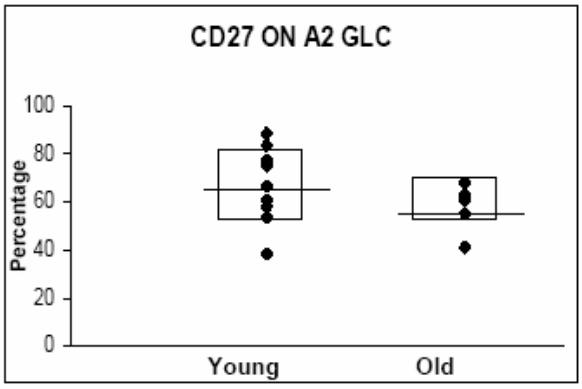

CD28 ON A2 NLV
Percentage
100
80
60
40
20
0
Young
Old

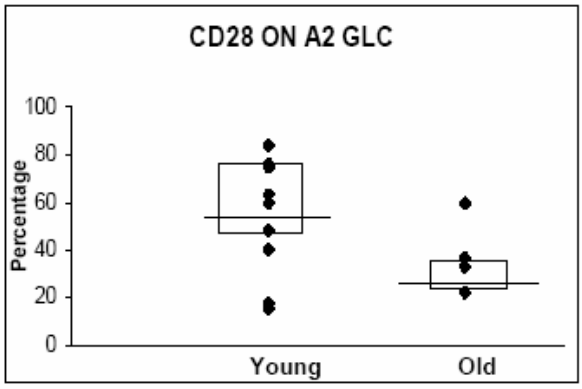

CD45RA ON A2 NLV
p = 0.01
Percentage
100
80
60
40
20
0
Young
Old

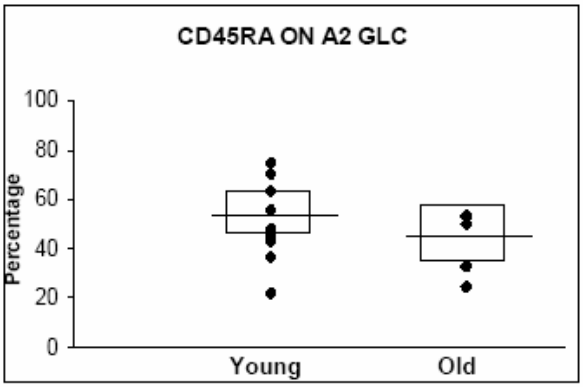

CD45R0 ON A2 NLV
Percentage
100
80
60
40
20
0
Young
Old

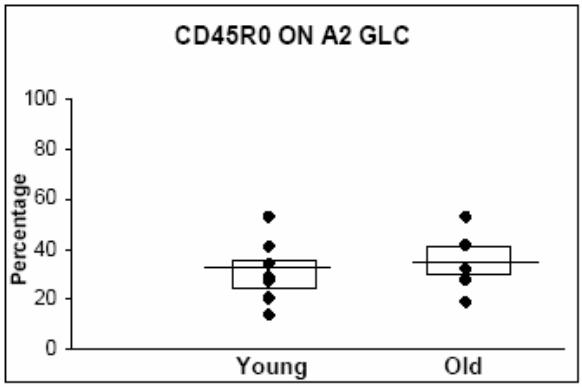

CD57 ON A2 NLV
Percentage
100
80
60
40
20
0
Young
Old

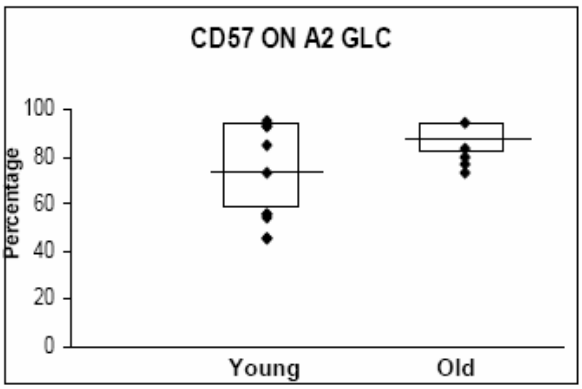

PERFORIN ON A2 NLV
Percentage
100
80
60
40
20
0
Young
Old

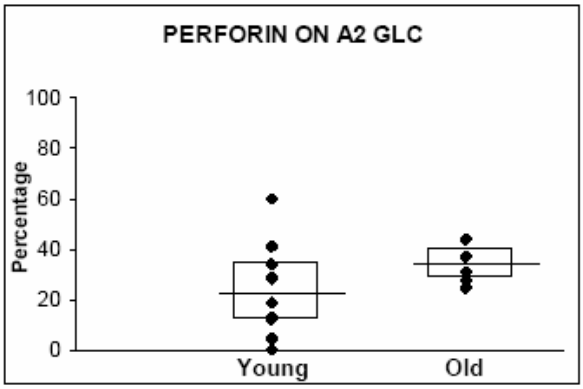

CD27 ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old


CD28 ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old


CD45RA ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old


CD45R0 ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old


CD57 ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old


PERFORIN ON A2 GLC
Percentage
100
80
60
40
20
0
Young
Old