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The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells

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Summary

CD4 T cells potentiate the inflammatory or humoral immune response through the action of Th1 and Th2 cells, respectively. The molecular basis of the differentiation of these cells from naive T cell precursors is, however, unclear. We found that GATA-3 was selectively expressed in Th2 cells. GATA-3 is expressed at a high level in naive, freshly activated T cells and Th2 lineage cells, but subsides to a minimal level in Th1 lineage cells as naive cells commit to their Th subset. Antisense GATA-3 inhibited the expression of all Th2 cytokine genes in the Th2 clone D10. GATA-3 directly activated an *IL-4* promoter-luciferase reporter gene in M12 cells. In transgenic mice, elevated GATA-3 in CD4 T cells caused Th2 cytokine gene expression in developing Th1 cells. Thus, GATA-3 is necessary and sufficient for Th2 cytokine gene expression.

Introduction

The discovery of the Th1 and Th2 subsets of CD4 T cells in both mouse and human (Mosmann et al., 1986; Romagnani, 1991) has provided the cellular basis for the diversity of T cell–dependent immune responses. Th1 cells produce IFN-γ, LTα (TNFβ), and IL-2 and promote the inflammatory and cellular immune response. In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and induce humoral immunity. In the course of infection and in pathologic conditions such as allergic and autoimmune diseases (Romagnani, 1994; Abbas et al., 1996) polarized Th1 and Th2 responses are found and are believed to be critical to the outcome of these conditions. Therefore, understanding the mechanisms of development of Th1 and Th2 responses should give insight into the pathogenesis and treatment of a variety of diseases.

The effector immune response results from the differentiation of naive CD4 T cells to Th1 or Th2 cells. This appears to be a multistep process, in which naive T cells may pass through an intermediate stage at which both Th1 and Th2 cytokines are produced (Abehsira-Amar et al., 1992; Kamogawa et al., 1993; Lederer et al., 1996; Nakamura et al., 1997). An individual naive CD4 T cell can differentiate into either Th1 or Th2 cells (Abehsira-Amar et al., 1992; Rocken et al., 1992; Kamogawa et al., 1993). The differentiation pathway followed is determined by the environment in which the naive T cells react to antigen stimulation. The most potent factors that influence Th1 and Th2 differentiation are IL-12 and IL-4, respectively (Seder et al., 1992, 1993; Hsieh et al., 1993; Kopf et al., 1993). IL-12 induces phosphorylation of Stat-4 in developing and differentiated Th1 cells but not in Th2 cells (Bacon et al., 1995; Jacobson et al., 1995; Szabo et al., 1995) and IL-4 activates Stat-6 in Th2 cells (Hou et al., 1994; Schindler et al., 1994). The activation of these two Stat proteins is essential for CD4 T cell subset development because Stat-4- or Stat-6-deficient mice are unable to generate Th1 and Th2 responses, respectively (Kaplan et al., 1996a; Thierfelder et al., 1996).

The molecular mechanisms by which antigen stimulation via T cell receptor (TCR) and cytokine signaling integrate to drive Th1 and Th2 effector cell differentiation are, however, poorly understood. The intracellular release of Ca^{2+} and activation of protein kinase C (PKC) induced by antigen stimulation via TCR induce the NF-AT and AP-1 transcription activities (Rao, 1994; Rao, 1991). Activation of the *IL-2* and *IL-4* promoters requires the coordinated binding of NF-AT and AP-1 to the NF-AT/AP-1 composite sites in the promoters (Rooney et al., 1995). However, since the IL-2 and IL-4 NF-AT/AP-1 composite sites are interchangeable without affecting the Th1/Th2 specificity of the promoters, it is likely that other transcription factors are responsible for determining promoter specificity (Rooney et al., 1995). AP-1 and NFAT are selectively activated in Th2 effector cells but show no difference in developing precursors, arguing that their selectivity relates to effector cytokines rather than the differentiation mechanism (Rincón et al., 1997b, 1997c). One possibility to explain specificity is that Stat-4 and Stat-6 may directly regulate the specificity of the cytokine gene promoters in Th1 and Th2 cells. However, while the activation of Stat-4 in Th1 effector cells requires IL-12 treatment (Szabo et al., 1995), IL-12 is not needed for the antigen-stimulated expression of Th1 cytokines (Magram et al., 1996). In contrast, in Th2 effector cells, antigen stimulation alone does activate Stat-6, but this activation appears to be a secondary autocrine effect of IL-4 (Lederer et al., 1996). Furthermore, activation of Stat proteins is a transient event that occurs rapidly upon stimulation, whereas the development of effector T helper cells takes several days (Abbas et al., 1996). Therefore, while it is apparent that Stats are essential early signal transducers in Th subset differentiation, they are unlikely to determine the Th phenotype directly. An alternative scenario of Th differentiation is that Stat activation and TCR signaling initiate a cascade of changes of gene expression that ultimately leads to the development of effector phenotypes of Th1 and Th2 cells.

In support of the second model, two transcription factors preferentially expressed in Th2 cells have been found to activate the *IL-4* gene promoter. First, the protein encoded by the proto-oncogene c-*maf* induced endogenous IL-4 production when cotransfected with the TCR-inducible NF-ATp into the B cell lymphoma M12 (Ho et al., 1996), indicating a cooperative role of these two factors. The transcription of the *IL-4* gene can be further enhanced by a newly identified factor, NIP45

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(Hodge et al., 1996). Second, the transcription factor NF-IL6 was also found to be required for the activation of the human *IL-4* promoter in a Th2 clone, via the positive regulatory element 1 (PRE-1) of the human *IL-4* promoter (Davydov et al., 1995). However, the selective expression of these two transcription factors was observed in fully differentiated Th2 cells. Their roles in the differentiation of Th2 cells from naive precursors are less clear. In addition, the regulation of the expression of other Th2 cytokine genes by these factors has not yet been demonstrated. Therefore, although much has been known about the regulation of the *IL-4* gene promoter, our knowledge on the molecular mechanisms leading to the development of the Th2 phenotype remains limited. We have recently shown that IL-6, which activates NF-IL6, can stimulate IL-4 production by CD4 T cells, providing a mechanism whereby APC could direct Th2 differentiation through the NF-IL6 family (Rincón et al., 1997a).

In the present study, we reasoned that developing Th1 and Th2 cells should express different sets of genes, some of which might direct the development of these cells from naive precursors. To identify these genes, we performed cDNA subtraction by representational difference analysis (RDA) (Lisitsyn et al., 1993; Hubank and Schatz, 1994) between normal Th1 and Th2 cells induced in vitro. We found that the transcription factor GATA-3 was the dominant species that was present in the RDA cDNA difference products, and that it was selectively expressed in differentiating and effector Th2 cells. Strikingly, GATA-3 is required for the transcription of all Th2 cytokine genes, and the loss of Th2 cytokine gene expression in Th1 cells is at least in part a consequence of the down-regulation of GATA-3.

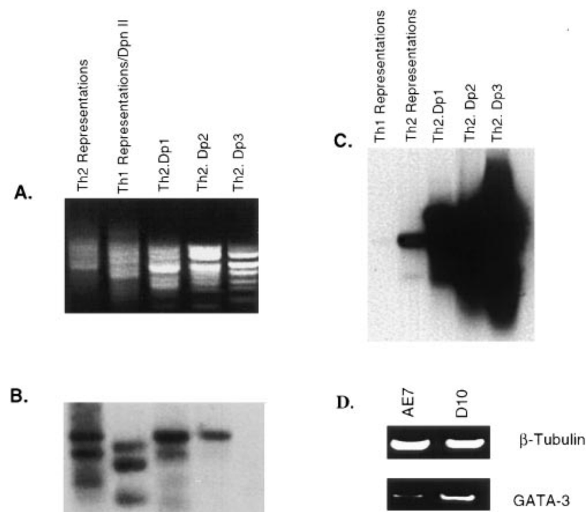

Figure 1. cDNA Representational Analysis of Gene Expression in Th2 Cells

(A) Naive CD4 T cells from B10A.5R pigeon cytochrome c TCR transgenic mice (Kaye et al., 1989) were induced to differentiate into Th1 and Th2 cells for 4 days. cDNA was derived from these cells and used for RDA with Th2 representations as the "testers" and the Th1 representations as the "drivers." The original representations and the difference products are shown. The faster migration of the drivers is due to the removal of the linkers.

(B) The testers, drivers, and the difference products were blotted and hybridized with a probe made from a 700 bp HindIII/BamHII γ-actin cDNA fragment (Gunning et al., 1983).

(C) A second blot was prepared and hybridized with probe from a full-length murine *GATA-3* cDNA (Ko et al., 1991).

(D) RT–PCR analysis of *GATA-3* gene expression in the Th1 clone AE7 and Th2 clone D10. Resting cells were stimulated with antigen (5 μg/ml pigeon cytochrome c peptide for AE7 and 1 μg/ml conalbumin peptide for D10) and AKR/J APC for 20 hr.

Results

RDA Shows That GATA-3 Is Selectively Expressed in Th2 Cells

To identify Th2-specific genes, we performed cDNA RDA (Hubank and Schatz, 1994) between Th2 and Th1 cells (Figure 1). Representations (see Experimental Procedures) of Th1 and Th2 cDNA were prepared from cDNA derived from Th1 and Th2 effector cells. RDA was performed with the Th2 representations as "testers" and an excess amount of Th1 representations as "drivers." After one round of subtraction, the first difference products (Dp1) showed some discrete bands. After subsequent subtractions, bands became more clear in the difference products, and no polydisperse DNAs were seen after three rounds of subtraction (Figure 1A). To show that genes common to both Th1 and Th2 cells were successfully subtracted, the DNA was Southern analyzed and hybridized to γ-actin probes (Figure 1B). The lowest band of γ-actin almost disappeared even after only one subtraction. After three rounds of subtraction, no γ-actin signals were detectable, demonstrating that subtraction was successful. Since the RDA products were small cDNA fragments of different genes, it was unproductive to recover all the genes represented in the final difference products by random cloning of the difference products. To overcome this problem and identify the full-length genes differentially expressed in Th2 cells, a Th2 cDNA library was constructed and screened with the third difference products (Dp3) as probes. After screening 7.5×10^5 recombinant colonies, 165 positive signals were identified, of which 67 (40.6%) were found to be identical to the transcription factor GATA-3 (Ko et al., 1991) by DNA sequencing.

To confirm that GATA-3 is preferentially expressed by Th2 cells, probes were made from *GATA-3* cDNA and hybridized to the original Th1 and Th2 representations and to the Th2 RDA difference products (Figure 1C). Strong signals were detected in original Th2 representations with only a very weak band being detected in Th1 representations. The GATA-3 signals became stronger after each subtraction, indicating that the fragments of this gene had been enriched. As in normal T cells, strong expression of GATA-3 was detected by RT–PCR in a Th2 clone D10 but only minimal expression in a Th1 clone AE7 (Figure 1D).

Kinetics of GATA-3 Expression during In Vitro Differentiation of Th1 and Th2 Cells Correlates with Subset Commitment

GATA-3 belongs to the GATA family of zinc finger proteins. Many members of this gene family function as regulators of cellular differentiation. For example, GATA-1 and GATA-4 are involved in erythrocyte and cardiac muscle cell development, respectively (Pevny et al.,

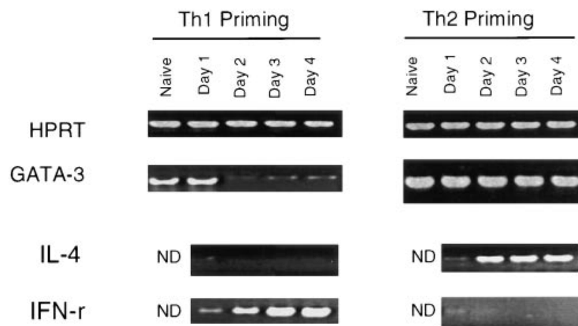

Figure 2. Time Course of *GATA-3* Gene Expression during In Vitro Differentiation of CD4 T Cells

Naive ($CD44^{low}CD45RB^{high}$) CD4 T cells from AKR/J mice were stimulated in vitro for differentiation. RNA was prepared at different time points for RT–PCR. HPRT was used as internal control to normalize the PCR templates. IL-4 and IFN-γ profiles are shown to indicate the differentiation status of the stimulated cells.

Figure 3. Reduction of GATA-3 Protein in Antisense *GATA-3* Transfectants

The transfectants were stimulated with APC and conalbumin peptide (1 μg/ml), and labeled with ^{35}S-methionine overnight. Equal number of cells were harvested for immunoprecipitation. GATA-3 proteins were captured by anti-GATA-3 mAb HG3–31 conjugated to agarose beads. Proteins were separated in a 12% polyacrylamide gel.

1991; Grepin et al., 1995). Therefore, we considered that GATA-3 may function in a similar way to determine the differentiation of Th2 cells. We approached this question by first examining the time course of GATA-3 expression as Th1 and Th2 cells differentiated in vitro (Figure 2). Consistent with a previous report that GATA-3 is expressed in mature CD4 thymocytes (George et al., 1994), we found that GATA-3 is also expressed in peripheral naive CD4 T cells. The level of expression was not altered after activation for one day using priming conditions for either Th1 or Th2 cells. However, while the expression level of GATA-3 remained at a high level during the entire time course of differentiation to Th2 cells, it was dramatically decreased after 2 days of priming for the Th1 response and remained at this low level in the Th1 lineage cells (Figure 2). To confirm the differentiation status of the in vitro–primed Th1 and Th2 cells, the cytokine mRNA expression patterns were analyzed by RT–PCR. In agreement with previous studies (Lederer et al., 1996; Nakamura et al., 1997), low levels of both *IL-4* and *IFN-γ* mRNA were detected after 1 day of priming for both Th1 and Th2 responses. Strong and skewed expression of these two cytokine genes was observed after 2 days of priming, by which time GATA-3 expression differed radically in these two populations, confirming that after 2 days, CD4 T cells have completed their "commitment" to the Th1 or Th2 lineages. For a detailed analysis of this issue see Nakamura et al. (1997). The kinetics of GATA-3 expression, therefore, correlated with the time course of Th1 and Th2 differentiation, which suggested that the regulation of GATA-3 expression may be a crucial event in the differentiation of CD4 T cell subsets.

GATA-3 Is Required to Maintain the Th2 Phenotype in the Prototypic Th2 Clone D10

The stable expression of GATA-3 in the Th2 lineage suggested that it may play a role in Th2 cytokine gene expression. In support of this notion, EL-4 and Jurkat cells, from which murine and human *GATA-3* were cloned, respectively, produce IL-4 and other Th2 cytokines (Klein et al., 1995; Lee et al., 1994), and potential GATA-3 binding sites are present in the promoters of all Th2 cytokine genes. Since GATA-3 deficiency causes embryonic lethality (Pandolfi et al., 1995) and *GATA-3*$^{-/-}$ ES cells can not reconstitute the T cell compartment in RAG-2-deficient mice (Ting et al., 1996), we sought to study the role of GATA-3 in regulating Th2 cytokine gene expression by expressing an antisense *GATA-3* construct in the Th2 clone D10. Two separate stable lines of anti-*GATA-3* transfectants, D10.3.4.1 and D10.3.4.2, were established from two independent transfections. GATA-3 expression was significantly reduced in both lines with the expression in D10.3.4.2 cells slightly higher than in D10.3.4.1 cells (Figure 3). As shown in Figure 4A, compared with the control transfectant D10.3, the expression of *IL-4*, *IL-6*, and *IL-13* mRNA in D10.3.4.1 cells was abolished and the expression of *IL-10* mRNA was also greatly inhibited. *IL-5* gene expression was apparently also inhibited, albeit to a lesser extent. In the second transfected line D10.3.4.2 (Figure 4B), expression of the *IL-6* and *IL-13* genes was also ablated by the antisense *GATA-3*, and *IL-4* message was greatly reduced but still weakly detectable. Just as in D10.3.4.1 cells, *IL-10* mRNA was also significantly reduced in this line, while inhibition of *IL-5* mRNA was not demonstrable. The Th1 cytokine IFN-γ was not expressed (Figures 4A and 4B), and the lineage-nonspecific IL-3 was equally expressed weakly in all three D10 lines (Figures 4A and 4B).

To quantitate the extent of inhibition of cytokine gene expression, we performed competitive RT–PCR (Reiner et al., 1994). Antisense *GATA-3* reduced *IL-4* mRNA to undetectable levels in D10.3.4.1 cells and by 86% in D10.3.4.2 cells and *IL-10* message by 87% and 75%, respectively, in these two cell lines. The effect on *IL-5* message was modest, being insignificant for D10.3.4.2 cells and 16% inhibition in D10.3.4.1 cells. *IL-6* mRNA was completely eliminated in both cell lines.

Similar inhibitory effects of antisense *GATA-3* on the levels of IL-4, -5, -6, and -10 in the supernatants of these transfectants were observed by ELISA (Figure 4D). Taken together, these data show that GATA-3 must be expressed above a threshold level to maintain the Th2 phenotype in differentiated Th2 cells.

Antisense *GATA-3* Transfectants Express Normal Level of TCR but Proliferate at Lower Rates in Response to Antigen Stimulation

GATA-3 binding sites have been found in the TCR δ and α gene enhancers and GATA-3 can transactivate these enhancers (Ko et al., 1991; Ho et al., 1993). To rule out the

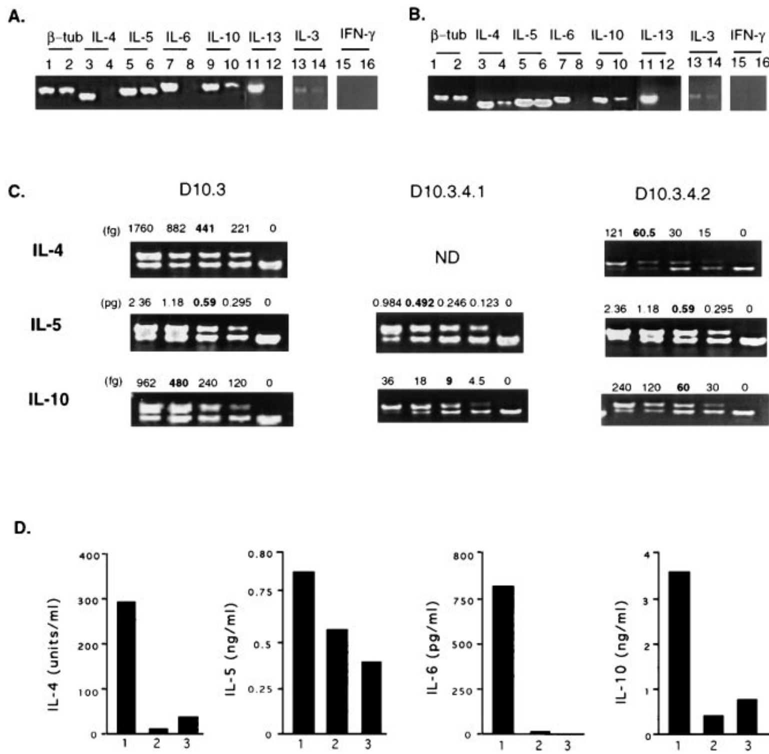

Figure 4. RT–PCR Analysis of Cytokine Gene Expression in D10 Transfectants

The transfectants were rested for 10–12 days after the last antigen stimulation and restimulated for 18–20 hr.

(A and B) Direct comparison between control D10.3 (lanes of odd numbers) and antisense *GATA-3* transfectants, D10.3.4.1 (A) or D10.3.4.2 (B) (lanes of even numbers).

(C) Competitive PCR (Reiner et al., 1994) for semiquantitation of cytokine gene expression. The numbers on the top of each panel represent the amount of competitive DNA used in the PCR. The amount of competitive cDNA that gave rise to the same intensity as the wild-type bands is shown in bold.

(D) Cytokine level in the supernatants of the transfectants. Column 1: D10.3; 2: D10.3.4.1; 3: D10.3.4.2.

possibility that the inhibition of cytokine transcription in the antisense *GATA-3* transfectants was due to inadequate stimulation of the cells caused by low TCR expression, TCR levels were analyzed by flow cytometry. The cells were stained with the clonotypic mAb 3D3, which recognizes both chains of the D10 TCR (Kaye et al., 1983). As shown in Figure 5A, TCR expression levels were not altered in the antisense *GATA-3* transfectants as compared with that of the control D10.3 cells. In fact, both D10.3.4.1 and D10.3.4.2 cells could respond to antigen stimulation as judged by proliferation (Figure 5B). However, the proliferation rates were consistently somewhat lower than the control transfectants. This decreased proliferation seems to be a secondary effect of the lack of autocrine IL-4 or other growth factors, because addition of culture supernatant from normal D10 cells could restore the growth of the antisense transfectants (data not shown). The inhibition of cytokine gene

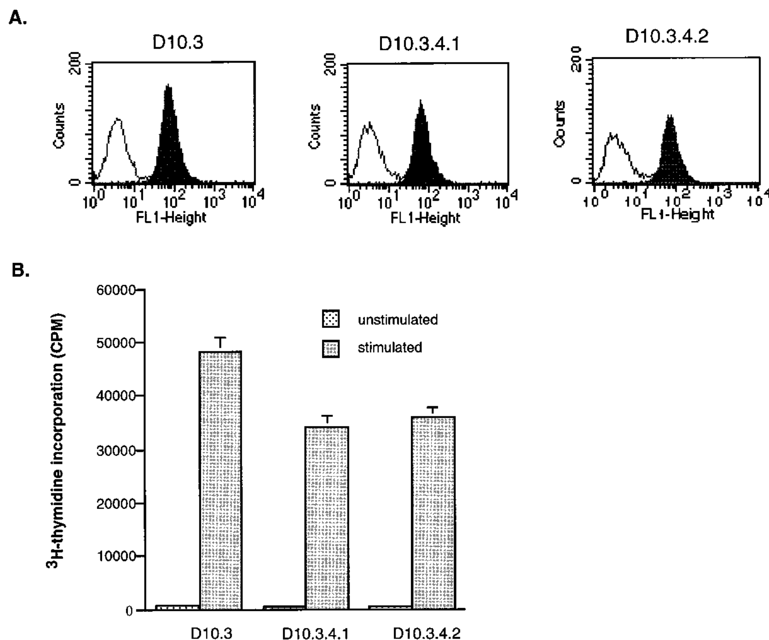

Figure 5. The Effect of Antisense GATA-3 on TCR Expression and Cell Proliferation

(A) The control D10.3 or antisense D10.3.4.1 and D103.4.2 cells were first incubated with the clonotypic mAb 3D3 (Kaye et al., 1983), then stained with anti-CD4-PE and FITC-conjugated goat anti-mouse IgG. $CD4^+$ cells were gated for flow cytometry analysis. The closed histograms show the TCR expression level. Open histograms are the negative controls (stained with FITC-conjugated goat anti-mouse IgG only).

(B) Proliferation assay was done as described in the Experimental Procedures. The incorporation of [^{3}H]thymidine of both stimulated (shaded columns) and unstimulated (stippled columns) cells are shown.

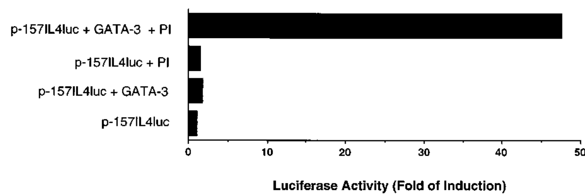

Figure 6. GATA-3 Transactivates the *IL-4* Promoter in M12 B Cell Lymphoma

The *IL-4* promoter reporter plasmid (p-157IL4luc) was cotransfected into M12 cells with or without a GATA-3 expression vector (R/mGATA-3). One day later, the cells were treated with PMA and ionomycin (PI) or left untreated as indicated for 24 hr. Cell extracts were measured for luciferase activities. Fold induction was shown as compared to the unstimulated (p-157IL4luc) cells.

expression in D10 cells by antisense *GATA-3* is therefore specific rather than a secondary result of the reduced capability to respond to antigen stimulation.

Ectopic Expression of GATA-3 Activates the *IL-4* Promoter in B Cell Lymphoma M12

To determine whether GATA-3 can directly transactivate the *IL-4* promoter, we constructed a reporter plasmid in which the firefly luciferase gene was driven by the −157 to +68 region of *IL-4* promoter (Todd et al., 1993; Hodge et al., 1995; Rooney et al., 1995). A *GATA-3* expression vector and the reporter construct were cotransferred into the B cell lymphoma M12, which does not express either *GATA-3* or *IL-4* genes. The induction of the luciferase activity in M12 cells was minimal with or without stimulation with PMA plus ionomycin in the absence of GATA-3 (Figure 6). GATA-3 alone did not activate the *IL-4* promoter in unstimulated cells. A dramatic induction of luciferase activity was, however, observed when the cells were treated with PMA plus ionomycin in the presence of GATA-3. GATA-3 appears therefore to be a potent transactivator of the *IL-4* promoter.

Constitutive Expression of GATA-3 Causes Th2 Cytokine Gene Expression in Th1 Precursors of *CD4-GATA-3* Transgenic Mice

The observation that GATA-3 expression was significantly down-regulated at the same time that naive T cells appeared to commit to the Th1 subset led us to speculate that the loss of Th2 cytokine expression in Th1 cells is caused by the reduced expression of GATA-3. To test this, we generated *GATA-3* transgenic mice in which GATA-3 expression was driven by the *CD4* promoter (Killeen et al., 1993) (Figure 7A). The expression of the *GATA-3* transgene in peripheral T cells was verified by RT–PCR (Figure 7B) using a 5′ primer derived from the sequence of the first exon of the *CD4* gene and the 3′ primer derived from the 5′ end of the *GATA-3* cDNA. Of the two transgenic lines used in this study, the level of transgene expression in line 25 was higher than in line 3 (Figure 7B). Overexpression of GATA-3 did not have any overt effects on lymphocyte development since both transgenic lines had normal profiles of thymocyte subpopulations ($CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$) and spleen cells (B cells, CD4 and CD8 T cells) (data not shown).

Naive CD4 T cells from the transgene-positive animals and negative littermates were differentiated in vitro to the Th1 subset by culturing in ConA plus IL-2, IL-12, and anti-IL-4 mAb for 3 days, a time at which the Th1 phenotype is fully manifested at the mRNA level in this system (Kamogawa et al., 1993; Nakamura et al., 1997; Rincón and Flavell, 1997). Interestingly, we found that the cells from transgenic mice grew slower than transgene-negative cells (Figure 7C). By day 3, there was an approximately 2-fold difference in the number of cells between the transgene-positive and -negative littermates. Consistent with our previous observations, only low levels of GATA-3 were present in the non-transgenic Th1 precursors (Figure 7D). GATA-3 expression was, however, greatly elevated in the transgenic samples. The higher expression of GATA-3 in Th1 precursors of line 25 than those of line 3 correlated with their expression levels of the transgene in resting T cells (Figure 7B). The same large amount of *IFN-γ* mRNA was detected in the Th1 precursors of both transgene-positive and -negative mice. In contrast, while no *IL-4* mRNA was detectable in the nontransgenic developing Th1 cells as expected, *IL-4* mRNA was detectable in the transgenic samples with the level in line 25 being higher than in line 3. Likewise, *IL-6* mRNA was barely detectable in the nontransgenic developing Th1 cells, but was detected at high level in transgenic line 25 and (to a somewhat lesser extent) in line 3. IL-5 and IL-10 were detected at low levels in the nontransgenic samples. A large increase in *IL-10* mRNA was observed in the transgenic samples with a greater increase in line 25 than in line 3. *IL-5* mRNA was increased in both transgenic lines although the effect was less dramatic than the effect on IL-4, 6, and 10, consistent with our antisense data in D10 cells (Figure 4). The same results were obtained with a third transgenic line. However, *IL-13* mRNA remained undetectable in both transgene-positive and -negative cells. The expression of lineage-nonspecific IL-3 was not affected by the *GATA-3* transgene (Figure 7D). Thus, ectopic expression of GATA-3 in developing Th1 cells causes the abnormal expression of all the Th2 cytokine genes.

Discussion

The molecular mechanisms underlying CD4 T cell subset differentiation have been under intensive study. For Th2 subset differentiation, much work has focused on the regulation of *IL-4* gene expression. In this study, we have identified the transcription factor GATA-3 to be necessary for the transcription of all the Th2 cytokine genes that we have tested in CD4 T cells. We have also shown that forced expression of GATA-3 in Th1 precursors is sufficient to alter the course of Th1 subset differentiation and that this causes abnormal expression of these same Th2 cytokine genes in these cells. Unlike most prior studies, which took the approach of analyzing specific promoter elements and their *trans*-activating factors, our study was based on a more general assumption that different sets of regulatory genes are utilized

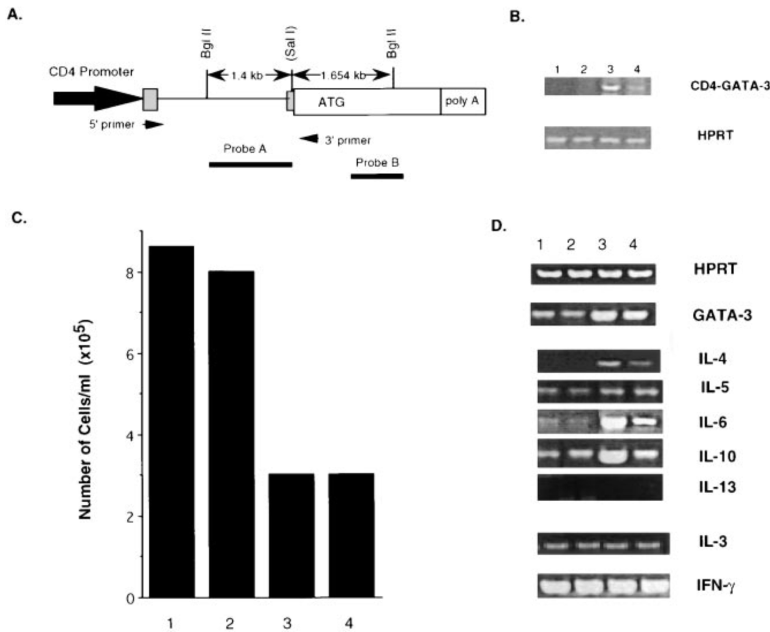

Figure 7. The Generation and Characterization of *CD4-GATA-3* Transgenic Mice

(A) Schematic representation of the CD4-GATA-3 construct. The single line represents the modified first intron of the *CD4* gene. Shaded boxes represent the first and second exons of *CD4* gene. The open box represents the full-length murine *GATA-3* cDNA. The positions of the probes used for screening transgene-positive mice and the primers for RT–PCR analysis of the expression of the transgene are also shown.

(B) RT–PCR was performed using the primers indicated in (A) to show the spliced mRNA species of the transgene.

(C and D) Naive CD4 T cells from transgene-negative (column/lanes 1 and 2) and -positive (column/lanes 3 and 4) littermates of line 25 and 3, respectively, were induced to differentiate in vitro to the Th1 subset for 3 days with ConA, IL-2, IL-12, and anti-IL-4 mAb. Cell number difference (C) and the expression of *GATA-3* and cytokine genes (D) are shown.

by the differentiating and/or differentiated Th2 versus Th1 cells. While this strategy could miss posttranscriptional events that might play important roles in differentiation, any process that leads to a difference in the steady state level of a given mRNA can be studied. We therefore embarked on the search for genes differentially expressed in Th2 cells by cDNA subtraction between Th2 and Th1 cells using RDA (Lisitsyn et al., 1993; Hubank and Schatz, 1994). We found that the transcription factor GATA-3 is the most predominant gene species selectively expressed in Th2 cells.

The time point we chose for RDA was 4 days after in vitro induction of Th1 and Th2 subsets from naive CD4 T cells. Although it is possible that genes turned on/off at earlier time points may be missing in the original cDNA pools, in this particular case, the striking difference in the expression of GATA-3 in Th1 and Th2 cells was already manifest by both day 2 and day 3 after primary stimulation. Specifically, in Th1 cells, GATA-3 expression was dramatically down-regulated on day 2 after stimulation. This change in GATA-3 expression correlated with the polarized expression of IFN-γ and the loss of IL-4 expression and, hence, with the susceptibility of the Th1 subset from *IL-4-TK* transgenic mice to killing by gancyclovir (Kamogawa et al., 1993; Nakamura et al., 1997). In other words, GATA-3 expression drops as Th1 cells "commit" to their lineage, suggesting that the loss of Th2 cytokine gene expression in Th1 cells is due to the diminished expression of GATA-3. Down-regulation of a particular transcription factor has been observed to be key to the differentiation of other hemopoietic lineages. For example, the CCAAT/enhancer binding protein C/EBPα is expressed at high level in myeloblasts but diminishes to low level in their terminal differentiation into polymorphonuclear cells (Scott et al., 1992).

Like the in vitro–induced Th2 cells, the long-term Th2 clone D10 also expressed a high level of GATA-3 in contrast to the Th1 clone AE7, which expressed low GATA-3. Such a pattern of high versus low expression in Th2 and Th1 cells, respectively, indicated that a relatively high level of GATA-3 is required for Th2 cytokine gene expression. To test this, we generated antisense *GATA-3* lines from D10 cells and showed that the transcription of all Th2 cytokine genes in these two lines was inhibited as a result of the decreased expression of GATA-3. Thus, a high level of GATA-3 is required for Th2 cytokine gene expression. Generally, GATA factors share highest homology in their DNA binding motifs (Yamamoto et al., 1990), so that different GATA factors often recognize overlapping DNA sequences. Consequently, the functional specificity of a particular GATA factor is achieved through specific expression in its host cells. Accordingly, selective expression of GATA-3 in Th2 cells is important for this transcription factor to be able to function as the specific regulator of Th2 cell differentiation. However, it remains unclear why a low level of GATA-3 is maintained in Th1 cells. Since GATA-3 is essential for T cell development (Hattori et al., 1996; Ting et al., 1996), a basal level of GATA-3 is perhaps required for maintaining the T cell status of the Th1 cells, for example, by maintaining TCR expression (Ko et al., 1991; Ho et al., 1993).

In trying to understand the mechanisms by which GATA-3 regulates Th2 cytokine gene expression, we searched the genomic sequences of all Th2 cytokine genes for potential GATA-3 binding sites. We found that GATA-3 binding sites are present in the promoter regions of all Th2 cytokine genes, suggesting that GATA-3 may directly activate these promoters. We confirmed this by showing that GATA-3 can activate the *IL-4* promoter in the B cell lymphoma M12. Unlike c-Maf, GATA-3 alone did not seem to activate the *IL-4* promoter in M12 cells without stimulation with PMA plus ionomycin, indicating that GATA-3 must act in cooperation with other activation-inducible factors such as AP-1 and NF-AT to activate the *IL-4* promoter. In a different study, it

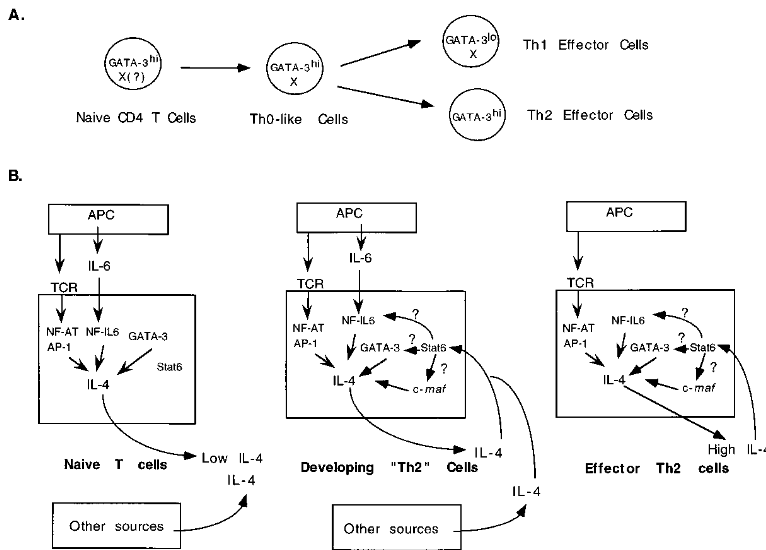

Figure 8. Schematic Illustration of the Differentiation Process of CD4 T Cell Subsets

(A) A general model for Th1 and Th2 cell development.

(B) Molecular events during Th2 differentiation.

was found that GATA-3 can also directly transactivate the *IL-5* promoter (D.-H. Zhang et al., submitted). It is also noteworthy that the susceptibility to inhibition by antisense *GATA-3* was different among the Th2 cytokine genes. Given the fact that GATA-3 can directly act on at least two (*IL-4* and *IL-5*), and probably all, of the Th2 cytokine gene promoters, the difference in susceptibility may reflect the difference in binding affinity of the GATA-3 sites in different Th2 cytokine genes. In other words, the expression of different Th2 cytokine genes requires a different threshold of GATA-3. Based on antisense inhibition and induction by GATA-3 elevation in transgenic mice, the level of GATA-3 required for different cytokine expression can be tentatively listed as IL-13>IL-6>IL-4>IL-10>IL-5. Obviously, the possibility cannot be excluded that the relatively high level of the remaining expression of IL-5 and IL-10 in the antisense transfectants is due to an alternative mechanism independent of GATA-3. Nevertheless, the differential requirement of GATA-3 for Th2 cytokine gene expression provides a potential explanation for the existence of intermediate CD4 T cells, between Th1 and Th2 extremes, that express different sets of cytokines (for review, see Mosmann and Sad, 1996).

To test if GATA-3 could divert the differentiation program of Th1 precursors to a Th2 program, transgenic mice were made where GATA-3 expression was driven in both subsets by the *CD4* promoter (Killeen et al., 1993). After stimulation under priming condition for the Th1 subset, the developing "Th1" population from the transgenic mice now expressed all Th2 cytokine genes except *IL-13*. These data, together with the down-regulation of GATA-3 in committed Th1 cells, demonstrate that the loss of Th2 cytokine gene expression in Th1 cells is at least in part due to their diminished GATA-3 expression. However, the forced expression of GATA-3 did not abort the program for Th1 cytokine gene expression as seen by the unaltered expression of the *IFN-γ* gene. This is consistent with a previous finding that hybrid cells derived from fusion of cloned Th1 and Th2 cells produced both Th1 and Th2 cytokines (Ho et al., 1996). Thus, it is reasonable to assume that other factors required for Th1 cytokine gene expression must be present in the Th1 lineage but are lost during Th2 subset development just as GATA-3 is lost in Th1 cells (Figure 8A). This hypothesis is further supported by our finding that, in the antisense *GATA-3* transfectants, no *IFN-γ* mRNA was induced (Figures 4A and 4B), and it is consistent with our previous general model for Th differentiation (Kamogawa et al., 1993; Nakamura et al., 1997).

The *IL-4* gene is expressed at a lower level in the *GATA-3* transgenic Th1 precursors than in effector Th2 cells, perhaps because other transcription factors such as c-Maf (Ho et al., 1996) are required for optimal expression of the *IL-4* gene in CD4 T cells. c-Maf is not detectable in developing Th2 cells but accumulates when Th2 effector cells have "completed" their differentiation (Ho et al., 1996). In addition, other differences in transcription regulation between Th1 and Th2 cells also exist, which may be important for optimal Th2 cytokine gene expression. For example, we found that AP-1 and NF-AT were preferentially activated in Th2 rather than Th1 effector cells (Rincón et al., 1997b), and JunB (Rincón et al., 1997b; Rooney et al., 1995) and JunD (Rooney et al., 1995) of the AP-1 complex were selectively expressed in Th2 cells. Because GATA-3 transactivation in M12 cells requires stimulation with PMA and ionomycin (Figure 6), which likely activate NF-AT and AP-1, GATA-3 may also synergize with these factors to induce *IL-4* gene expression in Th2 cells. In Th1 cells where these additional factors may be less readily available (Rincón et al., 1997a, 1997b), increased level of GATA-3 may therefore only induce suboptimal expression of *IL-4* gene. Alternatively, IFN-γ produced by the transgenic CD4 T cells could specifically inhibit the expression of the *IL-4* gene. Nevertheless, collectively, the data suggest that the differentiation of the Th2 subset may follow a model in which transcription factors cooperate in combination to define a cell type as seen in the differentiation of many other hemopoietic lineages (Ness and Engel, 1994).

To date, six transcription factors (Stat-6, c-Maf,

NF-IL6, NF-AT, AP-1, and now GATA-3) have been implicated in Th2 cell differentiation (Davydov et al., 1995; Rooney et al., 1995; Ho et al., 1996; Kaplan et al., 1996b; Rincón et al., 1997b, 1997c). Precisely how these factors interact remains to be elucidated. From the present study and a previous study on APC-produced IL-6 in Th2 differentiation (Rincón et al., 1997a), we believe that IL-6-activated members of the C/EBP family, such as NF-IL6 (Davydov et al., 1995), GATA-3 (expressed at high level in naive T cells), NF-AT, and AP-1 (Rooney et al., 1995) initiate IL-4 production in early activated naive T cells (Croft and Swain, 1995). The secreted IL-4 in turn activates Stat-6, which through an unkown mechanism might, for example, maintain a high level expression of GATA-3. As precursor cells commit to the Th2 lineage, high levels of c-Maf expression would be induced (Ho et al., 1996), perhaps by mechanisms involving GATA-3, to participate in high level expression of *IL-4* and perhaps other Th2 cytokine genes (Figure 8B). Obviously, these speculations need to be confirmed by future experiments.

Nevertheless, the present study shows that GATA-3 appears to be a key regulator of the collective activity of the transcription factors related to Th2 cytokine gene expression. It could therefore be a major target for modifying the immune responses in many immunological conditions. From a pharmaceutical viewpoint, the fact that merely lowering rather than abolishing GATA-3 expression is sufficient to change the phenotype of the CD4 effector cells may facilitate such intervention.

Experimental Procedures

Preparation of Naive CD4 T Cells and APCs

To isolate naive CD4 T cells, mice were kept in a clean environment for at least two weeks prior to sacrifice. CD4 T cells were prepared as described (Kamogawa et al., 1993). Naive (CD45RB^{high} CD44^{low}) cells were sorted on a Becton Dickinson FACStar plus. To prepare APCs, spleen cells were treated with mAb Y19, TIB105, GK1.5, and HB191 plus rabbit complement to remove T cells and NK cells, then γ-irradiated with 3000 rad.

In Vitro Differentiation of CD4 T Cells

Naive CD4 T cells (5×10^5/ml) were incubated with an equal number of syngeneic APC in Bruff's medium containing 5% FCS, 2.5 μg/ml Con A or 5 μg/ml pigeon cytochrome c peptide (in the case of pigeon cytochrome c TCR transgenic CD4 T cells), and 25 units/ml IL-2. For the induction of Th1 cells, 3.5 ng/ml IL-12 and anti-IL-4 mAb 11B11 were added to the culture; 1000 units/ml IL-4 and anti-IFN-γ mAb XMG1.2 were used for the induction of Th2 cells. Cells were stimulated for various days and harvested for further analysis.

cDNA Representational Difference Analysis

RDA for cDNA was performed as described (Lisitsyn et al., 1993; Hubank and Schatz, 1994). In brief, cDNA from the in vitro–induced Th cells was digested with Dpn II and ligated to the R-Bgl-12/24 adapters. Representations were made by PCR amplification of the R-ligated cDNA fragments for 20 cycles using the R-Bgl-24mer as primer. The R-linker sequences in Th2 representations were replaced with J-Bgl-12/24 adaptors. First subtractive hybridization was set up using 0.4 μg J-ligated Th2 representations as testers and 80 μg linker-removed Th1 representations as "drivers" (tester:driver = 1:100). An aliquot of the hybridization mixture was amplified by PCR for 10 cycles using the J-24mer as primers. The PCR products were then digested with mung bean nuclease (New England Biolabs) for 35 min at 30°C. The digested PCR products were further amplified for 18 cycles, and the products of this amplification are the first difference products (Dp1). The procedure was repeated twice using different primers and tester:driver ratios of 1:800 and 1:400,000.

cDNA Library Construction and Screening

Total RNA was isolated from in vitro–differentiated Th2 cells. cDNA synthesis and library construction were carried out using a ZAP Express/Gigapack III Gold Cloning Kit (Stratagene).

The Th2 library was screened using Th2.Dp3 or Th1.Dp3 as probes at 5–10 $\times 10^6$ cpm/ml. The screening was carried out in two steps. First, 2×10^4 colonies were plated in 20 150 mm dishes. Two separate plate lifts were prepared and differentially hybridized to Th2.Dp3 and Th1.Dp3 probes. The small number of colonies in each plate allowed the identification of single positive colonies without further screenings. Typically, colonies hybridized to Th2.Dp3 do not hybridize to Th1.Dp3, and few colonies hybridize to Th1.Dp3. Phagemids from Th2.Dp3-hybridizing colonies were pooled, and their cDNA inserts were recovered by a single restriction digestion and used as probes in the second step. In the second step, 7.5×10^5 colonies were screened with probes of Th2.Dp3 and the cDNA inserts recovered from the first step. Nonoverlapping colonies that hybridize to the Th2.Dp3 only were identified and further screened to obtain single colonies. Phagemid DNA of the Th2-specific colonies of both the first and second steps were sequenced using the 5′ T3 primer on an ABI automated sequencer and further studied.

RT–PCR

Total RNA was isolated from cultured cells using the Ultraspec RNA Isolation System-II (Biotecx). Reverse transcription was carried out using Superscript II RT (GIBCO–BRL). The primers and PCR conditions for HPRT and cytokine genes and competitive PCR were described previously (Reiner et al., 1994). The same PCR conditions were used for the analysis of other genes. Sequences of other primers used in this study are as following: GATA-3: GAAGGCAT CCAGACCCGAAAC and ACCCATGGCGGTGACCATGC; β-tubulin: ATGTT GCTCTCAGCCTCGGTGAAC and TGTCCATGAAGGAGGTG GAT GAG; IL-3: ATACCCACCGTTTAACCAGAACG and GATCTCGA ATGAAGACCCCTGGC; IL-6: GAACAACGATGATGCACTTGCAG and CCTTAGCCACTCCTTCTGTGAC; IL-13: CCACTACGGTCTCCA GCCTCC and TTGCCAGGCTGAGACCCTGAG; GM-CSF: GGCAAT TTC ACCAAACTCAAGGGC and CCAAGTTCCTGGCTCATTACGC.

Plasmids and Transgenic Mice

Murine *GATA-3* cDNA was a gift from Frank Grosveld and James D. Engel. The full-length *GATA-3* cDNA in pBlueScript SK was excised by EcoRI digestion and blunt-ended with T4 DNA polymerase I and cloned into the SalI site of plasmid CD4 promoter vector p37.1 (a gift of D. Littman), which expresses in both CD4 and CD8 T cells. The ligation produced clones with *GATA-3* in sense and antisense orientations with respect to the CD4 promoter. The *CD4-GATA-3* fragment was excised by NotI from the sense clone and used for construction of transgenic mice. The *CD4*-anti-*GATA-3* fragment was excised by NotI and cloned into the NotI site of pcDNA3 (Invitrogen). A clone in which the CMV promoter of pcDNA3 and the CD4 promoter were in the same orientation was used as the antisense *GATA-3* construct, pCD4αGATA3. The control plasmid pcDNA3.CD4 was created by cloning the NotI CD4 promoter fragment from p37.1 into pcDNA3 vector.

To make the IL-4 luciferase reporter construct pIL4–157Luc, the −157 to +68 IL-4 promoter fragment in the IL-4 CAT construct (a gift of L. Glimcher) was amplified by PCR with a 5′ primer linked to a HindIII adapter and a 3′ primer linked to an NheI adapter. The PCR fragments were digested with NheI and HindIII and cloned into the compatible sites of the luciferase plasmid pgl3 (Promega). The IL-4 promoter sequence in pIL4–157Luc was verified by sequencing.

Cell Culture and Transfection

Normal T cells and clones were grown in Bruff's medium plus 5% FBS. The Th1 clone AE7 was a gift of M. Lenardo and maintained by stimulation with AKR/J APC plus pigeon cytochrome c peptide (5 μg/ml) and 30–50 units IL-2/ml every two weeks. The Th2 clone D10 (a gift of C. A. Janeway, Jr.) were stimulated every 3–4 weeks with AKR/J APC and chicken conalbumin (Sigma) (100 μg/ml). The B cell lymphoma M12 was cultured in RPMI 1640 plus 5% FCS.

Transfection of D10 cells and M12 cells was done essentially as described (Todd et al., 1993; Ho et al., 1996). To establish stable transfectants, D10 cells transfected with pD4αGATA3 or pcDNA3. CD4 were allowed to recover for 48 hrs, then stimulated with antigen plus APC in medium containing 800 μg/ml G418 (GIBCO–BRL). The cells were stimulated every 1–2 weeks. After selection in G418 for 4–5 weeks, stable G418-resistant lines were established. Three independent transfections generated the stable antisense lines D10.3.4.1 and D10.3.4.2 and the control line D10.3. The cell lines were maintained in medium containing 200 μg/ml G418.

Immunoprecipitation

D10 transfectants were stimulated with AKR/J APC and conalbumin peptide (1 μg/ml) and labeled with 0.2 mCi/ml ^{35}S-methionine overnight. Cells were lysed and GATA-3 proteins were captured by agarose beads conjugated with anti-GATA-3 mAb HG3–31 (Santa Cruz Biotechnology) according to the manufacturer's instructions.

Proliferation Assay

D10 transfectants (5×10^4) were stimulated with APC in the presence or absence of antigen for 18 hr. Then 1 μCi [^{3}H]thymidine was added to the culture and allowed to be incorporated into the cells. After another 18 hr incubation, the cells were harvested and [^{3}H]thymidine incorporation was measured on a β counter (Beckman). Triplicates of each sample were measured and averages determined.

Luciferase Assay

One day after transfection, M12 cells were either treated or untreated with 50 ng/ml PMA plus 1 μM ionomycin for 18–24 hr. Cell extracts were prepared using the Luciferase Assay Kit (Promega). Equal amounts of extract protein were used for luciferase activity assay and the relative light units were measured in a luminometer (Lumat). Background was subtracted from the readings and fold induction of luciferase activity was calculated as compared with the unstimulated cells.

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