

## **An Instructive Component in T Helper Cell Type 2 (Th2) Development Mediated by GATA-3**

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### **Abstract**

Although interleukin (IL)-12 and IL-4 polarize naive CD4<sup>+</sup> T cells toward T helper cell type 1 (Th1) or Th2 phenotypes, it is not known whether cytokines instruct the developmental fate in uncommitted progenitors or select for outgrowth of cells that have stochastically committed to a particular fate. To distinguish these instructive and selective models, we used surface affinity matrix technology to isolate committed progenitors based on cytokine secretion phenotype and developed retroviral-based tagging approaches to directly monitor individual progenitor fate decisions at the clonal and population levels. We observe IL-4-dependent redirection of phenotype in cells that have already committed to a non-IL-4-producing fate, inconsistent with predictions of the selective model. Further, retroviral tagging of naive progenitors with the Th2-specific transcription factor GATA-3 provided direct evidence for instructive differentiation, and no evidence for the selective outgrowth of cells committed to either the Th1 or Th2 fate. These data would seem to exclude selection as an exclusive mechanism in Th1/Th2 differentiation, and support an instructive model of cytokine-driven transcriptional programming of cell fate decisions.

Key words: GATA-3 • instruction • stochastic • T lymphocytes • cytokine

### **Introduction**

Antigen-activated CD4<sup>+</sup> T cells differentiate into either Th1 or Th2 cells, producing proinflammatory or proallergic cytokines, respectively. The best understood mode of regulating Th cell differentiation is the cytokine environment during primary T cell activation, with IL-12 associated with Th1 development and IL-4 with Th2 development. However, it is still uncertain how these cytokines mediate their effect on differentiation (1). Coffman and Reiner (1) recently articulated three competing models, each compatible with the observed effects of cytokines on differentiation. In the instructive model, cytokine signaling precedes progenitor commitment; that is, cytokines direct each progenitor towards a defined fate through a program of molecular cues. In the selective model, progenitor commitment is independent of cytokines; that is, progenitor

cells stochastically commit to various fates, and cytokines subsequently act by favoring the selective outgrowth of a particular lineage. In a hybrid instructive-selective model, the ratio of progenitors initially committing to each fate may be altered by cytokines, but cytokine-driven selective outgrowth of committed cells is retained as a required mechanism of polarization.

An instructive model seemed compatible with known molecular processes in T cells, such as cytokine regulation of transcription factor expression and cytokine-dependent chromatin alterations in the IL-4 locus (2, 3). Further, the specific requirement of the transcription factors signal transducer and activator of transcription (Stat4) (4, 5) and Stat6 (6, 7) in Th1 and Th2 development, respectively, was generally interpreted to indicate an instructive role for cytokines in Th differentiation. However, the analysis of Stat4- and Stat6-deficient T cells was done on bulk populations and not performed specifically to distinguish instructive or selective models of development. Stat4 and Stat6 activation could either initiate an instructive program or alternately might

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simply mediate signals for selective outgrowth of committed Th1 or Th2 cells. Moreover, certain findings, such as monoallelic cytokine expression (8–10), a role of cell cycle in cytokine regulation (11–13), and Stat6-independent Th2 development (14–16) have provided an impetus to reconsider the issue of instructive versus selective differentiation.

The difficulty in distinguishing between these models by previous studies arises from their inability to directly track fates and measure relative outgrowth of individual progenitors. Initial bulk population analysis suggested that Th2 development was absolutely IL-4 dependent, consistent with either model (6, 7). However, recent results have modified this thinking (15–17). Th2 development has recently been reported to occur in Stat6-deficient animals (15, 16) and to be dependent on GATA-3 expression independently of IL-4 and Stat6 (17). GATA-3 expression, although augmented by IL-4 through Stat6, can also be achieved independently of IL-4, involving a Stat6-independent pathway of autoactivation. This feature of GATA-3 expression allows progenitors to acquire a stable, cytokine-independent phenotype in which GATA-3 directs the elaboration of downstream hallmarks of Th2 development that occur in response to IL-4. Importantly, ectopic GATA-3 expression in Stat6-deficient T cells induces all developmental components of the Th2 phenotype such as c-Maf expression, chromatin remodeling of the IL-4 locus, and Th2 cytokine gene expression (17). Further, Th2 commitment need not be absolutely IL-4 dependent, as other factors, such as TCR/CD28 signaling (18) and IL-12 (17, 19) can regulate GATA-3 expression. Thus, any signal that can regulate GATA-3 expression would also be expected to regulate Th2 development.

In this study, we have used two recently described technologies, cellular affinity matrix sorting and retroviral tagging, to determine whether GATA-3 functions to promote outgrowth of cells that have stochastically committed to IL-4 production (selection), or whether GATA-3 mediates programmatic alterations in gene expression without affecting outgrowth of uncommitted progenitors (instruction). To accommodate the known effects of GATA-3 expression in Stat6-deficient T cells, the selective model must adopt GATA-3, rather than Stat6, as a mediator of selective outgrowth of Th2 cells. However, in a direct test of this hypothesis, we found no evidence that GATA-3 influences selective outgrowth when expressed in Stat6-deficient T cells developing under any conditions. Rather, GATA-3 expression increased the proportion of IL-4-committed progenitors, even when developing in the presence of IL-12, a condition predicted by the selective model to inhibit the outgrowth of cells committed to the Th2 phenotype. These data exclude purely selective models of Th2 development and provide strong evidence that GATA-3 instructs Th2 development without inducing selective outgrowth.

## Materials and Methods

*Sorting of Live IL-4-secreting Cells by Cellular Affinity Matrix Technology.* In vitro cultures of DO11.10 TCR transgenic wild-type and DO11.10<sup>+</sup> × Stat6<sup>-/-</sup> CD4<sup>+</sup> T cells were stimulated

with OVA protein under neutralizing conditions in the presence of α-IL-4 (11B11, 10 μg/ml), α-IL-12 (Tosh, 10 μg/ml), and α-IFN-γ (H22, 10 μg/ml). 7 d after primary activation, live CD4<sup>+</sup>/IL-4-secreting cells were purified as described previously (17) with the following modifications. Cell cultures were restimulated with PMA/ionomycin for 2.5 h and subsequently labeled with the bifunctional Ab conjugate specific for CD45 (30-F11) and IL-4 (BVD6-24G2) for surface-capture of secreted IL-4. The cells were then diluted in medium and allowed to secrete IL-4 for 30 min at 37°C as described (17). IL-4-secreting CD4<sup>+</sup> T cells were identified by staining with a secondary mAb α-IL-4-PE (11B11-PE; BD PharMingen) and α-mCD4-TriColor (GK1.5-TC; Caltag). IL-4<sup>+</sup> and IL-4<sup>-</sup> cells were purified by FACS<sup>®</sup> sorting as described (20, 21).

*Retroviral Transduction.* DO11.10<sup>+</sup> × Stat6<sup>-/-</sup> CD4<sup>+</sup> T cells were activated for 36 h with OVA under Th1 (rmIL-12 [10 U/ml], and α-IL-4) or Th2 (α-IL-12, and α-IFN-γ and/or rmIL-4 [100 U/ml]) conditions. T cell cultures were then transduced with retroviral culture supernatants derived from the transient transfection of the Phoenix-ecotropic retrovirus packaging cell line (19). Percentages of retroviral-transduced cells were monitored for the expression of retroviral marker proteins green fluorescence protein (GFP) and hCD4 by FACS<sup>®</sup> analysis.

*T Cell Cloning.* DO11.10<sup>+</sup> × Stat6<sup>-/-</sup> CD4<sup>+</sup> T cells were activated with OVA protein under either Th1 or Th2 conditions and transduced with retroviral vectors as described in the text. Cells were rested in bulk culture to day 7 of activation and CD4<sup>+</sup>GFP<sup>+</sup> cells were purified by FACS<sup>®</sup> sorting. Purified T cells were cloned by single-cell deposition cloning by FACS<sup>®</sup> sorting into 96-well plates containing 5 × 10<sup>5</sup> irradiated BALB/c splenocytes, OVA protein (0.5 mg/ml), and rhIL-2 (50 U/ml) in 200 μl medium (DMEM supplemented with L-glutamine [0.2 mM], nonessential amino acids [10 μM each], sodium pyruvate [100 μM], β-mercaptoethanol [50 μM], and penicillin/streptomycin [100 U/ml each]). Cellular outgrowth was monitored between days 10–15 of plating, and individual clones were expanded by restimulation with irradiated BALB/c splenocytes, OVA protein, and rhIL-2 (50 U/ml).

*Identification of GFP-expressing Retrovirus and GATA-3-GFP-transduced T Cell Clones by PCR.* Genomic DNA derived from independent T cell clones were amplified for the detection of both the control GFP-expressing retrovirus (GFPRV) and the GATA-3-GFP integrated retroviral vectors by a two-step nested PCR technique. These PCR reactions were performed with the following primers: GFPRV, first set: 5'-CCTACATCGTGACCTGGGAAGCCTTGG, 5'-GTTCCGCTGCCTGCAAAGGGTCCG, nest: 5'-GGTCAAGCCCTTTGTACACCCTAAGCCTCC, 5'-CCTAGGAATGCTCGTCAAGAAGACAGGGC; GATA-3-GFP, first set: 5'-CCTACATCGTGACCTGGGAAGCCTTGG, 5'-GGTGAAGAGATGAGGACTGGAGTGGCC, nest: 5'-CCCTTTGTACACCCTAAGCCTCCGCC, 5'-GGAGGGTAAACG-GACAGAGGCCCC.

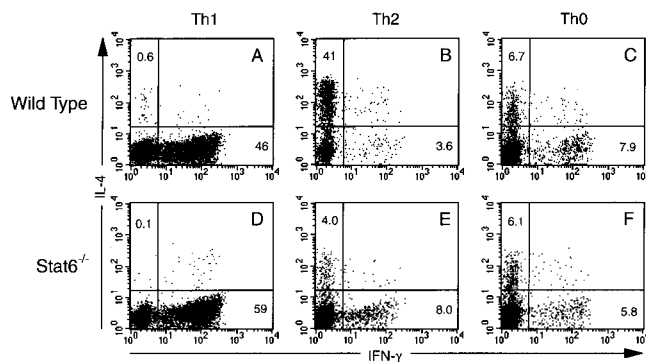
As controls, genomic DNA from nontransduced and single-transduced T cells from bulk culture populations were amplified in parallel PCR reactions. Clones used in the study were infected by one type of retrovirus, positive for either GFPRV or GATA-3-GFP, but not both.

## Results and Discussion

The observation that both IL-4- and IFN-γ-producing T cells can emerge under nonpolarizing conditions has been interpreted to suggest that a random, or stochastic

process generates the initial phenotype repertoire, motivating the selective model (1, 9, 11). Indeed, when naive progenitors are activated under conditions in which IL-12, IL-4, and IFN- $\gamma$  are all neutralized, a persistent population of cells was still observed that committed to the production of either IL-4 or IFN- $\gamma$  (Fig. 1 C). By the selective model, the increase in populations developing in fully polarizing conditions (Fig. 1, A and B) arise from the selective outgrowth of cells that stochastically commit even in the absence of these conditions (i.e., from the populations in Fig. 1 C). Moreover, this interpretation is consistent with persistence of a small IL-4-producing population even in Stat6-deficient mice (Fig. 1, E and F), which are proposed to represent committed progenitors whose phenotypes are fixed and awaiting selective outgrowth via the effects of polarizing cytokines.

However, the instructive and selective models differ in their predictions of how committed cells should respond to subsequent IL-4 exposure. In the selective model, commitment is permanent, with subsequent cytokine exposure altering cell growth, but not differentiation. In the instructive model, commitment results from cytokine-derived signals, which could be delivered even after initial activation. These predictions can be distinguished by testing responses of committed progenitors to the polarizing effects of IL-4. To test this experimentally, cells initially committed to an IL-4-producing phenotype or IL-4-nonproducing phenotype would need to be separated and analyzed independently for differentiation when exposed to IL-4 (Fig. 2 a). To separate IL-4-producing from IL-4-nonproducing committed cells, we used the novel cellular affinity matrix technology for purifying live cells based on their IL-4



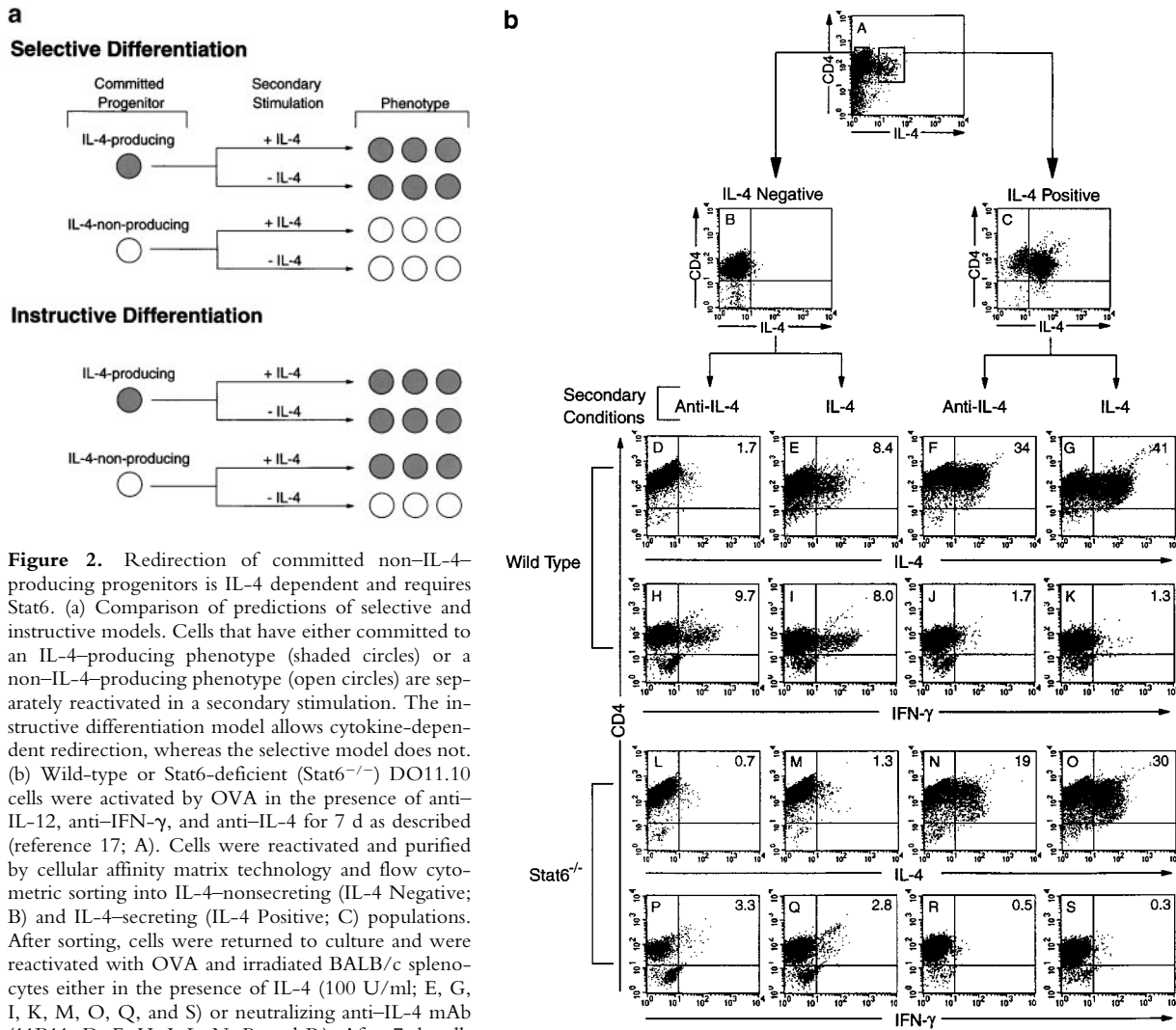
**Figure 1.** IL-4- and Stat6-independent commitment to IL-4-producing phenotype. DO11.10 TCR transgenic wild-type (A–C) or DO11.10 Stat6-deficient (Stat6<sup>-/-</sup>, D–F) T cells were stimulated with OVA (0.5 mg/ml chicken OVA) with the addition of IL-12 (10 U/ml) and 11B11 (10  $\mu$ g/ml; Th1; A and D), the addition of IL-4 (100 U/ml), anti-IFN- $\gamma$  (H22, 10  $\mu$ g/ml) and anti-IL-12 (Tosh, 10  $\mu$ g/ml; Th2; B and E), or with addition of anti-IL-12, anti-IFN- $\gamma$ , and anti-IL-4 (Th0; C and F), as described (reference 17). 7 d after activation, cells were restimulated with PMA and ionomycin for 4 h followed by analysis of IL-4 and IFN- $\gamma$  production by intracellular staining as described previously (references 20, 21, and 26). Gates for analysis excluded dead cells and quadrants were set based on isotype control stainings (reference 26). The percentages displayed indicate the frequency of cells positive for IL-4 or IFN- $\gamma$  within the live cell gates.

secretion (17). Wild-type and Stat6-deficient (Stat6<sup>-/-</sup>) DO11.10 naive T cells were activated in the presence of anti-IL-4, anti-IL-12, and anti-IFN- $\gamma$ . On day 7, cells were reactivated with PMA and ionomycin (Fig. 2 b, A), and CD4<sup>+</sup> T cells were sorted into IL-4-nonsecreting cells (Fig. 2 b, B) and IL-4-secreting cells (Fig. 2 b, C). Each population was then divided and restimulated either in the presence of IL-4 or anti-IL-4 mAb (11B11) for 7 d. Upon restimulation, IL-4 and IFN- $\gamma$  production by individual cells was measured by intracellular staining (Fig. 2 b, D–S).

Cells that had initially committed to IL-4 production (Fig. 2 b, C) retained this property at a frequency compatible with differentiated Th2 cells (Fig. 2 b, F and G). The frequency of IL-4-positive cells in both wild-type and Stat6-deficient populations was only slightly increased by exposure to IL-4 in secondary culture (Fig. 2 b, compare G to F and O to N). Cells that initially had not produced IL-4 and were not exposed to IL-4 in secondary culture remained negative for IL-4 production later (Fig. 2 b, D and L), as predicted by both models. However, wild-type cells that were initially IL-4 negative (Fig. 2 b, B), and were exposed to IL-4 in secondary culture showed a significantly increased frequency of IL-4-producing cells (compare Fig. 2 b, E to D), consistent with redirection to an IL-4-producing fate. This increase in IL-4 frequency was not observed in Stat6-deficient cells (Fig. 2 b, compare M to L), suggesting that the effect required an intact IL-4 signaling pathway. Thus, this effect cannot be explained as a continued stochastic generation of IL-4-producing cells, as the selective model would predict this to also occur in Stat6-deficient cells as well as in wild-type cells exposed to anti-IL-4 mAb. In addition, we observed only a minor population of cells committed to producing IFN- $\gamma$  in wild-type cells sorted for being IL-4 negative (Fig. 2 b, H and I). The percentage of this population in wild-type and Stat6-deficient cells was not influenced by either the presence or absence of IL-4 (Fig. 2 b, H to K and P to S). Thus, in these experiments, the expansion of IFN- $\gamma$ -producing Th1 cells was not seen, eliminating its potential influence on inhibiting Th2 development in Stat6-deficient T cells. In summary, IL-4 exposure appears to direct Th2 commitment even after the initial period predicted by the selective model to generate the stochastic fixed repertoire.

Due to the kinetics of gene activation, the frequency of IL-4 staining in activated Th2 cells is not 100%, but  $\sim$ 30–50%, consistent with previous reports (20–23). Thus, it is still possible to interpret the above results as consistent with the selective model by explaining the apparent redirection of cells in Fig. 2 as the IL-4-dependent outgrowth of Th2-committed cells that simply happened to not produce IL-4 at the time of sorting. Thus, to rigorously distinguish these aspects of selection from instruction, it is therefore necessary to track progenitor fates and frequencies as they expand in culture.

To do this, we developed a retroviral-based tagging system in which either a GFP retrovirus or a GATA-3-expressing retrovirus is used to mark cells by infection during the initial activation (Fig. 3 a). Instructive and selec-



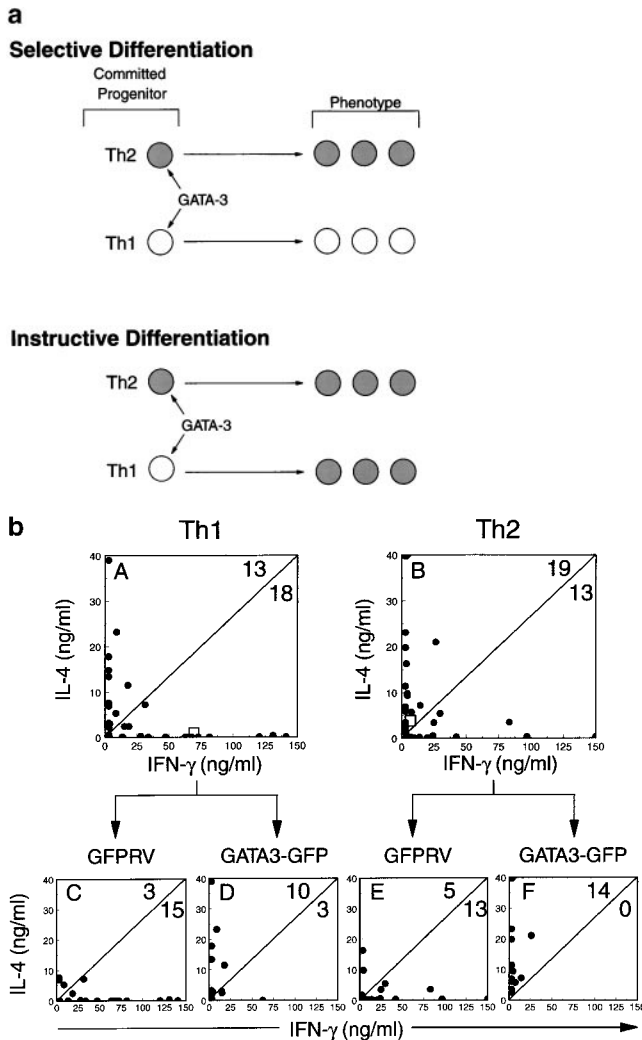
**Figure 2.** Redirection of committed non-IL-4-producing progenitors is IL-4 dependent and requires Stat6. (a) Comparison of predictions of selective and instructive models. Cells that have either committed to an IL-4-producing phenotype (shaded circles) or a non-IL-4-producing phenotype (open circles) are separately reactivated in a secondary stimulation. The instructive differentiation model allows cytokine-dependent redirection, whereas the selective model does not. (b) Wild-type or Stat6-deficient (Stat6<sup>-/-</sup>) DO11.10 cells were activated by OVA in the presence of anti-IL-12, anti-IFN- $\gamma$ , and anti-IL-4 for 7 d as described (reference 17; A). Cells were reactivated and purified by cellular affinity matrix technology and flow cytometric sorting into IL-4-nonsecreting (IL-4 Negative; B) and IL-4-secreting (IL-4 Positive; C) populations. After sorting, cells were returned to culture and were reactivated with OVA and irradiated BALB/c splenocytes either in the presence of IL-4 (100 U/ml; E, G, I, K, M, O, Q, and S) or neutralizing anti-IL-4 mAb (11B11; D, F, H, J, L, N, P, and R). After 7 d, cells were stimulated with PMA and ionomycin for 4 h followed by analysis of mCD4 expression and IL-4 (D to G and L to O) and IFN- $\gamma$  (H to K and P to S) production by intracellular staining. Analysis gates exclude dead cells. Quadrants were set based on isotype control staining as described (reference 26), and the percentage in the top right quadrant indicates the frequency of cells positive for cytokine production. These experiments were performed three times with consistent results.

tive models differ in how they predict progenitors will respond to tagging by the GATA-3-expressing retroviruses. To account for the known effects of GATA-3, the selective model predicts that cells committing to either IL-4-positive or IL-4-negative fates should each expand in response to GATA-3, but should not alter their respective fates (Fig. 3 a). In contrast, the instructive model predicts that, independent of initial commitment, progenitors tagged by GATA-3 may undergo redirection of fate toward IL-4 production. The control retrovirus expressing only the GFP tag is not predicted to alter fates in either model. In essence, the selective model predicts outgrowth of both GATA-3-expressing Th1 and Th2 clones, whereas the instructive model predicts that GATA-3 infected clones will be strongly skewed to a Th2 fate, with marked reduction in the numbers of GATA-3-infected Th1 clones.

To eliminate any bias, we blinded this tagging experiment to the identity of the retrovirus by infecting progeni-

tors with a mixture of control and GATA-3 retroviruses, both expressing GFP as the retroviral marker. The identity of the retrovirus infecting each clone was revealed by PCR only at the end of the experiment. Stat6-deficient progenitors were infected with GFP and GATA-3 retroviruses and activated in either Th1 or Th2 conditions. On day 7, single retrovirally infected CD4<sup>+</sup> cells were directly cloned into nonselecting conditions (i.e., in the absence of IL-12, IL-4, or IFN- $\gamma$ ). Clones were expanded and characterized for IL-4 and IFN- $\gamma$  expression by ELISA to assign phenotype (Fig. 3 b), and only then was the identity of the infecting retrovirus determined.

Cytokine production by all clones is presented in Fig. 3 b for progenitors activated in Th1 (Fig. 3 b, A) or Th2 (Fig. 3 b, B) conditions. The majority of clones exhibit cytokine production that is near to one of the cytokine axes, consistent with polarization observed in bulk populations. The phenotype of each clone was assigned based on its cy-



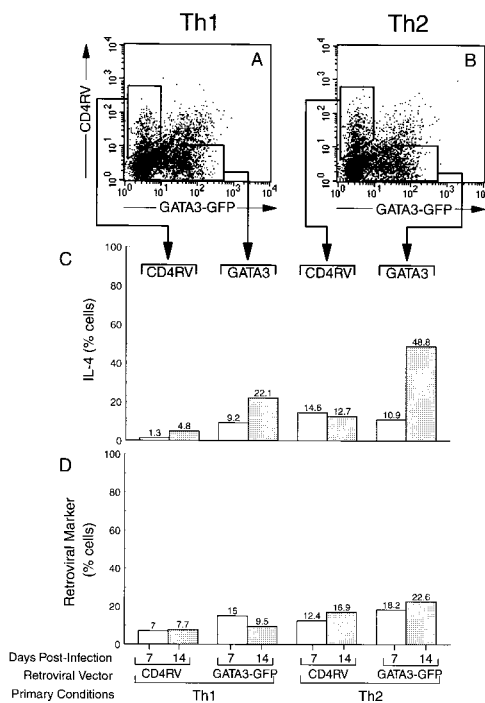
**Figure 3.** Clonal analysis of commitment in Stat6-deficient T cells infected with control or GATA-3-expressing retroviruses. (a) Comparisons of predictions of selective and instructive models. Progenitors committed to either an IL-4-producing (shaded circles) or a non-IL-4-producing phenotype (open circles) are infected with control retrovirus or GATA-3-expressing retrovirus. The selective model predicts GATA-3 to promote outgrowth of cells and not to affect their differentiation, whereas the instructive model predicts that GATA-3 should promote the differentiation of all progenitors to the IL-4-producing fate. (b) Stat6-deficient DO11.10 cells were activated with OVA under Th1 or Th2 conditions (reference 17). 36 h after activation, cells were infected with a mixture of two retroviruses, GFPRV and a GATA-3-expressing retrovirus (GATA3-GFP; reference 27). 7 d after activation, GFP-expressing CD4<sup>+</sup> T cells were purified and cloned by flow cytometric sorting and single-cell deposition into 96-well plates containing irradiated BALB/c splenocytes and OVA. After expansion, independent clones were restimulated on anti-CD3-coated plates for 24 h and supernatants analyzed for IL-4 and IFN- $\gamma$  secretion by ELISA (reference 28). The retrovirus infecting the clone was then determined by genomic PCR analysis. Cytokine production by individual clones derived from Th1 or Th2 conditions is presented in A and B. Cytokine production of clones segregated by the identity of the infecting retrovirus is presented in C–F.

tokine production being above or below the diagonal, after which the retrovirus identity was determined (Fig. 3 b, C–F). Stat6-deficient cells that were infected with control GFP retrovirus were strongly skewed to the Th1 pheno-

type, regardless of initial activation conditions (Fig. 3 b, C and E). The low number of IL-4-producing clones is compatible with the observed frequency of IL-4-positive cells in bulk populations (Fig. 1 E). However, Stat6-deficient clones infected with the GATA-3 retrovirus were markedly skewed to the Th2 fate, even in those clones whose progenitors were activated under Th1-inducing conditions (Fig. 3 b, D and F). Further, there was no apparent difference in the numbers of clones obtained under various conditions, arguing against any gross differences in selective outgrowth. Thus, the results of this retroviral tagging experiment are inconsistent with predictions of the selective model described above.

To try to accommodate for this last experiment, the selective model could be slightly revised to propose that the growth-promoting effects of GATA-3 can only be exerted in cells that are also committed to IL-4 production, and not in other cells. In this revised selective model, the decreased number of GATA-3-infected Th1 clones would be due to the inability of GATA-3 to promote outgrowth of non-IL-4-producing cells, rather than from the conversion of their phenotype from Th1 to Th2 fate. Although it may be conceivable to construct a selective mechanism to restrict the effects of GATA-3 in this way, importantly this possibility can be tested using a dual-marker retroviral system to simultaneously track both fate and expansion of committed cells over time. We carried out this approach using unique markers to distinguish two retroviruses, with the control retrovirus expressing hCD4, and GATA-3 retrovirus expressing GFP. In these experiments (Fig. 4), Stat6-deficient progenitors were infected with a mixture of control retrovirus and GATA-3 retrovirus in the same cultures, and activated under either Th1 or Th2 conditions. Cells were expanded together in bulk culture, and IL-4 production by individual cells was analyzed on day 7 and on day 14 after secondary stimulation, allowing direct measurement of the frequency and phenotype of cells over time.

On day 7 and day 14, cells were activated with PMA/ionomycin for 4 h and stained for intracellular production of IL-4. Cells were gated for expression of a single retroviral marker (Fig. 4, A and B), and the percentage of IL-4-positive cells in each gate was measured by intracellular IL-4 staining (Fig. 4 C). Last, the proportion of each retrovirally infected population is expressed as a percentage of the whole population (Fig. 4 D). Infection by the control virus had no effect on IL-4 production by cells over time, either in the Th1- or Th2-activated conditions (Fig. 4 C), as expected. In contrast, infection by the GATA-3 retrovirus (GFP-positive cells) markedly increased the percentage of IL-4-positive cells between day 7 and day 14. This effect occurred in both Th1 and Th2 conditions, and these data were highly reproducible in replicate experiments (Table I). Importantly, the percentage of IL-4-producing cells within the GATA-3 population increased even though the total percentage of GATA-3-infected cells remains constant over time, consistent with a continuing developmental effect. Finally, the prediction of the revised selective model is not observed; that is, we observe a constant ratio



**Figure 4.** Intrinsic GATA-3-mediated signaling induces IL-4 expression without promoting selective cell outgrowth. Stat6-deficient DO11.10 splenocytes were activated with OVA under Th1 or Th2 conditions (reference 17). 36 h after activation, cells were infected with a mixture of two retroviral vectors containing control retrovirus expressing human CD4 extracellular domain marker protein (CD4RV) and the GATA-3-expressing retrovirus expressing a GFP marker protein (GATA3-GFP; reference 17). 7 d after primary activation, each culture was analyzed for GFP, hCD4, and intracellular IL-4 by FACS<sup>®</sup> analysis (A and B). The remaining cells were restimulated with irradiated BALB/c splenocytes and OVA for an additional 7 d. After secondary stimulation, the cells were analyzed for expression of GFP, hCD4, and intracellular IL-4. Shown are FACS<sup>®</sup> analyses of T cell cultures developed under Th1 (A) or Th2 (B) conditions and analyzed for the expression of the retroviral markers GFP (bottom right polygon) and human CD4 (top left polygon). (C) T cells expressing intracellular IL-4 are calculated as a percentage of cells expressing the gated retroviral marker proteins from cultures analyzed 7 d (white bars) and 14 d (stippled bars) after primary activation. (D) T cells expressing the retroviral marker proteins (polygon gates, top panels) are expressed as a percentage of the total number of live cells. Data from replicate analyses are presented in Table I.

of cells infected with control or GATA-3 retrovirus over time in both conditions, rather than an increase in the proportion of GATA-3-expressing cells as predicted by the selective model.

These results strongly argue against a purely selective mechanism in GATA-3-dependent Th2 development. First, we observed an apparent redirection of fate caused by exposure of previously non-IL-4-producing cells to an IL-4-producing phenotype, inconsistent with the selective model. Second, we observed that GATA-3 strongly skewed the frequency of individual clones that commit to IL-4 production but did not exert selective outgrowth of clones already committed to the Th1 fate, as would be predicted by the selective model. Finally, using dual-tag retroviral infection, we directly measured the fate and expansion

**Table I.** Intrinsic GATA-3-mediated Signaling Induces IL-4 Expression without Promoting Selective Cell Outgrowth

Culture conditions	Analysis gait	Infecting retrovirus				
		CD4RV		GATA-3-GFP		
		Day 7	Day 14	Day 7	Day 14	
		%	%	%	%	
Expt. 2	Th1	IL-4	4.4	4.3	2.9	7.4
		Retroviral marker	2.4	1.3	4.4	5.4
	Th2	IL-4	8.2	5.7	7.6	22.5
		Retroviral marker	2.6	2.1	4.2	6.0
Expt. 3	Th1	IL-4	6.4	2.1	8.6	22.9
		Retroviral marker	11.7	4.6	7.6	10.8
	Th2	IL-4	5.2	12.1	11.0	56.1
		Retroviral marker	13.8	16.0	12.8	16.5

Data from two additional replicate experiments (as shown in Fig. 4) are represented as percentage of cells expressing the retroviral marker and percentage of cells within each retroviral marker gait that express IL-4 by intracellular staining. Expt., experiment.

of distinctly committed cells, which revealed an increasing frequency of IL-4 production in the absence of any apparent increase in size of this committed population.

Taken together, these results strongly support an instructive interpretation of recent findings regarding the transcriptional regulation of GATA-3-induced Th2 development. The initial production of IL-4 by small numbers of Stat6-deficient T cells is consistent with either the stochastic expression of GATA-3 in a small percentage of T cells or with the regulated expression of GATA-3 by additional factors, including the TCR/CD28 signaling pathway, as described recently (18). Importantly, either explanation is consistent with an instructive model for GATA-3-induced Th2 development. However, it is possible that IL-4 could exert some selective/growth-promoting effects on committed Th2 cells that may not completely involve Stat6-dependent GATA-3 regulation (24). For example, we observed a 10% increase in the proportion of IL-4-producing cells in both wild-type and Stat6-deficient Th2 cells after secondary exposure to IL-4 (Fig. 2 b, F to G and N to O). However, this observation may be consistent with either instructive or selective models; instructive, if IL-4 could augment expression of GATA-3 or other transcription factors through Stat6-independent pathways, which we have not excluded; selective, if IL-4 could alter cell survival or proliferation through a Stat6-independent pathway such as insulin receptor substrate (IRS)-1 (25). Regardless, our results exclude models exclusively requiring selection to explain GATA-3-induced Th2 development, and strongly support the existence of an instructive component in this process.

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