

# Altered IL-4 mRNA Stability Correlates with Th1 and Th2 Bias and Susceptibility to Hypersensitivity Pneumonitis in Two Inbred Strains of Mice<sup>1</sup>

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Previously, we have shown in a model of hypersensitivity pneumonitis that Th1-biased C57BL/6 mice are susceptible and Th2-biased DBA/2 mice are resistant to disease. We also showed that this was explained in part by differential regulation of IL-12 by IL-4. For these reasons, we postulated that C57BL/6 and DBA/2 mice differentially express IL-4. In this study, we show that C57BL/6 immune cells express Th2 but not Th1 cytokines at lower levels than DBA/2 cells. We also found that C57BL/6 splenocytes exhibit decreased mRNA stability of Th2 cytokines, relative to DBA/2 splenocytes. Stability of IL-2 and IFN- $\gamma$  were similar in the two strains of mice. Differences in Th2 cytokine mRNA stability between C57BL/6 and DBA/2 cells were not due to sequence polymorphism at specific regions of the IL-4/IL-13 locus. Furthermore, expression of Th1- and Th2-specific transcription factors T-bet and GATA-3, as well as the nuclear factor of activated T cells transcription factor, NFATc, was not significantly different between the two mice. Our data suggest that decreased mRNA stability of Th2 cytokines in C57BL/6 splenocytes may underlie the differential susceptibility to hypersensitivity pneumonitis between C57BL/6 and DBA/2 mice. Moreover, our results indicate that regulation of mRNA stability may serve as an important mechanism underlying Th1/Th2 immune polarization. *The Journal of Immunology*, 2002, 169: 3700–3709.

**H**ypersensitivity pneumonitis (HP)<sup>3</sup> is an inflammatory lung disease caused by repeated inhalation of an organic Ag (1, 2). Although exposure to these Ags is widespread, the number of individuals that actually manifest HP is quite low. Studies have demonstrated that only 5–10% of an exposed group develops clinical disease (2). As in humans, there exist strains of mice that are HP-resistant and HP-sensitive. We have previously categorized two such inbred mouse strains, C57BL/6 and DBA/2, as HP-susceptible and HP-resistant, respectively (3). We also showed that susceptibility to HP was explained in part by differential regulation of IL-12 by IL-4 (3). This observation suggested that Th1/Th2 polarization of the immune response following Ag exposure determines susceptibility to the disease.

Th1-biased CD4<sup>+</sup> T cells express high levels of IFN- $\gamma$ , IL-2, lymphotoxin, and TNF- $\alpha$ , which activate macrophages to orchestrate a robust cell-mediated immune response. By comparison, Th2-biased CD4<sup>+</sup> T cells express IL-4, IL-10, IL-5, and IL-13, and interact with B cells to generate strong humoral immune responses

(4, 5). Th1 and Th2 CD4<sup>+</sup> T cells each develop from the same naive precursor cells, and early observations suggested that they could be directed toward Th1 or Th2 lineage in vitro by the addition of exogenous cytokines (6). IL-12 induces T cells to secrete IFN- $\gamma$  and differentiate via the Th1 lineage, while IL-4 stimulates its own synthesis, and polarizes T cells to commit to the Th2 lineage. In addition, IL-12 and IL-4 have been shown to cross-regulate Th1 and Th2 CD4<sup>+</sup> T cells by inhibiting specific developmental pathways (7). For these reasons, it is widely accepted that IL-12 and IL-4 are “master signal” cytokines for Th1/Th2 polarization.

Previously, we have shown that the immunopathology of HP is more severe when the Th1/Th2 balance is shifted toward a Th1 response (8–10), and less severe when shifted toward a Th2 response (8). Because Th1-biased C57BL/6 mice and Th2-biased DBA/2 mice are susceptible and resistant to experimental HP, respectively, and IL-4 is a key signal for the development of Th2 immune responses, we hypothesized that IL-4 is differentially regulated and expressed among C57BL/6 mice and DBA/2 mice. In this report, we determined whether splenocytes and CD4<sup>+</sup> T cells from HP-sensitized and immunologically naive C57BL/6 and DBA/2 mice expressed different amounts of IL-4 protein and mRNA. Our findings suggest that the capability to express IL-4 and other Th2 cytokines is greater in DBA/2 cells than in C57BL/6 cells. There were no differences in expression of Th1 cytokines. We also found that this difference in expression of Th2 cytokines results at least in part from differences in IL-4 mRNA stability.

## Materials and Methods

### Mice

Six- to 8-wk-old female DBA/2 and C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Mice were housed in a pathogen-free environment at the Animal Care Facility at the University of Iowa (Iowa City, IA), and maintained on standard mouse chow and water ad libitum. All procedures used in this study were in compliance with Animal

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<sup>3</sup> Abbreviations used in this paper: HP, hypersensitivity pneumonitis; C<sub>t</sub>, threshold cycle; Act D, actinomycin D; UTR, untranslated region; CNS-1, conserved noncoding sequence-1; ARE, AU-rich element; *Tappr*, T cell airway phenotype regulator.

Welfare Act regulations, and with the Guide for the Care and Use of Laboratory Animals.

### Abs and reagents

Recombinant murine IL-2 was purchased from Roche Molecular Biochemicals (Indianapolis, IN), and recombinant murine cytokines IL-4, IL-12, and IFN- $\gamma$  were purchased from R&D Systems (Minneapolis, MN). mAbs against murine CD3 $\epsilon$  (145-2C11) and CD28 (37.51) were purchased from BD PharMingen (San Diego, CA). Hamster IgG1 (A19-3) and hamster IgG2 (Ha4/8) were obtained from BD PharMingen and used as isotype control Abs for CD3 $\epsilon$  and CD28, respectively. Monoclonal and polyclonal Abs against murine NFATc (7A6), GATA-3 (H-48), and T-bet (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Con A, actinomycin D (Act D), PMA, and ionomycin were all purchased from Sigma-Aldrich (St. Louis, MO). Neutralizing Abs directed against mouse IL-4, IFN- $\gamma$ , and IL-12 were purchased from R&D Systems.

### Cell culture

Spleens were aseptically removed from animals and then teased apart between the frosted edges of two glass slides. Cells were suspended in RBC lysis solution (1 mM KHCO<sub>3</sub>, 15.5 mM NH<sub>4</sub>Cl), washed in 1  $\times$  PBS, pelleted, filtered through a 70- $\mu$ m cell strainer (Falcon, Franklin Lakes, NJ), and resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (Life Technologies), 50  $\mu$ M 2-ME (Sigma-Aldrich), and 80  $\mu$ g/ml gentamycin. Cells were cultured with or without stimulation at 37°C in 5% CO<sub>2</sub> for the indicated time periods.

### T cell purification and in vitro differentiation

Single-cell suspensions were prepared from splenocytes depleted of RBCs. CD4<sup>+</sup> T cells were subsequently isolated to >97% purity with positive selection using anti-CD4 (L3T4) magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. For in vitro differentiation assays, CD4<sup>+</sup> T cells were stimulated for 2 days with 1  $\mu$ g/ml plate-bound anti-CD3 $\epsilon$  and 1  $\mu$ g/ml plate-bound anti-CD28 mAbs in the presence of 5 ng/ml IL-12 and 10  $\mu$ g/ml anti-IL-4 for Th1 differentiation, or in the presence of 10 ng/ml IL-4, 1  $\mu$ g/ml anti-IL-12, and 10  $\mu$ g/ml anti-IFN- $\gamma$  for Th2 differentiation. Three days after primary stimulation, cells were washed and then further cultured in the presence of IL-2 (20 U/ml), IL-12, anti-IL-4 (Th1 polarized), or IL-2 (20 U/ml), IL-4, anti-IL-12, and anti-IFN- $\gamma$  (Th2 polarized) for a total of 7 days. Fresh cytokines and Ab were added every 2 days. On day 7, cells were harvested and dead cells were removed by centrifugation through Fico-Lite density gradient (Atlanta Biologicals, Norcross, GA). Viable cells were washed twice in PBS, counted, and restimulated at 5  $\times$  10<sup>5</sup> cells/ml in the presence of 500 ng/ml ionomycin and 5 ng/ml PMA, or PBS control. Cells were harvested after 4 h for RNA isolation, while parallel cell culture supernatants were harvested after 24 h for ELISA. To assess GATA-3 and T-bet expression, T cells were polarized similarly.

### ELISA and ELISPOT

Murine IL-4, IL-13, and IFN- $\gamma$  were specifically detected in cell culture supernatants by use of ELISA DuoKits (IL-4 and IFN- $\gamma$ ) and an ELISA Quantikine kit (IL-13), all purchased from R&D Systems. Numbers of IL-4-producing cells were determined with an IL-4 ELISPOT kit from BD PharMingen following the manufacturer's instructions. Briefly, plates were precoated with anti-mouse IL-4 (clone B11B, 5  $\mu$ g/ml) overnight. Serial dilutions (2-fold) of splenocytes (from 2  $\times$  10<sup>5</sup>–5  $\times$  10<sup>4</sup>) were cultured in ELISPOT wells in the presence of media alone, 2.5  $\mu$ g/ml Con A, or 5 ng/ml PMA and 500  $\mu$ g/ml ionomycin for 20–24 h. For CD4<sup>+</sup> T cells, 2.5  $\mu$ g/ml anti-CD3 $\epsilon$  and 2.5  $\mu$ g/ml anti-CD28 mAbs, or isotype control Abs, were coimmobilized with 5  $\mu$ g/ml IL-4 capture Ab on the ELISPOT membranes overnight. Serial dilutions (2-fold) of purified CD4<sup>+</sup> T cells (from 5  $\times$  10<sup>4</sup>–1.25  $\times$  10<sup>3</sup>) were cultured in ELISPOT wells for 20–24 h. Biotinylated anti-mouse IL-4 (2  $\mu$ g/ml) was used to specifically detect captured IL-4. Spots were visualized with Avidin-HRP enzyme and 3-amino-9-ethyl-carbazole substrate. Results were recorded and analyzed with ImmunoSpot Series 1 Analyzer (CTL Analyzers, Cleveland, OH).

### Western blotting

For isolation of nuclear proteins, cells were harvested, washed two times in 1  $\times$  PBS, and then resuspended in ice-cold lysis buffer A (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl<sub>2</sub>) supplemented with protease and phosphatase inhibitors. Nuclei were incubated on ice for 15 min after which 0.5% Nonidet P-40 was added. Suspensions were gently vortexed for 15 s, and nuclei were pelleted at 4°C at 14,000 rpm for 30 s. Nuclei

were resuspended in lysis buffer C (50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% (v/v) glycerol) supplemented with protease and phosphatase inhibitors and then incubated on ice for 20 min with occasional vortexing. Debris was pelleted at 4°C at 14,000 rpm for 5 min and nuclear protein was transferred to a fresh tube, quantitated with Protein Assay Standard kit (Bio-Rad, Hercules, CA), aliquoted, and stored at –80°C. For each sample, 10  $\mu$ g of nuclear protein was fractionated on a 10% SDS-PAGE gel at 30 mA for 3 h. Proteins were transferred to nitrocellulose (ECL; Amersham, Arlington Heights, IL) for 30 min at 20 V on SemiDry Transfer Cell (Bio-Rad). Membranes were stained with Ponceau S (Sigma-Aldrich) to confirm equal loading, and then blocked with 5% milk in TBS with 0.1% Tween 20 (TTBS) for 1 h. Membranes were incubated for 1 h in the presence of primary Abs (diluted between 1/200 and 1/5000 in 5% milk in TTBS), washed four times with TTBS and then incubated for 1 h with the appropriate HRP-conjugated secondary Ab (diluted between 1/5000 and 1/10,000 in 5% milk in TTBS). Membranes were washed four times in TTBS. Immunoreactive bands were developed using ECL Plus chemiluminescent substrate (Amersham) and exposed to BioMax film (Eastman Kodak, Rochester, NY).

### Real-time RT-PCR

Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. RNA was quantitated using RiboGreen kit (Molecular Probes, Eugene, OR). Total RNA (1  $\mu$ g) was reverse transcribed to cDNA using RETROscript RT-PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting cDNA was subjected to PCR as follows. In a 0.2-ml PCR tube (Bio-Rad), 2  $\mu$ l of cDNA was added to 48  $\mu$ l of PCR mixture containing 160  $\mu$ M of each dNTP (Invitrogen, Carlsbad, CA), 3.0 mM MgCl<sub>2</sub> (Invitrogen), 1/15,000 SYBR Green I DNA dye (Molecular Probes), 0.2  $\mu$ M of each sense and antisense primer (Research Genetics, Huntsville, AL), and 2.5 U of Platinum Taq DNA Polymerase (Invitrogen). Amplification was then performed in an iCycler iQ Fluorescence Thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s 60°C, 20 s at 72°C, and 10 s at 3°C below the melting temperature for each amplimer. Fluorescence data were captured during the 10-s dwell to ensure that primer dimers were not contributing to the fluorescence signal generated with SYBR Green I DNA dye. Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by iCycler iQ software (Bio-Rad) and expressed as a function of threshold cycle (C<sub>t</sub>), the cycle at which the fluorescence intensity in a given reaction tube rises above background (calculated as 10  $\times$  mean SD of fluorescence in all wells over the baseline cycles). Specific primer sets used for murine cytokines and housekeeping gene are as follows (5' to 3'); IL-4 sense, GGTCTCAACCCAGCTAGT; IL-4 antisense, TGATGCTCTTTAGGCTTTCCA; IL-13 sense, ACAGCTCCCTGGTCTCTCA; IL-13 antisense, CCCCATTCACTACACATCA; IL-2 sense, AGCTTACAGCGGAAGCACA; IL-2 antisense, CTCCTCA GAAAGTCCACCAC; IFN- $\gamma$  sense, CGCTACACACTGCATCTTGG; IFN- $\gamma$  antisense, TGAGTCTATTGAATGCTTGG; hypoxanthine phosphoribosyltransferase sense, CCTCATGGACTGATTATGGAC; hypoxanthine phosphoribosyltransferase antisense, CAGATTCACCTTGGCTCATC. Primers were selected based on nucleotide sequences downloaded from the National Center for Biotechnology Information data bank and designed with software by S. Rozen and H. J. Skaletsky (1998 Primer3; code available at [http://www.genome.wi.mit.edu/genome\\_software/other/primer3.html](http://www.genome.wi.mit.edu/genome_software/other/primer3.html)).

### Relative quantitative gene expression

Relative quantitative gene expression was calculated as follows: for each sample assayed, the C<sub>t</sub> for reactions amplifying a gene of interest and a housekeeping gene were determined. The gene of interest C<sub>t</sub> for each sample was corrected by subtracting the C<sub>t</sub> for the housekeeping gene ( $\Delta$ C<sub>t</sub>). Untreated controls were chosen as the reference samples, and the  $\Delta$ C<sub>t</sub> for all experimental samples was subtracted by the  $\Delta$ C<sub>t</sub> for the control samples ( $\Delta\Delta$ C<sub>t</sub>). Finally, experimental mRNA abundance, relative to control mRNA abundance, was calculated by the formula 2<sup>– $\Delta\Delta$ C<sub>t</sub></sup>. Validity of this approach was confirmed by using serial 10-fold dilutions of template for all amplimers examined in this study. Using the 10-fold dilutions, the amplification efficiencies for each gene of interest and housekeeping amplimers were found to be identical.

### mRNA stability

Splenocytes were stimulated with 2.5  $\mu$ g/ml Con A for the times indicated and then treated with 5  $\mu$ g/ml Act D. Cells were harvested and RNA was isolated immediately ( $t = 0$ ) or at hourly intervals up to 3 h ( $t = 3$ ) after

addition of Act D. Cytokine stability was assessed by using real-time RT-PCR to quantify cytokine mRNA abundance at each time point, relative to cytokine mRNA abundance at  $t = 0$ .

### DNA sequencing

The 3' untranslated region (UTR) of murine *il4*, 839 bp of the proximal *il4* promoter, 214 bp of conserved noncoding sequence-1 (CNS-1) within the *il4/il13* locus, and a recently identified *il4* 3' distal enhancer element were amplified from genomic DNA from both C57BL/6 and DBA/2 mice. Specific primers used for amplification and sequencing are as follows (5' to 3'): *il4* promoter forward, GCTCCTGACCTCCACACTGATG; *il4* promoter reverse, TGACAAC TAGCTGGGGGTTGA; murine CNS-1 forward, CTCCGGATATCCCTGGAAGT; murine CNS-1 reverse, AGGCTGAGTGTCCCTGACCAT; *il4* distal 3' enhancer forward, TTAAACCATGCACACATGCTC; *il4* distal 3' enhancer reverse, AGGTCCTGATGCCACCTTAG; *il4* 3' UTR forward, CCTGGAAAGCCTAAAGAGCA; *il4* 3' UTR reverse, CCTTATGGCAAATGAAGTGTC. PCR product was purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA) and directly sequenced by the University of Iowa DNA Core.

### Statistics

Statistical analysis was done with unpaired (two-tailed) *t* tests. Values in figures are expressed as mean  $\pm$  SEM. Values of  $p < 0.05$  were considered significant and are indicated by an asterisk in figures.

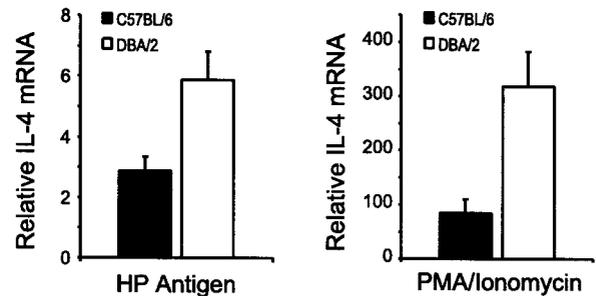
## Results

### Evidence of lower IL-4 expression in ex vivo-activated splenocytes and lungs of C57BL/6 HP-sensitized mice

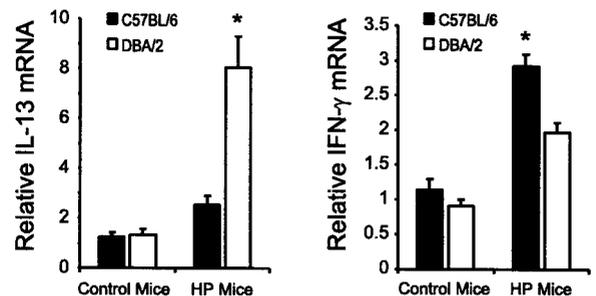
Th2-biased DBA/2 mice are resistant to HP (3) and HP-susceptible C57BL/6 mice become resistant to disease when the adaptive immune response to HP Ags is shifted in vivo toward Th2 (8). Furthermore, the cytokine IL-4 is a key potentiator of Th2 adaptive immune responses (11). For these reasons, we hypothesized that IL-4 is differentially expressed between C57BL/6 and DBA/2 mice and the expression of IL-4 would be inversely correlated with the development of HP. To test this hypothesis, we first quantified the expression of IL-4 mRNA in ex vivo-activated splenocytes from C57BL/6 and DBA/2 mice sensitized with HP Ag in a previously described model of experimental HP (3). Following 3 wk of Ag sensitization, splenocytes from HP-sensitized and saline control mice were isolated and activated ex vivo with the HP Ag, PMA and ionomycin, or media alone for 24 h. IL-4 mRNA expression was then assessed with real-time RT-PCR as described in *Materials and Methods*. As shown in Fig. 1A, splenocytes from HP-sensitized C57BL/6 mice expressed less IL-4 in response to both the HP Ag and PMA and ionomycin when compared with splenocytes from DBA/2 HP-sensitized mice.

In addition to examining IL-4 expression from ex vivo-activated splenocytes, we also attempted to define Th1/Th2 cytokine profiles in the lungs of HP-sensitized mice (Fig. 1B). IL-4 mRNA expression in the lungs of C57BL/6 and DBA/2 mice was undetectable after 3 wk of HP Ag sensitization. However, levels of another Th2 cytokine, IL-13, were 2.5 times lower in the C57BL/6 HP-sensitized lungs as compared with DBA/2 HP-sensitized lungs ( $p = 0.036$ ). When we measured IFN- $\gamma$  expression, a marker of Th1 polarization, mRNA levels were higher in the lungs of the C57BL/6 HP-sensitized mice ( $p = 0.030$ ). Taken together, the results in Fig. 1 suggest that splenocytes from HP-sensitized C57BL/6 mice express less IL-4 compared with splenocytes from HP-sensitized DBA/2 mice. Furthermore, these results confirm the previous observations that the immune response in the lungs of HP-susceptible C57BL/6 mice is relatively Th1-biased, and the immune response in the lungs of HP-resistant DBA/2 mice is relatively Th2-biased.

## A ex vivo Activation of Splenocytes



## B Total Lung RNA

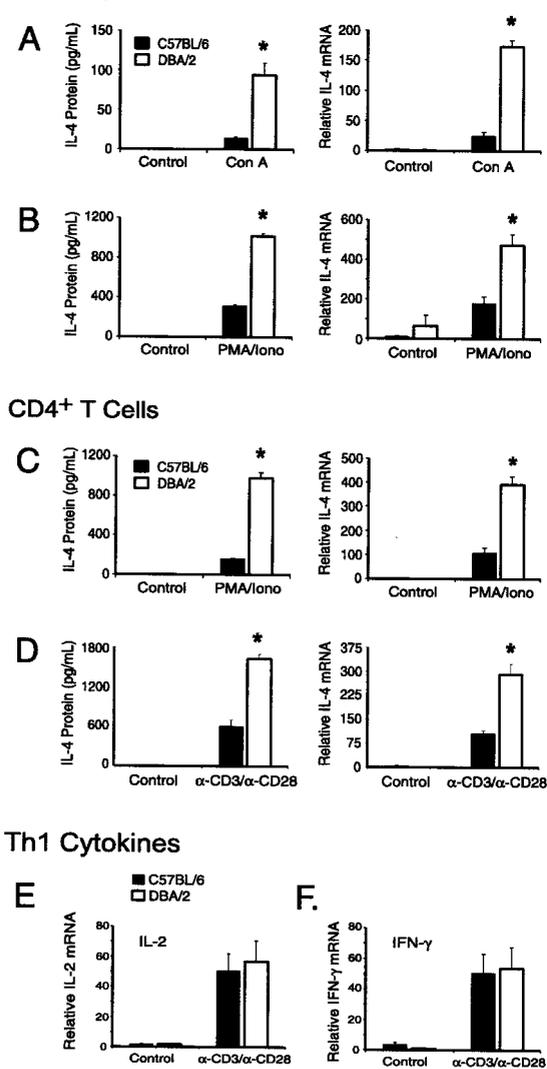


**FIGURE 1.** DBA/2 mice express higher levels of IL-4 during experimental HP. *A*, Splenocytes were isolated from HP-sensitized C57BL/6 and DBA/2 mice and activated ex vivo with HP Ag (left panel) or PMA and ionomycin (right panel) for 24 h. Relative IL-4 mRNA was quantitated with real-time RT-PCR, as described in *Materials and Methods*, and expressed as fold increase in IL-4 mRNA relative to unstimulated control cells. Data represent three replicate analyses of splenocytes pooled from three mice in each group. *B*, Total RNA was isolated from lungs of HP-sensitized C57BL/6 and DBA/2 mice and analyzed for the expression of IL-13 (left panel) and IFN- $\gamma$  (right panel) using real-time RT-PCR. Data are expressed as mean  $\pm$  SEM for three independent experiments (\*,  $p < 0.05$ ).

### Splenocytes and CD4<sup>+</sup> T cells from C57BL/6 mice express less IL-4 protein and mRNA

Because we found evidence that IL-4 mRNA is expressed at lower levels in HP-sensitized C57BL/6 splenocytes, relative to HP-sensitized DBA/2 splenocytes (Fig. 1A), we next quantified the expression of IL-4 mRNA and protein in activated splenocytes and CD4<sup>+</sup> T cells from immunologically naive C57BL/6 and DBA/2 mice. As shown in Fig. 2A, when splenocytes from C57BL/6 mice were stimulated with Con A for 24 h, they produced  $\sim 15$  pg/ml IL-4 protein and a 22-fold increase in IL-4 mRNA, relative to unstimulated control cells. In contrast, splenocytes from DBA/2 mice produced  $\sim 95$  pg/ml of IL-4 protein and a 175-fold increase in IL-4 mRNA when activated with Con A for 24 h. The differential expression of IL-4 from splenocytes was not limited to activation with Con A; when identical experiments were performed with PMA and ionomycin as the stimuli, the results closely mirrored the results with Con A. As shown in Fig. 2B, PMA and ionomycin-treated splenocytes from C57BL/6 mice secrete 350 pg/ml of IL-4 protein and express 200-fold increases in IL-4 mRNA, while DBA/2 splenocytes secrete 1000 pg/ml of protein and express 450-fold increases in mRNA. Flow cytometry verified that there were no significant differences in proportionate numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells present in splenocyte preparations obtained from each strain of mouse (data not shown). Furthermore,

## Splenocytes



**FIGURE 2.** Splenocytes and CD4<sup>+</sup> T cells from C57BL/6 mice produce less of the Th2-associated cytokine IL-4 relative to DBA/2 mice. *A*, Splenocytes were isolated from naive C57BL/6 and DBA/2 mice and cultured ( $2 \times 10^6$  cells/ml) in the presence of 2.5  $\mu$ g/ml Con A or PBS control for 24 h, after which cells and cell supernatants were harvested for analysis. IL-4 protein was measured directly from cell culture supernatants by ELISA, while real-time RT-PCR was used to quantitate relative IL-4 mRNA expression, as described in *Materials and Methods*. *B*, Same as *A* except splenocytes were activated with 5 ng/ml PMA and 500 ng/ml ionomycin for 24 h. *C*, Purified CD4<sup>+</sup> T cells were isolated with positive selection from naive C57BL/6 and DBA/2 mice and then stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin for 24 h. IL-4 protein and IL-4 mRNA was measured as described above. *D*, Same as *C* except CD4<sup>+</sup> T cells were activated with 1  $\mu$ g/ml plate-bound  $\alpha$ -CD3 and 1  $\mu$ g/ml  $\alpha$ -CD28 mAbs for 24 h. *E*, CD4<sup>+</sup> T cells from C57BL/6 and DBA/2 mice were isolated with positive selection and then stimulated for 24 h with 1  $\mu$ g/ml plate-bound  $\alpha$ -CD3 and 1  $\mu$ g/ml  $\alpha$ -CD28 mAbs. Total RNA was isolated at the end of the time period and relative expression of IL-2 was assessed with real-time RT-PCR. *F*, Same as in *E* except total RNA was analyzed for the expression of IFN- $\gamma$  mRNA with real-time RT-PCR. Data are expressed as mean  $\pm$  SEM for three independent experiments (\*,  $p < 0.05$ ).

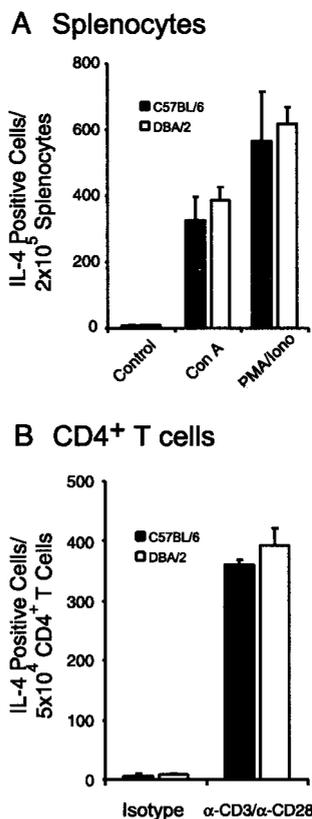
there were no detectable differences in cell viability among C57BL/6 and DBA/2 splenocytes and CD4<sup>+</sup> T cells, as measured by trypan blue exclusion at each stage of experimentation (data not shown).

To determine whether the differences in IL-4 production in our system were influenced by the presence of APCs (macrophages, dendritic cells, and B cells) in the splenocyte pool, we purified CD4<sup>+</sup> T cells and activated them with both PMA and ionomycin or immobilized  $\alpha$ -CD3 and  $\alpha$ -CD28 mAbs for 24 h. As shown in Fig. 2*C*, purified CD4<sup>+</sup> T cells from C57BL/6 mice produce significantly less IL-4 relative to DBA/2 CD4<sup>+</sup> T cells when treated with PMA and ionomycin (C57BL/6 mice express  $\sim$ 85% less IL-4 protein ( $p = 0.002$ ) and  $\sim$ 75% less IL-4 mRNA ( $p = 0.014$ ) as when compared with CD4<sup>+</sup> T cells from DBA/2 mice). Furthermore, when CD4<sup>+</sup> T cells from C57BL/6 mice are activated with plate-bound  $\alpha$ -CD3 and  $\alpha$ -CD28, they express  $\sim$ 65% less IL-4 protein ( $p = 0.005$ ) and  $\sim$ 73% less IL-4 mRNA ( $p = 0.019$ ) compared with DBA/2 CD4<sup>+</sup> T cells (Fig. 2*D*).

Because we observed marked differences in the level of IL-4 expression between C57BL/6 and DBA/2 CD4<sup>+</sup> T cells, we next determined if there were differences in the level of expression of the Th1-associated cytokines IFN- $\gamma$  and IL-2. To do this, we analyzed Th1 cytokine expression in purified CD4<sup>+</sup> T cells activated with immobilized mAbs against CD3 and CD28. We found no significant differences in the expression of either IL-2 ( $p = 0.783$ ) or IFN- $\gamma$  mRNA ( $p = 0.591$ ; Fig. 2, *E* and *F*) or protein (data not shown) between CD4<sup>+</sup> T cells from naive C57BL/6 and DBA/2 mice. These results show that activated CD4<sup>+</sup> T cells from C57BL/6 mice express lower levels of IL-4 mRNA and protein, as compared with CD4<sup>+</sup> T cells from DBA/2 mice (which we will hereafter refer to as the IL-4<sup>low</sup> and IL-4<sup>high</sup> phenotypes in C57BL/6 and DBA/2 mice, respectively). Furthermore, these results show that cytokine production by C57BL/6 and DBA/2 CD4<sup>+</sup> T cells differs for IL-4, but not for the Th1-associated cytokines IFN- $\gamma$  and IL-2.

*No difference in the number of IL-4-secreting cells in the spleens of naive C57BL/6 and DBA/2 mice*

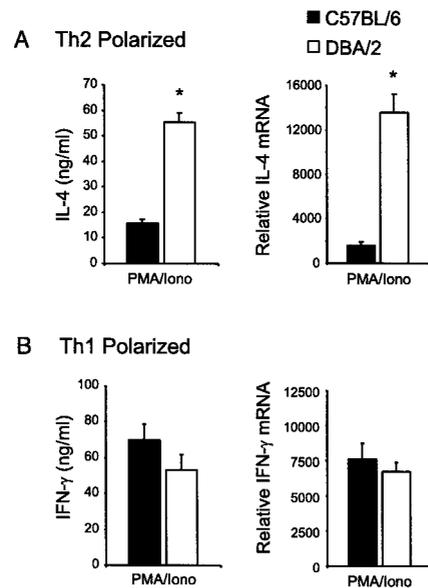
Because the IL-4<sup>high</sup> phenotype could be attributed to the presence of more Th2 memory CD4<sup>+</sup> T cells in the spleens of the DBA/2 mice (12), we next determined whether there were more IL-4-secreting cells in the DBA/2 cell preparations. Because our system examines IL-4 production in response to primary stimulation, relatively little IL-4 protein is expressed. Consequently, levels of IL-4 expression in our primary cell isolates were below the limits of detection for intracellular cytokine staining and FACS analysis. To circumvent the problem of low sensitivity, we used the ELISPOT assay, which has proven sensitive enough to specifically detect one cytokine-producing cell in a pool of 300,000 (13). When splenocytes (Fig. 3*A*) or CD4<sup>+</sup> T cells (Fig. 3*B*) were cultured in IL-4 ELISPOT plates, we found there were no significant differences in the number of IL-4-producing cells between C57BL/6 and DBA/2 mice after stimulation with Con A ( $p = 0.744$ ), PMA and ionomycin ( $p = 0.701$ ), or  $\alpha$ -CD3 and  $\alpha$ -CD28 ( $p = 0.322$ ). When we examined relative spot size between C57BL/6 and DBA/2 wells, an indicator of amount of IL-4 secreted per cell, we found that C57BL/6 spots were consistently smaller than the spots of DBA/2-positive cells (data not shown). These data suggest that there are approximately equal numbers of IL-4-secreting cells in the spleens of immunologically naive C57BL/6 and DBA/2 mice. Furthermore, these data and the data presented in Fig. 2 indicate that less IL-4 is secreted per cell by C57BL/6 cells relative to the DBA/2 cells. Thus, the presence of more Th2-associated memory CD4<sup>+</sup> T cells does not explain the IL-4<sup>high</sup> phenotype in DBA/2 mice.



**FIGURE 3.** No difference in the number of IL-4-producing cells between C57BL/6 and DBA/2 mice. **A**, Splenocytes were isolated from immunologically naive C57BL/6 and DBA/2 mice and activated in ELISPOT plates in the presence of 2.5  $\mu$ g/ml Con A or 5 ng/ml PMA and 500 ng/ml ionomycin for 20–24 h. Plates were developed and analyzed as described in *Materials and Methods*. **B**, CD4<sup>+</sup> T cells were isolated from C57BL/6 and DBA/2 mice and activated with  $\alpha$ -CD3 and  $\alpha$ -CD28, or isotype control mAbs that had been previously immobilized on an ELISPOT plate along with the IL-4 capture Ab. Cells were incubated for 20–24 h and plates were developed and data analyzed as described in *Materials and Methods*. Data are expressed as mean  $\pm$  SEM for three independent experiments.

*Addition of exogenous IL-4 does not rescue the IL-4<sup>low</sup> phenotype observed in C57BL/6 CD4<sup>+</sup> T cells*

Because IL-4 functions as an autocrine factor to stimulate its own synthesis, we tested whether addition of exogenous IL-4 could rescue the IL-4<sup>low</sup> phenotype and increase the expression of IL-4 in C57BL/6 CD4<sup>+</sup> T cells to levels approaching those observed in DBA/2 CD4<sup>+</sup> T cells. To do this, we isolated CD4<sup>+</sup> T cells from each strain of mouse, cultured the cells under conditions of Th2 polarization in the presence of IL-4, anti-IL-12 Ab, and anti-IFN- $\gamma$  Ab for 7 days (as described in *Materials and Methods*), and measured the production of IL-4 protein and mRNA after restimulation with PMA and ionomycin. As shown in Fig. 4A, under Th2-polarizing conditions, CD4<sup>+</sup> T cell cultures from C57BL/6 mice produce ~71% less IL-4 protein ( $p = 0.007$ ) and ~88% less IL-4 mRNA ( $p = 0.016$ ), relative to Th2-driven CD4<sup>+</sup> T cell cultures from DBA/2 mice. As controls, CD4<sup>+</sup> T cells from both C57BL/6 and DBA/2 mice were also polarized toward the Th1 immunophenotype in the presence of IL-12 and anti-IL-4 Ab for 7 days. When the expression of IFN- $\gamma$  was examined in Th1-polarized cultures, we found no significant difference between C57BL/6 and DBA/2 CD4<sup>+</sup> T cells (Fig. 4B). These results demonstrate that addition of exogenous IL-4 to cell cultures does not rescue the C57BL/6 IL-4<sup>low</sup> phenotype.



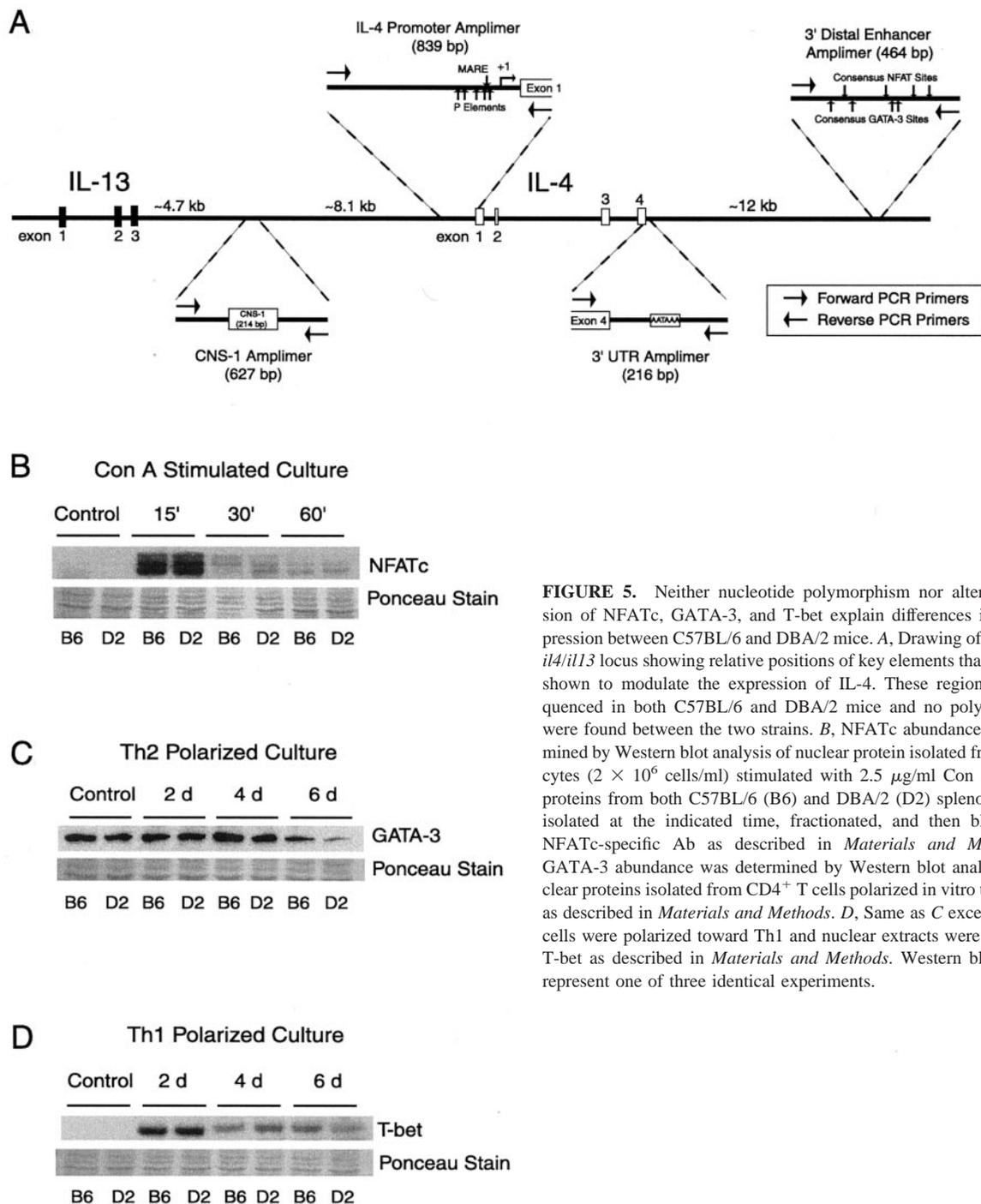
**FIGURE 4.** CD4<sup>+</sup> T cells from C57BL/6 mice do not polarize toward the Th2 immunophenotype to the same degree as CD4<sup>+</sup> T cells from DBA/2 mice. **A**, CD4<sup>+</sup> T cells from naive C57BL/6 and DBA/2 mice were polarized toward Th2 phenotype as described in *Materials and Methods*. After 7 days, cells were washed and then restimulated for 24 h (to assess protein release) or for 4 h (to assess mRNA expression) with 5 ng/ml PMA and 500 ng/ml ionomycin. IL-4 protein release (*left panel*) was measured with ELISA and IL-4 mRNA expression (*right panel*) was measured with real-time RT-PCR as described in *Materials and Methods*. **B**, CD4<sup>+</sup> T cells from naive C57BL/6 and DBA/2 mice were polarized toward Th1 immunophenotype as described in *Materials and Methods*. After 7 days, cells were washed and then restimulated for either 24 or 4 h with 5 ng/ml PMA and 500 ng/ml ionomycin. IFN- $\gamma$  protein (*left panel*) and mRNA (*right panel*) was measured as described above. Data are expressed as mean  $\pm$  SEM for three independent experiments (\*,  $p < 0.05$ ).

*No nucleotide sequence polymorphism at specific *il4/il13* loci or altered expression of NFATc, GATA-3, and T-bet in C57BL/6 and DBA/2 mice*

Differences in IL-4 mRNA expression between C57BL/6 and DBA/2 splenocytes and CD4<sup>+</sup> T cells might be attributed to nucleotide sequence polymorphism at specific regions of the *il4/il13* locus. To explore this possibility, the nucleotide sequence of several regions within *il4/il13* locus were examined, including the *il4* promoter, a 3' enhancer distal to *il4*, a 214-bp span of noncoding sequence conserved among humans and mice, and the 3' UTR of the *il4* gene (Fig. 5A).

Several critical elements in the proximal 250 bp of *il4* promoter have been shown to regulate the transcription of the *il4* gene. These elements include the NFAT family member binding elements P0, P2, and P3, the AP-1/NFAT binding elements P1 and P4 (14), and the c-Maf response element (15). To rule out the possibility that nucleotide polymorphism within the proximal *il4* promoter is responsible for the IL-4<sup>low</sup> phenotype in C57BL/6 CD4<sup>+</sup> T cells, we sequenced 839 bp of the *il4* promoter in both C57BL/6 and DBA/2 mice. Direct sequencing of a PCR product encompassing nucleotides -755 to +84 of the *il4* promoter revealed no difference between C57BL/6 and DBA/2 mice (data not shown).

A putative regulatory sequence located ~12 kb 3' to *il4* was identified with DNase hypersensitivity analysis (16). Furthermore, inducible binding of GATA-3 and NFATp to this distal enhancer element was demonstrated in Th2 cells, but not in Th1 cells, suggesting that this region may play a role in the regulation of *il4* gene expression. For these reasons, we sought to determine whether



**FIGURE 5.** Neither nucleotide polymorphism nor altered expression of NFATc, GATA-3, and T-bet explain differences in IL-4 expression between C57BL/6 and DBA/2 mice. **A**, Drawing of the murine *il4/il13* locus showing relative positions of key elements that have been shown to modulate the expression of IL-4. These regions were sequenced in both C57BL/6 and DBA/2 mice and no polymorphisms were found between the two strains. **B**, NFATc abundance was determined by Western blot analysis of nuclear protein isolated from splenocytes ( $2 \times 10^6$  cells/ml) stimulated with 2.5  $\mu\text{g/ml}$  Con A. Nuclear proteins from both C57BL/6 (B6) and DBA/2 (D2) splenocytes were isolated at the indicated time, fractionated, and then blotted with NFATc-specific Ab as described in *Materials and Methods*. **C**, GATA-3 abundance was determined by Western blot analysis of nuclear proteins isolated from CD4<sup>+</sup> T cells polarized in vitro toward Th2 as described in *Materials and Methods*. **D**, Same as **C** except CD4<sup>+</sup> T cells were polarized toward Th1 and nuclear extracts were probed for T-bet as described in *Materials and Methods*. Western blotting data represent one of three identical experiments.

there was nucleotide polymorphism at this inducible DNase hypersensitivity site. When the nucleotide sequence of a 464-bp PCR product spanning the putative 3' distal enhancer was examined, there were no differences between the C57BL/6 and DBA/2 mice (data not shown).

Another group of putative *il4* regulatory sequences was identified by searching for conserved sequence between the mouse and human genome at the *il4/il13* locus (17). One such element, CNS-1, when deleted in transgenic mice, led to a marked reduction in the frequency of Th2 cytokine-producing cells, suggesting that CNS-1 may be involved in modulating gene expression at the *il4/il13* locus. To rule out the possibility that there may be sequence variability between C57BL/6 and DBA/2 mice at CNS-1, the nucleotide sequence of a 627-bp PCR product spanning the critical

214 bp of CNS-1 was examined. The results of the sequencing analysis demonstrated that there are no differences in nucleotide sequence between the C57BL/6 and DBA/2 mice at CNS-1 (data not shown).

It is well-established that mRNA stability plays a central role in the control of gene expression, and that steady-state levels of several cytokines are in some instances determined by their half-lives (18). Cytokine mRNA stability is regulated, in part, by specific *cis*-acting determinants in the 3' UTR, known as AU-rich elements (AREs; Ref. 19). When the nucleotide sequence of a 216-bp PCR product spanning the entire *il4* 3' UTR was examined, we found no differences between C57BL/6 and DBA/2 mice (data not shown). The sequencing results demonstrate that there exists no nucleotide sequence polymorphism between C57BL/6 and DBA/2 mice at the

proximal *il4* promoter, putative 3' distal enhancer, CNS-1, and the *il4* 3' UTR. Although the sequence analysis comparing C57BL/6 and DBA/2 mice was by no means exhaustive, the results do suggest that within these putative and established regulatory regions, nucleotide sequence polymorphism does not account for the observed differences in IL-4 mRNA and IL-4 protein expression in these two strains of mice.

We next asked whether there were differences in the relative expression of three transcription factors that play an important role in modulating the expression of IL-4: NFATc, GATA-3, and T-bet. NFATc has been shown to transactivate the IL-4 promoter *in vitro* and *in vivo* (20), and deletion of NFATc in transgenic mice markedly reduces IL-4 production (21). For these reasons, we investigated whether there were differences in nuclear translocation of NFATc in mitogen-activated C57BL/6 and DBA/2 splenocytes. Using Western blot analysis, we assessed NFATc expression and translocation in nuclear extracts from C57BL/6 and DBA/2 splenocytes stimulated with Con A for 15, 30, and 60 min. We found no difference in the amount of NFATc translocated into the nucleus of C57BL/6 and DBA/2 splenocytes when activated with Con A (Fig. 5B). We also observed no difference in total cellular expression of NFATc between C57BL/6 and DBA/2 splenocytes (data not shown).

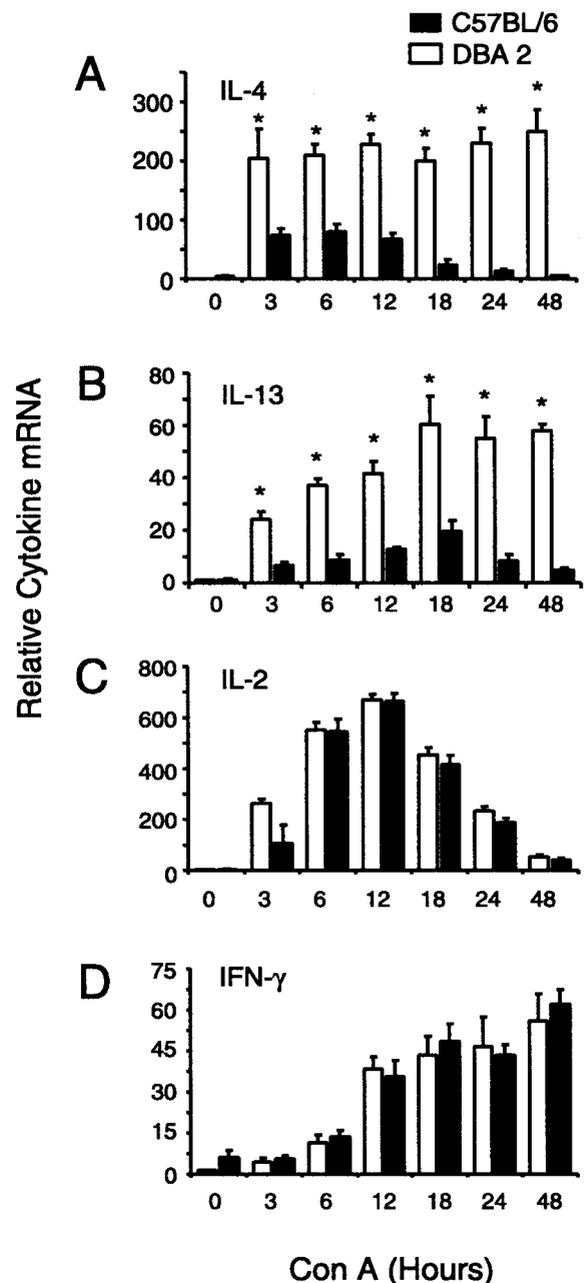
GATA-3 is another transcription factor that has been shown to modulate the expression of IL-4. GATA-3 is preferentially expressed in Th2 cells and absent in Th1 cells (22), and GATA-3 has been shown to transactivate the IL-4 promoter (23), bind an IL-4 3' distal enhancer (16), and coordinate chromatin remodeling at the *il4/il13* locus (24). For these reasons, we investigated whether GATA-3 was differentially expressed in Th2 polarized DBA/2 and C57BL/6 CD4<sup>+</sup> T cells. Western blot analysis of nuclear protein isolated from Th2-polarized C57BL/6 and DBA/2 CD4<sup>+</sup> T cells revealed no difference in GATA-3 expression between the two strains of mice (Fig. 5C). Interestingly, unstimulated CD4<sup>+</sup> T cells isolated from naive C57BL/6 and DBA/2 mice expressed detectable levels of nuclear GATA-3 protein; however, this observation is not completely unexpected, as GATA-3 transcripts have been specifically detected in Th0 cells (22, 25).

Another candidate transcription factor that may modulate IL-4 expression is T-bet, a recently identified member of the T-box family of transcription factors. T-bet is only found in Th1 cells and its activity and expression strongly correlate with IFN- $\gamma$  production (26). Furthermore, ectopic expression of T-bet in fully polarized Th2 cells converts them into Th1 cells, effectively repressing IL-4 while activating IFN- $\gamma$  secretion (26). For these reasons, we examined whether the IL-4<sup>low</sup> phenotype we observe in C57BL/6 cells could be due in part to higher expression of T-bet. To test this hypothesis, we polarized CD4<sup>+</sup> T cells from C57BL/6 and DBA/2 mice toward the Th1 immunophenotype and then assessed T-bet expression by Western blot analysis of fractionated nuclear protein. We found no difference in T-bet expression between C57BL/6 and DBA/2 Th1 cells (Fig. 5D). These results suggest that the IL-4<sup>high</sup> and IL-4<sup>low</sup> phenotype in DBA/2 and C57BL/6 CD4<sup>+</sup> T cells can neither be explained by selective expression of the Th1- and Th2-specific transcription factors T-bet and GATA-3, nor the T cell-associated transcription factor NFATc.

#### Kinetics of IL-4 and IL-13 mRNA expression differ between C57BL/6 and DBA/2 splenocytes

Having found that C57BL/6 splenocytes and CD4<sup>+</sup> T cells produce less Th2 cytokines, we next asked if there were also differences in the kinetics of mRNA expression between C57BL/6 and DBA/2 cells. To do this, we stimulated splenocytes from immunologically naive C57BL/6 and DBA/2 mice with Con A and eval-

uated IL-4 mRNA levels at regular intervals. Splenocytes from both strains (C57BL/6 and DBA/2) up-regulated IL-4 mRNA in response to stimulation, but the levels were significantly less in C57BL/6 cells. As shown in Fig. 6A, splenocytes from C57BL/6 mice exhibit a maximal increase in IL-4 mRNA expression of ~80-fold at 6 h poststimulation. This increase in expression is not sustained and drops back to near basal levels at 48 h poststimulation. In contrast, splenocytes from DBA/2 mice exhibit a maximal increase of IL-4 expression of ~200-fold at 3 h, the earliest time point analyzed. Furthermore, the 200-fold increase in IL-4 mRNA expression is sustained for the duration of the experiment (48 h).



**FIGURE 6.** Kinetics of cytokine mRNA expression in C57BL/6 and DBA/2 Con A-activated splenocytes. Splenocytes from naive C57BL/6 and DBA/2 mice were activated at  $2 \times 10^6$  cells/ml in the presence of 2.5  $\mu$ g/ml Con A. At the indicated time, total RNA was isolated from cells and relative IL-4 (A), IL-13 (B), IL-2 (C), and IFN- $\gamma$  (D) mRNA expression was assessed using real-time RT-PCR as described in *Materials and Methods*. Data are expressed as mean  $\pm$  SEM for three independent experiments (\*,  $p < 0.05$ ).

When the kinetics of mRNA expression of another Th2-associated cytokine, IL-13, was examined using the same total RNA samples, a pattern emerged similar to that observed for IL-4 mRNA kinetics. As shown in Fig. 6B, when stimulated with Con A, splenocytes from naive C57BL/6 mice exhibit a maximal 13-fold increase in IL-13 expression at 18 h poststimulation. Again, this expression was not sustained and the 13-fold induction dropped to near control levels as the time course approached 48 h. By comparison, splenocytes from DBA/2 mice exhibited a sustained increase in IL-13 mRNA expression as early as 3 h with a maximal ~50-fold IL-13 mRNA induction at 48 h poststimulation.

The mRNA expression kinetics of the Th1 cytokines, IL-2 and IFN- $\gamma$ , were also examined in the same total RNA samples analyzed in Fig. 6, A and B. We found no significant differences in either the kinetics or the overall magnitude of mRNA expression for IL-2 and IFN- $\gamma$  between C57BL/6 and DBA/2 splenocytes at any time point examined (Fig. 6, C and D). These results indicate that for the Th2 cytokines, IL-4 and IL-13, there is decreased mRNA expression in C57BL/6 splenocytes relative to the DBA/2 splenocytes. Furthermore, these data indicate that Th2 cytokine transcripts from C57BL/6 and DBA/2 mice differ in expression and/or stability.

#### IL-4 and IL-13 mRNA is less stable in C57BL/6 splenocytes

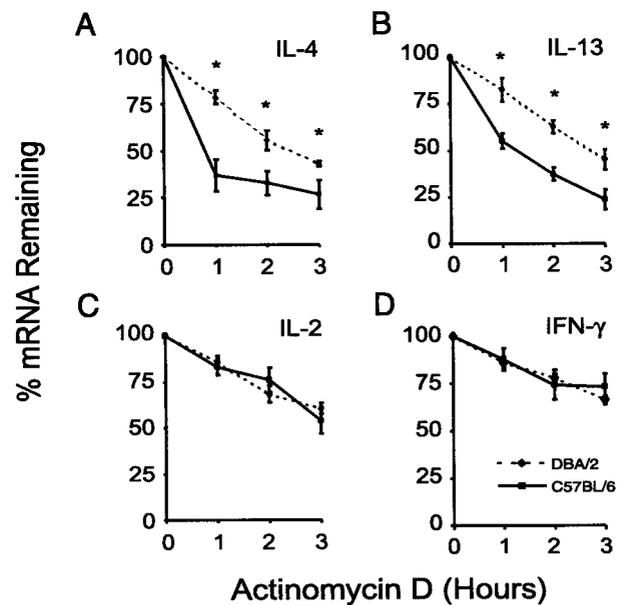
One mechanism that may account for the differences in steady-state levels of IL-4 protein and mRNA between C57BL/6 and DBA/2 mice is altered mRNA stability. It is well-established that mRNA stability plays a central role in regulating the expression of many genes (18). For this reason, differences in IL-4 cytokine mRNA stability between C57BL/6 and DBA/2 splenocytes were investigated. To examine mRNA stability of IL-4 and IL-13 (Th2 cytokines), as well as IFN- $\gamma$  and IL-2 (Th1 cytokines), Con A-activated splenocytes from naive C57BL/6 and DBA/2 mice were treated with Act D to block cellular DNA transcription. At hourly time points, total RNA was isolated from Act D-treated splenocytes and subjected to real-time RT-PCR to quantify relative levels of cytokine mRNA (as described in *Materials and Methods*).

As shown in Fig. 7A, when the mRNA stability of IL-4 was examined, we found that the half-life of IL-4 mRNA isolated from C57BL/6 splenocytes measured ~50 min. In contrast, when IL-4 mRNA stability was examined in DBA/2 splenocytes, the IL-4 mRNA half-life was ~135 min. Therefore, C57BL/6 IL-4 mRNA is less stable when compared with DBA/2 IL-4 mRNA. Similarly, when the stability of IL-13 was examined (Fig. 7B), we found that the half-life of IL-13 mRNA in C57BL/6 splenocytes was ~70 min, whereas the half-life of IL-13 mRNA in the DBA/2 splenocytes was ~160 min. Like IL-4 mRNA, the IL-13 mRNA from C57BL/6 cells is less stable than IL-13 mRNA from DBA/2 cells.

When Th1 cytokine mRNA stability was assessed as a control, no significant differences between the half-lives of IL-2 and IFN- $\gamma$  mRNA were detected in Con A-activated, Act D-treated splenocytes from C57BL/6 and DBA/2 mice. For both C57BL/6 and DBA/2 Con A-activated splenocytes, the half-life of IL-2 and IFN- $\gamma$  mRNA was estimated to be ~3 and >5 h, respectively (Fig. 7, C and D). These data suggest that altered IL-4 and IL-13 mRNA stability may provide a novel mechanism by which splenocytes regulate steady-state IL-4 and IL-13 mRNA levels. Furthermore, these data indicate that global regulation of Th2 cytokine mRNA stability may play a role in modulating the polarization of adaptive immune responses toward the Th2 immunophenotype.

## Discussion

The mechanisms responsible for susceptibility and resistance to HP are not well understood. We have previously demonstrated that



**FIGURE 7.** Cytokine mRNA stability in Con A-activated splenocytes from C57BL/6 and DBA/2 mice. Splenocytes from naive C57BL/6 and DBA/2 mice were isolated and cultured at  $2 \times 10^6$  cells/ml in the presence of 2.5  $\mu$ g/ml Con A for either 6 h (IL-4 and IL-2) or 18 h (IL-13 and IFN- $\gamma$ ). At the end of the Con A stimulation, Act D was added to a final concentration of 5  $\mu$ g/ml. At the indicated time, total RNA was isolated from cells and cytokine mRNA abundance for IL-4 (A), IL-13 (B), IL-2 (C), and IFN- $\gamma$  (D), relative to groups at  $t = 0$  (not treated with Act D), was assessed with real-time PCR. Data are expressed as mean  $\pm$  SEM for three independent experiments (\*,  $p < 0.05$ ).

Th1-biased C57BL/6 mice are susceptible to experimental HP, whereas Th2-biased mice are resistant to developing HP. In this report, we have examined altered expression of IL-4 as a possible mechanism underlying the Th1- and Th2-biased responses of C57BL/6 and DBA/2 mice, as well as the basis for their relative susceptibility and resistance to experimental HP. In this study, we provide evidence that splenocytes and CD4<sup>+</sup> T cells from C57BL/6 mice express less IL-4 mRNA and protein, relative to DBA/2 cells, and that lower IL-4 expression correlates with susceptibility to experimental HP. In a model of experimental HP, we observed 2- to 3-fold less IL-4 mRNA expressed in ex vivo-activated splenocytes isolated from HP-sensitized C57BL/6, as compared with splenocytes from HP-sensitized DBA/2 mice. When we looked for evidence of higher Th2 cytokine expression in lungs of HP-sensitized DBA/2 mice, we found ~3-fold more IL-13 mRNA in the lungs relative to the lungs of HP-sensitized C57BL/6 mice. We also found that splenocytes and CD4<sup>+</sup> T cells from immunologically naive C57BL/6 mice expressed less IL-4 protein and mRNA relative to cells from naive DBA/2 mice, in response to activation with polyclonal activators. By comparison, the Th1 cytokines IL-2 and IFN- $\gamma$  were not differentially expressed in C57BL/6 and DBA/2 CD4<sup>+</sup> T cells. When we explored mechanisms to explain the differences in IL-4 production, we found neither polymorphism in the *il4/il13* locus, nor differences in NFATc, GATA-3, and T-bet expression. We did find that IL-4 mRNA stability was decreased in the C57BL/6 splenocytes relative to the DBA/2 splenocytes. Similar results were also obtained for another Th2 cytokine, IL-13.

There are several examples of Th1- and Th2-bias among inbred strains of mice in the literature (27–31). In these reports, Th1-biased mice are resistant to intracellular parasites, while Th2-biased mice are susceptible. In other reports, Th1 mice are described

as resistant to models of allergic disease, while Th2 mice are highly susceptible to allergy and atopy. The reasons for bias toward Th1 or Th2 adaptive immune responses are not fully understood, but a significant portion of the underlying mechanism relates to genetic background (32–35). Recently, important contributions have been made toward understanding the genetics of Th1/Th2 bias in other inbred mice. Kuroda et al. (36) have reported that reduced expression and activation of STAT4 and decreased expression of IFN- $\gamma$  may account for the Th2 bias in BALB/c mice. In DBA/2 and C57BL/6 mice, we observed no difference in the expression of the Th1-associated cytokines IL-2 and IFN- $\gamma$ . Furthermore, differential production of IL-4 between C57BL/6 and DBA/2 mice is not likely due to altered expression and activation of STAT6, as early IL-4 expression in response to primary stimulation has been shown to be independent of STAT6 activity (25). In a second report (37), an evolutionarily conserved noncoding sequence was identified that when deleted, compromised the ability of mice to develop Th2 cells *in vitro* and *in vivo*, suggesting that within the sequence of CNS-1 there lie critical elements necessary for full activation and polarization of an adaptive Th2 immune response. These data support CNS-1 as a candidate for modulating Th2 immune polarization. However, when we examined the nucleotide sequence of CNS-1 in C57BL/6 and DBA/2 mice, we found no polymorphism to explain the differences in IL-4 production. Therefore, factors other than CNS-1 likely account for the observed differences in IL-4 production in our model. Lastly, a newly identified locus, termed *Tapr* (T cell airway phenotype regulator), was found to be associated with airway hyperreactivity (Ref. 38). *Tapr* encodes three membrane glycoproteins, called T cell Ig domain and mucin domain (TIM) proteins, which are expressed on the surface of both T cells and APCs. Importantly, two polymorphisms in *tim-1* were discovered in the BALB/c mouse that completely cosegregated with the airway hyperreactivity phenotype, and the authors concluded that the genotype at *tim-1* plays a major role in determining whether an animal will develop tendencies to develop either Th1 or Th2 adaptive immune responses. When the sequence of *tim-1* was examined in DBA/2 and C57BL/6 mice, it was discovered that these two strains share the same genotype (38). Therefore, differences in IL-4 expression between C57BL/6 and DBA/2 mice that we observed in our system are not likely explained by the contributions of *Tapr*.

Full Th2 polarization and maximal induction of IL-4 expression in CD4<sup>+</sup> T cells involves a number of well-defined events at both the level of chromatin remodeling and DNA methylation (16, 24, 39). When we attempted to polarize CD4<sup>+</sup> T cells toward the Th2 immunophenotype, we found that cells from both strains were able to significantly up-regulate IL-4 mRNA expression. C57BL/6 CD4<sup>+</sup> T cells increased IL-4 mRNA expression ~20-fold relative to unpolarized CD4<sup>+</sup> T cells, while DBA/2 CD4<sup>+</sup> T cells were able to increase IL-4 mRNA expression ~40-fold. This indicates that the disparity in IL-4 production between C57BL/6 and DBA/2 CD4<sup>+</sup> T cells had risen from 3- to 4-fold in primary cells (Fig. 2C) to ~7-fold in cells polarized toward Th2 for 7 days (Fig. 4A). This suggests that the increase in disparate IL-4 expression involves differences between the two strains that become magnified after successive rounds of cell division. In this regard, it is conceivable that factors related to IL-4 transactivation, such as transcription initiation or chromatin remodeling, contribute to the measured differences in IL-4 production in the two strains of mice. More work must be done to further clarify the role of IL-4 transcription initiation in our system.

Much work has been done to identify factors that regulate murine IL-4 expression (40), but to our knowledge this is the first report describing IL-4 mRNA stability as a mechanism which may

play a role in the polarization of an adaptive immune response. In this study, we provide evidence that IL-4 and IL-13 mRNA from Con A-activated C57BL/6 splenocytes are less stable than the IL-4 and IL-13 mRNA from DBA/2 splenocytes. When we examined the mRNA stability of two Th1-associated cytokines, IL-2 and IFN- $\gamma$ , we found no significant differences between C57BL/6 and DBA/2 splenocytes. Furthermore, sequencing of the IL-4 3' UTR revealed no nucleotide polymorphism to explain altered mRNA stability between C57BL/6 and DBA/2 splenocytes. The stability of mRNA transcripts plays a central role in regulating the expression of a number of genes. Located within the 3' UTR of many short-lived transcripts are sequences known as AREs. AREs are found in many cytokine messages, including IL-2, IL-4, and IL-13, and mediate both mRNA decay in resting cells and stability in activated cells (41). The mechanism by which AREs function, while complex and not completely understood, involves factors such as protein-RNA interactions and the RNA secondary structures. Because the 3' UTR of IL-2 contains AREs, and IL-2 mRNA stability is the same in both C57BL/6 and DBA/2 splenocytes, altered Th2 cytokine mRNA stability between these two strains may involve modulation by some additional mechanism, perhaps differential expression of Th1- or Th2-specific *trans*-acting factors. In addition to AREs, the activities of p38 MAP kinase and tristetrapolin have been shown to modulate the half-lives of short-lived transcripts (42–45). It is possible that p38 and tristetrapolin could modulate mRNA stability and that differential expression or activation of these molecules might account for altered Th2 cytokine mRNA stability in DBA/2 and C57BL/6 splenocytes.

In summary, we have demonstrated IL-4 is differentially regulated in Th1-biased C57BL/6 and Th2-biased DBA/2 mice. Based on the results presented in this study, we propose a model wherein altered expression of Th2 cytokines between C57BL/6 and DBA/2 mice establishes bias for Th1 vs Th2 adaptive immune responses in C57BL/6 and DBA/2 mice, respectively, and plays an important role in determining their relative susceptibility and resistance to experimental HP. Furthermore, our data suggest that modulation of Th2 cytokine mRNA stability may serve as an important novel mechanism that can determine Th1/Th2 immune polarization.

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