Ex Vivo Monitoring of Antigen-Specific CD4+ T Cells after Recall Immunization with Tetanus Toxoid

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To monitor antigen-specific CD4+ T cells during a recall immune response to tetanus toxoid (TT), a sequential analysis including ex vivo phenotyping and cytokine flow cytometry, followed by cloning and T-cell-receptor (TCR) spectratyping of cytokine-positive CD4+ T cells, was performed. Grossly, twice as many TT-specific CD4+ T-cell clones, ex vivo derived from the CCR7+/− CD69+ interleukin-2-positive (IL-2+) CD4+ subsets, belonged to the central memory (TCM; CD62L+ CD27− CCR7+) compared to the effector memory population (TEM; CD62L− CD27− CCR7−). After the boost, a predominant expansion of the TCM population was observed with more limited variations of the TEM population. TCR beta-chain-variable region (BV) spectratyping and sequencing confirmed a large concordance between most frequently expressed BV TCR-CDR3 from ex vivo-sorted CCR7+/− CD69+ IL-2+ CD4+ subsets and BV usage of in vitro-derived TT-specific CD4+ T-cell clones, further demonstrating the highly polyclonal but stable character of the specific recall response to TT. Taken together, ex vivo flow cytometry analysis focused on the CCR7+/− CD69+ IL-2+ CD4+ subsets appears to target the bulk of antigen-specific T cells and to reach an analytical power sufficient to adequately delineate in field trials the profile of the antigen-specific response to vaccine.

Phenotypic characterization of antigen-specific CD4+ T cells with distinct functional properties represents currently a major challenge in human applied experimental immunology. Ex vivo analysis of these cell populations is critical, since in vitro culture may alter composition and functional properties of the populations of interest. If major histocompatibility complex (MHC) class I tetramer/peptide complexes did greatly facilitate the analysis of antigen specific CD8+ T cells, the use of MHC class II tetramer/peptide has been hampered by the complexity of MHC class II tetramer production, by the usually low CD4+ T-cell precursor frequency, by an often-degenerated peptide epitope specificity, and by non-MHC class II molecule-restricted allelic expression (12, 15, 24, 35, 36). As an alternative approach to circumvent these difficulties, we here applied cytokine flow cytometry (9, 11), now extensively used to define ex vivo frequencies and phenotypic and functional characteristics of viral antigen-specific CD4+ T cells (8, 22, 43), coupled to T-cell-receptor (TCR) spectratype analysis. Indeed, characterization of the unique complementary determining region 3 (CDR3) sequence expression of CD4+ (or CD8+) T-cell clones appears to be a sensitive method for monitoring qualitative and quantitative T-cell responses during natural infection or vaccination (21, 23, 45, 48).

In the present study, tetanus toxoid (TT) was used as a model vaccine antigen that likely shares common functional characteristics with other nonviral antigens, such as a low frequency of specific CD4+ T cells in the periphery at steady state and a specific CD4+ T-cell response to multiple epitope peptides presented by MHC class II molecules (16, 36, 40). Using ex vivo cell phenotyping and cloning in combination with TCR spectratype analysis of cytokine-positive CD4+ T cells, we sought to establish experimental conditions to track ex vivo antigen-specific CD4+ T cells in conditions that are likely to reflect clinical situations such as vaccination, nonviral pathogen infections, or allergic diseases.

MATERIALS AND METHODS

Donor samples, cell preparation, and antigen stimulation. All donors (n = 4) were healthy subjects, immunized with TT at least 10 years previously according to current TT immunization guidelines. Each volunteer gave informed consent before immunization with a recall injection of TT vaccine (DiTe Anatoxal, Berna, Switzerland), and the study protocol was reviewed by the Ethical Review Board of the Faculty of Medicine, Lausanne, Switzerland. Peripheral blood mononuclear cells (PBMC) collected before (day 0) and at days 7, 14, 21, 35, 84, and 280 after the TT boost were isolated by centrifugation on a Ficoll-Paque gradient (Amersham Bioscience, Uppsala, Sweden). PBMC (4 × 10⁶/well) were stimulated for 16 h at 37°C in 5% CO2 in 24-well plates in RPMI 1640 medium (Sigma Chemical Co., Buchs, Switzerland) supplemented with 2 mM L-glutamine (Sigma), 1 mM nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 2 × 10⁻⁵ M 2-mercaptoethanol (Sigma), 1 mM kanamycin (Gibco-BRL/Life Technologies, AG, Basel, Switzerland), and 5% human serum (Swiss Red Cross, Bern, Switzerland) in the presence of 100 μg of purified TT (Aventis Pasteur, Lyon, France)/ml or 10 μg of Staphylococcus enterotoxin B (Fluka, Switzerland)/ml. After stimulation, the cells were centrifuged, and the supernatants were harvested and kept at −70°C for further use.

Immunofluorescence staining and cell sorting. Stimulated and nonstimulated PBMC were stained in phosphate-buffered saline buffer containing 0.5% bovine serum albumin (Fluka) and 1 mM EDTA, using the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated CD69 (FN50), CD45RO (UCHL1), and CD27 (M-T271); phycoerythrin-conjugated CD62L (DREG-56), HLA-DR, -DP, and -DQ (TU39); cytochrome-conjugated CD4 (RPA-T4); PerCP-Cy5.5-conjugated CD4 (SK3); APC-conjugated CD69 (FN50) and CD25 (2A3); and FITC- or APC-conjugated polyclonal goat anti-rat immunoglobulin (all from BD Bioscience, Basel, Switzerland). We also used phycoerythrin-conjugated CD8 (DK25; Dakocytomation, Zug, Switzerland), phycoerythrin/Texas Red-conjugated CD4 (SFCT2D11; Beckman Coulter, Nyon, Switzerland), and rat anti-human CCR7 antibody (3D12; rat immunoglobulin G2a). For cytokine expression, cells were first stained by using gamma interferon (IFN-γ) and/or interleukin-2 (IL-2) catch reagent according to the manufacturer’s protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by selected anti-
cell surface marker antibodies. Samples were analyzed on a FACScalibur, and the fluorescence intensity was determined by using CellQuest software (Becton Dickinson, Mountain View, CA). When indicated, labeled cells were sorted on a FACS Vantage SE (Becton Dickinson).

T-cell proliferation, T-cell cloning, and IL-2 production. Freshly prepared PBMC (3 × 10^6 to 4 × 10^6 cells) were plated in 96-well flat-bottom plates in complete RPMI 1640 medium, stimulated with TT at 50 μg/ml or phytohemagglutinin at 2 μg/ml or left unstimulated, followed by incubation for 5 days at 37°C in 5% CO2. Cells were harvested after 18 h of incubation with 1 μCi of [3H]thymidine (Hartmann Analytic GmbH, Braunschweig, Germany). [3H]thymidine incorporation was measured in a β-counter (Top Count; Packard Bioscience S.A., Zürich, Switzerland). T-cell cloning (0.3 cell/well) was performed as described earlier (3, 36). Clones with a stimulation index of >2 were further expanded and retested for specificity as described previously (4, 36). The IL-2 concentration in supernatants from PBMC stimulated with or without TT or Staphylococcus enterotoxin B was determined by using the IL-2-dependent CTL-L line assay.

T-cell receptor spectratype analysis and sequencing. CD4+ T-cell subpopulations of interest were first sorted in 0.5-ml tubes. The procedure for cDNA preparation and cDNA amplification was recently described in detail (7). cDNA amplification was performed with a minimal cell number of 80 and a maximum of 100 cells. PCR of the cDNA was performed using one side with a radio-labeled constant primer and on the other with a primer corresponding to the variable region of the TCR β-chain region, based on the procedure described by Malanka et al. (33). PCR conditions and primers used were as described previously (18). PCR products were applied to a 6% polyacrylamide-urea gel. Autoradiographies were performed on dried gels and, after scanning, each TCR-CDR3 band of the spectratype was quantified by densitometry using Instant-Imager electronic autoradiography software (Packard Bioscience). A separate CD3 PCR amplification was used as an internal control for cDNA quality and for migration reference. TCRs of T-cell clones were sequenced according to a standardized procedure. Briefly, PCR products were purified by using a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland). A sequencing reaction was performed by using BigDye terminator cycle sequencing kit (Applied Biosystems, Rotkreuz, Switzerland). Purified sequenced reactions (DyeEx spin kit; QIAGEN) were analyzed by using the sequencer ABI Prism 377 and Sequencher 4.1 software (Applied Biosystems).

RESULTS

Antigen-specific CD4+ T cells predominate within the CD69+ IL-2+ CD4+ T-cell subset. PBMC from four previously immunized donors (≥10 years) were collected prior to and after a recall immunization with TT. To delineate step-by-step potential, antigen-specific CD4+ T-cell enriched subpopulations, freshly prepared PBMC were first briefly stimulated with TT and then analyzed by four-color flow cytometry. Ex vivo, steady-state unstimulated CD4+ T cells encompassed the typical phenotype of naive (CD45RO−CCR7+; CD27+CCR7+), central memory (T CM; CD45RO−CCR7+; CD27+CCR7+), and effector memory (TEM; CD45RO−CCR7−; CD27−CCR7−) T cells (44) (Fig. 1A). Coexpression analysis of CD62L with CCR7 and CD27 cell surface markers further supported these data (not shown). As expected from primed donors, IL-2+ CD4+ T cells stimulated by a short exposure to TT were mainly found within the T CM (53.5) and TEM (23.2) populations (Fig. 1B, panel CD45RO/CCR7). However, activated cells (CD69+) were mainly present within the T CM population (CD45RO−CD27+CCR7+) and rarely within the CCR7− population. Coexpression of CD69 with HLA-DR, -DP, and -DQ molecules confirmed that these cells had been previously stimulated. In cells cultured for 16 h in the absence of antigen (Fig. 1C), <0.05% cells were IL-2+, and none displayed an activated phenotype.

To ascertain the level of antigen specificity of the above-defined CD4+ T-cell subsets, PBMC were sorted by four-color flow cytometry after stimulation with TT on the basis of IFN-γ and/or IL-2 secretion and then in combination with the early activation marker CD69, cloned, and analyzed for specificity by classical antigen proliferation (Table 1, donor 1, and Fig. 2A).
From the first step of this analysis, we could conclude that cytokine expression alone was unable to identify the core of TT-specific CD4+ T cells. Indeed, despite the high number of clones tested (212 and 96), the frequency of TT-specific T-cell clones in the IFN-γ-2%, as well as in the IL-2+ (3%), compartments was definitely low. This suggested that even a short in vitro antigen stimulation was able to induce a high, compartmentalized CCR7+ / H9253/H11001 clones in the IFN-γ-specific CD4+ cytokine expression alone was unable to identify the core of T-cell populations (range, 0 to 8%; median, 0.5%; Table 1). After double cytokine staining, only rare cells stained for both IL-2 and IFN-γ (0.06% in donor 3 [data not shown]). Nonetheless, whereas very few clones (n = 7) were derived from the IL-2+ IFN-γ+ double-positive population, most of them were antigen specific (n = 5 [71%]). Finally, we reproducibly failed to detect Th2 type IL-4+/CCR7+/CD69+/CD4+ T cells. Overall, from these cloning experiments we concluded that a marked proportion of TT-specific CD4+ T cells belonged to Th1, IL-2-expressing CCR7+/CD69+/CD4+ T-cell subsets, at steady state as well as postboost, although a significant but variable number of antigen-specific T cells could be detected among the IFN-γ-expressing CCR7+/CD69+/CD4+ population, depending on the donor. Pragmatically, to further track TT-specific CD4+ T cells, we concentrated our analysis on the IL-2-expressing CCR7+/CD69+/CD4+ T-cell populations.

**Longitudinal follow-up of TT-specific CD4+ T cells after recall immunization.** To evaluate the recently recognized functional and phenotypic heterogeneity of effector and memory compartments (1, 42), we longitudinally monitored the effect of a recall immunization with TT on the memory response of our volunteers. TT-specific proliferation was markedly enhanced by the TT boost and peaked between days 7 and 14 (Fig. 3A), with large variations between donors, from a 3.5- and 4.6-fold increase in donors 3 and 4, respectively, to a 12-fold increase in donor 2. IL-2 secretion of short-term-stimulated cells (Fig. 3B) followed grossly the same pattern. To get a better insight into the fate of TT-specific cell populations expanded upon the recall immunization, we specifically analyzed the response of TCM (CCR7+/CD69+/IL-2+CD4+) and TEM (CCR7+/CD69+/IL-2+CD4+) subsets. Compared to TEM subsets, a predominant (although variable) expansion of TCM cells was observed in all donors, which peaked at day 7 or 14 (Fig. 3C). In addition, analysis of CD27+CD45RO+, HLA-DR+, DP+, DO+CCR7+/IL-2+CD4+ T cells confirmed that TT-specific T-cell expansion at days 7 and 14 was mainly present within the TCM population (data not shown). Altogether, despite a limited set of observations, these data underlined the heterogeneity of the antigen-specific memory response prior to and after recall immunization.

**TCR repertoire analysis of TT-specific CD4+ T cells.** To further characterize the memory response and to validate our phenotypic approach of T-cell specificity as well, we next examined longitudinally TCR spectratypes of sorted TT-stimulated CCR7+/IL-2-CD69+CD4+ T cells from the four donors. We designed an algorithm to identify statistically relevant TCR spectratypes from the data, as well as a unique allele-specific TCR spectratype analysis, which was able to identify specific TCR spectratypes.

### TABLE 1. Antigen-specific clonal frequency of defined CD4+ T-cell subsets

| Donor and time point | Cell sorting phenotype |
|----------------------|-----------------------|
|                      | No. T-cell clones     |
|                      | No. TT specific       |
|                      | % TT specific         |
| Donor 1, day 0       | IFN-γ+/CD4+ 212       |
|                      | 44                    |
|                      | 2                     |
| IL-2−/CD4+          | 96                    |
|                      | 3                     |
| IFN-γ−/CD4+         | 192                   |
|                      | 1                     |
| IL-2−/CD4+          | 192                   |
|                      | 0                     |
| CD69+ IFN−/CD4+     | 104                   |
|                      | 6                     |
| CD69− IL-2+/CD4+    | 128                   |
|                      | 61                    |
| CD69− IFN−/CD4+     | 78                    |
|                      | 0                     |
| Donor 2, day 0       | CCR7+/CD69+ 178       |
|                      | 55                    |
|                      | 31                    |
| IL-2−/CD4+          | 19                    |
|                      | 2                     |
| CCR7−/CD69+         | 128                   |
|                      | 9                     |
| IL-2−/CD4+          | 109                   |
|                      | 3                     |
| CCR7+/CD69+         | 178                   |
|                      | 55                    |
|                      | 31                    |
| IL-2−/CD4+          | 19                    |
|                      | 2                     |
| CCR7−/CD69+         | 128                   |
|                      | 9                     |
| IL-2−/CD4+          | 109                   |
|                      | 3                     |
| CCR7+/CD69+         | 178                   |
|                      | 55                    |
|                      | 31                    |
| IL-2−/CD4+          | 19                    |
|                      | 2                     |
| CCR7−/CD69+         | 128                   |
|                      | 9                     |
| IL-2−/CD4+          | 109                   |
|                      | 3                     |
| CCR7+/CD69+         | 178                   |
|                      | 55                    |
|                      | 31                    |
| IL-2−/CD4+          | 19                    |
|                      | 2                     |
| CCR7−/CD69+         | 128                   |
|                      | 9                     |
| IL-2−/CD4+          | 109                   |
|                      | 3                     |

*An average of 60% cloning efficiency was obtained. The background frequencies for the cytokine-secreting subsets cultured in the absence of antigen were 0.0 to 0.05% and 0.0 to 0.02% for IFN-γ-/CD69− and IL-2-/CD69− gated CD4+ T cells, respectively.

*Phenotype of sorted and cloned T-cell populations, as described in Fig. 1.

*This value represents the number of growing wells that were tested in the proliferation assay.
donors described above, using 21 beta-chain-variable region (BV) families, before and after boost. When CD4+ T cells were selected ex vivo within the CCR7+/IL-2+ CD69+CD4+ T-cell populations, they expressed a defined and relatively limited set of TCR BV families prior to TT boost (day 0) (Fig. 4A). This was in contrast to the TCR BV spectratype analysis of a polyclonal, nonselected CD4+ T-cell population that displayed an expected Gaussian distribution (Fig. 4B, for BV3 and BV13). In the analysis of donor 4 as a representative example, 6 BV families (BV3, -7, -8, -9, -5.3, and -24) were detected at day 0. At day 14, a complex pattern of T-cell responses was obtained, including persistent, de novo-generated and/or reactivated, or abrogated responses. Overall, an increased BV and spectratype diversity was observed (detection of BV5.1, -2, -4, -11, and -18, novel bands in BV3 and BV8), whereas other BV disappeared (see BV7, -5.3, and -24). At day 35, the BV profile was further amplified compared to day 14. In part, the inconsistent presence or absence of some TCR BVs could be related to nonspecific selection during cell sorting, of activated bystander T cells, as demonstrated from the cloning experiments (Table 1). A bias due to the analysis of an insufficient number of cells is unlikely, since within a range of 80 to 1,000 sorted cells we did not find a correlation between the number of TCR-CDR3 bands and the number of sorted cells.

Since we had derived a large number of T-cell clones from sorted CCR7+/IL-2+ CD69+CD4+ T cells (Table 1), this allowed us to systematically correlate phenotypic and functional specificities (based on TT-specific T-cell proliferation and IL-2 production) with a particular TCR-CDR3 band visualized in the spectratype analysis. For instance, TT-specific T-cell clones D1.1, F8.2, and B6 from donor 2, which were restricted to BV9, corresponded precisely to a major BV9 TCR-CDR3 band detected in the originally sorted whole-cell population (Fig. 4C). Other examples are shown for three different clones of donor 1 (Fig. 4D). Globally, the BV usage profile of the four donors within the CCR7+/IL-2+ CD69+ CD4+ T-cell subsets was remarkably stable, as suggested by the
fairly constant number of TCR BV families used and by the persistence over time of most TCR-CDR3 from defined BV families (summarized in Table 2). Moreover, the stable expression of defined TCR-CDR3 in the ex vivo-sorted population was closely associated with both the frequency and BV usage of the in vitro-generated TT-specific T-cell clones, as demonstrated for donor 3 in Fig. 5A and B. Indeed, the BV usage profile of TT-specific T-cell clones (Fig. 5B) corresponded closely to the profile of the most stable BV families or subfamilies (BV1, BV2, BV3, BV4, BV5.1, BV5.3, BV6, and BV7) present in the long run in the originally sorted population (Fig. 5A). In contrast, we were practically unable to derive TT-specific T-cell clones from sporadically detected TCR-CDR3 and never from nondetected BV. Similar results were obtained with the other donors (Table 2). We next looked at potential differences in the diversity of BV usage between the T<sub>CM</sub> and T<sub>EM</sub> populations, postulating that a lesser degree of maturation and/or activation would favor a higher diversity (expressed as the total number of expressed TCR-CDR3) in the T<sub>CM</sub> population. However, TCR-CDR3 length polymorphism analysis of CCR7<sup>+</sup> CD69<sup>+</sup> IL-2<sup>+</sup> CD4<sup>+</sup> T<sub>CM</sub> cells and of CCR7<sup>+</sup> CD69<sup>+</sup> IL-2<sup>+</sup> CD4<sup>+</sup> T<sub>EM</sub> cells did not confirm such a hypothesis. Indeed, donors 2 and 4 showed a similar TCR-CDR3 repertoire diversity in both populations, whereas donor 3 had an even broader repertoire in TEM (Fig. 5C).

Finally, in vitro-generated TT-specific T-cell clones were analyzed for their unique signature designed during the rearrangement of the V-D-J fragments of the TCRs and leading to the formation of the CDR3 hypervariable region. Among a series of clones using the same BV, sequence analysis clearly revealed variability in their BJ usage and in their CDR3 sequences (Table 3), further diversifying the polyclonal response to TT.

Taken together, and within the limits of this approach, spectratype analysis strongly supported the sorting strategy devel-
opened in the present study and the observation that CD4+ T cells encountered within the CCR7+/− CD69+ IL-2− population were in large part antigen specific, with a predominance within the TCM subset. Moreover, it provided additional support to the cytokine flow cytometry and phenotypic approach to demonstrate the highly polyclonal, but over the long-term stable character of the recall response to TT, which was as diverse in the TEM as in the TCM compartments.

### DISCUSSION

The design of optimal strategies to improve vaccine efficiency is an ongoing challenge (32, 34). Specific CD4+ T cells found in naive, TCM, or TEM populations largely differ in their functional properties, such as antigen requirement for maximal efficiency, effector activity (level of cytokine secretion, costimulatory molecule expression), replicative activity, and/or life span (20, 29). Specific T-cell expansion of either population may therefore influence the protective efficacy of the pathogen-targeted, specific immune response. In the present study, we intended to define a pragmatic approach that could allow the ex vivo follow-up of non-viral-antigen-specific CD4+ T cells after vaccination, with the closest possible representation of the in vivo situation. To do this, we first defined the criteria that delineated ex vivo with a high degree of probability the antigen (TT)-specific CD4+ T-cell population and tracked this population longitudinally. We completed the analysis, and a posteriori validated our ex vivo phenotypic approach, by an antigen-specific CD4+ TCR repertoire analysis of ex vivo-derived TT-specific clones isolated from the subpopulations of interest (CCR7+/− CD69+ IL-2− CD4+). We observed from this sequential analysis a marked expansion, in all four donors, of the CD69+ IL-2− CD4+ TCM subset, which comprised a substantial proportion of TT-specific T cells. Nonetheless, a non-negligible number of TT-specific T-cell clones were also derived from the CCR7+/− CD69+ IFN-γ+ CD4+ T-cell subset of another donor (i.e., donor 3). This confirms that, upon priming, depending on the nature of the generated immune response, heterogeneous and variable pools of memory T cells could be generated, further diversified by recall immunization (30, 41). Indeed, although the recall immunization with TT induced a dominant population of antigen-specific IL-2-producing CD4+ T cells within the TEM subset, this does not exclude, depending on individual homeostatic constraints, the generation of a significant proportion of antigen-specific IFN-γ-producing CD4+ T cells within the TCM and TEM subset, results in line with recently published observations (19, 41, 47).

All time points included, the median frequency of TT-specific CD4+ T-cell clones was grossly twice as high in the CCR7+ CD69+ IL-2+ CD4+ T-cell subset (TCM; median, 43%) than in the CCR7− CD69+ IL-2− CD4+ T-cell subset (TEM; median, 20%). We cannot exclude differences in the ability of TCM and TEM cells to further expand in vitro because of differences in their replicative capacity and cannot totally rule out a transient CCR7 re-expression during the short-term stimulation, as well as a bias inherent to the assay, i.e., the exclusive study of the circulating compartment (44). Indeed, after immunization, some cells trafficking to lymph nodes may have disappeared from the periphery and therefore were not sorted at some defined bleeding time points (Fig. 5C). Nonetheless, a considerable expansion of the TCM population was observed after the boost, with a decline to the basal level as early as 3 weeks postimmunization, whereas variations of the TEM population were limited. This contrasts with the memory response in viral models (cytomegalovirus or hepatitis B virus), showing that antigen-specific, cytokine-secreting CD4+ T cells mainly expressed a TEM phenotype (8, 37). This difference may be related to the way a pathogen, as opposed to a polypeptide,
is detected by the immune system, and to the complex but distinct signals induced by such different triggers. Vaccines based on the use of live viral vectors such as modified vaccinia virus Ankara markedly stimulated an IFN-γ+/CD8+ T-cell response against pre-erythrocytic malaria antigens (2, 5). A similar strategy in the field of human immunodeficiency virus (HIV) induced a robust virus-specific, highly polyfunctional, mainly IL-2+ and IFN-γ+/CD8+ T-cell response (38). Preferential expansion of several memory T-cell populations with variable functional potentials, as reported in viral models (13, 14), may also be the consequence of multifactorial influences such as HLA class II molecule expression, T-cell epitope diversity, TCR avidity, TCR repertoire, cell homeostasis, interactions with antigenic environment, or vaccination type and schedule (reviewed in references 25, 28, and 46). In this respect, the variability observed in the proliferative capacity and IL-2 secretion (Fig. 3), as well as in the TCR-CDR3 and TCR BJ gene usage (Tables 2 and 3 and Fig. 5C) strengthens the heterogeneous character of the CD4+ T-cell recall response. The nature of the encountered antigen appears thus to largely orient the regulation of memory differentiation. Further experiments, such as fractionation of TCM and TEM subsets prior to antigen stimulation, are needed to definitively assess this hypothesis. Altogether, this certainly underlines the difficulty in defining a consensus pathway for T-cell memory generation and persistence and directly questions the monitoring of the T-cell response in vaccination trials, for instance.

Our phenotypic approach was reinforced by a molecular analysis of the T-cell repertoire, allowing a direct comparison of TCR BV spectratype polymorphism of the various ex vivo-sorted CD4+ T cells with TT-specific T-cell clone BV usage. We indeed observed a good correlation between largely persistent TCR BV present in the ex vivo-sorted populations at

TABLE 3. TCR sequencing of TT-specific T-cell clones revealed a highly diverse TCR repertoire, with preferential BV but diverse BJ family usage

| Donor | Time point (day) | Name | BV | BJ* |
|-------|-----------------|------|----|-----|
| 1     | 14              | A11.8| 5.1| 1.6 |
| 14    | 360             | F7.1 | 5.1| 1.1*|
| 2     | 14              | D1.1 | 9  | 1.6†|
| 14    | 280             | B6   | 9  | 1.4 |
| 3     | 0               | A2   | 2  | 2.1 |
| 84    | A8.2            | 2    | 1.2 |
| 14    | 1F12            | 5.1  | 2.2 |
| 14    | 5A7             | 5.1  | 2.7 |
| 14    | 5A2             | 5.1  | 2.5*|
| 84    | A8.1            | 5.1  | 2.5*†|
| 84    | H1.1            | 5.1  | 2.5†|
| 7     | C10             | 6    | 2.7 |
| 14    | 5A1             | 6    | 2.2 |
| 14    | 1F11            | 6    | 1.5*|
| 14    | 1C6             | 6    | 1.5*|
| 84    | H1.2            | 6    | 2.6 |

*Clones indicated by an asterisk (*) showed a different CDR3 hypervariable sequence; clones indicated by a dagger (†) were identical clones issued from different wells.
several time points and the TCR BV expressed by the in vitro-generated TT-specific T-cell clones (Table 2 and Fig. 5A and B). The polyclonal profile of the CCR7+/−CD69+/−IL-2+ TT-specific CD4+ T-cell repertoire confirmed earlier in vitro data (10). This contrasted with responses to viral antigens, which induced a restricted CD4+ T-cell response toward a dominant epitope (9, 14). This could be partly explained by the abundance of TT epitopes generated during antigen processing and their promiscuous presentation (36, 40). Interestingly, all TCR BV detected in the CCR7+/−CD69+/−IL-2+ CD4+ T-cell population were indeed present in CCR7+ CD69+ IFN-γ+ CD4+ T cells (data not shown), an observation in agreement with the concept of TCM as a reservoir of early differentiated memory T cells (41).

Most TT-vaccinated individuals are efficiently protected against Clostridium tetani infection. Our data suggest that this protection may be correlated with a highly diverse, but stable memory CD4+ T-cell response. This leads to an important remark regarding antigen-specific vaccination with peptide epitopes. If we postulate that a protective immune response is supported by a highly diverse T-cell response, vaccination protocols based on peptide epitopes might generate restricted CD4+ and/or CD8+ T-cell responses and subsequently impair the efficiency of the T-cell response. There are increasing examples in vaccination studies underlying the need for broad CD4+/CD8+ T-cell cooperation to get protective immunity (17, 49, 50) and consequently the need for efficient protocols to monitor the immune response. With regard to the B-cell and specific antibody response, with a T-dependent antigen such as TT, a close correlation between CD4+ T-cell frequency and antibody response is expected, as suggested from studies in immunodeficient human models (26, 27). However, the final development of the secondary B-cell response appears to be much more complex, and may involve not only CD4 T-cell-dependent antigen-specific stimulation but also vaccine-related, adjuvant-dependent innate stimulation, as well as lifelong B-cell polyclonal stimulation (6, 39).

Fine characterization and monitoring of T-cell responses to defined antigens are thus first-line objectives in the evaluation of many clinical situations involving antigen-specific immunotherapy or vaccination. For the CD4+ T-cell response, a fully characterized picture might be given by the use of a large range of specific MHC class II tetramer/peptide complexes and ex vivo specific analysis of the epitope restricted response. How much other strategies such as CD154 expression may improve this characterization warrants further analysis, since recent publications suggested that this methodology could reliably identify antigen-specific T cells (19). However, waiting for further developments, our approach appeared to be sufficiently sensitive to provide an overall evaluation of the antigen-specific CD4+ T-cell response satisfying practical issues of field trials. Only large field trials with sufficient statistical power will definitely give support to our concept, which is thus far limited to a longitudinal study of four subjects. However, because of the large number of epitopes present within TT, a heterogeneous response such as that observed here is reasonably expectable (16, 36). In addition, previous studies strongly support our observation, i.e., the demonstration of a broad clonal heterogeneous response to a recall response to TT, with a high frequency of specific precursors, as opposed to a rather oligoclonal T-cell response to primary antigens (HIV gp120 or HIV p66) (31). Taken together, as supported by cloning and TCR spectratyping, our phenotypic approach demonstrated that in this model of TT immunization a substantial proportion of antigen-specific CD4+ T cells were included within the CCR7+/−CD69+/−IL-2+ CD4+ T-cell subpopulations (up to 50%). Specific, broad-range CD4+ responses can thus be tracked in a highly feasible and collective manner. This approach appears thus far advantageous over an MHC class II tetramer/peptide complex strategy which, in the current stage of technology in field application, is limited to the analysis of selected and well-identified epitopes and therefore to restricted insights into the T-cell response.

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