Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load

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RESUME

Nous n’avons pas de connaissance précise des facteurs à l’origine de l’hétérogénéité phénotypique des cellules T CD4 mémoires.

Une troisième population phénotypique des cellules T CD4 mémoires, caractérisée par les marqueurs CD45RA⁺CCR7⁻ a été identifiée dans cette étude. Cette population présente un état de différenciation avancée, comme en témoigne son histoire de réplication, ainsi que sa capacité de prolifération homéostatique.

Les réponses des cellules T CD4 mémoires à différentes conditions de persistance et charge antigénique ont trois patterns phénotypiques différents, caractérisés par les marqueurs CD45RA et CCR7.

La réponse CD4 mono – phénotypique CD45RA⁺CCR7⁺ ou CD45RA⁻CCR7⁻ est associée à des conditions d’élimination de l’antigène (telle la réponse CD4 tétanos spécifique) ou à des conditions de persistance antigénique et de virémie élevée (telle la réponse HIV chronique ou la primo-infection CMV) respectivement.

D’autre part, les réponses T CD4 multi – phénotypiques CD45RA⁻ CCR7⁺ sont associées à des conditions d’exposition antigénique prolongée et de faible virémie (telles les infections CMV, EBV et HSV ou les infections HIV chez les long term non progressors).

La réponse mono – phénotypique CD45RA⁻ CCR7⁺ est propre aux cellules T CD4 secrétant de IL₂, définies également comme centrales mémoires, la réponse CD45RA⁻ CCR7⁺ aux cellules T CD4 secrétant de l’IFNγ et finalement la réponse multi-phénotypique aux cellules T CD4 secrétant à la fois de l’IL₂ et de l’IFNγ.

En conclusion, ces résultats témoignent d’une régulation de l’hétérogénéité phénotypique par l’exposition et la charge antigénique.
Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load

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The factors responsible for the phenotypic heterogeneity of memory CD4 T cells are unclear. In the present study, we have identified a third population of memory CD4 T cells characterized as CD45RA*CCR7* that, based on its replication history and the homeostatic proliferative capacity, was at an advanced stage of differentiation. Three different phenotypic patterns of memory CD4 T cell responses were delineated under different conditions of antigen (Ag) persistence and load using CD45RA and CCR7 as markers of memory T cells. Mono-phenotypic CD45RA*CCR7* or CD45RA*CCR7* CD4 T cell responses were associated with conditions of Ag clearance (tetanus toxoid-specific CD4 T cell response) or Ag persistence and high load (chronic HIV-1 and primary CMV infections), respectively. Multi-phenotypic CD45RA*CCR7*, CD45RA*CCR7* and CD45RA*CCR7* CD4 T cell responses were associated with protracted Ag exposure and low load (chronic CMV, EBV and HSV infections and HIV-1 infection in long-term nonprogressors). The mono-phenotypic CD45RA*CCR7* response was typical of central memory (Tcm) IL-2-secreting CD4 T cells, the mono-phenotypic CD45RA*CCR7* response of effector memory (Tem) IFN-γ-secreting CD4 T cells and the multi-phenotypic response of both IL-2- and IFN-γ-secreting cells. The present results indicate that the heterogeneity of different Ag-specific CD4 T cell responses is regulated by Ag exposure and Ag load.

Key words: Memory CD4 T cells / Phenotype / Heterogeneity

1 Introduction

Previous studies have clearly shown a great phenotypic and functional heterogeneity of memory T cells in both mice and humans (reviewed in [1-6]). A series of surface markers including CD45RO, CD45RA, CD28, CD27, CCR7, CD7 and CD57 have been used in order to define different populations of memory T cells and to correlate these markers with functional capacity and differentiation stage. These studies have led to the generation of different models of differentiation of memory CD4 and CD8 T cells. For instance, the marker combinations CD28*CD27*, CD28*CD27* and CD28*CD27* may represent memory CD8 T cells at an early stage, intermediate stage and advanced stage of differentiation, respectively [9-11]. The lack of expression of CD7 is characteristic of memory T cells at an advanced stage of differentiation [12-15]. The expression of CD57 defines CD8 T cells at a terminal stage of differentiation [16-19]. The combined use of CCR7 with CD45RA led to the identification of two phenotypically and functionally distinct subsets of memory T cells [20]. The lack of expression of CCR7 (CCR7) defines a population of effector memory T cells (Tem) that resides predominantly in the periphery, while the expression of CCR7 (CCR7) characterizes a population of central memory T cells (Tcm) that resides predominantly within the secondary lymphoid organs and serves as precursors of effector cells [20]. This latter study led to the identification of four and three subsets of CD8 and CD4 T cells, respectively [20].

Furthermore, the use of the above surface markers in studying memory CD4 and CD8 T cell responses has shown a great phenotypic heterogeneity in the memory T cell responses specific for Epstein-Barr virus (EBV), cytomegalovirus (CMV) and HIV-1 [16, 21-28]. These studies have been performed predominantly with CD8...
T cells, and the phenotypic heterogeneity observed in the different viral infections was proposed to result from skewed maturation or replication senescence of memory CD8 T cells during HIV-1 infection [21, 24] or an influence of the type of pathogen/virus on the specific development of distinct memory T cell populations [21].

Limited information is available on the phenotypic heterogeneity of memory CD4 T cell responses to human virus infections. Recent studies investigating the function of virus-specific CD4 T cells demonstrated a skewed representation of different populations of antigen (Ag)-specific CD4 T cells with a selective reduction in the proportion of helper (IL-2-secreting and proliferating) CD4 T cells in HIV-1 compared to CMV infection [27, 29]. In this regard, we recently provided evidence that the functional heterogeneity of Ag-specific CD4 T cells responses is influenced by Ag persistence/exposure and load (Harari et al., submitted). In the present study, we have investigated the factors that may be responsible for the phenotypic heterogeneity of Ag-specific CD4 T cell responses in humans by analyzing different models differing in Ag persistence/exposure and load. In particular, we have used a) tetanus toxoid (TT) as a model of memory T cell response where the Ag is cleared; b) the CMV, EBV, Herpes Simplex Virus (HSV) chronic infections as well as controlled HIV-1 infection in long-term nonprogressors (LTNP) as a model of repetitive Ag exposure and low Ag load; and c) chronic HIV-1 infection and acute CMV infection as a model of Ag persistence and high Ag load. Our results demonstrate the existence of a third population of memory CD4 T cells (CD45RA*CCR7+) with characteristics of terminally differentiated cells (i.e. short telomeres and a lack of proliferative capacity). In addition, we show that the phenotypic heterogeneity and maturation stage of memory CD4 T cells among the different models of immune responses can be explained by the Ag persistence and load and that Ag-specific CD4 T cells with the CD45RA*CCR7 phenotype are only generated in conditions of repetitive Ag exposure and low Ag load.

2 Results

2.1 Identification of three phenotypically distinct populations of memory CD4 T cells

The chemokine receptor CCR7 has been used in previous studies in combination with the CD45RA Ag as a tool to define different populations of human memory CD4 and CD8 T lymphocytes with different homing and functional capacities and at different stages of differentiation [20, 24, 26]. Using this approach two and three populations of memory CD4 and CD8 T lymphocytes have been identified, respectively [20]. The population with the CD45RA*CCR7 phenotype that defines the terminally differentiated memory CD8 T cell population (TEx) has not been described for CD4 T cells [20, 24, 26]. We therefore investigated the composition of the pool of memory CD4 T cells. Blood mononuclear cells were stained with antibodies to CD4, CCR7 and CD45RA. According to previous studies [20], the majority of blood CD4 T cells are CD45RA*CCR7+, i.e. naive cells, CD45RA*CCR7+ or CD45RA*CCR7− (Fig. 1). However, a small but well-defined fourth cell population with the CD45RA*CCR7+ phenotype was also consistently found among CD4 T cells (Fig. 1). The mean percentage of CD4+CD45RA*CCR7+ cells was 2.3±1.1 in a large group (n=43) of randomly selected blood donors (Fig. 1). Therefore, these results demonstrate the existence of a third population of memory CD4 T cells with the CD45RA*CCR7 phenotype. Among CD8 T cells, this phenotypically distinct cell population has been proposed to define a population with functional characteristics, e.g. poor proliferative capacity, of terminally differentiated cells [20, 24, 26].

2.2 Characterization of the replication history and proliferative capacity of phenotypically distinct populations of memory CD4 T cells

In order to provide insights into the differentiation stages of the different populations of memory CD4 T cells, we performed a series of experiments to evaluate the replication history of memory T cells and their ability to divide under conditions of homeostatic proliferation. According to previous studies [20], substantial differences in telomere length were observed among the different populations of CD4 T cells defined by the

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![Fig. 1. Phenotypic analysis of naive and memory blood CD4 T cell populations defined by the expression of CD45RA and CCR7. Four populations of CD4 T cells, CD45RA*CCR7+, CD45RA*CCR7+, CD45RA*CCR7− and CD45RA*CCR7−, were identified after staining with anti-CD4, -CD45RA and -CCR7. Cumulative data on the percentage of the CD4+CD45RA*CCR7− cell population in 43 randomly selected healthy subjects are also shown.](www.ej.de)
expression of CD45RA and CCR7. The overlaid histogram plots of telomere PNA fluorescence for each of the sorted populations indicates that CD45RA⁺CCR7⁺ CD4 T cells, i.e. naive cells, have longer telomeres compared to the CD45RA⁻CCR7⁺ (Tcm) and CD45RA⁺CCR7⁻ (Tem) memory CD4 T cell populations (Fig. 2A). These results are consistent with the higher number of cell divisions occurring in memory versus naive cells and with the previous observations of Sallustio et al. [20]. Of interest, the newly identified CD45RA⁻CCR7⁻ CD4 T cell population had a telomere length consistently (in three separate experiments) shorter than the other two populations of memory CD4 T cells (Fig. 2A). These results indicate that CD45RA⁺CCR7⁻ CD4 T cells are at an advanced stage of differentiation that is at least comparable to that of CD45RA⁻CCR7⁻ (Tem) CD4 T cells.

We then investigated the homeostatic proliferative capacity of the different populations of memory CD4 T cells. The ability to proliferate in the absence of Ag, i.e. homeostatic proliferation, is an important characteristic of memory T cells [30]. Blood mononuclear cells were stained with anti-CD45RA and anti-CCR7 antibodies. The different memory T cell populations were isolated by cell sorting, and homeostatic proliferation was assessed on the basis of their ability to proliferate in the presence of a cocktail of cytokines (IL-6, IL-7, IL-10, IL-15 and TNF-α) [30]. The population of CD45RA⁻CCR7⁺ memory CD4 T cells (Tcm), showed substantial homeostatic proliferative capacity, while both CD45RA⁺CCR7⁻ (Tem) and CD45RA⁻CCR7⁻ memory CD4 T cell populations proliferated poorly under these experimental conditions (Fig. 2B).

Previous studies [16-19] demonstrated that expression of the CD57 surface marker defines a population of T cells with poor replication capacity that is likely in an advanced stage of differentation. We therefore analyzed the expression of CD57 in the different subsets of CD4 T cells defined by the expression of CD45RA and CCR7. Consistent with telomere length, we observed that the level of CD57 expression increased progressively from CD45RA⁻CCR7⁺ (Tcm) to CD45RA⁺CCR7⁻ (Tem) cells and was highest in the CD45RA⁻CCR7⁻ population (Fig. 2C). Taken together, these results strongly support the hypothesis that memory CCR7⁻ CD4 T cells and particularly the CD45RA⁻CCR7⁻ cell population are at an advanced stage of differentiation.

Fig. 2. Replication history, homeostatic proliferation and CD57 expression in the blood CD4 T cell populations defined by the expression of CD45RA and CCR7. (A) Telomere length in the different populations of naïve and memory CD4 T cells. Blood mononuclear cells were stained with antibodies specific for CD4, CD45RA and CCR7 and sorted for CD45RA⁺CCR7⁺, CD45RA⁻CCR7⁺, CD45RA⁺CCR7⁻ and CD45RA⁻CCR7⁻ CD4⁺ cell populations. The sorted populations were assessed for telomere length using telomere-specific PNA followed by flow cytometry. Histograms of telomere channel fluorescence are shown. The black solid line corresponds to CD4⁺CD45RA⁺CCR7⁺ cells, the green to CD4⁺CD45RA⁻CCR7⁺ cells, the blue to CD4⁺CD45RA⁻CCR7⁻ cells and the red to CD4⁺CD45RA⁻CCR7⁻ cells. Dotted lines correspond to unstained controls of the sorted populations. (B) The three sorted populations of memory CD4 T cells were cultured in the presence of a panel of cytokines as described in the Methods. Cell proliferation was assessed by [3H]thymidine uptake. Data are expressed as stimulation index (SI). The mean SI of six independent experiments is shown. (C) Flow cytometry profiles and cumulative data of the expression of CD57 in the populations of CD4 T cells from six randomly selected donors.
2.3 Relationships between function, phenotype and differentiation of distinct populations of memory CD4 T cells

On the basis of previous observations [20] and the results shown in Fig. 2, it is clear that the three subsets of memory CD4 T cells are at different stages of maturation with CD45RA⁺CCR7⁺ cells (TDM) being the least differentiated, CD45RA⁺CCR7⁻ cells (TEM) at an intermediate stage and CD45RA⁻CCR7⁻ cells (TEFF) being the most differentiated. In order to better define the relationships between the function and phenotype of memory CD4 T cells, we analyzed the distribution of Ag-specific CD4 T cells within the different subsets identified by CD45RA and CCR7 in three models of memory CD4 T cell responses corresponding to conditions of a) Ag clearance (TT-specific responses), b) protracted Ag exposure and low Ag load (chronic CMV, EBV, HSV and controlled HIV-1 infections in LTNP) and c) Ag persistence and high Ag load (chronic HIV-1 and acute CMV infections). In particular, we analyzed the phenotype of IL-2- and IFN-γ-secreting Ag-specific CD4 T cells within the different subsets defined by CD45RA and CCR7 expression. For this purpose, blood mononuclear cells were stimulated with the specific Ag for TT, EBV, HSV and CMV and stained with CD4, CCR7, CD45RA and IFN-γ or IL-2 antibodies.

The distribution of TT-specific IL-2- and IFN-γ-secreting CD4 T cells within the different subsets of memory cells is shown in Fig. 3. Of interest, about 85% of TT-specific IL-2-secreting CD4 T cells were CD45RA⁺CCR7⁺, and the remaining cells were CD45RA⁺CCR7⁻ (Fig. 3). The large majority (>80%) of TT-specific IFN-γ-secreting cells were CD45RA⁺CCR7⁻ (Fig. 3). With regard to the different situations of protracted Ag exposure and/or low Ag load, i.e. CMV, EBV, HSV and HIV-1 in LTNP, Ag-specific IL-2-secreting CD4 T cells were mostly (80%) CD45RA⁺CCR7⁺, and a minority (20%) were CD45RA⁺CCR7⁻ (Fig. 4A). The IFN-γ-secreting CD4 T cells were either CD45RA⁺CCR7⁻ or CD45RA⁺CCR7⁺ (Fig. 4A). Cumulative data obtained from the analysis of a larger number of subjects are shown in Fig. 4B. Finally, the majority (about 94%) of HIV-1-specific IFN-γ-secreting cells were CD45RA⁺CCR7⁻ (Fig. 5A). Similarly, more than 90% of the CMV-specific IFN-γ-secreting CD4 T cells during primary CMV infection were also contained within the CD45RA⁺CCR7⁻ cell population (Fig. 5B). These results demonstrate substantial differences in the distribution of CD4 T cell populations with similar function within phenotypically distinct populations defined by the CCR7 and CD45RA. The composition of these phenotypically and functionally distinct memory T cell populations was substantially different among the different models of immune responses.

![Diagram](image-url)

**Fig. 3.** Distribution of Ag-specific IL-2- and IFN-γ-secreting cells in different populations of blood memory CD4 T cells defined by the expression of CD45RA and CCR7 in a model of Ag clearance. Flow cytometry profiles of the distribution of TT-specific IL-2- and IFN-γ-secreting CD4 T cells within CD45RA⁺CCR7⁺, CD45RA⁺CCR7⁻ and CD45RA⁻CCR7⁻ cell populations in one representative subject are shown. Blood mononuclear cells were stimulated with TT and analyzed for the expression of CD4, CCR7, CD45RA and IL-2 or IFN-γ. The cluster of events shown in red corresponds to the responder CD4 T cells, i.e. expressing IL-2 or IFN-γ, while the cluster of events in blue corresponds to the non-responder CD4 T cells. Cumulative data on the percentages of TT-specific IL-2- or IFN-γ-secreting CD4 T cells within CD45RA⁺CCR7⁺, CD45RA⁺CCR7⁻ and CD45RA⁻CCR7⁻ cell populations obtained in eight donors are also shown. At least 1 x 10⁶ events were analyzed.
Fig. 4. Distribution of Ag-specific IL-2- and IFN-γ-secreting cells in different populations of blood memory CD4 T cells defined by the expression of CD45RA and CCR7 in a model of repetitive Ag exposure and low Ag load. (A) Flow cytometry profiles of the distribution of CMV-, EBV-, HSV- and HIV-1 (in LTNP)-specific IL-2- and IFN-γ-secreting CD4 T cells within CD45RA⁺CCR7⁺, CD45RA⁺CCR7⁻ and CD45RA⁺CCR7⁻ cell populations in representative subjects. Blood mononuclear cells were stimulated with CMV, EBV, HSV or HIV-1 (in LTNP) and analyzed for the expression of CD4, CCR7, CD45RA and IL-2 or IFN-γ. The cluster of events shown in red corresponds to the responder CD4 T cells, i.e. expressing IL-2 or IFN-γ, while the cluster of events in blue corresponds to the non-responder CD4 T cells. (B) Cumulative data on the percentages of CMV-, EBV-, HSV- and HIV-1 (in LTNP)-specific IL-2- or IFN-γ-secreting CD4 T cells within CD45RA⁺CCR7⁺, CD45RA⁺CCR7⁻ and CD45RA⁺CCR7⁻ cell populations obtained in several donors. At least 1 x 10⁶ events were analyzed.
3 Discussion

Our study focused on memory CD4 T cells, and the results we report contribute to the explanation of the phenotypic heterogeneity of Ag-specific CD4 T cells, provide new insights into the different populations of memory CD4 T cells and their relationship with the stage of differentiation and help us to understand the factors that may influence the differences in the immune responses against different types of antigens/pathogens. The present study describes the identification of a third population of memory CD4 T cells defined by the CD45RA+CCR7− phenotype. This cell population appears to be at an advanced stage of differentiation on the basis of its replication history, i.e., shorter telomere, and its poor ability to respond to homeostatic proliferation as compared to the other two populations of memory CD4 T cells, i.e., CD45RA−CCR7+ and CD45RA+CCR7− cells. Therefore, similar phenotypically distinct populations of memory CD4 and CD8 T cells can be identified using the CD45RA and CCR7 surface markers [20, 24, 26].

Our results also provide insightful information into the relationships between the function and the phenotype of memory CD4 T cells and their stage of differentiation. The CD45RA+CCR7+ phenotype is typical of TCM cells and of cells secreting IL-2. Furthermore, it has been proposed that TCM cells represent long-lived memory T cells [20]. In support of this hypothesis, it was recently observed that hepatitis C virus (HCV)-specific CD4 T cells identified by MHC class II tetramers were indeed CD45RA+CCR7− in subjects who spontaneously resolved HCV viremia [31]. Similarly, influenza (Flu)-specific or Flu vaccine-specific CD4 T cells were also CD45RA+CCR7− [32]. Therefore, the CD45RA+CCR7− phenotype truly defines long-lived memory CD4 T cells and should be found in a situation of Ag clearance as is the case with the TT-specific CD4 T cell response. Our results indeed indicate that TT-specific CD4 T cells are mostly IL-2-secreting cells and CD45RA+CCR7−. TT is not a viral Ag. However, we wish to propose that similar memory CD4 T cell responses are also typical of virus infections such as influenza, respiratory syncytial virus, coronavirus, leukokoriome ningitis virus, etc., in which viruses are efficiently cleared after the acute phase of infection and, unlike CMV, do not have a latency reactivation pattern.

The CD4 T cell response in the model of protracted Ag exposure and/or low Ag load, i.e., chronic CMV, EBV, HSV and HIV-1 infection in LTNP, was functionally character-
ized by the presence of both IL-2- and IFN-γ-secreting CD4 T cells. These functionally distinct CD4 T cell populations were contained within three phenotypic populations: CD45RA+CCR7+, CD45RA+CCR7- and CD45RA-CCR7+. The IL-2-secreting cells were either CD45RA+CCR7+ or CD45RA+CCR7-, while IFN-γ-secreting cells were CD45RA+CCR7+ or CD45RA-CCR7-. Finally, the CD4 T cell response in the model of Ag persistence and high Ag load, i.e. chronic and progressive HIV-1 infection as well as acute CMV infection, was dominated by the presence of IFN-γ-secreting cells with the CD45RACCR7+ phenotype. Therefore, the three models of memory CD4 T cell responses are associated with either a mono-phenotypic response (CD45RA+CCR7+ for Ag clearance and CD45RA+CCR7- for Ag persistence and high Ag load) or a multi-phenotypic response (CD45RA+CCR7+, CD45RA+CCR7- and CD45RA-CCR7+ for repetitive Ag exposure and/or low Ag load).

The above results raise a series of issues. Firstly, how can it be explained that IFN-γ-secreting cells with the CD45RACCR7+ phenotype are found in memory CD4 T cell responses associated with protracted Ag exposure and/or low Ag load but not under conditions of Ag persistence and high load despite the fact that the memory CD4 T cells are exclusively composed of IFN-γ-secreting cells? On the basis of both the present data and previous studies on memory CD8 T cells, chronic stimulation with low Ag levels seems to be necessary for the appearance of memory CD4 and CD8 T cells with the CD45RACCR7+ phenotype. Secondly, it is important to underscore that substantial phenotypic and functional changes were observed within the CMV- and HIV-1-specific CD4 T cell responses under different conditions of Ag load. The mono-phenotypic CD45RA+CCR7- CD4 T cell response typical of conditions of Ag persistence and high Ag load (chronic and progressive HIV-1 infection) was not found in LTNP, where we found the multi-phenotypic CD4 T cell response typical of repetitive Ag exposure and low Ag load. Along the same line, a mono-phenotypic CD45RA+CCR7- CD4 T cell response characterized the CMV-specific CD4 T cell response during primary infection, whereas a multi-phenotypic response was associated with the CMV-specific CD4 T cell response during chronic CMV infection. An important variable between progressive and nonprogressive HIV-1 infection and between primary and chronic CMV infection is Ag load. Therefore, taken together our data indicate that the phenotypic as well as the functional heterogeneity of memory CD4 T cell responses is regulated by Ag persistence and Ag load. Thirdly, the phenotypic patterns shown for CD4 T cell responses within the different models of Ag persistence and load were consistent with those shown for CD8 T cells using CD45RA and CCR7 [24, 26]. In fact, multi-phenotypic CD8 T cell responses were only found under conditions of protracted Ag exposure and Ag load, e.g. chronic CMV and EBV infections [24, 26]. The mono-phenotypic CD45RA+CCR7- pattern appeared under conditions of Ag persistence or high Ag load, e.g. chronic HIV-1 infection and acute CMV infection, and the mono-phenotypic CD45RA+CCR7+ (dull) pattern was found under conditions of Ag clearance, e.g. influenza infection and LCMV infection in mice [32, 33]. Therefore, memory CD4 and CD8 T cell responses have common phenotypic patterns modulated by Ag persistence and Ag load. Fourthly, these different phenotypic patterns seem to reflect different stages of differentiation of memory CD4 T cells. Recent studies using CD27 and CD28 support this hypothesis [34]. However, additional studies will be needed to directly address the relationship between phenotype and differentiation stage. The results presented here support the hypothesis that the levels of Ag represent the critical factor influencing this heterogeneity.

4 Materials and methods

4.1 Study groups

The following study groups were included in the present study: a) blood from 43 HIV-negative subjects was obtained either from the local blood bank (Lausanne, Switzerland) or from lab co-workers; b) 7 subjects with progressive chronic HIV-1 infection, naïve to antiviral therapy, with CD4 T cell counts ≥250 cells/µl and plasma viremia ≥5000 HIV-1 RNA copies/ml [35]; c) 6 HIV-1-infected patients with nonprogressive disease, i.e. LTNP, as defined by documented HIV-1 infection for >14 years, stable CD4 T cell counts >500 cells/µl and plasma viremia <1000 HIV-1 RNA copies/ml; and d) 4 HIV-1-infected patients with primary CMV infection who have been previously described [36]. As previously reported [36], the four patients with primary CMV infection had detectable CMV DNA viremia at the time of phenotypic characterization performed in this study. Serostatus was not used as a strategy to identify subjects with potential CMV, EBV, and HSV CD4 T cell responses. The strategy used to select for the above responses was the routine Ag-specific stimulation of blood mononuclear cells from healthy blood donors. These studies were approved by the local institutional Review Board.

4.2 Determination of the relative telomere length

Sorted purified CD4+CD45RA-CCR7+, CD4+CD45RA-CCR7-, CD4+CD45RA+CCR7+ and CD4+CD45RA+CCR7- cells (2×10⁶ to 5×10⁷, purity >97%) were mixed with SupT1 cells in order to obtain 10⁶ cells per analysis. Cells were then
4.3 Homeostatic proliferation

Sorted purified CD4+CD45RA−CCR7+, CD4+CD45RA−CCR7+, CD4+CD45RA−CCR7+ and CD4+CD45RA−CCR7+ cells (2×10⁵ to 5×10⁶, purity >97%) were cultured in the presence of 25 ng/ml IL-6, IL-7, IL-10, IL-15 (BD systems, Minneapolis, MN) and TNF-α (Peprotech, UK) for 5 days. Cell cultures were then pulsed with [³H]thymidine (1 μCi per well) for 18 h [90].

4.4 FACS Analysis and sorting

Cryo-preserved cells were stored in liquid nitrogen were thawed and used for flow cytometry [27, 36]. Mononuclear cells preparations were stained for a panel of cell surface markers including CD4, CD45RA, CCR7, CD57 and CD69. Staining with rat anti-human CCR7 antibody from Becton Dickinson (BD, Franklin, NJ) was followed by goat anti-rat IgG(H+L)-FITC or -PE (Southern Biotechnologies Associates, Birmingham, AL). The following mouse anti-human antibodies were used in different combinations for cell surface staining and sorting: anti-CD4-PE, -PerCp Cy5.5 or -allophycocyanin (APC); anti-CD45RA-FITC or -CyChrome™ and anti-CD57-FITC (BD). Data were acquired on a FACScalibur™ system and analyzed using CellQuest™ software (BD). Cell sorting was performed on a FACSVantage™ (BD). The purity of the sorted cell populations was >97%. Flow cytometric analysis and cell sorting were performed as previously described [27, 36].

4.5 Intracellular cytokine staining (ICS)

Intracellular IFN-γ and IL-2 production was assessed as previously described [27]. Blood mononuclear cells (2×10⁶ to 4×10⁶ cells in 1 ml RPMI 1640 Gatumax-1 medium containing 10% inactivated fetal calf serum) were stimulated with 5 μg/ml HIV-1-p55 gag (Protein Sciences, Meriden, CT), 1 μg/ml CMV, EBV or HSV lysates (ABI, Columbia, MD), 100 μg/ml tetanus toxoid (Aventis Pasteur, Lyon, France) or 200 ng/ml Staphylococcal Enterotoxin B (Calbiochem, La Jolla, CA; positive control) for 16 h at 37°C in the presence of 0.5 μg/ml purified anti-CD28 antibody (BD) and 1 μg/ml GolgiPlug (BD). Cell surface staining was completed as described following the in vitro activation [27]. Cells were then permeabilized and fixed with FACS permeabilizing solution (BD) and labeled with anti-human IFN-γ-APC or IL-2-PE (PharMingen, San Diego, CA). The number of lymphocyte-gated events ranged between 150,000 and 600,000 in the flow cytometry experiments shown. With regard to the criteria for positive ICS, the background in the unstimulated controls never exceeded 0.02%. For an ICS to be considered positive, it had to have a background of less than 20% of the total percentage of cytokine-positive cells in the stimulated samples.

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