CD28$^-$ CD8$^+$ T cells do not contain unique clonotypes and are therefore dispensable

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1. Summary

Highly differentiated $CD28^-$ effector T cells which accumulate in a variety of diseases and also with increasing age contribute to inflammatory processes, limit immunological space and diversity, and are associated with immunological dysfunction and reduced responses to vaccination. Elimination of $CD28^-$ T cells has been suggested as a measure for immunological rejuvenation but may lead to the loss of important T cell specificities. Using T cells specific for the immunodominant CMV-derived epitope NLVPMVATV as a model, we show that the same clonotypes are present in $CD8^+CD28^+$ naïve/early memory and $CD8^+CD28^-$ effector T cells. Therefore, $CD28^-$ cells do not seem to contain clones which are not present in the residual population. The elimination of effector T cells would not lead to the loss of important specificities, as relevant clonotypes could be recruited and propagated from naïve or early memory T cell subsets in the case of exposure to pathogen.

2. Introduction

The costimulatory molecule CD28 is essential for productive activation of T cells as it amplifies the T cell receptor (TCR) signal [1]. CD28 is expressed constitutively on human T cells; however, T cell activation leads to a downregulation of CD28 expression [2] thus resulting in the occurrence of $CD28^-$, presumably replicative senescent T cells, after repeated antigenic stimulation [3,4]. $CD28^-$ T cells accumulate in HIV-infected individuals [5,6,7], in patients with inflammatory conditions such as rheumatoid arthritis [8], systemic lupus erythematosus [9] atherosclerotic artery disease [10] and inflammatory bowel disease [11,12], in persons with latent Cytomegalovirus (CMV) infection [13,14], and also during healthy aging [15,16]. In pathological conditions accumulation of $CD28^-$ T cells is believed to represent accelerated immunosenescence and exhaustion of the immune system [5,6]. $CD28^-$ T cells are considered to be detrimental as they are resistant to apoptosis [17,18], frequently autoreactive [19] and produce high levels of Interferon (IFN)-γ [17,20,21]. They may thus contribute to the development of inflammatory processes, which are known to be a predisposing factor for age-related diseases [22,23,24]. Elevated numbers of $CD8^+CD28^-$ T cells are part of the so-called "immune-risk phenotype", which has been identified as a predictor for 2-year-mortality in longitudinal studies on octo- and nonagenerians [25,26]. Therefore, the abundance of $CD8^+CD28^-$ T cells has been suggested as an indicator for immune dysfunction in old age. $CD28^-$ T cells are to a high degree clonally expanded [27,28] and occupy a lot of immunological space, thereby limiting the available space for naïve T cells and restricting the repertoire. High numbers of $CD28^-$ effector cells correlate with a poor response to influenza vaccination [29,30]. The number of highly differentiated CMV-specific T cells increases with age [14,31] and CMV-

seropositivity is associated with marked changes in the overall composition of the T cell compartment [13,32,33]. It has also been postulated that CMV-specific $CD28^-$ T cells are inhibitory for T cells of other specificities either directly or by perturbing antigen presentation by dendritic cells and macrophages [34], as the number of EBV-specific T cells increases with age only in CMV-seronegative but not in CMV-seropositive individuals [35].

Depletion of $CD4^+$ T cells in aged mice leads to the emergence of newly generated T cells that show a naïve phenotype and do not exhibit age-related defects in response to antigen [36]. In humans suffering from severe autoimmune diseases or leukemia immune cell ablation and autologous, hematopoietic stem cell transplantation lead to regeneration of a diverse, "rejuvenated" T and B cell system [37,38,39]. These results support the hypothesis that immunological space allows for the emergence of new, naïve T cells and it has been suggested that elimination of $CD28^-$ effector cells in diseased or aged persons could provide space for a variety of more "useful", less differentiated cells, thereby improving immune function [40,41,42]. The main criticism to this type of intervention is that removal of the $CD28^-$ T cell population might also eliminate T cell specificities that are needed for the control of infections. Therefore, it is crucial to ensure that T cell specificities that are not present elsewhere and are needed for the control of pathogens are preserved. We chose $CD8^+$ T cells specific for the immunodominant pp65-derived CMV epitope NLVPMVATV (CMV_{NLV}) as a model to investigate, whether the same T cell clonotypes are present in $CD28^+$ and $CD28^-$ T cell populations.

3. Materials and Methods

Blood donors

Peripheral blood was obtained from healthy, elderly, CMV-seropositive, HLA A*0201 positive donors (n=6; median age 70 years; range 69-83). Elderly donors were chosen because they have higher percentages of $CD8^+CD28^-$ T cells and a higher frequency of CMV-specific T cells than young adults. Only individuals without malignancies, acute diseases or advanced stages of severe chronic diseases, and persons without immunosuppressive therapy were included in the study. The study was approved by the local ethical committee and all participants gave their written informed consent. CMV-specific antibody levels were determined by enzyme-linked immunosorbent assay (Enzygnost® anti-CMV/immunoglobulin G; Dade Behring) according to the manufacturer's protocol. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (FicollHypac; Amersham Biosciences).

Isolation of T cell subsets

$CD8^+$ T cells were isolated using anti-CD8-coupled MultiSortMicrobeads, and the magnetic-activated cell sorting (MACS) system (Milteny Biotech) according to the specifications given by the manufacturer. $CD8^+CD28^+$ and $CD8^+CD28^-$ T-cells were enriched from $CD8^+$ T-cells after enzymatic removal of the magnetic particles. $CD8^+$ T cells were stained with a PE-conjugated anti-CD28 monoclonal antibody (BD Biosciences, USA) at a concentration of 5µl per 10^7 cells and incubated for 20min at 4°C. After washing the cells were incubated with anti-PE-coupled MicroBeads and $CD28^+$ and $CD28^-$ cell fractions were isolated following manufacturer´s instructions. In order to deplete the residual $CD28^+$ cells from the $CD28^-$ fraction the purification step with anti-CD28-PE and anti-PE Microbeads was repeated. $CD28^+$ and $CD28^-$ cell fractions were counted and purity was assessed by flow cytometry. Purity was > 90% for the $CD28^+$ and

>98% for the $CD28^-$ populations. PBMC were irradiated (30Gy) and used as antigen-presenting cells.

Cultivation of T cell subsets and enrichment of CMV_{NLV}-specific T cells

$CD8^+$, $CD8^+CD28^+$ or $CD8^+CD28^-$ T cells were cultivated in RPMI 1640 (Cambrex) supplemented with 10% FCS (Sigma-Aldrich) and 1% penicillin-streptamycin (Cambrex) at 37°C and 5% CO_2. T cells were stimulated for 14 days with 0.1µg/ml of the immunodominant peptide NLVPMVATV (Bachem) derived from the CMV-encoded protein pp65 in the presence of IL-2 (20ng/ml) and irradiated autologous PBMC (ratio $CD8^+$:PBMC 1:1). IL-2 (20ng/ml) was added every three days and cells were restimulated with peptide and irradiated autologous PBMC after 7 days. Percentages of CMV_{NLV}-specific T cells were determined prior to culture, after 7 days and after 14 days of stimulation by immunofluorescence surface staining with APC-coupled pentamers containing the CMV_{NLV} peptide (Pro5® MHC, Proimmune). Absolute numbers of CMV_{NLV}-specific T cells were calculated from total counts of viable cells and percentages of CMV_{NLV}-specific T cells measured by FACS. Population doublings were calculated using the formula $(\log(N)-\log(N_0))/\log(2)$.

After 14 days of cultivation CMV_{NLV}-specific T cells were purified using APC-conjugated CMV-pentamers, anti-APC-antibodies coupled with magnetic beads and MACS-technology. Purity of CMV_{NLV}-specific T cells was >95% in all cases.

Isolation of RNA and cDNA synthesis

RNA was isolated from CMV_{NLV}-specific T cells using the RNeasy Plus Mini Kit (QIAGEN) and cDNA-synthesis was performed using a Reverse Transcription system with Oligo(dT)-primers (Promega).

CDR3 spectratyping of V beta families

PCR fragments were amplified from cDNA derived from purified CMV_{NLV}-specific T cells for 24 V beta families (BV) and complementarity determining region (CDR3) spectratyping was performed as previously described [43,30]. The GeneScan 2.1 analysis software package (PE Applied Biosystems) was used to analyze the raw data utilizing the Local Southern method for fragment size estimation [30]. For each V beta family the occurrence of dominant clonal expansions was quantified by assigning scores for clonality and intensity as previously described [44]. Briefly, the clonality score (1= Gaussian distribution; 2= several peaks; 3= one peak) and the intensity score (0= < 500 RFU [relative fluorescence units]; 1= 500-3000 RFU; 2= 3000-8000 RFU; 3= >8000 RFU) were added and BV families with a total score of $\geq$5 were considered as predominant. This score equally weights monoclonal BV families with intermediate intensity (3+2) as well as oligoclonal BV families with high intensity (2+3). Both categories as well as monoclonal BV families with high intensity (3+3) were considered as predominant.

Bacterial cloning of TCR-sequences and sequence analysis

T cell receptor sequences of the BV8 and the BV13 families were amplified using forward primers specific for BV8 (5´-CGT TCC GAT AGA TGA TTC AGG-3´) or BV13 (5´-GTC GGC TGC TCC CTC CC-3´), respectively and a reverse primer (5´-CTG GGT CCA CTC GTC ATT CT-3´) located in the constant region of the TCR β-chain. PCR fragments were cloned into the pCR®-II-TOPO® vector (Invitrogen) via TA-cloning and the vector was transformed into chemically competent E. coli (One Shot TOP10, Invitrogen). Five positive clones were picked and plasmid DNA was extracted using standard procedures (QIAprep Spin Miniprep Kit, QIAGEN). TCR-sequences were determined by standard sequencing procedures (QIAGEN) and sequence data were analyzed using the Bioedit Sequence Alignment Editor 7.0.5.3.

4. Results

$CD8^+CD28^+$ and $CD8^+CD28^-$ T cells expand after stimulation with CMV_{NLV}-peptide

$CD8^+CD28^+$ (naïve/early memory) and $CD8^+CD28^-$ (effector) T cell populations were isolated and cultured in the presence of CMV_{NLV}-peptide and IL-2. CMV_{NLV}-specific T cells can be detected directly ex vivo using FACS-technology by staining with APC-coupled pentamers containing the CMV_{NLV}-peptide. Percentages of CMV_{NLV}-specific T cells are slightly higher in the $CD28^-$ effector population (4.79% ± 4.83) compared to the $CD28^+$ naïve/early memory population (2.62% ± 1.59). Upon cultivation and stimulation with CMV_{NLV}-peptide CMV_{NLV}-specific T cells within both populations proliferate and accumulate. After 14 days up to 90% of all viable cells are CMV_{NLV}-specific T cells in cultures started from the $CD28^+$ as well as the $CD28^-$ population indicating that both $CD28^+$ and $CD28^-$ T cells are capable to proliferate after antigenic stimulation in the presence of IL-2 (Figure 1A). In order to quantify the proliferative capacity for $CD28^+$ and $CD28^-$ T cell populations absolute T cell numbers and percentages of CMV_{NLV}-specific T cells were determined and the absolute number of CMV_{NLV}-specific T cells was calculated ex vivo and after 14 days in culture. The number of population doublings of CMV_{NLV}-specific T cells was determined. $CD28^-$ effector T cells proliferate slightly less than $CD28^+$ T cells (Figure 1B). CD28 expression is lost in a fraction of the $CD28^+$ population (5-33%). Reexpression of CD28 in the $CD28^-$ population is not observed during culture. Figure 1C shows expression of CD28 for the CMV_{NLV}-specific T cell population.

CMV-specific $CD8^+$ $CD28^+$ and $CD8^+$ $CD28^-$ T cells show the same V beta usage

CMV_{NLV}-specific T cells were further purified using APC-coupled pentamers and MACS-technology to achieve a purity of >95%. Spectratyping of the CDR3 (complementarity determining region 3) region of the T cell receptor (TCR) beta chain was performed as previously described [30,43]. The BV usage is very similar in expanded CMV_{NLV}-specific T cells derived from isolated $CD28^+$ or $CD28^-$ populations (Figure 2A). The BV families 8 and 13 are preferentially used as indicated by high relative fluorescence intensity (intensity score 2 or 3) and their TCR repertoire is highly restricted (clonality score=2 or 3). $CD28^+$BV8, $CD28^-$BV8 and $CD28^-$BV13 are monoclonal in 50% of the donors, $CD28^+$BV13 in 67% of the donors. Detailed analysis of the spectratypes revealed that the same individual peaks of identical size observed in $CD28^-$ CMV_{NLV}-specific T cells also occur in the $CD28^+$ population of the same donor (Figure 2B).

CMV-specific $CD8^+$ $CD28^+$ and $CD8^+$ $CD28^-$ T cells have the same TCR-sequences

To verify, that the same T cell clonotypes are present in the $CD28^+$ and the $CD28^-$ T cell populations the CDR3-region of the TCR of in vitro expanded and purified CMV_{NLV}-specific T cells was sequenced. TCR sequences of the BV8 and BV13 family were amplified from cDNA and were cloned into a bacterial vector. Plasmid-DNA was extracted and sequenced using standard procedures. Figure 3 shows the amino acid sequence of the antigen binding site for CMV_{NLV}-specific BV8 and BV13 T cells. In accordance with our previous data [44] the conserved sequence motifs “SVNEAF” and “SANYGYT” are detected within the BV8 family. In addition the related sequence motif “VVGGRYGYT” is also found. CDR3-sequences are also highly conserved in the BV13 family. The sequence motif “FQTGGYGYT” is found in 3 of the 4 donors. In addition the private BV13 clonotype “LSTGTSYGYT” is detected in one donor. In all cases dominant

clonotypes that are present in the expanded CMV_{NLV}-specific T cells derived from the $CD28^-$ population are also found in the $CD28^+$ fraction.

5. Discussion

$CD8^+$ T cells that lack the co-stimulatory molecule CD28 accumulate in elderly persons, particularly in those with latent CMV infection [14]. The accumulation of $CD8^+CD28^-$ T cells is associated with a decreased efficacy of vaccination [30] and is part of the immune risk phenotype, which is characterized by high $CD8^+$ and low $CD4^+$ cell numbers and poor proliferative responses of T cells to antigenic and unspecific stimulation [45]. CMV-seropositivity is also associated with the immune-risk phenotype [46].

$CD8^+CD28^-$ T cells are highly differentiated effector-memory T cells that lack important co-stimulatory receptors, such as CD27, CD28, CD126 and CD127, possess short telomeres and have alterations in TCR signaling [47,48,49,50,51]. They do not produce interleukin (IL)-2. $CD8^+CD28^-$ T cells display a potent cytotoxic potential but their proliferative capacity is limited [47,52]. Chronic antigenic stimulation and common γ-chain signaling mediated by IL-2 or IL-15 has been suggested to drive T cell exhaustion marked by the accumulation of $CD8^+CD28^-$ T cells [50,53]. We here demonstrate that CMV-specific $CD28^+$ and $CD28^-$ $CD8^+$ T cells can be efficiently expanded in vitro using autologous irradiated PBMC, CMV_{NLV}-peptide and IL-2. Proliferative capacity of the $CD28^-$ population is only slightly lower than of the $CD28^+$ population. These results are in concordance with previous reports showing that despite alterations in co-stimulation and TCR signaling, $CD8^+CD28^-$ T cells can still proliferate when stimulated in vitro with antigen and IL-2 or IL-15 [49], or antigen and 4-1BBL co-stimulation [54,55]. Similarly,

CMVpp65-specific $CD45RA^+CD27^-CD8^+$ T cells proliferate when stimulated by their cognate peptide in concert with either $CD4^+$ T cell help or IL-2, IL-15 or IL-21 [56].

We demonstrate that CMV_{NLV}-specific $CD8^+$ T cells derived from isolated $CD28^+$ or $CD28^-$ show the same overall BV usage. BV8 and BV13 are preferentially used by CMV_{NLV}-specific $CD8^+$ T cells as previously shown by us [44] and others [57,58,59,60,61,62]. Monoclonal expansions with the same CDR3 length additionally suggested the occurrence of identical clonotypes in the $CD28^+$ and $CD28^-$ T cells for both $BV8^+$ as well as $BV13^+$ cells. It has previously been shown that the repertoire of CMV-specific T cells is not altered by in vitro stimulation [58,59].

Sequence analysis confirmed this theory as the conserved CDR3 sequence motif “SANYGYT”, which has been identified as a dominant clonotype in healthy and HIV-infected adults [59,60,61,62], and “SVNEAF”, which has been described as a private clonotype in young adults [61,62], but is public in elderly donors [44] occur with similar frequency in BV8 cells whether they are $CD28^+$ or $CD28^-$. The CDR3-sequence motif “VVGGRYGYT” was found in the $CD28^+$ as well as the $CD28^-$ T cells of one additional donor again demonstrating that dominant clones are equally likely to expand from both populations upon stimulation. So far, a variety of different sequences has been described for the CDR3-region of CMV_{NLV}-specific $CD8^+$ T cells of the BV13 family, without the occurrence of a dominant clonotype [57,60,61,62]. However, in our study we clearly identify a dominant clonotype within the BV13 family (“FQTGAAYGYT”). This sequence is shared by 3 of 4 donors and the one private clonotype identified also shares the last 4 amino acids (YGYT). As for the $BV8^+$ T cells these clones occurred with identical frequency in the CMV_{NLV}-specific populations generated from either $CD28^+$ or $CD28^-$ cells. The so far not described predominant occurrence of a public CMV_{NLV}-specific clonotype in the $BV13^+$ cells may

reflect the restriction of the $CD8^+$ T cell repertoire in old age. It seems reasonable to assume that other peptides with different HLA-restriction show similar properties.

$CD28^-$ T cells and particularly $CD28^-$ CMV-specific T cells are believed to be detrimental. CMV-specific $CD8^+$ T cells from elderly donors exhibit a terminally differentiated phenotype and frequently occur as large expanded clones. It has been postulated that CMV-specific effector T cells occupy a lot of immunological space and are inhibitory for T cells of other specificities as the number of EBV-specific T cells increases with age only in CMV-seronegative but not in CMV-seropositive individuals [35]. Elimination of these cells might be useful [40] in order to generate space for more “useful” T cells. However, it is crucial that an efficient CMV-specific T cell response is maintained in order to control the virus. We show that the TCR-clonotypes found in the $CD28^-$ population are generally also present in the $CD28^+$ population. This demonstrates that the same T cell clonotype occurs at different differentiation stages at the same time, as previously shown also for $CD4^+$ T cells [63] , and that $CD28^-$ cells do not seem to contain clones not present in the residual population. This observation is corroborated by recent work performed by Iancu et al. [64]. Elimination of $CD28^-$ T cells in order to create more immunological space and consequently to allow the regeneration and rejuvenation of the aged T cell system would thus not lead to the loss of important specificities. Viral control would be intact due to the recruitment and propagation of the relevant clones from naïve or early memory T cell populations. Although we realize that this knowledge is unlikely to be translated into clinical practice in the near future, it may still be of relevance later on when due to steadily increasing life expectancy immunological exhaustion is becoming a realistic threat.

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Figure legends

Figure 1: Accumulation of CMV_{NLV}-specific T cells after 14 days of culture

A T cells were stained with PerCP–conjugated anti-CD8 antibody and APC-conjugated pentamers containing the CMV_{NLV}-peptide. A representative example is shown for the $CD8^+CD28^+$ and the $CD8^+CD28^-$ T cell fraction of one donor directly ex vivo and after 14 days of culture. Percentages of CMV_{NLV}-specific T cells are indicated.

B Population doublings of $CD8^+CD28^+$ and $CD8^+CD28^-$ CMV_{NLV}-specific T cells. Shown are mean values and standard error of mean (n=6). Population doublings were calculated as described in Materials and Methods. Significance was determined by Student´s t-test.

C T cells were stained with APC-conjugated CMV_{NLV}-pentamers, PerCP–conjugated anti-CD8 and PE-conjugated anti-CD28 antibodies. Gating on CMV_{NLV}-specific T cells was performed. A representative example is shown for the $CD8^+CD28^+$ and the $CD8^+CD28^-$ T cell fraction of one donor directly ex vivo and after 14 days of culture. Percentages of $CD28^+$ (left panel) or $CD28^-$ (right panel) of CMV_{NLV}-specific T cells are indicated.

Figure 2: Preferential expansion of BV8 and BV13 in $CD8^+$ T cells after cultivation with CMV_{NLV}-peptide

A Spectratyping of expanded, purified CMV_{NLV}-specific T cells was performed from PCR-products for 24 individual V beta families for 6 donors. Clonality and intensity scores were

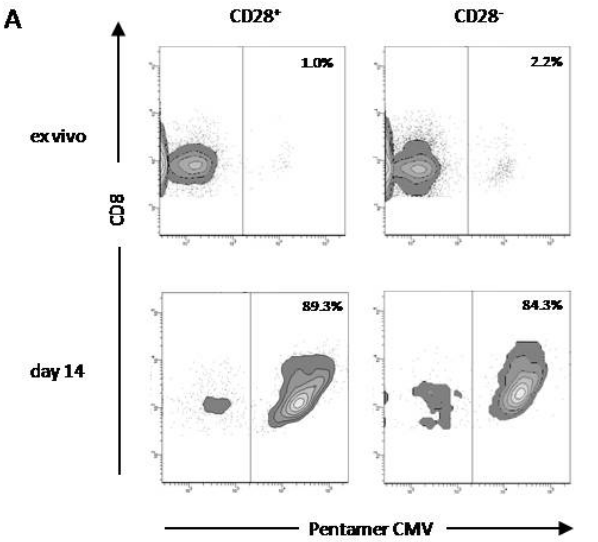
determined as previously described [44] and for each BV family the percentage of donors with a total score ≥5 is shown for the $CD28^+$ (black) and the $CD28^-$ (gray) T cell population.

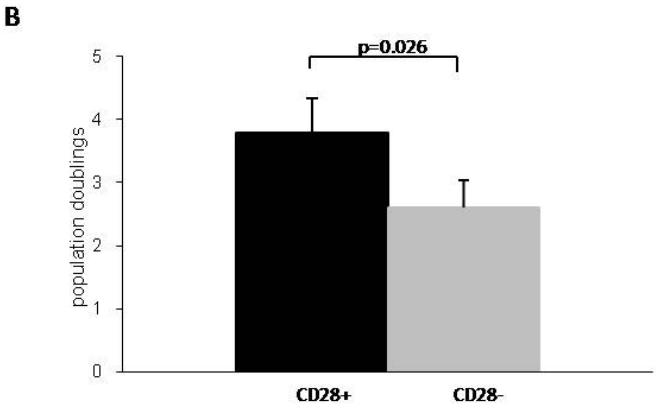
B Representative spectratyping result of the BV8 and the BV13 family for one donor. The same peaks can be observed in the $CD28^+$ (upper panel) and the $CD28^-$ (lower panel) population. The relative size of the CDR3-region is indicated below the peaks.

Figure 3: Sequence analysis of BV8 and BV13 CMV_{NLV}-specific $CD8^+$ T cells derived from isolated $CD28^+$ and $CD28^-$ T cells

PCR-products of BV8 and BV13 T cell receptor sequences were generated from cDNA of four donors and cloned into a bacterial vector. Five clones were picked for each donor and BV family and were sequenced. Predominant clonotypes within the $CD28^-$ population are shaded gray and the corresponding sequences are also marked gray for the $CD28^+$ population.

Figure 1


A
CD28+
CD28-
ex vivo
day 14
CD8
1.0%
2.2%
89.3%
84.3%
Pentamer CMV


B
p=0.026
population doublings
0
1
2
3
4
5
CD28+
CD28-

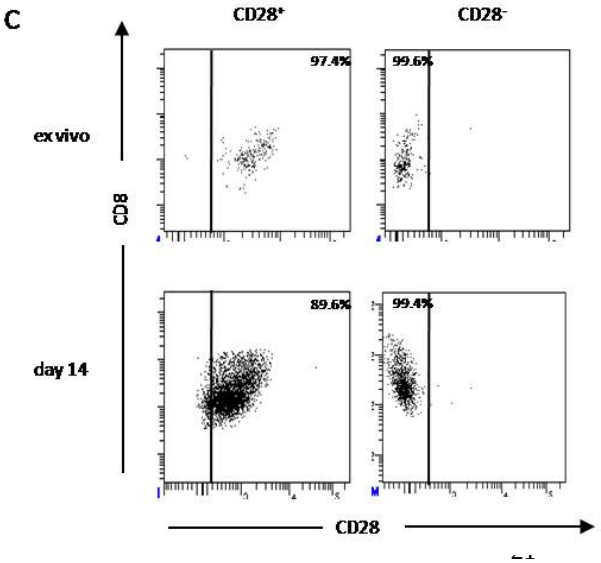
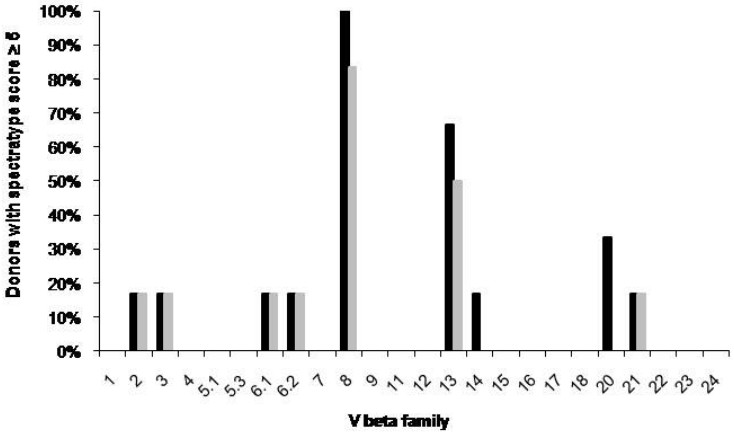

C
CD28+
CD28-
ex vivo
day 14
CD8
97.4%
99.6%
89.6%
99.4%
CD28

Figure 2

A


Donors with spectratype score ≥ 5
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5.1
5.3
6.1
6.2
7
8
9
11
12
13
14
15
16
17
18
20
21
22
23
24
V beta family

B

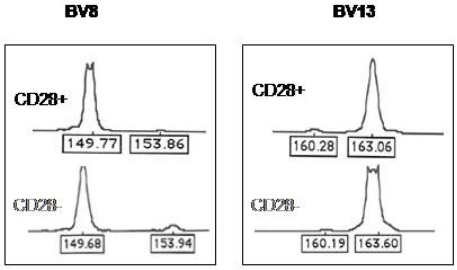

BV8
BV13
CD28+
149.77
153.86
CD28-
149.68
153.94
CD28+
160.28
163.06
CD28-
160.19
163.60

Figure 3

	BV8			BV13	
	CD28+	CD28−		CD28+	CD28−
1	CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ	CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ CASS~~**SVNEAF**~~~~~~~FGQ		CASS~~**LSTGTSIGIT**~~~FGS CASS~~**LSTGTSIGIT**~~~FGS CASS~~**LSTGTSIGIT**~~~FGS CASS~~**LSTGTSIGIT**~~~FGS	CASS~~**LSTGTSIGIT**~~FGS CASS~~**LSTGTSIGIT**~~FGS CASS~~**LSTGTSIGIT**~~FGS CASS~~**LSTGTSIGIT**~~FGS CASS~~**YNRNTEAF**~~~~FGQ
2	CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS	CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS CASS~~**VVGGRIGIT**~~~~FGS		CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS	CASS~~**FQTGAAIGIT**~~FGS CASS~~**LQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS
3	CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS	CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS CASS~~**SANIGIT**~~~~~~FGS		CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS	CASS~~**YDKQQLGF**~~~~FGQ CASS~~**LSTGTSIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS
4				CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CASS~~**FQTGAAIGIT**~~~FGS CAST~~**THWGTGSYERI**~~FGP	CASS~~**FQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS CASS~~**FQTGAAIGIT**~~FGS