

Selective Development of T Helper (Th)2 Cells Induced by Continuous Administration of Low Dose Soluble Proteins to Normal and β 2-Microglobulin-deficient BALB/c Mice

By Jean-Charles Guéry, Francesca Galbiati, Simona Smiroldo, and Luciano Adorini

From Roche Milano Ricerche, I-20132 Milano, Italy

Summary

Continuous administration of soluble proteins, delivered over a 10-d period by a mini-osmotic pump implanted subcutaneously, induces a long-lasting inhibition of antigen-specific T cell proliferation in lymph node cells from BALB/c mice subsequently primed with antigen in adjuvant. The decreased T cell proliferative response is associated with a down-regulation of the T helper cell (Th)1 cytokines interleukin (IL)-2 and interferon (IFN)- γ and with a strong increase in the secretion of the Th2 cytokines IL-4 and IL-5 by antigen-specific CD4⁺ T cells. This is accompanied by predominant inhibition of antigen-specific antibody production of IgG2a and IgG2b, rather than IgG1 isotype. Interestingly, inhibition of Th1 and priming of Th2 cells is also induced in β 2-microglobulin-deficient BALB/c mice, indicating that neither CD8⁺ nor CD4⁺ NK1.1⁺ T cells, respectively, are required. The polarization in Th2 cells is stably maintained by T cell lines, all composed of CD4⁺/CD8⁻ cells expressing T cell receptor for antigen (TCR) α/β chains, derived from BALB/c mice treated with continuous antigen administration, indicating that they originate from Th2 cells fully differentiated in vivo. This polarization is induced in BALB/c mice by continuous administration of any protein antigen tested, including soluble extracts from pathogenic microorganisms. Priming of Th2 cells is dose dependent and it is optimal for low rather than high doses of protein. Blocking endogenous IL-4 in vivo inhibits expansion of antigen-specific Th2 cells, but does not restore IFN- γ production by T cells from mice treated with soluble antigen, indicating the involvement of two independent mechanisms. Consistent with this, Th2 cell development, but not inhibition of Th1 cells, depends on non-major histocompatibility complex genetic predisposition, since the Th2 response is amplified in BALB/c as compared to DBA/2, C3H, or C57BL/6 mice whereas inhibition of the Th1 response is induced by soluble protein administration in any mouse strain tested. These findings support the hypothesis that continuous release of low amounts of protein antigens from pathogenic microorganisms may polarize the immune response toward a Th2 phenotype in susceptible mouse strains.

Differential lymphokine production allows the subdivision of mouse (1), rat (2) and human (3) CD4⁺ T cells into three major subsets: T helper 1 (Th1), characterized by secretion of IL-2 and IFN- γ ; Th2, selectively producing IL-4 and IL-5; and Th0, which are not restricted in their lymphokine production. The development of Th1 and Th2 cells is primarily influenced by the cytokine milieu during the initial phase of the immune response, in which IL-12 and IL-4, respectively, play decisive roles (4, 5).

The functional significance of CD4⁺ T cell subsets has been first clearly demonstrated in vivo by studying the immune response to infectious agents (1). In these situations,

development of the appropriate Th subset is particularly important since certain pathogens are most effectively controlled by either a cellular (Th1-type) or a humoral (Th2-type) immune response (1, 6-8). The ability of pathogens to stimulate preferentially either Th1 or Th2 responses depends on several factors including genetic background of the host, type of infecting organism, stage of infection and microbial load. High and low microbial loads have been shown to induce preferentially a Th2- or Th1-type immune response, respectively, which can be protective or deleterious depending on the pathogen (8, 9). Therefore, dominance of an inappropriate T cell response can exacerbate the disease, leading to the inability to eradicate the invading microorganism. For example, Th2 responses predominate in overwhelming infections such as lepromatous

J-C. Guéry's present address is INSERM U28, Hôpital Purpan, 31059 Toulouse, France.

leprosy (10), leishmaniasis (6), and helminthiasis (7). In the latter model, egg-stimulated Th2 cells have been shown to down-regulate Th1 responses and it has been hypothesized that the physical properties of eggs, whose protein shell continuously releases antigen, could influence T cell development (7, 11).

To analyze whether the continuous release of low dose protein antigen could selectively activate Th2 cells, which may represent the initial mechanism skewing the immune response against pathogens toward a nonprotective immunity, we have used mini-osmotic pumps to continuously deliver protein antigens to BALB/c mice. After continuous administration of soluble antigen, mice were primed with protein antigens in adjuvant and T cell responses analyzed. In the present paper, we show that continuous administration to BALB/c mice of any soluble protein antigen tested induces a long-lasting, antigen-specific unresponsiveness of CD4⁺ Th1 cells and a vigorous priming of CD4⁺ Th2 cells. Interestingly, continuous administration of low rather than high doses of antigen by mini-osmotic pumps is the most efficient in inducing Th2 cells. Crude soluble protein extracts from pathogens such as *Mycobacterium tuberculosis* or soluble leishmania antigen are also very effective in selectively inducing antigen-specific Th2 cells when delivered by mini-osmotic pumps to BALB/c mice. These results suggest that continuous release of soluble proteins by infectious microorganisms represents a critical stimulus for Th2 cell induction in susceptible mouse strains.

Materials and Methods

Mice. 2- to 3-mo-old BALB/c, DBA/2, C3H, and C57BL/6 female mice were purchased from Charles River Laboratories (Calco, Italy). H-2^b mice with disrupted β 2M genes (12) were back-crossed to BALB/c mice to obtain H-2^d β 2M^{-/-} mice (13). Expression of H-2^d was determined by cell surface staining for class II molecules and by Southern blot hybridization. β 2M^{-/-} mice were identified by lack of MHC class I expression as assessed by cell surface staining using FITC-SF1-1.1 (anti-K^d; PharMingen, San Diego, CA) mAb (14). β 2M^{-/-} mice on BALB/c background were used after seven back-crosses. H-2^d β 2M^{-/-} mice, at the fourth back-cross, were a kind gift of Dr. Faith B. Wells and Louis A. Matis (NCI, Frederick, MD).

Antigens. Hen egg-white lysozyme (HEL),¹ bovine RNase, OVA, human hemoglobin, and human transferrin were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant 65-kD heat shock protein (hsp) of *M. tuberculosis* was a kind gift of Dr. Ruurd van der Zee (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Tuberculin purified protein derivative (PPD) (Statens Serum Institut, Copenhagen, Denmark) was sequentially centrifuged for 10 min at 15,000 g and then for 1 h at 100,000 g to remove aggregates and insoluble material. A soluble extract from 1.5×10^8 *Leishmania major* promastigotes was prepared following a standard freeze-thaw procedure and kindly provided by Dr. G. Alber (Hoffmann-La Roche, Basel, Switzerland). All protein antigens were

filtered on 0.22- μ m membranes before use. Peptides were synthesized by the solid-phase method on phenylacetamidomethyl-polystyrene support using side-chain protection, coupling procedures and an automated apparatus (model 430A; Applied Biosystems, Inc., Foster City, CA). Crude peptides were purified by preparative HPLC on C18 reverse-phase column. The peptides showed correct amino acid ratios upon hydrolysis in 6 N HCl and the expected molecular ions in fast atom bombardment mass spectrometry. Sequences were confirmed by gas-phase microsequencing.

Delivery by Mini-Osmotic Pumps, Immunizations, and Anti-IL-4 Treatment. Mini-osmotic pumps (Alzet 2001; Alza Corp., Palo Alto, CA) were implanted subcutaneously in the dorsal flank through a 5-mm-long cut in the skin of the lumbar region and the wound was closed by metal stitches. The mean fill volume of pumps was \sim 220 μ l and the mean pumping rate \sim 1 μ l/h, delivering continuously for \sim 10 d. 12 d after pump implantation, mice were immunized subcutaneously into the hind footpads with the indicated amount of antigen emulsified in IFA or CFA containing H37Ra mycobacteria (Difco Laboratories, Inc., Detroit, MI).

For anti-IL-4 mAb treatment, mice were injected i.p. with 1 mg/mouse of protein G-purified 11B11 mAb (HB188; American Type Culture Collection, Rockville, MD) in PBS at the day of pump implantation, and then with 0.5 mg/mouse 4 and 8 d later. Control mice were injected with the same amount of isotype control LO-DNP-2 rat mAb, or with PBS only. LO-DNP-2 is a TNP-specific rat IgG1 mAb kindly provided by Dr. H. Bazin (University of Louvain, Brussels, Belgium).

T Cell Assays. For T cell proliferation assays, popliteal lymph nodes were removed, and 4×10^5 cells/well were cultured in 96-well culture plates (Costar Corp., Cambridge, MA) in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μ g/ml gentamicin (Sigma) with the indicated antigen concentrations. Cultures were incubated for 3 d in a humidified atmosphere of 5% CO₂ in air and were pulsed 8 h before harvesting with 1 μ Ci [³H]TdR (40 Ci/nmol; Radiochemical Centre, Amersham, UK). Incorporation of [³H]TdR was measured by liquid scintillation spectrometry. For cytokine production analysis, LNC cells (LNC) were cultured at 6×10^5 cells/well as indicated above. Supernatants from replicate cultures, usually four to six wells, were collected after 20–72 h and pooled for cytokine analysis. For blocking experiments, cultures were performed in the presence of 5 μ g/ml of GK1.5 anti-CD4 (TIB 207; American Type Culture Collection), or KT1.5 anti-CD8 (15) mAb. These mAb were purified from culture supernatant by affinity chromatography on a protein G-Sepharose column.

Cytokine Assays. For IL-2 determination, supernatants were harvested after 20 h of culture. IL-2 concentration was determined using the IL-2-dependent CTL line proliferation assay and mouse rIL-2 (PharMingen) to generate standard curves. Results were expressed as picograms per milliliter mouse rIL-2. IFN- γ , IL-4, and IL-5 were quantified by two-sites sandwich ELISA. For IFN- γ , polyvinyl microtiter plates (3012; Falcon Labware, Oxnard, CA) were coated with 100 μ l of AN-18.17.24 mAb (16) in carbonate buffer. After blocking, samples (50 μ l/well) diluted in test solution (PBS containing 5% FCS and 1 g/liter phenol) were incubated together with 50 μ l peroxidase-conjugated XMG1.2 mAb (17). After overnight incubation at 4°C, bound peroxidase was detected by 3,3'-5, 5'-tetramethylbenzidine (Fluka Chemical Corp., Ronkonkoma, NY), and absorbance read at 450 nm with an automated microplate ELISA reader (MR5000; Dynatech

¹Abbreviations used in this paper: HEL, hen egg-white lysozyme; hsp, heat shock protein; LNC, lymph node cells; PPD, tuberculin purified protein derivative; TCL, T cell line.

Laboratories, Inc., Chantilly, VA). For IL-4 and IL-5 determination two-sites ELISA was performed with paired mAb all purchased from PharMingen. For capture, the mAb were BVD4-1D11 or 11B11 (anti-IL-4), and TRFK5 (anti-IL-5). Samples were titrated in test solution and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with the biotinylated mAb BVD6-24G2 (anti-IL-4) or TRFK4 (anti-IL-5) in PBS containing 0.1% Tween 20 and 1% BSA (PBSA-Tw). After washing, the bound biotinylated antibodies were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch Laboratories, Avondale, PA) diluted 1:10,000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer, pH 9.6, (100 µl/well). The reaction was stopped by adding 50 µl/well NaOH 3 N and absorbance was read at 405 nm. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines and results expressed as cytokine concentration in nanograms or picograms per milliliter. Detection limits were 15 pg/ml for IFN-γ and IL-4, 3 pg/ml for IL-5.

In addition, IL-4 production was measured by using the IL-4-dependent indicator cell line CTL44 (18). Serial dilutions of culture supernatants were incubated in 96-well plates (Costar) with 5×10^3 CTL44 cells/well for 48 h. During the last 6 h of cultures, the cells were pulsed with 1 µCi [³H]TdR and thymidine incorporation measured as above. Standard curves were generated with purified recombinant mouse IL-4 (PharMingen) or IL-4 from H28 cell culture supernatant (a kind gift of Dr. E. Severinson, University of Stockholm, Stockholm, Sweden). The sensitivity of this assay was <3 pg/ml. Similar amounts of IL-4 were revealed by ELISA and bioassay.

Anti-HEL Antibody Determination. HEL-coated polyvinyl microtiter plates (3012; Falcon Labware) were incubated with serially diluted sera in PBSA-Tw for 90 min at 37°C. Plates were then washed and incubated for 1 h at 37°C with a mixture (100 ng/ml each) of anti-IgM, -IgG1, -IgG2a, -IgG2b, -IgG3 biotin-conjugated goat anti-mouse isotype-specific antibodies (Southern Biotechnology Associates Inc., Birmingham, AL). After washing, the bound anti-isotypic antibodies were revealed by an additional 1 h incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch Laboratories) diluted 1:20,000. The plates were washed again, incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer, pH 9.6 (100 µl/well) and absorbance read at 405 nm. Standard curves were generated using pooled anti-HEL sera and results expressed as arbitrary units per milliliter (U/ml; 1 U corresponding to 50% maximum OD). Anti-HEL IgG1, IgG2a, and IgG2b isotypes were determined as described above using the corresponding biotinylated isotype-specific antibodies. Standard curves were obtained using calibrated serum pools revealed with anti-isotypic developing reagents, and results expressed as units per milliliter.

Flow Cytometry. T cell lines were incubated with optimal concentrations of FITC- or biotin-conjugated antibodies for 30 min at 4°C in PBS containing 5% FCS, 0.1% sodium azide, and 1% normal rat serum to inhibit binding to FcR. The following mAbs (PharMingen) were used: FITC-L3T4 (anti-CD4), PE-53-6.7 (anti-CD8a), biotin-145-2C11 (anti-CD3-ε), and biotin-H57-597 (anti-TCRα/β). Cells were then washed and stained with PE-streptavidin (Southern Biotechnology). Analysis was performed on a FACScan® flow cytometer (Becton Dickinson and Co., Mountain View, CA). Data were collected on 5,000–10,000

viable cells as determined by forward light scatter intensity and propidium iodide exclusion, and analyzed using Lysis II software.

Results

Administration of Soluble HEL by Mini-Osmotic Pumps Readily Inhibits T Cell Proliferation in LNC from Mice Subsequently Primed with HEL-CFA. BALB/c mice were implanted subcutaneously with mini-osmotic pumps delivering continuously for ~10 d about 0.1 nmol/h of HEL or RNase. 12 d after pump implantation mice were primed into the hind footpads with HEL emulsified in CFA, and 8 d later antigen-specific T cell proliferative responses were measured in draining popliteal lymph node cells. Results in Fig. 1 show that delivery of soluble HEL inhibits, although not completely, HEL-specific T cell proliferation, whereas the response to PPD is unaffected. We also determined the

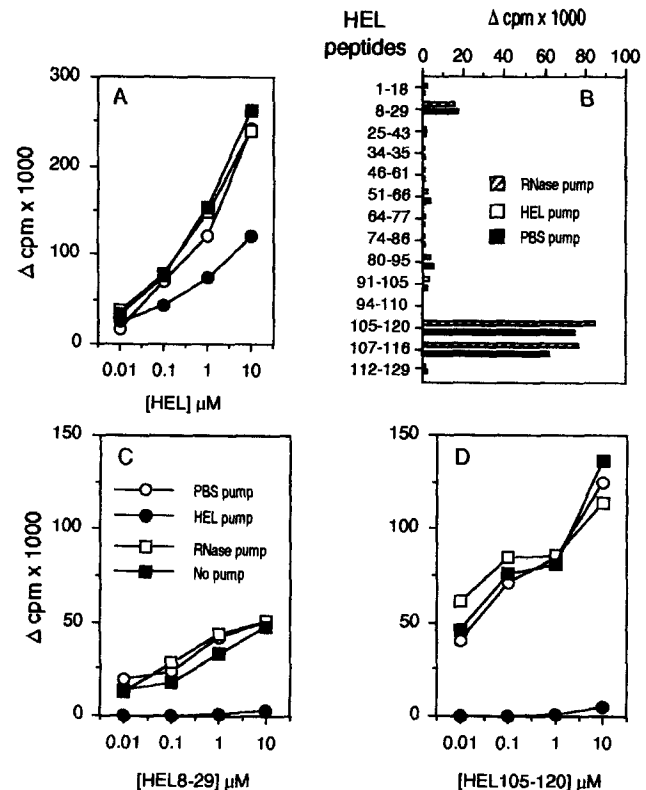


Figure 1. Administration of soluble HEL by mini-osmotic pumps inhibits T cell proliferation to dominant and subdominant HEL epitopes. BALB/c mice (three mice per group) were left untreated (■) or were implanted with a mini-osmotic pump containing PBS (○), 20 nmol RNase/mouse (□), or 20 nmol HEL/mouse (●). 12 d after pump implantation mice were primed into the hind footpads with HEL emulsified in CFA, and 8 d later pooled lymph node cells (4×10^5 cells/well) were cultured in triplicate with the indicated concentrations of HEL (A), HEL8-29 (C), HEL105-120 (D), or with 3 µM of a set of peptides spanning the entire HEL sequence (B). 3 d later cells were pulsed for 8 h with [³H]thymidine. Data are expressed as mean [³H]thymidine incorporation with background proliferation (usually between 2 and 10×10^3 cpm) subtracted (Δ cpm). Control proliferative responses to 10 µg/ml PPD were 234,068 (■), 272,797 (○), 228,034 (□), and 202,068 (●). Results are from one representative experiment out of eight performed.

T cell responses to dominant and subdominant HEL epitopes. The set of overlapping HEL peptides used defines a subdominant epitope included in the HEL sequence 12–29, and a dominant one included in HEL sequence 107–116 (Fig. 1 B). The T cell response to the former epitope is restricted by I-A^d molecules (19), whereas the latter is I-E^d-restricted (20). As shown in Fig. 1, administration of soluble HEL completely inhibits T cell responses to both subdominant and dominant HEL epitopes, as determined by lack of proliferation to peptides 8–29 and 105–120, respectively. This effect is due to the delivery of HEL and not to the pump implantation itself since no differences were observed between mice without pumps and mice implanted with pumps containing either PBS or RNase, an irrelevant protein antigen. Inhibition of HEL-specific T cell proliferation is long-lasting, as it is still present in LNC from mice implanted with pumps 6 mo before priming with HEL-CFA (data not shown).

Inhibition of Th1 and Priming of Th2 Cells by Continuous Protein Administration. The effect of continuous protein antigen administration on Th1/Th2 development in BALB/c mice was analyzed by antigen-specific production of IL-2, IFN- γ , IL-4, and IL-5 in cultures of LNC from protein-CFA-primed mice. As shown in Fig. 2, A and B, the HEL-specific production of both IL-2 and IFN- γ was profoundly inhibited in LNC from mice implanted with pumps containing 20 nmol HEL and subsequently primed with 0.3 nmol HEL-CFA, as compared to LNC from mice treated with PBS or RNase. Conversely, a very high production of IL-4 (up to 4.5 ng/ml) was found in culture supernatants of HEL-stimulated LNC from mice receiving soluble HEL, much higher than that induced in LNC from mice receiving PBS or RNase (124–322 pg/ml) (Fig. 2, C and D). Comparable results were found for IL-5. These effects are specific for the soluble antigen administered and are not restricted to HEL (Fig. 3). The increased IL-4 production in vitro by T cells from soluble antigen-treated mice is not a consequence of the inhibition of IFN- γ production, as demonstrated by the failure of XMG1.2 to enhance IL-4 production by T cells from control mice (not shown). Antigen-specific inhibition of Th1 and priming of Th2 cells is induced by any antigen tested, including RNase (Fig. 3, A–D), OVA (Fig. 3, E–H), human transferrin, and human hemoglobin (not shown). In all cases, continuous administration of soluble proteins induces inhibition of T cell proliferation associated with a strong reduction in the production of the Th1-associated cytokines (IL-2 and IFN- γ) and with a dramatic up-regulation of Th2-associated cytokines (IL-4 and IL-5).

Th2 cells favor the production of IgG1 and IgE isotypes, while IgG2a and IgG2b are associated with Th1 cells. Results in Table 1 show that in mice receiving soluble HEL delivered by mini-osmotic pumps the production of HEL-specific IgG2a/IgG2b isotypes is strongly inhibited (~100- to 1,000-fold decrease), whereas anti-HEL antibodies of IgG1 isotype are less affected (3- to 10-fold reduction), demonstrating inhibition of Th1 cell function in vivo.

T cell anergy has been shown to be a possible mecha-

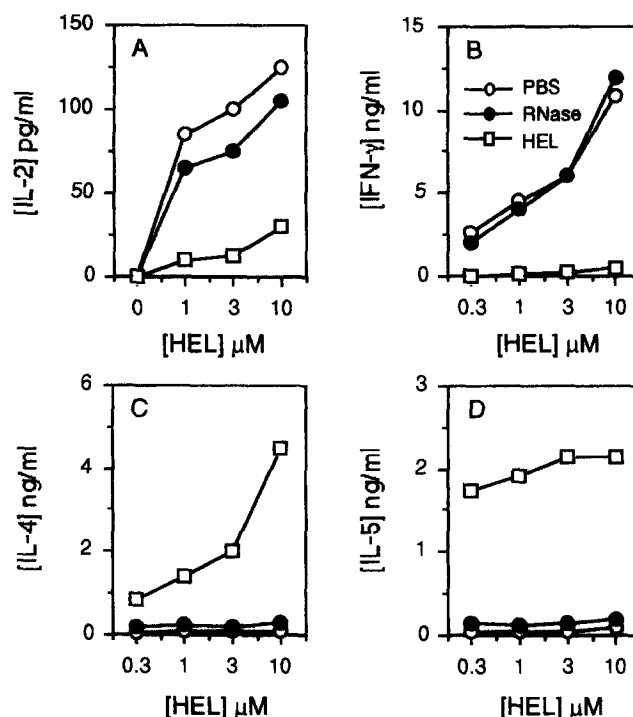


Figure 2. Inhibition of Th1 and stimulation of Th2 cells by continuous administration of soluble HEL. BALB/c mice were implanted with mini-osmotic pumps containing PBS (○), 20 nmol RNase/mouse (●), or 20 nmol HEL/mouse (□), and immunized subcutaneously 12 d later into the hind footpads with HEL in CFA (0.3 nmol/mouse). At day 21, pooled popliteal lymph node cells (6×10^5 cells/well) from two mice per group were cultured with the indicated concentrations of HEL, and supernatants assayed for cytokine production. IL-2 concentration (A) was determined in supernatants collected after 20 h of culture using a CTLL proliferation assay. IFN- γ (B), IL-4 (C), and IL-5 (D) production were assessed by two-sites sandwich ELISA in culture supernatants collected at 72 h. Results are from one representative experiment out of seven performed.

nism for induction of T cell unresponsiveness after administration of antigen in nonimmunogenic form (21). However, addition of IL-2 in vitro could restore neither the proliferative response nor IFN- γ production in LNC from mice treated with soluble antigen (data not shown).

Collectively, these results show that continuous administration by mini-osmotic pumps of any native protein tested results in an antigen-specific block of Th1 cells and in priming of Th2 cells. The inhibition of proliferative response and IFN- γ production is not reversed by IL-2 addition, suggesting that the mechanism of Th1 cell unresponsiveness does not involve anergy induction in vivo.

IL-4-producing Cells Are Fully Differentiated CD4⁺ Th2 Cells with Stable Cytokine Profile. To establish the cellular source of IL-4, we first tested the capacity of anti-CD4 mAb GK1.5 to prevent its production in vitro. As shown in Table 2, addition of GK1.5 to cultures of LNC from mice treated with soluble HEL abrogates the HEL-specific production of IL-4. No inhibition is observed in the presence of anti-CD8 mAb (KT1.5) or isotype control mAb (not shown). To analyze further the phenotype of IL-4-producing cells, T cell lines were generated from in vitro primary cultures of immune LNC from individual mice

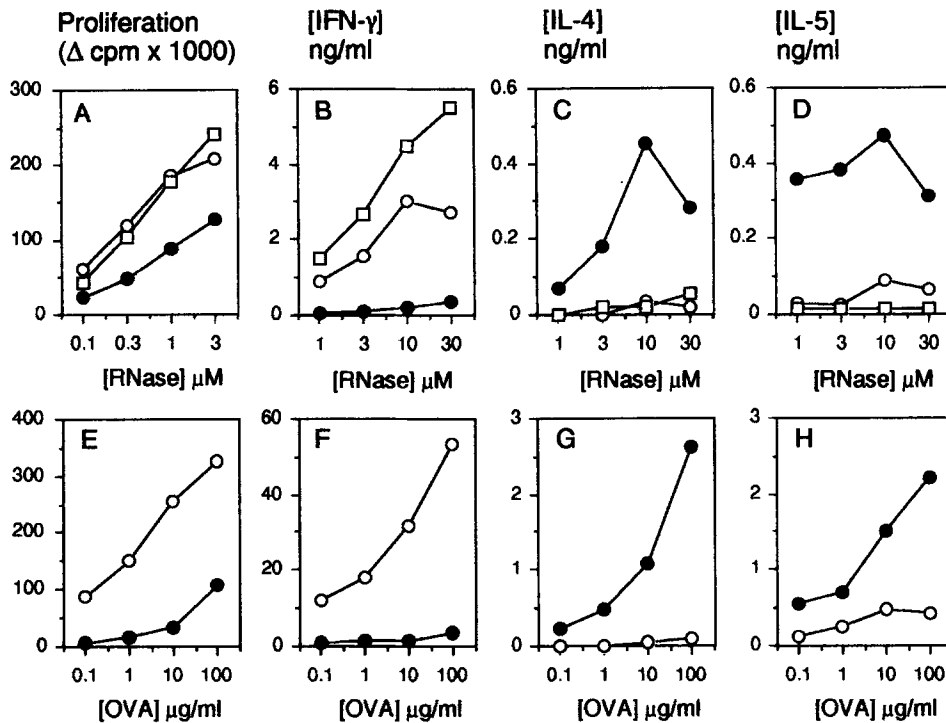


Figure 3. The polarization of T cell response from Th1 to Th2 phenotype is antigen specific and can be induced by any soluble protein tested. In A–D, BALB/c mice were implanted with mini-osmotic pumps containing PBS (○), 20 nmol RNase/mouse (●), or 20 nmol HEL/mouse (□), corresponding to ~300 µg protein. In E–F, BALB/c mice were implanted with mini-osmotic pumps containing PBS (○), or 400 µg OVA/mouse (●). 12 d after pump implantation, mice were immunized with 1 nmol RNase (A–D) or 15 µg OVA (E–F) in CFA. Immune LNC pooled from two mice per group were cultured at 4×10^5 cells/well (A and E) or 6×10^5 cells/well (B–D, F–H) with the indicated antigen concentrations. T cell proliferation (A and E) was measured as in Fig. 1. Antigen-driven IFN- γ (B and F), IL-4 (C and G) and IL-5 (D and H) production were assayed in 72 h culture supernatants by ELISA as in Fig. 2. Results are from two representative experiments out of six performed.

treated with continuous administration of PBS or of soluble HEL. After the initial 3-d culture period, LNC from all individual mice tested showed the expected cytokine profile (Fig. 4, A and B). Cells were then expanded in complete medium without addition of exogenous cytokines and restimulated after 7 d with mitomycin C-treated BALB/c spleen cells as APC and HEL. As shown in Fig. 4, C and D, cells from control mice produce high levels of IFN- γ and low amounts of IL-4. Reciprocally, the opposite cytokine profile is secreted by cells from mice treated with soluble HEL. After another 9 d in culture, lines were restimulated

with APC and antigen for 4 d, then expanded in medium containing IL-2 for 5 d before being tested for HEL-specific cytokine production. Results in Fig. 5 A show the dose-response curves of IFN- γ and IL-4 production for four representative T cell lines upon restimulation with HEL and APC. T cell lines (TCL) from control mice secrete intermediate to high levels of IFN- γ (2–80 ng/ml). IL-4 was produced by TCL Nos. 11, but was low or absent in culture supernatants from TCL Nos. 12 and 13, respectively. Conversely, IFN- γ production is undetectable in any TCL derived from mice treated with soluble HEL, whereas they all produce high levels (up to 3.5 ng/ml) of IL-4. All these T cell lines were composed of CD4⁺/

Table 1. Anti-HEL Antibody Isotypes in BALB/c Mice Treated with Soluble HEL

Soluble Ag	Anti-HEL antibody concentration			
	IgG	IgG1	IgG2a	IgG2b
RNase		<i>U/ml</i>		
		45,966	29,900	2,952
		18,500	16,090	409
HEL		26,173	23,723	339
		1,226	1,412	<1
		1,802	2,103	<1
		1,392	1,611	<1

BALB/c mice were implanted with pumps containing RNase or HEL (20 nmol/pump), and 12 d later immunized with HEL-CFA (3 nmole/mouse). After 9 d, mice were bled and sera analyzed for HEL-specific antibodies by ELISA. Results are expressed as serum antibody concentration in units per milliliter in individual mice. Results are from one representative experiment out of three performed.

Table 2. Antigen-induced IL-4 Production Is Blocked by anti-CD4 mAb

HEL	IL-4		
	no mAb	Anti-CD4	Anti-CD8
<i>µM</i>		<i>pg/ml</i>	
10	660	<15	540
1	381	<15	382
0	<15	<15	<15

BALB/c mice implanted with pump containing 20 nmol HEL/mouse were subsequently immunized with HEL-CFA (1 nmol/mouse). 9 d later, LNC were cultured with the indicated concentrations of HEL, in the presence of 5 µg/ml purified anti-CD4 (GK1.5) or anti-CD8 (KT1.5) mAb. IL-4 production was assessed in 72-h culture supernatants by ELISA as in Fig. 3. Results are from one representative experiment out of three performed.

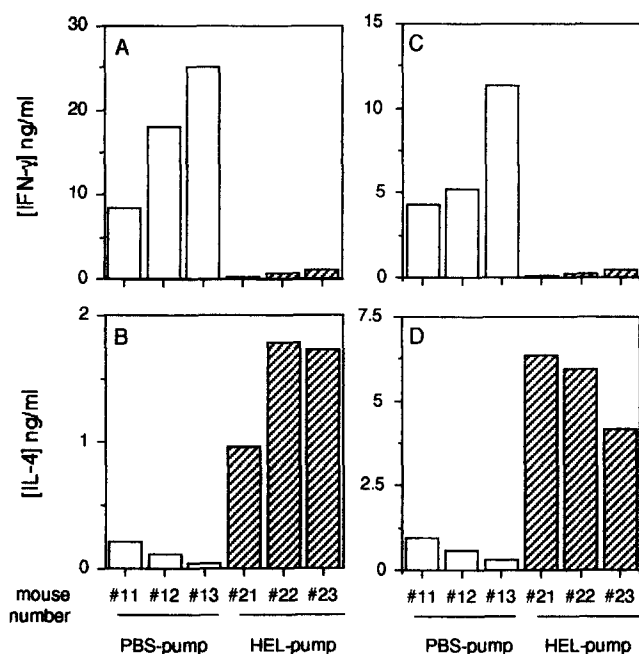


Figure 4. Generation of antigen-specific Th1 and Th2 cell lines. Immune LNC were obtained from individual BALB/c mice (three mice per group) implanted with pumps containing PBS or 20 nmol HEL/mouse, 12 d before immunization with HEL in CFA (0.3 nmol/mouse) and cultured at 6×10^5 cells/well in the presence of 10 μ M HEL. Culture supernatants from triplicate cultures were harvested at 72 h and tested for IFN- γ (A) and IL-4 (B) production by ELISA. In C and D, cells from the primary culture were expanded in complete medium and then tested at day 7 for cytokine production. They were cultured at 5×10^4 cells/well in the presence of mitomycin C-treated BALB/c spleen cells (3×10^5 /well) and 10 μ M HEL. Culture supernatants from triplicate cultures were harvested at 48 h and tested for IFN- γ (C) and IL-4 (D) production by ELISA.

CD8⁻ cells expressing similar amounts of TCR α/β and CD3 molecules as detected by FACS[®] analysis. A representative double staining with anti-CD4 and anti-TCR α/β mAb is shown for TCL Nos. 13 and 23 in Fig. 5 B.

Low Dose Soluble Proteins, including Extracts from Pathogens, Induce Antigen-specific Default Th2 Cell Development in BALB/c Mice. So far, experiments were performed with relatively high doses of protein antigen loaded in pumps (~ 300 μ g protein/pump) delivering ~ 30 μ g protein/d. Therefore, we next tested the effect of low dose soluble antigen administration on the polarization of Th subsets in BALB/c mice. As shown in Fig. 6, continuous administration of 400–4 μ g OVA per mouse results in a complete inhibition of IFN- γ production after restimulation of immune LNC with OVA in vitro. Conversely, induction of IL-4-secreting cells is dose dependent and it is maximum for low rather than high doses of protein. Administration of 4 μ g OVA/pump, corresponding to a protein delivery of 0.4 μ g/d, is still able to induce high levels of Th2-associated cytokines IL-4 and IL-5. As shown in Fig. 7, similar results are obtained with other protein antigens. In Fig. 7, A and B, HEL-specific production of IFN- γ was blocked by any dose of soluble HEL administered, whereas maximum induction of antigen-specific IL-4 production was observed

at the lowest HEL dose tested (0.8 nmol = 10 μ g/pump) corresponding to an HEL delivery of 1 μ g/d.

To address the relevance of these observations in the pathogenesis of chronic infectious diseases, we tested the ability of pathogen-derived crude soluble extracts administered by mini-osmotic pumps to induce Th2 development in BALB/c mice. PPD represents a model system in this respect, as it is derived from pathogenic microorganisms and T cell responses can be easily induced by immunizing mice with Freund's adjuvant containing heat-killed *M. tuberculosis*. Administration of soluble PPD by mini-osmotic pumps readily induces PPD-specific IL-4 production, highest at the lowest dose of PPD administered, while IFN- γ production is inhibited by any dose tested (Fig. 7, C and D). Continuous administration of soluble recombinant 65-kD hsp65, a major antigen of *M. tuberculosis* (22), to BALB/c mice also prevents IFN- γ production at both doses tested (100 and 10 μ g/pump), while IL-4 production is inversely related to the dose of soluble PPD administered, the lowest dose being the most efficient (Fig. 7, E and F). Similar results were obtained with soluble leishmania antigen (not shown).

Taken together, these data show that continuous administration of proteins at low doses efficiently induces default Th2 development in BALB/c mice. The inhibition of Th1 cell induction does not appear to be dependent on the magnitude of Th2 cell priming. Interestingly, a strong Th2 response is induced even by soluble PPD, an antigen mixture known to preferentially recall Th1-type cells (3), and by one of its purified components, hsp65.

Blocking Endogenous IL-4 Prevents Th2 Cells Expansion but Do Not Restore Th1 Cell Unresponsiveness. To address whether IL-4 is required in vivo for the expansion of antigen-specific Th2 cells, we examined the polarization of the T cell response in mice pretreated with soluble HEL in the presence of 11B11 mAb and then immunized with HEL-CFA. Results in Fig. 8 show that T cells from soluble antigen-treated mice subsequently injected with a control rat mAb (LO-DNP-2) have a reduced capacity to secrete IFN- γ , while IL-4 production is up-regulated upon restimulation. In contrast, administration of anti-IL-4 11B11 mAb at the time of soluble HEL delivery results in almost complete inhibition of IL-4 production by antigen-specific T cells (Fig. 8 B) and is not associated with a restoration of IFN- γ secretion (Fig. 8 A). Similar results were obtained using OVA as antigen (not shown). Taken together, these data demonstrate that blocking endogenous IL-4 inhibits completely the development of IL-4 producing Th2 cells, but does not reverse the block in IFN- γ -producing Th1 cells.

Selective Induction of Th2 Cells Depends on Non-MHC-linked Genetic Polymorphism. To address whether induction of Th2 cells in our model system is predictive of disease outcome after *L. major* infection, we compared the effect of continuous protein administration on Th2 development in the susceptible mouse strain BALB/c and in the resistant strains C3H, C57BL/6, and DBA/2. PPD was chosen because of its capacity to induce comparable T cell responses in different mouse strains. Results in Fig. 9 show that ad-

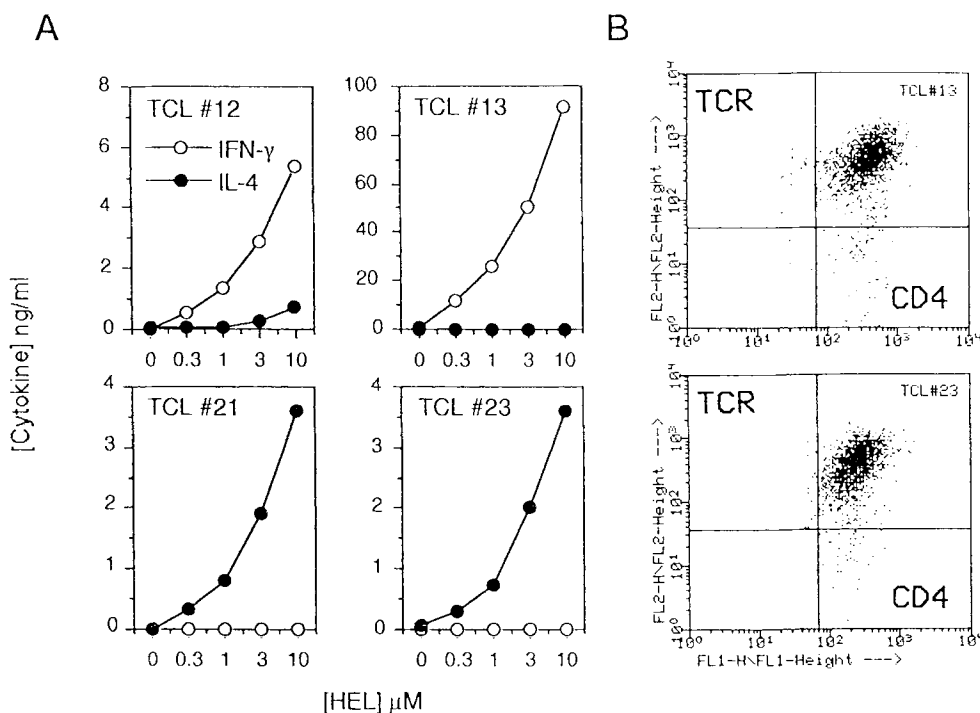


Figure 5. Th1 and Th2 cell lines are composed of TCR α/β , CD4⁺ Th2 cells. T cell lines generated as described in Fig. 6 were rested for 9 d before restimulation with APC and HEL. After another 4-d period, T cell lines were expanded with medium containing human rIL-2 (5 ng/ml) before being tested 5 d later for antigen-specific production of IFN- γ and IL-4 as in Fig. 4. Representative T cell lines from control (TCL#12 and #13) and HEL-treated (TCL#21 and #23) mice are shown in A. B shows cell surface staining of TCL Nos. 13 and 23 using anti-TCR-PE and anti-CD4-FITC mAb, performed after another round of rest and restimulation.

ministration of soluble PPD, for any dose tested, results in decreased PPD-specific IFN- γ production by LNC from both CFA-immunized BALB/c and C3H mice. Interestingly, this treatment leads to a clear-cut dose-dependent up-regulation of the production of the Th2-associated cytokine IL-4 in BALB/c mice, whereas this effect is much less evident in T cells from C3H mice. As shown in Fig. 10, similar results are obtained in the other strain tested. Again, a strong Th2 response is seen only in the susceptible BALB/c mice, as compared to DBA/2 and C57BL/6, whereas IFN- γ production is inhibited in all strains. Since BALB/c and DBA/2 share the same H-2 haplotype, selective induction of Th2 cells by soluble antigen administration appears to depend on non-MHC-linked genetic polymorphism.

Induction of Th1 Unresponsiveness and Th2 Priming Do Not Require CD8⁺ or CD4⁺ NK1.1⁺ T Cells, Respectively. CD8⁺ T cells have been shown to suppress T cell prolifer-

ation in oral tolerance (23), while CD4⁺ NK1.1⁺ cells are thought to produce the initial burst of IL-4 driving the differentiation of antigen-specific CD4⁺ T cells into the Th2 phenotype (24). Mice deficient for the $\beta 2M$ genes can be used to test the relevance of both cell types. These mice do not express MHC class I molecules, which results in the lack of positive selection of CD8⁺ T cells (25) and of the small population of CD4⁺ T cells expressing the NK1.1 marker (26, 27). $\beta 2M$ -deficient BALB/c mice (seventh back-cross generation) were implanted with pumps containing soluble HEL 12 d before HEL priming. Like normal BALB/c mice, $\beta 2M^{-/-}$ mice mount a strong proliferative response to HEL, associated with the production of IL-2 (not shown) and IFN- γ (Fig. 11 A), while the production of IL-4 (Fig. 11 B) and IL-5 (not shown) is relatively low. As shown for IFN- γ and IL-4 (Fig. 11, A and B), continuous administration of soluble HEL before immunization results in a complete reversion of the cytokine

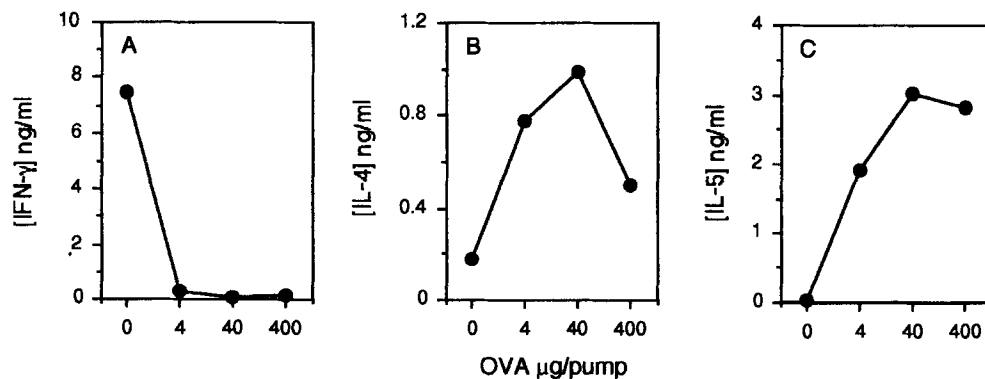


Figure 6. Continuous administration of low dose soluble protein can induce Th2 cell development. BALB/c mice were implanted with mini-osmotic pumps containing the indicated amounts of soluble OVA/mouse, 12 d before immunization with 50 μ g OVA in CFA. Pooled LNC from two mice per group were cultured at 6×10^5 cells/well in the presence of 30 μ g/ml OVA, and IFN- γ (A), IL-4 (B), and IL-5 (C) production were measured by ELISA in culture supernatants at 72 h as in Fig. 2. Results are from one representative experiment out of three performed.

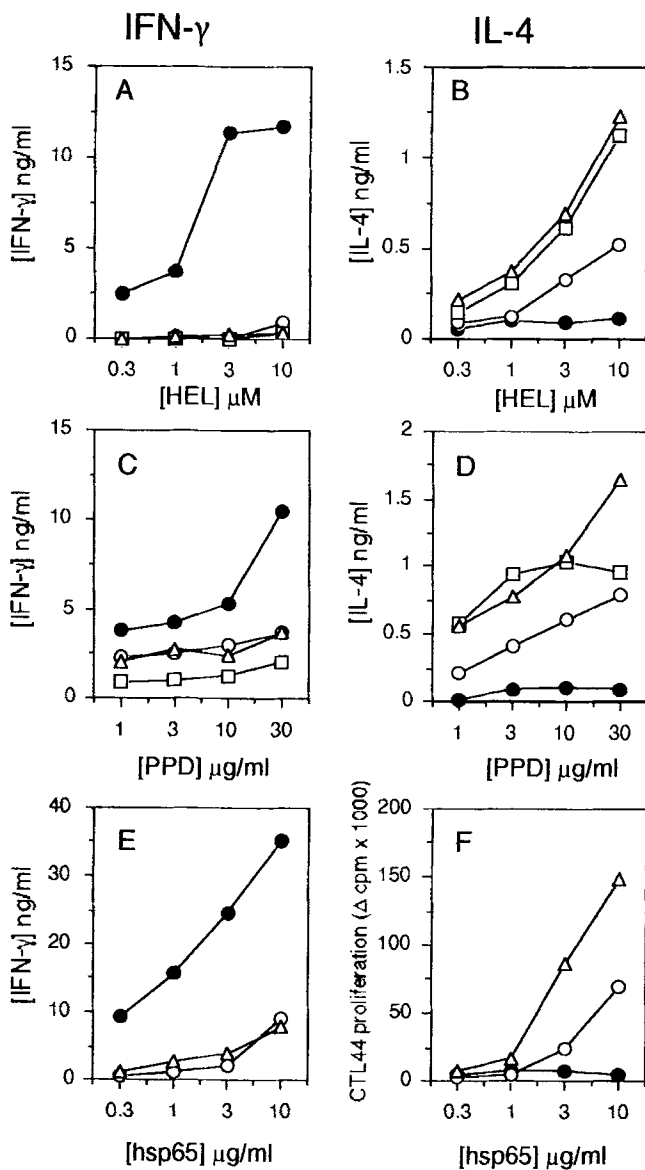


Figure 7. Continuous administration of soluble extracts from mycobacterium (PPD) or one of its purified components (hsp65) can readily induce Th2 cell response. In *A* and *B*, BALB/c mice were implanted with mini-osmotic pumps containing 20 (○), 4 (□), 0.8 (△) nmol soluble HEL/mouse, corresponding to 300, 60, and 10 μg/mouse, or PBS only (●). In *C* and *D*, mice were implanted with pumps containing PBS only (●), or 400 (○), 80 (□), 16 (△) μg soluble PPD/mouse. In *E* and *F*, mice were implanted with pumps containing PBS only (●) or 100 (○) and 10 (△) μg soluble hsp65/mouse. 12 d after pump implantation, mice were immunized with the following antigen in CFA: 1 nmol HEL (*A* and *B*), CFA only (*C* and *D*), or 10 μg hsp65 (*E* and *F*). 9 d later, pooled LNC from two mice per group were cultured at 6×10^5 cells/well in the presence of the indicated antigens. IFN- γ (*A*, *C*, and *E*) and IL-4 (*B* and *D*) production were measured by ELISA in culture supernatants at 72 h as in Fig. 2. In *F*, IL-4 production was assessed by proliferation of the IL-4-dependent CTL44 cell line. Data are expressed as [3 H]thymidine incorporation with background proliferation in the absence of antigen (7,228–8,489 cpm) subtracted. Results are from three representative experiments out of six performed.

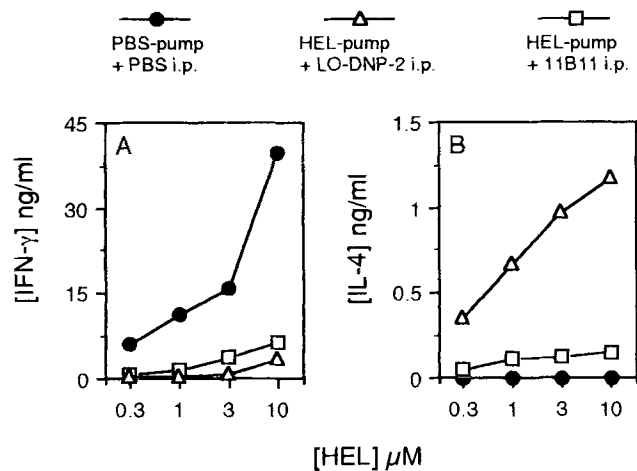


Figure 8. Blocking endogenous IL-4 *in vivo* inhibits Th2 cell induction but does not restore IFN- γ production. BALB/c mice (two mice per group) were implanted with pumps containing either PBS (●) or 1 nmol HEL/mouse (△, □). At days 0, 4, and 8 of the experiment, animals were injected intraperitoneally with PBS (●), anti-IL-4 11B11 mAb (□), or control LO-DNP-2 mAb (△). At day 12, mice were immunized with HEL in CFA (3 nmol/mouse) and the polarization of the T cell response tested 9 d later as in Fig. 2. Data are from one representative experiment out of three performed.

pattern produced by $\beta 2M^{-/-}$ immune LNC restimulated with antigen *in vitro*, comparable to that observed in normal BALB/c mice. Similar data were obtained using as antigen PPD (Fig. 11, *C* and *D*) and OVA (data not shown).

Therefore, these results demonstrate that inhibition of Th1 and priming of Th2 cells by continuous administration of either purified proteins or crude soluble protein extracts from pathogens does not require expression of MHC class I molecules, ruling out a role for CD8 $^+$ and CD4 $^+$ NK1.1 $^+$ T cells.

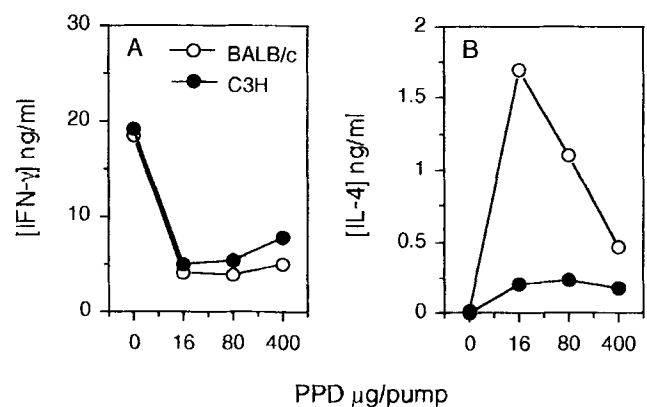


Figure 9. Genetic predisposition determines Th2 cell development in mice treated with continuous administration of soluble antigen. BALB/c or C3H mice were implanted with mini-osmotic pumps containing the indicated amount of soluble PPD in PBS, 2 wk before immunization with CFA only. Immune LNC, pooled from two mice per group, were restimulated *in vitro* with 30 μg/ml PPD. IFN- γ (*A*) and IL-4 (*B*) production was assessed in 72-h culture supernatants by ELISA. Data are from one representative experiment out of three performed.

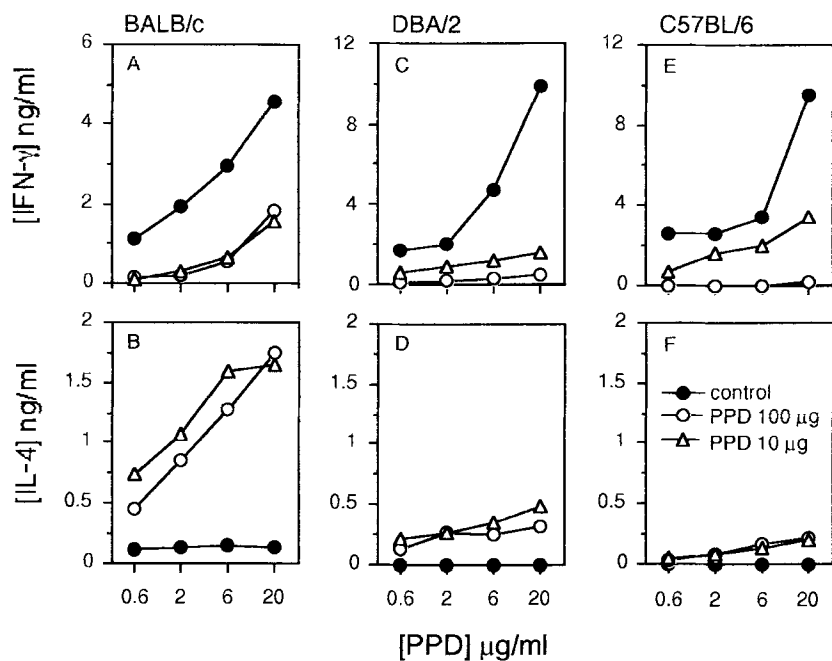


Figure 10. Non-MHC genes amplify the Th2 cell response in BALB/c mice. BALB/c (A and B), DBA/2 (C and D), or C57BL/6 (E and F) mice were implanted with mini-osmotic pumps containing 100 (\circ) or 10 (Δ) μg soluble PPD/mouse, or left untreated (\bullet). 3 wk later, they were immunized with 10 μg PPD in IFA. Pooled LNC from two mice per group were cultured in the presence of the indicated concentrations of PPD. IFN- γ (A, C, and E) and IL-4 (B, D, and F) production were measured in culture supernatants at 72 h as in Fig. 2. Data are from one representative experiment out of three performed.

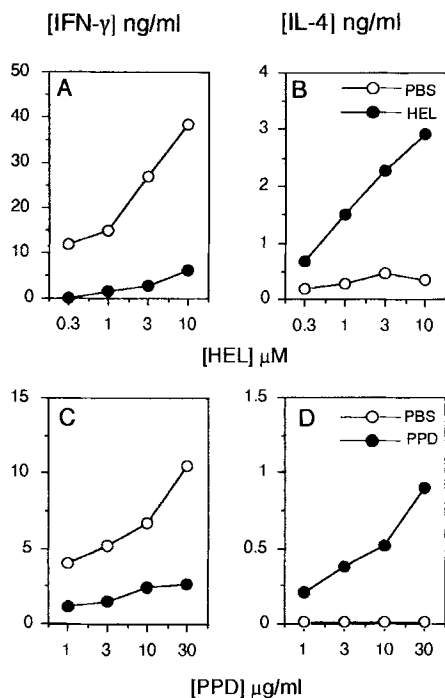


Figure 11. Inhibition of Th1 and priming of Th2 cells do not require $\text{CD}8^+$ or $\text{CD}4^+\text{NK}1.1^+$ T cells. In A and B, BALB/c mice (two mice per group) with disrupted $\beta 2\text{M}$ genes were implanted with mini-osmotic pumps containing PBS only (\circ) or 20 nmol HEL/mouse (\bullet). In C and D, $\beta 2\text{M}$ -deficient mice were implanted with pumps containing PBS (\circ) or 30 μg soluble PPD/mouse (\bullet). At day 12 mice were immunized with 1 nmol HEL in CFA (A and B) or CFA only (C and D). 9 d later, T cell responses to the indicated antigens were assessed. IFN- γ (A and C) and IL-4 (B and D) production were measured in culture supernatants at 72 h as in Fig. 2. Results are from two representative experiments out of five performed.

Discussion

Since the initial observations by Parish and Liew (28) on the dichotomy of the immune response and the demonstration by Mosmann et al. of Th1 and Th2 cells, defined by their different lymphokine production pattern (1), it has become increasingly clear that immune responses are often dominated by either type of IFN- γ or IL-4-producing $\text{CD}4^+$ T cells. For instance, protective immunity is associated with a Th1 rather than a Th2 pattern of cytokine production in lesions caused by *M. leprae* (10). Similarly, in most mouse strains, infection by the parasite *L. major* leads to a dominant Th1-type immune response and results in elimination of the pathogen. In contrast, BALB/c mice mount a predominantly humoral immune response, associated with induction of Th2-type lymphocytes producing IL-4 and IL-10, which is ineffective in controlling parasite growth (6, 29). This is not due to an intrinsic incapacity of leishmania-specific $\text{CD}4^+$ T cells in these mice to differentiate into Th1-type lymphocytes, because a protective Th1-type immune response could be obtained by blocking endogenous IL-4 (30) or by providing exogenous IL-12 (31). Actually, *L. major*-infected BALB/c mice contain antigen-specific Th1 cells whose functions are actively suppressed by the dominant Th2 population (32), but the initial mechanisms leading to the preferential activation of these Th2 cells are still unclear.

As a possible explanation, we hypothesized that infection by microorganisms would result in a continuous release of pathogen-derived proteins, skewing the immune response in BALB/c mice toward a Th2 phenotype. To test this hypothesis, we established an experimental model using mini-osmotic pumps as antigen delivery system. As shown in the present paper for defined protein antigens and crude solu-

ble extracts from pathogenic microorganisms, the continuous release of soluble proteins leads, in BALB/c mice, to a strong inhibition of antigen-specific Th1 cells and to the priming of Th2-type helper T cells. Selective priming of Th2 cells is associated with reduction of protein-specific T cell proliferation, in agreement with the observation that IL-4 production can be observed in the absence of T cell proliferation (33).

These results have implications for the pathogenesis of infectious diseases. First, the amount of IL-4 produced in our model (up to 5 ng/ml) is comparable to that secreted by immune LNC from *L. major*-infected mice (32). This is achieved in mice primed with protein emulsified in CFA which favors induction of Th1-type cells; adjuvants such as IFA or alum might promote even higher Th2 cell induction. Second, antigen-specific IL-4-producing Th2 cell lines could be easily obtained in the absence of exogenously added cytokines. These cell lines maintained their phenotype upon several weeks in culture showing that they were derived from Th2 cells fully differentiated in vivo. Third, priming of antigen-specific Th2 cells could be induced by any soluble antigen tested, including crude soluble extracts from pathogens such as *M. tuberculosis* or soluble leishmania antigen. Finally, Th2 priming, unlike Th1 inhibition, appears to depend on genetic predisposition, and it is not observed in the *L. major*-resistant C3H and C57BL/6 mouse strains.

Controversial results have been obtained by studying the effect of soluble antigen administration on Th1/Th2 cell development (34–37). In some instances, antigen-specific unresponsiveness induced by pretreatment with aqueous antigen selectively tolerized Th1 but not Th2 cells (34, 35), while in other reports both Th subsets were inhibited (36, 37). A common feature of all these studies is the modest or hardly detectable amount of IL-4 produced (38, 39). Aside from the fact that high dose soluble hapten–protein conjugates (35) or high molecular weight proteins such as deaggregated human γ -globulin (34, 36) or KLH (35) were used, none of these studies was carried out in BALB/c mice. Therefore, the lack of evidence for a strong priming of Th2 cells in these models could be easily explained by the mouse strains used, the high doses of antigen injected (>1 mg) and the mode of soluble antigen administration (single or repeated bolus injection). In fact, we observed an inverse correlation between the dose of antigen administered by mini-osmotic pumps and the level of IL-4 produced in vitro by immune LNC. Doses of 400–4 μ g protein/pump induced complete blockade of Th1 responses, but at lower doses reappearance of IFN- γ -producing cells was observed. Maximal IL-4 production was reached at protein doses between 100 and 4 μ g/pump, but a significant IL-4 production was still induced by doses <1 μ g/pump, corresponding to 100 ng/d (Guéry, J.-C., et al., unpublished observations). Due to the reappearance of IFN- γ -producing cells, which have been shown to inhibit Th2 cell proliferation in vitro (40), it is likely that induction of Th2 responses induced by very low doses of soluble protein is underestimated. The amount of pathogen-derived pro-

teins in the circulation is likely to be correlated with the type and load of infecting microorganisms. Only for high level of infection the threshold concentration necessary for Th2-priming would be reached. Below this threshold no Th2 development would occur, consistent with the observation that low level infection preferentially induces protective immunity in BALB/c mice (9).

Priming of Th2 cells in our model is dependent on the presence of endogenous IL-4, since administration of 11B11 mAb at the time of continuous antigen administration prevents Th2 cell expansion. Thus, the polarization of the response towards a Th2 phenotype in BALB/c mice could depend on the initial activation of IL-4-producing cells such as the CD4⁺NK1.1⁺ population (24). This is unlikely since similar results were obtained in β 2M-deficient BALB/c mice, lacking this cell population whose development depends on MHC class I expression (26, 27). The IL-4 required for Th2 priming in vivo rather appears to be produced by conventional antigen-specific class II-restricted CD4⁺ T cells. Th2 cells might develop after the initial activation of a rare population expressing particular phenotypic markers (e.g., LECAM-1^{dim}) and able to secrete significant amounts of IL-4 upon primary stimulation in vitro (41–43).

The APC type presenting soluble antigen may also play a role in selectively priming Th2 cells. Induction of experimental allergic encephalomyelitis, a Th1-mediated autoimmune disease, has been prevented by targeting the autoantigen to B cells (44), and this prevention has been recently found to be associated with the priming of antigen-specific Th2 cells (45). Therefore, antigen presentation by APC lacking appropriate costimulatory molecules such as B cells during a noninflammatory immune response could inhibit Th1 and induce Th2 development. IgD targeting on the B cell surface by bivalent antibody fragments results in B cell activation (46), which might be a prerequisite for priming of Th2 cells (45). However, Th2 development in our model is observed in the absence of any exogenous manipulation, e.g., administration of cytokines or anticytokine antibodies, or antigen targeting to any particular APC, and rather represents the default pathway of T helper cell differentiation in a particular mouse strain.

The preferential differentiation of antigen-specific CD4⁺ T cells into IL-4-producing cells might depend on the non-MHC-linked genetic predisposition of BALB/c mice to develop Th2 cells (47). Evidence for this is provided by the observation that continuous administration of soluble protein induces a strong Th2 response in BALB/c but not in DBA/2, C3H, or C57BL/6 mice, whereas the Th1 response is inhibited in any mouse strain tested. These data offer an explanation for the previous conflicting results describing selective inhibition of Th1 but not Th2 responses (34, 35) or inhibition of both Th subsets (36, 37) by pretreatment with soluble antigen. The commitment to Th2 cell differentiation in BALB/c mice may result from failure of IL-12 to induce phosphorylation of Jak2, Stat3, and Stat4 in Th2 cells, leading to extinction of IL-12 signaling (48).

In oral tolerance, induction of antigen-specific CD8⁺ suppressor T cells was found to be an important mechanism for T cell unresponsiveness (23, 49). This is not the case after continuous subcutaneous administration of antigen, since a similar inhibition of Th1 cells was obtained in normal BALB/c mice and in mice lacking CD8⁺ T cells. While induction of CD8⁺ suppressor T cells can be definitively ruled out, immunoregulation by antigen-specific Th2 cells could play a role in our model. Upon immunization with antigen in adjuvant, the primed Th2 population could then be preferentially expanded and influence further differentiation of naive Th cells in situ by the secretion of IL-4 and IL-10 (50).

Based on our present results, it appears that the polarization of the immune response towards a Th2 phenotype is mediated by a dual mechanism: induction of Th1 cell unresponsiveness and default development of Th2 cells. The different antigen dose dependence of these two events in

BALB/c mice suggests that, after continuous administration of soluble proteins, these two pathways may develop independently. This is further supported by two sets of experiments. First, blocking endogenous IL-4 during continuous administration of soluble antigen to BALB/c mice inhibits Th2 cell development, but does not reverse the inhibition of Th1 cells. Second, the inhibition of the Th1 response is unrelated to the development of a strong Th2 response, as indicated by data obtained in different mouse strains.

In conclusion, we have demonstrated that continuous administration of soluble antigen to BALB/c mice induces a polarization of the immune response towards a Th2 phenotype. This finding might have implications for understanding the pathogenesis of chronic infectious diseases mediated by organisms as diverse as helminths, protozoa, bacteria, and retroviruses, where infection is frequently accompanied by down-regulation of host defense and increased production of Th2-type cytokines (51).

J.-C. Guéry is supported by a Human Capital and Mobility fellowship from the European Community (ERBCHRXCT920008).

Address correspondence to L. Adorini, Roche Milano Ricerche, Via Olgettina 58, I-20132 Milano, Italy.

Received for publication 26 May 1995 and in revised form 18 September 1995.

References

1. Mosmann, T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145-173.
2. Fowell, D., A.J. McKnight, F. Powrie, R. Dyke, and D. Mason. 1991. Subsets of CD4⁺ T cells and their roles in the induction and prevention of autoimmunity. *Immunol. Rev.* 123: 37-64.
3. Del Prete, G.F., M. De Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani. 1991. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* 88:346-350.
4. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. *Immunol. Today.* 14:335-338.
5. Paul, W.E., and R.A. Seder. 1994. Lymphocytes responses and cytokines. *Cell.* 76:241-251.
6. Heinzl, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon γ or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169:59-72.
7. Pearce, E.J., P. Caspar, J.M. Grzych, F.A. Lewis, and A. Sher. 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J. Exp. Med.* 173:159-166.
8. Bancroft, A.J., K.J. Else, and R.K. Grencis. 1994. Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response. *Eur. J. Immunol.* 24:3113-3118.
9. Bretsher, P.A., G. Wei, J.N. Menon, and H. Bielefeldt-Ohmann. 1992. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*. *Science (Wash. DC)*. 257:539-542.
10. Yamamura, M., K. Uyemura, R.J. Deans, K. Weinberg, T.H. Rea, B.R. Bloom, and R.L. Modlin. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science (Wash. DC)*. 254:277-279.
11. Grzych, J.M., E. Pearce, A. Cheever, Z.A. Caulada, P. Caspar, S. Heiny, F. Lewis, and A. Sher. 1991. Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. *J. Immunol.* 146:1322-1327.
12. Zijlstra, M., E. Li, F. Sajjadi, S. Subramani, and R. Jaenisch. 1989. Germ-line transmission of a disrupted β 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature (Lond.)*. 342:435-438.
13. Wells, F.B., S.-J. Gahm, S.M. Hedrick, J.A. Bluestone, A. Dent, and L.A. Matis. 1991. Requirement for positive selection of $\gamma\delta$ receptor-bearing T cells. *Science (Wash. DC)*. 253: 903-905.
14. Guéry, J.C., and L. Adorini. 1995. Dendritic cells are the most efficient in presenting endogenous naturally processed self-epitopes to class II-restricted T cells. *J. Immunol.* 154: 536-544.
15. Tomonari, K., and S. Spencer. 1990. Epitope specific binding of CD8 regulates activation of T cells and induction of cytotoxicity. *Int. Immunol.* 2:1189.
16. Prat, M., G. Gribaudo, P.M. Comoglio, G. Cavallo, and S. Landolfo. 1984. Monoclonal antibodies against murine γ interferon. *Proc. Natl. Acad. Sci. USA.* 81:4515-4519.

17. Cherwinski, H.M., J.H. Shumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166:1229–1244.
18. Favre, N., and P. Erb. 1993. Use of the CTL44 cell line, a derivative of CTL/L cells, to identify and quantify mouse interleukin-4 by bioassay. *J. Immunol. Methods.* 164:213–220.
19. Gammon, G., H.M. Geysen, R.J. Apple, E. Pickett, M. Palmer, A. Ametani, and E.E. Sercarz. 1991. T cell determinant structure: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes. *J. Exp. Med.* 173:609–617.
20. Adorini, L., A. Sette, S. Buus, H.M. Grey, M. Darsley, P.V. Lehmann, G. Doria, Z.A. Nagy, and E. Apella. 1988. Interaction of an immunodominant epitope with Ia molecules in T cell activation. *Proc. Natl. Acad. Sci. USA.* 85:5181–5185.
21. Gaur, A., B. Wiers, A. Liu, J. Rothbard, and C.G. Fathman. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science (Wash. DC).* 258:1491–1494.
22. Kaufmann, S.H.E., U. Vöth, J.E.R. Thole, J.D.A. Van Embden, and F. Emmrich. 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur. J. Immunol.* 17:351–357.
23. Lider, O., L.M.B. Santos, C.S.Y. Lee, D.J. Higgins, and H.L. Weiner. 1989. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein II. Suppression of disease and in vitro response is mediated by CD8⁺ T lymphocytes. *J. Immunol.* 142:748–752.
24. Yoshimoto, T., and E.P. Paul. 1994. CD4^{pos}, NK1.1^{pos} T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179:1285–1295.
25. Zijlstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenisch. 1990. β 2-microglobulin deficient mice lack CD4⁻8⁺ cytolytic T cells. *Nature (Lond.).* 344:742–746.
26. Bendelac, A., N. Killeen, D.L. Littman, and R.H. Schwartz. 1994. A subset of CD4⁺ thymocytes selected by MHC class I molecules. *Science (Wash. DC).* 263:1774–1778.
27. Coles, M.C., and D.H. Raulet. 1994. Class I dependence of development of CD4⁺ CD8⁻ NK1.1⁺ thymocytes. *J. Exp. Med.* 180:395–399.
28. Parish, C.R., and F.Y. Liew. 1972. Immune response to chemically modified flagellin. III. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *J. Exp. Med.* 135:298–311.
29. Scott, P., P. Natovitz, R.L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med.* 168:1675–1664.
30. Sadick, M.D., F.P. Heinzl, B.J. Holaday, R.T. Pu, R.S. Dawkins, and R.M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. *J. Exp. Med.* 171:115–127.
31. Heinzl, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J. Exp. Med.* 177:1505–1509.
32. Powrie, F., R. Correa-Oliveira, S. Mauze, and R.L. Coffman. 1994. Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ T cells are important for the balance between protective immunity and pathogenic cell-mediated immunity. *J. Exp. Med.* 179:589–600.
33. Evavold, B.D., and P.M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science (Wash. DC).* 252:1308–1310.
34. De Wit, D., M. Van Mechelen, M. Ryelandt, A.C. Figueiredo, D. Abramowicz, M. Goldman, H. Bazin, J. Urbain, and O. Leo. 1992. The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes. *J. Exp. Med.* 175:9–14.
35. Burstein, H.J., C.M. Shea, and A.K. Abbas. 1992. Aqueous antigens induce in vivo tolerance selectively in IL-2- and IFN- γ -producing (Th1) cells. *J. Immunol.* 148:3687–3691.
36. Romball, C.G., and W.O. Weigle. 1993. In vivo induction of tolerance in murine CD4⁺ cell subsets. *J. Exp. Med.* 178:1637–1644.
37. Chu, E.B., M.V. Hobbs, D.N. Ernst, and W.O. Weigle. 1995. In vivo tolerance induction and associated cytokine production by subsets of murine CD4⁺ T cells. *J. Immunol.* 154:4909–4914.
38. Van Mechelen, M., D. De Wit, M. Ryelandt, S. Hjulström, M. Heynderickx, H. Bazin, J. Urbain, and O. Leo. 1995. Induction of Th2 responses to soluble proteins is independent of B cell tolerance status. *Int. Immunol.* 7:199–205.
39. Burstein, H.J., and A.K. Abbas. 1993. In vivo role of interleukin 4 in T cell tolerance induced by aqueous protein antigen. *J. Exp. Med.* 177:457–463.
40. Gajewski, T.F., and F.W. Fitch. 1988. Anti-proliferative effect of IFN- γ in immune regulation. I. IFN- γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* 140:4245–4253.
41. Bradley, L.M., D.D. Duncan, S. Tonkonogy, and S.L. Swain. 1991. Characterization of antigen-specific CD4⁺ effector T cells in vivo: immunization results in a transient population of Mel-14⁻, CD45RB⁻ helper T cells that secretes interleukin-2 (IL-2), IL-3, IL-4, and interferon- γ . *J. Exp. Med.* 174:547–559.
42. Gollob, K.J., and R.L. Coffman. 1994. A minority subpopulation of CD4⁺ T cells directs the development of naive CD4⁺ T cells into IL-4-secreting cells. *J. Immunol.* 152:5180–5188.
43. Macatonia, S.E., N.A. Hosken, M. Litton, P. Vieira, C.-S. Hsieh, J.A. Culpepper, M. Wysocka, G. Trinchieri, K.M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4⁺ T cells. *J. Immunol.* 154:5071–5079.
44. Day, M.J., A.G.D. Tse, M. Puklavec, S.J. Simmonds, and D.W. Mason. 1992. Targeting autoantigen to B cells prevents the induction of a cell-mediated autoimmune disease in rats. *J. Exp. Med.* 175:655–659.
45. Saoudi, A., S. Simmonds, I. Huitinga, and D.W. Mason. 1995. Prevention of experimental allergic encephalomyelitis in rats by targeting autoantigen to B cells: evidence that the protective mechanism depends on changes in the cytokine response and migratory properties of autoantigen-specific T cells. *J. Exp. Med.* 182:335–344.
46. Eynon, E.E., and D.C. Parker. 1993. Parameters of tolerance induction by antigen targeted to B lymphocytes. *J. Immunol.* 151:2958–2964.
47. Scott, B., R. Liblau, S. Degermann, L.A. Marconi, L. Ogata, A.J. Caton, H.O. McDevitt, and D. Lo. 1994. A role for

- non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity*. 1:1–20.
48. Szabo, S.J., N.G. Jacobson, A.S. Dighe, U. Gubler, and K.M. Murphy. 1995. Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity*. 2:665–675.
49. Miller, A., O. Lider, A.B. Roberts, M.B. Sporn, and H.L. Weiner. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc. Natl. Acad. Sci. USA*. 89:421–425.
50. O'Garra, A., and K. Murphy. 1994. Role of cytokines in determining T-lymphocyte function. *Curr. Opin. Immunol.* 6: 458–466.
51. Sher, A., R.T. Gazzinelli, I.P. Oswald, M. Clerici, M. Kullberg, E.J. Pearce, J.A. Berzofsky, T.R. Mosmann, S.L. James, and H.C. Morse. 1992. Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol. Rev.* 127:183–204.