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Antigen-Specific Central Memory CD4$^+$ T Lymphocytes Produce Multiple Cytokines and Proliferate In Vivo in Humans[1]

Muriel Stubbe, Nathalie Vanderheyde, Michel Goldman, and Arnaud Marchant[2]

The function of Ag-specific central (T_{CM}) and effector (T_{EM}) memory CD4$^+$ T lymphocytes remains poorly characterized in vivo in humans. Using CD154 as a marker of Ag-specific CD4$^+$ T cells, we studied the differentiation of memory subsets following anti-hepatitis B immunization. Hepatitis B surface Ag (HBs)-specific memory CD4$^+$ T cells were heterogeneous and included T_{CM} (CCR7$^+$CD27$^+$) and T_{EM} (CCR7$^-$CD27$^{+/-}$). HBs-specific T_{CM} and T_{EM} shared the capacity to produce multiple cytokines, including IL-2 and IFN-γ. Several years postimmunization, ~10% of HBs-specific memory CD4$^+$ T cells were in cycle (Ki67$^+$) and the proliferating cells were CCR7$^+$. These results suggest that the model of functional specialization of T_{CM} and T_{EM} cannot be applied to protein vaccine Ags and support the concept that T_{CM} are capable of self-renewal and contribute to maintain the pool of memory cells. *The Journal of Immunology,* **2006, 177: 8185–8190.**

The CD4$^+$ T lymphocytes represent critical targets for vaccines as they promote both cell-mediated and humoral immune responses (1). Understanding the differentiation and the functions of Ag-specific memory CD4$^+$ T lymphocytes is essential for the development of new or improved vaccines.

Memory CD4$^+$ T lymphocytes can be delineated into two subsets according to their homing capacity (2, 3). Central memory T cells (T_{CM})[3] express the chemokine receptor CCR7, a receptor for CCL21 and CCL19 allowing them to home to the T cell areas of lymphoid organs, whereas effector memory T cells (T_{EM}) do not express CCR7 and home to peripheral tissues. A model has been proposed which links these distinct homing properties to distinct functional programs. According to this model, T_{CM} have strong proliferative capacity but little effector functions, whereas T_{EM} produce effector cytokines but have limited proliferative capacity (2–4). The link between CCR7 expression and cytokine production by T lymphocytes remains controversial. Several reports in mice and humans have shown that T_{CM} produce effector cytokines and that the majority of cytokine-producing human CD4$^+$ T cells are actually detected in the CCR7$^+$ subset (5–8). In addition, it was recently shown that the T_{CM} pool contains committed cells that can differentiate into Th1 or Th2 cells under nonpolarizing conditions (9). The T_{CM}/T_{EM} model of T cell memory has been refined by the inclusion of the costimulatory molecules CD27 and CD28. A linear model of CD4$^+$ T cell differentiation was proposed, which involves a sequential loss of the expression of CCR7, CD27, and CD28 (10–14). Along this differentiation pathway, cells would progressively lose their proliferative capacity and increase their production of effector cytokines.

The persistence of memory T cells is related to their capacity to self-renew and continuously divide (3, 15, 16). The relative capacity of human T_{CM} and T_{EM} to self-renew in vivo remains poorly defined. In vivo deuterated glucose-labeling experiments indicate that CD4$^+$ T_{EM} have a higher turnover rate than T_{CM} (17). These results were confirmed by ex vivo BrdU-labeling experiments with the exception of a subset CXCR5$^-$ T_{CM} that has a higher rate of spontaneous proliferation than T_{EM} (9). Since the total pool of T_{EM} studied in these experiments is enriched in T cells specific for persistent microorganisms, their higher turnover rate may not reflect a higher capacity to self-renew but could rather be related to continuous Ag stimulation (10, 18, 19).

The role of memory subsets in Ag-specific CD4$^+$ T cell responses in humans remains poorly characterized. Studies of persistent viral infections indicate predominant T_{EM} responses in subjects with high viral loads and mixed T_{EM} and T_{CM} responses in subjects with protracted Ag exposure (10, 18–20). Studies of individuals immunized with the cleared Ag tetanus toxoid indicate mixed T_{CM} and T_{EM} responses, with IL-2 produced by both subsets and IFN-γ produced by T_{EM} (2, 18). The study of Ag-specific human CD4$^+$ T cells is classically based on the detection of cytokine-producing cells following short-term in vitro Ag stimulation. Because memory T cell subsets are reported to be specialized in the production of specific cytokines, individual cytokines do not allow the study of total populations of Ag-specific cells. We identified CD154, the ligand for CD40, as a reliable intracytoplasmic marker of Ag-specific T_{CM} and T_{EM}. Recent studies demonstrated that CD154 is a sensitive and specific marker of Ag-specific CD4$^+$ T cells producing IFN-γ, IL-2, or TNF-α (21, 22). In this study, we characterized the differentiation of human CD4$^+$ T cell memory subsets specific for a cleared Ag using CD154 as a marker of Ag-specific cells. We selected the anti-hepatitis B vaccine (HBVac), which contains a recombinant fragment of the hepatitis B virus surface Ag (HBs) and the alum adjuvant (23). This vaccine is highly immunogenic and induces persistent memory (24). Using

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[3] Abbreviations used in this paper: T_{CM}, central memory T cell; T_{EM}, effector memory T cell; HBVac, anti-hepatitis B vaccine; HBs, hepatitis B virus surface Ag; SEB, staphylococcal enterotoxin.

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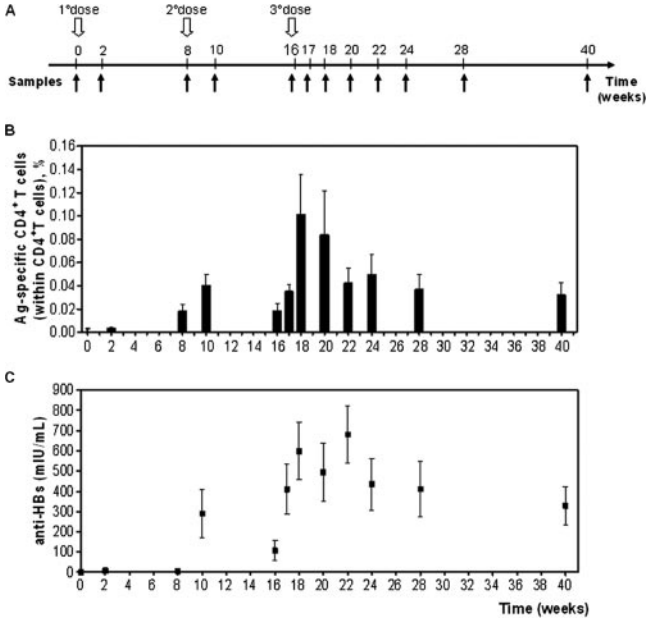

FIGURE 1. CD4$^+$ T cell response to primary HBVac. *A*, Healthy volunteers were immunized with HBVac. *B*, Ag-specific CD4$^+$ T cells were detected using intracytoplasmic CD154 expression and flow cytometry following short-term in vitro HBs stimulation. *C*, Anti-HBs Ab levels were measured by ELISA. Data are expressed as mean ± SEM. Three of 11 volunteers with frequencies of HBs-specific cells <0.03% CD4$^+$ T cells at the peak of the response were excluded from the analysis.

multiparameter flow cytometry, we demonstrate that Ag-specific T_{CM} are able to produce multiple cytokines, including IFN-γ, and have a high rate of proliferation in vivo.

Materials and Methods

Subjects

This study was approved by the Hôpital Erasme Ethics Committee. Healthy adults (20–30 years), naive for hepatitis B virus Ags, were immunized with the Engerix-B vaccine (gift from GlaxoSmithKline). Three doses (20 μg HBs) were given at 0, 8, and 16 wk. Blood was collected before and up to 40 wk after the last vaccine dose (Fig. 1*A*). A second group of volunteers who had been immunized with the same vaccine at least 2 years earlier was identified among laboratory staff (25–40 years). Those with a frequency of HBs-specific CD4$^+$ T cells above 1/1000 (6 of 20 volunteers tested) were recruited in the study.

Cell culture

PBMC were isolated using Lymphoprep (Nycomed) and cultured at 10^6/500 μl in RPMI 1640 with 10% FCS. Fresh cells were stimulated for 18 h with HBs (10 μg/ml; gift from GlaxoSmithKline), staphylococcal enterotoxin B (SEB, 2.5 μg/ml; Sigma-Aldrich) or medium alone but with no additional costimulation. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 16 h of culture.

Cell staining and flow cytometry analysis

After stimulation, Ag-specific cells were identified using intracellular staining of CD154. This method was recently described by Chattopadhyay et al. (21) and by Frentsch et al. (22). Using an in vitro model of toxic shock syndrome toxin-1 stimulation of Vβ2$^+$ CD4$^+$ T lymphocytes, we identified intracytoplasmic CD154 as a specific and sensitive marker of Ag-specific T_{CM} and T_{EM} (data not shown). The phenotype of HBs-specific cells was studied using the following conjugated Abs: CD4 PerCP or Pacific Blue, CD27 FITC or PE-Cy5, CD28 allophycocyanin, CD45RA FITC, CD45RO allophycocyanin, and HLA-DR FITC. Preliminary experiments indicated that brefeldin A interferes with the expression of CCR7 by activated CD4$^+$ T cells (data not shown). To avoid this interference, CCR7 was stained before cell stimulation. Using this method, the proportions of CCR7$^+$ and CCR7$^-$ CD4$^+$ T cells producing IL-2 and IFN-γ following SEB stimulation were similar to those detected in subsets purified by cell sorting with a FACSVantage (BD Biosciences; data not shown). After membrane marker staining, cells were permeabilized using FACS-Perm2 (BD Biosciences) and were stained using the following conjugated

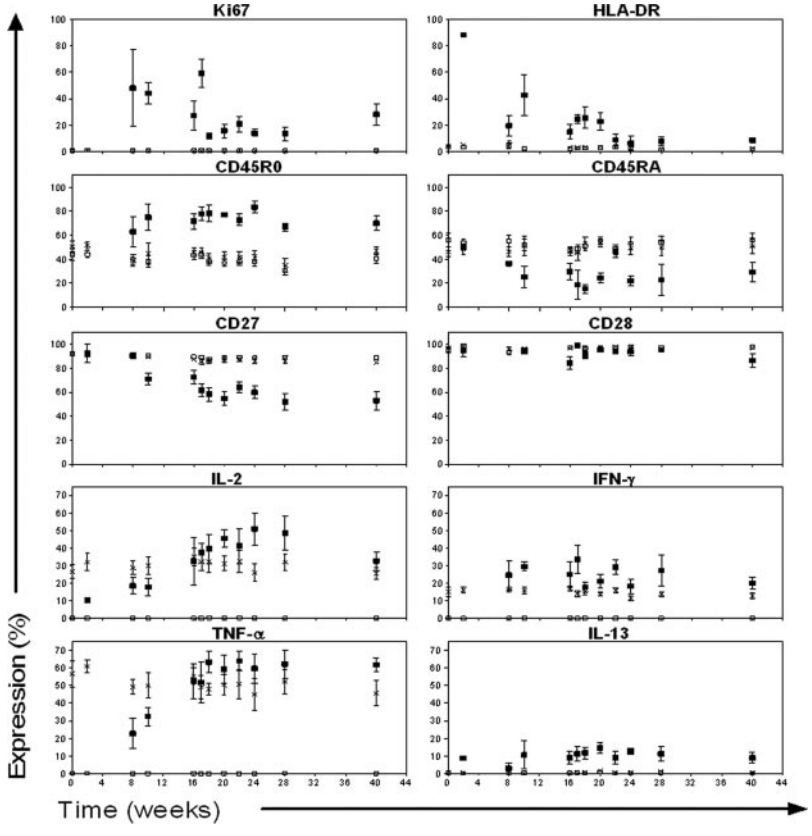

FIGURE 2. Activation and differentiation of CD4$^+$ T cells following primary HBVac. Healthy volunteers were immunized with HBVac. Expression of markers of activation and differentiation and production of cytokines were studied at the level of CD154$^+$CD4$^+$ T cells following HBs stimulation (■), CD154$^-$ CD4$^+$ T cells following HBs stimulation (□), and CD154$^+$ CD4$^+$ T following SEB stimulation (×). Data are expressed as mean ± SEM. Three of 11 volunteers with frequencies of HBs-specific cells <0.03% CD4$^+$ T cells at the peak of the response were excluded from the analysis.

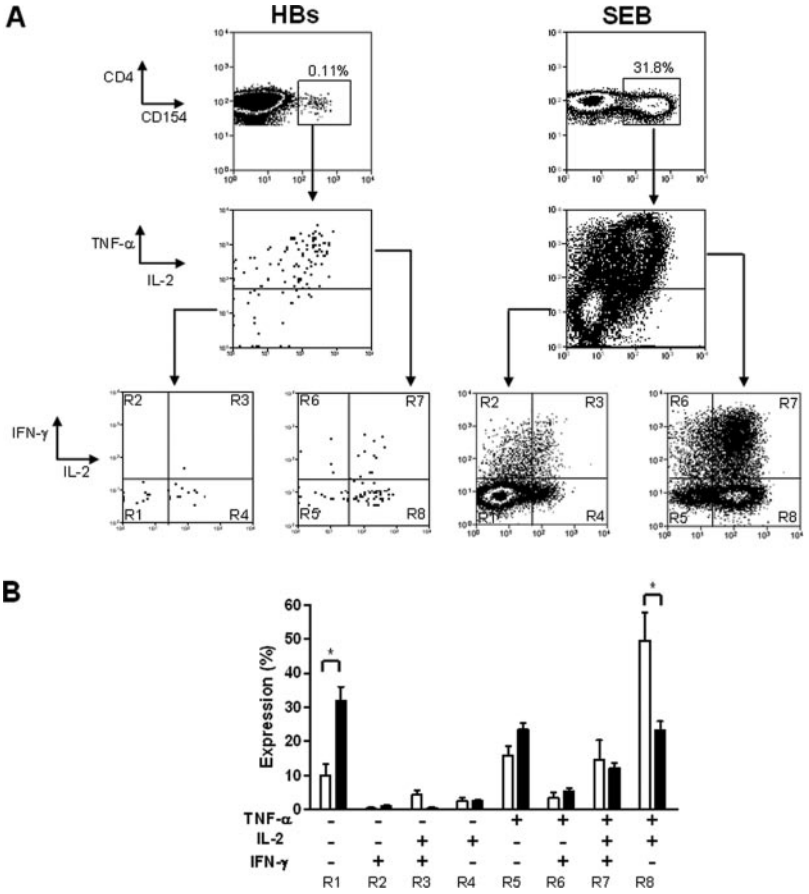

FIGURE 3. Cytokine production by HBs-specific $CD4^+$ T lymphocytes. $CD4^+$ T lymphocyte responses to HBs or SEB were analyzed in healthy donors who had been immunized with HBVac at least 2 years earlier ($n = 6$). $CD154^+CD4^+$ T cells were separated in eight subsets according to their capacity to produce TNF-α and/or IL-2 and/or IFN-γ. Dot plots from a representative donor are shown in *A*. *B*, The proportions (mean ± SEM) of the eight subsets measured in the six donors following HBs (□) or SEB (■) stimulation.

Abs: CD154 PE, allophycocyanin or PE-Cy5, IL-13 PE, IFN-γ FITC or PE, IL-2 PE or allophycocyanin, TNF-α allophycocyanin, and Ki67 FITC or PE. Ki67 expression by $CD154^+CD4^+$ T cells was used as a marker of cell cycle progression in vivo. Indeed, although SEB stimulation of PBMC for 18 h induced between 25 and 35% of $CD154^+CD4^+$ T cells, this in vitro stimulation did not influence the percentage of $CD4^+$ T lymphocytes expressing Ki67 (control mean ± SEM: 0.85 ± 0.1% vs SEB stimulated 0.91 ± 0.1%, $p = 0.48$). All Abs were obtained from BD Biosciences except CD27 PE-Cy5 (Beckman Coulter) and CCR7 PE (R&D Systems). Flow cytometry data for six parameters were obtained on a FACSCalibur using the CellQuest software (BD Biosciences), whereas data for nine parameters were obtained on a Cyan ADP LX9 using the Summit 4.2 software (DakoCytomation).

Serology

Anti-HBs Abs were detected in serum by ELISA using a commercial kit (DiaSorin).

Statistics

Frequencies of $CD154^+CD4^+$ T cells detected in unstimulated cultures were subtracted from stimulated conditions. Groups were compared using the paired Student's *t* test or the χ^2 test. Pearson's test was used to determine bivariate correlations. Analyses were performed using GraphPad Prism 4 for Windows (GraphPad).

Results

$CD4^+$ T cell response to primary anti-hepatitis B immunization

Primary immunization of seronegative adults with HBVac induced high frequencies of $CD4^+$ T cells expressing CD154 following short-term in vitro stimulation with HBs (Fig. 1*B*). No $CD154^+$ $CD4^+$ T cells were detected before immunization. The frequency of HBs-specific cells increased after each dose of vaccine and peaked 2 wk after the administration of the third dose (mean ± SEM, 1.01 ± 0.35/1000 $CD4^+$ T cells). Following this expansion phase, the HBs-specific cell population contracted and reached a plateau 12 wk postimmunization (0.37 ± 0.13/1000 $CD4^+$ T cells). The specificity of the changes was assessed by the stable frequencies of $CD154^+$ $CD4^+$ T cells following in vitro SEB stimulation (data not shown). Ab response to HBVac in these volunteers followed a similar profile to that of HBs-specific $CD154^+$ $CD4^+$ T cells (Fig. 1*C*). In addition, the areas under the curves of the two responses were significantly correlated ($r = 0.702$, $p = 0.035$; data not shown). During the primary phase of the response, high proportions of HBs-specific $CD4^+$ T cells expressed the proliferation marker Ki67 and were HLA-DR^+ (Fig. 2). This phenotype indicates in vivo activation of HBs-specific cells and was not observed on $CD4^+$ T cells that did not express CD154 following in vitro HBs stimulation or on cells that did express CD154 following SEB stimulation. During the memory phase of the response, very low proportions of HLA-DR^+ HBs-specific cells were detected. In contrast, high proportions of $Ki67^+$ cells were still detected up to 24 wk after the last vaccine dose. The expression of Ki67 by $CD154^+$ cells was not related to the in vitro short-term stimulation procedure (see *Materials and Methods*). This suggests that HBs-specific cells maintain the capacity to self-renew in vivo and will be studied in more detail in experiments presented below.

Soon after immunization, most HBs-specific cells were $CD45RO^+$ although ~20% of the cells expressed CD45RA (Fig. 2). The expression of CD27 by HBs-specific cells progressively decreased after the second and third doses of vaccine and reached a plateau thereafter. At steady state, ~50% of the cells were $CD27^-$. In contrast, HBs-specific cells remained $CD28^+$ during the entire follow-up, indicating an intermediate state of $CD4^+$ T cell differentiation (13, 25). The specificity of the differentiation

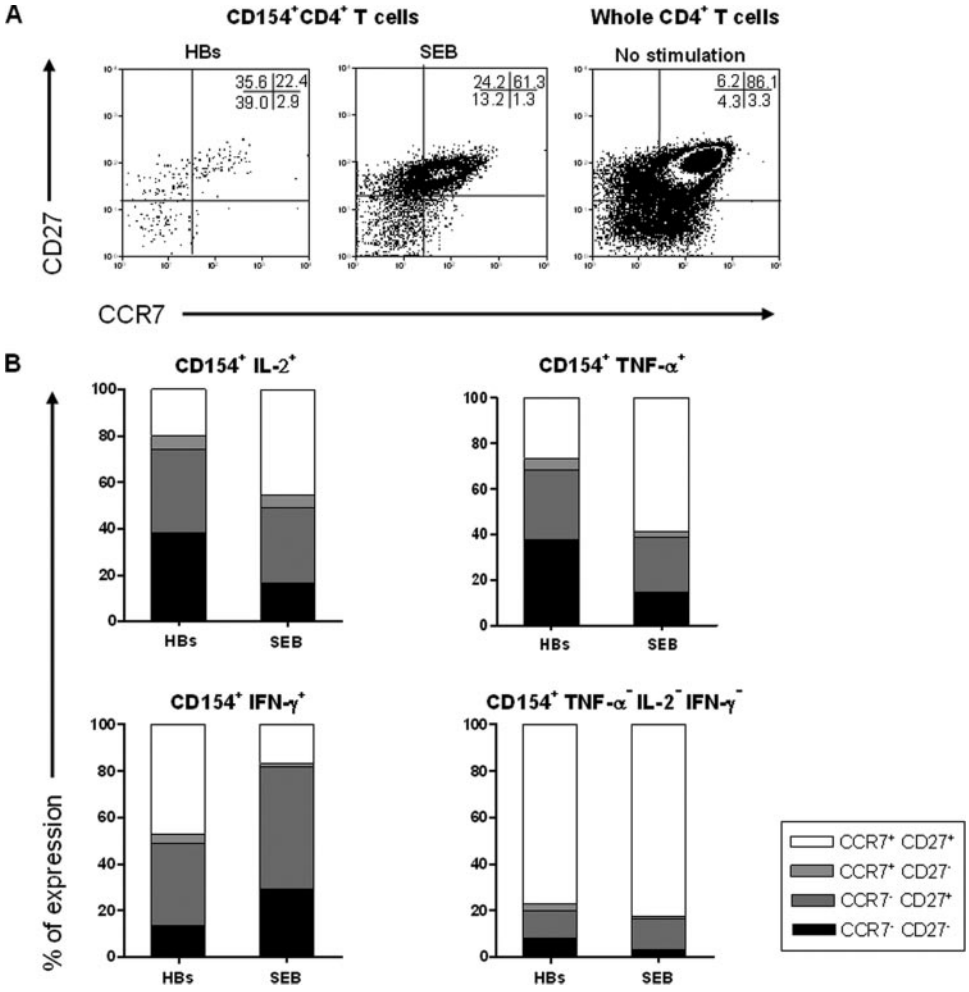

FIGURE 4. Differentiation of HBs-specific CD4+ T lymphocytes. CD4+ T lymphocyte responses to HBs or SEB were analyzed in healthy donors who had been immunized with HBVac at least 2 years earlier ($n = 6$). *A*, The expression of CD27 and CCR7 by HBs-specific, SEB-stimulated, or total CD4+ T cells was analyzed by flow cytometry. *B*, The proportion of the memory subsets in CD154+CD4+ T cells producing IL-2, TNF-α, IFN-γ, or none of these cytokines following HBs or SEB stimulation was analyzed. *A*, One donor representative of six and mean proportions of the six donors (*B*).

phenotype observed on HBs-specific cells was assessed by the different and stable phenotype of CD154− cells following HBs stimulation and of CD154+ cells following SEB stimulation.

The acquisition of the capacity to produce cytokines by HBs-specific cells followed two distinct profiles (Fig. 2). After the first dose of vaccine, ~20% of CD154+CD4+ T cells acquired the capacity to produce IFN-γ. Thereafter, the proportion of IFN-γ-producing cells remained stable during the entire follow-up. In contrast, the proportions of HBs-specific cells producing IL-2 and TNF-α continued to increase after the second and third doses of vaccine and maximum capacity to produce these cytokines was reached around 20 wk. At 40 wk, the majority of HBs-specific cells produced TNF-α and a large fraction were IL-2+. A small fraction of cells produced IL-13 after the first vaccine dose and this proportion remained stable during the follow-up. No cytokine production was detected in CD4+ T cells that did not express CD154 following HBs stimulation.

Cytokine production by HBs-specific memory CD4+ T cells

To study memory CD4+ T cells with no interference of recent Ag stimulation, we selected a population of volunteers who had been immunized with HBVac at least 2 years earlier. The phenotype and the cytokine-producing capacity of CD154+CD4+ T cells observed in these subjects following in vitro HBs stimulation were similar to those observed in the immunization cohort at 40 wk (data not shown). During the memory phase of the response, HBs-specific cells displayed a considerable heterogeneity in the cytokines produced. CD154+CD4+ T cells were separated in eight subsets according to their capacity to produce TNF-α and/or IL-2 and/or IFN-γ (Fig. 3). A fraction of CD154+ cells (10%) did not produce IL-2, IFN-γ, or TNF-α. The predominant cytokine produced by HBs-specific cells was TNF-α and the majority of IL-2+and IFN-γ+ cells were included in the TNF-α+ population. Cells producing only IFN-γ were undetectable. A similar profile was observed in CD154+CD4+ T cells following SEB stimulation. The frequency of cells that did not produce IL-2, IFN-γ, or TNF-α was higher than in the HBs-specific population (Fig. 3*B*). This difference is likely to be related to the presence of naive CD4+ T cells among the cells responding to SEB. Among the cells that produced cytokines following SEB stimulation, the same subsets were observed than among HBs-specific cells but the frequency of the TNF-α+IL-2+IFN-γ− subset was less represented (Fig. 3*B*).

HBs-specific cells were distributed in the three subsets of memory CD4+ T cells (CCR7+CD27+, CCR7−CD27+, and CCR7−CD27−) that were described by Fritsch et al. (13) in an in vitro model of polyclonal cell stimulation (Fig. 4*A*). The majority of HBs-specific cells were CCR7− and were distributed evenly among the CCR7−CD27+ and CCR7−CD27− subsets. Since CCR7+CD27− cells were virtually absent, all T_{CM} expressed CD27. The same memory subsets were detected among CD154+CD4+ cells following SEB stimulation and within the total population of CD4+ T cells but these two populations contained higher proportions of CCR7+CD27+ cells.

In keeping with previously published results, IFN-γ was predominantly produced by CCR7−CD4+ T cells following SEB stimulation, whereas CCR7+CD4+ T cells contributed more to

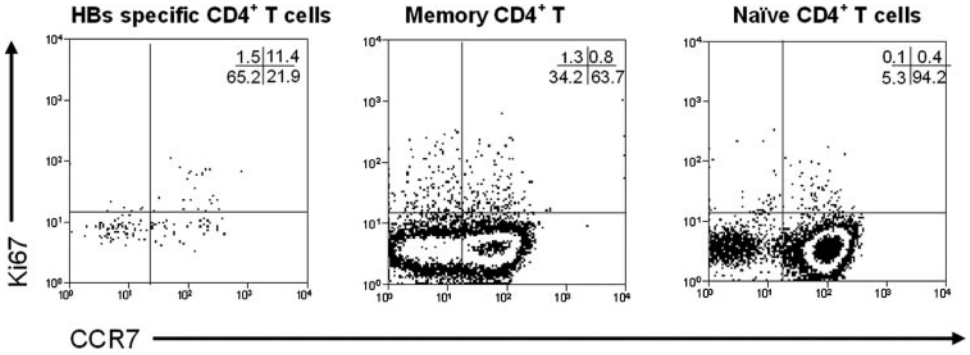

FIGURE 5. In vivo proliferation of HBs-specific $CD4^+$ T lymphocytes. The expression of Ki67 and CCR7 by HBs-specific $CD4^+$ T cells and total populations of $CD45RO^+$ memory and $CD45RO^-$ naive $CD4^+$ T cells were analyzed by flow cytometry in healthy donors who had been immunized with HBVac at least 2 years earlier ($n = 5$). One representative donor is presented.

IL-2 production (Refs. 7, 14, and 26 and Fig. 4*B*). A distinct pattern was observed among HBs-specific cells. The majority of $CD154^+$ cells that produced IL-2 were T_{EM}. A similar profile was observed for TNF-α-producing cells. In contrast, a large fraction of IFN-γ-producing cells were T_{CM}. Interestingly, almost all $CD154^+$ cells that did not produce IL-2, TNF-α, or IFN-γ were T_{CM}. No clear specialization of $CD27^+$ and $CD27^-$ T_{EM} was observed except for IFN-γ which was more dependent on $CD27^+$ than $CD27^-$ T_{EM}, in keeping with results obtained following polyclonal $CD4^+$ T cell activation (13).

In vivo proliferation of HBs-specific memory $CD4^+$ T cells

Results presented in Fig. 2 indicate that a high proportion of HBs-specific memory cells express Ki67. Similar results were obtained in donors immunized >2 years earlier (Fig. 5). In keeping with in vivo deuterated glucose and ex vivo BrdU-labeling experiments (9, 17, 27), higher frequencies of $Ki67^+$ cells were detected within the total pool of memory (mean ± SEM, 2.3 ± 0.6%) as compared with naive $CD4^+$ T cells (0.6 ± 0.1%, $p < 0.05$). Furthermore, within the pool of memory cells the proportion of $Ki67^+$ cells was higher in $CCR7^-$ T_{EM} (2.9 ± 0.8%) than in $CCR7^+$ T_{CM} (2.1 ± 0.6%, $p < 0.05$). In contrast, the HBs-specific cells expressing Ki67 were $CCR7^+$. Because the expression of Ki67 was not related to the in vitro short-term stimulation with HBs (see *Materials and Methods*), these results suggest a particular ability of HBs-specific T_{CM} to self-renew.

Discussion

Using CD154 as a marker of Ag-specific cells, we provide the first characterization of the activation and the differentiation of human $CD4^+$ T cells following primary immunization with a protein vaccine. CD154 was recently proposed as a marker of Ag-specific $CD4^+$ T cells (21, 22). We confirmed the sensitivity of this marker in our model since no IL-2, TNF-α, or IFN-γ production was detected in $CD154^-CD4^+$ T cells following in vitro HBs stimulation. During the primary phase of the response, HBs-specific $CD4^+$ T cells expressed markers of activation (HLA-DR) and proliferation (Ki67) and rapidly acquired a memory phenotype characterized by the expression of CD45RO by the majority of the cells and the loss of CD27 by ~50% of them. The acquisition by $CD154^+CD4^+$ T cells of the capacity to produce cytokines followed two distinct patterns. Maximum IFN-γ production was acquired early during the course of the response, whereas the proportions of $CD154^+$ cells producing IL-2 and TNF-α progressively increased following the second and third booster immunizations. These results contrast with the paradigm, derived from the study of persistent viral infections, that IL-2 production decreases during the course of $CD4^+$ T cell differentiation (10, 13, 18, 19, 25). In patients controlling the replication of persistent viruses, $CD4^+$ T cells produce IL-2 and IFN-γ, whereas active viral replication is associated with decreased capacity of virus-specific $CD4^+$ T cells to produce IL-2. Whether this phenomenon is related to chronic activation of cells by viral Ags or to cell differentiation per se remains unclear. Our results suggest that $CD4^+$ T cell differentiation is not necessarily associated with decreased production of IL-2 and increased production of IFN-γ. This concept is further supported by the analysis of the differentiation phenotype of IFN-γ- and IL-2-producing $CD4^+$ T cells. In keeping with previously published results, IFN-γ was predominantly produced by $CCR7^-CD4^+$ T cells following SEB stimulation, whereas $CCR7^+CD4^+$ T cells contributed more to IL-2 production (7, 14, 26). A distinct pattern was observed among HBs-specific cells. The majority of $CD154^+$ cells that produced IL-2 following HBs activation were T_{EM}. In contrast, a large fraction of IFN-γ-producing cells were T_{CM}. These results indicate that the population of HBs-specific cells that produces IL-2 is more differentiated than that producing IFN-γ. They also indicate that HBs-specific T_{CM} and T_{EM} share the potential to produce IL-2 as well as effector cytokines. These observations contrast with the classical model of functional specialization of T_{CM} and T_{EM} but are in keeping with results reported using polyclonal activators (2, 7). These conflicting results could be related to the specificity of the memory cells studied. The functional specialization of T_{CM} and T_{EM} could be different in the responses to cleared protein vaccine Ag and to persistent infections during which Ags continuously drive the differentiation of effector cells.

The high proportion of IL-2-producing $CD4^+$ T cells detected following HBVac immunization is in accordance with data reported recently indicating that protein vaccines induce uncommitted IL-2-secreting $CD4^+$ T cells, whereas infections induce more IFN-γ-secreting cells (28, 29). However, the large proportions of TNF-α-secreting cells observed following HBVac immunization indicate that $CD4^+$ T cells induced by protein vaccines also produce effector cytokines.

The use of CD154 as a marker of Ag-specific cells allowed us to identify a population of $CD4^+$ T cells that did not produce IL-2, IFN-γ, or TNF-α following HBs stimulation. The detection of this population further emphasizes the interest of CD154 in the monitoring of vaccine responses. Interestingly, most of these cells were T_{CM}. Their functional properties, including their capacity to produce other cytokines, remain to be determined.

During the memory phase of the response, ~50% of HBs-specific $CD4^+$ T cells had lost the expression of CD27 and all expressed CD28. This phenotype indicates an intermediate state of cell differentiation. According to the linear model of $CD4^+$ T cell differentiation, cells sequentially lose the expression of CD27 and CD28 (13, 25). Using IFN-γ and IL-2 production as markers of Ag-specific cells, studies indicate that during persistent viral infections, $CD4^+$ T cells

accumulate at different stages of differentiation, with CMV-specific cells displaying a late differentiation phenotype (CD27$^-$CD28$^-$), HIV-specific cells an intermediate stage of differentiation (CD27$^-$CD28$^+$), and EBV-specific cells an early differentiation phenotype (CD27$^+$CD28$^+$) (11, 12, 20, 25). Our results indicate that this model of differentiation could also apply to CD4$^+$ T cell responses induced by protein vaccines. No clear specialization of CD27$^+$ and CD27$^-$ T_{EM} was observed except for IFN-γ which was more dependent on CD27$^+$ than CD27$^-$ T_{EM}, in keeping with results obtained following polyclonal CD4$^+$ T cell activation (13). The functional properties of Ag-specific CD27$^+$ and CD27$^-$ memory CD4$^+$ T cells remain poorly characterized. Previous studies suggest that CD27$^-$ and CD27$^+$CD4$^+$ T cells differ in their capacity to stimulate Ig production by B cells (30, 31).

A substantial fraction of HBs-specific cells were in cycle during the memory phase of the response to HBVac. Interestingly, virtually all Ki67$^+$ cells expressed CCR7, suggesting a particular ability of the T_{CM} population to self-renew. An opposite pattern was observed in the total memory CD4$^+$ T cell population, where a higher proportion of Ki67$^+$ cells was detected in the T_{EM} pool than in the T_{CM} pool, confirming in vivo deuterated glucose experiments (17). This difference could be related to the enrichment of the total T_{EM} pool in cells specific for persistent microorganisms and to their chronic antigenic activation rather than to a different capacity to self-renew (18, 19). In vivo BrdU-labeling experiments in rhesus macaques suggest that Ki67 expression delineates cells that have undergone S phase in the previous 3–4 days (27). If these observations are applied to our model, the population of HBs-specific memory cells would renew every 30–40 days under steady-state conditions. These results are in line with the doubling times of CD4$^+$ memory T cells measured in vivo following deuterated glucose labeling (17). Because the frequency of HBs-specific memory CD4$^+$ T cells remain relatively stable over time and because the highest proliferative activity was detected among T_{CM}, these cells could represent a reservoir for shorter-lived T_{EM}. This model is supported by the observation that homeostatic cytokines stimulating the proliferation of T_{CM} also down-regulate their expression of CCR7 (4, 15).

In conclusion, using CD154 as a marker of Ag-specific cells, we demonstrated that following vaccination with a protein Ag, T_{CM} and T_{EM} share the capacity to produce multiple cytokines. These results indicate that the model of functional specialization of CD4$^+$ T cell memory subsets according to their homing properties is not universal and cannot be applied to all immune responses in humans. In contrast, this study provides the first in vivo evidence that T_{CM} specific for a cleared Ag have a higher capacity to self-renew than T_{EM}. Although we do not provide evidence for a lineage relationship between the two subsets, this study supports the model in which T_{CM} behave as memory stem cells contributing to the maintenance of the T_{EM} pool (3).

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Disclosures

The authors have no financial conflict of interest.

References

1. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787–793.
2. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
3. Lanzavecchia, A., and F. Sallusto. 2005. Understanding the generation and function of memory T cell subsets. *Curr. Opin. Immunol.* 17: 326–332.
4. Lanzavecchia, A., and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol.* 2: 982–987.
5. Debes, G. F., U. E. Hopken, and A. Hamann. 2002. In vivo differentiated cytokine-producing CD4$^+$ T cells express functional CCR7. *J. Immunol.* 168: 5441–5447.
6. Debes, G. F., K. Bonhagen, T. Wolff, U. Kretschmer, S. Krautwald, T. Kamradt, and A. Hamann. 2004. CC chemokine receptor 7 expression by effector/memory CD4$^+$ T cells depends on antigen specificity and tissue localization during influenza A virus infection. *J. Virol.* 78: 7528–7535.
7. Kim, C. H., L. Rott, E. J. Kunkel, M. C. Genovese, D. P. Andrew, L. Wu, and E. C. Butcher. 2001. Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest.* 108: 1331–1339.
8. Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher. 2002. Cutting edge: CCR7$^+$ and CCR7$^-$ memory T cells do not differ in immediate effector cell function. *J. Immunol.* 169: 638–641.
9. Rivino, L., M. Messi, D. Jarrossay, A. Lanzavecchia, F. Sallusto, and J. Geginat. 2004. Chemokine receptor expression identifies pre-T helper (Th)1, pre-Th2, and nonpolarized cells among human CD4$^+$ central memory T cells. *J. Exp. Med.* 200: 725–735.
10. Pantaleo, G., and A. Harari. 2006. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat. Rev. Immunol.* 6: 417–423.
11. Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A. B. Rickinson, A. J. McMichael, and M. F. Callan. 2003. Characterization of the CD4$^+$ T cell response to Epstein-Barr virus during primary and persistent infection. *J. Exp. Med.* 198: 903–911.
12. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, et al. 2002. Memory CD8$^+$ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8: 379–385.
13. Fritsch, R. D., X. Shen, G. P. Sims, K. S. Hathcock, R. J. Hodes, and P. E. Lipsky. 2005. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J. Immunol.* 175: 6489–6497.
14. Amyes, E., A. J. McMichael, and M. F. Callan. 2005. Human CD4$^+$ T cells are predominantly distributed among six phenotypically and functionally distinct subsets. *J. Immunol.* 175: 5765–5773.
15. Geginat, J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4$^+$ T cells. *J. Exp. Med.* 194: 1711–1719.
16. Grossman, Z., B. Min, M. Meier-Schellersheim, and W. E. Paul. 2004. Concomitant regulation of T-cell activation and homeostasis. *Nat. Rev. Immunol.* 4: 387–395.
17. Macallan, D. C., D. Wallace, Y. Zhang, C. De Lara, A. T. Worth, H. Ghattas, G. E. Griffin, P. C. Beverley, and D. F. Tough. 2004. Rapid turnover of effector-memory CD4$^+$ T cells in healthy humans. *J. Exp. Med.* 200: 255–260.
18. Harari, A., F. Vallelian, P. R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* 174: 1037–1045.
19. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4$^+$ T cells endowed with proliferative capacity. *J. Exp. Med.* 198: 1909–1922.
20. Yue, F. Y., C. M. Kovacs, R. C. Dimayuga, P. Parks, and M. A. Ostrowski. 2004. HIV-1-specific memory CD4$^+$ T cells are phenotypically less mature than cytomegalovirus-specific memory CD4$^+$ T cells. *J. Immunol.* 172: 2476–2486.
21. Chattopadhyay, P. K., J. Yu, and M. Roederer. 2005. A live-cell assay to detect antigen-specific CD4$^+$ T cells with diverse cytokine profiles. *Nat. Med.* 11: 1113–1117.
22. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4$^+$ T cells specific for defined antigens according to CD154 expression. *Nat. Med.* 11: 1118–1124.
23. Wiedermann, G., N. Scheiermann, P. Goubau, F. Ambrosch, M. Gesemann, C. De Bel, P. Kremsner, D. Paar, C. Kunz, P. Hauser, et al. 1987. Multicentre dose range study of a yeast-derived hepatitis B vaccine. *Vaccine* 5: 179–183.
24. Zanetti, A. R., A. Mariano, L. Romano, R. D'Amelio, M. Chironna, R. C. Coppola, M. Cuccia, R. Mangione, F. Marrone, F. S. Negrone, et al. 2005. Long-term immunogenicity of hepatitis B vaccination and policy for booster: an Italian multicentre study. *Lancet* 366: 1379–1384.
25. Appay, V., and S. L. Rowland-Jones. 2002. Premature ageing of the immune system: the cause of AIDS? *Trends Immunol.* 23: 580–585.
26. Fontenot, A. P., B. E. Palmer, A. K. Sullivan, F. G. Joslin, C. C. Wilson, L. A. Maier, L. S. Newman, and B. L. Kotzin. 2005. Frequency of beryllium-specific, central memory CD4$^+$ T cells in blood determines proliferative response. *J. Clin. Invest.* 115: 2886–2893.
27. Pitcher, C. J., S. I. Hagen, J. M. Walker, R. Lum, B. L. Mitchell, V. C. Maino, M. K. Axthelm, and L. J. Picker. 2002. Development and homeostasis of T cell memory in rhesus macaque. *J. Immunol.* 168: 29–43.
28. De Rosa, S. C., F. X. Lu, J. Yu, S. P. Perfetto, J. Falloon, S. Moser, T. G. Evans, R. Koup, C. J. Miller, and M. Roederer. 2004. Vaccination in humans generates broad T cell cytokine responses. *J. Immunol.* 173: 5372–5380.
29. Divekar, A. A., D. M. Zaiss, F. E. Lee, D. Liu, D. J. Topham, A. J. Sijts, and T. R. Mosmann. 2006. Protein vaccines induce uncommitted IL-2-secreting human and mouse CD4 T cells, whereas infections induce more IFN-γ-secreting cells. *J. Immunol.* 176: 1465–1473.
30. Tortorella, C., H. Schulze-Koops, R. Thomas, J. B. Splawski, L. S. Davis, L. J. Picker, and P. E. Lipsky. 1995. Expression of CD45RB and CD27 identifies subsets of CD4$^+$ memory T cells with different capacities to induce B cell differentiation. *J. Immunol.* 155: 149–162.
31. van Lier, R. A., M. O. Pool, P. Kabel, S. Mous, F. Terpstra, M. A. De Rie, C. J. Melief, and F. Miedema. 1988. Anti-CD27 monoclonal antibodies identify two functionally distinct subpopulations within the CD4$^+$ T cell subset. *Eur. J. Immunol.* 18: 811–816.