

Combining cytokine signalling with T-bet and GATA-3 regulation in Th1 and Th2 differentiation: a model for cellular decision-making

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Abstract

Differentiation of uncommitted T cells into Th1 and Th2 subpopulations depends on both intracellular events controlling expression of transcription factors T-bet and GATA-3 and interactions between cells mediated by cytokines, particularly IL4 and IFN γ . A great deal is known about the intracellular and extracellular events involved in Th1 and Th2 (Th) differentiation, but how these are integrated in T-cell populations or indeed why extracellular cytokine control is required after a decision has been made at a transcriptional level is not at all understood. We present a mathematical model of CD4⁺ T-cell differentiation that describes both intracellular and extracellular processes and the interactions between them. It shows how antigen stimulation in conjunction with cytokines and other extracellular signals gives rise to rapid, reversible and mutually exclusive expression of T-bet or GATA-3 due to feedback between the transcription factors and their signalling pathways. After transient signalling by APC, continued Th1 and Th2 differentiation is shown to require cytokine production by the proliferating T cells. Moreover, intercellular communication by T-cell-derived cytokines lowers the threshold of APC signals required for Th differentiation. This provides an explanation for enhanced Th differentiation by pre-existing memory T cells. The model also predicts that Th differentiation can be reversed at the single cell level before commitment by manipulating the cytokine environment. It suggests a mechanism for switching between Th1 and Th2 in the so-called irreversible state that may be developed as a novel therapeutic means of manipulating Th1 and Th2 responses.

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1. Introduction

Cell differentiation commonly involves making decisions between alternative maturation pathways. Typical examples in the immune system are the development of CD4⁺ and CD8⁺ T cells from double-positive thymocytes, and the maturation of Th1 or Th2 helper T cells from uncommitted precursors. Much recent discussion has focussed on whether the decision-making process is

one of instruction or selection. Under instruction, a completely neutral cell will embark upon a particular differentiation pathway according to the signals it receives. A selective process, on the other hand, operates at a population level where a subset of cells that have already committed to a differentiation pathway respond to signals that selectively promote their proliferation or survival. Some ingenious experimental models have been devised to address this question for CD4⁺ and CD8⁺ T-cell development and have tentatively concluded that a selective process is involved (Leung et al., 2001) but the issue for Th1 and Th2 maturation is still unclear (Coffman and Reiner, 1999; Farrar et al., 2001).

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T helper cell differentiation is controlled by a complex set of signals including the cytokines made by the T cells themselves. Th1 cells make interferon- γ and lymphotoxin and are important for protection against intracellular pathogens. Inappropriate Th1 responses have been implicated in inflammatory and autoimmune diseases (Seder and Paul, 1994; Liblau et al., 1995; O'Garra et al., 1997; O'Garra, 1998). Th2 cells make IL4, IL5 and IL13, which are required for IgE production and activate mast cells and eosinophils. They are strongly implicated in atopy and allergic inflammation (Romagnani, 1994). The decision to make a type 1 or type 2 response can be literally a matter of life or death and an enormous effort has been made over the past 10 years to elucidate the mechanisms involved. This has revealed a complex regulatory system involving cytokine signals between Th1, Th2 and antigen-presenting cells (APC) together with TCR activation and costimulation by APC. IL12 made by APC and IFN γ by natural killer (NK) cells promotes Th1 differentiation. IFN γ made by Th1 cells then drives further Th1 differentiation by acting on APC to produce more IL12 and by direct positive feedback onto the Th1 cells themselves (Trinchieri, 1995). At the same time, IFN γ inhibits differentiation of Th2 cells by enhancing expression of the IL12R β 2 chain (Rogge et al., 1997; Szabo et al., 1997). Th2 differentiation on the other hand is enhanced by IL4 and IL10 inhibition of IL12 production by antigen-presenting cells (Macatonia et al., 1995; Koch et al., 1996), and by IL4 inhibition of IL12R β 2 expression on T cells (Rogge et al., 1997; Szabo et al., 1997). The effects of IL4 on Th2 development are dominant over Th1-inducing cytokines due largely to the positive feedback effect of IL4 produced by Th2 cells (Hsieh et al., 1993; Seder and Paul, 1994). The positive and negative feedback of cytokines controlling Th1 and Th2 development has led to the proposal of both intuitive (Morel and Oriss, 1998) and mathematical models (Fishman and Perelson, 1993, 1994; Yates et al., 2000; Bergmann et al., 2002) to describe Th subset differentiation.

It is now clear that IFN γ and IL4 exert their effects on Th1 and Th2 differentiation by controlling the expression of transcription factors T-bet (Szabo et al., 2000) and GATA-3 (Zheng and Flavell, 1997). T-bet and GATA-3 are necessary for the development of CD4⁺ Th1 and Th2 effector phenotypes, respectively (Szabo et al., 2002; Pai et al., 2004). These transcription factors interact with each other and the cytokines that induce them through feedback loops (Ho and Glimcher, 2002) (Fig. 2a). IL4 binding to its receptor on Th precursor (Thp) cells activates the signalling factor STAT6, which then translocates to the nucleus and rapidly induces the expression of GATA-3 (Zheng and Flavell, 1997; Ouyang et al., 2000). Other signalling pathways such as CD28 (Rodriguez-Palmero et al., 1999) and OX40

(Ohshima et al., 1998) may also induce GATA-3. Expression of GATA-3 is followed by induction of the transcription factor c-MAF, a potent IL4 gene specific activator (Ho et al., 1996; Kim et al., 1999). This forms a positive feedback loop for IL4 induction of GATA-3 and Th2 differentiation. At the same time, GATA-3 induction inhibits Th1 differentiation both by increasing IL4 production, and by inhibiting the master Th1 transcription factor T-bet (Ho and Glimcher, 2002).

The interactions controlling Th1 development are perhaps less well characterized. IL12 and IFN γ signalling activate STAT4 and STAT1, respectively, which both contribute to expression of T-bet (Szabo et al., 2000; Lighvani et al., 2001). T-bet activates the IFN γ gene by chromatin remodelling, leading to secretion of IFN γ and increases expression of the IL12R β 2 chain, further enhancing both IFN γ and IL12 signals (Szabo et al., 1997; Mullen et al., 2001; Afkarian et al., 2002). It may also autoactivate, or sustain its own expression (Mullen et al., 2002), although this may require the presence of STAT1 signalling, suggesting that this autocrine reinforcement may be at the level of IFN γ signalling rather than at the transcriptional level (Afkarian et al., 2002; O'Shea and Paul, 2002). Either directly or indirectly, T-bet may also inhibit GATA-3 expression and IL4 production (Szabo et al., 2000). It therefore provides positive feedback for Th1 development and negative feedback for Th2 development. At a population level, the fate of T-cell differentiation therefore depends crucially on both the dynamics of GATA-3 and T-bet expression within individual T cells and the cytokines (particularly IFN γ and IL4) produced by the differentiating Th subsets themselves.

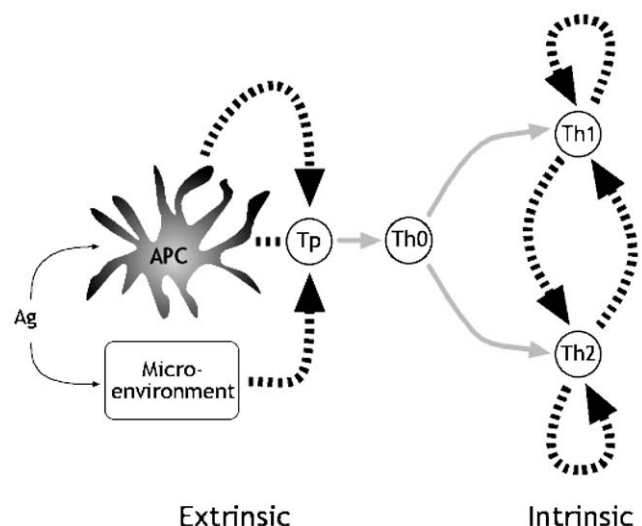


Fig. 1. Extrinsic and intrinsic cytokine interactions indicated by dashed arrows. Note that T-cell-derived cytokines can also influence APC activation status and/or other components of the microenvironment (e.g. IFN γ /IL12) (not shown).

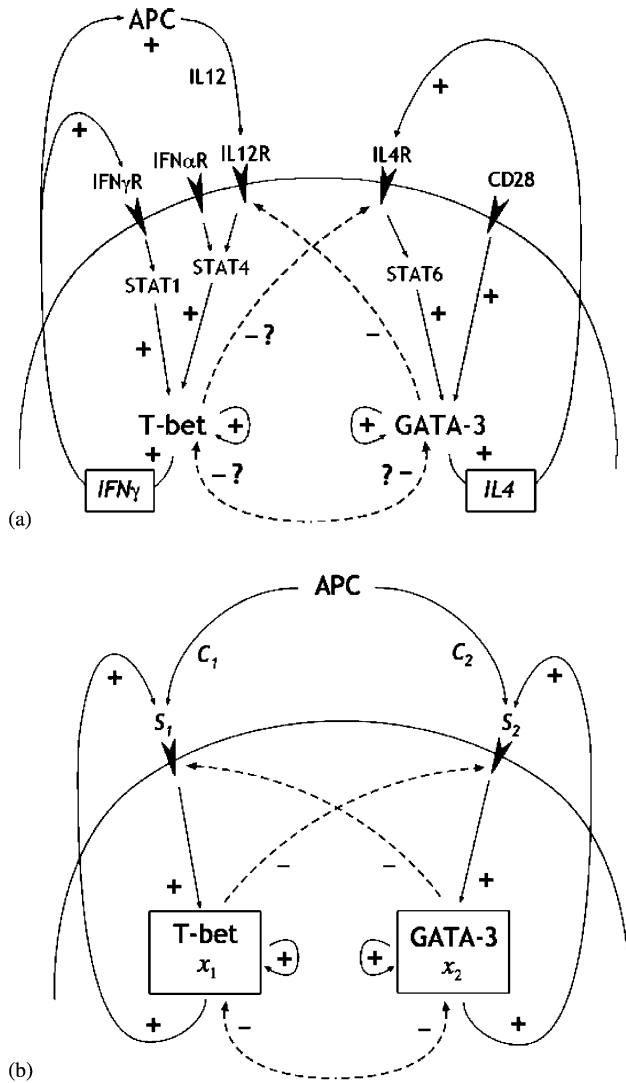


Fig. 2. (a) Schematic diagram of T-bet/GATA-3 interactions. (b) The simplified interaction scheme used in the model, derived from the interactions shown in (a).

Throughout this paper we use the concept of 'intrinsic' and 'extrinsic' signals (Figs. 1 and 2). Expression of T-bet and GATA-3 in an individual T cell is determined in part by *extrinsic* signals derived from sources other than the T cells, and in part by *intrinsic* signals from cytokines produced by the T cells themselves (Fig. 1).¹ *Extrinsic* signals made by dendritic cells or other accessory cells that promote T-bet expression include IL12 (Trinchieri, 1995) and IFN α (O'Shea and Visconti, 2000) and those that promote GATA-3 expression include IL4 (Yoshimoto et al., 1995; d'Ostiani et al., 2000), CD28 (Rodríguez-Palmero

et al., 1999); (Skapenko et al., 2001) and OX40 (Ohshima et al., 1998). Although expression of GATA-3 or T-bet does not seem to convey a selective proliferative advantage for Th2 or Th1 cells (Farrar et al., 2002) there is evidence that lineage commitment is coupled to cell division. In naïve T cells, the IFN γ and IL4/5/13 gene loci are relatively inaccessible to transcription factors. Changes in the chromatin structure and methylation of these genes may require several cell divisions, during which time some chromatin disassembly occurs mediated initially by antigen or cytokine-activated transcription factors such as NFAT and STAT but later by the sustained action of T-bet and GATA-3 (Miyatake et al., 2000; Rao and Avni, 2000). The need for cell division to attain progressively more rapid production of effector cytokines has been observed (Bird et al., 1998) and expression of IFN γ requires entry into S-phase of the cell cycle (Reiner and Seder, 1999). However, others have argued against a requirement for cell division for cytokine production as chromatin accessibility is acquired rapidly (Ben-Sasson et al., 2001). Nevertheless, all of these scenarios are consistent with epigenetic changes associated with the first 4 or 5 cell divisions leading to a reduction in the plasticity of a cell's cytokine secretion profile (Bix and Locksley, 1998; Grogan et al., 2001).

Despite the large amounts of experimental detail on the molecular basis of Th1 and Th2 differentiation there are still some key questions that have not yet been answered (Coffman and Reiner, 1999; Richter et al., 1999; Reiner, 2001; Farrar et al., 2002). Perhaps the most interesting is how the cytokine-mediated interactions between cells in a population are integrated with the signalling events and expression of transcription factors within individual cells. Put simply, if initial cytokine (IL12 and IL4) signalling together with costimulation (through, for example, CD28) at the time of antigen presentation by APC is sufficient to activate either GATA-3 or T-bet, why does this initial decision-making process have to be reinforced by cytokine feedback loops over several cell cycles to obtain a committed, polarized response?

In this study, a mathematical model of Th1 and Th2 differentiation is developed that allows gene regulation events occurring inside the cell to be integrated with intercellular signalling mediated by cytokines. The construction of the model is described in Appendix A. The model takes into account positive and negative feedback between GATA-3 and T-bet gene transcription and Th1 and Th2 cytokines acting between Th cells. This approach allows Th cell differentiation to be examined under different conditions in a way that is not feasible by experimentation. Although the model was developed specifically for T helper cell differentiation, the modelling framework can be applied more generally to populations of differentiating cells that

¹Note that this use of 'intrinsic' as a means of specifying T-cell-derived signals is slightly different to the notion of 'cell-intrinsic' encountered in the experimental literature (Murphy and Reiner, 2002) which refers to purely intracellular regulatory processes that operate in the absence of external signalling.

influence each other's state by cytokines or other extracellular signalling mechanisms.

2. Results and discussion

2.1. Dynamics of T-bet and GATA-3 expression in single cells

In order to understand how populations of Th1 and Th2 cells differentiate, it is necessary first to analyse how the *extrinsic* and *intrinsic* signals govern the behaviour of individual T cells. In this first section of the results, Eqs. (A.1a) and (A.1b) in Appendix A were used to analyse the behaviour of individual cells. The behaviour of Th cell populations is investigated in Section 2.2.

2.1.1. T-bet and GATA-3 systems function as bistable switches

To begin with, expression of GATA-3 in a single T cell responding to Th2 promoting signals was investigated. Fig. 3 shows schematically the steady-state expression of GATA-3 in a single T helper cell activated by antigen in the presence of extrinsic Th2 polarizing

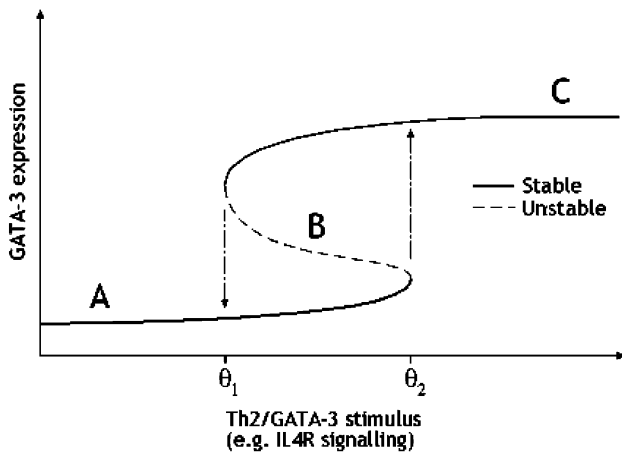


Fig. 3. A schematic diagram of the possible steady states of GATA-3 expression predicted by the model in the first few divisions following TCR activation, in the presence of varying levels of a continued extrinsic pro-GATA-3 signal. The quantity on the x-axis is S_2 , representing the sum of the external stimuli that induce expression of GATA-3, such as TCR signalling, IL4 or costimulation. We refer to these together as 'extrinsic' signals. Here, there is no Th1 stimulus and so T-bet expression remains low. Activated cells start with low levels of T-bet and GATA-3 expression. Increasing stimulation of GATA-3 by extrinsic signals up to a threshold level θ_2 increases GATA-3 levels slowly (region A). At the threshold, the cell rapidly approaches a state of high GATA-3 expression (region C) in which autoactivation of GATA-3 occurs at its maximum rate. In this state, the level of GATA-3 is relatively insensitive to fluctuations in the external stimulus. However if the stimulus is reduced below a lower threshold θ_1 the cell reverts to low level GATA-3 expression. In other words, continued extrinsic signalling at a level greater than θ_1 is required to sustain high levels of GATA-3 during the first few rounds of division.

stimuli but in the absence of any Th1 stimuli. At low levels of extrinsic Th2 promoting signals, the cell rapidly reaches a steady state with constant low levels of GATA-3 and T-bet expression. This may be interpreted as a Th0 state in which a recently activated cell is beginning to produce both Th1 and Th2 cytokines at low levels. This Th0-like state persists as the extrinsic Th2 promoting signal is increased until a critical threshold level is reached (θ_2) when the cell shifts quickly into a high level steady state of GATA-3 expression. We note that autoactivation of GATA-3 is essential for the existence of this state. The cell then remains in this state producing high levels of GATA-3 and no T-bet even if the initial Th2 signal is reduced below the original threshold level (θ_2). Only when the Th2 signal is reduced to a level (θ_1) well below the original activation threshold will GATA-3 expression fall back to the lower Th0 state. This simple model shows that the cell acts as a bistable switch, requiring a threshold of extrinsic Th2 signalling such as IL4 after TCR activation to reach a high level of GATA-3 expression. This is then insensitive to small reductions in the pro-Th2 signal. An entirely analogous picture holds for the relation between T-bet levels and the stimulation of T-bet production by IL12, IFN γ or IFN α , when GATA-3 levels are low and no Th2 stimuli are present. Biologically, this means that once a cell has been induced to express GATA-3 or T-bet by an initial Th2 or Th1 signal it will continue to express GATA-3 or T-bet at high levels even when the original extrinsic stimulus has been reduced. This may give an impression in experiments that Th2 and Th1 differentiation is irreversible, but this may not necessarily be true as shown in Section 2.2.4.

It is an important feature of the model that continued stimulation above the lower threshold level (θ_1) is required to maintain high steady-state expression of GATA-3 (or T-bet). If STAT-mediated or other Th2 or Th1 signalling is reduced below the lower threshold level (θ_1), the steady state characterized by high GATA-3 or T-bet expression is lost and the cell returns to a Th0 state. Note that a similar model describing bistable expression of GATA-3 is presented in Hofer et al. (2002), although the authors argue that once the high-level state of GATA-3 expression is reached it can be sustained solely by autoactivation of GATA-3 and so may be irreversible without subsequent modification of the rates of transcription or decay of GATA-3. We return to the issue of loss of reversibility in Section 2.2.6.

2.1.2. Expression of T-bet and GATA-3 is mutually exclusive

Experimental work has indicated that the Th1 and Th2 developmental programs are mutually suppressive at the level of cytokine gene expression (Grogan et al., 2001), although coexpression of T-bet and GATA-3 has

been observed under conditions of retroviral expression of T-bet in Th2-polarizing conditions (Afkarian et al., 2002). The dose-dependence and strength of any cross-inhibition of GATA-3 expression by T-bet and vice versa are critical for the behaviour of the model in this regard, and we chose parameter values to ensure that only one of these transcription factors can be expressed in high steady-state levels in any one cell at any time, reflecting the correspondence we make in the model between T-bet/GATA-3 expression and Th1/2 cytokine expression patterns during the early stages of the differentiation process. With these parameter choices, the model shows that a cell expressing high levels of GATA-3 maintained by continued signalling through the IL4R above the lower threshold level θ_1 is unable to increase T-bet expression *even* in the presence of a strong Th1 stimulus due to direct negative feedback of GATA-3 on T-bet and by downregulation of IL12R β 2 (Ouyang et al., 2000). This would give the appearance of irreversible commitment unless the original Th2 signal was reduced to a level well below that required to trigger the Th2 response in the first place (θ_2). Essentially, the same argument applies for Th1 responses.

One interesting outcome of the cross regulation between T-bet and GATA3 is that alterations of the parameters governing cross-suppression in disease could be responsible for inappropriate or mixed Th1 and Th2 responses. These parameters (γ_1 and γ_2) have not yet been determined experimentally but methods to measure them could be designed. It would also be interesting to compare these parameters in Balb/C mice, which have a predisposition to Th2 responses.

2.2. Th1 and Th2 differentiation of T-cell populations

With the conditions for high level T-bet and GATA-3 expression in individual cells established using Eqs. (A.1a) and (A.1b) (Section 2.1), the T-cell population model described by Eq. (A.3) was used to investigate Th1 and Th2 differentiation of T-cell populations. This combines the dynamics of GATA-3 and T-bet expression induced in single cells by *extrinsic* Th2 and Th1 signals with the action of T-cell-derived cytokines (i.e. *intrinsic* signals) on the whole population.

2.2.1. Extrinsic cytokine signals are required for Th1 and Th2 polarization

The roles of *extrinsic* and *intrinsic* signals in Th1 and Th2 differentiation of a population of T cells were analysed with the full model described by Eq. (A.3) in Appendix A. In the absence of extrinsic polarising signals from cytokines or co-stimulatory molecules such as IL12, IL4 and CD28, parameters are chosen such that stimulated proliferating T cells will remain in a Th0-like state and not express high levels of T-bet or GATA-3. This is consistent with numerous experiments that

suggest that optimal polarization of antigen-stimulated T cells into Th1 and Th2 populations requires extrinsic Th1 promoting (e.g. IL12) or Th2 promoting (e.g. IL4, OX40, CD28) signals derived from antigen-presenting or other accessory cells (for a review, see Ho and Glimcher (2002)). Thus, our model implies that intrinsic (T-cell derived) IFN γ or IL4 signals by themselves are not sufficient for Th1 or Th2 differentiation. Previous models of Th1/Th2 differentiation (Fishman and Perelson, 1993, 1994; Yates et al., 2000; Bergmann et al., 2002) implicitly impose the need for APC-derived signals for polarization, but in this study we model the transition from Th0 to Th1/2 status more explicitly. Note that the possibility of ‘pathogen-destruction feedback’ on the phenotype of the T helper response, as described in Bergmann et al. (2002), is not considered here. In our model, the information required for polarization comes from costimulation and cytokines present at the initiation of the response, before the development of effector T cells. The importance of cytokines is further highlighted in this model, in that blocking their action at any time during the first few divisions returns the population to a Th0-like state (Fig. 4). The model therefore predicts that Th1 and Th2 responses at this stage are reversible and that some other events or processes are required for irreversible commitment. This issue is examined more fully below (Sections 2.2.6 and 2.2.7).

An important feature of the requirement for both intrinsic and extrinsic signals in both the single cell and population models is that a threshold level of extrinsic signals from APC (or other accessory cells) is required to

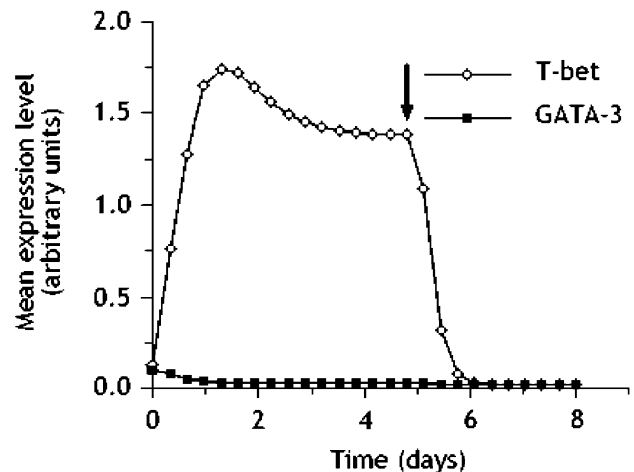


Fig. 4. Reversion to a Th0 phenotype in the absence of continued cytokine signalling. A population of cells was stimulated under strong Th1-inducing conditions (excess IL12) and the mean level of T-bet and GATA-3 expression over the cell population estimated as a function of time. Following the initial activation, T-bet increases rapidly and remains at a high level until cytokine signalling is blocked at day five (black arrow), when the population reverts rapidly to the Th0-like state with low levels of both T-bet and GATA-3.

generate a polarized T-cell population. This may be interpreted biologically as the impact of antigen-presenting cells and signals in the local microenvironment on Th1 and Th2 differentiation. The nature of this threshold was explored by stimulating T-cell populations in the presence of different levels of *extrinsic* Th2 signals that decay exponentially with a mean lifetime of 24 h. This might occur for example by apoptosis of antigen-presenting dendritic cells (Ludewig et al., 1995) and/or by down regulation of dendritic cell cytokine production. By varying the initial concentration of the *extrinsic* Th2 polarizing signal (defined in units of available cytokine molecules or costimulatory ligands per T cell) the threshold required for Th2 polarization was shown to be independent of the number of available T precursor cells. It is however dependent on choices of other parameters in the model as well as the initial distribution of T-bet and GATA-3 expression. In the simulations shown in Fig. 4, with an initial population of 32 000 T cells, the threshold level of extrinsic signaling required for Th2 differentiation was about five times the cytokine concentration produced by a population of polarized Th2 cells. In other words, with these parameter choices the threshold concentration of extrinsic cytokine or co-stimulatory signal required for polarization is about five times the concentration produced by the T cells themselves. Experimental determination of this factor would allow us to constrain the model parameters σ_2 (determining the relative contributions of STAT-mediated stimulation of GATA-3 levels and autoactivation to GATA-3 expression, which are set to be equal in this paper) and ρ_2 (the extrinsic signal level—or cytokine concentration—at which stimulation of GATA-3 is half-maximum).

If the *extrinsic* polarizing signal is present for a shorter time (that is, has a reduced half-life), for example by dendritic cell apoptosis or T-cell dissociation from the APC, the amplitude of the *extrinsic* signal required to achieve polarization must be increased in compensation. This can be thought of as the strength and duration of an APC signal and might include a “danger” signal. On the other hand, further increases in the magnitude or half-life of the signal above the threshold level have only a small effect on the time required for the population to reach the state of maximal GATA-3 expression due to the saturating nature of GATA-3 activation by cytokines or CD28.

An analogous process occurs for Th1 differentiation in the presence of an extrinsic Th1 polarizing signal such as IL12. In this case however there is stronger inhibition of T-bet production by GATA-3 than the converse due to a two-fold higher threshold of T-bet inhibition by GATA-3. This means that a twofold higher level of IL12 or other extrinsic signal is required to achieve a polarized Th1 population compared to the level of IL4 required for Th2 polarization. This asymmetry is in

keeping with experimental findings (Murphy et al., 1996; Park et al., 2000).

2.2.2. T-cell differentiation in the presence of mixed Th2 and Th1 extrinsic signals

T-cell responses to most antigens are not normally polarized exclusively into Th1 or Th2 responses but typically include components of both (Openshaw et al., 1995). The outcome of T-cell differentiation in the presence of mixed proportions of *extrinsic* Th2 and Th1 polarizing signals was therefore investigated. This approach can also be used to provide valuable information for therapeutic intervention with IL12 and IL4 cytokines or blocking antibodies. Fig. 5 shows the results obtained from the model by providing Th2 (IL4/CD28) and Th1 (IL12) signals together in varying ratios. The outcome of the response is indicated by the proportion of cells expressing high levels of T-bet (Th1) or GATA-3 (Th2) after 5 days.

In the presence of low levels of IL12 (3 arbitrary units/cell), a threshold value of approximately 40 arbitrary units/cell of IL4 is required to generate a Th2 polarized population compared to the 8 units/cell required in the complete absence of extrinsic Th1 signals (Section 2.2.1). In the presence of high levels of IL12 (3000 units/cell, Fig. 5B), doses of IL4 in the range 30–2000 units/cell are sufficient to negate the effects of IL12 and produce a Th0-like population but do not result in Th2 differentiation. Only when the IL4 dose exceeds approximately 3000 units/cell does a Th2 population develop. That is, the model predicts that in the presence of equal quantities of Th1 and Th2 polarizing signals (e.g. IL12 and IL4), much higher concentrations (150–300-fold) of both cytokines are required to achieve polarization than with either cytokine alone. At equal concentrations of extrinsic Th1 and Th2 polarizing signals above this threshold (3000 units per cell), the population of T cells uniformly expresses high levels of GATA-3, consistent with reports that IL4 signalling is dominant over IL12 (Nishikomori et al., 2000).

Various DC signals for Th2 differentiation have been proposed (Ohshima et al., 1998; Pulendran et al., 1999; Tanaka et al., 2000) but a Th2 response also appears naturally as a default pathway in the model described here. This is consistent with experiments in which Th2 responses arise in conditions under which IL12 or IFN γ are neutralized (Noben-Trauth et al., 2000; Ouyang et al., 1998).

2.2.3. Cytokine production by Th1 and Th2 cells acting on the whole population reduces the need for continued extrinsic (dendritic cell) instruction during Th1 and Th2 differentiation

A great deal of experimental work has been devoted to understanding how cytokines produced by Th1 and Th2 cells influence the differentiation of T-cell

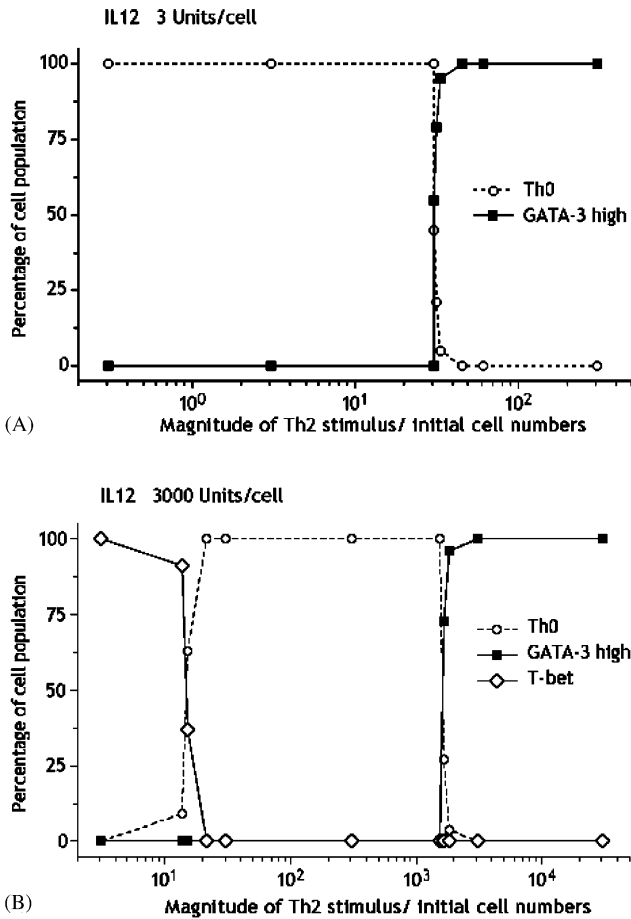


Fig. 5. Effect of combined Th1 and Th2 differentiation signals. To investigate how Th1 and Th2 signals interact to regulate Th differentiation, identical populations of cells were stimulated with extrinsic IL4 and IL12, which decay exponentially with a lifetime of 24 h. The percentage of cells classed as Th0, T-bet high or GATA-3 high after five days is shown when the initial dose of IL4 is varied in the presence of low levels of IL12 (3 arbitrary units/cell) (A) and high levels of IL12 (3000 arbitrary units/cell) (B). Cells were classed as having high or low levels of a transcription factor if its value was greater or less than the threshold value for autoactivation. The stimulus strength S_2 on the x-axis is expressed in units of initial stimuli divided by cell numbers. Mathematically, if the absolute level of an exogenous cytokine present in the culture can be represented by $C_0 \exp(-t)$, where C_0 is the initial dose, and N is the number of cells stimulated at $t=0$, the stimulus shown here is C_0/N . Measuring cytokine or costimulation levels in this way removes any dependence on the population size. In the presence of low levels of IL12, the population starts as Th0 and switches to Th2 (high GATA-3) when the Th2 signal is increased above 20 (A). In the presence of high levels of IL12, the population starts with high levels of T-bet (Th1) and switches first to Th0 when the Th2 signal is above 5. The population then remains in the Th0 state until the Th2 signal is increased to more than 500 when the cells switch to Th2 (high GATA-3) (B). Note that high levels of T-bet and GATA-3 are mutually exclusive, and that IL4 overrides IL12 signalling when both are present at equal, high doses.

populations (O’Garra, 1998). Less well understood is how these cytokines modify GATA-3 and T-bet expression in individual T cells responding to extrinsic (e.g. dendritic cell) signals. That is, the cross-talk between

events occurring within individual cells and the population as a whole. The role of intrinsic Th1 (IFN γ) and Th2 (IL4) signals on the polarization of T-cell populations was therefore investigated. Two numerical experiments were performed with identical populations of TCR activated T cells stimulated with a transient source of IL12 as described above (Fig. 6). In one experiment, the concentration of cytokines available to each cell was made proportional to the mean level of T-bet and GATA-3 expression in the whole population (see Appendix A). In effect, this means that cytokines produced by any one T cell are available to the whole T-cell population. In the other experiment, cytokines were considered to act exclusively through an autocrine mechanism. That is, the level of Th1 and Th2 cytokines sensed by each cell are directly related only to its own levels of T-bet and GATA-3 (IFN γ) and IL4). In this way, it is possible to distinguish between the action of intrinsic cytokine signals on individual cells (autocrine) and on the population as a whole (paracrine).

An important result emerged from these experiments (Fig. 6). When only autocrine cytokine signalling is allowed, a stronger or more sustained extrinsic signal (in this case IL12 from dendritic cells) is required to generate a completely polarized population than when the T-cell-derived cytokines are available to the whole population. In fact, a five-fold higher extrinsic signal was required to sustain a polarized response in the autocrine compared to the paracrine model (Fig. 5).

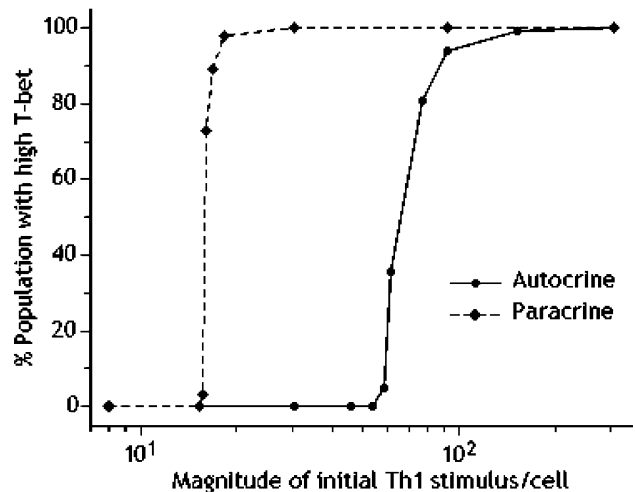


Fig. 6. Autocrine versus paracrine intrinsic cytokine signals. The threshold amount of IL12 required to polarize a population is higher when intrinsic cytokine signalling between cells is autocrine rather than paracrine. Populations were stimulated with a source of IL12 decaying with a lifetime of 24 h and the proportion expressing high levels of T-bet observed after five days. When cytokines are available to all proliferating cells (paracrine) a threshold dose of approximately 20 units/cell of IL12 is required to achieve Th1 polarization. This threshold is increased by a factor of five when cytokine signalling is autocrine.

Interestingly, when the variance of T-bet values over the population was increased, the amount of extrinsic signalling required to drive all the T cells into high levels of T-bet expression was also increased. These results show that the level of extrinsic (e.g. dendritic cell-derived) signal required by Th cells expressing very low levels of transcription factors to commit to Th1 or Th2 differentiation is very much reduced when cytokines produced by an individual T cell are available to all T cells in the population. This may be important in the later stages of Th differentiation when extrinsic signals from dendritic cells are reduced (Langenkamp et al., 2000). These simulations clearly highlight the importance of having both extrinsic signals from dendritic cells and other accessory cells AND intrinsic signals mediated by cytokines derived from Th1 and Th2 cells for optimal Th1 and Th2 differentiation.

2.2.4. Th1 and Th2 reversibility by manipulation of the cytokine environment—exploring the mechanism of T-bet/GATA-3 cross-suppression

The fates of differentiating T helper cells can still be influenced by cytokines several days after the priming event (Messi et al., 2003). The ability to reverse Th1 and Th2 differentiation would have important therapeutic benefits in a number of diseases such as immunity to parasites, inflammatory/autoimmune disorders and atopy, but this has proved difficult to achieve. In order to gain a better understanding of how Th1 and Th2 responses may be reversed, the model was used to investigate the plasticity of the response and the underlying mechanisms of cross-suppression.

To investigate reversibility of a Th1 response, T cells were first stimulated under saturating Th1-priming conditions generating a polarized Th1 population after 24 h. After 2.5 days, an extrinsic Th2 signal (e.g. IL4) was introduced for 24 h. The results obtained with varying doses of IL4 are shown in Fig. 7. Low doses of IL4 (<0.2 arbitrary units/cell) had no effect on the Th1 cells. At intermediate doses of IL4 between 0.2 and 50 units per cell, IL4 inhibited T-bet expression, and the Th1 cells reverted to Th0. High doses of IL4 (>50 units per cell) caused a switch to a Th2 population but only after transition through a Th0-like state. This switch occurred over approximately 24 h or 2 cell divisions. The transition through a Th0-like state en route to a Th2 state is required because T-bet expression, must first be decreased for a stable state of high level GATA-3 expression to exist at all. The transient Th0 state is stable if the IL4 concentration is high enough to abrogate T-bet expression, but insufficient to subsequently initiate GATA-3 production and drive the cell into a Th2 state. Interestingly, if the intrinsic cytokine signalling is autocrine rather than paracrine, the positions of the thresholds are unchanged but they are broadened. In this case, the width of the threshold is

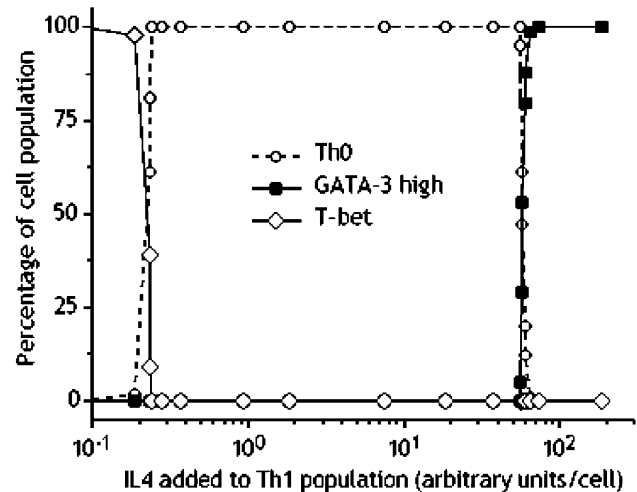


Fig. 7. Reversal of a Th1 population with a Th2 polarising signal such as IL4. Varying doses of IL4 were added to Th1-polarized populations after 2.5 days and the proportions of cells in the Th0, Th1 or Th2-like states were observed at day 6. Intermediate doses of IL4 induce reversion to a Th0 state but are not sufficient to fully switch the phenotype to Th2 (High GATA-3). Depending on the nature of T-bet/GATA-3 cross-suppression, additional blocking of IFN γ may also be required. The Th0 population switches to Th2 with higher IL4/cell concentrations (above 55 units/cell).

dependent on the distribution of the initial values of T-bet and GATA-3 expression and the amplitude of any noise that the levels of these factors are subject to. As a consequence, even a few T cells on an antigen-presenting cell in a localized microenvironment would commit to either Th1 or Th2 differentiation and local mixed populations would not occur. This implies that in responses where mixed Th1 and Th2 populations emerge, they would have to differentiate at distinct sites in a lymph node or nodes. Staining for cytokine (IL4 and IFN γ) production in T cell areas of lymphoid tissues could easily test this prediction.

Similarly, the model shows that Th1 cytokines such as IL12 can reverse a Th2 response in the same triphasic manner with the Th2 cells passing through a Th0 like state before differentiating to Th1 cells. In this case, however, the switch is far slower, even in the presence of saturating levels of IL12, taking 4 days (8 divisions). This is due to the stronger inhibition of T-bet expression by GATA-3 than the converse. In practice, other modifications to the cell dynamics (see below) may progressively reduce the plasticity of cells, implying that the Th2 state is effectively refractory to full conversion to Th1 status by addition of exogenous cytokines.

A consistent feature of reversing both Th1 and Th2 responses is the passage through a Th0 state. In the case of Th1 cells expressing high levels of T-bet, this arises from inhibition of Th2 (IL4) signalling thus preventing a stable state of high GATA-3 expression. T-bet levels have to decay sufficiently and the cells enter a quasi-Th0

state before Th2 signalling can increase GATA-3 levels and the stable Th2 state develop. Similarly, cells expressing high levels of GATA-3 cannot simultaneously express high levels of T-bet and the cells must pass through a quasi-Th0 state before switching to high T-bet expression. This mutual exclusion is a consequence of the cross inhibition between T-bet and GATA-3 as described above. Th1/Th2 plasticity is therefore a direct consequence of the ability of cells to revert to a Th0 state when the cytokine or costimulatory signalling necessary for stabilization of high T-bet or GATA-3 expression is reduced (Fig. 3).

Interestingly, this picture changes significantly if the architecture of cross-inhibition is altered so that T-bet and GATA-3 only inhibit each other indirectly by blocking extra-cellular signals such as IL12 or IL4 that induce their expression and not by direct inhibition of transcription. In this case, external Th2 signals (such as IL4) by themselves are unable to switch an established Th1 population to Th2 and blocking of Th1 signals is also required. Similarly, both Th1 cytokines and blocking of Th2 signals are required to switch a Th2 population to Th1.

These predictions from the model suggest simple experiments. Applying cytokines and blocking antibodies to recently stimulated cell populations should shed light on the internal wiring diagram of T-bet/GATA-3 regulation (Fig. 1). For example, a recent *in vitro* study showed that IL4 added 7 days after antigen stimulation could redirect non-IL4-producing CD4⁺ cells into an IL4-producing state without the requirement of blocking Th1 cytokines (Farrar et al., 2001). This finding is consistent with T-bet and GATA-3 cross-inhibition through both the direct and indirect pathways.

2.2.5. Reversal of Th2 and Th1 responses by ectopic expression of T-bet or GATA-3

Transfection and expression of T-bet in committed Th2 cells has been shown to convert them to a Th1 state with a concomitant increase in IFN γ production and inhibition of IL4 and IL5 expression (Szabo et al., 2000, 2002). In these experiments, IL2 was also suppressed, suggesting that this was not reversion to a Th0-like state but a genuine switch from Th2 to Th1. The switch was also independent of IFN γ and therefore not simply due to overproduction of IFN γ by the transfected cells and subsequent suppression of IL4. A similar picture was obtained when GATA-3 or STAT6 are ectopically expressed in Th1 cells, converting them to IL4-producers (Kurata et al., 1999; Lee et al., 2000).

The dynamics of this switch were studied using the model and compared with reversal of Th1 and Th2 responses by manipulating the extrinsic cytokine signal as described here. T cells were first stimulated with antigen in saturating Th1-inducing conditions. When a polarized Th1 population was generated, retroviral

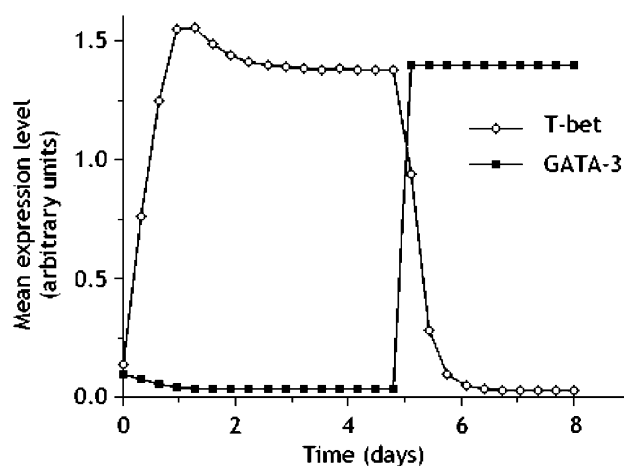


Fig. 8. Th1 and Th2 reversal by ectopic (retroviral) expression of T-bet or GATA-3. Naïve cells were stimulated with a source of IL12 decaying with a half-life of 8 h. Note that the mean level of expression of T-bet in the population ‘overshoots’ until maintenance of the high-level T-bet state is sustained by endogenous (intrinsic) cytokine production (2–5 days). At day 5 GATA-3 is ectopically expressed in all cells. After 24 h the mean level of T-bet expression in the cell population has decayed to background levels.

expression of GATA-3 was mimicked by imposing high levels of GATA-3 in all cells. The results showed that T-bet and GATA-3 were initially co-expressed but within 24 h (2 cell divisions) T-bet expression decayed to background levels (Fig. 8). Analogous behaviour was exhibited when high levels of T-bet were ectopically expressed in Th2 cells resulting in a purely Th1 population emerging after 24 h by way of a transient state of high T-bet and GATA-3 expression. We assume here that ectopic expression of either factor is not subject to cross-inhibition.

This result stands in contrast to the redirection of cell fates with cytokines as shown in Section 2.2.4 in which cells transit through a Th0-like state. Experimental observations of the speed and route of the transition between committed states under forced (ectopic) expression of transcription factors and treatment with cytokines would provide a further test of the validity of the model.

2.2.6. Irreversible commitment can occur through modification of transcription factor dynamics

It has been shown experimentally that after 4–5 cell divisions cytokine signalling may no longer be required to maintain cells in a high state of T-bet or GATA-3 expression and effector cytokine production. At this stage, reversibility is lost (Bird et al., 1998; Grogan et al., 2001). Irreversible commitment to Th1 or Th2 is associated with chromatin remodelling (Agarwal and Rao, 1998; Avni et al., 2002). The model as it stands does not predict this progressive loss of plasticity, as abrogation of extracellular signalling results in return to

an uncommitted (Th0) state. Although this is consistent with reversibility observed experimentally early on in the response it does not explain the irreversible commitment that occurs after 4–5 cell divisions. Chromatin remodeling may be involved but the model suggests an alternative mechanism by which irreversible commitment could occur later on in the response. A small (two-fold) increase in the maximal rates of transcription of T-bet or GATA-3 described by the parameters α_1 and α_2 (or a two-fold decrease in the rate of removal (μ) of these factors from the intracellular environment) would result in a state of stable high expression of T-bet (or GATA-3) in the complete absence of continued antigen activation and cytokine signalling (Fig. 9). Modification

of the T-bet/GATA-3 dynamics by increasing the maximum transcription rate or decreasing the rate of removal for example by stabilizing mRNA could be progressive and coupled to cell division. As commitment can be achieved through a small modification to the transcriptional dynamics, reversibility could potentially be restored by reducing the half-life of the transcription factors by for example degradation with caspases as described for GATA-1 (De Maria et al., 1999) or decreasing the transcription rate of GATA-3 and T-bet. Experimental manipulation of T-bet and GATA-3 transcription and degradation would confirm the model predictions and may lead to novel therapeutic interventions.

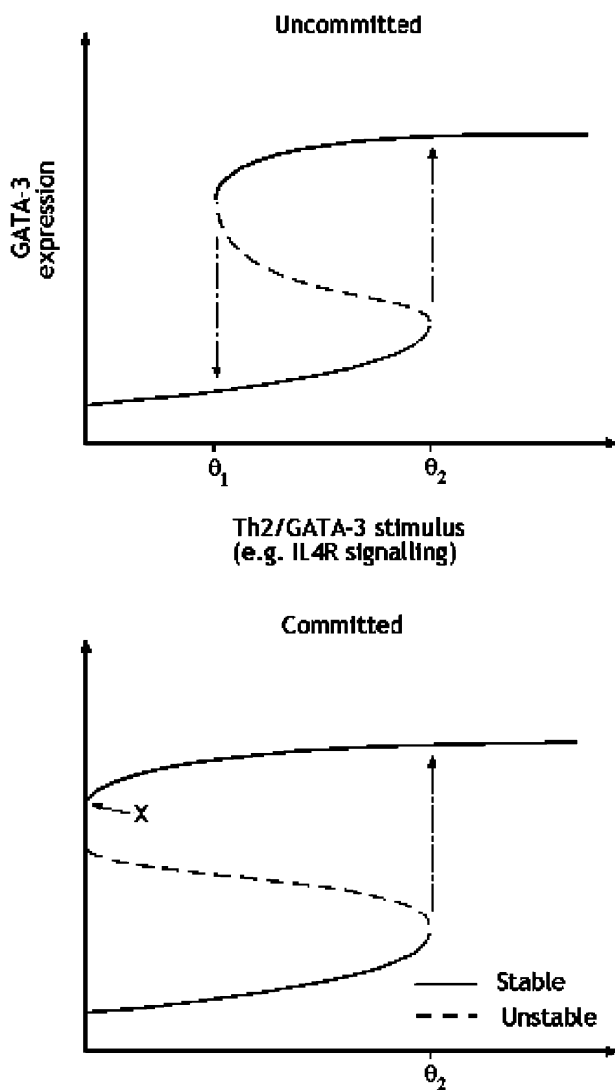


Fig. 9. Possible steady states of GATA-3 expression in committed and uncommitted cells. Modification of GATA-3 dynamics either by increasing the autoactivation rate of GATA-3 or decreasing its rate of removal results in the state of high GATA-3 expression becoming stable in the absence of any external signalling (point X). This modification of internal dynamics may occur through epigenetic modifications after several cell cycles.

3. Conclusion

The mathematical model described here was used to investigate the process of Th cell decision-making during Th1 and Th2 differentiation. The intracellular events that determine T-bet and GATA-3 expression at a single cell level were integrated with cytokine signals received by the differentiating Th1 and Th2 cells. In addition, the model distinguished between intrinsic signals from cytokines produced by the differentiating Th1 and Th2 cells ($\text{IFN}\gamma$ and IL4) and extrinsic signals from APC and the local microenvironment (IL12, IL10, CD80/86, etc.). It was shown that transient periods of TCR activation together with extrinsic Th2 (IL4/CD28) or Th1 (IL12) signals stimulate individual Th cells to enter steady states of high level but mutually exclusive T-bet or GATA-3 expression. Reversal of high level T-bet or GATA-3 expression can occur but only if the polarizing signal is reduced to a level well below that required to initiate the response. Experimentally, this could give the appearance of irreversible commitment if the polarizing signals could not be reduced to a sufficiently low level. An important feature of the model is that intrinsic signals from the cytokines ($\text{IFN}\gamma$ and IL4) produced by the differentiating Th1 and Th2 cells by themselves are not sufficient for Th polarization. This is in keeping with *in vitro* experiments, in which activated naïve T cells require exogenous cytokines or IL12 from APC to be polarized. Intrinsic signals can however lower the threshold of the response to extrinsic signals. Optimal polarization was shown to depend on both extrinsic and intrinsic signals. In particular, intrinsic cytokine signals can maintain Th1 and Th2 differentiation after the initial APC signals have diminished through for example dendritic cell apoptosis or migration of T cells away from APCs.

The picture that emerges from the model suggests that single cells require only a short instructive period during or immediately after Ag stimulation to begin autonomous progression towards a Th1 or Th2 state. This

instruction can take the form of the presence or absence of a variety of DC signals such as IL12 or CD80/86 ligation of CD28 and perhaps the duration of TCR signalling. However, this independent progress towards commitment at a single cell level can be enhanced and potentially redirected by additional cytokine signalling from other cells. In particular, intercellular signalling by cytokines (IFN γ and IL4) reduces the level of instruction signals from APCs needed to polarize a population and may also drive consensus among heterogeneous populations of recently stimulated T cells. Thus, T-cell-derived cytokines serve both to stabilize the internal cell states and amplify instructional signals from APCs.

The model shows that Th1 and Th2 differentiation could be reversed by appropriate manipulation of the extrinsic and intrinsic signals. Irreversible commitment could occur however by altering the rate of T-bet and GATA-3 transcription and/or degradation for example by permanent changes in the accessibility of cytokine gene loci. These epigenetic alterations would effectively lock the cell in one pattern of cytokine expression. The role of T-bet and GATA-3 transcription in commitment of Th1 and Th2 differentiation could be experimentally verified and may lead to novel therapeutic interventions in disease associated with inappropriate Th1 or Th2 responses.

Acknowledgements

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Appendix A

A.1. Methods: constructing the model

A mathematical model based on the interactions described in Fig. 2a was constructed to track the differentiation of naïve precursor T cells (Tp) following antigen stimulation. The Tp cells are assumed to express low levels of T-bet and GATA-3. T-cell receptor (TCR) activation induces the Tp cells to proliferate and become responsive to cytokines produced by the T cells themselves and by non-T-cell sources such as dendritic cells and NK cells. The combined level of Th1 inducing cytokines (e.g. IL12 and IFN γ) available to the cell is designated as S_1 , and Th2 inducing cytokines (e.g. IL4 and IL13) as S_2 . These variables also include the effect of other pathways that stimulate T-bet and GATA-3 production, such as signalling through IFN α , CD28 or the TCR. Note that this means that we do not explicitly model the STAT1, STAT4 and STAT6 signalling pathways independently. This simplified reaction scheme is shown in Fig. 2b. The concentrations of

T-bet within a single cell are denoted as x_1 and of GATA-3 as x_2 . The dynamics of T-bet and GATA-3 are modelled using a pair of rate equations, each of which can be expressed in words as:

The rate of change in T-bet (or GATA-3) expression equals the rate of increase in T-bet (or GATA-3) expression through external stimulation plus the rate of increase through autoactivation minus the rate of removal through natural decay or degradation.

The form of each of these terms was determined as follows.

A.1.1. Rate of increase in T-bet (or GATA-3) expression through external signalling

T-bet expression is increased by IL12 and (in humans) IFN α activation of STAT4 and IFN γ activation of STAT1 (Ho and Glimcher, 2002; Szabo et al., 2002). It may also be increased through TCR activation (Ho and Glimcher, 2002). In turn, T-bet itself induces the expression of IL12R β 2 (Szabo et al., 2000; Mullen et al., 2001). These pathways are represented in the model with a simple saturating rate of stimulation by pro-Th1 signals S_1 . The parameter ρ_1 is used to represent the level of signalling at which T-bet stimulation through this pathway is at half maximum and it is assumed that T-bet is transcribed at a maximal rate σ_1 in response to external signalling.

The rate of stimulation of T-bet through

$$\text{external signalling} = \sigma_1 \frac{S_1}{\rho_1 + S_1}.$$

Similarly, GATA-3 is stimulated at a maximum rate σ_2 by the IL4R-STAT6 pathway, and also through CD28/TCR signalling in a STAT6-independent manner as described above. Using the variable S_2 to represent the combined level of IL4 and CD28 signalling stimulating GATA-3 production,

The rate of stimulation of GATA-3 through

$$\text{external signalling} = \sigma_2 \frac{S_2}{\rho_2 + S_2}.$$

In addition, it is assumed that T-bet and GATA-3 are each transcribed at constant low background levels β_1 and β_2 , respectively.

A.1.2. T-bet and GATA-3 expression through autoactivation

GATA-3 stimulates its own production (Ouyang et al., 1998). The existence of an analogous pathway of T-bet production is controversial. Autoactivation in the absence of STAT4 signalling was reported by one study (Mullen et al., 2002), but Afkarian et al. (2002) argue that this autocrine stimulation occurs through an IFN γ -STAT1 mediated (i.e. an extracellular) pathway.

A more sophisticated model would treat the STAT1 and STAT4 (or $\text{IFN}\gamma$ and IL12) signalling pathways distinctly, but here we assume that autoactivation may occur at either the transcriptional level or through an exocrine ($\text{IFN}\gamma/\text{STAT1}$) pathway (these alternatives could be distinguished by inhibiting STAT1 signalling in developing Th1 cells in the absence of any other T-bet stimuli). If the T-bet stimulation is purely cytokine driven, the distinction between autoactivation and external stimulation of T-bet we describe in the model below can be interpreted as the relative contributions of cytokine signalling by the cell itself and from other cells in the proliferating population. Our key assumption, in either case, is that the autocrine pathway is inhibited by GATA-3 expression. Positive feedback on the expression of these key transcription factors, sustained at a single cell level, is the source of the bistability that is key to the model we present here.

Autoactivation is likely to be a multi-step process (Hofer et al., 2002), requiring at the very least three independent steps of transcription, translation and binding to the regulatory sequence of the gene. In the absence of knowledge of the relative rates of the steps in this chain of processes, we therefore model the autoactivation of these factors using Hill functions with exponent ≥ 3 . Autoactivation of T-bet and GATA-3 occurs at maximum rates α_1 and α_2 , and is half-maximum at the threshold levels κ_1 and κ_2 , respectively.

$$\text{Autoactivation rate of T-bet} = \alpha_1 \frac{x_1^n}{x_1^n + \kappa_1^n}$$

$$\text{Autoactivation rate of GATA-3} = \alpha_2 \frac{x_2^n}{x_2^n + \kappa_2^n}$$

The index n was chosen to ensure narrow threshold levels of transcription factors at which autoactivation is initiated. For example, with $n = 6$, the half-maximum autoactivation rate occurs at $x_i = 1$, 25% of the maximum autoactivation rate occurs at $x_i = 0.8$, and 75% at $x_i = 1.2$. The results discussed in this paper are robust for all choices of $n > 3$.

A.1.3. Removal of T-bet and GATA-3

We assume that free intracellular T-bet and GATA-3 are degraded at the same constant rate μ .

A.1.4. Cross-inhibition between T-bet and GATA3

Mutual inhibition of the Th1 and Th2 pathways has been observed in many studies of T helper differentiation at both the cytokine and transcriptional levels. For example, GATA-3, indirectly inhibits T-bet expression by downregulation of IL12R β 2 (Ouyang et al., 2000). It is also thought to directly inhibit T-bet expression (Ho and Glimcher, 2002). In the model presented here, dose-dependent GATA-3 inhibition of T-bet autoactivation (*direct inhibition*) and IL12R β 2 expression (*indirect*

inhibition) is manifest at a threshold level of GATA-3 denoted by the parameter γ_2 . Similarly, T-bet is assumed to inhibit both the STAT6 and STAT6-independent GATA-3 stimulatory pathways, as well as GATA-3 autoactivation. This suppression becomes apparent at a threshold level of T-bet expression given by the parameter γ_1 .

Cells cultured in the presence of both IL12 and IL4 give rise to a predominantly Th2-like population. This also occurs when IL12R β 2 is maintained by retroviral expression in the presence of exogenous IL4 and IL12, suggesting that it is due to dominance of IL4 signalling over IL12 (Nishikomori et al., 2000). This asymmetry is incorporated into the model through a small (2-fold) difference in the parameters that set the thresholds of suppression of IL4R and IL12R β 2 expression by T-bet and GATA-3 respectively (γ_1 and γ_2). The magnitude of this difference determines the time-scale of the development of a Th2 population under neutral stimulating conditions. The smaller the difference, the slower the development of the Th2 response.

Bringing together the assumptions and interactions described above, the following rate equations were used to describe the internal concentrations of T-bet (x_1) and GATA-3 (x_2) in a single cell:

$$f_1 = \frac{dx_1}{dt} = -\mu x_1 + \left(\alpha_1 \frac{x_1^n}{\kappa_1^n + x_1^n} + \sigma_1 \frac{S_1}{\rho_1 + S_1} \right) \times \frac{1}{(1 + x_2/\gamma_2)} + \beta_1, \quad (\text{A.1a})$$

$$f_2 = \frac{dx_2}{dt} = -\mu x_2 + \left(\alpha_2 \frac{x_2^n}{\kappa_2^n + x_2^n} + \sigma_2 \frac{S_2}{\rho_2 + S_2} \right) \times \frac{1}{(1 + x_1/\gamma_1)} + \beta_2. \quad (\text{A.1b})$$

A.1.5. Cytokine production by Th1 and Th2 cells

The levels of $\text{IFN}\gamma$ and IL4 in the intercellular environment during the first few rounds of cell division were taken to be proportional to the *total* levels of expression of T-bet and GATA-3, respectively, in the Th population. This follows from these assumptions:

- i During Th differentiation, the rates of production of $\text{IFN}\gamma$ or IL4 by a given cell are proportional to its levels of expression of T-bet or GATA-3, respectively.
- ii Rates of cytokine production and removal/binding are much faster than the rate of cell division. This implies that at any given time the cytokine concentration in the intercellular environment is proportional to its total rate of production.

Note that we only argue that this direct correlation between cytokine production and T-bet or GATA-3 expression holds immediately following TCR stimulation, during the first few rounds of division.

Experiments show that cytokine expression is dependent on activation, but that Th1/2 differentiation status can be maintained in its absence. We propose that cytokine signalling enhances polarization during the few rounds of division by feedback on T-bet/GATA-3 expression, until epigenetic changes ‘take over’ and Th1/2 differentiation becomes independent of the cytokine milieu.

A.1.6. Proliferation and differentiation of a CD4⁺ Th cell population

The Th1 and Th2 cell population density $\phi(x_1, x_2, t)$ is defined such that the number of cells expressing T-bet and GATA-3 in the ranges $[x_1, x_1 + \delta x_1]$ and $[x_2, x_2 + \delta x_2]$ at time t is $\phi(x_1, x_2, t) \delta x_1 \delta x_2$. Given the assumptions (i) and (ii) above, the levels of Th1 and Th2 cytokines (S_1 and S_2) that are available to each cell can be defined in terms of the exogenous (non-T cell) signals that stimulate T-bet and GATA-3 expression (C_i) together with the total levels of expression of T-bet or GATA-3 in the cell population:

$$S_i = \frac{C_i(t) + \int x_i \phi dx_1 dx_2}{\int \phi dx_1 dx_2}, \quad i = 1, 2. \quad (\text{A.2})$$

Here, C_i is the contribution of type 1 or type 2 cytokines and/or other signals from non-T helper cell sources. For example, IL12 will contribute to C_1 , and exogenous IL4 or stimulation of GATA3 through CD28 will contribute to C_2 . The C_i may also include direct stimulation of T-bet or GATA-3 by TCR stimulation. Note that normalization by total cell numbers allows for the limiting of access to cytokines by cell crowding. Given the internal dynamics described in Eqs. (A.1a) and (A.1b), it is straightforward to show (Cushing, 1998) that the time evolution of the cell density $\phi(x_1, x_2, t)$ is described by the structured population model

$$g \phi = \frac{\partial \phi}{\partial t} + \frac{\partial}{\partial x_1} (f_1 \phi) + \frac{\partial}{\partial x_2} (f_2 \phi), \quad \text{where} \\ f_1 = dx_1/dt, \quad f_2 = dx_2/dt. \quad (\text{A.3})$$

The quantity g is the rate at which cells divide. We assume that the cells divide uniformly every 12 h and therefore we choose g to be 2 days^{-1} . Making g a constant, independent of the x_i (T-bet and GATA-3 levels) and the S_i (extracellular signals), effectively decouples CD4⁺ T cell proliferation and differentiation, for which there is good experimental evidence. For example, transient exposure of CD4⁺ T cells to antigen initiates a program of up to seven cell divisions, while full development of a Th1 phenotype requires repeated TCR engagement with additional and continued cytokine signalling, normally through IL12 (Bajenoff et al., 2002).

It is known that costimulation, particularly through CD28, decreases the time spent between T-cell receptor

signalling by antigen and the first division as well as increasing the proportion of activated cells entering cycle (Gett and Hodgkin, 2000). Subsequent divisions occur relatively uniformly once every 12–18 h. This variability is not accounted for in our model, in part because it is assumed that all cells receive their stimulation at the same time and that any control of the rate of entry into the first cell cycle will be independent of the internal state (x_1, x_2) and so have no influence on Th1/2 differentiation. If the effect of antiproliferative signals such as CTLA-4 or TGF β were to be considered, these factors could be included in the proliferation function g through the signals S_1 and S_2 .

T cell-derived cytokines may be produced with different time-courses and have different polarizing capacities at given doses or stages in the developing response (Seki et al., 2004). However, we feel we are justified in grouping cytokines and other stimulatory signals together since we are concerned only with their net influence on the expression of T-bet or GATA-3, and use the population-averaged expression of these factors at any time as surrogate measures of extracellular cytokine levels. Treating these signals separately would clearly change the dose dependence of the interventions described in this paper, and modelling their timecourses would restrict the ‘windows’ available for each to used most effectively to manipulate a response. Importantly, decomposing the polarizing cytokine signals into more components would not alter the bistable switch behaviour of each arm of the model, but if the cross-suppression is restricted to certain components of S_1 and S_2 , the model may no longer predict the mutual exclusivity of T-bet and GATA-3 expression. Such coexpression has been observed experimentally (Afkarian et al., 2002).

A.1.7. Constraining parameters

The decay rate (μ of T-bet and GATA-3 governs estimates of the time required to reach maximum levels of transcription factor expression under saturating stimulation using Eqs. (A.1a) or (A.1b). Experimental evidence suggests that T-bet and GATA-3 can be detected soon after activation and reach maximum levels within 24 h of antigen stimulation (Grogan et al., 2001; Lighvani et al., 2001), and so an estimated value of 5 day^{-1} corresponding to a lifetime of 4–5 h was used. The stimulation rates σ_i and α_i were set to be comparable to each other, in the absence of experimental evidence to the contrary. Units of T-bet and GATA-3 levels were chosen such that σ_i and α_i are numerically equal to μ . This choice implies that equilibrium concentrations of T-bet and GATA-3 in a given cell at maximum levels of expression will be of order unity. In the absence of experimentally determined estimates, the values of the γ_i, κ_i and ρ_i were chosen to be equal, with the relative sizes of γ_1 and γ_2 reflecting the

Table 1

Parameter values for the model described in Eqs. (A.1a), (A.1b) and (A.3). Units of concentration of transcription factors are arbitrary

Parameter	Value	Meaning
σ_1, σ_2	5 day^{-1}	Maximum rate of stimulation of transcription of T-bet or GATA-3 through all external signalling pathways
γ_1	1	Level of T-bet at which inhibition of GATA-3 production is halved
γ_2	0.5	Level of GATA-3 at which inhibition of T-bet production is halved
α_1, α_2	5 day^{-1}	Maximum rate of auto-stimulation of T-bet or GATA-3 transcription
κ_1, κ_2	1	Level of T-bet or GATA-3 at which rate of auto-stimulation is half-maximum
μ	5 day^{-1}	Mean rate of removal or degradation of intracellular T-bet and GATA-3
ρ_1	1	Level of extracellular IL12/IFN γ at which stimulation of T-bet production is half-maximum. Units of cytokine concentration are chosen to match those of T-bet and GATA-3. That is, a population of cells expressing T-bet and GATA-3. That is, a population of cells expressing T-bet at maximal levels (≈ 1.8 with these parameter values) will correspond to an intracellular IFN γ concentration of 1.8.
ρ_2	1	Level of IL4 or CD28 signalling at which stimulation of GATA-3 production is half-maximum
β_1, β_2	0.05 day^{-1}	Baseline rate of transcription of T-bet or GATA-3 in resting naïve cells
g	2 day^{-1}	Rate of cell division

The degradation rate μ is estimated from T-bet/GATA-3 kinetics under optimal stimulation conditions (Grogan et al., 2001; Lighvani et al., 2001). See Section A.1.7 for a discussion.

differing cross-inhibitory potencies of IL4 and IL12 (Nishikomori et al., 2000). The parameter values used in the model are listed in Table 1.

A.1.8. Units of cytokine concentrations

The cytokine levels S_1 and S_2 are a measure of the available cytokines or costimulatory ligands *per cell* since the absolute levels are normalized by the total T-cell population size (Eq. (A.2)). Suppose an initial population of N_0 cells is stimulated with an exogenous source of IL12 with lifetime λ , then $C_1 = C_0 \exp(-\lambda t)$. Since the cells divide at constant rate g , the number of cells $N(t) = N_0 \exp(gt)$, and it follows that the IL12 available to each cell (that is, the exogenous contribution to S_1) will be $(C_0/N_0) \exp(-(\lambda + g)t)$. To remove effects of population size, exogenous cytokine levels are expressed throughout this paper as the value (C_0/N_0) —that is, as the initial quantity of cytokine available to each cell at the time of stimulation, rather than the absolute dose C_0 . The lifetime of the source (λ) is indicated in the text where necessary.

A.1.9. Solving the model

Eq. (A.3) was integrated numerically by the method of characteristics using a standard fourth-order Runge–Kutta algorithm. Using this technique reduces the master equation (Eq. (A.3)) to a set of $D \times 3$ coupled ordinary differential equations for the quantities $(\phi^{(i)}, x_1^{(i)}, x_2^{(i)})$, $i = 1 \dots D$, where D is the number of characteristics (or distinct initial cell states) used. The total initial population $\sum_i \phi^{(i)}(t = 0)$ was approximately 32 000 cells expressing low levels of T-bet and GATA-3 normally distributed with variance 0.05 over a square

patch of $D = 24 \times 24 = 576$ values of T-bet and GATA-3 expression $(x_1^{(i)}, x_2^{(i)})$ centred on the equilibrium levels of these variables in the absence of external stimulation (~ 0.2). All cells were stimulated with antigen at time $t = 0$ and begin to divide uniformly at the rate g . Other inputs to the model are the contributions to the cytokine or extracellular signalling environment from dendritic cells and/or other local microenvironmental sources, $C_1(t)$ and $C_2(t)$. At any time t , the population $(\phi^{(i)}(t), x_1^{(i)}(t), x_2^{(i)}(t))$ can be observed.

A.1.10. Modification of the cytokine environment

The cytokine levels S_1 and S_2 were calculated using Eq. (A.2). Blocking cytokine action with antibodies was mimicked by forcing these levels to zero. Artificial (retroviral) expression of transcription factors was simulated simply by overriding the normal intracellular dynamics and imposing particular values of x_1 or x_2 .

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