

The Role of IFN- γ in the Production of Th2 Subpopulations: Implications for Variable Th2-Mediated Pathologies in Autoimmunity¹

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It has become increasingly apparent in studies of mutant mice and observations of disease that cytokine production by fully committed effector T cells within the Th1 and Th2 phenotype can vary within each group. This can potentially influence the type and effectiveness of a given immune response. The factors responsible for inducing variable Th1 and Th2 subtype responses have not been well established. Using transgenic mice expressing the myelin basic protein-specific TCR, we demonstrate here that two distinct populations of Th2 cells that are characterized primarily by differential IL-4 and IL-5 expression levels can be generated depending upon the levels of IFN- γ present at the time of priming. We also demonstrate that populations expressing high levels of IL-4 relative to IL-5 vs those with intermediate levels of IL-4 relative to IL-5 are stable and possess distinct effector functions in an experimental autoimmune encephalomyelitis model. *The Journal of Immunology*, 2001, 167: 3074–3081.

The CD4⁺ T cell responses to Ag can be generally divided into two distinct types based on cytokine expression, designated Th1 and Th2 (1). Th1 cells express IFN- γ and lymphotoxin, whereas Th2 cells express IL-4, IL-5, and IL-6. TNF- α , GM-CSF, IL-10, and IL-13 can be produced by both helper types, with higher expression of TNF- α and GM-CSF in Th1 cells and higher expression of IL-10 and IL-13 in Th2 cells (2, 3). Upon infection by a particular type of pathogen, depending on the location of the infection and the genetic background of the host, a cascade of events is initiated that leads to a specific cytokine burst that subsequently influences Th cell commitment (2–6). It is becoming increasingly evident that the early conditions that lead to the initial priming of T cells can have long-lasting and permanent effects on the type of cytokines that these cells will eventually produce. For instance, it has been shown that specific stimulatory conditions occurring within the first 48 h of stimulation of naive CD4⁺ T cells can effectively modulate the production of IL-4 or IFN- γ , which are considered the hallmark cytokines of the Th2 and Th1 phenotypes, respectively (7). Importantly, however, fully polarized Th1 and Th2 responses are attained only after repeated stimulation in vitro or in vivo (8) with an intermediate Th0 phenotype, characterized by a mixed Th1/Th2 cytokine profile, being observed in early activation or certain in vivo infection situations (9–11).

In contrast to the abundance of studies of factors that influence Th1 or Th2 polarization, much less is known about the conditions that contribute to subpopulations within each group. Th1 and Th2 cells are broadly characterized by the cytokines that they express. However, within each group, there can be significant variation of the cytokines produced by individual T cells, suggesting that these cells may play differential roles in immunity and/or immunopathology. With respect to Th2 populations, it was initially thought that IL-4 production was an absolute requirement for the production of other Th2-type cytokines (12). However, studies analyzing mice with a disrupted IL-4 gene or using a blocking anti-IL-4 Ab have shown that the Th2 response is diminished, but not abrogated (13, 14). Indeed, IL-5 production was still observed in these cases with a somewhat attenuated, but still detectable, eosinophilia (13–15). This is in sharp contrast to IL-5-knockout mice, which do not develop eosinophilia when aeroallergen challenged or infected with the helminth *Mesocostoides corti* (15, 16). Other studies examining the pattern of IL-4 and IL-5 production, which are normally shown to be coexpressed, have indicated that these two cytokines can have dissociated expression patterns in vitro and in vivo (17, 18). Finally, growing evidence indicates that differential levels of IL-4 and IL-5 are observed in particular pathogenic infections and hypersensitivity reactions in humans (19, 20). Atopic asthma in humans is a predominantly IL-4 and IL-5 condition that correlates well with IgE (high IL-4) production and eosinophilia (high IL-5). However, many individuals afflicted with nonatopic asthma display an IL-5-mediated response without detectable IL-4 production in the bronchoalveolar lavage. This condition is characterized by eosinophilia, but normal levels of IgE (20).

In this study, we wanted to determine what factors contribute to the variable IL-4 and IL-5 phenotype of Th2 cells. Analyzing a wide array of factors, such as Ag dose, restimulation kinetics, and exogenous addition or inhibition of cytokines, we found that the presence or absence of IFN- γ in the initial Th2-polarizing conditions had a remarkable influence on the relative levels of IL-4 and IL-5 produced by in vitro-generated Th2 cell cultures. The cytokine profile of these subpopulations was stable over time and over several restimulations and was not reversible after commitment was established. Most importantly, we show that adoptive transfer of these effector Th2 subpopulations in an experimental

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autoimmune encephalomyelitis (EAE)³ model produced two disparate disease phenotypes, indicating that the relative levels of Th2 cytokines may have a strong influence on the nature of Th2-mediated diseases.

Materials and Methods

Mice

The establishment of myelin basic protein (MBP) *N*-acetylated (Ac)₁₋₁₁-specific TCR-transgenic mice has been described previously as well as the MBP-specific TCR-transgenic mice with a disrupted recombinase-activating gene (RAG)-1 (21). In brief, the mice were made by injection of C57BL/6 zygotes, with subsequent backcrossing with C57BL/10.PL (The Jackson Laboratory, Bar Harbor, ME) to incorporate the I.A^u restriction element. TCR- $\beta^{-/-}$ (22) and IFN- $\gamma^{-/-}$ (23) backcrossed to C57BL/6 were purchased from The Jackson Laboratory, and subsequently the H-2^u MHC was incorporated in homozygosity through crosses with C57BL/10.PL mice. Mice were kept under specific-pathogen-free conditions in individually ventilated cages (Thoren, Hazelton, PA) at the Skirball Institute Central Animal Facility (New York University Medical Center, New York, NY).

Disease evaluation

EAE was scored as previously described (24): level 1, limp tail; level 2, weak or partial leg paralysis; level 3, total hind leg paralysis; and level 4, hind leg paralysis and weak or partial front leg paralysis. All protocols involving mice handling were approved by New York University's institutional and animal care use committee.

Generation of MBP-specific Th cells

MBP-specific cells were obtained from spleens of MBP-specific TCR-transgenic, RAG1⁺ (referred to as T/R⁺) or T/R⁺ × IFN- $\gamma^{-/-}$ mice on the C57BL/10.PL genetic background. Spleen cells (~2 × 10⁶/ml) were cultured in the presence of 5 μ M MBP Ac₁₋₁₇ and IL-12 (BD PharMingen, San Diego, CA), IL-4 (BD PharMingen, San Diego, CA), or IL-4 and anti-IFN- γ Ab (BD PharMingen) to generate Th1, Th2, and Th2Hi5 cells, respectively. Cultures were restimulated 4 days after primary stimulation and every 7 days thereafter with irradiated (20 Gy) syngeneic TCR- $\beta^{-/-}$ or IFN- $\gamma^{-/-}$ (Fig. 5) splenocytes and MBP peptide (5 μ M).

Cytokine assays

Intracellular staining was performed according to the protocol recommended by BD PharMingen. Briefly, cells were harvested and treated with 10 ng/ml PMA (Sigma, St. Louis, MO) and 200 ng/ml ionomycin (Sigma) for 2 h, followed by 10 μ g/ml brefeldin A addition (Sigma) for 2 additional h. Cells were washed, and the cell surface was stained with anti-CD4 APC for 20 min at 4°C in staining buffer (PBS, 2% FBS, and 0.1% NaN₃). The cells were then treated for 5 min with lysis solution (BD PharMingen), washed, and incubated with 1× permeabilization solution (BD PharMingen) for 10 min. Cells were washed and incubated in staining buffer for 45 min with combinations of the following Abs: IL-4-FITC, IL-5-PE, and/or IFN- γ -PE (BD PharMingen). Cells were analyzed on a FACSCalibur instrument (BD Biosciences, Mountain View, CA).

The concentrations of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 (Quantikine IL-13 kit, R&D Systems, Minneapolis, MN), and IFN- γ in culture supernatants was determined by ELISA using Ab pairs and the protocol supplied by BD PharMingen unless otherwise noted.

Real-time PCR

Total RNA was extracted from Th1, Th2, and Th2Hi5 cultures using TRIzol (Invitrogen, San Diego, CA), and first-strand cDNA was synthesized using SuperScript II RT (Invitrogen). PCR was performed using the iCycler (Bio-Rad, Hercules, CA) and the SYBR Green fluorophore core reagents kit and protocol (PerkinElmer Applied Biosystems, Foster City, CA). The GATA-3 sequences used were 5'-CTACGGTGCAGAGGTATCC-3' (sense) and 5'-GATGGACGTCTTGGAGAAGG-3' (antisense). The c-Maf sequences used were 5'-TGTGTTACCGTTCGAGCTTT-3' (sense) and 5'-AGGTCGGAATTGTTTCATTGC-3' (antisense).

Competitive RT-PCR

Quantification of the cytokine transcripts for IL-1 α , IL-1 β , IL-4, IL-5, IFN- γ , and TGF- β 1 was performed as described previously (25) using the primer sequences supplied. Briefly, total RNA was isolated from Th1, Th2, and Th2Hi5 polarized cultures (described above) using Tri-Reagent (Molecular Research Center, Cincinnati, OH). cDNA was produced using SuperScript II RNase H⁻ reverse transcriptase according to the supplied protocol. Competitive PCR was subsequently performed using transcription-specific primers for the indicated cytokines and the plasmid pCCF-D as competitor. The relative amounts of cytokines in each of our samples were determined by comparing band intensities at varying pCCF-D input concentrations using hypoxanthine-guanine phosphoribosyltransferase as a standard.

Adoptive transfer experiments

Th populations were generated as described above for three stimulations on days 0, 4, and 8 for full polarization. On days 9 and 11, culture supernatants were taken and analyzed for IL-4, IL-5, and IFN- γ cytokine production by ELISA. On day 11, cells were washed, resuspended in PBS, and injected i.v. (5 × 10⁶ cells/mouse) into RAG1^{-/-} recipient mice on day 11 of culture. Weight and clinical score were monitored twice a week for the duration of the experiments.

Histology

Adoptively transferred mice with severe EAE were deeply anesthetized with ketamine/xylazine/acepromazine mixture and perfused through the left ventricle with ~100 ml PBS/0.5 mM EDTA, and CNS tissue was dissected in one piece and subsequently separated (spinal cords and cerebellum). Formalin-fixed tissue was paraffin embedded and sectioned to 7 μ m. The tissue was subsequently stained with H&E and analyzed using a Zeiss Axioplan microscope (Zeiss, New York, NY) and the SPOT RT camera and software system (Diagnostic Instruments, Sterling Heights, MI).

Results

The presence or absence of IFN- γ in Th2 polarized cells produces Th2 subpopulations with distinct IL-5 expression patterns

It has been previously reported that Th2 clones can express varying ratios of IL-4 and IL-5 (9, 17). We have confirmed these observations in T cell clones obtained under Th2 polarizing conditions from MBP Ac₁₋₁₁-specific splenocytes from TCR-transgenic mice (T/R⁺ mice). The majority of these clones produced high levels of IL-4 with widely varying levels of IL-5, with a few clones (~5%) producing low levels of IL-4 and high levels of IL-5. These clones were maintained and restimulated for long periods of time without alteration in their cytokine production program (L. Armstrong and J. Sampson, unpublished observations). To determine the factors that affect this variability we systematically analyzed a wide spectrum of known Th2 influences on total splenocyte cultures, such as Ag dose, time between stimulation, and cytokine addition or inhibition. Strikingly, it was found that the presence or the absence of IFN- γ during the initial stimulation of T/R⁺ splenocytes had the most dramatic influence on relative IL-4 and IL-5 cytokine production (Fig. 1). Initial stimulation of naive MBP-specific T cells under Th2 polarizing conditions (Ag and exogenously added IL-4) resulted in populations that expressed high IL-4 levels, low IL-5 levels, and no detectable IFN- γ . Under the same stimulatory conditions, except in this case, with the addition of neutralizing anti-IFN- γ Abs on day 0, we found that IL-4 levels were similar (if not slightly decreased) with a 2- to 3-fold augmentation of IL-5 levels. After three rounds of stimulation there was no detectable IFN- γ . High IL-5 levels were displayed by cells in which the anti-IFN- γ Ab was washed out of the cultures before the first restimulation (Fig. 1) as well as by cells grown under sustained Ab treatment, indicating that the critical event was the neutralization of IFN- γ at the time of naive T cell priming. Further phenotypic analysis of Th2 (high IL-4, low IL-5 producers) and

³ Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; Ac, *N*-acetylated; RAG, recombinase-activating gene; T/R⁺, myelin basic protein-specific TCR-transgenic mice with at least one normal RAG1 gene.

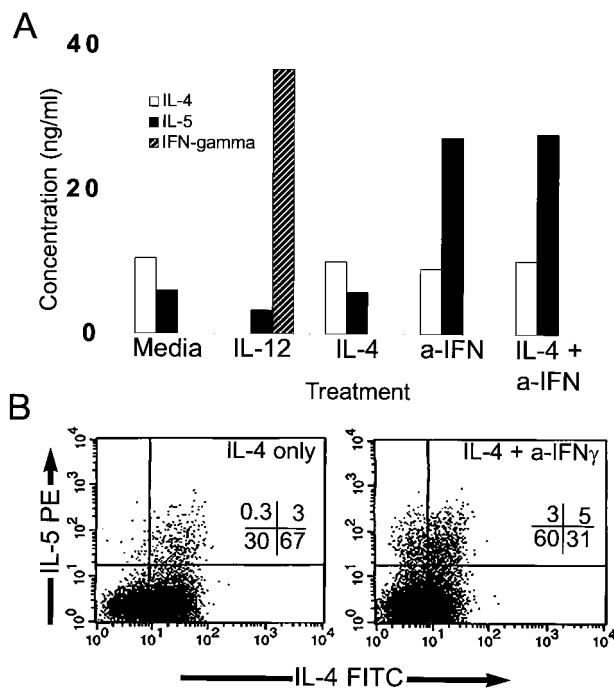


FIGURE 1. Neutralization of IFN- γ leads to higher IL-5 production by MBP-specific Th2 lymphocytes. Cytokine analysis of cultures grown under different conditions. *A*, Culture supernatants from primed MBP-transgenic splenocytes treated on day 0 with medium alone, IL-12 (100 U/ml), IL-4 (100 U/ml), anti-IFN- γ Ab (10 μ g/ml), or IL-4 plus anti-IFN- γ were harvested 24 h after two rounds of restimulation in vitro with syngeneic irradiated APCs ($\sim 2 \times 10^6$ /ml) and MBP Ac₁₋₁₇ peptide (5 μ M) and were analyzed by sandwich ELISA. *B*, Polarized T cell cultures treated as described above were harvested 24 h after the third stimulation and were stimulated for an additional 4 h with PMA and ionomycin for FACS analysis of IL-4 and IL-5 production by intracellular cytokine staining. The staining shows IL-4-FITC and IL-5-PE staining of gated lymphocytes based on forward and side scatter.

Th2Hi5 (high IL-4, high IL-5 producers) populations was performed to look for other cytokines that may be differentially expressed. We analyzed a wide array of cytokines (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and TGF- β 1) by ELISA, quantitative RT-PCR, and/or intracellular staining (Table I). Both Th2 and Th2Hi5 cells expressed Th2 cytokines, including IL-4, IL-5, IL-9, IL-10, and IL-13, with the only major difference found between the two populations being that Th2Hi5 populations had reproducibly higher levels of IL-5 expression compared with Th2 cultures. In addition, we analyzed various aspects of our populations after initial stimulation (24 and 72 h poststimulation), such as cell morphology and surface markers believed to be important in Th cell generation, activation, and function. These included a measure of cell size, as a correlate to cell activation, by forward scatter analysis and of TCR and coreceptor (CD4) expression levels, CD28, CTLA4, FAS, LFA-1, CD49d, and CD2 (Table I). As would be expected, both populations displayed an activated phenotype, which was confirmed by analysis of the activation markers CD69, CD44, and CD62 ligand (Table I). The results of these experiments showed a similar phenotype for all markers analyzed on Th2Hi5 cultures vs Th2 cultures.

Finally, we assessed the expression levels of the transcription factors GATA-3 and c-Maf in our various cultures (26, 27). Both GATA-3 and c-Maf are strongly associated with the Th2 phenotype, with c-Maf playing a significant role in IL-4 activation through c-maf response element sites in the IL-4 promoter (26).

GATA-3 consensus binding sites are found in both the IL-4 and IL-5 enhancer regions, and GATA-3 is considered to be a key regulator of the Th2 phenotype. Interestingly, GATA-3 seems to play a more striking role in IL-5 gene transcription in a luciferase reporter system (28), but recent evidence points to an enhancer element downstream of the IL-4 gene that may become available after initial activation and chromatin remodeling (29). Using quantitative PCR using transcript-specific primers we observed that GATA-3 and c-Maf were both strongly associated with the Th2 phenotype, as published, but there were no significant differences between Th2 and Th2Hi5 cultures (Fig. 2).

Th2 and Th2Hi5 populations display a stable phenotype over time, and differences in cytokine production are not due to kinetic differences in cell division

One of the hallmarks of the Th1 and Th2 populations is their ability to maintain their cytokine expression patterns over time. To test whether this was true for the Th2 and Th2Hi5 subpopulations as well, we generated Th2 and Th2Hi5 cells and maintained them over multiple additional rounds of stimulation in nonpolarizing conditions with irradiated APC and MBP peptide. The Th2 and Th2Hi5 phenotype, as measured by IL-4 and IL-5 production, was maintained over a long period of time, confirming the highly polarized Th2 nature of both subpopulations (Fig. 3) and the stable nature of clones with disparate IL-4 and IL-5 production levels. The fact that our Th2 cultures kept for long periods of time did not develop a Th2Hi5 profile was also an indicator that the enhanced proliferation that occurred in the absence of IFN- γ (30) was not responsible for the different IL-5 levels produced by Th2 and Th2Hi5 cells, as T cells in older Th2 cultures divided as many times as cells in younger Th2Hi5 cultures.

We wanted to further characterize the kinetics of IL-4 and IL-5 production in the two Th2 subpopulations, as recent data showed that cytokine production is regulated by cell cycle and number of cell divisions (31, 32). To address these issues we generated Th1, Th2, and Th2Hi5 populations and, after two restimulations in vitro, analyzed cytokine production at different time points. Fig. 4 shows that, beginning from the earliest time points, IL-4 and IL-5 productions are different in Th2 and Th2Hi5 cultures, further confirming that the two phenotypes are distinct from one another. These results indicate that cell cycle and the number of cell divisions do not play a major role in the Th2Hi5 phenotype observed.

T cell cultures from IFN- γ ^{-/-} mice show a phenotype similar to that of anti-IFN- γ Ab-treated cultures

Because the presence or the absence of IFN- γ in the milieu upon initial commitment of MBP-specific CD4⁺ cells to the Th2 phenotype greatly influenced relative IL-4 and IL-5 levels in subsequent stimulations, we wanted to determine whether this phenotype could be recapitulated in IFN- γ ^{-/-} mice. Using total splenocytes derived from anti-MBP TCR-transgenic IFN- γ ^{-/-} mice (T/R⁺ IFN- γ ^{-/-}), we observed a phenotype that closely matched the effects seen with the addition of anti-IFN- γ -neutralizing Ab in cultures that were stimulated with MBP plus exogenous IL-4 (Fig. 5). In addition, it was observed that Th1 polarized cultures (MBP plus IL-12 addition) from IFN- γ ^{-/-} mice also produced an enhanced IL-5 response similar to the response observed in Th2Hi5 cultures. These data confirm prior work on the effect of IL-12 in *Schistosoma*-infected IFN- γ ^{-/-} mice (33), support the necessity of IFN- γ for Th1 generation (23), and indicate that the lack of any IFN- γ in the initial priming conditions allows for the derepression of IL-5 production by the T cells. IL-4 levels, however, are affected very little by the presence or the absence of small amounts of endogenous IFN- γ .

Table I. Characterization of cytokine and cell surface markers for Th1, Th2, or Th2Hi5 populations

Cytokine ^a or Surface Marker ^b	Th1	Th2	Th2Hi5	Measured by
IL-2	–	–	–	ELISA
IL-3	+	++	++	ELISA
IL-4	–	+++	+++	ELISA, RT-PCR, IC stain ^d
IL-5 ^c	–	+	+++	ELISA, RT-PCR, IC stain
IL-6	–	–	–	ELISA
IL-9	–	++	++	ELISA
IL-10	–	++	++	ELISA
IL-13	–	++	++	ELISA
IFN- γ	+++	–	–	ELISA, RT-PCR, IC stain
TGF- β	–	–	–	RT-PCR
FSC	++	++	++	FACS analysis
CD4	++	++	++	FACS analysis
LFA-1	++	++	++	FACS analysis
VLA-4	+	+	+	FACS analysis
Fas	+	++	++	FACS analysis
CD28	+	++	++	FACS analysis
CTLA4	+	++	++	FACS analysis
CD69	++	++	++	FACS analysis
CD62L	–	–	–	FACS analysis
CD25	++	++	++	FACS analysis

^a Cytokine analysis was performed on fully polarized Th1, Th2, and Th2Hi5 populations 24 h after three rounds of stimulation in vitro. + and – represent relative levels of cytokine expression.

^b FACS analysis was performed at 72 h after initial stimulation, and + and – indicate a relative increase or decrease in fluorescent intensity from time 0; ++ indicates an ~2-fold up-regulation of surface expression or cell size.

^c Differences between Th2 and Th2Hi5 cultures.

^d IC, Intracellular cytokine; FSC, forward light scatter; VLA-4, very late Ag 4; CD62L, CD62 L-selectin.

Adoptive transfer of MBP-specific Th1, Th2, and Th2Hi5 populations into RAG1-deficient mice produces three distinct clinical phenotypes

Given the strong evidence for IFN- γ 's initial role in the generation of Th2 subpopulations with skewed IL-4 and IL-5 expression patterns, we next examined whether these populations exhibited any functional or pathogenic differences in vivo. To assess whether our populations possess differential effector functions, splenocytes from MBP-specific TCR-transgenic mice were cultured with peptide and IL-12 (Th1), IL-4 (Th2), or IL-4 plus anti-IFN- γ Abs (Th2Hi5) and restimulated twice on days 4 and 8 with irradiated splenocytes and peptide to polarize the populations fully. Cells were then injected into recipient RAG1^{-/-} mice at 5×10^6 cells/mouse on day 11 to ensure that the T cells were blasting and sufficiently polarized. The cultures were analyzed in vitro to confirm the skewed cytokine expression phenotype. The transferred Th1 cell populations induced severe EAE with very rapid onset (around day 7), leading to death ~3.5 wk after injection. The Th2 and Th2Hi5 populations, on the other hand, caused disease with delayed onset (days 16–20), but still exhibited a 100% incidence (Fig. 6A). The disease displayed by Th1- and Th2-transferred mice was characterized by the traditional ascending, progressive paralysis normally observed in classical EAE. This classical paralysis is characterized initially by hindtail weakness and paralysis that moves up the mouse through the hindlimbs and finally the forelimbs over time. However, the Th2Hi5-transferred mice showed a marked difference from the Th1 and Th2 recipient mice in the nature of disease that was manifested. The mice showed a delayed onset of disease, like the Th2 recipient mice, but exhibited a very rapid progression to death after initiation of paralysis. Instead of the classical ascending progressive paralysis observed in the Th1- and Th2-induced disease, the mice showed a marked decrease in their ability to keep themselves upright and would list, spin, or roll in their cages, indicating a severe disruption in their ability to maintain balance. These mice displayed a slight loss of tonus in the tail, but could maintain some tail responses throughout the disease

progression (Fig. 6B). We termed this type of EAE nonclassical, as it did not follow the well-established progression seen in most studies and, in addition, could not be scored according to rules established for normal EAE progression.

Discussion

Murine knockout studies and studies of the Th2 phenotype in vitro or in disease have shown that production of cytokines specific to this helper cell subset can be variable and independent of one another, which can have important in vivo consequences (14, 20,

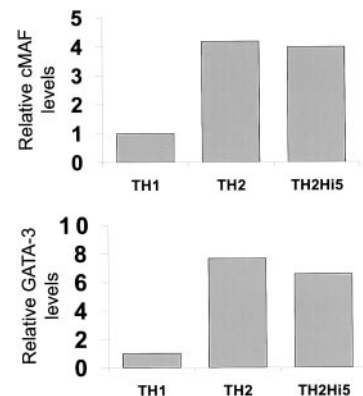


FIGURE 2. Expression of c-Maf and GATA-3 messenger RNA is similar for Th2 and Th2Hi5 populations. Th1, Th2, and Th2Hi5 cultures were generated as described in Fig. 1. Total RNA from the cultured cells was purified 24 h after the second restimulation. First-strand cDNA was generated, and quantitative RT-PCR was performed using SYBR Green and transcript-specific primers. The figure shows relative c-Maf and GATA-3 expression in Th1, Th2, and Th2Hi5 cultures. The bars represent levels of expression normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase and set arbitrarily at 1 for Th1 cells. The relative expression data are representative of two independent experiments for both c-Maf and GATA-3.

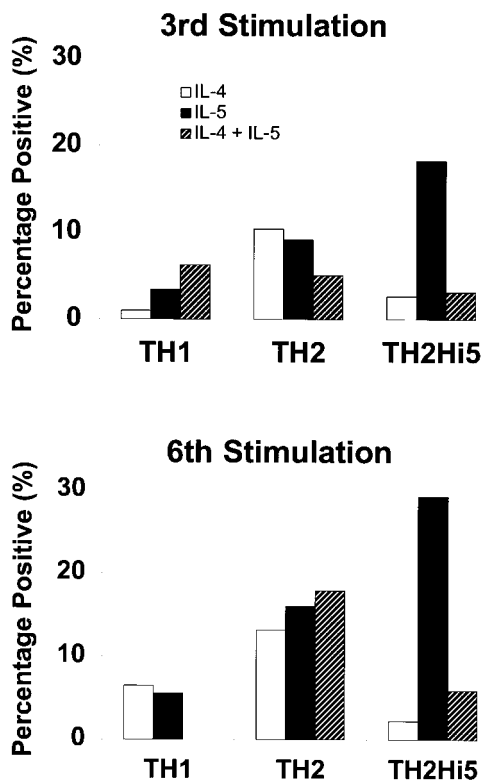


FIGURE 3. Th2 and Th2Hi5 cultures maintain their phenotypes after repeated stimulation. Splenocytes from T/R⁺ mice were stimulated in vitro with MBP peptide and IL-12 only, IL-4 only, or IL-4 plus anti-IFN- γ Ab, and harvested 24 h after three or six rounds of stimulation and analyzed by intracellular staining. The graph shows the percentage of CD4⁺ cells that stained positively for IL-4 only (□), IL-5 only (■), or both IL-4 and IL-5 double expressors (▨).

28, 34). In this study, we demonstrate that we can greatly influence the ratio of IL-5 to IL-4 production depending on the presence or the absence of small amounts of IFN- γ at the time of naive T cell priming. This is the first report showing IFN- γ 's influence on IL-5 expression. In addition, we determined that the Th2 and Th2Hi5 subpopulations are stable, irreversible, and have the ability to induce clinically different disease types in an EAE model.

Clones generated under Th2-polarizing conditions showed dramatic variations in the amounts of IL-4 and IL-5 they produced, with some even producing IL-5 almost exclusively, with very little IL-4 (<5% of the total clones). This phenomenon of dissociated IL-4 and IL-5 production has been observed in several settings (10, 34). Sewell and Mu (18) showed that immunization in the presence of pertussis toxin led to increase in IL-4, but no IL-5 production, and, conversely, immunization in the presence of cyclophosphamide led to an increase in IL-5, but no IL-4 production. Walker et al. (20) have shown that in nonallergic asthmatic patients, one can find cases where high eosinophilia (correlated with high IL-5 production) and low IgE (correlated with low IL-4 production) can be identified. Finally, many studies on IL-4^{-/-}, IL-4R^{-/-}, and STAT-6^{-/-} mice have shown definitively that IL-4 signaling is not necessary for the production of other Th2 cytokines, especially IL-5, and that in allergic models of asthma in BALB/c mice, eosinophilia and airway hyper-responsiveness are still present due to the influences of IL-5 and IL-13 (14, 35).

The variability of the IL-5:IL-4 ratio produced by our clones could be due to many factors, either intrinsic to the individual T cells or influenced by their surrounding environment, or both. However, considering that the cells were derived from TCR-trans-

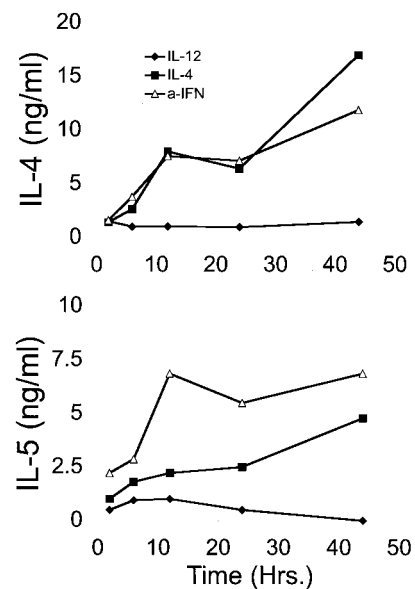


FIGURE 4. Differences in IL-4 and IL-5 expression in Th2 and Th2Hi5 populations are stable over time and are not due to cytokine release kinetics. Th1, Th2, and Th2Hi5 cultures were generated as described in Fig. 1. After the third stimulation supernatants were collected at 2, 6, 12, 24, and 48 h and analyzed by ELISA.

genic mice, intrinsic variations are expected to be reduced. To address this question we analyzed the roles of several factors that might influence the commitment of MBP-specific T cells. Among those, we showed that the absence or the presence of IFN- γ at the onset of polarization had a dramatic effect on the cytokine profile that would be programmed into our Th2 populations. Neutralizing Abs to IFN- γ are routinely used along with exogenous IL-4 addition for the generation of Th2 cultures in vitro, but a thorough analysis of differences between populations generated from IL-4

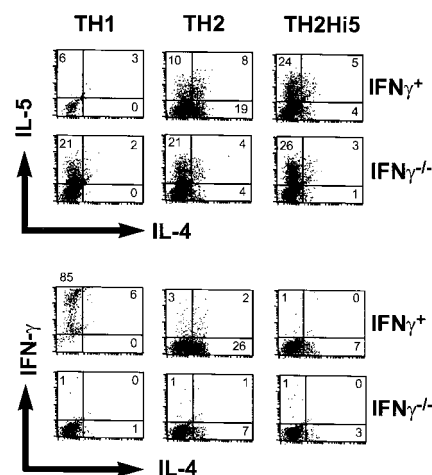


FIGURE 5. Cultures from MBP-transgenic mice with a disrupted IFN- γ gene display the same Th2Hi5 phenotype as anti-IFN- γ -treated cultures. Total splenocytes from T/R⁺ mice with normal or disrupted IFN- γ genes were initially stimulated side-by-side under the same conditions as in Fig. 1. IFN- γ wild-type and knockout cultures were restimulated with irradiated splenocytes from normal mice and IFN- γ -knockout mice, respectively. Twenty-four hours after three rounds of stimulation cells were harvested, and intracellular cytokine staining was performed. The plots for the various cytokines show populations that were gated on CD4⁺ lymphocytes.

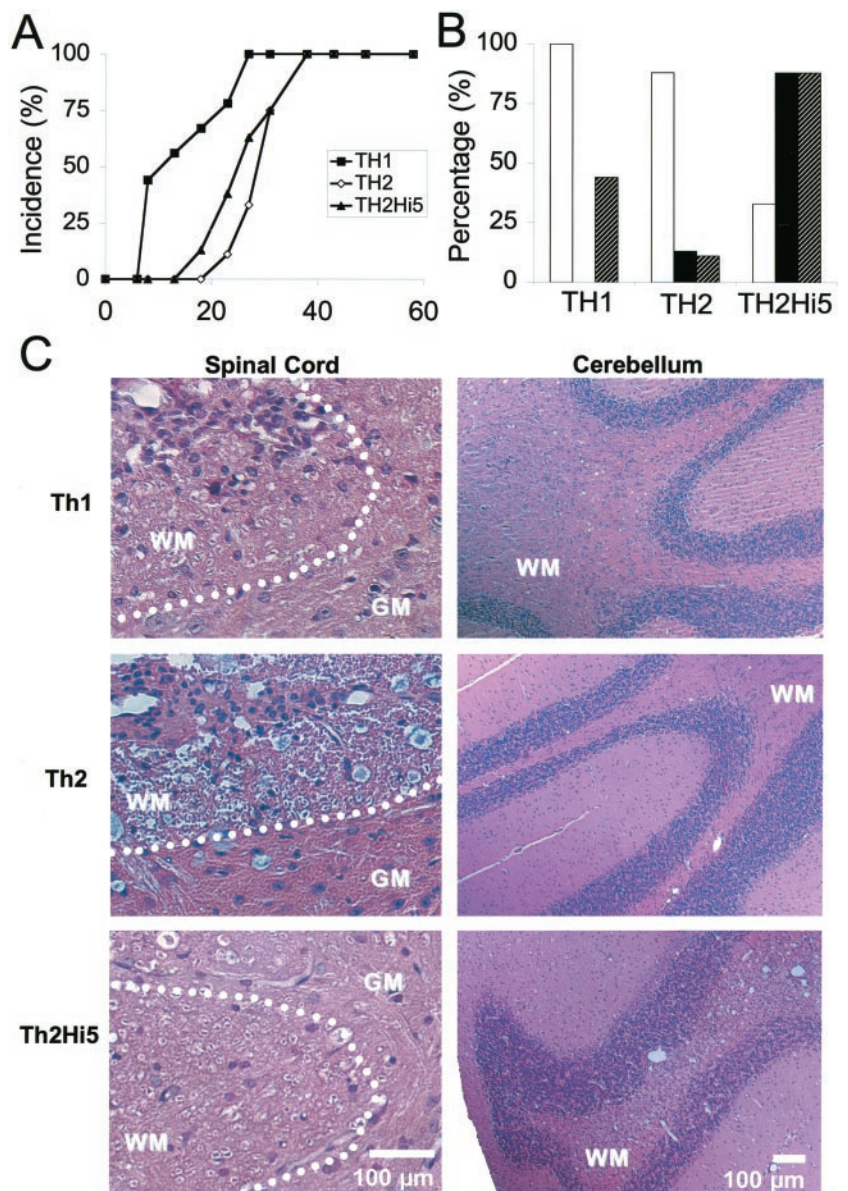


FIGURE 6. Clinical and histological analyses of EAE from adoptive transfer of Th1, Th2, and Th2Hi5 cultures into RAG1-deficient recipients. Four- to 6-wk-old RAG1^{-/-} recipient mice were adoptively transferred with 5×10^6 polarized Th1, Th2, or Th2Hi5 cultures generated as described in Fig. 1. Weight (not shown) and EAE incidence (A) were assessed approximately every 4 days, starting 7 days post-transfer. B, Summary of the severity and clinical signs of the EAE observed after 58 days. □, Percentage of mice from any given transfer group that manifested the classical ascending progressive form of EAE; ■, percentage of mice that manifested nonclassical or rolling EAE; ▨, percentage of mice that were moribund (L5) by day 58 for each group. This figure is representative of three experiments ($n = 5-6$ /group). C, Histological analysis of spinal cord and cerebellum from adoptively transferred mice with severe classical or nonclassical EAE. H&E staining with white matter (WM) and gray matter (GM) indicated and separated by a white dotted line.

addition only or IL-4 and anti-IFN- γ addition has not been performed. What is known is that IFN- γ is necessary, but not sufficient, for Th1 formation *in vitro* and the presence of IFN- γ in Th2 polarized cultures inhibits proliferation (30). Indeed, both Th2 and Th2Hi5 cells displayed a decreased ability to proliferate in response to IFN- γ , indicating that a functional IFN- γ R was present at similar levels on both Th2 subpopulations (data not shown). This inhibition of proliferation was not observed in Th1 cultures as would be expected from published work showing that IFN- γ 's effects are abrogated due to the down-regulation of IFN- γ R in these populations (36, 37).

The production of different Th2 subtypes is not a curiosity and the physiological role of IFN- γ in the generation of these subtypes *in vivo* could easily be imagined. During the life of a vertebrate, Ags enter the organism by a variety of routes. A different sequence of events is triggered by Ag entry through each of the different routes. Priming of T cells in the presence of IL-4 and small amounts of IFN- γ may lead to Th2 differentiation. IFN- γ may be present in the microenvironment due to its production by NK T cells, NK cells, or Th0 cells. NK cells and NK T cells are less frequent in the lymph nodes than they are in the spleen; thus, it

could be envisioned that a Th2 profile will develop when Ag makes its way systemically and T cells are primed in spleen. In contrast, Ag entry through the skin could lead to Th1 or Th2Hi5 priming in the draining lymph nodes depending upon the IL-12 levels produced by dendritic cells.

Our data indicate that both cell types, Th2 and Th2Hi5, have all the hallmarks of a Th2 population, and it is not just a matter of the Th2, as opposed to the Th2Hi5 cells, being "less Th2." They both express the signature Th2 cytokines, are stable over long periods of time, and are not reversible, the prototypical definition of Th2 type populations. In addition, the Th2-associated transcription factors GATA-3 and c-Maf were expressed at similar levels by Th2 and Th2Hi5 cells. c-Maf is an IL-4 specific transcription factor (26), and GATA-3 is thought to be a master regulator of Th2 vs Th1 cell differentiation. Naive T cells express low levels of GATA-3 and upon polarization GATA-3 expression either gets turned off in Th1 committed cells or is autocatalytically up-regulated in Th2 cells (27, 28, 38, 39). In our case, the fact that c-Maf and GATA-3 are up-regulated in both Th2 subpopulations is not surprising and points to other factors that may help fine-tune the specific cytokine response in Th2 cells. It also may be that subtle differences in

GATA-3 expression that are beyond the sensitivity limits of our assays can account for the different cytokine profiles of our two populations. Nevertheless, the 5- to 7-fold increase in GATA-3 levels in both our Th2 populations argue strongly in favor of them being true Th2 cells.

One of the most important features of the Th2 and Th2Hi5 populations is their ability to cause dramatically different types of pathology. Th2 cells have previously been shown to produce EAE in lymphopenic recipient mice (40), but the role of different Th2 cytokines has not been elucidated. Recently, Tran et al. (41) and Chu et al. (42) have reported that IFN- $\gamma^{-/-}$ mice are not only susceptible to EAE, but exhibit a more severe phenotype than wild-type mice. In our model, it is likely that the initial activation of MBP-specific cells in the presence or the absence of small amounts of IFN- γ produces encephalitogenic cells of either the Th2 or Th2Hi5 type that cause the classical or nonclassical forms of EAE, respectively. Indeed, the Th2Hi5 adoptively transferred cells are highly pathogenic and lead to 100% mortality as opposed to about 30% mortality in Th2 cell-transferred mice. Because IL-5 levels constitute the only difference that we could consistently detect among cytokines produced by Th2 and Th2Hi5 cells, it is conceivable that the two disease outcomes are related to the IL-5 levels produced by both subpopulations, although the effect of other molecules, such as chemokines, is also likely. Chu et al. (42) speculated that the severity of the IFN- γ -knockout EAE phenotype was due to a failure to suppress expansion of the activated T cell infiltrate, but the distinct clinical manifestation of our classical vs nonclassical disease indicates that actual infiltrate location could play a stronger role in the severity of the disease. Indeed, histological analysis of the CNS of adoptively transferred mice revealed a severe inflammation in the cerebella of Th2Hi5 transferred mice vs Th1 or Th2 (Fig. 6C). Spinal cords had inflammatory lesions in all three transfer groups, which could be expected because tail strength was somewhat diminished in the Th2Hi5 transferred mice, indicating that there was a reduced, but substantial, spinal cord component to the disease (Fig. 6C). Factors such as chemokines or chemokine receptors on both populations or in the CNS may play a role in determining the spatial distribution of the lesions. Tran et al. (41) demonstrated disparate production of several chemokines in IFN- γ and IFN- γ R knockout EAE in C57BL/6 vs IFN- γ -competent, susceptible SJL mice. In addition, Salazaar-Mather (43) elucidated the important role IFN- γ plays in chemokine up-regulation and recruitment of particular inflammatory components of the immune system.

Although the encephalitogenic clone from which the MBP-specific TCR-transgenic mice were made was a Th1 clone (24), naive T cells from specific pathogen-free-kept MBP-specific TCR-transgenic mice develop a Th2 cytokine profile upon stimulation with MBP peptide alone. This occurs over a wide range of peptide concentrations, with Th1 preponderance seen only upon stimulation with a very low concentration of MBP peptide. Moreover, the C57BL genetic background of our transgenic mice is considered Th1 prone. Thus, in the situation in which a T cell tenaciously develops a Th2 profile even in the presence of small amounts of IFN- γ , neutralization of IFN- γ leads to a high IL-5 production with no change or a small reduction in the IL-4 levels. How many T cells behave like the MBP-specific cells that we studied is not known. However, our preliminary observations on a DO 11.10 TCR-transgenic BALB/c system indicate that while the increase in IL-5 is smaller than that observed in the MBP TCR-transgenic system, the ratio between IL-4 and IL-5 production is altered by IFN- γ neutralization in a manner similar to that seen with MBP-specific T cells (data not shown).

The development of Th1 and Th2 type cells is a well-established phenomenon, and the modulation of those responses provides targets for therapeutic intervention. We have shown here that there are other levels of complexity within these phenotypes and that these subpopulations can have biological relevance.

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References

- Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
- Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.
- O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275.
- Constant, S. L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4⁺ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* 15:297.
- Fitch, F. W., M. D. McKisic, D. W. Lancki, and T. F. Gajewski. 1993. Differential regulation of murine T lymphocyte subsets. *Annu. Rev. Immunol.* 11:29.
- Lafaille, J. J. 1998. The role of helper T cell subsets in autoimmune diseases. *Cytokine Growth Factor Rev.* 9:139.
- Nakamura, T., Y. Kamogawa, K. Bottomly, and R. A. Flavell. 1997. Polarization of IL-4- and IFN- γ -producing CD4⁺ T cells following activation of naive CD4⁺ T cells. *J. Immunol.* 158:1085.
- Romagnani, S. 1994. Lymphokine production by human T cells in disease states. *Annu. Rev. Immunol.* 12:227.
- Bucy, R. P., L. Karr, G. Q. Huang, J. Li, D. Carter, K. Honjo, J. A. Lemons, K. M. Murphy, and C. T. Weaver. 1995. Single cell analysis of cytokine gene coexpression during CD4⁺ T-cell phenotype development. *Proc. Natl. Acad. Sci. USA* 92:7565.
- Kelso, A. 1995. Th1 and Th2 subsets: paradigms lost? *Immunol. Today* 16:374.
- Kamogawa, Y., L. A. Minasi, S. R. Carding, K. Bottomly, and R. A. Flavell. 1993. The relationship of IL-4- and IFN- γ -producing T cells studied by lineage ablation of IL-4-producing cells. *Cell* 75:985.
- Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362:245.
- Noben-Trauth, N., P. Kropf, and I. Muller. 1996. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science* 271:987.
- Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186:1737.
- Hogan, S. P., K. I. Matthaei, J. M. Young, A. Koskinen, I. G. Young, and P. S. Foster. 1998. A novel T cell-regulated mechanism modulating allergen-induced airways hyperactivity in BALB/c mice independently of IL-4 and IL-5. *J. Immunol.* 161:1501.
- Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, et al. 1996. IL-5-deficient mice have a developmental defect in CD5⁺ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 4:15.
- Bohjanen, P. R., M. Okajima, and R. J. Hodes. 1990. Differential regulation of interleukin 4 and interleukin 5 gene expression: a comparison of T-cell gene induction by anti-CD3 antibody or by exogenous lymphokines. *Proc. Natl. Acad. Sci. USA* 87:5283.
- Sewell, W. A., and H. H. Mu. 1996. Dissociation of production of interleukin-4 and interleukin-5. *Immunol. Cell Biol.* 74:274.
- Karlen, S., V. A. Mordvinov, and C. J. Sanderson. 1996. How is expression of the interleukin-5 gene regulated? *Immunol. Cell Biol.* 74:218.
- Walker, C., E. Bode, L. Boer, T. T. Hansel, K. Blaser, and J. C. Virchow, Jr. 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146:109.
- Lafaille, J. J., K. Nagashima, M. Katsuki, and S. Tonegawa. 1994. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* 78:399.
- Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itoharu, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, et al. 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. [Published erratum appears in 1992 *Nature* 360:491.] *Nature* 360:225.
- Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 259:1739.
- Baron, J. L., J. A. Madri, N. H. Ruddle, G. Hashim, and C. A. Janeway, Jr. 1993. Surface expression of α_4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* 177:57.

25. Prud'homme, G. J., D. H. Kono, and A. N. Theofilopoulos. 1995. Quantitative polymerase chain reaction analysis reveals marked overexpression of interleukin-1 β , interleukin-1 and interferon- γ mRNA in the lymph nodes of lupus-prone mice. *Mol. Immunol.* 32:495.
26. Ho, I. C., M. R. Hodge, J. W. Rooney, and L. H. Glimcher. 1996. The proto-oncogene *c-maf* is responsible for tissue-specific expression of interleukin-4. *Cell* 85:973.
27. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587.
28. Zhang, D. H., L. Yang, and A. Ray. 1998. Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. *J. Immunol.* 161:3817.
29. Agarwal, S., O. Avni, and A. Rao. 2000. Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. *Immunity* 12:643.
30. Gajewski, T. F., and F. W. Fitch. 1990. Anti-proliferative effect of IFN- γ in immune regulation. IV. Murine CTL clones produce IL-3 and GM-CSF, the activity of which is masked by the inhibitory action of secreted IFN- γ . *J. Immunol.* 144:548.
31. Gett, A. V., and P. D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl. Acad. Sci. USA* 95:9488.
32. Bird, J. J., D. R. Brown, A. C. Mullen, N. H. Moskowitz, M. A. Mahowald, J. R. Sider, T. F. Gajewski, C. R. Wang, and S. L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity* 9:229.
33. Wynn, T. A., D. Jankovic, S. Hieny, K. Zioncheck, P. Jardieu, A. W. Cheever, and A. Sher. 1995. IL-12 exacerbates rather than suppresses T helper 2-dependent pathology in the absence of endogenous IFN- γ . *J. Immunol.* 154:3999.
34. Jung, T., U. Schauer, C. Rieger, K. Wagner, K. Einsle, C. Neumann, and C. Heusser. 1995. Interleukin-4 and interleukin-5 are rarely co-expressed by human T cells. *Eur. J. Immunol.* 25:2413.
35. Cohn, L., R. J. Homer, H. MacLeod, M. Mohrs, F. Brombacher, and K. Bottomly. 1999. Th2-induced airway mucus production is dependent on IL-4R α , but not on eosinophils. *J. Immunol.* 162:6178.
36. Pernis, A., S. Gupta, K. J. Gollob, E. Garfein, R. L. Coffman, C. Schindler, and P. Rothman. 1995. Lack of interferon- γ receptor β chain and the prevention of interferon- γ signaling in Th1 cells. *Science* 269:245.
37. Bach, E. A., S. J. Szabo, A. S. Dighe, A. Ashkenazi, M. Aguet, K. M. Murphy, and R. D. Schreiber. 1995. Ligand-induced autoregulation of IFN- γ receptor β chain expression in T helper cell subsets. *Science* 270:1215.
38. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* 12:27.
39. Ranganath, S., W. Ouyang, D. Bhattacharya, W. C. Sha, A. Grupe, G. Peltz, and K. M. Murphy. 1998. GATA-3-dependent enhancer activity in IL-4 gene regulation. *J. Immunol.* 161:3822.
40. Lafaille, J. J., F. V. Keere, A. L. Hsu, J. L. Baron, W. Haas, C. S. Raine, and S. Tonegawa. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med.* 186:307.
41. Tran, E. H., E. N. Prince, and T. Owens. 2000. IFN- γ shapes immune invasion of the central nervous system via regulation of chemokines. *J. Immunol.* 164:2759.
42. Chu, C. Q., S. Wittmer, and D. K. Dalton. 2000. Failure to suppress the expansion of the activated CD4 T cell population in interferon γ -deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 192:123.
43. Salazar-Mather, T. P., T. A. Hamilton, and C. A. Biron. 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J. Clin. Invest.* 105:985.