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IL-12 Controls Cytotoxicity of a Novel Subset of Self-Antigen-Specific Human CD28$^+$ Cytolytic T Cells[1]

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Activated CD8 T cells develop cytotoxicity against autologous cells bearing foreign Ags and self/tumor Ags. However, self-specific cytolysis needs to be kept under control to avoid overwhelming immunopathology. After peptide vaccination of melanoma patients, we studied molecular and functional properties of T cell subsets specific for the self/tumor Ag Melan-A/MART-1. Ex vivo analysis revealed three Ag-specific effector memory (EM) populations, as follows: CD28-negative EM (EM28$^-$) T cells strongly expressing granzyme/perforin, and two EM28$^+$ subsets, one with high and the other with low level expression of these cytotoxic proteins. For further functional characterization, we generated 117 stable CD8 T cell clones by ex vivo flow cytometry-based sorting of these subsets. All EM28$^-$-derived clones lysed target cells with high efficacy. In contrast, EM28$^+$-derived clones were heterogenous, and could be classified in two groups, one with high and the other with low killing capacity, correlating with granzyme/perforin expression. High and low killer phenotypes remained surprisingly stable for several months. However, strongly increased granzyme expression and cytotoxicity were observed after exposure to IL-12. Thus, the data reveal a newly identified subset of CD28$^+$ conditional killer T cells. Because CD28 can mediate strong costimulatory signals, tight cytotoxicity control, as shown in this study through IL-12, may be particularly important for subsets of T cells expressing CD28. ***The Journal of Immunology,*** **2007, 178: 3566–3574.**

In response to antigenic challenge, T cells undergo complex programs of differentiation, which are still only partially defined. A better understanding of the regulation of T cell differentiation and function will most likely provide new perspectives for therapeutic interventions. To enhance immune protection against infection and cancer, improved therapies need to promote more robust (and longer lasting) T cell responses. Conversely, better control of transplant rejection and autoimmune disease depends on novel therapeutic interventions that block T cells efficiently. In any case, more potent therapies are also associated with increased risks (1–3), emphasizing the need for detailed knowledge of control mechanisms.

Immune activation induces multiple subsets of Ag-specific T cells with distinct properties (4–8). Despite specialization, many T cells remain receptive to environmental signals, allowing changes in differentiation and functional status. Some T cells develop effector functions, whereas others specialize for high proliferative potential, often essential for protective immune responses and immunological memory (9–11).

Human CD8 T cell responses against tumor Ags and persistent viruses are usually dominated by CCR7$^-$/CD45RA$^-$ effector memory (EM)[4] T cells. They are heterogenous, and contain subsets expressing various cytokines and/or cytolytic proteins such as granzymes and perforin (12–14). Whereas proliferating T cells continue to express CD28 and CD27, a large proportion of effector T cells down-regulates these costimulatory molecules (15, 16). Indeed, the majority of virus- and tumor-specific CD8 effector T cells are CD27 and CD28 negative, correlating with enhanced expression of effector proteins (cytokines, cytolytic proteins, NK receptors) (12–20).

Costimulation through CD28 strongly supports T cell activation (21). Nevertheless, a large fraction of human CD8 T cells does not express CD28. During recent years, it became increasingly clear that the pool of CD28-negative T cells primarily represents classical CD8 T cells with specificities against non-self and self Ags, similar to their CD28-positive counterparts (16, 18, 22). Indeed, specific clonotypes can often be observed in both CD28-positive and CD28-negative pools (our unpublished data). The latter can exert strong effector function despite that they lack CD28 expression. Based on current evidence, it seems likely that CD28 down-regulation represents a major control mechanism to avoid overwhelming T cell activation and immunopathology. CD28

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[4] Abbreviations used in this paper: EM, effector memory; DC, dendritic cell; Melan-A, melanoma Ag-A.

down-regulation appears to be the result of extended and prolonged T cell stimulation. Typically, this condition is also associated with immune attenuation controlled through multiple mechanisms (23–29).

A recent human trial unexpectedly revealed strong unfavorable side effects caused by small doses of an activatory CD28-specific Ab (1, 2), emphasizing the need for more detailed characterization of mechanisms controlling human $CD28^+$ T cells. In this study, we investigated the heterogeneity of Ag-specific EM CD8 T cells after peptide vaccination of melanoma patients. Ex vivo analysis revealed three Melan-A/MART-1-specific populations, as follows: CD28-negative EM ($EM28^-$) T cells strongly expressing granzyme/perforin, and two $EM28^+$ subsets, one with high and the other with low level expression of these cytotoxic proteins. For further functional characterization, we generated 117 stable CD8 T cell clones by ex vivo flow cytometry-based sorting of these subsets. $EM28^-$-derived clones efficiently killed specific target cells. In contrast, a large fraction of $EM28^+$-derived clones was unable to kill, correlating with poor granzyme/perforin expression. High and low killer phenotypes remained surprisingly stable, and correlated well with the results from ex vivo studies. However, strongly increased granzyme expression and cytotoxicity were observed after exposure to IL-12. Together, the data reveal the existence of a conditional killer subset of self Ag-specific T cells, controlled by IL-12, representing a novel checkpoint restricting cytotoxicity in humans.

Materials and Methods

Patients and peptide vaccination

As described previously (30), HLA-A*0201-positive patients with histologically proven metastatic melanoma of the skin expressing Melan-A/MART-1 (RT-PCR or immunohistochemistry) were vaccinated in monthly intervals. Vaccines were injected s.c. and contained 500 μg of CpG oligodeoxynucleotide 7909 (provided by A. Krieg Coley Pharmaceutical Group, Wellesley, MA), 100 μg of Melan-A analog $peptide_{26-35}$ ELAGIGILTV (Ludwig Institute for Cancer Research), and 300 μl of Montanide ISA-51 (IFA; Seppic), mixed altogether and prepared in a syringe as a stable emulsion.

Blood cells, HLA-A2/peptide multimers, and flow cytometry

Blood was withdrawn from patients 7–10 days after booster vaccination. Ficoll-Paque-centrifuged PBMC (1×10^7) were cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. PE-labeled HLA-A*0201/ELAGIGILTV peptide multimers (originally called tetramers) were prepared, as described (31, 32). Anti-CD8, anti-CD28, and goat anti-rat IgG^{APC} were purchased from BD Biosciences. Five color stains were done with HLA-A2/peptide multimers, anti-$CD28^{FITC}$, anti-$CD45RA^{PE\text{-}Texa\ Red}$ (Beckman Coulter), anti-$CD8^{APC\text{-}Cy7}$ reagents, and anti-CCR7 mAb (from M. Lipp, Max Delbrueck-Center for Molecular Medicine, Berlin, Germany), followed by goat anti-rat IgG^{APC} Ab. Briefly, $CD8^+$ T cells were enriched using a MiniMACS device (Miltenyi Biotec), resulting in >90% $CD3^+/CD8^+$ cells. Cells (10^6) were incubated with multimers (1 μg/ml, 60 min, room temperature) and then with Abs (30 min, 4°C). Intracellular content of granzyme B and perforin was measured in freshly isolated $CD8^+$ T cells without previous stimulation. In brief, after mAb staining, cells were fixed for 20 min at room temperature in PBS containing 1% formaldehyde, 2% glucose, and 5 mM sodium azide. Fixation and permeabilization were done with PBS, saponin (0.1%; Fluka), BSA (0.2%), and EDTA (50 μM), followed by staining with granzyme B^{FITC} (Hoelzel Diagnostika) or $perforin^{FITC}$ mAbs (Alexis) for 20 min at room temperature. Fluorescent isotype-matched Abs with irrelevant specificity were used as negative controls. A total of 5×10^5 $CD8^+$ T cells/sample was acquired with a FACSVantage SE machine, and data were analyzed with CellQuest software (BD Biosciences).

T cell sorting, cloning, and cytotoxicity assays

$CD8^+$ T cells from uncultured PBMC were prepared and stained with multimers and Abs, as described above. Subsequently, the $multimer^+$ $CD8^+$ T cell subsets were sorted by flow cytometry, cloned in Terasaki plates at 0.3 cell/well, and expanded with PHA and allogenic feeder cells in RPMI 1640 medium supplemented with nonessential amino acids, sodium pyruvate, penicillin-streptomycin, kanamycine, 2-ME, 5% human serum, and 150 U/ml rIL-2. Subsequently, they were periodically (3–4 wk) restimulated with PHA, irradiated feeder cells, and rIL-2. Lytic activity and Ag recognition were assessed in 4-h ^{51}Cr release assays, using ELAGIGILTV (Melan-$A_{26.35}$ analog) as peptide (32, 33) and T2 target cells (A^*0201^+/Melan-A^-). The redirected lysis assay was done with FcγR-expressing P815 mastocytoma target cells and anti-CD3 OKT3 mAb (34). The percentage of specific lysis was calculated as follows: 100 − ((experimental − spontaneous release)/(total − spontaneous release)). Cultures with rIL-12 (5 ng/ml; R&D Systems) were done with clones in absence of feeder cells, or with PBMC from healthy donors, in 5% human serum and 150 U/ml rIL-2. After 6, 24, 48, and 72 h of culture, cells were stained with anti-CD3 and anti-CD8 Abs, permeabilized, stained with Abs specific for granzyme B or perforin, and analyzed by gating on $CD3^+CD8^+$ cells.

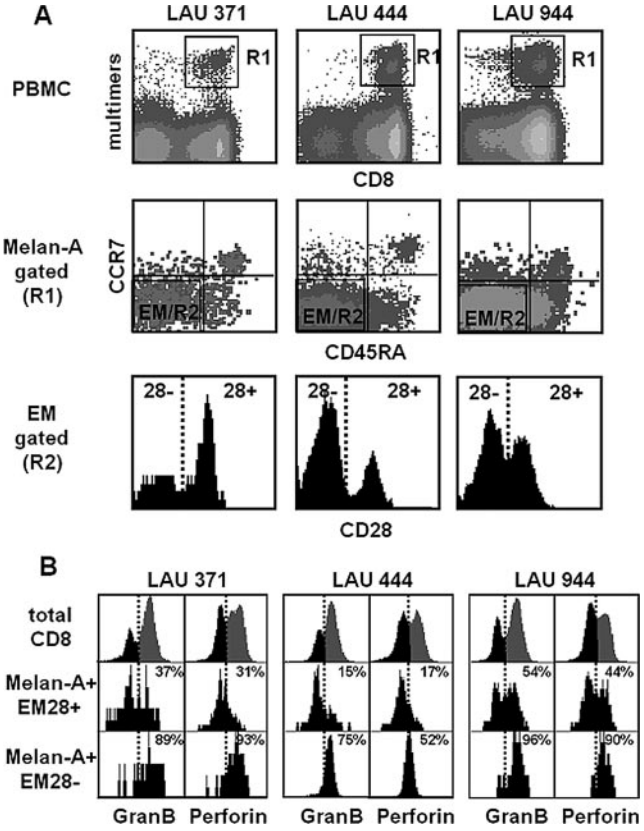

FIGURE 1. CD8 T cells responding to peptide vaccination are predominantly $CCR7^-/CD45RA^-$, with subsets distinguished by expression of CD28, granzyme B, and perforin. *A*, High frequencies of ex vivo detectable circulating CD8 Melan-A-specific T cells after vaccination with low doses of CpG 7909, Melan-A peptide, and IFA. In the three patients, LAU 371, LAU 444, and LAU 944, Melan-A $multimer^+$ T cells were found at 2.08, 3.00, and 1.25% of CD8 T cells, respectively (gate R1; *A*). These cells were dominantly $CCR7^-/CD45RA^-$ EM cells (30). Gating on EM cells showed that a fraction of these cells was CD28 positive, whereas the remaining were CD28 negative. *B*, Costaining with granzyme B and perforin-specific Abs revealed bimodal distributions, with large fractions of granzyme B and perforin-negative cells in total CD8 T cells and $EM28^+$, but not $EM28^-$ cells. PBMC were collected 7–10 days after vaccination, and analyzed ex vivo by multiparameter flow cytometry upon staining with A2/Melan-A multimers and Abs specific for CD8, CD45RA, CCR7, CD28, granzyme B, and perforin. Part of the data has been reported previously (30).

Cytokine production

$CCR7^+/CD45RA^+$, $EM28^+$, and $EM28^-$ ex vivo derived T cell clones (3×10^5 cells) were stimulated with 3×10^5 irradiated T2 target cells, in the presence or absence of Melan-A peptide (1 μg/ml), in a total volume

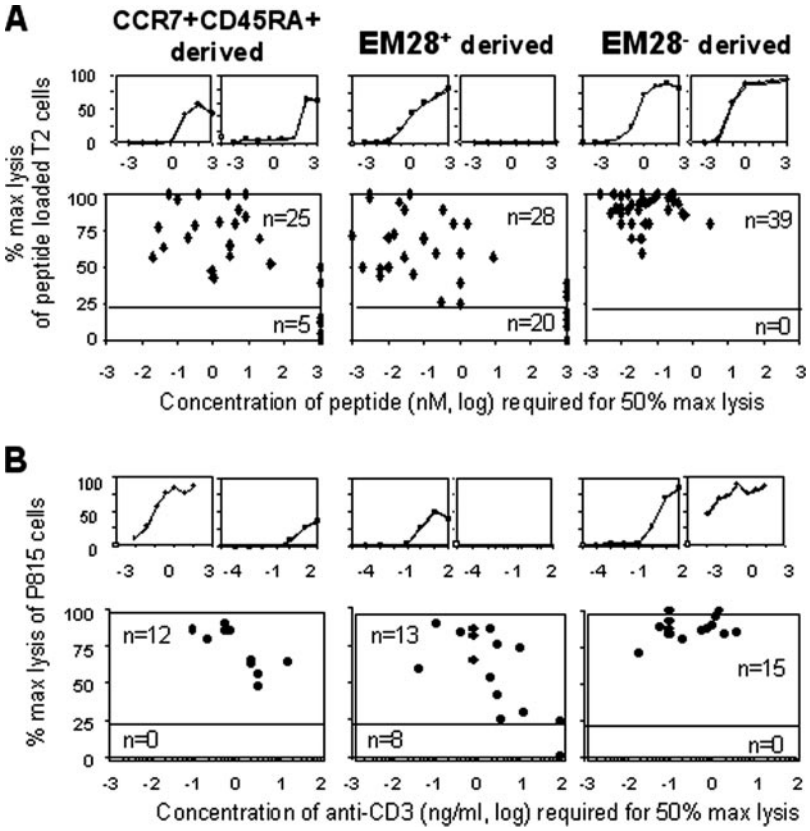

FIGURE 2. Differential cytotoxicity of T cell clones depending on in vivo differentiation stage. From circulating CD8 T cells of the three patients, 117 T cell clones were generated through ex vivo flow cytometric sorting, by gating on T cell subpopulations with distinct phenotypes. *A*, The six graphs on the *top* show representative cytotoxicity results of 3 × 2 individual clones derived from CCR7$^+$/CD45RA$^+$, EM28$^+$, and EM28$^-$ subsets, respectively, using T2 target cells in presence of titrated Melan-A peptide. The three figures below show for each of the three family of clones the percentage of maximal lysis (*y*-axis) and the concentration of peptide required for 50% maximal lysis (*x*-axis). Each data point represents the result from an individual clone. Clones with ≤25% maximal lysis/total number of clones tested are as follows: 5 of 30 CCR7$^+$/CD45RA$^+$-derived clones, 20 of 48 EM28$^+$-derived clones, and 0 of 39 EM28$^-$-derived clones. *B*, In parallel, redirected (i.e., anti-CD3 mAb-directed) cytotoxicity was measured against P815 target cells. *Upper panel*, Shows anti-CD3 mAb titration experiments with the same representative clones as in *A*. The lower three figures show maximal lysis (*y*-axis) of each clone, in relation to the concentration of cross-linking anti-CD3 mAb needed for 50% maximal lysis (*x*-axis). Clones with ≤25% maximal lysis/total number of clones tested are as follows: 0 of 12 for CCR7$^+$/CD45RA$^+$-derived clones, 8 of 21 for EM28$^+$-derived clones, and 0 of 15 for EM28$^-$-derived clones. Clones with 0% cytotoxic activity were grouped and symbolized as a single dot on the *right end* of the *x*-axis, to visualize them in the figures, despite that peptide/Ab concentrations for half-maximal lysis could not be determined.

of 1 ml. After 40 h, supernatants were collected and cytokine content was determined by FACS analysis with a CBA kit from BD Biosciences, according to the manufacturer's instructions.

Results

High percentages of Melan-A-specific EM (CCR7$^-$/CD45RA$^-$) T cells after peptide vaccination

As reported previously (30), we observed strongly increased frequencies of circulating Melan-A-specific T cells (Fig. 1*A*) after vaccination of advanced stage III/IV melanoma patients with low dose Melan-A/MART-1 peptide, CpG oligodeoxynucleotide 7909, and IFA. Between 1.25 and 3.00% of circulating CD8 T cells were positive when stained with HLA-A*0201/Melan-A multimers, as shown in Fig. 1*A* for the three patients (LAU 371, LAU 444, and LAU 944) analyzed in depth in the present study. The vast majority of Ag-specific T cells were negative for CCR7 and CD45RA, so-called EM cells (13, 30). These dominant EM cells were in part CD28 positive (EM28$^+$) and in part CD28 negative (EM28$^-$). Although the proportions of EM28$^+$ and EM28$^-$ cells varied between the three patients, both populations were present in all three patients (Fig. 1*A*).

Differences in granzyme B and perforin expression between EM28$^+$ and EM28$^-$ Melan-A-specific T cells

To determine the expression of effector proteins in vivo, we combined surface and intracellular staining. Gating on total CD8$^+$ T cells showed bimodal distributions, with fractions of cells expressing granzyme B and perforin in each patient (Fig. 1*B*). We also found bimodal distributions when gating on EM28$^+$ cells, as follows: T cells from the three patients expressed granzyme B at frequencies of 37, 15, and 54%, and perforin was expressed at 31, 17, and 44% (Fig. 1*B*). By sharp contrast, EM28$^-$ cells were predominantly positive for granzyme B and perforin. Thus, most EM28$^-$ T cells expressed cytolytic effector proteins, in contrast to EM28$^+$ T cells, which contained a substantial fraction of cells lacking such proteins in each patient.

A significant fraction of T cell clones derived from CD28$^+$ EM cells exhibits poor cytolytic activity

For functional analysis, we generated a total of 117 stable T cell clones from PBMC of the three patients, through FACS sorting of CD8$^+$ multimer$^+$ T cells gated according to expression of CCR7, CD45RA, and CD28. Individually sorted T cells were put in culture and stimulated with PHA and allogenic feeder cells to obtain T cell clones sorted ex vivo from the prominent CCR7$^-$/CD45RA$^-$ EM subpopulations, as follows: 48 clones were derived from EM28$^+$ cells, and 39 from EM28$^-$ cells. As controls, we also generated 30 clones derived from putatively naive CCR7$^+$/CD45RA$^+$ cells (35). All 117 clones were tested in cytotoxicity assays to measure percent specific lysis in relation to Melan-A peptide concentration used for target cell loading (Fig. 2). EM28$^-$-derived clones strongly killed peptide-loaded T2 cells, revealing a remarkable functional homogeneity (Fig. 2*A*). In contrast, cytotoxicity was heterogenous in the two other groups of clones, with a surprising absence or poor killing (≤25% specific lysis at high peptide concentrations) in a substantial fraction of EM28$^+$-derived clones, and some CCR7$^+$/CD45RA$^+$-derived clones (Fig. 2*A*). To examine whether poor killing was due to insufficient TCR triggering by MHC/peptide, we performed redirected lysis assays, using anti-CD3 Abs that bind to P815 target cells via FcγR mediating efficient T cell/target cell interaction. Nevertheless, there was still a large fraction of EM28$^+$-derived clones showing no or poor killing of P815 cells (Fig. 2*B*). Thus, in contrast to EM28$^-$-derived clones, many EM28$^+$-derived clones were poorly cytotoxic, even under conditions of strong T cell/target cell cross-linking.

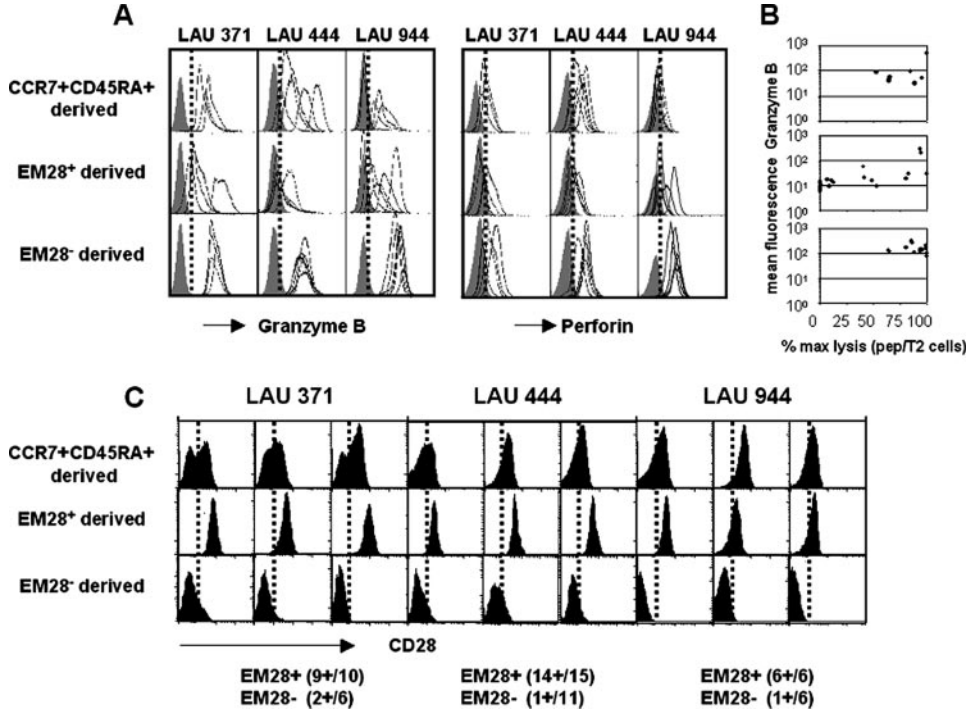

FIGURE 3. Differential expression of granzyme B and perforin among EM28$^+$ clones correlating with cytotoxicity. *A*, The three families of clones were permeabilized and stained with mAbs specific for granzyme B (*left*) and perforin (*right*). Filled histograms: stainings with isotype-matched control Ab. *B*, Maximal lysis of Melan-A peptide-loaded T2 cells (data from Fig. 2*A*) is plotted against mean fluorescence of granzyme B staining, revealing a statistically significant correlation of cytotoxicity and granzyme B expression ($p > 0.001$ for all clones, $p > 0.05$ for EM28$^+$-derived clones, NS for CCR7$^+$/CD45RA$^+$- and EM28$^-$-derived clones; ANOVA). *C*, Long-term maintenance of CD28 phenotype. T cell clones were stained with CD28-specific mAbs. The histograms show results from three clones for each group, demonstrating that most EM28$^+$- and EM28$^-$-derived clones expressed CD28 according to the ex vivo sorting that was initially performed for their generation. In contrast, CCR7$^+$/CD45RA$^+$-derived clones were heterogenous. A summary of results for CD28 expression by clones derived from the three patients, LAU 371, LAU 444, and LAU 944, is shown below the figure (clones expressing CD28/total clones).

The peptide titration experiments (Fig. 2*A*) show that EM28$^-$-derived clones required only low peptide concentrations for half-maximal lysis of T2 cells (mean concentration between 0.01 and 0.1 nM). In contrast, CCR7$^+$/CD45RA$^+$- and EM28$^+$-derived clones were more heterogenous, with the majority of CCR7$^+$/CD45RA$^+$-derived clones requiring >1 nM, and many of the EM28$^+$-derived clones >0.1 nM concentrations for half-maximal lysis. Importantly, the three different T cell subpopulations (CCR7$^+$/CD45RA$^+$, EM28$^+$, and EM28$^-$) were found in three of three patients, and sorting and cloning from blood samples obtained at multiple dates per patient reproducibly led to the generation of the described clones with high and low killing, indicating that these subpopulations coexist simultaneously in all three patients.

Low granzyme B and perforin expression in EM28$^+$-derived clones correlating with low cytotoxicity

To investigate whether poor killing was due to deficiencies in the expression of cytotoxic effector proteins, we performed intracellular staining of granzyme B and perforin (Fig. 3*A*). All EM28$^-$-derived clones expressed high levels of granzyme B. CCR7$^+$/CD45RA$^+$-derived clones exhibited lower values, and EM28$^+$-derived clones were in part very low and displayed a substantial heterogeneity between clones. The overall staining for perforin was lower, in part explained by the less efficient staining obtained with this mAb as compared with the granzyme B-specific mAb. Nevertheless, the pattern was comparable to granzyme B: practically all EM28$^-$-derived clones were perforin positive, in contrast to EM28$^+$ and CCR7$^+$/CD45RA$^+$-derived clones, which were partially perforin negative. The results correlated significantly with maximal cytotoxicity against T2 cells loaded with saturating peptide doses (1 μM; Fig. 2*A*), because strongly granzyme B-positive cells lysed T2 cells more efficiently than granzyme B low cells (Fig. 3*B*). A similar pattern was observed for perforin expression (data not shown). Together, the data demonstrate that all EM28$^-$-derived clones expressed high levels of cytotoxic proteins and exerted strong killing, and that CCR7$^+$/CD45RA$^+$-derived clones also killed relatively well. In contrast, EM28$^+$-derived clones were heterogenous with many that had poorly developed cytotoxic capacity.

Long-term in vitro maintenance of phenotype and function of T cell clones

The above described assessment of cytotoxicity was performed between 2 and 5 mo after the generation of T cell clones, whereby the clones were tested two to four times with intervals of in part >3 mo of culture. As frequently observed, cytotoxic activity was variable during the first 1–2 mo after cloning, and varied depending on the number of days after restimulation of clones (data not shown). Afterward, high or low killing became remarkably stable features of individual clones. Under these conditions, we also assessed CD28 expression in the clones cultured for 2–5 mo (Fig. 3*C*). The CCR7$^+$/CD45RA$^+$-derived clones were often CD28 positive or bimodal, i.e., they were

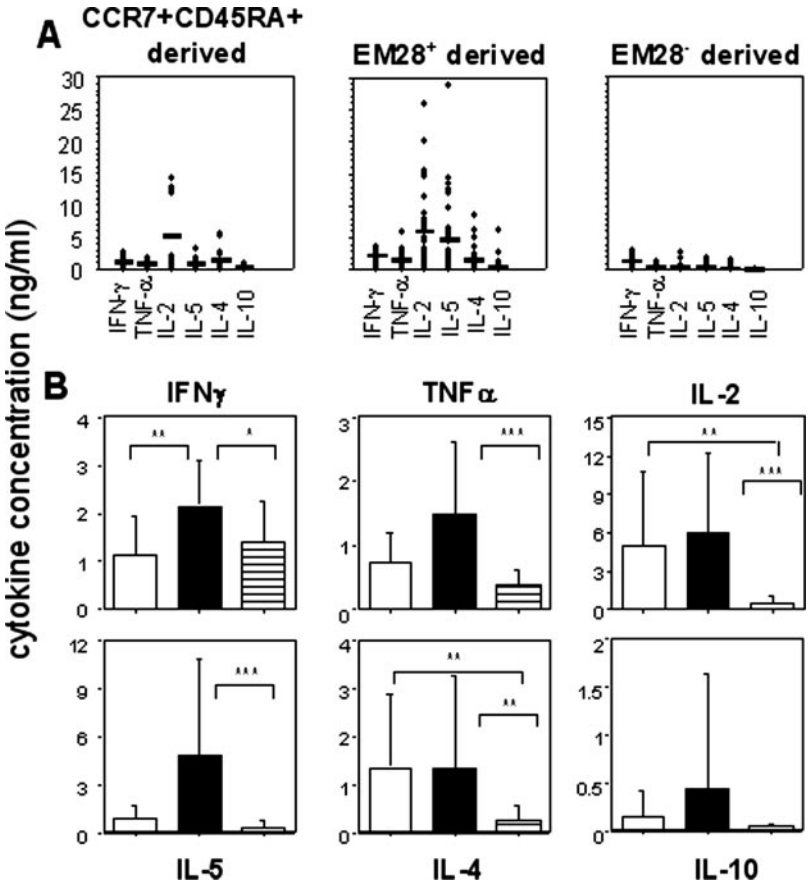

FIGURE 4. Cytokine secretion by T cell clones. Clones were stimulated with peptide-loaded T2 cells, and cytokine secretion was quantified with cytokine bead arrays. *A*, Concentration of cytokines secreted by individual CCR7$^+$/CD45RA$^+$, EM28$^+$, and EM28$^-$ T cell clones. Numbers of clones tested were 12, 31, and 24, respectively. *B*, Cytokine secretion comparison between T cell clones derived from CCR7$^+$/CD45RA$^+$ (□), EM28$^+$ (■), and EM28$^-$ (bars with horizontal lines) cells. Data represent means and SD. Only statistically significant differences between groups are depicted. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA, Student's *t* test).

partially CD28 positive and partially CD28 negative. In contrast, the majority of clones originating from EM28$^+$ PBMC expressed CD28, and clones from EM28$^-$ PBMC had no detectable CD28 expression. Thus, CD28 phenotype and cytotoxic function of EM-derived T cell clones remained stable, despite that they were maintained in culture for several months.

Increased cytokine secretion by EM28$^+$-derived T cell clones

To assess the ability of the clones to produce cytokines, we stimulated them with peptide-loaded T2 cells and measured cytokines in culture supernatants. IFN-γ was well detectable in all clones, with significantly higher levels of IFN-γ produced by EM28$^+$ cells (Fig. 4). More significantly, as compared with EM28$^-$- and CCR7$^+$/CD45RA$^+$-derived cells, EM28$^+$-derived clones were superior in the production of IL-2, IL-4, IL-5, and TNF-α. Therefore, EM28$^+$ cells strongly secreted IL upon short-term Ag stimulation. Cell viability was comparable in EM28$^+$- and EM28$^-$-derived clones (data not shown), indicating that differential IL production was not due to differences in cell survival. Among the six cytokines tested, only IL-10 was not significantly increased in EM28$^+$-derived clones as compared with the other populations. Finally, we addressed the question whether among EM28$^+$-derived clones, cytokine secretion would correlate inversely with killing. We found a trend toward stronger cytokine release by those cells with poor killing and low granzyme B expression. However, this was not statistically significant for any of the six cytokines tested (data not shown).

IL-12-induced granzyme B expression and cytotoxicity

To investigate whether T cells can acquire cytotoxic capacity, we stimulated them for 6 h with PMA and ionomycin. However, this did not up-regulate killing nor granzyme B/perforin expression (data not shown). A recent report indicated that murine CD8 T cells require IL-12 for the development of cytotoxicity (36). Therefore, we analyzed our T cell clones after incubation in medium supplemented with IL-12 for 3 days, and compared the results with clones that had been kept conventionally, i.e., similarly as for the experiments described above. Interestingly, IL-12 led to increased cytotoxicity (Fig. 5*A*), which was particularly enhanced in clones with initially low cytotoxicity (Fig. 5*B*). In parallel, we observed a significantly increased expression of granzyme B (Fig. 5*C*). Clones with initially strong killing also showed some degree of IL-12-mediated up-regulation of killing and granzyme B, which was, however, less pronounced than in clones with initially low cytotoxicity (Fig. 5, *B* and *C*). In contrast, we observed similar levels of perforin expression in presence and absence of IL-12. Kinetic studies showed that IL-12-driven granzyme B up-regulation was already detectable at 24 h and became strong at 48 h (Fig. 5*D*). Subsequently, we analyzed CD8$^+$CD3$^+$ PBMC of healthy donors, and again found IL-12-mediated up-regulation of granzyme B, but not perforin (Fig. 5*E*). This up-regulation was, however, weaker and slower (Fig. 5*F*) as opposed to clones, because the differences were smaller and became statistically significant only after 72 h (Fig. 5*D*). Together, the data demonstrate IL-12-driven up-regulation of granzyme B in CD8 T cells from circulating blood and in conditional killer clones. Finally, we determined whether IL-12 would also increase IFN-γ production by Ag-stimulated T cell clones cultured for 24 and 72 h. However, neither intracellular IFN-γ content nor the amount of IFN-γ secreted in culture supernatants was increased in cultures with vs without IL-12 (data not shown).

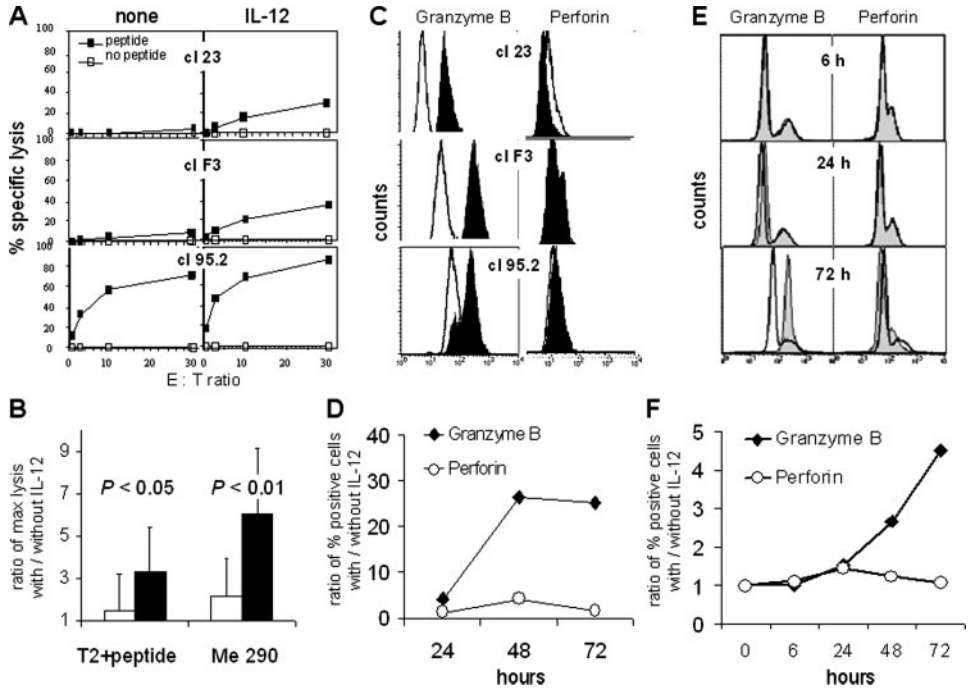

FIGURE 5. Induction of granzyme B expression and cytolysis by IL-12. *A*, Two conditional killer clones (clones 23 and F3) and one cytotoxic clone (clone 95.2) were analyzed in 4-h ^{51}Cr release assays, after 3-day culture in IL-2-containing medium as usual, in absence (*left*) or presence (*right*) of IL-12 (5 ng/ml). Target cells were T2 cells without peptide (open symbols) or loaded with Melan-A analog peptide (closed symbols). *B*, The ratio of maximal lysis with/without IL-12 incubation was calculated; compiled results from seven and four clones (with high and low initial cytotoxicity, □ and ■, respectively) are shown. *C*, In parallel to cytotoxicity assays, clones were permeabilized and stained with Abs specific for granzyme B (*left*) and perforin (*right*); results from clones after incubation with IL-12 (filled histograms) and without IL-12 (open histograms). *D*, Time kinetics showing IL-12-driven up-regulation of granzyme B, but not perforin (mean values of six conditional killer clones; data are ratios of percentage of granzyme B (circles)- or perforin (diamonds)-positive cells determined by intracellular staining after incubation with/without IL-12). *E*, PBMC from a representative healthy donor were cultured similarly and stained with Abs specific for granzyme B (*left*) and perforin (*right*); histograms are from $CD8^+CD3^+$-gated cells after incubation with IL-12 (filled histograms) and without IL-12 (open histograms). *F*, Time kinetics showing IL-12-driven up-regulation of granzyme B, but not perforin (values are ratios of percentage of positive cells after incubation with/without IL-12; mean values from $CD8^+CD3^+$-gated cells from PBMC of four healthy donors). Statistical comparisons of granzyme B expression with/without IL-12 are as follows: clones reached $p < 0.05$ already at 24 h, $CD8^+CD3^+$ PBMC only at 72 h (Student's *t* test).

Discussion

We analyzed subsets of human Ag-specific CD8 T cells, and determined functional differences in CD28-negative and CD28-positive EM cells. We found that the latter were composed of two subsets, one with high and the other with low cytotoxicity, correlating with their levels of granzyme B and perforin expression. Targeted cell sorting, followed by the generation of representative clones, revealed impressive stability of phenotypes, and poor cytotoxic potential in cells with low level expression of granzyme B. However, exposure to IL-12 led to strong up-regulation of granzyme B and cytolysis in these conditional killer T cells.

To assess the functional status of T cells, we primarily and systematically focused on expression of granzyme B and perforin (Figs. 1, 3, and 5). In addition, cytotoxicity was measured whenever possible (Figs. 2, 3, and 5). The fact that cytotoxicity correlated well with expression levels of granzyme B (Figs. 3 and 5) allowed us to draw conclusions about the in vivo status, despite that ex vivo functional testing for cytotoxicity of individual cells is not possible with currently available techniques. Recently, we have developed a novel assay for ex vivo measurements of cytotoxicity by small numbers of Ag-specific T cells (37). However, in most instances, it still remains impossible to perform ex vivo functional cytotoxicity tests with subsets of multimer$^+$ T cells. Nevertheless, we could test total Melan-A multimer$^+$ T cells from two patients (LAU 371 and LAU 944), and found that both reached 90–100% specific lysis of peptide-labeled T2 cells at E:T ratios $\geq$4. Interestingly, maximally reached levels of CD107a were much higher in patient LAU 944 (56%) than in patient LAU 371 (23%; data not shown), revealing a considerable difference of the fraction of lytic cells, and confirming the finding in this study that patient LAU 371 had many more nonlytic T cells than patient LAU 944.

Our results reveal a new mechanism for regulation of cytotoxicity in human CD8 T cells. Recent results in mice demonstrated that IL-12 (36, 38) and type I IFN (39, 40) play major roles in the control of T cell cytotoxicity. Interestingly, lytic and nonlytic murine T cells were similar in conjugate formation with Ag-bearing target cells, Ag-dependent calcium flux, and degranulation in response to TCR signaling. The only parameter significantly correlating with lytic activity was granzyme B content, which was much higher in T cells exposed to IL-12 (41), similar to our observations.

We also cultured T cell clones in presence of IFN-α, which did, however, not result in up-regulation of granzyme B and cytotoxicity (data not shown). At least three explanations may account for this, as follows: 1) additional signals to IFN-α may be required to induce cytotoxicity; 2) cytotoxicity of human T cell subsets other than the ones studied in this work may depend on IFN-α; or 3) IFN-α does not play this role in humans as it does in mice (39, 40).

Costimulation through CD28 leads to strong T cell activation (21), and can cause dramatic side effects particularly in humans (1, 2). T cell effector function, especially cytotoxicity, must be tightly controlled to avoid harmful extended cytotoxicity. As compared

with IL-2 (which we used for all T cell cultures), IL-12 signals through an entirely distinct system involving different Jak/STAT members and regulating different transcription factors and functions (42–45), emphasizing that distinct molecular mechanisms control T cell cytotoxicity. Bioavailability of IL-12 may be limited to specialized cellular and microenvironmental compartments. Mouse studies have shown that CD8α-expressing dendritic cells (DC) provide most of the IL-12 acting on CD8 T cells (38). Thus, the availability of IL-12 is most likely limited to the vicinity of activated DC in lymphoid tissues and inflammatory lesions. It may well be that the strong toxicity seen in patients after systemic treatment with IL-12 (46) is related to the capacity of IL-12 to induce cytotoxicity.

Our finding of increased cytokine production, especially IL-2, by CD28-positive T cells was not surprising, because T cells in early and intermediate ($CD28^+$), but not late ($CD28^-$) differentiation stages are known to produce IL-2 (47). Studies in mice have directly confirmed that absence of CD28-mediated costimulation impairs IL-2 production by virus-specific CD8 T cells (21, 48). Topp et al. (47) demonstrated that restoration of CD28 expression in CD28-negative CD8 human T cells reconstitutes Ag-induced IL-2 production. Interestingly, IL-2 production depended on intracellular signaling via CD28, and the loss of CD28 appeared to be the only critical blockade to IL-2 production by CD28-negative CD8 T cells. Triggering through other (costimulatory) molecules such as CD2, CD27, 41BB, or LFA-1 was not essential for IL-2 production (47).

Absence of lytic function may suggest partial tolerance (41). However, at least in mice, some nonlytic CD8 T cells have protective potential, for example, by IFN-γ secretion important for CD8 T cell-mediated tumor defense (49) and protection against some viruses (50, 51). There are further indications that CD28-positive T cells play central protective roles. CD28 expression and IL-2 production by CD8 T cells could be particularly critical in diseases associated with poor $CD4^+$ helper cell responses, as for example in HIV infection (52) and tumors (53). A recent study demonstrated that IL-2 expression was a feature of subsets of HIV-1-specific CD8 T cells, and that this property correlated with CD4 cell-independent proliferation of CD8 T cells (54). It has been reported that IL-2 production was primarily confined to the $CCR7^+/CD45RA^-$ central memory population, whereas IFN-γ production was more prominent among $CCR7^-/CD45RA^-$ EM cells (9, 13, 55). In our patients, we attempted to include the analysis of $CCR7^+/CD45RA^-$ central memory cells, but did not succeed because the vast majority of activated Melan-A-specific cells was $CCR7^-/CD45RA^-$ and only a very small fraction expressed low (borderline) levels of CCR7, which is a frequent observation in tumor Ag-specific T cells. However, IL-2 is also produced by a fraction of EM cells, as recently reported for HIV-1-specific CD8 T cells (54). In this study, we raised evidence for IL-2 production by tumor Ag-specific EM cells, and show that they are characterized by the expression of CD28. Whether the latter is also true for human virus-specific CD8 T cells remains to be determined.

Recent studies have demonstrated uncoupling of proliferative and effector function (56–58). Human CD8 T cells that proliferate in vivo are preferentially found in the IL-2-producing subsets (59, 60). It is conceivable that proliferation of Ag-specific CD8 T cells is a feature of protective immune responses (9–11, 61). In agreement with in vivo observations, in vitro proliferation is favored by CD28 stimulation, and CD28-positive T cells may sometimes grow better as compared with CD28-negative cells (22). Thus, CD28 triggering may be a central element in robust CD8 T cell responses, and may be critically missing in nonproductive T cell activation. The fact that solid tumors are B7 negative may be key to understand the usually poor protection mediated by T cells. Conversely, those human tumors that are the most susceptible to immunotherapy are B7 positive: striking therapeutic effects can be observed in leukemia patients through the graft-vs-leukemia effect (62), and possibly also in B cell lymphoma patients upon active specific immunotherapy (63, 64). However, CD28-negative CD8 T cells can also proliferate considerably, as we have observed for melanoma Ag-specific T cells in vivo and for the CD28-negative derived clones described in this work that proliferated almost as efficiently as their CD28-positive counterparts (65). It is important to further assess the still many open questions regarding, for example, time intervals and signaling pathways promoting successful T cell growth and function of the different subsets, and most importantly, to identify correlates of protection.

Although increasingly applied, ex vivo phenotyping of T cells with surface markers (such as CD45RA, CCR7, CD28, or CD27) is insufficient for the characterization of T cells with precisely defined differentiation and functional status. We used these markers to set an arbitrary grid for the classification and further characterization of T cells. Clearly, a classification based on functional properties would be preferable, but ex vivo assessment of T cell function is often not possible, and functionally important molecules are mostly intracellular, making it impossible to sort viable cells for further investigation. In general, a better understanding of the T cell system relies on a much more detailed characterization of cellular and molecular components and mechanisms involved in T cell responses. Cell surface markers are still a valuable tool, provided that they are not overinterpreted, e.g., as decisive for distinct immune functions. Despite several efforts, we have not (yet) been able to identify a cell surface marker (combination) specific for the newly identified conditional killer T cells, which would facilitate a more rapid and systematic investigation, including the cell sorting and further study of underlying mechanisms.

A puzzling finding is that T cell clones derived from presumably naive ($CCR7^+/CD45RA^+$) T cells were more strongly cytotoxic than $EM28^+$-derived clones. Thus, in vitro priming may favor differentiation toward cytotoxic effector cells, a conclusion that is compatible with several studies on in vitro primed CD8 T cells (13, 35, 66, 67). It will be useful to elucidate the responsible mechanisms. One possible reason may be that in vitro priming usually occurs through DC (providing IL-12), whereas in vitro stimulation of in vivo primed cells may often happen by nonprofessional APCs lacking IL-12 and unable to convert conditional killer cells to cytotoxic effector T cells. This view is compatible with an elegant mouse study that applied artificial APCs and showed that naive, but not memory CD8 T cells required IL-12 to acquire cytotoxic function (38). Alternatively, the conditional killer phenotype may be generated through other mechanisms that may occur in vivo (to some cells), but not during in vitro priming.

In our study, we also addressed the question whether killer and conditional killer T cells differed in their capacity to recognize Ag. The fact that all clones were positive in Ag-mediated killing assays and/or in Ag-triggered cytokine release assays demonstrated that they were capable of, and depended on, recognizing Melan-A peptide Ag specifically. In addition, nearly all of these clones also recognized $HLA\text{-}A2^+$ melanoma cells naturally expressing Melan-A (data not shown), suggesting that they could sense relatively low levels of Ag. We also found that peptide concentrations required for 50% maximal cytokine production were in a similar range for $EM28^+$- and $EM28^-$-derived clones (data not shown). Finally, we sequenced the TCRs (data not shown) of the clones to investigate whether some $EM28^+$-derived clones may have identical TCRs to cytotoxic and $EM28^-$-derived clones, which was, however, not the case.

Despite that the three patients had quantitative differences in the proportions of the studied T cell subsets, all three subsets were clearly present in the three patients, with substantial percentages of CD28-positive and CD28-negative cells, including $EM28^+$ conditional killer cells. Thus, our data suggest that the studied T cell subsets can regularly be found, at least for self/tumor Ag-specific T cells after patient vaccination with peptide and CpG 7909, emulsified in IFA. More recently, we studied T cells from a new patient (LAU 50) with a very strong spontaneous CD8 T cell response specific for the tumor-Ag NY-ESO-1 (>6% $multimer^+$ of circulating CD8 T cells!). Preliminary data indicate that the $EM28^+$ population of this patient also contained nonlytic cells expressing low levels of granzyme B and perforin ex vivo and in vitro (data not shown), suggesting that this occurs also in the absence of vaccination. Furthermore, we found such conditional killer T cells in anatomical compartments other than peripheral blood (68), i.e., in tumor-infiltrated lymph node cells. Nevertheless, due to the limited number of patients studied, it is too early to draw firm conclusions as to whether this new subset can be found frequently or whether it only exists due to exceptional conditions (of T cell activation) of some cancer patients.

Effector and memory T cells are heterogenous. It is important to elucidate the roles of the different subsets, and to identify how they are regulated. Some differentiation stages can hardly be induced in vitro. Therefore, in vivo/ex vivo analyses remain the gold standard. However, experimental approaches are limited in humans. A way to overcome such limitations is offered by the finding that selected T cell functions are robustly imprinted in vivo, to the extent that they are maintained in vitro over several months. Thus, specifically isolated T cell clones can serve as reliable models, if built on evidence as provided in this study, that the studied properties are similar in vivo.

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Disclosures

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