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Differentiation of human CD8 T cells: implications for *in vivo* persistence of $CD8^+CD28^-$ cytotoxic effector clones

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Abstract

CD8 T cells contain a distinct subset of $CD8^+CD28^-$ cells. These cells are not present at birth and their frequency increases with age. They frequently contain expanded clones using various TCRαβ receptors and these clones can represent >50% of all CD8 cells, specially in old subjects or patients with chronic viral infections such as HIV-1. Herein, it is shown that a large fraction of $CD8^+CD28^-$ cells expresses intracellular perforin by three-color flow cytometry, in particular when this subset is expanded. Together with their known ability to exert potent re-directed cytotoxicity, this indicates that $CD8^+CD28^-$ T cells comprise cytotoxic effector cells. With BrdU labeling, we show that $CD8^+CD28^-$ cells derive from $CD8^+CD28^+$ precursors *in vitro*. In addition, sorted $CD8^+CD28^+$ cells gave rise to a population of $CD8^+CD28^-$ cells after allo-stimulation. Moreover, *ex vivo* $CD8^+CD28^+$ cells contain the majority of CD8 blasts, supporting the notion that they contain the proliferative precursors of $CD8^+CD28^-$ cells. CD95 (Fas) expression was lower in $CD8^+CD28^-$ cells, and this subset was less prone to spontaneous apoptosis in *ex vivo* samples and more resistant to activation-induced cell death induced by a superantigen *in vitro*. Thus, the persistence of expanded clones *in vivo* in the $CD8^+CD28^-$ subset may be explained by antigen-driven differentiation from $CD8^+CD28^+$ memory precursors, with relative resistance to apoptosis as the clones become perforin$^+$ effector cells.

Introduction

In recent years a large number of TCR repertoire studies have uncovered oligoclonal T cell expansions in various diseases, including chronic viral infections such as HIV infection (1,2). These expansions also occur in apparently normal subjects. In a prior study we described clonal CD8 expansions in normal elderly human subjects (3). Clonal expansions were also found in normal young subjects and even children, among CD4 as well as CD8 cells, using more sensitive molecular assays (4–6). Several studies confirmed that CD8 clonal expansions were more prominent and more frequent in the elderly (3,5,7). Similar observations were made in old mice (8). In some human subjects individual clonal expansions have been followed for many years. Often, the expanded clones remain present for several years without much change in the percentage of cells (5,6,9–11). In these cases clonal 'exhaustion' does not appear to be common.

Expanded CD8 clones can be dramatic and take over >50% of the CD8 compartment (5,9,10). Recent studies with tetrameric MHC–antigen constructs have uncovered similarly large oligoclonal expansions of antigen-specific CD8 cells in response to viral infections (12). Usually these responses resolve, as after acute Epstein–Barr virus infection, but they may persist, as in the case of chronic HIV infection. The *in vivo* persistence of expanded CD8 clones in old subjects suggests chronic antigen exposure. The CD8 clones probably accumulate because of continuous production, perhaps combined with decreased elimination.

An early finding was that CD8 T cell clones that are expanded *in vivo* lack CD28 expression (3). Most $CD28^-$ T cells in the peripheral blood are $CD8^+$ TCRαβ T cells, although they may include double-negative $CD4^-CD8^-$ T cells, CD4 single-positive T cells and TCRγδ T cells. $CD8^+CD28^-$ TCRαβ

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T cells are not present at birth, but gradually increase in numbers with age, both in mice and in humans (3,5,7,13). Loss of CD28 expression is associated with a phenotype of antigen-experienced CD8$^+$ T cells: CD57$^+$CD11b$^+$CD27$^–$ CD49d(VLA4)$^+$ (13,14) with variable expression of CD45RO/RA (3,14). Co-expression of chronic activation antigens like CD38 and HLA-DR can occur, particularly in HIV-infected subjects (15–17). Acute activation antigens such as CD69, CD25 and CD71 are not usually expressed.

Stimulation of a T cell via the TCR and CD28 results in T cell responses such as proliferation, IL-2 secretion and activation of cytolytic effector function from memory precursor cytotoxic T lymphocytes (pCTL) (18). By contrast TCR stimulation without CD28 stimulation, for instance by antigen-presenting cells that lack CD80/86, can lead to anergy. CD8$^+$CD28$^–$ T cells characteristically give weak proliferative responses (13), but are perfectly capable of exerting cytotoxic effector function (18). Some authors attribute antigen non-specific suppressor functions in co-culture assays to a similar, if not identical subset (13,14,19–22).

The largely overlapping subset of CD8$^+$CD27$^–$ cells has been shown to contain intracellular granzyme B and perforin, express Fas ligand, and produce IFN-γ and tumor necrosis factor-α, and is thought to contain cytotoxic effector cells, while CD8$^+$CD27$^+$ cells comprise naive and memory CD8 subsets (14). *Ex vivo* CD8$^+$CD28$^–$ T lymphocytes are enriched in cytotoxic effector function in a re-directed cytotoxicity assay using anti-CD3-coated P815 cells as target cells (13). In HIV-infected patients CD8$^+$CD28$^–$ cells produce high levels of IFN-γ (23) and may express intracellular TIA-1 (24), but results vary on the expression of perforin (24,25).

It is not yet clear whether *all* CD8$^+$CD28$^–$ cells are cytotoxic effectors, whether they are functionally homogeneous or perhaps include a group of distinct maturational stages during differentiation of a CTL effector cell. Direct evidence of derivation from CD8$^+$CD28$^+$ precursors is still incomplete, especially since CD8$^+$CD28$^–$ and CD8$^+$CD28$^+$ T cell clones were reported to maintain their phenotype during *in vitro* culture (13). It is also not clear why these cells sometimes accumulate as persistent clonal expansions and other times not. This report documents long-term persistence of clones within an expanded CD8$^+$CD28$^–$ subset. As cytotoxic effector CD8$^+$ cells mature they appear to lose CD28 expression, acquire intracellular perforin and become relatively resistant to apoptosis.

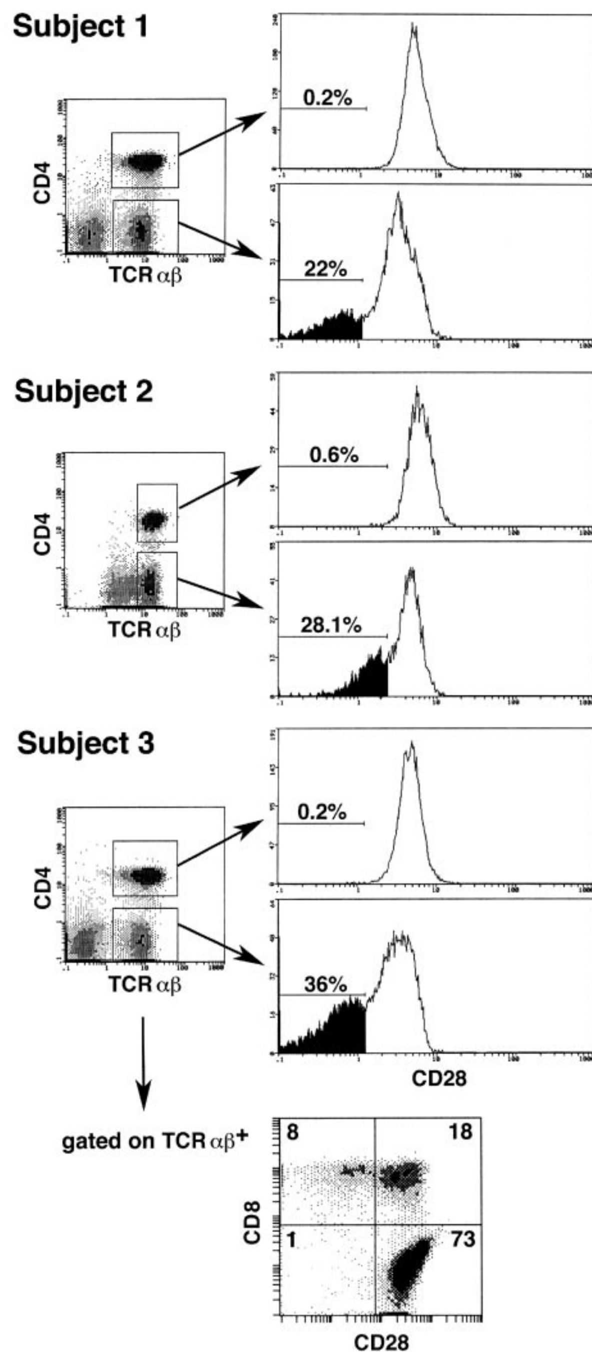

Fig. 1. Distinct CD8$^+$CD28$^–$ subset in normal adult subjects. Shown are three-color analyses with CD28–PE, CD4–biotin and anti-TCR$\alpha\beta$–FITC. The gated TCR$\alpha\beta^+$ CD4$^–$, but not the TCR$\alpha\beta^+$ CD4$^+$ cells, contain a variable percentage CD28$^–$ T cells.

Methods

Human subjects and samples

Peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypaque density centrifugation from heparinized blood according to an IRB approved protocol. The TCR repertoire of normal subjects 15 and 16 was previously described (3). Subject 15 presented with an increased CD8$^+$CD28$^–$ subset containing clonal expansions in 1993. In 1996 an asymptomatic renal adenocarcinoma was fortuitously discovered for which nephrectomy was performed. There was residual cancer in the contra-lateral kidney that has been followed by computed tomography scans since early 1997 without any change and without metastasis. Intravenous substance abusing (IVSA) patients, 25–58 years old (mean = 39.4; n = 16), were recruited from a methadone clinic. Eight of these patients were HVB and two were HVC sero-positive. Seven HIV-1$^+$ IVSA patients, 33–45 years old (mean = 36.8), were all positive for HVB and two had HVC. HIV-1$^+$ patients were all on HAART therapy, had 126–641 CD4 cells/μl blood and

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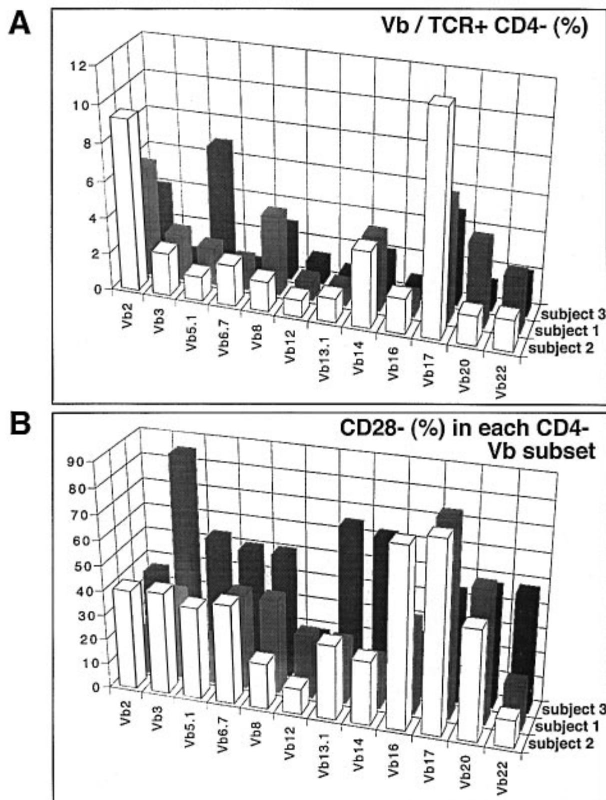

Fig. 2. Loss of CD28 expression differs among $CD4^-$ TCR V_β subsets. (A) The TCR V_β repertoire of $CD4^-$ cells from subjects 1–3 is shown. In subject 2, for example, there are two large subsets, $V_\beta2$ and $V_\beta17$. (B) The percentage of $CD28^-$ cells in each of the subsets shown in (A) is depicted. In subject 2, for example, $V_\beta16$ and $V_\beta17$, are particularly enriched in $CD28^-$ cells.

low viral loads by the bDNA assay. Twenty normal subjects of various ages were recruited. 'Old' subjects were 65–79 years old (mean = 71.6). 'Young' subjects were 24–49 years old (mean = 36.3).

Antibodies and staining

The following antibodies were used for staining in either two- or three-color protocols. In all cases the antibodies were titrated for optimal results. TCRαβ–FITC (PharMingen, San Diego, CA), CD57–FITC (PharMingen), CD56–FITC (Becton Dickinson, Mountain View, CA), CD94–FITC (PharMingen), CD95–FITC (Cal & Biological Labs), Annexin V–FITC (Trevigen), anti-perforin–FITC mAb δG9 (Ancell), CD4–biotin FFB2.3, CD8–biotin (PharMingen), CD28–PE (PharMingen), goat anti-mouse Ig–FITC $F(ab')_2$ (BioSource, Camarillo, CA), CD8–PE (Pharmingen), streptavidin–TriColor (Caltag), $V_\beta22$–FITC (Coulter-Immunotech), $V_\beta8$–FITC (Coulter-Immunotech), and $V_\beta2$ E2.2E7.2, $V_\beta3$ LE89, $V_\beta5.1$ IMMU157, $V_\beta5.2/3$ 4H11, $V_\beta6.7$ OT145, $V_\beta8$ MX3, $V_\beta9.1$ FIN9, $V_\beta11$ C21, $V_\beta12$ SV11, $V_\beta13.1$ H131, $V_\beta14$ CAS1.1.3, $V_\beta16$ TAMAYA1.2, $V_\beta17$ C1, $V_\beta20$ ELL1.4 and $V_\beta22$ IMMU546, all from the last international workshop on TCR antibodies (26).

To evaluate TCR V_β subsets, a two-color staining protocol

Table 1. Evolution of expanded TCR V_β subsets: subject 15

V_β subset followed	$V_\beta/CD3^+CD8^+$ (%)		
	11/1993	12/1996	8/1997
$V_\beta5.2$ ($CD8^+CD28^-$)	13.0	5.0	5.8
$V_\beta6.7$ ($CD8^{lo}CD28^-$)	10.4	9.6	8.8
$V_\beta8$ ($CD8^+CD28^-$)	12.0	6.3	5.2
$V_\beta22$ ($CD8^+CD28^-$)	11.5	10.2	13.9
Total	46.9	31.1	33.7

The phenotype given in brackets is the known phenotype of the expanded subset. The BV6S7 subset expressed distinctly lower levels of CD8.

was used. About 10^5–10^6 T cells were stained in microtiter wells with an anti-TCR V_β antibody for 30 min, then washed with staining buffer (HBSS, 5% FCS and 0.02% sodium azide), followed by staining with goat anti-mouse Ig $F(ab')_2$ labeled with FITC for 30 min, another wash with staining buffer and staining with anti-CD8–PE 1:50 dilution for 30 min. Washes were done by centrifuging at 2000 r.p.m. and flicking the plates to obtain dry pellets. Incubations were done at room temperature on a shaker in the dark.

For three-color staining a FITC-conjugated antibody (e.g. TCRαβ–FITC) and the biotinylated antibody (e.g. CD8–biotin) were used together for the first 30 min incubation, followed by a wash and then a 30 min incubation with the PE-conjugated antibody (e.g. CD28–PE) together with streptavidin–TriColor at a final dilution of 1:50. The cells were then washed and resuspended in wash buffer with 1% paraformaldehyde in a 0.5 ml volume for analysis on a Coulter Epics II cytofluorograph, calibrated with Coulter Standard-Brite beads and using Coulter XL software.

For cell sorting PBL were stained with TCRαβ–FITC, CD8–biotin and CD28–PE as above, and sorted on a FACS Vantage (Becton Dickinson).

For intracellular perforin staining the cells were first permeabilized with wash buffer containing 4% paraformaldehyde, 0.1% saponin and 10 mM HEPES, and then stained using the regular three-color staining protocol.

For annexin V–FITC staining, cells were first stained with CD8–biotin, followed by CD28–PE with streptavidin–TriColor, a wash with staining buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and then annexin V–FITC for 15 min at room temperature at a final dilution of 1:50 (in 50 μl), followed by dilution in 450 μl staining buffer with 1% paraformaldehyde. For staining with annexin V–biotin we stained as per the regular three-color protocol detailed above.

BrdU experiments

PBL (2×10^7) were cultured with 10 μg/ml phytohemagglutinin (PHA) and 40 U/ml IL-2 (Roche). After 3 days the cells were washed and resuspended at 10^6 cells/ml in fresh medium with PHA and IL-2, with or without 100 μM BrdU (Sigma, St Louis, MO). After 1 h the cells were washed and resuspended in media with IL-2. At the indicated time points thereafter cells were stained for CD8–biotin, CD28–PE and anti-BrdU–FITC

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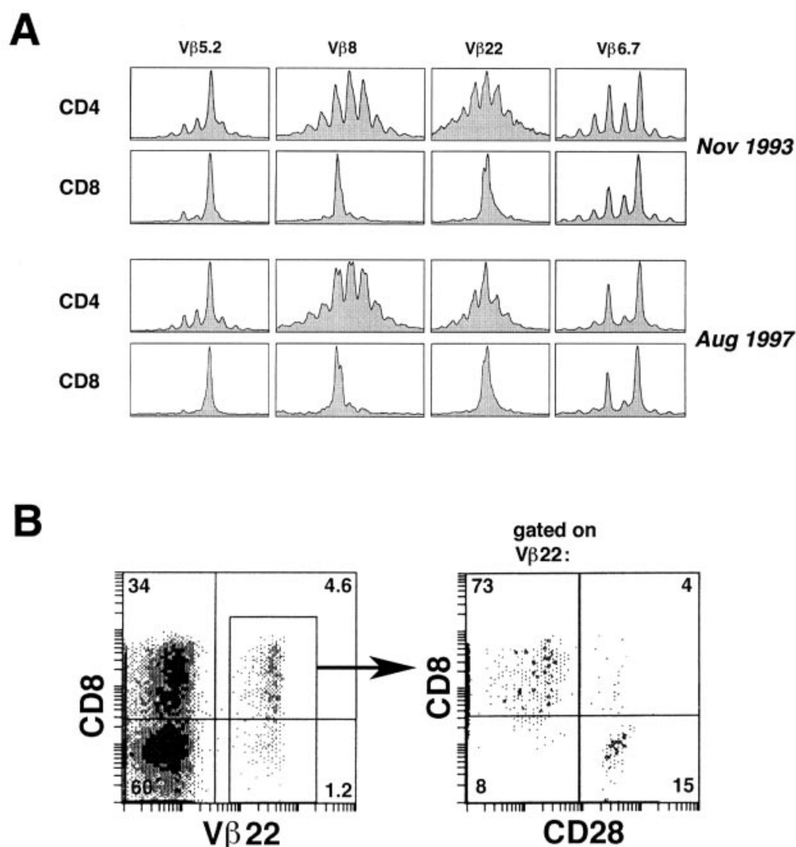

Fig. 3. The CD8$^+$CD28$^-$ TCR$\alpha\beta^+$ subset contains long-lived TCR oligoclonal V_β expansions. The indicated V_β subsets of subject 15 have been expanded for >4 years (Table 2). (A) CDR3 size distribution profiles for the indicated V_β subsets in CD4 and CD8 subsets. The V_β6.7 subset expresses low intensities of CD8 and CD4, and it was therefore impossible to get a clean separation of CD4 and CD8 cells using magnetic beads. (B) Three-color FACS of the expanded V_β22 subset with V_β22–FITC, CD8–biotin and CD28–PE. Similar results were obtained with other clonal expansions from several subjects.

as per Penit *et al.* (27) with modifications. An aliquot of 5×10^6 cells was first washed with 5 ml wash buffer (HBSS, 10% FCS and 0.02% sodium azide), then stained with CD8–biotin×30 min, then washed, stained with CD28–PE and streptavidin–TriColor for 30 min, washed, and fixed overnight in 1 ml 1% paraformaldehyde/0.5% Tween 20, at 4°C. The cells were then washed 4 times with 5 ml DNase I digestion buffer (20 mM Tris–HCl, 10 mM Mg^{2+}), centrifuged at 1000 r.p.m. for 10 min and resuspended in 100 μl buffer with 50 U DNase I for digestion at 37°C for 30 min. The cells were then washed with 5 ml stain buffer, resuspended in 100 μl, stained with 2 μl anti-BrdU–FITC (Pharmingen) on a shaker for 30 min at room temperature, washed and resuspended in 0.5 ml stain buffer for analysis. These experiments could not be extended beyond 48–72 h because of decreasing viability of the PHA-stimulated cells.

Cell cultures

PBL or CD4-depleted PBL were cultured with 200 ng/ml staphylococcal enterotoxin E (SEE), or with SEE pulsed BJAB lymphoblastoid cells (irradiated with 5000 rad), in RPMI with 10% FCS, supplemented with glutamine, penicillin/streptomycin and fungizone, for the indicated period of time. Where indicated, 40 U/ml of rhIL-2 (Boehringer Mannheim, Indianapolis, IN) was added on day 4 and 8 of culture. CD4 depletion was performed as follows. PBL were incubated with anti-CD4 at saturating concentrations for 30 min at room temperature, washed twice with sodium azide-free staining buffer, followed by a 30 min incubation with goat anti-mouse Ig F(ab′)$_2$-coated magnetic beads (Dynal, Oslo, Norway) at a 20:1 bead to cell ratio. Cells with bound magnetic beads were removed with a magnet. The remaining cells were washed and an aliquot was stained to assess the efficiency of CD4 cell removal which was usually >90%.

TCR β chain CDR3 analysis

The CDR3 length assay is described elsewhere in detail (6). Briefly, first-step PCR reactions were set up with selected V_β-specific primers paired with a C_β primer. The products were then subjected to a second step run-off reaction using a nested fluorophore-labeled anti-sense C_β primer, run on a 6% acrylamide sequencing gel and visualized on a 373A Applied Systems DNA sequencer with fluorescent size markers in the 80–380 bp range.

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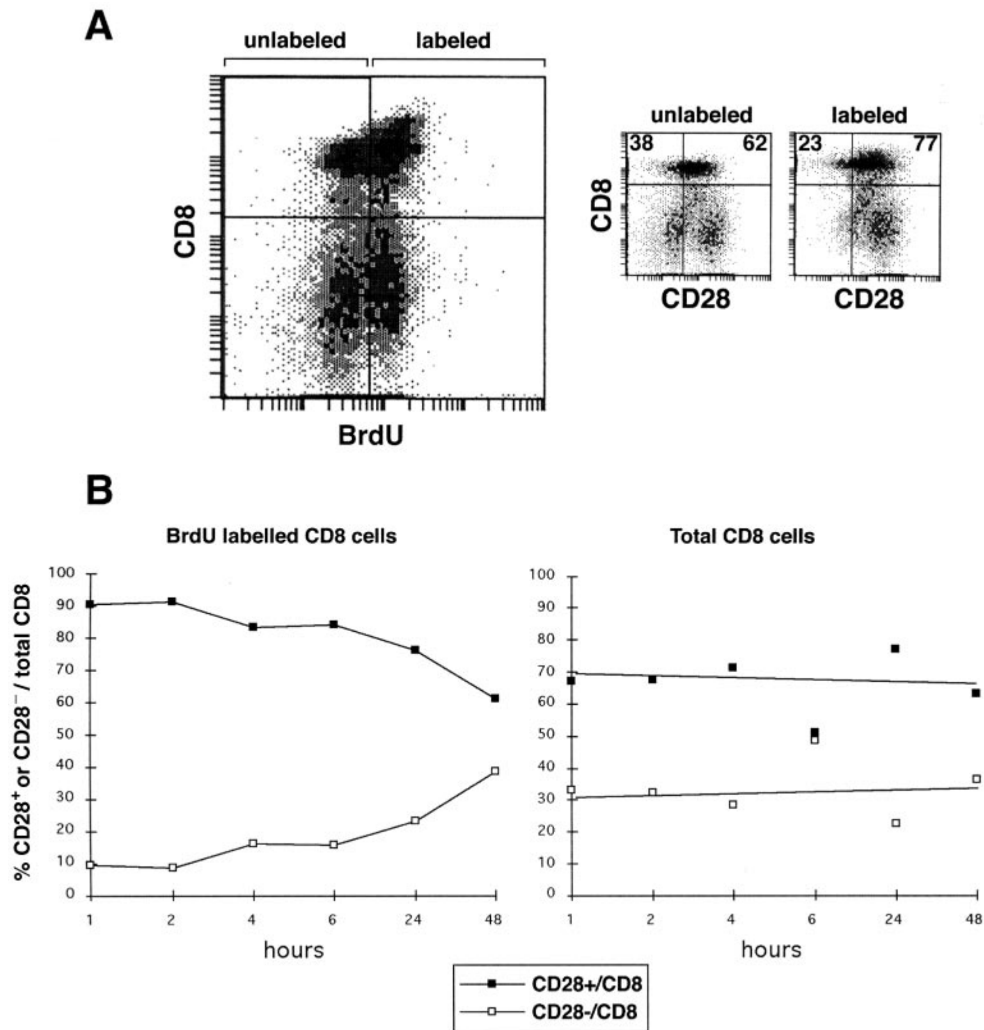

Fig. 4. $CD8^+CD28^+$ cells are direct precursors of $CD8^+CD28^-$ cells. Cycling PHA-stimulated PBL were followed by three-color staining after a 1 h BrdU pulse. (A) Three-color FACS with anti-BrdU–FITC: gated $BrdU^-$ or $BrdU^+$ cells were analyzed for CD8–biotin and CD28–PE staining 4 h after the BrdU pulse. (B) In a further experiment, $CD8^+CD28^+$ versus $CD8^+CD28^-$ subset distribution was followed over 48 h among $BrdU^+$ CD8 cells or among all CD8 cells. Similar results were obtained in two other experiments.

Results

TCR repertoire of $CD8^+CD28^-$ T cells

The peripheral blood of adults contains a distinct population of $CD4^-CD28^-$ cells. $CD28^-$ $TCR\alpha\beta^+$ cells were found nearly exclusively in the $CD4^-$ subset in three random normal subjects, aged 27–34 (Fig. 1). These $TCR\alpha\beta^+$ $CD4^-CD28^-$ cells were single-positive $CD8^+$ cells. Next we used the same three subjects to test CD28 expression in 12 V_β subsets gated on $CD4^-$ cells (Fig. 2). Expanded $CD8^+$ V_β subsets had previously been found to lack CD28 expression (3), but it was unclear to what extent non-expanded $CD8^+$ V_β subsets also contained $CD28^-$ cells. The size of the V_β subset did not correlate well with the extent of CD28 loss. Thus, two large V_β subsets in subject 2, $V_\beta17$ and $V_\beta2$, contained 75 and 40% $CD28^-$ cells respectively. However, the minor $V_\beta16$ subset in subject 2 and the small $V_\beta3$ subset in subject 3 also contained a majority of $CD28^-$ cells (Fig. 2). Therefore, V_β expansion was not a prerequisite for the $CD28^-$ phenotype. Overall, the prevalence of $CD28^-$ cells differed considerably from one V_β subset to the other.

Subject 15 (78 years old in 1997) was studied in detail because of a large $TCR\alpha\beta^+$ $CD8^+CD28^-$ subset. In subject 15, 58% of $TCR\alpha\beta^+$ cells were $CD8^+$ and 42% $CD4^+$. Greater than 84% of the TCR^+CD8^+ cells lacked CD28 (Figs 6 and 7). There were four V_β subset expansions that were followed over >4 years (Table 1), comprising together between 46.9 and 31.1% of total CD8 cells. As an example of their phenotype, the $V_\beta22$ subset consisted predominantly of $CD8^+CD28^-$ cells (Fig. 3B). In 1993 the $V_\beta5.2$ cells contained a single dominant sequence, assessed by sequencing $TCR\beta$ CDR3 regions (3). We used a PCR-based CDR3 length assay to detect restricted CDR3 length as a measure of oligoclonality. In each of the subsets examined the same peaks were present in 1993 and in 1997 (Fig. 3A). In sum, the $CD8^+CD28^-$ cells in subject 15 contain chronic oligoclonal expansions which could perhaps be driven by persistent antigens.

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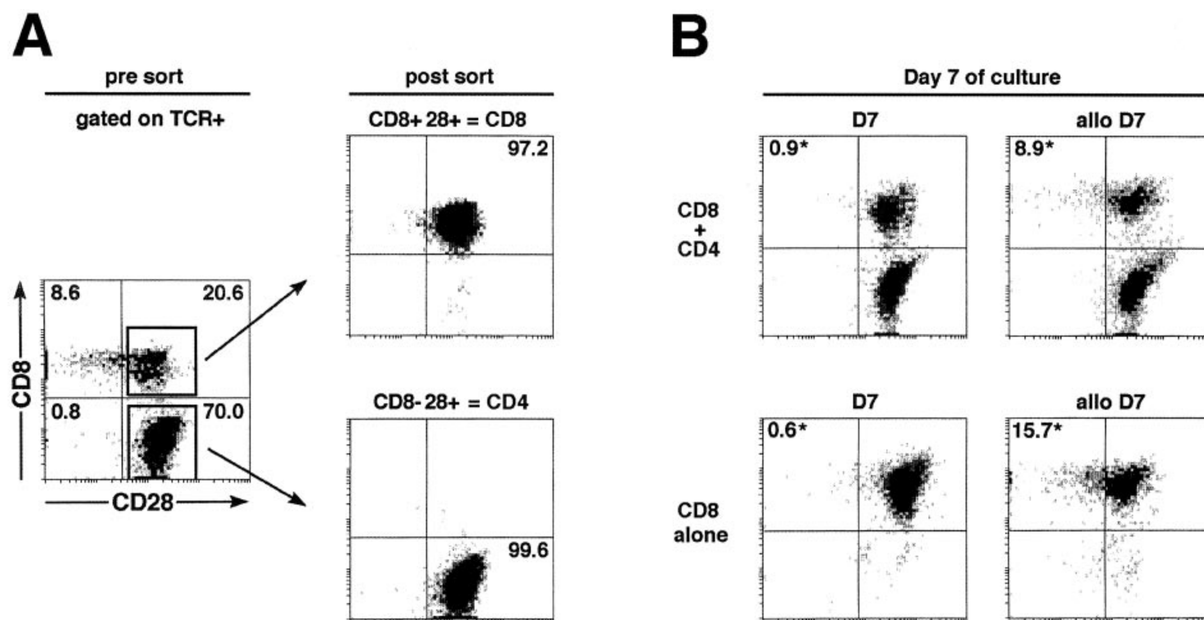

Fig. 5. Sorted CD8$^+$CD28$^+$ cells generate CD8$^+$CD28$^-$ cells *in vitro*. (A) Results and purity of sort for TCRαβ$^+$ CD8$^+$CD28$^+$ (CD8) and TCRαβ$^+$ CD8$^-$CD28$^+$ (CD4) cells. (B) Seven day culture of sorted CD8 + CD4 (at 1:1 cell ratio) or CD8 alone. Allogeneic stimulatory non-T cells (depleted of sheep red blood cell rosetting cells) were either added (1:1 cell ratio) or not. All cultures were replenished with medium containing 40 U/ml IL-2 twice a week. All histograms are CD8 versus CD28 staining in gated TCRαβ$^+$ cells. Percentages in respective quadrants are indicated. Percentages marked with an asterisk are percentages of CD8$^+$CD28$^-$ per total CD8$^+$ cells.

CD8$^+$CD28$^-$ T cells derive from CD8$^+$CD28$^+$ precursors

It has been proposed that CD8$^+$CD57$^+$ differentiate from CD8$^+$CD57$^-$ precursors (28,29) and that CD8$^+$CD28$^-$ cells derive from CD8$^+$CD28$^+$ precursors, because the CD8$^+$CD28$^-$ phenotype can be induced with *in vitro* stimulation of cord blood PBL (30) which typically contains only CD8$^+$CD28$^+$ T cells (13). To examine this question directly, BrdU-labeled T cells were used (Fig. 4). Among gated BrdU$^+$ cells, there was a gradual shift from a predominant CD8$^+$CD28$^+$ phenotype early in the culture to a CD8$^+$CD28$^-$ phenotype (Fig. 4B), demonstrating directly that CD8$^+$CD28$^-$ cells were generated from cycling CD8$^+$CD28$^+$ precursors. As a control, the ratio of CD28$^+$/CD28$^-$ cells in the total CD8 population remained constant, demonstrating that the switch from CD28$^+$ to CD28$^-$ was specific to BrdU$^+$ cells. Due to PHA stimulation, total cell numbers increased 2-fold during the observation period. Cell death was not observed by Trypan blue exclusion or forward scatter (FSC)/side scatter (SSC) light scatter characteristics. During the first 6 h of this experiment BrdU$^+$ CD8$^+$ cells contained on average 41.9% FSC^{hi} blast cells compared with 5.6% FSC^{hi} blasts in BrdU$^-$ CD8$^+$ cells. The BrdU$^+$CD8$^+$CD28$^+$ subset contained 2-fold more FSC^{hi} blasts than the BrdU$^+$ CD8$^+$CD28$^-$ subset. Thus, it is unlikely that BrdU$^+$ CD8$^+$CD28$^-$ cells were overgrowing the cultures. Rather, a fraction of BrdU-labeled CD8$^+$CD28$^+$ precursors were converted to a CD8$^+$CD28$^-$ phenotype over 48 h, providing clear evidence that CD8$^+$CD28$^+$ cells include precursors of CD8$^+$CD28$^-$ cells. Similar results were obtained in three independent experiments.

Additional evidence was obtained by sorting TCRαβ$^+$CD8$^+$ CD28$^+$ cells and culturing them to generate the TCRαβ$^+$CD8$^+$CD28$^-$ subset *de novo* (Fig. 5). Spontaneous differentiation was not observed, e.g. in medium with exogenous IL-2 or in the presence of CD4 cells. However, when allogeneic stimulator non-T cells were added to the cultures a subset of the CD8 population lost expression of CD28 (Fig. 5). This occured with or without the presence of added CD4 cells and required the presence of CD8$^+$CD28$^+$ cells as the latter could not be substituted by CD8$^-$CD28$^+$ cells (not shown). The allogeneic stimulating T-depleted PBL could be irradiated (3000 rad), or not, with similar results (not shown).

Finally, we found that gated TCRαβ$^+$ CD8$^+$CD28$^+$ cells obtained *ex vivo* from five normal subjects were enriched in distinct FSC^{hi} lymphoblasts when compared with TCRαβ$^+$ CD8$^+$CD28$^-$ cells (data not shown), suggesting that the CD8$^+$CD28$^+$ cells contain the proliferative precursors *in vivo*.

CD8$^+$CD28$^-$ cells are perforin$^+$ cytotoxic effectors

Stimulation of a T cell via the TCR and CD28 results in T cell responses such as proliferation, IL-2 secretion and activation of cytolytic effector function from memory pCTL (18). Given that CD8$^+$CD28$^-$ cells were the progeny of CD8$^+$CD28$^+$ cells, we asked whether CD8$^+$CD28$^-$ contained intracytoplasmic perforin granules. Cells permeabilized with saponin 0.1% were stained for CD8, CD28 and intracellular perforin. The CD28$^+$ cells, which include TCRαβ$^+$ CD4 and CD8 cells, were mostly negative for perforin staining (Fig. 6b and d). In contrast the majority of CD28$^-$ cells were perforin$^+$. Both CD8$^+$CD28$^-$ and CD8$^-$CD28$^-$ subsets contained perforin$^+$ cells. There were two intensities of perforin staining, a high-intensity cell type predominant in the CD8$^-$CD28$^-$ subset and an intermediate-intensity cell type predominant in the

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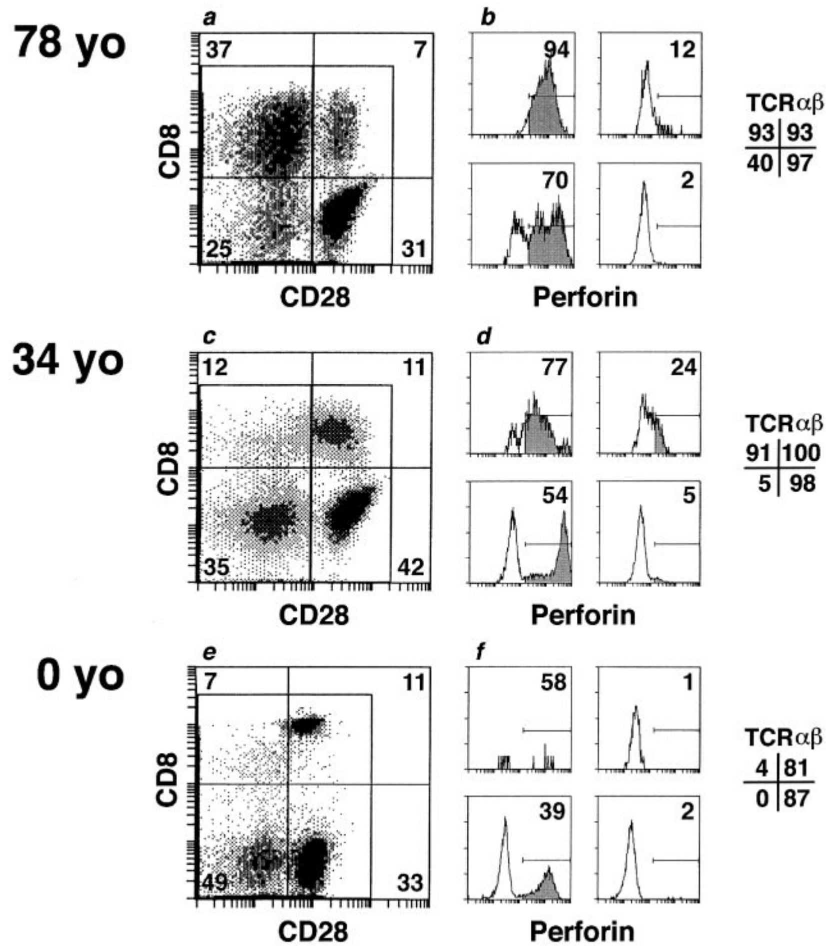

Fig. 6. Age-related changes in the CD8$^+$CD28$^-$ perforin$^+$ TCRαβ$^+$ subset. Three-color staining with CD28–PE, CD8–biotin and anti-perforin–FITC on saponin-treated permeable *ex vivo* PBL from cord blood (0 years old), a normal adult subject (subject 3; 34 years old) and an elderly subject (subject 15; 78 years old). Perforin staining (in b, d and f) for the four quadrants shown (in a, c and e). TCRαβ staining results are indicated on the far right for the four boxed quadrants (in a, c and e) from data obtained in a parallel three-color stain: CD28–PE, CD8–biotin and TCRαβ–FITC.

CD8$^+$CD28$^-$ subset. The perforin^{int} cells were TCRαβ$^+$ but the perforin^{hi} cells were TCRαβ$^-$ TCRγδ$^-$, CD56$^+$, CD16$^+$ NK cells (Fig. 6a–d and data not shown).

Perforin$^+$ CD8$^+$CD28$^-$ TCRαβ$^+$ were most prominent in subject 15. Most of the perforin$^+$ CD8$^+$ cells had lost expression of CD28 in this subject. By contrast, perforin$^+$ CD8$^+$CD28$^-$ were less prominent in other adults (Figs 6, 7 and 9) and completely missing in cord blood (Fig. 6), consistent with other results (13,31). The perforin$^+$ cells in normal cord blood had a CD8$^-$CD28$^-$ or CD8^{lo}CD28$^-$ phenotype and were invariably TCRαβ$^-$TCRγδ$^-$ NK cells (Fig. 6e and f, and data not shown). CD8$^+$CD28$^+$ cells containing low levels of perforin were uncommon, but clearly present (Fig. 6d and 7). It is possible that these are direct precursors of CD28$^-$ perforin$^+$ cells.

The size of the various CD28, CD8 and perforin defined subsets was examined in IVSA patients, that were either HIV$^+$ or HIV$^-$, and compared with normal young and old subjects, and with subject 15 (Fig. 7). HIV$^+$ IVSA patients had increased levels of both CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ cells compared with normal subjects or with HIV$^-$ IVSA patients. On average 55% of CD8$^+$CD28$^-$ cells contained perforin in all groups, including HIV$^+$ patients, but very few CD8$^+$CD28$^+$ cells were perforin$^+$. Unlike the HIV$^+$ patients, subjects 15, LB and Met43 had expansions of CD8$^+$CD28$^-$, but not CD8$^+$CD28$^+$ cells. Moreover, a larger fraction of the CD8$^+$CD28$^-$ cells was perforin$^+$. In these subjects there is a specific accumulation of perforin$^+$ CD8$^+$CD28$^-$ cells.

Expanded CD8+CD28– T cells are relatively resistant to apoptosis

A frequently used mechanism to limit the duration of an immune response is activation-induced cell death (AICD). Since clonally restricted CD8$^+$CD28$^-$ T cells persist *in vivo* for years, we asked whether CD8$^+$CD28$^-$ cells might be inherently resistant to apoptosis. Annexin V staining was performed in cultures of T cells stimulated with the superantigen SEE, which targets $V_\beta 8$ T cells. To check that efficient SEE stimulation had occurred, percentages of $V_\beta 8^+$ cells were measured. They increased from 2–5 to 10–15% over

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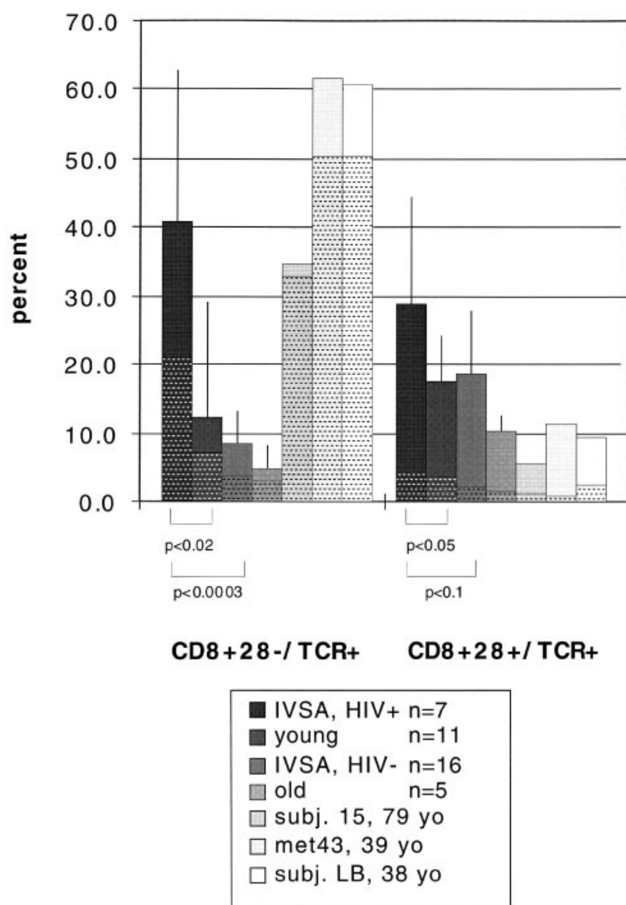

Fig. 7. CD8 subsets in different groups of patients. The mean percentages of $CD8^+CD28^-$ and $CD8^+CD28^+$ cells within PBL are given for the indicated groups with the SD of the mean and for three individual subjects. The fraction of $perforin^+$ cells in each subset is indicated by the stippled section of the bar. Indicated *P* values for comparison of means were obtained with a *t*-test. Met43 is an IVSA patient with chronic HVC infection. Met43, LB and subject 15 were not included in the analysis of their respective groups.

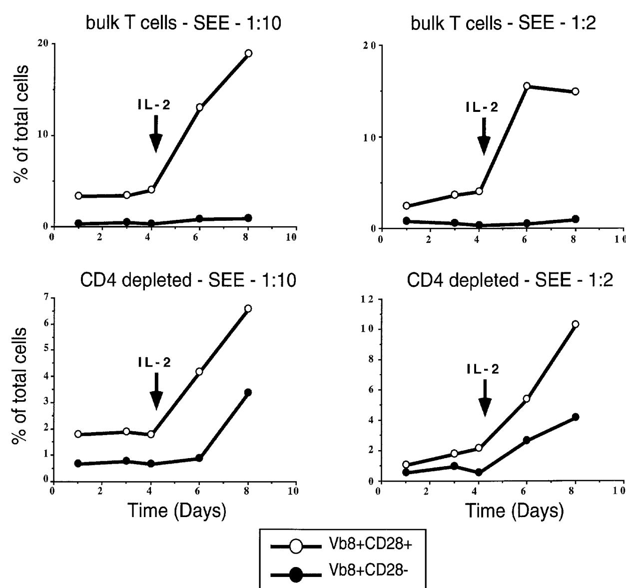

Fig. 8. Expansion of $V_\beta8$ T cells in response to SEE. Percentages of $V_\beta8$ $CD28^+$ and $V_\beta8$ $CD28^-$ cells at various time points after stimulation with SEE-pulsed, irradiated, BJAB lymphoblastoid cells, used at either a 1:10 or a 1:2 (B:T) ratio. Both bulk T cells and CD4-depleted T cells were stimulated and the cultures were supplemented with exogenous IL-2 at 40 U/ml on day 4. The efficiency of the CD4 depletion was >90%. During the 8 day observation period the total cell numbers increased ~3- to 5-fold, in particular after addition of IL-2. For annexin V staining on day 6 of this experiment, refer to Table 2 (Experiment 3).

the culture period. Both $V_\beta8^+$ $CD8^+CD28^+$ and $V_\beta8^+$ $CD8^+CD28^-$ T cells expanded during these cultures in relative percentages and in absolute cell numbers (Fig. 8). The percentage of annexin V^+ cells was next determined in $CD28^+$ and $CD28^-$ $V_\beta8$ T cells, and compared with bystander T cells not expressing $V_\beta8$ (Table 2). $V_\beta8^+CD28^+$ showed the highest levels and $V_\beta8^+$ $CD28^-$ showed background low levels of annexin V binding. Kinetic studies revealed that $V_\beta8^+$ $CD28^-$ cells remained relatively resistant to apoptotic cell death over 13 days of culture. This was independent of the cell donor origin or the addition of exogenous IL-2. When using PBL most of the proliferative response to SEE occurs among $CD4^+$ T cells (data not shown). CD8 cells were therefore isolated by negative sorting and stimulated with SEE. The same predominance of annexin V-binding cells among $V_\beta8^+CD28^+$ cells was observed in these CD8 enriched cultures (Table 2, Experiments 3 and 4). The same results were obtained when SEE was pulsed on to BJAB antigen-presenting cells (Table 2, Experiment 3), thus avoiding the presence of excess soluble SEE. Together, these results demonstrate that $CD8^+CD28^-$ cells activated with a superantigen were less susceptible to apoptosis than their $CD8^+CD28^+$ counterparts. A likely explanation is that proliferative $V_\beta8^+$ $CD8^+CD28^+$ precursor cells can have several different fates, including either apoptosis or differentiation in to $V_\beta8^+$ $CD8^+CD28^-$ cells that are more resistant to cell death. Similar results were obtained with another superantigen, *Mycoplasma arthritidis* mitogen (data not shown), which has a different specificity for $V_\beta17^+$ T cells.

In these experiments with superantigens, AICD is the likely mechanism of cell death. AICD requires TCR-mediated stimulation and cell death is Fas–Fas ligand mediated (32). To examine the spontaneous susceptibility of CD8 subsets to apoptosis, we first examined Fas (CD95) expression. In subject 15, $CD8^+CD28^-$ cells express CD95, albeit at levels that are consistently 2- to 5-fold lower than those of $CD8^+CD28^+CD95^+$ cells (mean channel fluorescence indicated with arrows in Fig. 9A). The intensity of CD95 expression was also decreased in other subjects, when comparing $CD8^+CD28^-$ and $CD8^+CD28^+$ cells (Fig. 9A). A lower level of Fas expression is consistent with a relative decrease in susceptibility to apoptosis.

In subject 15 CD8 cells, but not CD4 cells, undergo spontaneous apoptosis. In freshly isolated *ex vivo* PBL stained immediately after isolation without *in vitro* culture, annexin V^+ $CD8^+$ cells were abundant in subject 15, in particular in the $CD8^+CD28^+$ subset (53%), but also to a lesser degree (24%) in the $CD8^+CD28^-$ subset (Fig. 9A). Spontaneously annexin

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Table 2. CD28$^–$ phenotype correlates with resistance to AICD in superantigen-activated cultures

Experiment	Subject no.[a]	Culture conditions	Day analyzed	Annexin$^+$ cells (%) in each subset[b]		
				$V_\beta 8^-$ CD28$^+$	$V_\beta 8^+$ CD28$^+$	$V_\beta 8^+$ CD28$^-$
1	4	PBL + SEE + IL-2	0	<1.0	<1.0	<1.0
			6	1.5	18.0	2.0
			7	<1.0	5.5	1.5
			8	<1.0	2.7	<1.0
1	4	PBL + SEE	0	<1.0	<1.0	<1.0
			6	1.9	27.0	6.9
			7	<1.0	7.0	2.0
			8	<1.0	4.5	1.8
2	5	PBL + SEE	0	<1.0	<1.0	<1.0
			6	1.0	9.6	1.5
			7	9.6	24.7	<1.0
			9	7.5	17.7	3.0
			13	<1.0	1.1	<1.0
3[c]	7	PBL + SEE^{hi}	6	1.5	3.1	5.3
		PBL + SEE^{lo}	6	4.0	7.5	4.0
		CD8 + SEE^{hi}	6	6.1	14.0	2.3
		CD8 + SEE^{lo}	6	5.5	16.2	4.5
4	6	CD8[d] + SEE + IL-2 re-stimulated SEE (day 11)	14	22.9	34.5	11.0

[a]Normal subjects were nos 4 (49 years old), 5 (59 years old), 6 (44 years old) and 7 (24 years old).
[b]Percentages of $V_\beta 8$ cells increased from 2–5% on day 0 to 10–15% by end of the cultures. Total cell numbers increased by 2- to 10-fold over the culture periods.
[c]BJAB B lymphoblastoid cells were pulsed with 200 ng/ml SEE for 1 h, then washed twice and added at a ratio of 1:10 (B:T cells) or 1:2 for SEE^{lo} and SEE^{hi} respectively.
[d]PBL depleted of CD4 cells, from subject 6, were cultured with SEE and fed with IL-2 every 2–3 days. On day 7 a second CD4 depletion yielded a cell population with <2% CD4$^+$ cells. On day 11 the cells were re-stimulated with SEE to induce AICD and analyzed by three-color staining on day 14.

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V$^+$ cells were seen in smaller numbers in subject 16 and were undetectable in a younger subject (Fig. 9A). The same samples were examined for the relative presence of effector CD8 cells, demonstrating abundant perforin$^+$ CD8$^+$CD28$^–$ cells in subject 15 that were much more discrete in the other two subjects (Fig. 9B). Therefore, abundant effector CD8 cells correlated with increased spontaneous annexin V-binding CD8 cells.

In vivo there is a steady state in subject 15, with maintenance of a relatively high number of CD8$^+$ cells (~60% of all CD3$^+$ T cells). If CD8$^+$CD28$^+$ cells represent the subset with proliferative self-renewal potential and >50% of this subset undergoes immediate spontaneous apoptosis *ex vivo* (Fig. 9A), it follows that there must be a rapid rate of production of CD8 cells.

Discussion

Expansions of oligoclonal CD8$^+$CD28$^–$ cells

It is striking that many clonal expansions of human T cells described in healthy or diseased subjects have a CD28$^–$ phenotype (2). Herein, we show variable expression of CD28 within the CD8 TCR repertoire of normal young subjects as evidence that even small V_β subsets that are not dramatically expanded may be dominated by CD8$^+$CD28$^–$ cells and that the distribution of the CD8$^+$CD28$^–$ phenotype is quite variable. This is consistent with the CD28$^–$ phenotype being a marker for effector T cells stimulated by random antigens *in vivo*. Some clones may expand dramatically leading to expanded V_β subsets (3). However, small V_β subsets may also contain many CD28$^–$ cells (Fig. 2). In these cases, perhaps the clones undergo limited expansion, or clonal expansion is curtailed *in vivo* by AICD, or the clones may simply migrate in to tissues other than the blood.

In some elderly subjects there are chronic oligoclonal expansions within the CD8$^+$CD28$^–$ subset. CD8$^+$CD28$^–$ have shorter telomers than CD8$^+$CD28$^+$ cells, indicating a history of increased cell divisions (33,34). We found (i) that a large fraction of CD8$^+$CD28$^+$ cells is engaged in an apoptotic pathway, consistent with a high proliferation rate in this population, (ii) CD8$^+$CD28$^–$ cells originate from CD8$^+$CD28$^+$ precursor cells and (iii) CD8$^+$CD28$^–$ cells are relatively resistant to apoptosis compared with their CD8$^+$CD28$^+$ precursors. Therefore, CD8 cells that escape apoptosis may mature in to CD8$^+$CD28$^–$ perforin$^+$ effector T cells. Relative resistance to apoptosis among CD8$^+$CD28$^–$ cells, combined with increased production of antigen-driven CD8 clones, provides the best explanation for the accumulation of CD8$^+$CD28$^–$ clones in people like subject 15.

Which antigens might be at the origin of the CD8$^+$CD28$^–$ clones? Non-cytopathic viruses that may be chronically or intermittently productive and yet asymptomatic in humans include the herpes viruses I–VIII, HTLV-I and HTLV-II, HIV-1 and HIV-2, spumavirus, parvovirus B16, JC virus, and human endogenous retroviruses. Cytomegaloviruses in particular, but also other herpesvirus infections (35), have been shown to be associated with higher percentages of CD8$^+$CD57$^+$

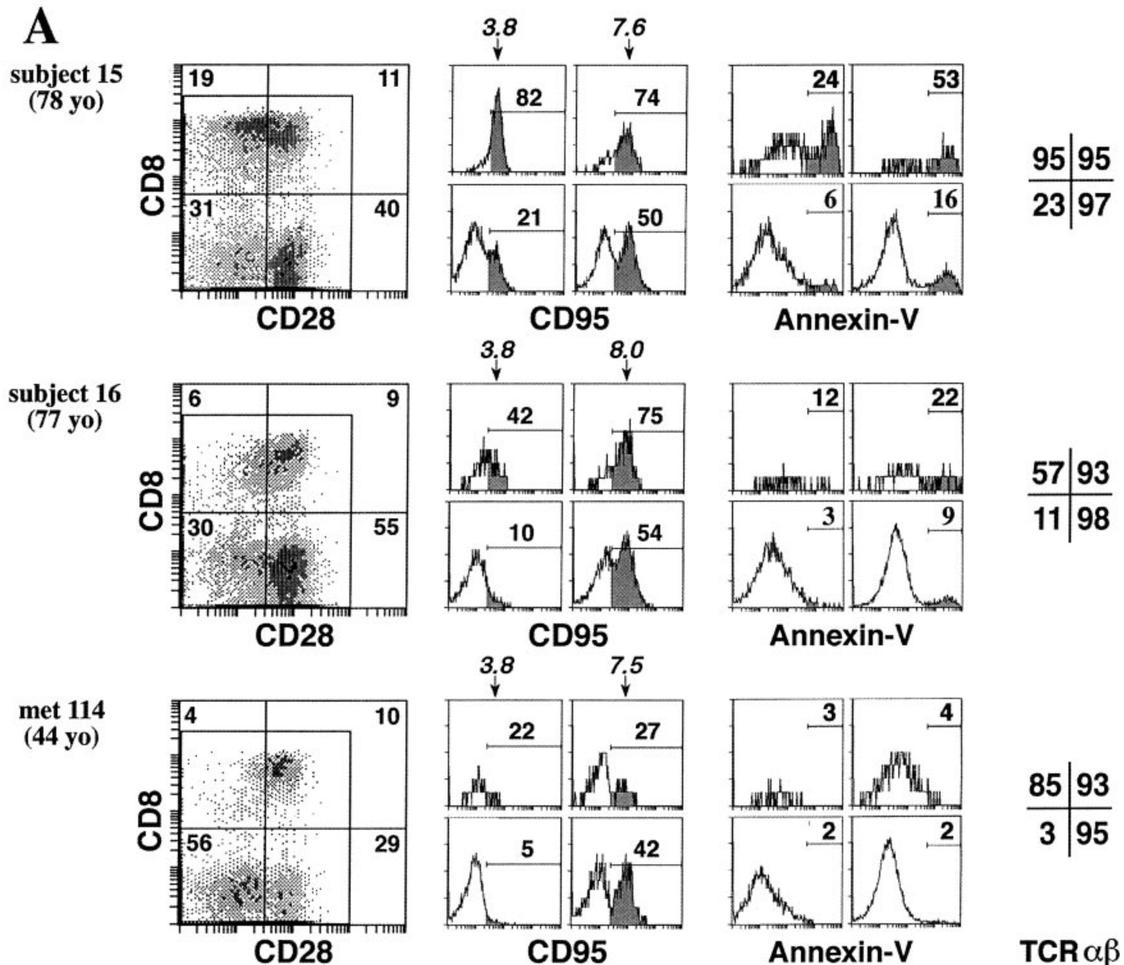
(CD28$^-$) cells (28,36), which include CMV-specific T cells (19). HTLV-I and HTLV-II seropositive IVSA subjects are known to have CD8 cells that proliferate spontaneously *ex vivo* and co-express NK markers CD16/CD56 (37), a phenotype which may overlap with the CD8$^+$CD28$^-$ subset.

Since there was no evidence of high antibody titers to the above listed viruses in subject 15 (data not shown), an alternative source for chronic antigenic stimulation was considered: an incidentally discovered renal cell carcinoma (RCC). A number of reports have described RCC-associated, serologically defined tumor antigens. Some of these appeared to be related to retroviral antigens. RCC are immunogenic and CTL may be effective in controlling tumor growth (38). In many patients *in situ* tumor-infiltrating lymphocytes contained oligoclonal expansions, but these were mostly not present in PBL (39–41). As the CD8$^+$CD28$^-$ expansion of subject 15 was present in the PBL several years prior to discovery of the RCC, it remains to be determined whether the CD8$^+$CD28$^-$ cells are related to the tumor.

CD8$^+$CD28$^-$ cells are cytotoxic effector cells with intracellular perforin

TCR$\alpha\beta^+$CD8$^+$CD28$^-$ cells were shown to include a population of cells with intracellular perforin, e.g. differentiated effector CTL. These cells are found in increased frequency in patients with acute and chronic viral infections, as exemplified by HIV-1 infected patients (Fig. 7). However, in such patients we found a general predominance of all types of CD8 cells, and the normal ratios of perforin$^+$ cells in the CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ subsets were preserved. Elderly subjects without major CD8 clonal expansions had low levels of the CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ subsets. However, three individuals (subject-15, LB and met-43) were identified with major expansions of CD8$^+$CD28$^-$ cells that were predominantly perforin$^+$ and yet the CD8$^+$CD28$^+$ subsets were not enlarged. This pattern clearly differs from what was seen in the chronic HIV-1-infected subjects. Thus, there are variations in differentiation patterns of CD8 cells. The data also suggest considerable heterogeneity among old subjects in the size of the CD8$^+$CD28$^-$ subset.

CD28$^{0/0}$ mice can reject tumors (42), give an acute graft versus host response (43), a delayed type hypersensitivity response and a CTL response (44). Therefore it is likely that other co-stimulatory molecules compensate for the loss of CD28. CD2/LFA-3 (18) and CD27/CD70 (45) have both been shown to co-stimulate maturation of a CTL response. Interestingly, CD27 expression is lost in concert with CD28 expression when normal human CD8 cells differentiate in to effector CTL



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(14). Perhaps it is crucial that co-stimulation be avoided at a late stage of CD8 cell maturation.

CD8$^+$CD28$^-$ T cells are relatively resistant to apoptosis

Loss of CD28 expression might serve to arrest a proliferative memory T cell response. AICD has been proposed as a mechanism to down-regulate a cellular immune response (32), and some authors claim that CD8 effector T cells are 'apoptosis-prone' (46) and lose expression of Bcl-2 (35). However, Kaneko *et al.* found that CD8$^+$CD28$^+$, but not CD8$^+$CD28$^-$ T cells, underwent apoptosis (47), using cells from patients with systemic lupus erythematosus and from normal controls. There were no differences in Fas and Bcl-2 expression, but CTLA-4 was only expressed in the CD8$^+$CD28$^+$ cells. Consistent with these data, we found CTLA-4 was not expressed on the CD8$^+$CD28$^-$ cells of subject 15, but only on CD8$^+$CD28$^+$ cells (data not shown). Thus CD8$^+$CD28$^+$ cells appear to be the subset that is tightly regulated and subject to AICD or CTLA-4-mediated down-regulation. These results agree with our findings of decreased apoptosis in the CD8$^+$CD28$^-$ relative to the CD8$^+$CD28$^+$ subset.

Zhang *et al.* (48) described murine antigen-specific CD8 T cells that escape AICD *in vivo*, in spite of the continued presence of antigen. These cells are anergic. They express activation antigens, including Fas and Fas ligand, and produce T_c2 cytokines (IL-4 and IL-10). The resistance of T_c2 cells to apoptosis has been studied in perforin$^{0/0}$ mice, where IFN-γ-producing T_c1, but not IL-5-secreting T_c2 CD8 cells, underwent Fas-mediated cytolysis (49). Moreover, T_c2 cells may express CD30 and produce soluble CD30 (49,50) which could interfere with CD30-mediated apoptosis. However, no expression of CD30 was found on the CD8$^+$ T cells from subject 15 (data not shown).

A major requirement for apoptosis is a cycling cell (18,51). CD28 co-stimulation and the presence of IL-2 are important in advancing T cells through the cell cycle. Although CD28 co-stimulation is thought of as anti-apoptotic, due to stimulation of increased levels of Bcl-X_L (52), this effect may not apply to AICD (53). Indeed, in some cells a CD28 co-stimulatory signal

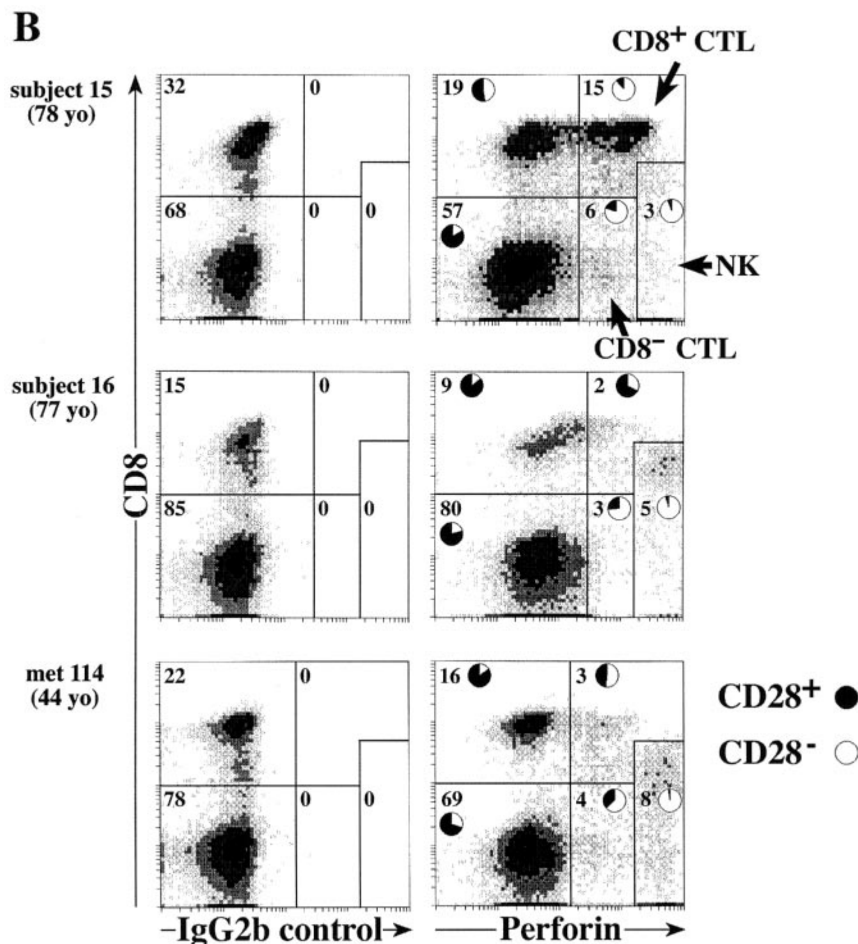

Fig. 9. Fas expression, spontaneous apoptosis and perforin staining in CD8 and CD28 defined subsets from three adult subjects. (A) *Ex vivo* three-color staining of PBL with CD28–PE, CD8–FITC and CD95–biotin; or CD28–PE, CD8–biotin and Annexin V–FITC; or CD28–PE, CD8–biotin and anti-TCRαβ–FITC. Peak fluorescence is indicated for the CD95 fluorescence in the CD8$^+$ subsets. (B) Three-color staining with perforin–FITC (or an isotype control antibody), CD8–biotin and CD28–PE. The perforin staining identifies three types of perforin$^+$ cells: CD8$^+$ CTL, CD8$^-$ CTL and TCRαβ$^-$ NK cells. Lack of TCRαβ$^+$ TCRγδ$^+$ cells defined the CD28$^-$CD8$^-$ and CD28$^-$CD8^{lo} as NK cells. The percentage of CD28 staining for each region is indicated in the pie diagrams (black, CD28$^+$; white, CD28$^-$).

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may facilitate cell death, as in double-positive thymocytes undergoing clonal deletion (54). It would seem important for a cytotoxic effector cell to avoid apoptosis. It undergoes repetitive antigen exposure when it serially kills one target cell after another (55) and repeated antigen exposures often result in AICD (32). Perhaps this is the reason for the loss of expression of CD28 and CD27 in cytotoxic effector cells.

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Abbreviations

AICD	activation-induced cell death
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
FSC	forward scatter
HAART	highly active anti-retroviral therapy
IVSA	i.v. substance abuse
PBL	peripheral blood lymphocyte
pCTL	precurosr CTL
PE	phycoerythrin
PHA	phytohemagglutinin
RCC	renal cell carcinoma
SEE	staphylococcal enterotoxin E
SSC	side scatter

References

1 Davis, M. M. and Buxbaum, J., eds. 1995. T-cell receptor use in human autoimmune diseases. *Ann. NY Acad. Sci.* 756:
2 Edinger, J. W. and Posnett, D. N. 1998. T cell antigen receptor repertoire in rheumatoid arthritis. In Paul, S., ed., *Pathogenic Autoimmune Reactions*. Humana Press, Totowa, NJ, p. 113–140
3 Posnett, D. N., Sinha, R., Kabak, S. and Russo, C. 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to 'benign monoclonal gammapathy'. *J. Exp. Med.* 179:609.
4 Hingorani, R., Choi, H., Akdkar, P., Gulwani-Akolkar, B., Pergolizzi, R., Silver, J. and Gregersen, P. K. 1993. Clonal predominance of T cell receptors within the CD8⁺ CD45RO⁺ subset in normal human subjects. *J. Immunol.* 151:5762.
5 Fitzgerald, J. E., Ricalton, N. S., Meyer, A. C., West, S. G., Kaplan, H., Behrendt, C. and Kotzin, B. L. 1995. Analysis of clonal CD8⁺ T cell expansions in normal individuals and patients with rheumatoid arthritis. *J. Immunol.* 154:3538.
6 Schwab, R., Szabo, P., Manavalan, J. S., Weksler, M. E., Posnett, D. N., Pannetier, C., Kourilsky, P. and Even, J. 1997. Expanded CD4⁺ and CD8⁺ T cell clones in elderly humans. *J. Immunol.* 158:4493.
7 Fagnoni, F. F., Vescovini, R., Mazzola, M., Bologna, G., Nigro, E., Lavagetto, G., Franceschi, C., Passeri, M. and Sansoni, P. 1996. Expansion of cytotoxic CD8⁺ CD28⁻ T cells in healthy ageing people, including centenarians. *Immunology* 88:501.
8 Callahan, J. E., Kappler, J. W. and Marrack, P. 1993. Unexpected expansions of CD8-bearing cells in old mice. *J. Immunol.* 151:6657.
9 Posnett, D. N., Gottlieb, A., Bussel, J. B., Friedman, S. M., Chiorazzi, N., Li, Y., Szabo, P., Farid, N. R. and Robinson, M. A. 1988. T cell antigen receptors in autoimmunity. *J. Immunol.* 141:1963.
10 Grunewald, J., Jeddi-Tehrani, M., DerSimonian, H., Andersson, R. and Wigzell, H. 1992. A persistent expansion in the peripheral blood of a normal adult male: a new clinical entity? *Clin. Exp. Immunol.* 89:279.
11 Morley, J. K., Batliwalla, F. M., Hingorani, R. and Gregersen, P. K. 1995. Oligoclonal CD8⁺ T cells are preferentially expanded in the CD57⁺ subset. *J. Immunol.* 154:6182.
12 McMichael, A. J. and O'Callaghan, C. A. 1998. A new look at T cells. *J. Exp. Med.* 187:1367.
13 Azuma, M., Phillips, J. H. and Lanier, L. L. 1993. CD28⁻ T lymphocytes. Antigenic and functional properties. *J. Immunol.* 150:1147.
14 Hamann, D., Baars, P. A., Rep, M. H. G., Hooibrink, B. and Van Lier, R. A. W. 1997. Phenotypical and functional separation of memory and effector human CD8⁺ T cells. *J. Exp. Med.* 186:1407.
15 Prince, H. E. and Jensen, E. R. 1991. Three-color cytofluorometric analysis of CD8 cell subsets in HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* 4:1227.
16 Plaeger-Marshall, S., Hultin, P., Bertolli, J., O'Rourke, S., Kobayashi, R., Kobayashi, A. L., Giorgi, J. V., Bryson, Y. and Stiehm, E. R. 1993. Activation and differentiation antigens on T cells of healthy, at risk, and HIV-infected children. *J. Acquir. Immune Defic. Syndr.* 6:984.
17 Kammerer, R., Iten, A., Frei, P. C. and Burgisser, P. 1996. Expansion of T cells negative for CD28 expression in HIV infection. Relation to activation markers and cell adhesion molecules, and correlation with prognostic markers. *Med. Microbiol. Immunol.* (*Berl.*). 185:19.
18 Azuma, M. and Lanier, L. L. 1995. The role of CD28 costimulation in the generation of cytotoxic T lymphocytes. *Curr. Top. Microbiol. Immunol.* 198:59.
19 Wang, E. C., Lehner, P. J., Graham, S. and Borysiewicz, L. K. 1994. CD8high (CD57⁺) T cells in normal, healthy individuals specifically suppress the generation of cytotoxic T lymphocytes to Epstein–Barr virus-transformed B cell lines. *Eur. J. Immunol.* 24:2903.
20 Vinci, G., Vernant, J. P., Nakazawa, M., Zohair, M., Katz, A., Henri, A., Rochant, H., Breton-Gorius, J. and Vainchenker, W. 1988. *In vitro* inhibition of normal human hematopoiesis by marrow CD3⁺, CD8⁺, HLA-DR⁺, HNK1⁺ lymphocytes. *Blood* 72:1616.
21 Yamashita, N., Nguyen, L., Fahey, J. L. and Clement, L. T. 1993. Phenotypic properties and cytotoxic functions of human CD8⁺ cells expressing the CD57 antigen. *Nat. Immun.* 12:79.
22 Autran, B., Leblond, V., Sadat-Sowti, B., Lefranc, E., Got, P., Sutton, L., Binet, J. L. and Debre, P. 1991. A soluble factor released by CD8⁺CD57⁺ lymphocytes from bone marrow transplanted patients inhibits cell-mediated cytolysis. *Blood* 77:2237.
23 Caruso, A., Licenziati, S., Canaris, A. D., Cantalamessa, A., Corulli, M., Benzoni, B., Peroni, L., Balsari, A. and Turano, A. 1996. Characterization of T cell subsets involved in the production of IFN-gamma in asymptomatic HIV-infected patients. *AIDS Res. Hum. Retroviruses.* 12:135.
24 Borthwick, N. J., Bofill, M., Gombert, W. M., Akbar, A. N., Medina, E., Sagawa, K., Lipman, M. C., Johnson, M. A. and Janossy, G. 1994. Lymphocyte activation in HIV-1 infection. II. Functional defects of CD28⁻ T cells. *AIDS* 8:431.
25 Boudet, F., Lecoeur, H. and Gougeon, M. L. 1996. Apoptosis associated with *ex vivo* down-regulation of Bcl-2 and up-regulation of Fas in potential cytotoxic CD8⁺ T lymphocytes during HIV infection. *J. Immunol.* 156:2282.
26 Posnett, D. N., Romagne, F., Necker, A., Kotzin, B. L. and Sekaly, R.-P. 1996. Summary: first human TCR monoclonal antibody workshop. *The Immunologist* 4:5.
27 Penit, C. and Vasseur, F. 1993. Phenotype analysis of cycling and postcycling thymocytes: evaluation of detection methods for BrdUrd and surface proteins. *Cytometry* 14:757.
28 Labalette, M., Salez, F., Pruvot, F. R., Noel, C. and Dessaint, J. P. 1994. CD8 lymphocytosis in primary cytomegalovirus (CMV) infection of allograft recipients: expansion of an uncommon CD8⁺ CD57⁻ subset and its progressive replacement by CD8⁺ CD57⁺ T cells. *Clin. Exp. Immunol.* 95:465.
29 Joly, P., Guillon, J. M., Mayaud, C., Plata, F., Theodorou, I., Denis, M., Debre, P. and Autran, B. 1989. Cell-mediated suppression of HIV-specific cytotoxic T lymphocytes. *J. Immunol.* 143:2193.
30 Arosa, F. A., Irwin, C., Mayer, L. F., de Sousa, M. and Posnett, D. N. 1998. Interactions between peripheral blood CD8 T

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lymphocytes and intestinal epithelial cells (iEC) result in the loss of CD28 expression. *Clin. Exp. Immunol.* 112:226.

31 Berthou, C., Legros-Maida, S., Soulie, A., Wargnier, A., Guillet, J., Rabian, C., Gluckman, E. and Sasportes, M. 1995. Cord blood T lymphocytes lack constitutive perforin expression in contrast to adult peripheral blood T lymphocytes. *Blood* 85:1540.

32 Lenardo, M. J. 1996. Fas and the art of lymphocyte maintenance [comment]. *J. Exp. Med.* 183:721.

33 Monteiro, J., Battliwalla, F., Ostrer, H. and Gregerson, P. K. 1996. Shortened telomers in clonally expanded CD28$^–$CD8$^+$ T cells imply a replicative history that is distinct from their CD28$^+$CD8$^+$ counterparts. *J. Immunol.* 156:3587.

34 Effros, R. B., Allsopp, R., Chiu, C. P., Hausner, M. A., Hirji, K., Wang, L., Harley, C. B., Villeponteau, B., West, M. D. and Giorgi, J. V. 1996. Shortened telomeres in the expanded CD28$^–$CD8$^+$ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* 10:F17.

35 Borthwick, N. J., Bofill, M., Hassan, I., Panayiotidis, P., Janossy, G., Salmon, M. and Akbar, A. N. 1996. Factors that influence activated CD8$^+$ T-cell apoptosis in patients with acute herpesvirus infections: loss of costimulatory molecules CD28, CD5 and CD6 but relative maintenance of Bax and Bcl-X expression. *Immunology* 88:508.

36 Wang, E. C. Y., Taylor-Wiedeman, J., Perera, P., Fisher, J. and Borysiewicz, L. K. 1993. Subsets of CD8$^+$ CD57$^+$ cells in normal healthy individuals: correlations with human cytomegalovirus (HCMV) carrier status, phenotypic and functional analyses. *Clin. Exp. Immunol.* 94:297.

37 Prince, H. E., Weber, D. M. and Jensen, E. R. 1991. Spontaneous lymphocyte proliferation in HTLV-I/II infection reflects preferential activation of CD8 and CD16/56 cell subsets. *Clin. Immunol. Immunopathol.* 58:419.

38 Schendel, D. J., Oberneder, R., Falk, C. S., Jantzer, P., Kressenstein, S., Maget, B., Hofstetter, A., Riethmuller, G. and Nossner, E. 1997. Cellular and molecular analyses of major histocompatibility complex (MHC) restricted and non-MHC-restricted effector cells recognizing renal cell carcinomas: problems and perspectives for immunotherapy. *J. Mol. Med.* 75:400.

39 Puisieux, I., Bain, C., Merrouche, Y., Malacher, P., Kourilsky, P., Even, J. and Favrot, M. 1996. Restriction of the T-cell repertoire in tumor-infiltrating lymphocytes from nine patients with renal-cell carcinoma. Relevance of the CDR3 length analysis for the identification of in situ clonal T-cell expansions. *Int. J. Cancer* 66:201.

40 Gaudin, C., Dietrich, P. Y., Robache, S., Guillard, M., Escudier, B., Lacombe, M. J., Kumar, A., Triebel, F. and Caignard, A. 1995. *In vivo* local expansion of clonal T cell subpopulations in renal cell carcinoma. *Cancer Res.* 55:685.

41 Angevin, E., Kremer, F., Gaudin, C., Hercend, T. and Triebel, F. 1997. Analysis of T-cell immune response in renal cell carcinoma: polarization to type 1-like differentiation pattern, clonal T-cell expansion and tumor-specific cytotoxicity. *Int. J. Cancer* 72:431.

42 Wen, T., Kono, K., Shahinian, A., Kiessling, R., Mak, T. W. and Klein, G. 1997. CD28 is not required for rejection of unmanipulated syngeneic and autologous tumors. *Eur. J. Immunol.* 27:1988.

43 Speiser, D. E., Bachmann, M. F., Shahinian, A., Mak, T. W. and Ohashi, P. S. 1997. Acute graft-versus-host disease without costimulation via CD28. *Transplantation* 63:1042.

44 Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B. and Mak, T. W. 1993. Differential T cell costimulatory requirements in CD28$^–$ deficient mice. *Science* 261:609.

45 Orengo, A. M., Cantoni, C., Neglia, F., Biassoni, R. and Ferrini, S. 1997. Reciprocal expression of CD70 and of its receptor, CD27, in human long term-activated T and natural killer (NK) cells: inverse regulation by cytokines and role in induction of cytotoxicity. *Clin. Exp. Immunol.* 107:608.

46 Wever, P. C., van der Vliet, H. J. J., Spaeny, L. H. A., Wolbink, A. M., van Diepen, F. N. J., Froehlich, C. J., Hack, C. E. and ten Berge, I. J. M. 1998. The CD8$^+$ granzyme B$^+$ T-cell subset in peripheral blood from healthy individuals contains activated and apoptosis-prone cells. *Immunology* 93:383.

47 Kaneko, H., Saito, K., Hashimoto, H., Yagita, H., Okumura, K. and Azuma, M. 1996. Preferential elimination of CD28$^+$ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis. *Clin. Exp. Immunol.* 106:218.

48 Zhang, L., Miller, R. G. and Zhang, J. 1996. Characterization of apoptosis-resistant antigen-specific T cells *in vivo*. *J. Exp. Med.* 183:2065.

49 Maggi, E., Giudizi, M. G., Biagiotti, R., Annunziato, F., Manetti, R., Piccinni, M. P., Parronchi, P., Sampognaro, S., Giannarini, L., Zuccati, G., *et al.* 1994. T_h2-like CD8$^+$ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J. Exp. Med.* 180:489.

50 Manetti, R., Annunziato, F., Biagiotti, R., Giudizi, M. G., Piccinni, M. P., Giannarini, L., Sampognaro, S., Parronchi, P., Vinante, F., Pizzolo, G., *et al.* 1994. CD30 expression by CD8$^+$ T cells producing type 2 helper cytokines. Evidence for large numbers of CD8$^+$CD30$^+$ T cell clones in human immunodeficiency virus infection. *J. Exp. Med.* 180:2407.

51 Boehme, S. A. and Lenardo, M. J. 1993. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur. J. Immunol.* 23:1552.

52 Boise, L. H., Minn, A. J., Noel, P. J., June, C. H., Accavitti, M. A., Lindsten, T. and Thompson, C. B. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. *Immunity* 3:87.

53 Van Parijs, L., Ibraghimov, A. and Abbas, A. K. 1996. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity* 4:321.

54 Amsen, D. and Kruisbeek, A. M. 1996. CD28–B7 interactions function to co-stimulate clonal deletion of double-positive thymocytes. *Int. Immunol.* 8:1927.

55 Griffiths, G. M. 1995. The cell biology of CTL killing. *Curr. Opin. Immunol.* 7:343.

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