Interleukin 4–producing CD4 T Cells Arise from Different Precursors Depending on the Conditions of Antigen Exposure In Vivo

By Gilles Foucras,* Laurent Gapin,‡ Christiane Coureau,* Jean M. Kanellopoulos,‡ and Jean-Charles Guéry*

*From the *Institut National de la Santé et de la Recherche Médicale (INSERM) U28, Institut Fédératif de Recherche 30, Hôpital Purpan, 31059 Toulouse Cedex, France; and ‡Unité de Biologie Moléculaire du Gène, INSERM U277, Institut Pasteur, 75724 Paris cedex, France*

Abstract

The precursor origin of T helper (Th) cell subsets in vivo has been difficult to study and remains poorly investigated. We have previously shown that chronic administration of soluble protein antigen induces selective development of antigen-specific CD4 Th2 cells in genetically predisposed mouse strains. To analyze the origin of effector T cells in this model, we designed a competitive polymerase chain reaction–based approach to track public BV-J rearrangement expressed by CD4 T cells specific for hen egg white lysozyme (HEL) in BALB/c mice. We show that public T cell clones are predominantly associated with type 1 or 2 effector Th cells recovered after primary immunization in complete or incomplete Freund's adjuvant, respectively. Conversely, continuous administration of soluble antigen, which induces strong memory Th2 response, is associated with a dose-dependent reduction of public clone size by a mechanism resembling clonal anergy. Thus, soluble HEL–induced Th2 cells do not express the public complementarity determining region 3 motifs characteristic of immunogenic challenge in the presence of adjuvant. These results demonstrate that there are multiple pathways of induction of Th2 responses depending on the condition of antigen exposure in vivo, i.e., clonal immune deviation versus recruitment of a different pool of precursor cells.

Key words: clonal expansion • antigen-specific public repertoire • CD4 T cell subsets • chronic antigen stimulation • clonal anergy

Introduction

CD4 T cells have been subdivided into different subsets according to their cytokine profiles (1–3). Mouse Th1 cells produce IL-2, IFN-γ, and TNF-β, whereas Th2 cells selectively secrete IL-4, IL-5, IL-10, and IL-13 (4). Th1 and Th2 cells not only differ by their cytokine secretion patterns but also by their effector functions in vivo, resulting in deleterious or protective immunity depending on the pathogens (5). To explain the Th1/Th2 differentiation paradigm, it has been initially hypothesized that the two subsets could be derived from either two different pools of precursor cells or a common precursor (6). Data obtained from limiting dilution analysis (7, 8) and studies in TCR-transgenic mice (9, 10) have clearly shown that naive CD4 T cells have the capacity to differentiate into either Th1 or Th2 effector cells and that this process is critically influenced by the cytokine milieu during the initial phase of T cell activation (11). Among cytokines, IL-12 and IL-4 have been shown to play a decisive role by driving the polarization of T cell responses toward the Th1 or Th2 phenotype, respectively (11–13).

To understand the mechanisms that govern the preferential growth and differentiation of normal CD4 T cell subsets in vivo, we have designed an immunization protocol that selectively induces Ag-specific Th2-type immune responses in BALB/c mice (14). Continuous administration in normal and β2 microglobulin–deficient BALB/c mice of any soluble protein Ag followed by immunization with the same Ag in adjuvant induces the selective development of Ag-specific Th2 cells, which depends on the production of endogenous IL-4 (14). Whereas Th1 cell unresponsiveness is observed in all strains tested after soluble Ag administration, Th2 development depends on a non-MHC–linked genetic polymorphism and is predictive of disease outcome after *Leishmania major* infection (15). Because in-

Address correspondence to J.-C. Guéry, INSERM U28, Hôpital Purpan, Place du Dr. Baylac, 31059 Toulouse, France. Phone: 33-5-61-77-92-96; Fax: 33-5-61-77-92-91; E-mail: Jean-Charles.Guery@purpan.inserm.fr

hibition of Th1 cells in this experimental model appears to be independent of induction of Th2 responses, we postulated that both populations may arise from different precursors in vivo. However, studying T cell responses to various Ag challenges in vivo is limited by the fact that the fate of Ag-specific T cells cannot be easily followed. We took advantage of the hen egg white lysozyme (HEL)[1]-specific T cell responses in BALB/c mice, mainly directed against two epitopes of the protein that involve public TCR Vβ repertoire usage in CD4 T cells specific for each determinant (16, 17). These public repertoires, found in every mouse, are characterized by specific BV-J rearrangements with conserved junctional sequences. Such characteristics allowed us to design molecular probes to follow and quantify HEL-reactive CD4 T cells bearing these particular CDR3 motifs.

In this paper, we analyze the clonal size and phenotype of public CDR3–bearing CD4 T cells after administration of the HEL protein either in adjuvant or soluble form to BALB/c mice. We show that continuous administration of low doses of soluble HEL induces a reduction of CD4 T cells expressing the public CDR3 motifs specific for both subdominant and dominant HEL epitopes, which can be correlated with downregulation of Th1 responsiveness. Therefore, the Th2 response that develops in these mice is not due to immune deviation of lymphocytes bearing the public CDR3 motifs toward IL-4–producing cells. This is not due to the incapacity of T cells that expressed the public rearrangements to develop along the Th2 pathway, as they are found in IL-4–producing cells from mice primed with HEL in IFA (HEL–IFA). Taken together, our data support the conclusion that public repertoire expression in polarized Th cell subsets is determined by the mode of protein Ag administration, suggestive that Th1 and Th2 cells originate from different precursor populations after in vivo priming.

Materials and Methods

Mice. BALB/c (H-2^d) mice were purchased from Centre d'Elevage R. Janvier and maintained in our animal facilities under pathogen-free conditions. 2–3-mo-old female mice were used in all experiments.

Ags and Immunization. HEL was obtained from Sigma Chemical Co. HEL peptides (purity >85%) were purchased from Neosystem. Mini-osmotic pumps (Alzet 2001; Alza Corp.) were implanted subcutaneously as previously described (14). 12 d after pump implantation, mice were immunized subcutaneously into the hind foot pads with 50 μg of HEL emulsified in IFA or CFA containing H37Ra mycobacterium (Difco Labs., Inc.). Draining popliteal LNs were removed 8–9 d after immunization.

T Cell Hybridomas. The BV8S2-J1S5 2E8 hybridoma, specific for HEL 107–116/I-E^d antigenic complexes, was obtained as previously described from HEL–CFA-immunized BALB/c mice (18). The 2E8 Vβ chain was sequenced as described elsewhere (16). The nucleotide sequence of the junctional region was identical to the previously published sequence of C6.2 T cell hybridoma (16), except that the first glycine codon in the CDR3 was GGT instead of GGG. The BV8S2-J2S7 T cell hybridoma LC21, specific for the HEL 12–25/I-A^d epitope, has been described elsewhere (17). The TCR β chain CDR3 regions of these two hybridomas were used to generate competitor plasmids as described below.

T Cell Assays. For cytokine production analysis, popliteal LN cells (LNCs), undepleted or depleted of $CD8^+$ cells, were cultured at 5 or 6×10^5 cells/well in 96-well culture plates (Costar Corp.) in synthetic HL-1 medium (HYCOR) supplemented with 2 mM l-glutamine (GIBCO BRL) and 50 μg/ml gentamicin (Sigma Chemical Co.) with the indicated Ag concentrations. Cultures were incubated for 3 d in a humidified atmosphere of 6% CO_2 in air. Supernatants from replicate cultures, usually three to four wells, were collected after 72 h and pooled for cytokine analysis. LNCs were depleted of CD8 cells by sequential incubation with KT1.5 mAb (19) culture supernatant and M-450 anti–rat IgG Dynabeads (Dynal), followed by magnetic cell separation. After 6 d of culture, 2×10^5 $CD4^+$ T cells, isolated as described below, were stimulated with 3×10^5 syngeneic spleen cells and Ag in complete medium. Complete medium was RPMI 1640 supplemented with 10% FCS (ATGC Biotechnologie), 1% pyruvate, 1% nonessential amino acids, 1% l-glutamine (GIBCO BRL), 50 μM 2-ME, and 50 μg/ml gentamicin (Sigma Chemical Co.).

Cytokine Assays. IFN-γ and IL-4 were quantified by sandwich ELISA as previously described (14). For IFN-γ, polyvinyl microtiter plates (Falcon 3912) were coated with 100 μl of AN-18.17.24 mAb (20) in carbonate buffer. After blocking, samples (50 μl/well) diluted in test solution (PBS containing 5% FCS and 1 g/liter phenol) were incubated together with 50 μl of peroxidase-conjugated XMG1.2 mAb (2). After overnight incubation at 4°C, bound peroxidase was detected by 3,3′-5,5′-tetramethylbenzidine (Fluka Chemie AG) after blocking with 50 μl of 1N H_2SO_4, and absorbance was read at 450 nm with an automated microplate ELISA reader (Emax; Molecular Devices). For IL-4 determination, sandwich ELISA was performed with paired mAbs, all purchased from PharMingen. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines, and results were expressed as cytokine concentration in nano- or picograms per milliliter. Detection limits were 15 pg/ml for IFN-γ and IL-4.

Flow Cytometric Analysis of Intracellular Cytokine Synthesis. LNCs were cultured as described above with 10 μM HEL. After 3 d of culture, cells were harvested, washed, and cultured for an additional 3 d in complete medium. Living cells separated on Ficoll gradient were resuspended at 10^6 cells/ml and stimulated with PMA (50 ng/ml; Sigma Chemical Co.) and ionomycin (0.5 μg/ml; Sigma) for 4 h at 37°C, with 10 μg/ml of brefeldin A (Sigma) added for the last 2 h. Cells were harvested, washed, and stained using biotinylated GK1.5 anti-CD4 mAb (PharMingen), followed by streptavidin–Cy-Chrome (PharMingen). At this step, cells were fixed with 2% paraformaldehyde (Fluka Chemie AG). Intracytoplasmic staining was performed as previously described (21). After washing and 10-min incubation in 0.5% saponin medium, cells were incubated for 30 min at room temperature with the appropriate concentration of FITC- or PE-conjugated, cytokine-specific mAb. The following mAbs were used: PE–11B11 anti–IL-4 (PharMingen), FITC–JES5-16E3 anti–IL-10 (PharMingen), and FITC-labeled XMG1.2 anti–IFN-γ (2). Cells were washed twice with saponin buffer and subsequently with PBS/FCS in the absence of saponin to allow mem-

[1]*Abbreviations used in this paper:* DCs, dendritic cells; HEL, hen egg white lysozyme; LNCs, lymph node cells.

brane closure. Data were collected on 2×10^4 $CD4^+$ T cells on an XL Coulter cytometer (Coultronics), and results were analyzed using CELLQuest™ software (Becton Dickinson).

CD4 T Cell Isolation and FACS® Sorting. After 4 or 6 d of culture, CD4 T cells were isolated by negative depletion using M-450 anti–rat IgG magnetic beads (Dynal). In brief, cells were washed and incubated for 30 min on ice with a cocktail of the following mAbs: KT1.5 anti-CD8, RA3-3A1 anti-B220, and M5-114 anti–MHC class II. After washing and incubation with M-450 anti–rat IgG beads under agitation, $CD4^+$ T cells were purified by magnetic depletion. The purity of the negatively selected $CD4^+$ T cells controlled by flow cytometric analysis after staining with FITC–GK1.5 anti-CD4 was ~95%.

For the detection of HEL-specific TCR β chain rearrangement in T helper subsets, purified $CD4^+$ T cells were stained for intracellular cytokine synthesis as described above and sorted using an Elite Coulter cell sorter (Coultronics). Sorted cells, usually $0.5–1 \times 10^6$, were pelleted, and DNA was extracted using the Simple Nucleic Acid Preparation (S.N.A.P) whole blood DNA isolation kit (Invitrogen BV).

TCRB Repertoire Analysis. The technique of repertoire analysis with Immunoscope has been described elsewhere (22). Total RNA was extracted from purified CD4 T cells using the TRIzol procedure (GIBCO BRL); cDNA was synthesized using an oligo$(dT)_{17}$ primer, murine moloney leukemia virus reverse transcriptase (Boehringer Mannheim), in the provided buffer and RNasin (Promega Corp.). PCR run to saturation was carried out in 50-μl reaction volume containing 2 U of Taq polymerase (Promega Corp.), 2.5 mM $MgCl_2$, 0.20 mM dNTP, 0.5 μl of each primer, and cDNA template in the provided buffer. Oligonucleotides for repertoire analysis have been described previously (17, 22, 23), except: BC130, 5′-TGCCTGAGCAGCCGCCTGAG-3′; BC145, 5′-CACTGATGTTCTGTGTGACAGGTTT-3′; BV8S2del, 5′-CATTATTCATATGGTGCTGGCATGAGAAAGGAGAT-3′; and BC130del, 5′-TGCCTGAGCAGCCGCCTGAGGGTCTCACCTTCTGGC-3′. 40 rounds of amplification, each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, were performed in a 9600 Perkin-Elmer thermocycler (Perkin-Elmer Applied Biosystems). In similar conditions, run-off reactions were performed on 2 μl of PCR product with a single Fam-labeled primer (CDR3, BJ1S5, or BC specific) at a final concentration of 0.1 μM. 10-μl final volumes were subjected to one to three cycles depending on PCR yield. 2-μl volumes of run-off products were run on a 6% polyacrylamide 8 M urea gel in an automated 373A DNA sequencer (Perkin-Elmer Applied Biosystems), together with size standard. The intensity of the various bands was then recorded and analyzed with Immunoscope software. To test the presence of the public rearrangement on the DNA of Th subsets isolated by cell sorting, semiquantitative PCRs were performed using AmpliTaq Gold™ DNA polymerase (Perkin-Elmer ABI) and the above-mentioned primers. PCR products were analyzed on 2% agarose gel stained with ethidium bromide. Specificity was confirmed by run-off reactions as above.

Public Clone Quantification. To estimate the frequency of $CD4^+$ T cells bearing clonotypic rearrangements, we used a fully quantitative PCR protocol, already described (22). In brief, it consists of the coamplification with the same primers of the cDNA mixed with a known number of copies of a competitor plasmid in which a 4-bp deletional specific sequence has been integrated. cDNA from LC21 (HEL 12–25/I-A^d) and 2E8 (HEL 107–116/I-E^d) T cell hybridomas bearing public CDR3 BV8S2-J2S7 and BV8S2-J1S5, respectively, were amplified with BV8S2del containing a 4-bp deletion and BJ-specific primers. The shortened BV8S2-J1S5 and BV8S2-J2S7 clonotypic sequences were then integrated into the plasmid pCR®2.1-TOPO using a TOPO TA cloning kit (Invitrogen Corp.). The recombinant clones with the deletion were screened by sequencing. The number of copies was estimated by OD determination. Aliquots of cDNA of interest and of competitor plasmid were amplified with BV8S2- and BJ-specific primers and then subjected to run-off reactions with primers specific for the public CDR3 motifs BV8S2-J2S7$^{HEL\ 12–25}$ (HEL 12–25/I-A^d–specific TCR β chain public rearrangement) and BV8S2-J1SS$^{HEL\ 107–116}$ (HEL 107–116/I-E^d–specific TCR β chain public rearrangement). Using the same procedure as for the repertoire analysis, the ratio of intensity of the two bands was plotted as a function of the number of copies of standard plasmid introduced before amplification. A regression calculation was performed to determine the equivalence point (ratio = 1), and thus the number of cDNA copies of the given gene before amplification. For normalization, the number of TCR β chain copies was quantified in the same samples using the same procedure (24). To generate the competitor Cβ plasmid, PCR products obtained using the BC130del and BC145 primers specific for a conserved region of Cβ1 and Cβ2 genes were cloned and screened as above. Run-off reactions were performed using the Fam-labeled BC 5′-TTGTCCTCCTCTGAAAGCCCATGG-3′ primer. Data were expressed as ratio of public CDR3 over TCR BC mRNA copies.

Results

Phenotype and Specificity of Soluble HEL–induced Th2 Cells. As previously shown, the continuous administration of soluble HEL to BALB/c mice results in the selective expansion of Ag-specific Th2 cells after subsequent in vivo challenge with Ag in CFA (14, 15). After HEL restimulation in vitro, immune LNCs from soluble Ag–treated or control BALB/c mice were analyzed at the single-cell level for the intracellular production of IFN-γ, IL-4, and IL-10. Results in Fig. 1 A show that IL-4–producing CD4 T cells selectively expand in mice implanted with HEL-containing mini-osmotic pumps but not in control untreated BALB/c mice. Among the IL-4–producing Th2 cells, 25% coexpress IL-10 (Fig. 1 A). Conversely, HEL-specific CD4 T cells that expand in HEL–CFA-immunized mice exhibit mainly a Th1 phenotype, as shown by their capacity to secrete IFN-γ.

In BALB/c mice, HEL-specific T cells are mainly directed against a dominant epitope included in the HEL sequence 107–116 presented by I-E^d molecules (25). A subdominant epitope, included in the HEL sequence 12–25, has also been characterized that is restricted by I-A^d molecules (17, 26). To analyze the specificity of Th2 cells, immune LNCs from control or soluble HEL–treated BALB/c mice were cultured with HEL for 6 d and then restimulated with the synthetic peptides HEL 12–27 and HEL 103–117. As shown in Fig. 1 B, an opposite cytokine profile is observed between the two groups of mice, indicating that both Th1 and soluble Ag–induced Th2 cells recognize the dominant and subdominant epitopes of the HEL pro-

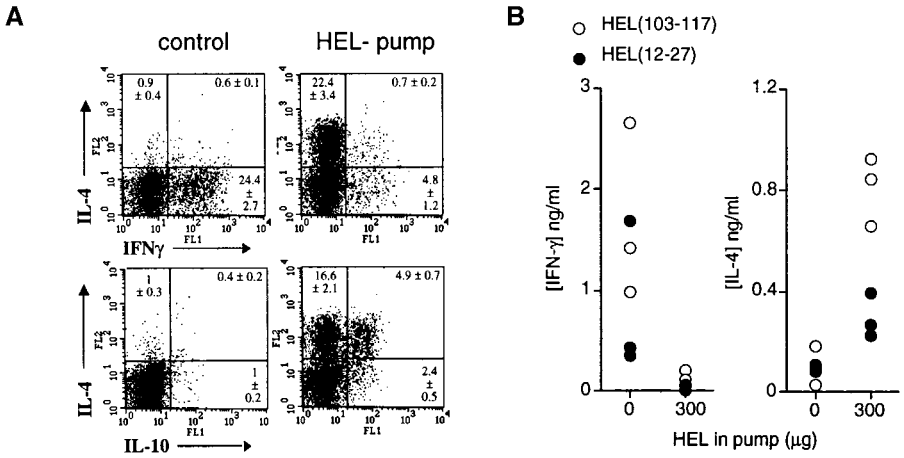

Figure 1. IFN-γ– and IL-4–producing CD4 T cells share common specificity. Popliteal LNCs from control or soluble Ag (HEL-pump)–treated BALB/c mice were stimulated with HEL (10 μM) in vitro. (A) After 6 d of culture, living cells were restimulated with PMA and ionomycin during 4 h in the presence of brefeldin A during the last 2 h. Intracytoplasmic staining for the indicated cytokines was performed and analyzed on CD4 T cells. (B) To analyze the reactivity against HEL 12–25 and HEL 103–117 epitopes, cells (10^5 per well) were stimulated with synthetic peptides (10 μM) in the presence of irradiated syngeneic splenocytes (3×10^5 per well). After 48 h, IFN-γ and IL-4 production was determined by ELISA in culture supernatants.

tein. Using a set of overlapping HEL peptides as described elsewhere (14), no other reactivity could be defined (data not shown).

The Public BV8S2-D1-J1S5 Rearrangement Is Associated with the Th1 Phenotype in HEL–CFA-immunized BALB/c Mice. It was previously shown that the T cell response against the immunodominant HEL 107–116 epitope in H-2^d mice involves a public BV8S2-J1S5 repertoire found in all animals with a specific CDR3 amino acid sequence, GTGNNQAP (16). Immune LNCs from HEL–CFA-primed mice secrete IFN-γ in response to both HEL protein and HEL 103–117 peptide. To analyze whether these IFN-γ–producing cells express the public rearrangement, we analyzed the presence of the public CDR3 motif (BV8S2-J1S5$^{HEL\ 107–116}$) on the DNA of HEL-specific CD4 T cells sorted on the basis of intracellular expression of IFN-γ. As shown in Fig. 2 A, DNA was extracted from highly purified (>95%) IFN-γ^- and IFN-γ^+ CD4 T cells from LNCs restimulated in vitro as described in Fig. 1 A and subjected to PCR using BV8S2- and BJ1S5-specific primers. As shown in Fig. 2 B, the rearranged BV8S2-J1S5 products are preferentially amplified from the DNA extracted from IFN-γ^+ but not IFN-γ^- CD4 T cells. Fig. 2 C shows the run-off reaction performed with CDR3-specific primers, demonstrating that the public CDR3 BV8S2-J1S5$^{HEL\ 107–116}$ motif is mainly expressed in IFN-γ–producing CD4 T cells.

Induction of Th1 Unresponsiveness Correlates with a Reduced Clonal Expansion of Public BV8S2-J1S5$^{HEL\ 107–116}$ CD4 T Cells in Soluble HEL–treated Mice. We next tested the effect of pretreatment with a wide dose range of soluble HEL on the expression of the public rearrangement in HEL-specific CD4 T cells. Using the PCR-based approach Immunoscope, we analyzed the CDR3 size distribution of TCRs bearing the BV8S2-J1S5 rearrangement as previously described (16). As shown in Fig. 3 A, the CDR3 size distribution among all BV8S2-bearing cells gives a set of seven peaks, each separated by three nucleotides in control and soluble HEL–injected mice. Conversely, a major expansion with a CDR3 size of eight amino acids is observed with BJ1S5 and public CDR3–specific primers in control animals immunized with HEL–CFA (Fig. 3 A). Interestingly, continuous administration of soluble HEL is associated with an increased heterogeneity in the CDR3 size in this BV-J combination due to a diminished expression of the BV8S2-J1S5$^{HEL\ 107–116}$ public rearrangement. Data from four individual mice per group are summarized in Fig. 3 B, where CDR3 BV8S2-J1S5$^{HEL\ 107–116}$–specific products are expressed as percent of the sum of the BV8S2-BC peaks. Therefore, continuous administration of low dose soluble

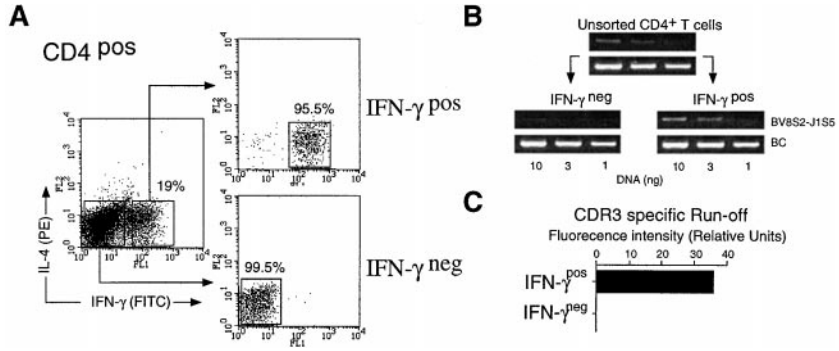

Figure 2. The public repertoire BV8S2-J1S5 specific for the dominant I-E^d–restricted HEL 107–116 determinant is associated with IFN-γ–producing cells. (A) After 6 d of in vitro stimulation, purified CD4 T cells from HEL–CFA-immunized mice were stained for intracytoplasmic synthesis of IFN-γ and IL-4 as described in Fig. 1 and isolated by FACS® as indicated. (B) Semiquantitative PCR for public rearrangement BV8S2-J1S5, normalized on constant region BC gene amplification, was performed on DNA from whole CD4, IFN-γ^-, and IFN-γ^+ cells. (C) Run-off reactions were performed on PCR products with a CDR3-specific fluorescent primer to confirm the presence of BV8S2-J1S5$^{HEL\ 107–116}$–specific sequence. Results are representative of three experiments performed.

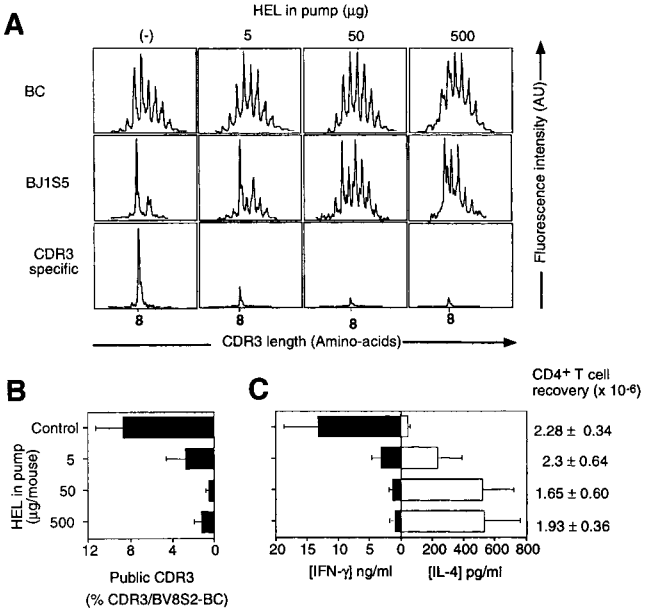

Figure 3. Immunoscope analysis reveals a dose-dependent loss of public repertoire BV8S2-J1S5$^{HEL\ 107–116}$ in soluble HEL–induced Th2 cells. Popliteal LNCs from mice untreated or treated with the indicated amounts of HEL in pump were stimulated in vitro with HEL (10 μM). (A) After 4 d, CD4 T cells were purified and subjected to RNA extraction. PCR amplification of BV8S2-BC rearrangements on cDNA and Immunoscope analysis were then performed as described in Materials and Methods. Data are from one representative mouse out of four analyzed per group. (B) Data from the Immunoscope analysis were expressed as ratio of the area under the public CDR3–specific peak by the sum of the CDR3 peaks obtained in the BC-specific run-off reactions. (C) Ag-specific IFN-γ and IL-4 production was measured in 72-h culture supernatants by ELISA. Data are expressed as mean ± SD of four mice per group. Results are representative of two experiments performed.

HEL results in a strong reduction of Ag-specific T cells expressing the public CDR3 BV8S2-J1S5$^{HEL\ 107–116}$ (Fig. 3 B), which can be correlated with the downregulation of Th1 response but not with the expansion of IL-4–producing cells (Fig. 3 C).

Induction of Th1 Cell Unresponsiveness by Soluble Ag Administration Correlates with a Reduction of the Clonal Size of Public Clones Specific for Both Dominant and Subdominant HEL Epitopes. The dominant 107–116 and subdominant 12–25 HEL epitopes have been shown to exhibit different requirements for Ag processing and presentation (17, 27). Therefore, we next tested whether T cells specific for these two determinants were similarly affected by systemic HEL administration. As has been shown for 107–116/I-E^d–reactive T cells, a public rearrangement, BV8S2-J2S7, characterized by a conserved junctional sequence GDRLGGYEQ, has been identified in CD4 T cells specific for HEL 12–25/I-A^d complexes (17). To quantify by competitive PCR analysis the clonal size of CD4 T cells expressing the two public CDR3 motifs, we designed two internal standard DNA plasmids that contain the TCR-β sequence corresponding to the public CDR3 motifs BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ with a four-nucleotide deletion in the BV8S2 gene segment. The competitive PCR amplifications were performed with BV8S2- and BJ-specific primers. Run-off reactions were subsequently done using CDR3-specific primers to reveal amplification of the relevant sequences only. For normalization, the number of TCR BC chain copies was determined in the same samples using a similar procedure. Data are expressed (Fig. 4) as ratio of CDR3/TCR BC mRNA copies and should correspond to the frequency of individual clones bearing such public rearrangements among all CD4 T cells. As shown in Fig. 4 A, administration of soluble HEL results in a dose-dependent and coordinated reduction of CDR3 copies for

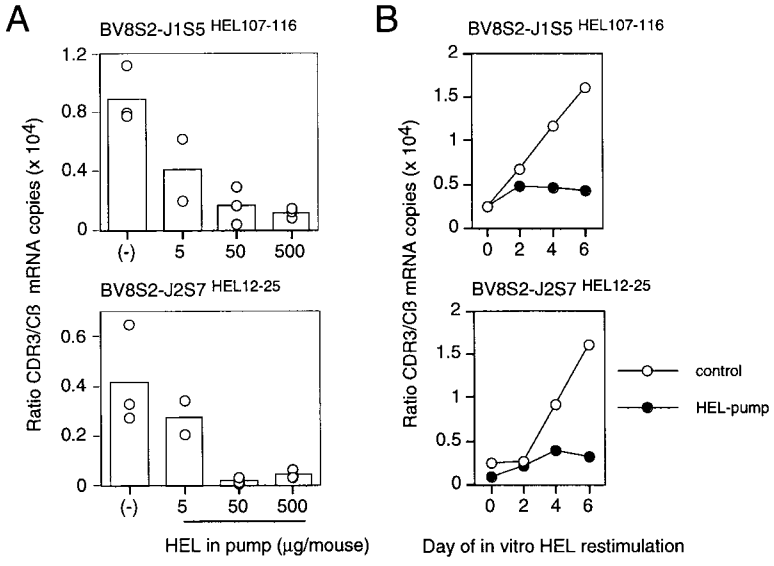

Figure 4. Soluble Ag administration induces anergy in public clones specific for dominant and subdominant HEL epitopes. CD8-depleted LNCs from mice pretreated with the indicated doses of soluble HEL in pump were stimulated in vitro as in Fig. 3. cDNA was prepared from CD4 T cells. Quantitative measurement of BV8S2-J2S7$^{HEL\ 12–25}$ and BV8S2-J1S5$^{HEL\ 107–116}$ mRNA copies was performed by competitive PCR (Q-PCR) as described in Materials and Methods. Sample variations were normalized on the number of TCR β chain mRNA copies. (B) To quantify public clone expansion during in vitro stimulation, pooled, CD8-depleted LNCs from control or soluble HEL–treated mice (150 μg in pump) were cultured with 10 μM of HEL and sampled at 48-h intervals for clonal frequency determination. Results are representative of three experiments performed.

the two public rearrangements analyzed. Although both public clones are still present in soluble Ag–induced effector T cell populations, their clonal expansion is reduced by up to 90% for the highest dose of soluble Ag administered as compared with untreated mice. These data are in agreement with the Immunoscope analysis and demonstrate that soluble Ag administration results in a reduced expansion of public T cell clones specific for dominant and subdominant HEL determinants. BV8S2-J1S5$^{HEL\ 107–116}$– and BV8S2-J2S7$^{HEL\ 12–25}$–specific products were barely detectable in resting splenic CD4 T cells or in CD4 T cells from mice immunized with CFA alone and represented a frequency of $\sim 10^{-6}$ (not shown).

To determine whether the mechanism(s) of clonal unresponsiveness in public CD4 T cells involved clonal anergy or lack of recruitment in vivo, we measured their frequency between days 0 and 6 after HEL restimulation in vitro. As shown in Fig. 4 B, the rearranged public CDR3 BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ are detected in LN CD4 T cells from HEL–CFA-immunized control and soluble Ag–treated mice at quite similar frequencies. In some experiments, BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ public clones were less frequent (twofold reduction) than in control mice, but this was inconsistently observed (not shown). HEL stimulation in vitro induces the expansion of public clones (up to 10-fold) in CD4 T cells from control mice. Conversely, the frequency of BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ CD4 T cells that are recruited in immune LNs of soluble Ag–treated mice increases moderately over time, indicating that public clones have an impaired capacity to proliferate in vitro. Taken together, these data suggest that systemic Ag administration might induce a state of functional unresponsiveness in public clones, correlated with the inability of HEL-reactive CD4 T cells to produce IL-2 (14) and IFN-γ (Fig. 1 and Fig. 3 B). Indeed, analysis of intracellular Th1-type cytokine synthesis in secondary T cells shows that the frequency of T cells able to secrete IFN-γ alone or in association with IL-2 is strongly inhibited in soluble HEL–treated mice (not shown).

The Development of BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ Public Clones Is Selectively Impaired in Soluble Ag–induced But Not IFA-induced Th2 Cells. The inability of public CDR3 CD4 T cells to differentiate toward the Th2 phenotype after soluble protein challenge could be explained by the fact that these populations are precommitted to develop toward the Th1 phenotype. Alternatively, the mode of protein Ag administration (e.g., localized delivery with adjuvant versus systemic soluble Ag release) might be critical in selecting a particular T cell repertoire and its associated Th phenotype. To address this issue, we next tested whether cells expressing the Th1-associated public repertoire could be induced to develop along the Th2 pathway using IFA, which has been shown to induce Th2 cell development (28). Data in Fig. 5 A show that after administration of HEL–IFA, CD4 T cells secrete a mixed cytokine profile upon primary in vitro stimulation. In contrast to HEL–CFA-primed mice, secondary T cells from HEL–IFA-immunized animals secrete low levels of IFN-γ and high levels of IL-4 (Fig. 5 B). These data are confirmed by the analysis of intracellular cytokine synthesis in cells restimulated by PMA/ionomycin after the primary culture (Fig. 5 D). CD4 T cells from IFA-primed mice are composed of a mixed population of cells that selectively secrete either IFN-γ (11%) or IL-4 (15%). In contrast, T cells from CFA-primed mice primarily express IFN-γ (18%). Analysis of public clone size was next performed in CD4 T cells that were stimulated with HEL for 4 d (Fig. 5 C). BV8S2-

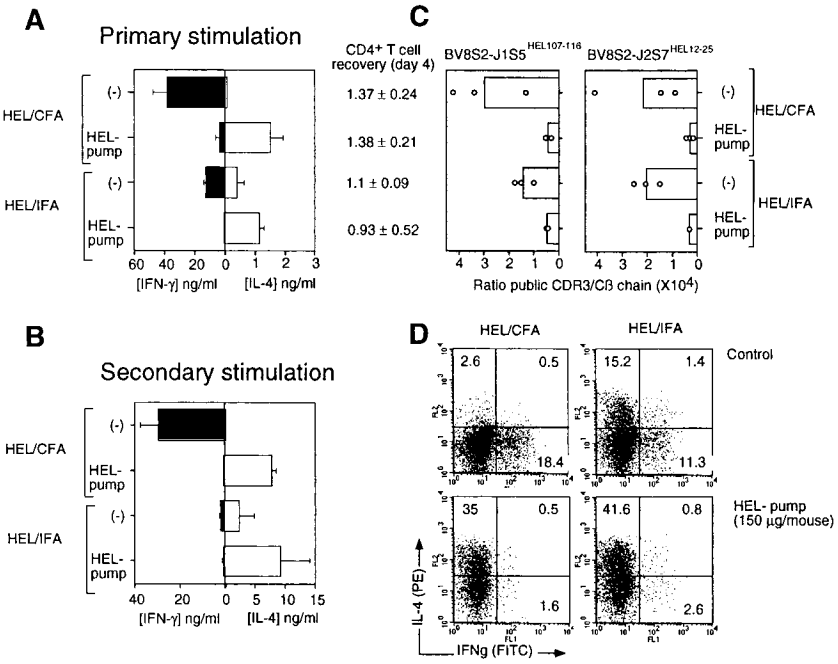

Figure 5. Public clones expand after immunization in CFA or IFA, but not in soluble Ag–treated mice. CD8-depleted LNCs from mice pretreated or not with soluble HEL and immunized with CFA or IFA were restimulated with HEL in vitro. (A) Ag-specific IFN-γ and IL-4 production was measured by ELISA in 72-h supernatant. (B) At day 6, CD4 T cells (10^5 cells per well) were restimulated with 10 μM of HEL and irradiated syngeneic spleen cells (3×10^5 cells per well). Cytokine production was analyzed by ELISA after 48 h. (C) At day 4, mRNA was extracted from purified CD4 T cells and reverse transcribed. Public clone frequency was determined by Q-PCR on cDNA. (D) Secondary CD4 T cells were stimulated with PMA/ionomycin, and intracellular synthesis of indicated cytokines was analyzed as in Fig. 1. Data are expressed as mean ± SD of three mice per group. Results are representative of three experiments performed.

J1S5$^{HEL\ 107–116}$– and BV8S2-J2S7$^{HEL\ 12–25}$–bearing CD4 T cells expand to a quite similar extent in mice primed with HEL–CFA or HEL–IFA.

Conversely, continuous administration of soluble HEL induces strong Th2 cell development in mice subsequently challenged with Ag emulsified in either CFA or IFA (Fig. 5). IL-4 production by primary and secondary CD4 T cells is always higher in soluble HEL–treated as compared with IFA-injected mice. Quantification of the clonal size of public CDR3 BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ indicates that the continuous administration of soluble Ag induces a strong reduction in public clone size that is independent of the type of adjuvant used for in vivo challenge (Fig. 5 C).

As shown above, selective expansion of IL-4–producing Th2 cells in HEL–IFA-primed mice is associated with the persistence of cells secreting IFN-γ. To demonstrate that public CDR3 BV8S2-J1S5$^{HEL\ 107–116}$– and BV8S2-J2S7$^{HEL\ 12–25}$–bearing cells are present in IFA-induced Th2 cells, we compared the expression of public CDR3 on CD4 T cells sorted according to the intracellular expression of IL-4 and IFN-γ. IFN-γ$^+$IL-4$^-$ CD4 T cells from mice immunized with HEL–CFA were sorted as in Fig. 2. The mixed population obtained from HEL–IFA-immune LNCs was sorted into IFN-γ$^-$IL-4$^+$ as shown in Fig. 6 A. The expression of the public CDR3 was analyzed at the DNA level by competitive PCR as described in Fig. 4. Data in Fig. 6 B demonstrate that IL-4–producing cells that expand in CD4 T cells from HEL–IFA-immune LNCs contain the BV8S2-J1S5$^{HEL\ 107–116}$ and BV8S2-J2S7$^{HEL\ 12–25}$ public clones with a frequency similar to that of INF-γ–producing cells from HEL–CFA-immune LNCs. These data demonstrate that these two types of adjuvant induce different Th phenotype acquisition in the same precursor population, indicating that HEL-specific CD4 T cells that express the public rearrangement are not precommitted to the Th1 phenotype.

Discussion

In this paper, we have examined the origin of Th2 cells induced in BALB/c mice after chronic administration of low-dose soluble Ag. We have studied the CD4 T cell responses to dominant and subdominant HEL epitopes for which public rearrangements of TCR β chain have been characterized (16, 17). At least two models could explain the effect of continuous administration of soluble protein in the selective development of Th2 cells. According to the first one, soluble Ag administration induces clonal deviation from the Th1 to the Th2 phenotype. Thus, systemic Ag presentation results in T cell priming but fails to induce IL-12, so that in the absence of this cytokine and in the presence of IL-4, the majority of HEL-reactive T cells develop along the Th2 pathway. The alternative model involves two populations of precursor cells that are differentially affected after soluble HEL administration. In this case, the dominant repertoire that normally expands after priming with Ag in adjuvant is rendered unresponsive after soluble Ag delivery. Concomitantly, this mode of Ag administration selectively induces different precursor T cell populations to develop along the Th2 pathway. Previous experiments have suggested that Th1 inhibition and Th2 priming by continuous delivery of soluble Ag were two independent mechanisms. For instance, Ag-specific Th2 cell development was observed in genetically predisposed mice, whereas Th1 cell development was blocked in any mouse strain tested (15). In addition, blocking Th2 cell development in BALB/c mice did not reverse the unresponsiveness in Th1 cells, indicating that these two phenomena may occur independently in the same mouse strain (14). Data in the present paper indeed support this hypothesis and provide direct evidence that soluble Ag–induced Th2 cell development is not due to clonal deviation, suggesting that in this model, effector Th cell populations rather originate from different precursors in vivo.

We directly demonstrate that the two public rearrangements BV8S2-J2S7$^{HEL\ 12–25}$ and BV8S2-J1S5$^{HEL\ 107–116}$ are predominantly associated with IFN-γ–producing CD4$^+$ T cells from HEL–CFA-primed mice. As we have quantified the number of public CDR3 clones among all TCR β chains, and because all T cells are likely to express the same number of TCR-β gene transcripts (29), this ratio should correspond to the frequency of public CDR3–bearing cells

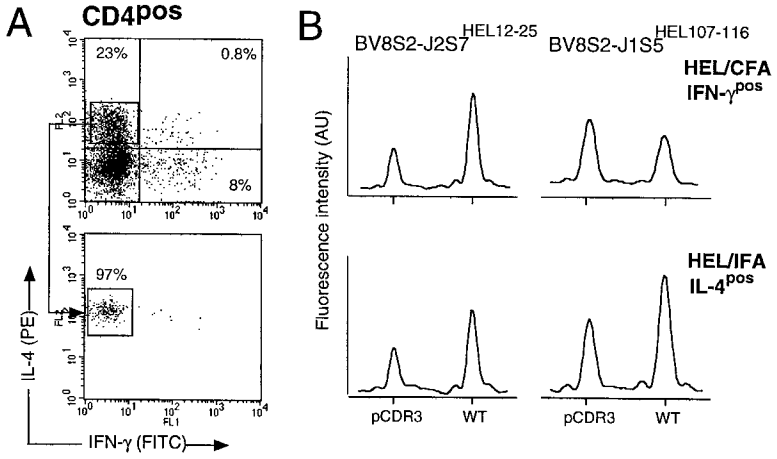

Figure 6. Public clones are recruited to a similar extent in adjuvant-guided IFN-γ– or IL-4–producing CD4 T cells. (A) Secondary Ag–specific CD4 T cells from mice immunized with HEL in IFA were stained intracellularly for IFN-γ and IL-4 synthesis and sorted according to their intracytoplasmic IL-4 cytokine expression. (B) After DNA extraction and Cβ normalization, competitive PCR was performed for the two public rearrangements with 20 or 30 copies of BV8S2-J2S7$^{HEL\ 12–25}$and BV8S2-J1S5$^{HEL\ 107–116}$ competitor plasmids, respectively. The DNA amount analyzed in both populations corresponded to 2×10^5 Cβ copies. Results are expressed as fluorescence intensity of CDR3-specific run-off products corresponding to the genomic sequence (wt) and competitor plasmid (pCDR3) as indicated. IFN-γ–sorted CD4 T cells from HEL–CFA-primed mice were prepared as described in Fig. 2.

among all CD4 T cells. The frequency of each public clone was calculated to be in the range of 10^{-5} to 0.5×10^{-4} of total helper T cells. Considering that at least 10^7 CD4 T cells are recruited in popliteal immune LNs (23), it means that for each public rearrangement, up to 500 CD4 T cells expressing a unique CDR3 sequence are present in the two lymphoid organs 9 d after HEL priming in adjuvant, a result in agreement with previously published estimates (23). However, higher clonal frequency of Ag-specific T cells in immune LNs has been reported by others (30, 31). Several reasons could explain this discrepancy. We have analyzed single TCR β chain rearrangements expressed most likely in individual T cell clones, because at least BV8S2-J2S7$^{HEL\ 12–25}$ T cells express an AV-J public rearrangement with a conserved CDR3 motif (17). In addition, 5 to 10 times more Ag in a different type of adjuvant was used in the other studies (30, 31). This difference could easily account for the higher number of Ag-specific T cell expansions observed (30, 31).

The clonal frequency of public clones in CD4 T cells from normal spleen or CFA-primed immune LNs was detectable but below 10^{-6}. The quantification of the two public rearrangements, BV8S2-J2S7$^{HEL\ 12–25}$ and BV8S2-J1S5$^{HEL\ 107–116}$, was highly specific to HEL priming and revealed a high frequency of $1–4 \times 10^{-4}$ after HEL restimulation in vitro. As public CDR3 clones are found almost exclusively in IFN-γ$^+$ cells that represent ~20% of CD4 T cells, the frequency of public clones in IFN-γ–secreting effector T cells can be estimated at 0.2% for each public rearrangement. Indeed, by quantitating the frequency of public CDR3 among TCR β chain DNA copies on IFN-γ–sorted cells, we calculated that each public clone tested represents 0.1–0.2% of Th1 effector cells (data not shown).

The data presented here indicate that the expansion of BV8S2-J2S7$^{HEL\ 12–25}$ and BV8S2-J1S5$^{HEL\ 107–116}$ public clones in vivo required Ag administration in adjuvant but not in soluble form, and that the type of adjuvant (CFA versus IFA) can influence the polarization of Ag-specific effector T cells. In agreement with recent work (32), priming in IFA, unlike CFA, promotes CD4 T cell development toward a mixed population of IFN-γ– or IL-4–secreting cells. This is not likely to involve qualitative differences in the APCs involved in Ag presentation in vivo, as we have previously shown that dendritic cells (DCs) isolated from immune LNCs of both IFA- and CFA-primed mice are the main APC type expressing peptide–class II complexes (33). Most likely the microbial products present in CFA could act in two ways: (a) by directly inducing IL-12 production by DCs (34) and (b) by stimulating a high frequency of mycobacterium-specific T cells that would in turn activate DCs to secrete IL-12 through CD40–CD40L interactions favoring the development of IFN-γ–producing Ag–specific CD4 T cells (35). The net result of the increased production of these two cytokines would be to maintain IL-12R β2 subunit expression on T cells and their subsequent development toward a stable Th1 phenotype (36, 37). Conversely, BALB/c T cells activated in a less inflammatory environment (IFA) may rapidly lose their capacity to respond to IL-12 signaling (38), favoring the expansion of Ag-specific CD4 T cells with a Th2 phenotype. Taken together, our data demonstrate that public clones specific for dominant and subdominant HEL determinants are recruited by both types of adjuvant in vivo and differentiate toward type 1 or type 2 effector T cells. Thus, CD4 T cells expressing these two public repertoires are not precommitted to Th1 development and probably derive from naive precursors that have the capacity to develop toward either IFN-γ– or IL-4–producing cells depending on the presence of mycobacterium products in the Ag depot.

Analysis of T cell anergy in vivo has relied so far on transgenic models in which a large number of T cells bearing the same TCR are exposed to high concentrations of soluble Ag (39, 40). Although this enables the tracking of the fate of clonotype-bearing T cells, the possibility of extrapolating these data to normal T cell populations has not been addressed. Our data are the first to analyze the mechanisms involved in soluble Ag–induced unresponsiveness in normal CD4 T cells in vivo. Preadministration of the soluble protein results in a strong decrease (up to 90%) of the Th1-associated CDR3 motifs in HEL-specific Th2 cells, as detected by quantitative PCR–based analysis. The loss of public CDR3 is not due to peripheral deletion or activation-induced cell death but rather appears to result from the induction of T cell anergy. Indeed, we show that although public CDR3–bearing T cells are present in immune LNCs from soluble Ag–treated mice, they poorly proliferate after in vitro challenge. Furthermore, the coordinated reduction in the size of BV8S2-J2S7$^{HEL\ 12–25}$ and BV8S2-J1S5$^{HEL\ 107–116}$ T cell clones correlates with the decreased production of IL-2 and IFN-γ by HEL-reactive CD4 T cells (14) and is dependent on the dose of soluble Ag administered. These data support the hypothesis that systemic Ag administration can induce tolerance in normal T cell populations in vivo by a mechanism resembling clonal anergy, similar to what has been shown in a TCR-transgenic adoptive transfer system (41, 42). Although Th2 cells obtained from mice pretreated with soluble HEL can produce IL-4 in response to the 12–25 and 103–117 epitopes, the frequency of public BV8S2-J2S7$^{HEL\ 12–25}$– and BV8S2-J1S5$^{HEL\ 107–116}$–bearing cells is strongly reduced in this T cell population as compared with Th1-type cells obtained from HEL–CFA-primed mice. Regardless of the exact mechanism involved, our data show that continuous release of soluble Ag induces effective reduction of the clonal size of public CD4 T cells specific to dominant and subdominant determinant of the protein. Therefore, the strong development of Th2-like memory cells induced by this mode of Ag administration is not due to a massive immune deviation of the dominant public repertoires toward the Th2 pathway.

The unexpected feature of the data presented in this paper is the demonstration that soluble Ag administration in vivo can simultaneously induce tolerance in some precursor T cells and probably prime for Th2 development a different pool of precursor cells in susceptible mouse strains. How can this selectively be achieved? What is the mecha-

nism that controls whether, after ligand–TCR interactions, two different outcomes may ensue tolerance or activation? One possible explanation is that differences in TCR affinity between the two precursor populations could contribute to the differential effect of soluble Ag administration. Indeed, several reports have demonstrated that the development toward the Th1 or Th2 phenotype of the same TCR-transgenic T cells can be regulated by varying the signal strength of an antigenic peptide during the initial phase of T cell priming in a neutral environment (43–45). This has been achieved by using altered peptide ligands (45) or by varying the density of epitopes presented by APCs (43, 44). In our model, Th1 and soluble Ag–induced Th2 cells at least share similar specificity: they both recognize epitopes contained in the HEL sequences 12–25 and 103–117. It is therefore tempting to speculate that after soluble Ag priming, a different TCR repertoire has been selected with a lower affinity for its ligand and/or with a difference in fine epitopic specificity (46). Conversely, the public clones that are induced when Ag is administered in adjuvant are likely to harbor high-affinity TCRs. Systemic Ag presentation may favor engagement of the inhibitory receptor for B7 molecules, CTLA-4, that has been shown to result, in the absence of IL-12, in the induction of functional T cell inactivation (42). Only high-affinity T cell clones would be sensitive to such a mechanism in vivo. Alternatively, it is possible that this mode of chronic Ag administration might favor the presentation of cryptic determinants by a restricted population of APCs, such as $CD8\alpha^-$ DCs (47). Selective Ag presentation by this professional APC would direct the development of CD4 T cells specific for these cryptic epitopes toward the Th2 pathway. Whatever the origin and specificity of soluble Ag–induced memory Th2 cells, their initial phase of differentiation is highly sensitive to the presence of proinflammatory cytokines in the microenvironment, as IL-12 coadministration results in an unipolar Th1 response in this model (37).

In conclusion, our study support the hypothesis that the mode of Ag administration may determine specific repertoire selection in effector CD4 T cells in vivo. Systemic Ag presentation in the absence of proinflammatory signals, such as IL-12, results in the selective priming of a specific CD4 T cell population that develop along the Th2 pathway in genetically predisposed mouse strains. The polarization toward the Th2 phenotype induced by soluble protein administration appears to be controlled by a balance between priming of Th2 precursor populations on the one hand and induction of functional unresponsiveness in the Th1 precursor populations on the other. Furthermore, we show that the clonal size of T cells expressing the two public CDR3 motifs is effectively reduced in soluble HEL–induced, IL-4–producing $CD4^+$ T cells but not in Th2 cells that develop after Ag priming in IFA. Therefore, we provide direct evidence that the induction of Th2 cell development by chronic antigenic stimulation is not due to large scale clonal deviation, indicating that IL-4–producing CD4 T cells may arise from a different pool of precursor cells depending on the conditions of Ag exposure in vivo. Understanding the cellular and molecular basis that drive the selective expansion of Th2 cells in this model system should have important implications in our comprehension of the genetic factors that influence the differentiation of distinct Th cell subsets in predisposed hosts in vivo.

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