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IL-15 preferentially enhances functional properties and antigen-specific responses of CD4+CD28^{null} compared to CD4+CD28+ T cells

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Summary

One of the most prominent changes during T-cell aging in humans is the accumulation of CD28^{null} T cells, mainly CD8+ and also CD4+ T cells. Enhancing the functional properties of these cells may be important as they provide an antigen-specific defense against chronic infections. Recent studies have shown that IL-15 does in fact play an appreciable role in CD4 memory T cells under physiological conditions. We found that treatment with IL-15 increased the frequency of elderly CD4+CD28^{null} T cells by the preferential proliferation of these cells compared to CD4+CD28+ T cells. IL-15 induced an activated phenotype in CD4+CD28^{null} T cells. Although the surface expression of IL-15R α-chain was not increased, the transcription factor STAT-5 was preferentially activated. IL-15 augmented the cytotoxic properties of CD4+CD28^{null} T cells by increasing both the mRNA transcription and storage of granzyme B and perforin for the cytolytic effector functions. Moreover, pretreatment of CD4+CD28^{null} T cells with IL-15 displayed a synergistic effect on the IFN-γ production in CMV-specific responses, which was not observed in CD4+CD28+ T cells. IL-15 could play a role enhancing the effector response of CD4+CD28^{null} T cells against their specific chronic antigens.

Key words: CD4+CD28^{null} T cells; chronic viral antigens; cytotoxicity; IL-15; Immunosenescence; memory T cells.

Introduction

Aging is characterized by a reduction in adaptive responses and has a profound impact on the T-cell compartment. Thymic involution implies a decreased output of naïve T cells, which is evident in peripheral blood and the lymph nodes (Sauce *et al.*, 2009; Appay *et al.*, 2010). In contrast, elderly donors display a marked increase in the proportion of highly differentiated effector and memory T cells owing to a lifetime of exposure to a variety of pathogens. One of the most prominent changes during T-cell aging in humans is the accumulation of CD28^{null} T-cells. Their accumulation is partially explained by their reduced susceptibility to apoptosis and their oligoclonal expansions against CMV and other chronic antigens (Vescovini *et al.*, 2004; Almanzar *et al.*, 2005; Derhovanessian *et al.*, 2009). Loss of CD28 expression is a hallmark of the age-associated decline of T-cell function. Because CD28 provides a pivotal role during T-cell activation, such as inducing cytokine production (IL-2) and promoting cell proliferation, lack of this costimulatory signal during activation results in a partial activation or even an anergic state of T cells (Godlove *et al.*, 2007). In this way, the accumulation of CD28^{null} T cells, particularly within the CD8 subset, is associated with a reduced overall immune response to pathogens and vaccines in the elderly (Saurwein-Teissl *et al.*, 2002). Despite CD4+ T cells are more resistant to age-related phenotypic and functional changes (Wikby *et al.*, 2002; Weinberger *et al.*, 2007), a progressive increase in the percentage of CD4+ T cells that lack CD28 expression is common with increasing age in healthy individuals (Goronzy *et al.*, 2007; Czesnikiewicz-Guzik *et al.*, 2008) and in patients with chronic infections and autoimmune diseases(Komocsi *et al.*, 2002; Fletcher *et al.*, 2005; Thewissen *et al.*, 2007). Thereby, CD4+CD28^{null} T cells can comprise up to 50% of the total CD4+ T-cell compartment in some individuals older than 65 years(Vallejo *et al.*, 2000). CD4+CD28^{null} T cells are functionally distinct from CD4+CD28+ T cells but exhibit similarities with CD8+CD28^{null} T cells. CD4+CD28^{null} T cells acquire expression of several receptors commonly associated with NK cells, secrete large amounts of IFN-γ, and express perforin and granzyme B, which convey cytotoxic capability to the cells (Appay *et al.*, 2002; van Leeuwen *et al.*, 2004).

Generation of long-term memory CD4+ and CD8+ T cells is dependent upon antigenic stimulation, but their survival is antigen-independent and requires peripherally produced cytokines, particularly those that use the common γ-chain for signaling, such as IL-15(Ku *et al.*, 2000). The importance of IL-15 in memory T cells was established by the characterization of IL-15 and IL-15Rα knockout mice which showed a striking lack of memory phenotype CD8+ T cells (Zhang *et al.*, 1998; Kennedy *et al.*, 2000). Furthermore, memory CD8+ T cells were increased in an IL-15-overexpressing mouse model (Fehniger *et al.*, 2001). Homeostatic proliferation of T cells can be one cause for the age-associated loss of CD28 expression, because CD8+ memory T cells in the presence of IL-15 alone, without TCR stimulation, lose CD28 expression and proliferate at a similar rate to CD8+CD28+ T cells (Chiu *et al.*, 2006). IL-15 not only potently induces the proliferation of memory CD8+ T cells but also augments their effector function by the induction of perforin expression and cytotoxic properties (White *et al.*, 2007). Because antigen-specific memory CD4+ T cells have distinct requirements for homeostatic regulation compared with CD8+ T cells, the role of IL-15 in the maintenance of antigen-specific memory CD4 T cells was missed by analyses of memory phenotype cells in early studies of IL-15$^{-/-}$ mouse models. Recent studies have shown that IL-15 does in fact play an appreciable role in CD4+ memory T-cell proliferation under physiological conditions (Purton *et al.*, 2007). IL-15 treatment promotes the proliferation of human memory CD4+ T cells *in vitro* and mouse Ag-specific CD4+ memory T cells *in vivo* (Geginat *et al.*, 2001; Lenz *et al.*, 2004). These findings demonstrate that in a normal environment, memory CD4+ T cells closely resemble memory CD8+ cells in their dependency on IL-15 for their homeostasis (Geginat *et al.*, 2001; Lenz *et al.*, 2004).

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As CD8+CD28^{null} T cells are highly influenced by IL-15, it is of great interest to determine the responsiveness of CD4+CD28^{null} T cells to this homeostatic cytokine, mainly because it has been postulated as a treatment of T-cell reconstitution in the elderly. We have demonstrated in this study that IL-15 may not only improve CD4+CD28^{null} T-cell expansion but also promote both their functional properties and their antigen-specific responses.

Results

Effect of IL-15 on CD28 expression in CD4+ T cells

We evaluated the possible effect of IL-15 on the expression of CD28 in CD4+ T cells from 10 elderly volunteers with percentages of CD28^{null} cells higher than 5% of total CD4+ T cells. Peripheral blood mononuclear cells (PBMC) were cultured in the presence and absence of the IL-15 for 7 days. Frequency of CD4+CD28^{null} T cells showed a significant increase in response to IL-15 treatment compared with cells cultured in medium alone, with a mean percentage of 15.8 ± 5.1% and 8.6 ± 3.3%, respectively (paired *T* test, $P = 0.00006$) (Fig. 1A). In contrast to that described in CD8+ T cells (Chiu *et al.*, 2006), IL-15 did not induce loss of CD28 expression in CD4+CD28+ T cells in individuals without CD4+CD28^{null} T-cell subset (data not shown). In spite of their defects in the proliferative ability (Appay *et al.*, 2002), CD4+CD28^{null} T cells may have increased owing to an enhanced proliferative response to IL-15 compared with that of CD28+. To analyze the effect of IL-15 on the proliferation of CD4+CD28^{null} T cells, PBMC were stained with CFSE and cultured in the presence of IL-15 for 7 and 14 days. When we compared the growth kinetics of both populations in response to the cytokine, we found that CD4+CD28^{null} cells effectively underwent cell division and expansion faster than did CD4+CD28+ cells. Figure 1B shows a representative experiment in which around 40% of the CD28^{null} cells have divided at least once after a 7-day culture with IL-15, whereas only 3% of CD28+ cells did. Differences in frequency of proliferating cells were maintained in the samples cultured for 14 days, but the number of division cycles was higher in CD28+ cells.

Differentiating CD4+ T cells first lose expression of CD27, and subsequently in a later phase, they lose CD28 (Amyes *et al.*, 2003; van Leeuwen *et al.*, 2004). To characterize the differentiation degree of the IL-15-generated CD28^{null} T cells, we analyzed the expression of CD27. All the CD4+CD28^{null} T cells present in the PBMC cultures both in medium alone and in the presence of IL-15 were also negative for CD27 (Fig. 1C). Moreover, depending on CD45RA and CCR7 expression, these CD27^{null}CD28^{null} cells can be divided into effector memory (EM; CD45RA−CCR7−) and the most differentiated effector memory RA (EMRA; CD45RA+CCR7−) subsets (Sallusto *et al.*, 1999). The increase induced by IL-15 was even more relevant in the EMRA (12.2% ± 8.4% in medium and 24.9% ± 12.5% with IL-15; paired T cells, $P = 0.005$) than in the EM cells (10.8% ± 4.4% in medium and 17.1% ± 8.3% with IL-15; paired T cells, $P = 0.005$) (Fig. 1D).

In conclusion, IL-15 increased the frequency of CD4+CD28^{null} T cells mainly in the highly differentiated subset EMRA by the preferential proliferation of these cells with respect to those expressing CD28.

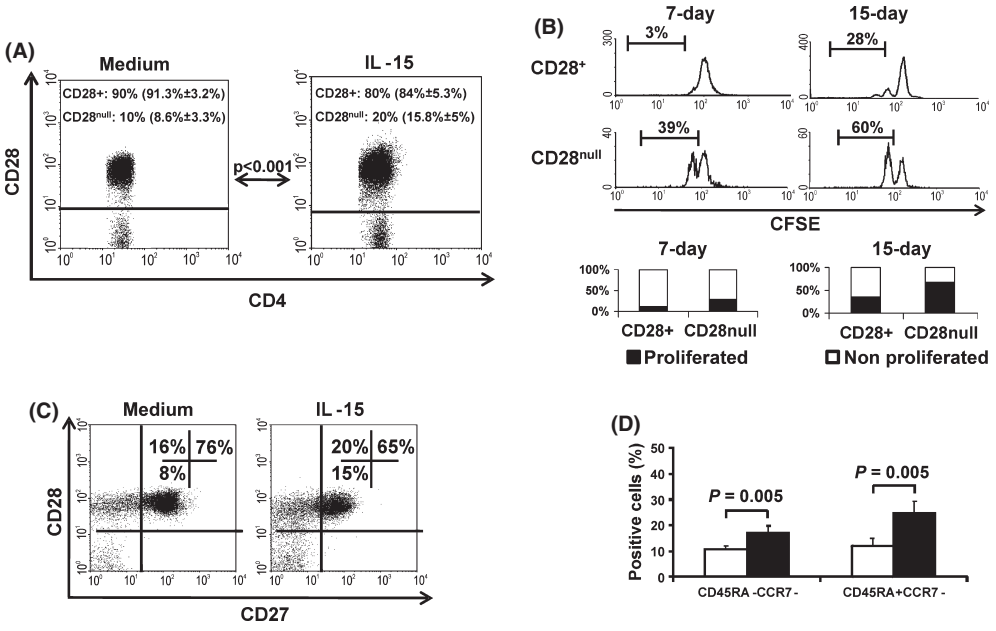

Fig. 1 Effect of IL-15 on the growth of CD4+CD28^{null} T cells in elderly individuals. (A) Frequency of CD4+CD28^{null} T cells in response to treatment with IL-15. Peripheral blood mononuclear cells (PBMC) from 10 elderly subjects were cultured in medium or in the presence of IL-15 (50 ng mL^{-1}) for 7 days and analyzed by flow cytometry. Cells were stained with CD3-FITC/CD28-PE/CD4-PerCP/CD45-APC, and 5×10^4 cells were acquired in each experiment. Dot plots show the frequencies of CD28+ and CD28^{null} cells in gated CD4+ T cells in this representative experiment, and the frequencies were averaged from all donors (mean ± SD). (B) Proliferative capacity of CD4+CD28+ and CD4+CD28^{null} T cells in response to IL-15. PBMC were labeled with CFSE (1.5μM) and cultured in the presence of IL-15 (50 ng mL^{-1}) for 7 and 15 days. CD4+CD28+ and CD4+CD28^{null} T cells were gated. Percentages of dividing cells were analyzed on days 7 and 15 of culture. Representative histogram plots of cells from one of five experiments are shown. Histograms depict the mean of the percentages of proliferated (black bar) and nonproliferated (white bar) cells in gated CD4+CD28+ and CD4+CD28^{null} T lymphocytes in the five experiments. (C) PBMC treated as described above were stained with CD3-FITC/CD28-PE/CD4-PerCP/CD27-APC. Dot plots show the expression of CD27 and CD28 in gated CD4+ T cells in this representative experiment. (D) Cells were stained with CD45RA-FITC/CD28-PE/CD4-PerCP/CCR7-APC and frequency of CD28^{null} cells in the EM (CD45RA-CCR7−) and EMRA (CD45RA+CCR7−) CD4+ T-cell subsets was analyzed. Histograms summarize the percentage of CD28^{null} cells in the indicated cell populations (mean ± SEM) after cells were cultured in medium (white bars) and in the presence of IL-15 (black bars). Paired *T* test was used to compare paired frequencies.

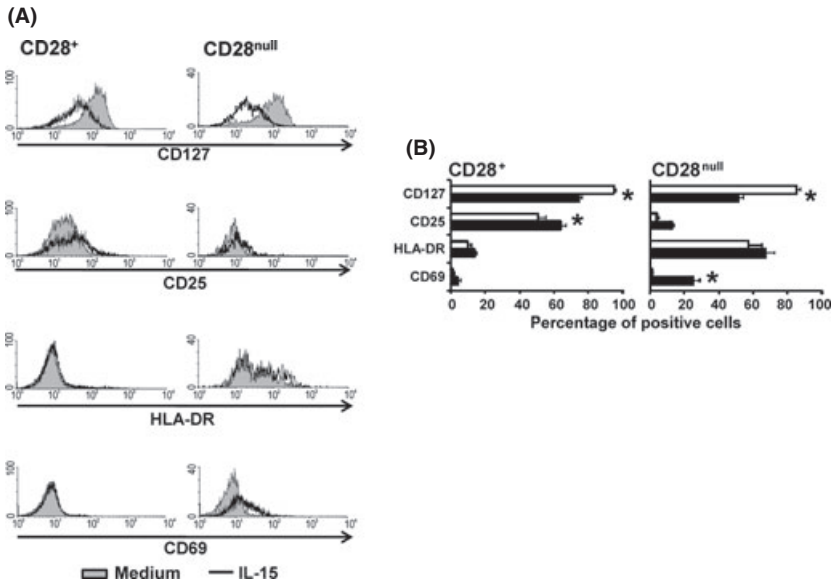

Fig. 2 Effect of IL-15 on the expression of activation markers. Results were representative for five elderly donors. Peripheral blood mononuclear cells were cultured for 18 h in the presence or absence of IL-15. Expression of CD127-PE, CD25-FITC, HLA-DR-FITC, and CD69-FITC was analyzed in CD4+CD28+ and CD4+CD28^{null} T cells by flow cytometry. (A) Histogram plots show a representative experiment of 5 performed. (B) Histograms represent percentage of positive cells (mean ± SEM) in CD28+ and CD28^{null} cells cultured in medium (white bars) and in the presence of IL-15 (black bars) cells. Paired *T* test was used to compare differences; significant differences were indicated (*$P < 0.05$).

Activation induced by IL-15

To further evaluate other differential effects of IL-15 on CD4+CD28^{null} T cells compared with CD28+, we studied the expression of several activation markers in response to the cytokine. Expression patterns of CD127, CD25, HLA-DR, and CD69 in CD4+CD28+ and CD4+CD28^{null} T cells cultured for 24 h in medium alone and in the presence of IL-15 are represented in Fig. 2A. IL-15 activation reduced CD127 expression in both CD28+ and CD28^{null} cells with respect to cells cultured in medium alone ($P = 0.005$ and $P = 0.002$, respectively) (Fig. 2B). CD25 was significantly increased only in CD28+ cells, whereas CD28^{null} cells showed low CD25 expression both basally and in response to IL-15. On the contrary, IL-15 induced higher expression of HLA-DR and mainly CD69 in CD28^{null} cells ($P = 0.027$) with no significant effect on CD28+ cells (Fig. 2B).

We then analyzed the expression of the specific IL-15R α-chain in CD4+CD28+ and CD4+CD28^{null} T cells to evaluate a possible mechanism for the different responsiveness of both subsets to IL-15. This receptor was barely expressed in resting CD4+ T cells and stimulation for 24 h with IL-15 did not induce up-regulation in CD4+CD28^{null} nor in CD4+CD28+ T cells (Fig. 3A). To investigate whether there was a difference in intracellular signaling after IL-15 interaction with its receptor, we analyzed the STAT-5 activation. As expected, phosphorylation of this signal factor was strongly induced by IL-15 in both subsets, but CD4+CD28^{null} showed significantly higher levels of pSTAT-5 with respect to CD4+CD28+ T cells (mean: 82.5% ± 6.5% vs. 69.6% ± 7.8%, paired *T* test, $P = 0.036$) (Fig. 3B).

IL-15 induced an activated phenotype in CD4+CD28^{null} T cells, with enhanced expression of CD69 but diminished CD127 expression. Moreover, the surface expression of IL-15R α-chain was not increased, but STAT-5 was preferentially activated.

IL-15 up-regulates granzyme B and perforin expression and cytolytic properties in CD4+CD28^{null} T cells

CD4+ T cells have not been classically considered as cytotoxic cells, although intracytoplasmic stores of granzyme B and perforin have been previously described in CD4+CD28^{null} T cells (Appay *et al.*, 2002). Because IL-15 is known to induce cytotoxic properties in NK and CD8+ T cells, we analyzed its influence on granzyme B and perforin expression in CD4+CD28^{null} T cells. After 5 h of culture with IL-15, expression of both proteins was up-regulated in CD28^{null} cells (Fig. 4A). Percentages and mean fluorescence intensity (MFI) of perforin-positive cells showed

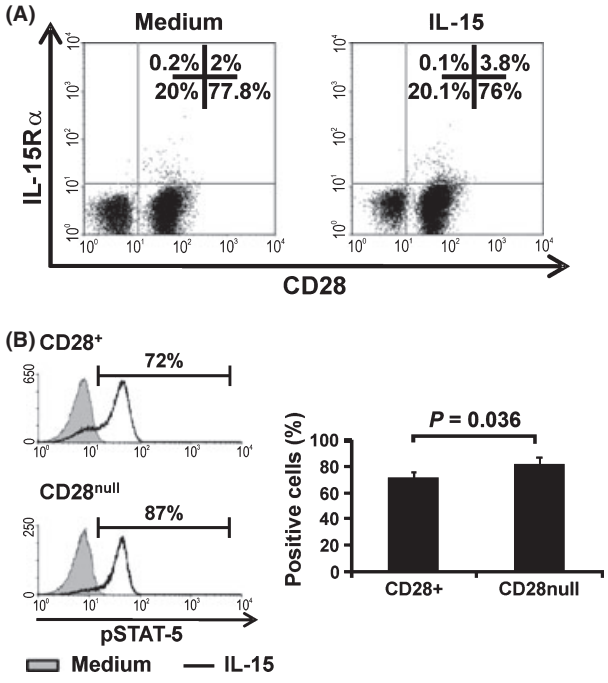

Fig. 3 Expression of IL-15Rα chain and activation of STAT-5 in response to IL-15. (A) Peripheral blood mononuclear cells from three elderly donors were cultured in medium or in the presence of IL-15 (50 ng mL^{-1}) for 24 h. Cells were stained with CD28-FITC/IL-15Rα-PE/CD4-PerCP, and 5 × 10^4 cells were acquired in each experiment. Dot plots show the frequencies of expression of IL-15Rα in CD4+ T cells related to CD28 expression in this representative experiment. (B) CD4+ T cells from 5 elderly subjects were isolated and stimulated with IL-15 for 15 min at 37 °C. Cells were surface-stained with CD28-PE and intracellularly stained with pSTAT5-AlexaFluor® 488. The percentages of pSTAT5-positive cells in CD4+CD28+ and CD4+CD28^{null} T cells in this representative experiment are expressed in each histogram plot. Histograms summarize the percentage of positive cells as mean ± SEM after the culture with IL-15 in the five experiments performed. Paired *T* test was used to compare paired frequencies.

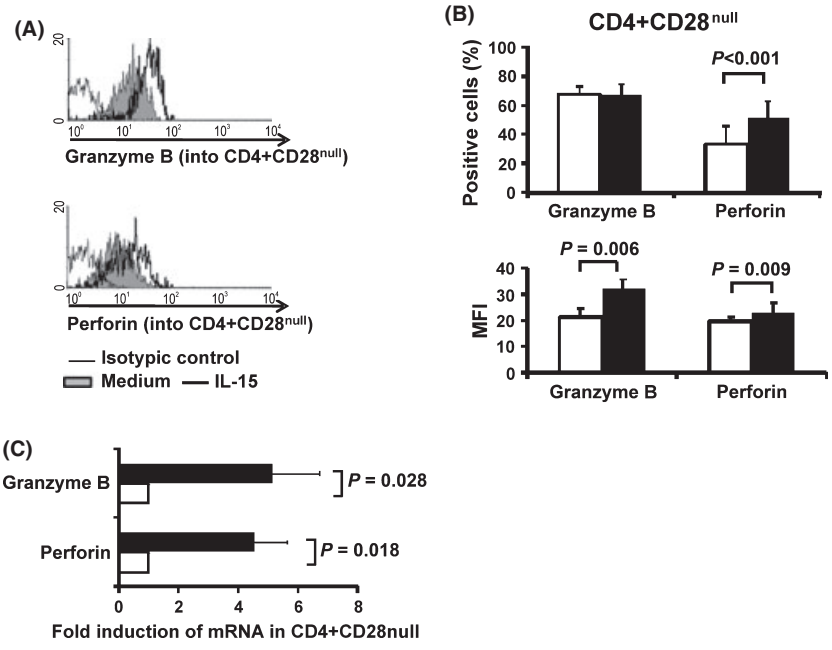

Fig. 4 IL-15 up-regulates granzyme B and perforin expression in CD4+CD28^{null} T cells. Granzyme B and perforin expression was studied by intracellular staining in 9 and 7 elderly donors, respectively, after 5 h of culture in medium in the absence or presence of IL-15 (50 ng mL^{-1}). (A) Histogram plots show a representative experiment. (B) Histograms represent the percentage and the MFI of positive cells (mean ± SEM) in CD28^{null} cells cultured in medium (white bars) and in the presence of IL-15 (black bars). (C) Granzyme B and perforin mRNA expression was determined by quantitative RT–PCR in isolated CD4+ T cells from six donors cultured for 5 h in medium (white bars) and in the presence of IL-15 (black bars) cells. Results were normalized with respect to mRNA expression in medium. Nonparametric Wilcoxon signed-ranks test (granzyme B) and paired *T* test (perforin) were used to compare differences.

significant differences in CD28^{null} cells treated with IL-15 compared with cells cultured in medium alone (P = 0.00028 and P = 0.009, respectively) (Fig. 4B). Similar results were found in the levels of expression (P = 0.006), but not in the percentage of cells expressing granzyme B (Fig. 4B). Quantification of granzyme B and perforin mRNA expression by RT–PCR in purified CD4+ T lymphocytes, with more than 15% of CD28^{null} cells, also showed higher expression following 5 h of culture with IL-15 (paired *T* test, P = 0.028 and P = 0.018, respectively) (Fig. 4C).

Lytic granules consisting of granzymes and perforin are contained in membrane-bound lysosomes coated with lysosome-associated membrane proteins (LAMPs). LAMPs are not usually present on the surface of T cells but are exposed only during active degranulation. CD107a (LAMP-1) expression has been described as a good candidate marker for the cytotoxic cellular activity (Aktas *et al.*, 2009). Isolated PBMC were cultured in medium or stimulated for 5 h with anti-CD3, IL-15, or both, and levels of surface CD107a were assessed in CD4+CD28^{null} T cells. Culture in medium alone or in the presence of IL-15 displayed no effect on degranulation (Fig. 5A). However, anti-CD3 stimulation induced expression of CD107a (mean CD107a+ 17.4% ± 4.9%), which was significantly increased with the addition of IL-15 (mean 24.5% ± 4.9%) (P = 0.015).

The effector function of CD4+CD28^{null} T cells (≥ 15% CD28^{null}) was examined in a redirected cytotoxicity assay against P815 cells previously coated with anti-CD3 mAbs. CD4+ T cells were isolated and cultured for 6 h with P815 cells in medium with or without IL-15. Cells treated with IL-15 showed cytotoxicity that was greater than that of medium-cultured cells at all target–effector cell ratios tested (from 1:1 up to 1:30) (Fig. 5B).

IL-15 showed an increasing effect on cytolytic properties of CD4+CD28^{null} T cells, both in the expression and storage of involved proteins and in the cytolytic effector functions.

IL-15 enhanced production of IFN-γ by CD4+CD28^{null} T cells in response to specific antigens

CD4+CD28^{null} T lymphocytes have been described as antigen-specific cells against chronic viral antigens, mainly in some autoimmune diseases (The-

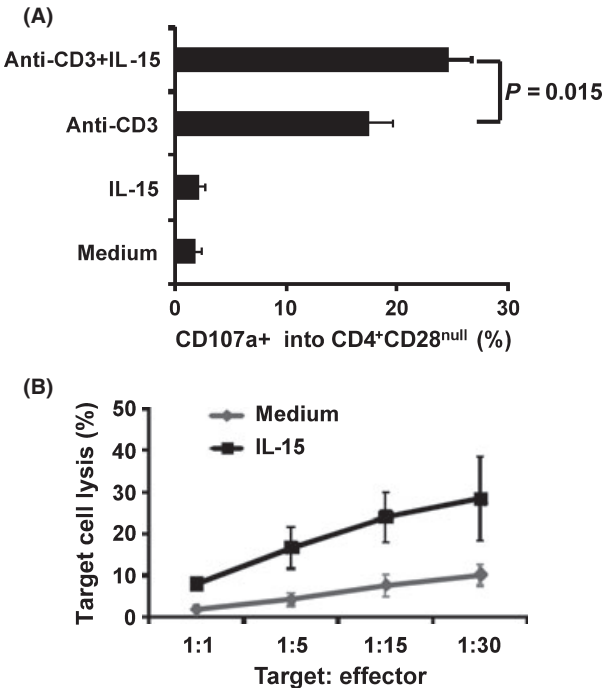

Fig. 5 Degranulation and redirected cytotoxicity induced by anti-CD3 and IL-15. (A) Peripheral blood mononuclear cells from six elderly donors were stimulated with anti-CD3 (1 μg mL^{-1}), IL-15 (50 ng mL^{-1}), and a combination of both for 5 h. Surface CD107a expression was analyzed in CD4+CD28^{null} T cells by flow cytometry. Histograms represent percentage of positive cells (mean ± SEM). Paired *T* tests were used to compare differences between medium and Il-15 and between anti-CD3 and anti-CD3 plus IL-15. (B) Cytotoxicity of purified CD4+CD28^{null} T cells from three elderly individuals in a redirected cytotoxicity assay was performed as described in Experimental procedures. Purified CD4+CD28^{null} T cells were cultured in medium or stimulated with IL-15 for 18 h, and cytotoxicity was measured against P815 cell line previously labeled with CFSE and coated with anti-CD3 (10 μg mL^{-1}). Data are shown as the percent lysis that was determined as [(%7-AAD staining sample – % 7-AAD staining of negative control) / (100 × %7-AAD staining of negative control)] × 100 (mean ± SEM).

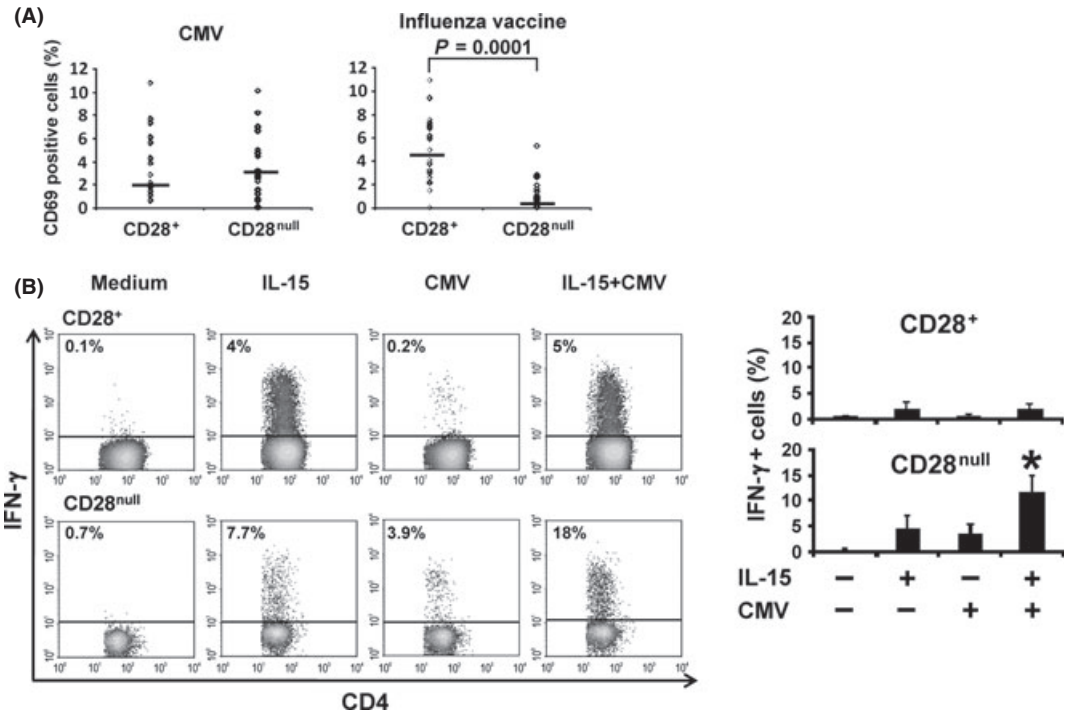

Fig. 6 CD4+CD28+ and CD4+CD28^{null} T-cell antigen-specific response to chronic and recent contact antigens. (A) CD69 expression in response to CMV antigens and to the influenza vaccine. Whole blood from elderly individuals were stimulated for 18 h with CMV (10^4 PFU mL^{-1}) or influenza vaccine (dilution 1/100), and CD69 expression was evaluated by flow cytometry. Dispersion plots summarize the percentage of CD69+ cells for CMV and influenza vaccine in the CD4+CD28+ and CD4+CD28^{null} subsets obtained from 20 elderly donors. Paired *T* test was used to compare paired frequencies. (B) Peripheral blood mononuclear cells were precultured for 18 h in medium or in the presence of IL-15 (50 ng mL^{-1}), washed, and cultured for 6 h in medium or with CMV (10^4 PFU mL^{-1}). The responder cells in the CD4+CD28+ and CD4+CD28^{null} T-cell subsets were analyzed for intracellular IFN-γ staining. Percentages of IFN-γ-positive cells in the indicated populations in this representative experiment are expressed on the dot plots. Histograms summarize the percentage of IFN-γ-positive cells in both subsets (mean ± SEM) from the five elderly donors tested. Paired *T* test was used to compare paired means (*represent $P < 0.05$ with respect all other conditions).

wissen *et al.*, 2007). In order to investigate this point in aging individuals, the response to chronic and to recent contact antigens was tested. We compared the induction of CD69 expression in response to CMV antigens and to influenza vaccine in CD4+ T cells with and without CD28 expression from a group of 20 elderly subjects (Fig. 6A). These elders were seropositive for CMV and had been recently vaccinated against the influenza virus. Their response to CMV did not induce differences in the median value of CD69-positive cells between the CD4+CD28+ subset at 2% (IR: 4.1%) and the CD4+CD28^{null} subset at 3% (IR: 3.65%) (Fig. 6A). However, there were statistically significant differences in response to influenza vaccine (Mann–Whitney *U* test, $P = 0.00013$), with 4.5% (IR: 10.9%) of CD4+CD28+ T cells expressing CD69 and only 0.67% (IR: 1.47%) of CD4+CD28^{null} T cells (Fig. 6A). To evaluate the effect of IL-15 on the antigen-specific response to chronic CMV infection, the cells were precultured for 18 h with IL-15 or in medium alone and then stimulated with an extract of CMV for 18 h. Both CD4+CD28+ and CD4+CD28^{null} T cells produced measurable levels of IFN-γ in response to IL-15 and to CMV; nevertheless, differences with cells cultured in medium alone were not statistically significant (Fig. 6B). CD4+CD28^{null} T cells pretreated with IL-15 and stimulated with CMV displayed the highest frequencies of IFN-γ-producing cells ($P < 0.05$, compared with all other stimulation conditions), showing a greater than additive effect and potentially a synergistic effect which was not observed in CD4+CD28+ T cells (Fig. 6B).

CD4+CD28^{null} T cells were mainly specific against chronic contact antigens, and pretreatment with IL-15 enhanced their antigen-specific responses.

Discussion

The effect of IL-15 was thought to be more pronounced in the CD8+ than in the CD4+ T-cell compartment. Nevertheless, to our knowledge, this is the first study demonstrating that IL-15 displays a striking effect on CD4+ T cells, with greater capacity for activating CD28^{null} cells than CD28+ cells. IL-15 increases the proliferation and frequency of CD4+CD28^{null} T cells, compared with CD4+CD28+ T cells. Moreover, activation is clearly induced by IL-15 on CD4+CD28^{null} T cells as shown by both expression of activation markers and an enhancing effect on their cytolytic properties. Pretreatment with IL-15 also enhances the IFN-γ production in the antigen-specific responses of CD28^{null} T cells with minimal effects on CD28+ cells. These effects of IL-15 may be important because the accumulation of CD28^{null} T cells is associated with reduced overall immune responses to pathogens and vaccines in the elderly(Saurwein-Teissl *et al.*, 2002) and IL-15 could potentially be useful in enhancing the antigen-specific response of CD4+CD28^{null} T cells.

A progressive increase in the percentage of T cells that lack CD28 expression is common with increasing age in healthy individuals (Goronzy *et al.*, 2007; Czesnikiewicz-Guzik *et al.*, 2008) and in patients with chronic infections and autoimmune diseases (Komocsi *et al.*, 2002; Effros *et al.*, 2005; Fletcher *et al.*, 2005; Thewissen *et al.*, 2007; Alonso-Arias *et al.*, 2009). These cells have a memory phenotype, are long-lived *in vivo*, display reduced diversity of the T-cell receptor (TCR), and have reduced division potential (Vallejo *et al.*, 1999, 2000; Appay *et al.*, 2002). At the same time, they express perforin and granzyme B, which confer cytotoxic

ability and the capability to produce large amounts of IFN-γ (Appay *et al.*, 2002; van Leeuwen *et al.*, 2004; Alonso-Arias *et al.*, 2011). The data suggest that the loss of CD28 expression is induced by repeated exposure to the same antigens such as CMV or other persistent viral infections; chronic CMV represents a model of antigen persistence with transient reactivations mainly in immunosuppressed individuals (Appay *et al.*, 2002; Goronzy & Weyand, 2005). The cause of loss of CD28 expression in T cells with age has been attributed to repeated antigenic stimulation (Valenzuela & Effros, 2002; Vallejo, 2005), and it is now accepted that CD28^{null} T cells have experienced past episodes of activation and cell cycling. However, the loss of CD28 can result not only from repeated TCR activation but also from homeostatic proliferation. IL-15-mediated proliferation, without antigenic stimulation, results in a stable loss of CD28 expression in CD8+ memory T cells in part through the induction of TNF-α secretion (Chiu *et al.*, 2006). In this study, we failed to demonstrate a similar effect of IL-15 on CD4+ T cells because IL-15 did not induce loss of CD28 expression in CD4+CD28+ T cells in our culture conditions. Despite this, we found a significant increase in the proportion of CD4+ T cells without CD28 expression, which was attributable to an enhanced proliferative response to IL-15 of the CD4+CD28^{null} T cells with respect to CD4+CD28+ T cells. IL-15 might be able to counteract the widely described defects in the proliferative ability of CD28^{null} T cells (Appay *et al.*, 2002), even in the most differentiated EMRA cells. The possible role of this cytokine in the maintenance of CD28^{null} T cells *in vivo* was previously suggested because CD8+CD28^{null} T cells also exhibited normal growth under IL-15 stimulation *in vitro* (Chiu *et al.*, 2006).

There are no differences in the serum levels of IL-15 between old and young controls (Gangemi *et al.*, 2005), and PBMC from aged donors produced IL-15 *in vitro* at levels similar to those found in the young (Tortorella *et al.*, 2002). However, IL-15 is overproduced in some inflammatory and autoimmune diseases, and it has been speculated that chronic exposure to this cytokine is likely the mechanism of the expansion of CD4+CD28^{null} T cell *in vivo* (Yamada *et al.*, 2007). In the case of rheumatoid arthritis, the best characterized disease in which IL-15 is of substantial clinical importance, the levels of the cytokine are elevated in the patients correlating with the disease severity (McInnes *et al.*, 1997). Simultaneously, the progression of the pathology is thought to be accompanied by the recruitment and rise of oligoclonal, autoreactive CD4+CD28^{null} T cells, suggesting the potential relevance of IL-15 in this expansion.

Nevertheless, the CD4+CD28^{null} population express activation markers with little or no evidence of proliferation (Appay *et al.*, 2002) and present a low activation threshold in response to TCR stimulation, which could be implicated in its predisposition to the breakdown of self-tolerance (Yung *et al.*, 1996). Similarly, we corroborated the activating effect of IL-15 on CD4+ T cells in short-term cultures. An activated phenotype was induced in both subsets of CD4+ T cells, with the most notable differences in the expression of CD69 and CD127 in the CD28^{null} cells. CD69 displayed a clear up-regulation, whereas the percentage of cells expressing CD127, the IL-7 receptor which is down-regulated on all human T cells after activation, was decreased by nearly half. Whereas IL-15 has minimal effect on the expression of other γ-chain cytokine receptors in CD28^{null} cells, such as IL-2 and IL-15 (Alves *et al.*, 2005), the expression of CD127 in CD8+ T cells is decreased after stimulation with IL-15. IL-15 binds to a trimeric receptor composed of the γ_c chain, the IL-2/IL-15 β-chain (CD122), and the unique IL-15R α-chain. IL-15Rα is barely expressed in naïve CD8+ T cells and completely down-regulated in cultures where IL-15 is present (Alves *et al.*, 2003). It has been shown that the IL-15R α-chain is dispensable on CD8+ T cells for IL-15-mediated proliferation (Dubois *et al.*, 2002). In CD4+ T cells, we found that an increase in receptor expression is not necessary to induce the activation of STAT5, the downstream signaling molecule used by IL-15. It has been postulated that CD4+ memory T cells rely on STAT5 considerably more than do effector CD4+ T cells (Purton *et al.*, 2007; Tripathi *et al.*, 2010). Accordingly, we have found the activating effect of IL-15 on STAT5 was more prominent on CD28^{null} than on CD28+ CD4+ T cells. IL-15 increased the cytolytic properties of CD4+CD28^{null} T cells and enhanced their antigen-specific responses. Granzyme B and perforin expression in CD4+ T cells is closely associated with the loss of CD28 on the cell surface. These CD4+CD28^{null} T cells resembled cytotoxic CD8+ T cells, because their cytotoxic capacity is mediated by TCR stimulation. In addition, they lack costimulatory molecule requirements (Appay *et al.*, 2002). Similar to our findings in CD4+CD28^{null} T cells, in CD8 T cells, IL-15 may regulate perforin expression at the level of transcription through STAT5 activation, and it augments anti-CD3-induced degranulation, in agreement with the induction of cytotoxic activity (Weng *et al.*, 2002). Although the role of CD4+ T cells as cytotoxic effector cells is not well known, the enhancing effector activity of IL-15 may have much impact, because CD4+CD28^{null} T cells were mainly specific against chronic contact antigens. IL-15 plays a critical role in the immune responses to early infection and chronic inflammation by amplifying the effects of proinflammatory cytokines on IFN-γ secretion (Smeltz, 2007). In this way, pretreatment with IL-15 enhanced the antigen-specific responses of CD4+CD28^{null}, increasing the frequencies of IFN-γ-producing cells, but exerted no synergistic effect on CD4+CD28+ T cells. IFN-γ production from CD4+CD28^{null} T cells is induced at much lower doses of anti-CD3 than in their CD28+ counterparts and is also independent of costimulation (Alonso-Arias *et al.*, 2011).

It has been hypothesized that CD4+CD28^{null} T cells might be playing a role in containing viral infections tropic for HLA class II cells, such as EBV in B cells, HIV-1 in activated CD4+ T cells, monocytes and dendritic cells, and CMV in endothelial cells, although the mechanism of this antigen presentation is unknown at this time. In the case of CMV infection, endothelial cells are poor antigen-presenting cells under normal conditions in a classical immune response because they lack costimulatory molecules. But because the CD28^{null} T cells do not require costimulation and present a low activation threshold, antigen presentation could be effective by nonprofessional cells such as endothelial cells. This hypothesis is supported by the fact that the class II pathway may be preferentially targeted because both EBV and CMV prevent normal MHC class I expression as part of their strategies of immune evasion (Alcami & Koszinowski, 2000).

In summary, our data show that IL-15 plays a role in enhancing activation, proliferation, and the effector response of CD4+CD28^{null} T cells against their specific antigens. This may be mainly relevant in the elderly immune system characterized by lower adaptive responses, and treatment with IL-15 has been proposed as a strategy to alleviate the effects of T-cell deficiencies and to improve the immune function.

Experimental procedures

Donors

Blood samples were obtained from elderly donors in the Hospital Universitario Central de Asturias and the Monte Naranco Hospital (Oviedo, Spain). The volunteers were not rigorously selected according to their health status in order to study a representative sample from the population. However, those volunteers with serious diseases such as cancer, chronic diseases (diabetes, autoimmune diseases), or congestive heart failure and those receiving ongoing treatment with immunosuppressive drugs were excluded from participation. Samples from 45 elderly donors (≥ 65 years, mean age: 76 ± 6 years) were analyzed in the study to determine whether they met the inclusion criteria of the presence of

CD4+CD28^{null} T cells at > 5% of total CD4+ T cells (n = 30). Informed consent was obtained from donors prior to participation in the study. The study was approved by the Hospital Central de Asturias (Oviedo, Spain) ethics committee.

Quantification of CD4+CD28^{null} T cells

The percentage of CD4+CD28^{null} T cells was determined in peripheral blood from the elderly participants by staining with anti-CD3 (FITC), anti-CD28 (PE) (eBioscience, San Diego, CA, USA), anti-CD4 (PerCP), and anti-CD45 (APC) (Immunostep, Salamanca, Spain). One hundred microliters of whole blood from elderly was stained with the combination of labeled monoclonal antibodies for 30 min at room temperature. Samples were red blood lysed with FACS Lysing Solution (BD Biosciences, San José, CA, USA), washed in PBS, and analyzed with CellQuest software in the FACSCalibur Cytometer (BD Biosciences). Appropriate isotype control monoclonal (mAbs) were used for marker settings. CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. BD Multicheck Control and Multicheck CD4 Low Control were used as quality controls.

Isolation of PBMC and cell cultures

Peripheral blood mononuclear cells were isolated from peripheral blood that had been anticoagulated with EDTA by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). Cultures were performed in RPMI 1640 medium containing 2×10^{-3}M L-glutamine and Hepes (BioWhitaker, Verviers, Belgium) and supplemented with 10% FCS (ICN Flow; Costa Mesa, CA, USA) and antibiotics. Cells were incubated at 37 °C and 5% carbon dioxide.

Stimulation with IL-15

Peripheral blood mononuclear cells (4×10^6 cells mL^{-1}) were cultured in the presence and absence of IL-15 (50 ng mL^{-1}) (Peprotech INC, Rockyhill, NJ, USA) for different times. Frequencies of CD4+CD28^{null} T cells were quantified after 7 days of culture with the same combination of labeled antibodies described earlier. Additional staining was performed with anti-CD27 (APC) (eBioscience), anti-CCR7 (Alexa Fluor 647) (BD Bioscience), and CD45RA (FITC) (Immunostep). To analyze an activated phenotype, cells were cultured for 18 h and then stained with anti-CD3 (APC), anti-CD28 (FITC or PE), anti-CD69 (FITC), (eBioscience), anti-CD4 (PerCP), anti-CD127 (PE), anti-IL-15R α-chain (eBioscience), anti-HLA-DR (FITC), and anti-CD25 (PE) (BD Bioscience). Frequencies of cells with intracytoplasmic stores of granzyme B and perforin in CD4+CD28^{null} T cells after 6 h of culture with IL-15 were measured. Cells were surface-stained for 30 min at room temperature, lysed and fixed with FACS lysing solution, permeabilized with BD FACS Permeabilizing Solution 2 (Perm II) (BD Bioscience), and stained with anti-granzyme B (FITC) or anti-perforin (FITC) (BD Bioscience) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

Proliferation assays

Peripheral blood mononuclear cells were resuspended in PBS at a final concentration of 5–10 $\times$ 10^6 cells mL^{-1} and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland, UK) for 10 min at 37 °C, washed with RPMI 1640 medium containing 2×10^{-3}M L-glutamine and Hepes twice, and cultured at 2×10^6 cells mL^{-1} in the presence of human recombinant IL-15 (50 ng mL^{-1}). The proliferative responses of CD4+ and CD8+ T cells were analyzed on days 7 and 14 by FACSCalibur after staining with anti-CD4 (PerCP) and anti-CD28 (PE).

mRNA quantification

CD4+ T cells from individuals with more than 15% CD4+CD28^{null} T cells were isolated (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany). mRNA was extracted using a Total RNA Isolation (Macherey-Nagel GmbH & CoKG, Düren, Germany) according to the manufacturer's instructions. Reverse transcription of mRNA isolated from each sample was carried out in a 20-μL final volume with the iScript cDNA Synthesis Kit (Bio-Rad, Life Science Research Group, Hercules, CA, USA) following manufacturer's instructions. The mixture was incubated at 25ºC for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min and stored at –80 °C until required for PCR.

Quantification of perforin and granzyme B expression was performed using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad) with the previously described primers (Morissette *et al.*, 2007). Briefly, a calibration curve was generated with serial dilutions of granzyme B and perforin and GAPDH external standards that allowed the quantification of cDNA samples, leading to the determination of mRNA relative units. Reactions without cDNA were always included as a negative control, and cDNA samples were quantified in duplicate. External granzyme B and perforin standards were serial dilutions of the cDNA obtained from CD8+ T cells stimulated for 3 h with anti-CD3 (1 μg mL^{-1}). Thermal cycling conditions began with 50 °C for 2 min and 94 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 1 min. Experimental samples were run in duplicate, and the replicate average value was recorded as the sample result.

STAT5 quantification

Isolated CD4+ T cells were stimulated with IL-15 recombinant protein (50 ng mL^{-1}) for 15 min at 37°°C. Cells were fixed using BD Cytofix Fixation Buffer (10 min at 37°°C), stained on the surface with anti-CD28 (PE), and permeabilized in BD Phosflow Perm Buffer III (30 min on ice). Cells were then washed twice in BD Pharmingen Stain Buffer and stained with 20 μL Alexa Fluor 488-conjugated phospho-Stat5 antibody for 1 h at room temperature. The cells were analyzed on a BD FACSCalibur flow cytometer.

Lysosomal degranulation assay

CD107a lysosome-associated membrane protein-1 (LAMP-1) expression was used to measure CD4+ T-cell degranulation. PBMC were incubated for 5 h in medium alone, IL-15 (50 ng mL^{-1}), anti-CD3 (1 μg mL^{-1}) (eBioscience), and IL-15 plus anti-CD3. After the first hour of culture, monensin (2 μM) (BD Biosciences), a protector of the cell degranulation, was added. Cells were then stained with anti-CD28 (FITC), anti-CD4 (PerCP), and CD107a antibody (Alexa Fluor 647) (eBioscience). All samples were analyzed with CellQuest software in the FACSCalibur Cytometer.

Redirected cytotoxicity assay

Peripheral blood mononuclear cells were separated from the blood of three elderly individuals with > 20% CD4+CD28^{null} T cells. Isolated CD4+ T cells (Miltenyi Biotec GmbH) were stained with anti-CD28-PE, and CD28^{null} cells were also isolated by negative selection with anti-PE

magnetic microbeads (Miltenyi Biotec GmbH). Cytotoxicity of T-cell subsets was determined as previously described (Lecoeur *et al.*, 2001). Briefly, purified CD4+CD28^{null} T cells from donors were cultured in medium with or without IL-15 for 18 h as a source of effector cells and resuspended at appropriate concentrations for the desired effector–target cell (E/T) ratio. Target cells (P815) were labeled with CFSE and washed three times in complete medium. The P815 cells were then incubated for 30 min on ice with 10 μg mL^{-1} of anti-CD3 mAb, resuspended in medium, and adjusted to 10^5 cells mL^{-1}. Effector cells were added to 100 μL target cells in round-bottom polystyrene tubes (BD Biosciences) to yield E/T ratios of 1:1, 1:5, 1:15, and 1:30, mixed by gentle tapping, incubated at 37 °C for 4 h, and stained with 5 μL 7-ADD (Immunostep) for 20 min before data acquisition. Percentage of lysis was determined as [(%7-AAD+ cells in test sample – % 7-ADD+ cells in negative control)/(100 × %7-ADD+ cells in negative control)] × 100.

Antigen-specific stimulation

Antigen-specific responses were analyzed in PBMC from CMV-seropositive individuals vaccinated with a trivalent influenza vaccine (Solvay Biologicals BV, Olst, Holland). CMV-infected cell lysate was prepared by infecting human embryonic lung fibroblasts with the AD169 strain of CMV. Viral titers in the supernatant were determined by standard plaque assays. The virus was inactivated by repeated freeze–thaw cycles. Heparinized venous blood samples were stimulated with CMV (10^4 PFU mL^{-1}) or a 1/100 dilution of the influenza vaccine (Solvay Biologicals BV) in 15-mL conical polypropylene tubes in a humidified 37 °C incubator for 18 h. Activation was assessed by surface staining with anti-CD69. The cells were also stained with anti-CD3, anti-CD4, and anti-CD28. Samples were red blood cell lysed with FACS lysing solution, washed in PBS, and analyzed with CellQuest software.

To quantify IFN-γ production, PBMC (4×10^6 cells mL^{-1}) were precultured for 18 h in medium or in the presence of IL-15 (50 ng mL^{-1}), washed, and cultured again in medium or with CMV (10^4 PFU mL^{-1}) for 6 h. Cultures for the detection of intracytoplasmic cytokines were treated after the first 2 h with the secretion inhibitor Brefeldin A (10 μg mL^{-1}) (Calbiochem, Darmstadt, Germany). After four additional hours, cells were treated with 2 mM EDTA for 15 min at room temperature, washed, and stained with antibodies against CD4 and CD28 at 4 °C. Intracellular staining of anti-IFN-γ (BD Biosciences) was performed as previously described for granzyme B and perforin.

Virological testing

Immunoglobulin G levels of CMV-specific antibodies were determined by enzyme-linked immunosorbent assay Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany) according to the manufacturer's specifications. Patient samples were quantified and interpreted by means of the calculation of the ratio (Cutoff Index = OD value of sample/cutoff value), whereby a ratio of 1.0 is equivalent to the cutoff value. Cutoff indexes > 1.1 were considered positive.

Statistical analysis

Comparisons between groups were performed with the nonparametric Wilcoxon signed-rank test when data were not normally distributed or with Student's *t* test for paired data. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc., Chicago, IL, USA). *P*-values of 0.05 or less were considered significant.

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