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Single-Cytokine-Producing CD4 Memory Cells Predominate in Type 1 and Type 2 Immunity

Alexey Y. Karulin, Maike D. Hesse, Magdalena Tary-Lehmann, and Paul V. Lehmann

The patterns of Ag-induced cytokine coexpression in normal, in vivo-primed CD4 memory T cells has remained controversial because of the low frequency at which these cells occur in effectively prevented direct ex vivo measurements. We have overcome this limitation by using two-color cytokine enzyme-linked immunospot assays and computer-assisted image analysis. We found CD4 memory cells that simultaneously expressed IL-2, IL-3, IL-4, IL-5, and IFN-γ to be rare (0–10%). This cytokine segregation was seen in adjuvant-induced type 1, type 2, and mixed immunity to OVA, in Leishmania infection regardless of the Ag dose used or how long after immunization the assay was performed. The data suggest that type 1 and type 2 immunity in vivo is not mediated by classic Th1 or Th2 cells but by single-cytokine-producing memory cells. The Journal of Immunology, 2000, 164: 1862–1872.

CD4 memory cells are thought to occur in at least two distinct subpopulations that can play opposing roles in infectious and autoimmune diseases by producing different cytokines (1, 2). Considerable progress has been made in understanding how naive T cells differenitized into memory cells that express either IFN-γ or IL-4 (reviewed in Refs. 3 and 4). Whether other cytokines are coexpressed by these memory cells when they reencounter Ag in vivo and exactly which ones they are is not presently known. Without knowing which cytokines are produced in a linked manner in individual memory cells and which cytokines are expressed independently, no conclusions can be drawn about how many distinct types of memory T cells exist beyond the IL-4/IFN-γ dichotomy; thus, it remains unclear how precise and versatile these memory cells are in implementing the individual effector functions induced by the individual cytokines. Because of the technical limitations that had made it intractable to resolve this question by direct measurements, several indirect approaches have been used, producing conflicting results.

The first studies performed with long-term T cell clones suggested that memory T cells express two sets of cytokines in a mutually exclusive fashion; Th1 cells produce IL-2 and IFN-γ, among other cytokines, while Th2 cells secrete IL-4 and IL-5 (1). Based on these data, the mainstream model emerged, according to which naive T cells (which do not produce cytokines or produce only IL-2) first differentiate into Th0 cells that coexpress type 1 and type 2 cytokines (5–8) and then further differentiate into the polarized Th1 or Th2 cells upon ongoing Ag stimulation. Subsequent studies of short-term clones showed considerable heterogeneity in cytokine profiles (9, 10), which suggested that T cells might not occur in distinct Th1 or Th2 subsets and that individual T cells can coexpress type 1 and type 2 cytokines in various combinations and ratios, a view that gave rise to the stochastic model of cytokine gene regulation (11). Studies performed later at the single-cell level using intracytoplasmic cytokine staining and dual-label cytokine hybridization also showed a high degree of heterogeneity in cytokine coexpression in cloned T cells (12–15). While T cell clones have the advantage of providing defined cell populations, the extent to which they are representative of memory T cells in vivo is not known. During tissue culture, T cells are continuously driven to cycle, they undergo changes of chromatin structure, and their DNA (including their cytokine genes) becomes demethylated (16–18). After 16–35 cell divisions, T cells undergo replicative senescence in vitro, and cells that survive in culture invariably reveal severe and multiple chromosomal abnormalities (reviewed in Ref. 19), all of which can affect their cytokine gene regulation. Therefore, it remains unclear whether T cells generated under the conditions of an immune response in vivo or during chronic T cell-mediated immune pathology have cytokine expression patterns like those of in vitro-expanded cells.

A different approach to the study of cytokine coexpression in memory cells relies on TCR-transgenic models. TCR-transgenic mice themselves show little immune competence (20); therefore, the priming and subsequent differentiation of the transgenic T cells has been primarily modeled in tissue culture. Unexpectedly, during the first 7 days in culture, the TCR-transgenic cells were found to express IL-2, IL-4, IL-5, and IFN-γ in an almost completely disassociated fashion, with each T cell expressing only one of these cytokine mRNAs. However, after further propagation in vitro, this phenotype was lost, and the transgenic cells started to coexpress these cytokine genes in apparently random combinations (21, 22). When in vitro-propagated TCR-transgenic cells were studied by intracytoplasmic cytokine staining, various coexpression patterns were seen including a high degree of “Th0-like” IFN-γ/IL-4 and IL-2/IL-4 coexpression (13, 23–25). Does, therefore, the expression of only one cytokine per cell characterize the initial phase of the T cell response in vivo, while cytokine coexpression subsequently prevails, and, if so, do the memory cells coexpress type 1 and type 2 cytokines in a mutually exclusive fashion, or do they express them in random combinations?

Studies of freshly isolated, nontransgenic T cells have been primarily confined to polyclonal mitogen stimulation. Depending on the cell populations tested and activation/culturing conditions chosen, cytokine expression and coexpression patterns of various sorts would differ.
were seen, which fit, respectively, the classic Th1/Th2 paradigm (26–28), the stochastic model (29–33), or a pattern of dissociated cytokine-expression (34–36). The reasons for these conflicting results might lie in the activation of T cells with different histories of Ag encounter and, perhaps more importantly, in the nonphysiological nature of the T cell-activating signal generated by the cross-linking of TCR by mitogens or by Abs (37, 38). During physiologic, MHC-restricted recognition of Ag, the TCR functions as a gate for the strength of the signal (39), frequently interacting with only a few MHC-peptide complexes on the surface of the APC (40, 41); the number of TCRs engaged and the kinetics of the engagement translate into different intracellular signaling patterns (39). The strength of this signal has been shown to affect cytokine coexpression (14), and different signal strengths can induce different functions in memory T cells (42). Therefore, it remains unclear whether the cytokine expression patterns seen in mitogen-stimulated/signal-enhanced T cells will be the same as those after physiologic recognition of Ag, and how the signal strength affects the coexpression of cytokines in T cells.

An understanding of cytokine gene regulation might help to predict the coexpression of cytokines in individual T cells. The data in this field as well are still controversial. Depending on the model and the stimulation/culture conditions used, evidence supporting both stochastic gene regulation (25, 43) and precisely controlled cytokine gene activation including allelic exclusion (44) has been obtained. Although the bulk of emerging evidence seems to favor regulated expression of individual cytokine genes, predictions about the coexpression of specific type 1 and type 2 cytokines in individual T cells in vivo cannot presently be made.

To address this controversy around the cytokine signature of normal, in vivo-differentiated memory cells, we pursued the measurement of cytokine coexpression in freshly isolated CD4 cells that had been physiologically activated by the nominal Ag. Building on previous efforts (45), we have developed two-color cytokine enzyme-linked immunospot (ELISPOT) assays in conjunction with computer-assisted image analysis to this end. After validating this approach, we measured the coexpression of key type 1 and type 2 cytokines early and late in the course of the immune response, in polarized response types, and in chronic autoimmune stimulation. We found single-cytokine-expressing CD4 memory cells to predominate under all these conditions.

**Materials and Methods**

Animals and immunizations

BALB/cByJ, BALB/cByJ Smn-Pkpc<sup>−/−</sup>J, C57BL/6J, and SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at Case Western Reserve University under specific pathogen-free conditions. Female mice were injected at 6–10 wk of age. OVA was purchased from Sigma (St. Louis, MO). OVA peptide 323–339 was purchased from Princeton Biomolecules (Columbus, OH). IFA was purchased from Life Technologies (Grand Island, NY), and CFA was prepared by mixing inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) at 1 mg/ml into IFA. Ags or peptides in PBS were mixed 1:1 with cells to predominate under all these conditions.

**Intracytoplasmic cytokine staining**

Intracytoplasmic staining was performed as described (15). Dual-staining for IL-2:IFN-γ and IL-4:IL-5 was achieved by combining JES6-5H4-PE/XMG1.2-FITC and TRFK5-FITC/11B1-biotin with streptavidin-PE, respectively (46), and the Th2 clone M33.25.6 (47) were provided by Dr. P. S. Heeger (Cleveland VA Medical Center, Cleveland, OH).

**Double-color cytokine ELISPOT assays**

Plates (ImmunoSpot M200; Cellular Technology Limited, Cleveland, OH) were coated overnight at 4°C with the cytokine-specific capture Abs specified below. The plates were then blocked with 1% BSA in PBS for 1 h at room temperature and washed four times with PBS. Subsequently, irradiated lymph node (LN) APC from naive, syngeneic mice were added (1 × 10<sup>6</sup> APC/ml with and without Ag: results are shown for the T cell-gated population. Results are shown for the stimulated cultures with the gates set using the nonstimulated cultures with relevant Abs and isotype controls. For the ELISPOT assays, the indicated numbers of T cells were plated per well with the numbers of APC constant at 1 × 10<sup>5</sup> cells per well. The SD shown is from triplicate wells. The least square approximation of the first order was used (Y = ax + b) to approximate these data. The correlation coefficients for all graphs shown (r) were >0.995 and b = 0 ± 2, showing that the graphs were linear and passed through the origin. The results are from one experiment that is representative of four performed. An example of two-color image analysis for B is provided in Fig. 2.

**FIGURE 1.** Coexpression of type 1 and type 2 cytokines in Th1 and Th2 clone as studied by intracytoplasmic staining and two-color ELISPOT analysis. Th1 clone SH10 (A and B) and Th2 clone M33 (C and D) were cultured with unirradiated, T cell-depleted, syngeneic LN cells from wild-type mice with and without Ag, and the induction of cytokine production was measured in parallel by intracytoplasmic FACS analysis or two-color ELISPOT analysis detecting IL-2 and IFN-γ (A and B) and IL-4 and IL-5 (C and D). For intracytoplasmic staining, 3 × 10<sup>5</sup> T cells/ml were cultured with 1 × 10<sup>6</sup> APC/ml with and without Ag: results are shown for the T cell-gated population. Results are shown for the stimulated cultures with the gates set using the nonstimulated cultures with relevant Abs and isotype controls. For the ELISPOT assays, the indicated numbers of T cells were plated per well with the numbers of APC constant at 1 × 10<sup>5</sup> cells per well. The SD shown is from triplicate wells. The least square approximation of the first order was used (Y = ax + b) to approximate these data. The correlation coefficients for all graphs shown (r) were >0.995 and b = 0 ± 2, showing that the graphs were linear and passed through the origin. The results are from one experiment that is representative of four performed. An example of two-color image analysis for B is provided in Fig. 2.

† Abbreviations used in this paper: ELISPOT, enzyme-linked immunospot; LN, lymph node.
FIGURE 2. Computerized image analysis of two-color ELISPOT assay. Enlarged image fragments of IFN-γ:IL-2 assay wells from Fig. 1B are shown to illustrate single-positive spots and several shades of double-positive spots (IFN-γ in red, IL-2 in blue). Unprocessed images of medium-control well (A) and of Ag-stimulated well (B) both containing 125 SH10 T cells and 10^5 APC/well are shown. Consecutive steps of image analysis are shown (C–F) with the image analyzer detecting red, blue, and double-colored spots separately by three different threshold settings. C. With the blue threshold active, single-color blue and all double-positive spots (which have blue as a part of their color composition) are detected and outlined, for better visibility highlighted with arrows. Similarly, under the red threshold (D), only red single-color and double-color spots are detected. E. The double-color threshold shown is a mathematical intersection of the two single-color thresholds. F. The final step of analysis, with single-color and double-color spots highlighted with artificial colors: blue, red, and green, for single-blue, single-red, and double-color spots, respectively. Counted spots are labeled with letter symbols of the corresponding color. To eliminate the assessment of partially overlapping red and blue spots as double-color spots, the image analyzer counts only double-color spots that are formed by the color mixture of concentric blue and red spots with single dense centers. Further detail on image analysis is provided in Materials and Methods.
53% of the T cells was found to produce only IFN-γ, 4% to secrete only IL-2, and 19% to secrete both cytokines (Fig. 1B; the image analysis of such two-color spots is shown in Fig. 2). The plot of the number of cloned cells plated per well against the number of single-positive and double-positive spots was linear and passed through the origin. Therefore, every cytokine-producing cell was directly visualized, even in the presence of $1 \times 10^6$ bystander cells. This close correlation between intracytoplasmic staining and ELISPOT assays was also seen in similar experiments performed on the Th2 clone M33 (Fig. 1, C and D): IL-4 and IL-5 were coexpressed by $\sim 50\%$ of the cloned cells. The numbers of single-positive and double-positive cells linearly decrease with the number of CD4 cells plated, consistent with the assay having single-cell resolution. In addition, these data show that, as far as the detection of the coexpression of IFN-γ/IL-2 and of IL-4/IL-5 in single T cells is concerned, the sensitivities of intracytoplasmic staining/FACS analysis and two-color ELISPOT analysis are comparable. The color resolution of two-color ELISPOT assays based on AEC (the red substrate for HRP) and NBT/BCIP (the blue substrate for AP) is at least as good as the color resolution of FITC and PE staining in two-color FACS analysis. Last, the use of two-color cytokine ELISPOT analysis confirmed that IFN-γ and IL-2, and IL-4 and IL-5, are coexpressed in long-term cultured T cells, an observation that led to the postulate that such Th1/Th2 cells would also occur in vivo.

When we studied the size distribution of Ag-induced IFN-γ spots generated by freshly isolated CD4 cells from the BALB/c mice primed with OVA$_{323-339}$ and those produced by cloned T cells, we found them to be comparable: both types of cells produced a wide spectrum of spots of various sizes with distributions close to Gaussian and very similar to those obtained by intracytoplasmic stainings (Fig. 3, A vs B). Although the size of the spots depends on several parameters including the kinetic of cytokine secretion, it is proportional to the net cytokine output per cell over the culture period (49). Therefore, such differences in spot sizes (amount of cytokine produced) within clones and freshly isolated cells reflects cell biology. (Similar results were obtained when comparing IL-2, IL-4, and IL-5 produced by clones and freshly isolated CD4 cells, data not shown.) Because cloned cells and freshly isolated T cells produced comparable amount of cytokines, single-cell sensitivity also seems to apply for freshly isolated memory T cells, which occurred at frequencies too low for intracytoplasmic staining to measure, thereby impeding direct comparison. The single-cell resolution of these measurements was further supported by the linear decrease in spot numbers seen when CD4 LN cells primed to produce either IFN-γ or IL-5 were serially diluted with unprimed spleen cells or were mixed in different ratios (Fig. 4). These data also showed that the cognate cytokine produced by type 1 or type 2 polarized memory T cells neither induced bystander cytokine production in the APC population nor inhibited IFN-γ/IL-5 produced by the memory cells.

**IL-2, IL-3, and IFN-γ are produced by different CD4 cells in type 1 immunity**

We chose to characterize more closely the immune response induced in BALB/c mice with a well-defined Ag, OVA, and its immunodominant peptide OVA$_{323-339}$. First, we immunized mice with the maximally immunogenic dose of OVA$_{323-339}$ peptide, 100 μg/mouse, in CFA, s.c. and tested the peptide-induced recall response of CD4 cells purified from the LN and spleens of these mice at various time points. Representative data are shown in Fig. 5 and are summarized in Table I. A classic type 1 (IFN-γ$^+$, IL-2$^+$, IL-3$^+$, IL-4$^-$, and IL-5$^-$) recall response was seen. Image analysis of data obtained in three independent experiments with 4–24 mice per experiment in which cells from each mouse were tested in triplicate wells and in serial dilutions (analyzing more than 600,000 peptide-induced cytokine spots) showed that 95 ± 4% of the spots in the IFN-γ/IL-2 assay were either red or blue (single-positive). Only about 5% of the spots appeared in various shades of purple indicating that only a minor fraction of Ag-specific T cells produced both cytokines simultaneously or switched cytokine production during the assays’ 24–48 h duration. This frequency range of double-positive spots was also seen when the primed T cells were tested in serial dilutions (data not shown). We performed single-color IFN-γ- and IL-2 assays in parallel to verify that we had detected all double-cytokine-producing T cells. The frequencies of cells producing IFN-γ and IL-2 in the single-color assays closely matched the sum of the frequencies of the single producers and double producers of IFN-γ/IL-2 detected in the two-color assay (within 5% error). These additional single-color data prove that the two-color analysis did not miss double-expressing cells. While we cannot exclude the possibility that some cytokine is being coexpressed below the detection limit of ELISPOT analysis, we can safely conclude that the secretion of the one cytokine detected was highly polarized.

Neither unimmunized nor control-immunized mice produced IL-2, IL-3, IL-4, IL-5, or IFN-γ when challenged with OVA or
OVA peptide. Although it has been suggested that naive cells can produce IL-2 (Refs. 50 and 51; a notion that is not unanimously agreed upon, see Ref. 21), their frequency must have been 1/1,000,000 the detection limit of the IL-2 ELISPOT assay as performed. Only after immunization were cells producing IL-2, IL-3, or IFN-γ detectable, suggesting that these cells have undergone Ag-driven clonal expansion and differentiation. Moreover, we found that these cytokine-producing T cells resided in the L-selectin, CD4 fraction (48), which corresponds to a memory phenotype. Therefore, the cytokine-producing cells that we detected after immunization appear to be memory cells that acquired their cytokine phenotype during an Ag-driven immune response in vivo.

Signal strength affects neither the induction of CD4 cell subsets that selectively express IL-2/IL-3/IFN-γ nor the coexpression of these cytokines

It has been suggested that the Ag dose (the density of MHC:nominal peptide complexes on the APC defining the extent of TCR ligation and, hence, the signal strength) affects both the postthymic differentiation of naive T cells along the type 1/type 2 pathway and the pattern of cytokine expressed by differentiated memory cells (42, 52, 53). To determine whether this also applies to adjuvant-driven CD4 cells differentiation in vivo, we immunized BALB/c mice with doses of OVA\textsubscript{323–339} peptide ranging from 0.01 to 100 μg/mouse, in CFA, and performed recall assays on the memory T cells induced, titrating the peptide dose from 0.01 to 40 μM. The 0.01-μg immunization dose did not induce a detectable cytokine recall response; it became detectable at 0.1 μg injected per mouse and reached the plateau at 10 –100 μg/mouse (Fig. 6A). The overall cytokine signature of these recall responses was unaffected by the immunization dose: induction of IL-2, IL-3, and IFN-γ was seen, with only marginal IL-4 and no IL-5 being produced. No IL-5 and only marginal IL-4 production was seen over the full range of peptide concentrations tested at recall (data not shown).

The dose-response characteristics for the activation of cells producing IL-2, IL-3, and IFN-γ were similar (Fig. 6, B–D). The concentration of the OVA peptide at which 50% of the maximally inducible cells become activated was 50\textsubscript{6} 15 nM for all three cytokines; this defines the functional avidity of these T cells for OVA\textsubscript{323–339} (48). Whereas the frequency of cytokine-producing cells increased with the peptide concentration, the fraction of double-positive cells stayed largely constant for given immunization doses varying between 1 and 8%. These data, shown for the day 21 time point in Fig. 6, are representative for all the time points tested (days 4, 10, 21, 42, and 91, data not shown). Therefore, the dissociated production of IL-2, IL-3, and IFN-γ was neither affected

\textbf{FIGURE 4.} Polarized and uninhibited production of IL-5 or IFN-γ in freshly isolated spleen cells. BALB/c mice were immunized with either OVA:IFA (A) or OVA:CFA (C) and their spleen cells were tested on day 21 in cultures containing either medium alone (the wells on the left) or OVA (all other wells) in a two-color cytokine ELISPOT assay: IL-5 in red, IFN-γ in blue. The cells from the primed mice were plated either undiluted, at 1 × 10^6 cells/well, or in two-fold serial dilutions with unirradiated naive BALB/c spleen cells, thus keeping the total cell number constant at 1 × 10^6 cells/well. The cell dilutions in A progress from the left to the right and, in C, from the right to the left. The number (×10^5) of cells derived from mice primed with either OVA:IFA or OVA:CFA is shown, respectively, in the upper right or lower left corner of each panel showing a representative well. B, The spleen cells from the mice primed with OVA:IFA and OVA:CFA were mixed such that the cell numbers correspond to the numbers shown in the panel above (A) and below (C) the image. Beneath each panel, in the respective colors, the mean ± SD of red or blue spots in quadruplicate wells is shown. The data are representative of two experiments performed with unseparated spleen cells and one each with purified CD4 cells and Th1/Th2 clones.
by the signal strength that induced the differentiation of the naive T cells nor by the signal strength that induced cytokine production in the memory cell. Similar results were obtained when purified CD4 cells were tested on different types of APC layers, irradiated or unirradiated spleen cells of BALB/cSCID mice, or spleen, LN, or thymic cells of naive BALB/c mice. Therefore, the presence or absence of B cells and the variation of other class II-positive cell types in these organs had no significant effect on the size and frequency of IFN-γ, IL-2, and IL-3 spots detected (data not shown).

In type 1 immunity, CD4 cells directly assume the IL-2, IL-3, and IFN-γ single-cytokine-expressing phenotype

It has been postulated that CD4 cells first differentiate into memory cells that coexpress type 1 and type 2 cytokines (Th0-type cells) in the course of the immune response and that only with chronic stimulation would these cells further differentiate into type 1 or type 2 cytokine-expressing memory cells. According to this model, the coexpression of type 1 and/or type 2 cytokines in individual T cells should be observed in vivo early in the course of response, whereas the cytokine expression pattern of the memory cells might become polarized over the course of the immune response. To address this possibility, we also studied OVA-peptide-induced CD4 memory cells soon after immunization. By the earliest time that we could detect a specific cytokine recall response, on day 4 after immunization, IL-2, IL-3, and IFN-γ single-positive cells were detected and none producing IL-4 or IL-5 were found. Therefore, at the population level, the cytokine response was type 1-polarized early on, and, at the level of individual memory cells, it was already mediated by single-cytokine-producing cells. Memory cells seem to assume this phenotype rapidly without first going through a prolonged state in which they coexpress cytokines.

Loss of dissociated cytokine expression in tissue culture

Transient segregation of cytokine mRNA expression has been reported for in vitro-primed D011.10 TCR-transgenic cells when using dual-label, in situ hybridization (21, 22). Two-color cytokine ELISPOT measurements yielded comparable results when the same D011.10 cells were tested after the second cycle of in vitro stimulation (Table I). We also observed that the frequency of double-cytokine-producing cells increased with further restimulations in vitro: after 42 days of cell culture, about 16% of the cytokine-producing, transgenic cells became IL-2/IFN-γ double-positive, starting to approximate the phenotype of T cell clones (Fig. 1). Moreover, also in confirmation of this previous report, we found that, with prolonged culture, the transgenic cells started to coexpress cytokine combinations like IFN-γ and IL-5 (close to 15% double-producers detected on day 42, Table I) that we have not seen directly ex vivo. This difference could be attributed to the continuous Ag/IL-2-driven proliferation that the T cells undergo in cell culture. Extensive cell cycling was shown to cause demethylation of cytokine genes and changes in chromatin structure (16), which makes cytokine genes more accessible and may favor cytokine coexpression. Our data suggest that such changes in cytokine expression may not readily occur in vivo, possibly because the T cells in vivo reach replicative senescence after 17–35 cell divisions (19).

FIGURE 5. Dissociated production of IL-2, IL-3, and IFN-γ in OVA-specific CD4 memory T cells from OVA:CFA-immunized mice. The recall response to OVA323–339 by CD4 cells freshly isolated from the spleen of a BALB/c mouse immunized 21 days earlier with OVA:CFA is shown: A–C, IFN-γ (red)/IL-2 (blue) assay; D–F, IFN-γ (red)/IL-3 (blue) assay. CD4 cells mixed with irradiated, naive LN APC were tested in the absence (A and D) and the presence of the OVA peptide (B and E). Two-color image analysis of B and E is shown in C and F, respectively. For image analysis, the legend to Fig. 2 applies. The wells shown have been selected from serial dilutions of CD4 cells for best visual representation of the data summarized in Table I and Figs. 6 and 8. The data are representative for three independent experiments in which 4–24 mice per group were tested individually at days, 4, 10, 21, 42, and 91 after immunization.
IL-4 and IL-5 produced by different CD4 cells in adjuvant-induced, type 2 immunity; induction of CD4 subpopulations that produce IL-2 but not IFN-γ

In contrast to immunizations with CFA, which tend to induce the classic aspects of polarized type 1 immunity (IFN-γ, IL-2, IL-4, IL-5), cytokine recall, delayed-type hypersensitivity, production of specific IgG2a but no IgE Abs), protein Ag injected in IFA typically results in polarized type 1 immunity (IL-2, IL-4, IL-5, IFN-γ; IgG1, IgE, IgG2a; no delayed-type hypersensitivity) (48, 54). The prevalence of the single-cytokine-producing T cell phenotype was also seen in the OVA:IFA-induced type 2 response (Table I and Fig. 7, A–F): ~95% of the Ag-specific CD4 cells produced IL-4 and IL-5 or IL-2 and IL-5 mutually exclusively. Despite the vigorous Ag-specific IL-2 recall response, there was no IFN-γ-induced. This dissociation of IL-2, IL-4, and IL-5, in the absence of IFN-γ, was seen over a wide range of Ag concentrations used for immunization and recall and using APC from different sources (LN, spleen, thymus of naïve mice, or spleen of SCID mice, data not shown). Therefore, the overall type 2-polarized cytokine profile of the IFA-induced response was the result of different T cells individually producing the individual cytokines comprising this profile.

In addition to the OVA:IFA-injected mice shown here, we observed the induction of high-frequency IL-2-producing memory cells in the complete absence of an IFN-γ-producing memory cells after injection of seven other protein Ags in IFA into six different mouse strains (data not shown).

IFN-γ immunization with OVA123–339 peptide results in a mixed cytokine profile in the absence of Th0 cells coexpressing IFN-γ and IL-4 or IFN-γ and IL-5

Unlike the immunizations with OVA protein in IFA, injections of BALB/c mice with OVA123–339 peptide in IFA resulted in mixed “Th0-type” response with IL-2, IL-4, IL-5, and IFN-γ production (48). Two-color assay performed on CD4 cells separated from spleens of these mice 3 wk after immunization showed that each of these cytokines was produced by different cells (Table I).
CFA, as indicated, and LN cells (13 recall response of a single representative mouse is shown. The data were re-numbers at any recall concentration, these data are not shown. SD for triplicate inserts. Because cells producing IL-4 and IL-5 were not detected in significant representative of three independent experiments. B–D, response 21 days later. A, the memory resets itself by generating new daughter cells. By comparing cytokine ELISPOT (24 or 48 h assay) and intracyto-

plasmic cytokine staining/FACS analysis (6 h assay) of cloned cells (Fig. 1) or freshly isolated CD4 cells from the LN after polyclonal stimulation with anti-CD3 mAbs or Con A (data not shown), we showed that, indeed, this is the case, as within this time frame proliferation did not occur. Moreover, by measuring this early cytokine response, we wanted to ascertain that the cytokines we measure were produced by the in vivo-differentiated memory cell, as opposed to their daughter cells, which may or may not inherit the cytokine commitment of the mother cell. Because differentiation of naive cells into memory/effector cells requires longer than this 24–48 h, the cytokines measured were produced by in vivo-primed memory cells (see above).

Unlike dual-label in situ hybridization, which measures mRNA levels in the T cell at a single time point, these ELISPOT assays integrate the production of both cytokines over the entire assay period (immediately after the Ag was added to the cells), accounting for possible differences in the kinetics of cytokine production, including switching from the production of one cytokine to the other. Instead of measuring mRNA (or measuring the accumulation of cytokine in the cytoplasm of pharmacologically treated cells), the ELISPOT approach detects the immunologically relevant secretion of the cytokine, thereby accounting for posttranscriptional and posttranslational regulation. The detection limit for the minimum amount of cytokine produced per cell was found to be comparable in intracytoplasmic staining and in ELISPOT (Fig. 1) for cloned T cells. Also, the distribution of cells according to the amount of cytokine produced per cell was similar with both methods (Fig. 3). However, when it came to detecting the few Ag-specific cells within the majority of non-Ag-specific cells, only the ELISPOT assay was suited for this purpose, as it proved to be sensitive enough to detect one cytokine-producing cell within a million non-cytokine-producing cells (Fig. 1). In contrast, when the frequency of cytokine-producing cells fell below 1:1000, they became undetectable by intracytoplasmic staining; therefore, ELISPOT was 1000 times more sensitive to detect cytokine produced by rare Ag-specific cells in freshly isolated populations.

Irrespective of how the immune response was induced (by infection or immunization with adjuvant), the Ag doses used for priming and recall, or the time point tested, the cytokines measured were mostly produced by different CD4 memory cells; because very similar results were obtained in all in vivo systems tested, the cumulative data obtained in multiple independent experiments for all models studied are summarized in Fig. 8 (they represent the frequency of cytokine coexpression measured in over 1 × 10⁶ individual CD4 cells). The dissociation of IFN-γ from IL-5, and IL-4 and IL-3 from IL-5, was virtually complete. The coexpression of the “type 1” cytokines IFN-γ, IL-2, and IL-3 or the “type 2” cytokines IL-2, IL-4, and IL-5 were confined to 4–6% of cytokine-producing CD4 cells. We also found that IL-4-producing memory T cells had been engaged in the absence of IL-5 in Leishmania-induced immunity and that IL-2 had been produced in the absence of IFN-γ in IFA-induced type 2 immunity (Table I).

The level of cytokine dissociation in individual cells that we observed in vivo was striking as it approximates the precision of allelic exclusion, e.g., 4% for TCR α-chains (55); thus, it was consistent with highly regulated cytokine gene regulation in T cells physiologically stimulated by Ag. Our data can also be explained within the framework of stochastic cytokine gene expression, provided that only a small fraction of the activated memory T cells is actually induced to produce cytokine. Moreover, one would have to postulate additional very rapid and precise selection mechanisms (11) to explain why there are essentially no IL-5-producing memory cells present in Leishmania-induced immunity and why
the nearly complete polarization of IL-4/IFN-γ and IL-5/IFN-γ is already seen at day 4 after immunization.

Alternatively, it is tempting to postulate that the differentiation and subsequent expansion of each of the individual cytokine-producing memory cell subpopulations is under independent instructive control. It is well-established that IL-12/IL-18 and IL-4/IL-13 are differentiation factors for the generation of memory cells expressing IFN-γ and IL-4 (3, 4). Recent evidence has emerged indicating that differentiation into IL-5-producing memory cells may have different differentiation requirements than IL-4 (56, 57), and this may apply for the other type 2 and type 1 cytokines as well. It was also demonstrated that the demethylation patterns of IFN-γ and IL-3 genes, which define their ability to be expressed upon T cell activation (16), are inherited in T cell lineages (17, 18). This observation might point toward the existence of memory cell sublineages with inherited commitment for expressing certain cytokine genes. Stringent control of the engagement and expansion of these single-cytokine-expressing memory cell lineages during the primary immune response would make the resulting composition of the memory cell effector functions finely tunable. When and where the Ag is reencountered for the second time, these memory cell lineages would express the individual cytokines to which they are precommitted. On the population level, they would create a cytokine microenvironment whose exact quality (which individual cytokine is produced and which is not) and magnitude (the population size of each single-cytokine-producing population) precisely execute the required combination of effector functions imprinted during the primary immune response.

Irrespective of the cytokine gene regulation mechanism underlying dissociated cytokine expression, the implications of these data in terms of immunobiology is that the different effector functions associated with the individual cytokines are each independently performed by the T cell system and, therefore, that cytokine-mediated effector functions of T cells are much more versatile and precise than anticipated. Dissociated expression of individual cytokines raises the repertoire of CD4 memory response types
FIGURE 8. Overall frequency of CD4+ cells coexpressing select cytokine pairs. The cumulative data from multiple experiments are shown for CD4+ memory cells induced by OVA protein or OVA123-339 in IFA or CFA and those induced in the course of Leishmania infection. The percentage of coexpressing cells from the total number of cytokine-producing CD4+ cells is shown, as specified in Table I. The mean values and SD were calculated from the data averaged from the individual experiments for each of these models and represent between 20,000 and 600,000 spots, depending on the individual cytokine combinations.

from two (IFN-γ/IL-4, Th1/Th2) to many discrete types, even within “type 1” and “type 2” immunity.

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References


