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Antigen-Reactive T Cell Enrichment for Direct, High-Resolution Analysis of the Human Naive and Memory Th Cell Repertoire

Petra Bacher,* Christian Schink,* Janka Teutschbein,† Olaf Kniemeyer,†,‡ Mario Assenmacher,* Axel A. Brakhage,† and Alexander Scheffold§

Ag-specific CD4+ T cells orchestrating adaptive immune responses are crucial for the development of protective immunity, but also mediate immunopathologies. To date, technical limitations often prevented their direct analysis. In this study, we report a sensitive flow cytometric assay based on magnetic pre-enrichment of CD154+ T cells to visualize rare Ag-reactive naive and memory Th cells directly from human peripheral blood. The detection limit of ∼1 cell within 105–106 permitted the direct enumeration and characterization of auto-, tumor-, or neo-Ag-reactive T cells within the naive and even memory CD4+ T cell repertoire of healthy donors. Furthermore, the analysis of high target cell numbers after pre-enrichment of rare Ag-specific T cells from large blood samples dramatically improved the identification of small subpopulations. As exemplified in this work, the dissection of the Ag-specific memory responses into small cytokine-producing subsets revealed great heterogeneity between pathogens, but also pathogen-related microsignatures refining Th cell subset classification. The possibility to directly analyze CD4+ T cells reactive against basically any Ag of interest at high resolution within the naive and memory repertoire will open up new avenues to investigate CD4+ T cell–mediated immune reactions and their use for clinical diagnostics.

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helper cells play a central role in the induction of immune responses against pathogens as well as in the development of immune-mediated diseases such as autoimmunity or allergy. T cells recognizing protective or disease-relevant Ags are usually rare, especially in the naive repertoire, but information about their frequency, phenotype, and functional capabilities has important diagnostic and/or prognostic value. Frequencies of Ag-specific T cells within the human naive repertoire have been estimated to range between 1 cell in 104–107 (1–3). In the memory repertoire, the frequencies strongly vary depending on the status of the immune reaction and are usually in the range between 10−3 and 5%. Recent efforts to define correlates of immune protection, for example, after vaccination, have revealed that multiparameter classifications are crucial, for example, for the identification of multifunctional CD8+ T cells (4–6). This imposes the need to analyze sufficient target events to be able to identify small subsets with high statistical significance.

Several flow cytometric methods for the direct detection of rare Ag-specific T cells have been described in the recent years, including peptide-MHC multimers, cytokine secretion, and expression of activation markers (7, 8). However, these technologies are limited by the number of cells per sample that can be acquired in reasonable time (105–106) as well as by the natural and/or technological background of the assay, which is typically between 0.01 and 0.1%. T cells occurring at lower frequency have traditionally been analyzed via indirect methods, such as long-lasting in vitro cultivation and/or population-based methods ([3H]thymidine incorporation, CFSE dilution, ELISA) or ELISPOT (9–14). These techniques provide only limited information about the relationship between frequency, phenotype, and function of the reactive cells.

Recent studies have shown that magnetic enrichment can be used to collect rare peptide MHC-multimer–labeled cells from large sample sizes, allowing direct cytometric detection of murine and human Ag-specific CD4+ and CD8+ T cells even in the naive repertoire (1, 15–22). However, the MHC-multimer technology is limited to the previous knowledge of MHC alleles and antigenic epitopes. Due to the very high diversity of MHC molecules and especially for complex pathogens, containing hundreds or thousands of different target epitopes, choosing the right peptide-MHC combinations to study Ag-specific T cell responses is quite challenging (23). Furthermore, the restriction to few antigenic epitopes gives only limited and preselected insight into the heterogeneous T cell populations specific for a certain Ag. In addition, the generation of functional peptide-MHC class II multimers for the detection of Ag-specific CD4+ T cells, especially for low-affinity autoreactive cells, is still difficult.

A more recent approach used libraries of polyclonal expanded naive or memory CD4+ T cells to determine the frequencies of Ag-specific T cells against naturally processed Ags (2). This method

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Abbreviations used in this article: AdV, adenovirus; ARTE, Ag-reactive T cell enrichment; GAD, glutamic acid decarboxylase; KLLH, keyhole limpet hemocyanin; MOG, myelin oligodendrocyte glycoprotein; WT-1, Wilms tumor Ag.
does not require knowledge of peptide-MHC combinations and allows detection of the total pool of Ag-specific T cells even for complex organisms in the memory as well as the naive CD4+ T cell compartment. However, a disadvantage of this technique is that it requires several weeks of in vitro cultivation and does not allow direct enumeration and characterization of the Ag-specific T cells, which implies the risk of potential phenotypic or functional changes introduced by in vitro manipulation.

To overcome the current limitations, we developed a method based on magnetic enrichment of CD4-reactive CD154+ (CD40L)–expressing T cells to access directly ex vivo the human Ag-specific naive and memory CD4+ T cell repertoire. CD154, which is specifically expressed by all Ag-activated CD4+ T cells shortly after TCR stimulation, is a reliable functional marker for Ag-specific CD4+ T cells irrespective of MHC allele or exact definition of the antigenic epitope (24–26). We developed an optimized magnetic Ag-reactive T cell enrichment protocol (ARTE) of CD154+ T cells, which allows reproducible and highly sensitive capture of rare Ag-specific CD4+ T cells prior to their phenotypical and functional in-depth characterization by multiparameter flow cytometry. The high sensitivity of our method provides direct access to the human naive T cell repertoire and, to our knowledge, enables the first characterization of human CD4+ T cells specific for an unrestricted pool of T cell epitopes from various auto- and tumor-associated Ags,directly ex vivo from peripheral blood of healthy donors. By performing functional in-depth characterizations, we dissected the Ag-reactive CD4+ T cell memory responses against various pathogens into small cytokine subsets and identified a high functional heterogeneity with additional pathogen-related patterns not necessarily associated with the dominant Th1, Th2, or Th17 functional in-depth characterization by multiparameter flow cytometry.

Materials and Methods

Blood donors and isolation of PBMCs

Buffy coats or leukapheresis products from healthy donors were obtained from the university hospital in Dortmund and Cologne. This study was performed according to established ethical guidelines, and all blood donors gave informed consent. PBMCs were separated by use of Ficoll–Hypaque (GE-Healthcare, Bio-Science) density gradient centrifugation.

Preparation of Aspergillus fumigatus lysate

The A. fumigatus strain ATCC 46645 (LGC Standards) was used for preparation of protein extract from mycelium. Conidia were inoculated at a concentration of 2 × 10^9 spores/ml in YPD medium and shaken for 20 h at 37°C with 200 rpm (27). Mycelium was recovered by filtration, washed with water, and stored at −70°C. Frozen mycelium was resuspended in saline (0.9% [w/v] NaCl) and disrupted in a microdismembrator (Sartorius) at 200 rpm for 10 min using glass beads. The extract was resuspended in 1× PBS buffer (2 mM MgCl2) and centrifuged for 20 min at 20,000 × g. Supernatant was stored in aliquots at −20°C until use.

Stimulation, isolation, and characterization of Ag-specific T cells

Stimulations were performed in RPMI 1640 medium (Milenyi Biotec), supplemented with 5% (v/v) AB serum (Lonza) and 2 mM L-glutamine (PAA Laboratories). For enrichment of recall Ag-specific T cells, 1 × 10^7 PBMCs were stimulated for 7 h with the following Ags: A. fumigatus-lysate (40 μg/ml), Candida albicans-lysate (20 μg/ml; Greer Laboratories), CMV-lysate (10 μg/ml; Siemens), tetanus-toxoid (10 μg/ml; Statens Serum Institute), or peptide pools of CMV pp65, adenovirus (AdV) Hexon (0.6 nmol/ml; both Miltenyi Biotec) in the presence of 1 μg/ml CD40 and 1 μg/ml CD28 functional grade pure Ab (both Miltenyi Biotec). For enrichment of auto-tumor-néo-Ag–specific T cells, 1 × 10^7 PBMCs were stimulated with keyhole limpet hemocyanin (KLH; 200 μg/ml; Immuc-
For expansion of T cell lines from the naive or memory repertoire, CD4+ T cells were isolated by negative selection using the naive CD4+ T cell isolation kit or the memory CD4+ T cell isolation kit (both Miltenyi Biotec), respectively. In brief, PBMCs from leukapheresis product were labeled with a mixture of biotin-conjugated Abs and antibiotics Microbeads for depletion of unwanted cell types. Sorted T cell subsets always reached a purity of >99%, as analyzed by flow cytometry. A total of $2 \times 10^6$ isolated naive or memory CD4+ T cells was stimulated with autologous CD3-depleted PBMCs as APCs in a ratio of 1:1 and the different Ags. Enrichment, cultivation, and restimulation of reactive CD154+ cells from both CD4+ T cell subsets were performed, as described above.

**Generation of Ag-specific T cell clones**

Ag-specific T cell clones were generated by single-cell sorting from *A. fumigatus* or AdV-stimulated and magnetically pre-enriched CD154+CD4+ T cells using a FACS Aria III cell sorter (Becton Dickinson Biosciences). Single cells were sorted into 96-well round-bottom plates with $1 \times 10^5$ well irradiated (20 Gy) autologous feeder cells in 200 μl TrisMACS medium (Miltenyi Biotec), supplemented with 15% AB serum, 2 μM 2-ME (Life Technologies), 2 mM glutamine, 300 IU/ml IL-2 (Proleukin), 100 IU/ml penicillin, 100 μg/ml streptomycin, and expanded polyclonal with 30 ng/ml anti-CD3 (OKT3; Miltenyi Biotec). After 7 d, 100 μl medium was replaced and 1 $\times 10^5$ irradiated feeder cells were added. An additional 7 d later, cells were split into 96-well flat-bottom plates and further expanded with *A. fumigatus* lysate or left unstimulated. CD154 expression was assessed after 4 wk by the single-cell sort by stimulating $5 \times 10^4$ expanded T cells/well with $5 \times 10^4$ autologous CD3-depleted PBMCs with or without Ag for 6 h. A total of 1 μg/ml brefeldin A was added for the last 4 h. Specificity of the expanded T cell clones was analyzed by intracellular cytokine staining.

**Statistics**

Statistical tests were performed using Prism software (GraphPad Software) using paired Student t test. For comparison of CD154+ cell recovery with or without column staining (Supplemental Fig. 1A), unpaired Student t test was used. Pearson’s correlation coefficient was used to calculate correlations. The p values <0.05 were considered statistically significant.

**Results**

CD154+ enrichment allows quantitative capture and highly sensitive enumeration and characterization of Ag-specific CD4+ T cells

CD154 is specifically expressed by all Ag-activated CD4+ T cells, irrespective of defined peptide-MHC combinations and without any bias toward a certain cytokine-producing subset (24, 25). CD154-expressing CD4+ T cells can readily be detected following 7-h stimulation of PBMCs with various viral (CMV, AdV), bacterial (tetanus toxoid), or fungal (*A. fumigatus*, *C. albicans*) recall Ags (Fig. 1A). However, it is also evident that, for certain Ags and/or donors, the frequencies of Ag-specific CD4+ T cells are often <0.1%, which is in general the limit of detection for standard flow cytometry. In particular, at frequencies <0.1%, the resulting low total number of positive cells per sample (e.g., <100–1000 cells for sample sizes of $10^5$–$10^6$ CD4+ T cells) restricts the further dissection into specialized subpopulations. This limits the applicability of this method for many clinically and scientifically relevant situations.

To enable the analysis of Ag-reactive T cells from larger sample sizes ($>10^5$ cells), as required for rare cell detection, we magnetically pre-enriched the Ag-specific CD154+ T cells before analysis. In this way, Ag-specific T cells from $10^6$–$10^7$ PBMC could be rapidly selected and subsequently analyzed within short time. As shown in Fig. 1B, in total PBMCs stimulated with *A. fumigatus* lysate, ~70 CD154+CD4+ T cells could be detected within 2 $\times 10^5$ acquired events. In contrast, when $1 \times 10^6$ PBMCs were stimulated and the CD154+ cells were enriched before the analysis, >2000 CD154+ T cells could be detected by acquiring the entire positive fraction with only ~5 $\times 10^3$ total events.

**FIGURE 1.** CD154+ enrichment increases the sensitivity of detection of Ag-specific CD4+ T cells. (A) Frequencies of CD154+ cells among CD4+ (bold) or total lymphocytes (italics) of one representative donor after stimulation of PBMCs with the indicated Ags. (B) Magnetic enrichment of CD154+ T cells combined with intracellular cytokine staining. PBMCs were stimulated with *A. fumigatus* lysate or left unstimulated. CD154+ expression was assessed among CD4+ T cells without enrichment (left plots) and after CD154+ enrichment (right plots). Numbers in brackets indicate the number of CD154+ cells that were re-acquired 2 $\times 10^5$ PBMCs (left plots) or obtained from 1 $\times 10^5$ PBMCs after enrichment (right plots). (C and D) Comparison of the CD154+ enrichment assay with standard flow counting using 1 $\times 10^6$ starting PBMCs (n = 20; 4 independent experiments were performed). (C) CD154+ cell numbers of nonstimulated or *A. fumigatus*-stimulated samples. To optimize the detection and quantification of CD154+ events among CD3+CD4+ T cells, cell aggregates (scatter area versus scatter height) and non-T cell lineages (CD14+, CD20+, dump) were excluded. (D) Signal-to-noise ratio was calculated based on CD154+ cell numbers in *A. fumigatus*-stimulated and nonstimulated PBMCs. Significance was determined using paired Student t test. (E and F) Detection limit of the CD154+ enrichment assay (n = 5; 2 independent experiments were performed). (E) The total number of enriched CD154+ cells from different starting cell numbers of *A. fumigatus*-stimulated or nonstimulated PBMCs is shown. (F) Signal-to-noise ratio was calculated based on CD154+ cell numbers in *A. fumigatus*-stimulated and nonstimulated PBMCs. Significance was determined using paired Student t test.
By performing all staining and washing steps directly on the magnetic columns, the protocol was optimized to result in minimal cell loss during processing (Supplemental Fig. 1A), a minimal intra-assay variability, and a linear correlation between isolated target cells and the input cell number (10^6–10^8 PBMCs) (Supplemental Fig. 1B). This shows that the enrichment technology allows reproducible and quantitative assessment of rare CD154+ T cells from large cell populations and enables to calculate back the frequency of Ag-specific CD4+ T cells based on the number of enriched CD154+ cells.

We further compared the background levels by acquiring the CD154+ cells of 10^6 PBMCs with and without pre-enrichment. Using an optimized staining and analysis strategy (see Fig. 1 legend), the background without enrichment ranged between 50 and 200 CD154+ cells per 10^6 counted PBMCs (Fig. 1C), that is, 0.02–0.07% among CD4+ T cells, given an average frequency of 30% CD4+ T cells in human PBMCs. In contrast, using the pre-enrichment strategy, the background was reduced by a factor of 10, whereas the number of CD154+ target cells was only half reduced. Despite a strong donor-to-donor variation, this resulted in a considerably improved signal-to-noise ratio of ∼5–50 fold with enrichment versus 2- to 10-fold without enrichment (Fig. 1D). Interestingly, at higher input cell numbers (up to 10^8), the number of isolated target cells increased linearly, whereas the proportion of background cells declined, resulting in an even superior signal-to-noise ratio at higher cell numbers (Fig. 1E, 1F).

These data show that magnetic pre-enrichment of CD154+ T cells allows rapid, quantitative, and reproducible analysis of rare Ag-specific T cells from large sample sizes and results in a strongly improved signal-to-noise ratio. The detection limit ranges between 10^2 and 10^3 for 10^7–10^8 starting cells, but basically the sensitivity of the assay depends on the number of input cells.

**CD154+ enrichment specifically identifies Ag-reactive T cells**

CD154 induction was dependent on MHC-peptide recognition, because an added HLA-DR-blocking Ab strongly inhibited CD154 upregulation on CD4+ T cells, but had no effect on CD4+ T cells activated by staphylococcal enterotoxin B or anti-CD3/CD28 (Supplemental Fig. 2A) nor on activated CD8+ T cells (not depicted). Furthermore, the CD4+ T cell responses against the Ag lysates but not against the pp65 peptide pool were prevented when APCs were fixed with formaldehyde before incubation with the Ags (Supplemental Fig. 2B), indicating that CD154 induction for native Ags is MHC class II and Ag processing dependent.

To further verify the specificity, the different recall Ag-activated CD154+ T cells were magnetically isolated, expanded for 14 d, and subsequently restimulated with autologous APCs and the indicated Ags. The expanded T cells showed high reactivity toward the inducing Ag (30–60%), as demonstrated by the upregulation of CD154 and expression of cytokines (Fig. 2A), whereas reactivity against irrelevant Ags was at background level.

In addition, T cell clones were generated via single-cell FACS sorting of CD154+ cells and expansion with anti-CD3 and IL-2. The cloning efficiency of the sorted CD154+ cells was ∼50% in each case (data not shown). Upon restimulation, 89% (107 of 120 clones) and 92% (110 of 120 clones) of initially A. fumigatus and AdV-stimulated clones, respectively, produced cytokines in an Ag-specific manner (Fig. 2B).

These data confirm the high specificity of the CD154+ enrichment assay and demonstrate that specific T cell lines or clones can rapidly be generated for all tested Ags.

**CD154+ enrichment reveals pathogen-related functional heterogeneity of Ag-specific CD4+ T cell responses**

After applying ARTE for different recall Ags, the frequencies of Ag-specific T cells were calculated from the total number of

![Image](https://example.com/image.png)

**FIGURE 2.** Specificity of expanded CD154+ T cells. (A) PBMCs were stimulated with the indicated recall Ags. CD154+ cells were isolated and subsequently expanded for 14 d with IL-2 and autologous feeder cells. Expanded cell lines were restimulated in presence of autologous APCs with and without Ags, as indicated, and reactive CD4+ T cells were determined by CD154 and TNF-α expression. Representative dot plot examples of one donor of five with percentage of reactive cells among CD4+ lymphocytes are shown. (B) T cell clones were generated from single CD154+CD4+ cells sorted by FACS from A. fumigatus lysate or AdV peptide pool-stimulated PBMCs. Cytokine expression was analyzed by intracellular staining upon Ag-specific restimulation. Dashed lines indicate the cutoff value of >5% cytokine producers. A total of 89.2% (107 of 120 clones) and 91.7% (110 of 120 clones) of the clones was specific for A. fumigatus and AdV, respectively.

![Image](https://example.com/image.png)
CD154+ cells obtained after enrichment relative to the total number of CD4+ cells applied on the column. The frequency range for the different recall Ags was between 1 cell within 10^3–10^2 CD4+ T cells, depending on the pathogen and the immune status of the donor (Fig. 3B, first diagram). The quality of an immune response is not only affected by the frequency or absolute number of the Ag-specific T cells, but also by their functional capacity, such as cytokine production. Dissection of pathogen-specific T cell responses into different functional T cell subsets therefore gives important insights into the induction of different functional capacities in vivo. A highlight of our method is that it allows the simultaneous assessment of the functional capacity of Ag-reactive T cells by multiparameter flow cytometric analysis, without long-term in vitro cultivation, thus directly reflecting the in vivo situation of the Ag-reactive T cells. Therefore, we used the CD154+ preselection to analyze the cytokine profiles of Ag-specific T cells reactive against the various recall Ags at high resolution.

FIGURE 3. Characterization of small cytokine-producing subsets within the total Ag-specific T cell pool. (A and B) Flow cytometric ex vivo analysis of cytokine-expressing subsets within Ag-specific CD4+ T cell responses. A total of 1 × 10^7 PBMCs was stimulated with the indicated Ags, and Ag-specific T cells were analyzed for cytokine expression using the CD154+ enrichment assay. Cells were gated on CD4+ lymphocytes, and percentages of cytokine-expressing cells among CD154+ T cells are shown. (A) Representative dot plot examples and (B) statistical analysis from several donors with indicated mean values (n = 5; two independent experiments were performed). Frequencies of Ag-specific T cells (first diagram) were calculated from the total number of CD154+ cells obtained after enrichment normalized to the total number of CD4+ cells applied on the column.
The power of our method is illustrated in Fig. 3, as follows: cytokines, which are not restricted to a certain T cell lineage, for example, IL-2 or TNF-α, occurred at high frequencies (up to 85%) in the Th cell responses against all analyzed Ags. However, most other cytokines, including the lineage-defining cytokines IFN-γ, IL-4, IL-17, and IL-22, which are important parameters for the classification of Th cell responses into Th1, Th2, Th17, or Th22 subsets, showed much higher variability and represented often only 1–10% of the total Ag-specific T cell pool, that is, 0.0001–0.1% for the Ags analyzed in this work (Fig. 3). As depicted in Fig. 3A, the magnetic preselection allows the collection of a number of Ag-reactive T cells, high enough to enable the precise identification of small cytokine-producing subsets, down to 1–10% within the Ag-reactive CD4+ T cell pool (Supplemental Fig. 3) (28).

Th cell responses against certain pathogens are often classified according to a certain dominant cytokine expression. For example, viral Ags have been shown to induce Th1 responses, extracellular pathogens, Th2 responses, and fungi Th17 responses. However, besides these dominant signatures, our refined analysis revealed a much higher heterogeneity in the functional cytokine repertoire of Ag-specific T cells. Consistent with previous data (29), the response against C. albicans was of a clear Th17 phenotype, with high levels of IL-17 and IL-22 producers (10–30%), but we also detected small populations of Th1 (IFN-γ(-) and Th2 (IL-4, IL-5, IL-13)-type cells that mainly did not coproduce IL-17 or IL-22 (data not shown). In contrast, in the T cell response against another fungal pathogen, A. fumigatus, the Th1 cytokine IFN-γ was dominant (10–30%), but also distinct populations of non-Th1 cytokines, such as IL-10 (4–6%), IL-17, and IL-22 (1–5%), as well as Th2 cytokines (IL-4 and IL-13, 1–5%), were present in most donors.

In contrast, the response against the vaccine Ag tetanus was less dominated by a single signature with intermediate levels of Th1 cells (IFN-γ, 10–40%) and quite variable frequencies of Th2 cells (IL-4, 2–30%; IL-5, 1–6%; IL-13, 1–20%). In some donors, we also observed a small population of IL-17 producers, but almost no IL-22 producers. However, even within the highly Th1-polarized antiviral immune responses against CMV and AdV, we found small differences with regard to non-Th1 cytokines. For example, we detected clear populations of IL-4 and IL-10 producers against CMV, whereas AdV-specific T cells also contained a small population of IL-17 and IL-22 producers that were completely absent in the response to CMV. Interestingly, despite this heterogeneity in the cytokine patterns against different pathogens, the distribution on the different T cell subsets was quite similar for different donors (Fig. 3B), indicating indeed the existence of pathogen-related cytokine signatures. Because most of the cytokines are restricted to small subpopulations, we use the term microsignatures to differentiate them from the classification according to the dominant Th cell signatures.

Taken together, our knowledge, our method enables for the first time to directly visualize the total ex vivo functional repertoire of complex pathogen-specific T cell responses on a single-cell level at high resolution. The refined subpopulation analysis reveals that, in addition to the major cytokine-producing subsets, the detection of rare cytokine-producing T cells allows to identify an unexpected broad complexity within the pathogen-specific CD4+ T cell responses.

**ARTE unmasks the naive T cell repertoire, and enables the direct visualization of autoreactive T cells even from healthy donors**

Until now, the cytometric analysis of Ag-reactive T cells using cytokines or activation markers as a readout was mainly restricted to the analysis of the memory T cell pool because the frequency of naive T cells was below the level of detection. The high sensitivity of our method prompted us to extend our study to the analysis of Ag-specific CD4+ T cell responses in the naive T cell repertoire. We therefore investigated the human T cell repertoire reactive against auto- and neo-Ags, which have to date escaped direct quantitative and qualitative characterization. We applied ARTE to analyze the CD4+ T cell responses of healthy donors against peptide pools of six different auto (MOG, GAD)-, tumor-associated (NY-ESO, WT-1), or neo-Ags (KLH, HIV Gag), and MP65, a major C. albicans Ag, as a positive control. Without enrichment, no CD154+ T cells against the auto- and neo-Ags were detectable within 5 × 10^5 acquired PBMCs, whereas MP65-reactive T cells could immediately be detected with a frequency of ∼1 in 10^5 CD4+ T cells (range 10^{-3}–10^{-5}) (Fig. 4A). However, following the enrichment from 10^8 stimulated PBMCs, reactive T cells against all Ags were clearly detected in all donors tested, with frequencies ranging from <10^{-6} to 10^{-4} (Fig. 4A, 4B). The frequencies of T cells differed between the various Ags, but, for a particular Ag, rather small donor-to-donor variations were observed. Surprisingly, we found similar frequencies (10^{-5}–10^{-4}) against the auto-Ags, MOG and GAD, and the neo-Ags, KLH and HIV Gag. However, the frequencies against the tumor-associated Ags NY-ESO and WT-1 were ∼10-fold lower (10^{-6}–10^{-5}). Further phenotypic analysis of the enriched CD154+ cells revealed that a large fraction, but not all of the T cells reactive against the auto-, tumor-associated, or neo-Ags indeed have a naive phenotype (40–60%, CD45RO+ CCR7+), whereas C. albicans MP65-specific CD4+ T cells were mainly memory cells (80–90%, Fig. 4C, 4D). These data demonstrate that the CD154+ enrichment assay can be used to monitor the human naive Ag-reactive T cell repertoire. The data also indicate that even in healthy donors a significant fraction of the self-reactive repertoire has a clear memory phenotype, which suggests a role for cross-reactivity against external Ags for priming of autoreactive T cells even in a healthy individual.

**Generation of Ag-specific T cell lines following CD154+ enrichment from the naive CD4+ T cell compartment**

To confirm the specificity of the CD4+ T cells reactive against auto-, tumor-associated, and neo-Ags within the naive, as well as in memory compartment, reactive cells were enriched from highly purified naive and memory CD4+ T cells (Supplemental Fig. 4A, 4B), expanded for 14 d, and tested for specificity by Ag restimulation. As shown in Fig. 5, expanded T cells from the naive as well as from the memory population specifically reacted against the inducing Ag. Interestingly, for the auto-, tumor-associated, and neo-Ags, but not for the recall Ag MP65, the frequency of specific T cells within the expanded T cell lines was higher for T cell lines generated from naive T cells versus memory-derived cell lines. This may indicate lower affinity or reduced proliferative potential of self-reactive T cells within the memory pool. However, these data clearly confirm our ex vivo phenotypic observations and demonstrate the applicability of ARTE for the direct ex vivo detection, enumeration, and characterization of Ag-specific T cell populations, undetectable by standard assays.

**Discussion**

The Ag-induced expression of CD154 has been described as a versatile tool for the direct ex vivo detection of CD4+ T cells specific for any pathogen or Ag of interest (24–26). We employed its high specificity in combination with a magnetic enrichment step of rare CD154+ cells from large cell samples. In this way, the sensitivity of detection is mainly restricted by the number of input.
cells, that is, for $10^7$–$10^8$ PBMCs we estimated a sensitivity of $10^{-5}$–$10^{-6}$. This allowed us to directly access extremely rare populations such as Ag-specific T cells within the naive CD4+ T cell repertoire as well as to dissect the Ag-specific memory repertoire at high resolution.

High resolution, that is, the characterization of minor subpopulations with high statistical precision, requires the collection of sufficient target events, allowing a proper statistical analysis. For example, to estimate a 10% subset of a population with a coefficient of variation $\pm 10\%$, at least 1000 CD154+ T cells have to be acquired (Supplemental Fig. 3) (28). The magnetic preselection allowed the collection of a number of Ag-reactive T cells, sufficient for the identification of small cytokine-producing subsets, down to 1–10% within the total reactive CD4+ T cell pool. The power of magnetic pre-enrichment has already been demonstrated for various rare cell populations (30–32) and has been employed for MHC-multimers to identify Ag-specific T cells even in the naive repertoire (1, 18–20). However, the method we describe in this work has several advantages over the MHC-multimer technology, which is restricted to a set of exactly defined peptide-MHC combinations. Therefore, the CD154+ enrichment technology fills an apparent gap in the Ag-specific T cell analysis toolbox, now also providing ex vivo access to the functional CD4+ T cell pool with a sensitivity comparable to the MHC-multimer enrichment approach, but without the need for in vitro expansion or restriction to a certain Ag or MHC allele.

We used this method to dissect the CD4+ T cell response against various pathogens into small but functionally distinct subpopulations. Many analyses restrict the characterization of pathogen-specific T cells to rather abundant cytokines such as IL-2, TNF-α, or IFN-γ, due to the limited access to Ag-specific T cell numbers. In this study, we show that many functionally important cytokines, such as IL-4, IL-5, IL-10, and IL-13, and for many pathogens, also IL-17 and IL-22 are produced only by $\sim 1$–10% of the total Ag-specific T cell pool and therefore may represent only 0.01–0.001% of the total CD4+ T cell compartment. These data dem-

**FIGURE 4.** Enumeration and characterization of CD4+ T cells reactive against auto- or neo-Ags. (A) A total of $1 \times 10^8$ PBMCs was stimulated as indicated, and CD154+ expression among CD4+ T cells was analyzed without enrichment (upper plots) and after performing the CD154+ enrichment assay (lower plots). Indicated are percentages of CD154+ cells among CD4+ and number of CD154+ cells after acquiring $5 \times 10^5$ PBMCs (upper plots) or obtained from $1 \times 10^8$ PBMCs after enrichment (lower plots). (B) Enumeration of rare Ag-specific CD4+ T cells in several donors using the CD154+ enrichment assay. The total number of enriched CD154+ T cells was determined using a single, live, nondump, CD3+CD4+ gating strategy, and background enriched from the nonstimulated control was subtracted. Depicted is the total number of CD154+ cells obtained after enrichment normalized to the total number of CD4+ cells applied on the column (MP65, n = 21; GAD, NY-ESO, n = 19; MOG, WT-1, n = 16; KLH, HIV Gag, n = 6). (C and D) Enriched CD154+ cells were ex vivo analyzed for phenotypic surface markers CD45RO and CCR7. Cells are gated on CD4+CD154+ lymphocytes. (C) Representative dot plot examples from one donor with percentages of cells among CD154+ cells and cell count (in brackets) and (D) statistical analysis for percentage of CD45RO−CCR7+ cells among the total number of CD154+ cells (n = 6; two independent experiments were performed). Background enriched from the nonstimulated control was subtracted.

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onstrate that the rather simplified classification of an immune response into Th1, Th2, or Th17 according to the few dominant lineage-defining cytokines can be refined by identification of the underlying cytokine microsignatures. Indeed, our analysis of the cytokine-producing subsets revealed an unexpected broad functional heterogeneity of T cells reacting against the different pathogens. Interestingly, the responses against a given pathogen were remarkably similar between different donors, indicating indeed the existence of pathogen-related cytokine signatures that can be identified only via high-resolution analysis.

One interesting finding was that the two fungal pathogens A. fumigatus and C. albicans elicit completely different patterns of Th cell responses. Although Th17 responses are often described as prototypic for antifungal immunity, we show that only C. albicans–reactive T cells produce mainly IL-17 and IL-22, whereas the response against A. fumigatus was rather dominated by IFN-γ–producing Th1 cells and also contained a small fraction of IL-10 producers. Interestingly, also a small fraction of A. fumigatus–specific Th17/22 cells was consistently present throughout all donors. The Th17 versus Th1 signatures might reflect the selective capability of C. albicans to elicit Th17 responses in vivo and in vitro, as it has recently been described (29, 33), or different infection routes of the two pathogens, that is, the lung for A. fumigatus versus gut and skin epithelia for C. albicans. However, it will be interesting to further analyze the origin of the A. fumigatus–reactive Th17 versus Th1 subset and to define which role the two subsets possess in the various antifungal immune responses, that is, allergies or invasive infections.

Also, in the antiviral CD4+ T cell responses, which are clearly dominated by a Th1 cytokine profile, we found a second, heterogeneous response of non-Th1 cytokines, specifically associated with a certain virus. In particular, IL-10 and IL-4 were found at low level against both viruses, but were more pronounced in response to CMV, whereas IL-22 and at lower level also IL-17 producers, which were consistently present in the AdV-specific immune response, were absent against CMV (<0.1%). This indicates that, besides the major Th subset-inducing capacity of certain classes of pathogens, for example, Th1 for viruses, different pathogens have additional capabilities to induce distinct cytokine microsignatures not necessarily related to the dominant signature. It will be interesting to identify the critical factors responsible for the induction of these cytokine microsignatures that all together constitute functional T cell immunity, such as the nature of the pathogen-associated signals, Ag dose, the site of Ag exposure, or the preparation of vaccine adjuvants, as well as potential cross-reactivity between species with different cytokine-inducing capabilities. Indeed, the importance of multiparameter characterization of the Ag-specific repertoire, for example, for prediction of immune protection, has been highlighted before by many studies (4–6). Thus, the possibility to directly visualize now in detail the broad heterogeneity of Ag-specific CD4+ T cell responses at high resolution will be an important tool to identify the underlying principles of subset heterogeneity and their use for diagnostic assays, for example, during immunotherapies.

Another important application of our technology was the analysis of Ag-specific T cells in the naive T cell compartment, which could to date not be directly assessed without restriction to selected peptides. Knowledge about Ag specificity in the naive repertoire may allow to predict the success of immunomodulation strategies, such as vaccination and tolerization, or to assess the risk for the development of immunopathologies. To our knowledge, the high sensitivity of the CD154 enrichment assay allowed us for the first time to visualize the full repertoire of CD4+ T cells reactive against various neo-, auto-, or tumor-associated Ags directly from PBMCs of healthy donors. We observed frequencies of reactive cells against these Ags in a range of <10^−6–10^−4, which is quite similar to the frequencies of naive CD4+ T cells specific for other naturally processed Ags determined by the T cell library approach (2). However, recent studies in humans, using MHC-multimer enrichment, detected frequencies of Ag-specific naive CD8+ and CD4+ T cells of 0.3–3.6 (1) and 0.2–10 (18) per million cells, respectively. The higher frequencies found in our study are most probably due to the fact that we did not determine frequencies of T cells specific for a single epitope. The peptide pools used in this study consist of overlapping 15-mer peptides (11-aa overlap), covering the complete protein sequence and all possible T cell epitopes (34). Therefore, the total frequency of Ag-specific T cells most likely reflects responses to multiple epitopes.

**Figure 5.** CD154+ enrichment allows generation of Ag-specific T cell lines from the naive CD4+ T cell repertoire. A total of 2×10^6 purified memory (A) or naive (B) CD4+ T cells was stimulated with CD3-depleted APCs and the indicated Ags. Enriched CD154+ cells were expanded for 14 d with IL-2 and autologous feeder cells. Expanded cell lines were restimulated in presence of autologous APCs with and without Ags, as indicated, and reactive CD4+ T cells were determined by CD154 and TNF-α expression. Representative dot plot examples of one donor of three with percentage of reactive cells among total CD4+ are shown.
Surprisingly, although a large number of the neo-, auto-, or tumor Ag-specific T cells was indeed in the naive state, as expected for healthy individuals, the reactive cells always contained a significant fraction of memory T cells. These are truly Ag-specific T cells because enriched cells from both T cell compartments gave rise to highly specific T cell lines following in vitro expansion. In healthy donors, memory T cells against auto-Ags and in particular against the neo-Ag KLH are most probably the result of cross-reactivity to external Ags. Accordingly, we observed a tendency that T cell lines against the neo- and auto-Ags were less efficiently generated from memory versus naive derived T cells, whereas pathogen-reactive T cell lines could efficiently be generated from memory T cells. This may be explained by lower affinity of T cells reactive against the cross-reactive versus the original priming Ag. Alternatively, this could be due to a reduced proliferative potential that may indicate an anergic state of the autoreactive memory T cells, although this would not apply for T cells reactive against neo-Ags like KLH or HIV gag. However, the contribution of cross-reactive memory T cells against neo- and auto-Ags to immune protection or the development of autoimmunity requires further investigation. Interestingly, the number of reactive cells against a certain neo- or auto-Ag was relatively constant between different donors, but differed between the various Ags, as described before for CD8+ T cells (1). Specifically, T cells reactive against the tumor-associated Ags NY-ESO and WT-1 were ~10-fold less frequent, but contained similar proportions of naive and memory T cells. Whether these differences result from differences in thymic negative selection or peripheral deletion or simply result from a lower number of available T cell epitopes remains to be analyzed. However, in the murine model, it has been shown that the size of a certain naive CD4+ T cell population is strongly affected by negative thymic selection with a stronger thymic deletion of smaller naive T cell populations (35). Furthermore, a correlation between naive precursor frequencies and the size and TCR diversity of the memory responses to different peptides has been reported (19, 35, 36). Because NY-ESO and WT-1 are used for antitumor vaccination, it would be interesting to study whether the frequency of precursors predicts the outcome of vaccination, as it has, for example, recently been shown for anthrax vaccination (18).

In summary, the CD154+ enrichment technology allows the visualization and quantification of functional Ag-specific T cells at unprecedented sensitivity, providing even access to the naive CD4+ T cell repertoire. Our results highlight the importance of dissecting the total Ag-reactive T cell pool into small, but functionally relevant subsets, giving a more precise picture of the functional T cell repertoire. The method allows quantification of various parameters with potential diagnostic or prognostic relevance within one single, short, and sensitive assay and can in principle be extended to any pathogen or Ag of interest. Because the absolute cell numbers available for a single analysis are low or require sample sizes that are difficult to obtain routinely from patients, as many parameters as possible should be measured in parallel. Therefore, recent developments, which have dramatically increased the number of parameters for cytometric analysis, will optimally synergize with the Ag-reactive T cell enrichment technology described in this work (37). The accurate and sensitive assessment of Th cell immunity provides new, exciting possibilities for basic and clinical research and represents a powerful diagnostoic tool for infections, autoimmunity, and allergies.

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Disclosures
P.B. and M.A. are employees of Miltenyi Biotec. A.S. works as a consultant for Miltenyi Biotec. Miltenyi Biotec provides materials and reagents suitable for performing the assays described in this paper and has filed IP rights for commercial use of the described method. The other authors have no financial conflicts of interest.

References
**Figure S1.** CD154⁺ enrichment assay allows accurate and reproducible quantification of rare antigen-specific T cells. (A) Performing intracellular staining directly on the magnetic columns strongly increases cell recovery. 1x10⁷ PBMCs were stimulated with *A. fumigatus* lysate and the recovery range of CD154⁺ cells following intracellular staining performed either directly on the magnetic column or after elution from the column was analyzed (n = 30). Horizontal bars indicate mean values and significance was determined using unpaired Student’s *t*-test. (B) Intra-assay variability of the recovered CD154⁺ cell numbers. Different PBMC starting cell numbers from two donors were stimulated in triplicates with *A. fumigatus* lysate and recovered CD154⁺ cell numbers were analyzed after performing the CD154⁺ enrichment assay. Pearson’s correlation coefficient was used to calculate correlations (p < 0.0001).
Figure S2. Specific induction of CD154⁺ expression following antigen stimulation. (A) Inhibition of the CD154 induction by HLA-DR blockade. PBMCs of CMV pp65-tetramer positive donors were stimulated with the indicated antigens in the presence or absence of anti-HLA-DR and the number of CD154⁺ T cells after enrichment (left) or the frequencies of CD154⁺ T cells without enrichment (right) were analyzed. Bars show mean percentages and SEM from 4 donors referring to samples without anti-HLA-DR. Two independent experiments were performed. (B) CD154 induction is antigen-processing dependent. Stimulation of isolated CD3⁺ T cells was performed under the indicated conditions and the number of CD154⁺ T cells after enrichment was analyzed. Bars indicate percentages of CD154⁺ cell numbers under the various conditions relative to the stimulation with live APCs + antigen from 3 different donors.
**Figure S3.** The precision of rare cell analysis is dependent on the number of acquired target cells. To describe the precision of flow cytometry data, the coefficient of variance (CV) can be calculated from the variance and the standard deviation (SD). For rare cell analysis, the approximations $SD = \sqrt{r}$ and $CV\% = 100/\sqrt{r}$ can be used, where $r$ is the number of positive events (28).

Using this approximation, the CV of a certain cytokine producing subset is illustrated, depending on the number of acquired CD154$^+$ target cells. For example, to obtain a CV of 10%, 100 CD154$^+$ cells must be acquired. If the cytokine producing cells are a subpopulation of 10% among all CD154$^+$ cells, 1000 CD154$^+$ cells need to be acquired.
**Supplementary Figure 4**  Bacher et al.

**A**

Non-sorted CD4+ T cells

Memory CD4+ T cells

Naive CD4+ T cells

**B**

Memory CD4+ T cells

Naive CD4+ T cells

**Figure S4.** Phenotypic and functional characterization of isolated naive and memory CD4+ T cells. (A) Phenotypic characteristics of non-sorted CD4+ T cells and isolated memory and naive CD4+ T cells subsets. (B) Cytokine expression by T cell subsets: Isolated memory and naive CD4+ T cells were stimulated with PMA/Ionomycin and analyzed for intracellular CD154+ expression and cytokine production. Data are representative of three independent experiments.