Cartography of gene expression in CD8 single cells: novel CCR7– subsets suggest differentiation independent of CD45RA expression

Running head: Gene expression profile of CD8+ T-cell populations

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Abstract

Understanding the distribution, function and lineage relationship of CD8+ T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations. However, the available data concerning the description of effector and memory CD8+ subsets in humans remains rather fragmentary since different studies favored the usage of distinct and restricted sets of cell-surface markers and functional parameters. We associated multiple markers to subdivide CD8+ T cells into fourteen different cell types several of which were not described previously, and evaluated the co-expression of eighteen genes simultaneously in individual cells from each subset. Our results show that each subset has a defined pattern of gene expression. Moreover, effector gene expression of CCR7- cells correlated only to CD27 expression levels and CD27/CD28 co-expression, but not with CD45RA/R0 phenotypes. Our findings thus describe new CD8+ cell subsets, allow the identification of relatively homogeneous CD8+ subpopulations, provide a predictable and precise correlation between particular cell-surface markers and CD8+ T-cell functional properties and identify effector cells present in both CCR7−CD45RA+ and CCR7−CD45R0+ compartments. The results also indicate that activated cells might modulate the expression of CD45RA/R0 asynchronously, rather than CCR7−CD45RA+ cells always issuing from CD45RA− precursors.
Introduction

CD8+ T lymphocytes play a key role in defense against cytosolic pathogens and tumors. Understanding the mechanisms through which the immune system controls such pathological situations to avoid disease depends upon the thorough characterization of all CD8+ T subpopulations and differentiation stages, from naïve precursors to fully mature effectors. For that purpose, the CD8+ T-cell compartment was subdivided into several different subsets with distinct properties. In humans, it has been established that expression of the lymph node homing receptor CCR7 can be used to separate both CD4+ and CD8+ CD45RA− T cells into two functionally distinct subsets: CCR7+CD45RA− and CCR7−CD45RA−, named “central memory” (T_{CM}) and “effector memory” (T_{EM}), respectively. Unlike CD4+ T cells, an additional CCR7− subset that expresses CD45RA (T_{EMRA}) can also be found in the CD8+ compartment. Since this population harbors cells expressing high perforin levels, it was suggested that the T_{EMRA} subset should correspond to a population of terminally differentiated CD27− effector cells, previously described by Hamann and collaborators1. These cells display a Vβ repertoire significantly different from naïve cells, containing oligoclonal expansions of particular TCR Vβ elements and also have shorter telomeres, suggesting that CD45RA−CD27− cells have been selected in vivo through antigen stimulation and evolved through extensive rounds of division2. The same authors had proposed an alternative classification for CD8+ T lymphocytes in which CD45RA and CD27 expression was used to identify naïve (CD45RA+CD27+), memory (CD45RA−CD27+) and effector (CD45RA−CD27−) CD8+ T cells in humans1. This classification, however, underestimates the complexity of the memory CD8+ T-cell subset revealed by the expression of CCR7. For instance, memory cells defined by the CD45RA−CD27+ phenotype would include both T_{CM} and T_{EM}, which were shown to enclose distinct functional specializations3,4. Furthermore, virus-specific CD27−CD8+ T cells may also express CD45RA5. Heterogeneity of the effector/memory compartments was shown to be further extended to CD28 differential expression. CD8+ T cells specific for several persistent human viruses were extensively characterized regarding their surface phenotype, perforin and granzyme A expression and ex vivo cytotoxic capacity. Based on these data, it was shown that co-expression of CD27 and CD28 could be used to distinguish three functionally different subsets of CD8+ T cells according to the progressive expression of effector functions: early (CD27−CD28+), intermediate (CD27−CD28+) and late (CD27−CD28−) differentiated cells5. This classification reflects, however, the differentiation status of antigen-experienced CD8+ T
cells, rather than discriminating effector and memory cells. Moreover, CD27 and CD28 expression does not allow distinguishing T<sub>CM</sub> from T<sub>EM</sub>, nor T<sub>EM</sub> from T<sub>EMRA</sub>. The prevailing data concerning the description of naïve, effector and memory CD8<sup>+</sup> T-cell populations in humans remains, thus, rather fragmentary. Manifestly, analysis of CD8<sup>+</sup> T cells including solely two or three parameters are not sufficient to reveal the whole heterogeneity of the antigen-experienced CD8<sup>+</sup> lymphoid compartment. The compound subsets are not clearly established, especially within the T<sub>EM</sub> and T<sub>EMRA</sub> compartments, and the correspondent differential roles and lineage relationships remain undisclosed. Interestingly, in different human chronic viral infections, as EBV, CMV, hepatitis C virus (HCV) and HIV-1, virus-specific CD8<sup>+</sup> T cells display distinct predominant phenotypes<sup>5-9</sup>. However, it is not clear whether the lack of certain CD8<sup>+</sup> T subpopulations results from a specific virus-induced blockage in differentiation or, instead, is a consequence of different clinical settings<sup>4,10</sup>. Thus, understanding the distribution, function and relationship of the CD8<sup>+</sup> T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations.

The present study aims to describe thoroughly the heterogeneity of the human CD8<sup>+</sup> T-cell compartment by establishing an accurate correlation between cell-surface phenotype and functional properties of each subset. In particular, this study characterizes the circulating human CD8<sup>+</sup> T-cell populations based on the simultaneous association of CCR7, CD45RA, CD27, CD28, CD11a and CD62L cell-surface markers. The association of these particular markers allowed the identification of 14 different CD8<sup>+</sup> T-cell subsets, some of which never previously described. We isolated individual cells of each subset and studied in each cell the expression of 18 different mRNAs coding for cytokines, chemokines, cytotoxic molecules and several receptors. This work describes new CD8<sup>+</sup> T-cell subsets, identifies homogeneous CD8<sup>+</sup> T cell sub-populations and allows a predictable correlation between cell-surface phenotype and <i>in vivo</i> function. Moreover, it reports the presence of effector cells both in T<sub>EM</sub> and T<sub>EMRA</sub> subsets and suggests an asynchronous modulation of CD45RA/CD45R0 expression after priming, rather than CCR7<sup>-</sup>CD45RA<sup>+</sup> cells always issuing from CD45RA<sup>-</sup> precursors.
Methods

Isolation of peripheral blood cells

Heparinized venous blood was obtained from healthy volunteers of both sexes with ages ranging from 22 to 56 years old, at Etablissement Francais du Sang (EFS) after informed consent following French ethical recommendations. PBMCs were isolated by centrifugation on Histopaque®-1077 Hybri-Max® density gradient (Sigma) and CD8+ T lymphocytes obtained using the Dynal® CD8 Negative Isolation Kit, which includes anti-CD4, CD14, CD16 (a and b), CD19, CD36, CD56, CDw123 and glycophorin A depleting antibodies. CCR7 depletion was performed by removing CCR7-labeled cells (R&D Systems) with anti-IgG Dynabeads® (Dynal). Resulting CCR7-CD8+ T cells were >98% pure.

Antibodies and reagents

Anti-human antibodies used were: fluorescein (FITC)-labeled anti-CCR7 (R&D Systems); phycoerythrin (PE)-labeled anti-CD27, allophycocyanin-cyanin 7 (APC-Cy7)-labeled anti-CD8α, biotinilated anti-CD27 and anti-CD28-PE-Cy7 (Ebiosciences); FITC-labeled anti-CD3 and CD8β-PE (Caltag); streptavidin-PE-Cy7, peridinin-chlorophyll-protein complex (PerCP)-Cy5.5-labeled streptavidin, CD62L-FITC, αβ-TCR-FITC, CD45R0-FITC, CD11a-PE, anti-CD4-biotin, CD28-biotin, γδ-TCR-biotin, CD3-PerCP-Cy5.5 and CD45RA-APC (Pharmingen).

Cell sorting and flow cytometry

Cells were double-sorted using a FACS Vantage upgraded to DiVa configuration and equipped with an automatic cell deposition unit (Becton Dickinson). Single-cells were collected as described. Cytofluorometric analysis was performed in a BD-LSR I flow cytometer.

Primers and quantitative multiplex RT-PCR

Procedures, primers and quantitative analysis for the simultaneous amplification of multiple genes in single cells were performed as described. The efficiency of amplifications for each gene and for each set of primers was calculated and proven to be maximal and uniform for all the genes. Competition was assessed and no interference was detected between the different primers and/or amplicons during multi-gene amplification.
Results

**Phenotypic characterization of the CD8⁺ T-cell subsets from the human peripheral blood**

We isolated αβ-TCR CD8⁺ peripheral blood lymphocytes (PBLs) after depletion of other minor cellular sets that can express CD8. The use of anti-CD56 and anti-CD16 in a depletion step should remove NK cells. We further ensured that our purified population did not contain NK cells by assessing the expression of CD3ε mRNA in all individual cells we studied (see below). Our depletion strategy should also remove the vast majority of NK-T cells since CD56− NK-T cells represent less than 0.09% of lymphocytes. We found that our purified CD8⁺ subset contained more than 99.9% αβ-TCR cells, whereas only 0.2 to 0% stained with anti-γδ-TCR (data not shown).

We subdivided CD8⁺ T-cell populations into four major subpopulations based on the expression of CCR7 and CD45RA, as reported (Figure 1A). Since CD45RA/R0 phenotypes are mutually exclusive, the populations labeled with CD45RA were the mirror image of CD45RO− sets and vice-versa (not shown). The co-expression of CD27, CD28, CD11a (the alpha chain of LFA-1), CD8β and CD8α were further evaluated within each subset. Both T N (CD45RA⁺CCR7⁺) and T CM (CD45RA⁻CCR7⁻) were quite homogeneous. Most co-expressed all these additional markers (Figure 1B), as well as CD8α and CD8β chains (not shown). In contrast, CCR7− subpopulations were very heterogeneous. Both T EM (CD45RA⁻CCR7⁻) and T EMRA (CD45RA⁺CCR7⁻) compartments contained T cells co-expressing CD28 and CD27 (double-positive, DP); expressing either one of these markers (single-positive, SP); or expressing none (double-negative, DN). Rare CD8α⁺β⁻ cells could be found within the CCR7− compartment, but they did not show preferential distribution amongst CD27/CD28 subsets (not shown). All these subsets were present in every donor, had the same characteristics in different donors (see below), but their frequency varied between individuals (Table 1).

*In vitro* activation of CD8⁺ T cells induces the down-regulation of CD27 and CCR7 and up-regulation of CD28 and CD11a. Accordingly, T N cells had the highest levels of CD27 and CCR7 (Figure 1A,C), as compared to T CM, while T EM⁻ and T EMRA-27SP expressed even lower levels of CD27 than T CM. CD28 and CD11a followed the opposite trend: CD28 expression was lower in T N than in all subsets of primed cells expressing this molecule and CD11a was up-regulated from T N to T CM < T EM < T EMRA (Figure 1D,E and Table 2).
However, within the T EM and T EMRA, cells expressing CD27 and/or CD28 (DP or SP) displayed similar expression levels of these markers, as well as identical high levels of CD11a (Figure 1). Therefore, CD27, CD28 and CD11a expression levels confirm the putative differentiation hierarchy T N < T CM < CCR7 CD8+ T cells, but do not allow further discrimination within the complex CCR7- compartment.

Besides these major subsets, we also identified minor CCR7-CD8+ T-cell subsets expressing high levels of CD27 (CD27\textsuperscript{high}) (Figure 1B, Table1). These subsets were present in both T EM and T EMRA compartments and included mostly CD27-SP cells, but could also harbor DP cells (Figure 1B). Independently of their additional CD28 or CD45RA phenotypes, the CD27\textsuperscript{high} cells expressed CD11a at higher levels than naïve or T CM cells (Table 2). In striking contrast to the other CD8+ subpopulations, the presence of CD27\textsuperscript{high} cells in the blood appeared to be transitory, as the representation of these subsets in the same donor varied significantly with time. It was previously reported that CD27 could be transiently up-regulated shortly after in vitro activation\textsuperscript{16}. The high CD11a expression and their transitory presence in the blood suggest CD27\textsuperscript{high} cells may be recently activated CD8+ T cells.

\textit{Correlation of CD62L expression with the CD27/CD28 phenotype}

CD62L plays a fundamental role on the migration of lymphocytes to secondary lymphoid organs. While T N and T CM CD8+ T cells are consistently CD62L+\textsuperscript{3}, only a fraction of CCR7- cells expresses this adhesion molecule\textsuperscript{3}. We have further investigated if CD62L expression was related to peculiar CCR7\textsuperscript{-}CD8+ T-cell subtypes (Figure 1F). We found a correlation to CD27/CD28 expression, but no differences between T EM/T EMRA subsets. Thus, independently of their CD45RA phenotype either CD27\textsuperscript{high}, DP and CD28SP cells contained abundant CD62L+ cells. CD27SP populations usually had low levels CD62L and in DN cells CD62L expression was even lower. It is unlikely that such low expression as found in DN cells might be sufficient to ensure migration to the lymph nodes, since we could never detect DN CD8+ T populations in lymph node cells (unpublished data).

Altogether, these observations revealed that T N and T CM are homogeneous populations with respect to all additional markers, whereas T EM and T EMRA are significantly heterogeneous, containing multiple subpopulations that likely cross a large spectrum of effector differentiation.

\textit{CD8+ T-cell subpopulations heterogeneity evaluated at a single-cell level}
The further addition of CD27 and CD28 to previous established CD45RA and CCR7 cell surface markers subdivided the CD8+ T-cell compartment into fourteen different subsets: T_N and T_CM (Figure 1), and 12 distinct CCR7⁻ subsets (Table 1). Whether this subdivision is sufficient to fully identify homogeneous populations with similar functional properties or if each of the individual CD8+ T-cell subsets can be yet heterogeneous, harboring cells with multiple functional potentialities is not known. To evaluate the homogeneity of CD8+ T-cell populations, we envisaged to isolate individual cells from each subpopulation and studied in each cell the simultaneous expression of genes coding for inflammatory chemokines, cytokines, cytotoxic molecules and several receptors described to be involved in CD8+ T-cell responses. To ensure the accuracy of single-cell sorting eliminating the possibility of including other cellular contaminants in the study, we have purified αβ-TCR CD8+ T lymphocytes prior to sorting, and further assessed in each cell the presence of the mRNA coding for CD3ε. Thus, the data presented here correspond exclusively to CD8+ T cells expressing the CD3ε mRNA.

We found that some genes (IL-2, IL-10 or MIP-1α/CCL3) were expressed in such low frequencies (less than 5%) that their impact on functional profiles could not be analyzed at single-cell level. Also some of these sub-populations were so rare (less than 0,05% of CD8 cell sets) that could not be sorted reliably. For this reason, we failed to characterize CD27high- DP cells and CD28-SP of the T_EMRA compartment. In addition, we could only collect T_EM CD28-SP from one single donor. For all remaining 11 populations we have characterized fifteen parameters in each individual cell in three independent donors. A representative donor is shown in Figures 2, 3.

**T_N cells lack effector functions, but express several receptors types**

As expected, the less activated CD8+ T-cell set was T_N (Figure 2A). These cells did not express mRNAs coding for chemokines, cytotoxic molecules or effector cytokines, such as TNF-α or IFN-γ. However, a small fraction of cells (<15%) generally expressed TNF-β (coded by the lta gene). In addition, about 40-50% of the cells expressed TGF-β1 and most expressed TGF-β receptor 2 (TGF-βR2). In addition to TGF-βR2, TGF-βR1 expression is also required for TGF-β1-induced signaling to occur. Co-expression of TGF-βR1 and TGF-βR2 was detected in more than 30% of the naïve cells. T_N population also contained the
highest frequency of cells expressing IFN-γR2, which determines the responsiveness to exogenous IFN-γ, and approximately one half of the cells expressed interleukin IL-10Rα (Figure 2A).

Cells expressing CD27\textsubscript{high} display the gene expression pattern more closely related to T\textsubscript{N} cells

Surprisingly, the CD8\textsuperscript{+} subpopulations most resembling T\textsubscript{N} were the T\textsubscript{EM}-CD27\textsuperscript{high} and T\textsubscript{EMRA}-CD27\textsuperscript{high} subsets (Figure 2B). These populations expressed TGF-β1 at the same frequency as naïve cells and TNF-β was expressed in a slightly lesser extent. In contrast, IFN-γR2 was no longer expressed, a finding we observed in all CCR7–CD8\textsuperscript{+} T-cell subsets. IL-10Rα frequencies increased up to 75% and perforin and granzymes were expressed in average by 20% and 10% of the cells, respectively. However, expression of perforin and either granzyme A (GZMA) or B (GZMB) was detected in separate cells, suggesting that cells expressing CD27\textsuperscript{high} are not cytotoxic. Remarkably, the mRNA coding for the inflammatory chemokine RANTES (also known as CCL5) was detected in 45-70% of CD27\textsuperscript{high} cells. Importantly, T\textsubscript{EM} and T\textsubscript{EMRA} CD27\textsuperscript{high} subsets had indistinguishable gene expression profiles, suggesting they might play similar functional roles in vivo.

T\textsubscript{CM}: the memory subset expressing the fewest effector functions

T\textsubscript{CM} cells, although expressing CCR7, displayed higher frequency of effector genes than CCR7–CD27\textsuperscript{high} subpopulations (Figure 2C). Three evident differences were noticed. First, the mRNA coding for GZMA was up-regulated, since it was expressed in up to 40% of the cells. However, only a very modest percentage of T\textsubscript{CM} cells (<15%) co-expressed this molecule along with perforin, indicating that only a small fraction of these cells can be cytotoxic. Secondly, expression of RANTES was also up-regulated and could be detected in more than 70% of the cells. Finally, expression frequency of the IFN-γR2 was down-regulated being detected but in a small fraction of cells. It must be noted that only T\textsubscript{N} and T\textsubscript{CM} subsets do express IFN-γR2 mRNA, all other sets of activated T cells lacking this molecule.

T\textsubscript{EM} and T\textsubscript{EMRA} harbor three hierarchically differentiated subsets
The gene expression pattern of all CCR7− subpopulations, with the exception of the previously described CD27high subsets, shows a degree of functional differentiation significantly higher than TCM cells (Figure 3). This was revealed by increased expression of RANTES, which is consistently expressed in more than 90% of the cells, perforin, GZMA and IL-10Rα, together with further expression of additional molecules.

Within CCR7− cells, the DP cellular subsets were those more closely resembling the TCM subset. As compared to TCM cells, virtually all TEM and TEMRA-DP now expressed RANTES, GZMA and IL-10Rα, and perforin was expressed in much higher frequencies ranging from 50 to 80% (Figure 3A). In a fraction of these cells, we detected for the first time expression of Fas-L (6-20%) and macrophage inflammatory protein (MIP)-1β, also named CCL4 (14-40%). Only very rare cells could score positive for IFN-γ or GZMB. Strikingly, the patterns of gene expression of TEM and TEMRA-DP populations were nearly overlapping, the sole difference concerning a slight increase on the frequencies of perforin and FAS-L expression in TEM-DPs. We were able to isolate CD28SP cells from a single donor and solely of the TEM compartment. Interestingly, this subset displayed a pattern of gene expression very similar to the DP subpopulations (Figure 3B), suggesting a close relationship.

The TEM and TEMRA CD27-SP subsets were more differentiated than the DP subsets (Figure 3C). Indeed, in addition to the genes already expressed at high frequency by DP cells, GZMB expression was up-regulated (10-50%), while the frequency of Fas-L expression shows a less striking increase (15-25%). Once again, we were surprised to notice an evident overlap between the expression patterns of TEM and TEMRA-CD27-SP subsets that was extended to all the molecules we have studied.

TEM and TEMRA-DN CD8+ T cells displayed the most differentiated gene expression profile (Figure 3D). Now, near all cells expressed perforin and GZMA, GZMB was expressed by most cells and Fas-L expression frequencies increased. In some donors, IFN-γ expression was also up-regulated, being detected in up to 30% of the cells. Again, we found no difference in gene expression profiles between TEM and TEMRA-DN CD8+ T cell sets.

Thus, the results obtained by single-cell multiplex RT-PCR clearly depict a hierarchy of T-cell differentiation status in antigen-experienced cells. Importantly, this hierarchy is defined by CCR7 expression, CD27 expression levels and CD27/CD28 co-expression, but does not correlate with expression of CD45RA.
Gene expression of TEM and TEMRA compounding subsets is similar at quantitative level

Since the gene expression profiles of all CCR7− T-cell subsets correlated to CD27/CD28 expression but not to CD45RA+/CD45RA− phenotype, we next investigated if we could distinguish TEM and TEMRA cell sets by a different amount of expressed mRNA molecules. This approach was possible because in the methodology used here the efficiency of reverse transcription was evaluated11. Therefore, we were able to directly quantify the number of mRNA molecules coding for distinct genes expressed by each individual cell. Moreover, our methodology also uses PCR reactions of identical efficiency for all genes, allowing the comparison of different gene expression levels11. We have thus quantified the expression of all genes in individual cells from different populations. As expected, not all the genes were expressed at the same level. Perforin and MIP-1β had the lowest number of mRNA molecules per cell, while RANTES and the receptors for TGF-β and IL-10R displayed the highest expression levels (not shown). Nevertheless, each gene was expressed quite similarly in all CCR7− CD27/CD28 cell subsets. This is exemplified for three different cells from each population and for two of the genes in Figure 4A. Moreover, gene expression levels were equivalent in TEM and TEMRA, since no significant differences for any of the genes were found when comparing the number of gene-specific mRNA molecules expressed per cell in TEM versus TEMRA subpopulations (Mann-Whitney test, p>0.05). In addition, in all genes we found an important correlation between the number of mRNA molecules per cell in TEM and TEMRA compounding subsets, which clearly illustrates that the mRNA expression levels of TEM and TEMRA cells have the same range and the same distribution (Figure 4B and data not shown). We thus conclude that CD45RA expression cannot discriminate CCR7−CD8+ T-cell subtypes neither at a qualitative nor at a quantitative level.

T-cell populations of the same phenotype have the same characteristics in different individuals

To evaluate if cell-surface phenotypes always correlated to peculiar functional profiles, we compared the gene expression profiles of three individuals. We found that within each phenotype, gene expression profiles were remarkably similar between all donors. Due to figure number and size limitations, we show in Figure 5 the pattern of gene expression from a single additional donor, focusing on the cytotoxic molecules and chemokines, since these molecules better define the signature of each CD8+ subtype. Comparison of the expression
profiles of different donors (Figures 3,4 and Figure 5) shows that each subpopulation has the same characteristics. Again, the profile of gene expression of CCR7− subpopulations correlates only to CD27/CD28 phenotype, with T_{EM} or T_{EMRA} cells of the same CD27/CD28 phenotype displaying the same characteristics. In conclusion, each cellular subset has equivalent characteristics in different donors.
Discussion

The human CD8+ T-cell compartment encloses several subpopulations with multiple functionalities, including naïve, effector and memory subsets. The prevailing data describing these subsets in the peripheral blood is unclear in several aspects mainly due to multiple analyses of CD8+ T cell subpopulations using different and limited sets of surface markers and functional properties. The present study aims to elucidate the ambiguous and missing data concerning the heterogeneity of the human CD8+ T-cell compartment. For this purpose, we used two approaches. Firstly, we performed a detailed characterization of the cell-surface phenotype of the circulating CD8+ T cell populations ex vivo, based on the simultaneous association of the most common and relevant cell-surface markers described in the literature, namely CCR7, CD45RA, CD27, CD28, CD62L and CD11a. These molecules are widely used to identify CD8+ T-cell subsets, but they are usually only partially associated1,3-5,14. The concurrent association of all these cell-surface markers allowed the identification of multiple CD8+ T cell subsets, several of which were never described previously. Importantly, we could directly compare the primed subsets within the CD45RA+ and CD45RA− compartments, whose precise differential functions remained unclear thus far. To ensure that the single cells we studied corresponded to classical αβ-TCR lymphocytes, only cells expressing the CD3ε mRNA were integrated in this study. A previous depletion step using a cocktail of antibodies that included CD4, CD16, CD19 and CD56 issued a population consisting of >99,9% αβ-TCR+ cells, where the vast majority of NK-T cells should also be absent. It remains however the possibility that very rare CD56− NK-T cells expressing CD8αα were still present in our subsets. Although we found some T cells expressing CD8αα in the CCR7− subsets of some donors, they were distributed similarly amongst the different subsets. Moreover, CD56− NK-T cells were described to constitute less than 0,09% of all lymphocytes13. Considering we study populations by characterizing individual cells, such rare cells could not have an impact on the general gene expression profiles we define here.

The second strategy concerned an approach to evaluate the heterogeneity of each one of these cellular subsets. We studied individual cells in each population and each cell was characterized for the expression of 18 different mRNAs involved in T-cell functions. Single-cell gene expression analysis allowed the assessment of functional heterogeneity inside each cellular subset and gave important insight concerning the differential function and differentiation of the various subpopulations. Furthermore, we also found that each one of
these particular phenotypes corresponded to specific patterns of gene expression, since each one displayed reproducible gene expression patterns in all the donors studied. Hence, the phenotypes we here describe apparently can be used to predict defined characteristics in CD8+ subpopulations in normal healthy individuals.

Our results show that the combination of CCR7, CD45RA, CD27 expression levels and CD28 permits to discriminate 14 CD8+ T-cell subsets. With the exception of CD27<sup>high</sup> cells, which display characteristics of recently activated populations, all remaining subsets could be found in all donors, albeit with different representations.

At single cell level, each cellular subset displayed a characteristic pattern of gene expression. In CCR7<sup>-</sup> cells, this pattern strongly correlated to expression of both CD27 and CD28, following a hierarchy of differentiation: CD27<sup>high</sup> < DP < CD28-SP < CD27-SP < DN. Surprisingly within each of these subsets, T<sub>EM</sub> subpopulations showed the same gene expression patterns, at both qualitative and quantitative level, as their counterparts T<sub>EMRA</sub>. These findings contradict the paradigm that T-cell differentiation necessarily leads to CD45RA loss, and that further maturation induces CD45RA re-expression in such way that effector cells should be present only in the T<sub>EMRA</sub> compartment. They rather suggest that, instead of T<sub>EMRA</sub> cells forcedly differentiate from T<sub>EM</sub> precursors, CD8<sup>+</sup> T cells may modulate or not CD45R0/RA expression after activation and/or may transit from a CD45RA to CD4R5R0 phenotype, and vice-versa. Indeed, we clearly identified fully differentiated cells co-expressing multiple “killer” genes in both CD45RA<sup>+</sup> and CD45RA<sup>-</sup> populations. Our results concerning the CD27<sup>high</sup> subpopulations also support that CD45RA isoform can be maintained after T cell activation. It has been previously reported that CD27 is transiently up-regulated after T-cell activation in vitro, the peak expression level occurring by 24 hours<sup>19,20</sup>. Our results strongly support that CD27 is also up-regulated following in vivo activation, since CD27<sup>high</sup> cells exhibit characteristics of recently activated cells: they were hardly detectable in some donors and their frequency in the same donor was not stable, suggesting that they may disappear with time. Furthermore, the gene expression profile of these CD27<sup>high</sup> subpopulations was very close to that of naïve cells. As major difference, an important fraction of CD27<sup>high</sup> cells expressed RANTES, a gene reported to be induced relatively early following in vitro activation and before cytotoxic genes. It was described to be already up-regulated 3 to 5 days after T cell activation<sup>21-23</sup>. However, “recently activated” CD27<sup>high</sup> cells with the same characteristics could be found in both T<sub>EM</sub> and T<sub>EMRA</sub> populations, indicating that primed populations may maintain CD45RA expression after activation. Other independent evidence suggests T<sub>EMRA</sub> populations can derive directly from naïve cells, since
their replication history may approach that of naïve T cells\textsuperscript{14}. It is possible that besides CD45R0/RA, activated T cells may alternatively maintain or lose CD28/CD27. We could detect recently activated CD27\textsuperscript{high} cells that co-expressed both molecules, as well as primed cells that were CD28-SP cells.

Since our results argue against the model of a mandatory T\textsubscript{EMRA} origin from T\textsubscript{EM} cells, it is adequate to review the experimental evidences leading to this notion. The CD45R0 phenotype was believed to be characteristic of primed cells, but the detection of CD45RA\textsuperscript{+}CD8\textsuperscript{+} cells with all characteristics of effector cells forced to review this issue. \textit{In vivo} analysis of antigen specific cells for persistent human viruses, commonly HCMV and EBV, showed that at early time points of acute infections epitope-specific CD8\textsuperscript{+} T cells were prevalent in the CD45R0\textsuperscript{+} subset, but in the chronic phase both CD45RA\textsuperscript{+} and CD45R0\textsuperscript{+} CD8\textsuperscript{+} T-cell subsets contain significant frequencies of cells with the same specificity\textsuperscript{24,25}. It was therefore assumed that after the primary response, some of the clonally expanded CD45R0\textsuperscript{+} virus-specific CTLs revert into a memory CD45RA\textsuperscript{+} phenotype\textsuperscript{25}. Nevertheless, this hypothesis was never fully confirmed. In the primary immune response to EBV infection, a frequency of 5-14\% of EBV-specific cells yet expresses the CD45RA isoform early after infection and the CD45RA/R0 distribution of individual clones was not investigated\textsuperscript{24}. Wills and collaborators analyzed the distribution of a single CMV-specific clone in only two donors and always found the same clone in both CD45RA/R0\textsuperscript{+} subsets, albeit at different frequencies. However a more extensive study investigating CD45 isotype expression and the TCR-V\textsubscript{\beta} usage showed that the dominance of the CD45 phenotype was extremely variable between individuals, as in some cases the immunodominant clone was predominantly CD45RA\textsuperscript{+} and in others CD45R0\textsuperscript{+}\textsuperscript{26}. Finally, the replication history of the CD45RA\textsuperscript{+} CD8\textsuperscript{+} T-cell subpopulations supports the idea that those cells can differentiate directly from the naïve pool and, thus, a CD45R0\textsuperscript{+} stage is not necessary\textsuperscript{14}. Furthermore, recent \textit{in vitro} studies failed to induce CD45RA re-expression in T\textsubscript{EM} cells, while T\textsubscript{CM} cells re-expressed CD45RA exclusively under cytokine influence, but never after T cell triggering. It is therefore clear that more thoroughly \textit{ex vivo} studies are required to determine the lineage relationships between these cell types, possibly through the evaluation of the distribution of multiple clones in several independent donors.

The possibility that CCR7\textsuperscript{+}CD45RA\textsuperscript{+}CD8\textsuperscript{+} T lymphocytes can issue directly from naïve cells\textsuperscript{14} is in apparent contradiction with other data suggesting that T\textsubscript{EMRA} corresponds to a terminal differentiation stage, in contrast to T\textsubscript{EM}, given the highest perforin content and reduced division capacity\textsuperscript{3,4}. It must be noted that in these later studies CD45RA\textsuperscript{+} and CD45RA\textsuperscript{-} cells were not subdivided based on CD27 and CD28 expression. Our results clarify
these apparent contradictory data. We show that $T_{EM}$ and $T_{EMRA}$ have different distribution of the compounding subsets DP, CD27-SP, CD-28SP and DN. DN cells, the most differentiated CD8$^+$ T-cell subset, are enriched in the CD45RA$^+$ compartment, explaining why CD45RA$^+$ cells appeared to harbor more differentiated cells in previous studies. These results emphasize the importance of the additional characterization of CCR7$^-$ cells through assessment of CD27 and CD28 co-expression.

We also show that each subset within $T_{EM}$ and $T_{EMRA}$ CD8$^+$ T-cell subpopulations is characterized by the acquisition of a particular effector function. Interestingly, this phenomenon appears to occur sequentially, in such a way that along the hierarchy of differentiation, the expression of each gene once induced is never lost in the subsequent differentiation stages. Hence, the DP subsets had high frequencies of cells expressing RANTES and GZMA, and relatively low perforin expression, whereas CD27SP cells maintained high frequencies of RANTES and GZMA, but now up-regulated perforin, some cells expressing GZMB. Subsequently, DN cells co-expressed high frequencies of RANTES, GZMA and perforin but also up-regulated GZMB at greater extent, and also Fas-L. These results demonstrate that cellular differentiation leads to a progressive co-expression of multiple “killer” mRNAs by the same cell. Since each of these molecules mediate killing by a different mechanism, their co-expression may occur to improve killer efficiency of individual cells. Indeed, association of granzymes and perforin is fundamental for perforin-mediated cytotoxicity$^{27}$ and FasL cytolysis alone is not very efficient$^{28}$. In humans, it was shown that different viral infections selectively induce a preferential differentiation of cells to distinct phenotypes$^5$. Generally, EBV induces DP cells, HIV CD27-SPs and CMV generates DN types. These differential phenotypes were attributed to a different capacity of lymphocytes to become fully activated. Nevertheless, all these infections induce major expansions of CD8$^+$ T lymphocytes in the acute phase and infection by EBV is well controlled by DP cells. Thus, an alternative possibility is that the immune response to each virus requires the generation of particular effector subsets. Actually, since all $T_{EM}$ and $T_{EMRA}$ populations (with the exception of CD27$^{high}$ subsets) co-express perforin and granzymes and are able to mediate cytotoxicity, it is possible that the panel of molecules expressed by each one CD8$^+$ T-cell subpopulation confers particular advantages for the control of each type of infections. Therefore, the predominance of a given phenotype amongst virus-specific CD8$^+$ T cells can result from the selection of the most advantageous CD8$^+$ T-cell subset in the control of each type of infection.
Acknowledgments

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References


Table 1. CD27 and CD28 co-expression in CD8⁺CCR7⁻ T cells

<table>
<thead>
<tr>
<th></th>
<th>CD27⁺CD28⁺</th>
<th>CD27⁺</th>
<th>CD28⁺</th>
<th>CD27⁻CD28⁻</th>
<th>CD27&lt;sup&gt;high&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DP</td>
<td>SP</td>
<td>DP</td>
<td>DP</td>
<td>SP</td>
</tr>
<tr>
<td>CD45RA⁻</td>
<td>50 (19-74)</td>
<td>19 (2-34)</td>
<td>6 (2-15)</td>
<td>15 (2-46)</td>
<td>6 (0.6-19) (1-13)</td>
</tr>
<tr>
<td>CD45RA⁺</td>
<td>17 (2-44)</td>
<td>32 (7-58)</td>
<td>3 (0.5-12)</td>
<td>40 (7-74)</td>
<td>2 (0.3-8) (0.4-17)</td>
</tr>
</tbody>
</table>

Results represent the distribution of different subpopulations expressing CD27 and/or CD28 within T<sub>EM</sub> and T<sub>EMRA</sub> compartments. They show the mean percentage of cellular subsets in 18 donors, with maximum and minimum values indicated in brackets. DP indicates double-positive; SP, single-positive; and DN, double-negative.
Table 2. Expression levels of different markers in CD8+ T cells

<table>
<thead>
<tr>
<th></th>
<th>CD27</th>
<th>CD28</th>
<th>CD11a</th>
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<tbody>
<tr>
<td>N</td>
<td>120</td>
<td>17</td>
<td>67</td>
</tr>
<tr>
<td>T_CM</td>
<td>109</td>
<td>31</td>
<td>195</td>
</tr>
<tr>
<td>T_EM</td>
<td>59</td>
<td>33</td>
<td>225</td>
</tr>
<tr>
<td>T_EMRA</td>
<td>50</td>
<td>22</td>
<td>237</td>
</tr>
<tr>
<td>CD27_{high}</td>
<td>147</td>
<td>37</td>
<td>240</td>
</tr>
</tbody>
</table>

Results show mean fluorescence intensity (MFI) of stainings for different cell-surface markers gated on positive populations in peripheral blood lymphocytes of one donor. Similar relationships were found in 10 other donors.
Figure Legends

Figure 1. Expression of CD27, CD28, CD11a and CD62L in CD8+ T peripheral blood lymphocytes. (A,B) CD8-enriched PBLs were simultaneously stained for CD8, CCR7, CD45RA, CD27 and CD28. CD8^high cells were then subdivided using CCR7 and CD45RA expression in naïve (T_N) central memory (T_CM) effector memory (T_EM) and effector memory CD45RA^+ (T_EMRA) cell subpopulations (A) and CD27/CD28 co-expression evaluated in each of these cell sets (B). (C-E) Comparison of CD27 (C), CD28 (D) and CD11a (E) expression levels on T_N cells (shadowed histograms) with: T_CM (dashed); T_EM; (thick line) T_EMRA (gray line). (F) CD8^+ cells depleted for CCR7 were simultaneously stained for CD8, CD62L, CD45RA, CD27 and CD28. T_EM and T_EMRA subpopulations gated on CD8^high were subdivided accordingly to their expression of CD27 and CD28 into CD27^high, CD28^CD27^ (CD28-SPs), CD28^CD27^ (DP) CD28^CD27^ (CD27-SP) and CD28^CD27^ (DN) cell sets. Results show CD62L expression in each of these sets in one representative donor.

Figure 2. Single-cell gene expression profiles of the less activated CD8+ T-cell subpopulations. TCR-αβ^+ CD8^+ PBLs were simultaneously stained for CD8, CCR7, CD45RA, CD27 and CD28. CD8^high T cells (A) naïve, (B) CD45RA^- (left) or CD45RA^+ (right) CCR7 CD27^high and (C) T_CM were single-cell sorted and the expression multiple genes was determined simultaneously in each individual cell. The accuracy of cell sorting was evaluated by the expression CD3ε mRNA, only wells positive for this molecule being depicted. Each horizontal line depicts the same individual cell, which is numbered. Each vertical line represents a different gene. Results show the profiles from a single representative donor out of the three we studied. The following genes ccl5, ccl4, tgb1, tnf, lta, ifng, prf1, gzm a, gzm b, faslg, tgfbr1, tgfbr2, ifngr2 and il10ra code, respectively, for RANTES, MIP-1β, TGF-β1, TNF-α, TNF-β, IFN-γ, perforin, granzyme A, granzyme B, FasL, TGF-β receptor 1, TGF-β receptor 2, IFN-γ receptor 2 and IL-10 receptor α.
Figure 3. Single-cell expression profiles of the most differentiated CD8+ T-cell subpopulations. TCR-αβ+ CD8+ PBLs were subdivided by their expression of CCR7, CD45RA, CD27 and CD28, and individual cells from each subset were sorted and studied as described in Figure 2. Each single cell corresponds to one horizontal line, whereas the expression of the several genes studied is depicted vertically. The representative gene expression patterns presented correspond to (A) DP, (B) CD28-SP, (C) CD27-SP and (D) DN CCR7-CD8+ T subsets. Each profile is from one representative donor out of three, with the exception of the minority CD28-SP set, that we could isolate only from this donor. The following genes ccl5, ccl4, tgfβ1, tnf, lta, ifng, prf1, gzmα, gzmβ, faslg, tgfbr1, tgfbr2, ifngr2 and il10ra code, respectively, for RANTES, MIP-1β, TGF-β1, TNF-α, TNF-β, IFN-γ, perforin, granzyme A, granzyme B, FasL, TGF-β receptor 1, TGF-β receptor 2, IFN-γ receptor 2 and IL-10 receptor α.

Figure 4. Quantitative assessment of mRNA expression in single cells. Single cells of each CD8+ T-cell subset were sorted and cells expressing each gene identified, as shown in Figure 2,3. In all cells that scored positive for the expression of any particular gene, the mRNA expression levels of that gene were further quantified. (A) Each graph shows three cells from each population. We show GZMA and RANTES amplification since these genes can be found in all cell sets, and thus their expression levels can be directly compared. (B) Correlation between the number of mRNA molecules expressed by every single cell of TEM and TEMRA compounding subsets. Individual cells expressing GZMA, IL-10Rα and RANTES from either TEM or TEMRA were ordered according to the expression level of that gene and attributed an ordinal point. The number of mRNA molecules associated to each ordinal point from TEM subsets was plotted against the correspondent ordinal point values of the equivalent TEMRA subset. The dots in the graphs represent the intersection of the values derived from the number of mRNA molecules expressed by one TEM cell (horizontal axis) and one TEMRA cell (vertical axis). Data is from one out of three donors.

Figure 5. The profile of gene expression of each CD8 subpopulation is the same in different donors. Results represent the profiles of gene expression of various CD8 subsets from a different donor than the one shown in Figures 2 and 3. Cell sets were isolated,
individual cells and genes were disposed as described in figures 2,3. To fit into a single figure, we show only majority populations and cytotoxic and chemokines mRNAs, since these genes by themselves clearly define the properties of each CD8 subtype.
Figures

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Cartography of gene expression in CD8 single cells: novel CCR7⁻ subsets suggest differentiation independent of CD45RA expression

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