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Immunodominant peptides from conserved influenza proteins – A tool for more efficient vaccination in the elderly?

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Immundominante Peptide von konservierten Influenzaproteinen – Eine Möglichkeit für eine wirksamere Impfung älterer Personen?

Zusammenfassung. Influenza-spezifische zytotoxische $CD8^+$ T-Zellen stellen einen wesentlichen Bestandteil bei der Überwindung einer Infektion dar, insbesondere in Hochrisikogruppen wie älteren Personen. Eine Aktivierung dieser Zellen durch eine Impfung wäre deshalb eine wichtige Voraussetzung um einen besseren Schutz für diese Altersgruppe zu erreichen. Deshalb haben wir die Häufigkeit, den Phänotyp und die Funktion von Influenza $M1_{58–66}$ Peptid-spezifischen $CD8^+$ T-Zellen in drei verschiedenen Altersgruppen (27 ± 1 Jahre; 46 ± 3 Jahre und 72 ± 2 Jahre) *ex vivo* und nach *in vitro* Stimulation untersucht. Bei Personen ab einem Alter von 38 Jahren stellten wir eine geringere Anzahl von $M1_{58–66}$-spezifischen $CD8^+$ T-Zellen fest. Diese Zellen exprimierten CD28, CD62L und waren entweder $CD45RA^{low}CD45RO^{low}$ oder $CD45RA^-CD45RO^+$, produzierten aber kein Perforin. Kein Unterschied zeigte sich beim Phänotyp von Influenza-spezifischen $CD8^+$ T-Zellen zwischen den drei Altersgruppen. Trotz der zu Beginn geringen Anzahl von $M1_{58–66}$-spezifischen $CD8^+$ T-Zellen in älteren Personen, konnte diese Population nach *in vitro* Stimulation mit dem $M1_{58–66}$-Peptid signifikant vergrößert werden. Diese $M1_{58–66}$-spezifische $CD8^+$ T-Zellen produzierten Perforin und hatten einen $CD45RO^+CD28^+CD62L^{+/-}$ Phänotyp. Unsere Ergebnisse zeigen, dass trotz einer geringen Anzahl $M1_{58–66}$-spezifischer $CD8^+$ T-Zellen bei älteren Personen, diese Zellen nach *in vitro* Stimulation mit einem Peptid gut expandieren und in Perforin-produzierende Effektor-T-Zellen differenzierbar sind. $M1_{58–66}$-Peptid oder andere immundominante Peptide aus konservierten Influenzaproteinen könnten somit einen wichtigen Bestandteil zukünftiger Influenzaimpfstoffe darstellen um älteren Personen einen verbesserten Schutz vor Influenza zu bieten, insbesondere beim Auftreten einer Influenza Pandemie.

Schlüsselwörter: $CD8^+$ T-Zellen, Influenza, Altern.

Summary. Influenza-specific $CD8^+$ T cells are important for the clearance of infection especially in high risk groups such as elderly persons. Activation of these cells by immunization might therefore be a useful tool for a better protection of this specific age group. We therefore analyzed the frequency, phenotype and function of $CD8^+$ T cells with specificity to the influenza $M1_{58–66}$ peptide in young, middle-aged and elderly persons *ex vivo* and after *in vitro* stimulation. Significantly lower numbers of $M1_{58–66}$-specific $CD8^+$ T cells were detected in the middle-aged and elderly compared to young donors. $M1_{58–66}$-specific $CD8^+$ T cells were either $CD45RA^{low}CD45RO^{low}$ or $CD45RA^-CD45RO^+$, expressed CD28 and CD62L and did not produce perforin. There was no difference in the phenotype of influenza-specific $CD8^+$ T cells between the three age groups. Despite the initially low numbers of $M1_{58–66}$-specific $CD8^+$ T cells in the older age groups, these cells could be expanded *in vitro* following peptide stimulation. They also acquired a $CD45RO^+CD28^+$ $CD62L^{+/-}$ phenotype and produced perforin. Our results demonstrate that although initially low in number, $M1_{58–66}$-specific $CD8^+$ T cells from elderly persons can be propagated and differentiated into perforin producing effector cells upon appropriate stimulation. $M1_{58–66}$ peptide or other immunodominant peptides derived from conserved influenza proteins could therefore be useful in future influenza vaccines in order to render elderly persons better protected against disease, in particular in the case of an influenza pandemia.

Key words: $CD8^+$ T cells, influenza, aging.

Introduction

Aging is associated with a decline in a large number of physiological functions, such as the immune function [1]. Elderly persons are therefore highly susceptible to infec-

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tions and malignant diseases compared to young persons. The incidence of infections caused by bacteria such as pneumococci and by viruses such as influenza or herpes zoster reactivation is high and the disease course severe [2]. Elderly persons suffering from opportunistic and recurrent pathogens or reactivation of quiescent diseases may also fail to respond to therapy. Aging affects humoral as well as cellular immunity. Due to the involution of the thymus, which is almost replaced by fat after 50, there is a dramatic reduction in the number of naïve T cells [3]. To compensate the loss of naïve T cells, the number of memory and effector T cells increases. Antigen-specific T cells undergo clonal propagation and acquire effector functions until a pathogen is eliminated from the body. A proportion of these cells undergoes apoptosis, while others are retained as memory cells. However, in persons with chronic infection, such as with the cytomegalovirus (CMV), dysfunctional $CD8^+CD28^-$ effector T cells accumulate [4].

Influenza is an important public health problem in the industrialized world with a high incidence especially in elderly people [5–7]. Huge efforts have been made throughout the last decades to launch influenza vaccination and to cover as many elderly persons as possible [8]. These efforts have, however, been hampered by a relatively low enthusiasm on the part of the elderly population to be vaccinated as well as by the failure of many vaccines on the market to induce a strong, long-lasting immune response in old age [9, 10]. A diminished response to influenza vaccination has been associated with latent CMV infection [11] and a high number of $CD8^+CD28^-$ T cells [12, 13]. Studies in aged mice and humans also suggested that low protection against influenza could be due to a decrease in the number of influenza-specific cytotoxic T lymphocytes (CTL) [14–16]. It is also well known that surface proteins of the influenza virus, namely Hemagglutinin (HA) and Neuraminidase (NA), which are the principal components of influenza subunit vaccines, change due to antigenic drifts and shifts which can render people unprotected [17–19]. In view of this fact as well as a possible shortage of vaccine in the case of pandemia, it seems reasonable to consider alternative vaccination strategies, in particular directed against conserved proteins of the influenza virus. In the present study, the frequency, phenotype and function of $CD8^+$ T cells that specifically recognize the epitope $M1_{58–66}$ of the matrix 1 protein (M1), which is one of the highly conserved proteins of the influenza virus, were studied before and after *in vitro* stimulation in young, middle-aged and elderly donors. We demonstrate that influenza $M1_{58–66}$-specific $CD8^+$ T cells can be propagated following peptide stimulation in elderly persons. This new insight may contribute to a better understanding of the immune response against influenza in old age and support the development of novel vaccination strategies.

Material and Methods

Volunteers

Peripheral blood samples were obtained from apparently well, healthy elderly donors (n = 29, male 13, female 16, age 72 ± 2 years, range 60–91), middle-aged (n = 13, male 6, female 7, age 46 ± 3, range 38–56) and young persons (n = 16, male 9, female 7, age 27 ± 1, range 22–32). All participants gave their written informed consent and the study was approved by the local ethical committee. All participants were in a good clinical condition, neither suffering from any severe acute or chronic illness nor taking any medication known to interfere with immune function. HLA typing was routinely performed. All donors described in this study were HLA-A2 positive.

Peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained by density gradient centrifugation (Ficoll-hypaque, Amersham Biosciences, Uppsala, Sweden) from freshly drawn venous blood. After washing, PBMCs were resuspended in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Gibco, Invitrogen Corporation, Paisley, Scotland, UK).

Tetramer and surface staining

3×10^6 cells were stained with influenza $M1_{58–66}$ tetramer (GILGFVFTL; ProImmune, Oxford, United Kingdom) in combination with anti-CD8, anti-CD45RA, anti-CD45RO, anti-CD28 or anti-CD62L (all antibodies were purchased from BD Pharmingen, San Jose, CA, USA) for 30 min at 4°C. Cells were washed twice with PBS and resuspended in PBS for flow cytometric analysis. All analyses were performed using a FACS Calibur flow cytometer utilizing CELLQuest software (BD Pharmingen).

Analysis of perforin production

Perforin expression was assessed by intracellular staining using the Cytofix/Cytoperm kit according to the specification given by the manufacturer (BD Pharmingen). 5 µL of FITC-conjugated anti-perforin antibody (BD Pharmingen) was added to permeabilized, unstimulated and influenza peptide-stimulated PBMC and incubated for 30 min at 4° C. Cells were washed twice with Cytowash buffer and once with PBS. Perforin production was measured using a FACS Calibur flow cytometer.

Analysis of IFN-γ production

$CD8^+$ T cells were isolated from young, middle-aged and elderly donors using a magnetic activated cell sorter (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). 2×10^5 $CD8^+$ T cells were added to each well of a PDVF-plate (96-well plate, Millipore, Bedford, MA) pre-coated with a mAb against IFN-γ (mAb 1-D1K, Mabtech, Nacka, Sweden; 10 µg/mL) and stimulated with 2 µg/mL of influenza $M1_{58–66}$ peptide for 12 h at 37° C. After incubation, cells were removed from the plate and the plate was washed with PBS/Tween 0.05%. The second antibody (Mab 7-B6–1-Biotin, Mabtech, Nacka, Sweden; 2 µg/mL) was added and the plate was incubated at 37° C for 2h. The plate was then washed as described above and incubated with streptavidin-ALP-PQ (1:1000, Mabtech, Nacka, Sweden) for 1h at room temperature. After this step the plate was washed three times with PBS/Tween 0.05% and three times with PBS, incu-

bated with 100 µL/well of BCIP/NBT alkaline phosphatase substrate (Moss, Inc.) for 5 min or until the development of spots at room temperature. Spots were measured using an ELISPOT reader (Carl Zeiss, Munich, Germany). Six duplicates were performed for each measurement and unstimulated cells were used for background correction.

Cell culture

2×10^6 PBMCs from young, middle-aged and elderly donors were stimulated with 2 µg/mL influenza $M1_{58–66}$ peptide (GILGFVFTL) (provided by the Fundacion Instituto de Inmunologia, Bogotá, Colombia), in the presence of IL-2 (20 ng/mL), for one week at 37° C, 5% CO_2. After incubation, cells were stained with influenza $M1_{58–66}$ tetramer (GILGFVFTL), anti-CD8, anti-CD45RA, anti-CD28 and anti-CD62L as described above. All analyses were performed using FACS Calibur flow cytometer utilizing CELLQuest software (BD Pharmingen).

Statistics

Student's t test for unpaired data was used for the comparison of groups. Mean ± SEM are shown for all analyses performed. *P* values of <0.05 were considered as statistically significant.

Results

Elderly persons have a decreased frequency of $M1_{58–66}$-specific $CD8^+$ T cells

The frequency of influenza $M1_{58–66}$-specific $CD8^+$ T cells was analyzed by tetramer technology in young, middle-aged and elderly persons (Fig. 1A, B). Although the frequency of $M1_{58–66}$-tetramer binding $CD8^+$ T cells was generally low, middle-aged and elderly persons still had significantly lower numbers than young controls.

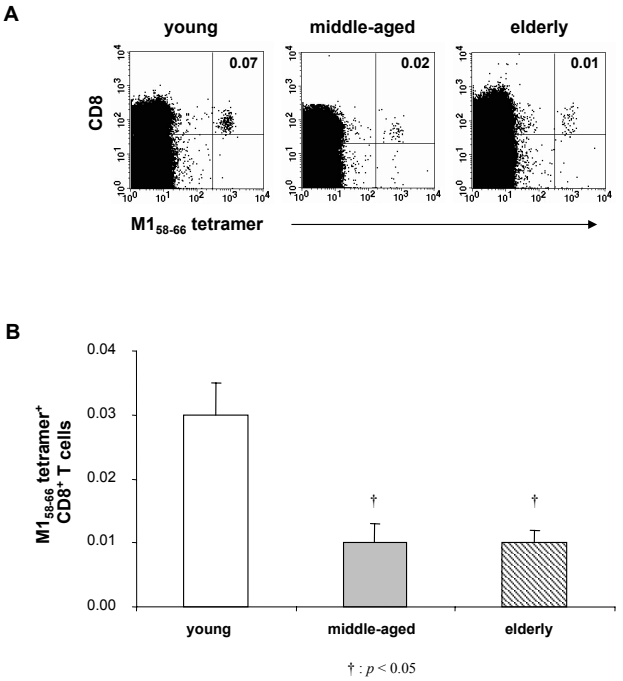

Fig. 1. The frequency of influenza $M1_{58–66}$-specific $CD8^+$ T cells is low in elderly persons
(A) A representative example of the staining of $M1_{58–66}$-specific $CD8^+$ T cells from a young, a middle-aged and an elderly person using tetramer technology. The numbers in the graphs indicate the percentage of $M1_{58–66}$-specific $CD8^+$ T cells in the lymphocyte gate. (B) The frequency of $M1_{58–66}$-specific $CD8^+$ T cells in young (n = 16), middle-aged (n = 13) and in elderly persons (n = 29) is shown. The bars represent the percentage of $M1_{58–66}$-specific $CD8^+$ T cells in each group. Data are presented as mean ± SEM. Significant differences are indicated as follows: † young vs. middle-aged / elderly, $p < 0.05$.

$M1_{58–66}$-specific $CD8^+$ T cells mainly display a memory phenotype

$M1_{58–66}$-specific $CD8^+$ T cells were then characterized for their surface expression of CD45RA, CD45RO, CD28 and CD62L. Three subpopulations were defined according to their expression of CD45RA and CD45RO (Fig. 2). Gate 1 contained cells that expressed CD45RA at high intensity, gate 2 cells that co-expressed CD45RA and CD45RO both at low intensity, and gate 3 cells that expressed CD45RO at high intensity but were CD45RA negative. In all three age groups $M1_{58–66}$-specific $CD8^+$ T cells were either $CD45RA^{low}CD45RO^{low}$ (young, 78.4% ± 6.6; middle-aged, 88.3% ± 6.5; elderly, 71.7% ± 8.2 of $M1_{58–66}$-specific $CD8^+$ T cells) or $CD45RA^-CD45RO^+$ (young, 23.4% ± 7.6; middle-aged, 11.2% ± 6.0; elderly, 22.0% ± 9.0 of $M1_{58–66}$-specific $CD8^+$ T cells) but only rarely $CD45RA^+$ (young, 1.4% ± 1.2; middle-aged, 1.1% ± 1.1; elderly, 1.0% ± 0.8 of $M1_{58–66}$-specific $CD8^+$ T cells). All $M1_{58–66}$ tetramer binding cells were considered 100%. These results demonstrate that influenza $M1_{58–66}$-specific $CD8^+$ T cells display an early memory cell phenotype. The phenotype of $M1_{58–66}$-specific $CD8^+$ T cells was further defined by the expression of the co-stimulatory molecule CD28, the homing receptor CD62L and the lack of perforin expression, demonstrating that the cells were able to home to lymph nodes and were not cytotoxic in an inactivated state (Fig. 2A). Comparing the three age groups, no significant difference was found in the expression of CD45RA, CD45RO, CD28 and CD62L within $M1_{58–66}$-specific $CD8^+$ T cells (Fig. 2B).

IFN-γ production by $CD8^+$ T cells decreases with age following stimulation with $M1_{58–66}$ peptide

We analyzed the number of IFN-γ producing $CD8^+$ T cells following *in vitro* stimulation with the influenza peptide $M1_{58–66}$ for 12 h by ELISPOT. $CD8^+$ T cells were purified from young, middle-aged and elderly persons. Our results show a decrease in the frequency of IFN-γ-producing $CD8^+$ T cells following peptide stimulation with increasing age (Fig. 3). Middle-aged and elderly persons had a significantly lower number of IFN-γ spot forming cells (sfc) than young persons.

$M1_{58–66}$-specific $CD8^+$ T cells acquire an effector phenotype during 7 days of in vitro stimulation

PBMCs from young, middle-aged and elderly persons were cultured for one week in the presence of the $M1_{58–66}$ influenza peptide and IL-2. Tetramer binding cells were then phenotypically analyzed by surface staining. During culture, $M1_{58–66}$-specific $CD8^+$ T cells acquired the fol-

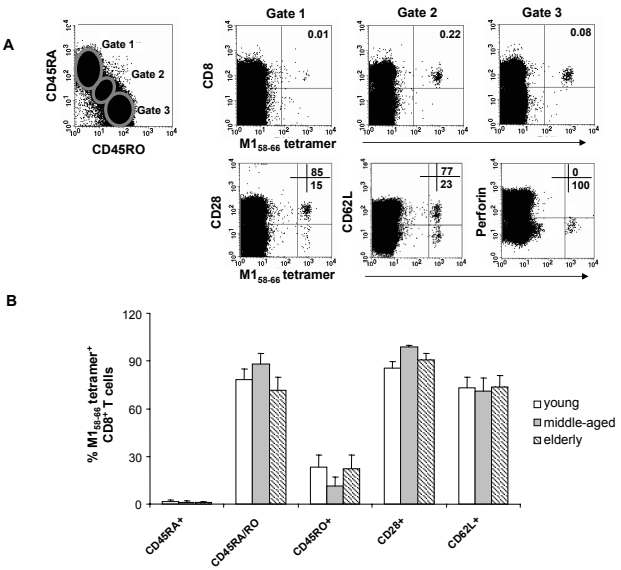

Fig. 2. $M1_{58–66}$-specific $CD8^+$ T cells from young, middle-aged and elderly persons have the same phenotype

Although the frequency of $M1_{58–66}$-specific $CD8^+$ T cells is low in elderly persons, their phenotype is unchanged. (A) Three subpopulations of $M1_{58–66}$-specific T cells were defined within the CD8 gate according to the expression of CD45RA and CD45RO: $CD45RA^+$ (gate 1), $CD45RA^{low}CD45RO^{low}$ (gate 2) and $CD45RO^+$ cells (gate 3). The distribution of $M1_{58–66}$-specific $CD8^+$ T cells within the three gates is shown for a young donor. The numbers in the graphs indicate the percentage of $M1_{58–66}$-specific $CD8^+$ T cells in: $CD45RA^+$ (gate 1), $CD45RA^{low}CD45RO^{low}$ (= early memory cells; gate 2) and $CD45RO^+$ cells (= memory cells; gate 3). The surface expression of CD28, CD62L and perforin on $M1_{58–66}$-specific $CD8^+$ T cells is also shown. The numbers in the graphs indicate the percentage of CD28, CD62L or perforin expressing / non-expressing cells within the $M1_{58–66}$-specific $CD8^+$ T cell population, which was considered 100%. (B) There is no difference in the expression of the surface molecules in $M1_{58–66}$-specific $CD8^+$ T cells from young (n = 16), middle-aged (n = 13) and elderly persons (n = 29). Bars represent the percentage of cells expressing CD45RA, CD45RA CD45RO, CD45RO, CD28 and CD62L within the $M1_{58–66}$-specific $CD8^+$ T cell population in young □, middle-aged ▩ and elderly persons ▨. $M1_{58–66}$-specific $CD8^+$ T cells were considered 100%. Perforin was not produced. Data are presented as mean ± SEM.

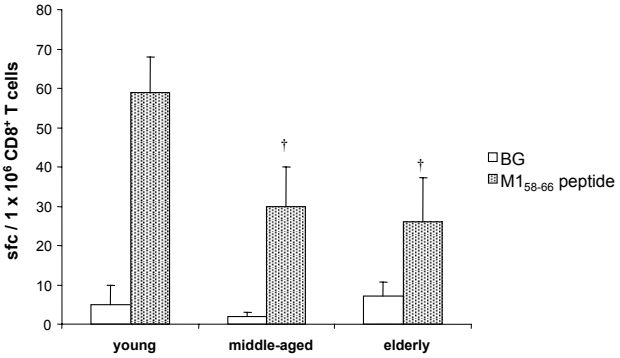

Fig. 3. Elderly persons have a decreased IFN-γ production following *in vitro* stimulation of $CD8^+$ T cells with $M1_{58–66}$ peptide

The IFN-γ-production in purified $CD8^+$ T cells from young, middle-aged and elderly persons was determined by ELISPOT following $M1_{58–66}$ peptide (2 µg/mL) stimulation for 12h. The number of spot forming cells (sfc) per 1×10^6 $CD8^+$ T cells for unstimulated cells (backgroung, BG, empty bars) and stimulated with $M1_{58–66}$ peptide (filled bars) is shown. Data from young (n = 5), middle-aged (n = 5), and elderly persons (n = 5) are presented as mean ± SEM. Significant differences are indicated as follows: † young vs. middle-aged / elderly, $p < 0.05$.

lowing phenotype: They exclusively expressed CD45RO and were CD45RA negative. They retained CD28 expression while the number of CD62L-expressing cells decreased (Fig. 4A, B). 100% of the $M1_{58–66}$-specific $CD8^+$ T cells also produced perforin following 7 days *in vitro* culture. These phenotypic changes were observed in cells from all age groups and indicate that the cells were capable of homing to disease-affected tissues and were cytotoxic following activation.

$M1_{58–66}$-specific $CD8^+$ T cells from elderly persons still have the capacity to proliferate upon peptide stimulation

Although the frequency of influenza $M1_{58–66}$-specific $CD8^+$ T cells was low, especially in elderly persons, they

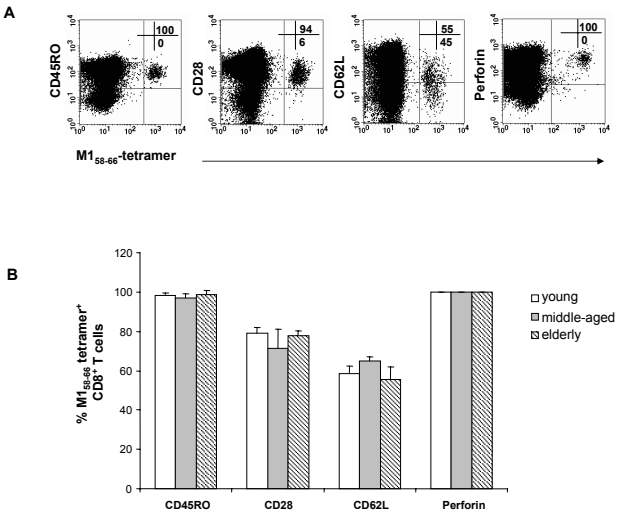

Fig. 4. $M1_{58–66}$-specific $CD8^+$ T cells acquire an effector phenotype following peptide stimulation

PBMCs from young, middle-aged and elderly persons were stimulated with influenza $M1_{58–66}$ peptide in the presence of IL-2 for one week. (A) A representative example of an elderly person following peptide stimulation is shown. The numbers in the graphs indicate the percentage of CD45RO, CD28, CD62L and perforin expressing cells within the $M1_{58–66}$-specific $CD8^+$ T cell population which was considered 100%. (B) No differences in the phenotype of expanded cells were found in young (n = 10) □, middle-aged (n = 8) ▩ and elderly (n = 18) persons ▨.

Bars represent the percentage of cells expressing CD45RO, CD28, CD62L and perforin within the $M1_{58–66}$-specific $CD8^+$ T cell population, which was considered 100%. Data are presented as mean ± SEM.

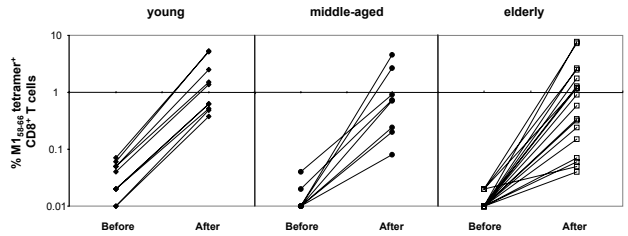

Fig. 5. Influenza-specific $CD8^+$ T cells can be propagated *in vitro*
The percentage of $M1_{58–66}$-specific $CD8^+$ T cells before and after stimulation with the influenza peptide $M1_{58–66}$ in young (n = 10), middle-aged (n = 8) and elderly persons (n = 18) is shown on a logarithmic scale.

could be propagated upon peptide stimulation *in vitro* (Fig. 5). Following $M1_{58–66}$ peptide stimulation for one week in the presence of IL-2, influenza-specific $CD8^+$ T cells divided rapidly and acquired an effector phenotype (Fig. 4). Elderly persons still had some variability in the magnitude of the response to the peptide which was not observed in the young group. Interestingly, this variability was already observed in persons above 35 years of age.

Discussion

Elderly persons have an increased susceptibility to viral infections such as influenza. Influenza is one of the major causes of acute respiratory infections especially in children and in the elderly. Influenza-specific $CD8^+$ T cells are important for the clearance of influenza virus during infection [20–22]. We report here that the frequency of $CD8^+$ T cells specific for the influenza $M1_{58–66}$ peptide, which is derived from the influenza matrix 1 protein, is significantly lower in middle-aged and elderly persons than in young ones. These results are in accordance with previous studies which have shown low numbers of fully functioning influenza-specific $CD8^+$ T cells in elderly persons [14, 23]. Although the frequency of influenza-specific $CD8^+$ T cells was lower in the middle-aged and in the elderly than in the young group, cells expressed a similar phenotype indicating that $M1_{51–66}$-specific $CD8^+$ T cells are generated and maintained within the central memory subset after virus clearance. The expression of the co-stimulatory molecule CD28 indicates that these cells can be activated and undergo clonal expansion after antigen exposition. In fact, $M1_{58–66}$-specific $CD8^+$ T cells were able to expand upon cognate peptide stimulation *in vitro* in young, middle-aged and elderly persons. Expanded $M1_{58–66}$-specific $CD8^+$ T cells acquired an effector phenotype and demonstrated cytotoxic capacity. Despite the initially low number of influenza-specific memory $CD8^+$ T cells in elderly persons, these cells have obviously still the potential to differentiate into cytotoxic effector cells and to divide, suggesting that elderly persons have the ability to recover from influenza virus infection when their T cells are appropriately stimulated. This suggests that vaccination with highly conserved antigens of the influenza virus might be a good strategy to increase protection against the virus, especially in elderly persons. Stimulation of cytotoxic T cells with immunodominant peptides, such as the $M1_{58–66}$ peptide used in this study, would be principally possible. However, as $M1_{58–66}$ can only be recognized by T cells in the context of HLA-A2, only 50% of the population possessing this phenotype could be targeted by this approach. Induction of $CD8^+$ T cell cytotoxicity by cross-priming with recombinant proteins, as suggested by our laboratory [24], would be another possibility. DNA vaccines might also be considered [25].

Although induction of cytotoxicity against highly conserved proteins would presumably not lead to full protection in the absence of antibodies against the influenza surface proteins, a mild disease course would most likely be assured. This would be of particular advantage in the case of pandemia, when it may be a problem to produce sufficient amount of vaccine in a short time or when a "new" virus proves to be extremely aggressive like during the 1918 pandemia [26].

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